Coboglobins: Oxygen-Carrying Cobalt-Reconstituted Hemoglobin and Myoglobin*

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Abstract. In this work we show that it is possible to prepare and study a cobalt-substituted hemoglobin—a coboglobin (Cb)—and that this reconstituted metalloprotein exhibits reversible oxygen binding. The effect of the protein environment on Co(II)-protoporphyrin IX is directly observed by esr measurements on deoxy- and oxy-Cb and by oxygen uptake measurements, all of which may be compared with similar measurements on the free metalloporphyrin. Reversing our point of view, we compare oxygen binding to Cb with that of hemoglobin, and thus investigate the relationship of the metal atom and metal-oxygen binding to such characteristics of the nature proteins as cooperative oxygen uptake.

The reversible binding of molecular oxygen to the heme-iron of hemoglobin and myoglobin has been examined extensively for several decades.[1,2] These studies, originally performed because of the intrinsic physiological significance of the reaction, are increasingly pursued with the aim of understanding the nature of protein function and mechanism, with hemoglobin of particular importance for its modeling of allosteric, or cooperative effects. However, despite the wealth of information which has been accumulated about the oxygenation process, the mode of binding of oxygen to the heme and the mechanism of the cooperative interaction of heme sites of hemoglobin during oxygenation remains unresolved.

We[3,4] and others[5] have recently shown that various planar Co(II) complexes can reversibly form 1:1 adducts of molecular oxygen. These adducts are further related to oxygen-carrying hemoproteins by the fact that they generally possess a coordinated nitrogen base in the sixth coordination site of the metal and the bound oxygen has become spin paired upon coordination. As the only synthetic systems known with such properties these adducts form obvious models for the natural systems. Since the cobalt adducts are paramagnetic, they may be studied by electron paramagnetic resonance (epr) which gives microscopic insights into their electronic and geometric structure which are not available for the diamagnetic oxy-Hb and oxy-Mb.[4,5]

In the course of these model studies, the reconstitution of globins with a cobalt (II)-porphyrin to form an oxygen-carrying, cobalt-metalloprotein suggested itself as an excellent means of introducing a *functional* paramagnetic probe into these proteins. The work of Antonini and co-workers on the placement of artificial iron porphyrins[6] as well as porphyrin[7] itself into globin was strong evidence to suppose that this approach might succeed.

As this preliminary communication describes, we have now found that the dimethyl ester of Co(II)-protoporphyrin-IX (CoP) reversibly forms a 1:1 oxygen adduct in organic solvents and in the presence of a nitrogen base. We have further taken globins from bovine hemoglobin and myoglobin and reconstituted them with CoP. These *coboglobins* (Cb), the cobalt analogs of hemoglobin and myoglobin, indeed function as oxygen-carrying proteins.

The systems described here make contact between a protein and a model reaction in direct and unique fashion because the same reaction of oxygen binding can be studied in the presence and absence of protein. The effect of the protein environment on CoP is directly observed by epr measurements on deoxy- and oxy-Cb and by oxygen uptake measurements, all of which may be compared with similar measurements on the free metalloporphyrin or its dimethyl ester. Alternatively, we may compare oxygen binding to Cb with that to Hb, and thus examine the relationship of the metal atom and metal–oxygen bond to functional characteristics of the native proteins, such as cooperative oxygen uptake by hemoglobin.

Materials and Methods. Protoporphyrin-IX dimethyl ester, Grade 1, lot 49B-0140 and hemin Type 3, lot 39B-0610, were purchased from Sigma and used without further purification. Whale skeletal muscle myoglobin, A grade, Lot 802091, was obtained from Calbiochem. Fresh bovine blood was a gift of Sol Hoffman. Sodium hydrosulfite was from Fisher Chemical. All other reagents were reagent grade.

The preparation of Co(II) protoporphyrin-IX dimethyl ester and its acid are fully described elsewhere.[8] Hemoglobin was prepared as described by Perutz.[9] The preparation of globin developed by Rossi-Fanelli, Antonini, and Caputo[10] was followed explicitly for both myoglobin and hemoglobin. Reconstitution of globin with heme is also detailed elsewhere.[6]

The reconstitution of coboglobin involves the addition of 1.5-fold excess of CoP in 0.04 ml of 10% pyridine in water and excess hydrosulfite to 1–2 ml of globin in 0.2 M phosphate buffer, pH 7.0. Within 5 min the mixture is placed on a small Biogel P-60 column to remove pyridine. The eluate is treated with DEAE-cellulose, De-52, batchwise, to remove excess free CoP. The product, which contains a significant amount of Co(III), is reduced with hydrosulfite. Oxy-Cb is produced upon aeration.

Epr spectra were taken at 77°K as previously described.[4a] Oxygenation curves were determined spectrophotometrically as described, for example by Rossi-Fanelli and Antonini.[11]

Results. Epr: Liquid-nitrogen-temperature epr spectra and spin-hamiltonian parameters for solid deoxy-CoP-(pyridine)$_1$, for deoxy-CoP dimethyl ester-(t-butyl pyridine)$_1$ in toluene, and for aqueous deoxy-coboglobin are extremely similar and are typical of low-spin Co(II) with the unpaired electron in the $d_z{}^2$ orbital and with axial or near axial symmetry.[4,5] All contain five-coordinated cobalt, as evidenced by observed hyperfine splittings from the nitrogen atom of a *single* axially coordinated base. Parameters for deoxy-coboglobin are: $g_\perp = 2.33$; $g_{11} \approx 2.0$; $A^{Co}{}_\perp \leqslant 10G$; $A^{Co}{}_{11} \approx 80G$; $A^N{}_{11} \approx 17G$.

In solutions with excess nitrogen base both deoxy-CoP monomers (10% pyridine–water) and deoxy-CoP dimethyl ester (organic media) apparently take on two bases giving spectra which are again similar, but quite different from the five coordinate spectra. Parameters for CoP dimethyl ester-(t-butyl pyridine)$_2$ in toluene–hexane are: $g_\perp = 2.23$; $g_{11} = 2.06$; $A^{Co}{}_\perp = 58.6G$; $A^{Co}{}_{11} = 52.8G$. The observation of both five- and six-coordinated porphyrins is similar

to the results of Walker[5e] with synthetic porphyrins and along with the observation of superhyperfine splittings in Cb from a single nitrogen atom, verifies the five-coordination of cobalt in coboglobin.   This latter splitting is undoubtedly from the nitrogen of the proximal histidine axially coordinated to the metal atom. Absence of observable effects from a second nitrogen indicates that the distal histidine is at best much more weakly coordinated.

The epr spectrum of deoxy-Cb is shown in Fig. 1A.   Often an additional broad signal occurs around g $\leqslant$ 2; this is believed to be aggregated cobalt porphyrin, presumably adsorbed to the protein (Fig. 2A).   This signal is well separated
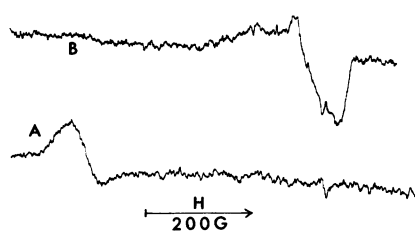

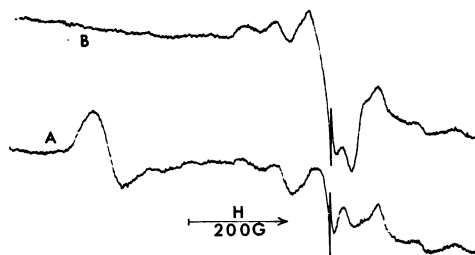FIG. 1. Epr spectra of (A) deoxy-coboglobin and (B) oxy-coboglobin.

FIG. 2. Epr spectra of (A) deoxy-coboglobin and (B) oxy-coboglobin showing CoP aggregate signal. Sharp spikes are from DPPH reference.

from the perpendicular region of the deoxy-Cb resonance and is invariant during cycles of oxygenation–deoxygenation.   Barely visible in this spectrum are the two lowest field[57] Co lines in the parallel region which when amplified give A^{Co}_{11} and A^{N}_{11}.

Oxygenation of CoP-dimethyl ester in organic media containing a variety of nitrogen bases produces the 1:1 molecular oxygen adduct of cobalt with typical epr spectra (Fig. 3A) and spin-hamiltonian parameters.[4,5]   The parameters for oxy-CoP-dimethyl ester in pyridine are $g_{\perp}$ = 2.08, g_{iso} = 2.02; $g_{\perp}$ = $^{1}/_{2}(3g_{iso} - g_{11})$ = 1.99; A^{N}_{11} = 17.5G, a^{Co}_{1so} = 11.8G, $A^{Co}_{\perp}$ $\approx$ 9G. However, we have observed that A^{Co}_{11} for a given N-base is solvent dependent, decreasing in more nonpolar solvents.   The spectrum of Fig. 3A was from a solution in toluene–heptane–pyridine 3:3:1.

CoP itself is not soluble in organic media.   Oxygenating solutions of CoP in 10% pyridine–water leads to loss of the monomer epr signal, presumably through oxidation to Co(III). The aggregate spectrum is unaffected and no new signal appears.

Upon admission of oxygen to deoxy Cb and freezing, the spectra of 1A and 2A change to those of 1B and 2B, respectively. The spectrum of deoxy-Cb vanishes and a new spectrum, that of oxy-Cb appears (1B), although in
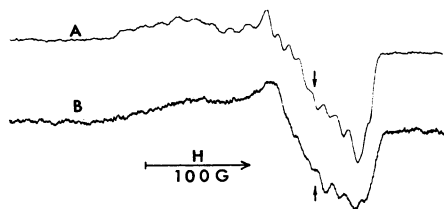

FIG. 3. Epr spectrum of (A) oxy-CoP-dimethyl ester–pyridine in heptane–toluene–pyridine 3:3:1 and (B) oxy-coboglobin. Arrows indicate g = 2.

the presence of aggregated porphyrin this signal is not distinct (2*B*).   The spectrum of oxy-Cb (3*B*) exhibits an envelope virtually identical with that of oxy-CoP dimethyl ester-pyr in an appropriate solvent (3*A*).   Thus, the electronic and geometric structure of the CoP adduct is not significantly altered by the environment of the protein "pocket" of Cb.   The oxygenation of Cb is completely reversible, at least over periods of weeks.   As many as five oxygenation-deoxygenation cycles have been performed on a given sample with no significant loss in signal and with no new signal being observed.   As expected, coboglobin-Mb from the parent myoglobin gives altogether similar results.

To better establish that the CoP has indeed been placed in the globin "cleft" and that nonspecific binding to the protein has not led to these results, the standard reconstitution has been carried out using CoP and hemoglobin.   In this circumstance, in which the protein already contains a full complement of heme, only the aggregated porphyrin epr spectrum is observed.   There are no spectral changes upon performing oxygenation–deoxygenation cycles, although the deoxy spectrum would be readily resolvable (Fig. 2) if present.

Oxygen binding: Epr experiments involve freezing the protein solution to nitrogen temperature.   The oxygenation of Cb in fluid solution may be followed by monitoring changes in the visible spectrum.   In deoxyhemoglobin, the porphyrin absorption with maximum at 555 nm is "split" upon oxygenation into two peaks at 541 and 578 nm.   This spectral change may be used to monitor oxygen uptake (Fig. 4*A*).   The same optical transition occurs at 545 nm in deoxy-Cb and upon oxygenation also "splits" with new peaks arising at 538 and 568 nm (Fig. 4*B*).   This spectral change for Cb is reversible as is that for Hb, and has also been used to monitor oxygen uptake.   A shift in the Soret
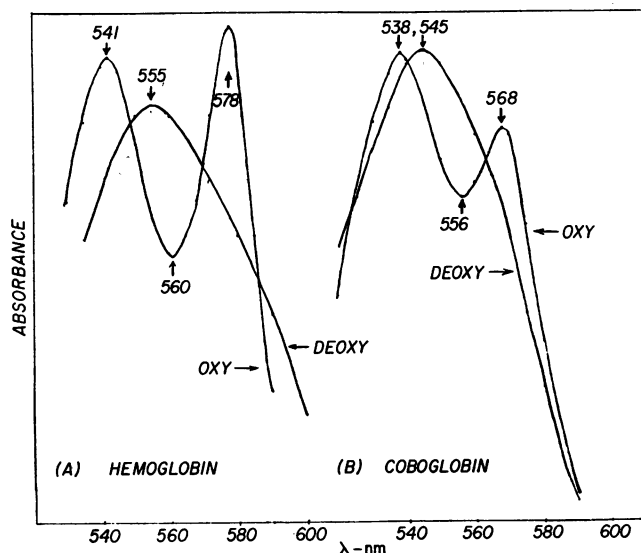

FIG. 4.   Visible spectra of oxy- and deoxy-hemoglobin and coboglobin.

peak from 423 to 402 nm accompanies oxygenation of Cb and Cb-Mb. This blue shift contrasts with the red shift from 412 nm to 430 nm in Hb.

Fig. 5 gives preliminary results for oxygen uptake measurements at 15°C, in 0.2 M phosphate buffer, pH 7. Oxygenation curves of native and reconstituted hemoglobin are indistinguishable (5A), indicating that the heme splitting, procedures employed can leave the globin undegraded. The curve is sigmoid, with $P^1/_2 = 7.5$ mm Hg. A Hill plot gives a straight line of slope $n = 2.5$, in acceptable accord with results of other workers.[1]
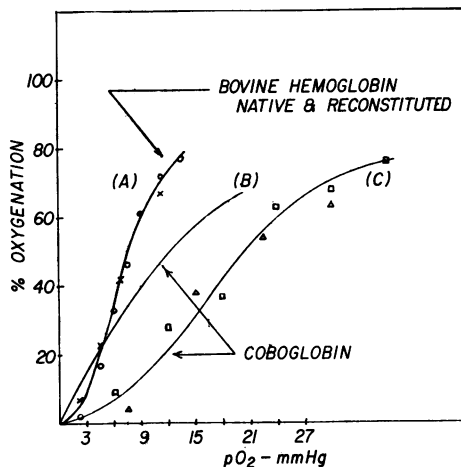

FIG. 5. Oxygen equilibrium curves: buffer 0.2 M phosphate, pH 7.0. C contained 0.3 M NaCl. Temperature 14–15°C. Samples: (A), hemoglobin, native (O) and reconstituted (X); (B), coboglobin from noncooperative reconstituted globin; (C), coboglobin from two samples of globin, that corresponding to □ being the same globin sample as gave the points X in curve A.

Curve 5B is a composite of several oxygenation curves for Cb reconstituted from globin preparations which showed noncooperative hyperbolic, oxygenation curves when reconstituted with hematin. This curve is also hyperbolic, with $P^1/_2 = 12.5$ mm Hg.

Curve 5C is the oxygenation curve for two Cb preparations reconstituted from globin samples which exhibit cooperativity when reconstituted with hematin. Although these are preliminary results, the curves appear cooperative, with $P^1/_2 = 20.5$ mm Hg. A Hill plot given a straight line with slope $n \approx 2.3$. Thus we find that the substitution of Co for Fe in native Hb reduces the strength of binding by a factor of only about 3. If these initial results are confirmed by further oxygenation experiments and through characterization of the protein, then we see that the substitution apparently has no significant effect on the cooperativity of binding.

CO binding: As a further comparison with hemoglobin, the binding of CO to deoxy coboglobin was investigated. At one atmosphere CO, no change in the 545 nm peak in the optical spectrum could be observed. When the vessel was flushed with air, the oxy-Cb spectrum returned quickly. Hence, little if any binding of CO occurs with Cb, in contrast to its strong interaction with Hb. CoP dimethyl ester also fails to bind CO, as demonstrated by epr and optical absorption methods.

Discussion. In this initial report we have shown that it is possible to prepare a cobalt-substituted hemoglobin—a coboglobin—and that this reconstituted

hemoglobin exhibits reversible and (apparently) cooperative binding. Cobo-globin-Mb, as expected, still exhibits the hyperbolic oxygenation curves of non-cooperative binding.

In accord with the consideration of the introduction, we first discuss the effect of the protein on the CoP and CoP–O_2 units. As mentioned above, when CoP is introduced into a globin to form deoxy-Cb, epr indicates a strong interaction with a protein residue, presumably with the proximal histidine. The epr signal is typical of a low-spin, planar Co(II) complex with a single nitrogen base axially coordinated.

Upon oxygenation the epr spectra indicate no significant differences between the oxy-Cb and oxy-CoP–dimethyl ester–pyr. Thus no specific interaction between oxygen and protein need be invoked. As discussed previously, the cobalt–oxygen adducts may be formally described as Co(III)-O_2^-, with roughly 80% of the odd electron residing on what may be considered a superoxide ion.[4a] This is highly analogous to the formalization Fe(III)O_2^- frequently used for oxy-Hb.[12] We further concluded that the cobalt–oxygen adducts are likely to be in the bent, Pauling structure, and the same conclusions apply to oxy-Cb.[3,4]

Our observation of a solvent dependence for A^{Co}_{11} of CoP–O_2–pyr complexes allows us to choose that solvent system which mostly closely mimics the environment in the hydrophobic globin pocket. Although the solvent heptane–toluene–pyridine 3:3:1 give excellent epr agreement, more thorough studies on the point are necessary.

Although the presence of protein has no major effect on the electronic structure of the oxygen adduct, it does produce a large effect on its stability. We consider the noncooperative Cb samples (*curve 5B*) for simplicity. Its equilibrium constant for binding is approximately 8×10^{-2} mm^{-1} Hg. From the negative results for oxygen binding to CoP dimethyl ester at the same temperature, assuming a 2% uncertainty in the results, $K^{CoP} \leqslant 1.3 \times 10^{-4}$ mm^{-1} Hg. Hence the protein environment increases the oxygen binding constant by $\geqslant 600$ over that in pyridine or toluene–pyridine solutions. The stability of oxy-CoP-dimethyl ester does depend, to some degree at least, on the nature of the nitrogen base axially coordinated to cobalt.[13] On the other hand, we find that CoP monomer in aqueous media is readily oxidized to the Co(III) form.

Considering the effect of the metal atom on the protein, we find that metal, substitution can leave a functional protein with strong oxygen affinity. Further, the cooperativity appears to be largely unaffected ($n \approx 2.3$) by the change. Thus the mechanism of cooperative oxygen uptake is apparently to some extent independent of metal atom: whatever may be the necessary properties for co-operativity, the properties of cobalt are sufficient. This result may be contrasted with those of Antonini, who found that substituting various unnatural iron porphyrins for the heme reduced cooperativity, and indeed eliminated cooperative interaction ($n = 1.0$) in the case of hematoporphyrin.[6]

Furthermore, whatever is the actual geometry of oxygen with respect to the heme plane in hemoglobin, the geometry of the Co–O_2 bond is compatible with cooperative oxygen bonding. We are pursuing single crystal epr studies of coboglobin and coboglobin-Mb in order to define the actual geometry of the

Co–O$_2$ linkage, both with respect to the heme plane and to the surrounding protein.[14] We are also conducting further oxygen binding studies in order to evaluate our preliminary indication of cooperativity in the Cb analog of hemoglobin and to extend those results to other features of the oxygenation reaction such as the Bohr effect.

Abbreviations: epr, electron paramagnetic resonance; CoP, Co(II)-protoporphyrin IX; Cb, coboglobin(s).

* A preliminary account of this study has been presented at the Symposium on Bioinorganic Chemistry, Blacksburg, Va., June 22–25, 1970.

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[14] For a similar study on nitrosylhemoglobin see Chien, J. C. W., *J. Chem. Phys.*, 51, 4220 (1969).