Closeout Notice Date: 21-JAN-1999

Project Number: G-33-E65
Center Number: 10/24-6-R6307-6A0
Project Director: BORKMAN, RAYMOND
Project Unit: CHEMISTRY
Sponsor: DHHS/PHS/NIH/NATL INSTITUTES OF HEALTH
Division Id: 3395
Contract Number: 2 R01 EY06800-06A3
Contract Entity: GTRC
Prime Contract Number:
Title: PHOTOCHEMISTRY & SPECTROSCOPY OF LENSES & LENS PROTEINS
Effective Completion Date: 30-APR-1998 (Performance) 30-JUL-1998 (Reports)

**Closeout Action:**

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<tr>
<th>Description</th>
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<tbody>
<tr>
<td>Final Invoice or Copy of Final Invoice</td>
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<td>Final Report of Inventions and/or Subcontracts</td>
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<td>Government Property Inventory and Related Certificate</td>
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<td>Classified Material Certificate</td>
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<td>Release and Assignment</td>
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**Comments:**

**Distribution Required:**

- Project Director/Principal Investigator: Y
- Research Administrative Network: Y
- Accounting: Y
- Research Security Department: N
- **Reports Coordinator:** Y
- Research Property Team: Y
- Supply Services Department/Procurement: Y
- Georgia Tech Research Corporation: Y
- Project File: Y

**NOTE:** Final Patent Questionnaire sent to PDPI
DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

APPLICATION
FOR CONTINUATION GRANT

VISA

REVIEW GROUP TYPE ACTIVITY GRANT NUMBER
TOTAL PROJECT PERIOD
From: 05/01/87 Through: 04/30/97
REQUESTED BUDGET PERIOD
From: 05/01/95 Through: 04/30/96

To be verified by applicant. Check information in Items 1 through 6. If incorrect, furnish correct information in Item 13.

1. TITLE OF PROJECT

PHOTOCHEMISTRY & SPECTROSCOPY OF LENSES & LENS PROTEINS

2a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR
(Name and address, street, city, state, zip code)

BORKMAN, RAYMOND P
GEORGIA INSTITUTE OF TECHNOLOGY
ATLANTA, GA 30332-0400

2b. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT

SCHOOL OF CHEMISTRY & BIOCHEM

2c. MAJOR SUBDIVISION

COLLEGE OF SCIENCES

3. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR BIOMEDICAL RESEARCH SUPPORT GRANT (See Instructions)

20 OTHER

4. APPLICANT ORGANIZATION (Name and address, street, city, state, zip code)

GEORGIA INSTITUTE OF TECHNOLOGY
GEORGIA TECH RESEARCH CORP
ATLANTA, GA 30332-0420

5. ENTITY IDENTIFICATION NUMBER

158063146A

6. TITLE AND ADDRESS OF ADMINISTRATIVE OFFICIAL

CONTRACTING OFFICER
GEORGIA INSTITUTE OF TECHNOLOGY
CENTENNIAL RESEARCH BUILDING
ATLANTA, GA 30332-0420

Complete the following (see instructions)

7. HUMAN SUBJECTS
If "YES," exemption no. or IRB approval date

x 7a. NO YES

4b. Assurance of compliance no.

8. VERTEBRATE ANIMALS
If "YES," IACUC approval date

x 8a. NO YES

8b. Animal welfare assurance no.

9. PERFORMANCE SITE(S) (Organizations and addresses)

School of Chemistry & Biochemistry
Georgia Institute of Technology
Atlanta, Georgia 30332-0400

10. COSTS REQUESTED FOR NEXT BUDGET PERIOD

10a. DIRECT $ 48,586
10b. TOTAL $ 68,020

11. INVENTIONS AND PATENTS (See instructions)

X NO YES "YES,"

Previously reported Not previously reported

TELEPHONE AND FAX INFORMATION

12. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Item 2a)

AREA CODE TELEPHONE NO. AND FAX NO.

Dr. R.F. Borkman
404 894-4024
404 894-7452

Janis L. Goddard
404 894-4817
404 894-6956

Janis L. Goddard
Contracting Officer

BITNET/INTERNET ADDRESS

raymond.borkman@chemistry.gatech.edu

13. USE THIS SPACE FOR CORRECTIONS TO ITEMS 1 THROUGH 6. INDICATE THE NUMBERS(S) WHERE ANSWERS APPLY.

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Wilful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 2a
(In ink. "Per" signature not acceptable.)

R.F. Borkman
DATE 2-23-95

15. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with the Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 12c
(In ink. "Per" signature not acceptable.)

Janis L. Goddard
DATE 2/24/95

PHS 2590 Optional (Rev. 9/91) Page 1
# Detailed Budget for Next Budget Period

**PERSONNEL (Applicant organization only)**

<table>
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<th>NAME</th>
<th>ROLE ON PROJECT</th>
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<th>DOLLAR AMOUNT REQUESTED</th>
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**CONSULTANT COSTS**

- 0

**EQUIPMENT (Itemize)**

- 0

**SUPPLIES (Itemize by category)**

- Chemicals ($1,340)
- Glassware & optical cells ($1,097)
- Chromatography solvents ($750)

- 3,187

**TRAVEL**

**SEE JUSTIFICATION**

- 3,400

**PATIENT CARE COSTS**

- Inpatient
- 0

- Outpatient
- 0

**ALTERATIONS AND RENOVATIONS (Itemize by category)**

- 0

**OTHER EXPENSES (Itemize by category)**

**PUBLICATION COSTS**

- 1,000

**SUBTOTAL DIRECT COSTS FOR NEXT BUDGET PERIOD**

- 48,586

**CONSORTIUM/CONTRACTUAL COSTS**

- Direct Costs: $0
- Indirect Costs: $0

**TOTAL**

- 0

**TOTAL DIRECT COSTS FOR NEXT BUDGET PERIOD (Enter on Page 1, Item 10a)**

- $48,586

**PHS 2590 (Rev. 9/91)**

(Form Page 2) Page __________
BUDGET JUSTIFICATION

SUPPLEMENTAL INFORMATION REGARDING ITEMS IN THE PROPOSED BUDGET FOR THE NEXT PERIOD WHICH REQUIRE EXPLANATION OR JUSTIFICATION. (See instructions)

TRAVEL:

Trip 1  Principle Investigator to attend ARVO meeting, Fort Lauderdale, Fl, May 14, 1995; ($900)

Trip 2  Principle Investigator to attend American Society Photobiology meeting, Washington, D.C., June 17, 1995 ($800)

Trip 3  Principle Investigator to attend European Society Photobiology meeting, Cambridge England, September 2, 1995, ($1,600)

CURRENT BUDGET PERIOD

<table>
<thead>
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<th>THROUGH</th>
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<td>5-1-94</td>
<td>4-30-95</td>
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The following pertains to your CURRENT PHS budget. This information may be used in determining the amount of support for the NEXT budget period.

A. CURRENT BUDGET

| TOTAL DIRECT COSTS | 77,325 | 0 |
| INDIRECT COSTS (As provided) | 19,212 | 0 |
| TOTALS | 96,537 | 0 |

PHS-2590 (Rev. 9/91) (Form Page 3) Page ______  Use Continuation Pages If Necessary C
OTHER SUPPORT
(Use continuation pages if necessary)

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or funding of the application.

Other support is defined as all funds or resources, whether Federal, non-Federal, or institutional, available to the principal investigator/program director (and other key personnel named in the application) in direct support of their research endeavors through research or training grants, cooperative agreements, contracts, fellowships, gifts, prizes, and other means.

Reporting requirements are: For each of the key personnel, describe (1) all currently active support and (2) all applications and proposals pending review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check “None.” Use continuation pages as needed to provide the required information in the format as shown below. Key personnel are defined as all individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project.

<table>
<thead>
<tr>
<th>Name</th>
<th>R. F. Borkman</th>
<th>Active</th>
<th>X</th>
<th>Pending</th>
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<td>p.i.</td>
<td>Borkman/Abraham</td>
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<td>Title</td>
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<td></td>
<td></td>
<td></td>
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<td>b. Your role on project</td>
<td>CO-PI</td>
<td>% Effort</td>
<td>10% no salary</td>
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<tr>
<td>e. Specific aims of project</td>
<td>R.F. Borkman (Georgia Tech) and E.C. Abraham</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>(Medical College of Georgia) are investigating the combined effect of UV radiation and sugar-induced glycation on animal lenses and lens protein extracts. UV and sugar insults are administered sequentially and Lens pigmentation and protein crosslinking are monitored. The aim is to see if there is synergism between UV and sugar.</td>
<td></td>
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<tr>
<td>f. Describe scientific and budgetary overlap</td>
<td>Some of the same experimental techniques are used in both projects, but there is no overlap in scientific aims or budget.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)</td>
<td>none needed.</td>
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1. SPECIFIC AIMS

This project is based on the premise that the living human lens absorbs UV radiation from sunlight during all or most of a person's lifetime and that radiation causes photochemical changes in the structural proteins of the lens. These photochemically altered crystallins experience different interactions with surrounding water and protein molecules in the cytoplasm resulting in protein aggregation and insolubilization, the net effect of which is a partially or completely opaque lens. The experiments described in the proposal are designed to detect and characterize photochemical reactions of the crystallins separately from other possible UV effects on the lens and to assess the potential of the photoproducts to initiate protein aggregation. Thus, our aims are:

To determine the chemical changes which occur in lens crystallin solutions exposed to monochromatic UV radiation in the 290-400 nm wavelength range for doses comparable to those received by the human lens under ambient conditions. Chemical changes which occur for low UV doses are of particular interest since damage to a small fraction of protein molecules in the living lens may initiate lens opacification. In our experiments, UV-induced changes in lens crystallins will be detected primarily by:

- SDS-PAGE, size exclusion HPLC, and electrospray mass spectrometry.
- Tryptic digestion followed by amino acid sequencing and FAB mass spectrometry of the tryptic peptides.
- Fluorescence and fluorescence quenching analysis of UV irradiated crystallin solutions.

To determine which of the UV-damaged populations may be important in causing protein aggregation and lens opacity. Can photochemically modified small oligomers, eg, monomers or dimers of gamma crystallin, initiate protein aggregation, or are high polymers required? This information will be obtained primarily by:

Measurements of turbidity in solutions containing defined populations of photochemically modified monomers or dimers or higher polymers of lens crystallins, mixed with normal crystallins.

Measurements of the temperature dependence of turbidity in solutions of photochemically altered crystallin proteins.
2. STUDIES AND RESULTS

During this first year of the new funding period, we have concentrated our efforts on: i.) Purchase and assembly of new photochemical irradiation and detection apparatus, and ii.) Photochemical experiments designed to detect interactions amongst individual crystallin proteins, including the possible role of α-crystallin as a "molecular chaperone" [Horwitz, 1992, 1993].

New apparatus purchased and put into use at the start of the funding period includes: 350 W mercury lamp and monochromator, programmable temperature bath/circulator, spectrophotometer interfaced to lab computer, and HPLC modifications. The UV irradiation set-up allows us to irradiate samples at monochromatic wavelengths from 290-400 nm, while maintaining sample protein solutions at fixed temperatures anywhere in the range 0-80 C. Changes in solution turbidity or chromophore absorption can be monitored throughout the UV and visible regions as a function of radiation dose and/or temperature. Since the spectroscopic data are obtained in situ while the thermal or UV insult is being delivered, kinetic data are obtainable. Thus, the effects of UV radiation, or elevated temperature, can be investigated separately or in combination. The state of aggregation of the resultant protein solutions can be checked after the UV/thermal treatment by measuring SDS-PAGE or size exclusion HPLC in separate experiments.

In work completed during the last grant funding period, we investigated the photochemical opacification of the bovine lens crystallin proteins individually, and noted important differences between them [Hott and Borkman, 1993], included in APPENDIX. β-crystallin and γ-crystallin solutions became opaque when exposed to UV radiation, while α-crystallin solutions remained transparent. Our original plan during the new funding period was to investigate the photochemical behavior of various combinations of the three crystallins in an effort to detect possible synergistic interactions amongst them. But, in the meantime, the important work of Horwitz appeared [1992, 1993] delineating the role of α-crystallin as a molecular chaperone. In light of these exciting new results, we have focussed our attention, initially, on the possible protective effect of bovine α-crystallin in photochemical opacification experiments on solutions of β-crystallin, γ-crystallin and other proteins.

Our important new finding, which has now been incorporated into a paper submitted for publication [Borkman, Knight, and Obi, 1995], and included in the APPENDIX, is that addition of stoichiometric amounts of α-crystallin to γ-crystallin solutions prior to UV photolysis afforded significant protection from opacification. Sample data are shown in Fig. 1 in the APPENDIX for 0.1 mg γ + 0.1 mg α in 1.0 ml of solution (panel A), 1.0 mg γ + 1.0 mg α in 1.0 ml of solution (panel B), and 10 mg γ + 10 mg α in 1.0 mg of solution (panel C). In each case, the opacity (absorbance at 600 nm) is greatly reduced when α-crystallin is present in 1:1 weight ratio. In other experiments (data not shown), we have studied the rate of UV induced γ-crystallin opacification as a function of added α-crystallin. The results indicate an optimum
α:γ ratio of 1:1 by weight. This corresponds to a molar ratio of 1:40, ie, one α-crystallin molecule (molecular weight 800,000) protected 40 γ-crystallin molecules (molecular weight 20,000) from UV aggregation at room temperature. This result is essentially identical to that found by Horwitz (1993) in thermal opacification experiments, and suggests that each subunit (αA2, αB2 chains) in the α-crystallin macromolecule can bind and protect one γ-crystallin molecule. Since the present experiments were done at room temperature, there is no evidence from our work that elevated temperatures are needed to activate the chaperone function of α-crystallin as has been suggested by some others [Rao and Raman, 1994].

Similar results were found for several other proteins, including aldolase, carbonic anhydrase, and enolase. In each case, addition of stoichiometric amounts of α-crystallin to these solutions afforded significant protection against UV induced opacification.

Irradiated solutions were analyzed by SDS-PAGE. γ-crystallin solutions were found to be highly crosslinked with the original 20 kDa material being converted to 40, 60 and > 100 kDa material. Solutions were centrifuged to separate any insoluble material formed by UV irradiation. The supernatant was found to be transparent following centrifugation. Thus, the major contribution to gamma crystallin UV opacification came from insolubilization, and added α-crystallin was able to prevent this insolubilization.

Photolyzed α-crystallin solutions analyzed by SDS-PAGE showed that much of the 20 kDa subunit material was converted to higher polymers greater than 100 kDa. Hence, calf alpha crystallin subunits were covalently aggregated by UV irradiation, but this did not result in insolubilization or solution opacity (Hott & Borkman, 1993). The fact that alpha crystallin was observed to crosslink, but not opacify, following UV irradiation suggested that this material may preferentially undergo intramolecular linking. Thus, the αA₂ and αB₂ polypeptide chains making up the α-crystallin macromolecule become photo-crosslinked to each other but the particle size remains at about 800 kDa. We next asked whether this photo-crosslinking would affect the ability of bovine α-crystallin to function as a molecular chaperone.

Three kinds of α-crystallin samples were prepared, by adjusting the dose of UV radiation delivered (Borkman & McLaughlin, 1995). First, no UV (normal α); second, α which was photo-crosslinked to the 50% level as detected by loss of 20 kDa subunits in SDS-PAGE; third, α which was photo-crosslinked to the 90% level as judged by SDS-PAGE. Each of these was used in subsequent chaperone experiments to determine if that α-crystallin sample could protect protein substrates (γ-crystallin, aldolase, liver alcohol dehydrogenase) from UV or thermal aggregation/opacification. Some typical data are shown in Fig. 2 for γ-crystallin, and all of the results are summarized Fig. 3 and in the pre-print (Borkman & McLaughlin, 1995) in the APPENDIX. The important conclusion is that UV photo-crosslinking of α-crystallin impaired its ability to function as a chaperone vis-a-vis both thermal and UV insults. But, the loss of chaperone function only
became large for large UV doses, ie, doses sufficient to produce 90% crosslinked α-crystallin. The α-crystallin which was 50% crosslinked by pre-treatment with UV still retained most of its chaperone capability as seen in Fig. 3. Thus, moderate UV damage seems to leave the chaperone function of α-crystallin intact; only extensive UV damage renders the chaperone inoperative (Borkman & McLaughlin, 1995).

3. SIGNIFICANCE

The human ocular lens is exposed to many forms of stress during an average person’s lifetime. This can result in cataracts. Until quite recently it was believed that the lens, being an avascular tissue, had little or no ability to protect itself from the deleterious effects of ambient UV radiation, chemical imbalances due to chronic diabetes, and other diseases and oxidative insults. The chemical effect of such insults can be to cause aggregation and insolubilization of the lens proteins. This ultimately results in an opaque lens or cataract. But, it now appears that nature provides some partial protection against protein aggregation in the form of the "molecular chaperone" α-crystallin. Horwitz showed that α-crystallin can prevent thermal aggregation of lens proteins by binding denatured protein intermediates. Our work demonstrates that α-crystallin may also be able to protect against UV induced lens protein aggregation by binding photochemical reaction intermediates. Thus, as long as the supply of chaperone molecules remains at a sufficiently high level, our lenses will be protected. This however raises the question as to whether the chaperone function of α-crystallin can be degraded by the same insults that affect the other lens proteins? In other words, does the chaperone wear out? The second aspect of our current work addresses this question. We have investigated the effect of pre-treatment of α-crystallin with UV radiation to ascertain the effect on chaperone function. Our data indicate that α-crystallin can withstand a relatively high level of photochemical damage and still perform the chaperone function, but ultimately, the ability to protect breaks down at high damage levels. This suggests that, clinically, it may be important in the future to be able to assess the state of a patient’s α-crystallin supply.

4. PLANS FOR NEXT YEAR

a.) Chaperone Studies: Since both heat and UV have now been documented as causes of lens protein aggregation preventable by the molecular chaperone α-crystallin, we plan studies of the combined effect of UV radiation and elevated temperature. Some previous reports have suggested that elevated temperatures are necessary to cause a molecular conformation change which then activates the α-crystallin chaperone function. If this is so, we expect to see more protection from a UV dose administered at high temperature (40C) than at low temperature (20C). A second aspect of UV chaperone function to be investigated is the nature of the complex which forms between α-crystallin and UV irradiated γ-crystallin. This can be elucidated using size exclusion HPLC as was done for the complex formed in thermal chaperone experiments (Wang &
Spector, unpublished). One expects to see loss of population in the molecular weight range associated with bare α-crystallin and growth of population at the molecular weight of the αγq chaperone complex. To date, no direct evidence for such binding is available for the UV aggregation case.

b.) **T. Measurements on UV Treated Proteins:** It is important to establish what kinds of photochemically produced species are responsible for initiating aggregation/opacification in solution, and in the intact lens. Thus, we will deliver sub-opacity-producing doses of UV to β- and γ-crystallin solutions, and measure the critical opacification temperature, Tc, of this modified protein compared to normal β- or γ-crystallin at the same concentration. The lens proteins βn, βl and various proteins of the γ family (γII, γIIIα, γIVα, etc) will be evaluated for their "aggregation potential" in this way. These photochemical intermediates will be further characterized chemically in the next phase of the experimental plan.

c.) **Characterization of Pre-Aggregated Lens Proteins:** We will use mass spectrometry to determine the masses of photochemical intermediates in pre-aggregated solutions of β- and γ-crystallin. Both the ESIMS and FAB versions of mass spectrometry are relevant. ESIMS will tell the molecular weight of the irradiated γ as it exists in solution. Is it monomer, dimer, trimer, etc? FAB will be used to determine the tryptic digest fragments present and to compared to normal, unirradiated γ-crystallin tryptic fragments.

*LITERATURE CITED IN REPORT*


Wang, K. and A. Spector (Unpublished Results).
This material has been removed by the Georgia Tech Library.

For more information, please contact Georgia Tech Library's Archives and Records Management Department.
5. HUMAN SUBJECTS -- None

6. VERTEBRATE ANIMALS -- None

7. PUBLICATIONS


CHECKLIST

Check the appropriate boxes and provide the information requested. Make this page the last page of the signed original of the application.

1. ASSURANCES/CERTIFICATIONS
The following assurances/certifications are made by checking the appropriate boxes and verified by the signature of the OFFICIAL SIGNING FOR APPLICANT ORGANIZATION on the FACE PAGE of the application. Descriptions of individual assurances/certifications begin on page 9 of Specific Instructions.

   a. Human Subjects (Complete Item 7 on the Face Page)
      [ ] Full IRB Review   [ ] Expedited Review
      Use of Human Subjects: [ ] Change   [X] No Change Since Previous Submission

   b. Vertebrate Animals (Complete Item 8 on the Face Page)
      Use of Vertebrate Animals: [ ] Change   [X] No Change Since Previous Submission

   c. Inventions and Patents (Complete Item 11 on the Face Page): none

   d. Debarment and Suspension: [X] No   [ ] Yes (Attach explanation)

   e. Lobbying
      With Federal appropriated funds: [X] No
      With other than Federal appropriated funds: [X] No   [ ] Yes
      (If "yes", see page 13, and attach Standard Form LLL, "Disclosure of Lobbying Activities," to the application behind the Checklist.)

   f. Delinquent Federal Debt: [X] No   [ ] Yes (Attach explanation)

   g. Misconduct in Science (Form PHS 6315): [X] Filed   [ ] Not Filed

      If filed, date of initial Assurance or latest Annual Report: 1/26/95

   h. Civil Rights
      Form HHS 441: [X] Filed   [ ] Not Filed

   i. Handicapped Individuals
      Form HHS 641: [X] Filed   [ ] Not Filed

   j. Sex Discrimination
      Form HHS 639-A: [X] Filed   [ ] Not Filed

   k. Age Discrimination
      Form HHS 680: [X] Filed   [ ] Not Filed

2. PROGRAM INCOME (See Instructions, Page 16)
All applications must indicate (Yes or No) whether program income is anticipated during the period(s) for which grant support is requested. [X] No   [ ] Yes. If "Yes" use the format below to reflect the amount and source(s) of anticipated program income.

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<th>Budget Period</th>
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3. INDIRECT COST
Indicate the applicant organization’s most recent indirect cost rate established with the appropriate DHHS Regional Office, or, in the case of for-profit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. Indirect costs will not be paid on foreign grants, construction grants, grants to Federal organizations and grants to individuals, and usually not on conference grants. Follow any additional instructions provided for Research Career Development Awards, Institutional National Research Service Awards, and specialized grant applications.

[ ] DHHS Agreement Dated:   [ ] No Indirect Costs Requested
[X] No DHHS Agreement, but rates established with Office of Naval Research   Date: 7/1/94

CALCULATION*
Enter proposed budget period:
Amount of base $48,586 × Rate applied 40 % = Indirect costs $19,434

*Check appropriate box(es):
[ ] Salary and wage base   [X] Modified total direct costs base
[ ] Other base (Attach explanation)

PHS 2590 (Rev. 9/91) (Form Page 8) Page 12
APPENDIX

BORKMAN  NIH  EY 6800-07

ANNUAL REPORT

MARCH 1, 1995
APPENDIX

Fig. 1. Absorbance at 600 nm versus time of irradiation at 295 nm for solutions of alpha and gamma crystallin: a. 10 cm path cell, 0.1 mg/ml gamma crystallin in buffer, —○—-, two runs are shown, and 0.1 mg/ml gamma plus 0.1 mg/ml alpha crystallin in buffer, —●—, two runs are shown; b. 1.0 cm path cell, 1.0 mg/ml gamma crystallin in buffer, —○—-, two runs, 1.0 mg/ml gamma plus 1.0 mg/ml alpha crystallin in buffer, —●—, two runs shown; c. 0.1 cm path cell, 10 mg/ml gamma crystallin in buffer, —○—, two runs, 10 mg/ml gamma plus 10 mg/ml alpha, —●—, two runs shown. All irradiations at room temperature with cell path length indicated.

Fig. 2. Thermal opacification data for 0.5 mg/ml γ-crystallin containing varying amounts of α-crystallin: 0.0 mg/ml, —□--; 0.25 mg/ml, —△--; 0.50 mg/ml, —△--; 1.0 mg/ml, —■--; and 2.0 mg/ml, —●—. Solution absorbance at 632 nm is plotted against the time of exposure to elevated temperature of 63°C. a. Normal α-crystallin. b. 50% crosslinked α-crystallin. c. 90% crosslinked α-crystallin.

Fig. 3. Summary of data on thermal and UV opacification of aldolase, HtrA2, and γ-crystallin in terms of the amounts of α-crystallin (0, 50, and 90% crosslinked) needed to significantly reduce aggregation/opacification.

REPRINTS and PREPRINTS


Borkman, R. F. and J. McLaughlin (1995) "The Molecular Chaperone Function of α-Crystallin is Impaired by UV Photocrosslinking" (Manuscript to be Submitted).
FIG. 1

A

B

C

UV TIME at 295 nm (min)

ABSORBANCE (600 nm)
CONCENTRATION DEPENDENCE OF TRANSMISSION LOSSES IN
UV-LASER IRRADIATED BOVINE \( \alpha \), \( \beta_H \), \( \beta_L \) AND
\( \gamma \)-CRYSTALLIN SOLUTIONS

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Abstract—Experiments with calf lens protein fractions in aqueous buffer solutions at room temperature showed that \( \beta_H \)–, \( \beta_L \)– and \( \gamma \)-crystallin fractions became opaque following ultraviolet exposure at 308 nm, while the \( \alpha \)-crystallin fraction remained transparent. Transmission loss, due to UV-irradiation, for all of the crystallin samples was studied in the concentration range of 0.1 mg/mL to 1.0 mg/mL, and for \( \alpha \)- and \( \gamma \)-crystallin, in the range up to 5 mg/mL. With increased concentrations of \( \beta_H \), \( \beta_L \), and \( \gamma \)-crystallin, the rate of opacification increased. However, with \( \alpha \)-crystallin, the loss of transmission was negligible for all of the concentrations and irradiation times studied. Opacification of the crystallins was accompanied by formation of higher molecular weight insoluble proteins as detected by SDS-PAGE.

INTRODUCTION

Ultraviolet light has been shown to cause opacity in calf lenses in vitro. In addition, other mammalian lenses have shown opacity after UV exposure in vivo.

In order to elucidate the changes occurring within the irradiated lens, various workers have studied the effects of UV irradiation on \( \alpha \)- and \( \gamma \)-crystallins. Investigation of the photolysis of \( \beta \)-crystallin has also been done, but to a lesser extent.

It has been ascertained that \( \gamma \)-crystallins became turbid when irradiated by 308 nm light (a wavelength present in sunlight and transmitted by the cornea), but that \( \alpha \)-crystallin remained clear under the same conditions. The loss of transmission was measured in \( \gamma \)-crystallin (20 kDa) because it acquires crosslinks when exposed to UV light and forms higher molecular weight species at 40 kDa, 60 kDa, 80 kDa, etc. \( \alpha \)-crystallin (800 kDa) also formed crosslinks between its 20 kDa subunit polypeptides but had no loss in transmission when irradiated. Because both of these crystallins are able to crosslink in solution, but their transmission results differ, it was proposed that \( \gamma \)-crystallin crosslinks occurred between the monomeric subunits, resulting in increased average particle size, whereas in \( \alpha \)-crystallin crosslinks formed only between subunits of a single 800 kDa oligomer and hence did not result in increased particle size. The situation for beta-high (\( \beta_H \))- and beta-low (\( \beta_L \))-crystallins was less clear.

The review article of Andley states that \( \beta \)-crystallins behaves like \( \alpha \) (and unlike \( \gamma \)) in that it does not form turbid solutions or precipitates upon UV-B exposure. Thus it was of interest to us to complete our study of the four crystallin fractions by including \( \beta_H \)- and \( \beta_L \)-crystallins in the present concentration study.

If crosslinking occurs between freely diffusing units, then one would expect the rate of crosslinking and opacification to increase with increasing protein concentration. However, for a protein that crosslinks mainly between subunits of a single oligomer, one would expect little or no concentration dependence and no opacification of the protein solution upon UV irradiation.

This work reports changes in optical transmission of crystallin solutions irradiated at 308 nm for a range of concentrations significantly lower than that in the intact lens. Our intent was to document the behavior of each individual crystallin solution, independently. Following the visible light transmission measurements, the samples are analyzed for changes in subunit molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Protein isolation. Lenses (courtesy of Brown Packing Co., Gaffney, SC) from approximately 3 month old calves were obtained immediately after death. The lenses were frozen at \(-5^\circ\)C until needed.

\( \alpha \)-, \( \beta_H \)- and \( \beta_L \)-crystallins were separated using Sephadex G-200 according to Bloemendal's procedure. The proteins were eluted with a buffer containing 1% ammonium bicarbonate (Fisher Chemical Co., Atlanta, GA) and 0.01 M \( \beta \)-mercaptoethanol (Sigma Chemical Co, St. Louis, MO), pH \= 8.1. The soluble fraction of a single lens homogenate was separated on a 2.5 \times 100 cm column at a flow rate of 0.5 mL/min for ca 30 h.

Separation of the \( \gamma \)-crystallin was performed according to Björk. Four lenses were homogenized with 10 mL of buffer (Tris-Cl 0.05 M, pH = 7.2, and 5 mM mercaptoethanol) and centrifuged. The supernatant was applied to a 5.0 \times 60.0 cm glass column (Sephadex G-75 also from Pharmacia) and eluted with 1300 mL of the above Tris buffer at a rate of 0.8 mL/min.

All of the separations were achieved in a 4°C cold box. After separation, the crystallins were lyophilized and stored at \(-5^\circ\)C until needed. The collected \( \gamma \)-crystallin was pooled and dialyzed in Spectravol 6000–8000 pore size membranes (Fisher Chemical Co.) prior to lyophilization.

Sample preparation. Stock solutions of the crystallins were made with 1 mg of the protein for every 1 mL of 0.01 M phosphate buffer, pH = 7.4. The solutions were stirred for 30 min, then centrifuged at 15000 rpm for 30 min, to remove any undissolved protein. The individual samples were made from the stock solution by dilution with phosphate buffer to a final concentration of 0.1, 0.3, 0.5, 0.7

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†Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; \( \beta_H \), beta-high; \( \beta_L \), beta-low; EDTA, ethylenediamine tetraacetic acid.
and 1.0 mg/mL. For a few experiments with α- and γ-crystallin, a higher concentration of 5.0 mg/mL was used.

**UV absorption measurements.** The absorbances of the crystallins in buffer solution were measured at 308 nm with a Shimadzu UV-3100 spectrophotometer prior to UV irradiation.

**UV irradiation and transmission measurements.** The irradiation and transmission measuring apparatus is shown in Fig. 1. A Lumonics excimer laser model 520 was operated at an average power of 0.5 W (at 10 Hz, 60 mJ per pulse) to irradiate the crystallin solutions. The wavelength of interest, 308 nm, was established using a mixture of 16 torr of 10% HCl, 40 torr of 99.997% Xe and helium (balance, to a final pressure of 50 psi) in the laser. We previously showed that under these irradiation conditions there was a negligible temperature rise (less than 1°C) in the protein solution. The irradiated solutions were monitored for loss of transmission utilizing a 5 mW helium–neon laser (model #105-1, Spectra-Physics Inc., Mountain View, CA). The beams of the excimer laser and He–Ne laser were made collinear via a beam splitter. The beams then passed through the sample, the collecting lens, a UV filter (Corning #2-61) and onto the photomultiplier detector (RCA 1P-28). Data collection was performed using an Omega DAS-16F interface board and Notebook software on an IBM PS/2 computer. Lens crystallin samples were irradiated for both 400 and 700 s at 25°C. A 300 s transmission baseline was taken before each irradiation was begun.

**SDS-PAGE.** SDS-PAGE was done using Bio-Rad's Mini-Protein II dual slab cell apparatus. The gels were made of 15% polyacrylamide and the SDS content was 0.1%. The gels were run at a constant voltage of 200 V. The stain used was Coomassie blue. The sample buffer contained: 50% water, 12.5% of a 0.5 M Tris (pH = 6.8) solution, 10% glycerol, 20% of a 10% (wt/vol) solution of SDS, 5% 2-β-mercaptoethanol and 2.5% of a 0.05% (wt/vol) solution of bromophenol blue. The samples were diluted 1:4 with sample buffer prior to application to the gel (per manufacturer's instructions).

**Centrifugation/insoluble protein samples.** Some irradiated crystallin samples were split into soluble and insoluble fractions via an Eppendorf centrifuge model 5415C at 10,000 rpm for 15 min. The insoluble fractions were washed with HPLC quality water (Fisher, Atlanta, GA) and centrifuged twice. For SDS-PAGE experiments, the insoluble fractions were dissolved in 100 μL of gel sample buffer. The soluble fractions were diluted 1:4 with sample buffer as previously stated.

**Protein assay.** A protein assay was performed on the supernatants of the irradiated and control samples using the Lowry method (Bio-Rad, Richmond, CA) to determine the soluble crystallin content. One hundred microliter aliquots of the samples were taken after centrifugation at 10,000 rpm for 15 min and mixed with 5 mL of the diluted dye reagent. Calibration curves of absorbance vs protein concentration were established using the unirradiated crystallins as standards. A Bausch and Lomb spectrophotometer model 20 was used to perform the absorbance measurements. Two samples were averaged for every data point.

**RESULTS**

The absorbances of the four crystallin fractions at 308 nm were measured prior to beginning the UV irradiation studies. For 1.0 mg/mL buffer solutions in 1.0 cm pathlength cells we obtained absorbances of: α, 0.002, βH, 0.01, βI, 0.01 and γ, 0.003 all in M⁻¹ cm⁻¹ units. Although these absorbances are quite low, the following data show that we were easily able to detect significant photochemical changes following UV exposures of only a few hundred seconds.

The transmission of the protein solutions, irradiated for 700 s at 25°C, is shown in Figs. 2-5 (note the initial baselines of 300 s before the UV source was turned on). With higher concentrations of βH-, βI- and γ-crystallins, the slopes of the transmission versus time curves became steeper. However, the transmission of the α-crystallin solutions changed very little, regardless of the concentration. Figure 6 shows a com-
posite of all four 1.0 mg/mL crystallins. Using this figure one can better compare the losses of transmission for the different proteins. A plot of the loss of transmission for the 0.5 mg/mL crystallins mimics the 1.0 mg/mL plot shown here.

Figures 7–10 are photographs of the crystallin solutions before and after 700 s of 308 nm irradiation at 25°C. The opacity of the irradiated \( \beta \text{H}, \beta \text{L} \) and \( \gamma \) solutions is easily seen, as is the appearance of insoluble precipitate, whereas the \( \alpha \) solutions remained transparent after irradiation. Upon centrifuging, the irradiated \( \beta \text{H}, \beta \text{L} \) and \( \gamma \) solutions became clear again (with precipitate at the bottom of the centrifuge tube). No insoluble material was seen after centrifuging the \( \alpha \)-crystallin samples.

Figure 11 shows the SDS-PAGE gels of the crystallin samples before and after irradiation. The data shown are for the entire samples, including any insoluble material that may have formed upon UV irradiation. Lanes 1, 4, 6 and 9 are the unirradiated \( \alpha, \beta \text{H}, \beta \text{L} \), and \( \gamma \) solutions, respectively. All of the dark control gels were intentionally overloaded in an attempt to clearly show any differences resulting from UV irradiation. (The gel of the unirradiated \( \alpha \) sample showed the presence of some residual \( \beta \text{H} \)-crystallin peptides, resulting from incomplete chromatographic resolution of the two crystallins.) Lanes 2, 5, 7 and 10 are the irradiated \( \alpha, \beta \text{H}, \beta \text{L} \), and \( \gamma \) samples. Note the loss of starting material in the 20–35 kDa range from each of the crystallins. There was also formation of high molecular weight material that did not enter the separating gel, but this is not noticeable in the figure.

In addition, gels were made for 400 s irradiated samples. The gels were practically identical to the 700 s irradiation gel and are therefore not shown. Essentially all of the starting 20–35 kDa material was found at higher molecular weights (>100 kDa) than the gel could resolve.

SDS-PAGE was also performed on separated soluble and insoluble \( \beta \) - and \( \gamma \) -crystallin material from irradiated samples and compared to unirradiated controls. Both samples showed a large amount of material above and below the molecular weight of the starting material in the insoluble irradiated fraction. The irradiated soluble fraction contained mostly...
original starting material (but of a less amount than in control samples) and some material above the gel limit of 100 kDa.

The data presented are for the concentration range of 0.1–1.0 mg/mL. However, the concentration range of 0.5–5.0 mg/mL was also explored with α- and γ-crystallins. In addition, a higher power output of the excimer laser, 2.0 W, was studied. With higher concentrations and greater UV power, the loss of transmission for γ-crystallin was much faster, whereas the loss rate for α-crystallin was still negligible.

All of the samples were irradiated until they reached a transmission level at which there was no further change (the lowest points in Figs. 2–5). In order to assure that α-crystallin had ample time to react, α-crystallin was irradiated for up to 1200 s, and still no transmission loss was seen.

In order to detect protein losses from the soluble crystallins, all of the samples were subjected to a Lowry protein assay. Every sample (α, β, βL, and γ) showed losses in the amount of soluble protein after irradiation, ranging from 10 to 50% of the starting concentration.

**DISCUSSION**

In attempting to make a connection between the optical transmission data and the SDS-PAGE crosslinking data obtained in the present work, it is important to clearly distinguish four possible classes of photochemical crosslinks in the crystallin proteins: (1) Link between two amino acids within a single polypeptide chain. (2) Link between amino acids located in two separate monomeric polypeptide chains. (3) Link between two separate polypeptide chains in a single oligomeric unit. (4) Link between a polypeptide chain in an oligomeric unit and another polypeptide chain in a second oligomeric unit. Type (1) could occur in any of the crystallin proteins, but it would not be likely to affect either the SDS-PAGE pattern (because it does not alter the subunit size) or the optical transmission. Type (2) could only occur in monomeric γ-crystallin. Type (3) could occur in α- or β-crystallin and would affect the SDS-PAGE pattern (crosslinking of subunits) but probably not the optical transmission. Type (4) could also occur in α and β, and in this case both the SDS-PAGE and the optical transmission would likely be affected.

From the transmission data presented (Figs. 2–5), one would expect that a photochemical reaction occurred in all of the samples except α-crystallin. However, the SDS-PAGE results in Fig. 11 show that the subunits of all of the crystallins, including α, were photochemically crosslinked upon exposure to UV. The gels show that for each crystallin fraction, there is a loss of concentration of the starting subunits (20–35 kDa range) and formation of higher molecular weight material at 100 kDa and above. (Previous work has demonstrated the presence of dimers and trimers of subunits, *e.g.* in γ-crystallin at 40 and 60 kDa, but these are not clearly seen in the gels in Fig. 11, because the UV dose here is greater, and most aggregates are of higher molecular weight.) Our data imply that the photo-crosslinking reactions in β- and
\[
\beta;\gamma;\alpha = (1.0):(0.3):(0.2).
\]
The computed tryptophan-only absorbances at 308 nm would lead to relative photochemical reaction rates of
\[
\beta;\gamma;\alpha = (1.0):(0.5):(0.25).
\]
These two sets of values are in reasonable agreement with each other and indicate that tryptophan is the dominant absorber in our samples at 308 nm. The actual observed ratio of photochemical reaction rates, taken from Fig. 6, is
\[
\beta;\gamma;\alpha = (1.0):(1.0):(0.04).
\]
Thus, the absorbances of the three crystallin fractions correctly predict \( \alpha \) to be the least reactive. But the remaining reactivity predictions do not agree quantitatively with our experimental rates. Thus, it seems that although absorbance may play some role in determining crystallin reactivity, it is probably not the most important factor in determining the relative photochemical reactivities of the four crystallin fractions.

The behavior of \( \beta \)-crystallins, which we observed in the present work, does not agree with the statement in the review article of Andley,\(^{18}\) which indicates that \( \beta \)-crystallins do not become insoluble upon UV exposure. The photographic data for \( \beta_1 \) crystallin in Fig. 9 show particularly clearly the precipitation of this protein following UV exposure.

The increase in the rate of opacification and crosslinking of \( \gamma \)-crystallin with an increase in protein concentration was
expected, as one proposed mechanism involves diffusional encounters between monomers in solution. The same mechanism might not be appropriate for α-crystallin (slower diffusion). Photo-crosslinking in α-crystallin may occur via mechanism above. This deduction is based on α-crystallin’s negligible loss of transmission, even though SDS-PAGE shows that most of the 20 kDa subunits are lost following UV.

We found βγ-crystallin’s transmission loss rates to be different from γ-crystallin’s loss rates. Nevertheless, both proteins ultimately reach the same point of opacity (βγ-crystallin’s transmission drops later, as seen in Fig. 6). The βγ-crystallin transmission loss was somewhat surprising because the quaternary structure of this family of proteins (a mixture of oligomeric structures) more closely resembles that of α-crystallin. The results suggest that a significant amount of crosslinking in βγ-crystallin may occur as in βL- and γ-crystallins. If crosslinking in βγ occurred only within a single oligomer unit, one would expect to see no change in the transmission of the sample, as in α-crystallin. One possibility is that both intra- and interoligomer crosslinking is occurring in βγ-crystallin.

The results from 400 s UV irradiations indicated that the crystallins did not begin to lose transmittance at these shorter times, even though they showed significant losses of subunits and formation of higher molecular weight species. One can infer that the loss of 20–35 kDa crystallin material to molecular weights above 100 kDa precedes significant loss of optical transmission. Loss of optical transmission presumably requires that the size of the light-scattering particles approaches the wavelength of visible light.

The Lowry protein assay was done to determine the loss of soluble protein. We observed losses in the soluble protein content for all of the samples, but these results are inconclusive because the dye used in the assay binds primarily to aromatic amino acids, which may be altered by UV radiation.

The role of the various crystallins in UV-damaged lenses is still being elucidated. Future studies must include mixtures of crystallins over a range of concentrations. The present data show that UV-radiation causes loss of transmission in the individual crystallins with γ being fastest and α slowest.

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Picosecond fluorescence decay of lens protein γ-II crystallin

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Picosecond fluorescence decay of lens protein γ-II crystallin

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Abstract

The fluorescence decay of tryptophan residues in the bovine lens protein γ-II crystallin has been measured in aqueous buffer solutions. Results were obtained as a function of emission wavelength, temperature, dissolved oxygen, and denaturing solvent. The protein displayed complex fluorescence decay which fit a biexponential model with a long component (ns) and a short component (few hundred ps). Measured fluorescence quantum yields data for γ-II crystallin allowed calculation of radiative and non-radiative rate constants. The radiative rate constant was consistent with that observed in other indole derivatives, while the nonradiative rate constant was quite large and accounted for the short lifetime in γ-II. The temperature dependence of the non-radiative decay in γ-II crystallin yielded a small activation energy of only 1–2 kcal/mol, compared to 4 kcal/mol for the reference compound NATA whose barrier is known to derive from the rotamer model.

Keywords: Fluorescence lifetimes; Lens proteins; Crystallins; Tryptophan; Non-radiative decay

1. Introduction

The decay of protein fluorescence is sensitive to changes in the environments of emitting fluorophores [1]. Thus, fluorescent probes near the binding sites of enzymes can serve as reporter groups for substrate or cofactor binding [2]. Similarly, changes in protein conformation can affect the emission properties of fluorescent tryptophan residues [3]. Examples involving dynamics of local and global motions in proteins have also been reported [4].

The crystallin proteins are responsible for maintaining the transparency of the ocular lens [5]. Hence, the light absorption [6], emission [7–10], Rayleigh [11,12] and Raman scattering [13] properties of the crystallins are important. Changes in the conformation and/or state of aggregation of the crystallins may accompany cataracts [14–17] resulting in loss of lens transparency. Complete X-ray structures are available for a few members of the gamma crystallin family [18,19], including bovine γ-II crystallin.

Photochemical reactions of lens crystallin proteins are of potential importance in the etiology of human cataracts and studies of the effect of...
UV radiation on whole lenses and lens protein fractions have appeared [20–27]. The aromatic amino acid tryptophan seems to play a key role in some types of lens photodamage [20,27]. Photochemical reactions of amino acids and proteins, may originate from excited singlet states, excited triplet states, and photoionized states. Photochemical reactions which originate from the lowest excited singlet states (S₁) of proteins compete in time with fluorescence emission. Hence measurement of fluorescence decay provides information on the rates of excited singlet state processes, including photochemical reactions. It has been suggested that protein photochemistry may stem from a photoionized state of tryptophan, but there has been controversy over whether ionization originates from the same precursor state as fluorescence or from a “pre-fluorescent state” [28–30]. Fluorescence lifetime measurements can aid in answering such questions.

In the present paper, we report measurements of the nanosecond and picosecond decay components of tryptophan residues in the bovine lens protein γ-III crystallin, including effects of temperature, dissolved oxygen, and denaturing solvent. None of these properties have been reported previously.

2. Materials and methods

Separation of the γ-crystallin was performed according to Björk [31]. Four lenses were homogenized with 10 mL of buffer (Tris-Cl 0.05 M, pH 7.2, and 5 mM mercaptoethanol) and centrifuged. The supernatant was applied to a 5.0 × 60.0 cm glass column (#XK50/60, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) packed with Sephadex G-75 (also from Pharmacia) and eluted with 1300 mL of the above Tris buffer at a rate of 0.8 mL/min. The separations were achieved in a 4°C cold box. After separation, the crystallin was lyophilized and stored at −5°C until needed. The collected γ-crystallin was pooled and dialyzed in Spectrapor 6000–8000 pore size membranes (Fisher Chemical Co.) prior to lyophilization.

Tryptophan monomer and N-acetyltryptophan amide (NATA) were used as standards for comparison with the lens crystallin protein. These were obtained from Sigma (St. Louis, MO).

For fluorescence decay measurements, samples were dissolved in 0.1 M pH 7.4 phosphate buffer to a level of about 1 mg/ml. Final concentrations of protein, Trp, and NATA were adjusted to give solutions having 295 nm absorbance of 0.1 units in a 1.0 cm path quartz cell. For some experiments, the protein was dissolved in 5 M guanidine hydrochloride solution in 0.1 M phosphate buffer also at pH 7.4. The lifetime measurements were done with samples in 1.0 cm path length quartz cells containing 1 ml of solution.

Steady state absorption and fluorescence emission spectra of the crystallin and reference solutions were routinely checked using a Shimadzu UV-3100 spectrometer and a Spex fluorolog-2 fluorimeter, respectively. All absorption and emission spectra were comparable to previous data from the literature [6,7] and are not reproduced here. The quantum yield of γ–II was determined relative to that of NATA by preparing solutions having equal absorbance of 0.1 at 295 nm and then comparing the areas under their corrected fluorescence emission curves excited at 295 nm. Under these conditions, absorption and emission by tyrosine residues in γ–II crystallin are negligible relative to that of the tryptophan residues.

To determine the possible effect of dissolved oxygen (O₂) on the measured fluorescence decays, some solutions were bubbled with oxygen or nitrogen gas for 30 minutes immediately prior to fluorescence lifetime measurements.

The apparatus for picosecond lifetime measurements has been described elsewhere [32]. A Coherent Antares 76-S mode locked YAG laser pumped rhodamine-6G in a Spectra Physics 375-00 dye laser with Spectra Physics 344 cavity dumper to produce a 3.8 MHz train of 10 ps pulses at 590 nm. The output of the dye laser was doubled with an angle tuned KTP crystal to produce radiation at 295 nm. The 590 nm light was removed from the excitation beam using a cutoff filter. The intrinsic polarization of the 295 nm beam was horizontal, and a half-wave plate was used to rotate the polarization to the vertical.
This beam was used to excite the samples. The instrument response function was determined by measuring the intensity profile of 295 nm radiation scattered from phosphate buffer solution containing a drop of milk. The full width at half maximum of this profile was typically 80 ps, as seen in Fig. 1.

Fluorescence decay was monitored at a 90° angle to the excitation beam at discrete emission wavelengths in the range 320–420 nm using a Nikon P250 monochromator between the sample and the Hamamatsu channel plate photomultiplier. A 310 nm cut-off filter was interposed between the sample and emission monochromator to remove any residual scattered exciting light. A Glan prism polarizer set at the magic angle of 54.7° was also placed between the sample and monochromator.

Photon counting rates were kept below 10,000 cps, and data were collected to yield 1000–10,000 counts in the maximum channel. This typically required 5–20 minutes per sample depending on the emission quantum yield. All measurements were done at room temperature of 23 ± 1°C except as noted. For temperature variation studies, a thermostated water–ethanol bath was circulated through a brass jacket surrounding the sample. Temperatures were stable to within ± 0.2°C.

At least three fluorescence decays were recorded under each set of experimental conditions, and numerical data presented are averages.

Fluorescence decay data were analyzed using the GLOBAL software package [33]. Decay functions containing one, two, or three exponential components were convoluted with the instrument response function and fitted to the experimental decay data. The quality of the fits was characterized in terms of the reduced chi-squared value, the distribution of residuals, and the autocorrelation function of the residuals. With these procedures, decay components as short as 20 ps could be recovered, but no decays shorter than 40 ps are reported in the present work. Although the GLOBAL software is capable of treating complex fluorescence decay situations involving excited state reactions and energy transfer, we were able to fit all of our fluorescence decay data as sums of single, double and triple exponentials.

3. Results

A typical decay trace, together with instrument response function and the autocorrelation function of the residuals to the least-squares fit, for γ-II crystallin in pH 7.4 phosphate buffer excited at 295 nm and detected at 320 nm and 23°C is shown in Fig. 1. This provides a graphic example of the typical quality of data and the fit using the GLOBAL least squares routine. The lifetimes for a two component fit to the data in Fig. 1 yielded: \( \tau_1 = 1.3 \text{ ns} \) and \( \tau_2 = 220 \text{ ps} \) with chi-squared of 1.4. A three-exponent model gave: \( \tau_1 = 1.5 \text{ ns} \), \( \tau_2 = 430 \text{ ps} \), and \( \tau_3 = 40 \text{ ps} \) with a chi-squared of 1.03. A single exponential fit was wholly inadequate as is clearly evident by the nonlinear nature of the decay in Fig. 1.

The γ-II crystallin protein showed no evidence for a rising component in the decay data (as would be indicated by a negative pre-exponential weighting coefficient \( \alpha_i \) among the fitting parameters). The decay for the reference N-acetyltryptophan amide (NATA) in phosphate buffer solution detected at 360 nm yielded a linear plot as shown in Fig. 2. This data was fit to a single exponential decay model with a lifetime of 2.9 ns and chi-squared of 1.00, in agreement with the

![Figure 1](image-url)
previous report of Petrich et al., [34]. The observed mono-exponential decay in NATA helped to establish that the complex decay and short lifetime components observed in γ-II crystallin were not the result of impurities in the solvent, or artifacts arising from the cuvette or optics. Complete numerical parameters for γ-II crystallin and for reference materials tryptophan and NATA are shown in Table 1 for 295 nm excitation and several emission wavelengths.

The decay of γ-II crystallin in buffer solution was adequately fit by a two-exponent decay model in most data sets (χ² = 1.1–1.5 range), but some decays were better fit by a three-exponential model which reduced χ² to the range 0.9–1.1. Typical results of the two-exponent fit are given in Table 1 as a function of monitored emission wavelength. When fit as a three-exponential decay, γ-II crystallin yielded a very short component τ₃ of order 40 ps in addition to the nanosecond and hundred-picosecond components recovered in the two-exponential model. Although there may be bona fide examples of protein fluorescence decay components as short as 40–70 ps in the literature [35,36], other authors have attributed such very short decays to Rayleigh or Raman scattered light [37]. Since a three-exponent model was only marginally justified by our data, we have not pursued this more complex analysis and present only results from the biexponential decay model.

The decay data for γ-II crystallin in Table 1 showed increasing lifetime as a function of detection wavelength. In Table 2 we indicate the degree of dependence of the fluorescence lifetime on monitored wavelength for both the long and short decays τ₁ and τ₂. In Table 2 the widths Δτ₁ and Δτ₂ express the variation in lifetime over the range of detection wavelengths from 320–400 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Detection wavelength (nm) b,c</th>
<th>320</th>
<th>340</th>
<th>360</th>
<th>380</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>τ₁ (α₁) (ns)</td>
<td>τ₂ (ns)</td>
<td>τ₁ (α₁) (ns)</td>
<td>τ₂ (ns)</td>
<td>τ₁ (α₁) (ns)</td>
<td>τ₂ (ns)</td>
</tr>
<tr>
<td>Gamma-II</td>
<td>1.3 (0.3)</td>
<td>0.22</td>
<td>1.5 (0.3)</td>
<td>0.25</td>
<td>1.8 (0.3)</td>
<td>0.30</td>
</tr>
<tr>
<td>Gamma-II/Gu</td>
<td>2.4 (0.6)</td>
<td>0.41</td>
<td>2.4 (0.6)</td>
<td>0.45</td>
<td>2.5 (0.6)</td>
<td>0.50</td>
</tr>
<tr>
<td>NATA</td>
<td>2.7 (1.0)</td>
<td>–</td>
<td>2.8 (1.0)</td>
<td>–</td>
<td>2.9 (1.0)</td>
<td>–</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.7 (0.5)</td>
<td>0.48</td>
<td>2.8 (0.7)</td>
<td>0.55</td>
<td>2.8 (0.8)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

a All samples in pH 7.4 phosphate buffer unless otherwise noted. The excitation wavelength was 295 nm.
b For each detection wavelength the longer decay component is τ₁. The α₁ value in parenthesis is the weighting factor for τ₁. For two components, α₁ + α₂ = 1.0, and thus α₂ is not shown.
c All lifetimes are in nanoseconds and are averages of at least three measurements. Estimated uncertainties are ±0.1 ns.
d Guanidine hydrochloride (5 M) solvent.
Table 2
Distribution of fluorescence lifetimes versus wavelength

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\tau_1$ (ns)</th>
<th>$\delta \tau_1$ (%)</th>
<th>$\tau_2$ (ns)</th>
<th>$\Delta \tau_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma$-II</td>
<td>1.8</td>
<td>72</td>
<td>0.30</td>
<td>37</td>
</tr>
<tr>
<td>$\gamma$-II/G-HCl</td>
<td>2.5</td>
<td>8</td>
<td>0.50</td>
<td>10</td>
</tr>
<tr>
<td>NATA $^d$</td>
<td>2.9</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.8</td>
<td>14</td>
<td>0.60</td>
<td>28</td>
</tr>
</tbody>
</table>

$^a$ All data for pH 7.4 phosphate buffer at 23°C. Lifetimes in columns labelled $\tau_1$ and $\tau_2$ were detected at 360 nm and are expressed in nanosecond units. Most decays were double exponentials with two lifetime components $\tau_1$ and $\tau_2$.

$^b$ The excitation wavelength was 295 nm in all cases.

$^c$ Guanidine hydrochloride solution, 5 M.

$^d$ Monoexponential decay.

expressed as a percent of the value at 360 nm. Native $\gamma$-II crystallin is seen to have the greatest inhomogeneity in its long lifetime component (72%), while its short component varied much less with detection wavelength (37%). The greatest homogeneity was found in NATA (10% variation in its single decay) and in $\gamma$-II crystallin in guanidine HCl, with 8% variation in the long decay component and 10% variation in the short decay component. Tryptophan was comparable but showed slightly greater inhomogeneity than the preceeding two examples.

The effect of the denaturing solvent 5 M guanidine hydrochloride on the fluorescence decay of $\gamma$-II crystallin excited at 295 nm and detected at 340 nm and 23°C is shown in Fig. 2. The decay in buffer solution was fit to a two component decay with $\tau_1 = 1.5$ ns (30%) and $\tau_2 = 250$ ps (70%). The decay rate in guanidine hydrochloride was significantly reduced relative to buffer solution, but the decay in guanidine hydrochloride still required a two-component fit, yielding $\tau_1 = 2.4$ ns (60%) and $\tau_2 = 450$ ps (40%). Thus, both the long and short lived decays increased upon protein denaturation, and the relative intensity of the long component increased at the expense of the short component. The data in Fig. 2, including the monoexponential decay of NATA for comparison, shows that denaturation of $\gamma$-II crystallin in guanidine hydrochloride caused the fluorescence decay to approach more closely to the free fluorophore limit exemplified by NATA, but that the transition was not complete. The fluorescence decays measured after one hour and twenty hours in guanidine hydrochloride were the same. Similar results were obtained at other detection wavelengths as shown in Table 1. It is noteworthy that the decay parameters for $\gamma$-II in guanidine hydrochloride were almost completely independent of detection wavelength as seen in Tables 1 and 2. This was definitely not the case for $\gamma$-II crystallin in buffer solution.

The fluorescence quantum yield of $\gamma$-II crystallin in pH 7.4 phosphate buffer solution was

Table 3
Temperature dependence of fluorescence lifetimes in NATA and $\gamma$-II crystallin

<table>
<thead>
<tr>
<th>Temp. (£C)</th>
<th>NATA $^b$ wavelength (nm)</th>
<th>Gamma-II $^c$ wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>330 350 380</td>
<td>330 350 380</td>
</tr>
<tr>
<td>0</td>
<td>3.6 3.7 3.9</td>
<td>1.7 0.28 1.9 0.27 2.8 0.42</td>
</tr>
<tr>
<td>10</td>
<td>- - -</td>
<td>1.6 0.29 1.8 0.29 2.7 0.39</td>
</tr>
<tr>
<td>20</td>
<td>2.8 2.9 3.1</td>
<td>1.5 0.25 1.7 0.28 2.5 0.35</td>
</tr>
<tr>
<td>30</td>
<td>- - -</td>
<td>1.4 0.22 1.7 0.28 2.5 0.31</td>
</tr>
<tr>
<td>40</td>
<td>1.8 1.9 2.0</td>
<td>1.4 0.20 1.6 0.25 2.2 0.25</td>
</tr>
<tr>
<td>50</td>
<td>- - -</td>
<td>1.3 0.19 1.5 0.21 2.1 0.24</td>
</tr>
</tbody>
</table>

$^a$ All values in nanoseconds. Decays measured in aqueous phosphate buffer solutions at pH 7.4. Excitation at 295 nm.

$^b$ Decays for NATA were monoexponential at all temperatures.

$^c$ Decays for $\gamma$-II Crystallin were all double exponentials and hence two values are given for each wavelength and temperature.
determined (relative to NATA with $\phi_F = 0.14$, [38]) to be $\phi_F = 0.040 \pm 0.005$. This value was in good agreement with the value of 0.043 reported by Mandal et al. [6], after correcting their tryptophan reference value to $\phi_F = 0.14$ of Robbins et al. [38], rather than the value of 0.20 which they used. Since, the radiative rate constant $k_R$ is

$$k_R = \phi_F / \langle \tau \rangle$$  \hspace{1cm} (1)

where $\langle \tau \rangle$ is the average lifetime, defined by

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i$$  \hspace{1cm} (2)

we calculated $k_R = (5.3 \pm 1.0) \times 10^7$ s$^{-1}$ using $\alpha_i$ and $\tau_i$ values from Table 1. This number is equal within experimental error to the value $(5.0 \pm 0.5)$ \times 10$^7$ s$^{-1}$, which we obtained for NATA, and with values in the literature ranging from $4.5 \times 10^7$ to $6.3 \times 10^7$ s$^{-1}$ for various indole derivatives [34,39]. We also measured the fluorescence quantum yield of $\gamma$-II crystallin in guanidine hydrochloride solution and found $\phi_F = 0.069 \pm 0.01$. Calculating $\langle \tau \rangle$ from eq. (2) and data in Table 1, eq. (1) yields $k_R = (4.7 \pm 1.0) \times 10^7$ s$^{-1}$ again in agreement with the value for NATA [34].

In order to determine if the rather fast decay observed for $\gamma$-II crystallin in buffer solution (Table 1) was the result of some thermally activated process, we measured the temperature dependence of the fluorescence decay. Similar measurements were performed on NATA in buffer solution for comparison. The data are shown in Table 3. The fluorescence decays in $\gamma$-II crystallin were adequately fit as double exponentials at all temperatures in the range 0–50°C. Following the analysis of Petrich et al. [34], we defined the non-radiative rate constant $k_{NR}$ by

$$k_{NR} = \tau^{-1} - k_R$$  \hspace{1cm} (3)

where $\tau$ is the experimental fluorescence lifetime and $k_R = 5.0 \times 10^7$ s$^{-1}$ is the radiative rate constant, assumed to be independent of temperature. Defined as in eq. (3), $k_{NR}$ includes the singlet-to-triplet intersystem crossing rate constant which has a value of $k_{ISC} = 3.3 \times 10^7$ s$^{-1}$ at 25°C [38]. Using $\tau$ values from Table 3, we calculated $k_{NR}$ for NATA and $\gamma$-II crystallin from eq. (3) at various temperatures and plotted $\ln(k_{NR})$ vs. $1/T$ as shown in Fig. 3. Data are given for NATA and $\gamma$-II for emission wavelengths of 330, 350 and 380 nm. For $\gamma$-II, the long- and short-lived decay components were plotted separately. The slopes of these lines, as seen in Fig. 3, appeared to be independent of emission wavelength within experimental error, and the average activation energies obtained for the nonradiative decay rate constants in NATA and $\gamma$-II were: $E_a = 4.2 \pm 1$ kcal/mol for NATA, $E_a = 1.2 \pm 0.2$ kcal/mol ($\tau_1$ component) and $E_a = 2.0 \pm 0.5$ kcal/mol ($\tau_2$ component) for $\gamma$-II crystallin. The value for NATA is in reasonable agreement with the number 5.6 ± 0.2 kcal/mol reported by Petrich et al. [34].

The effect of dissolved molecular oxygen ($O_2$) was investigated by bubbling oxygen or nitrogen gas through $\gamma$-II crystallin buffer solutions for 30 min prior to making lifetime measurements. Similar measurements were performed for tryptophan monomer in buffer solution as a reference. Nei-
ther O\(_2\)(g) nor N\(_2\)(g) bubbling had any measurable effect on the fluorescence decay of \(\gamma\)-II crystallin or on tryptophan monomer.

4. Discussion

According to one simple model [40], the degree of lifetime shortening in a protein (relative to a hydrophilic standard like tryptophan monomer or NATA) is a measure of the average degree of hydrophobicity of the emitting tryptophan residue(s) in the protein. On this basis, our data suggests that the Trp residues in \(\gamma\)-II crystallin exist in a highly hydrophobic environment. This is reasonable based on previous fluorescence spectral data and fluorescence quenching data [41].

The \(\gamma\)-II crystallin showed a pronounced increase in decay times as a function of monitored emission wavelength (Tables 1 and 2). This phenomenon is well known [36] and may be taken as a measure of inhomogeneity of the environment of the emitting tryptophan residues. The \(\Delta\tau\) values in Table 2 indicate that native \(\gamma\)-II crystallin possesses a much higher degree of emitter inhomogeneity than the standards or the denatured protein.

Our fluorescence quantum yield and lifetime data for \(\gamma\)-II crystallin show that the radiative rate constant is equal to that observed in other indole derivatives [34,39], and hence the short lifetimes in \(\gamma\)-II are due to efficient radiationless relaxation of the emitting tryptophan residues.

The relatively short fluorescence decay times recovered for \(\gamma\)-II crystallin in the present work are indicative of tryptophan residues in hydrophobic environments, and are thus consistent with previous fluorescence emission data [6], acrylamide quenching results [41], and the X-ray structure [18], which showed the four tryptophan residues of \(\gamma\)-II crystallin to be buried in the hydrophobic core of the protein. The lifetime of the longer component, \(\tau_1\), is quite short compared to many proteins, and the short-lived component, \(\tau_2\), is also relatively low compared to other proteins reviewed by Beechem and Brand [1]. This lifetime shortening could result from interactions between the indole rings of tryptophan and sulfur atoms at various sites [35]. The X-ray structure of \(\gamma\)-II crystallin shows that the protein is very rich in Cys, His, and Met residues. Trp-42 is in contact with Cys-74, Trp-64 is in contact with Cys-32, and, and Met-86 is in contact with Trp-125 [18].

In guanidine hydrochloride solution the lifetime components of \(\gamma\)-II crystallin were significantly lengthened relative to buffer solution. This suggests that at least some of the lifetime shortening in the native protein is conformation dependent. The fact that double exponential decay, with shortened lifetime components, was observed even in guanidine hydrochloride solution is consistent with a similar report by Grinvald and Steinberg [42] for several other proteins and the report that \(\gamma\)-II crystallin is particularly resistant to both chemical and thermal denaturation [43]. It is possible that some residual tertiary structure remains even in guanidine hydrochloride solution. An alternative possibility is that some of the lifetime shortening results from tryptophan interactions with neighboring groups in the primary structure of the protein, although this would not be the case for the sulfur atom interactions noted above since these do not involve sequence neighbor residues. The fact that the fluorescence decay in guanidine hydrochloride seems to be largely independent of monitoring wavelength suggests that the Trp residues in the denatured protein exist in homogeneous environments but that this environment is substantially different from that experienced by the indole ring in the reference compound NATA.

The activation energy which we observed for non-radiative relaxation in \(\gamma\)-II crystallin was smaller than that of NATA. The NATA activation energy has been attributed to a rotation barrier about the C\(-\)C bond according to the "rotamer model" [34]. Our data suggest that the significant lifetime shortening in \(\gamma\)-II crystallin stems from some interaction not present in the monomeric model NATA. This inactivation mechanism, however, must not require a major conformational charge involving an energy barrier greater than the 1–2 kcal/mol which we observed. This would be consistent with the close
proximity of several of the indole rings to sulfur atoms in the three-dimensional structure of the protein [18].

The idea of assigning the decay components of γ-II crystallin to individual tryptophan residues is an attractive one. For example, it is known that Trp-42 undergoes particularly rapid photochemical alteration upon exposure of the native protein to UV radiation [44,45]. This photochemical reaction could provide a fast deactivation channel for the excited singlet state of tryptophan and hence be responsible for the observed short-lived decay component of $\tau_2 = 250$ ps. However, studies of other proteins in which the individual tryptophan residues have been replaced by point mutations demonstrate the pitfalls of such assignments [37]. In addition, tryptophan monomer in solution is known to display double-exponential decay kinetics [34]. Since γ-II crystallin has four tryptophans, there are, in principle, eight or more possible decay components present in the protein. Many of these may be impossible to resolve under typical signal to noise conditions. Thus, it is dangerous to assign the γ-II decay components to particular tryptophan residues in the absence of more detailed information than is presently available.

The possibility of Trp-to-Trp electronic energy transfer in γ-II crystallin should be considered if the Trp residues are within a distance less than or equal to the Forster critical transfer distance, $R_0$. The value of $R_0$ for Trp-to-Trp energy transfer depends on the relative angular orientation of the indole rings and on the energy gap between their excited singlet states. Typically $R_0$ lies in the range of about 6–12 Å [36,46]. Examination of the X-ray structure [18] shows that none of the Trp–Trp distances in γ-II crystallin is less than 12 Å. Hence, Trp–Trp singlet energy transfer is not likely to be important.

The lack of fluorescence lifetime shortening by addition of O$_2$(g) at atmospheric pressure, and the failure to observe any fluorescence lifetime increase upon deoxygenation by N$_2$ purging, were not surprising. The fluorescence lifetimes of tryptophan and γ-II crystallin ($\leq 3$ ns) are too short to permit quenching by oxygen with an expected diffusion rate constant of $10^{10}$ M$^{-1}$ s$^{-1}$ and oxygen concentrations of ca. $5 \times 10^{-4}$ M for air-saturated water solutions at 23°C and atmospheric pressure [47].

Conclusions

The fluorescence of the lens structural protein γ-II crystallin decays much more rapidly than typical proteins. This rapid decay results from efficient non-radiative decay of the excited singlet state of one or more of the tryptophan residues in a nearly temperature independent process. Denaturing the protein in guanidine hydrochloride slows the decay rate by about a factor of two but the decay remains biexponential and significantly faster than in the monomeric model NATA.

Acknowledgments

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References

Picosecond Transient Absorption of Aqueous Tryptophan

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The primary photophysical and photochemical processes of aqueous tryptophan (Trp) are studied by picosecond transient absorption spectroscopy. Upon excitation of Trp at 292 nm, transient absorption appears within 1.5 ps in the whole visible region and remains for at least 400 ps. Under the present excitation conditions, the transient absorptions due to solvated electrons and the excited triplet state of Trp are relatively weaker, and the observed transient spectrum is predominantly due to excited singlet-state absorption, $S_t - S_i$.

Introduction

Tryptophan (Trp) plays an important role in the photochemical reactions of proteins exposed to UV irradiation. Photochemical reactions of Trp may cause conformational changes in proteins, resulting in enzyme inactivation and cataract formation. Thus, the photochemical and photochemical properties of Trp have been attracting much interest.

The photophysics and photochemistry of Trp have been studied by spectroscopic techniques, in gas phase and in liquid phase including proteins. Time-resolved absorption spectroscopy is a powerful technique for investigating the photophysics and photochemistry of Trp, since it can observe the transient absorption due to photoproducts. So far, flash photolytic studies have revealed two relaxation channels from the singlet excited state of Trp: (1) electron ejection to the solvent, yielding solvated electrons which absorb at about 700 nm and Trp radical cation which absorbs at about 580 nm, followed by the decay of Trp$^*$ to give Trp$\cdot$ neutral radical absorbing at about 510 nm; (2) intersystem crossing, yielding the triplet-state Trp which absorbs at about 430 nm. The early experiments were done with microsecond time resolution.

Fluorescence decay components of Trp are typically 0.5 ns (25%) and 3.0 ns (75%). A picosecond transient absorption study of aqueous Trp revealed that the electron ejection process takes place within 27 ps, which is consistent with other observations of the electron solvation process occurring on a subpicosecond time scale in water. The possibility was suggested that the electron solvation process takes place from a "nonrelaxed" excited state. However, experiments on the excitation wavelength dependence of the quantum yield of $e_{aq}$ generation revealed that the electron solvation takes place with the same quantum yield (0.075) even when Trp is excited at the long wavelength absorption edge (300 nm). This does not support the presence of a nonrelaxed state. Thus, the photochemical reaction channels of Trp are still unclear.

The initial state of these reactions is the singlet excited ($S_t$) state of Trp and the time scale is in picoseconds. Thus, these photochemical reactions must depend strongly upon the properties of the $S_t$ state of Trp. In spite of a number of spectroscopic studies of aqueous Trp, its $S_t$ absorption properties have not been characterized. In this article, we report the transient absorption spectra of aqueous Trp excited with a 1.5-ps pulse. The present experiments with weak excitation (0.05 photon/molecule at 292 nm) enabled us to observe the $S_t$ absorption spectrum under conditions where absorption due to $e_{aq}$ and $T_t - T_i$ were weak.

Experimental Methods

The apparatus for obtaining transient absorption was a computer-controlled double-beam spectrometer linked with a subpicosecond laser described previously. A 584-nm subpicosecond pulse was generated by a synchronously pumped, hybridly mode-locked dye laser (Rhodamine 6G/DODCI). An amplified pulse was produced by a three-stage amplifier (Kiton Red dissolved in methanol) pumped by a regenerative amplifier at 10-Hz repetition rate.

The pump pulse for transient absorption was generated by passing the amplified pulse through a 0.5-mm BBO crystal. The width of the pump pulse was estimated to be 1.5 ps by observing the rise signal due to $S_t - S_i$ absorption of trans-stilbene with the present transient absorption apparatus. The 292-nm pulse was separated with a dichroic beam splitter from the fundamental pulse (584 nm). The fundamental was used to generate a white light continuum as a probe pulse, by focusing it into a 1-cm cell containing D$_2$O/H$_2$O mixture (1:2). The angle of polarization between pump and probe pulses was set at 54.7°. To eliminate the remaining 584-nm light, we used a blue glass filter (Melles Griot, BG28) or sharp cutoff filter (Schott, RG630). The transmission of the blue glass filter made it difficult to measure transient absorption in the 560–590-nm region. The probe pulse was split into two parts by a half-mirror and then focused into two independent 25-cm spectrographs. One of the beams passed through the 2-mm light-path sample cell to monitor the absorbance change upon excitation, and the other beam was used as a reference. The pump pulse reached the sample cell along the same light path as the probe pulse, after passing through an optical delay. The signal was detected by a 512-channel photodiode (MCPD, Hamamatsu Photonics). To improve the signal-to-noise ratio, the excitation and the reference spectra were accumulated and then used to obtain the difference spectrum at each delay setting.

L-Tryptophan (Wako) was dissolved in 0.1 M phosphate buffer (pH 7.4). The concentration of Trp was 2.20 × 10$^{-3}$ M, giving 1.2 OD unit per 2-mm light path at 292 nm. It corresponded to 1.0 × 10$^{17}$ cm$^{-2}$/molecule as the absorption cross section. The excitation energy was 80 μJ per pulse with a 1.0-mm-diameter beam, indicating 1.5 × 10$^{-14}$ photons/cm$^2$. The pump pulse excited the sample at the long wavelength side of the absorption band (250–310 nm), and the excess energy was estimated to be about 2000 cm$^{-1}$ on the assumption that the origin lies at 310 nm. The Trp sample was flowed through the cell by a peristaltic pump so as to continually excite fresh Trp. The total amount of Trp solution was about 5 mL, and we observed no photolytic changes in the samples when measuring the ground-state absorption spectrum after the transient absorption experiments. All experiments were performed at room temperature of 22 ± 2°C.

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Transient Absorption of Aqueous Tryptophan

Results

Figure 1 shows transient absorption spectra of Trp after excitation at 292 nm with a 1.5-ps pulse. The spectra obtained at delay times of 3, 50, and 400 ps displayed similar features, showing the appearance of transient absorption in the entire visible region. The transient absorption spectra displayed a peak at ~430 nm and an absorption band at 560–590 nm. The former peak was apparently identical to that of the triplet state of Trp \(^3\)Trp, obtained by flash photolysis on the nanosecond–microsecond time scale, while the latter absorption was located in the wavelength region of \(\text{Trp}^*\) (580 nm) and/or \(\text{Trp}^*\) (510 nm). \(^{16-19}\) It should be noted that the three spectra in Figure 1 are similar, indicating no significant change in the transients from 3- to 400-ps delay.

The rise of the transient absorption is shown in Figure 2a. The decay is shown in Figure 2b and indicates that the transient absorption at 560 nm has an approximately 400-ps decay component which corresponds to about (15 ± 5%) of the total intensity. Both the decay time and fractional intensity are in agreement with fluorescence decay measurements, implying that the transient absorption is due to the excited singlet state of Trp. This assignment is also supported by the excitation power dependence shown in Figure 3. The linear relationship of the transient absorption at 560 nm to the excitation power at 292 nm indicates that the transient absorption is generated by single-photon absorption.

Discussion

The present experiment provides transient absorption for aqueous Trp, which covers the entire visible region. Possible assignments of this absorption include the singlet excited state of Trp \(S_1\), tryptophan radical cation \(\text{Trp}^+\), \(e_{aq}^-\) due to electron solvation, and \(^3\)Trp due to intersystem crossing. The neutral radical \(\text{Trp}^*\) is not likely to be observed on the picosecond time scale, because it is produced by the subsequent reaction of \(\text{Trp}^*\) in \(10^{-6}\) s. \(^{16}\) We know the absorption spectra of \(\text{Trp}^+\), \(e_{aq}^-\), and \(\text{Trp}^*\), while the absorption spectrum of the \(S_1\) state has not been obtained yet.

As described above, the transients at ~430 and 560–590 nm look like \(^3\)Trp and \(\text{Trp}^+\), respectively. If the transient at 560–590 nm is \(\text{Trp}^+\), it is produced by electron ejection. Accordingly, the absorption of the solvated electron \(e_{aq}^-\) has to be observed simultaneously. The solvated electron has an absorption maximum at about 700 nm, and the molar extinction coefficient is about 19,000 M\(^{-1}\) cm\(^{-1}\) at 700 nm, \(^{26}\) while the molar extinction coefficient of \(\text{Trp}^+\) is 2900 at 580 nm. \(^{17}\) The contributions of these absorbers to our spectrum can be estimated as follows: The concentration of the Trp sample is \(2.2 \times 10^{-5}\) M, corresponding to an absorption cross section of \(1.0 \times 10^{-12}\) cm\(^2\)/molecule, while the excitation photon density is calculated to be \(1.5 \times 10^{10}\) photons/cm\(^2\). Under the present excitation conditions, 5% of the Trp molecules are expected to be raised to the \(S_1\) state. Application of Lambert–Beer's law gives the following absorbance change due to the products:

\[
\text{OD}_{700\text{ nm}}(e_{aq}^-) = \epsilon_{700\text{ nm}}(e_{aq}^-)N\phi(e_{aq}^-) = 0.031
\]

\[
\text{OD}_{580\text{ nm}}(\text{Trp}^+) = \epsilon_{580\text{ nm}}(\text{Trp}^+)N\phi(e_{aq}^-) = 0.005
\]

In these calculations we used molar extinction coefficients of \(\epsilon_{700\text{ nm}}(e_{aq}^-) = 1.9 \times 10^4\) M\(^{-1}\) cm\(^{-1}\) \(^{26}\) and \(\epsilon_{580\text{ nm}}(\text{Trp}^+) = 2.9 \times 10^3\) L/mol/cm. \(^{17}\) The concentration of excited Trp was \(N = 0.05 \times 2.2 \times 10^{-3}\) M, the light path was \(L = 0.2\) cm, and the quantum yield of electron ejection was \(\phi(e_{aq}^-) = 0.075\) \(^{29}\).

The calculated absorption spectra of \(e_{aq}^-\) and \(\text{Trp}^+\), as well as the experimental transient absorption spectrum in Figure 4a. The data suggest that the long wavelength tail of the transient absorption at 640–720 nm is mainly due to \(e_{aq}^-\). On the other hand, the main band shorter than 640 nm is neither \(\text{Trp}^+\) nor \(e_{aq}^-\), since their calculated absorptions are only 10–20% of the observed intensity. The remaining absorption is presumably assignable to the \(S_0 \rightarrow S_1\) absorption of Trp, and we conclude that the transient absorption at 500–720 nm is mainly due to absorption of the \(S_1\) state (\(\text{Trp}^*\)).
TABLE I: Semiempirical PPP Calculation for Indole

<table>
<thead>
<tr>
<th>state energy/eV</th>
<th>transition wavelength/nm</th>
<th>oscillator strength</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S0 → S1</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>560 nm</td>
<td></td>
</tr>
<tr>
<td>4.36 (4.4)*</td>
<td>285</td>
<td>0.03</td>
</tr>
<tr>
<td>4.82 (4.6)*</td>
<td>255</td>
<td>0.06</td>
</tr>
<tr>
<td>5.98 (5.8)*</td>
<td>210</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>S1 → S2</td>
<td></td>
</tr>
<tr>
<td>1.62</td>
<td>750</td>
<td>0.006</td>
</tr>
<tr>
<td>2.01</td>
<td>630</td>
<td>0.009</td>
</tr>
<tr>
<td>2.24</td>
<td>550</td>
<td>0.003</td>
</tr>
<tr>
<td>2.61</td>
<td>500</td>
<td>0.03</td>
</tr>
<tr>
<td>3.07</td>
<td>400</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Using parameters from ref 27. * Experimental value from ref 28 in parentheses.

The absorption band at 430 nm is located near that of the absorption of 
Trp. Since the fluorescence from Trp has two components, 0.5 ns (25%) and 3.0 ns (75%),
and there are two triplet states in Trp, T1 (20–45 ns lifetime) and T2 (11–16 µs lifet ime),
the rise of 
Trp should occur on a nanosecond or even subnanosecond time scale. Accordingly, the transient absorption
at 400 ps might include a contribution from 
Trp absorption. However, a similar spectral feature was observed at 3 and 50 ps,
indicating that this transient absorption is not due solely to 
Trp. It is more reasonable to interpret the spectral feature at these
wavelengths mainly to S1 → S2 absorption. It should be noted that stimulated emission is often observed
in transient absorption measurements. The fact that the fluorescence spectrum of Trp is located in the 330–420 nm region
suggests that the observed transient absorption in this range might
contain a negative component due to stimulated emission. Thus, the peak at 430 nm attributable to S1 absorption may be larger
than shown in Figures 1 and 4a.

Figure 4. (a) Transient absorption spectrum of tryptophan in pH 7.4
phosphate buffer for times less than 10 ps. The predicted absorbances
of ε<sup>eq</sup> and ε<sup>Trp</sup> under the present 292-nm excitation conditions (0.05
photon/molecule) and a solvated electron quantum yield of 0.075 (see
text, ref 20) are also shown. (b) Measured absorption spectra of the S0
and S1 states of tryptophan. Semiempirical PPP calculated energies and
transition strengths of indole are shown as a stick spectrum.

That the transient absorption spectra shown in Figure 1 are
predominantly due to S1 absorption of Trp is supported by the
results of a semiempirical PPP calculation for indole. The
calculated band positions and relative intensities are given in
Table I and are indicated by the stick spectrum in Figure 4b. The
molar extinction coefficients for the experimental S1 absorption
can be estimated from Lambert–Beer's law and are also shown
in Figure 4b. The agreement between the measured transients
and the calculated PPP spectra for both S0 → S0 and S1 → S1
is quite reasonable. Shifting all of the calculated bands by 2000
cm<sup>−1</sup> to the red, a not unreasonable uncertainty in such a
calculation, substantially improves the agreement with experiment
for both the S0 → S0 and S1 → S2 transitions.

The singlet excited-state absorption properties of Trp have
been only briefly discussed in the literature. Bent and Hayon
assigned the 245-nm transient, which they observed at 0 ns with
a 15-ns laser excitation, to S1 absorption, and they assigned the
entire transient in the visible region to photoproducts. The
former assignment may be correct, but if the S1 absorption is
observable with their time resolution, the transient in the visible
region should have involved the S1 absorption presented here.
Since the S1 absorption is observable in the entire visible region,
spectral overlapping of the S1 absorption and the product
absorption could occur. For instance, the kinetic data by Mialloq
et al. may involve the contribution of the S1 absorption to
some extent, although we think their conclusion is reasonable.
Hirata et al. excited aqueous Trp with a 266-nm picosecond
pulse and reported a transient absorption spectrum at 100 ps,
which is quite different from that obtained here. Since 
Trp transient absorption was clearly seen in their work, the difference is
most likely due to a higher photon density resulting in two-photon absorption. However, this is difficult to assess since no
information on photon flux was given in their paper.

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during his 1991–92 Sabbatical leave. The authors are indebted to Prof. I. Ohmine of IMS for performing the PPP calculations
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References and Notes

Transient Absorption of Aqueous Tryptophan


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THE MOLECULAR CHAPERONE α-CRYSTALLIN INHIBITS

UV-INDUCED PROTEIN AGGREGATION†

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ABSTRACT

Buffer solutions of the lens protein \( \gamma \)-crystallin, and various enzymes from non-lens tissues, at neutral pH and 25\(^\circ\) C, became turbid upon exposure to UV radiation at 295 or 308 nm. Solid precipitate formed in the irradiated solutions, and SDS-PAGE analysis revealed extensive inter-chain crosslinking to produce high molecular weight aggregates compared to dark control solutions. When \( \alpha \)-crystallin was added to the protein solutions in stoichiometric amounts, UV irradiation did not cause turbidity, or turbidity developed less rapidly than in the absence of \( \alpha \)-crystallin. For example, 1.0 ml of solution containing 1.0 mg of \( \gamma \)-crystallin was protected from aggregation by addition of 1.0 mg of \( \alpha \)-crystallin. Under the conditions of our experiments, the molecular weights of \( \alpha \)- and \( \gamma \)-crystallin are about 800 kDa and 20 kDa, respectively. Thus, the amount of \( \alpha \)-crystallin needed to protect \( \gamma \)-crystallin from photochemical opacification corresponded to a molar ratio of \( \alpha:\gamma = 1:40 \). Alternatively, this ratio may be expressed in terms of subunits as \( \alpha:\gamma = 1:1 \), i.e., each of the 40 subunits in the \( \alpha \)-crystallin macromolecule can protect one \( \gamma \)-crystallin molecule from aggregation. Similar stoichiometries were observed for protection of the other proteins studied. Our evidence indicates that protection stems in part from screening of UV radiation by \( \alpha \)-crystallin but more importantly from a chaperone effect analogous to that seen in thermal aggregation experiments.
INTRODUCTION

It has been found that certain proteins can function as "molecular chaperones" (Ellis & van der Vies, 1991). These species prevent undesirable aggregation of denatured proteins (Schmidt & Buchner, 1991) and assist in their refolding to the native form (Buchner et al., 1991). The mechanism of protection involves binding of denatured substrate by the chaperone, for example, the chaperone GroEL binds denatured peptides within its central cavity (Braig et al., 1993).

Horwitz (1992; 1993) reported that the structural protein, α-crystallin, isolated from bovine lens, can function as a chaperone in that it prevents thermal aggregation of a number of enzymes as well as the lens structural proteins β- and γ-crystallin. Thus, it appears that α-crystallin plays a multiple role in the lens, and in some other tissues (Kato et al., 1991), serving both as a structural unit and as a protective agent preventing undesirable aggregation of proteins which could lead to lens opacity.

The mechanism by which α-crystallin protects proteins from aggregation is being investigated (Boyle et al., 1993; Takemoto, 1994), and model structures have been proposed (Tardieu et al., 1986; Augusteyn & Koretz, 1987; Wistow, 1993; and Carver et al., 1994). A relevant piece of information is the measured
stoichiometry of the protective interaction. In the case of thermal denaturation of α-/γ-crystallin mixtures, Horwitz (1993) found this stoichiometry to be about α:γ = 1:20 (mole/mole). γ-crystallin is a monomer of molecular weight 20 kDa (Wistow, et al., 1983). α-crystallin is an oligomer composed of subunits—the αA₂ and αB₂ chains, each of 20 kDa size (van der Ouderaa et al., 1974). α-crystallin particle sizes vary in the range 300-2000 kDa depending on conditions of temperature, pH, and ionic strength (Thomson & Augusteyn, 1988). Thus, at room temperature and neutral pH the molecular weight of α-crystallin is about 800 kDa, while at the higher temperatures used in the thermal aggregation/opacification experiments of Horwitz (1993), the α-crystallin effective particle size was estimated to be about 400 kDa. (It is possible that other conformational changes occur in α-crystallin at the 48–66°C temperatures used in the thermal aggregation work, but no evidence exists one way or the other.) Based on an α:γ mole ratio of 1:20 for protection at 66°C, it appears that each of the 20 subunits of the 400 kDa α-crystallin oligomer can bind (or in some way protect) one γ-crystallin molecule from aggregation. This suggests an open structure for α-crystallin, with most or all of the subunits accessible. The three dimensional structure of α-crystallin is not known, but various models have been proposed. A three layer model was first proposed by Tardieu et al. (1986). Augusteyn and Koretz (1987) suggested a micellar model. Wistow (1993) advanced a tetrameric arrangement of subunits in the structure. Most recently, Carver et al. (1994)
submitted a model more akin to other known chaperones, i.e., one with a hole in the center, lined with hydrophobic residues, where substrate binding is proposed to occur. The chaperone data seem to argue against any structure with a significant fraction of the $\alpha A_2$ and $\alpha B_2$ chains buried and hence inaccessible as binding sites. An open structure with most or all of the subunits accessible seems more likely.

Our laboratory has been interested in the photochemical aggregation of lens proteins. We have observed that $\beta$- and $\gamma$-crystallins in neutral buffer solutions at room temperature undergo photochemical crosslinking/aggregation and subsequent solution opacification when exposed to UV radiation. (Walker & Borkman, 1989; Li et al., 1990; Hott & Borkman, 1993). On the other hand, $\alpha$-crystallin solutions did not become opaque upon exposure to UV radiation under the same conditions (Hott & Borkman, 1993). Thus, when the thermal opacification results of Horwitz (1992; 1993) appeared, we began to investigate the possible role of $\alpha$-crystallin in protecting $\gamma$-crystallin and other proteins from UV induced aggregation.

The present paper reports photochemical opacification studies of mixtures of bovine $\alpha$- and $\gamma$-crystallin and $\alpha$-crystallin with some other enzymes in neutral pH buffer solutions. It is important to note that our photochemical work was carried out at 22-24° C, where $\alpha$-crystallin is more likely to retain its native tertiary and
quaternary structures than in thermal aggregation experiments performed at higher temperatures, typically in the 45-70° C range. Also, since in our experiments both α-crystallin, and the substrate protein, absorb significant UV radiation, we considered an additional possibility not present in thermal experiments: Namely, that protection of proteins from photo-aggregation might be the result of screening of the incident radiation by α-crystallin.

METHODS

Bovine α-crystallin was obtained from 3 month old calf lenses (courtesy of Brown Packing Company, Gaffney, SC) by size exclusion chromatography on Sephadex G-200 according to the procedure originally reported by Bloemendal (1981) and used in our previous work (Hott & Borkman, 1993). Some samples of bovine α-crystallin were also obtained from Sigma, St. Louis, MO. No differences were found between this and the material which we prepared ourselves. Separation of γ-crystallin was done by the procedure of Bjork (1961) also used in our previous work. Proteins were lyophilized and stored at -5° C until needed. The enzymes carbonic anhydrase, enolase, and aldolase were obtained from Sigma, St. Louis, MO and were used without further purification.

Stock solutions of proteins were prepared in 0.1 M phosphate buffer at pH 7.4. Protein concentrations were in the range 0.1 -
10.0 mg/ml, and we employed 1.0 cm path quartz cuvettes containing 1.0 ml of solution, except that the low and high protein concentration studies at 0.1 mg/ml and 10 mg/ml used a 10 cm path length quartz cell and a 0.1 cm path length quartz cell, respectively, to maintain constant absorbance in the three solutions. All solutions were irradiated at an ambient temperature of 22-24°C.

The absorbances of the protein solutions were measured, at 295, 308, 360, and 600 nm before, and at internals during, UV irradiation (or during heating, as discussed below) with a Milton Roy UV-visible spectrophotometer.

Some of the UV irradiation studies were done with a 1000 W xenon arc lamp with 10 cm water filter and a Bausch and Lomb high intensity grating monochromator. The monochromator was set to transmit 295 nm radiation with a spectral band pass of 10 nm. The UV output incident upon the sample cell under these conditions was 0.5 mW/cm² (5 W/m²) as measured with a Scientech Model 365 meter. Other UV irradiation studies were done with a Lumonics excimer laser model 520 using XeCl as the lasing medium to give an average power at 308 nm of 0.5 W in a 1 cm² cross sectional beam. Thus, the laser output at 308 nm was about 1000 times greater than that from the xenon lamp/monochromator system at 295 nm, i.e., the output ratio was \( \frac{O_{308}}{O_{295}} = 1000 \). However, since the absorbance of the protein solutions was greater at 295 than at 308 nm, the ratio
of the radiation flux actually absorbed at the two wavelengths was much less than the ratio of incident intensities. For example, for a 1.0 mg/ml solution of γ-crystallin in buffer the measured absorbance ratio was $A_{295}/A_{308} = 17$. Thus, the ratio of absorption rates at the two wavelengths was $I_{308}/I_{295} = 1000/17 = 60$. This ratio is reflected in the fact that typical irradiation times to achieve opacity with the laser were on the order of minutes, while with the xenon lamp they were on the order of hours. Because of the relatively high intensity of the laser source, we were concerned about possible heating of the irradiated solutions. Checks of solution temperatures indicated less than 1°C rise during a typical 15 minute laser irradiation.

Thermal opacification studies were performed by placing protein solutions in a thermostated water bath maintained at a fixed temperature in the range 55-70°C for times up to 10 minutes. Absorbance was monitored periodically during the heating period as described above.

SDS-PAGE was done using Bio-Rad's Mini-Protean II dual slab gel cell apparatus. The gels were made of 15% polyacrylamide and the SDS content was 0.1%. The gels were run at 200 volts and stained with Coomassie Blue. The sample buffer was: 50% water, 12.5% 0.5 M Tris (pH 6.8), 10% glycerol, 20% of a 10% (wt/vol) solution of SDS, 5% β-mercaptoethanol and 2.5% of a 0.05% (wt/vol) solution of bromophenol blue. The protein samples to be analyzed
were first diluted 1 : 4 with sample buffer prior to application to the gel. Protein solutions which were partially insolubilized by UV irradiation were centrifuged for 15 min at 10,000 rpm. The insoluble material was dissolved in 100 μl of sample buffer, and the soluble material was diluted 1 : 4 with sample buffer.

RESULTS

Figure 1 shows a plot of absorbance at 600 nm versus UV irradiation time at 295 nm and room temperature for: 1 mg/ml γ-crystallin, and for the same solution with 1 mg of α-crystallin added. UV irradiation at room temperature caused the γ-crystallin solution to become opaque, while the α-γ mixture remained transparent. Also shown in Fig. 1 is a plot of the hypothetical degree of opacification expected in a 1 mg/ml γ- plus 1 mg/ml α-crystallin solution based on screening of the UV intensity by α-crystallin. That is, we computed the fraction of radiation absorbed by α-crystallin and subtracted this from the total radiation intensity. The difference then represents the radiation intensity available for absorption by γ-crystallin. This is expected to result in a reduction in the number of photochemical events for γ-crystallin, and we refer to this phenomenon as the "UV screening mechanism". In Fig. 1, one sees that the screening mechanism can account for only about one third of the total protection afforded by α-crystallin.
Dark control and UV-irradiated solutions were examined by SDS-PAGE. UV-irradiated solutions were extensively crosslinked, with material in the 20-100 kDa molecular weight range and above, as our lab (Li et al., 1990; Hott & Borkman, 1993) and others (Mandel et al., 1988) have reported previously. SDS-PAGE results for the dark controls showed only bands characteristic of the starting materials, i.e., 20 kDa peptides for both α- and γ-crystallin. UV irradiated mixtures of α- and γ-crystallin also showed crosslinking, but the data did not reveal whether these links involved α-α, γ-γ, α-γ or some mixture of all three.

Experiments were done to assess the effect of protein concentration on the chaperone function. In Fig. 2 are UV opacification data for gamma crystallin in buffer solution at concentrations of 0.1, 1.0, and 10 mg/ml. As seen in Figs. 2 a, b, and c, all three γ-crystallin solutions became opaque following UV irradiation at 295 nm. Also seen is the effect of adding a stoichiometric amount of alpha crystallin to each solution. In each case, addition of α-crystallin significantly protected γ-crystallin from UV opacification. Two experimental runs are shown for each solution. There are some differences in the degree of opacification achieved in each solution and in the apparent degree of protection afforded, but there is no clear trend with respect to concentration.
In Fig. 3 are data on UV-induced opacification of solutions of aldose, carbonic anhydrase, and enolase. The irradiations were done with the excimer laser at a wavelength of 308 nm and room temperature. In each case, added α-crystallin is seen to prevent or reduce the degree of opacity produced in these solutions by exposure to UV radiation. For example, in Fig. 3a, one sees that 0.25 mg/ml of added α-crystallin afforded little protection against UV-induced opacification of aldolase, but that the level of protection was significant when 1-2 mg/ml of alpha was added. Analogous data were obtained for the other enzymes, seen in Fig. 3. Thus, the protective or chaperone effect of α-crystallin was not limited to the lens protein γ-crystallin but extended to other, non-lens proteins as well.

For comparison purposes, we plot in Fig. 4 results of thermal opacification experiments on the same enzymes, with and without added α-crystallin. The data for aldolase in Fig. 4a indicate that heating a 1.0 mg/ml solution to 60° C resulted in opacification, but that such opacification was largely prevented by addition of as little as 0.25 mg/ml of α-crystallin and completely prevented by addition of 1.0 mg/ml. Analogous results were obtained for carbonic anhydrase heated to 70°C except that more α-crystallin was needed to afford complete protection. In the case of enolase at 55°C, protection was significant, but incomplete, even at 2 mg/ml of α-crystallin, as seen in Fig.4.
Thermal opacification data were also obtained for γ-crystallin (data not shown). As an example, 0.4 mg/ml solutions of γ-crystallin, initially at room temperature, and containing varying amounts of α-crystallin were immersed into a 63°C water bath and the absorbance was monitored at 600 nm. The results showed that, in the absence of α-crystallin, solution opacification was complete within 10–15 minutes. When 0.2 mg of α-crystallin was added, the solution remained clear upon immersion in the 63°C bath.

Thermally aggregated solutions were examined by SDS-PAGE. The heated solutions contained only the 20 kDa starting peptides of α- and γ-crystallin. Thus, in contrast, UV treatment of γ-crystallin produced high molecular weight aggregates which survived treatment with the detergent SDS, but thermal aggregates of γ-crystallin did not survive SDS treatment. Interestingly, both types of aggregation were prevented by addition of α-crystallin.

In Fig. 5 are photochemical opacification data for 0.5 mg/ml γ-crystallin solutions containing varying concentrations of added α-crystallin. Higher levels of α-crystallin are seen to afford greater protection than lower concentrations. In Fig. 6, these results are plotted so as to display the dependence of γ-crystallin opacification on α-crystallin concentration. Examining the data in Fig. 6 suggests that a 0.5 mg/ml solution of γ-crystallin is protected from UV-induced aggregation by a minimum α-crystallin concentration of 0.5 ± 0.1 mg/ml. Since monomeric γ-crystallin has
molecular weight 20 kDa, and oligomeric α-crystallin is composed of 20 kDa subunit peptides, the suggestion is that each α-crystallin subunit can protect one γ-crystallin molecule from aggregation. Similar stoichiometries were observed for protection of other proteins from UV-aggregation by α-crystallin (Fig. 3), but the actual numerical stoichiometries were not evaluated.

An experiment was performed to test whether protein solutions which had been photo-aggregated could be de-aggregated by subsequent addition of α-crystallin. A 0.5 mg/ml γ-crystallin in buffer solution was irradiated at 308 nm with the excimer laser source for 15 minutes. The solution became opaque and solid precipitate formed in a similar fashion to that shown in Fig. 5, with an absorbance at 600 nm of about 0.45. α-crystallin was then added, to a concentration of 2 mg/ml, and the absorption at 600 nm, as well as the visual appearance of the sample, were monitored for one hour. The solution remained visually opaque, and the absorbance at 600 nm remained constant at 0.45 ± 0.05. The solution was then warmed to 40°C and incubated for an additional hour. There was no evidence for de-aggregation, and the absorbance remained constant during this period.

DISCUSSION

The data in Figs. 3 and 4 show that α-crystallin can protect proteins from both thermal and UV-induced protein
opacification/aggregation. The thermal case has been studied by (Horwitz, 1992, 1993). The results on protection from UV-induced opacity are new. The degree of protection afforded by \( \alpha \)-crystallin against thermal and UV-induced aggregation is seen to be of comparable magnitude. This conclusion does not agree with a preliminary report of Rao & Raman (1994) who found that \( \alpha \)-crystallin did not afford any significant protection against UV aggregation of \( \gamma \)-crystallin except at temperatures above 30\( ^{\circ} \)C.

\( \alpha \)-crystallin absorbs UV radiation at the same wavelengths as the substrate proteins. This is an additional factor to be considered, which is not present in thermal experiments, and may contribute to the protection phenomenon. We propose that the protection mechanism in UV aggregation is the sum of a screening effect plus a binding effect analogous to that in thermal experiments (Horwitz, 1992, 1993). Both protection mechanisms are operative, as indicated by the data in Fig. 1, which shows that UV screening can only account for about one third of the protection.

An important conclusion that can be drawn from the UV chaperone results is that \( \alpha \)-crystallin is able to bind the intermediates which occur in UV-irradiated proteins (free radicals?) as well as the intermediates which occur in heat treated proteins (random coil peptides?). The notion that the reactive intermediates are different in UV versus thermal aggregation experiments is supported by our SDS-PAGE results which show that UV
radiation leads to covalently linked aggregates which are not dissociated by SDS while heating leads to aggregates which are dissociated by SDS (non-covalent). Thus, it seems clear that different reaction intermediates are involved in UV versus heat aggregation but that α-crystallin can bind either type.

Comparison of the ability of α-crystallin to protect one protein versus another does not suggest any major differences amongst the proteins studied. Horwitz (1993) noted that the lens protein γ-crystallin was slightly more effectively protected than some other enzymes which he studied.

A conclusion that can be drawn from comparison of the UV and thermal opacification data stems from the fact that the former were all performed at room temperature, while the latter were done at elevated temperatures in the range 50-70° C. In the thermal chaperone experiments, the α-crystallin was heated above room temperature. The question arises as to whether this heating is necessary to activate the chaperone function. The UV experiments were done at room temperature and thus, the α-crystallin was not exposed to elevated temperatures, yet it still functioned as an effective chaperone. Thus thermal activation does not seem to be required. The same conclusion was reached in the chemical denaturation studies of Farahbakhsh et al. (1995) on insulin. A related point is that the quaternary structure of α-crystallin is known to be temperature sensitive. The macromolecule has a
molecular weight of about 800 kDa at 25°C (neutral pH and physiological ionic strength). At the elevated temperatures used in the thermal denaturation work, Horwitz (1993) estimated the particle size at 360 kDa. Considering the combined UV and thermal aggregation data, one sees that α-crystallin functions as an equally effective chaperone at 800 kDa and at 360 kDa. Thus, within this molecular weight range, the structural features needed for chaperone function are retained. Furthermore, the observed protection stoichiometry, for example for γ-crystallin, was α:γ = 1:1 regardless of whether α-crystallin was present in the 800 kDa or the 360 kDa form. This same point is supported by the results for insulin at room temperature (Farahbakhsh et al., 1995).

Most of our UV induced opacification experiments were done in the concentration range of a few mg/ml of both α-crystallin and substrate protein. The results obtained at lower (0.1 mg/ml) and higher (10 mg/ml) concentrations did not differ qualitatively from those at intermediate concentrations, but there are some variations in the apparent degree of protection by α-crystallin at the three concentrations, but there doesn't seem to be any clear trend in the data as a function of concentration. Signigicant protection was observed throughout the 0.1 - 10 mg/ml concentration range. It should be pointed out that our highest concentration, 10 mg/ml, is an order of magnitude less than that present in the intact ocular lens. The question of whether a chaperone effect operates in the intact lens has been addressed elsewhere (Boyle & Takemoto, 1994).
REFERENCES


FIGURE CAPTIONS

Figure 1: Absorbance at 600 nm versus time of irradiation at 295 nm for solutions: 1.0 mg/ml gamma crystallin in buffer, —O—; 1.0 mg gamma crystallin plus 1.0 mg alpha crystallin in 1.0 ml buffer, —●—. The broken line --●-- represents the expected degree of opacity based on the UV screening mechanism alone (see text). All irradiations at room temperature in 1.0 cm path quartz cell.

Figure 2: Absorbance at 600 nm versus time of irradiation at 295 nm for solutions of alpha and gamma crystallin: a. 10 cm path cell, 0.1 mg/ml gamma crystallin in buffer, —○—, two runs are shown, and 0.1 mg/ml gamma plus 0.1 mg/ml alpha crystallin in buffer, —●—, two runs are shown; b. 1.0 cm path cell, 1.0 mg/ml gamma crystallin in buffer, —○—, two runs, 1.0 mg/ml gamma plus 1.0 mg/ml alpha crystallin in buffer, —●—, two runs shown; c. 0.1 cm path cell, 10 mg/ml gamma crystallin in buffer, —○—, two runs, 10 mg/ml gamma plus 10 mg/ml alpha, —●—, two runs shown. All irradiations at room temperature with cell path length indicated.
Figure 3: Photo-opacification data for enzymes (1.0 mg/ml) and protection by addition of varying amounts of α-crystallin: 0.0 mg/ml, —O--; 0.25 mg/ml, —□--; 1.0 mg/ml, —○--; and 2.0 mg/ml, —●—. In each case, solution absorbance at 600 nm is plotted against the time of UV-irradiation at room temperature and 308 nm. a. Aldolase. b. Carbonic Anhydrase. c. Enolase.

Figure 4: Thermal-opacification data for enzymes (1.0 mg/ml) and protection by addition of varying amounts of α-crystallin: 0.0 mg/ml, —O--; 0.25 mg/ml, —□--; 1.0 mg/ml, —○--; and 2.0 mg/ml, —●—. In each case, solution absorbance at 600 nm is plotted against time at elevated temperature. a. aldolase, 60°C. b. Carbonic Anhydrase, 70°C. c. Enolase, 55°C.

Figure 5: Photo-opacification data for 0.5 mg/ml γ-crystallin with varying amounts of α-crystallin added: 0.0 mg/ml, —O--; 0.10 mg/ml, —□--; 0.25 mg/ml, —○--; 1.0 mg/ml, —■--; 2.0 mg/ml, —●—. Solutions irradiated at 308 nm, room temperature.

Figure 6: γ crystallin, 0.5 mg/ml, opacification detected at 600 nm versus concentration of added α-crystallin. Data taken from Fig. 5. Each solution was irradiated for 15 minutes at 308 nm and room temperature.
February, 1995

THE MOLECULAR CHAPERONE FUNCTION OF $\alpha$-CRYSTALLIN
IS IMPAIRED BY UV PHOTOCROSSLINKING$^\dagger$

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ABSTRACT

Buffer solutions of the lens protein γ-crystallin, and the enzymes aldolase and liver alcohol dehydrogenase became turbid and formed solid precipitate upon exposure to an elevated temperature of 63°C or to UV radiation at 308 nm. When α-crystallin was added to the protein solutions, in stoichiometric amounts, heat or UV irradiation did not cause turbidity, or turbidity developed much less rapidly than in the absence of α-crystallin. Hence, normal α-crystallin functioned as a "molecular chaperone", providing protection against both UV and heat induced protein aggregation. When α-crystallin was pre-irradiated with UV at 308 nm, its ability to function as a chaperone vis-a-vis both UV and heat induced aggregation was significantly impaired. A major effect of pre-irradiation of α-crystallin was to cause inter-peptide crosslinking amongst the αA₂ and αB₂ subunits of the α-crystallin macromolecule. In our experiments α-crystallin was exposed to UV doses which resulted in 0, 50, and 90% crosslinking as judged by SDS-PAGE. α-crystallin samples which were 50% and 90% crosslinked gave chaperone protection which was significantly reduced compared to unirradiated α-crystallin. The results are consistent with the notion that crosslinks in α-crystallin result in loss of chaperone binding sites.
stoichiometry $\alpha \gamma_{40}$, i.e., $\alpha = 800,000 \text{ Da} + 40(\gamma = 20,000) = 1.6$ million daltons. Such high mass particles exist in the intact lens in vivo, and efforts are underway to assess the importance of the $\alpha$-crystallin chaperone in vivo [Boyle & Takemoto, 1994]. It is worth noting that the stoichiometry of the $\alpha \gamma_n$ complex ($n \approx 40$) represents only a rough estimate. More experiments are needed to establish this quantity with precision.

Another important area which has not yet been addressed by previous workers is the kinetics of formation of the $\alpha$-crystallin chaperone complex. We propose to investigate chaperone complex formation and dissociation kinetics for the $\alpha \gamma_n$ complex. These studies are important since several previous reports have suggested that $\alpha$-crystallin chaperone complex formation is a temperature-dependent process requiring a phase transition or molecular rearrangement to facilitate substrate binding. We attack this question directly by measuring activation energies for both substrate binding and chaperone complex dissociation as follows:

1. What are the rates and activation energies for the chaperone-complex forming reactions:

$$\alpha \gamma^*_x + \gamma^* \rightarrow \alpha \gamma^*_{x-1},$$

where $\gamma^*$ is a heat or oxidatively denatured gamma crystallin molecule.

2. What are the rates and activation energies for the dissociation reactions:

$$\alpha \gamma^*_x \rightarrow \alpha \gamma^*_{x-1} + \gamma^*$$

We propose to answer these questions using a fluorescence energy transfer method which depends on observing emission from a fluorescent probe attached to $\alpha$-crystallin resulting from energy transfer from a second fluorescent probe attached to $\gamma$-crystallin. An analogous scheme has been reported in the literature for studying the kinetics of chaperone complex formation in the case of chaperone DnaK binding labeled-substrate peptide Cro* [Farr, et al, 1995], and we will compare our data on the kinetics of $\alpha$-crystallin chaperone binding with the data of Farr, et al on the DnaK chaperone to see if the former is unique.

C. PRELIMINARY STUDIES AND PROGRESS REPORT (This report covers the period from May, 1994 to January, 1997.)

1. Characterization of Photo-oxidative Damage in $\gamma$-Crystallin

In work completed during the last grant funding period, we investigated the photochemical opacification of the bovine lens crystallin proteins individually, and noted important differences between them [Hott and Borkman, 1993]. $\beta$-crystallin and $\gamma$-crystallin solutions became opaque when exposed to UV radiation, while $\alpha$-crystallin solutions remained transparent. We have also made efforts to characterize the chemical nature of photo-oxidation in $\gamma$-crystallin at the tryptic peptide and individual amino acid levels [Hott and Borkman, 1992],
analogous to the efforts of Dillon et al. [1987] to accomplish the same thing with α-crystallin. Both efforts have met with limited success, and both groups are now pursuing the use of mass spectrometry to elucidate the effects of photooxidation of α- or γ-crystallin at the peptide and amino acid levels.

We have found, using SDS-PAGE, that low UV doses suffice to produce measurable levels of gamma crystallin multimers such as γ*, γ-γ, and γ-γ-γ. γ* represents oxidatively-modified γ monomer. We have also detected polymers γn with n > 5 having molecular mass of about 100,000. The concentration of γ monomer decreased monotonically with photo-oxidation time. The level of dimer increased rapidly for short irradiation times, leveled off for intermediate times, and decreased gradually for long irradiation times. Thus, short irradiation times seem to favor dimers over higher polymers [Borkman and Knight, 1996].

Experiments with Dr. Kevin Schey at the Medical University of South Carolina are underway using ESIMS to characterize UV irradiated γn crystallin, and search for photo-dimers. We hope to obtain evidence for a dimer of molecular mass 2 x 20,000 = 40,000 daltons.

MALDI mass spectrometry is being used to analyze tryptic digest products of photo-oxidized γn crystallin. The UV dose was on the order of 20 J/cm² at a wavelength of 300 nm. In these experiments, we found evidence for photooxidation of methionine residues in the protein, as well as oxidation of tyrosine and tryptophan as reported at the 1996 ARVO meeting, Borkman & Knight [1996]. Some typical data on peptide mass changes following UV irradiation of calf γn crystallin in solution are shown in Table 1. One sees evidence for addition of either an oxygen atom, or a diatomic oxygen molecule in the first three lines of data, and evidence for a possible dimerization reaction in the fourth line of Table 1. Reported masses are ± 1 mass unit.

<table>
<thead>
<tr>
<th>Sequence Numbers</th>
<th>Before UV m/z</th>
<th>After UV m/z</th>
<th>Change m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>60-76</td>
<td>2088</td>
<td>2105</td>
<td>17</td>
</tr>
<tr>
<td>60-76</td>
<td>2088</td>
<td>2121</td>
<td>33</td>
</tr>
<tr>
<td>169-174</td>
<td>829</td>
<td>845</td>
<td>16</td>
</tr>
<tr>
<td>66-71</td>
<td>796</td>
<td>1593</td>
<td>797</td>
</tr>
</tbody>
</table>

Note: The first three lines of data correspond to O or O₂ addition; the last line is dimerization.
The question arises as to where in the peptide chain the oxidation occurs? The chart in Fig. 1 shows the segments of calf γ crystallin most affected by photooxidation, as detected by MALDI mass spectrometry of the tryptic digestate. All of the sequences which display marked changes (underlined segments in the chart) contain the amino acid methionine, and most contain tyrosine, and/or tryptophan. This is not surprising. In previous amino acid analysis of photo-oxidized gamma crystallin [Hott and Borkman, 1992] we found that methionine was most damaged, with 35% loss observed.

FIG. 1: MASS SPECTROMETRY RESULTS

Data show: Sequence of calf γ-II crystallin and segments most affected by UV photooxidation (underlined), 1 mg/ml, 25°C, 60 min at 300 nm.

1  GKITFYEDRGFQGHHCYECSSDCPNLQPYF
30  SRCNSIRVDSGCwmLYERPNYQGHQYFLRR
60  GDyPDyQQwmGFNDSIRSCRLPQHTGTF
89  RmRIyERDDFRGQMSEITDDCPSLQDRFHLTEV
122 SLNVLEGSWVLYEMPSYRGRQYLLRPGEYRR
154 vLDwGAmNAKVGSLRRVmDFy

Note: Oxidized sequences (underlined) contain Met, and several contain Trp or Tyr

2. Molecular Chaperone Studies

Following the important work of Horwitz [1992; 1993] implicating α-crystallin as a molecular chaperone, we began a study of the possible protective effect of bovine α-crystallin in photo-oxidative opacification. Our important new finding, which has now been incorporated into a paper [Borkman, Knight, and Obi, 1996], is that addition of α-crystallin to γ-crystallin solutions prior to photo-oxidation afforded protection from opacification. Data are shown in Fig. 2 a,b. The opacity was greatly reduced when α-crystallin was present, as seen in Fig. 2a. The results in Fig. 2b indicate that at a α:γ ratio of 1:1 by weight most of the protection was achieved. This corresponds to a molar ratio of 1:40, ie, one α-crystallin molecule (molecular weight of 800,000) protected 40 γ-crystallin molecules (molecular weight 20,000) from UV aggregation at room temperature. This result is similar to that found by Horwitz [1992; 1993] in thermal opacification experiments and suggests that each subunit (αA2, αB2 chains) in the α-crystallin
Fig. 2: Opacification of γ-crystallin solutions versus added α-crystallin. A.) Opacity versus UV dose for 0.5 mg/ml γ-crystallin with α-crystallin at concentrations of 0.0, 0.1, 0.25, 1.0, and 2.0 mg/ml. B.) Data replotted as opacity, after 15 min UV dose, versus added α-crystallin.

macromolecule can bind and protect one γ-crystallin molecule. Since the present experiments were done at room temperature, elevated temperatures were not needed to activate the chaperone function of α-crystallin, in contrast to the suggestion of Raman and Rao, [1994].

The kinetic data at 25°C indicated that an α:γ weight ratio of 1:1 afforded most of the available protection. We checked this stoichiometry using a molecular-weight-calibrated TSK-4000 HPLC column of dimensions 30 x 0.75 cm. The column was calibrated using blue dextran at 2000 kDa, thyroglobulin at 670 kDa, and gamma crystallin itself at 20 kDa. The column was used to detect the αγ₄₀ complex formed when an alpha-gamma mixture (1.0 mg/ml each) was irradiated with UV for 30 min at 300 nm (7 mW/cm²). Samples were centrifuged at 26,000g for 10 min and filtered through 0.2 micron Acrodisc before injection into HPLC. The HPLC elution buffer was 0.1 M phosphate with 0.3 M NaCl and 1.0 mM azide added. The flow rate was 1.0 ml/min. When the irradiation was done at 25°C, we observed loss of both the α- and γ-crystallin components in HPLC, but we were able to detect very little of the expected αγ₄₀ complex at this temperature. When the temperature was raised to 55°C, however, irradiation at 300 nm resulted in a complex which eluted at an apparent molecular weight of 1700 ± 200 kDa on the calibrated HPLC column--equal within experimental error to the predicted mass of the proposed αγ₄₀ complex, i.e., 800 + 40x20 = 1600 kDa. Some illustrative data are shown in Fig. 3, where one sees the peaks corresponding to α- and γ-crystallin prior to irradiation and the peak due to the complex αγ₄₀ after irradiation at 55°C. It should be mentioned that no complex could be detected in HPLC if the same alpha/gamma mixture was subjected to heat alone at 55°C. Thus, both radiation and heat were needed to form significant
amounts of HPLC-detectable chaperone complex [Knight and Borkman, 1996]. Our HPLC data provide support for the contention of Raman and Rao [1994] and of Das and Surewicz [1995] that \( \alpha \)-crystallin chaperone function is enhanced at elevated temperatures. Our own conclusion at present is that even though \( \alpha \)-crystallin protects \( \gamma \)-crystallin from UV opacification at 25°C (Fig. 3), the complex formed at this temperature is relatively weak and difficult to detect by HPLC.

All of the results based on HPLC studies of the alpha-gamma chaperone complex are summarized in the manuscript by Borkman and Knight [1997] which is included in the Appendix. It should be noted, however, that size exclusion HPLC in the molecular weight range around 2 million is not very precise, and more work is needed to establish the molecular weight and stoichiometry of the \( \alpha \gamma_n \) chaperone complex.

Protection from UV opacification by \( \alpha \)-crystallin was observed by us for several other target proteins, including aldolase, carbonic anhydrase, and enolase. In each case, addition of stoichiometric amounts of \( \alpha \)-crystallin to these solutions afforded significant protection against UV induced opacification. This is summarized in Borkman, Knight, and Obi [1996].

Irradiated solutions were analyzed by SDS-PAGE. \( \gamma \)-crystallin solutions were found to be highly crosslinked with the original 20 kDa material being converted to 40, 60 and > 100 kDa
material. Solutions were centrifuged to separate any insoluble material formed by UV irradiation. The supernatant was found to be transparent following centrifugation. Thus, gamma crystallin opacification came from insolubilization, and added α-crystallin prevented this insolubilization.

Photolyzed α-crystallin solutions analyzed by SDS-PAGE showed that much of the 20 kDa subunit material was converted to higher polymers greater than 100 kDa. Hence, calf α-crystallin subunits were covalently aggregated by UV irradiation, but this did not result in insolubilization or solution opacity [Hott & Borkman, 1993], nor did it result in any significant loss of chaperone activity, except at very high UV doses, as discussed in the next paragraph.

To investigate the effect of irradiation on chaperone function, three kinds of α-crystallin samples were prepared, by adjusting the dose of radiation delivered: First, dark control (normal α); second, α which was photo-crosslinked to the 50% level as detected by loss of 20 kDa subunits in SDS-PAGE; third, α which was photo-crosslinked to the 90% level as judged by SDS-PAGE. Each of these was used in subsequent chaperone experiments to determine if that α-crystallin sample could protect target proteins (γ-crystallin, aldolase, alcohol dehydrogenase) from UV or thermal aggregation/opacification. The results are summarized in our publication [Borkman & McLaughlin, 1995]. The important conclusion is that photo-crosslinking of α-crystallin impaired its ability to function as a chaperone vis-à-vis both thermal and UV insults. Moderate damage seemed to leave the chaperone function of α-crystallin intact; only extensive damage rendered the chaperone inoperative [Borkman & McLaughlin, 1995]. The effect of crosslinking which occurs with aging of α-crystallin was investigated by Sharma & Ortwerth [1995]. These authors found that crosslinked material lost a significant fraction of its chaperone activity relative to normal α-crystallin.

3. Preliminary Studies of Fluorescent Probe Binding to α- and γ-Crystallin

The cysteine residues of α-crystallin were labeled with the fluorescent probe FM (fluorescein -5-maleimide) and the cysteine residues of γ-crystallin were labeled with CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin). Both probes were obtained from Molecular Probes (Eugene, Oregon). Alpha crystallin at 5.0 mg/ml in 0.1 M phosphate buffer at pH 7.4 was reacted with a 10 fold excess of FM (FM stock solution 1.17 x 10^-2 M also in phosphate buffer) at 50°C overnight. Unreacted FM was quenched with 5 fold excess β-mercaptopethanol and removed by dialysis. A similar protocol was used for labeling gamma with CPM (stock solution 2.48 x 10^-3 M in dimethyl formamide). The protein concentration and degree of labeling for γ-CPM and α-FM were determined by measuring absorbances at 280 and 383 nm or 280 and 495 nm, respectively. The molar extinction coefficients (cm^-1 M^-1) used were:

\[
\begin{align*}
γ(280 \text{ nm}) & = 42,000 \\
\text{CPM}(280 \text{ nm}) & = 6,300 \\
\text{CPM}(383 \text{ nm}) & = 37,000 \\
α(280 \text{ nm}) & = 17,000 \\
\text{FM}(280 \text{ nm}) & = 16,500 \\
\text{FM}(495 \text{ nm}) & = 66,000 
\end{align*}
\]

The typical degree of labeling was about 1 CPM per γ molecule and 1 FM per two α subunits. This is consistent with the fact that gamma crystallins have 5-7 cysteines while αA, which comprises about 75% of the alpha, is the only alpha subunit with a cysteine and it has only one.
These probes were chosen because when they are brought into close contact (distances less than about 50 Å) energy transfer from CPM to FM occurs. Thus, in a dilute unreacted mixture of α-FM and γ-CPM, containing no chaperone complex, the two probes are far apart on the average and the fluorescence is the sum of the two independent probes, i.e., there is fluorescence at 480 nm coming from CPM and at 520 nm coming from FM. But, when chaperone complex, αγ, forms, the FM and CPM probes are brought into close proximity and energy transfer occurs. Under these conditions, one sees fluorescence from FM (hv' = 520 nm) even when exciting at the absorption wavelength of CPM (hv = 383 nm), according to:

\[
\text{CPM} + \text{hv} \rightarrow \text{CPM}^* \\
\text{CPM}^* + \text{FM} \rightarrow \text{CPM} + \text{FM}^* \\
\text{FM}^* \rightarrow \text{FM} + \text{hv}'
\]

Thus, the observation of enhanced FM fluorescence (and loss of CPM fluorescence) when the sample is excited at the CPM absorbance wavelength gives direct evidence for energy transfer which requires that γ-CPM and α-FM be in close proximity, i.e., that the αγ complex has formed.

Some typical data are shown in Fig. 4. A 1.0 ml sample of α-FM and γCPM (both 0.5 mg/ml) was placed in a thermally jacketed quartz cell and irradiated at 55° C for 30 min. The light source was an Oriel 350 W mercury vapor lamp fitted with a Spex 1681 B monochromator. The flux at 300 nm as measured by a Scientech 365 power meter was 7 mW/cm². The temperature was controlled to ± 0.5° C with a Neslab RTE-100LP circulator bath. The sample was allowed to cool to room temperature before fluorescence measurements were taken. An Oriel 1000 W xenon arc lamp and a Spex 1680 monochromator were used in the fluorescence apparatus. One sees growth of the fluorescence feature at 520 nm as chaperone complex formation proceeds. In addition, one sees a wavelength shift of the CPM emission from 480 nm to about 460 nm as a result of a change in the local environment of the CPM probe when γ-CPM binds to α-FM. During the next project period we plan to monitor the rate of growth of 520 nm fluorescence in mixtures of α-FM and γ-CPM exposed to photooxidizing conditions at various temperatures, and to use this data as a measure of the rate and extent (stoichiometry) of chaperone complex formation under various conditions. Similarly, the dissociation of previously formed αγ complex will be monitored by observing the reverse, i.e., loss of FM emission at 520 nm following decomposition of the complex. Finally, these same techniques can be used to obtain information relevant to the stoichiometry of the complex. These proposed experiments are described in more detail in the next sections.
Fig. 4: Fluorescence of labeled crystallins and chaperone complex. The solid curve is the fluorescence of an equimolar mixture (0.5 mg/ml each) of labeled α-FM-crystallin and γ-CPM-crystallin before UV exposure. The broken curve is the same mixture after UV irradiation at 300 nm and 55° C for 30 min. The fluorescence excitation wavelength was 395 nm in both spectra. The data were obtained by routing the fluorimeter output to a PC-type computer; see text.

D. RESEARCH DESIGN AND METHODS

1. Oxidative Aggregation of γ-Crystallin Solutions

a. Characterization of Small γ-Crystallin Aggregates

It is very clear from SDS-PAGE data reported by our lab [Li, et al, 1990 ] and others [Andley and Clark, 1989] that photooxidation of intact lenses or individual crystallins in solution results in formation of covalently crosslinked aggregates. Thus, one can detect monomers and small oligomers like γ, γ-γ, γ-γ-γ, as well as larger species γₙ, following irradiation [Borkman & Knight, ARVO, 1996]. We use γ-crystallin as a prototype for our photocrosslinking studies since such multimers have already been reported and because the mass spectrum of normal gamma-II crystallin monomer has been reported [Qin, et al., 1992]. We propose to detect and characterize the smaller species γ, γ-γ, and perhaps γ-γ-γ using ESIMS and MALDI mass spectrometry. The mass spectrometry experiments are to be done in collaboration with Dr. Kevin Schey of the Medical University of South Carolina. In the case of either method, we prepare and irradiate gamma crystallin solutions in our laboratories at the Georgia Institute of Technology, and send the irradiated samples, packed in dry ice, by overnight courier, to Dr. Schey for mass spectral analysis.
NIH EY-06800 -- FINAL REPORT

"Photochemistry and Spectroscopy of Lenses and Lens Proteins"

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REPORT DATED: DECEMBER, 1998

PERIOD COVERED:
(May 1, 1997 to April 30, 1998 represents a no-fund time extension period.)

[Signature]
Raymond F. Borkman

1-8-99
Date
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V. FUTURE PROJECT PLANS (NOT FUNDED)
A. SPECIFIC AIMS FOR MAY 1, 1987 -- APRIL 30, 1992

1. To determine average tyrosine-to-tryptophan inter-residue distances in each of the soluble lens protein fractions α-, β-, and γ-crystallin) and the gamma crystallin sub-fractions γII, γIII, γIV, for normal human and bovine lens proteins, via fluorescence and phosphorescence energy transfer experiments. The results are to be used to support or refute model protein structures for the proteins.

2. To determine the nature of the local environment and the degree of solvent exposure of the tryptophan residues in each of the soluble lens proteins listed in Aim (1) using acrylamide, iodide, and other fluorescence quenching experiments. The data will be used to evaluate model protein structures where possible.

3. To determine the anatomical distribution of protein damage in whole lenses caused by exposure to monochromatic UV laser radiation of various wavelengths. For a given wavelength we will determine damage is largely at the anterior cortex, nucleus, or posterior cortex. These studies will be performed for human and animal lenses of a range of ages, and some irradiations will be carried out in media containing possible protective drugs. UV altered lenses will be compared with normally aging and cataractous lenses from the literature.

4. To determine the molecular locations of UV induced damage sites within the lens crystallin proteins (ie, specific amino acid residues altered, particularly tryptophan residues) following exposure to monochromatic UV radiation of various wavelengths. The first protein to be studied will be calf γII-crystallin whose structure is known in detail from x-ray analysis.

5. To determine the nature and mechanism of action of UV laser and conventional radiation on whole lenses, and isolated lens proteins, by photochemical rate studies, ESR experiments, light scattering and turbidity measurements, and photoproduct analysis of irradiated proteins, peptides, and individual amino acids. One important aim is to elucidate the mechanism of action of 337.1 nm nitrogen-laser UV radiation on tryptophan.
B. RESULTS AND DISCUSSION FOR MAY 1, 1987 -- APRIL 30, 1992

**Aim 1:** Fluorescence and phosphorescence energy transfer experiments were carried out on the protein calf gamma-II crystallin, using the methods of Saito, et al., (1981). The total transfer efficiency from all fifteen tyrosines to the four tryptophans was determined from the data. This result compared very favorably with predictions based on Forster theory (1965) and the set of known Tyr-Trp inter-residue distances obtained from the xray structure of gamma-II crystallin determined by Blundell and coworkers (1981, 1983, 1984). Our work was published (Borkman and Phillips, 1985). This work illustrated the validity of the fluorescence energy transfer method for appraising the worth of model crystallin structures which might be proposed and for which no xray data are available.

**Aim 2:** Fluorescence quenching experiments using acrylamide and iodide as quenchers were carried out for α-, β-, and γ-crystallin (whole gamma fraction), and also for the gamma subfractions γ_{II}, γ_{III}, and γ_{IV}-crystallins. Studies were done in neutral pH buffer and in denaturing solvents. Stern-Volmer quenching constants, K_{SV}, were determined for each protein or protein fraction in each solvent. Our results were documented in two publications (Phillips, et al., 1986; Phillips and Borkman, 1988). Later publications from Chakrabarti's lab (Mandel and Chakrabarti, 1988) confirmed and extended our conclusions. The tryptophan residues in the lens proteins were determined to be buried and especially solvent-inaccessible in all of the crystallin fractions and proteins studied. This was consistent with conclusions reached from other spectroscopic methods (Mandel, et al., 1988) and from xray data (Wistow, et al., 1983; White, et al., 1989). Upon denaturation of the crystallins, the tryptophan residues were found to be highly exposed to solvent and K_{SV} values were ten times greater than in the native proteins. In the denatured crystallins, K_{SV} values were comparable to those of free amino acid tryptophan.

**Aim 3:** Whole bovine lenses *in vitro* were exposed to excimer laser radiation at wavelengths of 351, 337, and 308 nm. The irradiated lenses were characterized in terms of a photographic record and measurements of light scattering using a helium-neon laser as light source. Because data were available at three UV wavelengths, it was possible to construct an "action spectrum" for UV-induced lens damage analogous to that of Pitts (1974). That is, plots of lens opacity (based on degree of light scattering) versus irradiation wavelength were prepared. These plots revealed that 351 nm radiation had little or no effect on the lens, 337 nm radiation had an intermediate effect in which opacity, but not pigmentation, was produced, and 308 nm radiation caused the most opacification as well as a yellow-brown pigmentation not seen with 351 or 337 nm irradiation. Photos of the UV-irradiated lenses showed similarities to naturally occurring human cataracts. The results of this study were published (Li and Borkman, 1990).

**Aim 4:** To determine the molecular locations of UV-induced damage in lens proteins, we irradiated individual lens crystallins in their native states in buffer solution and then analyzed the irradiated protein and compared it to unirradiated control protein. The analysis consisted of enzymatic degradation with trypsin followed by peptide mapping with reverse phase HPLC. The first crystallin we examined in this way was calf γ_{II}-crystallin, a protein of known structure and sequence. Tryptic digestion and HPLC resulted in about twenty resolvable HPLC peaks in the peptide map, corresponding to the expected number of tryptic fragments based on the number of arginine and lysine residues in γ_{II}-crystallin. The four tryptophan residues in γ_{II} were each expected to be located in separate tryptic fragments, and this was confirmed by fluorescence analysis of the fragments collected from preparative HPLC.
The tryptic maps of irradiated and unirradiated γ II-crystallin were very similar, but there were some important differences. In particular, the absorbances in the 280-300 nm range of certain of the tryptic peptides in the irradiated material were significantly lower than in the dark control samples. This reduced UV absorbance occurred only in the four tryptic fragments containing tryptophan residues. We took this as preliminary evidence that some photochemical alteration of the tryptophans had occurred in the UV irradiated γ II protein. We confirmed this by changing the HPLC detector from absorbance mode to fluorescence mode, and the parameters were set specifically for the fluorescence characteristics of tryptophan, i.e., 350 nm detection and 295 nm excitation. This detection mode produced a much better signal-to-noise ratio in the tryptic map and eliminated all tryptic fragments from the map except the four Trp-containing peptides. These four peptide peaks were compared in the dark control and UV irradiated samples. The reduced fluorescence intensity in the four peaks of the UV irradiated material was used to quantitate the loss (photochemical destruction) of each of the four Trp residues in γ II-crystallin. The differences in photolysis rates were significant. The relative rates of destruction of the four Trp residues were tabulated and an effort was made to relate the photochemical reactivities to structural details of γ II-crystallin including nearest-neighbor amino acids, charged groups, acidic and basic functions, etc. Our work has been published (Tallmadge and Borkman, 1990). The results of similar experiments on α-crystallin were published by Dillon and coworkers (Dillon, et al., 1987). Again, important differences in the photolysis rates of the tryptophan residues were observed.

Later, the question of photochemical destruction of specific amino acid residues in lens proteins was revisited, and the loss of other amino acid residues besides tryptophan was investigated. These results were also published (Hott and Borkman, 1992).

C. LITERATURE CITED


II. REPORT ON GRANT FUNDED MAY 1, 1994 -- APRIL 30, 1997: EY-6800 6,7,8

A. SPECIFIC AIMS FOR MAY 1, 1994 -- APRIL 30, 1997

This project was based on the premise that the living human lens absorbs UV radiation from sunlight during all or most of a person's lifetime and that radiation causes photochemical changes in the structural proteins of the lens. These photochemically altered crystallins experience different interactions with surrounding water and protein molecules in the cytoplasm resulting in protein aggregation and insolubilization, the net effect of which is an opaque lens. The experiments were designed to detect and characterize photochemical reactions of the crystallins separately from other possible UV effects on the lens and to assess the potential of the photoproducts to initiate protein aggregation. Thus, our aims were:

To determine the chemical changes which occur in lens crystallin solutions exposed to monochromatic UV radiation in the 290-400 nm wavelength range for doses comparable to those received by the human lens under ambient conditions. Chemical changes which occur for low UV doses are of particular interest since damage to a small fraction of protein molecules in the living lens may initiate lens opacification. In our experiments, UV-induced changes in lens crystallins will be detected primarily by:

SDS-PAGE, size exclusion HPLC, and electrospray mass spectrometry.

Tryptic digestion followed by amino acid sequencing and FAB mass spectrometry of the tryptic peptides.

Fluorescence and fluorescence quenching analysis of UV irradiated crystallin solutions.

To determine which of the UV-damaged populations may be important in causing protein aggregation and lens opacity. Can photochemically modified small oligomers, eg, monomers or dimers of gamma crystallin, initiate protein aggregation, or are high polymers required? This information will be obtained primarily by:

Measurements of turbidity in solutions containing defined populations of photochemically modified monomers or dimers or higher polymers of lens crystallins, mixed with normal crystallins and measurements of the temperature dependence of turbidity in solutions of photochemically altered crystallin proteins.

**************************

** The additional goal of assessing the role of alpha crystallin as a molecular chaperone in lens crystallin photochemical reactions was added after the proposal for the grant of May 1, 1994 to April 30, 1997 was submitted and approved for funding. This goal was added in response to the discovery by Horwitz in 1992 of the molecular chaperone activity of bovine α-crystallin. This added aim in our proposal focussed on demonstrating the possible efficacy of alpha crystallin in protecting proteins from UV-induced aggregation, whereas Horwitz had demonstrated the role of α-crystallin in protecting proteins from thermal aggregation. We also proposed determining the stoichiometry of the αγ6 chaperone complex and investigating the kinetics of complex formation.
B. RESULTS AND DISCUSSION FOR MAY 1, 1994 -- APRIL 30, 1998

During the first year of the new funding period, we concentrated our efforts on: Purchase and assembly of new photochemical irradiation and detection apparatus and photochemical experiments designed to detect interactions amongst individual crystallins, including self-aggregation, and the possible role of α-crystallin as a "molecular chaperone" (Horwitz, 1992, 1993).

New apparatus purchased and put into use at the start of the funding period included: 350 W mercury lamp and monochromator, programmable temperature bath/circulator, spectrophotometer interfaced to lab computer, and HPLC modifications. The UV irradiation set-up allowed us to irradiate samples at monochromatic wavelengths from 290-400 nm, while maintaining sample protein solutions at fixed temperatures anywhere in the range 0-80 C. Changes in solution turbidity or chromophore absorption can be monitored throughout the UV and visible regions as a function of radiation dose and/or temperature. Since spectroscopic data are obtained in situ while the thermal or UV insult is being delivered, kinetic data are obtainable. Thus, the effects of UV radiation, or elevated temperature, can be investigated separately or in combination. The state of aggregation of the resultant protein solutions can be checked after the UV/thermal treatment by measuring SDS-PAGE or size exclusion HPLC in separate experiments.

In work completed during the last grant funding period, we investigated the photochemical opacification of the bovine lens crystallin proteins individually, and noted important differences between them (Hott and Borkman, 1993). β-crystallin and γ-crystallin solutions became opaque when exposed to UV radiation, while α-crystallin solutions remained transparent. Our original plan during the new funding period was to investigate the photochemical behavior of the crystallins, singly, and in various combinations of the three, in an effort to detect possible self-aggregation reactions for each crystallin and synergistic interactions amongst them. But, in the meantime, the important work of Horwitz appeared (1992, 1993) delineating the role of α-crystallin as a molecular chaperone. In light of these exciting new results, we devoted some effort to the possible protective role of α-crystallin in photochemical opacification experiments on γ-crystallin and other proteins as described below.

Photochemical Damage to γ-Crystallin Alone

Irradiated solutions of γ-crystallin were analyzed by SDS-PAGE. γ-crystallin solutions were found to be highly crosslinked with the original 20 kDa material being converted to 40, 60 and >100 kDa material. Solutions were centrifuged to separate any insoluble material formed by UV irradiation. The supernatant was found to be transparent following centrifugation. Thus, the major contribution to gamma crystallin UV opacification came from aggregation which resulted in reduced protein solubility. We have found, using SDS-PAGE, that UV irradiation times of 5 minutes or less sufficed to produce measurable levels of gamma crystallin multimers such as γ*, γ-γ, and γ-γ-γ. γ* represents photochemically-modified γ monomer, and γ-γ and γ-γ-γ are photodimer and phototrimer respectively. We have also seen polymers γₙ with n > 5 having molecular mass of about 100,000. The concentration of γ monomer decreased monotonically with UV irradiation time. The level of dimer increased rapidly for short irradiation times, leveled off for intermediate times, and decreased gradually for long irradiation times. Thus, short irradiation times seemed to favor dimers over higher polymers.
In light of the SDS-PAGE data, we concentrated some effort on characterization of photochemical damage in gamma crystallin in solution using electrospray (ESIMS) and matrix assisted laser desorption mass spectrometry (MALDI). The mass spectrometry experiments involved a newly initiated collaboration with Dr. Kevin Schey of the Medical University of South Carolina, and were aimed in part at characterizing UV irradiated γII crystallin, and searching for photo-dimers of approximate molecular mass 2 x 20,000 = 40,000 daltons.

MALDI mass spectrometry was used to analyze tryptic digest products of photo-oxidized γII crystallin. The UV dose was on the order of 20 J/cm² at a wavelength of 300 nm. We found evidence for photooxidation of methionine residues in the protein, as well as oxidation of tyrosine and tryptophan as reported at the 1996 ARVO meeting, Borkman & Knight [1996]. Typical data on peptide mass changes following UV irradiation of calf γII crystallin in solution are shown in Table 1. One sees evidence for addition of either an oxygen atom, or a

<table>
<thead>
<tr>
<th>Sequence Numbers</th>
<th>Before UV m/z</th>
<th>After UV m/z</th>
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Note: The first three lines of data correspond to O or O₂ addition; the last line is dimerization.

diatomoc oxygen molecule in the first three lines of data, and evidence for a possible dimerization reaction in the fourth line of Table 1. Reported masses are ± 1 mass unit.

The question arises as to where in the peptide chain the oxidation occurs? The chart in Fig. 1 shows the segments of calf γII crystallin most affected by photooxidation, as detected by MALDI mass spectrometry of the tryptic digestate. All of the sequences which display marked changes (underlined segments in the chart) contain the amino acid methionine, and most contain tyrosine, and/or tryptophan. This is not surprising. In previous amino acid analysis of photooxidized gamma crystallin [Hott and Borkman, 1992] we found that methionine was most damaged, with 35% loss observed.
FIG. 1: MASS SPECTROMETRY RESULTS

Data show: Sequence of calf γ-II crystallin and segments most affected by UV photooxidation (underlined), 1 mg/ml, 25°C, 60 min at 300 nm.

1  GKITFYEDRGFGHCHCECSSDCPNLQPYF
30 SRCNSIRVDSGCGwMYERPXYQGHQYFLRR
60 GDyPDyQQwmgGFNDSIRSCRLIPQHTGTF
89 RmRIyERDDFRGQMQEITDCPSLQDRFHLETV
122 SLNVLEGSWVLYEMPSYGRQYLLRPGEYRR
154 yLDwGAmNAKVGLRRVvmDFy

Note: Oxidized sequences (underlined) contain Met, and several contain Trp or Tyr

Role of α-Crystallin as Molecular Chaperone in the Photolysis of Proteins

One important finding, which has now been incorporated into a published paper [Borkman, Knight, and Obi, 1996], was that addition of α-crystallin to γ-crystallin solutions prior to UV photolysis afforded significant protection from opacification. We have studied the rate of UV induced γ-crystallin opacification as a function of added α-crystallin. The results at 25°C in Fig. 2 indicate that an α:γ weight ratio of 1:1 affords most of the available protection. This corresponds to a molar ratio of 1:40, i.e., one α-crystallin molecule protected 40 γ-crystallin molecules from UV aggregation. This stoichiometry is similar to that found in thermal opacification experiments. Similar results were found for several other proteins, including aldolase, carbonic anhydrase, and enolase. Addition of stoichiometric amounts of α-crystallin to these protein solutions afforded significant protection against UV induced opacification.

The kinetic data at 25°C indicated that an α:γ weight ratio of 1:1 afforded most of the available protection. We checked this stoichiometry using a molecular-weight-calibrated TSK-4000 HPLC column of dimensions 30 x 0.75 cm. The column was calibrated using blue dextran at 2000 kDa, thyroglobulin at 670 kDa, and gamma crystallin itself at 20 kDa. The column was used to detect the αγ40 complex formed when an alpha-gamma mixture (1.0 mg/ml each) was irradiated with UV for 30 min at 300 nm (7 mW/cm²). Samples were centrifuged at 26,000g for 10 min and filtered through 0.2 micron Acrodisc before injection into HPLC. The HPLC elution buffer was 0.1 M phosphate with 0.3 M NaCl and 1.0 mM azide added. The flow rate was 1.0 ml/min. When the irradiation was done at 25°C, we observed loss of both the α- and γ-crystallin
Fig. 2: Opacification of γ-crystallin solutions versus added α-crystallin. A.) Opacity versus UV dose for 0.5 mg/ml γ-crystallin with α-crystallin at concentrations of 0.0, 0.1, 0.25, 1.0, and 2.0 mg/ml. B.) Data replotted as opacity, after 15 min UV dose, versus added α-crystallin.

components in HPLC, but we were able to detect very little of the expected αγ₉ complex at this temperature. When the temperature was raised to 55°C, however, irradiation at 300 nm resulted in a complex which eluted at an apparent molecular weight of 1700 ± 200 kDa on the calibrated HPLC column—equal within experimental error to the predicted mass of the proposed αγ₄₀ complex, i.e., 800 + 40x20 = 1600 kDa. Some illustrative data are shown in Fig. 3, where one sees the peaks corresponding to α- and γ-crystallin prior to irradiation and the peak due to the complex αγ₉ after irradiation at 55°C. It should be mentioned that no complex could be detected in HPLC if the same alpha/gamma mixture was subjected to heat alone at 55°C. Thus, both radiation and heat were needed to form significant
Fig. 3: HPLC Data. The solid trace is an equimolar mixture of α- plus γ-crystallin at 55°C. The broken trace is the same mixture after irradiation at 300 nm.

Amounts of HPLC-detectable chaperone complex [Knight and Borkman, 1996]. Our HPLC data provide support for the contention of Raman and Rao [1994] and of Das and Surewicz [1995] that α-crystallin chaperone function is enhanced at elevated temperatures. Our own conclusion at present is that even though α-crystallin protects γ-crystallin from UV opacification at 25°C (Fig. 3), the complex formed at this temperature is relatively weak and difficult to detect by HPLC.

All of the results based on HPLC studies of the alpha-gamma chaperone complex are summarized in the manuscript by Borkman and Knight [1997, unpublished]. It should be noted, however, that size exclusion HPLC in the molecular weight range around 2 million is not very precise, and more work is needed to establish the molecular weight of the $\alpha\gamma_\alpha$ chaperone complex.

**Effect of Photolysis of α-Crystallin on its Chaperone Capacity**

Photolyzed α-crystallin solutions analyzed by SDS-PAGE showed that much of the 20 kDa subunit material was converted to higher polymers greater than 100 kDa. Hence, calf alpha crystallin subunits were covalently aggregated by UV irradiation, but this did not result in
insolubilization or solution opacity (Hott & Borkman, 1993). The fact that alpha crystallin was observed to crosslink, but not opacify, following UV irradiation suggested that this material may preferentially undergo intramolecular linking. Thus, the αA₂ and αB₂ polypeptide chains making up the α-cry stallin macromolecule become photo-crosslinked to each other but the particle size remains at about 800 kDa. We then asked whether this photo-crosslinking would affect the ability of bovine α-cry stallin to function as a molecular chaperone.

Three kinds of α-cry stallin samples were prepared, by adjusting the dose of UV radiation delivered. First, no UV (normal α); second, α which was photo-crosslinked to the 50% level as detected by loss of 20 kDa subunits in SDS-PAGE; third, α which was photo-crosslinked to the 90% level as judged by SDS-PAGE. Each of these was used in subsequent chaperone experiments to determine if that α-cry stallin sample could protect protein substrates (γ-crystallin, aldolase, liver alcohol dehydrogenase) from UV or thermal aggregation/opacification. The results are summarized in the paper by Borkman & McLaughlin (1995), and in the bar chart of Fig. 4. The important conclusion is that UV photo-crosslinking of α-cry stallin impaired its ability to function as a chaperone vis-a-vis both thermal and UV insults. But, the loss of chaperone function only became large for large UV doses, ie, doses sufficient to produce 90% crosslinked α-cry stallin. The α-cry stallin which was 50% crosslinked by pre-treatment with UV still retained most of its chaperone capability. Thus, moderate UV damage seems to leave the chaperone function of α-cry stallin intact; only extensive UV damage renders the chaperone inoperative (Borkman & McLaughlin, 1995).

![AMOUNT ALPHA NEEDED FOR PROTECTION](image)

**Fig. 4.** Summary of data on thermal and UV opacification of aldolase, HLAD, and γ-crystallin in terms of the amounts of α-crystallin (0, 50, and 90% crosslinked) needed to significantly reduce aggregation/opacification.
Studies of Fluorescent Probe Binding to α-Crystallin and Substrates

We adopt the fluorescence energy transfer method of Farr et al [1995] to monitor the amount of chaperone complex formation between α-crystallin and γ-crystallin. The cysteine residues of α-crystallin were labeled with the fluorescent probe FM (fluorescein-5-maleimide) and the cysteine residues of γ-crystallin were labeled with CPM (7-diethylamino-3-(4'-maleimidophenyl)-4-methylcoumarin). Both probes were obtained from Molecular Probes (Eugene, Oregon). Alpha crystallin at 5.0 mg/ml in 0.1 M phosphate buffer at pH 7.4 was reacted with a 10 fold excess of FM (FM stock solution 1.17 x 10^{-2} M also in phosphate buffer) at 50°C overnight. Unreacted FM was quenched with 5 fold excess β-mercaptoethanol and removed by dialysis. A similar protocol was used for labeling gamma with CPM (stock solution 2.48 x 10^{-2} M in dimethyl formamide). The protein concentration and degree of labeling for γ-CPM and α-FM were determined by measuring absorbances at 280 and 383 nm or 280 and 495 nm, respectively. The molar extinction coefficients (cm^{-1} M^{-1}) used were:

\[
\begin{align*}
\gamma(280 \text{ nm}) & = 42,000 \\
\text{CPM}(280 \text{ nm}) & = 6,300 \\
\text{CPM}(383 \text{ nm}) & = 37,000 \\
\alpha(280 \text{ nm}) & = 17,000 \\
\text{FM}(280 \text{ nm}) & = 16,500 \\
\text{FM}(495 \text{ nm}) & = 66,000
\end{align*}
\]

The typical degree of labeling was about 1 CPM per γ molecule and 1 FM per two α subunits. This is consistent with the fact that gamma crystallins have 5-7 cysteines while αA, which comprises about 75% of the alpha, is the only alpha subunit with a cysteine and it has only one. Glutathione reductase has ten Cys residues [Krauth-Siegel, et al., 1982; Pai and Schulz, 1983] and we propose to incorporate the CPM label following the same protocol used for labeling of γ-crystallin. Some of the Cys residues in glutathione reductase occur at the enzyme active site. Since our experiments do not assay enzyme activity, only protein aggregation/opacification, the fact that some labels may attach at the active site, hindering enzymatic activity, is not a problem.

The FM and CPM probes were chosen because when they are brought into close contact (distances less than about 50 Å) energy transfer from CPM to FM occurs. Thus, in a dilute unreacted mixture of α-FM and γ-CPM, containing no chaperone complex, the two probes are far apart on the average and the fluorescence is the sum of the two independent probes, i.e., there is fluorescence at 480 nm coming from CPM and at 520 nm coming from FM. But, when chaperone complex, αγn, forms, the FM and CPM probes are brought into close proximity and energy transfer occurs. Under these conditions, one sees fluorescence from FM (hv' = 520 nm) even when exciting at the absorption wavelength of CPM (hv = 383 nm), according to:

\[
\begin{align*}
\text{CPM} + \text{hv} & \rightarrow \text{CPM}^* \\
\text{CPM}^* + \text{FM} & \rightarrow \text{CPM} + \text{FM}^* \\
\text{FM}^* & \rightarrow \text{FM} + \text{hv}'
\end{align*}
\]

Thus, the observation of enhanced FM fluorescence (and loss of CPM fluorescence) when the sample is excited at the CPM absorbance wavelength gives direct evidence for energy transfer which requires that γ-CPM and α-FM be in close proximity, i.e., that the αγn complex has formed.
Some typical data are shown in Fig. 5. A 1.0 ml sample of α-FM and γCPM (both 0.5 mg/ml) was placed in a thermally jacketed quartz cell and irradiated at 55° C for 30 min. The light source was an Oriel 350 W mercury vapor lamp fitted with a Spex 1681 B monochromator. The flux at 300 nm as measured by a Scientech 365 power meter was 7 mW/cm². The temperature was controlled to ± 0.5° C with a Neslab RTE-100LP circulator bath. The sample was allowed to cool to room temperature before fluorescence measurements were taken. An Oriel 1000 W xenon arc lamp and a Spex 1680 monochromator were used in the fluorescence apparatus. One sees growth of the fluorescence feature at 520 nm as chaperone complex formation proceeds. In addition, one sees a wavelength shift of the CPM emission from 480 nm to about 460 nm as a result of a change in the local environment of the CPM probe when γ-CPM binds to α-FM. During the next project period we plan to monitor the rate of growth of 520 nm fluorescence in mixtures of α-FM and γ-CPM exposed to photooxidizing conditions at various temperatures, and to use this data as a measure of the rate and extent (stoichiometry) of chaperone complex formation under various conditions. Similarly, the dissociation of previously formed αγ, complex will be monitored by observing the reverse, ie, loss of FM emission at 520 nm following decomposition of the complex. Finally, these same techniques can be used to obtain information relevant to the stoichiometry of the complex.

Fig. 5: Fluorescence of labeled crystallins and chaperone complex. The solid curve is the fluorescence of an equimolar mixture (0.5 mg/ml each) of labeled α-FM-crystallin and γ-CPM-crystallin before UV exposure. The broken curve is the same mixture after UV irradiation at 300 nm and 55° C for 30 min. The fluorescence excitation wavelength was 395 nm in both spectra. The data were obtained by routing the fluorimeter output to a PC-type computer.
C. LITERATURE CITED


III. PUBLICATIONS SUPPORTED BY EY-6800: 1987 - 1998


IV. PRESENTATIONS SUPPORTED BY EY-6800: 1987 - 1998


R. F. Borkman, "Protein Photochemistry and Human Cataracts", Seminar, Department of Chemistry, University of Alabama, Tuscaloosa, AL, October 19, 1989 [Invited].


R. F. Borkman, J. Hott, and R. Church, "UV Aggregation of Lens Crystallins and Membranes", 9th International Congress of Eye Research (ICER) Meeting, Helsinki, FINLAND, July 31, 1990 [Contributed].

R. F. Borkman, "UV Photolysis of Tryptophan", Tennessee Technological University, Cookeville, TN, November 1, 1990 [Invited].

R. F. Borkman, "UV Photolysis of Tryptophan", Western Kentucky University, Bowling Green, KY, November 2, 1990 [Invited].


J. Hott, M. Walker, D. Li, and R. Borkman, "Studies of Lens Proteins Exposed to Ultraviolet Light", University System Symposium on Biomedical Sciences, Medical College of Georgia, May 17, 1991 [Contributed].


R. Borkman, "The Effect of UV Radiation on Calf Lens and Lens Protein", Department of Ophthalmology, Tokai University School of Medicine, Isehara, Kanagawa, Japan, January 17, 1992 [Invited].

R. Borkman, "Photochemistry of Calf Lens and Lens Protein", Department of Physico-Chemical Physiology, Osaka University Medical School, Osaka, Japan, February 5, 1992 [Invited].

R. Borkman, "The Effect of UV Radiation on Calf Lens and Lens Proteins", Department of Ophthalmology, Hamamatsu University School of Medicine, Hamamatsu, Japan, March 25, 1992 [Invited].

R. Borkman, "Tryptophan Photochemistry and Cataracts", Birmingham Southern College, Department of Chemistry and Biology, Birmingham, Alabama, November 18, 1992 [Invited].


R. Borkman, "Cataracts and the UV Photolysis of Lens Proteins", University of North Alabama, Florence, AL, October 14, 1993 [Invited].

R. Borkman and B. Obi, "Thermal and Photochemical Chaperone Effect in Mixtures of Alpha and Gamma Crystallin", CCRG Meeting, Wash., DC, November 9, 1993 [Contributed].

R. Borkman, "Alpha Crystallin Chaperone Inhibits UV Induced Protein Aggregation", ARVO Meeting, Ft. Lauderdale, Florida, May 14-20, 1995. [Contributed].


R. Borkman, "Cataracts and UV Photolysis of Lens Protein", Invited Seminar, Austin Peay State University, Clarksville, TN, February 1, 1996 [Invited]

R. Borkman and G. Knight, "Detection of Small Multimers of Gamma Crystallin", ARVO, Ft. Lauderdale, FL, April, 1996. [Contributed].

G. Knight and R. Borkman, "Properties of the Molecular Chaperone Complex αγₙ", ARVO Meeting, Ft. Lauderdale, FL, April, 1996. [Contributed].

G. Knight and R. Borkman, "Fluorescent Probes Yield Further Insight on the Properties of the Molecular Chaperone Complex αγₙ", American Society for Photobiology Meeting, Atlanta, GA, June, 1996. [Contributed].


G. C. Knight & R. F. Borkman, "Fluorescent Probes Detect the Molecular Chaperone Complex αγₙ", Georgia Academy of Science Meeting, State University of West Georgia, Carrolton, GA, April 25, 1997. [Contributed]

R. F. Borkman & G. C. Knight, "Temperature Dependence of Alpha Crystallin Chaperone Complex Formation with UV Denatured Lens Protein Gamma Crystallin", 25th Annual Meeting of the American Soc. for Photo-Biology, St. Louis, Mo, July 10, 1997 [Invited].
V. FUTURE PROJECT PLANS (NOT FUNDED)

T. Measurements on UV Treated Proteins:

It is important to establish what kinds of photochemically produced species are responsible for initiating aggregation/opacification in solution, and in the intact lens. Similar experiments using chemically oxidized lens proteins have been reported by Pande et al [1996]. Thus, we will deliver sub-opacity-producing doses of UV to β- and γ-crystallin solutions, and measure the critical opacification temperature, Tc, of the modified protein solutions compared to normal β- or γ-crystallin solutions at the same concentration. The lens proteins βH, βL and various proteins of the γ family (γII, γIIIa, γIVA, etc) will be evaluated for their "aggregation potential" in this way. The UV-produced photochemical intermediates will be characterized using mass spectrometry.

Kinetics of Formation of α-Crystallin Chaperone Complex

This project proposes a method, based on fluorescent labels, to elucidate the stoichiometry and kinetics of formation of the chaperone complex formed between α-crystallin and denatured protein substrates, S, where S is heated or oxidized γ-crystallin (20 kDa size) or glutathione reductase (100 kDa size). This allows assessment of the effect of protein size on the kinetics and stoichiometry of αSn complex formation. α-crystallin and S are to be prepared with bound fluorescent labels, F and C, (typically fluorescein and coumarin derivatives), and the complex, αSn, forms when S is heated or photo-oxidized in the presence of α-crystallin. The kinetics of this complex formation and dissociation are measured by monitoring the growth of F fluorescence which results from energy transfer from C which is excited directly by light absorption. The energy transfer only occurs when the donor and acceptor are within a critical distance of each other, i.e., when denatured S binds to α-crystallin. Analysis of the temperature dependence allows evaluation of rate constants for formation, kON, and dissociation, kOFF, and the activation energies, Eₐ on, and Eₐ off. Proposed experiments will allow us to distinguish between a concerted mechanism for αSn formation and a stepwise mechanism in which the α-crystallin chaperone is pre-activated by heating prior to binding S. Measuring kON for various n values in the αSn complex, will allow testing for cooperativity in the binding.
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In cataracts, lens proteins aggregate to form large, insoluble particles which scatter light and lead to vision loss. The protein α-crystallin can prevent such aggregation by serving as a molecular chaperone. This project uses a method, based on fluorescent labels, to elucidate the stoichiometry and kinetics of formation of the chaperone complex formed between α-crystallin and denatured protein substrates, S, where S is heated or oxidized γ-crystallin (20 kDa size) or glutathione reductase (100 kDa size). This allows assessment of the effect of protein size on the kinetics and stoichiometry of αS_n complex formation. α-crystallin and S are prepared with bound fluorescent labels, F and C, (typically fluorescein and coumarin derivatives), and the complex, αS_n, forms when S is heated or photo-oxidized in the presence of α-crystallin. The kinetics of this complex formation and dissociation are measured by monitoring the growth of F fluorescence which results from energy transfer from C which is excited directly by light absorption. The energy transfer only occurs when the donor and acceptor are within a critical distance of each other, i.e., when denatured S binds to α-crystallin. Analysis of the temperature dependence allows evaluation of rate constants for formation, k_{on}, and dissociation, k_{off}, and the activation energies, E_{on}^a, and E_{off}^a. Proposed experiments will allow us to distinguish between a concerted mechanism for αS_n formation and a stepwise mechanism in which the α-crystallin chaperone is pre-activated by heating prior to binding S. Measuring k_{on} for various n values in the αS_n complex, will allow testing for cooperativity in the binding. A second part of the project investigates mechanisms of lens opacification due to aggregation of lens crystallins under oxidative conditions. We test possible precursors of lens opacification by measuring critical opacification temperatures, T_c, of oxidation products such as the dimer of γ-crystallin.

School of Chemistry and Biochemistry
Georgia Institute of Technology
Atlanta, GA 30332-0400

KEY PERSONNEL. See instructions on Page 11. Use continuation pages as needed to provide the required information in the format shown below.

Name          Organization                   Role on Project
BORKMAN, Raymond F.   Georgia Inst. of Tech.                P.I.
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Appendix (Five collated sets. No page numbering necessary for Appendix.)

Number of publications and manuscripts accepted or submitted for publication (not to exceed 10)

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Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a, 3b.
# Detailed Budget for Initial Budget Period

## Direct Costs Only

### Personnel (Applicant organization only)

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<thead>
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<th>Name</th>
<th>Role on Project</th>
<th>Type of App. (months)</th>
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<th>Inst. Base Salary</th>
<th>Salary Requested</th>
<th>Fringe Benefits</th>
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**Subtotals**

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### Consultant Costs

- Fluorimeter ($29,000), PC-Type Computer ($2,000), Computer Interface ($1,500), Digital Plotter ($1,500)

- Quartz Optical Cells ($2,100), HPLC Columns ($2,000), Chemicals ($3,500), Glassware ($1,800), Laser Gases ($2,000), Electrical Cables & Connectors ($500), Lamps $1,100

**Supplies**

- Total: $34,000

### Travel

- PI and Postdoc to attend ARVO Meeting, 1999

**Travel**

- Total: $13,000

### Patient Care Costs

- Inpatient: None
- Outpatient: None

### Alterations and Renovations (Itemize by category)

- None

### Other Expenses (Itemize by category)

- Repair of Excimer Laser ($6,000), Publication Costs ($2,000)

**Other Expenses**

- Total: $8,000

### Subtotal Direct Costs for Initial Budget Period

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**Total Direct Costs for Initial Budget Period**

- Total: $121,499
## Budget for Entire Proposed Period of Support
### Direct Costs Only

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**Total Direct Costs for Entire Proposed Period of Support** (Item 8a, Face Page) $291,603

**Justification.** Follow the budget justification instructions exactly. Use continuation pages as needed.

**Personnel (Salaries increased 5% annually):**

**R. Borkman** -- The project PI is requesting summer salary of 25% of total annual salary.

**Grady Knight** -- Has been involved in the project as a PhD student to the present, and is expected to finish the degree in Spring, 1998. He has agreed to continue on the project as a Postdoctoral Associate. He has a publication in Exp. Eye Res., A Manuscript to be Submitted, two ARVO presentations, and a presentation at the American Society for Photobiology meeting. 12 Months annually requested.

(Continued)
BUDGET JUSTIFICATION CONTINUED

EQUIPMENT:

Fluorimeter -- A rapid scan fluorimeter is central to our measurements of kinetic parameters for formation and dissociation of the chaperone complex between fluorescent labeled alpha and substrate proteins. The only fluorimeter presently available to us is a home-built model dating from 1987 which has a fastest spectral scan time exceeding 5 minutes. We propose replacing the old fluorimeter with a Shimadzu model RF-5301PC fluorimeter. This model has rapid scan capability and allows scanning the complete fluorescence spectrum of the labelled chaperone complex every few seconds as required for our kinetic experiments.

PC computer--Needed to interface with our Milton Roy Spectronic 601 absorption spectrometer for measurement of absorbance versus temperature curves for the determination of critical opacification temperatures. An IBM or Dell with Windows software and pentium processor is anticipated.

Interface Board -- Needed to interface the analog output of the Spectronic 601 to the PC computer. An Omega DAS model is anticipated.

Digital Plotter -- Needed to present the data from the critical opacification temperature and fluorescence spectral experiments in hard copy form. An HP model 7475A or equivalent will be suitable.

OTHER EXPENSES

Laser Repairs -- Our Lumonics Excimer laser model 500 outputs pulses at 308 nm which are suitable for use in the proposed kinetic experiments on the labelled chaperone complex. The unit in the PI's lab was purchased in 1987 and is ten years old. Performance is often erratic. The unit needs to be reconditioned by a Lumonics Corp. technician so that it will provide reliable performance during the new grant period. $6,000 is requested for this purpose in year 1.

TRAVEL

Funds are requested for the PI and postdoctoral associate to attend the annual ARVO meeting in Ft. Lauderdale, FL, in years 1, 2, and 3, to present the results of our own research and to keep abreast of recent developments in the lens research field.

Funds are requested in year 3 for the PI to attend the ICER meeting to present the results of our own research and to keep abreast of lens research progress in the international community of lens scientists.
BORKMAN, Raymond F.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2. Photocopy this page or follow this format for each person.

NAME: Raymond F. Borkman

POSITION TITLE: Professor of Chemistry & Biochemistry

Georgia Institute of Technology

EDUCATION/TRAINING: (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
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<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
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<td>Univ. Calif. Riverside</td>
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<td>1966</td>
<td>Chemistry</td>
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RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Employment History:

Teaching Assistant, University of California, Riverside, 1962-63

NIH Predoctoral Fellow, University of California, Riverside, 1963-66

NSF Postdoctoral Fellow, The Johns Hopkins University, 1966-68

Assistant Professor, Georgia Institute of Technology, 1968-72

Sigma Xi Research Award (Georgia Tech) 1972

Associate Professor, Georgia Institute of Technology, 1972-78

Professor of Chemistry, Georgia Institute of Technology, 1978-95

NIH Vision B Study Section Consultant, August, 1991


Associate Chair and Professor, School of Chemistry & Biochemistry, Georgia Institute of Technology, 1995-present.

(Continued)
Recent Publications


OTHER SUPPORT

Borkman, R. F.

ACTIVE

None, except present NIH FY-6800-08

PENDING

PI--Borkman 7/1/98 - 8/31/00 5.0%
Georgia Institute of Technology $60,000
"Quantum Chemical Calculations Pertaining to the Barrier Properties of Polyesters"

The major goal of this project is to use ab initio molecular modeling methods to gain an understanding of the molecular factors important in controlling the gas permeability of polyester materials like polyethylene glycol terephthalate (PET).

OVERLAP

The project above involves theoretical modeling studies of non-biological systems, and hence there is no scientific overlap with the present NIH proposal pertaining to experiments on lens protein aggregation and molecular chaperone function.
RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: The PI has available a 1000 square foot laboratory in the Boggs Building (Chemistry and Biochemistry) with complete gas, water, and electrical utilities and computer network connections for performing the proposed experiments and providing desk space for up to four research associates.

Clinical: NA

Animal: NA

Computer: The PI's office and laboratory both have computer network connections and PC type computers for data analysis, report and publication preparation, and other clerical uses. Network servers provide internet and WWW access as well as access to campus mainframe computers if needed.

Office: The PI has a 200 square foot office for his sole use. This room in the Boggs Building is equipped with telephone, voice mail service, and computer network connection.

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Equipment available for use on the project in the PI's laboratory includes: Hussman double refrigerator for chromatography, column chromatography with ISCO fraction collector and strip chart recorder, SDS-PAGE mini-gel electrophoresis, home-built fluorimeter with 1000W xenon lamp, SPEX 1680 double monochromator, phototube and electronic detection, Lumonics model 500 excimer laser, Oriel 350W mercury lamp with SPEX 1681 monochromator for photochemistry, Spectronic 601 spectrophotometer, NESLAB RTE-100-LP circulating temperature controller, LKB model 2150 HPLC, Scientech 365 radiant flux meter.
INTRODUCTION TO REVISED APPLICATION

This Introduction addresses the review from the VISA-IRG meeting of June, 1997. The Summary Statement suggested some changes to improve the research plan. These suggestions have been incorporated, and responses to specific comments in the Summary Statement are indicated below:

1.) The critique indicated that the proposed fluorescence energy transfer method to measure the stoichiometry and kinetics of formation of the chaperone complex between denatured proteins and α-crystallin was the most important and innovative part of the proposal. Thus, this aspect is developed more fully in the revised proposal. A criticism was that some of the ideas and models for interpretation of the kinetic data were not sufficiently developed. We have remedied this in two ways:

a.) We have added experiments aimed at investigating the effect of substrate size on the kinetics of binding to the α-crystallin chaperone. That is, binding of substrate in the interior or cavity of the chaperone would be expected to be strongly dependent on substrate size, while binding at the chaperone surface would be much less size dependent. Thus, the previous proposal used only the 20 kDa protein γ-crystallin as a substrate while the revised proposal uses the additional substrate glutathione reductase with molecular weight 100 kDa. This protein was chosen as a test substrate partly because of its greater size relative to γ-crystallin, and partly because of its intrinsic importance as an antioxidant in the lens medium. The integrity of glutathione reductase in the lens needs to be maintained to spare the lens from oxidative damage. Our experiments will probe the kinetics of binding of photooxidized glutathione reductase to the α-crystallin chaperone. The new experiments are described on pages 13, 15, 23, 25, 28, and 33. It is noteworthy in this regard that Harding and coworkers have already shown that α-crystallin can protect glutathione reductase from inactivation by fructation. Thus, there is precedent for believing that oxidatively denatured glutathione reductase will be bound by α-crystallin.

b.) We have added experiments to distinguish between a stepwise and a concerted model for binding of denatured substrate by the α-crystallin chaperone. Data in the literature suggest that the chaperone activity of α-crystallin is enhanced by pre-treatment with heat, which presumably causes one or more phase transitions. In the revised proposal we consider two possible substrate binding mechanisms—stepwise, with a pre-activation step followed by a substrate binding step, and a concerted mechanism, where chaperone activation and substrate binding occur simultaneously. We propose to distinguish between the two mechanisms based on the results of a series of measured kinetic activation energies as described in the revised proposal on pages 13, 15, and 34.

2.) The reviewers were not enthusiastic about the potential for important new findings in our mass spectrometry experiments with Prof. Kevin Schey at the Medical University of South Carolina. Similar lack of enthusiasm for this part of the project was expressed in the last round of review. Thus, although mass spectrometry studies were a part of the grant funded in 1994, and we reported some preliminary findings in this area at the 1996 ARVO, this part of the project has now been deleted from the revised proposal.
3.) The critique agreed that the proposal budget was appropriate. The revised budget is the same as the previous one except for salary and fringe adjustments to reflect the new fiscal year rates and other minor changes. These modifications resulted in about a 1% budget reduction in the first year and a 1% budget increase over the total three year project. This is documented on pages 4, and 5 of the revised proposal. With the added experiments described in (1.) above, the deleted mass spectrometry work (2.) resulted in no net change in the project manpower or budget needs. (There was no provision for financial support of the mass spectrometry experiments in the original budget, at any rate, except for incidental sample mailing costs.)

4.) One critique comment pointed out that the Specific Aims of the past proposal were not summarized in the present proposal, and this has now been added on page 17.

5.) It was suggested that SDS-PAGE gels should be run with and without a reducing agent to test for the presence of disulfide crosslinks in the photooxidized proteins. This provision has now been added to the proposal on page 25.

6.) It was suggested that control experiments be included to demonstrate that the fluorescent labels added to the α-crystallin chaperone and to γ-crystallin and other protein substrates do not affect the behavior of the protein vis-à-vis self-aggregation or binding to α-crystallin. These needed experiments are now included on page 29 of the revised proposal.
A. SPECIFIC AIMS

This project is concerned with lens protein aggregation and its potential role in lens opacification and cataractogenesis. The proposed experiments involve lens crystallin proteins, α- and γ-crystallin, the enzyme glutathione reductase, and possibly other protein substrates in aqueous buffer solutions. The proteins are subjected to heat or photo-oxidizing conditions, and the stoichiometry and kinetics of formation of the molecular chaperone complex between protein substrates and α-crystallin is investigated. Our aims are as follows.

1. We will measure the stoichiometry and kinetics of formation of the chaperone complex, $\alpha S_n$, where $S$ is either γ-crystallin or glutathione reductase. We plan to measure the stoichiometry and the rates of complex formation for the reaction

$$\alpha + nS + uv \rightarrow \alpha S_n$$

as a function of temperature using a fluorescent probe method to evaluate the coefficient, $n$, the rate constant, $k_{on}$, and the activation energy, $E_a$, for complex formation. We will use $S = γ$-crystallin (20 kDa) and $S =$ glutathione reductase (100 kDa) to investigate the effect of substrate size on the kinetics of chaperone binding. Similarly, we propose to measure the rate of decomposition of the chaperone complex as a function of temperature so as to obtain the rate constant, $k_{off}$, and activation energy for complex dissociation.

$$\alpha S_n \rightarrow \alpha S_{n-1} + S$$

Both $k_{on}$ and $k_{off}$ and the corresponding activation energies are to be compared with data in the literature for the DnaK chaperone.

2. Two possible mechanisms for formation of $\alpha S_n$ are to be investigated— a stepwise mechanism wherein $α$-crystallin is pre-activated by heat prior to binding $S$, and a concerted mechanism where substrate binding occurs simultaneously with chaperone activation. Proposed experiments will distinguish the two possibilities.

3. The possibility of an allosteric effect (positive cooperativity) in the binding of substrate to $α$-crystallin will be determined by measuring reaction rates as a function of loading of the chaperone. That is, rates, $k_i$, will be measured for various $i$ values in the reaction

$$\alpha S_i + S + uv \rightarrow \alpha S_{i+1}$$

4. We will determine the effect of oxidative damage in crystallin solutions on the value of the critical opacification temperature of the solution, $T_c$. In particular we will ascertain which of the components present in oxidized crystallin solutions (monomers, dimers, high polymers) are most responsible for changes in the $T_c$ value.
B. BACKGROUND AND SIGNIFICANCE

i. General Introduction  The transparency of the normal ocular lens depends on the lens medium being homogeneous over distances comparable to the wavelength of visible light. Cataract or lens opacity results when the protein/water composition of the lens becomes inhomogeneous on this distance scale. Protein aggregation is one mechanism by which lens composition can become inhomogeneous and result in an opaque lens. As large protein aggregates form, the lens medium begins to consist of regions of high protein concentration (aggregated regions) alternating with regions of low protein concentration (water lakes). There are several possible mechanisms by which lens protein aggregation might occur, including: Glycation reactions of the Maillard type involving the lens proteins and excess sugar [Swamy, et al., 1993], chemical oxidation of lens proteins—for example by excess peroxide [Spector and Garner, 1981], by excess oxygen [Giblin, et al, 1988] or by UV or visible radiation [Li, et al, 1990; Ortwerth & Olesen, 1994].

This proposal focusses on protein aggregation experiments on calf α- and γ-crystallin and glutathione reductase solutions. Why these proteins? First, γ-crystallin undergoes aggregation and subsequent solution opacification as a consequence of a variety of insults, including: Heat, cold, chemical oxidation, and UV irradiation. Hence, the γ-crystallins are good models for aggregation leading to lens opacity in vivo. Also, γ-crystallin can be protected from aggregation by α-crystallin acting in its role of chaperone. In addition, the structures of several of the gamma crystallins are known from x-ray diffraction studies [Wistow, et al., 1983; White, et al., 1989]. Finally, the gamma crystallins have high Tc values meaning that they are likely to be important in lens opacification [Siezen, et al, 1985]. Glutathione reductase is chosen because it has a higher molecular weight than γ-crystallin, and we expect size to be a factor in the kinetics of substrate binding. That is, if binding of a substrate S occurs in a cavity in the interior of the α-crystallin chaperone, we would expect a lower binding rate constant and a larger activation energy for a large substrate compared to a small substrate. If binding occurs on the surface, size may not be a rate determining factor. Such size effects have not yet been investigated, but fortunately, it has already been shown that glutathione reductase can be protected from damage by the α-crystallin chaperone system [Blakaty and Harding, 1997]. Perhaps most importantly, glutathione reductase is of interest because it is the enzyme which maintains the anti-oxidant glutathione in the reduced state necessary for protecting the lens from oxidative damage. If this enzyme were to be deactivated by uv-aggregation or other causes, a significant mechanism of lens protection would be lost. It is possible that the α-crystallin chaperone can protect glutathione reductase from such inactivation, and we propose to investigate the kinetics and mechanism of this process. The use of α-crystallin in our studies is based on two facts: First, α-crystallin solutions, unlike γ-crystallin, do not opacify following heat or UV. Hence, alpha represents a contrasting situation to gamma, and it is important to understand these differences. In addition, α-crystallin displays some of the functions of a molecular chaperone. Molecular chaperones were reviewed by Braig, et al. [1993] and by Ellis and van der Vies [1991]. α-crystallin has been shown to protect proteins from aggregation due to: Heat [Horwitz, 1992; 1993], chemical oxidation [Wang & Spector, 1994; 1995], or UV [Raman & Rao, 1994]. We wish to understand how this protection occurs at the molecular level, and various model molecular structures have been proposed for α-crystallin [Augusteyn & Koretz, 1987; Tardieu, et al., 1986; Wistow, 1993; Carver, et al, 1994].
ii. **Alpha-Crystallin Chaperone Complex**  Horwitz [1992; 1993] discovered that calf α-crystallin can protect proteins from thermal aggregation by functioning as a molecular chaperone. It has since been shown that α-crystallin can prevent aggregation of proteins stemming from other insults. The mechanism of this protection involves formation of a complex in which the reactive intermediates (partially denatured substrate protein) are bound and maintained in the form of a soluble complex. It is possible that only a small fraction of the denatured proteins need be bound by chaperone in order to prevent lens opacity. That is, by reducing the concentration of denatured protein in the lens, nucleation of aggregates may be prevented or delayed. In the case of α plus γ crystallin we indicate the chaperone complex as αγ. Such complexes have been detected by several research groups including ourselves. Using TSK-4000 HPLC we detected a complex of molecular weight approximately $1.7 \pm 0.2$ million daltons following exposure of a mixture of α- and γ-crystallin to photo-oxidizing conditions in buffer solution. This molecular mass (though only a crude estimate) corresponds to that expected for an alpha-gamma complex of stoichiometry $\alpha \gamma_4$; i.e., $\alpha = 800,000 \text{ Da} + 40(\gamma = 20,000) = 1.6$ million daltons. Such high mass particles exist in the intact lens *in vivo*, and efforts are underway to assess the importance of the α-crystallin chaperone *in vivo* [Boyle & Takemoto, 1994]. More experiments are needed to establish the stoichiometry of $\alpha S_n$ complexes with precision.

An area which has not been addressed by previous workers is the kinetics of formation of α-crystallin chaperone complexes. We propose to investigate chaperone complex formation and dissociation kinetics for $\alpha S_n$ complexes, where S is a protein substrate of varying size, including γ-crystallin and glutathione reductase. These studies are important since some previous reports have suggested that pre-activation of α-crystallin by heating accelerates subsequent chaperone complex formation by inducing a phase transition or molecular rearrangement which facilitates substrate binding. But, no previous experiments have measured the temperature dependence of the actual binding rate constants. We attack this question by measuring the activation energies for substrate binding, assuming a concerted process, or alternatively a stepwise process. That is, the activation energy associated with substrate binding by the α-crystallin chaperone to form $\alpha S_n$ could involve a pre-activation of α-crystallin followed by substrate binding in a stepwise mechanism:

\[
\text{STEPWISE MECHANISM} \quad \alpha + \text{heat} \rightarrow \alpha^* \quad E_{a}^1
\]

\[
\alpha^* + S + \text{uv} \rightarrow \alpha S_n \quad E_{a}^2
\]

In this case, it would be possible to pre-activate α-crystallin by heating, prior to adding substrate, S, and an activation energy $E_{a}^1$ would be associated with this first step. The second step would be expected to have little or no activation energy, $E_{a}^2$, but this will also be determined in the proposal. The second possibility is a concerted binding reaction in which chaperone activation and substrate binding occur simultaneously:

\[
\text{CONCERTED MECHANISM:} \quad \alpha + S + \text{heat + uv} \rightarrow \alpha S_n \quad E_{a}^3
\]

Here, the activation energy barrier associated with substrate binding and with any needed rearrangement of the chaperone are combined into one step. The stepwise and concerted
possibilities can be distinguished using a series of temperature-dependence studies of the reaction rate constant as described later in the proposal. The use of uv radiation as the substrate denaturing agent in our proposed experiments is important in this regard, because this allows us to use temperature as an independent variable and thus determine kinetic activation energies. If heat were being used as the substrate denaturing agent (as in the original Horwitz experiments), then temperature plays a dual role and one could not readily separate the experimental activation energy into substrate-denaturation and chaperone-binding components.

We propose to measure rates of $\alpha S_n$ complex formation using a fluorescence probe method which depends on observing emission from a probe attached to $\alpha$-crystallin resulting from energy transfer from a second fluorescent probe attached to $S$. An analogous scheme has been reported in the literature for studying the kinetics of chaperone complex formation in the case of chaperone DnaK binding labeled-substrate peptide Cro* [Farr, et al, 1995], and we will compare our data on the kinetics of $\alpha$-crystallin chaperone binding with the data of Farr, et al on the DnaK chaperone to see if the former is unique.

### iii. Aggregation of Gamma Crystallin

The aggregation of calf $\gamma$-crystallin in solution to form water-insoluble particles has been noted often. Benedek and coworkers [Siezen, et al., 1985] established a possible connection between bovine "cold cataracts" and the critical opacification temperatures, $T_c$, of various members of the gamma family. This approach has progressed to the level of seeking anti-cataract agents based on inhibition of protein cryoprecipitation (lowering $T_c$) with chemical agents such as pantethine [Clark, et al, 1996]. Hott and Borkman [1993], and Chakrabarti and coworkers, [Mandel, et al, 1988] and Andley and Clark, [1989] reported on the photoaggregation of $\gamma$-crystallin by UV, or by visible light in the presence of photosensitzers. This photochemical aggregation results in detectable populations of monomers, $\gamma$, dimers, $\gamma_2$, and larger, covalently-bound aggregates. This is in contrast to thermal aggregation which does not involve covalently bonded oligomers. More recently, in connection with his discovery of the molecular chaperone role of $\alpha$-crystallin, Horwitz [1992, 1993] documented the heat-induced aggregation of gamma. This was followed by Spector and coworkers' [Wang and Spector, 1994; Wang and Spector, 1995] report of aggregation of $\gamma$-crystallin by chemical oxidation. Thus, it is quite clear that $\gamma$-crystallin forms water-insoluble aggregates following various insults and that this aggregation may be related to lens opacification in vivo. To date there have been no measurements of the change in $T_c$ accompanying photo-oxidation of lens protein solutions. The question which we address in this part of the proposal is: Which of the photochemical products of $\gamma$-crystallin are most important in seeding protein aggregation and how does each affect the critical opacification temperature, $T_c$, of a $\gamma$-crystallin protein solution? An answer to this question could form a basis for rational anti-cataract drug design, eg, $T_c$ lowering materials, but such design is not included in the present project.
C. PRELIMINARY STUDIES AND PROGRESS REPORT (This report covers the period from May, 1994 to September, 1997.)

Summary of Specific Aims from the Grant of May, 1994

This project is based on the premise that the living human lens absorbs UV radiation from sunlight during all or most of a person's lifetime and that radiation causes photochemical changes in the structural proteins of the lens. These photochemically altered crystallins experience different interactions with surrounding water and protein molecules in the cytoplasm resulting in protein aggregation and insolubilization, the net effect of which is a partially or completely opaque lens. The experiments described in the proposal are designed to detect and characterize photochemical reactions of the crystallins separately from other possible UV effects on the lens and to assess the potential of the photoproducts to initiate protein aggregation. Our aims are:

To determine the chemical changes which occur in lens crystallin solutions exposed to monochromatic UV radiation in the 290-400 nm wavelength range for doses comparable to those received by the human lens under ambient conditions. Chemical changes which occur for low UV doses are of particular interest since damage to a small fraction of protein molecules in the living lens may initiate lens opacification. In our experiments, UV-induced changes in lens crystallins will be detected primarily by:

SDS-PAGE, size exclusion HPLC, and electrospray mass spectrometry.

Tryptic digestion followed by amino acid sequencing and FAB mass spectrometry of the tryptic peptides.

Fluorescence and fluorescence quenching analysis of UV irradiated crystallin solutions.

To determine which of the UV-damaged populations may be important in causing protein aggregation and lens opacity. Can photochemically modified small oligomers, eg, monomers or dimers of gamma crystallin, initiate protein aggregation, or are high polymers required? This information will be obtained primarily by:

Measurements of turbidity in solutions containing defined populations of photochemically modified monomers or dimers or higher polymers of lens crystallins, mixed with normal crystallins.

Measurements of the temperature dependence of turbidity in solutions of photochemically altered crystallin proteins.
Progress Report

1. Characterization of Photo-oxidative Damage in γ-Crystallin

In work completed during the last grant funding period, we investigated the photochemical opacification of the bovine lens crystallin proteins individually, and noted important differences between them [Hott and Borkman, 1993]. β-crystallin and γ-crystallin solutions became opaque when exposed to UV radiation, while α-crystallin solutions remained transparent. We have also made efforts to characterize the chemical nature of photo-oxidation in γ-crystallin at the tryptic peptide and individual amino acid levels [Hott and Borkman, 1992], analogous to the efforts of Dillon et al. [1987] to accomplish the same thing with α-crystallin. Both efforts have met with limited success, and both groups are now pursuing the use of mass spectrometry to elucidate the effects of photooxidation of α- or γ-crystallin at the peptide and amino acid levels.

We have found, using SDS-PAGE, that low UV doses suffice to produce measurable levels of gamma crystallin multimers such as γ*, γ-γ, and γ-γ-γ. γ* represents oxidatively-modified γ monomer. We have also detected polymers γₙ with n > 5 having molecular mass of about 100,000. The concentration of γ monomer decreased monotonically with photo-oxidation time. The level of dimer increased rapidly for short irradiation times, leveled off for intermediate times, and decreased gradually for long irradiation times. Thus, short irradiation times seem to favor dimers over higher polymers [Borkman and Knight, 1996].

Experiments with Dr. Kevin Schey at the Medical University of South Carolina are underway using ESIMS to characterize UV irradiated γₙ crystallin, and search for photo-dimers. We hope to obtain evidence for a dimer of molecular mass 2 x 20,000 = 40,000 daltons.

MALDI mass spectrometry is being used to analyze tryptic digest products of photo-oxidized γₙ crystallin. The UV dose was on the order of 20 J/cm² at a wavelength of 300 nm. In these experiments, we found evidence for photooxidation of methionine residues in the protein, as well as oxidation of tyrosine and tryptophan as reported at the 1996 ARVO meeting, Borkman & Knight [1996]. Some typical data on peptide mass changes following UV irradiation of calf γₙ crystallin in solution are shown in Table 1. One sees evidence for addition of either an oxygen atom, or a diatomic oxygen molecule in the first three lines of data, and evidence for a possible dimerization reaction in the fourth line of Table 1. Reported masses are ± 1 mass unit.
TABLE 1: MASS SPECTRAL UV CHANGES

The sequences of calf γ-II crystallin most affected by 300 nm UV

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<th>Sequence Numbers</th>
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<td>66-71</td>
<td>796</td>
<td>1593</td>
<td>797</td>
</tr>
</tbody>
</table>

Note: The first three lines of data correspond to O or O₂ addition; the last line is dimerization.

The question arises as to where in the peptide chain the oxidation occurs? The chart in Fig. 1 shows the segments of calf γ-II crystallin most affected by photooxidation, as detected by MALDI mass spectrometry of the tryptic digestate. All of the sequences which display marked

FIG. 1: MASS SPECTROMETRY RESULTS

Data show: Sequence of calf γ-II crystallin and segments most affected by UV photooxidation (underlined), 1 mg/ml, 25° C, 60 min at 300 nm.

1

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GKITFYEDRGFGQHCYECSSDCPNLQPYF</td>
</tr>
<tr>
<td>SRCNSIRVDSGCwmLYERPNYQGHQYFLRR</td>
</tr>
<tr>
<td>GDyPDyQOwmGFNDISRSCRIPQHTGEF</td>
</tr>
<tr>
<td>RmRIyERDDFRQGQMSIITDDCSLQDRFHLTEV</td>
</tr>
<tr>
<td>SLNVLEGSWVLYEMPSYRGRQYLLRPGEYRR</td>
</tr>
<tr>
<td>yLDwGAmNAKVGSLRRVmdF</td>
</tr>
</tbody>
</table>

Note: Oxidized sequences (underlined) contain Met, and several contain Trp or Tyr
changes (underlined segments in the chart) contain the amino acid methionine, and most contain tyrosine, and/or tryptophan. This is not surprising. In previous amino acid analysis of photo-oxidized gamma crystallin [Hott and Borkman, 1992] we found that methionine was most damaged, with 35% loss observed.

2. Molecular Chaperone Studies

Following the important work of Horwitz [1992; 1993] implicating α-crystallin as a molecular chaperone, we began a study of the possible protective effect of bovine α-crystallin in photo-oxidative opacification. Our important new finding, which has now been incorporated into a paper [Borkman, Knight, and Obi, 1996], is that addition of α-crystallin to γ-crystallin solutions prior to photo-oxidation afforded protection from opacification. Data are shown in Fig. 2 a,b. The opacity was greatly reduced when α-crystallin was present, as seen in Fig. 2a. The results in Fig. 2b indicate that at a α:γ ratio of 1:1 by weight most of the protection was achieved. This corresponds to a molar ratio of 1:40, i.e., one α-crystallin molecule (molecular weight of 800,000) protected 40 γ-crystallin molecules (molecular weight 20,000) from UV aggregation at room temperature. This result is similar to that found by Horwitz [1992; 1993] in thermal opacification experiments and suggests that each subunit (αA2, αB2 chains) in the α-crystallin

![Graph A](image1)

![Graph B](image2)

Fig. 2: Opacification of γ-crystallin solutions versus added α-crystallin. A.) Opacity versus UV dose for 0.5 mg/ml γ-crystallin with α-crystallin at concentrations of 0.0, 0.1, 0.25, 1.0, and 2.0 mg/ml. B.) Data replotted as opacity, after 15 min UV dose, versus added α-crystallin.
macromolecule can bind and protect one γ-crystallin molecule. Since the present experiments were done at room temperature, elevated temperatures were not needed to activate the chaperone function of α-crystallin, in contrast to the suggestion of Raman and Rao, [1994].

The kinetic data at 25° C indicated that an α:γ weight ratio of 1:1 afforded most of the available protection. We checked this stoichiometry using a molecular-weight-calibrated TSK-4000 HPLC column of dimensions 30 x 0.75 cm. The column was calibrated using blue dextran at 2000 kDa, thyroglobulin at 670 kDa, and gamma crystallin itself at 20 kDa. The column was used to detect the αγ40 complex formed when an alpha-gamma mixture (1.0 mg/ml each) was irradiated with UV for 30 min at 300 nm (7 mW/cm²). Samples were centrifuged at 26,000g for 10 min and filtered through 0.2 micron Acrodisc before injection into HPLC. The HPLC elution buffer was 0.1 M phosphate with 0.3 M NaCl and 1.0 mM azide added. The flow rate was 1.0 ml/min. When the irradiation was done at 25° C, we observed loss of both the α- and γ-crystallin components in HPLC, but we were able to detect very little of the expected αγn complex at this temperature. When the temperature was raised to 55° C, however, irradiation at 300 nm resulted in a complex which eluted at an apparent molecular weight of 1700 ± 200 kDa on the calibrated HPLC column—equal within experimental error to the predicted mass of the proposed αγ40 complex, i.e., 800 + 40x20 = 1600 kDa. Some illustrative data are shown in Fig. 3, where one sees the peaks corresponding to α- and γ-crystallin prior to irradiation and the peak due to the complex αγn after irradiation at 55° C. It should be mentioned that no complex could be detected in HPLC if the same alpha/gamma mixture was subjected to heat alone at 55° C. Thus, both radiation and heat were needed to form significant

![Graph](image_url)

**Fig. 3:** HPLC Data. The solid trace is an equimolar mixture of α- plus γ-crystallin at 55°C. The broken trace is the same mixture after irradiation at 300 nm.
amounts of HPLC-detectable chaperone complex [Knight and Borkman, 1996]. Our HPLC data provide support for the contention of Raman and Rao [1994] and of Das and Surewicz [1995] that α-crystallin chaperone function is enhanced at elevated temperatures. Our own conclusion at present is that even though α-crystallin protects γ-crystallin from UV opacification at 25°C (Fig. 3), the complex formed at this temperature is relatively weak and difficult to detect by HPLC. All of the results based on HPLC studies of the alpha-gamma chaperone complex are summarized in the manuscript by Borkman and Knight [1997] which is included in the Appendix. It should be noted, however, that size exclusion HPLC in the molecular weight range around 2 million is not very precise, and more work is needed to establish the molecular weight and stoichiometry of the αγ chaperone complex.

Protection from UV opacification by α-crystallin was observed by us for several other target proteins, including aldolase, carbonic anhydrase, and enolase. In each case, addition of stoichiometric amounts of α-crystallin to these solutions afforded significant protection against UV induced opacification. This is summarized in Borkman, Knight, and Obi [1996].

Irradiated solutions were analyzed by SDS-PAGE. γ-crystallin solutions were found to be highly crosslinked with the original 20 kDa material being converted to 40, 60 and > 100 kDa material. Solutions were centrifuged to separate any insoluble material formed by UV irradiation. The supernatant was found to be transparent following centrifugation. Thus, gamma crystallin opacification came from insolubilization, and added α-crystallin prevented this insolubilization.

Photolyzed α-crystallin solutions analyzed by SDS-PAGE showed that much of the 20 kDa subunit material was converted to higher polymers greater than 100 kDa. Hence, calf α-crystallin subunits were covalently aggregated by UV irradiation, but this did not result in insolubilization or solution opacity [Hott & Borkman, 1993], nor did it result in any significant loss of chaperone activity, except at very high UV doses, as discussed in the next paragraph.

To investigate the effect of irradiation on chaperone function, three kinds of α-crystallin samples were prepared, by adjusting the dose of radiation delivered: First, dark control (normal α); second, α which was photo-crosslinked to the 50% level as detected by loss of 20 kDa subunits in SDS-PAGE; third, α which was photo-crosslinked to the 90% level as judged by SDS-PAGE. Each of these was used in subsequent chaperone experiments to determine if that α-crystallin sample could protect target proteins (γ-crystallin, aldolase, alcohol dehydrogenase) from UV or thermal aggregation/opacification. The results are summarized in our publication [Borkman & McLaughlin, 1995]. The important conclusion is that photo-crosslinking of α-crystallin impaired its ability to function as a chaperone vis-à-vis both thermal and UV insults. Moderate damage seemed to leave the chaperone function of α-crystallin intact; only extensive damage rendered the chaperone inoperative [Borkman & McLaughlin, 1995]. The effect of crosslinking which occurs with aging of α-crystallin was investigated by Sharma & Ortwerth [1995]. These authors found that crosslinked material lost a significant fraction of its chaperone activity relative to normal α-crystallin.
3. Preliminary Studies of Fluorescent Probe Binding to α-Crystallin and Substrates

The cysteine residues of α-crystallin were labeled with the fluorescent probe FM (fluorescein -5-maleimide) and the cysteine residues of γ-crystallin were labeled with CPM (7-diethylamino-3-[(4'-maleimidylyphenyl)-4-methylcoumarin). Both probes were obtained from Molecular Probes (Eugene, Oregon). Alpha crystallin at 5.0 mg/ml in 0.1 M phosphate buffer at pH 7.4 was reacted with a 10 fold excess of FM (FM stock solution 1.17 x 10^{-2} M also in phosphate buffer) at 50°C overnight. Unreacted FM was quenched with 5 fold excess β-mercaptoethanol and removed by dialysis. A similar protocol was used for labeling gamma with CPM (stock solution 2.48 x 10^{-2} M in dimethyl formamide). The protein concentration and degree of labeling for γ-CPM and α-FM were determined by measuring absorbances at 280 and 383 nm or 280 and 495 nm, respectively. The molar extinction coefficients (cm^{-1} M^{-1}) used were:

\[
\begin{align*}
\gamma(280 \text{ nm}) & = 42,000 \\
\text{CPM}(280 \text{ nm}) & = 6,300 \\
\text{CPM}(383 \text{ nm}) & = 37,000 \\
\alpha(280 \text{ nm}) & = 17,000 \\
\text{FM}(280 \text{ nm}) & = 16,500 \\
\text{FM}(495 \text{ nm}) & = 66,000
\end{align*}
\]

The typical degree of labeling was about 1 CPM per γ molecule and 1 FM per two α subunits. This is consistent with the fact that gamma crystallins have 5-7 cysteines while αA, which comprises about 75% of the alpha, is the only alpha subunit with a cysteine and it has only one.

Glutathione reductase has ten Cys residues [Krauth-Siegel, et al., 1982; Pai and Schulz, 1983] and we propose to incorporate the CPM label following the same protocol used for labeling of γ-crystallin. Some of the Cys residues in glutathione reductase occur at the enzyme active site. Since our experiments do not assay enzyme activity, only protein aggregation/opacification, the fact that some labels may attach at the active site, hindering enzymatic activity, is not a problem.

The FM and CPM probes were chosen because when they are brought into close contact (distances less than about 50 Å) energy transfer from CPM to FM occurs. Thus, in a dilute unreacted mixture of α-FM and γ-CPM, containing no chaperone complex, the two probes are far apart on the average and the fluorescence is the sum of the two independent probes, i.e., there is fluorescence at 480 nm coming from CPM and at 520 nm coming from FM. But, when chaperone complex, αγn, forms, the FM and CPM probes are brought into close proximity and energy transfer occurs. Under these conditions, one sees fluorescence from FM (hv' = 520 nm) even when exciting at the absorption wavelength of CPM (hv = 383 nm), according to:

\[
\begin{align*}
\text{CPM} + \text{hv} & \rightarrow \text{CPM}^* \\
\text{CPM}^* + \text{FM} & \rightarrow \text{CPM} + \text{FM}^* \\
\text{FM}^* & \rightarrow \text{FM} + \text{hv}'
\end{align*}
\]

Thus, the observation of enhanced FM fluorescence (and loss of CPM fluorescence) when the sample is excited at the CPM absorbance wavelength gives direct evidence for energy transfer which requires that γ-CPM and α-FM be in close proximity, i.e., that the αγn complex has formed.
Some typical data are shown in Fig. 4. A 1.0 ml sample of α-FM and γCPM (both 0.5 mg/ml) was placed in a thermally jacketed quartz cell and irradiated at 55°C for 30 min. The light source was an Oriel 350 W mercury vapor lamp fitted with a Spex 1681 B monochromator. The flux at 300 nm as measured by a Scientech 365 power meter was 7 mW/cm². The temperature was controlled to ± 0.5°C with a Neslab RTE-100LP circulator bath. The sample was allowed to cool to room temperature before fluorescence measurements were taken. An Oriel 1000 W xenon arc lamp and a Spex 1680 monochromator were used in the fluorescence apparatus. One sees growth of the fluorescence feature at 520 nm as chaperone complex formation proceeds. In addition, one sees a wavelength shift of the CPM emission from 480 nm to about 460 nm as a result of a change in the local environment of the CPM probe when γ-CPM binds to α-FM. During the next project period we plan to monitor the rate of growth of 520 nm fluorescence in mixtures of α-FM and γ-CPM exposed to photooxidizing conditions at various temperatures, and to use this data as a measure of the rate and extent (stoichiometry) of chaperone complex formation under various conditions. Similarly, the dissociation of previously formed αγₙ complex will be monitored by observing the reverse, i.e., loss of FM emission at 520 nm following decomposition of the complex. Finally, these same techniques can be used to obtain information relevant to the stoichiometry of the complex. These proposed experiments are described in more detail in the next sections.

Fig. 4: Fluorescence of labeled crystallins and chaperone complex. The solid curve is the fluorescence of an equimolar mixture (0.5 mg/ml each) of labeled α-FM-crystallin and γ-CPM-crystallin before UV exposure. The broken curve is the same mixture after UV irradiation at 300 nm and 55°C for 30 min. The fluorescence excitation wavelength was 395 nm in both spectra. The data were obtained by routing the fluorimeter output to a PC-type computer; see text.
D. RESEARCH DESIGN AND METHODS

1. Oxidative Aggregation of Protein Solutions

   a. Small γ-Crystallin Aggregates

   It is very clear from SDS-PAGE data reported by our lab [Li, et al., 1990] and others
   [Andley and Clark, 1989] that photooxidation of intact lenses or individual crystallins in solution
   results in formation of covalently crosslinked aggregates. Thus, one can detect monomers and
   small oligomers like γ, γ-γ, γ-γ-γ, as well as larger species γn, following irradiation [Borkman &
   Knight, ARVO, 1996]. We use γ-crystallin as a prototype for our photocrosslinking studies
   since such multimers have already been reported. Perhaps surprisingly, the crosslink(s) in γ-
   crystallin multimers do not appear to be due to disulfide bridges since these links were not
   cleaved when treated with mercaptoethanol in past experiments [Li, et al., 1990; Mandal, et al.,
   1988].

Experimental Details for Irradiation of γ-Crystallin or Glutathione Reductase

   Calf gamma crystallins will be prepared and purified as described previously [Tallmadge &
   Borkman, 1990]. Glutathione reductase will be purchased from Sigma, Inc. Solutions of 1.0 -
   10.0 mg/ml will be prepared in aqueous phosphate buffer at pH 7.4, and 1.0 ml samples will be
   irradiated in quartz cuvettes by a mercury arc lamp and monochromator combination at
   wavelengths near 300 nm and temperatures in the range 20 - 60° C. Although relatively little 300
   nm radiation is present in sunlight, and only a small fraction of this reaches the lens, nonetheless, it
   has been shown in the literature that the UVB component which we propose to use is one
   thousand times more effective in causing lens damage than UVA radiation [Pitts, 1974; Andley, et
   al., 1994]. The beam which impinges on the sample in our experiments has a flux of about 100
   W/m², and thus, irradiations of 1-30 minutes correspond to doses of about 1-20 J/cm², which is
   equivalent to about five hundred to several thousand hours of UV exposure from direct sunlight,
   ie, about one month of continuous exposure. Of course, humans are not exposed to sun
   continuously for 24 hours per day for a month, so that the proposed radiation levels would more
   likely correspond to a period of years rather than months. Thus, the proposed UV doses in our
   laboratory experiments are about equivalent to those received by humans, cumulatively, over a
   period of several years. The beam output is measured with a Scientech model 365 Radiant Flux
   Meter. Irradiated samples will be analyzed by SDS-PAGE to assess the degree of covalent
   crosslinking produced by photooxidation. SDS-PAGE will be done using Bio-Rad's Mini-
   Protean II dual slab gel cell apparatus. The gels are to be made of 15% polyacrylamide and an
   SDS content of 0.1%. The gels are run at 200 volts and stained with Coomassie Blue. The
   sample buffer is: 50% water, 12.5% 0.5 M Tris (pH 6.8), 10% glycerol, 20% of a 10% (wt/vol)
   solution of SDS, and 2.5% of a 0.05% (wt/vol) solution of bromophenol blue. The protein
   samples to be analyzed are first diluted 1:4 with sample buffer prior to application to the gel.
   Some samples will be pre-treated with mercaptoethanol to determine the amount of crosslinking
   attributable to disulfide bonds. Previous experiments [Li, et al., 1990] have indicated that less
   than 10% of the uv-induced crosslinks can be cleaved by mercaptoethanol.
b. \( T_c \) Measurements for \( \gamma \)-Crystallin Aggregates

Proteins in the living lens which have been altered by heat, oxidation, etc, may experience different interactions with each other and with water in the cytoplasm, resulting in a change in the opacification temperature for the lens. The critical opacification temperature, \( T_c \), is given by [Clark, et al., 1996]:

\[
\text{kT}_c = A [E_{pw} - (E_{ww} + E_{pp})/2].
\]

\( T_c \) depends on the interaction energies between protein and water, \( E_{pw} \), and between protein and protein, \( E_{pp} \), which are expected to change dramatically for protein dimers or trimers compared to the normal monomers present prior to oxidation. The dependence of \( T_c \) on water-water interaction, \( E_{ww} \), is expected to be independent of protein oxidation. Our aim is to detect changes in \( T_c \) of gamma crystallin solutions subjected to photo-oxidizing conditions. The gamma crystallins as a group have the highest \( T_c \) values of any of the crystallins, and hence they are expected to be the most important in determining overall lens opacity in the intact organ.

As a benchmark, we note that Pande, et al. [1996] reported that a solution of gamma crystallin containing 30% gamma dimers and 70% monomers had a measured \( T_c \) which was 35° C higher than a normal, 100% monomer solution. Thus, it appears that even relatively low concentrations of polymeric oxidation products in solution (perhaps just a few percent) can have a readily measurable effect on the solution \( T_c \) (an increase of several degrees).

We propose to produce solutions of small oligomers of gamma crystallin of the type \( \gamma \), \( \gamma^* \), \( \gamma-\gamma \), \( \gamma-\gamma^* \), \( \gamma^*-\gamma \), etc, by photochemical irradiation of gamma crystallin solutions, and to measure the change in \( T_c \) value for such solutions compared to dark controls. The symbol \( \gamma^* \) refers to a gamma crystallin molecule which has been photochemically altered, eg, by oxidizing one or more amino acid residues or by making an intra-molecular crosslink. We will use SDS-PAGE and/or size exclusion HPLC to characterize the composition of photooxidized solutions in terms of monomer, dimer, etc, content. For initial experiments, we propose to measure the \( T_c \) of the total photooxidized solution, to obtain an estimate of the magnitude of the \( T_c \) elevation. Subsequent experiments involve separation of the various polymer fractions so as to assess the importance of each in raising the \( T_c \) of the solution.

The choice of which fraction of gamma crystallin to use in the \( T_c \) measurements is important. Siezen et al [1985] measured \( T_c \) values for the fractions gamma II, III, and IV as well as a natural abundance mixture. There were substantial differences in \( T_c \) for each fraction, and all of the \( T_c \) values increased with protein concentration. The gamma-IV population had, by far, the highest \( T_c \) measured. Siezen et al [1985] reported a value of \( T_c = 18^\circ \) C at a concentration of 20 mg/ml, whereas the natural abundance mixture had \( T_c = 0^\circ \) C at a concentration of 20 mg/ml. Since it will be difficult for us to achieve concentrations of photooxidized protein solutions greater than about 10-20 mg/ml, and since we prefer to measure \( T_c \) values in the range above 0° C, we must select the gamma fraction with these ideas in mind. We would prefer to work with a natural abundance mixture of gamma crystallin, but if this proves difficult because of the restricted
Tc range cited above, we will do the experiments using the higher Tc gamma-IV fraction.

After measurements of Tc for solutions containing all of the photooxidation products, we will separate the polymeric components according to molecular weight and then repeat the Tc measurements. In this case, we would isolate: A monomer fraction containing normal, unreacted γ monomers, plus various forms of γ*; A dimer fraction with γ-γ plus various forms of γ-γ*. We only seek to distinguish the Tc values of monomers versus dimers versus trimers, without regard to internal changes, and thus no attempt will be made to separate γ-γ* from γ-γ type dimers. Internal changes may be important in a detailed analysis, but they are not likely to be as important in determining Tc as oligomer size.

Overall, we expect these studies to demonstrate that a relatively mild exposure to photooxidative conditions (comparable to typical human sunlight exposure over a period of several years) has the potential to generate small oligomers which can "seed" solution opacification. The implication for the whole lens is that once the Tc (cold cataract temperature) of the lens reaches that of the in vivo environment, a permanent cataract is present. To further estimate the relevance of the solution experiments for whole lenses, we propose parallel experiments on total lens soluble protein (TSP). Thus, we will prepare solutions of TSP and expose to the same photooxidizing conditions used for gamma crystallin solutions. The composition of the photooxidized mixture will be assessed using SDS-PAGE and size exclusion HPLC, and then the Tc of the solution will be measured and compared with dark control. Since the alpha crystallin component in such a mixture may exert a protective effect on the beta and gamma crystallin components in the mixture, through the chaperone effect [Wang & Spector, 1995; Borkman et al, 1996; Raman & Rao, 1994], such mixed solutions may display less rise in Tc than the gamma solutions. Such experiments lead naturally into the next phase of our project, the characterization of α-crystallin chaperone complexes.

Experimental Details

Size exclusion chromatography on Sephacryl S-100-HR polymer from Sigma, Inc., will be used to analyze and preparatively separate the various polymer fractions in a photooxidized solution of gamma crystallin. A typical elution buffer will be 0.05 M tris, and 1.0 mM azide, at pH 7.2. A column loading of 10 mg should suffice. Critical temperatures, Tc, for normal and photooxidized crystallin solutions will be determined by measuring the increase in absorbance (light scattering) at 360 nm as a function of temperature. Solutions of protein concentration in the range of 10-20 mg/ml are used for the Tc measurements, as suggested by Siezen et al [1985]. The solution to be studied, about 0.4 ml, is placed in a jacketed 1 cm pathlength quartz cell. The cell jacket is connected to a Neslab RTE-100LP temperature controller-circulator and placed in a Milton Roy 601 spectrophotometer. A PC computer with an analog-to-digital conversion board is interfaced to record the absorbance from the spectrophotometer and the temperature from the analog output of the circulating temperature bath. Thus, the computer stores and displays a plot of absorbance versus temperature. Resolution on the absorbance scale is about 0.001 units, and
resolution on the temperature scale is 0.05°C. During a run, the temperature is ramped through decreasing temperatures at a typical rate of 0.10 – 0.25°C/min over the range from 20 to 1°C. A stream of dry nitrogen is passed over the spectrophotometer cell to prevent water condensation on the cell at lower temperatures. The data is presented as a computer generated plot of absorbance versus temperature, and the Tc value is identified as the temperature at which the solution transmittance falls to 90% of its original value [Siezen, et al, 1985]. Parallel experiments are carried out for dark control and photooxidized γ-crystallin solutions and solutions of total lens soluble protein.

In some cases the photooxidized solution will be fractionated prior to measuring Tc. Since relatively high protein concentrations (10-20 mg/ml) are needed for Tc measurements, doing the separations by preparative column chromatography as described above, will result in a large dilution of the samples and the need to reconcentrate them. To avoid this difficulty, we will also try to separate the monomers from the dimers, trimers, and higher molecular weight polymers by dialysis. That is, using a suitable dialysis membrane, we will dialyze away the monomer fraction of the photooxidized solution leaving the original concentrations of dimers, trimers, etc. Fisher Scientific has dialysis tubing which may be suitable: Spectra/Por 6 can be ordered with cutoffs of 25 kDa or 50 kDa (Spectrum #132550 and 132540). This would allow the monomer (20 kDa), and then the dimer (40 kDa), respectively, to be fractionated from the photooxidized mixture. The Tc of these fractionated solutions will then be measured.

2. Characterization of the Chaperone Complexes αS_n

    a. Stability and Stoichiometry of αγ_n

As reported in our Progress Report and Preliminary Studies, we have estimated the stoichiometry of the αγ_n chaperone complex in two ways: First using the kinetics of complex formation (Fig. 2 and text) and second, by estimating its molecular weight from the observed retention volume on a calibrated HPLC column (data of Fig. 3). Both methods are approximate but lead to an estimated molar ratio of γ:α = 40:1. The stoichiometry of the chaperone complex of glutathione reductase (gr) with α-crystallin was estimated by Blaktytny and Harding [1997] to be about 1:1 by weight, corresponding to a molar ratio of gr:α = 8:1.

We can use our proposed fluorescent labeling/energy transfer method to determine the stoichiometry of the complexes as follows. Starting with a fixed concentration of fluorescein labeled α-crystallin (α-FM), say 0.5 mg/ml, we will add coumarin labeled γ-crystallin (γ-CPM) or coumarin labeled glutathione reductase, say 0.3 mg/ml. This mixture is then divided into thirds. One third is heated to 70°C, one third is irradiated with UV, and the final third is retained as a no-heat, no-UV control. After heat/UV the fluorescence of the three solutions is measured with 395 nm excitation. The fluorescence intensity at 520 nm due to energy transfer from γ-CPM to α-FM should increase in the heated and UV irradiated solutions but should remain constant in the control solution. The experiment is then repeated for increased concentrations of added γ-CPM of 0.4, 0.5, 0.6, ..., 1.0 mg/ml. The composite emission spectrum (sum of α-FM plus γ-CPM) will continuously change as increasing amounts of γ-CPM are added to the existing α-FM
solution. (A detailed analysis of the expected changes with concentration of an energy transfer pair has been provided by Saito, et al. [1981], and we have used these methods previously to analyze energy transfer in gamma-II crystallin, [Borkman and Phillips, 1985].) When the α-FM chaperone is saturated with denatured γ-CPM, the growth of α-FM fluorescence will level off and remain constant with further addition of γ-CPM. At this point the fluorescence from γ-CPM at 480 nm will begin to grow due to the accumulation of free γ-crystallin in the solution. The concentration of added γ-CPM where these two end points occur corresponds to the saturation stoichiometry of the αγn complex. To define this stoichiometry more precisely, we can repeat the experiments with a finer grid of γ-CPM concentrations, eg, steps of 0.05 mg/ml. The experiment yields the number of γ-crystallins bound to α-crystallin per concentration of γ-crystallin added. The procedure is parallel for CPM labeled glutathione reductase. Experiments will be done to ensure that the FM and CPM labels in α- and γ-crystallin and glutathione reductase do not interfere with the normal aggregation of denatured γ-crystallin or its binding to α-crystallin. This will be done by repeating experiments of the type summarized in Figs. 2 and 3 using labeled α- and γ-crystallin. We do not anticipate a problem here, because, although the labels are known to bind to the Cys groups of the proteins, we and others have previously shown that photochemical aggregation of γ-crystallin does not involve disulfide crosslinks [Li et al., 1990; Mandal, et al., 1988].

Another approach to determining the stoichiometry of αSₙ complexes is to prepare the αSₙ complex, and collect it using preparative HPLC. The properties of the collected complex will then be investigated by decomposing it into its component parts and determining the composition. Thus, the αSₙ complex will be subjected to various conditions of dilution, temperature, and pH in order to cause decomposition, and the products of decomposition will subsequently be analyzed by HPLC, to determine the molar ratio α/S in the complex. This

\[ αSₙ \rightarrow α + nS \]

analysis can be done by HPLC as stated (basically the reverse of the complex formation experiment in Fig. 3), or by taking advantage of the fluorescent probe method. That is, if the αSₙ complex is prepared with the fluorescent-probe labeled species α-FM and γ-CPM (or gr-CPM), the decomposition of the complex can be monitored by observing the change in fluorescence properties of the solution. This experiment represents the reverse of the complex forming reaction monitored in Fig. 4. That is, as the complex decomposes, emission from the energy acceptor α-FM will fall, and this information can be used to assess the amounts of decomposition products and hence the stoichiometry of the original complex. The stability toward thermal decomposition of αSₙ can also be determined in this way.
b. Proposed Experiments on Kinetics of $\alpha S_n$ Formation

It is perhaps surprising that to date no studies of the kinetics of $\alpha S_n$ complex formation have been reported. Such rates have been measured and reported in the literature for other chaperones [Farr, et al, 1995], but there are no measurements of the rate constants, $k_x$, associated with $\alpha$-crystallin chaperone complex forming reactions

$$ S + \alpha S_x \longrightarrow \alpha S_{x+1}, \quad k_x, \quad E_x $$

Nor are there measurements of the temperature dependence of the rate constants for these reactions. Such information can be used to calculate activation energies, $E_x$, for binding of the target protein ($S = \gamma$ or $\sigma$) by the chaperone ($\alpha$). Questions to be answered are: What is the effect of the size of $S$ on the binding rate constant and activation energy, and does the binding occur in a concerted or stepwise mechanism? Another question of considerable interest is: Does binding of a first or second or third substrate molecule by the $\alpha$-crystallin chaperone facilitate binding of subsequent substrate molecules, i.e., is there an allosteric effect (positive cooperative effect)? Again, evidence for an allosteric effect in chaperone binding reactions has been obtained for other chaperone systems [Yifrach & Horovitz, 1995]. The fact that we and others have seen relatively sharp HPLC peaks associated with $\alpha$-crystallin chaperone complexes like $\alpha \gamma_n$ suggests that there may be positive cooperativity in substrate binding by the $\alpha$-crystallin chaperone. That is, if the binding affinity was the same for each substrate molecule (all $k_x$ equal), we would expect to see a wider distribution of molecular weights than typically observed in HPLC [Wang & Spector, 1994; 1995; Knight & Borkman, 1996; 1997]. We would expect to see $\alpha \gamma_n$ varying over a wide size range of say $n = 10 - 40$, instead of the apparently narrow range observed. This observation could be explained if the binding propensity of the chaperone increased as more and more substrates are bound. In this case, we would tend to see each alpha particle "fill up" with a full complement of substrate, and hence a corresponding narrow range of $n$. This corresponds to the rate constants, $k_n$, for the above reactions increasing with the value of $x$ such that, for example, $k_{10} \ll k_{20}$. This would mean that if the $k_{10}$ and $k_{20}$ reactions were competing for a substrate, the bigger complex would win. This favors formation of larger complexes over smaller ones and results in a narrow size distribution. We propose to measure the rate constants, $k_x$, the activation energies, $E_x$, and to test for possible cooperativity in substrate binding by the $\alpha$-crystallin chaperone using the energy-transfer-fluorescent-probe methodology which we have developed.

To see how we propose to accomplish these aims, recall from Fig. 4 that when labeled chaperone $\alpha$-FM and labeled substrate (e.g., $\gamma$-CPM) form a complex, the progress of the reaction can be monitored by observing the growth of emission of the FM fluorescence acceptor $I_A$ at 520 nm. As seen in Fig. 4, the 520 nm FM fluorescence occurs superimposed on the broad CPM fluorescence which extends from about 450 nm to about 550 nm. To analyze the growth of FM fluorescence at 520 nm, it will be necessary to draw a baseline from about 500 nm to about 550 nm, representing the CPM background emission, and then measure the intensity at 520 nm relative to this baseline. The hypothetical data in Fig. 5 indicate, schematically, the expected form of fluorescence growth as a function of added insult. One sees that the intensity of 520 nm
emission, \( I_A \), is expected to increase until all of the labeled \( \alpha \)-crystallins are saturated with labeled substrate, \( \gamma \)-CPM, at which point the emission intensity levels off to a constant value as shown. This kind of plot can be used to determine the stoichiometry of the complex. But, such a plot does not yield the actual rate of substrate binding by the chaperone but rather reflects only the rate of input of the insult in the form of added heat or photo-oxidation. To measure the actual rates of the binding reactions, it is necessary to apply the insult over a relatively short period of time and then monitor the growth of the \( I_A \) emission following the insult. In our proposed experiments, the insult will be photooxidation, and we will monitor the rate of \( I_A \) emission growth following a brief period of UV exposure. (It would be possible to do experiments where heat was added in a pulsed fashion by a temperature jump method or a pulsed laser heating method, but we do not pursue these experiments in the present proposal which focusses on photooxidation.)

Fig. 5: Hypothetical emission intensity at 520 nm from \( \alpha \)-FM energy acceptor versus UV dose. Upper curve, continuous irradiation, lower curve, pulsed irradiation of a mixture of \( \alpha \)-FM-crystallin and \( \gamma \)-CPM-crystallin in solution photolyzed at 300 nm, with fluorescence excitation at 395 nm.

In this way, we can also study the effect of temperature as an external variable simply by performing the photo-oxidation experiments at various solution temperatures so as to obtain activation energies for the binding reactions. To proceed, we consider the data in Fig. 5 and note that if the UV insult were to be applied as a series of pulses instead of in a continuous fashion, the
plot would look like a stairway with a rapid increase in $I_A$ occurring after each pulse and then leveling off until the next pulse, and so forth. The actual rate of the substrate binding reaction is just the rate of growth of $I_A$ after each pulse. Note that this rate can be studied as a function of solution temperature so as to obtain activation energy. Also note that as more pulses of UV are added, the chaperone complex becomes more and more saturated, and hence as we proceed up the stairway, the $k_x$ values measured correspond to successively higher $x$ values and any cooperativity will be revealed by increased values of $k_x$ as $x$ increases. The early pulses measure the rate constants for binding of the first few substrates molecules, $k_1 - k_{10}$, etc, and the later pulses measure the rate constants for the nearly saturated complex, $k_{30} - k_{40}$. As we have said, these rates may be the same or they may increase or decrease with $x$ if positive or negative cooperativity is involved. We can adjust the duration and intensity of the UV pulses so as to determine how much reaction occurs after each. In this way we adjust the number of "steps" in the stairway (resolution of the parameter $x$ in the rate constants $k_x$). We can also adjust the rate of reaction following a pulse, by altering temperature and reactant concentration, so as to cause the reaction rate to occur on a more convenient time scale for measurement. Thus, the proposed rate measurements will not require the fast timing techniques of flash photolysis. Under appropriate conditions the "pulses" can be nothing more than brief (few minutes) exposures to a conventional mercury arc light source, and the change in the fluorescence spectrum following each "pulse" can be detected with a rapid scan fluorimeter interfaced to a computer as described in the Experimental Details.

An important consideration in the planning of the kinetic experiments is the expected rate of the chaperone-substrate binding reaction since this will determine the type of apparatus needed to measure the rate constants. We consider two extreme case scenarios. First, Witt and coworkers [Farr, et al, 1995], found the second order rate constant for the reaction

$$\text{Cro}^* + \text{Dnak} \rightarrow \text{Chaperone Complex}$$

to be in the range 8-200 $\text{M}^{-1}\text{sec}^{-1}$ for temperatures from 15-37°C, corresponding to an activation barrier for binding of 26 kcal/mole. This very slow rate permitted these workers to easily measure the reaction rate in a conventional fluorimeter. Clearly, by lowering the temperature even further, the reaction rate would be further slowed. At the other extreme, the fastest possible reaction rate between substrate and chaperone in aqueous solution corresponds to a diffusion controlled reaction with zero activation energy and a second order rate constant of about $1 \times 10^8 \text{M}^{-1}\text{sec}^{-1}$ at 25°C. Even with this very large, hypothetical rate constant, which is $10^2$-$10^8$ times faster than observed for the Dnak chaperone, the rate of substrate/chaperone binding reaction would only be about $5 \times 10^4 \text{M sec}^{-1}$, for $\alpha$- and substrate concentrations of 0.1 mg/ml, and the reaction half-life under these conditions can be estimated to be 0.1 millisecond. This is a bit too fast to measure with a fluorimeter linked to a computer as we propose, but it is very unlikely that the rate would indeed be this fast. An activation energy of 6 kcal/mole for the substrate binding reaction of $\alpha$-crystallin (only one-fifth of that measured by Farr et al. [1995] for the Dnak chaperone) would reduce the rate constant to about $1 \times 10^5 \text{M}^1\text{sec}^{-1}$ and the reaction half-life would increase to 1 second at 25°C, putting the experimental rate within range of our proposed apparatus, since the time needed to perform a wavelength scan in a modern fluorimeter is on
the order of seconds. At all events, the measurements could certainly be done with a pulsed UV laser excitation source such as the excimer laser in our laboratory which emits at 308 nm and a computer interfaced fluorimeter. But, it seems unlikely that these extreme conditions will prevail and we should be able to make the kinetic rate measurements in the fashion of Witt and co-workers [Farr, et al, 1995]. We will compare our data on rate constants and activation energies with those obtained by Witt for the Dnak chaperone.

c. Effects of Substrate Size on Kinetics of α-Crystallin Chaperone Binding

Two substrates, γ-crystallin (20 kDa) and glutathione reductase (100 kDa) will be used initially to probe the effect of substrate size on the kinetics of binding to α-crystallin chaperone. Other substrates could be added later, particularly if we encounter technical problems with γ-crystallin or glutathione reductase. A possible substitute for glutathione reductase would be β-galactosidase, molecular weight 116 kDa [Fowler and Zabin, 1970]. The requirements for such substrates are that they have available Cys residues, as binding sites for attachment of the fluorescent probes, and that they be bound by α-crystallin. Glutathione reductase has 10 Cys residues [Krauth-Siegel, et al., 1982], and β-galactosidase has 17 Cys residues [Fowler and Zabin, 1970]; thus, either is acceptable for labeling purposes. The α-crystallin chaperone has shown itself to be quite promiscuous in binding an assortment of substrates, including: γ- and β-crystallin, glutathione reductase, alcohol dehydrogenase, enolase, aldolase, carbonic anhydrase, insulin, and others.

Why might substrate size be important in the kinetics of binding to α-crystallin? Various scenarios are possible. An obvious case is the situation where binding occurs in a cavity or cleft in the α-crystallin macromolecule. If the cavity were small, larger substrates might not fit so easily as smaller substrates and the rates of the former would be slower than for the latter. Also, kinetic activation energies would be larger for large substrates than for small substrates. On the other hand, if substrate binding were to occur on the surface of α-crystallin, one would not expect such a pronounced size effect, and the activation energies for substrate binding might be more nearly equal. These considerations can also be related to possible binding mechanisms as discussed in the next section. Binding in a cleft or cavity would be more consistent with a stepwise mechanism where the role of the pre-activation step (and associated activation energy) could be to open or enlarge the cavity opening so as to accommodate the substrate. This would not be necessary for surface binding, and hence surface binding might more likely proceed by a concerted mechanism with a lower activation energy. Unfortunately, the three dimensional structure of α-crystallin is not available (although some model structures have been proposed) to aid us in constructing rational molecular models for substrate binding. Nonetheless, we suggest that the simple experiments proposed here, comparing binding rates and activation energies for smaller versus larger substrate proteins, will provide useful information and ideas for future experiments on substrate binding to the α-crystallin chaperone. No such kinetic experiments have been reported to date although α-crystallin has been shown to be effective in protecting a number of substrates of varying sizes from heat, oxidative damage, or glycation damage.
d. Distinguishing Stepwise vs Concerted Mechanisms of Chaperone Binding

The stepwise and concerted binding mechanisms were outlined in the Background and Significance section at the beginning of the proposal. The stepwise mechanism invokes a first step in which α-crystallin chaperone, by itself, is pre-activated by heat so as to prepare it for the subsequent step of binding the denatured substrate protein. If the α-crystallin has been pre-activated in this way, it is reasonable to assume that the actual binding step might proceed with little or no additional activation energy. In a concerted mechanism, binding and substrate rearrangement occur simultaneously in a single reaction step. For many chemical reactions this is the favored reaction path because the simultaneous forming of new bonds with the breaking of old bonds results in a lower activation energy than in a stepwise process.

How can the two mechanisms be distinguished from each other experimentally? The key is that pre-activation of α-crystallin can be done by heating a solution of α alone, in the absence of any substrate, and this should enhance the subsequent reaction rate. That is, samples of α-crystallin will be pre-heated to various temperatures T1 in the range 35-80° C and then mixed with substrate and irradiated with UV at various temperatures, T2, usually in the 5-55° C range. As described below in the section on Experimental Details, the outcome of such a kinetics experiment will be the value of a chaperone binding rate constant k(T1, T2) obtained at pre-temperature T1, and reaction temperature T2. Rates will be measured for various values of T1, for a fixed value of T2, and a plot of log(k) vs 1/T1 will yield the activation energy E_a1 associated with the pre-activation step. Similarly, plotting log(k) vs 1/T2 with T1 held fixed, will yield the activation energy E_a2 for the substrate binding step. An observation of E_a1 > E_a2 ≈ 0 would be consistent with a stepwise reaction mechanism with a significant pre-activation step. An observation of 0 = E_a1 < E_a2 would suggest that pre-activation was unimportant and that the mechanism is concerted. If E_a1 ≈ E_a2 this is indicative of a stepwise mechanism and that the same chaperone activation is occurring before and after mixing of chaperone and substrate but that there is little or no activation energy associated with the actual substrate binding step. To summarize: A stepwise mechanism is supported by: i.) Pre-heating α alone increases the subsequent rate of S binding. ii.) After pre-heating α, the binding reaction (step 2) has a low or zero activation energy. iii.) Heating α and S together produces the same apparent activation energy as pre-heating α alone. A concerted mechanism is supported by: i.) Pre-heating α has little or no effect on the subsequent binding reaction rate and the activation energy for the latter is large. ii.) Heating α and S together increases the binding rate, but heating α alone does not.

Experimental Details

Labeled crystallins α-FM, γ-CPM, and gr-CPM (or other Cys containing protein) will be prepared as described above in the Progress Report and mixed together in buffer solution at the concentration desired for the experiment (somewhere in the range 0.05-1.0 mg/ml) and stored at 4° C in the dark. (For some experiments, the α-FM will have already been pre-activated by heat treatment as described in Section d. above.) A 1.0 ml aliquot of the chaperone-plus-substrate solution will be placed in a quartz fluorescence cell and brought to the temperature of the
particular experiment (range of 5-55°C), using a circulating water bath jacket around the fluorescence cell. We have previously verified that temperatures of 55°C and below do not result in chaperone complex formation with γ-crystallin, except in the presence of UV radiation. The UV radiation at 300 nm will then be turned on and the sample fluorescence monitored at the FM emission wavelength of 520 nm while exciting at the CPM absorption wavelength of 395 nm. Thus there are three radiation wavelengths involved in the experiment: A UV photolysis "pulse" at about 300 nm is input periodically, a 395 nm fluorescence excitation beam is input continuously, and the fluorescence emission spectrum is recorded in rapid scan mode after each UV pulse. The fluorescence excitation and emission are intrinsic to the fluorimeter, while the UV photolysis pulse is input from an external source. This is provided either by a mercury arc/monochromator or by an excimer laser operated with XeCl as the lasing gas and emitting 10 nsec pulses at 308 nm [Li & Borkman, 1990]. The fluorimeter's emission intensity and wavelength outputs are stored in a PC-type computer. The time resolution of this arrangement is about 1-10 milliseconds depending on the sensitivity scale chosen. Preliminary experiments will be used to determine the appropriate conditions of: reactant concentrations, solution temperature, and UV flux so as to produce a convenient reaction rate as judged by the rate of change in the rapid scan fluorescence spectrum. If the reaction rate is too fast, the UV flux, reactant concentration, and/or solution temperature will be reduced so as to sufficiently reduce the reaction rate to a range for convenient measurement. In these experiments we must always verify that the reaction continues on at a measurable rate after the UV "pulse" has ended so as to ensure that we are measuring the actual rate of the binding reaction and not merely the rate of UV input. Three possible cases are depicted in the hypothetical data of Fig. 6. The upper trace shows a case where the reaction has already stopped at the time the UV pulse ends, and this trace therefore yields no useful data on chaperone binding rates. The middle trace shows a case where the reaction continues on after the UV pulse has terminated; this data can be used to extract a binding rate constant. The lower trace shows a case where the reaction is too slow to measure on this time scale.

It is anticipated that most measurements can be made with the mercury arc lamp as source. If unexpectedly fast reaction rates occur, we will use our excimer laser as the UV source. The fluorimeter, which has a response time of 1 millisecond should still be fast enough for detection of the fluorescence growth even when using excimer laser photolysis. Rate measurements will be made for temperatures in the range of 5-55°C, and activation energies will be determined by plotting logarithm of reaction rate versus reciprocal temperature according to the Arrhenius form. Reactant concentrations will be varied so as to confirm that the rate is first order with respect to substrate concentration and second order overall and to determine the rate constant from the reaction rate versus concentration data.
Fig. 6: Hypothetical kinetic data on growth of concentration of $\alpha\gamma_\alpha$ chaperone complex. $I_A$ is the intensity of $\alpha$-FM-crystallin emission. The UV "pulse" occurs from time zero to the position of the vertical line in the figure. Examples of three hypothetical reaction rates are shown as discussed in the text.

The rate data will be analyzed according to the model (for initial rates, corresponding to small UV dose):

$$\alpha + S \rightarrow \alpha S$$

with the rate given by

$$\text{Rate} = k_i [\alpha]^i [S]^j$$

where the bracketed quantities are molar concentrations and the order parameters $i$ and $j$ are determined by the dependence of rate on reactant concentration. The value of $k_x$ ($x = 1-10$ range) will be determined from the slope of a plot of initial reaction rate versus [S] while holding $[\alpha]$ fixed. For example, such a plot will be linear for a reaction which is first-order in gamma crystallin concentration. Rate constants for subsequent binding steps, $k_x$, $x = 20-30$ range, will be obtained in like fashion from data taken at higher degrees of complex saturation (higher up the "ladder" in Fig. 5). Thus, each of the $k_x$ values will not be measured individually, but rather, ranges of $x$ will be grouped together as indicated, $k_{1-10}$, $k_{20-30}$, etc., and evaluated. This should suffice to reveal any cooperative effect.
E. HUMAN SUBJECTS

None.

F. VERTEBRATE ANIMALS

None

G. LITERATURE CITED


BORKMAN, Raymond F.


H. CONTRACT ARRANGEMENTS

None

I. CONSULTANTS

None
CHECKLIST

□ NEW application. (This application is being submitted to the PHS for the first time.)

X REVISION of application number: EY-6800-08

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

□ COMPETING CONTINUATION of grant number:

(This application is to extend a funded grant beyond its current project period.)

□ SUPPLEMENT to grant number:

(This application is for additional funds to supplement a currently funded grant.)

□ CHANGE of principal investigator/program director.

Name of former principal investigator/program director:

□ FOREIGN application or significant foreign component.

1. ASSURANCES/CERTIFICATIONS

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications begin on page 27 of Section III. If unable to certify compliance where applicable, provide an explanation and place it after this page.

□ Human Subjects; □ Vertebrate Animals; □ Debarment and Suspension; □ Drug-Free Workplace (applicable to new [Type 1] or revised [Type 1] applications only); □ Lobbying; □ Delinquent Federal Debt; □ Research Misconduct; □ Civil Rights (Form HHS 441 or HHS 690); □ Handicapped Individuals (Form HHS 641 or HHS 690); □ Sex Discrimination (Form HHS 639-A or HHS 690); □ Age Discrimination (Form HHS 680 or HHS 690); □ Financial Conflict of Interest.

2. PROGRAM INCOME (See instructions, page 20.)

All applications must indicate whether program income is anticipated during the period(s) for which grant support is requested. If program income is anticipated, use the format below to reflect the amount and source(s).

<table>
<thead>
<tr>
<th>Budget Period</th>
<th>Anticipated Amount</th>
<th>Source(s)</th>
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3. INDIRECT COSTS

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office, or, in the case of for-profit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal. This is to be based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent DHHS Guide for Establishing Indirect Cost Rates, and submitted to the appropriate DHHS Regional Office or PHS Agency Cost Advisory Office. Indirect costs will not be paid on foreign grants, construction grants, grants to Federal organizations, grants to individuals, and conference grants. Follow any additional instructions provided for Research Career Awards, Institutional National Research Service Awards, and specialized grant applications.

□ DHHS Agreement dated:

□ DHHS Agreement being negotiated with ______________ Regional Office.

X No DHHS Agreement, but rate established with Office of Naval Research Date 7/15/96

CALCULATION* (The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information. Supplying the following information on indirect costs is optional for for-profit organizations.)

a. Initial budget period: Amount of base $97,499 x Rate applied 49.1% = Indirect costs (1) $48,646

b. Entire proposed project period: Amount of base $291,603 x Rate applied 49.1% = Indirect costs (2) $148,086

(1) Add to total direct costs from form page 4 and enter new total on Face Page, Item 7b.
(2) Add to total direct costs from form page 5 and enter new total on Face Page, Item 8b.

*Check appropriate box(es):

□ Salary and wages base  □ Modified total direct cost base
□ Off-site, other special rate, or more than one rate involved (Explain)
□ Other base (Explain)

Explanation (Attach separate sheet, if necessary):

4. SMOKE-FREE WORKPLACE

Does your organization currently provide a smoke-free workplace and/or promote the nonuse of tobacco products or have plans to do so?

□ Yes  □ No  (The response to this question has no impact on the review or funding of this application.)

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