Initial-rate kinetics of the flavin reductase reaction catalysed by human biliverdin-IX β reductase (BVR-B)

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The initial-rate kinetics of the flavin reductase reaction catalysed by biliverdin-IX β reductase at pH 7.5 are consistent with a rapid-equilibrium ordered mechanism, with the pyridine nucleotide binding first. NADPH binding to the free enzyme was characterized using stopped-flow fluorescence quenching, and a K_d of 15.8 μ M was calculated. Equilibrium fluorescence quenching experiments indicated a K_d of 0.55 μ M, suggesting that an enzyme–NADPH encounter complex (K_d 15.8 μ M) isomerizes to a more stable ‘nucleotide-induced’ conformation. The enzyme was shown to catalyse the reduction of FMN, FAD and riboflavin, with K_m values of 52 μ M, 125 μ M and 53 μ M, respectively. Lumichrome was shown to be a competitive inhibitor against FMN, with a K_i of 76 μ M, indicating that interactions with the isoalloxazine ring are probably sufficient for binding. During initial experiments it was observed that both the flavin reductase and biliverdin reductase activities of the enzyme exhibit a sharp optimum at pH 5 in citrate buffer. An initial-rate study indicated that the enzyme obeys a steady-state ordered mechanism in this buffer. The initial-rate kinetics in sodium acetate at pH 5 are consistent with a rapid-equilibrium ordered mechanism, indicating that citrate may directly affect the enzyme’s behaviour at pH 5. Mesobiliverdin XIII α , a synthetic biliverdin which binds to flavin reductase but does not act as a substrate for the enzyme, exhibits competitive kinetics with FMN (K_i 0.59 μ M) and mixed-inhibition kinetics with NADPH. This is consistent with a single pyridine nucleotide site and competition by FMN and biliverdin for a second site. Interestingly, flavin reductase/biliverdin-IX β reductase has also been shown to exhibit ferric reductase activity, with an apparent K_m of 2.5 μ M for the ferric iron. The ferric reductase reaction requires NAD(P)H and FMN. This activity is intriguing, as haem cleavage in the foetus produces non- α isomers of biliverdin and ferric iron, both of which are substrates for flavin reductase/biliverdin-IX β reductase.

Key words: bilirubin, biliverdin reductase, ferric reductase, haem degradation.

INTRODUCTION

The first step in the haem catabolic pathway in adult humans involves the specific cleavage of haem by haem oxygenase (EC 1.14.99.3) (both haem oxygenase-1 and -2) at its α -methene bridge to form the linear tetrapyrrole biliverdin-IX α . This is followed by the NAD(P)H-dependent reduction of biliverdin-IX α catalysed by biliverdin-IX α reductase (BVR-A) (EC 1.3.1.24) to form bilirubin-IX α . There is now considerable interest in this pathway, as it has been suggested that bilirubin-IX α may act as a physiologically important antioxidant. Reactions of bilirubin-IX α involving free radicals or toxic oxygen reaction products have been well documented [1]. Unconjugated bilirubin-IX α scavenges singlet oxygen with high efficiency [2], reacts with superoxide anion [3] and peroxy radicals [4], and serves as a reducing substrate for peroxidases in the presence of hydrogen peroxide or organic hydroperoxides [5,6]. It has been shown that both free and albumin-bound bilirubin-IX α are efficient co-antioxidants for α -tocopherol, inhibiting plasma and low-density lipoprotein lipid peroxidation [7], and that bilirubin-IX α is a significantly more effective protector of human ventricular myocytes than several known physiological antioxidants, including vitamin C [8]. That this antioxidant activity is physiologically important is indicated by the finding that there is an inverse relationship between plasma bilirubin-IX α levels and the risk of coronary artery disease [9,10].

Yamaguchi and co-workers [11] presented the first evidence for the existence of a second form of biliverdin reductase (BVR-B), capable of reducing the IX β , IX γ and IX δ isomers of biliverdin, but not the IX α isomer. They subsequently described the purification of four isomers of BVR from human liver cytosolic fractions [12]. All four isomers of BVR were found to be monomers, with molecular masses of approximately 21 kDa (isoenzymes I and II) and 34 kDa (isoenzymes III and IV). The relationship between the two chromatographically separable forms of BVR-B (isoenzymes I and II) is currently unclear. The function of BVR-B in the adult is uncertain, as adult bile contains 95–97% bilirubin-IX α . However, the proportions of bilirubin isomers found in human foetal bile are 87% -IX β , 6% -IX α , 6% -IX δ and 0.5% -IX γ [13]. It is possible that the foetus produces predominantly the -IX β isomer of bilirubin, since this isomer does not undergo internal hydrogen bonding, unlike bilirubin-IX α , and consequently has a much higher solubility. Because of its low solubility, bilirubin-IX α must first be conjugated to glucuronate before it can be excreted into the bile. This conjugation reaction is catalysed by UDP-glucuronyltransferase (EC 2.4.1.17), an enzyme that is not expressed at significant levels until the first week after birth. It has been shown that bilirubin-IX β can be excreted directly into the bile without the need for conjugation to glucuronate [14].

It was initially reported that neither BVR-A nor BVR-B displayed any other oxidoreductase activities [11]. However, it

Abbreviations used: BVR, biliverdin reductase; BVR-A, biliverdin-IX α reductase; BVR-B, biliverdin-IX β reductase; FR, flavin reductase.

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Figure 1 Structures of substrates and inhibitors of FR/BVR-B

(a) FAD; (b) riboflavin; (c) FMN; (d) lumichrome; (e) biliverdin-IX β ; (f) mesobiliverdin-XIII α .

was subsequently shown that BVR-B is in fact identical to flavin reductase (FR; EC 1.6.99.1) [15]. FR is widely distributed in human tissues, but is most abundant in erythrocytes. It has been variously referred to as erythrocyte NADPH dehydrogenase, diaphorase and met-haemoglobin reductase. It has been shown that FR can provide free reduced flavins for the reduction of met-haemoglobin; however, under normal conditions NADH:cytochrome b_5 reductase (EC 1.6.2.2) probably acts as the physiological reducing activity for met-haemoglobin [16]. Intriguingly, FR/BVR-B was first isolated as 'green haem binding protein', although the nature of the tetrapyrrole has not been completely characterized [17,18].

FRs from many bacteria have been well characterized. Their functions are related to bacterial bioluminescence, chorismate synthesis and iron metabolism. The best characterized FR is the NAD(P)H:flavin oxidoreductase (EC 1.6.8.2) from *Escherichia coli*. This enzyme is capable of reducing riboflavin, FMN and FAD by NADH, or riboflavin and FMN by NADPH [19]. This enzyme also displays ferric reductase activity, and this activity is responsible for the reduction and mobilization of ferric iron from ferrisiderophores [20].

The cloning and overexpression of human liver BVR-B/FR [21] has allowed a detailed kinetic study of the FR activity of the enzyme to be carried out. The structural requirements of the enzyme's flavin binding site, which also binds various isomers of biliverdin (O. Cunningham, D. Lightner and T. J. Mantle,

Figure 2 Initial-rate kinetics of the FR reaction at pH 7.5

(A) FR activity as a function of NADPH concentration in the presence of 5 ($\circ$), 10 ($\bullet$), 25 ($\square$), 50 ($\blacksquare$), 100 ($\triangle$), 150 ($\blacktriangle$) and 200 ($\times$) μM FMN. (B) FR activity as a function of FMN concentration in the presence of 5 ($\circ$), 10 ($\bullet$), 25 ($\square$), 50 ($\blacksquare$) and 100 ($\triangle$) μM NADPH. The results are presented as double-reciprocal plots. The lines were drawn using the kinetic parameters $1/v$ and K/v determined from the least-squares fits of the initial-rate data to a hyperbola. All data points represent the mean $\pm$ S.E.M. of three measurements.

Table 1 Kinetic constants for FR at pH 7.5

Data were plotted using the MacCurve Fit program. Reciprocal data were fitted to a line using $1/V_{\text{max}}$ and K/V_{max} parameters calculated from the initial-rate data fitted to a Michaelis-Menten hyperbola. The kinetic constants were calculated by the method of Florini and Vestling [23]. k_{cat} values were estimated using a molecular mass of 22 000 Da.

Flavin	K_m (μM)	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	k_{cat} (s^{-1})
FMN	52.8 ± 3.9	0.21 ± 0.01	0.099
Riboflavin	53.2 ± 12.6	0.29 ± 0.07	0.107
FAD	125.3 ± 31.4	0.24 ± 0.06	0.088

unpublished work), have been probed in the present study and it has been shown that, like the *E. coli* enzyme, human BVR-B/FR catalyses the reduction of ferric iron. The FR reaction mechanism is shown to be buffer- and pH-dependent, obeying a rapid-equilibrium ordered mechanism in phosphate buffer at pH 7.5 and a steady-state ordered mechanism in citrate buffer at pH 5.

Figure 3 Product inhibition of the FR reaction by NADP^+

(A) NADP^+ as a competitive inhibitor of FR. The FR activity was measured as a function of NADPH concentration using $150 \mu\text{M}$ FMN in the presence of 0 ($\circ$), 5 ($\bullet$), 10 ($\square$), 25 ($\blacksquare$), 50 ($\triangle$), 75 ($\blacktriangle$) and 100 ($\times$) μM NADP^+ . (B) NADP^+ as a mixed inhibitor of FR. The FR activity was measured as a function of FMN concentration using $25 \mu\text{M}$ NADPH in the presence of 0 ($\circ$), 10 ($\bullet$), 25 ($\square$), 50 ($\blacksquare$) and 100 ($\triangle$) μM NADP^+ .

EXPERIMENTAL

Biochemical and chemical reagents

NADPH, NADH, NADP^+ , FMN, lumichrome, riboflavin, FAD, ferrozine, ferric chloride and nitrilotriacetic acid were obtained from Sigma. Mesobiliverdin- $\text{XIII}\alpha$ was kindly donated by Professor David Lightner (University of Nevada, Reno, NV, U.S.A.). Other reagent-grade chemicals were obtained from Merck and BDH.

FR assay

FR activity was determined at 25°C from the decrease in absorbance at 340 nm due to the oxidation of NADPH. Under standard conditions the cuvette contained 100 mM potassium phosphate, $\text{pH } 7.5$, and variable concentrations of NADPH and the flavins indicated. The reaction was initiated by the addition of enzyme in each case. Lumichrome was dissolved in DMSO to a final concentration of 1 mM (control reactions were carried out in the presence of the appropriate concentration of DMSO to ensure that it had no effect on enzyme activity). Riboflavin was prepared in concentrated NaOH (5 M), which was immediately

Figure 4 Inhibition of FR by lumichrome

(A) Lumichrome as a competitive inhibitor of FR. The FR activity was measured as a function of FMN concentration using $25 \mu\text{M}$ NADPH in the presence of 0 ($\circ$), 25 ($\bullet$), 50 ($\square$), 75 ($\blacksquare$) and 100 ($\triangle$) μM lumichrome. (B) Lumichrome as a mixed inhibitor of FR. The FR activity was measured as a function of NADPH concentration using $150 \mu\text{M}$ FMN in the presence of 0 ($\circ$), 25 ($\bullet$), 50 ($\square$) and 75 ($\blacksquare$) μM lumichrome.

titrated to $\text{pH } 7$ with 5 M HCl and diluted into assay buffer to give a final stock concentration of 10 mM . FMN and FAD were made up fresh daily as 10 mM stocks in assay buffer.

When the initial-rate kinetics were performed under acidic conditions, appropriate blank rates were determined at each substrate concentration.

Ferric reductase assay

Ferric reductase activity was determined at 25°C from the increase in absorbance at 562 nm due to the formation of the Fe^{2+} -ferrozine complex ($\epsilon_{562} 28 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The reaction mixture contained, in a final volume of 2 ml , 100 mM potassium phosphate, $\text{pH } 7.5$, $50 \mu\text{M}$ NADPH, $150 \mu\text{M}$ FMN, 2 mM ferrozine and various amounts of a ferric chloride/nitrilotriacetic acid complex. Reactions were initiated by the addition of enzyme in each case.

Determination of K_d for NADPH

An Applied Photophysics stopped-flow multi-mixing fluorimeter (SX17) was used to monitor the binding of NADPH to FR

Figure 5 Inhibition of FR by mesobiliverdin-XIII α

(A) Mesobiliverdin-XIII α as a competitive inhibitor of FR. The FR activity was measured as a function of FMN concentration in the presence of 25 μ M NADPH and 0 ($\circ$), 0.2 ($\bullet$), 0.4 ($\square$), 0.6 ($\blacksquare$) and 0.8 ($\triangle$) μ M mesobiliverdin-XIII α . (B) Mesobiliverdin-XIII α as a mixed inhibitor of FR. The FR activity was measured as a function of NADPH concentration in the presence of 150 μ M FMN and 0 ($\circ$), 0.2 ($\bullet$), 0.6 ($\square$), 1.0 ($\blacksquare$) and 1.5 ($\triangle$) μ M mesobiliverdin-XIII α .

(2.5 μ M). All data were analysed using software supplied by the manufacturer. Equilibrium fluorescence quenching studies were carried out using a Perkin-Elmer luminescence spectrophotometer (LS 50B), and data were processed using the Perkin-Elmer FL WinLab scan application. Quenching of FR fluorescence (3 μ M) was achieved by the sequential addition of NADPH from a 0.1 mM stock solution. All data were corrected for the 'inner filter effect' as described by Levine [22].

RESULTS

The experiments described here were carried out under aerobic conditions, where reduced flavins are rapidly reoxidized by oxygen; indeed, it is possible to measure a 'flavin oxidase' activity for human BVR-B/FR by monitoring oxygen depletion in an oxygen electrode in the presence of NADPH and FMN (O. Cunningham, unpublished work). The structures of all substrates and inhibitors used in this kinetic study are shown in Figure 1. The reactions reported here were monitored by following the oxidation of NADPH at 340 nm. FR activity was determined at pH 7.5 as a function of NADPH concentration at several fixed

Figure 6 NADPH binds to the free enzyme

The quenching of FR (final concentration 2.5 μ M in 100 mM KH_2PO_4 , pH 7.2) fluorescence was measured at various concentrations of NADPH using stopped-flow fluorimetry. (A) Representative trace of FR fluorescence (excitation at 280 nm, emission at 340 nm) after rapid mixing with NADPH (10 μ M). The pre-mixing conditions are indicated. (B) Quenching of FR at 5, 10, 15 and 20 μ M NADPH was measured and k_{app} (s^{-1}) was calculated at each concentration. A plot of k_{app} (s^{-1}) as a function of NADPH concentration allowed the determination of the encounter K_d for NADPH.

Figure 7 Equilibrium protein fluorescence quenching studies

Sequential additions of NADPH (from a 0.1 mM stock solution) were made to a cuvette containing 3 μ M FR in 100 mM KH_2PO_4 , pH 7.5. The fluorescence intensity was measured at 350 nm after each addition. All measurements were corrected for the 'inner-filter' effect as described by Levine [22]. The change in fluorescence after addition of each NADPH concentration was plotted as a function of NADPH concentration to allow the calculation of the equilibrium K_d for NADPH.

Scheme 1 Proposed reaction mechanism for FR

E denotes enzyme. The asterisk (*) represents the nucleotide-induced isomerized enzyme form with a dissociation constant of $0.55 \mu\text{M}$. It is distinguished from the native enzyme form, which has an 'encounter' K_i of $15.8 \mu\text{M}$. The k_1 and k_{-1} rate constants shown were calculated from the results of the stopped-flow fluorescence quenching experiments shown in Figure 6.

Figure 8 Initial-rate kinetics of the FR reaction in citrate buffer, pH 5

(A) FR activity as a function of NADPH concentration in the presence of 5 ($\circ$), 10 ($\bullet$), 25 ($\square$), 50 ($\blacksquare$), 100 ($\triangle$) and 150 ($\blacktriangle$) μM FMN. (B) FR activity as a function of FMN concentration in the presence of 5 ($\circ$), 10 ($\bullet$), 25 ($\square$), 50 ($\blacksquare$), 100 ($\triangle$) and 150 ($\blacktriangle$) μM NADPH. All data points represent the mean $\pm$ S.E.M. of three measurements.

concentrations of FMN (Figure 2A), and as a function of FMN concentration at several fixed concentrations of NADPH (Figure 2B). Initial velocities showed typical hyperbolic depend-

Figure 9 Initial-rate kinetics of the FR reaction in acetate buffer, pH 5

(A) FR activity as a function of NADPH concentration in the presence of 5 ($\circ$), 10 ($\bullet$), 25 ($\square$), 50 ($\blacksquare$), 100 ($\triangle$) and 150 ($\blacktriangle$) μM FMN. (B) FR activity as a function of FMN concentration in the presence of 5 ($\circ$), 10 ($\bullet$), 25 ($\square$), 50 ($\blacksquare$), 100 ($\triangle$) and 150 ($\blacktriangle$) μM NADPH. All data points represent the mean $\pm$ S.E.M. of three measurements.

ence on the varied substrate concentration. In Figure 2(B) the lines appear to intersect on the ordinate axis, which is indicative of a rapid-equilibrium ordered mechanism. Identical experiments were repeated using both riboflavin and FAD in place of FMN, and the kinetic constants obtained are summarized in Table 1. The primary plots with FAD and riboflavin as the variable substrate, like those with FMN, exhibited no intercept effect with increasing levels of NADPH.

To allow discrimination between various possible ternary mechanisms, product-inhibition studies were conducted. When the enzyme activity was determined as a function of NADPH concentration in the absence or in the presence of NADP^+ , the double-reciprocal plots showed typical competitive kinetics, with a K_i of $4.89 \mu\text{M}$ for NADP^+ (Figure 3A). Product inhibition by NADP^+ using FMN as the variable substrate resulted in double-reciprocal plots that intersected to the left of the y-axis, indicating a mixed pattern of inhibition (Figure 3B). This suggests that the first product to be released is the reduced flavin, followed by NADP^+ .

Furthermore, lumichrome is both a competitive inhibitor of FMN (K_i $48.57 \mu\text{M}$) and a mixed inhibitor of NADPH (Figure 4). As the K_i for lumichrome is almost identical to the K_m calculated for FMN and riboflavin, it is clear that the isallox-

Table 2 Buffer and pH effects on the kinetic constants for FR

Data were plotted using the MacCurve Fit program. Reciprocal data were fitted to a line using $1/V_{\max}$ and $K/V_{\max}$ parameters calculated from the initial-rate data fitted to a Michaelis–Menten hyperbola. The kinetic constants were calculated by the method of Florini and Vestling [23]. k_{cat} values were estimated using a molecular mass of 22000 Da.

Buffer	K_{mNADPH} (μM)	K_{mFMN} (μM)	$V_{\max}$ ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	k_{cat} (s^{-1})
Phosphate, pH 7.5	0.97 ± 0.13	52.76 ± 3.92	0.21 ± 0.01	0.099
Citrate, pH 5	3.08 ± 0.08	14.50 ± 0.18	0.81 ± 0.07	0.30
Acetate, pH 5	0.32 ± 0.85	47.40 ± 0.79	0.81 ± 0.08	0.30

azine ring structure is sufficient for binding to FR, but not for catalysis.

As discussed previously, FR also has BVR-B activity. Mesobiliverdin-XIII α is a synthetic biliverdin that binds to FR, but is not reduced to the corresponding rubin. Inhibition studies were carried out using FMN as the variable substrate at saturating concentrations of NADPH with several concentrations of mesobiliverdin-XIII α . Figure 5(A) indicates that mesobiliverdin-XIII α acts as a potent inhibitor of FR activity (K_i 0.59 μM), and that this inhibition appears to be competitive. Inhibition studies using NADPH as the variable substrate resulted in a mixed pattern of inhibition (Figure 5B). This suggests that the enzyme has a single pyridine nucleotide binding site, and that flavin and biliverdin compete for a second site.

Stopped-flow studies monitoring the quenching of protein fluorescence showed that NADPH can bind to the free enzyme, and does so with an initial-encounter K_d of 15.8 μM (Figure 6). However, a K_d of 0.55 μM was calculated from equilibrium protein fluorescence quenching experiments (Figure 7). This suggests that the initial binding of nucleotide to the enzyme is followed by isomerization to a 'nucleotide-induced' conformation. These results indicate that NADPH binds to the free enzyme, followed by FMN. A mechanism for FR at pH 7.5 is proposed based on these results (Scheme 1).

Initial experiments showed that the FR reaction has an optimum at pH 5. An initial-rate study was carried out using FMN and NADPH as substrates for the enzyme at pH 5 in 100 mM citrate buffer. FR activity was determined at pH 5 as a function of NADPH concentration at several fixed concentrations of FMN (Figure 8A), and as a function of FMN concentration at several fixed concentrations of NADPH (Figure 8B). Initial velocities showed typical hyperbolic dependence with the varied substrate concentration. Unlike the results shown in Figure 2(B), the lines do not appear to intersect on the ordinate axis, suggesting that the enzyme follows a steady-state ordered mechanism in citrate buffer at pH 5. To ensure that this was not simply a buffer effect, the same initial-rate study was repeated using acetate buffer at pH 5. Figure 9 shows the results of these experiments, and indicates that the enzyme appears to follow a rapid-equilibrium ordered mechanism under these conditions. All the kinetic constants calculated from these results are summarized in Table 2. Equilibrium fluorescence binding studies indicated that the K_d for NADPH increased from 0.55 μM at pH 7.5 to 8.5 μM in citrate at pH 5.

The ability of FR to act as a ferric reductase was also investigated. Initial studies demonstrated that FR can reduce ferric iron and that this reaction requires both NADPH and FMN. An apparent K_m of 2.56 μM for ferric iron was observed.

DISCUSSION

The FR reaction follows a sequential mechanism, and at pH 7.5 NADPH clearly binds to the free enzyme [k_1 (rate of association of the complex), $9.6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; k_{-1} (rate of dissociation of the complex), 152 s^{-1}], yielding an encounter K_d of 15.8 μM . The enzyme–NADPH complex appears to isomerize to a more stable conformation with an equilibrium K_d of 0.55 μM . We have not explored the magnitude of the isomerization constants k_2 and k_{-2} further in the present work (see Scheme 1). Flavin binds to the enzyme–NADPH complex, which rapidly isomerizes to the enzyme–NADP $^+$ –FMNH $_2$ complex. As NADP $^+$ exhibits competitive kinetics with NADPH as the variable substrate, it appears that FMNH $_2$ dissociates from the enzyme first, followed by NADP $^+$. The k_{cat} for the reaction is very low (approx. 0.1 s^{-1} at pH 7.5 for all three flavin substrates tested), and we suggest that this reflects the dissociation of FMNH $_2$. This is consistent with a rapid-equilibrium ordered mechanism, which is indicated by the primary plot shown in Figure 2(B). The initial-rate kinetics in citrate buffer at pH 5 show that the k_{cat} increases, and that this is coupled with a decrease in K_{mFMN} and an increase in K_{mNADPH} . These changes are consistent with the change in pattern for the primary plots, which no longer converge on the $1/v_0$ axis, and suggest a steady-state ordered mechanism for the enzyme at pH 5 in citrate buffer. This pattern is dependent on the buffer used, and a similar effect is not observed when the enzyme is assayed in acetate buffer, pH 5. Under these conditions the k_{cat} for the reaction is similarly increased. However, the K_m values for both substrates remain the same as those seen with phosphate buffer, pH 7.5 (see Table 2). It seems likely that citrate causes a conformational change in the enzyme, as it has been shown that the equilibrium dissociation constant for NADPH in citrate at pH 5 is 8.5 μM , compared with a value of 0.55 μM calculated in phosphate buffer at pH 7.5.

We have shown that synthetic biliverdins with propionate groups at positions C-8 and C-12 are good substrates for human BVR-A, but are not substrates for BVR-B (O. Cunningham, A. Dunne, D. Lightner and T. J. Mantle, unpublished work). Mesobiliverdin-XIII α contains propionate groups at C-8 and C-12 and is not a substrate for BVR-B. However, it is a potent inhibitor, exhibiting competitive kinetics against FMN and mixed inhibition against NADPH, consistent with the hypothesis that human BVR-B/FR has one pyridine nucleotide site and that various flavins and verdins can bind productively at a second site. In the case of biliverdins with C-8/C-12 propionates, such as mesobiliverdin-XIII α , the binding, although tight, is non-productive.

Human BVR-B/FR, like the *E. coli* FR, exhibits ferric reductase activity. It is intriguing that an enzyme functionally coupled to haem cleavage should be capable of reducing both the linear tetrapyrrole and the ferric iron produced. It is still unclear how haem cleavage to produce non- α isomers of biliverdin is accomplished in the foetus, and this aspect needs to be addressed.

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