The Reaction of Fe(III) Myoglobin Fluoride with Reducing Agents

By RAYMOND P. COX

Institute of Biochemistry, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

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The reaction of reducing agents with Fe(III) myoglobin fluoride from sperm whale was studied at alkaline pH values. The rate of reduction by dithionite was indistinguishable from the rate of ligand dissociation even when the values of the rate constants for both were only $1.0 \times 10^{-3} \text{ s}^{-1}$ (at pH 10.6). Reduction by the reduced Methyl Viologen radical ion and reduced Safranin was faster than the rate of dissociation, providing evidence that these reductants can donate electrons to the iron centre via a pathway involving an (undetectable) liganded Fe(II) intermediate.

Fe(III) myoglobin can form complexes with a variety of charged ligands that have low affinities for the Fe(II) species (Antonini & Brunori, 1971). There are two possible pathways for the irreversible reduction of such compounds:

where Mb is myoglobin and L is the ligand. [The bound water molecule ionizes with a pK of 9.0. If unliganded Mb·Fe(III) participates in reaction (1) it is assumed to be in rapid equilibrium with Mb·Fe(III)OH₂ or Mb·Fe(III)OH[−] (Sweigart & Bern, 1974).]

A study of the reduction by dithionite of a number of derivatives of Fe(III) myoglobin (Cox & Hollaway, 1977) suggested that the fluoride derivative differed from the other compounds studied (those with cyanide, azide, thiocyanate and imidazole) in that the only pathway of reduction involved prior dissociation of the ligand. Fe(III) myoglobin fluoride, with the ligand in position, was not detectably reduced by dithionite; even at pH 8.9 the rate of reduction was not distinguishable from the rate of dissociation of the ligand, even though the value of both rate constants was only $5 \times 10^{-3} \text{ s}^{-1}$ at this pH.

This suggested the possibility that in the undissociated complexes the only pathway of electrons to the iron atom of myoglobin from dithionite (actually the SO₂^{·−} radical ion (Lambeth & Palmer, 1973; Cox & Hollaway, 1977) lies through the dissociable ligand; it is noteworthy that the derivatives showing evidence for direct reduction had a potential pathway of electron conductance in their $p\pi$ -orbital systems.

Further investigation of the reaction of reducing agents with Fe(III) myoglobin fluoride is reported here.

Experimental

Sperm-whale myoglobin was from Sigma Chemical Co., St. Louis, MO, U.S.A. Fe(III) myoglobin fluoride was prepared as described previously (Cox & Hollaway, 1977) with 0.2M-Tris, -sodium borate or -glycine buffers containing 0.5M-KF. Sodium dithionite was from Merck, Darmstadt, Germany. Reactions were followed in degassed buffer solutions with a Perkin-Elmer 356 dual-wavelength spectrophotometer. The temperature was 23–24°C. The kinetics of all reactions reported were pseudo-first-order.

All other conditions were as reported previously in a paper that also contains control experiments and theoretical justifications of the conditions used (Cox & Hollaway, 1977).

Results

The effect of increasing the pH in decreasing the values of the dissociation rate constant of fluoride from Fe(III) myoglobin (Blanck *et al.*, 1961) means that the study of reduction reactions at alkaline pH provides a sensitive test for the occurrence of low rates of direction reduction. This is because reduction by a pathway involving prior dissociation of the ligand can be no faster than the dissociation rate. Unfortunately the relatively low affinity of Fe(III) myoglobin for fluoride at alkaline pH means that high ionic strengths are necessary if a reasonably high proportion of myoglobin is to have the ligand bound, and it proved difficult to obtain accurate estimates of the dissociation rate constant, k_{off} ($=k_{+1}$ in eqn. 1), from the effect of the fluoride concentration on the rate of the association reaction under these conditions. However, the value of the association rate constant for the azide ion between pH 8.0 and pH 10.0

Table 1. *Interaction of fluoride with Fe(III) myoglobin from sperm whale*

Reactions were measured in 0.2M-sodium borate buffer containing 0.5M-KF. The rate constant for dissociation (k_{off}) was measured from the rate of displacement of fluoride by azide. The value of the association rate constant (k_{on}) was calculated from the values of k_{off} and the rate of formation of the complex in the presence of 0.5M-KF.

	pH ... 8.0	9.0	10.0
$k_{\text{off}} (\text{s}^{-1})$	1.8×10^{-2}	4.0×10^{-3}	1.5×10^{-3}
$k_{\text{on}} (\text{M}^{-1} \cdot \text{s}^{-1})$	5.6×10^{-1}	7.6×10^{-2}	1.0×10^{-2}

Fig. 1. *Effect of pH on reactions of Fe(III) myoglobin fluoride*

The medium contained 0.5M-KF and 0.2M-sodium borate adjusted to various pH values by the addition of NaOH. (The exact pH of the solution was measured immediately after the experiment.) ○, Displacement of fluoride by NaN₃ (final concentration 50mM); △, reduction by sodium dithionite (final concentration 20mM). Reactions were followed by measuring $A_{605}-A_{540}$. The final myoglobin concentration was 26μM. The k values are on a logarithmic scale.

is more than $10^3 \text{M}^{-1} \cdot \text{s}^{-1}$ (Duffey *et al.*, 1966). This is so much greater than the value of the association rate constant for fluoride (Table 1) that k_{off} for fluoride can be measured by the rate of displacement of fluoride by azide when the concentrations of the two ligands are such that the final equilibrium position is overwhelmingly in favour of the azide derivative.

Fig. 1 shows the effect of pH on both the rate of formation of Fe(III) myoglobin azide after the addition of azide to a solution containing Fe(III) myoglobin fluoride, and the rate of reduction of the fluoride derivative by dithionite [in both cases only the slow phase of the change is shown; there was a rapid phase occurring within the time necessary for the addition of reagents and stirring (<5s) corresponding to the amount of Fe(III) myoglobin with water or OH⁻ bound]. It was confirmed that under

Fig. 2. *Effect of redox mediators on the rate of reduction of Fe(III) myoglobin fluoride in the presence of 20mM-sodium dithionite*

The reaction medium contained 0.5M-KF and 0.2M-Tris buffer, adjusted to pH8.0 with HCl. The reaction was started by adding a small volume of Fe(III) myoglobin fluoride (final concentration 8μM) to a solution containing 20mM-sodium dithionite and various concentrations of Methyl Viologen (□), Safranine (○) or anthraquinone-2-sulphonate (△). The reaction was followed by measuring A_{432} (reference wavelength 456nm).

the conditions of the experiment the value of the association rate constant for azide was several orders of magnitude greater than that for fluoride, and that the equilibrium position was overwhelmingly in favour of the azide form. As expected, the rates were independent of the concentrations of azide or dithionite.

It has already been shown by rapid-wavelength-scanning stopped-flow spectrophotometry that the reduction of Fe(III) myoglobin fluoride by dithionite at pH6.4 does not involve any detectable intermediates before the final product, unliganded Fe(II) myoglobin (deoxymyoglobin), is formed (Cox & Hollaway, 1977). This was confirmed during the present experiments for the reaction at pH10.5; a sequence of spectra taken during the course of the reaction showed clear isosbestic points.

The close agreement between the rate of reduction and the rate of dissociation of fluoride provides further support for the idea that reduction occurs predominantly by a pathway involving prior ligand dissociation. Reduction by a direct pathway cannot occur at a rate greater than $2 \times 10^{-4} \text{s}^{-1}$ at pH10.6; this corresponds to a value of the second-order rate constant of less than $50 \text{M}^{-1} \cdot \text{s}^{-1}$ for the reaction with the $\text{S}_2^{\cdot -}$ radical ion, assuming the value for the

equilibrium constant for the dissociation of $\text{S}_2\text{O}_4^{2-}$ given by Lambeth & Palmer (1973).

The relatively slow reaction of Fe(III) myoglobin fluoride with dithionite at alkaline pH values means that it is possible to investigate the reactions of other reducing agents used as mediators in the presence of an excess of dithionite, which will remove any residual oxygen and under suitable conditions will keep the mediator reduced so that pseudo-first-order kinetics are observed, even at low mediator concentrations.

Fig. 2 shows the effect of the addition of Methyl Viologen (1,1'-dimethyl-4,4'-bipyridylum ion), Safranin and anthraquinone-2-sulphonate in the presence of 20 mM-sodium dithionite on the rate of reduction of Fe(III) myoglobin fluoride at pH 8.0. The redox potential of all these mediators (Clark, 1960) is much lower than that for myoglobin [E'_0 at pH 8.0 = +40 mV (Brunori *et al.*, 1971)] under these conditions. The addition of anthraquinone-2-sulphonate had very little effect on the rate of reduction, but Safranin and especially Methyl Viologen gave an appreciable stimulation. The rate constant for the reaction with reduced Methyl Viologen (presumably the radical cation) was $7 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. No intermediate compounds were detected during the course of the reduction.

Discussion

The results presented here suggest that the mechanism for the reduction of Fe(III) myoglobin derivatives suggested by Cox & Hollaway (1977) for the reaction with dithionite, i.e. electron transfer via the dissociable ligand, may not apply to all reducing agents. Reduced Methyl Viologen and reduced Safranin appear to be able to donate electrons to the iron atom of Fe(III) myoglobin with fluoride bound, whereas dithionite is able to do this only at a very low rate, if at all. However, it is noteworthy that the rate of reaction with Methyl Viologen radical cation seems low for what is

normally an extremely reactive reductant, suggesting that electron transfer via the fluoride ion cannot be excluded in this case. An alternative explanation for the results is that Methyl Viologen radical cation is able to donate electrons to the iron centre through the body of the molecule, in a reaction that is much less favourable with SO_2^{2-} , either because of the higher reactivity of Methyl Viologen radical cation or because of the charge distribution on the protein surface. A comparison of the reaction of dithionite and reduced Methyl Viologen with other myoglobin derivatives may help to distinguish between these possibilities.

None of the present results conflicts with the general idea that, unless a suitable ligand provides a pathway for electrons to the iron centre, myoglobin is an unreactive redox reagent compared with haem proteins involved in biological electron transfer. This property has obvious importance in lowering the rate of oxidation of the physiologically significant Fe(II) forms.

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