CHAPTER 1

HISTORICAL: PIONEERING WORK ON HORSERADISH AND YEAST CYTOCHROME *c* PEROXIDASES

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INTRODUCTION

Although examples of what is now known as enzyme action were described in the early nineteenth century (see Paul, 1963; Saunders et al., 1964), the beginning of the modern era of peroxidase research may be defined as starting with the studies of Bach and Chodat (1903). They demonstrated that their enzyme isolated from horseradish was a true peroxidase, not an oxygenase.

Peroxidase research was almost exclusively on the horseradish enzyme until the 1940s. Today the annual research publications on horseradish peroxidase (HRP) still outnumber the total for all other peroxidases. Discovery dates for some other well-known peroxidases are yeast cytochrome *c* peroxidase and chloroperoxidase, 1940 (although further work did not begin for another 25 years); myeloperoxidase, 1941; and lactoperoxidase, 1947. The majority of the known peroxidases have been discovered since 1970.

In the 1920s and 1930s a controversy raged about the true nature of enzymes, with Richard Willstätter insisting upon the role of metal ions, from his work with horseradish peroxidase, and James Sumner claiming that enzymes were pure proteins, from his work on jackbean urease and other enzymes. Thus, from the controversy between a Nobel Prize winner and a future winner, it was established that there are metalloenzymes and also pure proteins that are enzymes.

The classic work of Michaelis and Menten (1913) interpreted observed enzyme kinetics in terms of an enzyme–substrate complex, a lock-and-key hypothesis. This became the the accepted explanation for rectangular hyperbolic kinetics. The asymptotic limit was ascribed to saturation of the enzyme binding site by substrate.

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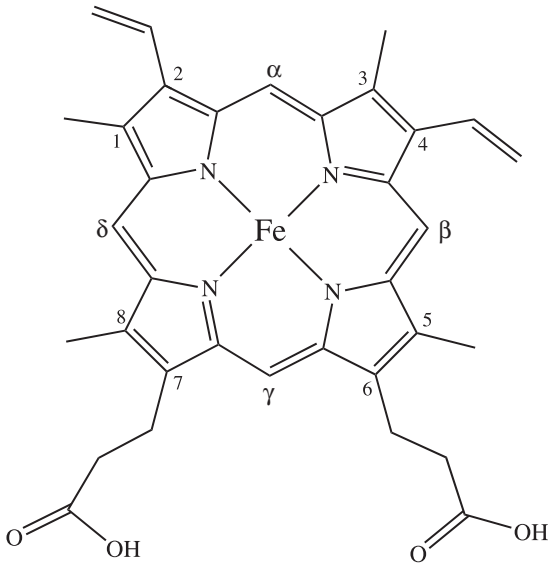


Figure 1.1. Iron protoporphyrin IX (heme *b*).

Therefore, it took time and many investigations, as exemplified by the work of Philip George (1952, 1953a, 1953b), to establish that horseradish peroxidase formed covalent compounds between its heme iron and fragments of hydrogen peroxide, not loose complexes with the entire hydrogen peroxide molecule. What is now known as Compound I was discovered by Hugo Theorell (1941). Theorell was awarded the Nobel Prize in Medicine in 1955 for his work on oxidative enzymes. The discovery of Compound II occurred earlier (Keilin and Mann, 1937). Compound II of HRP is the first enzyme intermediate to have been identified.

Free radical formation in peroxidase reactions violated another perception about enzymes, namely that they were designed to prevent free radical formation, all deemed to be physiologically harmful. Heme peroxidases, their compounds and complexes, are highly colored, especially in the near ultraviolet (Soret) region of the spectrum, a property that offers many advantages over colorless proteins in designing experimental work.

Both yeast cytochrome *c* peroxidase and horseradish peroxidase have ferriprotoporphyrin IX, also called heme *b*, as their prosthetic group, a simpler heme than is found in most mammalian peroxidases (Fig. 1.1). The intensity of research on these two enzymes makes them ideal topics for an introduction to peroxidase and catalase enzymes.

TECHNIQUES AND INSTRUMENTATION

The following is a brief discussion of dominant techniques used in peroxidase research. In addition to standard biochemical procedures, many of the techniques used in inorganic chemistry provide essential information about metalloenzymes.

Chromatography

Although botanists utilized chromatographic techniques as early as 1900, it was the development of a host of gels, resins, and polymers with a wide range of properties that made chromatography the key to successful enzyme isolation and purification. Thus, the late Karl-Gustav Paul (1958) used carboxymethylcellulose chromatography to both simplify and improve the purification of horseradish peroxidase. Paul described enzymology in terms of the chromatographic era and the prechromatographic era.

Amino Acid Sequencing

Frederick Sanger won the Nobel Prize in Chemistry in 1958 for determining the primary amino acid sequence of insulin. His pioneering work was followed rapidly by the sequencing of many proteins. The first primary sequence of a peroxidase was that of horseradish (Welinder, 1976). This was followed by the determination of the yeast cytochrome *c* peroxidase sequence (Takio et al., 1980; Bosshard et al., 1991). Today amino acid sequences are often deduced indirectly from nucleic acid sequences using the genetic code.

Site-Directed Mutagenesis

The 1993 Nobel Prize in Chemistry was awarded to Kary Mullis and Michael Smith for development of the techniques required for site-directed mutagenesis. Yeast cytochrome *c* peroxidase was one of the first enzymes to be cloned and undergo mutagenesis. A mutant enzyme is a new molecule, for which all the techniques that were applied to the wild-type enzyme may be required for characterization.

UV-Visible Spectrophotometers

In today's biochemical laboratories use of sophisticated spectrophotometers is routine. At the end of World War II the best commercial spectrophotometer was the Beckman DU using a car battery as the most stable power supply. One can see the limitations placed on earlier researchers. A paper that I found especially valuable when I started research on horseradish peroxidase was from David Keilin's laboratory where they used a Zeiss microspectroscope and a Hartridge reversion spectroscope (Keilin and Hartree, 1951). Spectrophotometry is especially relevant to peroxidase research because of the intense and different coloring of native enzyme, Compound I, and Compound II. Indeed, transformations in peroxidase reactions provide a direct measure of the number of electrons being transferred.

Spectroscopic Techniques Developed by Physicists

The following four spectroscopic techniques invented by physicists are described in some detail in Chapters 8 and 9. Of the four, only Mössbauer spectroscopy has remained largely in the hands of physicists.

Nuclear Magnetic Resonance (NMR). Discovered by Konrad E. Bloch and Edward M. Purcell in 1946, it led to their Nobel Prize in Physics in 1952. All modern chemical and biochemical laboratories have NMR facilities, although full utilization of the power of NMR requires the knowledge of specialists. In peroxidase research these include Gerd La Mar, Isao Morishima, James Satterlee, and Nigel Veitch. Nobel Prizes in Chemistry were awarded to Richard R. Ernst (1991) for the development of two-dimensional NMR, and to Kurt Wüthrich (2002) for his contributions to three-dimensional protein structural determinations in solution by NMR.

Electron Spin Resonance (ESR). Described by E. Zavoisky in 1945. At an anniversary celebration in Russia, both American and Russian scientists had words with a Nobel Prize committee member saying that Zavoisky deserved a prize, unfortunately to no avail. ESR spectroscopy is widely used, but the work of Ronald Mason deserves special mention.

Mössbauer Spectroscopy. Named after its discoverer, Rudi Mössbauer, who published a description in 1957 and received the Nobel Prize in Physics in 1961. Use of this technique relevant to peroxidases has been conducted by Peter Debrunner, Vincent Boi Han Huynh, Eckard Münck, and Alfred Trautwein.

Resonance Raman Spectroscopy. Raman spectroscopy is named after Chandrasekhara Venkata Raman who discovered it in 1928 and received the Nobel Prize in Physics in 1930. Like infrared spectroscopy, Raman spectroscopy measures transitions between vibrational levels. Resonance Raman spectroscopy is a post-World War II development. In it a laser is used to cause electronic as well as vibrational transitions with enhancement of the latter. Specialists include Teizo Kitagawa, Giulietta Smulevich, Thomas Spiro, and James Terner.

Crystal Structures

The use of X-ray diffraction to elucidate the structures of simple salt crystals and crystals of small molecules was developed by physicists and led to Nobel Prizes in Physics to Max von Laue in 1914 and to the father-and-son team of William Henry Bragg and William Lawrence Bragg in 1915. However the structures of macromolecules were too complicated for the techniques developed by von Laue and the Braggs. The use of heavy metal ions to provide a marker and the determination of primary amino acid sequences provided the necessary breakthroughs, and chemists led the way. The first protein crystal structure determined by X-ray diffraction was that of myoglobin (Nobel Prize in Chemistry to John Kendrew and Max Perutz in 1962).

The first published peroxidase crystal structure was that of yeast cytochrome *c* peroxidase (Poulos et al., 1980). That of horseradish peroxidase was not published until much later (Gajhede et al., 1997), and was aided by comparisons to the crystal structure of peanut peroxidase (Schuller et al., 1996). X-ray analysis of peroxidase crystal structures is now widespread. The groups headed by Thomas Poulos and Ignacio Fita deserve special mention because of the extent of their contributions.

Rapid Reaction Kinetics

Steady-state enzyme kinetic studies are often performed in a conventional spectrophotometer where the dead time (time elapsed between start of the reaction and experimental observations) is one second or greater. By using a plunger to drive the solutions in two syringes through a mixing chamber, the dead time can be reduced to milliseconds. The first example was an accelerated flow apparatus (Chance, 1940a, 1940b), in which the two syringes were pushed manually. A mechanical stop was introduced, thus creating a stopped flow apparatus (Gibson, 1954). For many years the Durrum–Gibson stopped flow apparatus was the commercial instrument of choice. Reaction traces could be obtained only at one wavelength at a time.

I had long desired a stopped flow with rapid spectral scan capabilities. A commercial instrument became available that had a rapidly rotating prism. I wrote to Jui Wang at Yale, who possessed one of the instruments, asking his opinion. His reply, dated 1969, said that the apparatus was fine for preliminary work but was unsatisfactory for quantitative studies. I waited eight more years until the Union Giken stopped flow with rapid scan became available, and possessed the first instrument sold outside Japan. The instrument uses a fixed grating for spectral measurements. Now there are several reliable commercial instruments that combine stopped flow, rapid scan, and sequential mixing capabilities.

The time required to mix two solutions uniformly limits the time at which accurate measurements can be started. The ingenious relaxation techniques, developed principally by Manfred Eigen, eliminated the mixing step. Eigen shared the Nobel Prize in Chemistry in 1967. If a reversible reaction has a finite enthalpy of reaction, then a temperature jump will shift the equilibrium position. The rate of the shift is a function of both the forward and reverse rate constants, and the time required to reach the new equilibrium position is called the relaxation time. Discharge of a high voltage capacitor through the solution can cause the temperature rise. Reaction rates in the microsecond range can be measured using the temperature jump technique (Czerlinski and Eigen, 1959). In principle, the relaxation times, if not the individual rate constants, of all of the steps in a reversible enzyme reaction could be measured in a single T-jump experiment. To the best of my knowledge this has not yet been accomplished. With the use of lasers, reaction times in the femtosecond range and faster can now be measured.

Mass Spectrometry

Developments in mass spectrometry have led to its use as a valuable tool for analysis of biopolymers (McLafferty, 2008). The technique appears to be underused by peroxidase researchers; there are few examples (Donald et al., 2003; Choudhary et al., 2006).

SUMMARY AND CONCLUSIONS

Nearly all of the contributions outlined above were made after World War II. One can only marvel at the patient accomplishments of earlier scientists who paved the way.

The peroxidases are extremely potent catalysts of oxidation. One of the first stopped flow apparatuses I used was machined from a block of polypropylene. Horseradish peroxidase and its substrates started to eat it. There is a recent report that horseradish peroxidase will degrade single-walled carbon nanotubes (Allen et al., 2008). The potency of peroxidase catalysis deserves more emphasis.

A property that has perhaps been overemphasized is the nonspecificity of peroxidase catalysis. The requirements for an oxidizing substrate are highly specific: a molecule of the general formula ROOH that enters the heme cavity to donate an oxygen atom to iron(III). Specific reducing substrates appear to choose one of three routes to donate electrons: at the δ-heme edge, the γ-heme edge, or through the heme propionate side chains. Look at the natural reducing substrates of specific peroxidases. Three of them are included in the name: ascorbate peroxidase, manganese peroxidase, and yeast cytochrome *c* peroxidase. Lignin peroxidase utilizes veratryl alcohol, chloroperoxidase from *C. fumago* favors monochlorodimedone. Catalases utilize hydrogen peroxide as both oxidizing and reducing substrate. Salivary peroxidase utilizes thiocyanate. *Mycobacterium tuberculosis* catalase-peroxidase reacts very well with the antituberculosis drug isoniazid (but perhaps the past tense is more appropriate). Myeloperoxidase reacts with chloride but subsequent reactions may occur through chloramines; taurine is a favored substrate. Similarly, thyroid peroxidase reacts with iodide but further reaction may occur via sulfenyl iodides. There appears to be a physiological symbiosis between enzyme (peroxidase or catalase) and reducing substrate. Peroxidases continue to evolve and new ones to be discovered. Chlorite dismutase is a recent example. A detailed story of the peroxidases and catalases follows, not only of heme peroxidases, but glutathione peroxidases and the vanadium peroxidases.

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