Electron transfer between horse ferritin and ferrihaemoproteins

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Reactions of reduced horse spleen ferritin with horse and *Saccharomyces cerevisiae* ferricytochromes *c*, cow ferricytochrome *b₅*, sperm-whale metmyoglobin and *Pseudomonas aeruginosa* ferricytochrome *c*-551 were investigated by u.v.-visible spectrophotometry. In all cases the reduced ferritin reduced the ferrihaemoproteins. The rates of reduction varied from $< 0.2 \text{ M}^{-1} \cdot \text{s}^{-1}$ for metmyoglobin to $1.1 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ for horse ferricytochrome *c* (0.1 M-phosphate buffer, pH 7.4, at 25 °C). We conclude that the mechanism of ferrihaemoprotein reduction involves long-range electron transfer through the coat of ferritin and that such electron transfer is rapid enough to account for the rates of iron release observed by other workers in reductive release assays.

INTRODUCTION

There are a wealth of structural and functional data available for the iron-storage protein ferritin, but despite this its mechanisms of iron uptake and release remain unclear (Clegg *et al.*, 1980; Ford *et al.*, 1984; Crichton & Charlotteaux-Wauters, 1987). *In vitro*, and probably *in vivo*, redox reactions are associated with iron deposition and mobilization. The purpose of the present paper is to report interprotein long-range electron-transfer reactions of ferritin that may be relevant to its physiological redox reactions.

Some insight into the mechanisms of iron deposition and mobilization is provided by the three-dimensional structure of ferritin (Ford *et al.*, 1984). The ferritin molecule consists of 24 subunits, each with an M_r of approx. 20000. These form a protein shell that surrounds a space of approx. 8 nm (80 Å) diameter in which the non-haem iron core of ferritin is laid down. Channels through the coat, formed by the subunit interfaces, allow iron to enter and leave the core.

Iron deposition is proposed to occur by the iron entering the core as Fe^{2+} , binding to the protein or to the growing Fe^{3+} -containing mineral that forms the non-haem iron core, and being oxidized to Fe^{3+} (Clegg *et al.*, 1980; Ford *et al.*, 1984; and references therein). The recent discovery of a monomeric Fe^{3+} centre in the coat of human ferritin may require this proposal to be modified to include the Fe^{2+} being oxidized before its attachment to the mineral core (Lawson *et al.*, 1989). Such a ferroxidase centre bears some resemblance to the mechanism of iron uptake proposed by Crichton & Roman (1978). However, the central role of the redox step remains. Iron mobilization appears to follow the reverse process with the release step after reduction of iron to Fe^{2+} . Although Fe^{3+} chelators can remove iron, they do so slowly and are probably not physiologically important (Clegg *et al.*, 1980; Theil, 1983).

Two general schemes for the reductive iron mobilization process can be envisaged. Firstly, the reductant enters the core and reduces the Fe^{3+} at short range, perhaps at the same time chelating the Fe^{2+} produced (Clegg *et al.*, 1980). Thioglycolic acid and flavins have been proposed to function in this way (Funk *et al.*, 1985; Jones *et al.*, 1978). Secondly, the reductant may remain outside the protein and pass electrons into the core. This involves electron transfer over a range of 2–2.5 nm (20–25 Å).

The latter possibility has attracted attention since the discovery of bacterial analogues of ferritin, known as bacterioferritin, that contain iron in haem groups in the protein coat in addition to the iron in the non-haem iron core (Stiefel & Watt, 1979; Yarov *et al.*, 1981; Moore *et al.*, 1986). Watt *et al.* (1988) have directly investigated the possibility of long-range electron transfer with ferritin using a reaction system involving the oxidation of Fe^{2+} -containing ferritin by mitochondrial cytochrome *c*, flavodoxin and blue copper proteins. Although they did not report the overall rates of reaction, Watt *et al.* (1988) showed convincingly that long-range electron transfer occurred to produce oxidized ferritin similar to that obtained from oxidation with O_2 or $[\text{Fe}(\text{CN})_6]^{3-}$.

We have built on the work of Watt *et al.* (1988) to measure the rates of reaction of reduced horse spleen ferritin with mitochondrial cytochrome *c*, myoglobin, cytochrome *b₅* and a bacterial cytochrome *c*-551. Our results, reported herein, confirm that long-range electron transfer occurs and shows that the rate of transfer does not depend simply on the redox potential of the oxidant.

MATERIALS AND METHODS

Proteins

Horse spleen ferritin was obtained as a solution (25 mg/ml) from Sigma Chemical Co. Its average content of Fe^{3+} determined by atomic absorption spectroscopy was 0.125 mg/mg of protein. This is the equivalent of approx. 1100 Fe^{3+} ions per ferritin molecule. Reduced ferritin was prepared under N_2 by reaction of holoferritin with an excess of $\text{Na}_2\text{S}_2\text{O}_4$ at pH 7.4 (0.1 M-sodium phosphate buffer) at 20 °C for 30 min. Excess $\text{Na}_2\text{S}_2\text{O}_4$ was removed by gel filtration through Sephadex G-25 under N_2 . The content of Fe^{2+} determined as $[\text{Fe}(\text{bipy})_3]^{2+}$ was 0.125 mg/mg of protein.

Horse heart cytochrome *c* and sperm-whale myoglobin were obtained from Sigma Chemical Co. and used without additional purification, and *Saccharomyces cerevisiae* iso-1 cytochrome *c*, *Pseudomonas aeruginosa* cytochrome *c*-551 and bovine liver cytochrome *b₅* were prepared by standard methods (Parr *et al.*, 1976; Reid & Mauk, 1982). Solutions of the ferrihaemoproteins were deoxygenated by repeated degassing and flushing with O_2 -free N_2 .

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Instruments

Atomic absorption spectra were obtained with a Varian atomic absorption spectrometer, the Spectra AA-20, equipped with a PSC-56 programmable sample changer and a GTA-96 graphite tube atomizer.

Optical spectra and kinetic measurements were recorded with a Hitachi 557 spectrophotometer.

Redox reactions

A 2 ml portion of stock solution of reduced ferritin [containing 2.5 mg of protein/ml (0.125 mg of Fe/mg of protein) in 0.1 M-phosphate buffer, pH 7.4] was placed in a sample cuvette under N_2 and the cuvette sealed with a rubber Suba-Seal (Aldrich Chemical Co.). Samples of the anaerobic ferrihaemoprotein stock solutions and additional buffer solution were added to the cuvette to give a final volume of 3 ml and haemoprotein concentrations in the range 20–250 μM . Optical spectra of the resulting mixtures were recorded as a function of time. Reaction half-lives were obtained from single-wavelength measurements as a function of time. After the reactions had proceeded for 1–2 h (4 h in the case of myoglobin), excess $Na_2S_2O_4$ was added to reduce the haemoproteins completely. Haemoprotein concentrations were determined by using the following absorption coefficients (Antonini & Brunori, 1971; Lemberg, & Barrett, 1973; Ozols & Strittmatter, 1964): ϵ_{551}^{red} 31 $mm^{-1} \cdot cm^{-1}$,

ϵ_{550}^{red} 29 $mm^{-1} \cdot cm^{-1}$, ϵ_{557}^{red} 31 $mm^{-1} \cdot cm^{-1}$ and ϵ_{434}^{red} 115 $mm^{-1} \cdot cm^{-1}$ for cytochrome *c*-551, cytochrome *c*, cytochrome *b₅* and myoglobin respectively.

To ensure that the ferritin reduced the ferric protein through interprotein electron transfer and not from the leakage of Fe^{2+} from the core of ferritin, control experiments with $K_3Fe(CN)_6$ were carried out (Watt *et al.*, 1988). In this procedure 2 ml of the reduced ferritin stock solution was placed in a cuvette and portions of a freshly prepared solution of $K_3Fe(CN)_6$ were added to give a final $K_3Fe(CN)_6$ concentration of 0.01 M. Optical spectra over the range 500–800 nm were obtained for the resulting solutions as a function of time. Comparable spectra were obtained with solutions of 0.01 M- $FeSO_4$ and 0.01 M- $K_3Fe(CN)_6$ at pH 6.5.

RESULTS

Preparation of reduced ferritin

Reduction of the iron core of ferritin by dithionite followed by column chromatography on Sephadex G-25 under N_2 , to remove excess reductant, yielded a product containing the same amount of iron as the starting oxidized ferritin. Addition of $K_3Fe(CN)_6$ to the reduced ferritin did not result in the formation of Prussian Blue (Robin, 1962), indicating that the Fe^{2+} was retained by the ferritin. Partial and complete oxidation of the reduced core by O_2 or ferrihaemoproteins did not lead to iron release.

Reduction of ferricytochrome *c*-551 by ferritin

The reduction of ferricytochrome *c*-551 by reduced ferritin was monitored spectrophotometrically over the wavelength range 350–630 nm. The spectrum of the ferrocytochrome *c*-551 produced was indistinguishable from that of ferrocytochrome *c*-551 obtained by reduction with ascorbic acid.

The rate of the reaction between ferricytochrome *c*-551 and reduced ferritin was determined from the time-dependence of the absorbance change at 551 nm; immediately after mixing of the two proteins the absorbance increased. Values of reaction half-lives ($t_{1/2}$) were obtained from the time-course profiles at various concentrations of cytochrome *c*-551. These showed a linear inverse dependence on protein concentration, indicating that the reaction is second-order, and from the data of Fig. 1 the second-order rate constant for the reduction of cytochrome *c*-551 was found to be $0.9 \times 10^2 M^{-1} \cdot s^{-1}$.

Reduction of other ferrihaemoproteins by ferritin

The rates of ferritin-induced reduction of the horse and yeast cytochrome *c*, cytochrome *b₅* and myoglobin were measured in the same way as described above for cytochrome *c*-551. Only myoglobin presented difficulties because the reaction is very

Fig. 1. Time course for the reduction of 60 μM *Ps. aeruginosa* ferricytochrome *c*-551 by 10.41 μM reduced ferritin under N_2 and at pH 7.4 (0.1 M-phosphate) and 20 °C, monitored by the change in absorbance at 551 nm

The inset shows the concentration-dependence of $t_{1/2}^{-1}$ for the reduction of ferricytochrome *c*-551.

Table 1. Rates of reduction of ferrihaemoproteins

The charge and E_m were measured at pH 7.4, and the rate of reduction of $[Fe(EDTA)]^-$ at pH 7.0.

Protein	Charge	E_m (mV)	Electron self-exchange rate ($M^{-1} \cdot s^{-1}$)	Rate ($M^{-1} \cdot s^{-1}$) of reduction with:		References
				Ferritin	$[Fe(EDTA)]^-$	
Cytochrome <i>c</i>	+8.4	262	1×10^3	1.1×10^3	2.57×10^4	Gupta <i>et al.</i> (1972); Hodges <i>et al.</i> (1974); Moore & Pettigrew (1990)
Cytochrome <i>c</i> -551	-2.0	260	1×10^7	9.0×10^1	4.2×10^3	Coyle & Gray (1976); Moore <i>et al.</i> (1980); Dixon <i>et al.</i> (1989); Moore & Pettigrew (1990)
Cytochrome <i>b₅</i>	-14.2	0	2.6×10^3	1.1×10^2	2.85×10^3	Reid & Mauk (1982); Mathews (1985); Dixon <i>et al.</i> (1990)
Myoglobin	+7	50	—	< 0.2	22	Antonini & Brunori (1971); Lim & Mauk (1985)

slow. Thus for this protein only an upper limit to the rate was obtained. The rate constants are given in Table 1.

DISCUSSION

Comparison of electron-transfer second-order rate constants

The rates of ferrihaemoprotein reduction with ferritin compare well with those for reduction with $[\text{Fe}(\text{EDTA})]^-$ (Table 1). The rates for each protein are about 10-fold faster with $[\text{Fe}(\text{EDTA})]^-$, but the order of the rates for each reductant is similar, with myoglobin giving the lowest and cytochromes the highest rate. $[\text{Fe}(\text{EDTA})]^-$ was selected for comparison because it is a well-characterized reductant with some similarity to ferritin: it contains high-spin Fe^{2+} with at least four oxygen ligands, has an overall negative charge and possesses a redox potential of approx. 100 mV at pH 7 (Schwarzenbach & Heller, 1951). The redox potential of the core of horse ferritin is approx. -190 mV (Watt *et al.*, 1985) and, according to the theory of Marcus & Sutin (1985), the difference between these two potentials corresponds to a factor of approx. 3×10^2 in rate assuming all other factors are the same. Thus the reduction reactions with ferritin are slower than the comparable reactions with $[\text{Fe}(\text{EDTA})]^-$ by a factor of 10^3 – 10^4 -fold when corrected for the redox potential difference.

A large difference in rate is expected for the two reductants because $[\text{Fe}(\text{EDTA})]^-$ is smaller than the ferritin coat. Thus electron transfer takes place over a much shorter distance for $[\text{Fe}(\text{EDTA})]^-$ than it does with ferritin. The significance of the $[\text{Fe}(\text{EDTA})]^-$ comparison with ferritin comes in the analysis of the individual proteins. For example, reduction of metmyoglobin is slow in both cases, and reduction of ferricytochrome *c* is faster than reduction of the other cytochromes in both cases. This suggests that, as far as the ferrihaemoproteins are concerned, the mechanisms of electron transfer may be similar for both reductants. Therefore we have analysed the reactions using the scheme described by Marcus & Sutin (1985).

In this scheme electron transfer is proposed to take place in a non-covalently bound complex of the two proteins. The overall rate of reaction is then determined by the following parameters: (1) the redox driving energy (the difference between the donor and acceptor redox potentials); (2) the work needed to form the complex; (3) the work involved in protein conformational changes accompanying electron transfer and the electron-transmission properties of the reactants (e.g. how readily electrons move through the organic matrix and how far they have to travel). These factors are incorporated into the protein self-exchange rates.

Marcus & Sutin (1985) have shown that the following relationship applies to many redox reactions where the equilibrium constants are small and work terms can be ignored:

$$k_{12} = (k_{11}k_{22}K_{12})^{\frac{1}{2}} \quad (1)$$

where k_{12} is the rate for the cross-reaction between reactants 1 and 2, K_{12} is the equilibrium constant for the cross-reaction and k_{11} and k_{22} are the reactant self-exchange rates. Thus a comparison of the ferritin reactions with two haemoproteins, given in Table 1, yields the following relationship:

$$\frac{(k_{12}^A)^2}{(k_{12}^B)^2} = \frac{(k_{11}^F \cdot k_{22}^A \cdot K_{12}^{F,A})}{(k_{11}^F \cdot k_{22}^B \cdot K_{12}^{F,B})} \quad (2)$$

where the superscript F refers to ferritin, and superscripts A and B refer to two of the ferrihaemoproteins.

The comparison of cytochromes *c* and cytochrome *c*-551 is particularly instructive. Here the redox driving energies are the same, the structures of the proteins are very similar and neither

has a significant redox state conformation change (Moore & Pettigrew, 1990). Eqn. (2) then becomes:

$$\frac{(k_{12}^{\text{cyt.c}})^2}{(k_{12}^{\text{cyt.c-551}})^2} = \frac{(k_{22}^{\text{cyt.c}})}{(k_{22}^{\text{cyt.c-551}})} \quad (3)$$

From the self-exchange rates given in Table 1 the expected value of $k_{12}^{\text{cyt.c}}$ is calculated to be 10^{-2} times that of $k_{12}^{\text{cyt.c-551}}$. However, the observed value of $k_{12}^{\text{cyt.c}}$ is 10 times that of $k_{12}^{\text{cyt.c-551}}$. The difference between these rates is largely due to the complex-formation work terms, which we have omitted from this analysis. These are favourable for cytochrome *c*, because the reactants are oppositely charged, but unfavourable for cytochrome *c*-551.

The work terms are also unfavourable to a very great extent for the reaction of ferritin with cytochrome *b₅* and this, together with the decreased redox driving energy for the cytochrome *b₅* reaction compared with the cytochrome *c* reactions, should have produced a much lower rate of electron transfer. From eqn. (2) and the self-exchange rates and redox potentials in Table 1 we calculate that the expected rate of reaction between cytochrome *b₅* and ferritin should be approx. 10^{-2} times the rate of the cytochrome *c* reaction and approx. 10^{-4} times the rate of the cytochrome *c*-551 reaction. However, the observed cytochrome *b₅* rate with ferritin is comparable with that of cytochrome *c*-551, reversing the $[\text{Fe}(\text{EDTA})]^-$ trend, and only 10^{-1} times the cytochrome *c* rate. This indicates strongly that the cytochrome *b₅*-ferritin reaction is anomalous, with some feature of the system favouring electron transfer. This could be the formation of a complex, which may be only short-lived, particularly suited for electron transfer. Given the earlier proposal (Moore, 1985) concerning the possible physiological relevance of the cytochrome *b₅*-ferritin reaction, this system needs to be explored further.

Although cytochrome *b₅* could be a physiological partner of ferritin, since both proteins have a similar tissue distribution (Moore, 1985), neither of the cytochromes *c* is a likely physiological partner, since they are mitochondrial proteins and ferritin is extramitochondrial, and the myoglobin reaction is unlikely to be significant *in vivo* because it is so slow.

Interestingly, the difference in redox potential between the haem of apobacterioferritin and the core of holobacterioferritin is approx. 250 mV (Watt *et al.*, 1986), which is comparable with the difference in redox potential between cytochrome *b₅* and the core of ferritin of approx. 190 mV. It may be in both cases that the haem accepts electrons from the growing core when the incoming Fe^{2+} is oxidized to Fe^{3+} . If this is so, the marked decrease in the redox potential of the haem of bacterioferritin once the core is partially loaded (Watt *et al.*, 1986) could act as a control to ensure that some of the core iron remained in the Fe^{2+} state, or even to limit the growth of the core itself.

Long-range electron transfer in ferritin

The observed rate constants for the oxidation of reduced ferritin show that electron transfer across the protein coat of ferritin can occur relatively rapidly. Electron transfer over distances of 2–3 nm (20–30 Å) does not require the mediation of electron-transfer hop centres, though these can assist the transfer (Williams, 1969; Marcus & Sutin, 1985; Moore & Pettigrew, 1990). Iron located in the channels or subunits of ferritin could act as electron hop centres. Thus the Fe^{3+} centre identified by Lawson *et al.* (1989), a monomeric iron atom bound to side chains in human H-chain ferritin, may be present to assist electron transfer by decreasing the distance for each individual electron hop. However, it is not certain that such centres are present in all ferritins, and the indications are that they are absent from the ferritin used for the present study (Levi *et al.*, 1989). An alternative mediator for the transmission of electrons

through the protein coat is an, as yet, unidentified fluorescent group present in all ferritins and bacterioferritins (Maruyama & Listowsky, 1982; Moore *et al.*, 1986; F. H. A. Kadir, F. K. Al-Massad & G. R. Moore, unpublished work).

Whether the electron transfer involves an intermediary hop centre or is direct into, and out of, the core is secondary to the question of whether the rates are high enough to implicate long-range electron transfer in the uptake and release of iron. The data of Jones *et al.* (1978) concerning the rates of iron release resulting from reduction of the core of horse spleen ferritin by dihydroflavins address this point. These workers report rates of $30\text{--}310\text{ M}^{-1}\cdot\text{s}^{-1}$ for a range of dihydroflavins with redox potentials of -128 to -280 mV . These redox potentials provide a smaller driving force than for the cytochrome reactions described in the present paper. However, assuming that the rates of iron release are limited by the rate of electron transfer, we estimate that for a driving force of 200 mV the rate of iron release would be approx. $10^3\text{ M}^{-1}\cdot\text{s}^{-1}$. This is similar to the rates of electron transfer observed by us and indicates that long-range electron transfer is rapid enough to account for the rates of iron release observed by Jones *et al.* (1978).

We thank the Wellcome Trust for providing financial support for our work on ferritin, and the Science and Engineering Research Council, who help support the Centre for Metalloprotein Spectroscopy and Biology at the University of East Anglia via its Molecular Recognition Initiative.

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Received 17 December 1990/27 March 1991; accepted 9 April 1991