

# *A Lifetime of Playing with Enzymes*

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I was born and raised in a small town in southeastern Kansas called Hepler. I attended grammar school and high school in Hepler, and the student body of the high school was approximately 100. The United States entered World War II during my sophomore year, and accelerated programs allowed me to skip my senior year, so I enrolled in college in the summer of 1943. I was 16 years old. I also volunteered for the United States Air Force cadet training program when I was 16. I enrolled in Valparaiso University in Indiana in the fall of 1943; however, my first year at Valpo was not terribly productive. Although I was enrolled in college, I thought I would be called to serve in the Air Force at any moment. Thus, I was not very interested in classwork.

I did get called up in January 1945, did basic training in Biloxi, Missouri, and was shipped to Mesa, Arizona, for “on the line training.” Soon after I arrived at Mesa Field, the war in Europe ended, and the cadet training program was subsequently canceled. I was transferred to Scott Field near Belleville, Illinois, and mustered out of the Air Force in late November 1945. I returned to Valparaiso University, determined to make good grades, and started thinking about my future.

I took two courses in my junior and senior years at Valpo that were decisive in my future planning. I took a biochemistry course in my junior year, followed by a microbiology course in my senior year.

I became very interested in bacterial enzymes, especially in their wide range of biological activities, and I decided to go to graduate school and learn more about bacterial diversity. After finishing my undergraduate work in 1947 at Valparaiso University, I enrolled for graduate training in microbiology at the University of Kansas. However, my career in scientific research got off to a rather rocky start. I was rather naïve, and instead of searching for the graduate program that would be a good fit for my potential research interests, I applied only to the University of Kansas, my home state institution. I enrolled in the microbiology department in the fall of 1947. Three major professors dominated the department: Professors Noble Sherwood, M.D., E. Lee Treece, Ph.D., and Cora Downs, Ph.D.

Dr. Sherwood was a well known immunologist, and Dr. Downs specialized in viral diseases. Neither the Sherwood program nor Downs program interested me. I was left with Dr. Treece, who was interested primarily in teaching but agreed to let me work under his direction on a project of my own choosing. I decided to attempt the isolation of a thermophilic organism capable of degrading cellulose. As I look back, I was joining the green movement long before it became the thing to do.

It was very easy to get mixed cultures of organisms that efficiently degraded filter papers; however, the isolation of a single organism capable of degrading cellulose became the major problem. I used standard methods in my attempt to isolate a thermophile capable of degrading cellulose but was not successful. Dr. Treece offered little advice, and I admitted to myself that I better come up with a different project.

At this time, Dr. Sherwood, having reached the age of 65, retired as head of the department, and a new head, Dr. Robert Guthrie, was appointed. For me, Guthrie was like a breath of fresh air: he immediately appointed two young assistant professors, and I became interested in working with one new appointee (Professor Francis Jarvis) who had trained in microbial biochemistry at the University of Wisconsin.

I applied to change research directors, and things went smoothly but only for a short period of time. Sherwood, Downs, and Treece told Chancellor Deane W. Malott that Guthrie was unsuitable as head of microbiology, and Malott asked Guthrie to resign, which he did.

I led a student delegation to Malott's office and asked that he not accept Guthrie's resignation. We were unsuccessful. Guthrie left the University of Kansas and became well known for his development of a test used on new babies to detect phenylketonuria.

Two weeks after our visit to Chancellor Malott, I was informed by the new head of the department, Treece, that I would not be reappointed to my assistantship. Soon afterward, Malott left Kansas to become chancellor at Cornell University but was almost forced to resign from that position when the *New Yorker* magazine reported that he had plagiarized his inaugural address at Cornell. I felt some justice had been done.

It was clear that I had to leave the University of Kansas, but to go where? I had read papers by I. C. (Gunny) Gunsalus, and it was obvious that he was doing the kind of microbial biochemistry that I was interested in doing. Gunsalus was listed as a faculty member at Indiana University, but my letter of inquiry was forwarded to Severo Ochoa's laboratory at the New York University School of Medicine, where Gunny was spending his sabbatical.

I received a hand-written reply from Gunny informing me that he was moving to the University of Illinois and telling me that I should apply for admission to the graduate school. Although I had considered my firing at the University of Kansas a great tragedy, it turned out to offer me a great opportunity. Not only was Gunny a great research director, but he was a great friend and motivator.

The big project in Gunny's lab in the fall of 1950 was the isolation and characterization of the "acetate-replacing factor." Some strains of lactobacilli are unable to convert pyruvate to acetate and must have acetate supplied in their growth media. Small amounts of liver extract could supply a growth factor (a new vitamin?) that replaced the acetate requirement for growth. In collaboration with Lester J. Reed at the University of Texas, the factor was isolated and named lipoic acid.

Because the acetate-replacing factor had to be involved in the oxidation of pyruvate to acetate, Gunny gave me the job of isolating pyruvate and  $\alpha$ -ketoglutarate dehydrogenases and to work on the role of lipoic acid in these two reactions. Gunny knew that initial work in Severo Ochoa's lab had prepared partially purified *Escherichia coli* extracts and had shown that two fractions (the A and B fractions) were required for the oxidation of pyruvate.

At this point, Ochoa's lab had stopped working on pyruvate dehydrogenase, so I jumped at the chance to work on the project. In addition to producing highly purified A and B fractions, I also purified two components involved in the oxidation of  $\alpha$ -ketoglutarate dehydrogenases (fractions A' and B). The purified B fractions appeared identical and were required for the oxidation of both keto acids.

After the identification of the acetate-replacing factor as lipoic acid, I was supplied with small amounts of lipoic acid, and in my first experiments, I tested the B fraction for activity with lipoic acid and discovered that  $\text{NAD}^+$  served as an electron acceptor for the oxidation of reduced lipoic acid. Both B fractions, one from the pyruvate dehydrogenase preparations and the other the  $\alpha$ -ketoglutarate fraction B, had identical lipoic acid dehydrogenase activity (1).

My next project was to test the pyruvate fraction A for lipoic transacylase activity. I incubated acetyl-CoA with reduced lipoic acid and showed that my purified pyruvate fraction A component catalyzed the transfer of the acetyl group from coenzyme to reduced lipoic acid (2, 3).

This experiment was done late in the evening, and I wanted Gunny to know about what I considered to be exciting results. I found out that Gunny was playing poker with Roger Adams and friends, so I called, and Gunny rushed over to the laboratory.

This work essentially ended my tenure in Gunny's laboratory. I had identified two activities associated with the  $\alpha$ -keto acid dehydrogenases, and it was time to write my doctoral thesis and move on. I found it difficult to part with Gunny. My work in his lab had been so much fun and so exciting that I really did not want to leave.

Gunny suggested that I do postdoctoral work either with Fritz Lipmann at Harvard Medical School or with Severo Ochoa. He assured me the both Fritz and Severo would welcome me in their labs. After much thought, I decided on the Lipmann lab and moved to Boston in September 1953.

Fritz wanted me to continue work on pyruvate oxidation and assigned me the job of rediscovering the enzyme he had discovered in a *Lactobacillus* species that converted pyruvate to  $\text{CO}_2$  and acetyl phosphate. This was an important discovery because it led Fritz to the concept of "high-energy" phosphate bonds.

Lipoic acid was not involved in the *Lactobacillus* reaction. The *Lactobacillus* species Lipmann had worked on in Europe was lost in his transfer to the United States; however, Fedor Lynen volunteered to help to rediscover the organism and shipped me about 30 *Lactobacillus* cultures. I tested them and found a *Lactobacillus delbrueckii* species that had the Lipmann acetyl phosphate activity. I purified the *L. delbrueckii* enzyme and showed that it had the distinct activity of producing acetyl phosphate directly (4). After one year with Lipmann, I was offered an assistant professorship at the Massachusetts Institute of Technology (MIT) by John Buchanan.

In those days, affirmative action and search committees were unknown: you just got picked for an offer. Lipmann did not want me to take the MIT offer, and I then decided to stay with Lipmann and take over David Novelli's role as a chief lieutenant in the Lipmann laboratory. However, after I spent one more year in the Lipmann lab, Konrad Bloch arranged an offer of an assistant professorship in the chemistry department at Harvard University.

Lipmann cautioned me against accepting the offer, but I considered it promising and something I should accept. Konrad was persuasive in his argument that biochemistry would be expanding in the chemistry department, and he was sure that additional tenured positions would be added. Unfortunately, this idea turned out not to be true. I moved to Harvard in the fall semester of 1955, and my first teaching assignment was to organize and teach a laboratory course.

My first independent research at Harvard included examining an acetate-requiring mutant of *E. coli* that had been isolated by Bernie Davis. We quickly showed that this mutant lacked a functional lipoic dehydrogenase (5).

Anne Gounaris also discovered a new pyruvate oxidase in the Davis *E. coli* mutant. The question we then asked was why this oxidase did not provide the acetate for the growth of the dehydrogenase mutant. Some answers came from the discovery that pyruvate oxidase was activated by lipids or, alternatively, by brief proteolytic hydrolysis by trypsin (6–8).

Robert Williams, a graduate student at Harvard, moved with me to the University of Illinois at Urbana-Champaign and obtained crystals of pyruvate oxidase (9). The mechanism of activation of pyruvate oxidase by proteolytic digestion was traced to the release of a small peptide from the C-terminal end of the protein, and in turn, this finding led to the identification of the lipid-binding site in the enzyme (10, 11).

Only the reduced form of pyruvate oxidase could bind the lipid membranes and become activated; however, oxidative growth kept the oxidase in an oxidized form. Under



FIGURE 1. In 1200 B.C., a blue-purple dye was discovered and quickly became a favorite. This dye, Tyrian purple, also known as royal purple, was a major commercial item in Asia Minor and was very expensive. The dye was produced by shellfish of the murex family.

partially anaerobic conditions, the mutant did not require acetate for growth. These observations explained the problem of why the oxidase did not provide acetate for growth of the mutant enzyme when the mutant was grown aerobically.

As a new assistant professor, I was determined to initiate a research program that I could claim as my own. In preparing for lectures in my new “comparative biochemistry” course, I became very interested in biological halogenation reactions, especially because large numbers of halogenated compounds had been isolated from marine sources (about 2000) and also because of the growing number of reports describing the isolation of antibiotic molecules containing chlorine atoms.

Harold Raistrick, an English chemist, loved to isolate, crystallize, and determine the structure of natural products. He described a chlorinated product produced in large quantities by a soil fungus, *Caldariomyces fumago* (12). The product, caldariomycin (2,2-dichloro-1,3-dicyclopentane), was produced in gram/liter quantities. I considered *C. fumago* to be a likely source for a chlorinating enzyme, and I persuaded Paul Shaw to join my group as a postdoctoral fellow and work with me on examining the fungus as a source of a halogenating enzyme. The field of enzymatic halogenation had remained fallow for many, many years.

### A Brief History of Tyrian Purple

In 1200 B.C., a blue-purple dye was discovered and quickly became a favorite. This dye, Tyrian purple, also known as royal purple, was a major commercial item in Asia Minor and was very expensive. The dye was produced by shellfish of the murex family (Fig. 1). It was highly

prized because it would adhere to cloth and was nonfading.

God commanded Moses to require Jews to use the dye to color the edges of their shawls with this murex dye so that they could recognize dawn and start their morning prayers. The dye was reported to be worth more than silver and pearls. Cleopatra requested that Anthony give her the dye instead of pearls.

Tyrian purple was first produced in large amounts by ancient Phoenicians and exported to various Mediterranean ports. Pliny the Elder, in his ninth book of *Naturalis Historia*, described the preparation of the dye.

The dye is obtained from the mucous secretion from the murex shellfish hypobranchial gland (Murexidae family). The shellfish glands are incubated for three days in seawater and then boiled, and the insolubles are removed. The murex glands contain both the organic substrate and enzymes for production of the dye.

Tyrian purple was synthesized later by Franz Sachs and Richard Kempf in 1903 and identified by Paul Friedlander in 1909 to be 6,6'-dibromoindigo (13). Archeologists have identified dye production sites that contain large quantities of murex shells. The production of the indigo dye represents a very early biotech project using an enzymatic process to produce a valuable product. The next report on an enzymatic halogenation reaction, about 3000 years later, was the paper Shaw and I published on the detection of a chlorinating enzyme in *C. fumago* cells (14).

We detected chloroperoxidase (CPO) activity in our first experiments using crude extracts prepared from *C. fumago*. In these early experiments, we incubated  $^{36}\text{Cl}$  with potential precursors of caldariomycin and examined the incubation mixtures for a  $^{36}\text{Cl}$ -labeled organic compound. We identified  $\delta$ -chlorolevulinic acid as a product formed from  $\beta$ -keto adipic acid in these crude reaction mixtures (15, 16). Subsequent work at Harvard established the peroxidative nature of the halogenation reaction (17), and work on CPO continued upon my move to the University of Illinois in 1960.

Harvard graduate student Jon Beckwith initiated studies on the biosynthesis of caldariomycin (18) and established the structure by synthesis. In radioactive labeling studies, Beckwith found that shikimic acid was an efficient precursor of caldariomycin and later showed that extracts of *C. fumago* catalyzed the peroxidative chlorination of a precursor of caldariomycin, 1,3-cyclopentanedione, in a two-step chlorination reaction (19, 20).

CPO, a heavily glycosylated protein, was purified and crystallized by my first University of Illinois graduate student, David Morris (21). Soon after Morris started purifying CPO, we discovered that washed *C. fumago* mycelium

did not contain CPO. Obviously, CPO was being secreted into the culture medium, and all of our previous work had relied upon the small amount CPO adhering to the mycelium. The culture filtrates provided a much better source of the enzyme.

CPO is a typical heme enzyme. It contains ferric protoporphyrin IX as its prosthetic group and is composed of 299 amino acid residues. The x-ray crystallographic structure of CPO was determined by Thomas L. Poulos and co-workers (22). The heme group is buried in the center of the protein and is connected to solvent by a narrow channel that allows small molecules (with a maximum linear dimension of 10 Å) access to the interior of the enzyme. The distal ligand to the heme iron is a glutamic acid residue at position 183; the proximal ligand is a thiolate donated by Cys<sup>29</sup>; and the mechanism of the chlorination was shown to be electrophilic, in which chloride ion was oxidized to a  $\text{Cl}^+$  species (23).

In addition to halogenation reactions, CPO catalyzes a variety of one- and two-electron oxidations. CPO catalyzes *N*-demethylation and dealkylation of alkylamine reactions (24), the dimerization of phenols, and the stereospecific hydroxylation of alkyl and benzylic compounds and chiral sulfoxidations (25). CPO also catalyzes the oxidation of alcohols to aldehydes, aldehydes to acid, and amines to nitroso compounds and oxidizes 2-alkynes to chiral alcohols via propargylic oxidation (26–28).

Starting in the early 1960s, we started intensive work on the mechanism of CPO reactions. A spectrophotometric assay using monochlorodimedone greatly enhanced routine assays of the enzyme (29) because prior assays depended on the utilization of radioactive chloride by the enzyme. Monochlorodimedone has proved invaluable in the detection of other halogenases (30). We found that CPO utilized chloride, bromide, and iodide (but not fluoride) in halogenation reactions. The mechanism of action of antithyroid agents, such as propylthiouracil, was developed by showing that these agents were substrates for CPO (31).

F. S. Brown discovered that CPO committed suicide in reactions containing only hydrogen peroxide and chloride ion. This was the first report of an enzyme committing suicide (32) and provided evidence for an ionic electrophilic substitution mechanism for the chlorination reaction (23).

In contrast with horseradish peroxidase, CPO catalyzes the dismutation of hydrogen peroxide and peroxy acids with oxygen evolution. CPO efficiently catalyzes the evolution of oxygen from peroxide substrates and approaches beef liver catalase in terms of turnover number. This cat-

alase reaction allowed us to make a contribution to the chemistry of the green intermediate (Compound I) formed in the reaction of peroxidases with hydrogen peroxide.

In collaboration with J. C. Martin's lab, we showed, in the dismutation of peroxy acid by CPO, that the oxygen atoms in the evolved oxygen were derived from two separate peroxy acids (34), clearly indicating that the green intermediate in the dismutation reaction contained one oxygen atom that subsequently reacted with a second peroxy acid to form oxygen. This initiated our detailed studies on the chemistry of Compound I (35–39), in which we identified the structure of horseradish peroxidase and CPO Compound I as oxyferryl porphyrin  $\pi$ -cation radicals (40–42).

All reactions catalyzed by CPO are ping-pong reactions in which Compound I is formed in the initial reaction and subsequently reacts with a second substrate to return to the native enzyme. Kelath Murali Manoj showed that CPO Compound I reacts with chloride ion and generates a diffusible  $\text{Cl}^+$  intermediate that can react with nucleophilic centers to form the chlorinated derivatives (43).

In related studies on enzymatic halogenation reactions, we found that horseradish peroxidase utilizes chlorite for chlorination reactions (44–47). In this reaction, we detected the formation of a short-lived intermediate that rapidly decomposed to Compound I and a chloride ion. We postulated that this intermediate contains an  $-\text{OCl}$  ligand sitting on the ferryl protoporphyrin IX prosthetic group of horseradish peroxidase. CPO also catalyzes the chlorite chlorination reaction (48).

In 1972, the National Science Foundation provided us with a grant to study the occurrence of halogenated compounds in marine organisms. This grant included utilization of the Alpha Helix research ship for the collection and analysis of marine species. At that time, there were approximately 2000 different known halogenated compounds, practically all of marine origin, so we were confident of having a successful Alpha Helix expedition.

Robert White, a graduate student in my laboratory, developed a spectrophotometric assay for the rapid determination of organic halogens in lipid extracts of marine species, and we examined hundreds of samples collected on the Alpha Helix expedition. We found that brominated compounds were the more prevalent compounds in marine organisms and were very abundant in marine red algae (49–52). *Bonnemaisonia hamifera*, a red alga, synthesizes large amounts of 1-bromo-2-heptanone and 1,1-dibromo-2-heptanone (50). The production of the brominated ketones by *B. hamifera* is seasonal, and during the

high season, brominated heptanones can reach concentrations as high as 3% of the dry weight of the organism.

It is interesting to note that *B. hamifera* is considered a delicacy in Hawaii, and the raw algae is added to salads when seasonal high levels of the brominated ketones are present. The addition of the red algae to green salads probably is not a good idea because the brominated ketones can be powerful alkylating agents.

We isolated and characterized a new bromo compound from *Laurencia intricata* (53) and developed a biosynthetic route for the halogenated sesquiterpenes found in *L. intricata*. In addition to isolating a bromoperoxidase from red algae, we isolated a bromoperoxidase from green algae (54) and characterized its catalytic properties (55) and oxidation states (56).

### A Brief Detour from Halogenating Enzymes

In 1973, I asked Renato Dulbecco for permission to join his lab for my sabbatical leave. I was interested in doing something quite different from my usual research activities, and I thought that the work in Dulbecco's lab on simian virus 40 was very interesting.

On my arrival at the Imperial Cancer Research Fund in London, I introduced myself to Renato and said that I had great news for him: I told him that he was going to win a Nobel prize in the near future. It brought forth a peculiar facial reaction in Renato, and I am sure that he was thinking, "Something is wrong with this guy." I then explained that soon after I joined Fritz Lipmann's lab, he won a Nobel prize; likewise, when I was with Konrad Bloch, he won a Nobel prize; and after my sabbaticals in Munich and Oxford, both Fedor Lynen and Hans Krebs won Nobel awards. It turned out that my prediction was right: two years later, Renato was awarded the Nobel prize.

During my stay at the Imperial Cancer Research Fund, I collaborated with Robert Carroll, and we were the first to discover that SV40 T antigen binds to DNA (57). Later, upon my return to Illinois, we examined the binding of single-stranded DNA to T antigen (58), and we discovered that T antigen catalyzed the oligo(dT)-dependent hydrolysis of ATP (59). Donald Giacherio, a graduate student in my lab, pursued the T antigen story and showed that T antigen carried out a specific DNA-unwinding activity (60).

### Molecular Biology Studies on the Synthesis and Expression of CPO

The synthesis and secretion of CPO turn out to be under catabolite control. When fructose serves as a source of carbon for growth, relatively large amounts of CPO accumulate in the growth medium. Steven R.

Blanke developed both semi-continuous and continuous flow bioreactors for the production of 1–1.5 g/liter CPO (61).

In our early work on CPO, we routinely grew the fungus on glucose and only later discovered that all of the glucose was first converted to organic acids that accumulated in the culture medium, and only then did CPO production commence using the organic acids as a source of energy for growth. Milton J. Axley found that control of CPO expression was at the level of mRNA production. Fructose induces and glucose represses CPO mRNA levels (62).

Mark Nuell, Guo-Hua Fang, and Axley isolated and sequenced the CPO gene (63, 64), and a comparison of the amino acid sequence and the gene showed that the mature CPO that accumulates in the CPO cultures contains deletions at both the N- and C-terminal ends. A 21-amino acid secretion signal peptide is removed from the N terminus, and a 52-amino acid peptide is cleaved from the C terminus (65).

We were very interested in expressing recombinant CPO in *E. coli*. Qin Zong, Pawel A. Osmulski, and I developed a high-pressure assisted reconstitution of a recombinant CPO (66). An expression vector containing a T7 promoter and an OmpA signal sequence followed by the DNA sequence of mature CPO was used to transform *E. coli*. This construct gave a high-level expression of an apo-CPO when induced with isopropyl thiogalactopyranoside. The recombinant apo-CPO represented about 2% of the total *E. coli* protein but proved very difficult to convert to the holoenzyme.

Maximum yields of the holoenzyme were obtained when the apoenzyme was dissolved in medium containing iron protoporphyrin IX, calcium ions, and oxidized glutathione. The holoenzyme preparation was first subjected to high pressure and then incubated at atmospheric pressure and room temperature for refolding. Holoenzyme was produced at about the 5% level. These experiments showed that glycosylation was not a mandatory requirement for refolding. Later, a more efficient expression system for the production of recombinant CPO using *Aspergillus niger* was developed by Xianwen Yi in collaboration with Ana Conesa and Peter J. Punt (67).

### Active-site Mutants of CPO

Cytochrome P450cam mutants indicated that the proximal heme thiolate ligand was essential for enzyme activity. Our similar studies with CPO mutants indicated that the thiolate ligand (Cys<sup>29</sup>) could be replaced with a histidine residue and retain most of the chlorination, peroxidation, epoxidation, and catalase activities (68). *C. fumago*,

the fungus that produces wild-type CPO, was used for the expression of the Cys-to-His mutant. The gene transformation required that the mutant gene replace the normal chromosomal copy. This gene replacement was maximized by using constructs that promoted double-cross-over events.

All attempts to produce CPO mutants of the distal Glu<sup>183</sup> failed. Similarly, all attempts to produce CPO knock-outs also failed, probably due to the fact that the complementary DNA strand to the CPO gene encodes an essential protein in *C. fumago*. Apparently, the Cys mutant can be tolerated on the complementary strand but not mutations at Glu<sup>183</sup>.

We turned to the *A. niger* expression system for the replacement of Glu<sup>183</sup> with a histidine residue. These experiments were successful (67). The E183H mutants had diminished activity in terms of their chlorination, peroxidation, and catalase activities, but surprisingly, their epoxidation activity was increased by more than two-fold.

His<sup>148</sup> is adjacent to and hydrogen-bonded to Glu<sup>183</sup>. Early experiments showed that chemical modification of His<sup>148</sup> destroyed the halogenation activity of CPO (69). Much later, in a paper entitled “Chloroperoxidase, a Janus Enzyme,” we showed that modification of His<sup>148</sup> did not affect the ability of CPO to catalyze one-electron oxidations (70). We concluded that all two-electron oxidations carried out by CPO occur at the heme active site of the enzyme and require the participation of His<sup>148</sup>, whereas the one-electron oxidations use a different face of the enzyme, where the oxidations occur outside the buried heme active site, presumably at the surface of the enzyme.

### Asymmetric Epoxidations Catalyzed by CPO

The discovery that Cys<sup>29</sup> contributed a thiolate ligand to the CPO iron heme prosthetic group suggested that CPO is related to the cytochrome P450 family and further suggested that we should explore oxygen insertion reactions catalyzed by CPO. In collaboration with Eric N. Jacobsen's lab, Eric J. Allain in my lab found that CPO catalyzed highly enantioselective epoxidation of disubstituted alkenes (26). The incubation of CPO, hydrogen peroxide, and *cis*- $\beta$ -methylstyrene produced the stereospecific formation of the corresponding *cis*-epoxide in 100% yield and 96% enantioselectivity.

A large number of olefins were screened for epoxidation, and we found that a number of *cis*-disubstituted alkenes bearing alkyl substituents were good substrates for the chiral epoxidation reaction. In an olefin-screening process, we identified 2-methyl-1-alkenes as a general class of substrates with high turnover and high enantioselective epoxidation (71). It became apparent that, in those

olefins giving high enantioselective products, a terminal methyl group was an important substituent that allowed both high turnover and enantioselectivity. Epoxidation of several monosubstituted olefins gave low catalytic turnover with poor to moderate enantioselectivities (72).

Size was an important factor in the epoxidation reaction. Substrates containing nine linear carbon atoms were substrates, presumably because they were small enough to enter the channel that allowed them an approach to the heme active site. Substrates containing 10 or more linear carbons were not substrates. Overall, the results indicated that methallyl alkenes and styrenes can function as good substrates.  $K_m$  values for the methallyl substrates are in the millimolar range, and the  $V_{max}$  values reach turnovers of 200/min. Hammett plot data are consistent with the formation of a radical as opposed to a carbocation intermediate in the rate-determining step in the epoxidation of substituted styrenes (73).

### The Lazarus Effect

We became interested in the possibility of using CPO in the production of pseudoephedrine. The commercial source of this important cold remedy is a Chinese plant and is imported in large quantities. As discussed above, *cis*- $\beta$ -methylstyrene is an excellent substrate for CPO, and the epoxidation product can be converted by the addition of methylamine to form pseudoephedrine.

Amoco Oil Company indicated that it could produce large quantities of *cis*- $\beta$ -methylstyrene, and we visualized the development of a competitive process for the large-scale synthesis of pseudoephedrine. Amoco supplied us with a sample of its *cis*- $\beta$ -methylstyrene, and unfortunately, we found that the Amoco product was contaminated with trace amounts of allylbenzene (<1%).

When CPO was incubated with the *cis*- $\beta$ -methylstyrene/allylbenzene mixture, CPO was rapidly and quantitatively converted to a green enzyme species that lacked enzyme activity. Further study showed that the reaction of CPO with allylbenzene produced small amounts of allylbenzene oxide (80 eq) before becoming inactivated (74).

Upon standing, the green inactive CPO slowly returned to the native CPO. The reactivation was temperature-dependent: at 5 °C, reactivation took about 3 days, and at 25 °C, the half-life for restoration of activity was 5.8 h. Mass spectral analysis of the green enzyme revealed a new heme species, having a mass spectrum consistent with the addition of allylbenzene plus an oxygen atom to the heme prosthetic group. The Mössbauer and EPR studies identified that the green heme resulted from the *N*-alkylation of the heme (75). Because we knew that CPO was dead but could return to life, we termed this the "Lazarus Effect."

### Directed Evolution Experiments with CPO

The suicide inactivation of CPO by allylbenzene was not unique; similar results were obtained when 4-pentenoic acid, allyl bromide, or allyl alcohol was incubated with CPO. These results suggested that all terminal unsaturated substrates mediated the suicide inactivation reaction. We reasoned that it might be possible to prevent the inactivation via mutation.

Gyan P. Rai investigated the isolation of directed evolution mutants that would be resistant to inactivation by primary olefins (76). Rai prepared plasmid vectors containing error-prone copies of the CPO gene and a hygromycin-resistance marker gene, and these vectors were used to transform *C. fumago* spheroplasts for the production of mutant libraries. The mutant libraries were screened for their ability to resist mechanism-based inactivation by allylbenzene. Four generations of PCR-based random mutagenesis produced mutants that were completely resistant to the suicide inactivation reaction. These results suggested that directed evolution could be used to produce CPO mutants that could be valuable for the synthesis of chiral intermediates. Directed evolution also was used to isolate mutants with enhanced epoxidation and chlorination activity (77).

### Chiral Synthons via CPO Catalysis

Seeing that CPO could be produced in very large amounts, we explored its utilization for the large-scale synthesis of chiral compounds. Frederick J. Lakner demonstrated the use of CPO as an effective catalyst for the efficient synthesis of gram quantities of (*R*)(-)-mevalonolactone (78). All chemical syntheses of this mevalonolactone have many steps, low overall yield, and moderate enantiomeric excess, and they require expensive starting materials.

In contrast, CPO catalyzes the epoxidation of 3-methyl-3-butenol, and the resulting epoxide is converted to the mevalonolactone in two easy steps. Similarly, CPO can function as a chiral epoxidation catalyst in the large-scale synthesis of (*R*)-dimethyl 2-methylaziridine-1,2-dicarboxylate, a potential  $\alpha$ -methylamino acid synthon (79).

We had high hopes that these demonstrations of the large-scale synthesis of chiral compounds could be adopted by the pharmaceutical industry and used for the synthesis of chiral synthons. Although the use of CPO for this purpose has not yet developed, we continue to believe that CPO could be useful in the chiral synthesis of precursors of chiral drugs.

Alkynes are important intermediates for the synthesis of a variety of complex molecules, and the chemical ox-

dation of alkyne triple bonds has been widely investigated. However, there are only a few examples of propargylic oxidations. CPO catalyzes the enantioselective oxidation of 2-alkynes to aldehydes in the presence of hydrogen peroxide or *t*-butyl hydroperoxide via the intermediate formation of the propargylic alcohol (80). Acetylene is a poor substrate (57% enantioselectivity and 7% yield); however, substituted acetylenes give high yields of asymmetric alcohols in the range of 91–94% yields and 80% enantioselectivity.

### Biosynthesis of Methyl Chloride

The most abundant halohydrocarbon in the upper atmosphere is methyl chloride. The annual production of methyl chloride is estimated to be  $5 \times 10^6$  tons. In early searches for the biological synthesis of halogenated marine compounds, we found that marine algae were capable of producing methyl chloride. In one of our last collecting trips, we traveled to the Hopkins Marine Station in Pacific Grove, California, with the intention of screening marine plants for the synthesis of methyl chloride.

After our first dive off the Marine Station beach, we pulled the boat on shore with some difficulty through a big growth of ice plant. I suggested to Ann Wuosmaa that she check to see if ice plant produced any methyl chloride. Much to our surprise, ice plant produced abundant amounts of methyl chloride, and subsequent investigations found that ice plant extracts contained an enzyme that catalyzes the formation of methyl chloride using *S*-adenosyl-*L*-methionine as the methyl donor (81).

Other halophytic plants also are good sources of the enzyme. *Batis maritima*, an abundant halophytic plant, was collected in large amounts and served as the source for the isolation of the enzyme. Xinhai Ni isolated homogeneous methyl chloride transferase preparations that allowed the cDNA cloning of the gene (82). Ni then expressed the methyl chloride transferase in *E. coli* (33). When grown in high salt concentrations, the transformed *E. coli* cells evolved methyl chloride from the culture medium.

Since our discovery of CPO, many haloperoxidases have been discovered (see review in Ref. 30). Many of these halogenating enzymes use quite different mechanisms for the attachment of a halogen to a carbon atom. Vanadium haloperoxidase, non-heme iron  $\alpha$ -ketoglutarate halogenases, flavin-dependent non-metallohalogenases, and *S*-adenosyl-*L*-methionine-dependent chlorinases are examples (30).

Usually, Mother Nature evolves a chemical reaction and preserves it in new evolving types of reactions. Hemoglobin is a good example. There are many different hemoglo-

bins, but they all contain the same iron protoporphyrin prosthetic group.

The utilization of thyroxine as a metabolic regulator is another example of a preserved reaction. The formation of thyroxine must have first been developed in amphibians and continued in the evolution of land mammals, even though there are large land masses that are deficient in iodine. In contrast, chemical halogenation reactions occur via several different reaction mechanisms using unrelated chemistry.

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