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BIS-MYOGLOBIN RECONSTITUTED WITH COVALENTLY BONDED SYMMETRIC HEMES

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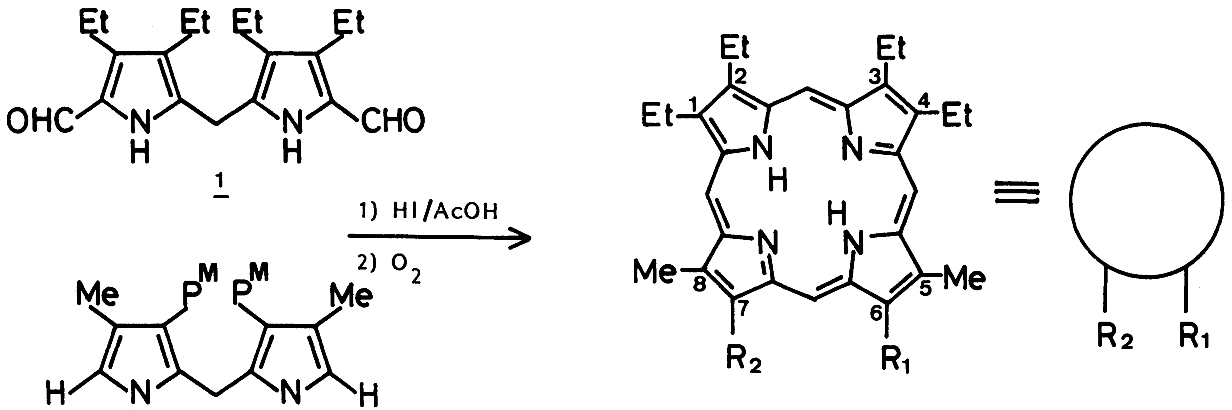
Two symmetric hemins were covalently bonded with polymethylene diamine. Reconstitution of bis-hemin with sperm whale apomyoglobin afforded bis-myoglobin(bis-Mb). Absorption spectra and paramagnetic ^{1}H-NMR spectrum of bis-Mb were compared with monomeric myoglobin reconstituted with symmetric heme.

The protein-protein interaction at exact distances has attracted much attention with biological respect to electron and energy transfer.$^{1)}$ Recent photochemical investigations on Zn substituted cytochrome c and cytochrome b_5 complex have provided more accurate mechanism of electron transfer between different heme enzymes.$^{2)}$ Model systems of the protein-protein complex at fixed distances are required to elucidate biological functions in the chemical sense. The present paper reports synthesis of bis-hemin from symmetric hemes and bis-Mb reconstituted with the bis-hemin.

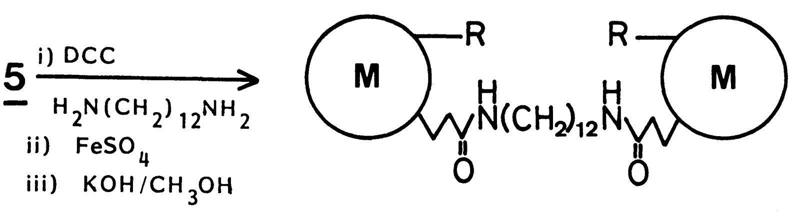
^{1}H-NMR studies have indicated that naturally occurring protoheme and its derivatives such as mesoheme and deuteroheme are incorporated in apoprotein in two different manners, namely normal or reverse orientation, upon reconstitution.$^{3)}$ Rotation around the Cα-Cγ axis of normally orientated heme gives the reversed one. Synthesis of a symmetric porphyrin is required to avoid the problem of orientation for hydrophobic alkyl substituents of prosthetic hemins. Symmetric porphyrin, dimethyl ester of 1,2,3,4-tetraethyl-5,8-dimethylporphyrin-6,7-dipropionic acid 3 was obtainable from 3,3',4,4'-tetraethyl-5-methyl-5'-bromo-2,2'-dipyrromethene hydrobromide in 38% yield.$^{4)}$ In this work, alternative synthesis from coupling of 3,3',4,4'-tetraethyl-5,5'-diformyldipyrromethane 1 and 3,3'-dimethoxycarbonylethyl-4,4'-dimethyl-2,2'-dipyrromethane 2 gave 3 in improved yield(56%).$^{5)}$ Purification by chromatogaphy on a alumina gel column and recrystallization from methanol and chloroform gave purplish red crystals. Hydrolysis of 3 in 4 M-HCl afforded mixture of diacid 4, monoacid 5 and diester 3. The mixture was chromatographed on silica gel. Eluant with CH_2Cl_2-CH_3OH(4:1) gave monoacid 5 in 25% yield.$^{6)}$ The diacid 4 was obtainable from elution with CH_3OH. Bis-porphyrin dimethyl ester 6 was prepared by condensation of the monoacid 5 with 1,12-dodecamethylenediamine using N,N'-dicycklohexylcarbodiimide. The dimethyl ester of bis-porphyrin diamide 6



3 ; $R_1 = R_2 = CH_2CH_2CO_2CH_3$

4 ; $R_1 = R_2 = CH_2CH_2CO_2H$

5 ; $R_1 = CH_2CH_2CO_2H$
$R_2 = CH_2CH_2CO_2CH_3$



6 ; M = H_2 , R = $CH_2CH_2CO_2CH_3$
7 ; M = Fe , R = $CH_2CH_2CO_2CH_3$
8 ; M = Fe , R = $CH_2CH_2CO_2H$

was purified by preparative thin layer chromatography on a silica gel plate developed with $CHCl_3$-CH_3OH (vol 95:5) and successive chromatography on alumina gel with $CHCl_3$. After removal of the solvent, recrystallization from CH_2Cl_2-CH_3OH afforded dimethyl ester of bis-porphyrin diamide 6 as deep red crystals in 20% yield.7) The mass spectrum of 6 exhibits a strong peak of its half molecular weight due to the doubly charged ion. Bis-porphyrin 6 was converted to the diester of bis-hemin 7 by refluxing 6 in the mixture of acetic acid and pyridine(50:1) containing ferrous sulfate and sodium chloride for 2 h. The reaction mixture was poured into the mixture of $CHCl_3$ and H_2O. The water layer was extracted with $CHCl_3$. The combined $CHCl_3$ solution was treated with 5% HCl and washed with aqueous NaCl. The solution was condensed to dryness. Crystallization from $CHCl_3$-hexane gave the diester of bis-hemin 7 in 59% yield as dark reddish brown crystals.8)

Hydrolysis of 7 with aq KOH and methanol afforded the bis-hemin 8 in 60% yield.9) The bis-hemin was incorporated into sperm whale apomyoglobin in a 0.1 M phosphate buffer solution(pH 7.0). The reconstituted bis-Mb was purified by dialysis, centrifuge, and successive chromatography on Sephadex G-25 and CM-52 columns.10) Molecular weight of bis-Mb was qualitatively determined as 30000 by elution chromatography of Sephadex G-75 with reference proteins, human hemoglobin(64500) and sperm whale myoglobin(17800).11) This result indicates that each hemin is combined with one molecule of apomyoglobin. The bis-metMb(Fe^{3+}) thus obtained can be reduced to the deoxy form(Fe^{2+}) by an enzymic method.[12] Figure 1 shows absorption spectra of met form, deoxy, oxy and carbonyl addcuts of bis-Mb. These absorption maxima are almost identical with those[11] of the corresponding forms of monomeric Mb reconstituted with iron complex of 4. It was noticed that the oxy form of bis-Mb showed fast autoxidation to the met form relative to the monometric myoglobin.

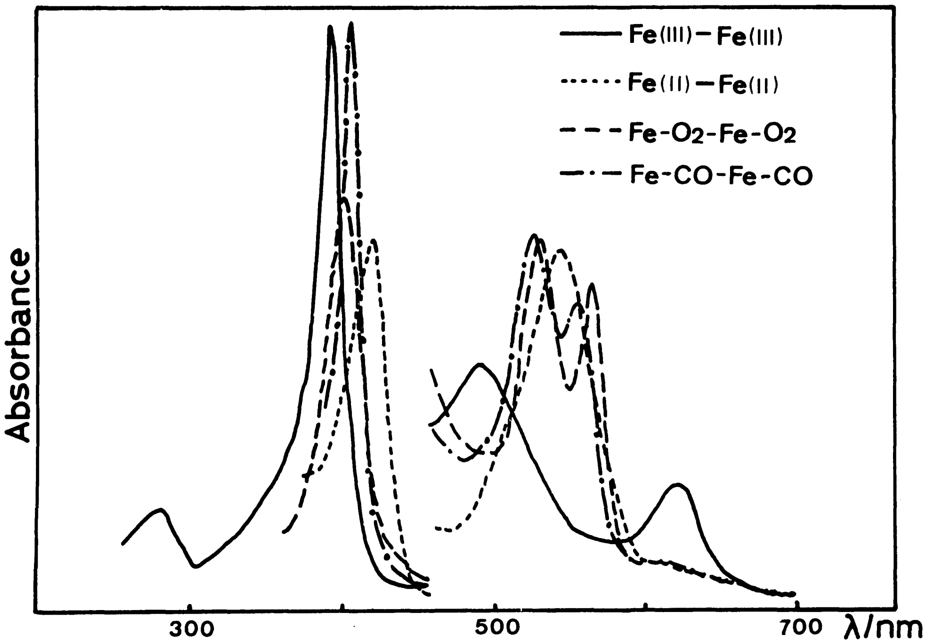


Fig. 1. Absorption spectra of the various forms of bis-Mb in 0.1 M KPB buffer, pH 7.0.

The X-ray crystallographic study of myoglobin has shown that the 6,7-propionic acid groups of prosthetic heme constitute different polar interaction involving hydrogen network with the polar amino acid residues.[13] When one of two propionic acids is condensed with amine to link the second heme, the -$CH_2CH_2CO_2H$ and -CH_2CH_2CONH- groups interact with the polar amino acid residues in two different manners. Consequently an acid or amide group of each heme can be situated at position 6 or 7 of the native heme in myoglobin. Therefore, the bis-Mb has three possible diamide links between the half molecules; through 6-6, 7-7, and 6-7 positions(Fig.2). No difinite conclusion for the sites of diamide linkage between

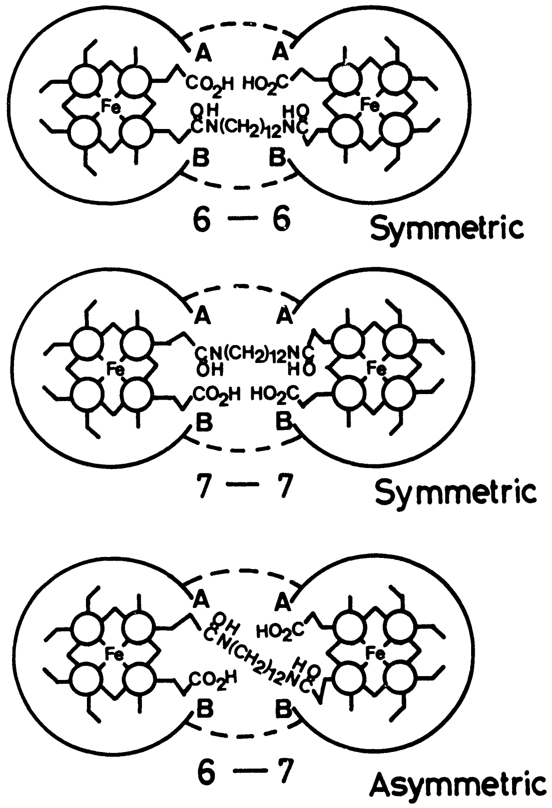


Fig. 2. Schematic representation of bis-myoglobin. Broken circle denotes apomyoglobin.

the two sub-units has been establised yet. It is noted that paramagnetic ^{1}H-NMR of bis-Mb in low-spin state shows simple signals of methyl groups at 12.1(8-CH_3) and 25.2(5-CH_3) ppm. It is most likely that the two subunits are bonded through 6-6 or 7-7 links. The present study indicates that further studies of the protein-protein complexes are promising and they are now under progress to elucidate the structure and function of bis-Mb in full detail.

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Method of synthesis was shown, but no characterization of 3 was described.

5) Diester 3; ^{1}H-NMR($CDCl_3$ TMS) δ -3.7(br s, 2H, NH), 1.93(t, 12H, $-CH_2C\underline{H}_3$), 3.37(q, 4H, $-CH_2C\underline{H}_2CO_2CH_3$), 3.63(s, 12H, 5,8-$CH_3$, 6,7-$CH_2CH_2CO_2C\underline{H}_3$), 4.10 (t, 8H, four $-C\underline{H}_2CH_3$), 10.05(s,4H, meso-H),; Vis($CHCl_3$) λ_{max}(log ε) 400 nm (5.22), 498(4.12), 53(3.98), 593(3.05), 621(3.68); IR(KBr) 3320, 1740 cm^{-1}.

6) Mono acid 5 ; NMR($CDCl_3$) δ 1.88(t, 12H, 1,2,3,4-$C\underline{H}_2CH_3$), 3.27(t, 4H, 6,7-$-CH_2C\underline{H}_3$), 3.53(s, 6H, 5,8-CH_3), 4.13(m, 12H, 1,2,3,4-$C\underline{H}_2CH_3$, 6,7-$C\underline{H}_2CH_2$-CO_2CH_3), 9.87, 9.93(s,4H,meso-H); Vis($CHCl_3$) λ_{max} (log ε) 400nm(5.25), 499 (4.17), 533(4.02),567(3.87), 595(3.20), 621(3.74); IR(KBr) 3320, 1749, 1710 cm^{-1}.

7) Bisporphyrin diester 6; NMR(270 MHz, $CDCl_3$), δ -3.78(s, 4H, NH), 0.93-1.25 (m, 24H, $-NH(C\underline{H}_2)_{12}-NH-$), 1.93(t, 24H, 1,2,3,4,-$CH_2C\underline{H}_3$), 3.27, 3.32(t, 4H, $-CH_2C\underline{H}_2CO_2CH_3$),3.65, 3.67, 3.69(s, 18H, 5,8-CH_3, $-CH_2CH_2CO_2C\underline{H}_3$),4.14(m,16H), four $-C_2\underline{H}_2CO_2CH_3$), 3.65, 3.67, 3.69(s, 18H, four $-CH_3$, two $-CH_2CH_2CO_2C\underline{H}_3$), 4.11(m, 16H, eight $-C\underline{H}_2CH_3$), 4.64(m, 8H, two $-C\underline{H}_2CH_2CO_2CH_3$), 5.21(br, 2H, amide NH), 10.04, 10.07, 10.09(s, 8H, ; meso-H); Vis($CHCl_3$) λ_{max}(log ε) 400nm(5.48), 499(4.37), 534(4.21), 566(4.02), 595(3.21), 620(3.86).

8) Bis-hmin chloride 7; Vis($CHCl_3$) λ_{max}(log ε) 380 nm(5.18), 508(4.13), 536(4.14), 573(3.66), 637(3.84); IR(KBr) 1740, 1660, 1640 cm^{-1}.

9) Bis hemin 8; IR(KBr) 3320, 1710, 1630 cm^{-1}.

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