Effect of Pressure on the Formation and Deoxygenation Kinetics of Oxymyoglobin. Mechanistic Information from a Volume Profile Analysis

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Abstract: The effect of pressure on the formation and deoxygenation kinetics of oxymyoglobin was studied by using temperature-jump and stopped-flow techniques, respectively. The corresponding volumes of activation are $+5.2 \pm 0.5$ and $+23.3 \pm 1.8$ cm^3 mol^{-1}, which result in a reaction volume of -18.1 ± 2.3 cm^3 mol^{-1} for the system Mb + O$_2$ $\rightleftharpoons$ MbO$_2$. The latter was also measured directly from the pressure dependence of the equilibrium constant and resulted in a reaction volume of -19.3 ± 1.5 cm^3 mol^{-1}. A volume profile analysis indicated that bond breakage during deoxygenation proceeds according to a dissociative mechanism with almost complete Mb–O$_2$ bond cleavage in the transition state. This suggests that almost no bond formation with the heme center occurs in the transition state during the oxygenation of myoglobin. The volume increase observed during this process is ascribed to hydrogen-bonding effects accompanied by desolvation as the oxygen molecule finds its way through the protein. The results are discussed in reference to related studies reported in the literature.

The application of high-pressure techniques in mechanistic studies of inorganic and organometallic systems has received increasing attention from kineticists in recent years.[2-5] The large volume of data now available enable a detailed interpretation for various types of reactions. Especially in the case of substitution processes,[2,6] these studies have contributed significantly toward a better understanding of the intimate mechanism.

Our own activities in this area have encouraged us to apply such techniques to systems of biochemical interest.[7,8] In general, we know that processes involving bond formation should be accelerated by the application of pressure in those cases where bond formation is not accompanied by major changes in electrostriction that could possibly counteract this effect. Rather surprisingly, Hasinoff[9] reported a *positive* volume of activation, i.e., a decrease in rate constant with increasing pressure, for the formation of oxymyoglobin (MbO$_2$), a process that involves bond formation. In contrast, he found a *negative* volume of activation for the formation of MbCO, which is in line with that expected for a bond-formation process. Recent interest[10-16] in the binding of O$_2$ and CO to hemoglobin and myoglobin has emphasized[16] the significant difference between the binding of these molecules on the basis of the high-pressure kinetic data.[9] In addition, several studies have now clearly indicated that the binding of O$_2$ and CO

involves different activation barriers and rate-determining steps.[17-19] These activities have encouraged us to reinvestigate the pressure dependence of the formation of MbO$_2$. In addition, we have also studied the pressure dependence of the reverse reaction, i.e., the release of O$_2$, as well as of the overall equilibrium constant. The activation and reaction volume data obtained in this way enable us to construct a volume profile and to comment on the intimate nature of the reaction mechanism.

Experimental Section

Materials. Unless otherwise stated, aqueous 0.005 M 2-amino-2-(hydroxymethyl)-1,3-propandiol buffer (Tris; Sigma Chemicals) was used in this study. The acidity was adjusted by adding hydrochloric acid (Titrisol Merck) to give a pH of 8.5 ± 0.1 and a conductivity of 240 ± 10 μS cm^{-1}.

The ionic strength of all solutions was adjusted prior to any measurement to 0.1 M with sodium chloride (Merck) unless otherwise stated.

Lyophilized sperm whale myoglobin, partly in the metmyoglobin form, was purchased from Sigma. The heme content of the protein was determined according to the method of De Duve.[20] A 3-mL aliquot of an alkaline pyridine solution [100 mL of pyridine (Merck), 30 mL of a 1 M sodium hydroxide solution (Titrisol; Merck), and water added to give a total volume of 300 mL] was mixed with 1 mL of a myoglobin solution (ca. 0.2%). After the resulting Fe(III) was reduced with a few crystals of sodium dithionite (Merck), the absorbance of the Fe(II)–heme–pyridine complex was measured at 557 (ϵ = 32 000 M^{-1} cm^{-1}) and 525 nm (ϵ = 16 000 M^{-1} cm^{-1}) and the iron content calculated (total iron content, 0.26%).

Myoglobin was purified by the following procedure:[21] 250 mg of the crude protein were dissolved in 6 mL of buffer and reduced with sodium dithionite, followed by gel filtration over equilibrated PD-10 columns (Pharmacia; prepacked with Sephadex G-25 medium). The resulting solution (8 mL) was loaded onto an equilibrated column packed with DEAE ion-exchange cellulose (Whatman DE-23). The initial main fractions having absorbance ratios A(582 nm)/A(544 nm) > 1.04[10,22] were collected and stored in a deep freezer at -25 °C until they were used as an oxymyoglobin stock solution for subsequent measurements.

Instrumentation. UV/vis measurements at ambient pressure were performed on a Shimadzu UV-250 or a Hitachi U-3200 dual-grating spectrophotometer. Spectra under high pressure were obtained from a

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

pressure, MPa	correction factor,[b] %		
	MbO$_2$[c]	Mb[d]	H$_2$O[e]
5			0.25
50	1.7 ± 0.2	1.2 ± 0.2	2.10
100	3.2 ± 0.2	2.2 ± 0.2	3.94
150	4.3 ± 0.2	3.3 ± 0.3	5.58

[a] Experimental conditions: $T = 25$ °C; [Tris buffer] = 5 mM; pH = 8.2–8.5; [total Mb] = (0.3–1.2) × 10^{-5} M. [b] Correction factor is expressed as $\Delta V/V$(at 0.1 MPa) (see Results and Discussion); mean value of between four and six measurements. [c] Measured for a completely oxygenated solution. [d] Measured for a completely deoxygenated solution. [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*; Springer Verlag: Berlin, 1980; New Series Group IV, Volume 4.

Zeiss DMR 10 spectrophotometer equipped with a high-pressure cell previously described in the literature.[23] Temperature-jump studies were conducted on a Messanlagen Studiengesellschaft Göttingen temperature-jump spectrometer equipped with a high-pressure cell.[24] Relaxation traces were recorded on a Nicolet 1090 A Explorer digital oscilloscope. Stopped-flow kinetics were carried out with a Dionex stopped-flow apparatus for reactions at ambient pressure and a high-pressure stopped-flow instrument described elsewhere.[25] Data handling and evaluation were performed either on an Apple IIe or on an Atari Mega ST4 computer using own customized software[26] and Lotus 1-2-3 spreadsheet capabilities. Conductivities were measured with a WTW LF91 conductometer. The pH values were determined on a Metrohm 632 pH meter equipped with a Sigma glass calomel electrode specially designed for use with Tris buffers.
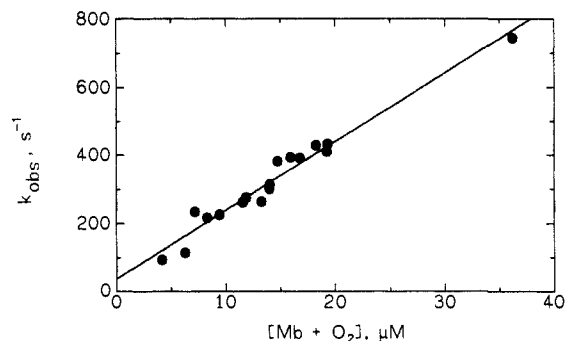
Procedures. A. Protein Concentrations. The myoglobin stock solution was diluted with appropriate amounts of buffer to give working solutions for subsequent investigations. Myoglobin levels were determined by using the absorbance peak maxima in the Soret region at 434 nm ($\epsilon = 115\,000$ M^{-1} cm^{-1}) for deoxymyoglobin and 418 nm ($\epsilon = 128\,000$ M^{-1} cm^{-1}) for oxymyoglobin.[27]

B. Equilibrium Studies. Oxymyoglobin solutions were deaerated in a Schlenk flask sealed with a septum, followed by gentle purging of the solution with argon for 20 min. After that, the spectrum showed the formation of the equilibrium myoglobin–oxymyoglobin mixture. By raising the argon pressure, the solution was directly pressed through a tube into the evacuated quartz high-pressure cuvette, which was sealed gastight with a septum.[28] Deoxymyoglobin solutions were prepared by adding a small excess of sodium dithionite to degassed oxymyoglobin solutions under air-free conditions.

C. Kinetic Measurements. Equilibrium solutions for temperature-jump (T-jump) measurements were prepared by the above mentioned method and transferred to the T-jump cell or the high-pressure T-jump cell in an oxygen-free, argon-flushed glovebag with a gastight Hamilton syringe. Dithionite solutions for stopped-flow kinetics were prepared by dissolving crystalline sodium dithionite in deaerated, argon-saturated buffer in a sealed Schlenk flask under an argon atmosphere. By increasing the Argon pressure, the solution was pressed directly into the gastight stopped-flow syringe (Hamilton). The other drive syringe was filled with an oxymyoglobin working solution.
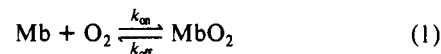
Results and Discussion

Preliminary Observations. The UV/vis absorption spectra of deoxymyoglobin (Mb) and oxymyoglobin (MbO$_2$) were investigated as a function of pH and pressure. The absorbance maxima are independent of pH at ambient pressure within the range (4.9–9.0) investigated. This is in agreement with similar findings reported in the literature[29,30] and indicates that there are no

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Figure 1. Plot of k_{obs} versus ([Mb]$_e$ + [O$_2$]$_e$) for the reaction Mb + O$_2$ → MbO$_2$. Conditions: $T = 20$ °C; $\Delta T \approx 3$ °C; [total Mb] = 1 × 10^{-5} M; pH = 7.0 (phosphate buffer).

acid–base equilibria involved in the investigated pH range. No significant shift in λ_{max} is observed for solutions of Mb on increasing the pressure to 200 MPa. In the case of MbO$_2$, a slight red shift from 417 to 419 nm is observed. The extinction coefficients do reveal a substantial pressure dependence when the absorption spectra of Mb and MbO$_2$ are recorded in an optical cell with a fixed optical path length. The observed changes are partly due to the compressibility of the solvent (water) and the pressure sensitivity of the Mb and MbO$_2$ components.[11,31-33] The latter may include structural changes within the protein usually referred to as pressure denaturation[8] and the formation of open and closed crevice structures.[34] We have expressed the observed absorbance increases in terms of a correction factor in Table I, i.e., the percentage with which the measured absorbance must be corrected to compensate for the compressibility of the solvent and the system. This correction factor is of fundamental importance in the determination of the equilibrium constant at elevated pressure (see further discussion). The correction factors for MbO$_2$ and Mb are significantly smaller than for pure water due to the effects referred to above. We also found very similar correction factors for water by measuring the pressure dependence of the absorbance of a methyl orange solution at pH = 9 and 465 nm.

Kinetic Measurements. The oxygenation of Mb, as indicated in eq 1, follows a relatively simple rate law as given in eq 2.

$$\text{Mb} + \text{O}_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{MbO}_2 \qquad (1)$$

$$-\text{d}[\text{Mb}]/\text{d}t = \text{d}[\text{MbO}_2]/\text{d}t = k_{on}[\text{Mb}][\text{O}_2] + k_{off}[\text{MbO}_2] \qquad (2)$$

Earlier studies have demonstrated that the oxygenation rate constant, k_{on}, can be determined by using T-jump and flash photolysis techniques, whereas the deoxygenation rate constant, k_{off}, can be determined directly by using stopped-flow techniques.[9,10]

Under the conditions of a T-jump experiment, i.e., a small deviation from the equilibrium situation, the relaxation rate constant can be expressed as in (3), where the subscript e refers

$$k_{obs} = \tau^{-1} = k_{on}([\text{Mb}]_e + [\text{O}_2]_e) + k_{off} \qquad (3)$$

to the equilibrium position prior to the temperature jump. The values of [Mb]$_e$ and [O$_2$]$_e$ were estimated in the following way: The absorption spectrum of an equilibrium mixture of MbO$_2$/Mb,O$_2$ was recorded inside the normal- and high-pressure T-jump cells. The degree of saturation, Y, is defined in eq 4, where [Mb]$_t$

$$Y = [\text{MbO}_2]/[\text{Mb}]_t \qquad (4)$$

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Table II. Summary of Rate Data for the Oxygenation of Mb as a Function of Concentration and Pressure[a]

pressure, MPa	$([Mb]_e + [O_2]_e)$, M	τ^{-1},[b] s^{-1}	k_{on}[c] $\times 10^{-7}$, M^{-1} s^{-1}	$\overline{k_{on}} \times 10^{-7}$, M^{-1} s^{-1}
5	7.84×10^{-6}	180	2.30	2.52 ± 0.22
	10.07×10^{-6}	247	2.45	
	11.15×10^{-6}	279	2.50	
	17.47×10^{-6}	495	2.83	
50	7.84×10^{-6}	160	2.04	2.11 ± 0.33
	10.07×10^{-6}	173	1.72	
	11.15×10^{-6}	241	2.16	
	17.47×10^{-6}	440	2.52	
100	7.84×10^{-6}	143	1.82	1.79 ± 0.21
	10.07×10^{-6}	156	1.55	
	11.15×10^{-6}	191	1.71	
	17.47×10^{-6}	359	2.06	
150	7.84×10^{-6}	124	1.58	1.58 ± 0.29
	10.07×10^{-6}	130	1.29	
	17.47×10^{-6}	327	1.87	
$\Delta V^{\ddagger}_{on}$, cm^3 mol^{-1}				$+8.0 \pm 0.6$

[a] $[Mb]_t = (0.8-1.2) \times 10^{-5}$ M; pH = 8.5; [Tris buffer] = 5 mM; T = 22 °C; $\Delta T \approx 3$ °C; ionic strength = 0.1 M. [b] Mean value of at least five kinetic runs. [c] Calculated from $\tau^{-1}/([Mb]_e + [O_2]_e)$; see Results and Discussion.

represents the total concentration of myoglobin, i.e., $[Mb]_t = [Mb] + [MbO_2]$. It can be determined from the recorded spectrum by using eq 5, where $A(Mb)$, $A(MbO_2)$, and A_e are the absor-

$$Y = [A(Mb) - A_e]/[A(Mb) - A(MbO_2)] \qquad (5)$$

bances of pure Mb, MbO$_2$, and the equilibrium mixture, respectively, at a particular wavelength (usually 418 nm). With these equations, the values of $[Mb]_e$ and $[MbO_2]_e$ can be calculated. Finally, the value of $[O_2]_e$, which cannot be measured directly inside the T-jump cells, was calculated from the overall equilibrium constant for reaction 1 given in eq 6. For the latter

$$K = [MbO_2]_e/([Mb]_e + [O_2]_e) = k_{on}/k_{off} \qquad (6)$$

calculation, an ambient pressure value of 1.1×10^6 M^{-1} was assumed for K, based on available literature data[27,35] and our own measurements (see further discussion).

It follows from eq 3 that a plot of τ^{-1} versus $([Mb]_e + [O_2]_e)$ should be linear, with intercept k_{off} and slope k_{on}. A typical example of such a plot for kinetic data at ambient pressure is presented in Figure 1, from which it follows that the slope can be determined fairly accurately, i.e., $k_{on} = (2.0 \pm 0.1) \times 10^7$ M^{-1} s^{-1}. Unfortunately, the scatter usually observed in such data does not allow an accurate estimation of the intercept, $k_{off} = 38 \pm 16$ s^{-1}, and a more direct method must be employed. The value of k_{on} is in good agreement with those reported in the literature for the oxygenation of horse and sperm whale myoglobin.[9,10,29,36-39] The intercept in Figure 1 is indeed small and k_{on} can thus be calculated directly from $\tau^{-1}/([Mb]_e + [O_2]_e)$.

The pressure dependence of k_{on} was studied at various $([Mb]_e + [O_2]_e)$ and some typical results are summarized in Table II. Plots similar to that shown in Figure 1 were found at all investigated pressures. The above assumption that k_{off} is small was made to estimate k_{on} as a function of pressure. In these calculations it was assumed that $([Mb]_e + [O_2]_e)$ is independent of pressure, i.e., the overall equilibrium constant in (6) was assumed to be independent of pressure.[9] A recalculation of the data following the determination of the pressure dependence of K (see forthcoming discussion) indicated that the corrected K values only slightly affected the values of $([Mb]_e + [O_2]_e)$ and, therefore, k_{on}, at elevated pressure. The so calculated value of $\Delta V^{\ddagger}_{on}$ is +5.2 $\pm$ 0.5 cm^3 mol^{-1} (see Figure 2) for $10^7 k_{on}$ values of 2.52 $\pm$ 0.22,
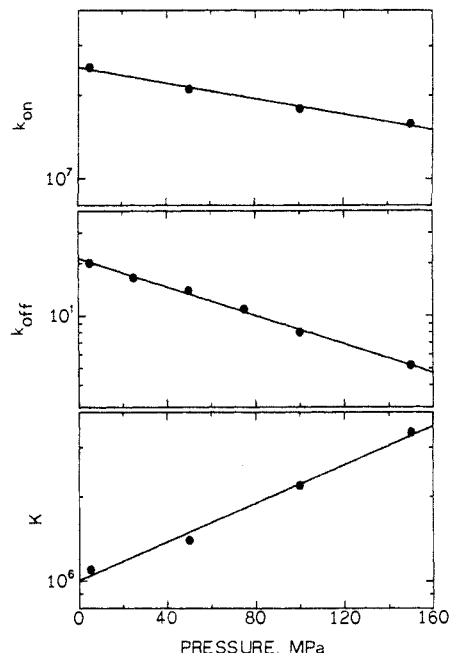
Figure 2. Logarithmic plot of k_{on}, k_{off}, and K versus pressure for the system Mb + O$_2$ (k_{on}) $\rightleftharpoons$ MbO$_2$ (k_{off}). Data taken from Tables III (corrected), V, and VI.

Table III. k_{off} as a Function of Temperature for the Reaction[a] MbO$_2$ → Mb + O$_2$

T, °C	k_{off},[b] s^{-1}
15.0	7.12 ± 0.06
20.0	12.3 ± 0.2
25.0	22.4 ± 0.3
30.0	36.0 ± 0.7
35.0	63.3 ± 2.0
40.0	98.5 ± 1.1
45.0	155 ± 6
$\Delta H^{\ddagger}$, kJ mol^{-1}	76.1 ± 0.9
$\Delta S^{\ddagger}$, J K^{-1} mol^{-1}	$+36 \pm 3$

[a] Experimental conditions: $[MbO_2]$ = 6 μM; ionic strength = 0.1 M (NaCl); $[Na_2S_2O_4]$ = 16.5 mM; pH = 8.5 (before mixing). [b] Mean value of at least six kinetic runs.

2.18 ± 0.31, 1.95 ± 0.20, and 1.82 ± 0.27 M^{-1} s^{-1} at 5, 50, 100, and 150 MPa, respectively. The obvious reason for the small correction lies in the selected experimental conditions, since $[O_2]_e \gg [Mb]_e$ so that a shift in the equilibrium position under pressure hardly affects the concentration expression. The plots of ln k_{on} versus pressure are linear within the experimental error limits, and the quoted volumes of activation are close to the value of +7.8 $\pm$ 1.3 cm^3 mol^{-1} reported by Hasinoff[9] using a flash photolysis technique. It follows that the T-jump and flash photolysis techniques reveal very similar $\Delta V^{\ddagger}_{on}$ values for the oxygenation of myoglobin.

The pressure dependence of the deoxygenation reaction was studied by use of a stopped-flow technique.[10] In this procedure dithionite was used to rapidly reduce oxygen, which results in a shift in equilibrium (1) to the left and the rate-determining step becomes the release of oxygen, i.e., k_{off}. The reaction was studied under an excess of dithionite to ensure that released oxygen is rapidly reduced and the observed rate law simplifies to $k_{obs} = k_{off}$. The observed rate constant was found to be independent of the dithionite (1–33 mM) and oxymyoglobin (0.5–12.5 μM) concentrations over the indicated ranges. In general, a large excess of Na$_2$S$_2$O$_4$ is required to ensure that the free oxygen in the test solution is rapidly reduced and does not affect the rate-determining deoxygenation step. Under optimized conditions, i.e., $[Na_2S_2O_4]$ = 0.0326 M, $[MbO_2]$ = 6 $\times$ 10^{-6} M, [Tris buffer] = 5 $\times$ 10^{-3} M, and T = 25 °C, the average value of k_{off} calculated from 36 kinetic runs was 21.8 $\pm$ 0.5 s^{-1}. This is in close agreement with

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Table IV. k_{off} as a Function of Pressure for the Reaction[a] $MbO_2 \rightarrow Mb + O_2$

	k_{off},[b] s⁻¹				
5 MPa	25 MPa	50 MPa	75 MPa	100 MPa	ΔV^*_{off}, cm³ mol⁻¹
18.7 ± 1.2	17.8 ± 2.3	13.8 ± 0.3	10.8 ± 1.0	7.7 ± 0.8	23.5 ± 2.4
19.2 ± 1.8	16.8 ± 0.9	14.1 ± 1.1	11.3 ± 1.4	8.6 ± 1.5	20.6 ± 1.3
18.7 ± 0.8	14.7 ± 0.5	13.4 ± 0.4	9.4 ± 1.4	7.1 ± 0.5	24.6 ± 2.2
20.5 ± 1.6	16.9 ± 1.1	15.0 ± 0.6	12.9 ± 0.6	8.9 ± 0.9	20.0 ± 2.5
21.0 ± 1.0	15.5 ± 1.0	13.4 ± 0.7	10.7 ± 0.1	7.9 ± 0.2	24.0 ± 1.8
22.1 ± 0.8	18.3 ± 0.6	14.8 ± 0.9	12.0 ± 1.1	7.5 ± 1.2	26.8 ± 2.9
20.5 ± 0.3	15.8 ± 1.4	13.2 ± 1.5	9.9 ± 0.9	8.2 ± 0.3	23.8 ± 1.2
20.2 ± 1.2	16.5 ± 1.2	13.4 ± 0.7	10.5 ± 1.1	8.0 ± 0.9	23.8 ± 0.6
mean value[c]					23.4 ± 2.2
mean value[d] 20.1 ± 1.2	16.5 ± 1.2	13.9 ± 0.7	10.9 ± 1.1	8.0 ± 0.6	23.3 ± 1.4

[a] Experimental conditions: T = 25 °C; [Tris buffer] = 5 mM; [MbO₂] = 7.7 μM; ionic strength = 0.1 M (NaCl); [Na₂S₂O₄] = 16.5 mM; pH = 8.5 (before mixing). [b] Mean value of between four and six kinetic runs for each quoted rate constant. [c] Mean value of ΔV^*_{off}. [d] Mean value of k_{off}.
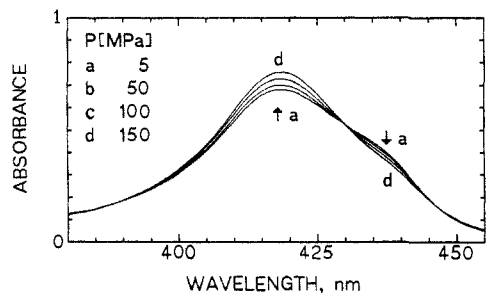

Figure 3. Effect of pressure on the absorbance spectrum of Mb/MbO₂. Conditions: [total Mb] = 0.6–1.5 μM; [Tris buffer] = 5 mM; T = 25 °C; pH = 8.0–8.5; optical path length = 1 cm; ionic strength = 0.1 M (NaCl).

the value of 23.6 ± 2.2 s⁻¹ reported by Armstrong and Sykes.[10] The temperature and pressure dependence of k_{off} was studied in the range 15–45 °C and 0.1–100 MPa, respectively. The rate constants obtained from two series of measurements for the temperature dependence and eight series of measurements for the pressure dependence are summarized in Tables III and IV, respectively. The corresponding plots for the determination of ΔH^*, ΔS^*, and ΔV^* are linear within the quoted error limits and their values are included in the tables. The average value of ΔV^*_{off} calculated from the individual series of ΔV^*_{off} values is in close agreement with that obtained from the average k_{off} values, such that ΔV^*_{off} = 23.3 ± 1.8 cm³ mol⁻¹ (see Figure 2). It follows that deoxygenation is accompanied by a significant increase in volume, which is in line with that expected for a bond-breakage process.

Equilibrium Measurements. The equilibrium constant K for eq 1 can be calculated from the equilibrium concentrations as demonstrated in eq 4–6 or from the ratio of the k_{on} and k_{off} values. The k_{on} value of (2.5 ± 0.2) × 10⁷ M⁻¹ s⁻¹ (Table II) and the k_{off} value of 21.8 ± 0.5 s⁻¹ result in a K value of (1.15 ± 0.12) × 10⁶ M⁻¹, which is in good agreement with the quoted literature values.[27,35] At elevated pressure, equilibrium 1 is shifted to the right, as illustrated by the increase in [MbO₂] as shown by the spectral changes in Figure 3. The change in equilibrium concentrations are related through eq 7. For this purpose approximately 20

$$\Delta[O_2] = \Delta[Mb] = -\Delta[MbO_2] \qquad (7)$$

absorbance measurements were recorded at and close to the absorption maximum of MbO₂ (i.e., 418 nm) and used to estimate the equilibrium position. The extinction coefficients used were 1.28 × 10⁵ and 7.65 × 10⁴ M⁻¹ cm⁻¹ for MbO₂ and Mb, respectively, at 418 nm. The experimental absorbance values were corrected for the compressibility of the system by using the values obtained for MbO₂ in Table I, since the equilibrium position is such that mainly MbO₂ is present under the employed conditions. In total, 12 series of measurements were performed, and the average values of K as a function of pressure are summarized in Table V along with the values estimated from the kinetic measurements. Plots of ln K versus pressure are linear within the experimental error limits (Figure 2) and the estimated reaction

Table V. Pressure Dependence of K for the Equilibrium[a] $Mb + O_2 \rightleftharpoons MbO_2$

	K^b × 10⁻⁶, M⁻¹			
5 MPa	50 MPa	100 MPa	150 MPa	ΔV, cm³ mol⁻¹
1.10	1.35	2.35	3.84	-22.1 ± 2.4
1.10	1.58	2.74	3.29	-19.6 ± 2.6
1.10	1.46	2.15	2.90	-16.9 ± 0.6
1.10	1.61	2.25		-18.6 ± 1.3
1.10	1.49	2.07	3.15	-17.8 ± 0.8
1.10	1.44	2.00	3.74	-20.5 ± 2.7
1.10	1.38	2.09	3.82	-21.3 ± 2.7
1.10	1.32	2.08	3.87	-21.7 ± 3.2
1.10	1.27	1.96	2.98	-17.6 ± 2.2
1.10	1.56	2.40	3.05	-17.8 ± 1.5
1.10	1.33	1.91	2.84	-16.5 ± 1.5
1.10	1.40	2.02	3.51	-19.7 ± 2.2
mean value[c]				-19.2 ± 1.9
mean value[d] 1.10	1.4 ± 0.1	2.2 ± 0.2	3.4 ± 0.4	-19.3 ± 1.2
K^e × 10⁻⁶, M⁻¹ 1.25	1.57	2.44	3.50[f]	-18.1 ± 1.2

[a] Experimental conditions: T = 25 °C; [Tris buffer] = 5 mM; pH = 8.5. [b] Spectrophotometrically determined values. [c] Mean value of ΔV. [d] Mean value of K. [e] Kinetically determined values, $K = k_{on}/k_{off}$. [f] Extrapolated value from lower pressure data.

volumes, ΔV, are included in Table V. The average value of ΔV determined from the individual values of ΔV for each of the 12 series of data is close to the value obtained from the average value of K. In addition, the thermodynamically determined values of K are in excellent agreement with those calculated from the values of k_{on} and k_{off} in Tables II and IV, respectively. ΔV can also be estimated from ΔV^*_{on} and ΔV^*_{off} according to eq 8 and has the

$$\Delta V = \Delta V^*_{on} - \Delta V^*_{off} \qquad (8)$$

value -18.1 ± 2.3 cm³ mol⁻¹ when the corrected value for ΔV^*_{on} (due to the pressure dependence of K) is employed. Our values for ΔV differ significantly from the value of -2.9 ± 0.2 cm³ mol⁻¹ reported by Hasinoff.[9] No details of how he estimated the value of K at elevated pressure are given, and we can therefore not account for the apparent discrepancy. The fact that our ΔV values, determined either spectrophotometrically or kinetically, are in such good agreement with each other underlines the validity of our measurements. By way of comparison, we estimated ΔV for the oxygenation of hemoglobin from the equilibrium constants reported in the literature[32] (10⁻⁴K = 3.86, 4.17, and 7.25 M⁻¹ at 0.1, 10, and 100 MPa) to be -14.2 cm³ mol⁻¹. It should be kept in mind that such spectrophotometric measurements at elevated pressure are usually subjected to large error limits since small spectral changes can result in large changes in K. Therefore, utmost precision is required during such measurements. In addition, complications may arise when different correction factors are used to correct the observed absorbance measurements for the compressibility of the solvent. We have in our calculations adopted the correction factors determined for the compressibility of MbO₂
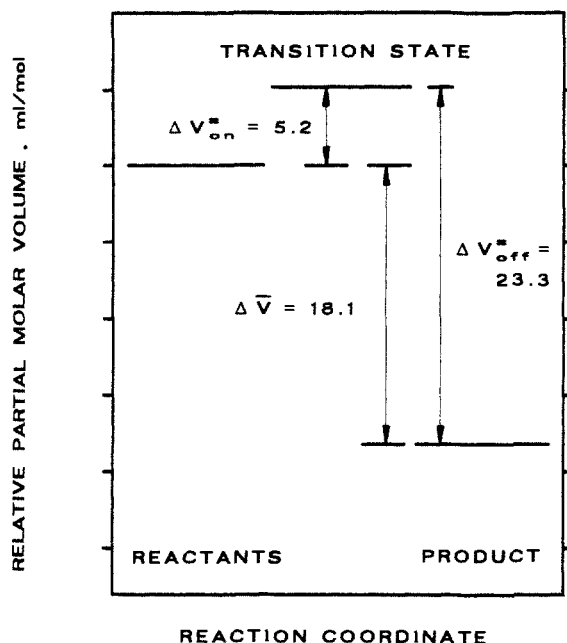
Figure 4. Volume profile for the reaction $Mb + O_2 \rightleftharpoons MbO_2$.

Table VI. Summary of Rate and Activation Parameters for the Reaction of Myoglobin with CO and O_2[a]

reaction		parameters	ref
$Mb + CO \rightarrow MbCO$	k	$= (3.8 \pm 0.2) \times 10^5 \ M^{-1} \ s^{-1}$	9
	$\Delta H^{\ddagger}$	$= 17.1 \ kJ \ mol^{-1}$	9
	$\Delta S^{\ddagger}$	$= -81.1 \ J \ K^{-1} \ mol^{-1}$	9
	$\Delta G^{\ddagger}_{298}$	$= 41.0 \ kJ \ mol^{-1}$	9
	$\Delta V^{\ddagger}$	$= -8.9 \pm 0.1 \ cm^3 \ mol^{-1}$	9
$Mb + O_2 \rightarrow MbO_2$	k	$= (1.3 \pm 0.3) \times 10^7 \ M^{-1} \ s^{-1}$	9
	k	$= (2.5 \pm 0.2) \times 10^7 \ M^{-1} \ s^{-1}$	b
	$\Delta H^{\ddagger}$	$= 23.0 \ kJ \ mol^{-1}$	9
	$\Delta S^{\ddagger}$	$= -30.0 \ J \ K^{-1} \ mol^{-1}$	9
	$\Delta G^{\ddagger}_{298}$	$= 32.2 \ k \ J \ mol^{-1}$	9
	$\Delta V^{\ddagger}$	$= +7.8 \pm 1.3 \ cm^3 \ mol^{-1}$	9
	$\Delta V^{\ddagger}$	$= +5.2 \pm 0.5 \ cm^3 \ mol^{-1}$	b
$MbO_2 \rightarrow Mb + O_2$	k	$= 21.8 \pm 0.5 \ s^{-1}$	b
	k	$= 23.6 \pm 2.2 \ s^{-1}$	10
	$\Delta H^{\ddagger}$	$= 76.1 \pm 0.9 \ kJ \ mol^{-1}$	b
	$\Delta S^{\ddagger}$	$= +36 \pm 3 \ J \ K^{-1} \ mol^{-1}$	b
	$\Delta G^{\ddagger}_{298}$	$= 65.4 \pm 1.8 \ kJ \ mol^{-1}$	b
	$\Delta V^{\ddagger}$	$= 23.3 \pm 1.8 \ cm^3 \ mol^{-1}$	b
$Mb + O_2 \rightleftharpoons MbO_2$	K	$= (0.92 \pm 0.07) \times 10^6 \ M^{-1}$	b
	ΔH°	$= -43 \ kJ \ mol^{-1}$	c
	ΔS°	$= -66 \ J \ K^{-1} \ mol^{-1}$	c
	ΔG°_{298}	$= -33 \ kJ \ mol^{-1}$	c
	$\Delta \bar{V}$	$= -19.3 \pm 1.5 \ cm^3 \ mol^{-1}$	b

[a] Data at 25 °C. [b] This work. [c] Extrapolated from kinetic data.

(Table 1). These values not only include the compressibility of the solvent but also the pressure dependence of the Mb and MbO_2 molecules in terms of induced denaturation and other possible structural changes. Although these effects seem to be minor according to the values in Table II, calculations demonstrated that they can markedly affect the values of K and the corresponding value of $\Delta \bar{V}$. Again these complications do not affect the kinetically determined values.

At this point it is appropriate to comment on the pressure sensitivity of Mb, MbO_2, and proteins in general. It is well established[7,8] that proteins undergo a pressure-induced denaturation, i.e., they undergo changes similar to that observed at high temperatures or in the presence of particular chemicals. Various effects have been suggested to account for the dissociation of protein aggregates.[8] The absorption spectrum of Mb at 600 MPa[33] is very similar to that reported[13] for the denaturation of Mb at low pH, which involves bond breakage with the proximal histidine (F8). Similarly, the observed spectral changes for MbO_2 can also be interpreted in terms of denaturation.[33] Another important observation is that the compressibility of proteins is extremely low (between 5 and 15 Mbar^{-1}).[7] A typical value for Mb is 9 Mbar^{-1},[40] which is significantly smaller than for liquid water. It is therefore concluded that the main pressure effects observed up to 200 MPa are due to small localized conformational changes in the protein. In this respect the possibility of open and closed crevice structures has been considered.[33]

Volume Profile and Mechanistic Interpretation. A volume profile for the reaction in (1) is presented in Figure 4, from which it follows that the partial molar volume of the transition state is significantly higher than either the reactant or product states. The significantly more positive $\Delta V^{\ddagger}_{off}$ value compared to $\Delta V^{\ddagger}_{on}$ does underline the importance of bond breakage during the deoxygenation reaction. This trend is also observed in the values of $\Delta S^{\ddagger}$, where $\Delta S^{\ddagger}_{off}$ is significantly more positive than $\Delta S^{\ddagger}_{on}$, as summarized in Table VI. The oxygenation and deoxygenation reactions also exhibit significantly different activation enthalpies and free energies, indicating that oxygenation is the kinetically favored reaction step. This also accounts for the negative values of ΔH° and ΔG° for the overall equilibrium constant.

The magnitude of $\Delta V^{\ddagger}_{off}$ reported in this study is in close agreement with similar values reported for bond breakage involving the release of a neutral molecule. For instance $\Delta V^{\ddagger}$ for the dissociation of CO has the following values for the quoted systems: $HRu_3(CO)_{11}^-$, $+21.2 \pm 1.4$;[41] $Ru_3(CO)_{10}(CO_2CH_3)P(OCH_3)_3^-$,

$+24.5 \pm 2.0$;[41] $Mn(CO)_5Cl$, $+20.6 \pm 0.4$[42] $cm^3 \ mol^{-1}$. Similarly, a $\Delta V^{\ddagger}$ of $+20 \pm 2 \ cm^3 \ mol^{-1}$ was recently reported for the release of H_2 from $H_3Ru_3(\mu_3\text{-}COCH_3)(CO)_9$.[43] In view of the comparable partial molar volumes of CO ($35 \ cm^3 \ mol^{-1}$), H_2 ($29 \ cm^3 \ mol^{-1}$) and O_2 ($28 \ cm^3 \ mol^{-1}$), determined from the density of the liquid gases at their boiling points, the value of $\Delta V^{\ddagger}_{off}$ is in close agreement with these data. It follows that deoxygenation can be visualized as a limiting dissociative mechanism, such that the $Mb-O_2$ bond is almost completely broken in the transition state.

In terms of the partial molar volume of O_2 and the overall reaction volume, it follows from eq 9 that $\bar{V}(MbO_2) - \bar{V}(Mb) \approx$

$$\Delta \bar{V} = \bar{V}(MbO_2) - \bar{V}(Mb) - \bar{V}(O_2) \qquad (9)$$

9 $cm^3 \ mol^{-1}$, i.e., MbO_2 is only slightly larger than Mb and the oxygen molecule is almost completely taken up by the myoglobin molecule on a volume basis. In this respect we must keep in mind that $\bar{V}(MbO_2)$ and $\bar{V}(Mb)$ will be very large numbers since we are dealing with large molecules,[40] such that the mentioned volume difference may result from only a minor structural change.

The positive value found for $\Delta V^{\ddagger}_{on}$ does not fit our general conceptions of a bond-formation process. The fact that $\Delta V^{\ddagger}_{on}$ approximately equals the volume difference $\bar{V}(MbO_2) - \bar{V}(Mb)$ indicates that the total volume increase on the myoglobin molecule occurs during the activation process. In this way, the volume collapse on going from the transition state to the product state equals the partial molar volume of O_2. This means that O_2 is only weakly bonded in the transition state in terms of bond formation (oxygenation) and almost not bonded anymore in the transition state in terms of bond breakage (deoxygenation). The question remains to account for the volume increase on going from the reactant to the transition state. In this respect we must emphasize the completely different result observed for the binding of CO, for which $\Delta V^{\ddagger}_{on} = -8.9 \pm 0.1 \ cm^3 \ mol^{-1}$, i.e., significant bond formation is present in the transition state, or less structural modifications occur during the binding of CO.

Various authors have pointed at the fundamental difference between the binding of CO and O_2 on a heme unit.[7,9,12,14,16-19,33] It is generally accepted that the overall binding process involves multiple barriers for both ligands. In the case of CO, the ligand rapidly equilibrates with the distal pocket and Fe–CO bond

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22 *J. Am. Chem. Soc., Vol. 112, No. 1, 1990*

Projahn et al.

formation is rate determining. In contrast, the rate-determining step for the binding of O_2 involves the movement through the protein to the iron center. From the available structural data on Mb, MbO_2, and MbCO, some additional important conclusions can be drawn. The Fe(II) atom is displaced 42, 18, and 10 pm from the porphyrin plane toward the proximal histidine (F8) for Mb, MbO_2, and MbCO, respectively.[44-47] This means that a substantial change in this displacement occurs during oxygenation and carbonylation of Mb. Furthermore, it is generally accepted[7,45,48,49] that O_2 is bound with an angle of $115°$, whereas CO is bound almost perpendicular to the porphyrin plane. In addition, neutron diffraction data have indicated that the oxygen molecule in MbO_2 is hydrogen bonded to the distal histidine (E7),[46] whereas no evidence for such hydrogen bonding could be found in the case of MbCO.[47,50] It has been shown from IR data that hydrogen bonding does occur in the latter case at low pH.[12] The deuterium isotope effect reported for the oxygenation of Mb and deoxygenation of MbO_2[10] is also in line with the hydrogen bonding of O_2 to the distal histidine. It is also interesting to note that no significant pH dependence was reported for the oxygenation of Mb,[14,51] whereas a significant acceleration at lower pH was found for the carbonylation reaction.[13]

Another important factor concerns the electronic structure and spin state of the complexes. During the binding of O_2 and CO to Mb, the paramagnetic five-coordinate high-spin Fe(II) species is converted to a diamagnetic six-coordinate low-spin Fe(II) complex.[7,14,16,52] Such a high-spin to low-spin conversion is accompanied by a significant decrease in volume of between 5 and 15 cm^3 mol^{-1}.[7,52-54] It is understandable that the smaller low-spin Fe(II) center can fit better into the porphyrin pocket and thus move more into the plane. Thus, it follows that the spin change during the binding of O_2 and CO to Mb should contribute a decrease in volume in both cases. However, a more detailed analysis of the spin states in MbO_2 and MbCO, taking into account that O_2 is in the triplet state and CO in a singlet, suggests a net spin change of 1 for the formation of MbO_2 compared to a net spin change of 2 for the formation of MbCO.[16] This would require a larger decrease in volume in the latter case and a correspondingly more negative volume of activation, which is in agreement with the experimental results. However, in this way we still cannot account for the overall positive volume of activation for the binding of O_2 to Mb.

It has been suggested[7,9] that the difference in ΔV^* for the binding of CO and O_2 results from a difference in the binding geometries of these ligands i.e., the way they fit into the heme

pocket. These must be significantly different, especially in terms of the hydrogen bonding with the distal histidine mentioned above. In this respect it is important to note that the manner in which CO and O_2 find their way through the protein to the heme iron differs substantially.[19] During oxygenation, hydrogen bond formation with the distal histidine plays an important role during the activation process. Such bond formation may be accompanied by significant desolvation and a corresponding increase in volume. This step is then followed by rapid bond formation with the Fe(II) center, during which the change in spin and the movement of the Fe(II) center into the porphyrin plane occur. Magde and co-workers[55] recently reported evidence for two very different configurations of the geminate reactant pair: a fast-reacting form, in which the ligand (O_2) remains within a few angstroms of the iron atom, and a slow-reacting form, in which the ligand wanders throughout the pocket. These findings are well in line with the above-outlined arguments in favor of a hydrogen bond formation process. In this way we can account for the positive volume of activation (due to desolvation) and the completely different behavior from that observed for the binding of CO. Furthermore, this is also in line with our suggestion, based on the reported volume profile, that the volume increase occurs prior to the binding of O_2 to the Fe(II) center. In the case of carbonylation, CO directly binds to the Fe(II) center such that bond formation accompanied by the change in spin state and movement of the metal atom into the porphyrin plane account for the negative volume of activation. The large positive volume of activation reported for the deoxygenation process must in a similar way reveal information on the way the dioxygen ligand dissociates from the metal center and escapes from the heme pocket. Such data could be of fundamental meaning for molecular dynamics calculations dealing with such processes.[56]

We conclude that the differences observed in the activation parameters (especially ΔV^*) for the binding of O_2 and CO to Mb result from different activation barriers for these ligands to reach the heme iron.[17-19] In the case of O_2, movement up the heme pocket is rate determining, whereas in the case of CO, this ligand rapidly equilibrates with the heme pocket followed by rate-determining Fe–CO bond formation. This study has provided detailed insight into the overall volume profile for the oxygenation process and has revealed the very significant volume increase during the deoxygenation process. To throw further light on the mechanistic differences for the binding of CO and O_2 to Mb it will be necessary to construct a volume profile for the carbonylation process. i.e., ΔV^*_{off} and ΔV must be measured. In addition, an extension to other biological oxygen carriers such as hemerythrin and hemocyanine, and various model complexes, may also improve our understanding of such processes. Investigations along these lines are presently underway in our laboratories.

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