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Some Redox Properties of Myohemerythrin from Retractor Muscle of *Themiste zostericola*[†]

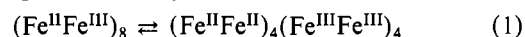
Patricia C. Harrington, Barry B. Muhoberac, David C. Wharton, and Ralph G. Wilkins*

ABSTRACT: Distinct semimetmyohemerythrin species are produced by one-electron oxidation of deoxymyohemerythrin and one-electron reduction of metmyohemerythrin. The former, (semimetmyo)_O, changes (≥90%) to the latter, (semimetmyo)_R, with $k = 1.0 \times 10^{-2} \text{ s}^{-1}$, $\Delta H^\ddagger = 15.1 \text{ kcal mol}^{-1}$, and $\Delta S^\ddagger = -17 \text{ eu}$. Oxidation of (semimetmyo)_O by $\text{Fe}(\text{CN})_6^{3-}$ rapidly produces an unstable metmyohemerythrin form which converts to the final metmyohemerythrin with $k = 4.6 \times 10^{-3} \text{ s}^{-1}$, $\Delta H^\ddagger = 16.8 \text{ kcal mol}^{-1}$, and $\Delta S^\ddagger = -13 \text{ eu}$. The two met forms react at the same rate with N_3^- , but the unstable form

reacts very rapidly with $\text{S}_2\text{O}_4^{2-}$ in contrast to stable metmyohemerythrin. (Semimetmyo)_R or a mixture of metmyohemerythrin and deoxymyohemerythrin equilibrate very slowly to a mixture containing all three species. The rate constants for disproportionation and comproportionation are $0.89 \text{ M}^{-1} \text{ s}^{-1}$ and $9.4 \text{ M}^{-1} \text{ s}^{-1}$, respectively. EPR spectra near liquid He temperatures and optical absorption spectra have been used to characterize and measure the rates at 25 °C, pH 8.2, and $I = 0.15 \text{ M}$. The comparative behavior of octameric and monomeric protein is discussed.

Hemerythrin (Hr)¹ is an easily isolable respiratory protein from marine worms, whose properties have been well characterized and for which a good deal of structural data is available (Hendrickson, 1978; Kurtz et al., 1977; Loehr & Loehr, 1979; Stenkamp & Jensen, 1979). Each subunit of hemerythrin contains two nonheme irons. The deoxy form contains both irons in oxidation state +2 and interacts reversibly with oxygen to give the oxy form in which the Fe/O₂ stoichiometry is 2:1. Both deoxy and oxy forms are easily oxidized to a met species containing irons only in the oxidation state +3. This is no longer O₂ sensitive but does react with a number of anions to form adducts with distinctive spectra

(Keresztes-Nagy & Klotz, 1965; Meloon & Wilkins, 1976; Olivas et al., 1979). In a series of recent studies, two distinctive semimet forms have been characterized, namely, (semimet)_O from one-electron oxidation of deoxyhemerythrin and (semimet)_R from one-electron reduction of methemerythrin (Harrington et al., 1978; Babcock et al., 1980; Bradić et al., 1980; Muhoberac et al., 1980; Harrington & Wilkins, 1981). These semimet species undergo a number of reactions which arise from the presence of both iron(II) and iron(III) in the molecule, including a remarkable intramolecular disproportionation (eq 1). Hemerythrin exists in a number of oli-



gomer forms—octameric in the coelomic fluid of a number of sipunculids (including *Phascolopsis gouldii*, *Themiste zostericola*, and *Themiste dyscritum*), trimeric in the coelomic

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¹ Abbreviations used: Hr, hemerythrin; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance.

fluid of *Phascolosoma lurco* (Addison & Bruce, 1977) and *Phascolosoma agassizii* (Liberatore et al., 1974), and monomeric in the retractor muscle of *Themiste zostericola*. Each monomer unit has a molecular weight of $\sim 13\,500$. Most of our redox and semimet characterization studies have been carried out with octameric protein from *P. gouldii* and *T. zostericola*. In this paper, we describe some redox reactions of monomeric protein (myohemerythrin) from *T. zostericola* and the properties of the derived semimet forms. The EPR spectra at liquid helium temperatures (Muhoherac et al., 1980) have been invaluable for interpreting the spectral changes observed, the latter being used for monitoring the kinetics. There are interesting differences in the behavior of the corresponding species from monomer and octamer. This is particularly the case with the disproportionation reaction, where an intramolecular mechanism analogous to (1) is not feasible with myohemerythrin.

Materials and Methods

The marine worms *T. zostericola* were obtained from Pacific Biomarine Supply, Venice, CA. Metmyohemerythrin was obtained directly from the retractor muscles by omitting the azide step in the procedure described by Klippenstein et al. (1972). Typically, 70–80 mg (although on one occasion 160 mg) of pure metmyohemerythrin resulted from 100 worms. The protein was stored in small portions in phials at -20°C . Refreezing of unused material from one phial was not employed. All the protein from one consignment of worms was used generally within 2 months. All procedures used have been previously described (Bradić et al., 1980; Harrington & Wilkins, 1981). Production of (semimetmyo)_R and deoxy-myohemerythrin by irradiation of a mixture of metmyohemerythrin (0.1 mM), riboflavin (3–5 μM), and EDTA (5 mM) by a 300-W projector lamp required the same lengths of time (90 s and 30 min, respectively) as used for met-hemerythrin (Bradić et al., 1980). Spectral and kinetic measurements were performed on a Beckman 24 recording spectrophotometer and a Gibson-Durrum stopped-flow apparatus interfaced with an OLIS data collection system. Most of the kinetic plots were first order for 3–4 half-lives, and an excess of one reagent was used to ensure first-order conditions. All EPR spectra were recorded on the frozen solutions at 10 K, as the first derivative, by using a Varian E-4 X-band spectrometer equipped with an Air Products Model LTD-110 helitran for work at liquid helium temperatures. All data refer to pH 8.2, $I = 0.15\text{ M}$, and 25°C . Protein concentrations are based on a molecular weight of 13 500.

Results

Kinetics of Reaction of Deoxy- and Oxy-myohemerythrin with $\text{Fe}(\text{CN})_6^{3-}$ and Characterization of Intermediate Species. When deoxy-myohemerythrin is treated with $\text{Fe}(\text{CN})_6^{3-}$ in a stopped-flow apparatus, three phases can be observed. Thus at 550 nm, there is (stage 1) an extremely rapid increase in absorbance ($\Delta A = 900\text{ M}^{-1}\text{ cm}^{-1}$) which is complete within mixing times, even when concentrations of reactants as low as 20 μM are used. On the basis that this is a second-order reaction [in analogy to the behavior of the octameric forms (Bradić et al., 1980)], the second-order rate constant is $\geq 3 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$. The very rapid absorbance increase is followed by an absorbance decrease ($\Delta A_{550} = 400\text{ M}^{-1}\text{ cm}^{-1}$), the rate of which can be easily measured by stopped flow (stage 2). The pseudo-first-order rate constant is proportional to the concentration of $\text{Fe}(\text{CN})_6^{3-}$ in excess, and from a number of kinetic measurements using 0.1–3.0 mM oxidant, a second-order rate constant of $5.7 \times 10^2\text{ M}^{-1}\text{ s}^{-1}$ is obtained. This step

Table I: Rate Constants for Reactions of Octameric and Monomeric Hemerythrin Species from *T. zostericola* at 25°C , pH 8.2, and 0.15 M

reactants	product	$k(\text{monomer})$ ($\text{M}^{-1}\text{ s}^{-1}$)	$k(\text{octamer})$ ($\text{M}^{-1}\text{ s}^{-1}$)
Redox			
deoxy, $\text{Fe}(\text{CN})_6^{3-}$	(semimet) _O	$\geq 3 \times 10^6$	10^5 ^a
(semimet) _O , $\text{Fe}(\text{CN})_6^{3-}$	*met ^b	5.7×10^2	no ^{c,d}
met, $\text{S}_2\text{O}_4^{2-}$	(semimet) _R	1.1×10^6 ^e	4.7×10^5 ^{e,f}
(semimet) _R , $\text{Fe}(\text{CN})_6^{3-}$	met	$\geq 6 \times 10^6$	4×10^5 ^a
(semimet) _R , $\text{S}_2\text{O}_4^{2-}$	deoxy	2.5×10^{-4} ^g	1.1×10^{-3} ^{g,h}
(semimet) _R , (semimet) _R	deoxy + met	0.9	2.2×10^{-3} ^{g,h}
deoxy, met	(semimet) _R	9.4	no ^c
Anation Adducts			
(semimet) _O , N_3^-	semimet- N_3^-	rapid ⁱ	3.2×10^2 ^h
*met ^b , N_3^-	met- N_3^-	14	no ^c
met, N_3^-	met- N_3^-	11	1.4 ^h
(semimet) _R , SCN^-	semimet- SCN^-	1.1×10^{-3} ^g	1.9×10^{-3} ^{g,h,j}
Conformational			
(semimet) _O	(semimet) _R	1.0×10^{-2} ^{g,h}	no ^c
(semimet) _R	(semimet) _O	$\leq 10^{-3}$ ^{g,i}	no ^c
*met	met	4.6×10^{-3} ^{g,m}	no ^{c,n}

^a Bradić et al., 1980. ^b Unstable form. ^c Not observed. ^d For *P. gouldii* at pH 6.3, $k = 1 \times 10^2\text{ M}^{-1}\text{ s}^{-1}$. ^e Reaction of SO_2^- radical. ^f Harrington & Wilkins, 1979. ^g s^{-1} . ^h Harrington & Wilkins, 1981. ⁱ Difficult to measure rate constant because rapid $\text{O} \rightleftharpoons \text{R}$ change. ^j At low concentration of SCN^- (1 mM) monitor disproportionation, at high SCN^- concentration (100 mM) rapid adduct formation. ^k $\Delta H^\ddagger = 15.1\text{ kcal mol}^{-1}$, $\Delta S^\ddagger = -17\text{ eu}$. ^l Estimated from k_2/K_2 ; $K_2 \geq 10$. ^m $\Delta H^\ddagger = 16.8\text{ kcal mol}^{-1}$, $\Delta S^\ddagger = -13\text{ eu}$. ⁿ The reaction is observed with *P. gouldii* at pH 6.3.

is followed by a much slower first-order further decrease in absorbance (stage 3) for which $\Delta A_{550} = 390\text{ M}^{-1}\text{ cm}^{-1}$. The first-order rate constant is independent of a wide variation in $\text{Fe}(\text{CN})_6^{3-}$ concentration, ranging from 0 (conditions obtained by using initially a 2:1 mole ratio of $\text{Fe}(\text{CN})_6^{3-}$ to deoxy-hemerythrin) to 3.0 mM. Its determination at temperatures between 8 and 25°C allowed the estimation of activation enthalpies and entropies. The kinetic data for all stages are collected in Table I. Similar rate constants are obtained from observations at other wavelengths (360, 425, and 475 nm), but those at the longest wavelength are free of $\text{Fe}(\text{CN})_6^{3-}$ background absorbance and possible interference.

The absorbance change in stage 1 is very similar to that observed very soon after 1 mol of $\text{Fe}(\text{CN})_6^{3-}$ is added to 1 mol of deoxy-myohemerythrin. Stage 1 is likely therefore to have produced the (semimetmyo)_O form by a one-electron oxidation. This is confirmed by EPR measurements at liquid helium temperatures on this immediate product of the reaction of equimolar (0.3 mM) mixtures of deoxy-myohemerythrin and $\text{Fe}(\text{CN})_6^{3-}$ (Figure 1). Freezing of the product must be carried out quickly (within 15 s after mixing) because of the instability of the (semimetmyo)_O form (see below and Figure 1). The g values of 1.95, 1.72, and 1.69 for (semimetmyo)_O are identical with those of the octamer (Muhoherac et al., 1980). The absorbance change which accompanies stage 2 is the same as that obtained quickly after the addition of 2 mol of $\text{Fe}(\text{CN})_6^{3-}$ to deoxy-myohemerythrin. The product of stage 2 thus appears to be a species in which both irons are trivalent. This is supported by the absence of an EPR signal

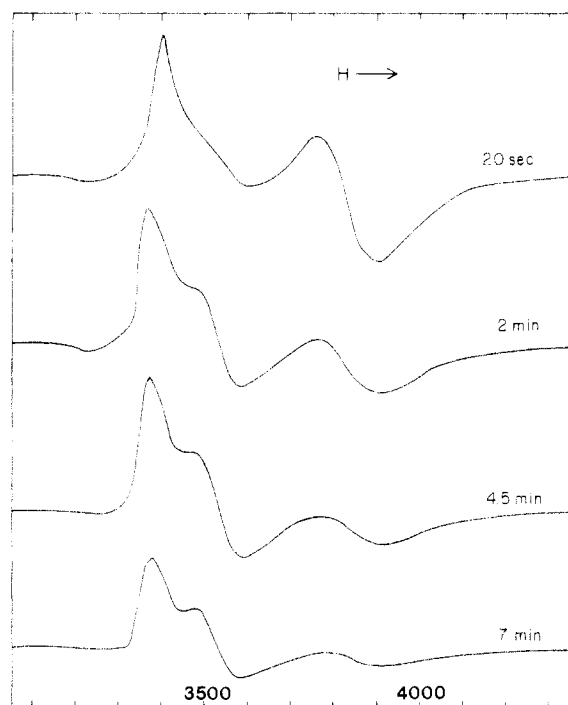


FIGURE 1: EPR signals at various times after mixing 0.3 mM $\text{Fe}(\text{CN})_6^{3-}$ and 0.3 mM deoxymyohemerythrin at 25 °C, pH 8.2, and $I = 0.15 \text{ M}$ [(semimetmyo) $_O \rightarrow$ (semimetmyo) $_R$]. Instrument settings: microwave power, 5 mW; field modulation, 3.2 G; receiver gain, 3.2×10^3 ; microwave frequency, 9.218 MHz; time constant, 0.3 s; field centering, 3500 G; field range, $\pm 1000 \text{ G}$; scanning speed, 8 min. The temperature was 10 K.

due to the semimet form in the product of a mixture of deoxymyohemerythrin (0.2 mM) and $\text{Fe}(\text{CN})_6^{3-}$ (1.2 mM) left for 2 min. This allows time for the completion of stage 2 but for stage 3 to have progressed only $\sim 30\%$. The spectra of the products of stages 2 and 3 are shown in Figure 2. The spectrum of the final product is identical with that obtained from the product of the workup of the muscle protein (metmyohemerythrin) and is very similar to methemerythrin prepared from the coelomic fluid protein. Stage 3 is thus identified with the transformation of an unstable form of metmyohemerythrin to a stable one. The isosbestic point at 390 nm is maintained during the change. The two metmyohemerythrins have distinctive behavior toward $\text{S}_2\text{O}_4^{2-}$ ion. The unstable one reacts rapidly within a few seconds to give ($\sim 90\%$) deoxymyohemerythrin, whereas stable metmyohemerythrin with $\text{S}_2\text{O}_4^{2-}$ gives a rapid absorbance decrease ($\sim 30\%$ total absorbance change at 420 nm), followed by a much slower absorbance decrease over $\sim 240 \text{ min}$, before deoxymyohemerythrin is formed (see next section). In contrast, both forms of metmyohemerythrin react at similar rates with N_3^- ion (Table I) to give a metmyohemerythrin-azide adduct.

Oxymyohemerythrin reacts with excess $\text{Fe}(\text{CN})_6^{3-}$ to give metmyohemerythrin in a manner similar to that of deoxymyohemerythrin. There is a rapid loss of the oxy peak at 500 nm (reaction is complete within the mixing times with 0.05 mM concentrations of reactants). This reaction is followed by second and third stages of the same type and magnitude of absorbance changes as observed with deoxymyohemerythrin. The second-order rate constant for stage 2 is $156 \text{ M}^{-1} \text{ s}^{-1}$, and the first-order rate constant for the met-met interconversion is $5 \times 10^{-3} \text{ s}^{-1}$. When oxymyohemerythrin is treated with $\text{Fe}(\text{CN})_6^{3-}$ (1:1 mole ratio), the immediate product is (semimetmyo) $_O$, and this transforms to (semimetmyo) $_R$ ($k = 9 \times 10^{-3} \text{ s}^{-1}$) and subsequent disproportionation products as shown by EPR and spectral monitoring.

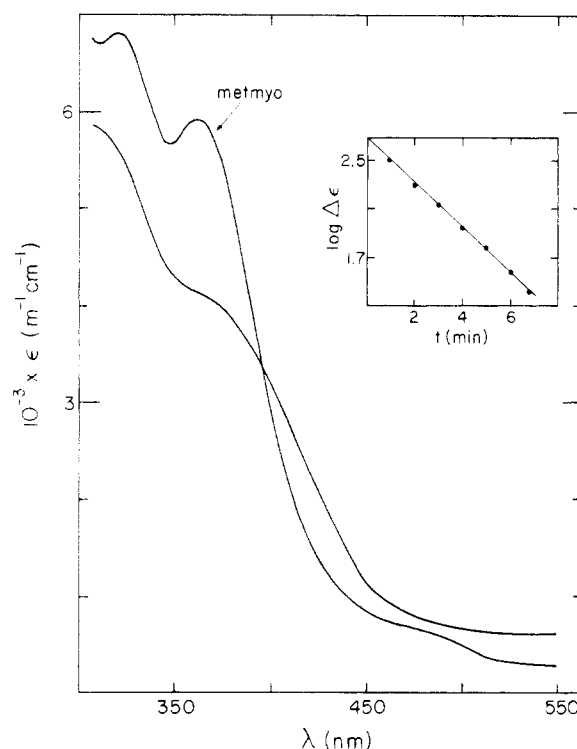
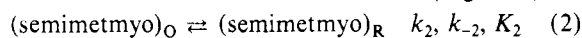


FIGURE 2: Spectra of unstable and stable forms of metmyohemerythrin. Deoxymyohemerythrin (0.12 mM) was mixed with $\text{Fe}(\text{CN})_6^{3-}$ (0.22 mM) at 11 °C, and at the end of redox reaction ($\sim 1 \text{ min}$), the spectrum (of unstable metmyohemerythrin) was run. The solution was left for 30 min for the final metmyohemerythrin spectrum. The inset shows the first-order plot for met-met transformation at 25 °C, pH 8.2, and $I = 0.15 \text{ M}$. $\lambda = 550 \text{ nm}$.

Properties of Semimetmyo Forms. The product of one-electron oxidation of deoxymyohemerythrin, (semimetmyo) $_O$, is rapidly reduced by $\text{S}_2\text{O}_4^{2-}$ to deoxymyohemerythrin. With SCN^- (0.1 M), (semimetmyo) $_O$ rapidly forms an adduct, the peak of which at 450 nm intensifies over hours from $\epsilon = 2.5 \times 10^3$ to $4.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. This latter change is unattended by any change in the character or strength of the EPR signal ($g = 1.92, 1.82$, and 1.57) generally attributed to semimet- SCN^- complex. However, over a period of time, a new signal appears with g values of 2.11 and 2.03, which is strikingly similar to those of certain HIPIP iron-sulfur proteins (Palmer et al., 1967). The growth of this signal may possibly be related to the intensification of the peak seen in the optical spectrum at 450 nm. An adduct with N_3^- is rapidly formed. The (semimetmyo) $_O$ species undergoes spontaneous spectral changes. At 375 nm, there is a rapid absorbance decrease ($k \approx 0.1 \text{ s}^{-1}$) and increase ($k = 9 \times 10^{-3} \text{ s}^{-1}$). At 475 and 500 nm, the faster change is barely observed, and the bulk of the absorbance decrease is at the slower rate ($k = 1.0 \times 10^{-2} \text{ s}^{-1}$). The spectrum at the end of this stage is (semimetmyo) $_R$ (Figure 3). EPR monitoring confirms that an $O \rightleftharpoons R$ change has occurred with a rate constant $\sim 10^{-2} \text{ s}^{-1}$ (Figure 1):



The EPR spectrum indicates that there is, at the most, 10% of the (semimetmyo) $_O$ form remaining at equilibrium (2), i.e., $K_2 \geq 10$. The observed first-order rate constant for the approach to equilibrium approximates to k_2 , and $k_{-2} \leq 0.1k_2$. The values of k_2 , k_{-2} , ΔH^\ddagger_2 , and ΔS^\ddagger_2 are recorded in Table I. Equilibration according to (2) is followed by a very slow diminution in the EPR signal as disproportionation occurs, but there is only a slight spectral change (see below).

The (semimetmyo) $_R$ form is most conveniently produced by photochemical reduction of metmyohemerythrin in the pres-

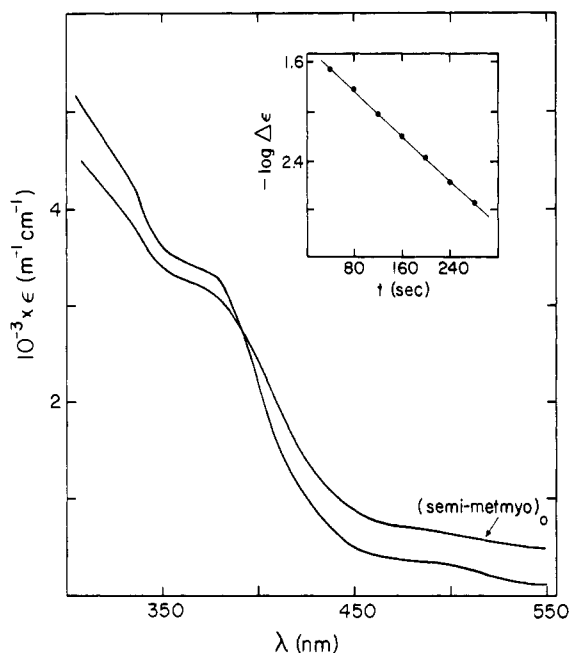
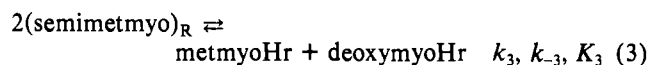


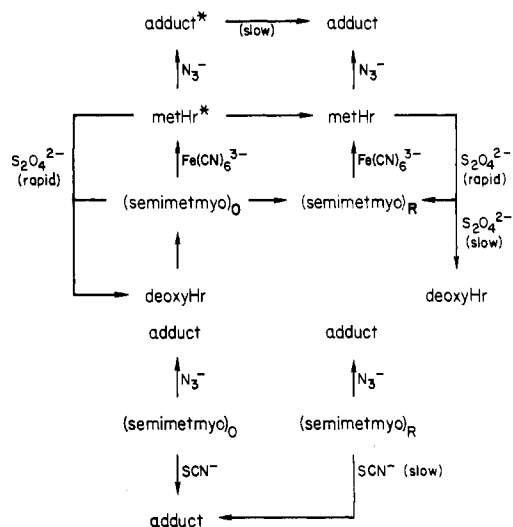
FIGURE 3: Spectra of (semimetmyo)_O and (semimetmyo)_R. Deoxy-myohemerythrin (0.14 mM) was mixed with Fe(CN)₆³⁻ (0.14 mM) at 7 °C. The spectrum was run immediately and was mainly (semimetmyo)_O (some transformation occurs). The solution left for 7 min at 25 °C and spectrum rerun (semimetmyo)_R. The inset shows the first-order plot for transformation at 25 °C, pH 8.2, and $I = 0.15$. $\lambda = 550$ nm.

ence of riboflavin and EDTA (Massey & Hemmerich 1977; Babcock et al., 1980). Its electronic and EPR spectra resemble closely those of the octameric analogue (Babcock et al., 1980; Muhoberac et al., 1980). It reacts rapidly with Fe(CN)₆³⁻ (reaction is complete within 1–2 ms with 0.1 mM oxidant) to give metmyohemerythrin with no sign of an intermediate met form similar to that observed when (semimetmyo)_O is oxidized. It reacts rapidly with N₃⁻ but *slowly* with SCN⁻ ion with a first-order rate constant ($k = 1.1 \times 10^{-3} \text{ s}^{-1}$) independent of a SCN⁻ concentration change from 0.7 to 16.0 mM. There is hardly any effect of a further increase of [SCN⁻] to 100 mM. There is an attendant EPR change from the (semimetmyo)_O signal (obtained by freezing ~1 min after adding SCN⁻) to that of a (semimetmyo)–SCN⁻ adduct at the completion of the reaction. This latter can also be obtained (rapidly) by adding SCN⁻ to (semimetmyo)_O. Both have identical g values of 1.92, 1.82, and 1.57 (broad). The (semimetmyo)_R form is also obtained from the first (fast) stage of the reduction of metmyohemerythrin by dithionite. The rate of this stage is [S₂O₄²⁻]^{1/2} dependent and can thus be interpreted as reduction by SO₂⁻ radical (Table I) (Harrington et al., 1978). The slow reduction of (semimetmyo)_R by dithionite to deoxymyohemerythrin is complete in ~4 h as judged from the completion of absorbance changes at 420 nm and from the amount of oxymyohemerythrin produced by adding oxygen to the solution. The EPR signal of (semimetmyo)_R in the presence of dithionite disappears within 60 min! The absorbance change at 420 nm is first order for 3 half-lives, and the rate constant ($k = 2.5 \times 10^{-4} \text{ s}^{-1}$) is independent of a protein concentration change of 0.13–0.33 mM and an S₂O₄²⁻ change of 1–10 mM.

The EPR signal due to (semimetmyo)_R decreases very slowly on standing in solution, and this is ascribed to the spontaneous disproportionation reaction:



Scheme I: Reactions of Myohemerythrin (Hr) Derivatives Examined in This Study



At equilibrium, the amplitude of the EPR signal indicates $K_3 = 0.19$. The amounts of deoxymyohemerythrin (from intensity of the oxy peak) and metmyohemerythrin (from the amplitude of the signal for the slower reaction with N₃⁻) in the equilibrium mixture are in reasonable agreement with those expected from the value of K_3 . The same equilibrium EPR spectrum can be generated by mixing equal and appropriate concentrations of metmyohemerythrin and deoxyhemerythrin. From the magnitude of two EPR signals measured during the course of the disproportionation and comproportionation reactions, values of k_3 and k_{-3} of $1.4 \text{ M}^{-1} \text{ s}^{-1}$ and $7.4 \text{ M}^{-1} \text{ s}^{-1}$ and $K_3 (=k_3/k_{-3}) = 0.19$ could be calculated by using equations for a $2A \rightleftharpoons B + C$ scheme (Graven, 1956; Frost & Pearson, 1961). There is very little difference in the spectra of (semimetmyo)_R and a mixture of met and deoxy. This equivalence is observed when fresh protein is used and prevents the easy determination of the kinetics of (3) by spectral methods. However, with slightly aged protein, there are small spectral changes accompanying (3), and by use of these, it is found that the rates in both directions conform very well with second-order reversible kinetics, with values of $k_3 = 0.9 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-3} = 9.4 \text{ M}^{-1} \text{ s}^{-1}$. The values of K_3 derived from the kinetics, 0.10, is in excellent agreement with that assessed from spectral examination of the equilibrated solutions.

Discussion

There is a common polypeptide folding pattern for myohemerythrin and hemerythrin [for reviews, Hendrickson (1978), Kurtz et al. (1977), Loehr & Loehr (1979), and Stenkamp & Jensen (1979)]. In addition, the spectra of derivatives of myohemerythrin resemble closely those of the octamer. The binuclear iron site is therefore likely to be similar in both forms. The myohemerythrin reactions studied in the present work are shown in Scheme I. They resemble, with some important exceptions, those of the octamer. Distinctive semimetmyohemerythrin forms can be produced in ways similar to those used with octameric hemerythrin from *T. zostericola* and *P. gouldii* (Babcock et al., 1980; Bradić et al., 1980; Harrington & Wilkins, 1981).

The (semimetmyo)_O results from one-electron oxidation of deoxymyohemerythrin by Fe(CN)₆³⁻. The EPR (Figure 1) and optical (Figure 3) spectra resemble those of octameric (semimet)_O from *T. zostericola* (Babcock et al., 1980; Harrington & Wilkins, 1981). Both monomeric and octameric (semimet)_O forms react rapidly with N₃⁻, SCN⁻, and S₂O₄²⁻

ions. However, (semimetmyo)_O is *directly* oxidized by Fe(CN)₆³⁻ to a met form whereas, with the octamer, the corresponding oxidation is indirect, via intramolecular disproportionation (Bradić et al., 1980). The immediate metmyohemerythrin product of direct oxidation slowly changes in one step to normal metmyohemerythrin (Figure 2). Both met forms lack an EPR spectrum and have similar reactivity toward N₃⁻ ion (Table I). The unstable met species, however, resembles (semimetmyo)_O in being rapidly reduced by S₂O₄²⁻ ion, whereas the final met product is reduced in a slow multiphasic fashion (see below). The (semimetmyo)_O form changes readily to (semimetmyo)_R, a transformation which occurs to the extent of ≥90% (Figure 1) and has not been observed with octamer. The EPR change parallels the slower (and much more significant) spectral change, and the rate constants obtained by using these two monitors are close and assigned to the (semimetmyo)_O → (semimetmyo)_R transformation (Table I). The activation parameters for this change are remarkably close to those for the *met–met transformation (Table I), and this suggests that the two processes have a common basis. We have suggested that the iron site in (semimet)_O is similar to that in deoxy whereas the iron site in (semimet)_R resembles that of met (Babcock et al., 1980). If then, the rapidly produced metmyohemerythrin form resembles (semimetmyo)_O, its change to the stable met will involve an iron site change similar to that of (semimetmyo)_O to (semimetmyo)_R. The two processes might therefore have similar rate parameters, if the iron coordination changes are a controlling factor in the kinetics. These considerations depend on the fact, for which there is every indication, that ligands coordinated to the irons in the met form differ, at least in part, from those in the deoxy species (Kurtz et al., 1977; York et al., 1980).

The destruction of the oxy peak at 500 nm when oxymyohemerythrin is treated with Fe(CN)₆³⁻ is complete within mixing times with 0.05 mM solutions. If the reaction proceeds through the deoxy form, as has been substantiated with the octamer (Bradić et al., 1980), then this places a lower limit of ~10⁷ M⁻¹ s⁻¹ for the second-order oxidation rate constant for deoxymyohemerythrin. The immediate product is (semimetmyo)_O as shown by EPR, with conversion to (semimetmyo)_R in the absence of Fe(CN)₆³⁻ and oxidation by Fe(CN)₆³⁻ to metmyohemerythrin via the unstable met form. The observed rate constants are similar to those obtained when deoxymyohemerythrin is the starting material.

The (semimetmyo)_R form, as well as arising from (semimetmyo)_O, is also produced by one-electron reduction of metmyohemerythrin using either S₂O₄²⁻ or reduced riboflavin generated photochemically (Massey & Hemmerich, 1977; Babcock et al., 1980). The EPR and optical spectra of (semimetmyo)_R from these three sources are respectively similar. The interpretation of the reactions of (semimetmyo)_R with SCN⁻ and S₂O₄²⁻ ions poses real problems. The independence of the respective first-order rate constants on the concentration of these reagents establishes that a change involving the protein is dominating the reactions. The behavior of (semimetmyo)_R and (semimet)_R toward SCN⁻ is in striking contrast. At millimolar concentrations of SCN⁻, the disproportionation of (semimet)_R is monitored, and only at high concentrations of SCN⁻ (0.1 M) is an adduct rapidly formed (Harrington & Wilkins, 1981). The (semimetmyo)_R form reacts slowly with SCN⁻, and even in 0.1 M concentration no adduct is rapidly produced. The mixed oxidation state is retained, and the EPR and electronic spectra of the product resemble those formed rapidly when (semimetmyo)_O is mixed

with SCN⁻. We interpret the first-order reaction of (semimetmyo)_R with SCN⁻ as a conformational change to another form which reacts rapidly with SCN⁻. This other form *could* be (semimetmyo)_O since the estimated rate constant for (semimetmyo)_R to (semimetmyo)_O conversion (≤10⁻³ s⁻¹) is consistent with the SCN⁻ reaction rate constant of 1.1 × 10⁻³ s⁻¹.

The reaction of (semimetmyo)_R with S₂O₄²⁻ is even more difficult to understand. The absorbance changes are complete in ~4 h, which is a significantly shorter time than for the *complete* reduction of (semimethemerythrin)_R (Harrington et al., 1978). If the reduction of (semimetmyo)_R was disproportionation controlled as it appears to be with (semimet)_R (Harrington et al., 1978; Harrington & Wilkins, 1981), it would be expected to be a second-order reaction with a second-order rate constant about half of the value of *k*₃ (Harrington & Wilkins, 1981). The observed behavior is obviously much more complex than this, the absorbance changes being best described as *first order* for 2 to 3 t_{1/2}'s. The first-order rate constant is independent of protein and dithionite concentrations, but the value varies with protein sample (fresh, aged and batch of worms) and we have had more problems of reproducibility of behavior with dithionite reduction of semimetmyohemerythrin than any other reaction we have so far studied. In addition, the EPR behavior is curious. There is an intensification of the normal (semimetmyo)_R EPR signal (Muhoherac et al., 1980) for ~10 min and then a slower decrease to a zero signal, complete within ~1 h. At this point, absorbance measurements on the solution alone, and after opening to oxygen, indicate that the formation of deoxymyohemerythrin is incomplete. The (semimet)_R form appears to have changed to another species without an EPR spectrum, which is still reduced by dithionite. The monomeric protein may also be more sensitive to attack by the vigorous reducing agent than the octameric form, and this may be a contributing cause to the complexity. Addition of sulfite, the primary product of oxidation of dithionite, which is formed in very small amounts during these reactions, does not modify the observed behavior.

The mode of disproportionation of (semimetmyo)_R contrasts nicely with that of the similar species produced from octameric *T. zostericola* hemerythrin. Whereas the disproportionation of (semimet)_R [and (semimet)_O] is complete and clearly first order, arising from an intramolecular electron transfer (Babcock et al., 1980; Harrington & Wilkins, 1981), the disproportionation of (semimetmyo)_R is kinetically complex and incomplete (*K*₃ = 0.19). The identical equilibrium mixture of metmyo-, (semimetmyo)_R-, and deoxymyohemerythrin can also be attained by mixing metmyohemerythrin and deoxymyohemerythrin. Somewhat limited kinetic data, which we hope to augment, indicate second-order reversible disproportionation and comproportionation rate processes with rate constants shown in Table I. The value of the rate constant for reaction of metmyohemerythrin with deoxymyohemerythrin is interestingly close to that for the reaction of metmyohemerythrin with deoxymyoglobin (2.0 M⁻¹ s⁻¹ at pH 6.3 and 7.0; Bradić et al., 1979). The free energy changes in both cases are similar and small and therefore not an important contributing factor. The presence of the heme ring near the surface of myoglobin (Antonini & Brunori, 1971) does not therefore apparently aid the electron transfer between these relatively small molecules (*M_r*'s of 13 500 and 18 000 for myohemerythrin and myoglobin, respectively). It is difficult to compare the intramolecular (for the octamer, *k* = 2.2 × 10⁻³ s⁻¹; Harrington & Wilkins, 1981) and intermolecular (for

the monomer, $k \approx 1 \text{ M}^{-1} \text{ s}^{-1}$) electron transfer rate constants. This is related to the general problem of comparing intra- and intermolecular catalyses which result in different reaction order processes. It can be estimated that the half-life for disproportionation of 6 mM concentrations of myohemerythrin is about the same as that for octameric hemerythrin. This suggests that the orientation of the two semimetmyohemerythrin molecules in an adduct or transition complex might resemble that for two adjacent monomer units within the octamer where the distances between binuclear iron units are $\sim 28 \text{ \AA}$ (Harrington & Wilkins, 1981). It is apparent that, quite surprisingly, the behavior of monomeric protein, particularly in its reduction reactions, is more complicated than that of the octameric form.

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Structure of Fragment E Species from Human Cross-Linked Fibrin[†]

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ABSTRACT: Fragments E₁, E₂, and E₃ are plasmic derivatives of fibrin encompassing the NH₂-terminal region of the molecule. The first two species, but not the third, can bind to fragment DD, forming a (DD)E complex, and therefore probably contain binding sites involved in the polymerization of fibrin. For localization of these sites the structure of the fragments was determined by establishing the NH₂- and COOH-terminal boundaries of the molecules and using the published amino acid sequence of fibrinogen. Fragment E₁ encompasses Gly- α 17 to Lys- α 78, Gly- β 15 to Lys- β 122, and Tyr- γ 1 to Lys- γ 62, this representing the intact NH₂-terminal region of fibrin. Fragment E₂ is an asymmetric molecule which

is lacking the sequence Gly- β 15 to Lys- β 53 in one β -chain remnant. This fragment E₂ also lost Lys- β 122 from the COOH terminal of the β chain as compared with fragment E₁. These cleavages did not affect the ability of fragment E₂ to bind to fragment DD. Fragment E₃ was heterogeneous, the main species encompassing Val- α 20 to Lys- α 78, Lys- β 54 to Leu- β 120, and Tyr- γ 1 to Lys- γ 53. Thus, the loss of the binding function involved in the formation of fibrin clot was associated with the removal of small fragments from all three polypeptide chains: α 17-19 (Gly-Pro-Arg), β 15-53 from the remaining half of the molecule, β 121 (Leu), and γ 54-58 (Thr-Ser-Glu-Val-Lys).

The study of binding sites on the fibrin molecule involved in polymerization of monomers and formation of a clot indicated that the NH₂-terminal region of the parent molecule participates in this reaction. The cleavage of fibrinopeptide

A was requisite to express the binding site since intact fibrinogen or fibrinopeptide A containing fibrinogen derivatives were nonreactive (Heene & Matthias, 1973; Kudryk et al., 1973; Kudryk 1974; Laudano & Doolittle, 1978, 1980; Matthias & Heene, 1973; Matthias et al., 1973; Olexa & Budzynski, 1979b, 1980). Two lines of evidence indicated that the location of this binding site is on the α chain in the amino acid sequence following fibrinopeptide A. First, peptides containing the amino acid sequence which corresponds to the sequence α 17-19, bound to fibrin and inhibited polymerization of fibrin monomer (Laudano & Doolittle, 1978, 1980).

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