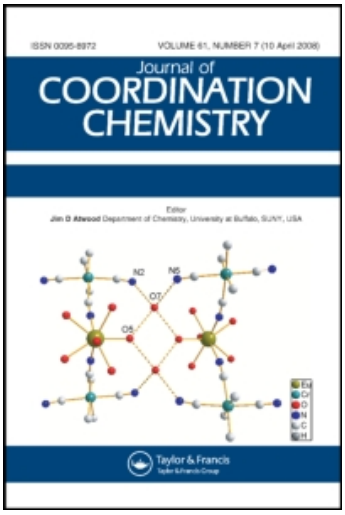

Journal of Coordination Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455674

RECONSTITUTION OF APOMYOGLOBIN WITH TRANSITION METAL COMPLEXES CONTAINING SYNTHETIC MACROCYCLIC LIGANDS

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To cite this Article Kildahl, Nicholas K. , Kolis, Joseph W. , Beckett, Joanne , Holland, Gary F. and Patz, Michael A.(1983) 'RECONSTITUTION OF APOMYOGLOBIN WITH TRANSITION METAL COMPLEXES CONTAINING SYNTHETIC MACROCYCLIC LIGANDS', Journal of Coordination Chemistry, 12: 4, 259 — 271
To link to this Article: DOI: 10.1080/00958978308073856
URL: http://dx.doi.org/10.1080/00958978308073856

J. Coord. Chem., 1983, Vol. 12, pp. 259-272
0095-8972/83/1204-0259 $18.50/0

RECONSTITUTION OF APOMYOGLOBIN WITH TRANSITION METAL COMPLEXES CONTAINING SYNTHETIC MACROCYCLIC LIGANDS

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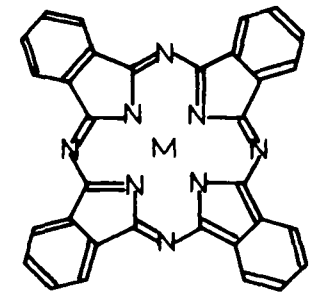
(Received May 23, 1982; in final form September 7, 1982)

Reconstitution of apomyoglobin has been attempted with six transition metal complexes containing synthetic macrocyclic ligands and has been successful in several cases. The success of reconstitution has been judged by electronic absorption spectroscopy and ANS fluorescence analysis of solutions of the reconstituted proteins. The most dramatically successful reconstitution was achieved with the complex [Fe TAAB $(An)_2$] $(BF_4)_2$, (An = accetonitrile, TAAB = [b,f,j,n] p1,5,9,13] -tetraazacyclohexadecine) which undergoes a color change from maroon-red to deep blue when exposed to apomyoglobin. The electronic spectrum of the blue solution corresponds with the known spectrum of FeTAAB$(meIm)_2^{2+}$ (MeIm = N-methylimidazole).

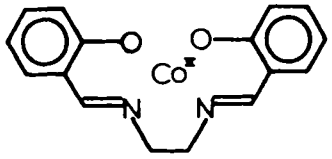
INTRODUCTION

Since the first successful reconstitution of a heme apoprotein with a specie other than heme itself,[1] an extensive research effort has shown that a wide variety of metalloporphyrins may be readily incorporated within the heme pockets of these proteins. To date, porphyrin compexes of Co,[1-11] Mn,[12-17] Zn,[18-20] Cu,[21] Ni,[21] Ag,[21] Cr,[21] Ru,[21] and Re[21] have been successfully incorporated and the resulting reconstituted proteins characterized by a variety of physical methods. In addition, the successful reconstitution of apomyoglobin with zinc and magnesium pyrochlorophyllides has been recently achieved.[22] However, despite the great number of synthetic macrocyclic ligands which is now available,[23] only a few attempts to incorporate metal complexes of such ligands within the heme pocket of hemoglobin or myoglobin have thus far been made.[24-28] Emphasis on porphyrin complexes is understandable considering the ubiquity of the porphyrin macrocycle in nature; nonetheless, it is important to extend these studies to synthetic macrocyclic systems. In particular, it is of interest to examine the influence of the protective protein matrix on the oxygen-binding capabilities of such systems, some of which have the ability to reversibly bind oxygen in the absence of the protein.[29,30]

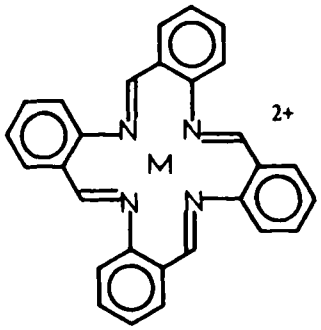
Accordingly, we have undertaken a research program with the twin goals of successfully reconstituting heme apoproteins with metal complexes containing synthetic macrocyclic or pseudo-macrocyclic ligands and determining the effect of the protein environment on the reversible oxygen-binding abilities of these complexes. This paper reports the results of our attempts to achieve the first goal. Specifically, we have attempted to reconstitute sperm whale apomyoglobin with the metal complexes shown in Figure 1. We chose to work with myoglobin, rather than hemoglobin, because it can be readily obtained from commercial sources and because it is readily "dehemed" by proven procedures. We chose the above complexes for our initial attempts either because of the structural similarity between the synthetic and porphyrin macrocycles (I-III, V, VI) or because of the known oxygen-binding ability of the complex (IV). We have shown, primarily *via* electronic absorption spectral analysis and ANS-fluorescence analysis



I, M = Fe^{II}
II, M = Co^{II}
III, M = Mn^{II}



IV



V, M = Co^{II}
VI, M = Fe^{II}

FIGURE 1 Metal complexes.

(vide infra) that complexes II, III, and VI are readily incorporated into the heme pocket of apomyoglobin. Our results for complexes I and V allow us to conclude only tentatively that we have achieved successful reconstitution. Finally, despite numerous attempts to reconstitute apomyoglobin with complex IV, we have thus far been unsuccessful.

EXPERIMENTAL

Reagents and Materials. Iron(II) phthalocyanine (FePc), I, and cobalt(II) phthalocyanine (CoPc), II, were purchased from Eastman Organic Chemicals, and used as received. Sperm whale myoglobin (Mb) was obtained from U.S. Biochemical Corporation, and stored at $-10°$. Manganese(II) phthalocyanine (MnPc), III, and magnesium 8-anilino-1-naphthalene sulfonate (ANS) were purchased from Fisher Scientific Company and used as received. Heme, obtained from Aldrich, was used as received and stored at $-10°$. Sephadex G-25 (Medium) was obtained from Pharmacia Fine Chemicals, and treated as

described below. Dialysis tubing (1.0 in flat diameter) was purchased from Scientific Products, and prepared as described below. The solvents, pyridine, 2, 6-lutidine, *N, N*-dimethylformamide (DMF), acetonitrile, dimethylsulfoxide (DMSO) and 2-butanone (MEK), used in removing the heme group from myoglobin, were all of reagent grade and were used without further purification. $K_2HPO_4 \cdot 3H_2O$ and $NaHCO_3$, used in preparing buffer solutions, were of reagent grade.

Physical Methods. Infrared spectra were obtained in nujol mulls and/or KBr pellets using either a Perkin-Elmer 457 (4000-250 cm^{-1}) or a Perkin-Elmer 397 grating IR spectrometer (4000-400 cm^{-1}). Low temperature electronic spectra were obtained with a Perkin-Elmer 323 recording spectrophotometer, equipped with a Forma Scientific Masterline constant temperature bath and circulator. Matched 1 cm quartz cells were supplied by Precision Cells Inc. Fluorescence spectra in the region 400-600 nm were measured using an Aminco-Bowman spectrofluorometer equipped with an EMI 9785B photomultiplier tube and a 150 watt Xenon arc excitation source. Spectra were recorded on a Leeds and Northrup Speedomax G strip chart recorder. EPR spectra were recorded on a Varian E-9 spectrometer operating at X-band frequencies. Magnetic field strength was measured with a Magnion NMR-type gaussmeter, and freqeuency was measured with the same counter equipped with a 5255A frequency converter. All measurements were performed on frozen solutions at or near liquid N_2 temperature (196 K). The pH measurements were carried out using an Instrumentation Laboratory Inc., Porto-Matic pH meter, model 175, and an Ingold combination pH electrode. Finally, the inert atmosphere glove chamber used in synthesis and in sample preparation was supplied by Kewaunee Scientific Equipment Corporation. Inert atmosphere was achieved by continuously flushing the box with the head gas from a liquid nitrogen tank.

Buffers. All buffer solutions were prepared using tap distilled H_2O and stored in Nalgene plastic bottles at 5°. Phospate buffers (0.01M, pH6; 0.025M, pH7; 0.10M, pH6.8) were prepared using $K_2HPO_4 \cdot 3H_2O$ and adjusted to pH using 6 N HCl,[8] or as described in *Buffers* by Calbiochem.[31] Bicarbonate buffer was prepared by dissolving 50 mg $NaHCO_3$ in 1 dm^3 of distilled H_2O.

Syntheses. 1. *(N,N'-Bis(salicylaldehyde)ethylenediminato)cobalt(II); (Co^{II} salen), IV.* IV was synthesized according to the procedure of Appleton[32] and was characterized by infrared absorption spectroscopy.[33] 2. *4b, 5, 15b, 16-tetrahydrodibenzo[3, 4: 7, 8][1, 5]diazocino[2, 1-b : 6, 5-b]diquinazoline-11, 22-diium tetrafluoroborate; (TAAB·$2HBF_4$).* This compound was prepared as red crystals from fresh *o*-aminobenzaldehyde and 48% tetrafluoroboric acid, according to published procedures.[34] The product was characterized by its infrared spectrum.[34] 3. *($Co^{II}TAAB(BF_4)_2$); V.* Synthesis of V was performed under a nitrogen atmosphere, using a Schlenk manifold, by a modification of, a published procedure.[34] Cobalt acetate tetrahydrate (0.24g, 1 mmole) and TAAB·$2HBF_4$ (0.59g, 1 mmole) were added to 40 cm^3 of degased ethanol. The resulting mixture was refluxed with stirring for 30 minutes, during which time the mixture changed from orange to dark green in color and green crystals began to deposit. Half of the solvent was removed *in vacuo*, and the green crystalline product was isolated by filtration in air, washed with ether, and dried *in vacuo*. The product, V, was characterized by infrared spectroscopy.[35] 4. *Iron(II) acetate.* Reagent grade iron powder (10g) was slurried with 200 cm^3 of glacial acetic acid, under an N_2 atmosphere. The mixture was heated at reflux for 15 hours, during which time white iron(II) acetate formed in the reaction mixture. The mixture was cooled to room temperature, still under N_2, and transferred to an N_2-filled glove bag. The white product was isolated by filtration, washed copiously with

ether to remove all traces of acetic acid, thoroughly dried *in vacuo*, and quickly transferred in capped vials to an inert atmosphere glove chamber, where it was stored and utilized in the synthesis of VI *(vide infra)*. 5. *([Fe^{II}TAAB(An)$_2$] (BF_4)$_2$); VI.* In an inert atmosphere glove chamber, 2.5 mmole (0.44g) iron(II)-acetate and 2.2 mmole (1.47g) TAAB·2HBF_4 were slurried with 50 cm^3 of acetonitrile. The mixture was stirred at reflux for two hours, during which time the color of the solution changed from bright orange, chatacreristic of TAAB·2HBF_4, to deep maroon-red, characteristic of VI. After 2 hours the mixture was filtered through celite ro remove unreacted iron(II) acetate and the filtrate was reduced in volume *in vacuo* by a factor of two. Dropwise addition of diethyl ether, with stirring, effected precipitation of dark red (almost black) crystals of VI. The product was isolated by filtration, washed with ether, and N_2 dried. Recrystallization was from acetonitrile, with addition of ether. The product was characterized by its IR[36,37] and electronic absorption[38] spectra.

Preparation of Sephadex and Dialysis Tubing. G-25 medium Sephadex (16g) was slurried with 150 cm^3 of distilled water and heated on a steam bath for 2 hours. The Sephadex slurry was then loaded into a separation column ($\sim$25 cm by $\sim$5 cm^2), which was subsequently kept at 5° at all times. Cellulose dialysis tubing was cut into 12 or 15 cm strips, which were placed in a beaker containing distilled water and heated for 2 hours. After this time, the hot water was decanted and replaced with fresh cold distilled H_2O. Prior to use in dialysis, one end of a strip was tied with dental floss. After filling, the top of the tube was tied similarly.

Preparation of Apomyoglobin (AMb). Stock aqueous solutions of apomyoglobin having concentrations of ca. 1×10^{-3}M were prepared by the acid-butanone method[39,40] from commercially available sperm whale myoglobin. Concentrations of AMb were determined by measuring the absorbance of a fivefold or tenfold dilution of the stock solution at 280 nm (ϵ = 15,800 cm^2/mmole).[7]

Procedure for Reconstitution of AMb with metal complexes I – VI. The following general procedure, based on published methods,[1,4,8] was used in all reconstitution attempts. All of the manipulations were carried out at 5° under nitrogen atmosphere, unless otherwise noted.

A Sephadex separation column, prepared as described above, was equilibrated with 250 cm^3 of deoxygenated 0.01M phospate buffer (pH6). The column and buffer solution in the column reservoir were subsequently kept free of O_2 by maintaining a continuous purge of N_2 through the column reservoir. A 50 to 500% molar excess of metal complex was then dissolved in 15 cm^3 of an organic solvent/H_2O mixture, which had been previously deoxygenated by purging with N_2 for 20 minutes and cooled in an ice bath to 5° (the specific solvent mixture used for each complex is given in Table I). All metal complex solutions were prepared in either an inert atmosphere glove bag or glove box. The solution of AMb prepared above was filtered to remove a small quantity of denatured protein. 5 cm^3 of metal solution was then added carefully to 5 cm^3 of aqueous AMb, with gentle agitation, and the resulting solution was placed immediately on the cold Sephadex column and eluted with .01M phospate buffer (pH 6). Typically two colored bands developed on the column during elution, one moving rapidly down the column, the other more slowly. Following the collection of an initial 20 cm^3 of eluant, ten 5 cm^3 fractions were collected. The first colored band was usually collected in fractions 2-5 and the second in fractions 7-9. Fractions 1, 6, and 10 were typically colorless. Following elution, the colored fractions were characterized by electronic absorption spectroscopy (to detect the presence of both protein and metal complex), epr spectroscopy where

appropriate, and dialysis against ANS followed by fluorescence analysis *(vide infra)* in order to establish whether or not reconstitution had been successful. In almost all cases, electronic absorption spectra showed the presence of both protein and metal complex in fractions 3-5. The protein concentration in these fractions was readily measured by recording the absorbance at 280 nm of tenfold dilutions of aliquots of the fractions. Dilutions were made using cold distilled H_2O. Quantitative measurement of protein concentration in the fractions was necessary in order to perform the ANS fluorescence experiments *(vide infra)*. After each reconsitution attempt, the separation column and associated plastic attachments were soaked overnight in 6M HCl before reuse to remove all traces of pyridine or 2, 6-lutidine.

Dialysis against ANS; Fluorescence Analysis. It has been previously shown[41] that the anion of magnesium 8-anilino-1-naphthalenesulfonate (ANS) binds specifically at the heme pocket of both apomyoglobin and apohemoglobin. Upon binding, the weak fluorescence of ANS at 515 nm (quantum yield = 0.004, excitation wavelength – 365 nm) intensifies substantially and shifts to 475 nm (quantum yield = 0.98, excitation wavelength = 365 nm). ANS therefore serves as a convenient tool for probing the occupancy of the heme pocket. All solutions containing reconstituted AMb were thus equilibrium dialyzed against ANS and subsequently fluorescence analyzed in order to determine whether or not successful reconstitution of AMb with metal complex had been achieved.

A 10^{-4} solution of ANS in 0.1M phosphate buffer (pH 6.8) was prepared as follows. ANS (.0621g, 10^{-4} mole) was slurried with 300 cm^3 of buffer. The slurry was heated and stirred until complete dissolution occurred, giving a pale green solution. This solution was cooled to room temperature and diluted with an additional 700 cm^3 of buffer solution. The resulting solution was transferred to a Nalgene bottle and stored at 5°.

Dialyses of aqueous solutions of AMb and reconstituted Mb (abbreviated RMb where R = I-VI) against aqueous ANS were carried out by the following procedure. Equimolar solutions ($\sim 10^{-6}$ M) of AMb and RMb were prepared by appropriately diluting solutions whose concentrations were measured using the protein absorbance at 280 nm. In the case of RMb, the elution fraction containing the highest protein concentration was routinely used. 4 cm^3 of each solution was transferred to a dialysis bag. The bags were then placed in separate flasks, each containing 100 cm^3 of 10^{-4} M ANS in 0.1M pH 6.8 phosphate buffer solution. The solutions of AMb and RMb were equilibrium dialyzed against the ANS solution for 24 hours at 5° with constant stirring.

Following equilibrium dialysis, fluorescence spectra of the following solutions were examined in the range 420-600 nm (excitation was at 365 nm); 1. AMb which had been dialyzed against ANS; 2. RMb which had been dialyzed against ANS; 3. Aqueous ANS. Equivalence of the spectra of solutions 1 and 2 indicated unsucessful reconstitution; equivalence of the spectra of solutions 2 and 3 indicated successful reconstitution.

RESULTS AND DISCUSSION

The results of attempts to reconstitute AMb with complexes I-VI will be discussed for each complex in turn. In general, either 3 or 4 experimental methods were used for each metal complex to establish whether or not reconstitution had been successfully achieved. Simultaneous movement of protein and colored complex down the Sephadex column indicated an intimate association between complex and protein, since in the absence of such association, relatively low MW metal complexes (MW < 1000) would be expected to elute much more slowly than high MW proteins (MW $\sim$ 17,500). Indeed, in most cases, two colored bands were observed, the first moving down with protein (collected in frac-

tions 2-5), and the second lagging considerably behind (fractions 7-9). The latter band was due to excess unreacted metal complex, as established by electronic spectral measurements. It should be noted that although simultaneous movement of metal complex and protein indicates intimate association between the two, it does not necessarily indicate that the metal complex is bound to the protein *via* the proximal histidine in the heme pocket. Electronic spectra of elution fractions containing both protein and metal complex were examined and compared with spectra of the metal complex in the absence of protein. Shifts in metal complex band positions resulting from the presence of protein were interpreted to indicate protein-complex binding. With the exception of complex VI, this method served to substantiate only that metal complex was bound in some way to the protein. Again, no information as to the location of the metal complex within the protein was obtainable by this method, except for the case of complex VI, where electronic spectra clearly indicated that the metal complex was bound at the heme site. Electron paramagnetic resonance spectra of frozen solutions of RMb were examined for those complexes containing cobalt and manganese (complexes I, III-V). In no case was an epr spectrum observed. Finally, the fluorescence spectra of aqueous solutions of RMb which had been dialyzed against ANS were measured and compared with corresponding spectra of solutions of AMb which had been dialyzed against ANS, and ANS solution which had not been exposed to RMb or AMb. As outlined above, ANS binds relatively strongly in the heme pocket of heme apoproteins, and when so bound, exhibits a strong fluorescence at 475 nm. In contrast, free ANS exhibits only weak fluorescence at 515 nm. Consequently, observation of only weak fluorescence at 515 nm in ANS-dialyzed RMb solutions indicates occupation of the heme pocket by metal complex, and hence a successful reconstitution. On the other hand, strong fluorescence at 475 nm indicates either that metal complex was never bound at the heme site, or that it was displaced by ANS during dialysis. Of the four experimental approaches used to test the success or failure of reconstitution, the fluorescence probe is the only one which unambiguously establishes binding of the metal complex at the heme site.

Reconstitution Attempts. Complex I. Elution of a mixture of 5 cm^3 of a 2.9×10^{-3} M solution of I in 4/1 pyridine/H_2O (intensely blue in color) and 5 cm^3 of $\sim 1 \times 10^{-3}$ M AMb on a Sephadex column yielded two distinct blue-green bands. The first band was

TABLE I
Organic Solvent/H_2O Mixtures

Complex	Solvent/H_2O (V/V composition)	
I	pyridine/H_2O	(4/1)
II	pyridine/H_2O	(4/1)
	pyridine/H_2O	(2/3)
III	pyridine/H_2O	(4/1)
IV	pyridine/H_2O	(4/1)
	DMF/H_2O	(4/1)
	DMF/H_2O	(1/2)
	2,6-lutidine/H_2O	(1/1)
V	DMF/H_2O	(2/3)
	DMF/H_2O	(1/4)
VI	DMF/H_2O	(1/4)

collected in fractions 1–4. Electronic spectra of these fractions indicated the presence of both protein (λ_{max} = 280 nm) and I (λ_{max} = 674 nm). The second colored band was collected in fractions 7 and 8; similar analysis showed only pyridine absorbtions in the UV (i.e., the absence of protein) and absorptions due to I in the visible (λ_{max} = 668 nm). A second reconstitution attempt using 2/3 pyridine/H_2O to dissolve I gave identical results.

It is well-known that the position of the MLCT band in low spin complexes of Fe(II) is strongly dependent on the donor strength of the axial ligand(s).[42] Specifically, the band shifts to longer wavelength as the strength of the axial ligand increases. Data indicating this shift for complex I are given in Table II. It is to be noted that λ_{max} shifts about 7 nm to the red when imidazole replaces pyridine as the axial ligand. Visible spectral data for elution fractions 3, 4, 7, and 8 are also included in Table II. The wavelength of maximum absorption for fractions 3 and 4, which contain both I and protein, occurs some 6 nm to longer wavelength than the corresponding absorption for fractions 7 and 8, in which I, but no protein, is present. Presumably in these fractions, pyridine occupies the axial sites

TABLE II
Electronic Spectral Data for Metal Complexes and Reconstituted AMb

Specie	Axial Ligand	Solvent	λ_{max}(nm), Visible	λ_{max}(nm), UV
I	DMSO	DMSO	652 656	
	py	py	655 657	
	Im	DMSO	662 660	
I + AMb				
Fraction 3			673	280
Fraction 4			673	280
Fraction 7			668	
Fraction 8			668	
II	DMSO	DMSO	664	
	py	50% py/H_2O	669	
	Im	DMSO	676	
II + AMb				
Fraction 3			681	280
Fraction 4			681	280
Fraction 8			676	
III				
III + AMb			660, 520, 365	280
$Mn^{III}Pc(OH^-)_{py}$			716, 645, 513 (Ref 45)	
VI	An	An	665 522 490 (sh)	
	DMF	1/4 DMF/H_2O		
	MeIm	An	591, 550 (sh) 594, 555 (sh)	
VI + AMb	Im	phosphate buffer	595, 560 (sh)	

in I. This 6 nm shift to longer wavelength in fractions 3 and 4 is entirely consistent with axial coordination of I by imidazole, and indicates that I has indeed been incorporated at the heme site.

Attempts to confirm reconstitution *via* ANS fluorescence analysis were unsuccessful. We therefore conclude that if I does bind at the heme site, it is completely replaced by ANS during dialysis.

Complex II. Elution of a mixture of 5 cm^3 of a 2.9×10^{-3} M solution of II in 4/1 pyridine/H_2O (intensely green in color) and 5 cm^3 of $\sim 1 \times 10^{-3}$ M AMb resulted in two distinct green bands. The first band was collected in fractions 2-4, and the second in fractions 7-8. Electronic spectral analysis indicated the presence of both II and protein in fractions 2-4, but only II and pyridine in fractions 7-8. The relevant data are collected in Table II. Again, it is to be noted that λ_{max} for II occurs about 5 nm to longer wavelength in fractions 2-4 than in fractions 7-8. Table II contains electronic spectral data for II in the presence of various axial ligands, showing that λ_{max} shifts to the red as the donor strength of the axial ligand increases. The data therefore indicate that II in fractions 2-4 is coordinated by a stronger axial ligand than in fractions 7-8, and hence that II has been successfully incorporated into the heme pocket.

Further support for successful reconstitution is provided by the results of ANS fluorescence experiments. The fluorescence spectrum of reconstituted protein dialyzed against ANS shows only weak fluorescence at 500 nm. The intensity of the peak is the same as that of an equimolar aqueous solution of ANS. The absence of strong fluorescence at 475 nm in the reconstituted protein solution indicates occupation of the heme sites by II, and hence a successful reconstitution.

Finally, we made several attempts to observe EPR spectra of frozen phosphate buffer solutions of IIMb, but without success. We are currently unable to explain the absence of an EPR spectrum.

Complex III. Elution of a mixture of 5 cm^3 of a forest-green 2.9×10^{-3} M solution of III in 4/1 pyridine/H_2O and 5 cm^3 of $\sim 1 \times 10^{-3}$ M AMb yielded two distinct green bands, the first being collected in fractions 3-5 and the second in fractions 7-9. Electronic spectral analysis of fractions 3-5 proved conclusively that both III (actually, $Mn^{III}Pc(OH)$, *vide infra*) and protein were present in an intimate mixture in the first colored band. A typical spectrum is shown in Figure 2. In contrast, the electronic spectra of the second set of colored fractions showed the presence of pyridine and $Mn^{III}Pc(OH)$ (*vide infra*), but no protein (see Figure 2). The results of Sephadex elution and electronic spectral analysis thus indicate complex formation between III, or a derivative thereof, and AMb.

In order to establish the incorporation of III into the heme pocket of the protein, we dialyzed the reconstituted protein solution against ANS and recorded the fluorescence emission of the protein solution. The spectrum consists of a single weak band at 515 nm, with the same intensity as that of an equimolar aqueous solution of ANS. There is no evidence of the strong fluorescence at 475 nm characteristic of ANS in the heme pocket. We thus conclude that ANS was effectively blocked from entering the heme pocket due to the presence of III. The fluorescence experiment proves definitively that reconstitution with III was successful.

It is appropriate at this point to discuss more fully the electronic spectral results described above. Although we have implied up to this point that it is III which is incorporated into AMb and bound at the heme site, this is apparently not the case. Phthalocyanine and porphyrin complexes of Mn(II) have long been known to be susceptible to one-electron oxidation at the metal center,[43,44] and in fact, Mn^{II}PPIX (PPIX = protoporphyrin

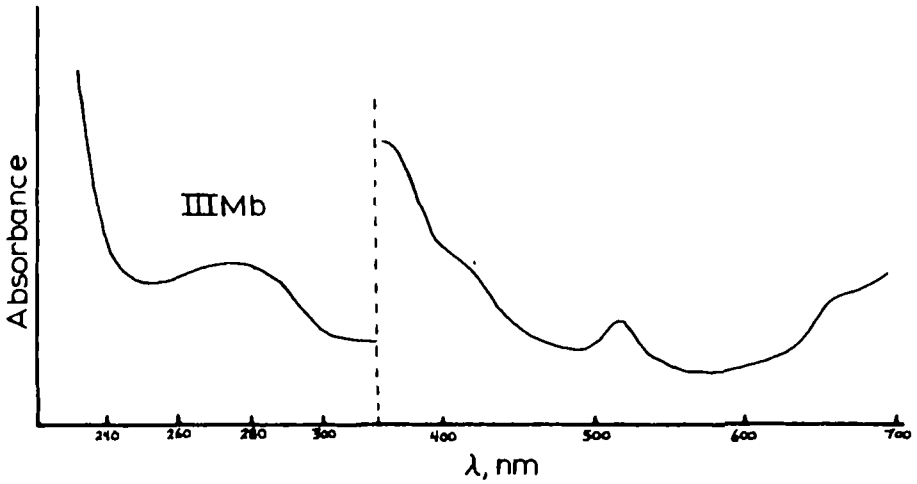


FIGURE 2 Electronic spectra of IIIMb.

IX) is irreversibly oxidized when incorporated into apohemoglobin.[17] In view of these results we felt it possible that, rather than incorporating III, we had in fact incorporated a $Mn^{III}Pc$ derivative, formed from III during the reconstitution procedure. We therefore compared the visible spectrum of the reconstituted protein with the spectrum of $Mn^{III}(Pc)\ (OH)^-$,[44,45] the most likely product of oxidation of III in acqueous solution ($Mn^{III}(Pc)\ (O_2)^-$ is an alternate, but less likely, possibility).[30] We find that the spectra are in remarkable agreement. This indicates that we have actually reconstituted apomyoglobin with $Mn^{III}(Pc)\ (OH)^-$, which presumably coordinates to the proximal imidazole *via* the open axial site. To further support the proposed oxidation, we examined the EPR spectrum of reconstituted protein in frozen phosphate buffer solution. The complete absence of a typical Mn(II) signal is completely consistent with oxidation to Mn(III) having occurred.[46]

Complex IV. Despite numerous attempts to incorporate IV at the heme site in AMb, using DMF, pyridine/water mixtures or 2, 6-lutidine/water mixtures of varying compositions (see Table I), we were unable to achieve successful reconstitution. Electronic spectral analysis of colored fractions indicated the complete absence of protein.

Complex V. Elution of a mixture of 5 cm^3 of a 5×10^{-3} M solution of V in 4/1 DMF/H_2O (intense forest-green in color) and 5 cm^3 of $\sim 1 \times 10^{-3}$ M AMb yielded two distinct colored bands. The first band, collected in fractions 2-5, was yellow-gold in color, and the second band, collected in fractions 7-10, was green. Electronic spectral analysis showed the simultaneous presence of V and protein in fractions 2-5, and the absence of protein in fractions 7-9. Further, the spectra of fractions 2-5 are similar to the spectrum of V in acetonitrile containing imidazole. Some representative spectra are shown in Figure 3. Electronic spectral results therefore indicate that reconstitution to form VMb was successful.

Unfortunately, quantitative ANS fluorescence experiments could not be carried out on this system. The TAAB ligand absorbs strongly in the region of 280 nm, thereby rendering impossible a quantitative measure of protein concentration in solutions containing VMb. To confirm incorporation of V into the heme pocket of the protein, we attempted to measure the epr spectra of frozen phosphate buffer solutions of VMb.

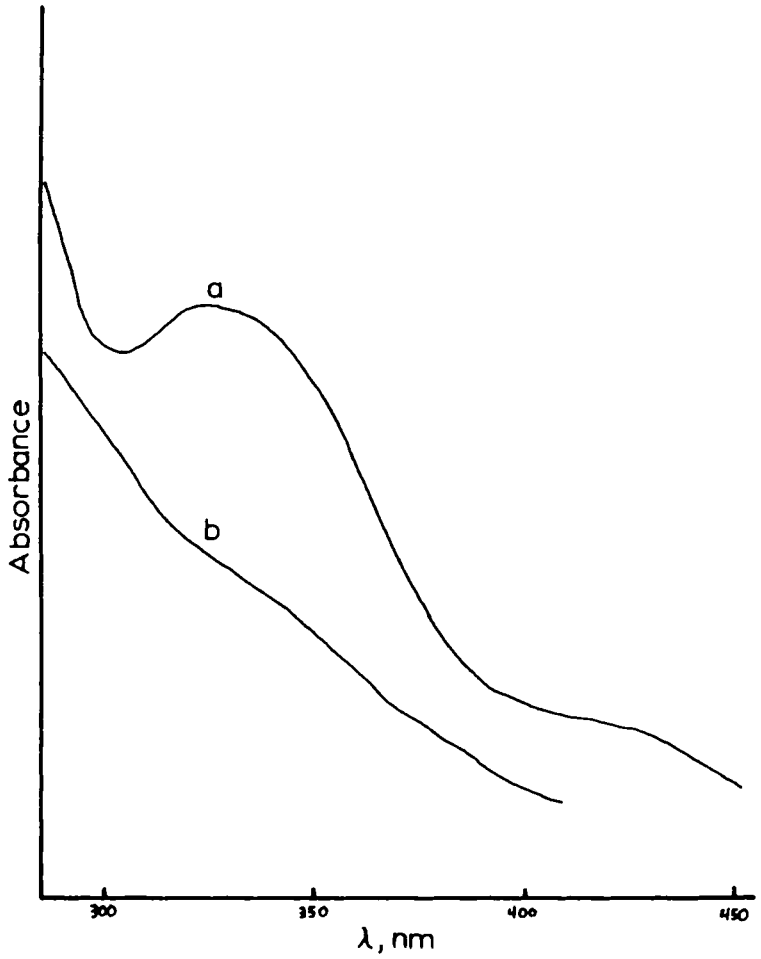


FIGURE 3 Electronic spectra of V; a = VMb, b = V + imidazole in acetonitrile.

However, these attempts were unsuccessful. We felt that the absence of an epr signal might indicate that the cobalt(II) center had been oxidized to cobalt(III) either before or during the reconstitution procedure. However, intense epr signals from fractions 7-9, characteristic of the $Co^{II}TAAB^{2+}$ species,[47] proved that oxidation could not have occurred prior to reconstitution. In addition, electronic spectra were consistent with the presence of Co(II) in VMb. Thus our uniform inability to observe epr signals from Co(II)-containing reconstituted proteins remains puzzling.

Complex VI. The behaviour of this complex upon exposure to AMb was very dramatic. Addition of 5 cm^3 of a maroon-red, 3.3×10^{-2} M solution of $Fe(TAAB)(An)_2(BF_4)_2$ in 1/4 DMF/H_2O to 5 cm^3 of $\sim 1 \times 10^{-3}$ M AMb resulted in an immediate color change to violet blue. Elution of the mixture on a Sephadex column resulted in two distinct colored bands. The first band, collected in fractions 2-5, was intensely blue in color, whereas the second band, collected in fractions 7-10, was the maroon-red characteristic of $Fe^{II}TAAB$ coordinated axially by relatively weak ligands such as CH_3CN and DMF[38] (λ_{max} = 520, 560 (sh)). Interestingly, the electronic spectra of the solutions in fractions 2-5 all

exhibited a band at 595 nm, with a shoulder at 560 nm. This spectrum agrees very closely with that reported for the *bis*-methylimidazole complex of $Fe^{II}TAAB^{2+}$ in acetonitrile solution (λ_{max} at 591 nm, with a shoulder at 550 nm).[38] Appropriate spectra are shown in Figure 4, and the spectral data are included in Table II. Experiments in our laboratories indicate that formation of *bis*-methylimidazole $Fe^{II}TAAB$ from the corresponding *bis*-acetonitrile complex is thermodynamically very favorable, even in acetonitrile solvent.[48] In fact, we have observed that even at [MeIm]/[FeTAAB] ratios as low as 0.5, formation of the *bis*-methylimidazole specie occurs. $Fe^{II}TAAB^{2+}$ therefore has a strong affinity for imidazole-type ligands, and in this way is analogous to naturally occurring hemes. Due to the close correspondence of the spectra of VIMb with that of *bis*-(MeIm)-$Fe^{II}TAAB^{2+}$, it is tempting to speculate that VI has induced both the proximal and distal histidine residues to coordinate, thereby changing the conformation of the protein in the vicinity of the heme pocket. As we have no further evidence supporting this hypothesis, however,

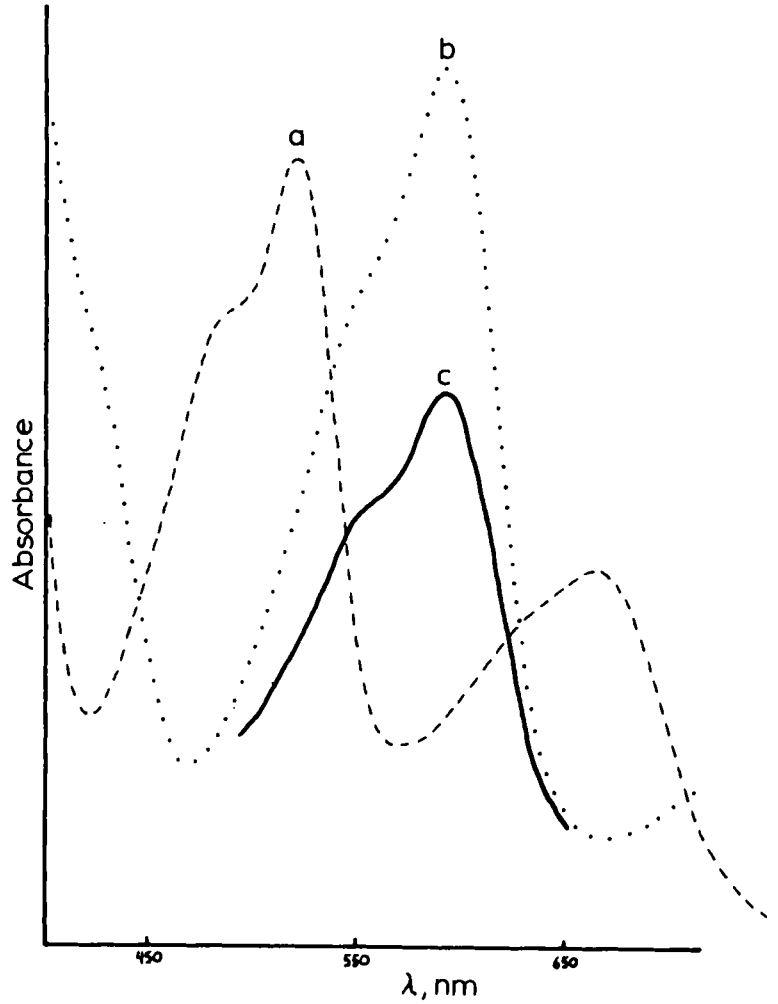


FIGURE 4 Electronic spectra of VI; a = VI in acetonitrile (axial acetonitrile), b = VI + imidazole in acetonitrile, c = VIMb.

it must remain speculation at this point. As for complex V, the presence of aromatic rings in VI precluded quantitative ANS fluorescence experiments. However, the electronic spectral data show conclusively that VIMb has indeed been formed.

Due to the hydrophobic nature of the heme pocket, it seems surprising that dicationic species such as V and VI can reside there. However, there is a precedent for incorporating cationic species in the heme pocket. Dori and coworkers successfully reconstituted apohemoglobin with a monocationic macrocyclic ligand complex of Fe(III) and showed that the resulting reconstituted protein exhibited oxygen binding capabilities.[24] Further, the monoanionic ANS specie apparently enters the heme pocket with little difficulty,[41] although it may exist there in the form of ion pairs with the Mg^{2+} cation.

In conclusion, we have shown that both charged and uncharged metal complexes containing synthetic macrocyclic ligands may be successfully incorporated within the heme pocket of apomyoglobin. Of the complexes tried, VI ($FeTAAB^{2+}$) shows the greatest affinity for the imidazole ligand of proximal histidine, and exhibits the most dramatic spectral change upon incorporation. Interestingly, the TAAB macrocycle is very similar to the protoporphyrin IX ring of heme, both in electronic structure and size, so that the match between VI and AMb is apparently quite good. Having demonstrated that reconstitution of heme proteins with non-porphyrin macrocyclic ligand complexes is possible, we now plan to investigate the effects of the protein environment on the oxygen-binding abilities of the complexes.

ACKNOWLEDGEMENTS

We would like to thank Dr. R. Gagné for suggesting the use of a liquid N_2 tank to maintain an inert atmosphere in the glove box, Professor B.M. Hoffmann for suggesting that we attempt reconstitution of AMb with MnPc and Cynthia E. Atkins for synthesizing Fe(TAAB) $(An)_2$ $(BF_4)_2$.

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