

Synthesis of the Myoglobin Active Site

(hemoglobin/oxyheme/heme-oxygen bond/neighbor group)

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Communicated by Bruno Zimm, June 8, 1973

ABSTRACT A simple heme-imidazole compound, having the same geometry as the heme-imidazole complex in myoglobin, has been synthesized. This compound, ferropyrroperphyrin-*N*-[3-(1-imidazolyl)propyl]amide, reversibly binds oxygen in the solid state or when dissolved in a polystyrene film. These results suggest that the principal factors governing reversible oxygen binding are the electronic nature of the base (imidazole), neighboring-group effects of the basic group, and immobilization of the heme group.

Myoglobin and hemoglobin reversibly bind oxygen at the heme iron (1). Because the ability to reversibly bind oxygen is lost when the apoprotein is removed from the heme, it has been concluded that the protein is required for stability of the oxygen complex (1, 2). Chemical experience suggests that a simple reaction such as oxygen binding should depend only upon the local environment at the iron atom (3). Consequently, the function of myoglobin should be achieved with a simple heme compound if the local geometry and electronic (4-7†), and solvent environment in the synthetic compound duplicated that in myoglobin (8). We report here studies in a nonaqueous environment.

We therefore began with the published coordinates of the imidazole and heme portion of myoglobin (8, 9), assumed that the Fe^{II} myoglobin would have a planar heme (10), and prepared this "site" model (Fig. 1). It was soon apparent that attachment of a five-atom connection (shown within the dotted line in Fig. 1) between atoms A and B, results in a conformation that maintains the myoglobin geometry‡. We have synthesized several compounds incorporating this geometry, the simplest of which (*I* in Fig. 2) is reported here.

Synthesis

Pyrroperphyrin XV (14) (II), Calbiochem., was esterified by the diazomethane method (15*b*); the ester was purified by chromatography on silica gel with benzene-chloroform elution to produce a pure methyl ester (III). This compound moved as a single spot on thin-layer chromatography and had nuclear magnetic resonance (NMR) (16) and infrared

Abbreviation: NMR, nuclear magnetic resonance.

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† The importance of basicity of the ligands on the metal to the reversible oxygen bonding to cobalt has been thoroughly documented (4-7).

‡ Side chains containing imidazole have previously been attached to hemins (11-13). However, these compounds had either too few (5) atoms between A and B, resulting in strain in the iron-imidazole bond (11-13), or too many atoms (13). The neighboring group effect discussed here is best achieved by the model in Fig. 1.

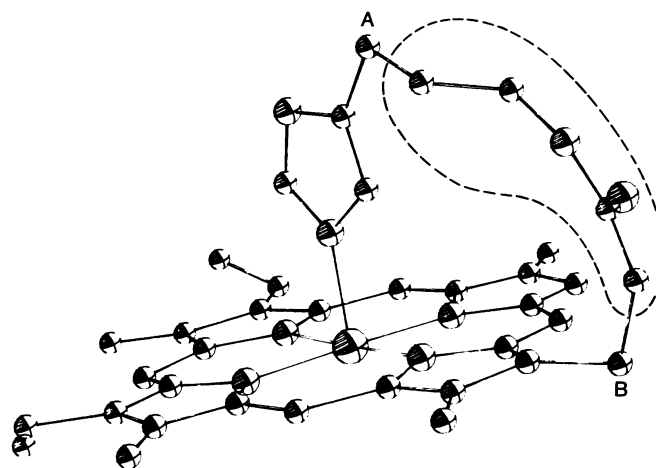
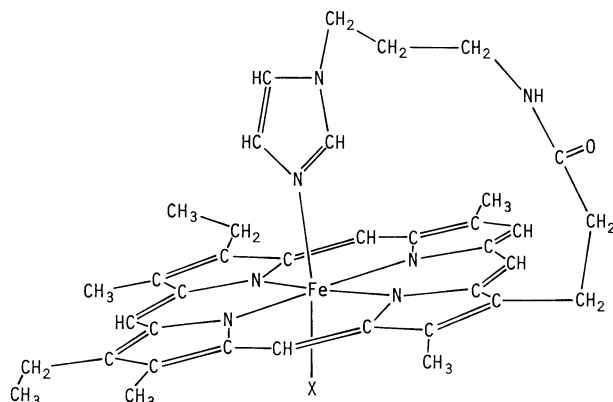


FIG. 1. The myoglobin site and proposed covalent connections to maintain its geometry without the apoprotein.

(17) spectra consistent with the assigned structure. The ester was hydrolyzed in 20% aqueous hydrochloric acid (15*c*) to the free acid (II). It was treated with excess thionyl chloride at room temperature (25°) to produce the acid chloride (IV), which was evacuated to remove thionyl chloride and used without purification. The acid chloride (IV) from 25 mg of pyrroperphyrin was treated at room temperature for 0.5 hr with 0.5 g of 1-(3-aminopropyl)imidazole prepared as described by Schwan (18) in a dry solvent composed of 2 ml of chloroform, 1 ml of acetone, 1 ml of pyridine. Chromatography on silica gel with a 4:1 chloroform-methanol eluent afforded 30 mg of pyrroperphyrin-*N*-[3-(1-imidazolyl)propyl]-amide V having visible absorptions at 619, 569, 530, and 498 nm and the NMR listed in Table 1. Comparison of the NMR of V with those of deuteroporphyrin and mesoporphyrin in Table 1 leaves no doubt about the structure (V).

This porphyrin (V) was converted into the corresponding hemin (VI) (see Fig. 2) by insertion of iron by the usual HOAc-FeSO₄ procedure (15*d*). The pyrrohemin amide (VI), dissolved in a benzene-chloroform mixture, gave the typical hemin spectrum (Fig. 3*A*). This solution was reduced with aqueous sodium dithionite at pH = 7 (15*e*) to produce the spectrum (Fig. 3*B*) of the heme (I), which probably has a weakly bound imidazole in the fifth and a water molecule in the sixth positions on the iron (15*f*; §). Addition of carbon

§ Treatment of wet benzene-chloroform solution of I with oxygen caused oxidation, producing a spectrum like that in Fig. 3*A*.



- I, X = no substituent or H₂O, Fe^{II}
 VI, X = Cl, Fe^{III}
 VII, X = CO, Fe^I
 VIII, X = O₂, Fe^{III}

FIG. 2. Structure of pyrroheme-N-[3-(1-imidazolyl)propyl]amide and its derivatives.

monoxide for a few minutes converted the spectrum to that of Fig. 3C, typical of a strongly bound imidazole—CO heme spectrum (15f). Passage of argon through this solution for an hour did not change this spectrum, revealing an extraordinary affinity of I for carbon monoxide. The carbon monoxide complex (VII) in chloroform revealed a strong CO absorption at 1963 cm⁻¹ (Cary 90), compared to 1970 cm⁻¹ for the pyridine carbon monoxide heme and 1951 cm⁻¹ for the carboxyhemoglobin (19). When this solution was evaporated to leave a thin film of VII, the CO absorption in the solid was at 1950 cm⁻¹.

Oxygen binding of solid I

The benzene-chloroform solution of VII was transferred to a cuvette filled with carbon monoxide and evaporated under a stream of carbon monoxide so as to leave a thin layer of solid on the cuvette window. The visible spectrum of this solid (Fig. 4A) is identical to that of the CO complex in solution. Although the carbon monoxide could not be removed by passage of argon over the solid at 25°, it was removed when

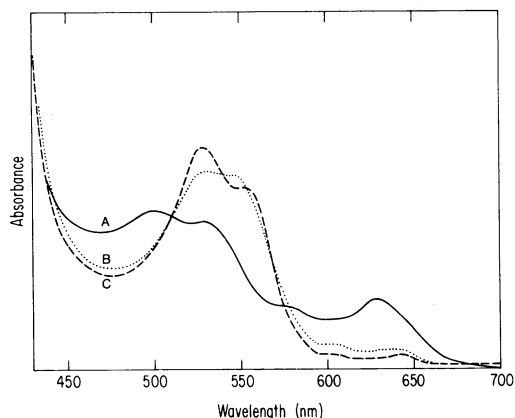


FIG. 3. Visible spectra in CHCl₃-benzene solvent of: (A) hemin (VI); (B) Heme (I); (C) Heme-CO complex (VII). Not shown are the Soret bands at: VI, 388 nm; I and VII, 412 nm.

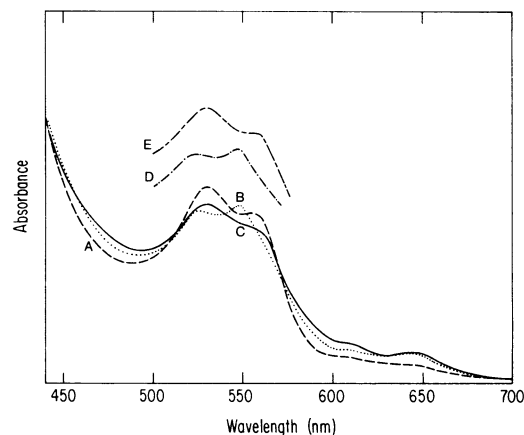


FIG. 4. Visible spectra of solid pyrroheme-N-[3-(1-imidazolyl)propyl]amide and its derivatives. Structures are shown in Fig. 2. (A) CO complex (VII); (B) after heating 30 min at 80° under argon (I); (C) after passing in oxygen for 5 min at 25° (VIII); (D) after passing argon over C for 3 hr; (E) after passing CO over D for 30 min.

the sample was heated to about 80° in an argon stream for 30 min. The resulting spectrum (Fig. 4B) has a larger 550-nm band than did I in solution (Fig. 3B), indicating that the five coordinate iron was probably bound more strongly to the imidazole in the solid. Passage of oxygen over this solid for a few minutes produced the spectrum in Fig. 4C, indicating formation of the oxyheme. Passage of argon over the solid gave the spectrum of Fig. 4D, identical to that of Fig. 4B, and subsequent treatment with carbon monoxide produced the spectrum of Fig. 4E, indicating regeneration of the carbon monoxide complex (VII). In all these treatments, neither the Soret band at about 400 nm nor the 650-nm region indicated any oxidation of the heme.

Similar results were obtained with a polystyrene film obtained by addition of a polystyrene solution to the carbon monoxide heme solution before evaporation.

A comparison of the spectra of Fig. 4A-E with the corresponding spectra of reconstituted hemoglobins and myoglobins containing mesoheme and deuteroheme is shown in Table 2.

The remarkable similarities of the spectra of deuterohemoglobin derivatives and those of our pyrroheme amide (I) (Table 2) make it quite clear that the simple heme reversibly binds oxygen in the solid state. A compound similar to I but having a pyridine nitrogen bound to iron did not bind oxygen (Chang, C. K., unpublished).

Reversibility of oxygen binding

A chloroform solution containing CO complex (VII), prepared from 5.9 mg (8.9 μmol) of the pyrrohemin amide (VI), was evaporated in a 25-ml flask fitted with vacuum stopcocks. (Total flask gas volume was 48.3 ml.) Evaporation was done so as to leave a thin film of red solid over the entire inner surface of the flask. This solid had a visible spectrum very similar to that shown in Fig. 4A even after pumping at 0.02 torr pressure for 1 hr at 25°. However, pumping at this pressure for 0.5 hr at 90° changed the spectrum of the solid to that of Fig. 4B. A 1.38-ml, 1-atmosphere, aliquot of an argon-oxygen mixture having relative peak heights of mass spectra at 40 and 32 of 2.95:1.00 was admitted to the evacuated flask

TABLE 1. Nuclear magnetic resonance of porphyrins (in CDCl₃)

	Methene H	Ring CH ₂	Ring—CH ₂ —CH ₂ —C ^O	Ring H	Ring ethyl
Deuteroporphyrin (ref. 16)	9.75	3.57 3.59 3.42 3.47	4.25 (t) 3.18 (t)	8.88	—
Mesoporphyrin (ref. 16)	9.83 9.88	3.45 3.47 3.50 3.51	4.30 (t) 3.21 (t)	—	3.93 (q) 1.79 (t)
Pyrroporphyrin amide (V)	9.45 9.54 9.74 9.82	3.40 3.42 3.52 3.54	4.41 (t) ~3.2 (t)	8.74	3.85 (q) 1.79 (t)

O
 \parallel
 $\text{—C—NH—CH}_2\text{—CH}_2\text{—CH}_2\text{—N}$
 $\text{3.34 (m) 2.21 (m) } \sim \text{3.2 (t) 6.35}$
 6.54

TABLE 2. Visible spectra of hemochromes having alkyl side chains

	Reduced		CO			O ₂			Met (pH 7)		
	S	β-α	S	β	α	S	β	α	S	β	α
Sperm-whale Mb ^a	434 (123.8)	556 (14.9)	426 (187)	540 (17)	578 (15.1)	418 (129.5)	545 (152)	582 (15.2)	410 (148)	500 (9.6)	630 (4.7)
Deutero Mb ^b	421 (90)	544 (10.4)	409 (187)	528 (12.2)	554 (7.8)		532 (11.1)	565 (7.1)	393 (116)	496 (7.9)	620 (3.1)
Meso Mb ^a	422 (117.5)	546 (13)	409 (111)	528 (15.3)	556 (12.2)				394 (102)	492	—
Deutero Hb ^c	421 (115)	544 (11.3)	408 (200)	528 (12.2)	556 (9.3)	403 (116)	532 (12.1)	565 (9.1)	394 (116)	500 (7.1)	620 (2.8)
Meso Hb ^c	421 (115)	550 (12)	410 (210)	532 (13.9)	560 (12.6)		543 (12.8)	568 (10.6)	396 (144)	495 (8.9)	620 (3.8)
3-(1-Imidazolyl) propyl pyrrohemamide (I, VI, VII, VIII)	413	522-548	412	528	555	409	530	564	389	500	628

Mb, myoglobin; Hb, hemoglobin. The numbers in parentheses are extinction coefficients, ε_{mM}.

^a Gibson, Q. H. & Smith, M. H. (1957) *J. Physiol.* **136**, 27P.

^b Rossi-Fanelli, A. & Antonini, E. (1957) *Arch. Biochem. Biophys.* **72**, 243-246.

^c Antonini, E., Brunori, M., Caputo, A., Chiancone, E., Rossi-Fanelli, A. & Wyman, J. (1964) *Biochim. Biophys. Acta* **79**, 284-292.

(16 μmol O₂). The spectrum became very similar to that of Fig. 4C. After 50 min, a 1.33-ml aliquot of the gas over the solid was removed and analyzed in a mass spectrometer. The mass 40 to mass 32 ratio was 4.13:1.00 (11 μmol O₂ in gas phase). A 1.33-ml aliquot of CO was admitted to the flask. After 30 min, the spectrum of the solid was taken (similar to that of Fig. 4A), and the gas in the flask was again analyzed and found to have a mass 40 to mass 32 ratio of 2.89:1.00 (16 μmol O₂ in the gas phase).

The spectral changes in this experiment were identical to those in the previous series. There was 5 μmol or 56% of 1 mol of oxygen per mol of heme absorbed and displaced by CO at an oxygen concentration of 5.5 mm of Hg.

Conclusions

These results show that the electronic nature of the proximal base is very important, that immobilization of the heme to prevent bimolecular heme oxidation (Chang, C. K., unpublished; 20;[†]) is necessary, and that neither the protein nor the distal imidazole are required for reversible oxygen binding.

However, neighboring group effect (21), which accrues from having the imidazole held in position either by the protein in

[†] This series of spectra (Fig. 4A-E) are very similar to the spectra reported by J. H. Wang (3) for heme dissolved in a mixture of polystyrene and 1-β-phenethylimidazole. We have repeated his experiment and obtained his results.

myoglobin (9) and hemoglobin (22) or by our seven-atom side chain in (I), has an enormous effect upon the binding of the sixth ligand (CO or O₂)**. This effect makes the myoglobin function much simpler than has been assumed. This compound and its very stable complexes afford a means of determining the geometry of the heme-CO and heme-O₂ complexes.

That we can duplicate the function of myoglobin with such a simple compound suggests that other kinds of protein catalysis can also be duplicated with protein-free small molecules.

We thank Drs. Herbert Diekmann, Yoshihiko Ito, and David Eaton for advice and assistance in synthetic procedures, and the National Institutes of Health for support (Grant USPHS HE 13581).

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** The neighboring group effect has been alluded to in the elegant model study of J. H. Wang (3b). He refers to the importance of "an imidazole group, snugly coordinated to the Fe⁺⁺-ion of the haem."

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