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Heme-linked ionization and ligand binding produce identical changes of proximal heme stereochemistry in reduced horseradish peroxidase

Evidence for existence of two protein conformations

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The visible and near infrared magnetic circular dichroism spectra of chemically reduced horseradish peroxidase at neutral and alkaline pH values and 5-coordinate protoheme-(2-methylimidazole) at pH 9.1 were compared at 4.2 K with those of photolysis products of their carbon monoxide complexes. From the results obtained we concluded that: (i) there are two protein conformations of HRP which determine the geometry of the Fe-N(His) bond; (ii) the transition from one conformation (heme stereochemistry) to another can be induced by either heme-linked ionization or ligand binding; (iii) a trigger mechanism for switching between two conformations has to exist.

Horseradish peroxidase; Protoheme-(2-MeIm); Carbon monoxide; Photolysis; Heme stereochemistry; Magnetic circular dichroism

1. INTRODUCTION

Theorell was the first who observed the presence of a heme-linked ionization group in reduced HRP [1]. Later, the changes of the heme group properties following ionization of a group with a pK value of 7.2 were registered and studied by optical spectroscopy [2], pH-stat titration [2], MCD [3], RR scattering [4] and ^{1}H-NMR [5] methods. The linkage of the heme spectral characteristics with ionization of amino acid residues is a common feature for plant peroxidases but the pK values depend on the species and they are quite different for various isoenzymes from the same source [6].

In this paper we compare at 4.2 K the visible and the near infrared MCD spectra of neutral and alkaline forms of reduced HRP in their equilibrium and non-equilibrium conformations generated by chemical reduction of oxidized enzyme at room temperature before freezing of the sample and by low-temperature photolysis of their CO complexes, respectively. We demonstrate for the first time that such different perturbations as heme-linked ionization and binding of a ligand to the heme iron produce identical changes in the proximal heme stereochemistry. The photolysis experiments for CO-protoheme-(2-MeIm) are described and compared with those for HRP.

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Abbreviations: HRP, horseradish peroxidase; Mb, myoglobin; His, histidine; 2-MeIm, 2-methylimidazole; MCD, magnetic circular dichroism; RR, resonance Raman

2. MATERIALS AND METHODS

HRP (Worthington, R.Z. 3.0), protohemin (Sigma, type I) and 2-MeIm (a kind gift from Dr G.D. Rudkovskaya) were used without further purification. Enzyme was dissolved in 0.2 M sodium phosphate (pH 6.0 and 6.8) or glycine-phosphate (pH 10.2) buffers. The 5-coordinate model compound was prepared from protohemin in 0.05 M borate buffer (pH 9.1) by adding 2-MeIm to a concentration of 0.1 M: To obtain transparent

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glasses for low-temperature measurements the stock solutions were mixed with a purifed glycerol (1:1.5, v/v). Ferric HRP and protohemin were reduced by a few grains of sodium dithionite (Merck) after deoxygenation of solutions under moist helium. CO forms were prepared by equilibration of the reduced samples with CO at 1 atm. All measurements were carried out in a contact-type quartz cell with a light path of 1 mm. The MCD measurements and photolysis experiments were performed at 4.2 K as described earlier [7] by using a home-made dichrograph fitted with an electromagnet and equipped with a helium cryostat.

3. RESULTS

Fig.1 shows the MCD spectra at 4.2 K of reduced HRP and metastable photoproducts of CO-complexes at pH values of 6.0 and 10.2. The shaded region outlines the spectra of the photolysis products at both pH 6.0 and 10.2 and of the equilibrium reduced enzyme at pH 10.2. Some of the differences observed in the MCD intensity can be partially explained by the errors in the measurements due to light depolarization as a result of strains and cracks in the glassy samples. The three spectra are very similar over the entire spectral region and differ drastically from those of the equilibrium reduced enzyme at pH 6.0. We have not observed any detectable distinctions between the MCD spectra of reduced HRP prepared at pH 6.0 and pH 6.8 or in 0.02 and 0.2 M phosphate buffer. Thus only two types of spectra could be observed for reduced HRP and the metastable photoproducts at the various pH and ionic strength values examined.

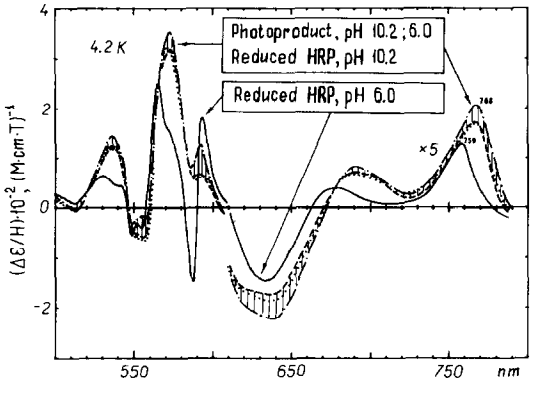

Fig.1. MCD spectra of carbon monoxide photoproducts and reduced preparations of HRP at 4.2 K. Photoproducts at pH 6.0 (···) and 10.2 (– – –); reduced HRP at pH 6.0 (——) and 10.2 (–·–·–). Enzyme concentration, 3.2×10^{-4} M; magnetic field, 1.45 T.

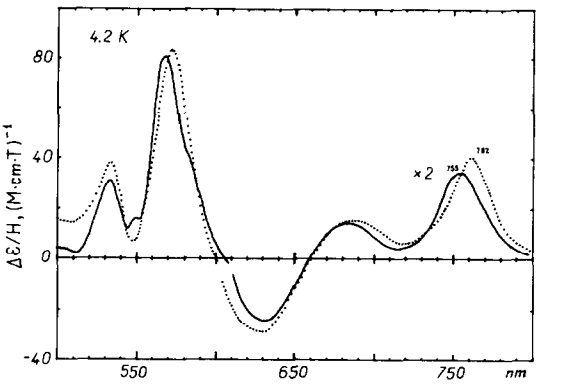

Fig.2. MCD spectra of protoheme-(2-MeIm) (solid line) and photolysis product of its carbon monoxide complex (dotted line) at 4.2 K. Heme concentration, 1×10^{-3} M; magnetic field, 1.45 T.

Fig.2 displays the result of photolysis experiment for protoheme-(2-MeIm). On lowering the temperature, the high-spin 5-coordinate model heme compound was partially and reversibly converted into the low-spin form [3,8]. The content of the low-spin species depended on porphyrin substituents, 2-MeIm concentration and the freezing rate and did not exceed 10% in our experiment. The low-spin forms lack bands in the near infrared region while in the visible MCD their presence appears only as a shoulder at 550 nm. Negligible contribution of the low-spin form in the MCD at cryogenic temperatures is due to the fact that its MCD is temperature independent, while the MCD intensity of the high-spin form increases by a factor of about 70 on cooling the sample from 300 to 4.2 K.

4. DISCUSSION

At room temperature, no differences have been observed in the transient time-resolved 7-ns RR spectrum for the photoproducts of HRP-CO at neutral pH values [10,11] as compared with the reduced enzyme including the Fe-His stretching mode, whose frequency was shifted from 244 to 241 cm^{-1} by heme-linked ionization of reduced HRP [4]. Our experiments suggest that in the RR studies at room temperature the photoproducts have presumably fully relaxed on the 7-ns time scale. Similar nanosecond relaxation has been observed in the photolysis experiments for MbCO

[12,13], but unrelaxed photoproducts were detected on the 25-ps time scale at room temperature [14]. What's more the shift of the Fe-His stretching mode between deoxy-Mb and the carbon monoxide photoproduct undetectable in the time-resolved 35-ps experiment at room temperature [15] was observed for the metastable photoproduct at 4 K [16].

Fig.2 shows that at 4.2 K the stereochemistry of the photoproduct remains unrelaxed even for protein-free heme bound with 2-MeIm. For the CO-protoheme-(2-MeIm) photoproduct the high-spin bands at ~680 and ~760 nm are red shifted relative to their equilibrium positions on the values typical for the protein bound heme [7,17]. Earlier the correlation was established between the energies of these bands and those of the Fe-pyrrols and Fe-His bonds. Therefore after photolysis at cryogenic temperatures the geometry of the Fe-porphyrin and the Fe-His bonds in the metastable photoproduct can be considered to be similar to that in a ligand bound heme.

From our results we concluded that: (i) there are no changes in a geometry of the Fe-His bond or they are very small upon ligand binding to the heme of alkaline HRP; (ii) the ligand binding to neutral HRP is accompanied with a significant rearrangement of the proximal heme stereochemistry; (iii) heme-linked ionization and ligand binding produce identical changes in the geometry of the Fe-His bond in neutral HRP; (iv) there are no detectable distinctions between the photoproducts for neutral and alkaline HRP. The latter observation suggests that the pH-dependent isomerization of the Fe-CO bond observed in CO-HRP [18,19] either does not affect the Fe-His bond or only one of two possible isomers is stablized at low temperatures.

Alkaline HRP is the first example known to us where no red shift of the near infrared bands were observed in the photoproduct at cryogenic temperature as compared with the equilibrium preparation. We interpret this fact as the existence of a rigid correlation between the geometry of the Fe-His bond and the protein conformation. The energy of conformation rearrangement required for the heme stereochemistry changes is so high that the Fe-His bond remains unchanged on ligand binding. The conformation 'pressure' on the proximal heme stereochemistry of neutral HRP is weaker than that on alkaline HRP and ligand binding is followed by changes of the Fe-His bond geometry and of the protein conformation [20].

In conclusion, (i) the ferrous HRP can exist in two conformations, two tertiary structures that determine the proximal heme stereochemistry; (ii) the transition between these two conformations can be induced by either heme-linked ionization or ligand binding to the heme; (iii) a trigger mechanism for switching between the two conformations most probably exists. The contribution of conformation to the proximal heme stereochemistry of hemoproteins and a possible role for the two conformations observed in the catalysis of HRP are discussed elsewhere [21,22].

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