Articles

Formation and Deoxygenation Kinetics of Oxyhemerythrin and Oxyhemocyanin. A Pressure Dependence Study

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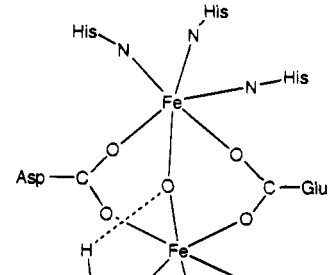
The kinetics of the formation and deoxygenation of oxyhemerythrin and oxyhemocyanin was investigated by *T*-jump and stopped-flow methods as a function of pressure. For hemerythrin the activation volumes $\Delta V^{\ddagger}_{on} = +13.3 \pm 1.1$ cm^3 mol^{-1} and $\Delta V^{\ddagger}_{off} = +52.2 \pm 0.7$ cm^3 mol^{-1} were obtained. These values give a reaction volume $\Delta \bar{V} = -39 \pm 2$ cm^3 mol^{-1} for the hemerythrin/dioxygen system. With these volume data a volume profile was constructed. A comparison with the volume profile for the same reaction of myoglobin shows their similarity but with the difference that the volume data for hemerythrin are twice as large as for myoglobin. In contrast hemocyanin did not show a pressure-dependent uptake of dioxygen. The pressure dependence of the deoxygenation of oxyhemocyanin could not be measured. The reaction mechanisms for the formation and deoxygenation of the oxygen carrier proteins are discussed in a comparative way.

Introduction

In addition to the biochemical interests in understanding the mechanisms of the reversible dioxygen uptake by the oxygen carrier proteins, there is interest in these reactions because of the goal to synthesize small transition metal complexes for the transport and activation of dioxygen.[2-5] Such model complexes can also be used to clarify the nature of the active site in the proteins and to elucidate the structure−reactivity relationship in terms of the coordination chemistry involved. Three kinds of oxygen carrier proteins are known, which are based on an iron porphyrin system (myoglobin, Mb, or its tetramer hemoglobin), a binuclear iron system (hemerythrin, Hr), and a binuclear copper system (hemocyanin, Hc) as the active site. The M_r values for the monomer units of these proteins are 16 kDa (Mb), 13.8 kDa (Hr), and 78 kDa (arthropod Hc).

In contrast to the large number of biological systems in which myoglobin (or its tetramer hemoglobin) and hemocyanin are used as oxygen carriers, the respiratory protein hemerythrin is comparatively rare and found in only a few marine phyla.[6] This protein is found as an octamer (molecular weight 108 kDa) in *Themiste zostericola* consisting of eight almost identical subunits (molecular weight 13.5 kDa).[7-9] In the retractor muscle of *T. zostericola* it is found as a monomer (myohemerythrin, molecular weight 13.9 kDa). The subunits in general do not exhibit any cooperativity, in contrast to that found in hemoglobin, although some difference in the rate and equilibrium constants for the oxygenation and deoxygenation of the octamer and monomer have been reported.[8,10] It has been known for almost 3 decades that each monomer consists of a binuclear iron center, capable of binding one oxygen molecule.[11] More recently, studies involving the application of X-ray diffraction, EXAFS and Raman techniques have yielded information on the structure of the active center of HrO_2 as shown in **1**.[3,5,6,9,12,13] According



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to these results, oxygen binds in an unique manner as a hydroperoxo ligand to one of the iron atoms of the binuclear Fe(III) active site, which can be formally described as a met-peroxo species.[9] It follows that protonation and H-bonding must be responsible for this unique binding mode. Detailed studies of deoxyHr have indicated that the two Fe(II) centers are

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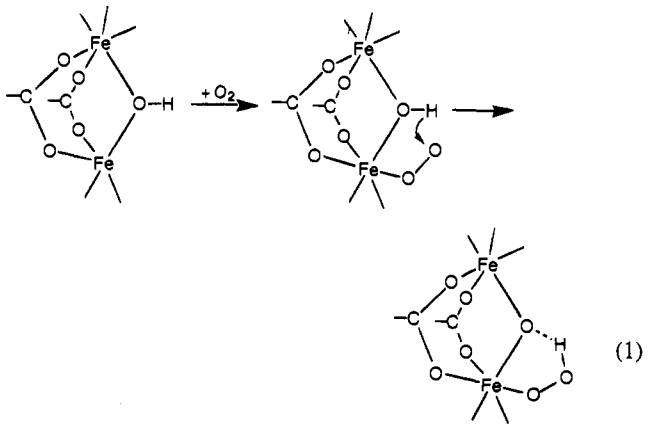
hydroxo bridged, and that one iron atom is pentacoordinated with an unoccupied coordination position.[3,6,12,13] The structural data available for the oxygenation of hemerythrin suggest the overall reaction mechanism as is outlined in eq 1. Hemocyanins



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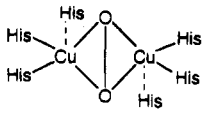
are large multisubunit proteins (molecular weight 450–9000 kDa) found freely dissolved in the blood of many invertebrates.[14,15] They contain different subunits which generally exhibit cooperativity.[14,15] For the hemocyanin of the arthropod *Panulirus interruptus* (spiny lobster) three nonidentical subunits (*a*, *b*, and *c*) were identified.[16] From spectroscopic investigations and the recent X-ray analysis of the oxyhemocyanin of *Limulus polyphemus* (horseshoe crab) the active site can be described by a binuclear copper(II) complex, the copper ions being coordinated to six histidines and bridged by peroxide as shown in **2**.[17–19] The X-ray crystal structure has been reported



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for the deoxy form of *P. interruptus*.[20] The overall reaction for the reversible uptake of dioxygen can be formulated as in eq 2.[14,15,21]

$$Hc + O_2 \rightleftharpoons HcO_2 \qquad (2)$$

In recent years, various groups have demonstrated very successfully that investigations at elevated pressure can reveal valuable information on thermodynamic and kinetic parameters. In this way pressure has become a diagnostic parameter in the elucidation of reaction mechanisms.[22–27] It can in many cases supply additional information and in some cases unique and crucial information to assist in the assignment of the intimate reaction mechanism. We have in the past, in collaboration with others,[28–30] investigated the effect of pressure on the reaction of myoglobin and model compounds with small molecules such as O_2, CO, and isocyanides, in an effort to contribute to the understanding of the underlying reaction mechanisms. In this respect it is very interesting to note that the volume profile for the oxygenation of myoglobin is rather exceptional since a positive volume of activation was reported for the formation of oxymyoglobin, i.e. a bond formation process that is accompanied by a volume increase instead of the usually expected volume decrease.[31,32] This observation was interpreted in terms of conformational changes in the protein structure prior to actual bond formation.[28] Hydrogen bonding between the coordinated oxygen and the distal Histidine E7 was also suggested to play an important role in this respect.[30]

During oxygenation of hemerythrin, H-bonding is also important for the stabilization of the product as illustrated in **1** and eq 1.[33] The X-ray crystal structures reported for oxy- and deooxyhemerythrins have shown that the Fe–Fe distance slightly decreases from 3.32 to 3.27 Å after uptake of dioxygen.[13] For hemocyanin, H-bonding is not important for the stabilization of the bound dioxygen.[34] However, the change in the oxidation state of the copper ions during the uptake or loss of dioxygen leads to significant changes in the geometry of the active site,[14] which should be quite sensitive to the variation of pressure. Recent work on a model Cu(I) dimeric complex clearly demonstrated the pressure sensitivity on the binding of oxygen.[35] Analysis of the pressure dependence is therefore expected to throw more light on the nature of the intimate mechanism and allow a detailed comparison of the myglobin, hemerythrin, and hemocyanin systems. The results obtained in this study are discussed with reference to all the available kinetic and structural information on hemerythrin and hemocyanin.

Experimental Section

Materials. Analytical reagent grade chemicals were used throughout. An aqueous solution of 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris, Sigma) was used as a buffer, since the pH value of Tris buffers is nearly pressure independent.[36] It is known, that several anions and cations influence the kinetics of hemerythrin.[10] Therefore all buffers were adjusted to the pH of the protein samples (8.5) and the ionic strength was adjusted to $I = 0.10$ M with sodium sulfate. The sulfate ion apparently does not react with the protein.[10] For hemocyanin the ionic strength was adjusted to $I = 0.10$ M with sodium chloride.

The batch of worms *T. zostericola* and hemolymph of *P. interruptus* were obtained from Pacific Biomarine Supply, Venice, California. The purified oxyhemerythrin solutions (Octamer) and oxyhemocyanin

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subunit *a* and subunit mixture of *b* and *c* were prepared at the Department of Chemistry, University of Newcastle Upon Tyne, England. The purification processes are described in the literature.[16,37] The stock solutions of oxyhemerythrin of concentration 8.8×10^{-4} M were also adjusted to pH 8.5 and to an ionic strength of 0.10 M (sodium sulfate). Samples were stored at −25 °C until they were required for experiments. Oxyhemerythrin solutions were obtained by dilution of stock samples with an appropriate amount of Tris buffer. Protein concentrations were determined by measuring the absorbance of the HrO_2 peak at 500 nm ($\epsilon = 2200$ M^{-1} cm^{-1}). All protein concentrations were expressed in terms of the monomeric unit, i.e. one binuclear iron site (molar mass 13.5 kDa).[10,34] Oxyhemocyanin samples of concentration 2.3×10^{-4} M were also stored at −25 °C until they were used for experiments. Solutions for the *T*-jump experiments were obtained by dilution and contained 3×10^{-5} M hemocyanin, 50 mM Tris buffer, and 5 mM edta, with the pH adjusted to 8.7 and an ionic strength of 0.10 M (NaCl). Protein concentrations of HcO_2 were determined by measuring the absorbance at 337 nm ($\epsilon = 1.8 \times 10^4$ M^{-1} cm^{-1}).[38]

Instruments. UV−vis measurements were conducted on either a Shimadzu UV-2100 spectrophotometer, a dual-grating Hitachi U-3200 spectrophotometer, or a HP 8452 diode-array spectrophotometer. Spectra of samples under high pressure were recorded on a Zeiss DMR 10 spectrophotometer equipped with a high pressure cell previously described in the literature.[39] Temperature-jump studies were conducted on a Messanlagen Studiengesellschaft Göttingen temperature-jump spectrometer equipped with a high-pressure cell.[40] Traces were recorded on a digital storage oscilloscope Nicolet 1090 A Explorer (2 MHz). In addition, the temperature jump apparatus was interfaced to a 486 PC equipped with a transient recorder card[41] (Dr. Strauss Systemelektronik, Gundelsheim, Germany). Data were evaluated further by a kinetic software package (KINFIT routines, On-line Instrument Systems, Bogart, GA). Stopped-flow experiments at ambient pressure were performed on a Dionex system. High-pressure-stopped-flow kinetic measurements were performed on a high-pressure stopped-flow unit described elsewhere.[42] The pH values were determined on a WTW pH-meter 535 equipped with a Sigma glass electrode specially designed for use with Tris buffers.

Procedures. A. Temperature Jump. For preparation of equilibrium solutions 4 mL of an oxyhemerythrin or oxyhemocyanin working solution were transferred to a glass finger and the oxygen content monitored via an oxygen sensitive electrode. The glass finger was airtight even when the oxygen electrode is in place. Argon may be bubbled over the solution via cannules. When the desired oxygen concentration was reached, the solution was directly pressed through a tube into the evacuated quartz-window sample cell. This cell is also under an inert atmosphere and may be closed without oxygen access. It was then placed into the high-pressure cell of the *T*-jump instrument.

B. Stopped-Flow. Dithionite solutions for stopped-flow kinetics were prepared[28] by dissolving crystalline sodium dithionite in deaerated, argon-saturated buffer. The buffer solution was degassed by at least five freeze−pump−thaw cycles. As the argon pressure, was increased the solution was pressed directly into the gastight stopped-flow syringe. The other drive syringe was filled with an oxyhemerythrin working solution.

Results and Discussion

Preliminary Investigations. Based on our earlier experience obtained with the oxygenation and carbonylation reactions of myoglobin,[28−30] some preliminary investigations were performed with hemerythrin. Deoxyhemerythrin exhibits no characteristic bands in the UV−vis region, whereas oxyHr exhibits a characteristic absorption at 500 nm ($\epsilon = 2200$ cm^{-1} M^{-1}).[8] Variations in the spectrum of oxyhemerythrin were studied as a function of pH and pressure. In the pH range 5.8 to 9.0 no variation of the characteristic bands was observed, which is in agreement with that reported elsewhere [8,10,43−45] and indicates that no acid−base equilibria in the vicinity of the active site occurs in this pH range. A pillbox cuvette[46,47] was employed to study the pressure dependence of the oxyhemerythrin bands. The latter are rather broad bands and do not exhibit a significant shift for pressures up to 150 MPa. A small decrease in the absorption coefficient of ca. 1% was observed at the highest employed pressure, which could as in the case of oxymyoglobin[48−51] indicate small structural changes in the protein under such conditions. Such effects have been discussed in terms of pressure-induced denaturation in the literature.[52] Spectrophotometric changes observed for the effect of pressure on an equilibrated solution of deoxy- and oxyhemerythrin were too small to determine the pressure dependence of the oxygenation equilibrium constant, since the absorption coefficient of the HrO_2 band is only 2200 M^{-1} cm^{-1} and results in very minor absorbance changes. However, our earlier studies[28,30] demonstrated that reliable data can be obtained for the equilibrium constant from kinetic data for the oxygenation and deoxygenation reactions as a function of pressure. Samples of oxyhemocyanin showed an irreversible absorption decrease at 337 nm of about 5% during 1 h at a pressure of 140 MPa.

***T*-Jump Measurements.** It has been shown in earlier work that the oxygenation kinetics of both the hemerythrin octamer and monomer can be described by a single step process (eq 3),[10] for which the kinetic and thermodynamic parameters differ

$$Hr + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} HrO_2 \qquad (3)$$

slightly for the two forms. The rate law for this scheme is given in eq 4, and the expression for the observed first order rate

$$-d[Hr]/dt = d[HrO_2]/dt = k_{on}[Hr][O_2] + k_{off}[HrO_2] \qquad (4)$$

constant,[31,34] determined with a *T*-jump relaxation method, is given in eq 5, where the subscript e refers to equilibrium

$$k_{obs} = \tau^{-1} = k_{on}([Hr]_e + [O_2]_e) + k_{off} \qquad (5)$$

concentrations prior to the temperature jump.[53,54] Some typical kinetic traces as a function of pressure are shown in Figure 1. In these traces the solid lines represent the values calculated on the basis of a first-order fit with deviations as indicated obtained directly from the experimental data. The kinetic data are summarized as a function of selected concentrations and pressure in Table 1. The equilibrium concentrations were calculated from

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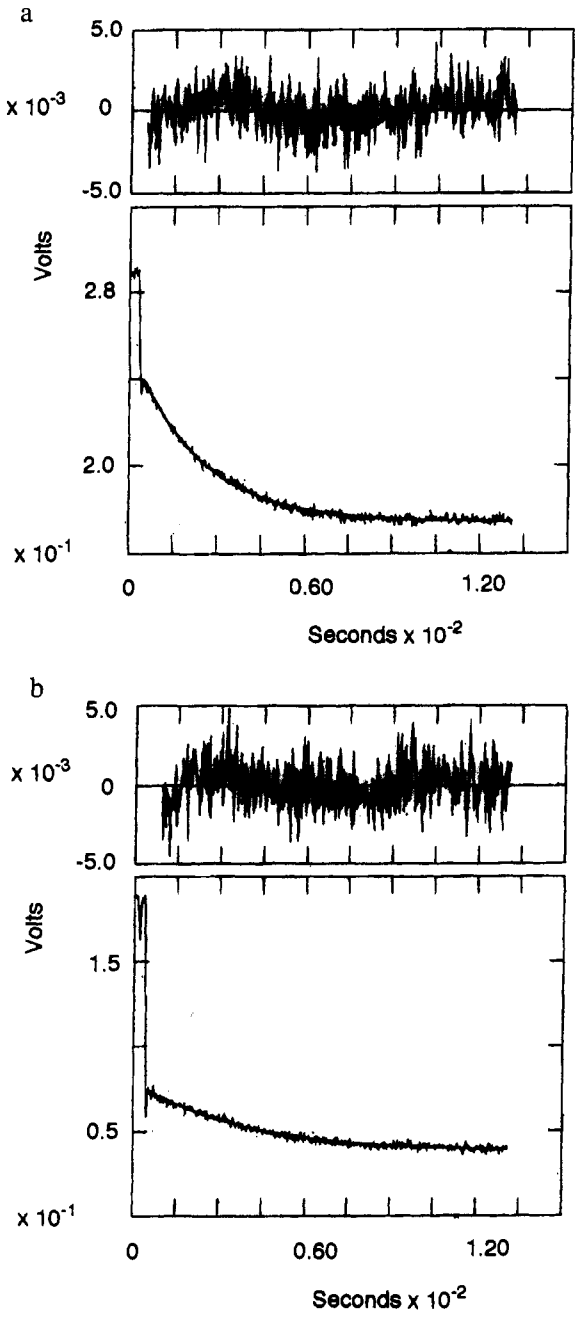

Figure 1. Plots of temperature jump voltage signal vs time and the appropiate residuals plots for reaction 3: (a) 5 MPa; (b) 150 MPa. Experimental conditions see Table 1.

eq 6, where $[Hr]_{Tot}$ and $[O_2]$ were measured directly and K was

$$K = \frac{[HrO_2]}{[Hr][O_2]} = \frac{[Hr]_{Tot} - [Hr]}{[Hr][O_2]} = k_{on}/k_{off} \qquad (6)$$

assumed to be independent of pressure and equal to 9×10^4 M^{-1} according to literature values.[10,34] The data were plotted according to eq 5 as a function of pressure as shown in Figure 2, from which it follows that k_{on} can be determined rather accurately in this way. However, the intercepts of these plots exhibit too large error limits for an accurate determination of k_{off}. The experimental value of k_{on} (included in Table 1) at 5 MPa is in close agreement with the value of $(7.5 \pm 0.7) \times 10^6$ M^{-1} s^{-1} reported for this reaction at atmospheric pressure.[10] Similar values were also reported for hemerythrin originating from *Phascolopsis gouldii*, viz. 1.2×10^7 and 7.4×10^6 M^{-1} s^{-1}.[34] Due to the small intercepts in Figure 2, k_{on} can also be calculated directly from $\tau^{-1}/([Hr]_e + [O_2]_e)$, and the agreement between the k_{on} values is rather good (see Table 1). A similar procedure for the analysis of the data was adopted in our earlier study of the oxygenation of myoglobin.[28] The relationship between $\Delta V^{\ddagger}$ and the rate constant is given by $\Delta V^{\ddagger} = -RT\{\delta(\ln k)/\delta p\}$ and $\Delta V^{\ddagger}$ is therefore obtained from the slope of a plot of ln k vs p. The pressure dependence of k_{on} can be clearly seen from the data in Table 1. The decrease in k_{on} with increasing pressure results in a $\Delta V^{\ddagger}_{on}$ value of $+13.8 \pm 0.8$ cm^3 mol^{-1} when calculated from the average k_{on} values at each pressure. Alternatively, a value of $+14 \pm 2$ cm^3 mol^{-1} is obtained from the slopes of the lines in Figure 2. A recalculation of the equilibrium concentrations taking into account the pressure dependence of K (see further discussion) resulted in only minor changes in $([Hr]_e + [O_2]_e)$, and therefore the k_{on} values were changed by only a small amount. A value of $\Delta V^{\ddagger}_{on} = +13.3 \pm 1.1$ cm^3 mol^{-1} was estimated from $k_{on} = 7.6 \pm 0.3$, 6.4 ± 0.4, 4.9 ± 0.4, and 3.5 ± 0.5 M^{-1} s^{-1} at 25 °C and 5, 50, 100, and 150 MPa, respectively, using the pressure dependent K values. The small effect obviously results from the selected conditions, since when $[O_2] \gg [Hr]$ the equilibrium can only shift to a small extent under pressure.

The oxygenation kinetics of hemocyanin (**2**) can be described by the same rate law (eq 4) and expression for the first order rate constant (eq 5) as for hemerythrin by substituting Hr by Hc. In contrast to hemerythrin or myoglobin we found that the measured rate constants k_{on} were independent of pressure (k_{on} at 5 and 140 MPa $= 5 \times 10^7$ M^{-1} s^{-1}) for hemocyanin subunit *a* with the result that $\Delta V^{\ddagger}_{on}$ is close to zero. Subunit mixture *b/c* showed a slight pressure dependence, rate constants are slightly larger at 140 MPa as compared to 5 MPa, leading to a negative but close to zero $\Delta V^{\ddagger}_{on}$ value. Unfortunately, the effect is very small and data were difficult to reproduce, mainly because of the slow denaturation of the protein at high pressure as mentioned above.

Stopped-Flow Measurements. The deoxygenation of oxyhemerythrin, i.e. dissociation of O_2, can be studied using stopped-flow techniques. For this purpose an excess of $Na_2S_2O_4$ is used to rapidly reduce the oxygen concentration in equilibrium (2), and k_{off} is then the rate-determining step. The characteristic absorption of HrO_2 at 500 nm ($\epsilon = 2200$ M^{-1} cm^{-1}) was used to follow this process. The observed rate constant, $k_{obs} = k_{off}$ under these conditions, was found to be independent of dithionite concentration $(8-33) \times 10^{-3}$ M and the HrO_2 concentration ($2 \times 10^{-5} - 2 \times 10^{-4}$ M). A typical value of 29 ± 3 s^{-1} was found at $[S_2O_4^{2-}] = 0.033$ M, $[HrO_2] = 8 \times 10^{-5}$ M and 20 °C, which is in close agreement with values reported in the literature, viz. 66 and 82 s^{-1} at 25 °C.[10] Similar values (43.1 and 51 s^{-1})[34] have been reported for the deoxygenation of *P. gouldii* hemerythrin, which is also an octamer. The pressure dependence of this reaction was studied at 17 and 23 °C in the range 5−100 MPa, for which the data are summarized in Table 2. The activation volume $\Delta V^{\ddagger}$ was estimated in the usual way (see Figure 3), and is included in Table 2. The deoxygenation reaction is characterized by a large positive $\Delta V^{\ddagger}$ value. The deoxygenation reaction of oxyhemocyanin ($k_{off} = 410$ s^{-1}, pH = 8.9, 25 °C) for subunit *a* is too fast to be measured on the available high pressure stopped-flow instrument (dead time of ca. 15 ms), and therefore no data on the pressure dependence of k_{off} could be obtained.

The values of k_{on} and k_{off} for oxygenation/deoxygenation of hemerythrin as a function of pressure can be used to estimate the equilibrium constant ($K = k_{on}/k_{off}$, see eq 6) for reaction 3 as a function of pressure. This results in the values 9.7×10^4, 2.2×10^5, 4.9×10^5, and 8.2×10^5 M^{-1} at 5, 50, 100, and 150

Table 1. Summary of Rate Data for the Oxygenation of Hemerythrin (*Themiste zostericola,* Octamer) as a Function of Concentration and Pressure[a]

pressure, MPa	$10^6[O_2]_e$, M	$[Hr]_e + [O_2]_e$, M	τ^{-1},[b] s^{-1}	$10^6 k_{on}$,[c] $M^{-1} s^{-1}$	$10^6 k_{on}$,[d] $M^{-1} s^{-1}$	$10^6 k_{on}$,[e] $M^{-1} s^{-1}$
5	316	317	2398	7.6	7.6 ± 0.2	7.4 ±0.3
	181	183	1399	7.7		
	147	150	1073	7.2		
	75	80.7	624	7.7		
50	316	317	2000	6.3	6.2 ± 0.4	6.2 ± 0.3
	181	183	1215	6.6		
	172	174	1026	5.9		
	147	150	915	6.1		
	147	149	863	5.8		
	75	80.7	524	6.5		
100	316	317	1550	4.9	4.7 ± 0.4	4.9 ± 0.3
	172	174	907	5.2		
	147	150	658	4.4		
	147	149	665	4.5		
	131	134	578	4.3		
	75	80.7	397	4.9		
150	316	317	1045	3.3	3.4 ± 0.5	3.2 ±0.1
	172	174	550	3.2		
	147	150	507	3.4		
	147	149	488	3.3		
	141	144	452	3.1		
	131	134	464	3.5		
	75	80.7	317	3.9		
$\Delta V^{\ddagger}_{on}$, cm^{-3} mol^{-1}					+13.8 ± 0.8	+14 ± 2

[a] Experimental conditions: pH = 8.5, [tris buffer] = 0.005 M, T = 22 °C, ΔT = 3 °C; ionic strength = 0.1 M ($NaSO_4$); λ = 365 nm. [b] Mean value of at least three kinetic runs. [c] Calculated from $\tau^{-1}/([Hr]_e + [O_2]_e)$; see Discussion. [d] Mean values from *c*. [e] Slope of a plot of τ^{-1} vs $([Hr]_e + [O_2]_e)$; see Figure 1.

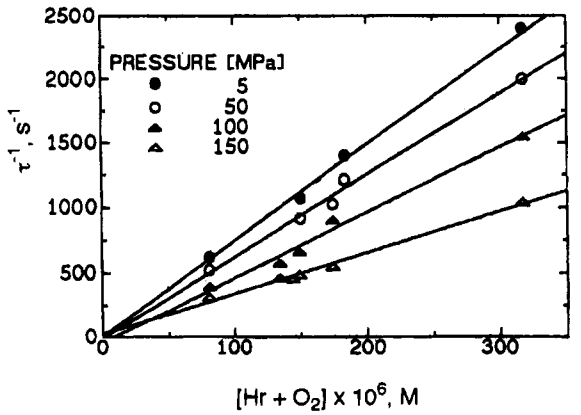

Figure 2. Plot of reciprocal relaxation times vs $([Hr]_e + [O_2]_e)$ as a function of pressure for the reaction Hr + O_2 ⇌ HrO_2. For experimental conditions: see Table 1. The lines represent the best fit to the data.

Table 2. Summary of Rate Data for the Deoxygenation of Hemerythrin as a Function of Pressure at 17 and 23 °C[a]

pressure, MPa	temp, °C	k_{off},[b] s^{-1}	$\Delta V^{\ddagger}$, cm^3 mol^{-1}
5	17	19.3	
	23	40	
25	17	14.1	
	23	26.1	
50	17	8.39	
	23	15.7	
75	17	5.47	
	23	9.1	
100	17	3.48	
	23	6.9	
	17		44.0 ± 0.8
	23		52.2 ± 0.7

[a] Experimental conditions: $[Na_2S_2O_4]$ = 0.033 M; $[HrO_2]$ = 8 × 10^{-5} M; [Tris buffer] = 0.05 M; ionic strength = 0.1 M (Na_2SO_4), pH = 8.5. [b] Mean value from at least five kinetic runs.

MPa, respectively. A plot of ln K vs pressure results in $\Delta\bar{V}$ = −36 ± 2 cm^3 mol^{-1}, which is in good agreement with the value

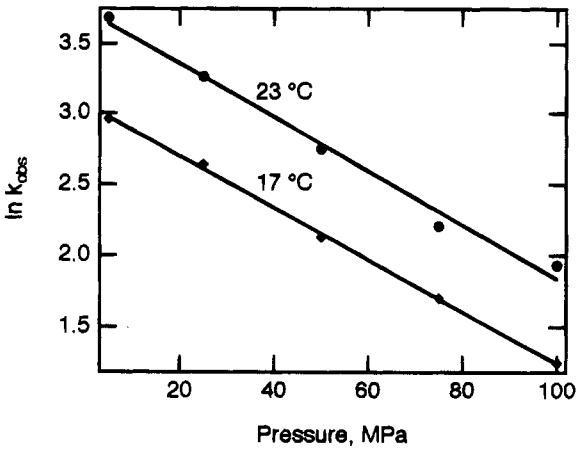

Figure 3. Plot of ln k_{obs} vs pressure at 17 and 23 °C for the reaction Hr + O_2 ⇌ HrO_2. For experimental conditions, see Table 2. The lines represent the best fit to the data.

calculated from the activation volume data at 23 °C, viz. $\Delta\bar{V}$ = $\Delta V^{\ddagger}_{on} - \Delta V^{\ddagger}_{off}$ = (+13.3 ± 1.1) - (+52.2 ± 0.7) = −39 ± 2 cm^3 mol^{-1}.

Interpretation of Volume Profile. Activation volumes are generally interpreted in terms of intrinsic volume changes ($\Delta V^{\ddagger}_{intr}$) resulting from changes in bond lengths and bond angles, and solvational changes ($\Delta V^{\ddagger}_{solv}$) resulting from changes in electrostriction on producing the transition state.[52,55,56] The activation and reaction volume data can be used to construct the overall volume profile for reaction 3 in Figure 4. This profile is very similar to the one reported for the oxygenation of myoglobin,[28] with the one difference that all volume parameters are almost double that found for myoglobin. In the remainder of the discussion we will focus on the interpretation

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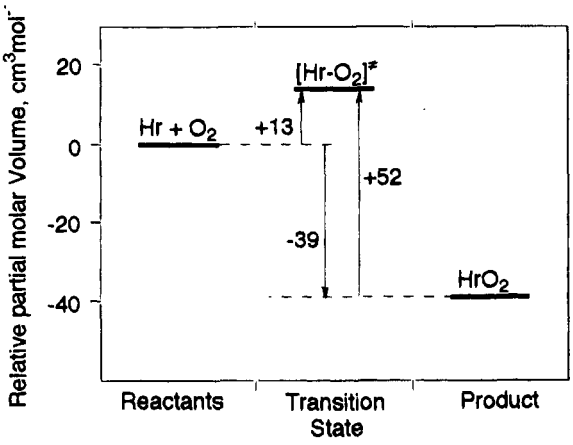

Figure 4. Volume profile for the reaction Hr + O_2 ⇌ HrO_2.

of the volume profile in Figure 4 in comparison with that reported for the related myoglobin system.

Bond formation processes are usually characterized by a decrease in volume,[22-27] i.e. a negative volume of activation. In contrast, the oxygenation of both hemerythrin and myoglobin is accompanied by positive $\Delta V^{\ddagger}$ values, although the structures of these proteins and iron active sites are completely different. The overall reaction volumes are considerably negative, in line with a bond formation process.[22-27] Bond breakage in both systems is accompanied by positive $\Delta V^{\ddagger}_{off}$ values and an overall increase in volume, in line with our general expectations for such processes.[28,29] It follows that the pressure dependence of the on reaction is not typical for a bond formation process and must be related to other effects. In the case of myoglobin, it was suggested[28,29] that the rate-determining step for oxygenation involves the movement of O_2 through the protein pocket, during which desolvation of this molecule and a gate-effect of the protein, which are both accompanied by a volume increase, play an important role. Recent molecular modelling studies in which CO binding to Mb was simulated suggested that there are multiple approaches for small molecules to access the active site.[57] The general behavior of most CO molecules was described as a diffusive hopping from one cavity to the other. The barriers between the cavities are reduced transiently by motions of the protein backbone coupled with fluctuations in side-chain orientations ("breathing motion" of the protein). This can also account for the positive activation volume found for the reaction of myoglobin with oxygen. Furthermore, if hemerythrin behaves very similar to myoglobin, a possible explanation for the finding that all volume parameters for the reaction of Hr with dioxygen are twice as large as compared to Mb may be related to the fact that the distances from the iron active site to the surface of the protein are estimated to be about twice as large in Hr as in Mb (3.8 Å in Mb and 6.3 Å in Hr).[58] For Mb the large volume collapse following the transition state was ascribed to Fe−O_2 bond formation, change in spin state from high to low of the iron center, and H-bonding of O_2 with the distal histidine (E7). It follows that conformational changes (breathing) of the protein are required during the bond formation process to account for the unusual $\Delta V^{\ddagger}_{on}$ found for myoglobin and hemerythrin.[59]

The $\Delta V^{\ddagger}_{on}$ value of +13.1 ± 1.1 cm^3 mol^{-1} found for the oxygenation of hemerythrin may partly be ascribed to the desolvation of oxygen during its entrance into the protein. However, the value is too positive to be solely accounted for in this way if we compare it with the value of + 5 cm^3 mol^{-1} found for the oxygenation of myoglobin.[28,32] The value of $\Delta V^{\ddagger}_{on}$ is such that it suggests some form of dynamic "breathing" motion of the protein that momentarily causes an opening up and enables the entrance of oxygen into the protein. During the binding of O_2, electron transfer from the metal centers results in the reduction of O_2 to O_2^{2-} and oxidation of Fe(II) to Fe(III). The significant volume decrease that occurs following the transition state can therefore be ascribed to the oxidation of both Fe(II) centers to the significantly smaller Fe(III) species. Furthermore, the formation of the Fe−O_2 and O_2−H bonds will also result in an overall volume collapse. Thus various factors can contribute towards the overall volume collapse following the transition state.

These mentioned contributions are reversed during the deoxygenation reaction, since bond breakage and reduction of Fe(III) to Fe(II) will all contribute to the large volume increase. For the release of a molecule such as O_2 a volume increase as large as +20 cm^3 mol^{-1} can be expected on the basis of data available for the dissociation of CO and H_2.[60] It follows that the $\Delta V^{\ddagger}$ value of +52 ± 1 cm^3 mol^{-1} at 23 °C for the deoxygenation of oxyhemerythrin can be accounted for in terms of the various contributions referred to above. The fact that we are dealing with a binuclear iron center may partially account for the fact that $\Delta V^{\ddagger}_{off}$ is ca. two times as large as that reported for the deoxygenation of the mononuclear oxymyoglobin center.[28]

The overall reaction volume of −39 ± 2 cm^3 mol^{-1} is also nearly double that reported for the oxygenation of myoglobin,[28] and mainly results from the large contribution of $\Delta V^{\ddagger}_{off}$. By combining this value with the partial molar volume of O_2 (viz. 28 cm^3 mol^{-1} [61,62]) in eq 7, it follows that $\bar{V}(HrO_2) - \bar{V}(Hr) =$

$$\Delta\bar{V} = \bar{V}(HrO_2) - \bar{V}(Hr) - \bar{V}(O_2) \qquad (7)$$

−11 cm^3 mol^{-1}. This value indicates that HrO_2 is slightly smaller than Hr, which means that an overall volume decrease occurs to give HrO_2 a more compact structure as was found in the X-ray structures.[13]

A volume profile for the formation and deoxygenation of Hc was not possible since k_{off} was too fast to be measured with the available high pressure stopped-flow instrumentation and the reaction of hemocyanin with dioxygen, k_{on}, was found to be pressure independent. This is in so far rather surprising because we did expect to find a negative activation volume. It is expected that binding of dioxygen should result in a volume decrease.[25] Studies on the reaction of a binuclear copper complex with dioxygen showed a negative activation volume of −21 ± 1 cm^3 mol^{-1},[35] and earlier studies on the reaction of a mononuclear copper(I) complex with dioxygen showed a negative activation volume of −22 ± 2 cm^3 mol^{-1}.[63] The most likely explanation for the absence of a significant pressure dependence is that, similar to the cases of Mb and Hr, some volume increase due to desolvation and/or structural changes on the protein must partially compensate the expected volume

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decrease for the binding of dioxygen. The overall effect is a pressure independent process. But the situation is somewhat different for Hc compared to Hr and Mb. Although the copper active site is 20 Å buried, X-ray crystal structure information has indicated a path of well-ordered water molecules to within 5 Å of the closest copper, with a partial obstruction at 10 Å.[64,65] It is possible that dioxygen is using this route to access the active site. In this case the entering dioxygen molecule must displace solvent molecules along the route which could account for the absence of a significant pressure dependence. Efforts to obtain the overall reaction volume from equilibrium measurements (eq 2) under pressure were also unsuccessful due to the slow denaturation of the protein under such conditions.

Investigations under pressure using nano- and picosecond techniques[29] have contributed to the understanding of the role of the protein in the myoglobin system. Such measurements have resulted in further information on the four stage mechanism generally applied to account for such fast kinetic measurements.[59,66-68] Similar measurements are required to gain further insight into the details of the oxygenation and deoxygenation of hemerythrin and hemocyanin.

In summary, the results of this study have indicated some important agreement and differences that occur in the oxygenation and deoxygenation kinetics of hemerythrin, hemocyanin and myoglobin. The positive value of $\Delta V^{\ddagger}_{on}$ for the oxygenation of hemerythrin is ascribed to partial desolvation of dioxygen and a dynamic opening up of the protein to enable the uptake of dioxygen. The subsequent oxidation of the metal centers results in the overall strong volume decrease. The fact that this volume collapse is almost double that observed for the oxygenation of myoglobin may indicate similar structural features in oxyhemerythrin and oxymyoglobin, viz $Fe^{III}(O_2H)$-Fe^{III} and $Fe^{III}O_2^-$, respectively. Therefore a description of the bonding mode as $Fe^{III}O_2^-$ or even $Fe^{III}O_2H$ (H atom from histidine E7), which are also discussed in the literature,[8,69] may be more appropriate for oxymyoglobin.

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