MÖSSBAUER EFFECT IN PEROXIDASE

Yutaka Maeda, Takenobu Higashimura

Research Reactor Institute, Kyoto University, Osaka, Japan

and Yuhei Morita

Research Institute for Food Science, Kyoto University, Kyoto, Japan

Received September 28, 1967

Mössbauer spectroscopy provides valuable information on the electronic configuration of heme-iron and the surrounding ligand field strength in heme-proteins complementary to that derived from magnetic susceptometry and electron spin resonance (ESR) spectroscopy. Quite recently, the method has been applied on some biological materials containing iron atom, and some investigations have been reported on heme-proteins such as hemoglobin, myoglobin, catalase and cytochrome c (Gonser et al. (1964); Bearden et al. (1965); Lang and Marshall (1966); Caughey et al. (1966)), but not yet on peroxidase.

This communication presents an outline of the experimental results on Mössbauer effect in Japanese-radish peroxidase a (JRP-a) and some of its derivatives enriched artificially with ^{57}Fe in order to obtain any information complementary to that derived from the ESR study of this enzyme (Morita and Mason (1965)).

MATERIALS AND METHODS JRP-a was purified by the method of Morita and Kameda (1957) and recrystallized three times in ammonium sulfate solution, the R. Z. value being 3.48. Apo-peroxidase was prepared by the acid-acetone treatment of Gjessing and Sumner (1943). Protohemin chloride enriched to 68% with ^{57}Fe was synthesized from protoporphyrin dimethylester and $^{57}Fe_2O_3$, purchased from Oak Ridge National Laboratory, by the method of ferrous

sulfate-acetic acid (Falk (1964)) and purified by column chromatography on Celite 545 using a solvent system of pyridine-chloroform-isooctane-water (Caughey and York (1962); Caughey et al. (1966)). The incorporation of protohemin enriched with ^{57}Fe into apo-peroxidase was performed in borate buffer solution, pH 9.1, and the synthesized peroxidase was purified by passing through a DEAE-Sephadex A-25 column to remove excess hematin and by-products and then through a Sephadex G-25 column to remove salts. The desalted solution was concentrated under reduced pressure at low temperature. The concentrated solution of peroxidase or its derivatives was poured into a Lucite absorber cell, 5 mm thick and 15 mm in diameter, sealed with vacuum grease and put into a liquid nitrogen bath to freeze rapidly. The cell was then mounted on a cell-holder in a low temperature Dewer. The holder was in good thermal contact with a refrigerant reservoir holding liquid helium ($4.2^{o}K$), liquid nitrogen ($77^{o}K$) or dry ice-acetone ($195^{o}K$), and the surrounding helium gas ensured the heat exchange with the absorber. For the measurements at intermediate temperature the holder was detached from the reservoir in vacuo, so that the sample was able to be set at any desired temperature by a heating wire equipped with a temperature regulator. The source used in the present experiment consisted of 30 mCi of ^{57}Co diffused into metallic Pd, which was attached to a velocity transducer of a Mössbauer spectrometer* to provide Doppler shift. The spectrometer was of the conventional linear velocity type, in conjunction with a multichannel analyzer operating in multiscaler mode. The velocity scale was calibrated absolutely from an independent Mössbauer run using a thin metallic iron absorber, taking the center of symmetry of this spectrum as the velocity zero. The velocity was determined to an accuracy of $\pm$ 0.03 mm/sec.

RESULTS AND DISCUSSION The Mössbauer spectra of a frozen solution of JRP-a dissolved in water, 0.85 ml of 2.2 mM solution, at four sample temperatures are shown in Fig. 1. Two Mössbauer parameters, i.e., isomer shift

* ELRON #5011 Mössbauer Resonance Analyzer, Elron Electric Ind., Ltd., Haifa, Israel.

and quadrupole splitting are summarized in Table I together with those for various derivatives. For free enzyme a pair of absorption lines could be observed and they became asymmetric at the lower temperatures. At 4.2°K, the pair of absorption lines disappeared almost completely, while weak hyperfine splitting lines appeared. These results are very similar to those obtained on dehydrated metmyoglobin by Caughey et al. (1966), and on methemoglobin by Lang

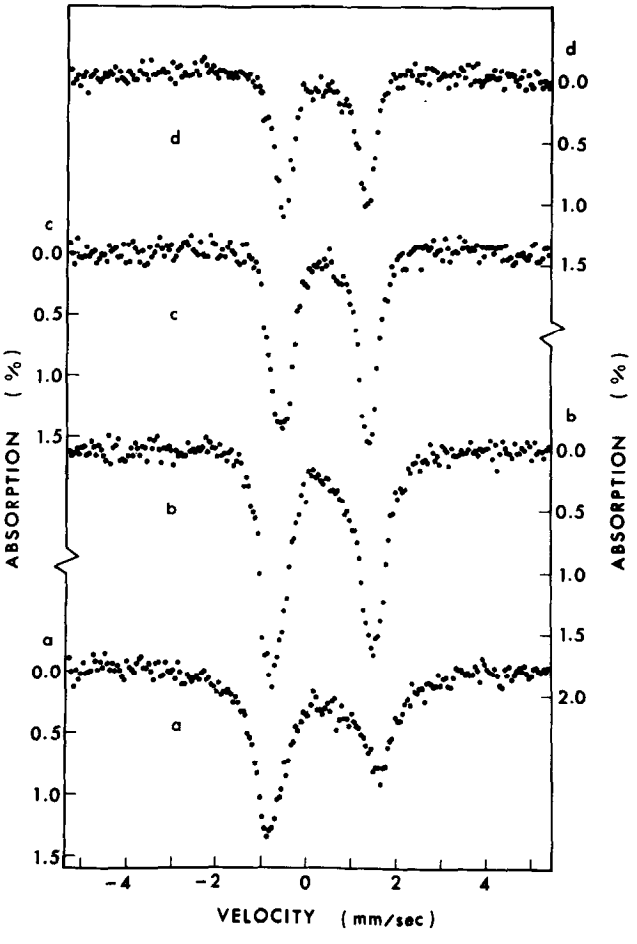

Fig. 1. Mössbauer absorption spectrum of JRP-a at (a) 77°K, (b) 120°K, (c) 195°K and (d) 243°K.

---

and Marshall (1966), although the quadrupole splitting for peroxidase was found to be highly temperature-dependent. The widely split pair of quadrupole lines and relatively small isomer shift at 77°K was compatible with low spin ferric

Table I. Isomer Shift and Quadrupole Splitting for Japanese-radish Peroxidase a and its Derivatives

Material	Temperature (°K)	Quadrupole splitting (mm/sec)	Isomer shift* (mm/sec)
Free peroxidase	243	1.84 ± 0.06	0.35 ± 0.06
	195	1.92	0.36
	120	2.28	0.34
	77	2.49	0.38
	4.2	hyperfine structure	
Hydroxide	243	1.84	0.27
	195	2.38	0.16
	120	2.36	0.22
	77	hyperfine structure	
Azide	195	2.22	0.29
	120	2.22	0.31
	77	hyperfine structure	
Cyanide	195	1.61	0.11
	120	1.71	0.13
	77	1.81	0.15

* Isomer shifts include the second-order Doppler shift due to the temperature difference between source and absorbers. The temperature of source was 293°K.

iron. As stated in the ESR study, JRP-a may be in an equilibrium of high and low spin states, and the Mössbauer spectra shown in Fig. 1 should be attributed to this low spin state of heme-iron at neutral pH, although the temperature dependence of quadrupole splitting cannot be understood until some contribution of high spin component will be considered. Hydroxide, cyanide and azide compounds of JRP-a exhibited similar absorptions characteristic to the low spin ferric iron, as expected from the ESR study. The parameters are summarized in Table I. Among them, the absorption lines for cyanide compound were sharp and quadrupole splitting was smaller than that for free enzyme, while the lines for azide and hydroxide compounds were diffused and the quadrupole splittings were somewhat larger. In azide compound hyperfine splittings could be easily

observed at lower temperatures. These results were also very similar to those for the corresponding compounds of methemoglobin. As demonstrated in ESR study by Ehrenberg (1962) and Morita and Mason (1965), azide and hydroxide compounds of metmyoglobin and peroxidase exhibited sharp ESR absorptions with anisotropic g-value centered around g = 2. On the other hand, cyanide compound of metmyoglobin and peroxidase, and probably free ferricytochrome c exhibited almost no observable or very broad ESR absorption around g = 2. The broad Mössbauer absorption lines of hydroxide and azide compounds are consistent with their critical ESR absorptions with relatively slow spin-lattice relaxation, while the sharp Mössbauer absorption lines of cyanide compound should be attributable to its faster relaxation. On the basis of the ligand field theory, it can be concluded that the latter group has a structure which is more symmetric about the axis perpendicular to the heme plane than the former group. That is, in a regular octahedral field five 3d electrons go into a lower triplet t_{2g} (d_{xy}, d_{xz}, d_{yz}) for the low spin ferric compounds, and a reduction of the symmetry of field removes the degeneracy of the t_{2g} state and lifts the d_{xz} and d_{yz} orbitals above the d_{xy} orbital. Therefore, the first excited level in the cyanide compound lies lower than in the azide and hydroxide compounds. It follows that the quadrupole splitting of the cyanide compound is small and temperature-dependent. Moreover, it should be noted that the mixing of one of t_{2g} orbitals with the vacant π-antibonding orbital of CN^- causes the partial electron transfer, reducing the quadrupole splitting of this compound.

Further studies on other derivatives such as fluoride, reduced compounds and hydroperoxide compounds are now in progress, and will be reported elsewhere.

ACKNOWLEDGMENT Protoporphyrin dimethylester was partly supplied by Dr. S. Sano, Kyoto University, to whom thanks are due.

REFERENCES

Bearden, A. J., Moss, T. H., Caughey, W. S., and Beaudreau, C. A., Proc. Nat. Acad. Sci., U. S., 53, 1246 (1965).

Caughey, W. S., Fujimoto, W. Y., Bearden, A. J., and Moss, T. H., Biochemistry, 5, 1255 (1966).
Caughey, W. S., and York, J. L., J. Biol. Chem., 237, PC2414 (1962).
Ehrenberg, A., Arkiv Kemi., 19, 119 (1962).
Falk, J. E., "Porphyrin and Metalloporphyrins", p 133, Elsevier Publishing Co., Amsterdam, 1964.
Gjessing, E. C., and Sumner, J. B., Arch. Biochem., 1, 1 (1943).
Gonser, U., Grant, R. W., and Kregzde, J., Science, 143, 680 (1964).
Lang, G., and Marshall, W., J. Mol. Biol., 18, 385 (1966), Proc. Phys. Soc., 87, 3 (1966).
Morita, Y., and Kameda, K., Mem. Research Inst. Food Sci., Kyoto Univ., 12, 14 (1957).
Morita, Y., and Mason, H. S., J. Biol. Chem., 240, 2954 (1965).