Project Title: Enzymatic Oxidation and Oxygen Activation

Project No: G-33-636

Principal Investigator: Dr. Sheldon W. May

Sponsor: National Science Foundation

Agreement Period: From 1/1/75 Until 6/30/77

*24 months budget period plus 6 months for submission of required reports, etc.

Type Agreement: Grant #NSF-74-20630

Amount: $50,000 - NSF Funds (G-33-636)
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$55,561 - Total

Reports Required: Annual Letter Technical; Final Report

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Assigned to: School of Chemistry

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RA-3 (6-71)
SPONSORED PROJECT TERMINATION SHEET

Date 3/17/83

Project Title: Enzymatic Epoxidation & Oxygen Activation
Project No: G-33-686
Project Director: Dr. Sheldon W. May
Sponsor: National Science Foundation

Effective Termination Date: 10/31/79
Clearance of Accounting Charges: 1/31/80

Grant/Contract Closeout Actions Remaining:

- [ ] Final Invoice and Closing Documents
- [x] Final Fiscal Report Acct (FCTR)
- [x] Final Report of Inventions
- [ ] Govt. Property Inventory & Related Certificate
- [ ] Classified Material Certificate
- [ ] Other

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Project File
Other May
FIRST ANNUAL TECHNICAL LETTER

NSF Grant BMS74-20830

January 1, 1975 - December 31, 1975

Enzymatic Epoxidation and Oxygen Activation

Submitted by

Sheldon W. May

Principal Investigator

School of Chemistry
Georgia Institute of Technology
Atlanta, Ga. 30332
I. Publications and Presentations since start of project.

The following is a list of our publications and presentations since the initiation of work on this project on January 1, 1975. Appropriate copies of abstracts, preprints and reprints are attached to this report, and others will be forthcoming as they become available.


"Enzymatic Epoxidation of Olefins", Sheldon W. May, Symposium on Chemistry of Coenzymes and Coenzyme Analogs, Chicago, Ill., September, 1975 (invited presentation), [Symposium was under NSF sponsorship]

"Analysis of the Enzymatic Epoxidation of trans, trans-dideutero-1,7-octadiene using Partially Relaxed NMR - A Direct Test of the 'Oxenoid' Mechanism", Sheldon W. May, S. L. Gordon and M. S. Steltenkamp, submitted to JACS.

"Stereochemical Analysis of the Epoxidation Reaction Catalyzed by an Enzyme System of Pseudomonas oleovorans", S. W. May, R. D. Schwartz and M. S. Steltenkamp, in preparation.

The following review of our work on liquid-membrane-enzyme systems, which has considerable relevance to our work on this project, has also been completed.

"Liquid Membrane Encapsulated Enzymes," Sheldon W. May and Norman N. Li, Biomedical Applications of Immobilized Enzymes and Proteins (T. Chang, ed.) Plenum, N. Y., in press

*Although this paper presents results directly relevant to this proposal, the actual experimental work was completed after the submission of the proposal but before the project was funded. Thus, a formal acknowledgement of NSF support was not included in this paper.
In addition, I have accepted an invitation to present a paper at the Symposium on Separation and Encapsulation by Liquid Membranes at the Centennial ACS Meeting, New York, April, 1976.

II. Summary of Research Progress

Mechanistic Studies on Enzymatic Epoxidation - In a highly significant development which we did not foresee at the outset of this project, we have successfully synthesized trans, trans-dideutero-1,7-octadiene (I) and employed this substrate to directly probe the mechanism of enzymatic oxygen activation. The rationale behind these experiments is as follows: Following the original suggestions of Hamilton, it is now widely assumed that monooxygenase reactions involve the generation of an electrophilic "oxenoid" active oxygen species by transfer of two electrons to oxygen prior to, or concurrent with, the transfer of oxygen to the substrate. If this notion is correct, then organic systems which are known to undergo oxenoid-type reactions - particularly the peracid epoxidation of olefins which is the most well-studied of all such systems - should be good model systems for monooxygenase reactions. Perhaps the most distinguishing characteristic of peracid epoxidations is that they involve cis addition of oxygen, and thereby preserve the original cis-trans configuration of the olefin. Thus, an examination of the configuration of the epoxide product formed enzymatically from (I), provides the first direct test of the "oxenoid" mechanism in an enzymatic reaction (note that simple internal olefins are enzymatically unreactive.)

The epoxide products formed enzymatically from (I) were isolated and examined via relaxation techniques using Fourier Transform NMR. We were
extremely surprised to discover that the product was a mixture of 70% cis and 30% trans deuterated 7,8-epoxy-l-octene. Thus, the enzymatic reaction had proceeded primarily with inversion of configuration, a result completely unexpected on the basis of current notions regarding the mechanisms of monooxygenase reactions. To our knowledge, these results represent the first clear and unequivocal demonstration that a "classical" monooxygenase reaction - epoxidation of simple olefins - cannot involve the simple addition of an "oxenoid" species.

In order to further clarify the pathway involved in this reaction we have completed a detailed analysis of the stereoselectivity of the enzymatic epoxidation reaction. One of the most exciting aspects of this reaction is our discovery that R(+)-7,8-epoxy-l-octene is formed from octadiene in high optical purity, a clear example of enzymatic asymmetric synthesis [S. W. May and R. D. Schwartz, J. Amer. Chem. Soc. 96, 4031 (1974)]. (Note that optically active simple epoxides cannot be prepared by any chemical means whatsoever to any reasonable extent). We have now found that 1,2-7,8-diepoxyoctane produced enzymatically from octadiene, via the intermediacy of the R(+) monoepoxide, is also primarily the (R,R) isomer. In sharp contrast, the same diepoxide produced enzymatically from (R,S)-7,8-epoxy-l-octene is a mixture of (R,R) and (S,S) isomers. Thus, our results demonstrate that the presence of an asymmetric center at one end of the molecule profoundly alters the direction of oxygen attack during epoxidation at the other end of the molecule.

The significance of these results is twofold. On the one hand, they supply information about the topography of the active site region and the mode of substrate binding, and suggest that a similar study with dienes of varying carbon chain length would be particularly informative.
This type of information is essential if the highly unusual specificity patterns we have observed with this system [S. W. May, et al., Biochim Biophys Acta 403, 245 (1975)] are to be rationalized. But perhaps more significantly, our stereochemical results prove that oxygen attack at carbon number two of the double bond occurs from only one face of a given substrate molecule. Since the results with the deuterated substrate, described above, show that the configuration at carbon number one is not maintained during epoxidation, a stepwise addition of oxygen to the olefin seems to be indicated. Among the possibilities which we are now considering are mechanisms involving radical or cationic intermediates, or the prior formation of a glycol which closes in only one direction to give the epoxide product.

We have also continued our exploration of a third aspect of the enzymatic epoxidation reaction - the effect of chemical structure on substrate reactivity and inhibitor potency. A homologous series of alkyl imidoester inhibitors have been synthesized in order to allow an evaluation of the effect of carbon chain length on the binding at the active site. The reactivities of cyclic, internal, conjugated, and aromatic olefins as well as dienes of various carbon chain length, have been explored. Taken together, our results reveal that hydrophobic interactions at the active site critically affect the binding and reactivity of molecules in this enzyme system. The high stereoselectivity of this reaction is clearly in line with this conclusion, since binding must occur in such a way as to allow oxygen attack from only one face of the olefin. By considering the effect of an asymmetric center at one end of the molecule on epoxidation at the other end in substrates with varying carbon chain length, together with specificity results, we should be able to develop a good picture of the
mode of substrate binding at the active site.

**Highly Active Epoxidizing Preparations** - We have begun to develop procedures for the production of cell-free preparations with high epoxidizing activity from cells of *P. oleovorans*. In this, we are taking advantage of a unique characteristic of this system - the fact that epoxide products accumulate in high yield even with crude whole cells systems and are metabolized only slowly, if at all [S. W. May and B. J. Abbott, *J. Biol. Chem.*, **248**, 1725 (1973); S. W. May and R. D. Schwartz, *J. Am. Chem. Soc.*, **96**, 4031 (1974)]. Thus, active cell-free preparations would allow one to easily carry out the epoxidation of organic molecules on a preparative scale, and their ready availability would be very useful in synthetic chemistry. For example, we have produced optically active mono- and diepoxides with high optical purity on a preparative scale with such preparations, a feat impossible to accomplish using any known chemical epoxidizing agent. Aside from the interest in such procedures as a standard synthetic tool, the monoepoxides from simple olefins are potentially useful in the synthesis of pheremones, and the diepoxides may be useful as crosslinking agents in the synthesis of stereoregular polymers or as chemical modification reagents in protein chemistry. As another example, with the enzymatic system we can selectively epoxidize a terminal olefin functionality in the presence of more highly substituted double bonds. Since it is not possible to attain this type of selectivity with chemical agents, the use of these enzymes in steroid derivatization or drug synthesis would be a direct example of exploitation of oxygenase specificity in an advanced, high technology process.

To date, we have succeeded in producing cell-free preparations which produce epoxides on the gram-per-liter scale. We are now attempting to fractionate these crude preparations further in order to be able to supply
the synthetic chemist with a well-defined "epoxidizing reagent" which can
be used without special "biological precautions" (aseptic conditions,
care in handling "live" cells) with which he is unfamiliar.

Enzymatic Studies - Due to difficulties in using procedures published
by others, we encountered some delay in initiating our studies with
purified enzymes. We have now successfully developed our own procedure
for the isolation of rubredoxin which we have used to obtain a large
quantity of the purified enzyme. We have successfully immobilized ru-
bredoxin on Sepharose and are currently characterizing the conjugate
as to spectral properties, electron transfer ability and catalytic activity.
To our knowledge, this represents the first example of an immobilized
non-heme iron protein. All of the alkyl- and alkenyl-Sepharose conjugates
described in our original proposal are now in hand. We are currently
proceeding with the ligand-specific chromatography work using fractions
from the high-activity cell-free preparations, described above, which
we have developed. We have also obtained some very interesting results
from Isoelectric Focusing experiments with purified rubredoxin, which
have revealed that the pI of rubredoxin is below 5.0. Thus, this protein
is highly negatively charged in neutral solution, suggesting that the
"epoxidase", if it indeed complexes with rubredoxin, is comparatively
basic. We plan to make use of this information in the design of new
inhibitors and substrates in order to test for which protein is the
true "oxygenase" in this system.

Microenvironmental Effects on Oxygenase Catalysis - For the past
several years, we have had an ongoing research program aimed at examining
the effects of the hydrophobic microenvironment on a few well characterized
enzymes. These studies have involved the incorporated enzymes into hydro-
carbon-based liquid-membrane emulsions. [S. W. May and N. N. Li, Biochem. Biophys. Res. Commun. 47, 1179 (1975); Enzyme Engineering, 2, 77 (1974); Biomed Applications Immob. Enzym. Prot. (T. Chang, ed.) in press]. Since oxygenases are known to be membrane-bound within the cell, and react with hydrophobic substrates, it seemed likely to us that these enzymes might exhibit enhanced physical stability or radically altered reactivity in a hydrophobic microenvironment. The fact that our results with the epoxidation system of *P. oleovorans* have revealed the unusual sensitivity of this reaction to hydrophobic interactions at the active site led us to choose this oxygenase system for initial studies with the liquid membrane system.

During the past several months, we have developed a cofactor recycling system suitable for use with the epoxidation system within the liquid membrane emulsion. The details of this work are described in a publication which is in press [S. W. May and L.M. Landgraff, Biochem. Biophys. Res. Commun., in press] and a copy is attached to this report. We now plan to proceed with an examination of the effects of a multiphase microenvironment on oxygenase catalysis.
ANNUAL TECHNICAL LETTER

NSF Grant PCM76-20830

May 1, 1977 - April 30, 1978

Enzymatic Epoxidation and Oxygen Activation

Submitted by
Sheldon W. May
Principal Investigator

School of Chemistry
Georgia Institute of Technology
Atlanta, Georgia 30332
I. PUBLICATIONS AND PRESENTATIONS SINCE START OF PROJECT.

The following is a list of our publications and presentations since the last report. Appropriate copies of abstract, preprints and reprints have been sent to NSF as they have become available.


"Interaction of Protocatechuate-3,4-dioxygenase with Fluoro-Substituted Hydroxybenzoic Acids and Related Compounds," S.W. May, R.S. Phillips and C. D. Oldham, Biochemistry, accepted for publication (1978).


"Enzymatic Production of Saturated Ketones from Allylic Alcohols," S.W. May and M.S. Steltenkamp, in preparation.
II. SUMMARY OF RESEARCH PROGRESS

Preparation and Properties of Cobalt (II) Rubredoxin - In what we regard as a highly significant development which we did not foresee at the outset of this project, we have recently succeeded in replacing the native iron atoms of rubredoxin from Pseudomonas oleovorans with cobalt, to give cobalt rubredoxin containing two atoms of cobalt per protein molecule. This represents the first example of the chemical substitution of the metal in an iron-sulfur protein and thus a number of properties of the cobalt enzyme were examined. Cobalt rubredoxin was carefully characterized as to metal content, and competitive ligation studies established that the apoenzyme binds cobalt preferentially over iron. The spectral properties of cobalt rubredoxin are fully consistent with the presence of two Co(II) atoms in rubredoxin-type binding sites, exhibiting d-d transitions and charge transfer bands of the intensity and position predicted from data with model compounds. The CD and resonance Raman spectra were also compared for both native and cobalt rubredoxin. As judged from difference spectra, cobalt rubredoxin interacts with rubredoxin reductase, and mediates reduction of cytochrome c in the presence of NADH and reductase, although it is less efficient in this regard than native rubredoxin. Strikingly, cobalt rubredoxin is much more stable than the native enzyme toward denaturants and metal dissociation, and no evidence for nonequivalence of the two cobalt binding sites was obtained.

From our point of view, the preparation of cobalt rubredoxin is highly significant for several reasons. In the first place, P. oleovorans rubredoxin differs from those of the anaerobes in that an extra thiol group is located near each metal binding site. It has thus been an important goal to probe for two "reactive," non-metal protected sulphydryls using selective chemical modification, in order to test for a possible role in electron transport or catalysis. To date, this has been impossible due to the lability of the second iron in (2Fe) rubredoxin, which precludes selective modification of two sulphydryls per protein molecule. In sharp contrast, with (2Co)-rubredoxin we have been successful in demonstrating the presence of two highly reactive sulphydryl groups, and have prepared cobalt rubredoxin with only these sulphydryls modified, on a preparative scale. The modified enzyme was isolated and shown to be still active in electron transport to cytochrome c, and we are currently using this modified rubredoxin in experiments designed to probe for the involvement of sulphydryl groups in the oxygen activation process.

A second very significant aspect of our preparation of cobalt rubredoxin arises from the fact that cobalt is known to be a highly useful environmental probe for alterations in coordination geometry at the active sites of metallo-proteins. The particular suitability of cobalt for such purposes due to its paramagnetism and the sensitivity of its visible spectrum, especially in the d-d transition region, to changes in coordination geometry. Thus, with cobalt rubredoxin in hand, we have a very powerful handle for looking at the interaction of rubredoxin and "epoxidase" during the catalytic cycle of oxygenation, a goal which has been particularly elusive with this enzyme system due to the lack of strong visible absorption bands such as those found in P-450-containing systems.
**Substituent Effects on Epoxidation** - In the course of our continuing analysis of the specificity, stereochemistry, and mechanism of enzymatic epoxidation, we were particularly interested in evaluating the effects of allylic alcohol substituents for comparison with chemical systems. In the case of peracid epoxidations, allylic alcohol substituents lower the reactivity of an olefin, whereas increased reactivity is often observed for such compounds in transition metal-catalyzed epoxidations by alkyl hydroperoxides. In both cases, there is a striking *syn* directive effect on the stereochemistry of chemical epoxidation, which might be ascribed to a complexation effect in the transition metal systems. We have now discovered that with the enzyme system, the presence of an allylic alcohol substituent so decreases the reactivity of a double bond toward epoxidation that such compounds are instead converted to their corresponding saturated ketones. We have analyzed this ketonization reaction from a mechanistic viewpoint, and have scoped the synthetic potential of this system for the production of saturated ketones on a preparative scale. In our view, these results are highly significant for they represent another point of direct comparison between an enzymatic oxygenation reaction and well-understood, defined chemical systems. In general, I think it is fair to say that our continuing work on the detailed specificity, regioselectivity and stereochemistry of enzymatic epoxidation has produced the only direct detailed comparison between an oxygenase system operating on simple substrates and corresponding chemical reagents. Such comparative information is essential to defining the detailed pathway of enzymatic oxygen activation.

**Laser Raman Studies** - As proposed in our original application, we have begun using laser Raman spectroscopy to probe the molecular details of non-heme iron involvement in oxygen activation. Essential to the success of such experiments is the availability in our hands of rubredoxin, cobalt rubredoxin, "epoxidase," and protocatechuate-3,4-dioxygenase, and the rationale behind these experiments is outlined in detail in our original grant application. To date, these experiments have been going extremely well. We have obtained Raman data for both native and cobalt rubredoxin, and have completed a detailed study on both native protocatechuate-3,4-dioxygenase and its complexes with potent competitive inhibitors. Our results have provided detailed information regarding the ligation of the non-heme iron atom and the mode of interaction of inhibitors and iron at the active site of this oxygenase. We will now be proceeding to a study of ES\*O\(_2\) complexes with protocatechuate-3,4-dioxygenase, and of analogous complexes with the protein components of our enzymatic epoxidation system.

**Affinity Chromatography Studies** - We are continuing to attempt to use affinity chromatography as a powerful tool in mechanistic and component interaction studies with the epoxidation system. We have been using both the immobilized rubredoxin conjugates which we have prepared and characterized as well as various alkyl-Sepharose conjugates in these studies. To date, we have successfully used hydrophobic chromatography for purification of the difficult-to-isolate reductase component, but due to the steric restrictions of multipoint attachment we have been unable to use immobilized
rubredoxin for this purpose. Our approach with this latter conjugate will now be to attach rubredoxin by a limited number of bonds to the support in order to enhance its usefulness as a ligand in affinity chromatography studies with epoxidase. As set forth in our original proposal, we are hopeful that these studies will not only lead to more facile purification procedures, but will also provide invaluable information regarding the component interactions in this system. We are particularly excited about the prospect of utilizing immobilized cobalt rubredoxin, which has much enhanced stability, in such studies.
PART I—PROJECT IDENTIFICATION INFORMATION

1. Institution and Address
   Georgia Institute of Technology
   Administration Bldg.
   Atlanta, GA 30332

2. NSF Program
   Biochemistry

3. NSF Award Number
   PCM74-20830-A02

4. Award Period
   From 1/1/75 To 10/31/79

5. Cumulative Award Amount
   $116,000

6. Project Title
   Enzymatic Epoxidation and Oxygen Activation

PART II—SUMMARY OF COMPLETED PROJECT (FOR PUBLIC USE)

The goal of this project was to examine the specificity, stereochemical and mechanistic aspects of oxygenation by a 3-component bacterial enzyme system known to catalyze both the methyl group hydroxylation of alkanes and the epoxidation of olefins. This enzyme system is one of the very few monooxygenases described which is known to contain functional non-heme-iron at the active site. Thus, it represents a poorly understood class of monooxygenases.

In the course of these studies, mechanistic studies with specifically deuterated olefins were carried out to demonstrate that the epoxidation reaction does not proceed with retention of configuration. Complimentary stereochemical studies were also carried out to demonstrate the stereospecificity of this reaction. By combining this information, a 2-step mechanism is proposed. Furthermore, biochemical characterization of the enzymes themselves, and the first reported preparations of cobalt-substituted and immobilized rubrodoxins were achieved. Finally, related reactions such as secondary alcohol dehydrogenation were discovered and described.

Taken together, these results have advanced our understanding of how this non-heme-iron oxygenase enzyme system functions, and thus have expanded our basic knowledge of oxygenase biochemistry.

PART III—TECHNICAL INFORMATION (FOR PROGRAM MANAGEMENT USES)

1. ITEM (Check appropriate blocks)
   NONE ATTACHED PREVIOUSLY FURNISHED TO BE FURNISHED SEPARATELY TO PROGRAM
   a. Abstracts of Theses
   b. Publication Citations
   c. Data on Scientific Collaborators
   d. Information on Inventions
   e. Technical Description of Project and Results
   f. Other (specify)

2. Principal Investigator/Project Director Name (Typed)
   Sheldon W. May

3. Principal Investigator/Project Director Signature

4. Date
   3/8/83
INSTRUCTIONS FOR FINAL PROJECT REPORT
( NSF FORM 98A)

This report is due within 90 days after the expiration of the award. It should be submitted in two copies to:

National Science Foundation
Division of Grants and Contracts
Post-Award Projects Branch
1800 G Street, N.W.
Washington, D.C. 20550

INSTRUCTIONS FOR PART I

These identifying data items should be the same as on the award documents.

INSTRUCTIONS FOR PART II

The summary (about 200 words) must be self-contained and intelligible to a scientifically literate reader. Without restating the project title, it should begin with a topic sentence stating the project's major thesis. The summary should include, if pertinent to the project being described, the following items:

- The primary objectives and scope of the project.
- The techniques or approaches used only to the degree necessary for comprehension.
- The findings and implications stated as concisely and informatively as possible.

This summary will be published in an annual NSF report. Authors should also be aware that the summary may be used to answer inquiries by nonscientists as to the nature and significance of the research. Scientific jargon and abbreviations should be avoided.

INSTRUCTIONS FOR PART III

Items in Part III may, but need not, be submitted with this Final Project Report. Place a check mark in the appropriate block next to each item to indicate the status of your submission.

a. Self-explanatory.
b. For publications (published and planned) include title, journal or other reference, date, and authors. Provide two copies of any reprints as they become available.
c. Scientific Collaborators: provide a list of co-investigators, research assistants and others associated with the project. Include title or status, e.g. associate professor, graduate student, etc.
d. Briefly describe any inventions which resulted from the project and the status of pending patent applications, if any.
e. Provide a technical summary of the activities and results. The information supplied in proposals for further support, updated as necessary, may be used to fulfill this requirement.
f. Include any additional material, either specifically required in the award instrument (e.g. special technical reports or products such as films, books, studies) or which you consider would be useful to the Foundation.