Articles

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Active-Site Chemistry of Hemerythrin: Kinetic Studies on the Reduction of Metmyohemerythrin from *Themiste zostericola* and the Mechanism for Met and Deoxy Interconversion

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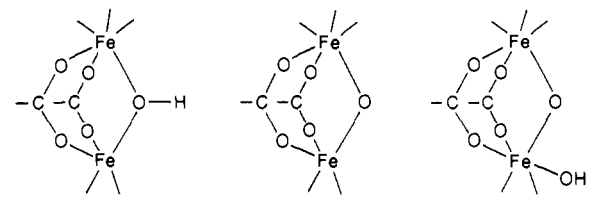
Three stages are observed in the 2-equiv reduction of the binuclear Fe(III,III) active site of *Themiste zostericola* metmyohemerythrin. At pH 8.2 the first stage is dependent on the concentration of reductant, and second-order rate constants k_1 (M^{-1} s^{-1}; 25 °C) are as follows: $[Co(sep)]^{2+}$, 1.4×10^3; $[Co(9\text{-}aneN_3)_2]^{2+}$, 33; $[V(pic)_3]^-$, 4.4×10^3; and (from an earlier study) dithionite, 1.1×10^6 ($I = 0.15$ M (Na_2SO_4)). The second stage, which also consumes 1 mol of reductant, is (at pH 8.2) independent of reductant ($k_2 = 4 \times 10^{-3}$ s^{-1}). This step is believed to involve an isomerization of the kind Fe(II,III) → Fe(III,II), followed by rapid reduction. The third stage, having a relatively small absorbance change, may correspond to an isomerization of the Fe(II,II) state. Similar behavior is observed at pH 6.3, with some interplay of redox and isomerization in the second stage of reaction with the positively charged reactants. The mechanism that we propose takes into account recent structural information and previous kinetic results for the deoxymyo → metmyo conversion. This involves, for reduction of met, retention of the protein structure characteristic of methemerythrin until the third stage, when the protein adjusts to the stable deoxy form. A previous study has shown that the oxidation of the deoxy form involves the same number of stages, the last being attributed to formation of a stable met form. One possibility is that these slow isomerization processes involve interconversion of μ-oxo and μ-hydroxo forms.

Introduction

The chemistry and biochemistry of the O_2-binding respiratory protein hemerythrin found in sipunculid, annelid, two brachiopod, and two priapulid marine invertebrates has been featured in recent reviews.[1-3] The protein is readily available from sipunculid worms, in this study *Themiste zostericola*. An octamer form is present in the coelomic fluid, each subunit of which contains a binuclear Fe active site. Kinetic studies on the reduction of the octamer met Fe(III,III) to the deoxy Fe(II,II) form have already been reported by us[4] and by the Wilkins group.[5,6] The overriding impression from these studies is the complexity of the chemistry relating to such redox interconversions with 1-equiv reagents. Smaller amounts of a monomer, which has five extra amino acids (118 in all; mol wt 13 900), but which is otherwise very similar to the octamer subunit, are obtained from the retractor muscle. It is the reactivity of this form (met → deoxy) that we now turn to, in an attempt to clarify mechanistic aspects of the interconversions.

Recently there have been major advances in understanding structural aspects of the hemerythrin active site. Information available suggests that monomer and octamer forms from different hemerythrin sources have the same active-site structure. From X-ray crystallography[7] and EXAFS,[8,9] it has been proposed that the deoxy form has the structure shown in A, with histidine residues coordinated in five positions. In A the Fe(II)'s are weakly antiferromagnetically coupled,[10,11] and evidence from crystallography (3.9-Å resolution) and EXAFS studies is not consistent with a μ-oxo bridge (the Fe atoms are too far apart), but is acceptable for a μ-hydroxo bridge. Strong antiferromagnetic coupling is observed for the met ($J = -134$ cm^{-1}) and oxy ($J = -77$ cm^{-1}) forms,[10] in support of a μ-oxo structure. Resonance Raman[12] and kinetic[13] studies on the binding of O_2 in H_2O and D_2O solvent are consistent with the presence of a coordinated hydroperoxo ligand H-bonded to the μ-oxo group. Recent X-ray crystallography studies[14] on methemerythrin from *Themiste dyscritum* (pH ≤6.5) have indicated a structure in which one of the Fe(III) atoms is octahedral and the other is trigonal bipyramidal (B). It has been demonstrated that solvent OH^- coordinates to the 5-coordinate Fe to give C in a slow acid–base equilibrium ($t_{1/2} \sim 1$ min at 25 °C),[15] the pK_a of which is 7.8 for *Phascolopsis gouldii*. In the case of *T. zostericola* differences in UV–vis spectra are consistent with similar changes. At pH >9 resonance Raman studies have detected the hydroxomet form C.[16] X-ray crystallography,[14] EXAFS,[8,9] Mössbauer,[10] and resonance Raman studies[17] on the met form support a μ-oxo-bridged structure, features of which are Fe–O(oxo) = ca. 1.75



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Å, Fe–Fe = ca. 3.3 Å, and an Fe–O–Fe angle of 135°. Here we seek to apply this structural information to redox interconversions of the myo (monomer) form. Because of the existence of different met structures B and C at pHs 6.3 and 8.2, respectively, we saw the need to investigate reactions of the met form at both these pHs.

Experimental Section

Preparation of Protein. Metmyohemerythrin (hereafter MetmyoHr) was isolated directly from the retractor muscles of *T. zostericola* marine worms (Pacific Bio-Marine Laboratory, Venice, CA) by using a modified version of the procedure described by Klippenstein et al.[18] (dialysis against NaN_3 was omitted). Purification was by Sephadex G-75-120 (Sigma Chemicals) gel-filtration chromatography. Concentration of the protein was achieved by ultrafiltration using Amicon CF-25 cones. Protein was dialyzed for approximately 15 h at 4 °C against buffer of the required pH. Concentrations were determined from absorption measurements at 333 nm (ϵ 6500 M^{-1} cm^{-1}),[19] which is an isosbestic point for the acid–base forms B and C.

Rigorous air-free conditions were employed in all studies. Protein samples were deoxygenated by dialysis against deareated buffer. Nitrogen gas (BOC, White Spot), further purified by bubbling through solutions of Cr^{2+}, was used in all experiments.

Preparation of Complexes. These were obtained by literature procedures and purified to known UV–vis peak positions λ/nm (ϵ/M^{-1} cm^{-1}): $[Co(sep)]Cl_3 \cdot H_2O$, where sep denotes the sepulchrate cage ligand 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]eicosane, 472 (109), 340 (116);[20] $[Co(9\text{-}aneN_3)_2]Cl_3 \cdot 3H_2O$, where 9-aneN$_3$ is 1,4,7-triazacyclononane, 458 (100), 333 (89);[21] chromium(II) chloride, $CrCl_2 \cdot 6H_2O$, 715 (4.8);[22] tris(1,10-phenanthroline)iron(III) perchlorate, $[Fe(phen)_3](ClO_4)_3 \cdot H_2O$, which was determined by Zn/Hg reduction to $[Fe(phen)_3]^{2+}$, peak at 510 (10 900).[23] Other complexes were generated in solution by addition of the ligands to solutions containing the required metal in picolinic acid (2-carboxypyridine) (Sigma Chemicals), 1,10-phenanthroline (Aldrich Chemical Co.) in 10-fold excess, or 15-aneN$_4$ (Strem Chemicals) in 3% excess: tris(picolinato)vanadium(II), $[V(pic)_3]^-$, λ 660 nm (ϵ 3800);[24] tris(1,10-phenanthroline)chromium(II), $[Cr(phen)_3]^{2+}$;[25] $[Cr^{II}(15\text{-}aneN_4)(H_2O)_2]^{2+}$, where 15-aneN$_4$ is the macrocyclic ligand 1,4,8,12-tetraazacyclopentadecane, λ 540 nm (ϵ 36.5).[26] Solutions of V^{2+} were prepared from vanadyl sulfate, $VOSO_4$ (BDH), by electrochemical reduction at a Hg-pool cathode.[27]

Solutions of $[Co(sep)]^{2+}$ and $[Co(9\text{-}aneN_3)_2]^{2+}$ were generated from the 3+ ions by controlled potentiometric reduction at a Hg-pool cathode using a Chemical Electronics Ltd. (Washington, Tyne & Wear) potentiostat, Model DD505U. To avoid interference from chloride (as Cl_2) during electrolysis, the Cl^- salt of $[Co(9\text{-}aneN_3)_2]^{3+}$ was converted to the sulfate by dissolving in concentrated H_2SO_4 and adding ethanol to precipitate the mixed SO_4^{2-}/HSO_4^- salt. The product was recrystallized from a minimal volume of 0.5 M H_2SO_4. Amounts of HSO_4^- were determined by titration against standard 0.1 M NaOH (BDH; Convol). Reductant concentrations were determined by addition of excess $[Fe(phen)_3]^{3+}$ and measuring the absorbance of $[Fe(phen)_3]^{2+}$ at 510 nm.[27] Sodium dithionite, $Na_2S_2O_4$ (BDH, G.P.R.), was determined by titration against potassium ferricyanide, $K_3[Fe(CN)_6]$ (BDH AnalaR), peak at 420 nm (ϵ 1010 M^{-1} cm^{-1}). We leave open the extent of protonation (and charge) in the case of dithionite, about which little seems to be known.

Buffers. A solution of 0.050 M tris(hydroxymethyl)aminoethane (Tris) (Sigma Chemicals), to which 1.0 M H_2SO_4 (BDH, AnalaR) was added, was used as buffer over the range pH 8.0–9.2. The buffer 2-morpholinoethanesulfonic acid (Mes) (Sigma Chemicals) in a 0.050 M solution adjusted with 1.0 M NaOH (May and Baker) was used to buffer at pH 6.3. All buffer solutions were adjusted to an ionic strength of 0.150 ± 0.001 M with anhydrous Na_2SO_4 (BDH, AnalaR). The pH of solution was determined by using a Radiometer (PHM62) pH meter with a Russell (CWR/322) glass electrode.

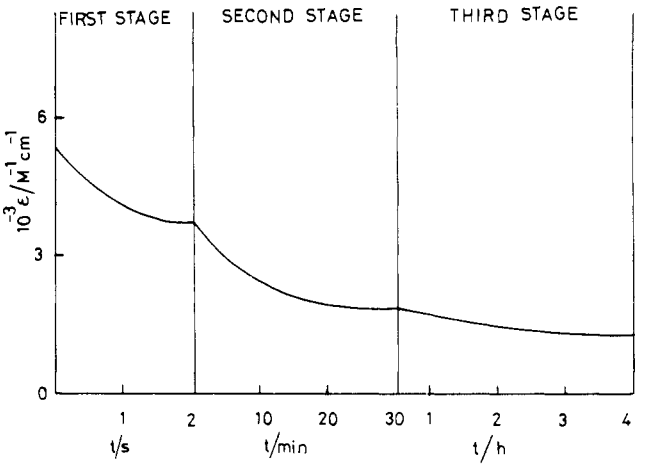

Figure 1. Changes in absorbance at 360 nm with time for the $[Co(sep)]^{2+}$ (1.6×10^{-3} M) reduction of *T. zostericola* metmyoHr (1.6×10^{-5} M) at 25 °C, pH 8.2, and I = 0.15 M (Na_2SO_4).

Kinetics. Fast stages, $k_{obsd} > 10^{-2}$ s^{-1}, were monitored by using a Dionex D-110 stopped-flow spectrophotometer complete with logarithmic amplifier. The output was stored digitally on a Datalab DL901 transient recorder, which was interfaced to a Commodore PET 2001-16K computer. Absorbance traces were displayed on a Textronix 5150 storage oscilloscope. For those runs that reached a satisfactory absorbance (A_∞) value, a simple program was used to obtain first-order rate constants (k_{obsd}) from the slope of ln ($A_t - A_\infty$) against time graphs. Such plots were linear to at least 3 half-lives. Where stable A_∞ values (for the first stage) were not observed due to the incidence of the second stage, the Guggenheim method was used to determine k_{1obsd}.[28]

Slower stages were monitored by conventional UV–vis spectroscopy on either a Perkin-Elmer 554 or Lambda 5 instrument. A standard consecutive reaction treatment[29] was employed where appropriate, i.e. for k_{2obsd} and k_{3obsd}.

Treatment of Data. Unweighted linear least-squares programs were used.

Results

The reduction of metmyohemerythrin was studied at pH 6.3 and 8.2. In each case three stages were identified (e.g. Figure 1), the first requiring the stopped-flow method and the latter two conventional spectrophotometric studies.

First Stage. First-order rate constants k_{1obsd} were obtained with $[Co(sep)]^{2+}$, $[Co(9\text{-}aneN_3)_2]^{2+}$, and $[V(pic)_3]^-$ as reductants. In all cases linear dependences on reductant concentrations were observed

$$k_{1obsd} = k_1[\text{reductant}] \quad (1)$$

as illustrated in Figure 2, and second-order rate constants k_1 could be obtained directly. Values of k_1 (M^{-1} s^{-1}) are as follows. At pH 8.2: $[Co(sep)]^{2+}$, $(1.4 \pm 0.2) \times 10^3$; $[Co(9\text{-}aneN_3)_2]^{2+}$, 33 ± 3; $[V(pic)_3]^-$, $(4.4 \pm 0.2) \times 10^3$. At pH 6.3: $[Co(sep)]^{2+}$, $(2.2 \pm 0.1) \times 10^3$; $[Co(9\text{-}aneN_3)_2]^{2+}$, 49 ± 6; $[V(pic)_3]^-$, $(3.0 \pm 0.8) \times 10^5$. A comparison with the rate constant already reported for dithionite[30] is given in Table II. We did not choose to explore the effects of pH in greater detail since these appear to be similar to those observed for the octamer.[4] The first stage of the reaction with $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$ was not studied.

Second and Third Stages. Rate constants were obtained from a standard consecutive treatment. Values of k_{2obsd} and k_{3obsd} with different reductants are listed in Tables III and IV. At pH 8.2 values of k_{2obsd} were found to be independent of the identity and concentration of reductant giving $k_2 = (3.9 \pm 0.2) \times 10^{-3}$ s^{-1}. At pH 6.3, with $[Co(sep)]^{2+}$ and $[Co(9\text{-}aneN_3)_2]^{2+}$ as reductants (Figure 3), saturation kinetic behavior was observed for k_{2obsd} with

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Table I. Rate Constants k_{1obsd}(25 °C) for the First Stage of Reduction of *T. zostericola* metmyoHr (1.9×10^{-5} M) with $[Co(sep)]^{2+}$, $[Co(9\text{-}aneN_3)_2]^{2+}$, and $[V(pic)_3]^{-}$[a]

reductant	pH	10^3[reductant], M	k_{1obsd}, s^{-1}
$[Co(sep)]^{2+}$	8.2	5.76	8.16
	8.2	4.61	6.03
	8.2	3.46	4.29
	8.2	2.30	2.36
	8.2	0.58	0.80
	6.3	5.86	12.9
	6.3	4.69	10.0
	6.3	3.52	7.53
	6.3	2.34	4.82
	6.3	1.17	2.11
$[Co(9\text{-}aneN_3)_2]^{2+}$	8.2	6.20	0.205
	8.2	4.47	0.146
	8.2	3.10	0.101
	8.2	2.23	0.084
	6.3	4.70	0.231
	6.3	2.82	0.129
	6.3	2.35	0.098
	6.3	0.94	0.045
$[V(pic)_3]^{-}$	8.2	0.49	2.16
	8.2	0.34	1.42
	8.2	0.25	1.10
	8.2	0.15	0.66
	6.3	0.20	60.5
	6.3	0.10	32.6

[a] Buffers were Tris for pH 8.2 and Mes for pH 6.3; I = 0.15 M (Na_2SO_4).

Table II. Summary of Second-Order Rate Constants k_1(25 °C) for the First Stage of Reduction of *T. zostericola* metmyoHr (I = 0.15 M (Na_2SO_4))

		k_1, M^{-1} s^{-1}	
	$E°$, V	pH 8.2	pH 6.3
$[Co(sep)]^{2+}$	−0.30[a]	1.4×10^3	2.2×10^3
$[Co(9\text{-}aneN_3)_2]^{2+}$	−0.40[b]	33	49
$[V(pic)_3]^{-}$	−0.41[c]	4.4×10^3	3.0×10^5
dithionite	−0.66[d]	1.1×10^6[f]	

[a] Reference 20. [b] Reference 21. [c] Reference 24. [d] Value indicated for SO_2^-: Mayhew, J. *Eur. J. Chem.* **1978**, *85*, 535. [e] Reference 26. [f] Reference 30.

limiting rate constants of 3.9×10^{-3} and 6.9×10^{-3} s^{-1}, respectively. At the same pH, with negatively charged reactants no dependence on the concentration of reductant was observed (Figure 3), and k_{2obsd} corresponds to k_2. Values of 10^3k_2: $[V(pic)_3]^-$, 2.3 ± 0.2 s^{-1}; dithionite, 2.2 ± 0.2 s^{-1}. These latter two values are within experimental error the same. It was more difficult to obtain rate constants k_{3obsd} (Table IV) for the third stage due to the small absorbance change, the duration of experiments (O_2 sensitivity), and tendency to get some (generally faint) cloudiness of solutions. In spite of allowances made, a difference of a factor of 4–5 in rate constants was obtained. For all reactions for which rate constants are quoted quantitative production of deoxymyoHr was observed; $\Delta\epsilon$ = 450 M^{-1} cm^{-1} at 390 nm for the third stage. No dependence on reductant concentration was observed. With $[V(pic)_3]^-$ and dithionite the same rate constants (k_3) were obtained: (1.58 ± 0.10) × 10^{-4} and (2.0 ± 0.2) × 10^{-4} s^{-1} at pH 6.3 and 8.2, respectively. At pH 8.2, with the positively charged reductants rate constants 10^4k_3 for $[Co(sep)]^{2+}$ (9.3 s^{-1}), $[Co(9\text{-}aneN_3)_2]^{3+}$ (7.4 s^{-1}), and $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$ (3.9 s^{-1}) are different (Table IV). With these reductants at pH 6.3 cloudiness was observed during the third stage, which excluded quantitative measurements.

Additional experiments with $[Co(sep)]^{2+}$ (1.6×10^{-3} M) and dithionite (1.5×10^{-3} M) in which the protein concentration was varied over a wider range (0.8–4.80) × 10^{-5} M gave no significant variation of rate constants k_2 and k_3 at pH 6.3 and 8.2.

Stoichiometry of Different Stages. Consumption of reductant for each stage of the reaction was determined from spectrophotometric measurements using 2×10^{-5} M metmyoHr and (2–5)

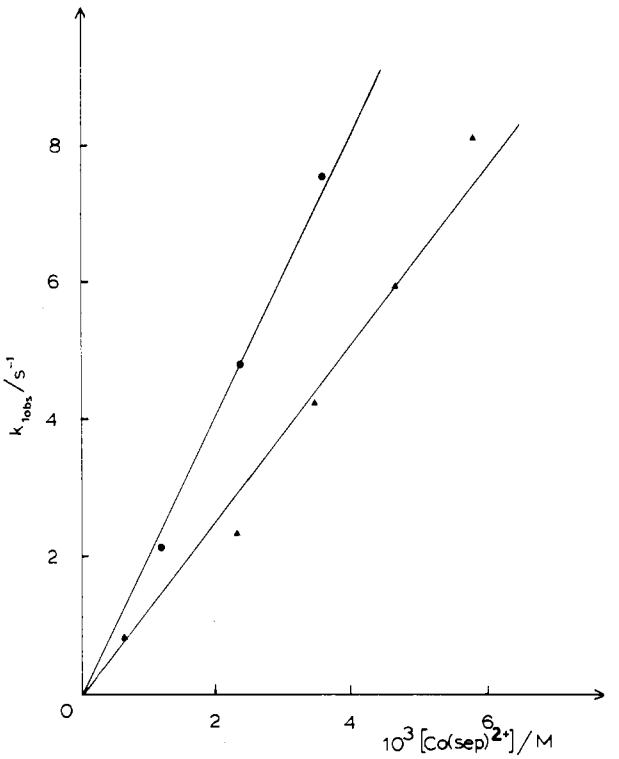

Figure 2. Dependence of first-order rate constants k_{1obsd} (25 °C) for the first stage of the $[Co(sep)]^{2+}$ reduction of *T. zostericola* metmyoHr (1.9×10^{-5} M) on concentration of reductant at pH 8.2 (▲) and 6.3 (●) and I = 0.15 M (Na_2SO_4).

Table III. Rate Constants k_{2obsd}(25 °C) for the Second Stage of Reduction of *T. zostericola* metmyoHr ((1.6–5.0) × 10^{-5} M) with $[Co(sep)]^{2+}$, $[Co(9\text{-}aneN_3)_2]^{2+}$, $[V(pic)_3]^-$, $[Cr(15\text{-}aneN_4)]^{2+}$, and Dithionite[a]

reductant	pH	10^3[reductant], M	10^3k_{2obsd}, s^{-1}
$[Co(sep)]^{2+}$	8.2	1.60	4.1
	8.2	0.16	4.0
	6.3	6.00	3.4
	6.3	4.5	3.2
	6.3	2.80	2.96
	6.3	1.60	2.85
	6.3	1.10	2.22
	6.3	1.00	1.89
	6.3	0.57	1.54
	6.3	0.40	1.20
$[Co(9\text{-}aneN_3)_2]^{2+}$	8.2	8.00	3.9
	8.2	1.60	4.1
	8.2	1.60	3.9
	6.3	6.63	5.50
	6.3	6.00	5.45
	6.3	5.00	5.25
	6.3	4.00	4.81
	6.3	1.60	3.40
	6.3	1.00	2.72
	6.3	0.80	2.56
	6.3	0.63	1.86
	6.3	0.50	1.55
$[Cr(15\text{-}aneN_4)]^{2+}$	8.2	1.00	3.9
	8.2	0.33	3.8
$[V(pic)_3]^-$	8.2	1.00	3.7
	8.2	0.33	3.9
	6.3	4.31	2.25
	6.3	3.10	2.31
	6.3	1.72	2.07
	6.3	0.86	2.43
dithionite	8.2	1.50	3.7
	8.2	0.75	3.9
	6.3	2.00	2.00
	6.3	0.50	2.36

[a] Buffers were Tris at pH 8.2 and Mes at pH 6.3; I = 0.15 M (Na_2SO_4).

Table IV. Rate Constants k_{3obsd}(25 °C) for the Third Stage of Reduction of *T. zostericola* metmyoHr ((5.0–1.6) × 10^{-5} M) with $[Co(sep)]^{2+}$, $[Co(9\text{-}aneN_3)_2]^{2+}$, $[V(pic)_3]^-$, $[Cr(15\text{-}aneN_4)]^{2+}$, and Dithionite[a]

reductant	pH	10^3[reductant], M	$10^4 k_{3obsd}$, s^{-1}
$[Co(sep)]^{2+}$	8.2	1.60	9.3
	8.2	0.16	9.2
$[Co(9\text{-}aneN_3)_2]^{2+}$	8.2	8.00	7.2
	8.2	1.60	7.5
$[Cr(15\text{-}aneN_4)]^{2+}$	8.2	4.74	3.9
	8.2	1.00	4.0
$[V(pic)_3]^-$	8.2	1.00	1.97
	8.2	0.33	1.75
	6.3	1.00	1.56
dithionite	8.2	1.50	1.97
	8.2	0.75	2.14
	6.3	2.00	1.68
	6.3	0.50	1.52

[a] Buffers were Tris for pH 8.2 and Mes for pH 6.3; I = 0.15 M (Na_2SO_4).

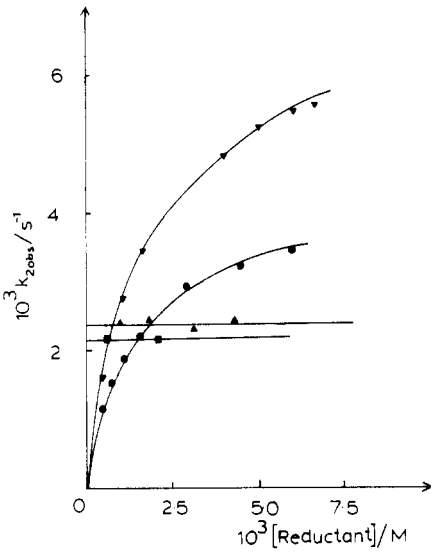

Figure 3. Variation of first-order rate constants k_{2obsd}(25 °C) for the second stage of the reduction of *T. zostericola* metmyoHr (1.6 × 10^{-5} M) on concentration of reductants $[Co(sep)]^{2+}$ (●), $[Co(9\text{-}aneN_3)_2]^{2+}$ (▼), $[V(pic)_3]^-$ (▲), and dithionite (■) at pH 6.3 and I = 0.15 M (Na_2SO_4).

× 10^{-4} M $[Cr(phen)_3]^{2+}$ as reductant ($\Delta\epsilon$ at 850 nm for one-electron oxidation is 3650 M^{-1} cm^{-1}).[4] Results indicated that 1 equiv of reductant (0.94 ± 0.10) was consumed in the first stage, followed by a further 1 equiv (1.01 ± 0.18) in the second; k_{2obsd} = 4.0 × 10^{-3} s^{-1} at pH 8.2. There was no apparent variation in the stoichiometry with pH (6.3 and 8.2). The larger error for the second stage is attributed to the longer duration of experiments. Semi-metmyo forms have been characterized previously.[30] No clearly defined consumption of $[Cr(phen)_3]^{2+}$ was observed in the third stage.

Spectra. UV–vis spectra were recorded at pH 8.2 (Figure 4) for the products at the end of the first, second, and third stages with dithionite as reductant. Addition of only 1 equiv of dithionite to metmyoHr (0.30 × 10^{-4} M) generated the spectrum of the product of the first stage of reduction (B). Addition of 2 equiv generated initially the first-stage product (B), and further reaction yielded the product generated in the second stage (C). A slow absorbance change then occurred, generating a spectrum of deoxymyoHr (D). Spectra are the same at pH 6.3 as at pH 8.2 except in the case of spectrum B when minor differences were noted (spectrum B′) (Figure 4).

Discussion

It has been shown that the first stage of the reduction of metmyoHr consumes 1 equiv of reductant, indicating that the

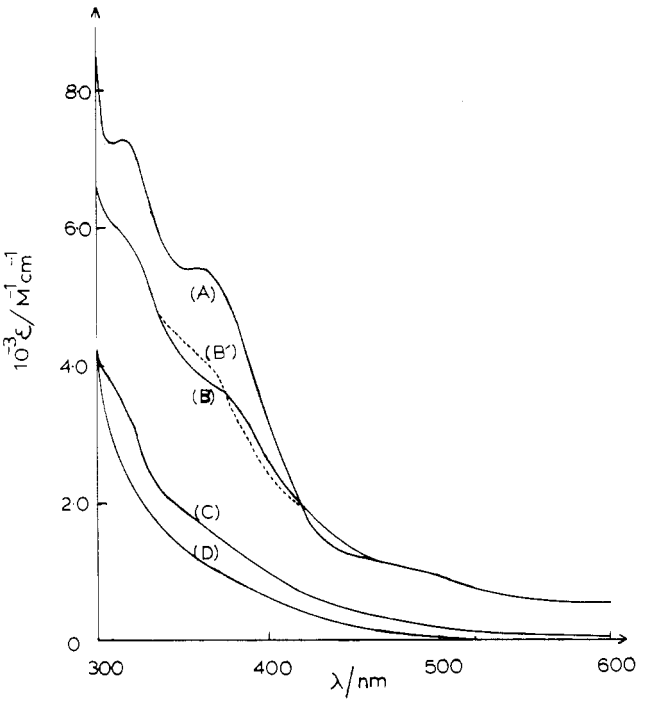

Figure 4. Spectra observed at different stages in the reduction of *T. zostericola* metmyoHr (0.3 × 10^{-4} M): (A) spectrum for metmyo; (B) spectrum for (semi-metmyo)$_r$; (C) spectrum after isomerization and further (rapid) 1-equivalent reduction; (D) spectrum after isomerization to deoxy. All spectra were obtained with dithionite as reductant at pH 8.2, except spectrum B′, which was obtained at pH 6.3.

initial product is a semi-metmyo form, as proposed by Wilkins and colleagues.[30] Second-order rate constants k_1 (Table II) vary with the identity of the reductant and in the case of the positively charged reductants show a mild dependence on pH as observed with the octamer.[4] With the negatively charged reductant $[V(pic)_3]^-$ a much larger effect of pH is observed, possibly (in part) due to the more favorable electrostatics. The relatively slow interconversion of acid and base forms B and C of metHr, which have distinctive UV–vis spectra, has been noted. If this slow equilibrium was influential in the present studies, biphasic kinetics would be obtained in the first stage. No such behavior is observed. Therefore, as in the case of the octamer, it is concluded that the 6-coordinate and not the 5-coordinate Fe(III) is reduced in the first stage.[31] This interpretation requires that coordination at the variable 5/6-coordinate Fe(III) site does not affect the reactivity at the 6-coordinate Fe(III).

At pH 8.2 further reduction of semi-metmyoHr occurs with a rate constant that is independent of the concentration (6-fold variation) and identity of the reductant, consistent with an intramolecular process (k_2 = 4.0 × 10^{-3} s^{-1}). Stoichiometric measurements have indicated that the second reducing equivalent is consumed in this stage. The rate constant agrees closely with that observed in the case of the octamer (3.7 × 10^{-3} s^{-1}), suggesting a common rate-controlling process. In the octamer case Wilkins et al. have suggested long-distance (~30 Å) intramolecular electron transfer between subunits for this process.[5,6] Such an interpretation now seems unlikely, in view of the similarity of rate processes in the reduction of monomer and octamer forms. At pH 6.3, with negatively charged reductants, first-order rate constants k_{2obsd} are again independent of the concentration and identity of the reductant, and k_2 (2.0 × 10^{-3} s^{-1}) agrees exactly with the value observed for the octamer.[4] With the positively charged reductants however saturation kinetic behavior is observed (Figure 3). We have difficulty in explaining this, particularly as the limiting rate constants are not the same, and neither agrees with the rate constant(s) obtained for $[V(pic)_3]^-$ and dithionite.

(31) Maroney, M. J.; Lauffer, R. B.; Que, L.; Kurtz, D. M., Jr. *J. Am. Chem. Soc.* **1985**, *107*, 6445.

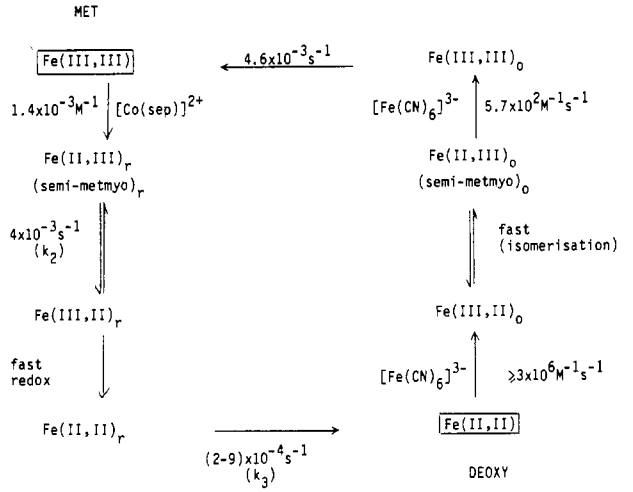

Figure 5. Reaction scheme proposed, with stable met and deoxy forms enclosed. The data for the deoxy to met conversion are from ref 30. Conformationally different states generated on reduction (r) and oxidation (o) are indicated.

Clearly, some interplay of redox and isomerization is implied, one possibility being that direct reduction parallels the isomerization step. It is possible that association of the positive complexes on the protein (metmyo has a charge of 3- at pH ~7 from its amino acid composition) may influence the rate of isomerization at this stage.

It has been demonstrated in the stoichiometry experiments with $[Cr(phen)_3]^{2+}$ that both Fe(III)'s are reduced on completion of the second stage (~30 min). The third stage of reaction, with a smaller absorbance change (Figure 1), may represent a further isomerization step, in this case involving the Fe(II,II) state. EPR measurements ~1 h after commencement of the reaction have indicated a less than expected paramagnetism,[30] and that on exposure to O_2 formation of oxyHr is incomplete. It might appear therefore that the deoxy form capable of binding O_2 is generated in this third stage. Variations in the rate constant k_3 are possibly related to specific effects of the kind noted previously for 1- common anions.[32]

A full mechanism for interconversion of the met and deoxy forms of myoHr must consider the following: (i) the two 1-equiv Fe(III) → Fe(II) reduction steps; (ii) the possibility of an Fe(II,III) to Fe(III,II) isomerization, since the two Fe atoms are not identical; (iii) the conversion of μ(O) → μ(OH) (assumed to be bridging); (iv) the loss of the terminal OH^- of the met form (present at pH 8.2) on reduction to deoxy. At pH 8.2 and 6.3 three stages are involved, and (iv) does not appear to feature in the changes observed. We assume that it accompanies one of the other stages as a relatively fast non-rate-determining process. The observation of the slow (third stage) Fe(II,II) isomerization is important, particularly as Wilkins and colleagues[30] have identified a third stage in the oxidation of deoxymyo with $[Fe(CN)_6]^{3-}$ and assigned this to an Fe(III,III) isomerization. Together these observations lead us to suggest a mechanism for interconversion (Figure 5), in which the conformations of the semi-metmyo forms closely resemble the met and deoxy states, respectively, from which they are generated. Slow structural changes are possible in the ultimate stages of the reduction and oxidation of met and deoxy leading to formation of the stable states. Such changes may involve slow μ-oxo to μ-hydroxo interconversion, but this is not supported by NMR studies on the semi-met forms.[31]

The mechanism proposed is able to account for the different intermediate spectra and different routes observed for the oxidation of deoxy and the reduction of met forms, which has been a difficulty also in explaining the corresponding interconversions of the octamer.[4] A point to note in the $[Fe(CN)_6]^{3-}$ oxidation of deoxy is that the first two stages are both first order in oxidant concentration.[30] There is therefore no evidence for a slow isomerization step, Fe(III,II) → Fe(II,III), and if this is present, then it must occur as a rapid sequel to the first redox stage, as indicated in Figure 5.

Similar processes are envisaged for the octamer, but a notable difference (with *T. zostericola* at least) is that no isomerization of the Fe(III,III) and Fe(II,II) forms has yet been detected. These possibilities will be discussed further in a subsequent paper on the oxidation of the deoxy octamer form.[33]

Although we have chosen to describe the deoxymyo form as having a μ-hydroxo ligand, this may not be precisely the situation. In structure A we have in fact adopted the formulation indicated in ref 7 but note that an unsymmetrically bound (or monodentate) hydroxo group is also possible. This structural feature requires clarification and may be crucial to the mechanism proposed (Figure 5).

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

Registry No. $[Co(sep)]^{2+}$, 63218-22-4; $[Co(9\text{-}aneN_3)_2]^{2+}$, 89637-25-2; $[V(pic)_3]^-$, 76298-57-2; $[Cr(15\text{-}aneN_4)]^{2+}$, 70833-04-4; dithionite, 14844-07-6.

(32) Bradič, Z.; Conrad, R.; Wilkins, R. G. *J. Mol. Biol.* **1977**, *252*, 6069.

(33) Armstrong, G. D.; Sykes, A. G., to be submitted for publication.