the limiting process if the solution is well stirred.

Acknowledgment. We appreciate the support and encouragement of Michael Perlsweig, Joseph Strakey, and John Williams. This work was supported by the Assistant Secretary for Fossil Energy, Office of Coal Research, Advanced Environment Control Division of the U.S. Department of Energy, under Contract No. DE-AC03-76SF00098 through the Pittsburgh Energy Technology Center, Pittsburgh, PA.

Registry No. NHAS, 26640-26-6; SO_3^{2-}, 14265-45-3; HSO_3^-, 15181-46-1; NO, 10102-43-9.

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Reactions of O_2 with Hemerythrin, Myoglobin, and Hemocyanin: Effects of D_2O on Equilibration Rate Constants and Evidence for H-Bonding

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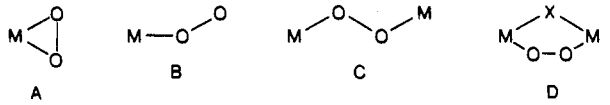
Received January 17, 1986

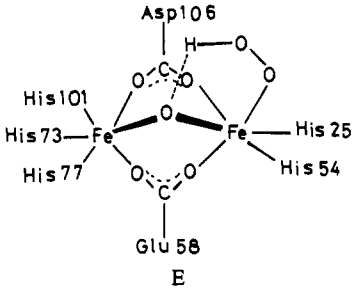
On and off rate constants associated with the binding of O_2 to hemerythrin (octamer from *Phascolopsis gouldii*), myoglobin (sperm whale), and hemocyanin (monomer from *Panulirus interruptus*) have been determined by using the temperature-jump and stopped-flow methods. When H_2O is replaced by D_2O, the following effects are observed: hemerythrin, k_{on} unchanged, k_{off} 19% decrease; myoglobin, k_{on} 17% decrease, k_{off} 16% decrease; hemocyanin, k_{on} and k_{off} unchanged. These effects are consistent with H-bonding effects in the case of the oxy forms of hemerythrin and myoglobin but not in the case of hemocyanin. The results obtained support the recently proposed structure for binding of O_2 at the hemerythrin active site.

Introduction

Dioxygen is known to bind to a metal (or metals) in different ways as illustrated in A–D.[1] The O_2 in A is assigned a peroxo



O_2^{2-} formal oxidation state. In B a superoxo O_2^- state is found, while for C and D both the superoxo and peroxo states are known. It has been established that B is relevant to O_2 binding in myoglobin (and hemoglobin), with the implication that the Fe(III)–superoxo description applies.[2,3] From vibrational spectroscopy[4,5] and EXAFS studies[6] the μ-peroxo structure (D) is relevant in the case of hemocyanin. Recently, from EXAFS,[7] X-ray crystallography,[8] and resonance Raman spectroscopy,[9] it has been proposed that hemerythrin binds O_2 in a novel and at present quite unique manner as illustrated in E. Although



E

somewhat different, a μ-hydroperoxo ligand is known to be present in the dicobalt(III) complex $[(en)_2Co(\mu\text{-}O_2H,NH_2)Co(en)_2]^{4+}$, which at present remains an isolated example.[10] Since the peroxo description applies to E,[11] it appears that protonation and H-bonding are responsible for the O_2 taking up its unusual orientation, when sideways-on binding (to one Fe) or bridging (to two Fe's) might otherwise have been expected. In order to confirm (or disprove) this feature of the oxyhemerythrin active site, we set out to study the effect of replacing solvent H_2O by D_2O on the kinetics of O_2 binding. To help assess the data, it was also decided to carry out similar studies with myoglobin and hemocyanin. It is known in the case of oxymyoglobin that there is H-bonding of the bound O_2 to the distal histidine,[2,12] whereas no H-bonding has been reported for the symmetrical binding of O_2 to hemocyanin.[5] Monomer forms were used except in the case of hemerythrin, where it is known that there is no cooperativity effect between the different subunits. Also the resonance Raman work referred to was with the octamer from *Phascolopsis gouldii*. A comparison of rate constants for the octamer and monomer forms of hemerythrin from *Themiste zostericola* has been made in a previous temperature-jump study from this group.[13] The protein sources were those for which X-ray crystallographic or EXAFS information is available. Thus *P. gouldii* hemerythrin (Hr), sperm whale myoglobin (Mb),[2] and *Panulirus interruptus* hemocyanin (Hc)[14] were selected for investigation.

Experimental Section

Protein. Methemerythrin from the marine worm *P. gouldii*, obtained from the Marine Biological Laboratory, Woods Hole, MA, was supplied by Professor D. M. Kurtz. Oxyhemerythrin (2 mL, 1 mM) was prepared by dialysis against 0.05 M Tris/H_2SO_4 at pH 8.55 containing sodium dithionite (BDH) (1 g/L) and purified by Sephadex G-100 gel filtration chromatography (column 1000 × 1.5 cm) to remove excess reductant and denatured protein. Sperm whale myoglobin (Sigma Chemicals) (50 mg), mostly in the met form, was dissolved in 10 mL of Tris/HCl (5 mM, pH 8.4), reduced with sodium dithionite, and then dialysed against the same

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buffer (1 L). Oxymyoglobin was purified by Whatman DE-23 ion-exchange chromatography (200 × 25 cm), the final elution being with 5 mM Tris/HCl at pH 8.40. The initial major band (~80%) was collected, and fractions having ratios $A_{582}/A_{514} > 1.04$ were pooled and used for kinetics.[15]

Oxyhemocyanin from *P. interruptus* was provided by Dr. H. J. Bak as a lyophilized sample of pure subunit a. The protein was dissolved in 12 mL of distilled water and dialyzed against Tris/edta buffer (see next section) at pH 8.75. Protein with $A_{280}/A_{337} < 5.5$ was used for kinetics (personal communication of Dr. H. J. Bak). Under the conditions employed the protein should remain as monomer units.[16] At pH <8.5 with Ca^{2+} present, formation of the hexamer occurs (hence the use of edta in the present studies).

Protein concentrations were determined by UV–vis absorption measurements using peak positions [λ/nm (ϵ/M^{-1} cm^{-1})] of 500 (2×10^6),[17] 418 (1.33×10^5),[14] and 280 (1.04×10^5)[15] for oxyhemerythrin (expressed as monomer), oxymyoglobin, and oxyhemocyanin, respectively.

Buffers. Prior to kinetic measurements, protein samples were dialyzed into Tris buffer (tris(hydroxymethyl)aminomethane, Sigma Chemicals) under conditions appropriate to each particular study. Thus with oxyhemerythrin, pH 8.55 (Tris/H_2SO_4), I = 0.10M (anhydrous Na_2SO_4, BDH AnalaR), was chosen to replicate resonance Raman studies;[9] with oxymoglobin, pH 8.50 (Tris/HCl), I = 0.10 M (NaCl, BDH AnalaR), was used to replicate neutron diffraction studies;[12] and with oxyhemocyanin, pH 8.75 (25 mM Tris with the addition of 5 mM edta), I = 0.10M (NaCl), was appropriate to ensure that the protein remained as monomer.[16] The disodium salt of edta, ethylenediaminetetracetate (Aldrich Chemicals), was used.

Where D_2O buffers were required, the appropriate volume of acid was diluted with 10 mL of D_2O (Aldrich 99.8%, Gold Label) and solid Tris added to give the required pD. The solution was rotary evaporated until dry and then redissolved in 10 mL of D_2O. Appropriate dilutions and additions of electrolyte were carried out to produce, in general, 100 mL of solution in Tris buffer, I = 0.10 M.

The D_2O buffer exchange was performed by using Sephadex G-25 gel filtration chromatography. Protein (0.5 mL, ca. 1 mM) was loaded onto the column (15 × 0.75 cm), previously equilibrated with buffer in D_2O. Eluted protein was then dialysed for 36 h against ca. 15 mL of buffer in D_2O at 4 °C prior to kinetic runs. Concentration of the protein solution was routinely performed by using the ultrafiltration technique (Amicon CF-25 cones).

The determination of pH (or by the same procedure pD) was performed using a Radiometer PHM62 pH meter combined with a Russell CWR 322 glass electrode.

Time Required for Exchange. Of particular importance to this study is the time required to effect D for H atom exchange. With myoglobin the proton on the distal histidine and with hemerythrin the proton on the hydroperoxo group, which most likely originates from the μ-hydroxo bridge of the deoxy protein, are believed to be relevant.[9] In the resonance Raman studies on hemerythrin, exchange occurred within the 30 min required for exchange of H_2O for D_2O using column chromatography, and results were in agreement with those obtained for solutions prepared by dialysis over 36 h. In the present studies 2 days were required for protein dialysis. No variations in kinetic results were observed over a further 2 days required to collect such data. Over longer periods the oxy protein samples undergo some partial conversion to met protein.[18]

Kinetics. Temperature-jump studies were carried out on a Messanlagen Studiengesellschaft (Göttingen, West Germany) apparatus. A temperature jump of 3 °C (using a previously calibrated cell)[19] was obtained by applying a voltage discharge to a solution initially at 22 °C. Temperature accuracy was ±0.2 °C. Kinetics were monitored at 365, 436, and 337 nm for hemerythrin, myoglobin, and hemocyanin, respectively.

Equilibration rate constants for the on and off processes relating to O_2 binding are as defined in (1), where P is the deoxy protein. Free O_2

$$P + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PO_2 \qquad (1)$$

was in at least 40-fold excess over concentration of available protein binding sites. Thus all hemerythrin concentrations are expressed according to the number of monomeric binuclear units. In all experiments only a single relaxation process was observed. Output was stored on a Tektronix 564B storage oscilloscope, and relaxation times (τ) were calculated from the slopes of ln ($A_t - A_\infty$) versus time graphs, which were linear to at least 4 half-lives. Each rate constant reported is the average of at least three separate determinations.

Solutions for temperature-jump studies were prepared by exposing protein to a stream of O_2 (or N_2) until (with careful mixing) the free concentration was at the required level. A Beckman Model 0260 oxygen analyzer complete with O_2 sensor cathode was used to determined O_2 concentrations. Calibrations were made against distilled water at 22 °C. At 1 atm pressure (101 kPa), the concentration of O_2 is 8.80 parts per million. Solutions were syringed into the temperature-jump cell, the 3-mm-diameter entry of which was then plugged to avoid equilibration with the atmosphere.

Stopped-flow dissociation experiments to determine k_{off} were performed on a Dionex D-110 spectrophotometer equipped with a logarithmic amplifier. Measurements were at the following wavelengths (nm): hemerythrin, 500; myoglobin, 436; hemocyanin, 360. The absorbance was stored digitally on a Datalab DL901 transient recorder, and the output was displayed on an oscilloscope. The recorder was interfaced to a Commodore PET2001-16K microcomputer, and rate constants (k_{diss}) were calculated from the slopes of ln ($A_t - A_\infty$) vs. time graphs by using a simple program.

Dithionite was used as a scavenger for O_2, thus enabling k_{off} to be determined directly. Applicability of the method requires the reaction to be independent of dithionite concentration,[20] and the condition [$S_2O_4^{2-}$] >> [P] was adopted therefore. Experiments were performed with the oxygenated protein in one drive syringe and dithionite in the other. Both solutions had buffer and ionic strength adjusted to the same (required) values. All such experiments were performed at 25.0 ± 0.1 °C with dithionite under rigorous air-free (N_2) conditions. Dithionite concentrations were obtained by spectrophotometric determination against the $K_3[Fe(CN)_6]$ (BDH, AnalaR) peak at 420 nm (ϵ = 1010 M^{-1} cm^{-1}).

Treatment of Data. Relaxation times were weighted according to τ^2 in the linear least-squares programs used.

Table I. Reciprocal Relaxation Times (τ^{-1}) at 25 °C for the Equilibration of *P. gouldii* Hemerythrin with Oxygen, at pH 8.55 (Tris/H_2SO_4), I = 0.10 M (Na_2SO_4), and λ = 365 nm

solvent	$10^5[Hr]_T$, M	$10^5[O_2]$, M	$10^5[Hr]$, M	$10^{-3}\tau^{-1}$, s^{-1}
H_2O	13.1	65.6	0.07[a]	7.63
H_2O	13.1	50.6	0.10[a]	6.46
H_2O	13.1	39.4	0.12[a]	4.73
H_2O	13.1	24.4	0.20[a]	3.22
H_2O	13.1	15.0	0.32[a]	1.82
D_2O	12.5	59.4	0.06[a]	8.28
D_2O	12.5	41.0	0.09[b]	4.90
D_2O	12.5	33.1	0.11[b]	3.84
D_2O	12.5	17.2	0.21[b]	1.79

[a] Concentration of deoxyHr at equilibrium with calculated $K = 2.7 \times 10^5$ M^{-1} from Table V (each binuclear unit carrying one O_2).

Table II. Reciprocal Relaxation Times (τ^{-1}) at 25 °C for the Equilibration of Sperm Whale Myoglobin with Oxygen, at pH 8.50 (Tris/HCl), I = 0.10 M (NaCl), and λ = 418 nm

solvent	$10^5[Mb]_T$, M	$10^5[O_2]$, M	$10^6[Mb]$, M	$10^{-3}\tau^{-1}$, s^{-1}
H_2O	2.70	24.0	0.12[a]	5.42
H_2O	2.70	20.9	0.13[a]	4.75
H_2O	2.70	15.9	0.18[a]	3.73
H_2O	2.70	15.0	0.19[a]	3.49
H_2O	2.70	11.7	0.24[a]	2.69
H_2O	2.70	6.9	0.40[a]	1.81
D_2O	3.70	23.4	0.16[b]	4.43
D_2O	3.70	20.0	0.19[b]	3.70
D_O	3.70	16.9	0.22[b]	3.24
D_2O	3.70	10.0	0.37[b]	2.23
D_2O	3.70	4.7	0.79[b]	1.14

[a] Concentration of deoxyMb at equilibrium calculated by using $K = 9.7 \times 10^5$ M^{-1} from Table V. [b] Concentration of deoxyMb at equilibrium calculated by using $K = 10.0 \times 10^5$ M^{-1} from Table V.

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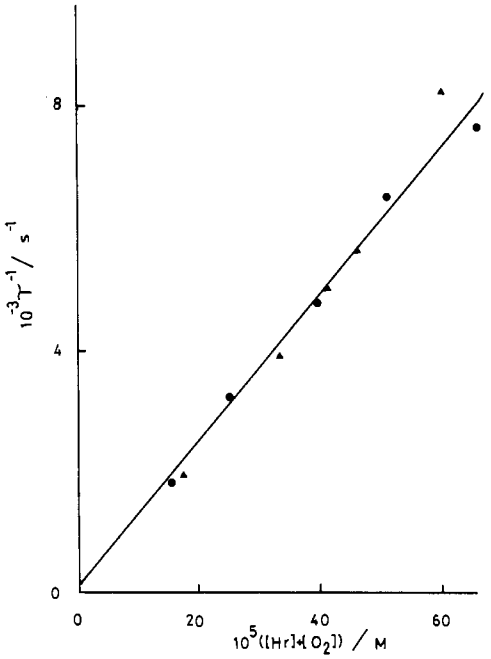


Figure 1. Dependence of temperature-jump reciprocal relaxation times for the equilibration of hemerythrin (*P. gouldii*) and O_2 on equilibrium concentrations of the reactants at 25 °C, I = 0.10 M: (●) in H_2O; (▲) in D_2O.

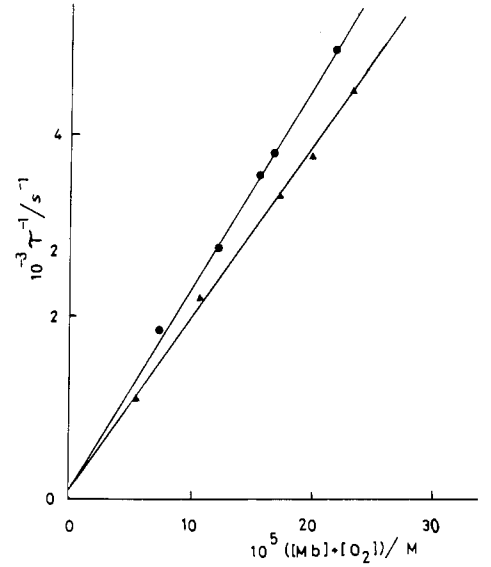


Figure 2. Dependence of temperature-jump reciprocal relaxation times for the equilibration of myoglobin (sperm whale) with O_2 on equilibrium concentrations of the reactants at 25 °C, I = 0.10 M: (●), in H_2O; (▲), in D_2O.

Results

Reciprocal relaxation times (τ^{-1}) from temperature-jump studies on the equilibration of hemerythrin with O_2 in H_2O and D_2O are given in Table I. Similar listings for myoglobin and hemocyanin are given in Tables II and III, respectively. The linear dependence of τ^{-1} on the sum of the free concentrations of deoxy protein and oxygen [P] + [O_2], (2), is illustrated in Figures 1–3 for hemerythrin, myoglobin, and hemocyanin, respectively. The intercepts are in all cases small and do not give a reliable estimate for k_{off}. From the slopes $10^{-7}k_{on}$ (M^{-1} s^{-1}) values obtained are as follows: hemerythrin, 1.2 ± 0.1 (H_2O), and 1.2 ± 0.1 (D_2O); myoglobin, 2.3 ± 0.05 (H_2O), 1.9 ± 0.1 (D_2O); hemocyanin, 5.7 ± 0.2 (H_2O), 5.7 ± 0.2 (D_2O). Rate constants k_{diss}, for the dissociation of the oxy proteins with dithionite as scavenger (Table IV), are independent of the concentrations of protein (5-fold variation) and dithionite (1.2–8.8 mM) and provide an accurate means of determining k_{off}. Values of k_{off} (s^{-1}) obtained are as follows:

$$\tau^{-1} = k_{on}([P] + [O_2]) + k_{off} \quad (2)$$

Table III. Reciprocal Relaxation Times (τ^{-1}) at 25 °C for the Equilibration of *P. interruptus* Hemocyanin with Oxygen, at pH 8.75 (Tris/edta), I = 0.10 M (NaCl), and λ = 337 nm

solvent	10^5[Hc]$_T$, M	10^5[O_2], M	10^5[Hc],[a] M	$10^{-3}\tau^{-1}$, s^{-1}
H_2O	3.15	13.6	0.04	7.49
H_2O	3.15	10.2	0.05	6.10
H_2O	3.15	8.2	6.07	5.02
H_2O	3.15	6.2	0.09	4.10
H_2O	3.15	3.6	0.15	2.50
D_2O	2.81	13.6	0.04	7.87
D_2O	2.81	12.6	0.04	6.67
D_2O	2.81	9.3	0.05	5.41
D_2O	2.81	4.9	0.10	3.09

[a] Concentration of deoxyHc at equilibrium calculated by using K = 5.7 × 10^5 M^{-1} from Table V (each binuclear unit carries one O_2).

Table IV. Rate Constants k_{diss} (25 °C) for the Dissociation of Oxy Proteins with Sodium Dithionite as a Scavenger for O_2[a]

protein	solvent	10^5[protein], M	10^3[$S_2O_4^{2-}$], M	k_{diss}, s^{-1}
HrO_2	H_2O	1.02	1.77	41.0
HrO_2	H_2O	1.02	8.85	44.3
HrO_2	H_2O	2.55	1.77	44.6
HrO_2	D_2O	2.50	6.65	34.8
HrO_2	D_2O	2.50	1.33	34.8
HrO_2	D_2O	1.00	6.65	35.5
HrO_2	D_2O	1.00	1.33	34.9
MbO_2	H_2O	0.41	6.10	21.1
MbO_2	H_2O	0.08	6.10	24.5
MbO_2	H_2O	0.08	1.21	25.1
MbO_2	D_2O	0.23	5.45	19.4
MbO_2	D_2O	0.23	1.08	21.5
MbO_2	D_2O	0.05	5.45	17.8
HcO_2	H_2O	1.58	4.00	111
HcO_2	H_2O	1.58	2.00	90.2
HcO_2	D_2O	1.40	4.00	99.2
HcO_2	D_2O	1.40	2.00	105

[a] Conditions are as in Tables I–III.

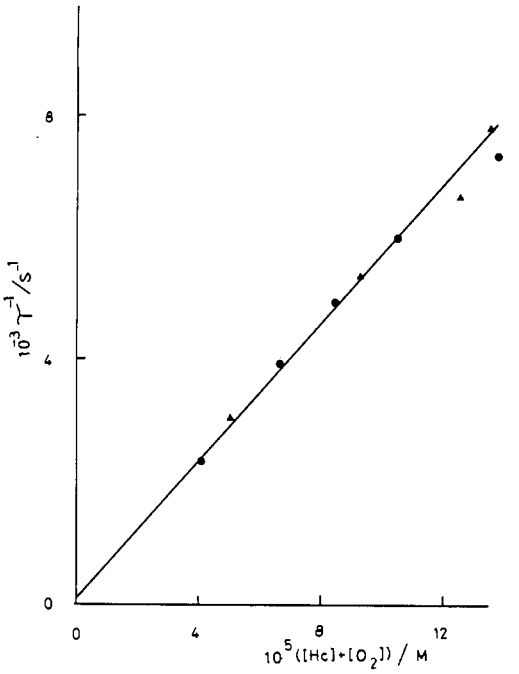


Figure 3. Dependence of temperature-jump reciprocal relaxation times for the equilibration of hemocyanin (*P. interruptus*) with O_2 on equilibrium concentrations of the reactants at 25 °C, I = 0.10 M: (●) in H_2O; (▲) in D_2O.

Table V. Summary of Rate Constants (25 °C Unless Otherwise Stated) for Formation (k_{on}) and Dissociation (k_{off}) of Oxy Forms of Hemerythrin, Myoglobin, and Hemocyanin

protein	solvent	$10^{-7}k_{on}$, M^{-1} s^{-1}	k_{off}, s^{-1}	$10^{-5}K$, M^{-1}	conditions
		This Work			
Hr(octamer *P.g.*)	H_2O	1.2	43.1	2.8	pH 8.55; I = 0.10 M (Na_2SO_4)
	D_2O	1.2	35.0	3.4	
Mb(sperm whale)	H_2O	2.3	23.6	9.7	pH 8.5; I = 0.10 M (NaCl)
	D_2O	1.9	19.6	9.7	
Hc(monomer *P.i.*)	H_2O	5.7	100	5.7	pH 8.75; I = 0.10 M (NaCl)
	D_2O	5.7[a]	101	5.6	
		Previous Studies			
Hr(octamer *T.z.*)[b]	H_2O	0.75	82	0.91	pH 8.2; I = 0.10 M (Na_2SO_4)
Hr(monomer *T.z.*)[b]	H_2O	7.8	315	2.5	
Hr(octamer *P.g.*)[c]	H_2O	0.74	51	1.5	pH 8.2; I = 0.15 M (Na_2SO_4)
Mb(sperm whale)[d]	H_2O	1.9	11	17	pH 7.0; I = 0.10 M (KNO_3)
Hc(hexamer *P.i.*)[e]	H_2O	3.1	60	5.2	pH 9.6; I = 0.10 M (NaCl)
Hc(aggregate *B.u.*)[f]	H_2O	0.78	70	1.11	pH 8.2; I = 0.10 M (NaCl)

[a] $10^{-7}k_{on}$ = 4.6 M^{-1} s^{-1}, k_{off} = 400–1500 s^{-1} from ref 21. [b] Octamer and monomer from *T. zostericola*.[13] [c] Octamer from *P. gouldii*.[20] [d] Kinetic studies at 21.5 °C.[22] [e] Reference 21. [f] *B. undatum* (R-state).[29]

hemerythrin, 43.1 ± 2.5 (H_2O), 35.0 ± 2.1 (D_2O); myoglobin, 23.6 ± 2.2 (H_2O), 19.6 ± 1.6 (D_2O); hemocyanin, 100 ± 10 (H_2O), 101 ± 7 (D_2O). In the case of the hemerythrin reaction in H_2O, it was observed that k_{on} and k_{off} do not change on decreasing the pH to 6.3.

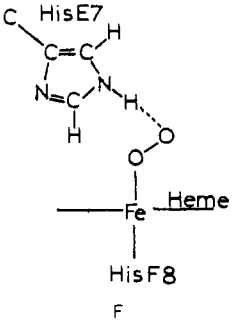
A summary of the effects of D_2O on k_{on} and k_{off} is given in Table V. Other relevant determinations of k_{on} and k_{off} are also noted. Rate constants previously obtained for *P. gouldii* hemerythrin were obtained at I = 0.015 M by using the stopped-flow method, which is less accurate for k_{on}.[20] A previous study on monomeric hemocyanin from *P. interruptus* gave k_{on} in satisfactory agreement with present values (Table V) but did not yield an accurate value for k_{off}.[21] Results reported for myoglobin at pH 7.0 (Table V)[22] are in satisfactory agreement with those now obtained.

Discussion

It was decided to work with octameric hemerythrin in order to relate present studies with previous work. Oxygen-binding experiments on *P. gouldii* hemerythrin have indicated noncooperativity with a Hill coefficient of 1.1.[22] Such a result is consistent with individual subunits behaving independently and yielding only a single rate-determining step.[12] As in earlier work on *T. zostericola*, a single uniphasic relaxation process is obtained.[12] Similar behavior is observed for myoglobin and monomeric hemocyanin, respectively. The monomer form of *T. zostericola* hemerythrin has also been studied, and these and other results are included in Table V.[12] Studies on the dissociation of oxy forms using dithionite as O_2 scavenger also gave uniphasic behavior.

The O_2-binding proteins have different active site structures and are now known to bind O_2 in quite different ways, an observation which is at first surprising for the binuclear entities. The most unusual of these is hemerythrin, where binding as in E has recently been suggested. Vibrational spectroscopy studies[22-24] are consistent with a peroxo assignment and formation of a binuclear Fe(III) unit.[23-25] Also ^{18}O labeling has indicated a nonequivalence of the two O atoms,[26] and structures as in A, C, or D are unlikely, therefore. From Mössbauer spectroscopy[27] it has been suggested that apparently equivalent Fe atoms in deoxyhemerythrin become nonequivalent on binding O_2. An X-ray diffraction study on *Themiste dyscritum* (2.2-Å resolution)[8] and EXAFS measurements (*P. gouldii*)[7] have been sited in support of E. The recent resonance Raman studies on *P. gouldii* hemerythrin[9] have indicated that replacement of H_2O by D_2O solvent brings about a shift in the Fe–O_2 and O–O stretching frequencies, consistent with replacement of H- by D-bonding. Of interest in the present kinetic study is the observation that k_{off} decreases by 19%, but k_{on} remains unchanged upon deuteration. It is concluded that protonation and H-bonding do not contribute to the activation process for k_{on}, but for O_2 release from E there exists an appreciable barrier. Whereas a μ-hydroxo ligand is believed to be present in deoxy Hr, giving weak coupling of the Fe(II)'s,[28] in the oxy form a higher antiferromagnetic coupling constant (J = −77 cm^{-1}) is observed, consistent with a μ-oxo structure.[27] We believe therefore that the proton of the μ-hydroxo group is transferred to the bound O_2, and this is supported by rates of O_2 binding to deoxyHr, which are unaffected by changes in pH from 6 to 9.[11] Because D_2O has no effect on k_{on}, it is possible that the prime act of O_2 binding is accomplished without the H^+ transfer assuming any importance.

The magnitude of the effect of deuteration on k_{off} is of interest when compared to that observed for myoglobin.[12] X-ray crystallographic and neutron diffraction studies have indicated that O_2 in myoglobin is H-bonded to the distal histidine (E7) as in F.[2]



F

This histidine is present in most myoglobins (as well as hemoglobins) where it helps stabilize the bound O_2. As can be seen from the results obtained, deuteration produces a decrease in both k_{on} (17%) and k_{off} (16%). The effect on k_{off} is very similar to that observed for hemerythrin.

X-ray crystallographic (3.8-Å resolution),[14] EXAFS,[6] and vibration spectroscopy studies[4,5] on hemocyanin have been reported. Evidence obtained is consistent with O_2 binding to both Cu atoms (separation of 3.5 Å) as peroxide as in D, with accompanying change in oxidation state from Cu^I_2 to Cu^{II}_2. There is no evidence that the bound peroxide is influenced by H-bonding. Consistent with this, both k_{on} and k_{off} remain unchanged on when H_2O is replaced by D_2O. The rate constant k_{on} is 1 order of magnitude larger than previous values obtained for the aggregate of (mollusc) hemocyanin in the R-state.

The rate constants k_{on} in H_2O (Table V) are also of interest because of their magnitude and similarity. Evidence has been obtained for coordination numbers less than six for Fe(II) in deoxy

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myoglobin and for one of the Fe(II)'s in deoxyhemerythrin and for a reduced coordination number for the Cu(I)'s in deoxyhemocyanin. For large molecules such as these in which there is limited access of substrate (O_2) to the active site, formation rates are fast and probably approach the diffusion-controlled limit, consistent with addition at an unoccupied coordination position. The similar magnitudes of equilibrium constants (M^{-1}) in H_2O at 25 °C for myoglobin (9.7×10^5), the monomeric form of hemocyanin (5.7×10^5), and octameric hemerythrin (2.5×10^5) are also noted (Table V).

To summarize, the studies described here add support to the active site structure E, which has been proposed for oxyhemerythrin, with an unusual and at present quite unique form of O_2 binding. The kinetic results obtained for myoglobin and hemocyanin in H_2O and D_2O are consistent with known O_2-binding properties of these proteins.

Acknowledgment. We thank the U.K. Science and Engineering Research Council for a postgraduate studentship to G.D.A.

Registry No. O_2, 7782-44-7; D_2, 7782-39-0.

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Reactions of the Metallodrug Auranofin [(1-Thio-β-D-glucopyranose-2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold] with Biological Ligands Studied by Radioisotope Methodology

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Received February 21, 1986

The metallodrug auranofin [(1-thio-β-D-glucopyranose-2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold] is an orally active drug in the treatment of rheumatoid arthritis. The reactivity of auranofin with purified bovine serum albumin (BSA), transferrin, histidine-rich glycoprotein, and metallothionein was studied by using a triple-label radioisotope methodology developed for this purpose. Auranofin was labeled specifically in the triethylphosphine with tritium, in the glucose ring with carbon-14, and with the radionuclide gold-195. The sensitivity of radioisotope detection allowed investigation of ligand-exchange reactions in aqueous solution at physiologically relevant concentrations of drug and biomolecule. By triple-label radioisotope methodology, linked with gel filtration chromatography, it was possible to follow the fate of the gold ion and both ligands in the same experiment. BSA reacted with auranofin to replace the thiosugar ligand and form (triethylphosphine)gold-S-BSA. This complex underwent thiol exchange with cysteine and glutathione but did not react with histidine, methionine, or chloride ion. BSA was coordinated to gold through the thiol of Cys-34 and did not dissociate the phosphine ligand of auranofin. No reaction was observed for auranofin with transferrin or histidine-rich glycoprotein, prototypic metal carrier proteins in the serum that bind essential metals through tyrosine and/or histidine side chains. Metal-free rabbit liver metallothionein I reacted with auranofin to dissociate the thiosugar and phosphine ligands. These studies provide insights into the relationship of structure to efficacy for the metallodrug.

Introduction

Although metals have been used medicinally for centuries, discovery of the antitumor activity of *cis*-diamminedichloroplatinum(II)[1] and the success of this coordination compound as an anticancer drug[2] has stimulated renewed interest in metal complexes as drugs. The metallodrug auranofin [(1-thio-β-D-glucopyranose-2,3,4,6-tetraacetato-*S*)(triethylphosphine)gold] was recently introduced for the treatment of rheumatoid arthritis. There is little doubt that other metallodrugs will be developed in a variety of therapeutic areas as the field of inorganic medicinal chemistry continues to grow.

It is important to realize that the etiology of rheumatoid arthritis is not well understood and the biological target site(s) that are responsible for the activity of gold metallodrugs are not known. Gold metallodrugs used in the treatment of rheumatoid arthritis are illustrated in Figure 1. These compounds contain gold in the +1 oxidation state. Au(I) is generally coordinated by two ligands in an approximately linear geometry. As suggested by the physical properties of their ligands, Solganal (with thioglucose ligands) and Myochrysine (with thiomalate ligands) are both extremely water-soluble while auranofin (with tetraacetylthioglucose and triethylphosphine ligands) is sparingly soluble in water. Auranofin is an orally active drug while Solganal (gold thioglucose) and Myochrysine (gold sodium thiomalate) are active only when injected.[4-8]

The biological metabolism of metal-ligand complexes is not like the enzymatic modification, degradation, and excretion of organic drugs. Metal-ligand complexes may exchange some or all of their original ligands for biological ligands on contact with biological fluids. In the study of inorganic drug action, knowledge of ligand-exchange reactions in the biological system and the structures of metal-biomolecular complexes can provide a basis for hypotheses about the mechanisms of biological activity. The reactivity of metal complexes also provides critical insights into structure-activity relationships (SAR) to suggest a logical chemical basis for the synthesis of more effective compounds.

Studies of metal-biomolecule complexes usually rely on spectroscopy as a primary research tool.[3] However, spectroscopic methods can be limited by the availability of sufficient quantities of the *biological* material. Millimolar solution concentrations (milligram quantities of biological molecules) are usually required. The detection limits of spectroscopic techniques can also make studies in aqueous solution difficult for lipophilic compounds like auranofin.

In this report we describe the development of a new triple-label radioisotope technique that makes it possible for the first time to follow the ligands and the gold ion of auranofin in the same experiment. We use the technique to investigate the reactivity of auranofin with the purified proteins bovine serum albumin, transferrin, histidine-rich glycoprotein, and metallothionein in

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