folded in their best folded state. The dry protein or a protein crystal so incompletely hydrated that there is no interstitial liquid–water phase provides the reference state. Fully hydrated crystals or the protein in solution will have a larger heat capacity insofar as the protein is unfolded. Thus CGN shown by nmr studies to be about 20% unfolded in state A[33] has a heat capacity in solution about 30% higher than the anhydrous or 10% wet crystals studied by Hutchens, Cole, and Stout.[34] α-Chymotrypsin, on the other hand, is fully folded in state A by the nmr measurement and has the same heat capacity in the dry solid as in solution. This use of the heat capacity as a measure of unfolding is very useful. Its application to the kinetics data on unfolding and refolding have shown that the unfolding of chymotrypsin in transition I occurs after the formation of the activated complex.[5,35]

It is possible to carry out van't Hoff studies of higher precision than was obtained in this work. However, baseline uncertainties make it nearly certain that the best of such data cannot be used to determine the form of the relationship between heat capacity and temperature. It appears that we must depend on heat-capacity calorimetry[16] for this relationship. Our raw data are included in Table VI so that more accurate heat capacity and enthalpy values can be computed when the heat-capacity expression is established. Nevertheless the data now available suggest that the enthalpy changes obtained by our method are reliable and that the average ΔC_p° values we can calculate on the assumption that this quantity is independent of temperature are sufficiently accurate estimates of the average heat capacity change to be of considerable use in estimating the amount of random coil in state A and in state B. Such applications of the data reported in this paper will be made in a subsequent paper of this series.

Acknowledgment. One of us (D. F. S.) was recipient of the Minnesota Mining and Manufacturing Co. Fellowship in Chemistry during part of this work. We wish also to express our gratitude to Dr. R. Biltonen for his useful suggestions and criticisms during the course of this work. We are indebted to Mrs. Meredith Falley for assistance in preparing purified RNase.

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Studies of Heme Proteins. II. Preparation and Thermodynamic Properties of Sperm Whale Myoglobin[1]

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Contribution from the Laboratory for Biophysical Chemistry, Chemistry Department, University of Minnesota, Minneapolis, Minnesota 55455. *Received February 9, 1970*

Abstract: Preparations of sperm whale myoglobin have been found to contain a tightly bound contaminant which yields abnormally large negative values for enthalpy and entropy of O_2 and CO binding. Characteristics of the contaminant are described. A simple procedure for removing the contaminant is reported. The standard enthalpy changes for O_2 and CO obtained with purified myoglobin are -18.1 ± 0.4 kcal/mol and -21.4 ± 0.3 kcal/mol, while the standard entropy changes were -60 ± 1 and -65.7 ± 0.8 eu/mol, respectively, with a 1 Torr standard state. The van't Hoff enthalpy value for oxygen binding has been confirmed by calorimetric measurements. The results are in good agreement with those of Theorell but not with more recent data, and suggest that some studies of myoglobin have been complicated by contamination.

The use of myoglobin for quantitative studies of oxygen binding, carbon monoxide binding, and linkage mechanisms has been restricted by inconsistencies in the values of the enthalpy and entropy of ligand binding reported by Theorell[2] and by Rossi-Fanelli and Antonini,[3] and by the nonlinearity of van't Hoff plots. Both situations suggest that myoglobin preparations may still be unreliable. The best preparative procedure has appeared to be that of Yamazaki, *et al.*,[4] in which myoglobin is prepared directly in the Fe^{II} form.

Having developed a spectrophotometric method for determining the ligand-binding isotherm at the required level of precision,[5] a study of the nonlinear van't Hoff plots for myoglobin led us to the discovery of an as yet unknown substance or substances which as a result of their interaction with the protein alter the ligand-binding equilibria.[6,7] In this paper we dis-

(1) Publication No. 59 from this laboratory. Request reprint by number. This study was supported by the Office of Naval Research, Department of Defense (Nonr 710(55)), the Air Force Office of Aerospace Research (AF AFOSR 1222 67), and the United States Public Health Service (A.M. 05853).
(2) H. Theorell, *Biochem. Z.*, **268**, 73 (1934).
(3) A. Rossi-Fanelli and E. Antonini, *Arch. Biochem. Biophys.*, 77, 478 (1958).
(4) I. Yamazaki, K. Yokota, and K. Shikama, *J. Biol. Chem.*, **239**, 4151 (1964).
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(6) M. Keyes, Ph.D. Dissertation, University of Minnesota, 1968.
(7) M. Keyes and R. Lumry, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 27, 895 (1968).

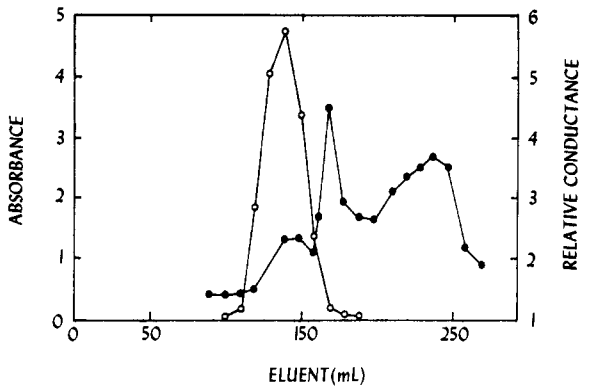

Figure 1. The elution pattern for Yamazaki myoglobin chromatographed on G-25 Sephadex. The relative conductance (●) as well as the absorbance at 250 mμ (○) of the eluent were measured.

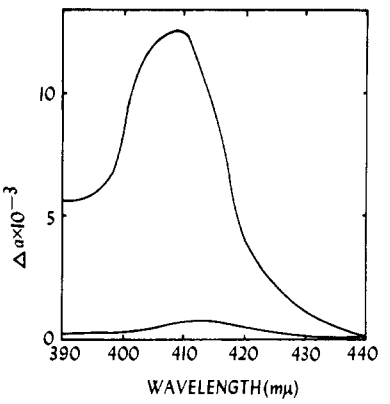

Figure 2. The change in absorptivity in the Soret region from 390 to 440 mμ for Yamazaki MbO_2 when 0.5 *M* KBr is added. The concentration of protein is 7.2×10^{-6} *M* and the concentration of THAM sulfate is 1.0×10^{-3} *M*. The lower plot represents the change in absorptivity *vs.* the wavelength in millimicrons for MbO_2 when 0.5 *M* NaCl is added. All other conditions are the same.

cuss the contamination problem and the determination of the thermodynamic changes on binding of oxygen and carbon monoxide to sperm whale myoglobin.

Experimental Section

Materials. Chemicals were reagent grade from Mallinckrodt Chemical Works except for Trizma brand THAM base from Sigma Chemical Co., "enzymic grade" ammonium sulfate from Mann Research Laboratories, and research grade CO from Matheson Co., Inc. Technical grade oxygen was used since it gave identical binding results as research grade. In our experience reagent grade ammonium sulfate contained impurities which were apparently strongly bound by MbO_2. These were revealed by interference in determination of the molar extinction coefficients by iron analysis.[8]

Myoglobin Preparation.[8] The procedure of Yamazaki, *et al.*, for horse MbO_2 was followed except that DEAE Sephadex was substituted for DEAE cellulose. The first component eluted from the Sephadex column is ferri-Mb and the second is MbO_2, which we will call "Yamazaki MbO_2." Hartman, *et al.*,[9] have shown that Yamazaki MbO_2 is electrophoretically identical with the major component from the preparation of Edmundson and Hirs.[10] The remaining components of MbO_2 remain tightly bound to DEAE Sephadex at this pH and ionic strength (pH 8.5, 0.001 *M* THAM sulfate).

The contaminant of Yamazaki MbO_2, hereafter known as substance K, can be removed by gel chromatography using G-25 Sephadex. Furthermore, substance K probably has an oxidized and reduced form, since it is much easier to remove in a solution of MbO_2 containing a strong reducing agent. The following procedure has been used effectively to remove substance K. The Yamazaki MbO_2 solution at pH 8.0 is degassed quickly but not necessarily completely since the purpose of this step is only to prevent peroxide production from O_2 and ascorbate. Sodium ascorbate is added to approximately 0.01 *M*. The solution is then degassed with agitation at 5-min intervals for a total of 60 min to complete the removal of dissolved gases. Apparently during this process the major contaminants are reduced. The resulting solution was exposed to the air, allowed to reequilibrate with O_2, and then applied immediately to a G-25 Sephadex column (2.5 × 40 cm) which had been equilibrated at a temperature of 5° with 1.0 ± 10^{-3} *M* THAM sulfate at pH 8.5. The resulting chromatographic pattern is shown in Figure 1. There was no indication of the accelerated production of ferri-Mb which could have occurred because of hydrogen peroxide production. The absorbance at 250 mμ and the conductance of the eluent were measured (Figure 1). Substance K is present in the first peak after the peak which absorbs at 250 mμ and contains the MbO_2.[6] Samples of MbO_2 obtained from the front of the MbO_2 peak during chromatography with G-25 Sephadex will be referred to as "K-free" Mb. The justification for this term is provided in the following section.

All protein solutions discussed in this paper contain 0.001 *M* THAM sulfate at pH 8.5 ± 0.1 unless another buffer and pH are specifically stated.

Detection and Properties of Substance K. The presence of substance K can be detected by changes in the affinity of Mb for CO or O_2. For example, at 20° and pH 8.4, the value of the association constant for binding CO to Mb in the absence of substance K is about one-half of the value of this constant when Mb is saturated with substance K. It is, however, inconvenient as a general procedure to detect the presence of substance K in this manner, since a sufficiently precise determination of the association constant of Mb to CO takes approximately 6–8 hr.

A much quicker procedure has been developed which uses changes in spectrum of MbO_2 due to the addition of KBr to MbO_2 solutions. The difference between the spectrum of Yamazaki MbO_2, *i.e.*, contaminated protein, in the presence of 0.5 *M* KBr and 1.0×10^{-4} *M* THAM sulfate and the spectrum of the same MbO_2 solution in the absence of KBr is shown in Figure 2. The maximum in this difference spectrum is at about 405–410 mμ, well displaced from the Soret band in the direct spectrum of MbO_2 which is located at 418 mμ. No change in the ultraviolet or visible spectrum at longer wavelengths than the Soret band was observed. One might suspect that the change in the spectrum when KBr is added to this high concentration is due to the binding of Br^- to the iron ion of ferri-Mb present as a minor component in Yamazaki MbO_2, rather than to a change in the amount of substance K bound. However, two observations show this cannot be the explanation. First, with the addition of KBr no difference in the spectrum of Yamazaki MbO_2 is observed in other regions of the spectrum where large changes are observed when any ligand is bound to ferri-Mb. Second, because K-free MbO_2 requires a longer time to prepare than Yamazaki MbO_2, it contains a larger fraction of ferri-Mb. Nevertheless, no difference spectrum is observed when K-free MbO_2 is compared with and without KBr. Moreover, the second observation is inconsistent with the possibility that the spectral change is due to THAM or sulfate ion binding as well as Br^- binding, since both K-free MbO_2 and Yamazaki MbO_2 have THAM sulfate buffer present at the same concentration. Solutions of MbO_2 obtained from chromatography on G-25 Sephadex which were thought to be free of substance K were routinely checked for the presence of substance K by this "KBr difference spectrum" method. If any difference in the spectrum was observed with addition of KBr, the solution was discarded.

One observation indicated that the spectral changes obtained with KBr can also be obtained with NaCl added to an MbO_2 solution containing substance K. Figure 2 shows that the difference spectrum obtained with 0.5 *M* NaCl is very nearly the same in shape as that obtained with 0.5 *M* KBr. Quantitative comparisons of optical densities were not possible since the amounts of substance K in the two preparations was not determined.

The tests of ligand affinity and spectral change with KBr were developed with material collected at the extreme leading edge

(8) In this paper Fe(II) myoglobin, oxymyoglobin, carbonylmyoglobin, and Fe(III) myoglobin will be referred to as Mb, MbO_2, MbCO, and ferri-Mb, respectively.

(9) K. Hartman, E. Eylar, D. Ray, L. Banaszak, and F. Gurd, *J. Biol. Chem.*, **241**, 432 (1966).

(10) A. Edmundson and C. Hirs, *Nature (London)*, **190**, 663 (1961).

of the MbO_2 chromatographic peak in experiments with no ascorbate ion present. To improve the yield the ascorbate method was developed. By ligand affinity and KBr tests there are no differences in the materials prepared by the two methods.

Although the identity of substance K is not yet known, several properties have become apparent. A solution of substance K has high conductivity compared with the eluent of the G-25 chromatography and is thus presumably ionic. It must be less than 5000 mol wt or no separation would be possible with G-25 Sephadex. The material is concentrated during the chromatographic procedure and can be added back in excess to saturate K-free MbO_2.

A group of experiments also leads to the tentative conclusion that substance K can be oxidized and reduced and that only the oxidized form is tightly bound to MbO_2. Exposure of Yamasaki Mb to low pressures of O_2 causes an increase in the KBr difference spectrum when the material is subsequently resaturated with O_2. Low pressures of oxygen are known to cause increases in the rate of oxidation of Mb and the formation of free radicals.[11] Furthermore, addition of $NaBH_4$ causes reduction of the KBr difference spectrum. Thus oxidation and reduction of the Mb solution with O_2 and $NaBH_4$, respectively, are consistent with the proposal that substance K has an oxidized and a reduced state.

Ligand-Binding Measurements. The apparatus and general procedure used in these experiments have been described elsewhere.[5,6] The apparatus was encased in a thermostatted enclosure which fitted into the cell compartment of a Cary 11 spectrophotometer modified to provide exact replacement of this enclosure unit.

To measure temperature, a thermistor was attached to the outside of the optical cell at a point corresponding to the inside surface in contact with the solution during shaking. It was found by measurement of the temperature inside and outside of the optical cell that the difference in temperature was less than 0.01°.

Oxidation causes appreciable error in the calculation of the degree of oxygenation of myoglobin above 25°. Although a correction can be made to eliminate this error, the amount of oxidation was reduced in many experiments where "K-free" Mb was used by adding sodium ascorbate. The concentration of sodium ascorbate was always less than one-half of the protein concentration and careful comparison experiments showed no difference in spectral or ligand-binding properties.

The calorimetric results reported here were obtained with an LKB Flow microcalorimeter (Model 10700-1).[12] Sodium ascorbate was added to a concentration of about one-half the protein concentration. The solution of MbO_2 was evacuated in a glass apparatus in the usual manner.[6] A glass adapter was used for transferring the Mb solution from the glass apparatus to the tubing of the flow calorimeter. Flow of the Mb solution was facilitated by application of nitrogen under pressure and the protein solution was mixed in the cell of the calorimeter with an air-equilibrated buffer solution. There appeared to be no leakage from the glass apparatus and the oxidation of the sample determined spectrophotometrically after the experiment was negligible. The only major error which might have escaped detection in these experiments is premature leakage of oxygen to the Mb solution through the tubing before mixing. Since this error would reduce the observed value of $\Delta H°$, the value which we report must be considered a lower limit for the true value.

Results

The equilibrium between CO and myoglobin was measured spectrally as a function of temperature. From the change in the spectrum in the visible region accompanying the addition of small amounts of CO (typically, pressures were less than 1 Torr), the fraction of Mb in the form of MbCO could be calculated. From the fraction of MbCO the equilibrium constant for ligand binding to myoglobin could be obtained.[5]

Figure 3 shows the van't Hoff plots for the binding of CO to three different kinds of Mb preparations. Mb samples obtained by pooling the fractions from the entire MbO_2 peak eluted during chromatography (remixed G-25 Mb) gave points which lie along the curved line obtained with Yamazaki Mb. K-free Mb and K-free Mb with substance K added were also studied. The addition of substance K was accomplished by mixing equal amounts of the high-conductance conductivity portion of the eluent from G-25 chromatography and 10^{-5}–10^{-6} *M* G-25 Mb solution. The resulting solution was judged to contain Mb saturated with substance K since the calculations of the equilibrium binding constant gave consistent results and the van't Hoff plots of the resulting data gave straight lines.

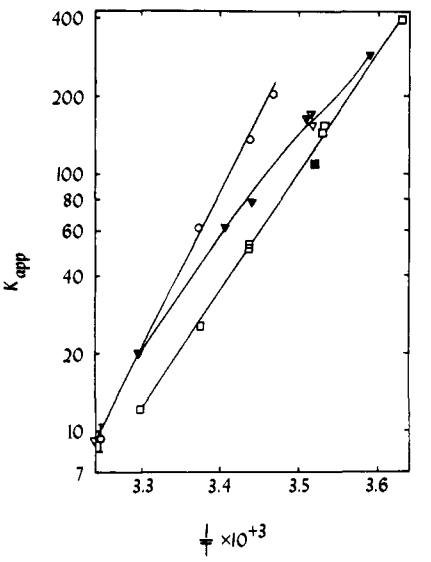

Figure 3. The van't Hoff plots for the binding of carbon monoxide to different Mb preparations are shown in this figure. The ○ and □ represent the data obtained by using substance K saturated and K-free Mb, respectively. The ▼ are experiments using Mb preparations obtained by remixing the entire Mb peak from the G-25 Sephadex column. The ▽ represent experiments with Yamazaki Mb. The ■ represent an experiment with Yamazaki Mb in 0.5 M NaCl. The ▽ represents experiments with Yamazaki Mb in 0.02 *M* total phosphate at pH 7.0. All other experiments were performed with a solution containing 1.0×10^{-3} *M* THAM sulfate at pH 8.5.

The lower straight line in Figure 3 is drawn through the points obtained for K-free Mb, while the upper line is drawn through the points obtained with Mb saturated with substance K. The curvature of the middle line which was obtained with both Yamazaki Mb and K-free Mb remixed with high-conductivity eluent is due to the difference in temperature dependence of contaminated and K-free material or to a change in the concentration of the two kinds of Mb with temperature. This middle line does not yield meaningful values for the thermodynamic quantities since it applies to a mixture, but the linear van't Hoff plots for K-free Mb and for Mb saturated with substance K can be reasonably expected to give reliable values of $\Delta H°$ (Table I).

Figure 4 shows the van't Hoff plots for the binding of oxygen to Yamazaki Mb and K-free Mb. The data for the binding of oxygen to Yamazaki Mb yield a curved line, while the data for K-free Mb yield a straight line, the same pattern as was observed for CO binding.

The values of the thermodynamic quantities obtained by suitably weighted least-squares analysis of the van't Hoff data are given in Table I. The errors given are the standard errors. Also included is the value of $\Delta H°$ obtained for oxygen binding calorimetrically.

(11) P. George and C. Stratman, *Biochem. J.*, 51, 418 (1952).

(12) The authors wish to thank Dr. F. Schaub of the LKB Company for making the flow calorimeter available and Dr. S. Rajender for her assistance.

Table I. Thermodynamic Changes in Ligand Binding to Fe^{II} Myoglobin[a]

Type of myoglobin	Ligand	$\Delta H°$,[b] kcal/mol	$\Delta S°$,[b] eu	$\Delta H°$,[c] kcal/mol	$\Delta S°$,[c] eu
K-free (van't Hoff)	CO	−21.4 ± 0.3	−65.7 ± 0.8	−21.2	−38.5
Fully contaminated (van't Hoff)	CO	−25.9 ± 0.6	−79.0 ± 3.0	−25.7	−52.0
K-free (van't Hoff)	O_2	−18.1 ± 0.4	−60.0 ± 1.0	−17.9	−33.0
K-free (calorimetric)	O_2	−19.0 ± 1.0			

[a] pH 8.5; 0.001 *M* THAM buffer. [b] Standard state of 1 Torr. [c] The standard state for the two columns on the right of the table is unit mole fraction of the ligand in an organic solvent. See text. The adjustment is made using the thermodynamic changes for the process X (gas) ⟷ X (organic solvent); these are $\Delta F°$ (27°) = 8.0 ± 0.2 kcal/mol; $\Delta H°$ = −0.2 ± 0.4 kcal/mol; $\Delta S°$ = −27.2 ± 0.2 eu/mol (F. W. J. Roughton and R. L. J. Lyster, *Hvalrodets Skrifter*, **No. 48**, 185 (1968)).

Table II. Comparison of the Literature Values of the Thermodynamic Changes to Those Reported in This Paper

Ref	Protein preparation	Ligand	$\Delta F°$[a]	$\Delta H°$[a]	$\Delta S°$[a]
Theorell[b,c]		O_2	0.0	−17.5	−58
Rossi-Fanelli and Antonini[d,e]		O_2	−0.2	−13.0	−44
Keyes and Lumry[f]	G-25Mb	O_2	−0.1	−18.1	−60
Theorell[c,d]		CO	−1.3	−22	−69
Keyes and Lumry[f]	G-25Mb	CO	−1.7	−21.4	−65.7
Keyes and Lumry[f]	Saturated with substance K	CO	−2.2	−25.9	−79
Keyes and Lumry	Yamazaki	O_2	−0.8	−14.0	−44

[a] Standard state is 1 Torr. [b] pH 7.4; 0.067 *M* phosphate buffer; T = 27°. [c] Reference 2. [d] pH 7.45; 0.05 *M* THAM buffer; T = 27°. [e] Reference 3. [f] pH 8.5; 0.001 *M* THAM buffer; T = 27°.

The inadvertent observations with NaCl subsequently shown to produce the same spectral change as KBr served to link spectral changes in the Soret region to differences in CO affinity of Yamazaki Mb and K-free Mb since, as shown in Figure 4, addition of 0.5 *M*

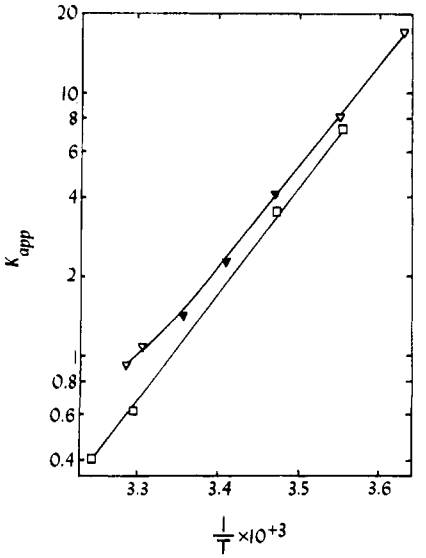

Figure 4. The van't Hoff plot for the reaction of oxygen with Yamazaki and Keyes Mb. The symbols have the same notation as in Figure 3.

NaCl changes the CO affinity of Yamazaki Mb to that of K-free Mb. Thus for NaCl and probably also for KBr the spectral changes take place concomitant with changes in CO affinity.

Calorimetric experiments give a value of −19 ± 2 kcal/mol for the $\Delta H°$ for the binding of oxygen to K-free Mb at 25°. A similar experiment with Yamazaki Mb proved unsuccessful because of oxidation during the experiment. In the latter experiment ascorbate was not added since its presence would reduce the amount of substance K bound to the Mb.

Discussion

The linearity of the van't Hoff plots obtained with K-free Mb and the agreement between the calorimetric and van't Hoff values of $\Delta H°$ obtained with this material suggest that the $\Delta H°$ values obtained by the van't Hoff method are correct. Furthermore, the literature values for the thermodynamic quantities for the reaction of Mb with CO and O_2 can be correlated with our values for different preparations of Mb. Table II shows the literature values for the thermodynamic quantities as well as the values obtained in this study. The rough values of the thermodynamic quantities for Yamazaki Mb were obtained by drawing a straight line through the points above 20° shown in Figure 2. Note that the values of the thermodynamic quantities which Theorell obtained agree with those for K-free Mb while the values for the same thermodynamic quantities obtained by Rossi-Fanelli and Antonini[3] agree with those for Yamazaki Mb using data for temperatures of 20° and higher. The correlation of literature values with those reported in this paper can be explained on the basis of the presence or absence of substance K in the preparations of Mb. In Theorell's preparative method ferri-Mb was reduced to Mb with $Na_2S_2O_3$. The excess $Na_2S_2O_3$ was removed by electrodialysis. A reasonable conclusion to be drawn is that

if his original preparation had substance K present, that contaminant was removed by his reduction–electrodialysis procedure. On the other hand, Rossi-Fanelli and Antonini[8] using enzymic reduction of human Mb obtained much lower values of $\Delta H°$. It is possible that their treatment reduced the Mb but left substance K unaffected as is consistent with the low estimate of $\Delta H°$ which is obtained if only the higher temperature points in the curved van't Hoff plot of Figure 3 are used.

The discovery of substance K has given an explanation for the differences in literature values for the thermodynamic quantities for binding O_2 to Mb as well as a self-consistent explanation for our data. Mb has been studied for many years without adequate attention to purity and it is probable that some values of physical measurements determined heretofore are in error.

The thermodynamic data shown in Table II reaffirm the similarity of CO and O_2 as ligands and show that the higher affinity for CO is due to a more negative value of $\Delta H°$. They also show that the horse heart preparation used by Theorell and our sperm whale preparations are identical with respect to enthalpy and entropy changes. This result is not a necessary consequence of the fact that both proteins perform the same function *in vivo*. Beetlestone and Irvine[13] show that the nearly constant value for the free energy of ionization of the water molecule at the sixth ligand position of a large number of ferrihemoglobins is associated with a very wide range of enthalpy and entropy changes. In warm-blooded in contrast to cold-blooded animals evolutionary experiments to be successful need only satisfy a free-energy requirement. There is no requirement that the enthalpy and entropy changes in a process remain fixed and we should not anticipate that the latter quantities and perhaps even the functional mechanism will be found to be conserved when proteins having the same function but from different organisms are compared

The quantitative similarity of the binding parameters for CO and O_2 suggest a similarity in the bonding of the ligand to heme iron However, large fractions of the bonding enthalpy and entropy may be due to changes in the protein–water system so that as yet the enthalpy changes cannot be converted into formation about the metal–ligand bond. No enthalpy data have been reported for metal–porphyrin complexes but it is interesting to compare the behavior of the chlorocarbonylbis(triphenylphosphine)iridium(I) complexes studied by Vaska and Ibers[14] and their coworkers with the behavior of Mb. Like Mb, in the absence of O_2 or CO the metal has a coordination number of 5. The chloro complex binds both ligands reversibly but in different ways. In the case of oxygen there is a bond between each oxygen atom and the metal. The thermodynamic changes are: $\Delta G°_{20} = -7.7$ kcal/mol; $\Delta H° = -17.1$ kcal mol; and $\Delta S° = -31.0$ eu. These quantities were measured in chlorobenzene and have been correct for the cratic entropy loss. The standard state for oxygen is thus unit mole fraction in the organic solvent. Because CO and O_2 produce large entropy losses in water just as do hydrocarbons,[15] this standard state is the most useful in making comparisons between heme proteins and between heme proteins and small complex ions, when the purpose of the comparisons is to detect contributions from the protein. In the last two columns of Table I are listed standard enthalpy and entropy changes for the Mb reactions computed for a standard state of the ligand of unit mole fraction in an organic solvent, *e.g.*, carbon tetrachloride or ethanol, within the precision with which the corrections are given in the literature.[16] With this standard state the entropy change is that associated with changes in the complex ion rather than desolvation of the ligand and unmixing of ligand and solvent.

The entropy loss in binding O_2 to the irridium complex equals that for O_2 binding to Mb (Table I), and is attributed by Vaska not only to the loss of two rotational degrees of freedom but also to a tightening of the complex ion as a whole; for CO binding: $\Delta G° = -6.4$ kcal mol, $\Delta H° = -10.8$ kcal mol, and $\Delta S° = -14.0$ eu. CO forms a single bond to iridium. According to Vaska[14] CO does not produce the same tightening of the complex ion as does O_2 binding. The iridium complexes are thus not very good models for Mb and we are left with no guide in evaluating the significance of the entropy changes for the binding processes of Mb.

There is considerable variation in the enthalpy and entropy values which have been reported for ligand binding to Hb, sufficient to suggest that there is some variability in this quantity among some species, although it must be noted that none of the literature data take the binding or the variation in binding of diphosphoglyceric acid or equivalent phosphate compound into consideration. However, the best values for the average enthalpy change/mole of oxygen bound appear to be the calorimetric values of Roughton and Splittgerber and Gill. Roughton's value is -13.2 kcal for ox hemoglobin at pH 9.1;[17] the value of Splittgerber and Gill is -13.4 kcal[18] for human Hb at pH 7 after correction for the contribution from the Bohr effect. The correction is negligible at pH 9.1. The only free-energy values which can be considered reliable are those obtained by gasometric procedures[19] and the best χ^2 values have been obtained with sheep hemoglobin. Combining the sheep free-energy changes with the calorimetric enthalpy change, $\Delta H° = -13.3$ kcal/mol, we can estimate the average entropy change per O_2 bound as -23 eu/mol. The average free-energy change, $\overline{\Delta F}°$, is -7.3 kcal/mol. Using the values for oxygen binding to Mb given in Table I we can estimate the differences between Mb and Hb for 4 mol of oxygen bound as $\Delta\Delta F° \approx -4$ kcal, $\Delta\Delta H° = -18.3$ kcal, and $\Delta\Delta S° \approx -40$ eu. These numbers are illuminating. Note first that although only a change

(13) J. Beetlestone and D. Irvine, *J. Chem. Soc.*, 5090 (1964); *ibid.*, 3271 (1965).

(14) Reviewed by L. Vaska, *Accounts Chem. Res.*, 1, 335 (1968). See also J. McGinnety, R. Doedens, and J. Ibers, *Inorg. Chem.*, 6, 2243 (1967). These complexes form "adducts" with other diatomic molecules. See also J. McGinnety, H. Payne, and J. Ibers (*J. Amer. Chem. Soc.*, 91, 6301 (1969)) for related iridium and rhodium complexes. Even better models for Mb are the *N,N'*-ethylene(acetylacetoniminate) ligand–cobalt (II) complexes (A. Crumbliss and F. Basolo, *ibid.*, **92**, 55 (1970); B. Hoffman, D. Diemente, and F. Basolo, *ibid.*, **92**, 61 (1970)) but the thermodynamic changes on ligand binding have not yet been determined.

(15) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).

(16) H. S. Stephen and T. Stephen, "Solubilities of Inorganic and Organic Compounds," Macmillan, New York, N. Y., 1963.

(17) F. W. Roughton, *Biochem. J.*, **29**, 2604 (1935).

(18) A. G. Splittgerber, Ph.D. Dissertation, University of Colorado, 1968.

(19) See Roughton and Lyster, footnote *c*, Table I.

of 4 kcal in the free energy of oxygen binding is produced by the different protein environment, 18 kcal of enthalpy change and 40 eu of entropy change are required to produce this free-energy change. Hence the free-energy difference is a deceptive underestimate of the true differences in behavior of the proteins. We have already mentioned the similar situation which appears in the formation of alkaline methemoglobin.[13] The considerable compensation of the enthalpy differences by the entropy differences, which are much more accurate thermodynamic measures of the differences between the two proteins in oxygen binding, produces a small net free-energy difference. Only the free-energy difference is of importance to the physiologist, at least the physiologist interested in warm-blooded animals. For others the enthalpy and entropy differences should be a matter of major importance. In particular one might hope to get some idea of the relative extent of conformation changes from the entropy difference, though obviously not from the free-energy differences. If so, the larger entropy change in myoglobin might indicate the larger change in conformation. This reasoning may be oversimplified but it is nonetheless quite possible that there are larger changes in Mb than in Hb. Changes on oxygenation of Mb attributable to conformation change have been detected in epr studies; apparently not in studies of Hb.[20] Also a large linkage system consisting of the heme group, two xenon-binding sites, and the site of substance K has been established in Mb.[6,7] It is possible that Mb can be as complex in its behavior as Hb.

In another place[21] we have shown that the binding of zinc and mercuric ions to Mb alters the oxygen affinity. It is an attractive possibility that substance K is also a metal ion so that a systematic attempt to identify that contaminant might profitably start with a survery of metal ions Amiconi, *et al.*,[22] cite unpublished results of Amiconi, Antonini, Brunori, and Magnusson for oxygen binding to whale myoglobin as follows: $\Delta F^\circ = -8.0$ kcal/mol; $\Delta H^\circ = -14.8$ kcal/mol; $\Delta S^\circ = -23$ eu/mol based on a 1 *M* standard state for oxygen. With a 1-Torr standard state $\Delta H^\circ = -18.4$ kcal/mol and $\Delta S^\circ = -62$ eu. The latter quantities are in excellent agreement with Theorell's values and with our own (*cf.* Table II). The former group of authors have determined the enthalpy and entropy changes for oxygen binding to *N*,*N*′-ethylene-(acetylacetoniminate)ligand–cobalt(II) in pyridine (*cf.* ref 14). They report: $\Delta F^\circ = -6.45$ kcal/mol; $\Delta H^\circ = -15.0$ kcal/mol; and $\Delta S^\circ = -29$ eu/mol for an oxygen standard state of 1 *M* in pyridine. These quantities when compared with those of Mb of Amiconi, Antonini, Brunori, and Magnusson demonstrate a considerable similarity in oxygen binding. The validity of this comparison can be tested by thermodynamic studies of carbon monoxide binding to the cobalt complex.

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(20) R. Shulman, S. Ogawa, K. Wüthrich, T. Yamone, J. Peisach, and W. Blumberg, *Science*, **165**, 251 (1969).

(21) M. Keyes and R. Lumry, submitted for publication.

(22) G. Amiconi, G. Tauzher, G. Costa, M. Brunori, and E. Antonini, submitted for publication.