Oxygen Binding to Myoglobins and Their Cobalt Analogues

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Abstract: Thermodynamic measurements for O_2 binding to native myoglobins and their cobalt analogues are discussed for proteins from three aquatic mammals: sperm whale, grey whale, and sea lion; reference is also made to studies on horse myoglobin. Emphasis is placed on the differences between O_2 binding by the Fe and Co forms of the same protein. By comparing these differences within the set of myoglobins, and between proteins and the Fe and Co "picket-fence" porphyrin models of Collman et al., we are able to draw inferences that would be impossible with, say, the direct comparison of myoglobin and the Fe-porphyrin model, or Co-myoglobin and the Co-porphyrin model. In particular, such comparisons confirm the fact that the cobalt-substituted myoglobins are true analogues of the natural proteins. They also suggest that there can be significant differences between oxygen binding in the proteins and the porphyrin models: similar affinities are achieved by different enthalpy-entropy compensation effects.

Introduction

One of the fundamental goals of hemoprotein studies is to understand the mechanisms by which a protein environment modifies the reactivity of the heme prosthetic group. The intrinsic importance and ease of study of hemoglobin and myoglobin have made the oxygen-binding reaction perhaps the touchstone of metalloprotein investigations.

Myoglobins form a particularly convenient group of proteins, since they are monomeric, and thus show a simple association reaction with O_2. Shire[2] summarized the results obtained for various myoglobins from different sources and noted that the oxygen affinities represented by the half-saturation oxygen pressure, $P_{1/2}$, are very similar for most of the myoglobins, both from the land and from marine mammals, investigated at room temperature. In 1970, Hoffman and Petering[3] successfully demonstrated that the cobalt protoporphyrin IX substituted myoglobin and hemoglobin are capable of reversible oxygenation, and later discussed the binding properties of cobalt myoglobins from two different species, finding that the two proteins achieve similar modifications in prosthetic group reactivity (similar O_2 affinity, ΔG) through different balance in enthalpy and entropy (different ΔH, ΔS).[4]

Recently, Collman et al.[5-8] synthesized a series of model compounds, i.e., the iron and cobalt "picket fence" porphyrins (FeTPivPP(*N*-MeIm) and CoTPivPP(*N*-MeIm), iron and cobalt mesotetra($\alpha,\alpha,\alpha,\alpha$-*O*-pivalamidophenyl)porphyrin). Both the iron and cobalt systems bind dioxygen with affinities that are surprisingly close to the values obtained for the oxygen-carrying iron and cobalt proteins, respectively. They concluded[8] that special interaction between the protein and the bound oxygen is not needed to explain the oxygen affinities of these hemoproteins; the case of carbon monoxide was believed to be different.[9] The discrepancies in thermodynamic quantities between the models and the proteins were discounted because these authors felt that the published protein data were too scarce and of too low accuracy. Oxygen binding studies on proteins are necessarily performed over a rather narrow temperature range, and the calculated (ΔH, ΔS) values are subject to large correlated errors; a more negative ΔH is compensated for by a more negative ΔS, and the reverse.[6]

In order to understand better the effects of a protein environment, we have undertaken a coordinated study of a series of myoglobins and the corresponding series of their cobalt analogues, and discuss here proteins from three aquatic mammals: sperm whale, grey whale, and sea lion; results for horse-heart myoglobin are also discussed. By performing each series of measurements in a single laboratory, we eliminate the possibility of different systematic errors in different measurement schemes. We also place heavy emphasis on the differences between O_2 binding by the Fe and Co forms of the same protein. By comparing these differences within the set of myoglobins, and between proteins and model, we are able to draw inferences which would be impossible with, say, the direct comparison of Mb[9] and the Fe-porphyrin model, or CoMb and the Co-porphyrin model. Such comparisons confirm the fact that the cobalt-substituted myoglobins are true analogues of the natural proteins, and also indicate that there can be significant differences between oxygen binding in the proteins and the porphyrin models.

Materials and Methods

Ferrous myoglobin samples were either prepared directly from muscle extract or from ferrimyoglobin preparations that were reduced by a modified dithionite reduction technique.[2,11,12] The methods of preparation yielded similar oxygen binding results.[2] Apoproteins are prepared by the acid-acetone method,[13] and direct formation of CoMb by reconstitution with cobalt(II) protoporphyrin IX followed procedures described elsewhere.[14-16]

The oxygen binding of FeMb was studied in Bloomington by following optical changes from 500 to 600 nm with a Cary 14 spectrophotometer equipped with a thermostated cell holder, using a tonometer modified[2] from the design of Keyes et al.[11] Oxygen binding to cobalt-substituted myoglobins was studied in Evanston by following optical spectral changes in the Soret region (400-500 nm) upon oxygenation using a Beckman Acta III spectrophotometer equipped with thermostated cell holder and an Aminco constant temperature bath, as described before.[16-18] Solution conditions are noted under Results.

The equilibrium constant K and the Hill coefficient n were determined from a least-squares fit to the Hill equation:[19]

$$\ln\left(\frac{y}{1-y}\right) = \ln K = n \ln P_{O_2} \qquad (1)$$

where P_{O_2} is the partial pressure of oxygen above the solution; y is the fractional saturation of the protein by O_2; and K is the binding constant expressed in units of $Torr^{-1}$. The slopes of the lines, n, were generally 1.00 ± 0.07, as expected for monomeric noninteracting proteins, and results are typically reported as $P_{1/2}$, the half-saturation oxygen pressure in Torr.

Results

The cobalt-substituted myoglobins prepared from California grey whale and sea lion myoglobins have spectral properties essentially identical with those of sperm whale and horse heart.[4] Similarly, the native myoglobins from all four species have essentially identical spectra.[2] Isosbestic behavior and

0002-7863/79/1501-7394$01.00/0

Table I. Thermodynamic Quantities Calculated for the Native Iron and Cobalt-Substituted Myoglobins and Model Compounds[a]

oxygen binding species[b]	$\Delta H°$, kcal/mol		$\Delta S°$, eu		$\Delta G°$, kcal/mol, 25 °C		$P_{1/2}$, Torr, 25 °C	
	Fe	Co	Fe	Co	Fe	Co	Fe	Co
myoglobins								
California grey whale	−15.3 ± 0.2	−9.4 ± 0.5	−51.2 ± 0.6	−40 ± 1.5	~0	2.5	0.94	64
California sea lion	−17.1 ± 0.8	−10.4 ± 0.5	−57 ± 3	−43 ± 1.5	~0	2.4	0.71	57
sperm whale[c]	−19.1 ± 1.0	−13.3 ± 0.5	−63 ± 3	−53 ± 1.5	~0	2.4	0.82	57
horse heart[c,d]	−21.0	−11.3 ± 0.5	−70	−46 ± 1.5	~0	2.4	1.1	57
models[d]								
(M)TPivPP(*N*-MeIm)[e]	−15.6 ± 0.2	−13.3 ± 0.9	−51 ± 1	−53 ± 3	~0	2.6	0.49	61
(M)P(DME)(*N*-MeIm)[f]		−11.8		−59		5.7		1.7×10^4

[a] Standard state: O_2 pressure, 1 Torr. [b] The buffer for all the native myoglobins is pH 8.6–8.8 Tris buffer, 0.01–0.025 M, unless otherwise marked. For the cobalt-substituted myoglobin, it is pH 6.9, ionic strength $I = 0.1$, phosphate buffer. [c] Data for CoMb taken from ref 4. [d] The native myoglobin data are taken from ref 21; buffer conditions are pH 7.4 phosphate, 0.067 M. Note that ref 20 incorrectly labels values for the $[O_2] = 1$ M standard state as being for $P_{O_2} = 1$ Torr standard state. [e] Solid state; ref 8. [f] P(DME) stands for protoporphyrin IX dimethyl ester. Measured in toluene; ref 24.

complete reversibility of the spectral properties were observed during the spectrophotometric oxygen binding measurements. This demonstrated the absence of side reactions such as oxidation or denaturation.

The half-saturation pressures, $P_{1/2}$, for the oxygen binding to the grey whale and sea lion CoMb were obtained in the temperature range 4–30 °C. Figure 1a gives the van't Hoff plots of ln $P_{1/2}$ vs. T^{-1}:

$$\ln P_{1/2} = \frac{\Delta G}{RT} = \frac{\Delta H}{RT} - \frac{\Delta S}{R}$$

Solid lines result from least-squares fit to the data, and the figure includes the fitted lines from an earlier study of sperm whale and horse cobalt myoglobins.[4] The standard enthalpies and entropies (1 Torr standard state) obtained from these plots are given in Table I. Yonetani and co-workers have also measured O_2 binding to sperm whale CoMb,[14] obtaining somewhat different thermodynamic values. By employing only results from this laboratory, we are able to consider a set of thermodynamic parameters that has been obtained through a single set of procedures for protein handling and for measurement of O_2 binding.

The corresponding van't Hoff plots for the FeMb from the three aquatic mammals are given in Figure 1b, with the standard enthalpies and entropies so obtained presented in Table I. The results for sperm whale Mb are in agreement with those of Keyes et al.[20] Results for horse-heart Mb, reported by Theorell,[21] are included in Table I for completeness. These results for horse Mb are very different from those obtained later in Evanston;[4] since they were not remeasured in Bloomington, the conclusion reached below will largely rest on the results for the aquatic mammals.

Collman et al.[5,6] have noted that considerable uncertainties may be associated with thermodynamic measurements over the necessarily limited temperature range available for work with proteins. However, by relying on data for FeMb and for CoMb obtained in the same laboratory and with the same apparatus, it is possible to usefully compare the thermodynamic values for proteins of different species. As noted, the only exception to this is our use of the values for horse FeMb.[21] This approach further permits us to discuss in a consistent fashion the differences between the oxygen binding behavior of Fe and Co in the several myoglobins. In short, the results in Table I can properly be used to compare O_2 binding among the different Mb and CoMb, and to compare among species the differences in O_2 binding by a Mb and its cobalt analogue.

The first feature of note is that all the natural systems have roughly the same free energy of binding at ~25 °C, that is the same affinity for O_2; the same applies to the cobalt proteins, although with a reduced affinity. Thus, $\delta(\Delta G) = \Delta G^{Co} - \Delta G^{Fe}$, the difference in the free energy of O_2 binding by a Mb and its Co analogue, is independent of the protein. Note that

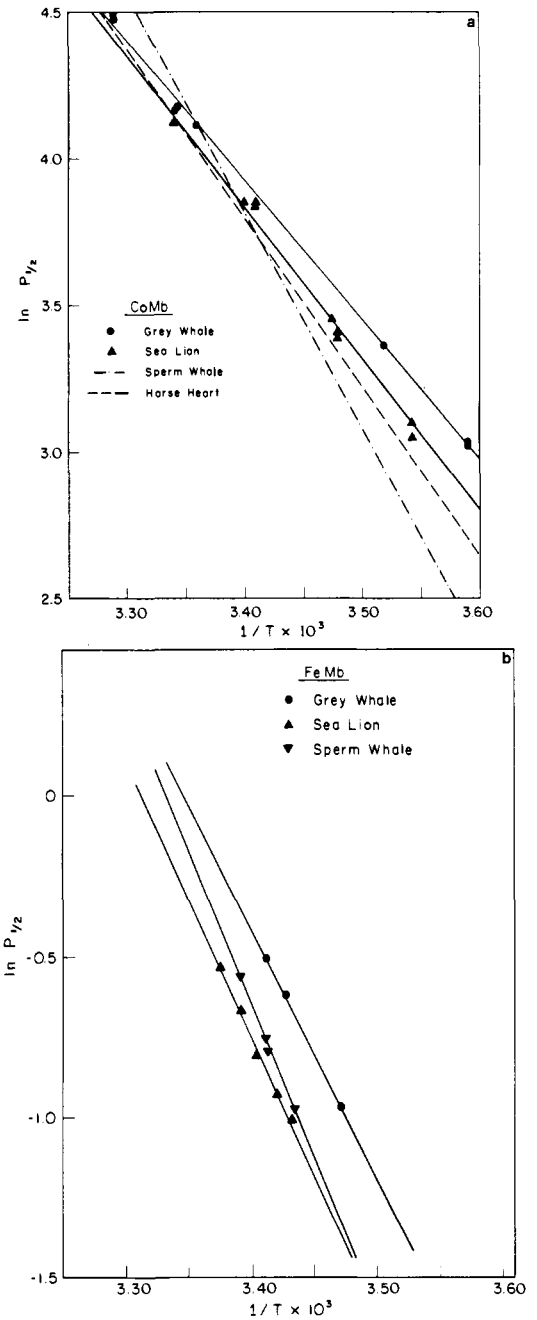

Figure 1. Plots of ln $P_{1/2}$ vs. T^{-1}: (a) cobalt-substituted myoglobins; dashed lines for the sperm whale and horse proteins are from results (Table I) from ref 4; (b) the native iron myoglobins. Solution conditions of both sets of proteins are given in Table I, which presents the derived thermodynamic quantities.

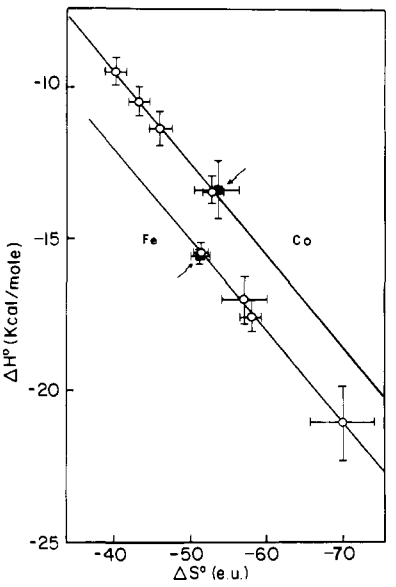

Figure 2. Plots of ΔH vs. ΔS for the several FeMb and CoMb. Data are from Table I. The points corresponding to the model compounds (■) are indicated by arrows.

Table II. Comparison of the Thermodynamic Quantities for the Co and Fe Myoglobins[a]

oxygen binding species	$\delta(\Delta H°) = \Delta H°(\text{Co}) - \Delta H°(\text{Fe})$	$\delta(\Delta S°) = \Delta S°(\text{Co}) - \Delta S°(\text{Fe})$	$\delta(\Delta G°) = \Delta G°(\text{Co}) - \Delta G°(\text{Fe})$
grey whale	5.9 ± 0.7	11.2 ± 2.1	2.5
sea lion	6.7 ± 1.3	14 ± 4.5	2.4
sperm whale	5.7 ± 1.5	10 ± 4.5	2.4
models	2.3 ± 1.1	−2.1 ± 4	2.6

[a] Definitions are discussed in the text. Raw data are obtained from Table I. Nature of the models is also given in Table I. Enthalpies in kcal/mol; entropies in eu; standard state, 1 Torr O_2.

systematic errors in either or both Co and Fe protein results would change only the size of $\delta(\Delta G)$, but would not influence the conclusion that it is protein independent.

A second feature emerges from Figure 2, which plots ΔH vs. ΔS for O_2 binding to both the natural and cobalt-substituted proteins. As has been found for a variety of processes involving proteins and small electrolytes in water solutions,[22] for each metal there is a linear relationship between the enthalpy and entropy changes upon O_2 binding. Interestingly, the slopes of the lines for the Fe and Co proteins, the so-called compensation temperatures, are effectively the same, within experimental errors: $T_c \simeq 300 \pm 20$ K. Also included in Figure 2 are the data reported by Collman et al.[8] for the "picket fence" model systems. These cobalt and iron porphyrin models fall in line with the cobalt and iron proteins, respectively. This is a necessary result of the models having oxygen affinities comparable to those of the proteins at room temperature (~300 K), which is essentially the compensation temperature.

Not only do the free energies for O_2 binding to the two metals exhibit a constant difference, but the same is true, for the Mb from the aquatic mammals (am), of ΔH and ΔS, separately. Table II shows $\delta(\Delta H) = \Delta H^{\text{Co}} - \Delta H^{\text{Fe}}$ and $\delta(\Delta S) = \Delta S^{\text{Co}} - \Delta S^{\text{Fe}}$ for the myoglobins and the only models for which the data for the (Co,Fe) pair are available. Figure 3 plots these $\delta(\Delta H)$ vs. their respective $\delta(\Delta S)$. The points for the three proteins all cluster together around the value $\delta(\Delta H) \simeq 6$ kcal/mol and $\delta(\Delta S) \simeq 12$ eu and are indistinguishable within experimental error. Again, this clustering of points is independent of any systematic bias in the values for either or both the FeMb or the CoMb.

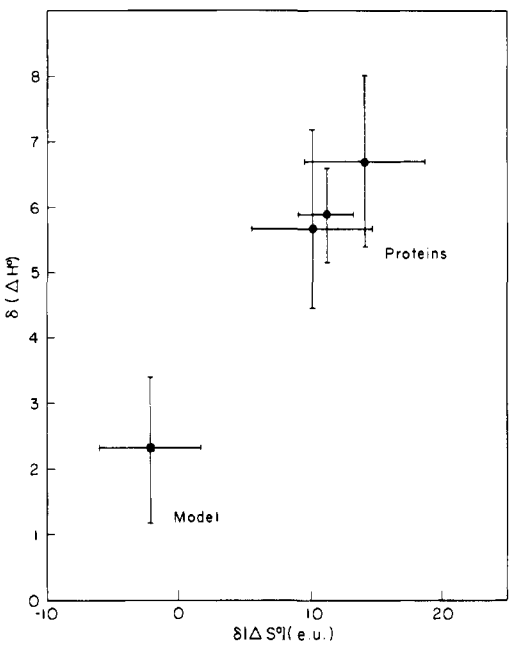

Figure 3. Plots of $\delta(\Delta H)$ vs. $\delta(\Delta S)$ for the proteins (●) and the picket fence model system (■). Data are from Table II. As described in the text, the feature of significance is a clustering of protein points in a locus removed from the model system point.

On the other hand, the figure also shows that even when the estimated experimental errors are considered, the porphyrin models do not behave similarly to the proteins. The $\delta(\Delta H)$ of these "picket fence" models is around one-third of the protein value, while their $\delta(\Delta S)$ is virtually zero, compared to ~12 eu in the case of proteins. The horse protein also differs from the models. Such differences would also appear to be outside any possible, unnoticed, systematic errors of the kind discussed by Collman et al.[8] These considerations indicate that the nature of the enthalpy-entropy compensation is different in protein and model, although the same balance (affinity, ΔG) is reached.

Discussion

The range of enthalpies and entropies for O_2 binding to these several natural and cobalt-substituted monomeric oxygen binding proteins reflects the degree to which chemical properties of the prosthetic group are influenced by the apoprotein. The existence of data from a number of proteins, with both Fe and Co as the sites for O_2 binding, allows a number of insights into the nature of these influences.

The constant free energies of binding, ΔG^{Fe} (25 °C) for the native iron proteins and ΔG^{Co} (25 °C) for the cobalt-substituted analogues (Table I), are in accord with the observation that, for a homeostatic organism, the affinity (free energy) is of primary importance; since the organism's temperature is held constant, the relative contributions of ΔH and ΔS are of secondary consequence. However, variation in the latter pair reflects the different behavior of the proteins.[22]

Considering the (Fe,Co) pairs, all three of the thermodynamic quantities, $\delta(\Delta G)$, $\delta(\Delta H)$, and $\delta(\Delta S)$, are effectively constants for the am myoglobins, independent of protein. An obvious conclusion to be drawn from this observation is that the cobalt-substituted myoglobins are indeed precise analogues of the native proteins. The heme environment of one protein will alter ΔH and ΔS from the values for another. However, differences between heme environments are equally reflected in the Fe and Co proteins. There appears to be an intrinsic difference between the thermodynamics of O_2 binding by cobalt and iron porphyrins incorporated in these myoglobins: $\delta(\Delta H) \simeq 6$ kcal/mol, $\delta(\Delta S) \simeq 12$ eu, and $\delta(\Delta G)$ ($T = 298$)

$\simeq$ 2.5 kcal/mol. In addition, if we write G^M and G^{MO_2} as the absolute free energy of a deoxy- or oxymyoglobin, then the free energy of O_2 binding is $\Delta G^M = G^{MO_2} - G^M$ and $\delta(\Delta G) = \Delta G^{Co} - \Delta G^{Fe} = (G^{CoO_2} - G^{FeO_2}) - (G^{Co} - G^{Fe})$, where the first parenthesis represents the free-energy difference between oxy-CoMb and oxy-Mb and the second, the free-energy difference between CoMb and Mb. Similar equations hold for enthalpy and entropy. The constancy of $\delta(\Delta G)$, $\delta(\Delta S)$, and $\delta(\Delta H)$ suggests that the thermodynamic differences between Mb and CoMb and between MbO_2 and $CoMbO_2$ are also constant.

A corollary to this conclusion is that it should be equally proper to draw inferences concerning protein influences upon O_2 binding by use of comparisons with either cobalt or iron porphyrins as model compounds. This is of some importance because it is relatively easy to measure solution O_2 binding by a five-coordinate cobalt porphyrin, but difficult to do so for iron porphyrins; in the presence of an excess of nitrogenous base, a typical ferrous porphyrin is six-coordinate, and one can only readily measure the thermodynamics of the replacement of a base by O_2.[23] Cobalt porphyrins are readily prepared in the five-coordinate state, with a single axial base, and thus it is possible to directly observe the addition of O_2, just as occurs in myoglobins.[24]

The "picket fence" porphyrins of Collman et al. are the only model compounds for which data for oxygen binding to a five-coordinate metal are available for both Fe and Co.[8] Comparisons of the $\delta(\Delta S)$ and $\delta(\Delta H)$ of the model system with those in the proteins reveal a substantial difference in the oxygen binding environments of proteins and model. In particular, the difference between $\delta(\Delta S) \simeq 12$ eu for the am proteins and $\delta(\Delta S) \simeq 0$ eu for the model system is intriguing. Collman et al.[5,8] stated that the essentially unchanged ΔS of binding of *all* their models ($\Delta S = 53 \pm 3$ eu, standard state of 1 Torr) can be completely accounted for, on statistical mechanical grounds, by the entropy change calculated for the loss of translational and rotational entropy of bound O_2. The positive $\delta(\Delta S)$ found in the protein then argues that there must be other processes involved, and that metal exchange makes a greater difference in the proteins than in the models. This could be one quantitative measure that nature has achieved selectively for the central metal ion in these proteins.

The exact values of $\delta(\Delta S)$ and $\delta(\Delta H)$ reported here are not particularly important, for they are subject to possible systematic differences in the measurements of the FeMb and CoMb. Nevertheless, these results do indicate that if one looks in proper detail, the oxygen-binding properties of a prosthetic group incorporated within an apoprotein can differ significantly from those of the model. Such a conclusion would also arise from looking to nonmammalian O_2 carriers;[25] indeed, we would expect a larger group of proteins to exhibit a wide range of behaviors. The protein is more than a mere heme container.

Acknowledgments. This work was supported by Grants HL-13531 (B.M.H.) and HL-05556 (F.R.N.G.) from the National Heart, Lung, and Blood Institute.

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Communications to the Editor

A 360-MHz Photo-CIDNP Study of Bovine Pancreatic Phospholipase A_2. Observation of a pH-Dependent Conformational Change

Sir:

The photo-CIDNP[2] technique, recently applied to biological macromolecules,[3-6] has proven to be quite useful in the assignment of tryptophan, tyrosine, and histidine residues in the ^1H NMR spectrum of proteins. Selective nuclear spin polarization induced in a small number of surface residues results in a great simplification of the photo-CIDNP difference spectrum compared with the normal NMR spectrum. This has allowed us to detect a pH-dependent conformational change in the N terminus of bovine phospholipase A_2. The CIDNP results are compared with ^{13}C NMR titration data of the same enzyme in which the N-terminal alanine residue has been replaced by 90% enriched L-[3-^{13}C]alanine.

Pancreatic phospholipase A_2 hydrolyzes the fatty ester bond at the 2 position of naturally occurring 3-*sn*-phosphoglycerides in the presence of Ca^{2+} ions.[7] The enzyme is secreted as a single chain precursor which can be converted into the active enzyme by tryptic cleavage of the Arg^{-1}-Ala^1 bond which makes Ala^1 the new N-terminal amino acid.[8,9] In the porcine enzyme the α-NH_3^+ group is thought to interact with a carboxylate situated at the end of a hydrophobic cleft. This interaction in the active enzyme induces a special site, the so-called interface recognition site (IRS) which is responsible for micellar binding. The ionization state of the α-ammonium group regulates this binding.[10,11]

According to Slotboom et al.[12] we have substituted the

0002-7863/79/1501-7397$01.00/0