A more complete picture is given by Monte Carlo methods. The largest such calculation to data has been the study of solvated lysozyme done by Hagler and Moult.[32] In their treatment, a large number of water molecules surrounding the entire protein were included, a set of configurations was randomly generated, and their energy was calculated by using empirical force functions. The contribution of each configuration to the total energy was weighted by the Boltzmann factor. Hagler and Moult were able to show that this procedure correctly predicts the crystallographic R factors for a small peptide in an aqueous crystal. For lysozyme it appears that one or two layers of water molecules are strongly localized at the protein surface, especially near side chains. Water molecules further away from the surface than about 4 Å are not localized but are very much like "bulk" water. Clementi et al., in a Monte Carlo study relevant to the present work, calculated the solvent structure around a Zn^{2+} ion[33] and also around Zn plus 27 nearby residues for the active site of HCAB.[34] Their work indicates one or two strongly localized layers of waters near Zn both for the isolated ion and the active site.

The next logical step needed to follow a treatment such as that of Clementi et al. is calculation of the electrostatic potential in the enzyme active site using solvent coordinates obtained from the Monte Carlo calculations. Computer programs for such electrostatic calculations are not yet available for carrying this out, but we can make a preliminary estimate on the maximum amount by which the dipoles of sovlent molecules can modify the in vacuo active site potential assuming that only about one laye of water is localized. The rotational and translational average of the dipole of a molecule of bulk water is obviously zero. Most of the correction, therefore, will come from the single layer of water molecules at the protein surface and then only if the molecules are rotationally oriented as well as translationally fixed. The maximum possible effect will occur when each molecule dipole is at right angles to the isopotential contour lines. Our model consists of a central point charge surrounded by a cage of six water molecules. Three point charges are used to represent the atoms of each water molecule. The "oxygens" are placed 3 Å from the central point charge. The symmetry axis of the water passes through the charge; i.e., the dipoles of the waters are fully aligned. Polarization and charge transfer are neglected. Figure 8 compares the potential field emanating from central charges of magnitude 0.1, 0.5, and 1.0 unit surrounded by six waters. As can be seen, there is little change in the contours for central charges much greater than 0.1. In the case of protein potentials this would lead us to expect that the potential produced by a charged residue would not be affected very much by one shell of strongly oriented water. Only in regions of low-field strength would the contribution from oriented water dipoles be significant, and in that case the dipoles would not be strongly oriented. Thus, in general, we do not expect solvent alone to make gross changes in those in vacuo maps for which charged residues are included. Counterions, on the other hand, might make a strong difference.

Acknowledgment. The authors wish to thank Eric Henry and Dr. C. S. Wright for patient instruction in the use of HYDRO and the Rao and Rossmann routine. We are also grateful to Dr. D. M. Hayes, for providing his Mulliken point charge library. This work was supported by the National Institutes of Health (Grant GM 26462).

Supplementary Material Available: The primary sequence for HCAB and HCAC including the recent revisions of Henderson et al. (Table II); the matrix transform to rotate and translate coordinates from the HCAB frame to the HCAC frame (Table III); assumed hydrogen bond networks involving serine, threonine, tyrosine, and neutral histidine (Table IV); description and list (Table V) of the point charge library for amino acids (22 pages). Ordering information is given on any current masthead page.

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The Reactions of Semi-Met Forms of Hemerythrin

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Abstract: The properties of $(\text{semi-met})_O$, produced by one-electron oxidation of deoxyhemerythrin, and $(\text{semi-met})_R$, produced by one-electron reduction of methemerythrin, are described. Both forms react with F^-, Br^-, SCN^-, CN^-, and N_3^- to give, with each anion, a semi-met adduct. Full kinetic data are given for the formation of the azide adduct from met, $(\text{semi-met})_O$ and $(\text{semi-met})_R$ forms of hemerythrin at pH 6.3 and 8.2 and 25 °C. The stability of the met-azide form is much higher than that of the semi-met form, mainly arising from a much smaller dissociation rate constant. The rate parameters for the disproportionation of the two semi-met forms at pH 8.2 and 25-35 °C were determined, and the role of disproportionation in reactions of the semi-met forms with O_2, $Fe(CN)_6^{3-}$, and $S_2O_4^{2-}$ is delineated. The processes are discussed in terms of the octomeric structure of the protein, and it is concluded that disproportionation results from an intramolecular electron transfer involving a rate constant of 2.7×10^{-3} s^{-1} over distances of 28-30 Å, between each (binuclear) iron unit. Data are for protein from *Themiste zostericola* and (limited) from *Phascolopsis gouldii* and *Themiste dyscritum*.

Hemerythrin occurs in the erythrocytes of certain marine worms in a polymeric, usually octameric, form. Each subunit, mol wt ~13 500, contains two linked nonheme irons, but there is still a question whether amino acids or oxy bridging is involved.[1-4] The form with both irons in the oxidation state +2 (deoxy) interacts rapidly and reversibly with oxygen.[5] It is easily oxidized to the met form containing irons only in the +3 oxidation state. This is no longer O_2 sensitive but does react with a number of anions

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to form adducts with a wide range of stabilities.[6-8] A detailed study of the dithionite reduction of methemerythrin[9] and the ferricyanide oxidation of deoxyhemerythrin[10] has led to the appearance of two distinct semi-met forms of hemerythrin, $(semi\text{-}met)_R$ and $(semi\text{-}met)_O$, respectively, in both of which formally one of the binuclear irons is +3 and the other +2. These have different electronic[11] and EPR[12] spectra. Both semi-met forms of the protein from the sipunculid *Themiste zostericola* change slowly to the same spectrum which is identical with that of an equimolar mixture of met- and deoxyhemerythrin.[11] At the same rate the EPR spectra disappear.[12] This behavior is the result of a first-order disproportionation (≥85% complete) which occurs within the octamer.

$$(Fe^{II}Fe^{III})_8 \rightarrow (Fe_2^{II})_4(Fe_2^{III})_4 \quad (1)$$

The disproportionation, aside from being a fascinating intramolecular electron-transfer process, is important because it controls a number of redox reactions of the semi-met forms and therefore plays an important role in the interconversion of the met, oxy, and deoxy forms.

Some properties of the semi-met forms at pH 8.2 using protein from *Themiste zostericola* were described in a recent communication.[11] In this paper we detail and discuss those and other properties of the semi-met forms at pH 6.3 and 8.2. The bulk of the work concerns protein from *Themiste zostericola*, but some limited data are provided for protein from *Phascolopsis gouldii* and *Themiste dyscritum* for which the octameric structures are known.[13,14]

Experimental Section

Materials. The marine worms *P. gouldii*, *T. zostericola*, and *T. dyscritum* were obtained respectively from Marine Biological Laboratory, Woods Hole, MA, Pacific Biomarine Supply, Venice, CA, and Oregon Institute of Marine Biology (courtesy of Dr. R. C. Terwilliger). Oxyhemerythrin was obtained from the coelomic fluid of the worms[15-17] in which form it was stored in a freezer. The material from *T. zostericola* after about 6–8 weeks storage gave poor kinetics traces for some of the reactions studied. Reprecipitation with $(NH_4)_2SO_4$ gave material which behaved the same as the fresh protein. After 10–12 weeks the protein was discarded. A number of the reactions, including the disproportionations, have been repeated many times with different samples of *T. zostericola* with quite consistent results. Methemerythrin, $(semi\text{-}met)_R$, and deoxyhemerythrin were prepared as described previously.[10] The $(semi\text{-}met)_O$ form was produced by mixing equimolar solutions of $Fe(CN)_6^{3-}$ and deoxyhemerythrin (expressing the concentration of the latter on the basis of the monomeric unit, mol wt 13 500). The azide adduct of either semi-met form was prepared by adding azide ion to semi-met as soon as it had been prepared and in sufficient concentration (10.0 mM) to ensure completion of formation within seconds. All other materials used were chemically pure and were either commercially available or prepared by literature methods.

Measurements. All preparations and reactions of deoxy and semi-met forms were carried out with scrupulous exclusion of O_2 using cells with serum caps and gas-tight syringes. Kinetics and spectrophotometry measurements utilized a Beckman 24 recording spectrophotometer and a Gibson-Durrum stopped-flow apparatus. Unless otherwise specified all reactions were run at 25 °C and I = 0.15 M, with added Na_2SO_4. Experiments at pH 6.3 used 0.05 M Mes and at pH 8.2, 0.05 M Tris was employed. The analysis of the products of the semi-met reactions was carried out as follows (characteristics of *Themiste zostericola*): deoxyhemerythrin was determined as the oxy species ($\epsilon_{500\,nm} = 2.05 \times 10^3\ M^{-1}\ cm^{-1}$) by passing O_2 over the solution; when SCN^- was also present in solution, the oxy form autoxidized to met-SCN^- and absorbance of this was used as a further check; methemerythrin was analyzed as the thiocyanate at 452 nm ($\epsilon = 4.4 \times 10^3\ M^{-1}\ cm^{-1}$) or azide at 446 nm ($\epsilon = 3.5 \times 10^3\ M^{-1}\ cm^{-1}$) adduct. In most of the kinetics studies the concentration of protein used was 0.05–0.3 mM (always on the basis of the monomer (mol wt 13 500)). The spontaneous changes of the semi-met forms were followed directly at a number of wavelengths (360, 400, 420, 450 nm) or by loss of EPR signal.[12] The rate of loss of the semi-met form could also be monitored by reacting samples stored for various times in one syringe with N_3^- ion contained in the other syringe.[9] The amplitudes of the rapid and slower reactions at 450 nm could be assigned to the semi-met and met forms, respectively. The reactions of the semi-met forms with O_2 were monitored at 500 nm. Reactions of met and semi-met forms with N_3^- ion and of the azide adducts formed were monitored at 450 nm, near the absorbance peaks for the adducts. Because of the spontaneous disproportionation of the semi-met forms, the reaction of $(semi\text{-}met)_R$ with N_3^- was most conveniently studied by reacting methemerythrin (20 μM) in one syringe with a mixture of $S_2O_4^{2-}$ (1.0 mM) and N_3^- (0.1–1.0 mM) in the other syringe of a stopped-flow apparatus. For the corresponding reaction of $(semi\text{-}met)_O$, deoxyhemerythrin (20 μM) was mixed with a $Fe(CN)_6^{3-}$ (0.1 mM), N_3^- (0.1–1.0 mM) mixture.[10] In both cases, the semi-met formed rapidly (within about 5–10 s) and the reaction with N_3^- was virtually complete before further reactions of semi-met or semi-met-N_3^- adducts with $S_2O_4^{2-}$ or $Fe(CN)_6^{3-}$ occurred.[9,10] When more than one monitoring method or wavelength was used, consistent rate data were obtained. Second-order reactions were carried out with pseudo-first-order conditions, and, in general, excellent first-order traces were obtained often up to 3–4 $t_{1/2}$'s. The rate constants were generally accurate to ±3% while values of ΔH^* and ΔS^* were ±1 kcal mol^{-1} and ±3, eu, respectively.

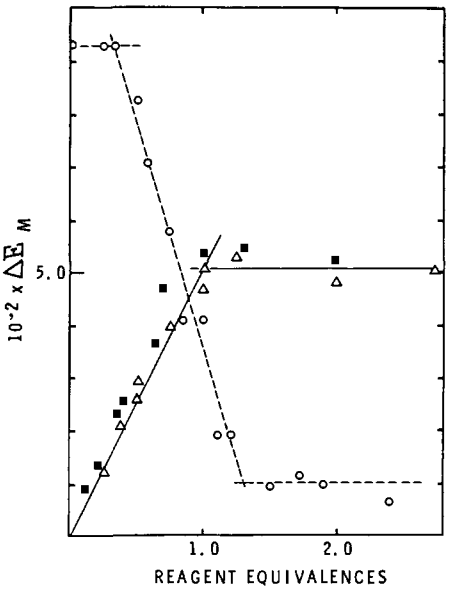

Figure 1. Spectral titrations of deoxyhemerythrin (△) and disproportionated product (■) with $Fe(CN)_6^{3-}$ (λ = 550 nm) and of methemerythrin (○) with $S_2O_4^{2-}$ (λ = 420 nm). Readings were taken within 5–10 s of addition of redox reagent, and several separate titrations were performed. Reagent equivalences are in electrons added or removed per single Fe_2^{III} or Fe_2^{II} unit of the protein (*Themiste zostericola*).

Results

Reactions were studied at pH 6.3 and 8.2. These are pHs for which we have a considerable amount of data, and they are close to the limits of the pH range which can be conveniently studied with this protein. There was, even then, a tendency for turbidity to develop at pH 6.3 during the slower reactions of the reduced forms of hemerythrin. The results refer mainly to protein from *Themiste zostericola*. Some more limited data using hemerythrin from the other sipunculids are included at the end of this section.

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Table I. Characteristics for Reactions of Semi-met Forms of Hemerythrin (*Themiste zostericola*) at 25 °C

reactant	pH	k, s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, eu	product[a]
		(semi-met)$_R$			
spontaneous	8.2	2.2×10^{-3}	20.6	−1	$^1/_2$(met,deoxy)
	6.3	1.2×10^{-3}			$^1/_2$(met,deoxy)
O_2	8.2	2.1×10^{-3} [b]			$^1/_2$(met,oxy)
F^-, Br^-, SCN^-	6.3				semi-met adduct
CN^-	8.2				semi-met adduct
SCN^-	8.2	1.9×10^{-3} [c]			$^1/_2$(met SCN$^-$, deoxy)
$Fe(CN)_6^{3-}$	8.2	4×10^5 [d]			met
	6.3	$>>10^5$ [d]			met
$S_2O_4^{2-}$	8.2	1.1×10^{-3} [e]	23.3	+6	deoxy
	6.3	7.0×10^{-4} [e]			deoxy
		(semi-met)$_O$			
spontaneous	8.2	2.7×10^{-3}	22.9	+6	$^1/_2$(met,deoxy)
	6.3	1.1×10^{-3}			$^1/_2$(met,deoxy)
O_2	8.2	2.1×10^{-3} [b]			$^1/_2$(met,oxy)
F^-, Br^-, SCN^-	6.3				semi-met adduct
CN^-	8.2				semi-met adduct
$Fe(CN)_6^{3-}$	8.2	1.3×10^{-3} [f]	21.9	+1	met
	6.3	5.5×10^{-4} [f]			met
$S_2O_4^{2-}$	8.2	4×10^5 [d,g]			deoxy

[a] See text. [b] Independent $[O_2]$, 0.16–0.52 mM. [c] In low concentration (1 mM) SCN^- monitors disproportionation; at high concentrations (0.1 M) adduct formation occurs. [d] M^{-1} s^{-1}. [e] Data relate to second phase of reaction.[9] [f] Independent $[Fe(CN)_6^{3-}]$.[10] [g] Rate constant for reaction with SO_2^-.

Production of Semi-Met Forms. There was a rapid reduction of methemerythrin by dithionite and a rapid oxidation of deoxyhemerythrin by $Fe(CN)_6^{3-}$ to produce species which were only slowly further reduced or oxidized, respectively. This allowed the easy determination of the stoichiometry of the rapid changes by appropriate spectral titrations. These were characterized as a one-electron reduction or one-electron oxidation, respectively (Figure 1). The immediate redox products were therefore designated as, respectively, (semi-met)$_R$ and (semi-met)$_O$ forms. We were also able to produce the (semi-met)$_R$ form from methemerythrin by using two other reducing systems. Light irradiation of an anaerobic mixture of methemerythrin, riboflavin, and EDTA[18-20] produced a biphasic loss of absorbance (e.g., at 420 nm). Within 60–90 s (with our conditions[10]), the semi-met stage was reached. Reduction to the deoxy form required a further 30–45-min light exposure. The reduced form of methyl viologen also reduced methemerythrin rapidly to the semi-met form. The properties of the semi-reduced form produced by the three methods were the same.

A number of oxidants were tried at pH 8.2 in place of $Fe(CN)_6^{3-}$ in order to produce (semi-met)$_O$ from deoxyhemerythrin. The reagents CoEDTA$^-$, $S_2O_8^{2-}$, $NO(SO_3)_2^{2-}$, $Co(phen)_3^{3+}$, and $Co(terpy)_2^{3+}$ reacted quite slowly with deoxyhemerythrin, even when used in relatively large concentrations (≥10 mM). Two oxidants, $IrCl_6^{2-}$ and *m*-chloroperbenzoic acid, reacted rapidly with protein, but there was little, if any, attack at the iron site as shown by a lack of absorbance decrease at 420 nm. The oxidation by $Co(terpy)_2^{3+}$ of deoxyhemerythrin from *Phascolopsis gouldii* was much faster (k = 0.62 M^{-1} s^{-1} at pH 8.2) than that from *Themiste zostericola*. Consequently, with a large excess (40 mM) of oxidant, the usual two stages seen in the formation of met when $Fe(CN)_6^{3-}$ was used could be separated, i.e., the production ($t_{1/2}$ = 28 s at pH 8.2) of an intermediate, presumably (semi-met)$_O$, which was further oxidized to met at a disproportionation-controlled rate ($k = 9 \times 10^{-4}$ s^{-1} at pH 8.2). The absorbance changes at 525 nm of the two stages corresponded to the production of one and two molecules, respectively, of $Co(terpy)_2^{2+}$ (ϵ = 740). All the experiments with oxidants were plagued by the development of turbidity in the solutions because of the high concentrations of oxidants which needed to be used. In addition, there was marked decomposition of the colored oxidants in the presence of protein, which either did not occur or was much less pronounced in the absence of protein.

Table II. Reaction Parameters for Interaction of Met- and Semi-methemerythrin (*Themiste zostericola*) with Azide Ion at 25 °C

species	pH	k_f, M^{-1} s^{-1}	k_d, s^{-1}	K,[a] M^{-1}	K,[b] M^{-1}
met	6.3	6.8	$\leq 5 \times 10^{-4}$	$\geq 1.4 \times 10^4$	$>10^4$
	6.3[c]	7.6	$<5 \times 10^{-5}$	$\geq 1.4 \times 10^5$	1.0×10^6
	8.2	1.4	$\leq 10^{-4}$	$\geq 1.4 \times 10^4$	$>10^4$
(semi-met)$_R$	6.3	2.8×10^3	~0.15	$\sim 2 \times 10^4$	$>10^5$
	8.2	33	0.01	3.3×10^3	4×10^3
(semi-met)$_O$	6.3	1.1×10^3	0.3	3.6×10^3	5×10^3
	8.2	3.2×10^2	0.2	1.6×10^3	1.2×10^3

[a] k_f/k_d. [b] Spectrally from amplitude of traces. [c] Values for *Phascolopsis gouldii*.[7]

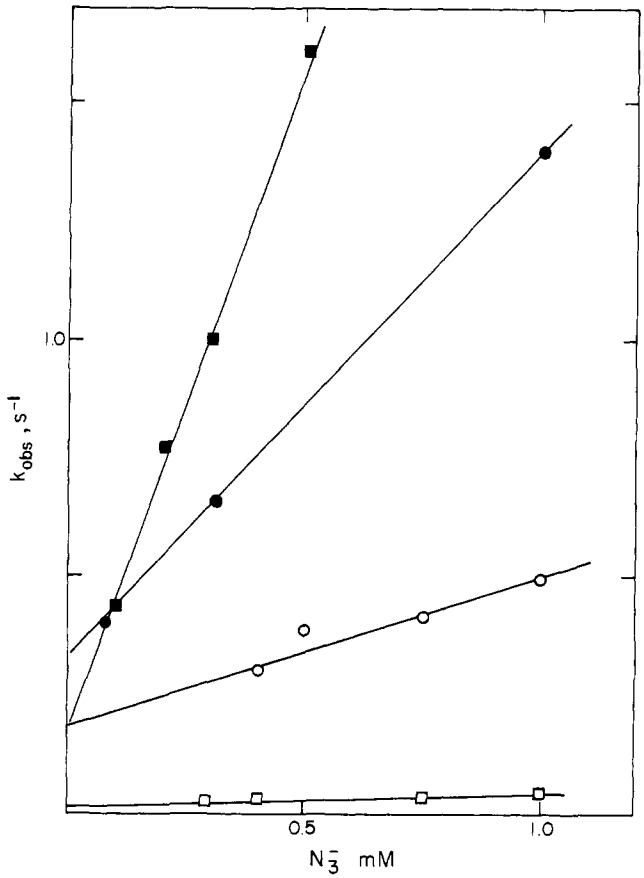

Figure 2. Plot of k_{obsd} (s^{-1}) vs. $[N_3^-]$ (mM) at 25 °C for the interaction of semi-met forms of *Themiste zostericola* hemerythrin: (semi-met)$_O$, pH 6.3 (●), pH 8.2 (○); (semi-met)$_R$, pH 6.3 (■), pH 8.2 (□).

Properties of Semi-Met Forms. The spectra of the semi-met forms differed substantially at pH 8.2.[11] This difference persisted at 6.3. The spectrum of (semi-met)$_O$ at pH 6.3 was the same as that at pH 8.2, whereas that of (semi-met)$_R$ at the two pHs differed markedly ($\epsilon_{350} = 2 \times 10^3$, $\epsilon_{400} = 8.8 \times 10^2$, $\epsilon_{450} = 4 \times 10^2$ at pH 6.3 and $\epsilon_{350} = 3 \times 10^3$, $\epsilon_{400} = 1.3 \times 10^3$, $\epsilon_{450} = 4.3 \times 10^2$ M^{-1} cm^{-1} at pH 8.2). A variety of reactions of the semi-met forms were investigated, and these are listed in Tables I and II. A number of anions formed adducts with the semi-met forms but the kinetics of binding of the semi-met forms with only N_3^- ion were determined, spectrally by stopped-flow. (Semi-met)$_O$ was produced in situ by mixing deoxyhemerythrin with a $Fe(CN)_6^{3-}$,

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N_3^- mixture and $(semi\text{-}met)_R$ by mixing methemerythrin with a $S_2O_4^{2-}$, N_3^- mixture. The semi-met forms were rapidly generated by this procedure, and their subsequent reactions with N_3^- ion were much faster than their disproportionation or further reactions of the azide. The observed first-order anation rate constants, k_{obsd}, were plotted against $[N_3^-]$, in excess, and are shown in Figure 2. From the slopes and intercepts, values of k_f and k_d in the process (2) could be calculated since for this reaction, the rela-

$$\text{semi-metHr} + N_3^- \rightleftarrows \text{semi-metHr-}N_3^- \qquad k_f, k_d, K \quad (2)$$

tionship (3) holds. Similar measurements[7] on the rates of reaction

$$k_{obsd} = k_f[N_3^-] + k_d \quad (3)$$

of methemerythrin with N_3^- were also carried out for comparative purposes. All data obtained, as well as that for methemerythrin from *Phascolopsis gouldii*,[7] are included in Table II. It is difficult to obtain accurate values for k_d from the intercepts in Figure 2 and impossible to give other than upper limit values for k_d for reaction of met forms, since the latter intercepts are near zero. Therefore only approximate values or lower limits for the formation constants could be obtained kinetically ($K = k_f/k_d$), but these checked quite well or were consistent with values obtained by analysis of the amplitudes of the spectral changes with differing N_3^- ion concentrations and with the assumption that only one N_3^- is bound per dinuclear iron unit, as is observed with met.

The azide adducts resulting from either semi-met form have very similar electronic and EPR spectra. The azide adducts obtained from $(semi\text{-}met)_R$ and from $(semi\text{-}met)_O$ reacted with $Fe(CN)_6^{3-}$ in a similar biphasic manner at pH 6.3 with the first phase a second-order oxidation by $Fe(CN)_6^{3-}$ with elimination of coordinated azide ($k = (7\text{–}9) \times 10^2\ M^{-1}\ s^{-1}$) and the second phase a reaction of methemerythrin, resulting from the first phase, with N_3^- ion ($k = 3.5\text{–}3.9\ s^{-1}$ at $[N_3^-] = 9$ mM).[10] The behavior toward H_2O_2 of the azide adducts from both semi-met forms was also similar. The oxidation of semi-met-N_3^- adducts to methemerythrin by H_2O_2 was examined at 380 nm (an isosbestic point for methemerythrin and methemerythrin azide). The second-order rate constant was 0.7–1.0 $M^{-1}\ s^{-1}$ (pH 8.2, 25 °C). It was difficult to separate kinetically the subsequent reaction of azide with methemerythrin from this first stage, but the approximate rate constant obtained was close to that obtained in separate studies of the reaction of azide ion with methemerythrin.

The only reactions investigated where the azide adducts prepared from the two semi-mets appeared to differ was in their dissociative behavior (k_d in Table II) and in their reduction by dithionite ion. Reduction of $(semi\text{-}met)_O$-N_3^- with dithionite was a first-order process independent of azide and dithionite concentrations ($k = 2.5 \times 10^{-3}\ s^{-1}$, pH 8.2) leading to facile production of deoxyhemerythrin. The reduction of $(semi\text{-}met)_R$-N_3^- on the other hand appeared to be slightly slower ($k = 2.0 \times 10^{-3}\ s^{-1}$, pH 8.2) and to be biphasic, with a small residual slow reaction which had only a small absorbance change. However, the $(semi\text{-}met)_R$-N_3^- preparation doubtless contained some met-N_3^- adduct (from disproportionation during the irradiation and subsequent manipulations), and this is reduced quite slowly by dithionite. Its presence may cause the additional feature not observed with $(semi\text{-}met)_O$-N_3^- which can be prepared almost free of met-N_3^-.

Addition of 0.1 M solutions of Br^- and F^- ions at pH 6.3 and 0.01 M solutions of CN^- at pH 8.2 to $(semi\text{-}met)_O$ and $(semi\text{-}met)_R$ hemerythrin resulted in the formation of adducts within 3–5 min. Reaction of 0.1 M solutions of SCN^- with both semi-met forms occurred within seconds at both pHs. In all adducts the anions were replaced by N_3^- ions to yield the (semi-met)-N_3^- adduct. The F^- ion was replaced more slowly than was Br^-, SCN^-, or CN^- and all substitutions by N_3^- were much slower than the interaction of (unliganded) semi-methemerythrins with N_3^- ion. Spectrally, the product of interaction of any specific anion with the $(semi\text{-}met)_O$ and $(semi\text{–}met)_R$ formed appeared identical. Spectral characteristics of the semi-met anionic adducts (in $M^{-1}\ cm^{-1}$) were for N_3^- ($\epsilon_{315} = 4.4 \times 10^3$; $\epsilon_{470} = 2.4 \times 10^3$), SCN^- ($\epsilon_{450} = 1.8 \times 10^3$), Br^- ($\epsilon_{375}^{sh} = 2.6 \times 10^3$), F^- ($\epsilon_{355}^{sh} = 3.0 \times 10^3$), and CN^- ($\epsilon_{360}^{sh} = 3.2 \times 10^3$). The values for the Br^-, F^-, and CN^- are only approximate because of the presence of some met-anion adduct formed from disproportionation during anation. Except for the azide derivative *all* semi-met species lack a peak in the 315–330-nm region.

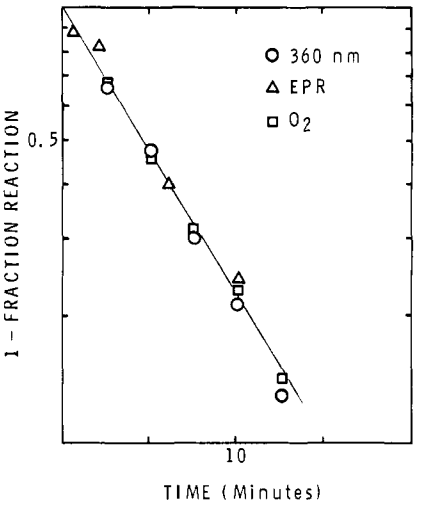

Figure 3. Semilog plots for reactions of $(semi\text{-}met)_O$ with O_2 (□) and for spontaneous disproportionation spectrally (○) and by decrease of EPR signal (△). $(Semi\text{-}met)_O$ was prepared by addition of stoichiometric amounts of $Fe(CN)_6^{3-}$ to deoxyhemerythrin (*Themiste zostericola*) at pH 8.2 and 25 °C.

The products of disproportionation of the semi-met forms were determined by a variety of methods, including analysis of the Fe_2^{II} entity with O_2 and of the Fe_2^{III} entity by SCN^- and N_3^- ions. The amount of the $(Fe^{II}Fe^{III})$ unit could be assayed, uniquely, from the strength of the EPR signal (sample frozen at liquid He), there being no EPR spectra associated with the Fe_2^{II} and Fe_2^{III} entities. All these analyses indicated ≥95% conversion of $(semi\text{-}met)_O$ and ≥85% conversion of $(semi\text{-}met)_R$ into a common disproportionated species containing $(Fe_2^{II})_4(Fe_2^{III})_4$ units. The lower value for $(semi\text{-}met)_R$ is quoted because of consistently, slightly lower values for deoxy found in the disproportionated product compared with that from $(semi\text{-}met)_O$. In addition, a small EPR signal remained after $(semi\text{-}met)_R$ was left for a considerable time (35 min at room temperature) whereas the EPR disappeared completely in the same time when $(semi\text{-}met)_O$ was used.[12] The disproportionation of $(semi\text{-}met)_O$ can therefore be considered as an irreversible reaction, whereas that of the $(semi\text{-}met)_R$ probably has ~10–15% contribution from the reverse step.

The spontaneous change of the semi-met forms to products was a first-order process for at least 3–4 half-lives with similar rate constants resulting from using a number of wavelengths in the 360–450-nm region for observation, as well as from the decrease of EPR signal (Figure 3). There was no indication of $(semi\text{-}met)_O$–$(semi\text{-}met)_R$ interconversion during the disproportionation. The rate constant was unchanged when the protein concentration was increased from 40 to 380 μM. Changes in concentrations of species involved in the production of the semi-met forms ($Fe(CN)_6^{3-}$, $Fe(CN)_6^{4-}$, EDTA, riboflavin, $S_2O_4^{2-}$) and changes in the concentration and nature of buffer were without effect on the rate constant. The rate constants for disproportionation of both semi-met forms at 25–35 °C yielded Arrhenius plots shown in Figure 4 and activation parameters in Table I. The disproportionation was markedly accelerated by the addition of imidazole. For $(semi\text{-}met)_R$ at pH 8.2, the observed first-order disproporationation rate constant, k (s^{-1}), was given by eq 4, and

$$k = 0.0023 + 0.9[\text{imidazole}] \quad (4)$$

a similar effect was observed for $(semi\text{-}met)_O$. The disproportionation of both semi-met forms was suppressed by the addition of anions, e.g., 0.1 M concentrations of SCN^-, Br^-, or F^- at pH 6.3 and lower (mM) concentrations of N_3^- at pH 6.3 and 8.2 or CN^- at pH 8.2. The EPR signal of the semi-met azide adduct remained unchanged at pH 8.2 over many hours.[12] Addition of

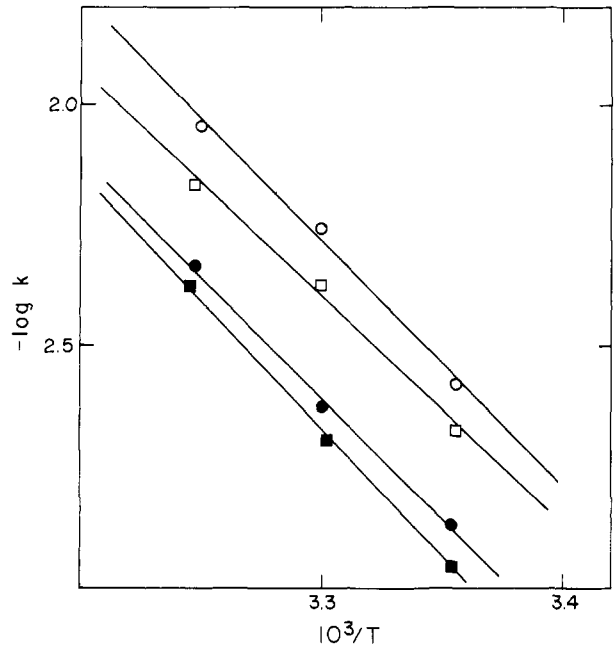

Figure 4. Arrhenius plots for first-order disproportionation of (semi-met)$_O$ (○) and (semi-met)$_R$ (□) and for first-order reactions of (semi-met)$_O$ with $Fe(CN)_6^{3-}$ (●) and (semi-met)$_R$ with $S_2O_4^{2-}$ (■). All data at pH 8.2 using *Themiste zostericola*.

only mM concentration of SCN^- to semi-met forms at pH 8.2 produced little adduct, and so disproportionation occurred and was followed by reaction of SCN^- with the $(Fe_2^{II})_4(Fe_2^{III})_4$; SCN^- thus monitors the disproportionation.[11] The observation also shows the much greater stability at pH 8.2 of the met than the semi-met adducts with SCN^- and of the semi-met-N_3^- adduct compared with the SCN^- one.

There were no direct rapid reactions of (semi-met)$_R$ or (semi-met)$_O$ with oxygen. Rather, disproportionation occurred and a portion of the product species subsequently reacted rapidly with the reagent, e.g.

$$(Fe_2^{II})_4(Fe_2^{III})_4 + 4O_2 \rightarrow (Fe_2^{II}\cdot O_2)_4(Fe_2^{III})_4 \quad (5)$$

This was shown by the first-order rate constants for disproportionation being unchanged in the presence of different concentrations of O_2 (Figure 3, also Figure 2 in ref 11), and from analysis of the products (Table I). The reactions of (semi-met)$_R$ with dithionite and of (semi-met)$_O$ with $Fe(CN)_6^{3-}$ were also disproportionation controlled but with rate constants half the corresponding values for disproportionation (Table I). This factor persisted at temperatures other than 25 °C (Figure 4) and in the presence of imidazole which markedly accelerated both processes. The activation parameters for these redox reactions are included in Table I. The oxidation of (semi-met)$_R$ by $Fe(CN)_6^{3-}$ and reduction of (semi-met)$_O$ by SO_2^- were both rapid second-order reactions (Table I).

The spectra of disproportionated products were similar whether (semi-met)$_O$ or (semi-met)$_R$ was the starting material. A solution of X μM disproportionated species $(Fe_2^{II})_4(Fe_2^{III})_4$ simulated closely the behavior of that of a mixture of 1 part of X μM $(Fe_2^{II})_8$ and 1 part of X μM $(Fe_2^{III})_8$. The titration of disproportionated product with $Fe(CN)_6^{3-}$ resembled closely that of deoxyhemerythrin (Figure 1). The biphasic reaction of disproportionated product with $Fe(CN)_6^{3-}$ gave rate constants of 3.6×10^5 M^{-1} s^{-1} and 1.1×10^{-3} s^{-1} at pH 8.2 and 25 °C to compare with those for deoxy of 2.0×10^5 M^{-1} s^{-1} and 1.2×10^{-3} s^{-1} with the same conditions.[10] The rate constants for reaction of disproportionated product and deoxyhemerythrin (using *Phascolopsis gouldii*) with O_2, in excess, were identical. The rate constants for reaction of disproportionated product with N_3^- and dithionite ion were close to those for the corresponding reactions of methemerythrin.

A sequence of redox and disproportionation reactions, (6) and (7), was carried out with the following rate constants and absorbance changes resulting. In these schemes O represents a

$$(Fe_2^{II})_8 \xrightarrow{O} (Fe^{II}Fe^{III})_8 \xrightarrow[\Delta\epsilon_{550}^M = 280]{k = 2.3 \times 10^{-3}\ s^{-1}} (Fe_2^{II})_4(Fe_2^{III})_4 \xrightarrow{O}$$
$$(Fe^{II}Fe^{III})_4(Fe_2^{III})_4 \xrightarrow[\Delta\epsilon_{550}^M = 150]{k = 1.8 \times 10^{-3}\ s^{-1}} (Fe_2^{III})_6(Fe_2^{II})_2 \xrightarrow{O}$$
$$(Fe^{II}Fe^{III})_2(Fe_2^{III})_6 \xrightarrow[\Delta\epsilon_{550}^M = 80]{k = 1.5 \times 10^{-3}\ s^{-1}} (Fe_2^{II})(Fe_2^{III})_7 \xrightarrow{O}$$
$$(Fe^{II}Fe^{III})(Fe_2^{III})_7 \quad (6)$$

$$(Fe_2^{III})_8 \xrightarrow{R} (Fe^{II}Fe^{III})_8 \xrightarrow[\Delta\epsilon_{420}^M = 270]{k = 2.0 \times 10^{-3}\ s^{-1}} (Fe_2^{II})_4(Fe_2^{III})_4 \xrightarrow{R}$$
$$(Fe_2^{II})_4(Fe^{II}Fe^{III})_4 \xrightarrow[\Delta\epsilon_{420}^M = 150]{k = 1.7 \times 10^{-3}\ s^{-1}} (Fe_2^{II})_6(Fe_2^{III})_2 \xrightarrow{R}$$
$$(Fe_2^{II})_6(Fe^{II}Fe^{III})_2 \xrightarrow[\Delta\epsilon_{420}^M = 90]{k = 1.5 \times 10^{-3}\ s^{-1}} (Fe_2^{II})_7(Fe_2^{III}) \xrightarrow{R}$$
$$(Fe_2^{II})_7(Fe^{II}Fe^{III}) \quad (7)$$

stoichiometric addition of $Fe(CN)_6^{3-}$, i.e., 8, 4, 2, and 1 mol, respectively, corresponding to Fe_2^{II} present, while R represents a short irradiation (90 s) in the presence of EDTA and riboflavin. Both O and R are rapid reactions compared with the subsequent disproportionations.

Protein from Other Sipuncula. The (semi-met)$_R$ and (semi-met)$_O$ forms from *Phascolopsis gouldii* could be prepared at pH 8.2 by the same methods as employed for *Themiste zostericola*. They had correspondingly similar electronic[10,11] and EPR[12] spectra. When left standing for long times, the EPR signal decreased only by about 20% and the amount of deoxy species formed (by disproportionation) was consistent with this value. The reactions of the (semi-met)$_O$ with $Fe(CN)_6^{3-}$ and of (semi-met)$_R$ with $S_2O_4^{2-}$ were both redox reagent independent, and complete oxidation and reduction via disproportionation was evident. In analogy with the behavior of semi-methemerythrin from *Themiste zostericola*, the disproportionation rate constants for reaction of (semi-met)$_O$ and (semi-met)$_R$ for hemerythrin from *Phascolopsis gouldii* could be estimated from the redox reactions as 3.4×10^{-3} s^{-1} and 1.3×10^{-3} s^{-1} at pH 8.2, respectively. Complete disproportionation of the semi-met forms of hemerythrin from *Themiste dyscritum* (prepared as described for *Themiste zostericola*) occurred in solution at pH 8.2. The spontaneous disproportionation of (semi-met)$_R$ was first order at λ = 400 and 420 nm, and the rate constant (2.3×10^{-3} s^{-1}) was consistent with the value for reduction by $S_2O_4^{2-}$ (1.2×10^{-3} s^{-1}). Similarly, the first-order rate constant for disproportionation of (semi-met)$_O$ 9×10^{-4} s^{-1} was about twice the value for first-order reaction of (semi-met)$_O$ with $Fe(CN)_6^{3-}$, 5×10^{-4} s^{-1} (at λ = 360 and 550 nm). However, observations of the spontaneous change of (semi-met)$_R$ at 360 nm indicated a slower non-first-order process. This may arise from an impurity (the EPR spectrum indicated this) showing up at 360 nm or may result from a conformational change concomitant with the disproportionation. Nonetheless, semi-met forms of hemerythrin from both species disproportionate completely (in the case of *Phascopsis gouldii*, redox-induced) and at rates comparable to those for semi-met from *Themiste zostericola*.

Discussion

Production of Semi-Met Forms. The rapid reduction of methemerythrin and rapid oxidation of deoxyhemerythrin both involve one-electron transfers (Figure 1) and lead to distinctive forms, namely, (semi-met)$_R$ and (semi-met)$_O$, respectively. The initial additions of dithionite to methemerythrin resulted in loss of dithionite (at 315 nm) but not of the iron(III) sites (at 420 nm). This may have arisen from dithionite reacting preferentially with traces of oxygen, although these would need to be absorbed by the protein, since such relatively large amounts of free O_2 (~0.03 mM) are certainly not present. More likely, reaction at the protein away from the sites occurred in this initial stage, just as is observed, to a much greater degree, in the reduction of methemerythrin by e_{aq}^- and CO_2^- radicals.[21] Such behavior has been noted in titration

(21) P. C. Harrington and R. G. Wilkins, *J. Biol. Chem.*, **254**, 7505 (1979).

by dithionite of bacterial ferredoxin[22] and cytochrome P450.[23] The lag in the reaction at the iron sites was not observed when the milder reagent $Fe(CN)_6^{3-}$ was used (Figure 1). We presume that the mechanism involved in the photolytic generation of $(semi\text{-}met)_R$ is that which is believed to operate in the reduction of flavoproteins and other proteins by similar methods, i.e.[18-20]

$$\text{flavin}_{ox} \xrightarrow{h\nu,\ \text{EDTA}} \text{flavin}_{red}H_2 \quad (8)$$

$$\text{methemerythrin} + \text{Flavin}_{red}H_2 \rightarrow \text{semi-methemerythrin} + \text{flavinH}^\circ \quad (9)$$

$$2\text{flavinH}^\circ \rightleftarrows \text{flavin}_{ox} + \text{flavin}_{red}H_2 \quad (10)$$

Consistent with this mechanism, reaction 9 was measured independently and shown to be rapid ($k = 1.4 \times 10^5\ M^{-1}\ s^{-1}$ at 25 °C and pH 8.2).

We were much less successful in producing $(semi\text{-}met)_O$ by using oxidants other than $Fe(CN)_6^{3-}$. Since our primary aim was to prepare $(semi\text{-}met)_O$ free of other hemerythrin derivatives, when the behavior of $Fe(CN)_6^{3-}$ was not duplicated the examination was discontinued. Most of the oxidants reacted very slowly with deoxyhemerythrin so that substantial amounts of the intermediate $(semi\text{-}met)_O$ could not accumulate because of its subsequent, relatively fast, disproportionation-controlled oxidation. Partial success was obtained only in the observation of biphasic oxidation of deoxyhemerythrin (*Phascolopsis gouldii*) by $Co(terpy)_2^{3+}$ where fast second-order buildup of an intermediate was followed by a slow, oxidant-independent reaction, with a first-order rate constant close to that obtained[10] with $Fe(CN)_6^{3-}$. It is clear that we were fortunate in our choice of the latter to produce the semi-met form. It is a *rapid* one-electron oxidant of the deoxy but not semi-met species, and its use leads to little denaturing of the protein. In its behavior it has the characteristics of a site-directing reagent.

Properties of Semi-Met Forms. The spectra of $(semi\text{-}met)_O$ at pH 6.3 and 8.2 are similar. Markedly different spectra of methemerythrin at these pH are ascribed to an acid–base equilibrium involving aqua- and hydroxyiron(III) species.[6,24] If this assignment is correct, then with only one iron(III) in the binuclear site of $(semi\text{-}met)_O$, the relevant ionization constant ($pK = 7.8$ with methemerythrin)[24] is shifted outside the pH 6.0–8.5 range. The different spectra for $(semi\text{-}met)_R$ at pH 6.3 and 8.2 indicates that distinct pH-related species exist for $(semi\text{-}met)_R$.

The semi-met forms undergo a variety of reactions arising from the presence of both iron(II) and iron(III) in the binuclear site (Tables I and II). A comparison of the parameters for reaction of N_3^- ion with met-, $(semi\text{-}met)_O$-, and $(semi\text{-}met)_R$-hemerythrin is very informative (Table II). The variation of the formation constant (K) for reaction of $(semi\text{-}met)_R$ with N_3^- at pH 6.3 and 8.2 but not for interaction of $(semi\text{-}met)_O$ with N_3^- at those pHs is consistent with the spectral observation alluded to above that indicated that the $(semi\text{-}met)_R$ but not the $(semi\text{-}met)_O$ species differed at the two pH values. The data at pH 6.3 suggest and that at pH 8.2 confirm that the order of values of K are met $\gg$ $(semi\text{-}met)_R >$ $(semi\text{-}met)_O$. This sequence suggests that N_3^- might be a bridging group in methemerythrin but only bound to the one Fe(III) in the semi-met forms. This idea is reinforced by consideration of rate constants for reaction of the three species. This *much* lower value for k_d for dissociation of azide from the met adduct as compared with those from the semi-met azide complexes is reminiscent of the behavior of bidentate compared with unidentate ligands in simple metal complexes.[25] Spectroscopic data have not yet clarified the mode of binding of azide to methemerythrin although only one terminal nitrogen is involved in bonding.[1,3] The different values for k_f for met and semi-met

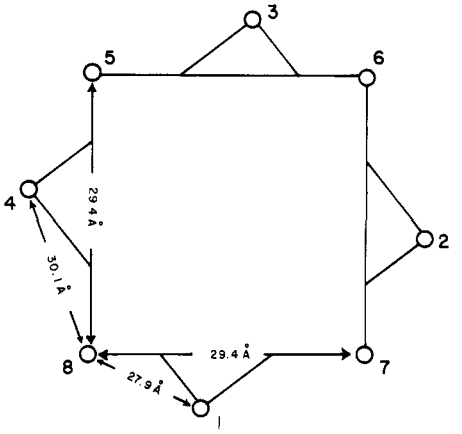

Figure 5. Binuclear iron positions and separated distances in octameric hemerythrin B from *Phascolopsis gouldii*. The open circles represent the iron units at the corners of an almost regular square antiprism, with 24.18 Å between layers.[13]

reactions with N_3^- is unexpected and may arise from charge differences or partial opening up of the iron site in transforming from the met- to the semi-methemerythrin form.

It is believed that the detailed geometry, and even the nature of the ligands attached to the irons, in the binuclear site is quite different in the deoxy and met forms. Some type of bridging in the met form is probably missing in the all iron(II) species.[1] The coordination geometry around $(semi\text{-}met)_O$ would be like that of deoxy (e.g., perhaps no bridging group) and would be easily formed from, and able to revert to, the deoxy species. It might then be anticipated to be difficult to convert $(semi\text{-}met)_O$ to met directly with external reagents. Conversely, the $(semi\text{-}met)_R$ is easily produced from, and converted to, the met species (since they would have similar iron sites) but is reduced to deoxy with difficulty. The differences between the $(semi\text{-}met)_O$ and $(semi\text{-}met)_R$ forms are reduced when N_3^- is added to either, since examination of spectra, EPR, and most chemical properties indicated that a very similar (semi-met)-N_3^- adduct results. More limited spectral data indicate that SCN^-, Br^-, F^-, and CN^- also produce a similar anionic adduct from the two semi-met forms. The EPR[12] and chemical properties of the (semi-met)-N_3^- adduct suggest that the conformation more closely resembles that of $(semi\text{-}met)_R$- than $(semi\text{-}met)_O$-hemerythrin. All semi-met species (except the azide adduct) lack the 315–330-nm peak which has been ascribed to a Fe^{III}–O–Fe^{III} entity.[1] Extremely low values for the disproportionation constant of mixed-valence compounds have been associated with substantial charge delocalization in the molecule.[26] On this basis, one might suggest that the iron valencies are localized in semi-methemerythrin, i.e., a class II system in the Robin–Day classification.[27] This assignment has also been suggested on the basis of near-infrared spectral data.[28]

The Disproportionation Process. With use of a variety of spectral analyses it has been shown that disproportionation of $(semi\text{-}met)_O$ is complete and that of $(semi\text{-}met)_R$ is ≥90% complete. The product of disproportionation resembles closely that of a mixture of met- and deoxyhemerythrin. It is concluded that disproportionation is an intramolecular redox process *within* the octameric framework of hemerythrin for the following reasons. (a) The rate process is nicely first order for several half-lives and the first-order rate constant is independent of a tenfold change of protein concentration. (b) There are only slight differences in the rate constant and activation parameters for disproportionation of the two semi-met forms at pH 8.2, and a change of pH by 2 units only reduces the rate slightly (Table I) with small effects expected for such an intramolecular process. (c) The

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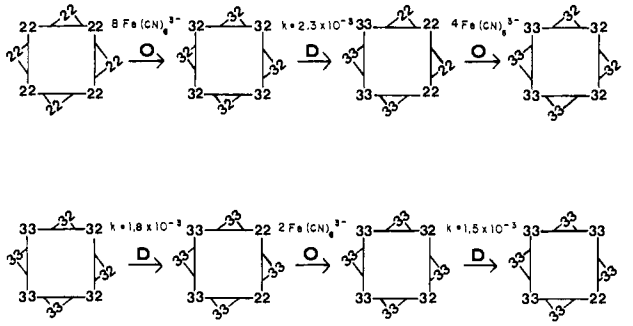

Figure 6. Sequential oxidation (O) and disproportionation (D) of octameric hemerythrin. (Semi-met)$_O$ was prepared by the addition of 1 equiv of $Fe(CN)_6^{3-}$ for each deoxy unit. After each disproportionation the indicated equivalences of $Fe(CN)_6^{3-}$ were added and subsequent disproportionation measured (k in s^{-1}). Fe_2^{II}, $Fe^{II}Fe^{III}$, and Fe_2^{III} units are represented by 22, 32, and 33. Data are for *Themiste zostericola* at pH 8.2 and 25 °C.

reaction persists with a variety of medium conditions and is therefore unlikely to be a small molecule-mediated process. (d) It is extremely unlikely that the octameric structure would break down to a monomeric form prior to, or during, disproportionation.[29] Disproportionation of the semi-met forms therefore results from electron transfer from one of the binuclear sites in the protein to another, a process which must be considered in terms of the detailed octameric structure. In *Themiste dyscritum* and *Phascolopsis gouldii* the oligomer contains eight identical subunits packed as a square donut with four subunits in each of two layers.[13,14] The eight iron binuclear centers are at the corners of an almost regular square antiprism, with any iron pair 28–30 Å distant from two adjacent pairs in the same layer and about the same distance from two adjacent pairs in the other layer. Figure 5 indicates the distances for octamer from *Phascolopsis* gouldii,[13] but a very similar structure pertains for *Themiste dyscritum*[14] also. The nearly equal distances of the four adjacent binuclear sites to each binuclear site allows complete intramolecular electron transfer from four sites to another four sites in the octamer. This is illustrated in the first disproportionation (D) shown in Figure 6 for the (semi-met)$_O$ form. Although the disproportionation behavior of protein from *Themiste zosteriocola* has been most extensively investigated, disproportionation is also observed with the two proteins whose structures are known and is completely promoted by redox reagents and at rates similar to those observed with *Themiste zostericola*. Moreover there is no reason to expect the octameric structure of hemerythrin from *Themiste zostericola* to be radically different from that of the other proteins, especially since the amino acid sequences for octameric *Themiste zostericola* and *P. gouldii* are very similar[30] as are the iron sites.[17]

It is the beautifully symmetrical structure shown in Figure 5, we believe, which allows complete disproportionation, stoichiometric redox reactions controlled by disproportionation, and effective sequential redox and disproportionation reactions of the octamer (eq 6). After oxidation of the four Fe_2^{II} units in $(Fe_2^{II})_4(Fe_2^{III})_4$ by the stoichiometric addition of $Fe(CN)_6^{3-}$, the resulting species disproportionates with almost the same rate constant and half the absorbance change as with $(Fe^{II}Fe^{III})_8$. This suggests that the four $Fe^{II}Fe^{III}$ units in $(Fe^{II}Fe^{III})_4(Fe^{II})_4$ resulting from the oxidation are more likely to be adjacent, as pictured in Figure 6. If they were in diagonally opposite positions, say 8 and 6 in Figure 5, the rate constant for electron transfer over these much greater distances (~42 Å) might be expected to be very much less. This latter problem could be circumvented by electron transfer between adjacent 32 and 22 units thus converting a 32 unit, originally diagonal to another 32 unit, to an adjacent position. Thus the sequence pictured in Figure 6 may be the (favored) one of a number which are statistically possible. We picture the species with two Fe_2^{II} sites, after oxidation, giving rise to two $Fe^{II}Fe^{III}$ units which are adjacent. It is difficult to be certain whether $(Fe^{II}Fe^{III})(Fe_2^{III})_7$ is oxidized directly by $Fe(CN)_6^{3-}$ or by an intermolecular disproportionation. The experimental data for a reduction and disproportionation sequence (eq 7) indicate here also that disproportionation is hardly slowed whether there are eight, four or two $Fe^{II}Fe^{III}$ units within the octamer. Apparently, it is quite difficult to reduce the remaining $Fe^{II}Fe^{III}$ unit, and this leads to very slow reduction over hours by dithionite of about 10–15% material.[9] This phenomenon may also arise from some denaturing of the active site.[17]

The first-order rate constant for loss of (semi-met)$_R$ by disproportionation is within experimental error twice that for loss of (semi-met)$_R$ by reaction with dithionite. A similar relationship exists between disproportionation of (semi-met)$_O$ and reaction with $Fe(CN)_6^{3-}$ (Table I). The sequence shown in (6) and (7) indicates that one $Fe^{II}Fe^{III}$ unit is regenerated (from Fe_2^{III} with dithionite and Fe_2^{II} with $Fe(CN)_6^{3-}$) for every two lost by disproportionation. Provided that the successive disproportionation rate constants are close (as indicated), the disproportionation-controlled redox reactions will be uniphasic and have rate constants close to half of those of disproportionation. The factor of 2 being a statistical one should persist at other temperatures, and this is shown in Figure 4. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for disproportionation of (semi-met)$_O$ and for reaction with $Fe(CN)_6^{3-}$ are within 1 kcal mol^{-1} and 5 eu of each other, while those for disproportionation of (semi-met)$_R$ and for reaction with $S_2O_4^{2-}$ (more difficult to measure accurately because of problems in determining the completion of reaction[9]) only vary by slightly higher values (Table I). The rate constant for disproportionation of both semi-met forms is twice that for reaction with $S_2O_4^{2-}$ ion in the presence of imidazole. The latter has a marked accelerating effect on both processes, although we have no explanation for the catalysis. These observations lend further support to the interpretations embodied in Figure 6 in a striking manner and show the importance of the disproportionation process in the interconversion of the met and deoxy forms of the protein. The absence of a direct reaction of the semi-met forms with O_2 may reflect the difficulty of a one electron reduction of O_2 to O_2^- ion.[31] The easier conversion of O_2 to O_2^{2-} occurs in the reaction of the fully reduced deoxyhemerythrin which forms an adduct with O_2, considered to exist as the $Fe_2^{III}{\cdot}O_2^{2-}$ species.[1]

There is currently much effort being expended at understanding electron transfer in metal complexes and in biological material.[32] Experimental data for electron-transfer rates over long distances in proteins are scarce, however, and the semi-methemerythrin disproportionating system appears to be a rare genuine example of slow electron transfer over distances of 28–30 Å. Iron site and conformational changes may also be involved and the electron-tunneling distances from coordinated histidyl imidazoles may be reduced from the 30-Å value. The first-order electron-transfer rate constant is some 5–8 orders of magnitude smaller than those for electron transfer involving redox (usually heme) proteins for which reaction distances of 15–20 Å appear established.[32]

Acknowledgment. We thank Drs. Lucia Babcock and Z. Bradič for some preliminary studies and Dr. Peterson for a sample of pure sodium dithionite. We are also very grateful to Dr. Wayne Hendrickson for the information embodied in Figure 5, to the authors of ref 28 for a preprint, and to Dr. Robert C. Terwilliger for collecting and sending the *Themiste dyscritum* worms to us. The work was supported by Grant HL 17828 from The Division of Blood Diseases and Resources, National Institutes of Health.

(29) The behavior of semi-metmyohemerythrin (using protein from the muscle of *Themiste zostericola*) is quite different from that of the octameric form. Reaction of semi-metmyohemerythrin[12] with dithionite ion is a *second-order* reaction. The rate constant (24 M^{-1} s^{-1} at pH 8.2) is independent of dithionite concentration and a bimolecular disproportionation is invoked.

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