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MINI-REVIEW

Ferric reductases or flavin reductases?

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Assimilation of iron by microorganisms requires the presence of ferric reductases which participate in the mobilization of iron from ferrisiderophores. The common structural and catalytic properties of these enzymes are described and shown to be identical to those of flavin reductases. This strongly suggests that, in general, the reduction of iron depends on reduced flavins provided by flavin reductases.

Keywords: flavin, iron, reductases

Introduction

Iron is an essential element for virtually all living organisms. It participates in a large number of biological processes (e.g. storage and activation of molecular oxygen, electron transport, etc.) and is a cofactor of enzymes of intermediary metabolism (Crichton 1991). In aerobic environments the stable form is Fe^{3+}, which has little solubility in neutral aqueous solutions (10^{-17} M at physiological pH). To solubilize iron and override the iron limitation, most aerobic and facultative anaerobic microorganisms excrete in the growth medium highly efficient and specific low molecular mass soluble Fe^{3+}-chelating agents, named siderophores (Neilands 1981; also covered in: Winkelman 1991). Transfer of iron into the intracellular compartment is mediated by specific membrane receptors and transport systems which recognize the iron–siderophore complexes (Winkelmann & Huschka 1987, Crichton 1991). While an impressive number of studies have genetically established the siderophore uptake pathways and their regulation (Braun *et al.* 1987, Crosa 1989), less is known about the mechanisms by which iron is released from ferrisiderophores and thus made available for biosynthesis of iron proteins within the microbial cell. However, there is increasing evidence for the intracellular reduction of the ferric complex being one of the major pathways during liberation of iron. In particular, cytosolic ferrisiderophore reductase activities have been detected and studied in a large number of bacteria (Crichton 1991). Since siderophores have a much lower affinity for Fe^{2+}, such a process might allow the transfer of iron to iron-requiring enzymes. However, the corresponding enzymes have generally been incompletely purified and characterized. Very recently, two ferrisiderophore reductases, isolated from *Escherichia coli* and *Pseudomonas aeruginosa*, have been identified as flavin reductases and shown to utilize reduced flavins as mediators for the electron transfer to the ferric complex (Hallé & Meyer 1992a,b, Covès & Fontecave 1993). In this paper we review the current knowledge on flavin reductases and ferric reductases, and make the proposition that ferric reductases are flavin reductases.

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Flavin reductases

NAD(P)H: flavin oxidoreductases (flavin reductases) catalyze the reduction of flavins, flavin mononucleotide (FMN), flavin adenine dinucleotide

(FAD) or riboflavin, by reduced pyridine nucleotides, NADPH or NADH. Flavin reductases have been isolated in pure form from the luminous marine bacteria *Beneckea harveyi* (Jablonski & DeLuca 1977, Michaliszyn *et al.* 1977) and *Photobacterium fischeri* (Jablonski & DeLuca 1977), from microorganisms such as *E. coli* (Fontecave *et al.* 1987), *Bacillus subtilis* (Hasan & Nester 1978a,b), *Entameba histolytica* (Lo & Reeves 1980) and *P. aeruginosa* (Hallé & Meyer 1992a,b), and from human erythrocytes (Yubisui *et al.* 1977, 1979). A FMN reductase has been demonstrated in rat liver mitochondria (Ulvik & Romslo 1981).

All purified enzymes have several characteristics in common. Most flavin reductases are cytosolic enzymes. They all consist of one polypeptide chain of a rather small size (in general between 10 and 40 kDa). The visible spectrum of the protein gives no evidence for a chromophore and excludes the presence of flavins in the isolated enzyme. When added, FMN or FAD does not bind tightly and the enzyme thus should not be classified as a flavoprotein. Rather, the polypeptide chain displays an active site in which both the reduced pyridine nucleotide and the flavin can transiently bind, allowing a rapid electron transfer to proceed.

In general, there is no specificity for the flavin electron acceptor, although differences are observed in terms of the affinity of the enzyme for the various flavins, with K_m values found between 1 and 40 μM, in the case of bacterial enzymes. Flavin reductases are divided in three groups: one including enzymes specific for NADH, one specific for NADPH, and the third one for enzymes accepting both NADPH and NADH as electron donors.

Several reductases may be present in a given microorganism. This has been shown in the case of *B. harveyi* (Watanabe & Hastings 1982). Recently, we discovered a second flavin reductase in *E. coli*, purified it and identified it as the sulfite reductase (Covès *et al.* 1993a). This large enzyme has the capacity to catalyze the reduction of free flavins by NADPH, but its affinity for the substrates is rather low. Very preliminary results seem to indicate that the hemoglobin-like protein (HMP) of *E. coli* also carries a flavin reductase activity (J. Covès & M. Fontecave, personal communication).

The only flavin reductase whose gene has been cloned, sequenced and mapped is the enzyme from *E. coli* (Spyrou *et al.* 1991). This gene has been named *fre*. The sequence contains the AGGTG motif which has previously been suggested to form part of a pyridine nucleotide binding site in a number of NAD(P)H binding proteins. Moreover, we and others found striking sequence homologies with *lux*G, an uncharacterized open-reading frame so far found in the *lux* operons of three different species of luminescent bacteria, *V. harveyi*, *V. fischeri* and *Photobacterium leiognathi* (Swartzman *et al.* 1990a,b, Lee *et al.* 1991). This strongly suggests that *lux*G encodes the flavin reductase of these microorganisms.

In general, flavin reductases have been found to be associated with other biological processes requiring free reduced flavins. The reductase from human erythrocytes was discovered for its activity during reduction of the iron center of methemoglobin and has thus been also named methemoglobin reductase (Yubisui *et al.* 1977, 1979). This has been at the origin of the therapeutic administration of riboflavin to patients with hereditary methemoglobinemia, whose erythrocytes lack the NADH-cytochrome *b*5 reductase, the major methemoglobin reductase. Very recently, it has been shown that this flavin reductase is identical to the so-called heme-binding protein present in erythrocytes and liver (Quandt *et al.* 1991). This protein has a high affinity for protohemin and binding of protohemin completely inhibits reductase activity (Xu *et al.* 1992). These new results thus bring into question long-held beliefs as to the catalytic function of the protein. The reductases from *B. subtilis* and *E. coli* provide the reduced flavins required for the activation of chorismate synthase (Hasan & Nester 1978a,b, Ramjee *et al.* 1991). The enzyme from *B. harveyi* and from other luminescent bacteria provides reduced flavins as substrates of the light emitting luciferase reaction (Hastings *et al.* 1985). Luciferase catalyzes the oxidation of reduced FMN by oxygen in the presence of a long-chain aldehyde with the emission of light. On the basis of the great efficiency of reduced flavins during the reduction and mobilization of iron from ferritins, the enzyme from *B. harveyi* has been used as a reducing system to study mechanisms of ferritin iron reduction (Sirivech *et al.* 1974, Jones *et al.* 1978). The flavin reductase from *E. coli* was discovered as a component of a complex multiprotein system that catalyzes the transformation of an inactive form of ribonucleotide reductase into an active enzyme, containing a radical on Tyr-122 (Fontecave *et al.* 1987). The function of the flavin reductase is to reduce the non-heme ferric center of ribonucleotide reductase (Fontecave *et al.* 1989). The tyrosyl radical is then generated during the reaction between the reduced iron center and molecular oxygen. In this context it has been suggested that the flavin reductase might provide a mechanism for regulating ribonucleotide reductase

and DNA synthesis. Later, it was found that the flavin reductase from *E. coli* had a more general ferric reductase activity (Covès & Fontecave 1993). It catalyzes the reduction of ferric citrate, ferrisiderophores and ferritins during reactions that absolutely required the presence of free flavins and might provide a mechanism for the release of iron from Fe(III)-storage or Fe(III)-carrier systems (Figure 1). An extract from an *E. coli* mutant lacking an active *fre* gene has a greatly diminished capacity to reduce ferrichrome (Covès & Fontecave 1993). This result supports the importance of flavin reductase for iron reduction under physiological conditions. Sulfite reductase also activates ribonucleotide reductase in the presence of free flavins by a similar mechanism and has a ferrisiderophore reductase activity (Covès *et al.* 1993a,b). The enzyme from *P. aeruginosa* was first isolated as a ferrisiderophore reductase and later characterized as a flavin reductase (Hallé & Meyer 1989, 1992a,b). It should be noted that polyclonal antibodies raised against this enzyme did not present cross-reaction with the flavin reductase from *E. coli* (Hallé & Meyer 1992a).

It is thus striking that the flavin reductases have in common the ability to catalyze electron transfers from NAD(P)H to biological ferric complexes and raise the question whether ferric reductases, in general, are in fact flavin reductases.

Ferric reductases

In this paper, we define ferric reductases as enzymes catalyzing the reduction of non-protein ferric complexes, such as ferric citrate, ferrisiderophores or synthetic complexes such as ferricyanide or Fe(EDTA). We thus do not include the various enzymes transferring electrons to protein-bound iron (cytochromes, transferrin, etc.).

Both soluble and membrane-bound ferric reductases have been detected in a large number of bacteria including *Mycobacterium smegmatis* (Brown & Ratledge 1975), *Bacillus megaterium* (Arceneaux & Byers 1980), *Agrobacterium tumefaciens* (Lodge *et al.* 1982), *Azotobacter vinelandii* (Huyer & Page 1989), *Pseudomonas fluorescens* (Hallé & Meyer 1989), *B. subtilis* (Gaines *et al.* 1981), *Spirillum itersonii* (Dailey & Lascelles 1977) and *Staphylococcus aureus* (Lascelles & Burke 1978). However, only a limited number of enzymes have been purified to homogeneity and fully characterized. This is the case for the three cytosolic ferrisiderophore reductase activities of *E. coli*. The two first enzymes have been unambiguously identified as flavin reductases. One resides in the product of the *fre* gene, the second in the sulfite reductase and the third is the HMP (Andrews *et al.* 1992, Covès & Fontecave 1993, Covès *et al.* 1993a). As mentioned above the ferric reductase of *P. aeruginosa* has been obtained in pure form and shown to be a flavin reductase (Hallé & Meyer 1992a,b). Ferric reductases have also been purified in *Rhodopseudomonas sphaeroides* (Moody & Dailey 1985) and from *Neisseria gonorrhoeae* (Le Faou & Morse 1991). They have reported molecular masses of 32 and 25 kDa, respectively. These values are very close to that of the *E. coli* flavin reductase (26 kDa). Both require a flavin for optimal activity. They show similar behavior during DEAE chromatography and have a strong affinity for phenyl Sepharose. It thus appears very likely that these two ferric reductases are closely related to or even identical to the flavin reductase. Furthermore, the partially purified enzyme from *B. subtilis* is in all probability identical to the flavin reductase independently isolated from the same microorganism (Gaines *et al.* 1981). If one excepts the activity from *B. megaterium* and *M. smegmatis* for which there is not enough good data to conclude to a requirement for free flavins, all ferric reductases described so far display activity only in the presence of a free flavin and all utilize a reduced pyridine nucleotide (NADPH or NADH) as the electron source.

One striking property of ferric reductases is the total lack of substrate specificity. They usually catalyze the reduction of a large variety of iron complexes. It is remarkable that a given microorganism has the capacity to also reduce ferrisiderophores which do not support its own growth. In any case, ferric reduction is strongly inhibited by molecular oxygen (Figure 1).

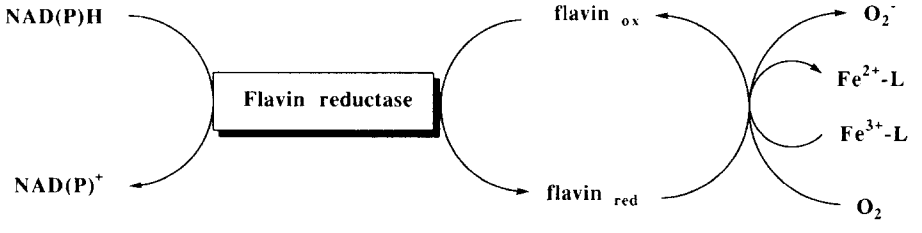

Figure 1. Enzymatically reduced free flavins transfer their electrons to ferric complexes or oxygen.

Most enzymes consist of a single polypeptide chain and are found to be located within the cytoplasm. However, iron reduction in *S. itersonii* and *S. aureus* was found to be associated with the respiratory chain (Dailey & Lascelles 1978, Lascelles & Burke 1978). Membranes of *E. coli* were shown to contain a ferrichrome reductase activity (Fischer *et al.* 1990).

From several studies it seems that the expression of ferric reductases is constitutive. It was similar in cells grown under aerobic and anaerobic conditions and was not affected by mutations in iron-uptake genes or mutations in ferric uptake regulation (Fischer *et al.* 1990). In general, this activity is not regulated by iron since equal reduction capacity is measured in extracts from iron-enriched or iron-deficient cells.

Finally, very few ferric reductase activities have been studied in eukaryotic organisms. One example is the plasma membrane-bound ferric reductase from the yeast *Saccharomyces cerevisiae*. The enzyme has been partially purified and shown to be specific for NADPH and to require FMN or FAD for activity (Lesuisse *et al.* 1990). In that case again the enzyme might not be a flavoprotein as previously suggested but rather a flavin reductase. Its gene has been cloned and sequenced (Dancis *et al.* 1992). The sequence has significant similarity to the sequence of the plasma membrane NADPH-binding cytochrome *b*558. The function of the reductase is to reduce ferric iron external to the cell, followed by a transmembrane movement of ferrous iron to the interior of the cell. It was shown that a mutant of *S. cerevisiae* lacking the externally-directed reductase activity was deficient in the uptake of ferric iron and was extremely sensitive to iron deprivation (Dancis *et al.* 1990). An NADH:sideramine oxidoreductase has also been observed in *Neurospora crassa* (Ernst & Winkelmann 1977).

Plants have the ability to acquire iron from certain microbial ferrisiderophores present in the soil (Crowley *et al.* 1987) or from ferriphytosiderophores (graminaceous plants). It has been suggested that again a reduction of iron can take place at the root surface and thus that a ferric reductase is an intimate component of iron assimilation in plants. However, no specific ferric reductase has been isolated from plants yet. On the other hand, several studies have reported that nitrate reductase, the first enzyme of the nitrate assimilatory pathway in higher plants, could catalyze the reduction of ferric citrate and a variety of ferrisiderophores (Castignetti & Smarrelli 1986). This enzyme is a flavoprotein containing FAD as a prosthetic group and does not seem to require free flavins for ferric reduction. Much more experimentation is needed to determine whether nitrate reductase or other ferrisiderophore reductases are involved in iron assimilation in plants.

Conclusions

Living organisms contain ferric reductases probably to allow an adequate supply of essential iron to cells. These enzymes have been carefully studied only very recently, in particular in bacteria. It appears now quite clearly that most ferric reductases are flavin reductases, a family of enzymes also poorly characterized. One exception might be the ferric reductase activity carried by the flavoprotein nitrate reductase. Reduced free flavins are well-adapted to iron reduction probably because (i) they are small molecules (when compared with flavoproteins for example), (ii) they have very low redox potentials (below -0.2 V) and (iii) they are able to transfer their two electrons stepwise, due to the relative stability of the semiflavin state. That free flavins play the role of electron transfer mediators during reduction of iron is consistent with the lack of substrate specificity of ferric reductases and the strong inhibition by oxygen, which competes with ferric iron for electrons. The reaction between enzymatically reduced flavins and oxygen results in the production of harmful superoxide and hydroxyl radicals (P. Gaudu & M. Fontecave, personal communication). An important question remains whether free flavins and oxygen are available within the cell at concentrations which allow the expression of such flavin or ferric reductase activities. Flavins have been extensively studied but only in the context of flavoproteins. Protein-free flavins might have other unexpected important functions.

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