Mössbauer Spectroscopic Evidence for the Electronic Configuration of Iron in Horseradish Peroxidase and Its Peroxide Derivatives*

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ABSTRACT: The Mössbauer spectra of horseradish peroxidase and its peroxide derivatives have been studied. Optical spectra of frozen solutions prepared under the same conditions as the Mössbauer samples have been used to confirm that concentrated frozen samples of compounds I and II were obtained in nearly pure form.

The data show that there is a major change in iron electronic configuration on going from the resting enzyme to the primary or secondary compounds (compounds I and II) formed when the protein interacts with peroxide. This change is compatible with the formation of an Fe(IV) configuration. There is no major change in Mössbauer spectra on going from compound I to II, so that the extra oxidizing equivalent of compound I is probably not found in a Fe(V) configuration, but instead must be localized at a site other than that of the iron itself.

Horseradish peroxidase (HRP,[1] donor:H_2O_2 oxidoreductase, EC 1.11.1.7) is of special interest for biophysical studies of heme proteins, because of the possible existence of oxidation states of the iron higher than Fe^{3+}. The green compound I (HRP-I), formed rapidly on reaction of Fe(III) HRP with hydrogen peroxide or alkyl hydroperoxides (Theorell, 1941), has two oxidizing equivalents above the Fe^{3+} state (Chance, 1952; George, 1953). The red compound II (HRP-II), formed more slowly from compound I (in absence of substrate), retains one oxidizing equivalent above Fe^{3+} (George, 1953; Yonetani, 1966). The existence of these extra oxidizing equivalents suggested a possible Fe(V) configuration for compound I and an Fe(IV) configuration for compound II (George, 1956; George and Irvine, 1957). Magnetic susceptibility studies (Theorell and Ehrenberg, 1952) were compatible with this interpretation. However, other structures, involving a combination of free radicals, and the more typical Fe(III) configuration (for review, see Saunders *et al.*, 1964) or a low-spin Fe(II) configuration with the additional oxidizing equivalents on the iron-bound ligand or porphyrin ring (Peisach *et al.*, 1968), have also been suggested. At least some of these alternatives are equally compatible with all previous data. This paper reports the results of Mössbauer experiments designed to distinguish these possibilities experimentally.

Mössbauer spectroscopy is sensitive to the electronic configuration immediately surrounding the iron nuclei. In this way it is an especially useful technique for judging if the electronic charge actually localized on the iron is affected by changes in formal valence state, such as occur in going from HRP to HRP-I and HRP-II. That is, it is a way to check if the formal valence changes are reflected in the occupation of molecular orbitals of substantial iron d character. From our results we conclude that in this sense there is some validity to the idea of an Fe(IV) state in compound II. There is a fundamental change in iron configuration in going from HRP to HRP-II, and it is compatible with a net loss of electrons from the d shell. However, the Fe(V) state postulated for compound I probably does not exist: the electronic configurations of the iron in HRP-I and HRP-II appear essentially the same.

The basic chemical and physical interpretations of the Mössbauer spectral parameters are fully discussed in several reviews (Fluck *et al.*, 1963; Goldanski, 1963; Wertheim, 1964).

Experimental Section

HRP was prepared according to Paul (1958) and only chromatographic fraction C from the CMC column was used. The prosthetic group was split off and the apoprotein was isolated (Theorell and Maehley, 1950). HRP was then reconstituted using ^{57}Fe protoheme which had been prepared by the method of Caughey (Caughey *et al.*, 1966) with the following modifications.

To ensure the absence of water during the reconstitution of protoheme from protoporphyrin IX and ^{57}Fe, 25 ml of acetic anhydride was added to a solution of 300 mg of protoporphyrin IX in 250 ml of redistilled glacial acetic acid. This was heated to boiling and 300 mg of powdered NaCl was added. Hot [^{57}Fe]ferrous acetate was then added dropwise until the fluorescence disappeared, by using a syringe fitted with a filter to avoid additions of undissolved iron powder. Then 3 g of tetraethylammonium chloride dissolved in 20 ml of redistilled glacial acetic acid was added. The system was oxidized by a stream of dry air; 200 ml of glass-distilled water was added and the precipitate formed was collected by centrifugation. The yield was 229 mg.

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[1] Abbreviations used are: HRP, horseradish peroxidase; JRP, Japanese radish peroxidase.

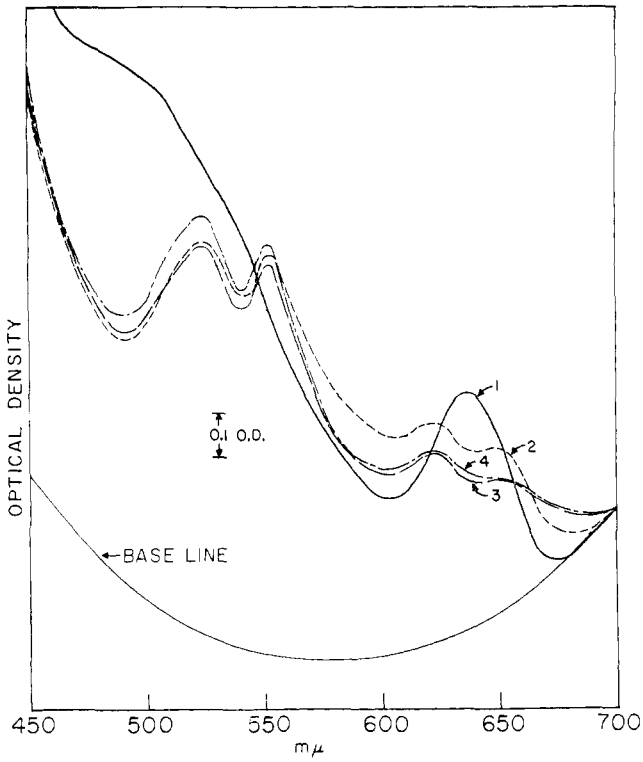

FIGURE 1: Optical spectra of HRP and EtOOH-treated HRP at pH 8.9 and 77°K. Curve 1, untreated 1.7 mM HRP. Curve 2, the same treated with twofold molar excess of EtOOH and frozen in 70 sec. Curve 3, the same, after thawing for 90 sec and refreezing. Curve 4, the same, after thawing for 7 min and refreezing.

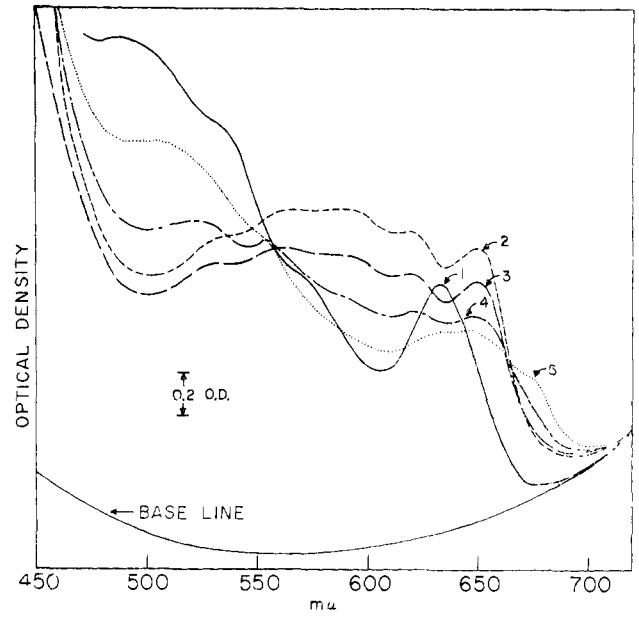

FIGURE 2: Optical spectra of HRP and EtOOH-treated HRP at pH 3.5 and 77°K. Curve 1, untreated 1.7 mM HRP. Curve 2, the same treated with a twofold molar excess of EtOOH. Curves 3, 4, and 5, the same, after thawing for 1, 3, and 12 min, respectively, and refreezing.

Instead of Celite chromatography, further purification was done by countercurrent distribution. The solvent system was made by shaking 2.5 volumes of pyridine, 2.0 volumes of glass-distilled water, and 1.25 volumes of chloroform in a separatory funnel and allowing the layers to separate. Then five volumes of upper phase and five volumes of lower phase were mixed with two volumes of methyl alcohol, shaken, and allowed to separate again. The upper and lower phases of this system were used in the countercurrent distribution. Typically, about 70 mg of ^{57}Fe protoheme were obtained in a fraction with absorption maxima (as pyridine hemochromogen) at 418, 515, and 557.5 mμ.

The visible and ultraviolet absorption bands of the reconstituted HRP, just before the first Mössbauer experiments, were at 640 (0.31), 496 (1.00), 402 (9.55), and 275 (2.70) mμ. The relative absorbancies are given in parentheses. All Mössbauer samples were frozen solutions. Samples were checked spectrophotometrically before and after the Mössbauer experiments to verify that freezing and thawing, and exposure for 10 hr to a 20-mCi Mössbauer source, did not damage the protein. There were no detectable changes in the optical absorption.

Compound II was made by mixing 8 μg of 240 mM ethyl hydroperoxide (EtOOH) (twofold molar excess) with 0.5 ml of 2 mM HRP in 0.15 M borate buffer (pH 9.3) (Theorell *et al.*, 1952). The color change from brown to green was instantaneous; after a few seconds the green color gave way to red. Four samples, prepared in the same way but frozen in liquid nitrogen 70, 100, 150, and 180 sec after mixing were studied. An aliquot of the material examined at room temperature 5 min after mixing showed absorption bands at 420 (0.85), 528 (0.07), and 555 (0.08) mμ, with relative absorbancies in parentheses. At the conclusion of the Mössbauer experiments the optical absorption of an aliquot of the thawed material was examined; the only difference was a small change in absorbance at 500 mμ, indicating the presence of about 5% HRP in the sample. It was found that compound II did not completely revert to HRP even after 8 hr at 20°. Optical spectra of a frozen solution (77°K) of EtOOH-treated HRP, made exactly as the Mössbauer samples, were also taken to verify that the highly concentrated frozen material actually contained principally the compound II characterized in the literature by experiments at room temperature. The spectra, shown in Figure 1, display all the features of compound II (Chance, 1949; Blumberg *et al.*, 1968) for times considerably longer than the time required to freeze the Mössbauer samples to 77°K. Samples of compound II allowed to stand at 20° for 24 hr showed the Mössbauer spectrum of the original unreacted HRP.

Compound I (two independent samples) was made by mixing a similar twofold molar excess of EtOOH with 0.5 ml of 2 mM HRP, but in citrate buffer at pH 3.5. At this pH compound I is stabilized in the reaction system relative to compound II (Theorell *et al.*, 1952). Samples were frozen in liquid nitrogen at about 70 sec after mixing. Because of the rapid decay of HRP-I to HRP-II, no room temperature optical spectra could be taken of HRP-I. However, frozen solutions of HRP-I, prepared under the same conditions as the Mössbauer sample, were examined spectrophotometrically at 77°K. The spectra are shown in Figure 2. The sample is more than 90% HRP-I, as judged from published spectral data (Chance, 1949; Blumberg *et al.*, 1968). In order to ascertain independently that compound I was the principal component of the system

TABLE I: Mössbauer Parameters for HRP and Its Peroxide Derivatives.

Compound (°K)	Quadrupole Splitting (ΔE)	Isomer Shift (δE)
HRP		
205	1.87 ± 0.03 mm/sec	+0.18 ± 0.03 mm/sec
77	1.96	+0.25
4.6	Too broad to determine parameters.	
HRP II		
205	1.37 ± 0.02	−0.02 ± 0.02
77	1.36	+0.03
4.6	1.42	+0.03
HRP I		
77	1.20 ± 0.02	0.00 ± 0.02

[a] All isomer shifts are measured relative to the center of the Fe metal spectrum.

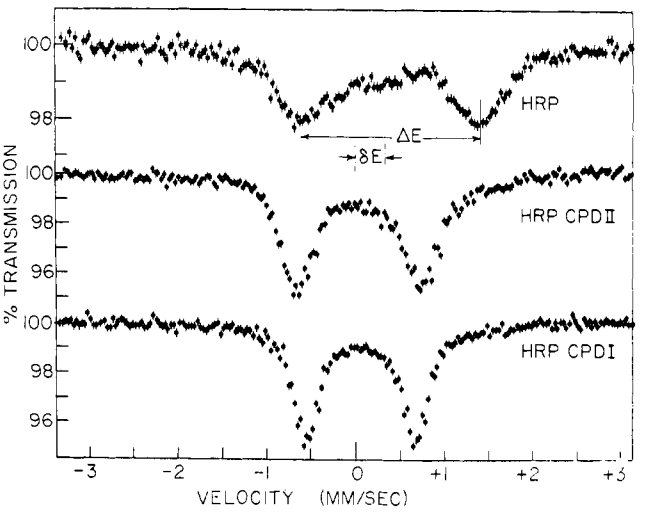

FIGURE 3: Mössbauer spectra of HRP and HRP I and II at 77°K.

for the freezing times used, the room temperature kinetics of the samples were studied by observing the absorption at 650 mμ as a function of time after mixing. These experiments at 25° indicated a time of about 130 sec for the disappearance of one-half of the compound I in the initial mixture. Thus at 70 sec, the time when our samples were completely frozen, a *lower limit* of 75% of the original compound I was in the solution. The actual fraction of compound I is probably considerably higher than this, as the reaction rate must be slowed down as the sample is chilled, even before complete freezing.

The Mössbauer apparatus used for these measurements has been described previously (Bearden *et al.*, 1965a). To ensure that the samples were actually at the measured temperature of the sample holder and monitor resistor, ^{4}He-exchange gas was sealed in the sample chamber.

Results

The Mössbauer spectral results at 77°K are shown in Figure 3. The numerical results are tabulated in Table I. The quadrupole splitting, ΔE, is measured from the splitting of the absorption lines; the isomer shift, δE, is the energy difference between the center of the sample Mössbauer spectrum relative to the center of an iron metal spectrum.

Samples of compounds I and II allowed to stand for 24 hr at 20° gave Mössbauer spectra very similar to those obtained with unreacted HRP. The fact that all of the Mössbauer absorption changes in going from HRP to compound II indicates that there is a negligible amount, if any, of the ^{57}Fe heme which is not specifically bound to the protein in an enzymatically active conformation. Difficulty with nonspecifically bound heme was experienced in previous attempts at reconstituting iron proteins with the Mössbauer isotope (Caughey *et al.*, 1966).

Discussion

All of the high-spin ($S = 5/2$) Fe(III) heme proteins examined so far by Mössbauer spectroscopy have very similar quadrupole splittings, isomer shifts, and temperature-dependent line broadenings (Lang and Marshall, 1966; Moss *et al.*, 1968). The fact that HRP shares these distinctive Mössbauer spectroscopic features of the other proteins is in good correlation with its previously reported magnetic and optical spectrophotometric similarities to them (for review, see Saunders *et al.*, 1964).

The large difference between the Mössbauer spectra of compound II and that of unreacted HRP indicates a fundamental difference in the iron configuration. The symmetry of the charge cloud, as reflected by the quadrupole splitting, has obviously changed. Further, since no hyperfine splitting or line broadening is observed even at 4°K, either a ground state with ground level $S_z^2 = 0$ has been formed, or the relaxation time of the electronic spin in the new state has become very much shorter (Bradford and Marshall, 1966).

As well as the change in quadruple splitting and line broadening, there is a decided change in the isomer shift in going from resting HRP to HRP II. Total s electron density and isomer shifts are a function of the occupancy of the d shell, because the d electrons tend to shield the nucleus from the s electrons (Walker *et al.*, 1961; Wertheim and Herber, 1962; Gallagher *et al.*, 1964, 1966). The change toward less positive values of the isomer shift in going from HRP to compound I or II can be explained by assuming a charge configuration with less d electron density than is found in the resting Fe(III) enzyme. Though covalent effects make unambiguous interpretations of Mössbauer isomer shifts difficult (Shulman and Sugano, 1965), the data are certainly compatible with long-standing ideas that the extra oxidizing equivalent of HRP compound II represents an actual loss of charge from the iron, creating an Fe(IV)-like state. Recent susceptibility measurements on the spectrophotometrically analogous complexes of cytochrome peroxidase also point toward an Fe(IV) state (T. Iizuka, M. Kotani, and T. Yonetani, to be published).

The similarity of Mössbauer spectra of compound I with those of compound II suggest that there is no difference in iron configuration between these two compounds comparable with that between them and unreacted HRP. Kinetic experiments (Experimental Section) and frozen solution optical spectra (Figure 2) show that there was in fact a large proportion of

compound I in our sample, so that the null result cannot be due to conversion of compound I into compound II. For compound I, the data thus indicate that the charge associated with the second oxidizing equivalent (above Fe^{3+}) is not localized in orbitals associated primarily with the iron. It should be noted that though the effective moment, $\mu = 3.99$ BM (Theorell and Ehrenberg, 1952; Ehrenberg, 1963), of compound I is very near that expected for the orbital singlet Fe^{5+} ionic state, it would also be compatible with the measured susceptibility of compound II (Theorell and Ehrenberg, 1952; Ehrenberg, 1963) plus that of an independent free radical.

Though the difference between the quadrupole splittings of compounds I and II is too small to be attributed to a major change in iron configuration, it could indicate a perturbation of the symmetry of the iron charge cloud due to a change on the porphyrin ring associated with a free radical (Brill and Williams, 1961). Changes in quadrupole splittings of this magnitude have been observed between model heme compounds with saturated (methyl) or unsaturated (ethyl) groups on the 2,4 porphyrin positions (Bearden *et al.*, 1965b).

Our data are not easily compatible with the scheme presented by Peisach *et al.* (1968) for the electronic configurations of HRP and its derivatives. These authors propose a $3d^5$, $S = {}^3/_2$, Fe(III) configuration for compound I, which is reduced to a $3d^6$, $S = 0$, Fe(II) configuration on passing to compound II. The fact that the Mössbauer spectra of compounds I and II are nearly identical cast doubt on the major change in iron electronic configuration predicted by these authors on *converting* compound I into II.

Independent experiments similar to those of this work have recently been reported for the related protein, Japanese radish peroxidase (Maeda, 1968). Resting HRP and JRP differ in Mössbauer parameters, as they differ in magnetic properties (Morita and Mason, 1965). However, the JRP Mössbauer data for compounds I and II, including the close similarity of these two forms and their fundamental difference from the resting enzyme, are identical with our findings for HRP.

In summary, the Mössbauer data indicate a major change in iron configuration in going from resting enzyme to compound I and II. The change is compatible with the proposed Fe(IV) state for compound II. There is no major change in Mössbauer parameters in going from compound I to compound II, indicating that the second oxidizing equivalent above Fe^{3+} is not localized on the iron.

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