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A Calorimetric Study of the Binding of Carbon Monoxide to Myoglobin†

Stephen A. Rudolph, Stephen O. Boyle, Carlton F. Dresden, and Stanley J. Gill*

ABSTRACT: The heats of binding of carbon monoxide to both horse and sperm-whale myoglobin have been measured using a gas–liquid microcalorimeter. At pH 8.00 it was found that $\Delta H = -19.2$ kcal mole^{-1} for the reaction Mb(aq) + CO(g) → MbCO(aq) for horse Mb and $\Delta H = -23.9$ kcal mole^{-1} for sperm-whale Mb. Extensive dialysis before reaction changed these values to -18.1 and -20.7 kcal mole^{-1}, respectively. The observed heats were the same in both Tris and phosphate buffers, indicating no release or uptake of protons during the reaction. Since the equilibrium constants for these reactions are similar the differences in ΔH are for the most part entropy compensated. The unitary entropy changes for the reactions in aqueous solution at 25° are $\Delta S_u = -14.5$ and $\Delta S_u = -27.0$ cal deg^{-1} mole^{-1} for undialyzed horse and sperm-whale myoglobins, respectively. The differences in ΔH between dialyzed and undialyzed samples is believed to be due to the binding of an unidentified small molecule, as suggested by Lumry and coworkers (M. H. Keyes, Ph.D. Thesis, University of Minnesota, 1968).

Myoglobin, the oxygen binding heme protein found almost universally distributed in muscle tissue, has been under intensive study for the past 40 years. In addition to revealing the function of this protein as a "storehouse" of oxygen, these studies have also done much to uncover the relationships between the structure and function of myoglobin. However, thermodynamic investigations of the interaction of myoglobin with O_2 and CO have generally not gone beyond the measurement of equilibrium constants. Equilibrium measurements have been made on the Mb–O_2[1] reaction for many species and in a few cases also for the Mb–CO reaction (see review articles by Rossi-Fanelli *et al.*, 1964, and Antonini, 1965). In the cases where equilibrium measurements were made as a function of temperature, values of ΔH are available by the van't Hoff method (Theorell, 1934; Rossi-Fanelli and Antonini, 1958; Rossi-Fanelli *et al.*, 1959; Keyes, 1968). However, these data are sketchy (particularly for MbCO) and there is some disagreement as to the correct values. We therefore decided to measure the heat of the Mb–CO reaction by direct calorimetry in order to complete the thermodynamic picture. This study also afforded us the opportunity of testing a unique gas–liquid reaction calorimeter (described below) which is now being used to study similar reactions with hemoglobin.

The Mb–CO reaction was chosen rather than the physiological Mb–O_2 reaction because earlier calorimetric experiments showed that consistent results were not obtainable with O_2. Unlike hemoglobin, Mb is subject to fairly rapid oxidation, particularly at low O_2 concentrations. Horse and sperm-whale myoglobins were chosen because of their ready availability and the existence of a large body of data on their physical and chemical properties, including amino acid sequence and X-ray diffraction studies.

Experimental Section

Materials. Horse and sperm-whale myoglobin were obtained as salt-free, crystalline powders from Seravac, Ltd., and were stored dry at −6°. The absorption spectra of these materials showed them to be primarily in the met or oxidized state. Preparation for use in calorimetric runs was carried out in ice-cold solutions as follows. Dry myoglobin (300–600 mg) was dissolved in 20 ml of the chosen buffer. This solution was centrifuged at 10,000*g* for 10 min to remove small amounts of insoluble material. In experiments where dialyzed protein was used, the Mb was dialyzed against several 2-l. changes of buffer for a total of 18–24 hr. The clear solution was then transferred to a graduated vessel having a 10-mm spectrophotometric cell with a 9-mm quartz spacer at the bottom and a three-way stopcock at the top. Dissolved oxygen was removed from the solution by bubbling with pure nitrogen for 20 min. Reduction of the Mb^+ was then carried out by the method of Rossi-Fanelli *et al.* (1957) using NADH, Methylene Blue, and cytochrome *c* reductase. An alternate method for reduction was found by simply adding ascorbic acid to a concentration of 0.01 M to the Mb^+ solution and bubbling with

† From the Department of Chemistry, University of Colorado, Boulder, Colorado 80302. *Received November 15, 1971.* This work was supported by grants from the National Science Foundation and National Institutes of Health.

* Author to whom correspondence should be addressed.

[1] Abbreviations used are: Mb, reduced or ferromyoglobin; Mb^+, oxidized or metmyoglobin; MbO_2, oxymyoglobin; MbCO, carbon monoxymyoglobin.

pure nitrogen for 20 min. After 6–8 hr at 4°, this method gave material that was spectrally identical with that produced by the enzymic reduction. Spectra were measured in the vessel in which the reduction had been carried out with a 1-mm optical path using a Cary Model 17 spectrophotometer. The reduced material gave a single absorption peak between 500 and 650 nm at 558–560 nm. Concentrations were estimated using ϵ_{560} = 13,000 M^{-1} cm^{-1} (Antonini, 1965). The Mb solutions used in the calorimeter were from 0.7 to 1.4 mM. pH measurements were made with a Beckman digital pH meter and combination electrode.

Calorimeter. The heat measuring apparatus used in these experiments is a highly sensitive differential microcalorimeter designed to measure heats of reaction of gaseous ligands with substances in solution. It is possible to measure both heat evolution (or uptake) and gas uptake simultaneously. The calorimeter design was developed initially by Gill,[2] Splittgerber (1968), and Boyle (1969) and more recently modified for increased sensitivity in the work reported here.

Figure 1 shows a cross section of this calorimeter. The principle of operation is that heat is fed to the reference cell (a copper or aluminum slug), R, by means of a nominal 100-ohm heater wire wound around it and a constant current power supply. This creates a thermal gradient between R and the sample cell, S. The gradient is sensed by a multijunction thermopile, T_m, whose output is fed to a Keithley Model 147 Nanovolt null detector. The Keithley output then goes to a pulsing device designed and built by Albert (1969, 1972) which provides constant current pulses of $^1/_{60}$-sec duration at a maximum rate of 30 sec^{-1}. The actual pulsing rate is proportional to the input to the pulser. The pulser output is fed to a nominal 100-ohm heater wire wrapped around the sample cell, S. Thus, the heat supplied to S is proportional to the thermal gradient between S and R. The pulse size is normally chosen to provide a convenient pulse rate (~20 sec^{-1}) under the conditions of the experiment. In the experiments described here, the pulse size was 8–20 μcal. Under steady-state conditions, the heat supplied to R will induce a constant pulse rate, known as the base line. When a reaction is initiated, the pulse rate will change to compensate for the heat evolution or uptake of the reaction. The rate of heat evolution or uptake is then given by the difference between this pulse rate and the base line. The total number of pulses is counted by a pulse counter and may be recorded at any time with a Hewlett Packard Model 561B digital recorder. The number of pulses is normally recorded each time some given volume of gas is taken up by the solution (usually 0.01 ml). The volume of gas reacted is measured with a sensitive differential manometer. It is thus possible to follow the thermal course of the reaction dynamically instead of simply obtaining an overall heat for the reaction going to equilibrium.

The heat input to R will also establish thermal gradients between the cells and the shields. These gradients are sensed by thermopiles T_{TS} and T_{BS}. Their output is fed to a pair of Beckman Model 14 dc amplifiers which drive proportional power supplies (Albert, 1969). The power supply outputs are fed to nominal 300-ohm heater wires wound around the two shields. This arrangement provides very close control of the thermal environment inside the calorimeter. The entire calorimeter is enclosed in a massive copper jacket and is immersed in a 25-l. water bath. The heat flow into the calorimeter makes it slightly hotter than the water bath, creating a thermal gradient across thermopile T_B. The output from T_B goes to a

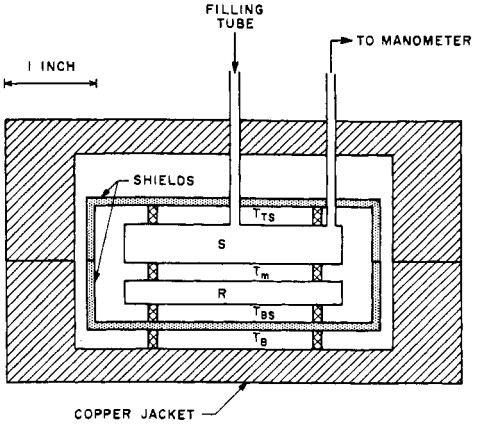

FIGURE 1: Cross-sectional view of the gas–liquid microcalorimeter. Heater wires for cells S and R are wound around the circumference of the cells in grooves (not shown). Heater wires for the top and bottom shields are wound into grooves machined into the top and bottom surfaces of the shields, respectively. The thermopiles are comprised of thin nylon rings with 200–250 turns of constantan wire, each half-turn plated with copper.

Hewlett Packard Model 419A DC null voltmeter which drives a proportional power supply (Albert, 1969). The power supply output is fed to three 100-ohm knife heaters immersed in the water bath, thus providing heat to the bath proportional to the thermal gradient between it and the calorimeter. This maintains the temperature difference between the bath and the calorimeter at approximately 0.001°.

For the initial experiments the sample cell, S, had been constructed of stainless steel and had a volume of approximately 15 ml. It is provided with small stainless steel tubes for filling, gas exchange, and manometer connection. A more thermally responsive cell was incorporated into the calorimeter during the latter stages of this work. It is made of gold-plated copper and gives improved time response but results equivalent to the stainless steel cell. A tight-fitting Teflon tube is inserted in the center tube of the cell for introduction of the liquid sample. It is then sealed with a Swagelok fitting. The side tube is connected to the manometer and by means of a three-way stopcock it may also be used for the introduction of the gas sample.

The procedure for a calorimetric run is described below. The cell is thoroughly flushed with a dry, inert gas (argon or nitrogen in these experiments) and loaded with 4–6 ml of myoglobin solution by pumping under pressure of the same inert gas. The entire calorimeter is rocked in the water bath by a motor to effect stirring of the sample. About 30–60 min is required for the system to reach thermal equilibrium and for the establishment of a stable base line. After the base line has been established, about 2–4 ml of carbon monoxide which has been equilibrated with water at the temperature of the calorimeter is injected into the sample cell, through the side tube. The center tube is opened during this procedure to allow displacement of the inert gas. The reaction then begins, displacing both the base line and the manometer level. It should be noted that although the reaction of carbon monoxide with myoglobin is very rapid in solution (Gibson, 1959), the rate in the calorimeter will be determined by the rate of diffusion of the gas into the solution. This is relatively slow, the reaction taking from 15 to 45 min to reach completion. In general, the pulse rate returned to within ±2% of the original base line. In a few experiments, large base-line shifts were observed;

[2] Unpublished work.

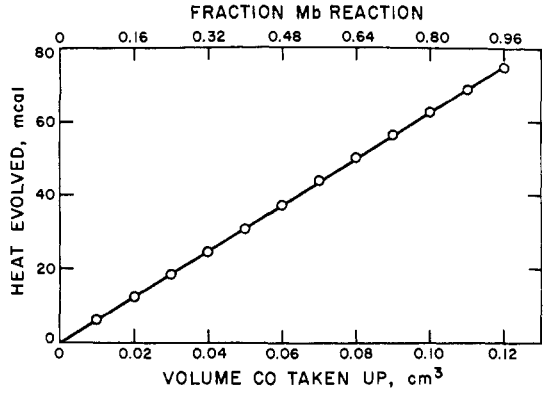

FIGURE 2: Results of a typical calorimetric run. Experimental conditions are 0.71 mM horse Mb in 0.10 M Tris-HCl, pH 8.00, 24.2°. The straight line is a least-squared fit giving $\Delta H = -18.7$ kcal mole^{-1}. Each point corresponds to the uptake of 0.01 ml of CO, which under the experimental conditions corresponds to the formation of 3.36×10^{-7} mole of MbCO.

results from these runs were discarded since it was not possible to determine at what point during the course of the reaction the shift had occurred. Molar heats of reaction were calculated from the raw data, which comprise a table of heat pulses as a function of gas volume reacted, using a Nova computer (Digital General Corp.).

The only correction applied to the observed data was that for heat leakage between R and S. This was measured by perturbing the base line with known amounts of electrical heat from an independent heater inserted through the filling tube into the cell S, and comparing the observed base-line shift with the heat input. This difference was found to vary inversely with the amplification of the thermopile output. At the gain setting used in these experiments (1 μV) the correction factor was found to be 1.095 for the stainless steel cell. The calorimetric efficiency was thus 90.5%. For the gold-plated copper cell, more efficient thermopile placement was used, giving a correction factor of 1.018 and a calorimetric efficiency of 98.2%.

Results and Discussion

The results of all calorimetric experiments are summarized in Table I. The values are averages of at least five runs and are shown with their standard deviations of the mean. All experiments were performed at pH 8.00 using either Tris-HCl or potassium phosphate buffers. As expected, the observed heats of reaction were indifferent to the buffer used, confirming the observation (Theorell, 1934; Rossi-Fanelli and Antonini, 1958) that ionizable groups do not play a significant role in this reaction at this pH.

Figure 2 shows the results of a typical calorimetric run. The fraction-reacted scale at the top is somewhat arbitrary since it is not possible to get data at the very beginning and very end of the reaction. However, CO uptake was followed corresponding to 96% of the total myoglobin present. It should be noted that some runs showed slight inhomogeneity, with the last 20–30% of the reaction giving values of $\Delta H = 2$–3 kcal mole^{-1} less exothermic than the initial 70–80% of the reaction. In these cases the final points were discarded, since the initial part was in good agreement with our other results. This observation may indicate the existence of a weaker ligand binding fraction of Mb. Heterogeneity in myoglobin preparations has been observed and described in detail by Theorell and Åkeson (1955) and by Åkeson and Theorell (1959).

TABLE I: Observed Heats for the Reaction: Mb(aq) + CO(g) → MbCO(aq).[a]

	ΔH (kcal mole^{-1})
Sperm-whale Mb (undialyzed)	-23.9 ± 0.5
Sperm-whale Mb (dialyzed)	-20.7 ± 0.4
Horse Mb (undialyzed)	-19.2 ± 0.3
Horse Mb (dialyzed)	-18.1 ± 0.2

[a] All measurements at pH 8.00. Values shown are averages of at least five calorimetric runs with standard deviations of the mean.

Significant differences in the heats of reaction were observed when the protein was subjected to extensive dialysis before reaction. In the case of sperm-whale Mb this amounts to 3.2 kcal mole^{-1} less heat evolution after dialysis. It is interesting to compare this observation with results obtained by Lumry and his coworkers (Keyes, 1968). They found that sperm-whale Mb prepared by the method of Yamazaki *et al.* (1964) had a low molecular weight ionic species bound to it which they called substance K. Substance K was removable by column chromatography on Sephadex G-25 and its presence had significant effects on the thermodynamics of the Mb–CO reaction. While the effect on the free energy of the reaction by substance K was found by Keyes (1968) to be only 0.5 kcal mole^{-1}, its effect on the heat of reaction was 4.5 kcal mole^{-1}. They found by the van't Hoff method, $\Delta H = -21.4$ kcal mole^{-1} for the Mb–CO reaction in the absence of substance K and $\Delta H = -25.9$ kcal mole^{-1} in the presence of substance K. These values compare quite well with the values determined calorimetrically in this work for the dialyzed and undialyzed samples, respectively. This would indicate that substance K may have been present in our commercially prepared samples of sperm-whale Mb and was removed upon dialysis. However, the situation is complicated by the observation by Keyes (1968) that substance K may exist in both an oxidized and reduced form, only the oxidized form of which binds tightly to the protein. Since almost all of our myoglobin preparations contained a molar excess of ascorbic acid, if substance K were present it would probably have been at least partially in the reduced form. This may account for the difference between the heats of reaction for the dialyzed and undialyzed preparations being somewhat smaller in our work than that observed by Keyes (1968). In the case of horse Mb, the difference in ΔH between dialyzed and undialyzed preparations is only 1.1 kcal mole^{-1}. This may indicate that horse Mb does not contain substance K or that its effect on ΔH is much smaller than in the case of sperm-whale Mb. It is also possible that substance K was removed or inactivated in the course of the commercial preparation.

It appears that the heat of reaction of horse Mb with CO is from 2.6 to 4.7 kcal mole^{-1} more endothermic than that of sperm-whale Mb under similar conditions, depending on whether dialyzed or undialyzed preparations are compared. The value for undialyzed horse Mb ($\Delta H = -19.2$ kcal mole^{-1}) may be compared to that obtained by Theorell (1934), $\Delta H = -22.3$ kcal mole^{-1}. This was obtained by applying the van't Hoff method to the equilibrium between MbO_2 and MbCO

and adding the ΔH for this reaction to that obtained for the reaction between Mb and O_2. However, Theorell's value of ΔH for the Mb + O_2 reaction ($\Delta H = -17.5$ kcal mole^{-1}) is 4.5 kcal mole^{-1} more exothermic than that found by Rossi-Fanelli and Antonini (1958) for horse Mb. This difference would bring Theorell's value of ΔH down to -17.8 kcal mole^{-1} for the Mb + CO reaction, which is in good agreement with our calorimetrically determined ΔH.

The difference in ΔH for the reaction of horse and sperm-whale myoglobins with CO is not due primarily to a difference in affinity for the ligand, as can be seen from Table II. Values of $\Delta G°$ vary by only 1.0 kcal mole^{-1} for a 4.7 kcal mole^{-1} change in ΔH. The differences in ΔH are compensated by differences in ΔS for the two species. Although both horse and sperm-whale Mb have 153 amino acids, there are 19 differences in their amino acid sequence (Edmundson, 1965; Dautrevaux *et al.*, 1967; Hermans and Lu, 1967). Since the binding of the heme group to the protein has such profound effects on the heme's ligand binding properties it is not surprising that changes in the protein sequence are reflected in the thermodynamics of ligand binding.

The high affinity of Mb for CO is a result of the large exothermic ΔH; the entropy effect is in all cases unfavorable. The fourth column of Table II shows the unitary entropy, ΔS_u, for each reaction. This was calculated from $\Delta S_u = \Delta S + 7.98$ (Kauzmann, 1959). ΔS_u represents the entropy changes resulting from interactions between the Mb and CO molecules and changes in interactions between these molecules and water molecules. Entropy changes due to purely statistical effects (cratic entropy) are not included.

The data of Theorell (1934) and of Keyes (1968) was obtained at pH 7.45 and 8.5, respectively. All the calorimetric results presented in this paper were obtained at pH 8.00. This presents no difficulty, however, since the ligand binding properties of Mb are virtually independent of pH in this range (Theorell, 1934; Rossi-Fanelli and Antonini, 1958). This is also true of the effects of ionic strength. Where binding constants were measured at a temperature other than 25°, these have been corrected to 25° by using the calorimetric values of ΔH.

TABLE II: Thermodynamic Parameters for the Reaction Mb(aq) + CO(aq) → MbCO(aq) at 25°.

	$\Delta G°$ (kcal mole^{-1})	ΔH^a (kcal mole^{-1})	ΔS (cal deg^{-1} mole^{-1})	ΔS_u (cal deg^{-1} mole^{-1})
Sperm-whale Mb (undialyzed)	-10.5^b	-20.9	-35.0	-27.0
Sperm-whale Mb (dialyzed)	-10.0^b	-17.7	-25.8	-17.8
Horse Mb (undialyzed)	-9.50^c	-16.2	-22.5	-14.5

[a] Corrected for the heat of solution of CO in H_2O, $\Delta H = -3.0$ kcal mole^{-1}. [b] Calculated from equilibrium data of Keyes (1968). [c] Calculated from equilibrium data of Theorell (1934).

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