Conversion of Oxy- into Methemerythrin in the Presence of Anions*

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The interaction of oxyhemerythrin (HrO₂) with cyanate and azide ions (X⁻) is biphasic. The rapid loss of HrO₂ is monitored at 500 nm using stopped flow. The dependence of the pseudo-first order rate constant on [HrO₂], [X⁻], and [O₂] can be interpreted by the scheme

\[ HrO₂ ⇌ Hr + O₂ \quad K_1 \]
\[ Hr + X⁻ \rightarrow HrX⁻ \quad k_1, k_2, K_2 \]

At 25°C, pH = 6.3 and I = 0.15 M for X⁻ = CNO⁻, \( k_1 = 59 \text{ M}^{-1} \text{s}^{-1} \) and \( k_2 = 0.019 \text{ s}^{-1} \); and for X⁻ = N₃⁻, \( k_1 = 1.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) and \( k_2 = 0.10 \text{ s}^{-1} \). The values of \( K_1, K_2 \) from kinetics are in good agreement with those obtained from spectral and \( [O₂] \) determinations of equilibrated mixtures of Hr, CNO⁻, and N₃⁻. The second step has been analyzed separately by flow observations of the small spectral changes accompanying addition of X⁻ to deoxyhemerythrin. The slower, stoichiometric formation of methemerythrin cyanate or azide has also been studied and second order rate constants for reaction of HrO₂ with X⁻ determined (N₃⁻, 0.34 \text{ M}^{-1} \text{s}^{-1} \); and for X⁻ = CNO⁻, 8.3 \times 10^{-3} \text{ M}^{-1} \text{s}^{-1} \); and for X⁻ = F⁻ (0.12 \text{ M}^{-1} \text{s}^{-1} \); and for X⁻ = SCN⁻, 0.08 \text{ M}^{-1} \text{s}^{-1} \) at 25°C and pH = 6.3). These are compared with those for accelerated formation of the met form adduct of the met form, in addition to peroxide (1, 8). The reaction may be represented as:

\[ \text{Fe}^{III}(\text{O}_2^{-})_8 + 8\text{X}^- \rightarrow \text{Fe}^{III}(\text{N}_3^{-})_8 + 8\text{O}_2^- \] (1)

with any charges on the protein being omitted and the oxyhemerythrin being formulated as the peroxo species. The peroxide and azide groups may be associated with one or both irons (9). Only a few details of the kinetics for the catalysis have been reported. The second order reaction is acid-catalyzed in the pH range 6 to 9. The N₃⁻ reaction is polyphasic, the catalysis by F⁻ and NO₃⁻ uniphasic (1).

In principle, these are attractive reactions to investigate. The stoichiometry is clean (8), and specific spectral changes accompany the transformations. We have investigated the detailed kinetics for autooxidation of Golfingia gouldii oxyhemerythrin in the presence of a number of anions, and compared the results with those for oxyhemoglobin (4-7) and oxymyoglobin (10).

**Materials and Methods**

Oxyhemerythrin crystals were prepared from the coelomic fluid of worms of the species Golfingia gouldii, purchased from Marine Biological Laboratories, Woods Hole, Mass., by the method of Klotz et al. (11). Since such crystals usually contained as much as 10% met, these were converted either completely to methemerythrin (8, 12, 13) or reduced to the deoxy form by dialyzing oxyhemerythrin (8, 12, 13) or reduced to the deoxy form by dialyzing oxy- or methemerythrin solution against a slight excess of sodium dithionite in the appropriate buffer saturated with nitrogen gas. The remaining free dithionite was removed by dialyzing against a deoxygenated buffer solution. Freshly prepared solutions of oxyhemerythrin were obtained either by exposing a buffered oxymyoglobin solution to air or by bubbling through oxygen until the desired free oxygen concentration was attained. Several batches of worms were used, prepared over a 30-month period, with no apparent difference in kinetic behavior (see below). All chemicals used were sodium or potassium salts. The azide, cyanate, and thiocyanate salts were recrystallized, the others were Baker analyzed reagents and were used without further treatment. The buffer Mes⁻·H₂O was obtained from Calbiochem, and the buffers Mes⁻, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Tris, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were Sigma products.

∥ The abbreviation used is: Mes, 2-(N-morpholino)ethanesulfonic acid.

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Adjustment of pH was made with NaOH or H$_2$SO$_4$ solutions. Nitrogen gas (O$_2$ free, <5 ppm, Matheson Co.) was bubbled through Cr$^{3+}$ solution prepared by reduction of Cr$^{3+}$ with zinc amalgam.

**Kinetic Measurements** — All kinetic experiments were carried out using a Gibson-Durrum stopped flow spectrophotometer. 7110 with a 2-cm observation cell, or in a 1-cm cell in a Cary 14 or Beckman 24 recording spectrophotometer. Thermostating control was to ±0.1°C. The majority of the runs were at 25°C, pH 6.3, provided by 0.05 to 0.05 M Mes, and f = 0.15 M using Na$_2$SO$_4$ or K$_2$SO$_4$. The rapid reaction between deoxyhemerythrin and CNO$^-$ or N$_3^-$ was monitored at 380, 460, or 550 nm where there are small absorbance decreases on adduct formation. Protein concentrations of 80 to 100 μM were used. The rapid interactions between HrO$_2$ and CNO$^-$ or N$_3^-$ and between HrCNO$^-$ and O$_2$ were monitored at 380 and 500 nm where a change of [HrO$_2$] is recorded. Protein concentrations of 30 to 50 μM were used. The slow formation of the met derivative was also monitored at 370 to 580 nm or at 500 nm using 50 μM protein. The formation of met azide and thiocyanate was also followed at 450 nm (one of their absorption peaks) with similar results. Manipulation of solutions in the stopped flow apparatus was as described previously (14). The equilibrium constant $K_{1}$, $K_{2}$ was determined spectrally by recording (using the digital read-out of the Beckman 24) the absorbance at 380, 360, and 500 nm of mixture of HrO$_2$ with different concentrations of cyanate ion and oxygen as soon as mixing has occurred, before significant met formation occurred. The corresponding equilibrium between HrO$_2$ and N$_3^-$ ions was examined using the stopped flow traces. A conventional spectrophotometer could not be used since met formation occurs quickly and is accompanied by too large a relative absorbance change. The rapidly established equilibrium between HrO$_2$ and CNO$^-$ or N$_3^-$ ions was also examined by determining free oxygen concentration changes with a Beckman 76 oxygen sensor. The oxygen meter was calibrated at 25°C against air-saturated distilled water ([O$_2$] = 0.24 mm at 25°C and an oxygen partial pressure of 145 mm Hg). Hemerythrin concentration was determined routinely via the oxyhemerythrin derivative (5) using the digital read-out of the Beckman 24) the absorbance at 380, 460, and 500 nm where there are small absorbance decreases on adduct formation. Protein concentrations of 30 to 50 μM were used.

### RESULTS

Addition of the anions N$_3^-$, CNO$^-$, SCN$^-$, HCO$_3^-$, NO$_2^-$, F$^-$, and Cl$^-$ to oxyhemerythrin leads to quantitative production of methemerythrin under all concentrations and pH values studied. Spectral examination of the product shows that the anion is incorporated in the met species. The spectral characteristics of methemerythrin azide, cyanate, thiocyanate, fluoride, and chloride have been previously reported (8). The formate adduct has shallow absorption peaks at 480, 360, and 340 nm (ε = 6.1 × 10$^3$, 6.2 × 10$^3$, and 6.2 × 10$^3$) and the nitrite complex at 480 (sh), 377, and 330 nm (ε = 7.7 × 10$^3$, 5.4 × 10$^3$, and 5.9 × 10$^3$). The kinetics of the conversion of oxy- to methemerythrin in the presence of anions was studied with concentrations of anions and free oxygen in excess of that of the protein. When CNO$^-$ ion was added to HrO$_2$, there was a rapid diminution of the oxyhemerythrin peak at 500 nm, the extent of which depended on the concentration of CNO$^-$ and O$_2$. This could also be observed visually by the marked bleaching of the rose color of HrO$_2$. This rapid change termed Reaction I was followed by a very much slower conversion to the met-cyanate. Reaction II, which could be monitored on a spectrophotometer (Fig. 1). A similar biphasic spectral course was observed in the presence of N$_3^-$ ions, but the time separation was less marked than with the cyanate system. With all other anions studied the two step mechanism:* $\text{HrO}_2 + \text{X}^- \rightarrow \text{HrX}^- + \frac{1}{2} \text{O}_2$ (2) $\text{Hr} + \text{X}^- \rightarrow \text{HrX}^- \quad k_2$ (3) The rate parameters for the first step have been measured previously (14), and a rapid equilibrium compared with Step 3 can be assumed. It is then easily shown that (3): $\frac{1}{2} \ln \frac{[\text{HrX}_2]_e}{[\text{HrX}_2]_i} = - \frac{k_2}{k_1} \ln \frac{[\text{HrX}]_e}{[\text{HrX}]_i} - \frac{k_2}{k_1} \ln \frac{[\text{HrX}]_e}{[\text{HrX}]_i} + k_2 \ln \frac{[\text{HrX}]_e}{[\text{HrX}]_i}$ (4) where subscripts $t$, $e$, and $i$ refer to times zero, $t$, and at equilibrium. Values of $k_2$ and $K_1 k_2 k_3$ can be calculated from the intercepts and slopes of Fig. 2 and using the value of $K_1$ ($k_1 / k_2 k_3$) previously determined (14) values of $k_2$ and $K_1$ can be estimated. These are shown in Table I at pH 5.3 and 6.3 for X$^- = N_3^-$ and CNO$^-$. The reaction was also investigated in the opposite direction by studying the rate of production of HrO$_2$ when a mixture of Hr and CNO$^-$ ion (containing appreciable amounts of HrCNO$^-$) in one syringe was treated with oxygen solution in

* Hr represents the binuclear Fe$^{3+}$ species in the monomeric unit of deoxyhemerythrin. Hr$^+$ represents the corresponding binuclear Fe$^{3+}$ species present in methemerythrin. Anionic adducts of deoxy- and methemerythrin and oxyhemerythrin are thus represented as HrX$^-$, Hr$^+$X$^-$, and HrO$_2$, respectively. Hb = hemoglobin.
another syringe in the stopped flow. For this situation, Equation 4 still applies. Low values of \([\text{CNO}^-]/[\text{O}_2]\) had to be used to promote a significant extent of formation of \(\text{HrO}_3\), and the resultant rate constants (Table I) were less accurate. The experimental points for \(k_{\text{obs}}\) fitted quite well the plot of Fig. 2.

The overall equilibrium constant \(K_{1}K_{2}\) for Reaction I shown in Equation 5

\[
\text{HrO}_3 + X^- \rightleftharpoons \text{HrCNO}^- + O_2 \quad K_{1}K_{2}
\]

could also be determined both spectrally and by oxygen monitoring. This presented no difficulties with \(X^- = \text{CNO}^-\) since the subsequent reaction (Reaction II) was sufficiently slow so that spectra and oxygen concentrations could be measured on equilibrated mixtures of \(\text{HrO}_3\) and \(X^-\) before its onset. In the spectral treatment (15), Equation 6 applies:

In Fig. 2, plots of \(k_{\text{obs}}, s^{-1}\) versus \([X^-]/[O_2]\) for (fast) Reaction I, at pH 6.3, \(T = 25^\circ\). Left ordinate for \(\text{CNO}^-\): \(A = 200\). Right ordinate for \(N_3^-\): \(A = 0.04\); \(A = 0.67\); \(A = 1.2\) mm free \(O_2\) for \(X^- = N_3^- (I = 0.5\); \(x = 0.24\) mm; \(x = 0.67\) mm free \(O_2\) for \(X^- = \text{CNO}^-\). These plots correspond to reaction of a \(\text{HrCNO}^-\) mixture with \(O_2\) (\(I = 0.15\)).

![Figure 2](http://www.jbc.org/)

\[
\frac{1}{k_1} + \frac{1}{k_2} + \frac{1}{K_{1}K_{2}[O_2]} = \frac{1}{k_{\text{obs}}}
\]

FIG. 3. Plots of \((\epsilon_3 - \epsilon_1)^{-1}\) versus \((\text{CNO}^-)^{-1}\) for (fast) Reaction I, at pH 6.3, \(I = 0.15\), \(T = 25^\circ\). \(\epsilon = 0.50\) nm; \(\epsilon = 0.38\) nm; \(\epsilon = 0.36\) nm. Total protein = 0.13 mm, \([O_2] = 0.24\) mm.

**TABLE I**

<table>
<thead>
<tr>
<th>Reactants</th>
<th>pH</th>
<th>(k_1 M^{-1}s^{-1})</th>
<th>(k_2 s^{-1})</th>
<th>(K_{1}K_{2} (M^{-1}))</th>
<th>(k_{\text{obs}} (s^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{HrO}_3 + N_3^-)</td>
<td>5.3</td>
<td>(1.9 \times 10^3)</td>
<td>(0.1)</td>
<td>(1.9 \times 10^{11})</td>
<td>(0.01)</td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>(1.5 \times 10^3)</td>
<td>(0.15)</td>
<td>(1.6 \times 10^{10})</td>
<td>(0.017)</td>
</tr>
<tr>
<td>(\text{HrO}_3 + \text{CNO}^-)</td>
<td>5.3</td>
<td>(4.8 \times 10^6)</td>
<td>(0.019)</td>
<td>(3.4 \times 10^9)</td>
<td>(0.021)</td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>59</td>
<td>(0.019)</td>
<td>3.1 (10^9)</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td></td>
<td></td>
<td></td>
<td>0.044</td>
</tr>
<tr>
<td>(\text{HrCNO}^- + \text{O}_2)</td>
<td>6.3</td>
<td>97</td>
<td>(0.011)</td>
<td>(9 \times 10^9)</td>
<td>0.0041</td>
</tr>
<tr>
<td>(\text{Hr} + \text{N}_3^-)</td>
<td>6.3</td>
<td>50</td>
<td>(0.70)</td>
<td>71 (30)</td>
<td></td>
</tr>
<tr>
<td>(\text{Hr} + \text{CNO}^-)</td>
<td>6.3</td>
<td>7.7</td>
<td>(0.30)</td>
<td>26 (30)</td>
<td></td>
</tr>
</tbody>
</table>

* From measurements of \([O_2]\) in equilibrated solution before Reaction II occurs.
* Approximate values only since difficult to estimate intercept of \(k_{\text{obs}}\) versus \([X^-]/[O_2]\) plot.
* Pre-equilibrium constant from data for Reaction II.
* From oscilloscope trace amplitudes.
tions (Fig. 5). This behavior can be interpreted with the following scheme:

\[ \text{HrO}_2 + X^- \rightleftharpoons \text{HrX}^- + \text{O}_2 \quad K_1 K_2 \quad (5) \]

\[ \text{HrX}^- + \text{O}_2 \rightleftharpoons \text{Hr}^{+}X^- + \text{O}_2^{2-} \quad k_3 \quad (7) \]

or

\[ \text{HrX}^- + \text{O}_2 \rightleftharpoons \text{Hr}^{+}X^- + \text{O}_2^{2-} \quad k_4 \quad (8) \]

The first step is a rapid pre-equilibrium and \( k_{\text{obs}} \) is given by:

\[ k_{\text{obs}} = \frac{a[X^-]}{[O_2] + K_1 K_2[X^-]} \quad (9) \]

with \( a = k_3 \) or \( k_4 K_1 K_2 \). This rate law explains the main features of the kinetics depicted in Figs. 4 and 5—i.e. a limiting \( k_{\text{obs}} \) independent of \([X^-] \) when \( K_1 K_2[X^-] > [O_2] \); this limiting value is, however, dependent on \([O_2] \). Obviously higher concentrations of \( X^- \) are needed to produce this limiting rate as the oxygen concentration is increased. At low \([\text{anion}], K_1 K_2[X^-] < [O_2] \) and \( k_{\text{obs}} = a[X^-] \), now independent of \([O_2] \). Inverting Equation 9,

\[ \frac{1}{k_{\text{obs}}} = \frac{1}{a[X^-]} + \frac{K_1 K_2}{a[O_2]} \quad (10) \]

Plots of \( k_{\text{obs}}^{-1} \) versus \([X^-]^{-1} \) should be linear, with slopes independent of \([O_2] \) and intercepts inversely proportional to \([O_2] \). Plots of \( k_{\text{obs}}^{-1} \) versus \([O_2]^{-1} \) should be linear with slopes independent of \([X^-] \) and intercepts inversely proportional to \([X^-] \).

Demonstrations of the correctness of these predictions are shown in Fig. 6 (for \( X^- = \text{CNO}^- \) ) and Fig. 7 (for \( X^- = \text{N}_3^- \)). From plots such as these, the values of \( k_3, k_4, \) and \( K_1 K_2 \) for \( X^- = \text{NB}^- \) at a variety of \( pH \) values are summarized in Table II, and a linear dependence of \( k_3 \) or \( K_1 K_2 k_4 \) on \([H^+] \) is indicated.

The plots of \( k_{\text{obs}} \) versus \([\text{NB}^-] \) were only slightly curved at the highest \( pH \), and the derived value for \( K_3 K_4 \) is only approximate. This arises because the value of \( K_3 K_4[\text{NB}^-] \) is less than \([O_2] \) even at the highest azide concentrations used, and \( k_{\text{obs}} = a[\text{NB}^-] \) (Equation 9). The small value of \( K_3 K_4 \) is also indicated by a constant absorbance for the slow reaction change with different \([\text{NB}^-] \) at \( pH \) 8.5, whereas at lower \( pH \), absorbance changes for the slow reaction increase with increasing \([\text{NB}^-] \), since substantial amounts of the colorless \( \text{HrNB}^- \) are formed in the rapid pre-equilibrium Equation 5. The slow reaction of \( \text{HrO}_2 \) and \( \text{CNO}^- \) ion was investigated only at \( pH \) 7.3 and 6.3 because of the very long half-lives involved, with concommi-
FIG. 7. Plots of $10^{-9} \times k_{\text{obs}}$, s versus $10^{-9} \times [O_2]^{-1}$, for (slow) Reaction II, pH 6.3, $I = 0.15$ M, and $T = 25^\circ$. $\bigcirc$, 10 mM; $\bullet$, 20 mM; $\triangle$, 90 mM; $\bigtriangleup$, 100 mM azide.

**TABLE II**

Rate and pre-equilibrium constants for slow formation of metaazide (Reaction II) at 25° and $I = 0.15$ M

| Values from $k_{\text{obs}}$ versus $[N_3^-]$ plots. |
| --- | --- | --- | --- |
| $pH$ | $10^9 \times k_1$ | $k_3$ | $10^9 \times K_{K_2}$ |
| 8.0 | 0.50 | $\approx 20$ | $\approx 0.026$ |
| 8.0 | 2.3 | 6 | 0.39 |
| 7.5 | 2.8 | 21 | 0.13 |
| 7.0 | 8.8 | 19 | 0.46 |
| 6.3 | 34 | 21 | 1.6 |
| 5.5 | 450 | 19 | 24 |

* Values from mean results of three $k_{\text{obs}}$ versus $[O_2]$' plots.

It is clear that $K_1 K_2 [X^-] < [O_2]$ in Equation 9 in all conditions, and that $k_{\text{obs}} = a[X^-]$. The second order rate constants $a$ ($= k_3$ or $k_4 K_2$) are recorded in Table III for all anion catalysis. The effect of pH and temperature on these values was determined for the $NO_3^-$ and $F^-$ systems. First order dependence for $a$ on $[H^+]$ was noted in Table III. Enthalpies of activation values were 21.9 and 22.9 kcal mol$^{-1}$; and entropies of activation were $+12$ and $+15$ e.u., respectively.

**TABLE III**

Second order rate constants for slow formation of met derivatives from $HrO_2$ and ligands at 25° and $I = 0.15$ M

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_4$</th>
</tr>
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<tbody>
<tr>
<td>$N_3^-$</td>
<td>6.3</td>
<td>0.34</td>
<td>14</td>
</tr>
<tr>
<td>7.6</td>
<td>0.028</td>
<td></td>
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</tr>
<tr>
<td>CNO$^-$</td>
<td>6.3</td>
<td>0.080</td>
<td>80</td>
</tr>
<tr>
<td>7.3</td>
<td>0.0062</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>6.3</td>
<td>0.0083</td>
<td>3</td>
</tr>
<tr>
<td>7.0</td>
<td>0.00014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI$^-$</td>
<td>7.3</td>
<td>0.00033</td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>5.3</td>
<td>3.5</td>
<td>500</td>
</tr>
<tr>
<td>6.3</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.3</td>
<td>0.026</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.3</td>
<td>0.0022</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F$^-$</td>
<td>5.3</td>
<td>2.0</td>
<td>300</td>
</tr>
<tr>
<td>6.3</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.3</td>
<td>0.0094</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Imidazole 8.0 0.004

* Rate constant for reaction of HX (see text).

with all other anions studied, only one reaction was observed forming the met. No fast spectral changes were observed when $HrO_2$ was mixed with SCN$^-$, NO$_3^-$, or $F^-$ ions in a stopped flow apparatus. Using anion and oxygen concentrations in excess of protein, excellent pseudo-first order plots were obtained, often to three to four half-lives. The rate constant $k_{\text{obs}}$ was independent of $[O_2]$ and was directly proportional to anion concentration, the $k_{\text{obs}}$ versus $[X^-]$ plot extrapolating to the origin. Curvature in these plots was not evident.

**DISCUSSION**

**Rapid Interaction of Oxyhemerythrin with $N_3^-$ and CNO$^-$ (Reaction I)** There is very good agreement for the values of $K_1 K_2$ for Equation 5 using various methods (Table I). Since $K_2$, is pH-independent from 6.8 to 9.0 (13, 17, 18), there is a linear dependence of $K_2$, on $[H^+]$ (Table II) for $X^- = N_3^-$, and a similar dependence for CNO$^-$ from limited data. The dependence of $K_2$ on $[H^+]$ resides mainly in a proton-assisted $k_2$ step for the $N_3^-$ and (possibly CNO$^-$) reactions. If the reactive species are therefore assumed to be HN$^+$ and HCNO (which appears more likely than a proton associated with the protein (14, 19)), the calculated second order rate constants and equilibrium constants for reaction with Hr are $6.4 \times 10^8$ M$^{-1}$ s$^{-1}$ and $6.0 \times 10^8$ M$^{-1}$ s$^{-1}$ and $-6 \times 10^9$ M$^{-1}$ and $10^9$ M$^{-1}$, respectively. The values

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of $k_1$, $k_2$, and $K_e$ for the reactions of Hr with $\text{N}_2\text{O}_5^-$ and CNO$^-$, measured directly, differ significantly from those (above) for the same step, measured indirectly (Table I). It appears that deoxyhemerythrin produced as a stationary state intermediate in Step 2 behaves differently than deoxyhemerythrin, prepared separately. We have no convincing explanation for this. In the concentrations used in our studies ($\geq 30 \mu M$ protein) oxy-, met-, and (presumably) deoxyhemerythrin were present in the octameric form at all pH values (20, 21). In the steady state (low) concentrations of Hr in Equations 2 and 3, protein would be monomeric if the octamer, monomer equilibrium could adjust sufficiently rapidly, which is unlikely (20). The steady state Hr may not equilibrate with water to form an aquated species or may have a different conformation than "equilibrated" deoxyhemerythrin. This behavior is reminiscent of that of hemoglobin although the parallelism may be unrelated. Sudden removal of CO from the hemoglobin-CO adduct produces a form of hemoglobin which recombines with CO much more rapidly than the "normal" form (3, 29). We examined the reaction of CNO$^-$ with "fresh" deoxyhemerythrin, by mixing oxyhemerythrin with CNO$^-$/S$_2$O$_8^{2-}$ in the stopped flow apparatus. A rapid decrease in absorption at 500 nm due to the formation of Hr ($t_{1/2} \approx 10$ ms (14)) was followed by a slower smaller absorption decrease due to the interaction of Hr with CNO$^-$ ion. The rate constant for the latter process agreed well with that obtained from "prepared" deoxyhemerythrin and not the "steady state" material.

The coordination of the iron pairs in the subunit of methemerythrin from Themiste dyscritum has been recently deduced from a 2.8 Å resolution electron density map of the protein (23). Each iron is coordinated with five amino acids from protein side chains, two of which are shared and thus form bridges between the ions. There is a third bridging position, possibly the site of O$_2$, H$_2$O, or anions in the met species. Each iron is octahedrally coordinated (23). We believe that the anions which coordinate to the deoxy form also attach directly to iron, rather than binding to secondary sites (1), because of the relative sluggishness of the formation and dissociation processes.

**Conversion of Oxyhemerythrin into Methemerythrin (Reaction II) — A very slow autooxidation of oxyhemerythrin occurs in the presence of chloride ions (8, 24). At neutral pH and in 0.3 M KCl, the half-life for conversion is 18.5 h at 25° with some denaturing. All other anions accelerated the production of methemerythrin in an overall second order process with rate constants $k_3$ shown in Table III.

Anions$^3$ catalyze the conversion of oxyhemoglobin into the corresponding met form in a uniphasic process (4). The reactivity order $N_2O_5^- > SCN^- > OCN^- > F^- > Cl^-$ at pH 5.0 resembles somewhat that found for hemerythrin, but the rates are some 10$^6$ to 10$^9$ times slower. This apparently resides in much larger activation energies (27 to 32 kcal mol$^-$1) (5) than with our systems (23 kcal mol$^-$1). Both autooxidations are subject to acid catalysis (see Tables II and III) with a rate law (Fe = HrO$_2$ or HbO$_2$)

$$
\text{Rate} = k_3 [\text{Fe}[X^-][H^+]^{-1}
$$

(12)

The reactions with hemoglobin and hemerythrin can be interpreted in terms of a nucleophilic displacement of superoxide (4) or peroxide from a protonated intermediate. The acid dependence could arise from protonation of the coordinated peroxide or anion. The second order rate constant for reaction of HX ($k''_3$, Table III) can be calculated from the expression

$$
k''_3 = k_3 (1 + k_{\text{aq}}^* [H^+]^{-1})
$$

(13)

where $K_{\text{aq}}$ is the acid dissociation constant of HX (20). Some contribution of the oxyhemerythrin to the acid dependence is indicated by the small acceleration by acid in the presence of the nonbasic chloride ion. With cyanate and azide catalysis, we can determine the pH invariant rate constant, $k_3$ (Table III), for the alternative path which is an oxidation of the Fe$^{3+}$ anion adduct by molecular oxygen. With autooxidation in the presence of HCO$_3^-$, NO$_3^-$, and F$^-$ ions, Mechanism 8 is still a viable alternative to Equation 7 but because no deviations from second order behavior are observed, or rapid interactions detected, $[O_2] \gg K_c [X^-]$ in Equation 9, $K_c K_e < 2 \times 10^{-3}$ M$^{-1}$, and only lower limits for $k_3$ can be assessed (Table III). In this mechanism the function of the anion is to eliminate oxygen from the coordination sphere of the iron and allow electron transfer from the two irons, now in the bivalent state, to the oxygen. The outer sphere reduction of O$_2$ by one Fe$^{3+}$ ion to produce O$_2^-$ and Fe$^{3+}$ is expected to be very slow and a rate-determining step in which O$_2$ is directly reduced to O$_2^-$ by two Fe$^{3+}$ ions is favored (27). The binuclear Fe$^{3+}$ entity in each subunit of hemerythrin provides the two Fe$^{3+}$ centers. Recent data on the autooxidation of oxyhemoglobin (MbO$_2$) support a mechanism in which the oxidation of oxyhemoglobin by molecular oxygen is the slow step (10).

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