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RECONSTITUTED SPERM WHALE MYOGLOBINS WITH IRON AND COBALT COMPLEXES OF CHLORINS

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Reconstituted myoglobins (Mb) with iron and cobalt complexes of chlorins were prepared for the first time. The Fe(II)-Mb's form oxy-adducts, but the reversibility of oxygen binding is rather poor. The Co(II)-Mb's prepared under anaerobic conditions bind O_2 reversibly. Reconstitution under aerobic conditions leads to Co(III)-myoglobins; Co(II) proteins derived therefrom undergo instantaneous oxidation back to the Co(III) derivatives.

The reconstitution of hemoglobin and myoglobin (Mb) with chemically modified prosthetic groups provides a clue for a better understanding of these hemoproteins.[1] The natural prosthetic group, protoheme, has been modified in two ways; modification of side chains[2] and metal substitution.[3] Especially important are the cobalt- and more recent rhodium-substituted Mb's.[3,4] We wish to report here the preparation and properties of reconstituted Mb's with iron and cobalt complexes of chlorins.[5] This study is along the line of research interest in the alteration of hemoprotein function by changing the prosthetic groups.[6]

Iron(III) complexes of pheophorbide-a, pyropheophorbide-a, mesopheophorbide-a, and mesopyropheophorbide-a (1, 2, 3, and 4, respectively; M = Fe(III), X = Cl) were prepared.[7] Combination of these Fe(III)-chlorin complexes or dihydrohemins with sperm whale apomyoglobin followed by standard purification procedures[2] gave relatively stable reconstituted Mb's, which were readily reduced with dithionite to the ferrous deoxy states to form oxy-adducts upon contact with O_2;[8] the reversibility, however, was rather poor and the oxy-adducts underwent more or less facile autoxidation to the met-forms especially at *lower* O_2 pressures.[9] The O_2 affinities in terms of P_{50} (oxygen pressure at half oxygenation) were approximate values due to poor reversibility; P_{50} = 0.4-1.0 torr for all of Fe(II)-Mb's derived from 1, 2, 3, and 4. These P_{50} values are similar to those for reconstituted Mb's with the corresponding heme derivatives (5 and 6);[10,11] P_{50} = 0.57 and 0.81 torr, respectively. This similarity may indicate on one hand that the heme pocket in apomyoglobin is flexible enough to bind chlorin prosthetic groups. On the other hand, however, the enhanced rates of autoxidation of the oxy-adducts cast a more fundamental question regarding whether the chlorin-reconstituted Mb's are precise analogs of the native protein or more properly regarded as denatured protein deriva-

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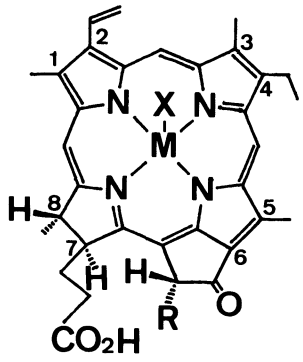

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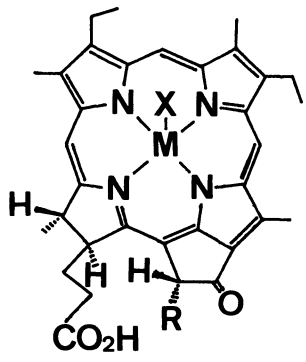
2; R=H



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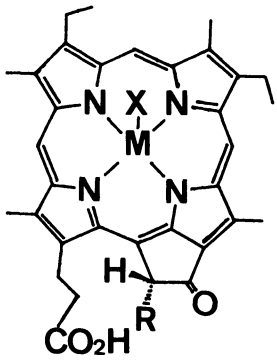
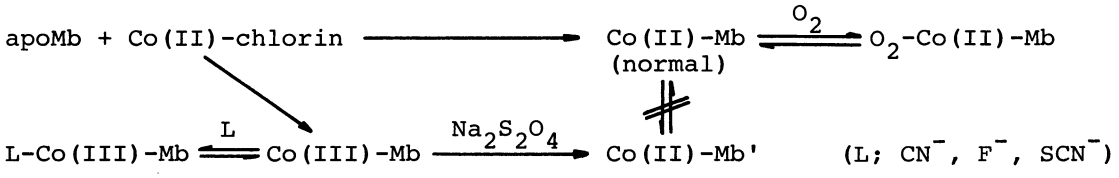


5; R=CO_2H
6; R=H

tives. In order to get deeper insight into this point, we have also studied the cobalt systems.

Insertion of cobalt into free base chlorins afforded Co(II)-chlorin complexes (1, 2, 3, 4; M = Co(II), X = none).[12] Complexes 3 and 4 of the meso type were combined with apomyoglobin by essentially the same procedure as for the preparation of coboglobins.[13] To an aqueous buffer solution (pH 7.0, 7 mL) of apomyoglobin (0.2 mM; 1 M = 1 mol dm^{-3}) at 0°C under nitrogen was added a solution (0.7 mL) of 3 or 4 (1.5 equiv.) in water-pyridine (1:1 by volume) saturated with $Na_2S_2O_4$. The mixture was stirred for 1 min at 0°C and quickly chromatographed on a column of Sephadex G-25 with 10 mM phosphate buffer (pH 6.0) as eluant. The protein fraction was further purified by chromatography on a CM-52 column. Elution with 0.1 M phosphate buffer (pH 7.0) afforded the oxygenated protein (O_2-Co(II)-Mb). The formulation as the oxy-adduct was confirmed by its deoxygenation. Introduction of nitrogen gas into a solution of O_2-Co(II)-Mb led to the deoxy derivative (Co(II)-Mb) and the spectral change associated with this interconversion[14] was similar to that for the corresponding cobalt porphyrin derivatives.[13] That the present Co(II)-Mb's bind O_2 reversibly was evidenced by repeated oxygenation-deoxygenation cycles.

The presence of pyridine and a reductant ($Na_2S_2O_4$) under anaerobic condition was essential for the preparation of reversible oxygen carrier. Aerobic reconstitution or anaerobic one without pyridine or $Na_2S_2O_4$ led to Co(III)-Mb's (λ_{max} for the one derived from 4; 434, 550, 602, and ca. 660 nm) after chromatographic manipulation as above. The Co(III) ion could be reduced *readily* with $Na_2S_2O_4$ to afford the Co(II) derivative, the electronic spectrum of which was essentially the same as that of the above or normal Co(II)-Mb. Surprisingly, the Co(II) derivative thus formed showed no ability to bind O_2; exposure to O_2 resulted in an instantaneous oxidation back to the Co(III) derivative. These observations indicate that Co(III)-chlorins may be incorporated into the heme pocket in a significantly different manner from that of Co(II)-chlorins and that the Co(II) derivative (Co(II)-Mb') derived from Co(III)-Mb *memorizes* the structural characteristics of the original Co(III) protein; there is no interconversion between normal Co(II)-Mb and Co(II)-Mb'. In a marked contrast, Co(III) globins having cobalt porphyrin prosthetic groups are reduced to Co(II) globins capable of binding O_2 reversibly.[3]



The Co(III) globin is known to be reduced with $Na_2S_2O_4$ only very slowly. Furthermore, it does not bind external anions, except for a very slow binding of CN^-.[15] These observations suggest that the Co(III) binds both proximal and distal imidazole groups as in internal hemichrome, a plausible intermediate in acid denaturation of oxyMb.[16,17] On the other hand, the present Co(III) protein undergoes almost instantaneous reduction with $Na_2S_2O_4$ (vide supra). It is also readily ligated by anions such as CN^-, F^-, and SCN^- (λ_{max} for the cyano-adduct; 452, ca. 600, 639, and ca. 680 nm). These observations are not consistent with an internal hemichrome formulation for the present Co(III)-Mb. It is a possibility that polar interaction of the *upward-oriented* propionic acid group at 7-position with a polar amino acid residue (His FG2 in the case of native Mb) prevents coordination of the distal imidazole, but this remains to be clarified.

This study may be summarized as follows. Combination of apomyoglobin and Co(II)-chlorin complexes under appropriate conditions affords *normal* Co(II) protein as far as reversible O_2 binding is concerned. Reconstituted Mb's derived from Fe(III)-chlorin complexes can also be regarded as native Mb analogs. The similarity of the O_2 affinities of the heme- and dihydroheme-Mb's indicates that the orientation of the 7-propionic acid group, *in plane vs. upward*, is not an important factor governing the O_2 affinity. This is consistent with our finding made on a modified heme having methyl group instead of propionic acid at 7-position.[10] The situation is quite different for the Co(III) and Co(II) proteins derived therefrom, where the upward-oriented propionic acid seems to induce a considerable change in protein conformation.

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2) H. Ogoshi, K. Kawabe, S. Mitachi, Z. Yoshida, K. Imai, and Y. Tyuma, Biochim. Biophys. Acta, 581, 266 (1979); K. Kawabe, K. Imaizumi, K. Imai, I. Tyuma, H. Ogoshi, T. Iwahara, and Z. Yoshida, J. Biochem., 92, 1703 (1982) and references cited therein.
3) B. M. Hoffman, "The Porphyrins," ed by D. Dolphin, Academic Press, New York (1979), Vol. VII, Chapter 9.
4) Y. Aoyama, K. Aoyagi, H. Toi, and H. Ogoshi, Inorg. Chem., in press (1983).

5) An apoMb complex having pyrochlorophilide(Mg) as prosthetic group is known: S, G. Boxer and K. A. Wright, J. Am. Chem. Soc., 101, 6791 (1979); K. A. Wright and S. G. Boxer, Biochemistry, 20, 7546 (1981); S. G. Boxer, A. Fuki, K. A. Wright, B. A. Katz, and N. H. Xuong, Proc. Natl. Acad. Sci. U. S. A., 79, 1121 (1982).

6) For a recent approach to the manipulation of Mb redox property by the attachment of redox-active inorganic groups, see: R. Margalit, I. Pecht, and H. B. Gray, J. Am. Chem. Soc., 105, 301 (1983).

7) Monomethyl or dimethyl esters of hemin chlorides (1-4) were prepared by heating a solution at 80°C of the corresponding free base chlorin (20 mg) in acetic acid (50 mL) containing pyridine (1 mL), $FeSO_4$ (100 mg), and NaCl (100 mg). Found: C, 60.41; H, 5.20; N, 7.94%. Cacld for $C_{36}H_{37}ClN_4O_5Fe \cdot H_2O$ (1 + $2CH_3$ - 2H + H_2O): C, 60.59; H, 5.33; N, 7.85%. Found: C, 63.98; H, 5.43; N, 8.94%. Calcd for $C_{34}H_{35}ClN_4O_3Fe$ (2 + CH_3 - H): C, 64.05, H, 5.34; N, 8.97%. Found: C, 60.49; H, 5.49; N, 7.76%. Calcd for $C_{36}H_{39}ClN_4O_5Fe \cdot H_2O$ (3 + $2CH_3$ - 2H + H_2O): C, 60.42; H, 5.59; N, 7.83%. Found: C, 63.62; H, 5.72; N, 8.80%. Calcd for $C_{34}H_{37}ClN_4O_3Fe$ (4 + CH_3 - H): C, 63.85; H, 5.63; N, 8.76%. Hemin chlorides monomethyl and dimethyl esters were hydrolyzed in methanol containing 1% KOH under reflux for 1 h to give 1-4.

8) As in Mb's having heme prosthetic groups, λ_{max} for the Soret bands undergo red-shifts in going from Fe(III) through O_2-Fe(II) to Fe(II) derivatives; e.g., 405 (Fe(III)), 414 (O_2-Fe(II)), and 425 nm (Fe(II)) for reconstituted Mb with 2.

9) Cf. W. J. Wallace, R. A. Houtchens, J. C. Maxwell, and W. S. Coughey, J. Biol. Chem., 257, 4966 (1982).

10) Y. Aoyama, K. Okuda, K. Aoyagi, and H. Ogoshi, submitted to FEBS Letters.

11) Found: C, 60.69; H, 5.20; N, 7.81%. Calcd for $C_{36}H_{37}ClN_4O_5Fe \cdot H_2O$ (5 + $2CH_3$ - 2H + H_2O): C, 60.59; H, 5.33; N, 7.85%.

12) Purified by means of chromatography on alumina followed by recrystallization from dichloromethane-n-hexane. Found: C, 65.31; H, 5.71; N, 9.06%. Calcd for $C_{34}H_{37}N_4O_3Co \cdot H_2O$ (4 + CH_3 - H + H_2O): C, 65.27; H, 5.96; N, 8.96%.

13) T. Yonetani, H. Yamamoto, and G. V. Woodraw, III, J. Biol. Chem., 249, 682 (1974).

14) λ_{max} for Mb derived from 4: 422, 534, 573, 620,and ca. 650 nm for the oxy-form and 405, 560, and 623 nm for the deoxy-form.

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