Studies on Cobalt Myoglobins and Hemoglobins

INTERACTION OF SPERM WHALE MYOGLOBIN AND GLYCERA HEMOGLOBIN WITH MOLECULAR OXYGEN*

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The pH dependences of the electron paramagnetic resonance (EPR) spectrum and oxygen affinity of cobaltous porphyrin-containing myoglobin (CoMb) have been examined. The hyperfine structures of the EPR spectrum of oxy-CoMb undergo small, reversible pH-dependent changes with pH values of 5.33, 5.55, and 5.25 ± 0.05 for proto-, meso-, and deuter-CoMb's, respectively, whereas deoxy-CoMb does not exhibit any pH dependence of its EPR spectrum. The partial pressure of oxygen at half-saturation of proto-CoMb decreases from 26 to 42 Torr on lowering the pH from 7.0 to 4.8.

For comparison, we have prepared cobaltous porphyrin-containing monomeric Glycera hemoglobin (CoHb (Glycera)), in which the histidyl residue of myoglobin is replaced by a leucyl residue, and examined the equilibria and kinetics of its oxygenation and EPR spectrum. CoHb (Glycera) has exhibited a very low oxygen affinity (p50 = 7 × 10^6 Torr at 5°) and a large dissociation rate constant (more than 8 × 10^8 s^-1 at 5°). The EPR spectrum of oxy-CoHb (Glycera) was affected by neither pH nor replacement of H2O with D2O. Low temperature photodissociation studies by EPR and spectrophotometry have shown that the photolyzed form of the ligated hemoglobin (Glycera) is similar to its deoxy form, in contrast to myoglobin which gives a new intermediate state as the photolyzed form.

These differences between CoMb and CoHb (Glycera) are interpreted with relation to the possible role of the distal histidyl residue in CoMb.

The chemical substitution of ferrous protoporphyrin IX with cobaltous porphyrins in myoglobin and hemoglobin has allowed the detailed examinations of the mode of interaction between the molecular oxygen, the prosthetic group, and the apoprotein moiety in these oxygen-carrying hemoproteins by various methods [3-9], especially by EPR technique [1, 4]. Oxy-CoMb' exhibits the EPR spectrum of an essentially axial symmetry which is derived from the substantial transfer of the electron spin density from the cobaltous ion to the bound oxygen [1, 4]. Yonetani et al. [4] reported that the hyperfine structure of the EPR spectrum of oxy-CoMb is substantially narrowed upon replacement of H2O with D2O, and proposed the formation of a hydrogen bond between the bound oxygen and the histidyl group. Low temperature photodissociation study has indicated that the photolyzed form of oxy-CoMb exhibits a different EPR signal from that of the deoxy or oxy forms [4, 5]. Light absorption spectra [10] and the Mössbauer spectrum [11] of the photolyzed product of carbon monoxide-FeMb or oxy-FeMb at 4.2 K have indicated that the photolyzed product is distinguishable from deoxy-FeMb.

In order to investigate the interaction of the distal histidine with the bound oxygen, we have examined the effect of pH on the oxygen affinity and on the EPR spectrum of CoMb. In addition, as a further probe to elucidate the role of the distal histidine, we have prepared CoMb (Glycera) in which the distal histidyl residue of Mb is replaced by a leucyl residue [12]. We have studied the photodissociation of oxy-CoMb (Glycera) and carbon monoxide-FeMb (Glycera) near the temperature of liquid helium, as well as their oxygenation properties and the characteristics of their EPR spectra.

EXPERIMENTAL PROCEDURES

Cobalt porphyrins and CoMb were prepared according to the methods of Yonetani et al. [2]. Whole blood of Glycera dibranchiata was a gift of Dr. E. A. Padlan (National Institute of Arthritis, Metabolism and Digestive Disease, Bethesda, Md.). The hemolysate, obtained by freezing and thawing [12], was gel-filtered on a Sephadex G-25 column which was equilibrated with 0.01 M phosphate buffer, pH 6.0. The filtrate was applied to a Sephadex G-75 column which was equilibrated with the same buffer. After removal of Hb, the column was washed with 0.01 M phosphate buffer, pH 6.8. The major component of Glycera Hb was eluted by 0.01 M phosphate buffer, pH 7.0. Examination by gel filtration on a Sephadex G-100 column and by cellulose acetate gel electrophoresis showed that the major fraction thus obtained corresponded to the component which was called "monomer major" by Seamonds et al. [13].

The abbreviations used are: CoMb, cobalt myoglobin (sperm whale); FeMb, iron myoglobin (sperm whale); CoHb, cobalt hemoglobin; FeHb, iron hemoglobin.

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ApoHb (Glycera) was prepared by the 2-butane method (14). For preparation of CoMb (Glycera), the same method used for CoMb (2) has been successfully employed. Thus synthesized CoMb (Glycera) showed the same molecular characteristics as FeHb (Glycera) on a Sephadex G 100 column and on cellulose acetate gel electrophoresis.

Light absorption spectra were measured by a Cary 118C recording spectrophotometer. Oxygenation equilibrium measurements were performed by the method of Imai et al. (15) with an on-line PDP 11/40 computer as described previously (8, 9, 16). The MetHb reductase system described by Hayashi et al. (17) was used for the measurements of FeHb (Glycera). Temperature-jump relaxation technique was used for the measurements of kinetics of the oxygenation as described previously (3). A temperature-jump of 4.5°C was applied at 0.5°C with a discharge of 18 kV using the air-saturated solution of CoMb (Glycera). The relaxation process was analyzed as described by Yamamoto et al. (5).

EPR measurements were performed by a PDP 11/40 on-line Varian E-109 x-band EPR spectrometer with 100 kHz field modulation equipped with an Air-Product Helitran transfer line (LTD-3-110) to maintain sample between room temperature and 6 K. Low temperature spectrophotometric measurements were carried out with a dual beam spectrophotometer equipped with a double Dewar flask for liquid helium studies (5), which was constructed at the Johnson Foundation, University of Pennsylvania. The photolysis experiments were performed after illuminating the spectrophotometric and EPR samples with white light from a Spectral reflectometer source (Labsorde, OH-150SR) at cryogenic temperature (<10 K).

Mb or Hb (Glycera) was dissolved in 0.1 M phosphate buffer between pH 6 and 8 and 0.1 M acetate buffer below pH 6. Above pH 8, pH was adjusted by addition of 0.1 N NaOH.

RESULTS

pH Dependence of CoMb

Fig. 1 shows the light absorption spectra of oxy proto-CoMb at pH 4.5 and 7.1 at a partial oxygen pressure of 650 Torr. Upon lowering the pH value, the α- and β-bands are shifted toward lower wavelength by 5 and 3 nm, respectively. This pH dependence has been found to be reversible with a pK value of 5.6 at 20°C. Meso- and deutero-CoMb have also exhibited similar pH-dependent spectral changes. Proto-CoMb was found to be more stable than meso- and deutero-CoMb in the acidic pH region. Deoxy-CoMb's exhibited no detectable change in their absorption spectra between pH 4.5 and 9.

Fig. 2 illustrates the EPR spectra of oxy proto-CoMb at pH 4.5 and 7.1 at 77 K. The hyperfine structure of the EPR spectrum exhibited a small reversible change similar to that observed by light absorption spectra (Fig. 1). This small change could be amplified by a second derivative display as shown in Fig. 2B. Fig. 2C exhibits the pH dependence of the ratio of the EPR intensities at 3312 and 3308 oersteds (Oe) (I3312/3308 Oe) for the EPR spectrum of oxy proto-CoMb. The pH value of this change is estimated to be 5.3. Oxy meso- and deutero-CoMb also showed pH dependences of the oxy-EPR spectrum similar to that of proto-CoMb, having pK values of 5.55 and 5.25, respectively. The deoxy-EPR spectra of CoMb's showed no pH-dependent change between pH 4.8 and 8.5.

The oxygen affinity of CoMb was found to be pH-dependent below neutral pH. In Fig. 3, the observed value of pO2 (partial oxygen pressure at half-saturation in Torr) of proto-CoMb at 15°C is plotted against pH. The pK value of the pH dependence of its oxygen affinity was estimated to be 5.4. The oxidation of the cobalt ion or the formation of the met form during the oxygenation measurement below pH 5.5 was estimated to be between 6 and 10%.
Equilibrium and Kinetics of Oxygenation of CoHb (Glycera) - The oxygen affinity of proto-CoHb (Glycera) was found to be about 7 x 10^7 Torr at 4°C in 0.1 M phosphate buffer, pH 7.4, whereas proto-FeHb (Glycera) exhibited a p50 value of 1.6 Torr at 5°C. A temperature-jump study of oxygenation of proto-CoHb (Glycera) at 5°C gave a relaxation time of less than 7 μs, which is the dead time of the instrument. Thus, we estimate the dissociation rate constant (k_diss) to be larger than 8.5 x 10^4 s^-1, which is remarkably greater than found in other cobalt porphyrin-containing hemoproteins (3, 9).

EPR Spectrum of CoHb (Glycera) - Deoxy-CoHb (Glycera) exhibited an EPR spectrum similar to that of deoxy-CoMb. The EPR spectrum of oxy-CoHb (Glycera) gave a somewhat different hyperfine structures from that of oxy-CoMb, as shown in Fig. 4, A and B. The EPR parameters of oxy- and deoxy-CoHb (Glycera) are listed in Table I together with those of CoMb (4).

As reported previously (4) and duplicated here, oxy-CoMb dissolved in buffered D2O exhibits the distinctly narrowed hyperfine structure (Fig. 4A). However, measurement in D2O had no effect on the EPR spectrum of oxy-CoHb (Glycera) (Fig. 4B). In addition, no pH effect was observed with the EPR spectrum of oxy-CoHb (Glycera) between pH 4.5 and 9.

Low Temperature Photodissociation of Ligated Hb (Glycera) - Low temperature spectrophotometric examinations have indicated that the absorption spectrum of oxy meso-CoHb (Glycera) is readily changed to that of the deoxy form upon illumination at 4.2 K, indicating the breakage of the bond.

### Table I

| Protein, state, and porphyrin | Effective g values | Hyperfine coupling constants | Reference |
|-------------------------------|--------------------|-----------------------------|-----------|
| Oxy Meso-CoMb (Glycera)       |                   |                             |           |
| Proto-                        | 2.32              | 2.03                        | 6.2       | 77       | 17         | This paper |
| Meso-                         | 2.32              | 2.03                        | 6.2       | 77       | 17         | This paper |

* g value is estimated from the peak to peak line width of the signal.

** g value has been determined by K band measurement.

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**Fig. 3.** The pH dependence of p50 (Torr) for oxygenation of proto-CoMb at 15°C.

**Fig. 4.** A, EPR spectra of oxy meso-CoMb in buffered H2O (top) and D2O (bottom) at 77 K. pH and pH* are 7.0. B, EPR spectra of oxy meso-CoHb (Glycera) in buffered H2O (top) and D2O (bottom) at 77 K. pH and pH* are 7.0.
between the cobaltous ion and the oxygen molecule, as observed in oxy-CoMb (4, 5). The spectrum of the deoxy form reverts to that of the original oxy form upon increasing the temperature above 20 K.

Fig. 5 illustrates the change in the EPR spectra of oxy meso-CoHb (Glyceru) and oxy meso-CoMb upon repeated illumination at 6 K. As reported previously (4) and also shown here, the EPR spectrum of the illuminated product of oxy meso-CoMb, exhibiting extrema at $g = 3.87$ and around $g = 1.9$, resembles the spectrum of neither the deoxy nor the oxy form (4). On the other hand, the photolyzed compound of oxy meso-CoHb (Glyceru) (Curve A) exhibited an EPR signal at $g = 2.33$ which closely resembles that of the deoxy form.

Fig. 6 illustrates the EPR spectrum of the illuminated product of oxy meso-CoHb (Glyceru) recorded at a low microwave power (10 microwatts) level at 6 K. Besides the EPR signal from the remaining oxy form around 3.2 kOe, the hyperfine and superhyperfine structures of the EPR spectrum of the illuminated product of oxy meso-CoHb (Glyceru) are clearly shown around 3 kOe. Although some of the superhyperfine and hyperfine structures overlap with the remaining oxy signal, the coupling constants of these fine structures are the same as those of the deoxy form. The EPR spectrum of the illuminated product is considered to be indistinguishable from that of the deoxy form except for the signal from the residual nonphotolyzed oxy form. Proto-CoHb (Glyceru) gives the EPR spectrum of the illuminated product similar to that of meso-CoHb (Glyceru), but it is photodissociated to a lesser extent. The EPR spectrum of the photolyzed form reverted to that of the original oxy form upon increasing the temperature above 20 K.

The photodissociation of the carbon monoxo complex of the native Glyceru hemoglobin (carbon monoxo-FeHb (Glyceru)) was also examined by low temperature spectrophotometry. Upon illumination, the absorption spectrum of carbon monoxo-FeHb (Glyceru) changed to a spectrum with a small absorption band at 774 nm, which is also observable with deoxy-FeHb (Glyceru). The absorption band at 774 nm of the photodissociated form disappears with raising the temperature up to 50 K due to the recombination of carbon monoxide, whereas the same band of the deoxy form remains unchanged under the same conditions.

**DISCUSSION**

Our preliminary attempt to measure the absorption spectrum and oxygen affinity of FeMb below pH 6 showed a formation of a considerable amount of MetMb even in the presence of the MetMb reductase system (17). As reported previously (2) and examined here, proto-CoMb was far more resistant to autoxidation. EPR measurements were performed below 77 K where CoMb is considered to be more stable than at ambient temperature. Furthermore, MetCoMb is not EPR visible. Thus, we have focused our examination of the pH dependence of CoMb mainly on the EPR measurements.

The EPR spectrum of oxy-CoMb has indicated that the electron spin density delocalizes substantially from the cobalt ion to the bound oxygen, and the paramagnetic center of oxy-CoMb is mainly the bound oxygen (1, 4). The values of $A_{1}$, $A_{2}$, and effective $g$ values of oxy-CoMb (Glyceru) show that the situation on the electron spin delocalization in oxy-CoHb (Glyceru) is similar to that in oxy-CoMb. The pH dependence of the EPR spectrum of oxy-CoMb indicates the interaction of the bound oxygen with a proton dissociable group. Taking into account the pK values of the pH dependence, the most probable candidate of this proton dissociable group is considered to be the distal histidine (E-71) in CoMb. The pH-dependent change of the light absorption spectrum of oxy-CoMb, which coincides with that of the EPR spectrum, also shows the existence of the heme-linked proton dissociable group in oxy-CoMb. As reported previously (4), measurement in $D_{2}O$ exhibits the distinct effect on the EPR hyperfine lines of oxy-CoMb. On the other hand, measurement in $D_{2}O$ has no effect on the EPR spectrum of oxy-CoMb (Glyceru). In addition, the effect of pH on the EPR spectrum of oxy-CoMb (Glyceru) is found to be absent. These indicate that the paramagnetic center (principally the bound oxygen) is not interacting with a neighboring exchangeable hydrogen atom. In Glyceru Hb, the position usually occupied by the distal histidyl group in vertebrate myoglobins and hemoglobins is occupied by a leucyl residue (12), which is incapable of forming a hydrogen bond to the bound ligand. Thus we can confirm further our previous proposal (4) that the hydrogen bond is formed between the bound oxygen and the distal histidyl group in CoMb. The order of the pK values of this pH dependence of the EPR spectrum is deuterol $\leq$ proto- $\leq$ meso-CoMb. This order coincides with
that of $A_{540}$ values of the EPR spectrum of their oxy form (4) and with that of the oxygen affinity of these CoMb near 0° (2), but does not correspond to the order of the electron-withdrawing characteristics of the porphyrin substituents.

Deoxy-CoHb (Glycera) exhibits the same EPR spectrum as deoxy-CoMb, suggesting that the interaction between the cobalt ion and the proximal histidine is the same between deoxy-CoHb (Glycera) and deoxy-CoMb. Oxy-CoHb (Glycera) exhibits a somewhat different EPR spectrum from oxy-CoMb (Fig. 4). The $g$ value of oxy meso-CoHb (Glycera) ($g = 2.07$) is smaller by 0.01 unit (15 G higher magnetic field) than that of oxy meso-CoMb ($g = 2.08$). The line shape of the hypfine structure of the $g_z$ component is also different from that of CoMb. These differences suggest that the orientation of the bound oxygen molecule to the porphyrin plane in CoHb (Glycera) is somewhat different from that in CoMb. This may be a reflection of the steric difference between Mb and Hb (Glycera) in the distal site of the prosthetic group, especially due to the lack of the distal histidine group in Hb (Glycera). The pH-dependent change of the oxygen affinity for CoMb also exhibits a $pK$ value of 5.4 which is close to that of the EPR spectrum. Fig. 3 indicates that the oxygen affinity of CoMb is also related to the proton dissociable group, most likely the distal histidine (E-7) in CoMb. This fact is considered to be additional evidence for the formation of the hydrogen bond between the bound oxygen and the distal histidyl group. These pH-dependent properties of CoMb might be interpreted as follows: on lowering the pH, the $\delta$-nitrogen of the imidazole ring of the distal histidine is protonated which could weaken the hydrogen bond between the $\epsilon$-nitrogen and the bound ligand. Thus, the oxygen affinity, the light absorption, and EPR spectrum of CoMb are equally affected by pH changes.

The oxygen affinity of CoHb (Glycera) is about 50 times lower than that of CoMb at 5°, just as FeHb (Glycera) exhibits a lower oxygen affinity than FeMb. It has been pointed out by Yamamoto et al. (3) that the $k_{off}$ values depend both on the nature of metal (i.e. cobalt or iron) and on the nature of the environments of the metallocorphyrin (i.e. protein moiety), while $k_{on}$ values are in the same order of typical biological macromolecular-ligand interactions. The estimated $k_{off}$ value of oxygenation of CoHb (Glycera) is at least 20 times larger than that of CoMb (3). The extremely low oxygen affinity of CoHb (Glycera) is attributed to its large $k_{off}$ value. As shown by the EPR spectra of deoxy-CoHb (Glycera) and deoxy-CoMb, the mode of the interaction between the cobalt ion and the proximal histidine is very similar between these two cobalt hemoproteins. However, the orientation of the bound dioxygen molecule relative to the heme plane in CoHb (Glycera) might be somewhat different from that in CoMb due to the difference in the environment of the distal histidine site as discussed above. The large value of $k_{on}$ may be due to the lack of the hydrogen bond between the bound oxygen and apoprotein in CoHb (Glycera). Thus the low ligand affinity in Glycera Hb might be reflection of the absence of the distal histidine group as proposed by Padlan and Love (12).

The fact that the photolyzed form of carbon monooxy-FeHb (Glycera) gives the same near infrared absorption band as its deoxy form and that the photolyzed form of oxy-CoHb (Glycera) also shows the same EPR spectrum as the deoxy form support a conclusion that the photolyzed form of the ligated Hb (Glycera) is the deoxy form. Iizuka et al. (10) have shown that the photolyzed form of carbon monooxy-FeMb at low temperature exhibits the absorption band at 777 nm whereas that of deoxy-FeMb is found at 758 nm. The photolyzed form of oxy-CoMb gives an EPR signal different from the deoxy form (4, 5). The photolyzed form has been considered to have the ligated conformation, in which the bonding between the exogenous ligand (CO or O$_2$) and the metal ion is broken by irradiation of light. Mössbauer spectra of the photolyzed forms of the ligated FeMb or FeHb (human) have also been reported to be somewhat different from their deoxy compounds (11).

Difference in the nature of the heme pocket is thought to yield the deoxy form as the photolyzed product of the ligand Hb (Glycera). The substitution of leucine 58, which is incap-

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**Fig. 7.** Schematic diagram of the mode of the interaction among oxygen, cobaltous ion, and apoprotein of Mb and Hb (Glycera) for deoxy, oxy, and photolyzed forms.
ble of forming a hydrogen bond to the bound ligand, has a very large effect on the nature of this hemoprotein, as discussed above. The appearance of the deoxy form upon illumination of the ligated Hb (Glycera) is considered to be due to the lack of a hydrogen bond between the bound oxygen and the distal group. A schematic diagram of the heme vicinity of Mb and Hb (Glycera) is shown in Fig. 7. In the case of Mb, the bound oxygen would form a hydrogen bond with the distal histidyl residue. In cooperation with another conformation change occurring during the ligation, the metal is pulled up into the porphyrin plane at ambient temperature (4). At 4.2 K, illuminating the sample dissociates the ligand from the metal, but the protein conformation remains unchanged in the ligated form (10). This photolyzed product is spectrophotometrically distinguishable from the deoxy form (4, 5, 11). In Hb (Glycera), because of the lack of the hydrogen bond, the metal ion is presumably still somewhat out of the porphyrin plane even in the ligated state. Thus, the photolyzed product is expected to be the same as deoxy form. Therefore, the chemical nature of the photolyzed product of the ligated Hb or Mb may depend on the mode of the interaction between the bound oxygen and apoprotein moiety.

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REFERENCES
1. Hoffman, B. M., and Petering, D. H. (1970) Proc. Natl. Acad. Sci. U. S. A. 67, 637-643
2. Yonetani, T., Yamamoto, H., and Woodrow, G. V. III (1974) J. Biol. Chem. 249, 682-690
3. Yamamoto, H., Kayne, F. J., and Yonetani, T. (1974) J. Biol. Chem. 249, 691-694
4. Yonetani, T., Yamamoto, H., and Iizuka, T. (1974) J. Biol. Chem. 249, 2168-2174
5. Iizuka, T., Yamamoto, H., Kotani, M., and Yonetani, T. (1974) Biochim. Biophys. Acta 351, 182-195
6. Woodruff, W. H., Adams, D. H., Spiro, T. G., and Yonetani, T. (1975) J. Am. Chem. Soc. 97, 1695-1698
7. Padlan, E. A., Eaton, W. A., and Yonetani, T. (1975) J. Biol. Chem. 250, 7069-7073
8. Imai, K., Yonetani, T., and Ikeda-Saito, M. (1977) J. Mol. Biol. 109, 83-97
9. Ikeda-Saito, M., Yamamoto, H., Imai, K., Kayne, F. J., and Yonetani, T. (1977) J. Biol. Chem. 252, 620-624
10. Iizuka, T., Yamamoto, H., Kotani, M., and Yonetani, T. (1974) Biochim. Biophys. Acta 371, 126-139
11. Spartalian, K., Lang, G., and Yonetani, T. (1976) Biochim. Biophys. Acta 428, 381-390
12. Padlan, E. A., and Love, W. E. (1974) J. Biol. Chem. 249, 4067-4078
13. Seamonds, B., Forster, R. E., and Gotlieb, A. J. (1971) J. Biol. Chem. 246, 1700-1706
14. Yonetani, T. (1967) J. Biol. Chem. 242, 5000-5013
15. Imai, K., Merimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M. (1970) Biochim. Biophys. Acta 196, 189-198
16. Imai, K., and Yonetani, T. (1977) Biochim. Biophys. Acta 490, 164-170
17. Hayashi, A., Suzuki, T., and Shin, M. (1973) Biochim. Biophys. Acta 310, 309-310
18. Stryer, L., Kendrew, J. C., and Watson, H. C. (1964) J. Mol. Biol. 8, 96-104
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