This difference may be also associated with the difference in the active sites of both electrodes.

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(33) The electrochemical reduction of NO_2^- by the $[Mo_2Fe_6S_8]$/GC at −1.00 V was too slow to analyze the reaction products.

Registry No. DMF, 68-12-2; NO_3^-, 14797-55-8; NO_2^-, 14797-65-0; $(Bu_4N)_4[MoFe_3S_4(SPh)_3(O_2C_6Cl_4)]_2$, 134817-97-3; C, 7440-44-0; NH_3, 7664-41-7; N_2, 7727-37-9; NO, 10102-43-9; $(Bu_4N)_3[Mo_2Fe_6S_8(SPh)_9]$, 68197-68-2; $NaNO_3$, 7631-99-4; $NaNO_2$, 7632-00-0; N_2O, 10024-97-2; Bu_4NBr, 1643-19-2; H_2, 1333-74-0; NH_2OH, 7803-49-8; Bu_4NNO_3, 1941-27-1; CD_3CN, 2206-26-0; CH_3CN, 75-05-8; $(Bu_4N)_2[MoF_3S_4(SPh)_3(O_2C_6Cl_4)(CD_3CN)]$, 134847-22-6.

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Volume Profile Analysis of the Formation and Dissociation of Carboxymyoglobin. Comparison with the Corresponding Oxymyoglobin System

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The effect of pressure on the decarbonylation kinetics of carboxymyoglobin was studied by using stopped-flow techniques. The corresponding volume of activation is $\Delta V^{\ddagger}_{off} = -3.8 \pm 1.6$ cm^3 mol^{-1}. The reaction volume was calculated by using pressure-dependent k_{on} values reported in an earlier study to be -4.1 ± 0.8 cm^3 mol^{-1}. It was also measured directly from the pressure dependence of the equilibrium constant, which resulted in a reaction volume of -3.0 ± 0.6 cm^3 mol^{-1}. A comparison of the volume profiles for the reactions of myoglobin with CO and O_2 reveals that the reactions proceed according to two different mechanisms. Bond formation is rate-determining for CO, whereas entry into the protein is rate-determining for O_2. The results are compared to related studies reported in the literature.

Introduction

In recent years the application of high-pressure kinetic techniques has significantly assisted the mechanistic interpretation of reactions in inorganic and organometallic chemistry.[1-3] High-pressure techniques have also been applied to the study of biochemical and bioinorganic systems.[2,4] In this respect the interest in reactions of small molecules (isonitriles, CO, NO, O_2) with the oxygen transport protein myoglobin has increased in recent years. Various methods have been applied to this system in an effort to improve the understanding of the functional reaction paths of this protein, viz. flash photolysis,[5-7] flash photolysis at low temperatures,[8-10] X-ray diffraction,[11-16] neutron diffraction,[17,18] IR/Raman spectroscopy,[9,19,20] cyclic voltammetry,[21] ^{1}H NMR spectroscopy,[21-26] and protein engineering.[27-30] It is commonly accepted that the mechanisms of binding are totally different for oxygen and carbon monoxide. These reactions show different activation barriers (with different conformational states) and different rate-determining steps.[9,29-34] In two recent studies[35,36] we have investigated the reactions of O_2, CO, and isonitriles with myoglobin and some model compounds via the application of high-pressure techniques. A volume profile treatment for the formation and dissociation of oxymyoglobin enabled us to comment

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on the intimate mechanisms of these reactions. The pressure effects at ambient temperature were attributed to contributions of bond formation and cleavage, solvation effects, changes in the spin state of the porphyrin iron, and changes in the conformational states of the protein, in agreement with the results of other studies.[9,31,32] We have now completed our work on the myoglobin system with an investigation of the dissociation reaction of CO from carboxymyoglobin (MbCO) in this study. The data obtained here enable us to construct a volume profile for the reaction with CO and to compare it with that for the myoglobin–oxygen system.

Experimental Section

Materials. Reagent grade chemicals were used throughout this study. Unless otherwise stated, a 0.005 M aqueous solution of 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris, Sigma) was used as buffer. The pH value was adjusted to 8.5 ± 0.1 and the conductivity to 240 ± 10 μS cm^{-1} by titration with hydrochloric acid (Titrisol, Merck). In some experiments a 0.1 M phosphate buffer solution (Merck) was used at pH 7.0 but only at ambient pressure. It is not appropriate to use a phosphate buffer at elevated pressure, since the pH value of this buffer system strongly depends on pressure.[37] In contrast, the pH of Tris buffer is almost pressure independent.[37] Unless otherwise stated, the ionic strength was adjusted to 0.1 M prior to any experiment with the aid of sodium chloride (Merck).

The heme content of the sperm whale myoglobin used was determined according to the method of De Duve[38] as described before.[34] The total iron content was 0.26%. The myoglobin used was purified by chromatographic methods described in the literature.[35,39] Fractions with an absorbance ratio of A(582 nm)/A(544 nm) > 1.904 were collected and stored at −25 °C as an oxymyoglobin stock solution. Microperoxidase MP-11 (Sigma), sodium dithionite, $K_3[Fe(CN)_6]$, and *n*-heptane (Merck) were used without further purification.

Solutions of CO and NO in water or aqueous buffer solutions were prepared by equilibrating the solutions with CO gas (99.97% by volume, Messer Griesheim) or NO gas (99.8% by volume) for 2 h at 1 bar.

Instruments. UV/vis spectrophotometry at ambient pressure was conducted on a Shimadzu UV-250 or a Hitachi U-3200 spectrophotometer. UV–vis spectra at elevated pressure were recorded in the high-pressure cell[40] of a Zeiss DMR-10 spectrophotometer. Stopped-flow experiments were executed on a Dionex stopped-flow apparatus at ambient pressure and on a homemade high-pressure stopped-flow instrument.[41] Data acquisition and evaluation was performed either on an Apple IIe or on an Atari Mega ST 4 computer using our own customized application software[42] or spreadsheet capabilities. Conductivities were measured on a WTW LF-91, and pH values, with a Metrohm 632 pH meter. A calomel glass electrode specially designed for Tris buffer solutions (Sigma) was used in these measurements.

Procedures. 1. Protein Concentrations. Working solutions were obtained by diluting the myoglobin stock solution with the appropriate amount of buffer. Myoglobin concentrations were determined by monitoring the absorbance maxima at 422 nm (ϵ = 187 000 L mol^{-1} cm^{-1}) for carboxymyoglobin and 434 nm (ϵ = 115 000 L mol^{-1} cm^{-1}) for deoxymyoglobin (i.e. the five-coordinate species).[43]

2. Stopped-Flow Kinetics. Solutions for stopped-flow measurements were degassed via at least five freeze–pump–thaw cycles. Solutions of carboxymyoglobin were obtained by injection of the calculated stoichiometric amount of CO-saturated buffer into the myoglobin working solutions. Solutions of microperoxidase were prepared by dissolving an appropriate amount of microperoxidase and sodium dithionite in degassed buffer.

Results and Discussion

Preliminary Investigations. The UV/vis absorption spectra of deoxymyoglobin and carboxymyoglobin were investigated as a function of pH and pressure. The spectra are independent of pH in the range from 6 to 9, which is in agreement with that reported in the literature.[43-45] Similarly, the spectra of microperoxidase do not show a pH dependence in the same range.[46-49] At elevated pressure, microperoxidase and MbCO only show a slight red shift of the absorbance maxima, but this phenomenon is generally found for UV/vis absorption bands of solutes in isotropic media at elevated pressure. The increase in absorbance of MbCO and microperoxidase at elevated pressure is mainly attributed to the compressibility of the solvent,[51,52] although structural changes of the protein under pressure may also play an important role.[52-54] We have therefore taken these effects into account in terms of a correction factor for the absorbance of MbCO, as in our earlier study;[35] see Table I.

Table I. Correction Factors to Account for the Compressibility of the Solvent and System[a]

	corr factor,[b] %		
P, MPa	MbCO[c]	Mb[d]	H_2O[e]
5			0.25
50	2.4 ± 0.2	1.2 ± 0.2	2.10
100	4.4 ± 0.2	2.2 ± 0.2	3.94
150	5.9 ± 0.2	3.3 ± 0.3	5.58

[a] Experimental conditions: temperature 25 °C, [Tris buffer] = 5 mM; pH = 8.5. [b] Correction factor is expressed as $\Delta\bar{V}/\bar{V}$ (at 0.1 MPa)—see Results and Discussion; mean value of between 4 and 6 measurements. [c] Measured for a completely carbonylated solution. [d] Measured for the five-coordinated species. [e] Reported for pure water in the literature: Beggerow, G., Schäfer, K., Hellwege, K.-H., Eds. High Pressure Properties of Matter. In *Numerical Data and Functional Relationships in Science and Technology*, Landolt-Börnstein New Series Group IV; Springer Verlag: Berlin, Heidelberg, 1980; Vol. 4.

In some preliminary investigations we also tried to determine the volume of reaction for equilibrium 1 by dilatometric and

$$Mb + CO \rightleftharpoons MbCO \quad (1)$$

density measurements. The partial molar volume of a substance is usually calculated from density measurements with the aid of eq 2, where ρ_s represents the solvent density, and ρ, M, and c

$$\bar{V} = \frac{1}{\rho_s}\left[M - \frac{\rho - \rho_s}{c}\right] \quad (2)$$

represent density, molar mass, and concentration of the substance under investigation, respectively. The partial molar volumes of O_2 and CO are rather similar[35,36] (28 and 35 cm^3 mol^{-1}, respectively). It follows that the measured partial molar volumes of carboxy- and deoxymyoglobin should reveal a volume difference in these protein species due to conformational changes in the bound state. The volume of reaction can subsequently be calculated from eq 3, i.e. by substracting the partial molar volumes of the reactants from the partial molar volume of the product.

$$\Delta\bar{V} = \bar{V}_{MbCO} - \bar{V}_{Mb} - \bar{V}_{CO} \quad (3)$$

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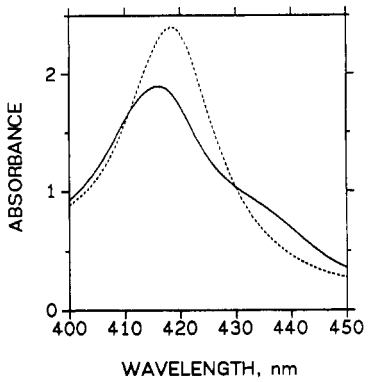

Figure 1. Spectra of carboxymyoglobin and five-coordinate microperoxidase recorded with a 0.88-cm tandem cuvette before mixing (---) and after mixing (—). Conditions: [MbCO] = 3 μM; [microperoxidase] = 30 μM; temperature 25 °C; 5 mM Tris buffer; pH = 8.4 (before mixing).

Unfortunately, the molar masses of myoglobin and carboxymyoglobin are approximately 500 times the molar mass of CO. Therefore, even an experimental error of 1% (which is in fact very good and can not be reduced further) does not result in any meaningful value for the volume of reaction. The partial molar volume of CO was easily measured as $\bar{V}_{CO} = 33 \pm 1$ cm^3 mol^{-1}, and the values for $\bar{V}_{Mb}$ and $\bar{V}_{MbCO}$ were found to be 12396 ± 261 and 12204 ± 227 cm^3 mol^{-1}, respectively. The individual experiments result in a $\Delta\bar{V} = -45 \pm 188$ cm^3 mol^{-1}, which demonstrates the above prediction.

In dilatometry the volume change during a reaction can be measured directly.[57] In this study the two compartments of a Carlsberg dilatometer were filled with 15 cm^3 of a CO-saturated buffer and with 5 cm^3 of deoxymyoglobin solution of the calculated stoichiometric concentration, respectively. The two reactants were mixed under *n*-heptane, and the deviation of the heptane level in the capillary was monitored with a cathetometer. The detailed procedure is described elsewhere.[55,56] The dilatometer was accurately thermostated ($T = 25.0 \pm 0.005$ °C). Since the solubility of CO in water is fairly small (0.99 mM), the absolute volume differences that may be achieved are also very small. Although we could observe a decrease in volume during the experiment (the heptane level in the capillary dropped), which means a negative volume of reaction, the deviation within a series of experiments ($\Delta\bar{V} = -225 \pm 188$ cm^3 mol^{-1}) did not allow an accurate prediction of the reaction volume.

Kinetic Studies. We[36] and other groups[7,31] have already reported in detail on the pressure dependence of reaction 1 (see also Table IV), but no high-pressure data are presently available for the reverse reaction, i.e. the dissociation of CO from MbCO. We therefore thoroughly investigated this reaction as a function of temperature and pressure using stopped-flow techniques. Similar to our work on the dissociation reaction of O_2 from MbO_2,[35,57] we used a "scavenger" for CO. Various reagents are suggested in the literature to study the dissociation of CO from MbCO, namely NO,[43,58] $K_3[Fe(CN)_6]$,[43,59,60] and microperoxidase.[61,62] Only microperoxidase acts as a scavenger for CO, whereas $K_3[Fe(CN)_6]$ oxidizes the heme iron and NO competes with CO for free binding sites (Fe binds NO more strongly and faster than CO). In some preliminary experiments we found that either NO or $K_3[Fe(CN)_6]$ used under our experimental conditions resulted in only minor absorbance signal changes, whereas the application of microperoxidase resulted in absorbance changes of about $\Delta A \approx 0.25$ units at $\lambda_{obs} = 438$ nm (see Figure 1). Microperoxidase (MP-11) reacts much faster with CO than myoglobin, and the binding constant is much larger ($k_{on} = 2 \times 10^7$ M^{-1} s^{-1}, $K = k_{on}/k_{off} = 2 \times 10^9$ M^{-1}).[61,62] Although spectra of MbCO and iron(II) microperoxidase (iron(III) microperoxidase does not bind O_2) exhibit a partial overlap in the Soret region,[46-48] the dissociation of CO from MbCO can easily be followed by monitoring the increase of absorbance of the five-coordinate deoxymyoglobin species between 430 and 440 nm (see also Figure 1). However, the rate of this reaction is relatively slow, which resulted in a poor signal-to-noise ratio for the sweep time under consideration (100–300 s). It was not possible to monitor the reaction at elevated pressure in a static instrument, since the time required to fill and pressurize a pillbox cell is about 10 min. For this reason we were forced to use the stopped-flow technique, although this method is not the best one for the time scale stated. In addition, the rate of the reaction investigated exhibited only a weak dependence on pressure.

Table II. Activation Parameters for the Reaction MbCO → Mb + CO

no.	$\Delta G^{\ddagger}$, kJ mol^{-1}	$\Delta H^{\ddagger}$, kJ mol^{-1}	$\Delta S^{\ddagger}$, J mol^{-1} K^{-1}
1	81.2	68.2 ± 1.6	−43 ± 5
2	80.7	59.8 ± 2.3	−70 ± 8
3	81.4	69.3 ± 2.8	−41 ± 9
4	81.3	60.0 ± 1.5	−72 ± 5
mean value	81.2 ± 0.3	64.3 ± 5.3	−57 ± 17

In contrast to the usually quite high degree of accuracy accomplished in kinetic studies at elevated pressure, the effects listed above resulted in relatively large error limits for the calculated rate constants and activation parameters and in considerably high deviations of the individual experiments.

It is known that iron(II) microperoxidase in alkaline medium exhibits a very slow reaction with the CO bound to the heme iron.[62] We investigated the reaction of microperoxidase (50 μM) with a CO-saturated buffer solution and found a rate constant $k_{obs} = (2.7 \pm 1.7) \times 10^{-3}$ s^{-1} at the pH used for our experiments (8.5). Hence this reaction is 1 order of magnitude slower than the dissociation reaction of CO from MbCO, but it has to be taken into account in terms of a drift in the infinity absorbance value.

The reaction was investigated under pseudo-first-order conditions, i.e. with an excess of microperoxidase. The observed rate constant is independent of carboxymyoglobin and microperoxidase concentrations in the range used (1.5–13.9 and 6.3–167 μM, respectively), but we did observe a dependence of the rate constant on the spectral bandwidth similar to that reported in the literature.[61] We varied the spectral bandwidth from 0.1 to 5 nm. Evidently, the dissociation of CO is facilitated by photoinduced dissociation when the sample is irradiated by a high light intensity. We eliminated this effect by reducing the slit width until the rate constant of the dissociation reaction did not show any variation. For our instrumental setup, the rate was constant at a slit width below 0.6 mm.

Under optimized conditions ($[Na_2S_2O_4] < 1$ mM; $[Mb]_{tot.} = [MbCO] \approx 1.5$–14 μM; [MP] ≈ 50–150 μM; slit width 0.4 mm, $\lambda_{obs} = 438$ nm) the average from six series of experiments with eight measurements each was $k_{obs} = 0.021 \pm 0.002$ s^{-1} at $T = 20$ °C and pH = 8.5. These results are in good agreement with the values found by Sharma and Ranney[61] (0.0175 s^{-1}) and La Gow and Parkhurst[58] (0.015 s^{-1}) and with earlier results for horse myoglobin (0.017[43] and 0.04 s^{-1} [60]) or Aplysia myoglobin (0.02 s^{-1} [59]), which were determined in part via other methods (reaction of MbCO with NO and $K_3[Fe(CN)_6]$). The temperature and pressure dependence of the dissociation reaction was investigated in the range between 4 and 46 °C and 0.1 and 100 MPa, respectively. The plots for the determination of $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta V^{\ddagger}$ are linear within the error limits stated. From the series of Table II it is apparent that $\Delta G^{\ddagger}$ and $\Delta H^{\ddagger}$ show large positive values, whereas $\Delta S^{\ddagger}$ always shows a negative sign. In contrast to these findings, the reaction with O_2 shows a *positive* entropy of activation.[35] The pressure dependence of the reaction was measured

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Table III. k_{on}, k_{off}, K, and K_{exp} as a Function of Pressure at 25 °C for the Equilibrium Mb + CO ⇌ MbCO

P, MPa	$10^{-5}k_{on}$,[a] M^{-1} s^{-1}	$10^{4}k_{off}$,[b] s^{-1}	$10^{7}K$[d]	$10^{7}K_{exp}$
5	5.2	308	1.69	1.69
50	6.0	354	1.70	1.87
100	7.5	384	1.95	1.96
150	9.2	438[c]	2.10	2.03
ΔV, cm^3 mol^{-1}			−4.1 ± 0.8	−3.0 ± 0.6

[a] From ref 36; $\Delta V^{\ddagger}_{on}$ = −10.0 cm^3 mol^{-1}. [b] Typical series of data; $\Delta V^{\ddagger}_{off}$ = −5.8 cm^3 mol^{-1}. [c] Extrapolated from lower pressure data. [d] Calculated from kinetic data ($K = k_{on}/k_{off}$).

at three different temperatures (T = 20, 25, and 28 °C), and we could not detect a significant change in the activation volume in this temperature range. As an example, Figure 2 shows some experimental series that were recorded at the different temperatures stated; the slopes of the least-squares lines are apparently equal. The average from 23 series of measurements was $\Delta V^{\ddagger}_{off}$ = −3.8 ± 1.6 cm^3 mol^{-1}. It follows that the dissociation of CO from MbCO is accompanied by a small volume *decrease*. This result is in contrast to the expectations, since bond cleavage is usually accompanied by an *increase* in volume. In earlier work,[35] we found a large positive volume of activation for the deoxygenation reaction of MbO_2.

Equilibrium Studies. The equilibrium constant for equilibrium 1 was calculated from the ratio of the k_{on} and k_{off} rate constants and determined experimentally by a measurement of the equilibium concentrations. These procedures have been described elsewhere.[35] The values of k_{on}, k_{off}, and K are summarized as a function of pressure in Table III and compared with the K_{exp} values that were obtained experimentally from the pressure dependence of the equilibrium constant (see Figure 3). The plots of ln K versus pressure P are linear; the so calculated volumes of reaction are summarized in Table III.

It is apparent, that the experimentally determined volume of reaction agrees well with the volume of reaction calculated from the kinetic data. $\Delta\bar{V}$ may also be calculated according to eq 4

$$\Delta\bar{V} = \Delta V^{\ddagger}_{on} - \Delta V^{\ddagger}_{off} = -10.0 - (-3.8) \qquad (4)$$

by subtracting the average volumes of activation for the on and off reactions. In this way we find $\Delta\bar{V}$ = −6.2 cm^3 mol^{-1}, which is in reasonable agreement with the experimental value. It should be noted that spectrophotometric determinations at elevated pressure are usually subjected to quite large experimental errors, since small absorbance changes due to either the instability of the baseline of the spectrophotometer or small geometrical changes in the high-pressure optical cell may result in large changes in K. In addition, the correction factors for the compressibility of the solvent have to be determined as accurately as possible. Our

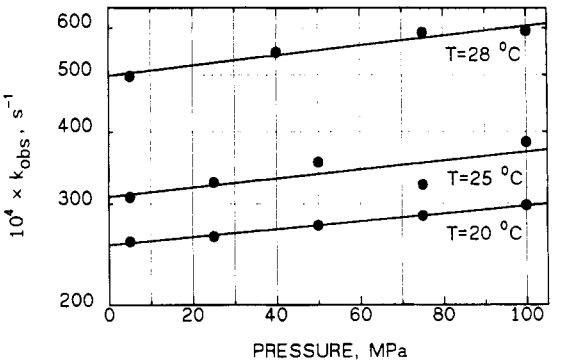

Figure 2. Logarithmic plots of k_{obs} versus pressure at various temperatures for the reaction MbCO ⇌ Mb + CO. Conditions: [MbCO] = 3 μM; [microperoxidase] = 30 μM; λ_{obs} = 438 nm; 5 mM Tris buffer; pH = 8.4 (before mixing).

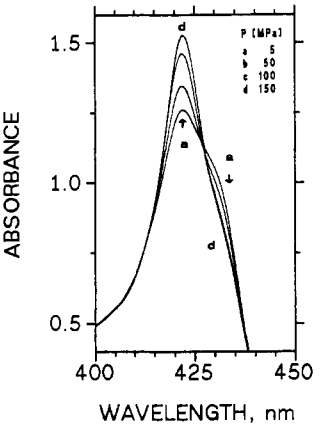

Figure 3. Effect of pressure on the absorbance spectrum of Mb/MbCO. Conditions: [Mb_{tot}] = 8–12 μM, temperature 25 °C; 5 mM Tris buffer; ionic strength 0.1 M (NaCl); pH = 8.4

Table IV. Summary of Rate and Activation Parameters for the Reaction of Myoglobin with CO and O_2

param[a]	Mb + CO ⇌ MbCO	ref	Mb + O_2 ⇌ MbO_2	ref
k_{on}, M^{-1} s^{-1} [b]	5.2×10^5	36	2.5×10^7	35
	3.8×10^5	7	1.3×10^7	7
k_{off}, s^{-1} [b]	0.021	*c*	21.8	35
	0.017	61, 62	23.6	57
$K°$, M^{-1} [b]	1.69×10^7	*c*	0.92×10^6	35
	2.5×10^7	59, *d*	1.06×10^6	43
$\Delta H^{\ddagger}_{on}$, kJ mol^{-1}	+17.1	7	+23.0	7
	+29.8	43, *f*	+23.1	43, *f*
$\Delta H^{\ddagger}_{off}$, kJ mol^{-1}	+62.1	*c*	+76.1	35
	+70.1	43, *f*	+79.8	43, *f*
$\Delta H°$, kJ mol^{-1}	−45	*e*	−43	*e*
	−40.3	43, *f*	−62.5	43
$\Delta S^{\ddagger}_{on}$, J mol^{-1} K^{-1}	−81.1	7	−30	7
$\Delta S^{\ddagger}_{off}$, J mol^{-1} K^{-1}	−61	*c*	+36	35
$\Delta S°$, J mol^{-1} K^{-1}	−20	*e*	−66	*e*
$\Delta G^{\ddagger}_{on}$, kJ mol^{-1} at 25 °C	+41	7	+32.2	7
$\Delta G^{\ddagger}_{off}$, kJ mol^{-1} at 25 °C	+80.2	*c*	+65.4	35
$\Delta G°$, kJ mol^{-1} at 25 °C	−39.2	*e*	−33.2	*e*
$\Delta V^{\ddagger}_{on}$, cm^3 mol^{-1}	−10.0	36	+5.2	35
	−8.9	7	+7.8	7
	−9.2	31	+4.6	31
$\Delta V^{\ddagger}_{off}$, cm^3 mol^{-1}	−3.8	*c*	+23.2	35
$\Delta\bar{V}$, cm^3 mol^{-1}	−6.2	*c*	−18	35

[a] At 25 °C. [b] At 0.1 MPa. [c] This work. [d] For Aplysia Mb at 20 °C. [e] Extrapolated from kinetic data. [f] Horse Mb.

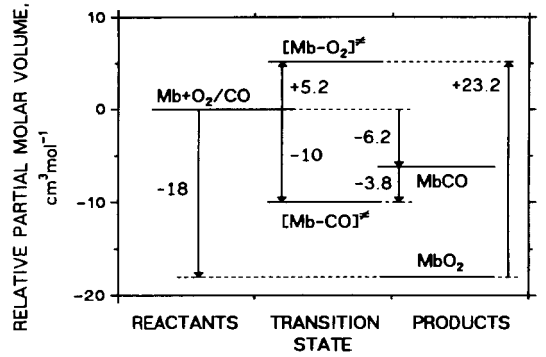

Figure 4. Comparison of the volume profiles for the reactions Mb + O_2 ⇌ MbO_2 and Mb + CO ⇌ MbCO.

correction factors in Table I not only include the effect of the increase in absorbance at elevated pressure but also include possible small structural changes of the protein. The effect of pressure on the spectra of Mb and MbCO has been discussed in detail by us[35,36] and other groups.[9,50,51,63] Although the processes that occur during studies of proteins at high pressures have not completely been resolved, it appears that the observed effects can be contributed to conformational changes of the protein, at least at relatively low pressures (<200 MPa). This view is supported by the relatively low compressibility of myoglobin (approximately 9 $Mbar^{-1}$)[9,64] as compared to water (ca. 50 $Mbar^{-1}$).

Volume Profile and Interpretation. The volume profiles for reaction 1 and the analogous reaction with oxygen[35] are combined in Figure 4. Table IV summarizes the rate and equilibrium constants and the activation parameters for both reactions in comparison with literature data. It is generally accepted that bond formation of myoglobin with small ligands may be described by a four-stage mechanism,[5,18,34] as indicated by the scheme in (5).

$$\text{Mb} + \text{L} \rightleftharpoons [\text{Mb}\,||\,\text{L}] \rightleftharpoons [\text{Mb L}] \rightleftharpoons \text{MbL} \qquad (5)$$

free ligand	protein separated pair	contact pair	bound ligand

$$\text{Mb} + \text{L} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{MbL} \qquad (6)$$

Our high-pressure studies at ambient temperature in the millisecond and second time frames only result in new insight and understanding of the rate-determining step in the overall equilibrium shown in (6). These type of measurements were recently criticized by Frauenfelder and co-workers[9] on the basis of low-temperature–high-pressure investigations at <160 K in 75% glycerol solutions. Such experimental conditions are however according to our opinion irrelevant for the study of biological processes that occur at ambient temperature in aqueous solution. A comparison of the reaction and activation parameters for both the association and dissociation reactions of O_2 and CO leads to important conclusions concerning the overall course of the reactions.

From the data of Table IV, it is apparent that the enthalpy ($\Delta H^{\ddagger}$) and free enthalpy ($\Delta G^{\ddagger}$) of activation are significantly different for the association and dissociation of the two different ligands. In contrast, these parameters are very similar for O_2 and CO, which results in a similar reaction enthalpy ΔH° (−45 kJ mol^{-1} for CO and −43 kJ mol^{-1} for O_2) and free reaction enthalpy ΔG° (−39 and −33 kJ mol^{-1} for CO_2 and O_2, respectively). Indeed these parameters are strongly negative and very similar. It follows that the association reaction is strongly favored for both ligands.

In contrast to the enthalpies, the entropies of activation differ significantly, not only for O_2 and CO but also for the on and off reactions. The only reaction that results in an increase in entropy is the deoxygenation reaction of MbO_2; all other reactions under consideration proceed with a decrease in entropy. It may be concluded that fundamentally different transition states must occur during the dissociation reactions. This conclusion is supported by the reported activation volumes.

The volume profile for the reaction with oxygen differs completely from that for the reaction with CO. First we will discuss the on reactions of these two small ligands. For the reaction of myoglobin with oxygen, we found an increase in volume (+5 cm^3 mol^{-1}) during oxygen uptake followed by a drastic volume decrease (−23 cm^3 mol^{-1}) after passing through the transition state.[35] In contrast, the activation volume for CO uptake of −10 cm^3 mol^{-1}, which has been confirmed by several groups,[7,31,36] may be explained in terms of a "late" transition state that closely resembles the product state. Here the rate-determining step is bond formation accompanied by a spin change of the heme iron from high spin to low spin. The measured activation volume of −10 cm^3 mol^{-1} is actually too small (in terms of absolute magnitude), because the individual contributions of bond formation and spin change should result in a much more negative value. However, it should be kept in mind that the entering of CO into the protein is expected to be accompanied by desolvation, i.e. a volume increase, which will partially offset the expected negative value. Furthermore, it is also likely that conformational changes occur in the protein during bond formation with CO which result in a volume increase during and after bond formation. The contraction of the porphyrin core of about 120 pm and the movement of the iron center into the porphyrin plane (300 pm) only results in a volume change of approximately −0.2 to −0.3 cm^3 mol^{-1}.[52] It follows that solvational and conformational effects *before* the transition state result in an activation volume that is too small (i.e. a volume decrease that is too small) and logically results in a volume *increase after* the transition state has been passed. Similar conformational changes also occur during the oxygenation reaction but in terms of the reaction coordinate and transition state much "earlier" than for CO i.e. during the entry of the O_2 molecule into the protein.[14,36] The protein therefore plays a crucial role, and it is appropriate to think in terms of a protein "gate" that the ligand has to pass on entering the protein. The volume of activation found for this reaction (ca. +5 cm^3 mol^{-1}) approximately corresponds to the partial molar volume difference between MbO_2 and Mb of ca. 9 cm^3 mol^{-1}, as can be calculated from eq 7.

$$\Delta\bar{V} = \bar{V}(MbO_2) - \bar{V}(Mb) - \bar{V}(O_2) \qquad (7)$$

This means that the opening up of the protein corresponds closely to the situation achieved on complete binding of O_2. It follows that the volume difference between the transition and product states (24 cm^3 mol^{-1}) approximately equals the partial molar volume of O_2 (28 cm^3 mol^{-1}) in terms of these numbers. Hence the overall volume of reaction is −19 cm^3 mol^{-1}. The high activation volume for the deoxygenation reaction is therefore the sum of all contributions resulting from bond cleavage and conformational changes in the protein, which occur during the escape of the molecule from the protein. In an earlier study,[36] we demonstrated that ligand escape from the protein is always accompanied by a large volume increase for all ligands investigated (+11.7 cm^3 mol^{-1} for CO, +12.6 cm^3 mol^{-1} for O_2, and +9.6 cm^3 mol^{-1} for methyl isocyanide). These results also support the statement that the protein matrix acts as a gate that strongly controls the entrance and escape of ligands in a crucial way. Case and Karplus[65] state that the energy barriers for a "rigid" protein are so high (>400 kJ mol^{-1}) that a thermally controlled reaction with these ligands would be slow enough to be negligible. For this reason conformational changes must play an important role.

In this work we found that the escape of CO from MbCO (as for the association reaction) results in an activation volume of ca. −4 cm^3 mol^{-1}. This finding seems to stand in contrast with the statements outlined above. However, a detailed discussion of the off reaction may well account for the negative volume of activation observed.

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(65) Case, D. A.; Karplus, M. *J. Mol. Biol.* **1979**, *132*, 343.

First of all we will compare the similarities and differences of MbO_2 and MbCO. They both have the same protein matrix, porphyrin, and central metal atom, a low-spin (0 μ_B) Fe(II). The porphyrin iron binds O_2 under an angle of 115° [15] and exhibits a hydrogen bond to histidine E7.[17] This results in a sterically favored orientation of the oxygen molecule in the porphyrin pocket. In contrast, CO does not show any hydrogen bonding,[18] and its favored linear bonding geometry is not possible due to the histidine E7. The X-ray structure of MbCO shows that the heme "pocket" is widened significantly compared to deoxymyoglobin. For example, the distal histidine moves ca. 140 pm during the binding process.[16] During the dissociation of O_2 from MbO_2, the Fe–O bond and the hydrogen bond to histidine E7 are broken, the heme iron changes its spin from low to high, and the protein "expands" via conformational changes. At the transition state, this process is nearly complete as demonstrated by the large positive volume of activation (+24 cm^3 mol^{-1}).

Contrary to this process, steric tension should first be released during cleavage of the Fe–CO bond. The quantum yield for the photolytical cleavage of the Fe–CO bond is nearly 100%;[5,36] i.e. once the bond has been broken, the ligand nearly always "escapes" into the surroundings. In comparison to O_2, CO must be bound much stronger to the heme iron, since the rate of dissociation of CO is 3 orders of magnitude slower than that of O_2 (see Table IV). This indicates that the Fe–CO bond exhibits stronger σ-donor/π-acceptor characteristics than the Fe–O_2 bond. It follows that Fe–CO bond breakage will be accompanied by a decrease in steric tension and a slight volume collapse due to reorganization of the protein pocket as CO leaves the iron coordination site. If the transition state is relatively "early" during bond *cleavage*, this will result in a volume decrease, i.e. a negative volume of activation (experimental value −4 cm^3 mol^{-1}). By analogy, these arguments support a "late" transition state during Mb–CO bond *formation*. The large negative entropy of activation for the CO off reaction also supports this interpretation. Following this decrease in volume, the completion of bond cleavage is accompanied by the low-to-high spin shift and leads to a volume increase during ligand escape.

As a whole, a total reaction volume of −6 cm^3 mol^{-1} is obtained, inspite of the unfavorable steric situation of bound CO. For both O_2 and CO the outlined effects lead to an overall negative reaction volume; the difference in the reaction volumes ($\Delta\bar{V}(O_2) - \Delta\bar{V}(CO)$) is −13 cm^3 mol^{-1}. The arguments in the above section lead to the conclusion that the mechanism of ligand binding must be the determining factor to account for this difference, which in total means a larger volume decrease for O_2. The reasons for this may be the different bonding characters for O_2 and CO, the bonding angles, hydrogen bonding effects, and conformational changes resulting from these factors. In terms of bonding character it may also be appropriate to think of the bonding modes as Fe^{III}–O_2^- and Fe^{II}–CO, respectively, which would further account for the significantly different volume profiles reported in this paper.

In summary, the reactions of oxygen and carbon monoxide proceed according to two different mechanisms. Whereas the entry of the ligand into the protein is the rate-determining step for the binding of oxygen, bond formation is the crucial factor for carbon monoxide. Hence, entry and "migration" through the protein is rate-determining for O_2, as indicated by the rate constant of the bond formation reaction, but for CO these processes are only of minor importance. Bond cleavage, however, is the rate-determining step during the dissociation reaction for both ligands, and here the transition state for oxygen strongly resembles the product state, i.e. Mb + O_2. CO is however still tightly bound in the transition state. The negative volume of activation for Mb–CO bond *cleavage* corresponds to the positive volume of activation for bond *formation* between Mb and O_2, in that the sign of the activation volumes for both reactions is contrary to that generally expected. As outlined above, the reason for these effects is related to the bonding mechanism of both ligands, which at different locations of the reaction coordinate can result in different contributions from conformational changes.

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Contribution from the Department of Chemistry and Laboratory for Molecular Structure and Bonding, Texas A&M University, College Station, Texas 77843

New Di- and Trinuclear Complexes of Ruthenium with Octahedra Joined on Faces or Edges. 3.[1] New Trinuclear Compounds of the Type $[(R_3P)_2ClRuCl_3RuCl_3RuCl(PR_3)_2]^n$ (n = 0, +1): Structures, EPR Spectroscopy, Electrochemistry, and Molecular Orbitals

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We report here the preparation and characterization of four new compounds of the type $(R_3P)_2ClRuCl_3RuCl_3RuCl(PR_3)_2$ as well as a cationic oxidation product of one of them. The new compounds that have been crystallographically characterized are as follows: $Ru_3Cl_8(PEt_3)_4$ (**3**), monoclinic, space group $P2_1/n$, a = 11.041 (5) Å, b = 14.484 (5) Å, c = 12.939 (4) Å, β = 102.92 (3)°, V = 2106 (3) Å^3, Z = 2, Ru–Ru = 2.862 (1) Å; $Ru_3Cl_8(PMe_3)_4$ (**4a**), monoclinic, space group $P2_1/n$, a = 13.292 (3) Å, b = 7.128 (1) Å, c = 16.667 (5) Å, β = 109.16 (2)°, V = 1491.6 (6) Å^3, Z = 2, Ru–Ru = 2.828 (1) Å; $Ru_3Cl_8(PMe_3)_4 \cdot C_6H_6$ (**4b**), triclinic, space group $P\bar{1}$, a = 9.038 (4) Å, b = 13.253 (4) Å, c = 7.503 (2) Å, α = 101.31 (2)°, β = 100.00 (3)°, γ = 97.09 (3)°, V = 856.2 (5) Å^3, Z = 1, Ru–Ru = 2.842 (0) Å; $[Ru_3Cl_8(PEt_3)_4][SbF_6]$ (**5**), monoclinic, space group $P2_1/a$, a = 14.558 (5) Å, b = 31.81 (2) Å, c = 15.049 (6) Å, β = 93.95 (3)°, V = 6952 (9) Å^3, Z = 6, average Ru–Ru = 2.906 (3) Å. In addition, $Ru_3Cl_8(PMe_2Ph)_4$ (**2**) and the PMe_3 (**6**) and PBu_3 (**7**) homologues of **5** are reported. Together with the previously reported $Ru_3Cl_8(PBu_3)_4$ (**1**), this gives four such molecules, one (with PMe_3) known in two crystal forms. A detailed SCF–Xα–SW study of these species is reported and compared with previous results for $[Ru_3Cl_{12}]^{4-}$. The electrochemistry of all four of these molecules and of **5** as well as the EPR spectrum of **5** are presented and discussed. From the electrochemistry as well as by reactions with $AgSbF_6$, Cp_2Co, and Na/Hg, it has been shown that besides the neutral molecules the +1, −1, and −2 ions also exist, although only the first has so far been isolated and characterized as compound **5**.

Introduction

In 1980, the first trinuclear complex consisting of three octahedra sharing faces in a linear array was reported.[2] This was the $[Ru_3Cl_{12}]^{4-}$ ion, and its electronic structure was later investigated in detail by means of SCF–Xα–SW calculations.[3] The

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