Substitution of murine ferrochelatase glutamate-287 with glutamine or alanine leads to porphyrin substrate-bound variants

Ricardo FRANCO^{*1}, Alice S. PEREIRA^{*1}, Pedro TAVARES^{*1}, Arianna MANGRAVITA^{*}, Michael J. BARBER^{*†}, Isabel MOURA[‡] and Gloria C. FERREIRA^{*†¶2}

^{*}Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, Tampa, FL 33612, U.S.A., [†]Institute for Biomolecular Science, University of South Florida, Tampa, FL 33612, U.S.A., [‡]Centro de Química Fina e Biotecnologia, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825-114 Monte de Caparica, Portugal, and [¶]H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, FL 33612, U.S.A.

Ferrochelatase (EC 4.99.1.1) is the terminal enzyme of the haem biosynthetic pathway and catalyses iron chelation into the protoporphyrin IX ring. Glutamate-287 (E287) of murine mature ferrochelatase is a conserved residue in all known sequences of ferrochelatase, is present at the active site of the enzyme, as inferred from the *Bacillus subtilis* ferrochelatase three-dimensional structure, and is critical for enzyme activity. Substitution of E287 with either glutamine (Q) or alanine (A) yielded variants with lower enzymic activity than that of the wild-type ferrochelatase and with different absorption spectra from the wild-type enzyme. In contrast to the wild-type enzyme, the absorption spectra of the variants indicate that these enzymes, as purified, contain protoporphyrin IX. Identification and quantification of the porphyrin bound to the E287-directed variants indicate that approx. 80 % of the total porphyrin corresponds

to protoporphyrin IX. Significantly, rapid stopped-flow experiments of the E287A and E287Q variants demonstrate that reaction with Zn²⁺ results in the formation of bound Zn-protoporphyrin IX, indicating that the endogenously bound protoporphyrin IX can be used as a substrate. Taken together, these findings suggest that the structural strain imposed by ferrochelatase on the porphyrin substrate as a critical step in the enzyme catalytic mechanism is also accomplished by the E287A and E287Q variants, but without the release of the product. Thus E287 in murine ferrochelatase appears to be critical for the catalytic process by controlling the release of the product.

Key words: haem biosynthesis, protoporphyrin IX, tetrapyrrole biosynthesis.

INTRODUCTION

Ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) catalyses the terminal step of haem biosynthesis, which is the insertion of ferrous iron into protoporphyrin IX [1,2]. Synthesized in cytosolic ribosomes as a precursor protein prior to import and mitochondrial processing, eukaryotic mature ferrochelatase is associated with the inner mitochondrial membrane, with the active site facing the mitochondrial matrix [2]. In animals there is a single gene for ferrochelatase, with the human gene having been mapped to chromosome 18q21.3 [3,4].

The three-dimensional structure of the *Bacillus subtilis* ferrochelatase at a resolution of 1.9 Å (1 Å = 0.1 nm) represents the first structure to be determined for any ferrochelatase [5,6]. The structure is comprised of two similar domains, each with a four-stranded parallel β -sheet flanked by α -helices. The two domains associate to form a cleft, in which conserved residues, proposed to be critical in the binding of substrates and in the proper functioning of the enzyme, are located [5]. In contrast to the bacterial and yeast ferrochelatases, which appear to be devoid of cofactors, vertebrate and *Drosophila melanogaster* ferrochelatases are metalloenzymes with a [2Fe-2S] cluster [7–10]. Despite having no direct catalytic role, the iron–sulphur cluster is essential for activity of the enzyme [11]. A recent study reported the crystallization of *D. melanogaster* ferrochelatase [12]; however, no three-dimensional structure, other than that of *B. subtilis*

ferrochelatase, has been published for mammalian or any other ferrochelatase. Because of the presence of an iron–sulphur centre in the *D. melanogaster* ferrochelatase, this particular three-dimensional structure should have some distinctly different structural features from those of *B. subtilis* ferrochelatase.

The ferrochelatase-catalysed reaction involves binding of the metal and porphyrin substrates to the enzyme, distortion of the porphyrin ring and metal–ligand exchange with the release of the haem product [1,2,5,6]. One of the proposed contributions of the ferrochelatase protein moiety to the catalytic mechanism is forcing the distortion of the porphyrin macrocycle, which in turn should facilitate the exposure of the nitrogen lone pair of electrons to the incoming metal ion [1,2]. Indeed, resonance Raman spectroscopic studies on the binding of mesoporphyrin IX to yeast ferrochelatase led Blackwood et al. [13] to advocate an ‘allosteric mechanism, in which a conformational change that distorts the porphyrin towards the transition state geometry is induced by metal binding at an adjacent site’. However, resonance Raman studies on the porphyrin interactions with the wild-type and variant forms of the murine ferrochelatase indicated that, at least for some porphyrins, the macrocycle distortion occurs even without metal binding [14]. In addition, based on the recent determination of the X-ray structure of the *B. subtilis* ferrochelatase–*N*-methylmesoporphyrin complex, Lecerof et al. [6] proposed that the mechanism leading to the macrocycle distortion involves fixing of the porphyrin substrate

¹ Present address: Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825-114 Monte de Caparica, Portugal.

² To whom correspondence should be addressed, at the Department of Biochemistry and Molecular Biology (e-mail gferreir@hsc.usf.edu).

through rings B, C and D and the tilting of ring A. Further, the authors suggested that the metal ion enters the macrocycle via the strongly tilted ring A [6].

Murine mature ferrochelatase glutamate-287 (E287) is a conserved residue in all known ferrochelatase sequences. Significantly, K_m values of the porphyrin and metal substrates of bacterial extracts, harbouring a recombinant yeast ferrochelatase mutant in which the corresponding glutamate (i.e. E314) was substituted by alanine, were lower and identical, respectively, to those of bacterial extracts harbouring wild-type ferrochelatase. The value of V_{max} for the bacterial extracts harbouring the mutant ferrochelatase was lower than that of the wild-type ferrochelatase-containing cells [15]. These results led the investigators to propose that E287 might act as a base in the abstraction of the *N*-pyrrole proton(s) [15]. In this study we examine the effects of substituting E287 of recombinant murine ferrochelatase with either glutamine (Q) or alanine (A). The E287-directed mutant enzymes, in addition to exhibiting lower enzymic activities than that of the wild-type ferrochelatase, maintain the porphyrin substrate tightly bound during the purification procedure. Further, although metallated porphyrin is formed upon the addition of the metal substrate to the E287 mutant enzymes, the metallated product remains bound to the enzyme, indicating that a glutamate is required at position 287 of murine ferrochelatase for proper functioning of the enzyme and product release.

EXPERIMENTAL

Materials

Restriction enzymes and Vent_R DNA polymerase were obtained from New England Biolabs and used according to the supplier's instructions. Oligonucleotide primers were synthesized by Cybersyn (Lenni, PA, U.S.A.) and Gibco-BRL. The Chameleon[™] double-stranded, site-directed mutagenesis kit was a product from Stratagene. Sequenase and sequencing kit were from the U.S. Biochemical Corp. [α -³⁵S]dATP was from Dupont/NEN Research Products. Acrylamide and gel reagents were purchased from Bio-Rad. The bicinchoninic acid protein-assay reagents were obtained from Pierce. Ni²⁺-nitrilotriacetate agarose and the QIAquick PCR purification kit were purchased from Qiagen. All of the porphyrins were purchased from Porphyrin Products (Logan, UT, U.S.A.). All other chemicals were from Fisher or Sigma and were of the highest purity available.

Mutagenesis and construction of His-tagged wild-type and mutant ferrochelatase plasmids

Plasmid pGF42, which contains the murine mature ferrochelatase wild-type cDNA sequence under the control of the alkaline phosphatase promoter [16], was purified from its *Escherichia coli* DH5 α host cells. Single-stranded pGF42 DNA was prepared according to the alkaline lysis method [17] and used as a template for site-directed mutagenesis, according to the instructions provided with the Chameleon mutagenesis kit from Stratagene. The mutagenic oligonucleotide for E287Q was 5'-CC AGT GAC CAC ATT CAG ACG CTC TAC G-3' (the nucleotide substitution is underlined). The construction of the E287A-encoding plasmid was based on a PCR site-directed mutagenesis method, which involved three PCRs. The reaction mixture for PCR1 contained 0.2 ng/ μ l pGF42 DNA, 200 μ M of each dNTP, 0.6 μ M FC25 primer (5'-ATA TGT CGA CTC ACC ACC ACC ACC ACA CCA CCA AAC CCC AAG CT-3'), 0.6 μ M mutagenic primer (5'-TAG AGC GTC GCA ATG TGG TCA-3', the

nucleotide substitution is underlined) and 0.5 units of Vent_R DNA polymerase in reaction buffer. The reaction mixture for PCR2 contained 0.2 ng/ μ l pGF42 DNA, 200 μ M of each dNTP, 0.6 μ M pBR-SphI primer (5'-AGC TGA CTG GGT TGA AGG CT-3'), 0.6 μ M mutagenic primer (5'-T GAC CAC ATT GCG ACG CTC TA-3', the nucleotide substitution is underlined) and 0.5 units of Vent_R DNA polymerase in reaction buffer. The reaction mixture for PCR3 contained 0.24 ng/ μ l and 0.22 ng/ μ l of gel-purified PCR1 and PCR2 products, respectively, 200 μ M of each dNTP, 0.6 μ M FC25 and pBR-SphI primers and 0.5 units of Vent_R DNA polymerase in reaction buffer. All three PCRs underwent one cycle of 94 °C for 4 min, 55 °C for 2 min and 72 °C for 7 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 2 min, 72 °C for 7 min and extension for 10 min at 72 °C. Once gel-purified, the 1.4 kb fragment was digested with *Sph*I and *Bsp*120I and the corresponding purified gel fragment was ligated into pGF42 previously digested with *Sph*I and *Bsp*120I. *E. coli* strain DH5 α was transformed with the ligation reaction. Clones obtained after the mutagenesis procedures were screened using DNA sequencing according to the dideoxynucleotide chain-termination method [18]. The resulting expression plasmids containing the mutations E287Q and E287A were named pAP4 and pAM6, respectively.

The pGF47 plasmid was constructed from pGF42 and contained those nucleotides encoding a 374-amino acid protein corresponding to the murine mature ferrochelatase, from threonine-1 to leucine-369, preceded by five N-terminal histidine residues. pGF42 was used as a template for the PCR. The reaction mixtures (100 μ l) contained 1 ng of pGF42 DNA template, 200 μ M dNTPs, 0.5 μ M of each primer and 0.5 units of Vent_R DNA polymerase in reaction buffer. One of the primers (5'-ATA TGT CGA CTC ACC ACC ACC ACC ACA CCA CCA AAC CCC AAG CT-3') encoded five histidine residues, the first six N-terminal amino acids of mature ferrochelatase (TTKPKQA) and contained the sequence for a *Sal*I restriction site at its 5' end, whereas the second primer (5'-ATC TCG AGA TCT GCA GGG TCA CAG CTG TTG GC-3') encoded the last six C-terminal amino acids of ferrochelatase (FTSQQL). A total of 25 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min were followed by an extension of 7 min at 72 °C. The amplified DNA fragment was purified (with the QIAquick PCR purification kit) and digested with *Sal*I and *Bam*HI. The 220 bp generated fragment was gel-purified and subcloned into pGF42 previously digested with *Sal*I and *Bam*HI. An aliquot of the ligation reaction mixture was used to transform *E. coli* strain DH5 α , and the pGF47 plasmid was identified upon DNA sequencing [18].

To construct pGF56, an expression plasmid encoding the histidine-tagged E287Q ferrochelatase mutant, pAP4 was digested with *Mfe*I and *Sph*I, and the E287Q-mutation-containing DNA fragment was ligated into pGF47 previously treated with *Mfe*I and *Sph*I.

Overproduction and purification of wild-type and mutant ferrochelatase forms

The mutant and wild-type enzymes were overproduced in the *E. coli* DH5 α host strain harbouring the different expression plasmids (pAP4 and pAM6) and purified to homogeneity according to the method described previously [16]. The purified protein was subsequently concentrated in a stirred cell with a YM 30 Diaflo ultrafiltration membrane (Amicon) and subjected to gel-filtration chromatography on a Superdex 200 column (2.6 cm $\times$ 45 cm; Pharmacia) to minimize the amount of cholate. The purified protein was eluted with buffer H (20 mM Tris/HCl,

pH 8, 0.5 M NaCl and 10 % glycerol). Alternatively, the E287Q variant was overproduced in *E. coli* DH5 α cells harbouring pGF56 and purified to homogeneity using nickel-chelate agarose chromatography. Briefly, the DH5 α cells harbouring the E287Q variant were grown and induced for the production of recombinant protein as described previously [16]. The induced cells were then harvested by centrifugation. All purification procedures were carried out at 4 °C unless otherwise stated. The cells were resuspended in buffer H containing 10 μ g/ml PMSF, lysed in a French-press cell (two passages at 12000 p.s.i., 82800 kPa) and subjected to ultra-centrifugation at 100000 *g* for 45 min. The resulting supernatant was loaded on to a Ni²⁺-nitrilotriacetate column (1.5 cm $\times$ 10 cm), previously equilibrated with buffer H. The column was washed with buffer H until the eluate presented a value A_{280} of <0.1, followed by 4–5 column volumes of buffer H with 50 mM imidazole. Elution of the E287Q protein was accomplished with buffer H containing 100 mM imidazole. The fractions containing E287Q were pooled and concentrated using a stirred cell with a YM 30 Diaflo ultrafiltration membrane. To remove imidazole and a high-molecular-mass protein contaminant, the concentrated protein was subjected to gel-filtration chromatography on a Superdex 200 gel column connected to a Perkin-Elmer HPLC system, using buffer H as the elution buffer. The eluted protein fraction was then concentrated as described above and kept in liquid nitrogen until further use.

Porphyrin solutions

Porphyrin solutions were prepared by dissolving 5 mg of the powder reagent in 50 μ l of 2 M NH₄OH and 500 μ l of 20 % Tween-20 solution. Typically, stocks of approx. 2 mM were prepared by adding 4 ml of water to the above solution. The final concentrations were determined spectrophotometrically as described previously [16].

SDS/PAGE, determination of protein concentration and enzyme activity assay

Protein purity was estimated by SDS/PAGE [19] and was never less than 95 %. Protein-concentration determination was performed as described previously [16]. Briefly, 15 % acrylamide and 1.5 mm-thick gels were used in SDS/PAGE and protein concentrations were determined by the bicinchoninic acid assay using BSA as standard, following the manufacturer's instructions. The enzymic activity was determined using the pyridine haemochromogen, as described in [20].

Spectroscopic methods: UV-visible, CD and stopped-flow spectra

Absorption spectra were recorded using a 1 cm path-length quartz cell and a Shimadzu UV 2100U dual-beam spectrophotometer at 25 °C. The porphyrin stock solutions were made in water with 0.4 % Triton X-100 and 4 mM NH₄OH. The ZnCl₂ stock solution used in the titration of the E287Q mutant was made in HPLC-grade water.

CD spectra were obtained using a Jasco J710 spectropolarimeter calibrated for both wavelength maxima and signal intensity using an aqueous solution of δ -10-camphorsulphonic acid [21]. UV spectra (6–13 μ M enzyme) were recorded over the wavelength range 200–270 nm using a cylindrical cell of 0.1 cm path length and a total volume of 300 μ l. All spectra were obtained at 25 °C and are corrected for buffer contributions.

Rapid-scanning stopped-flow kinetic experiments were performed using an RSM spectrophotometer from OLIS, equipped with a stopped-flow mixer from OLIS with an optical path length of 1.5 mm. The dead time of the stopped-flow unit was approx.

2 ms. Scans covering the range 350–450 nm were collected at a rate of 1000 scans/s. For 50 s and 300 s reactions, the collected scans were averaged to yield 30 scans/s and 5.2 scans/s, respectively, so that the data files were reduced to a manageable size. The measurements were performed at 20 °C, with the temperature of the stopped-flow syringes and observation cell maintained with an external circulating-water bath. The concentrations of enzyme and ligands were 2-fold greater than the final concentrations present in the observation cell. Time courses at 423 nm were analysed by fitting to equations for 1–3 exponentials with the SIFIT program (OLIS), where A_t is the absorbance at time t , a_i is the amplitude of each phase, k_i is the observed rate constant for each phase and c is the final absorbance (eqn 1). Qualities of fit were judged by analysis of the calculated residuals and by the Durbin–Watson value [22].

$$A_t = \sum_{n=1}^2 a_i e^{-k_i t} + c \quad (1)$$

Reversed-phase HPLC and quantification of the bound porphyrin

To identify the chemical nature and abundance of the E287Q-bound porphyrin, the purified E287Q was treated with acidified acetone, following a well-established method for the extraction of non-covalently bound haem [23]. The red supernatant generated from the extraction procedure was analysed by reversed-phase HPLC (Merck-Hitachi D-7000 system). The separation was carried out using a modification of the method of Guo et al. [24]. Essentially, the separation was performed on a LiChroCART RP-18 column (250 mm $\times$ 4 mm, 5 μ m particle size; Merck). The flow rate was 1 ml/min using as the mobile phase a mixture of 88 % methanol (HPLC grade, Merck), 10 % water (Milli-Q grade) and 2 % 1 M ammonium acetate, pH 5 [24]. Elution was monitored by visible absorbance spectroscopy at 410 nm [24]. Protoporphyrin IX, mesoporphyrin IX and Zn-protoporphyrin IX, either alone or in a mixture, were used as standards in the determination of the retention times of the different porphyrins.

RESULTS AND DISCUSSION

Mutagenesis of the conserved E287 residue

Murine mature ferrochelatase E287 is a perfectly conserved residue among all known ferrochelatase sequences and is at the active site of the enzyme, as demonstrated by the three-dimensional structure of the *B. subtilis* ferrochelatase [5,6]. To evaluate the potential role of E287 in maintaining the catalytic competence of ferrochelatase, in this study E287 was replaced with either Q or A, so that the carboxy group could be eliminated at that position. CD spectra in the far-UV region (200–270 nm) indicated no significant difference in the spectral pattern between the purified wild-type and E287Q and E287A mutant enzymes, suggesting that no gross conformational change was introduced through the mutations of the glutamate residue (results not shown). Since purified E287A and E287Q exhibited approx. 1.2 % and 0.4 % of the wild-type enzyme's activity (results not shown) respectively, the presence of the side-chain carboxy group at position 287 appears to be critical for enzyme activity.

E287Q- and E287A-porphyrin complexes

The absorbance spectrum of wild-type ferrochelatase exhibits an absorbance maximum at 280 nm and, in addition, an absorption band at 330 nm and prominent shoulders at 440 and 525 nm, as

Figure 1 Absorbance spectra of (A) E287Q, free protoporphyrin IX and wild-type ferrochelatase with bound protoporphyrin IX and (B) E287A, E287Q and wild-type ferrochelatase with bound protoporphyrin IX

(A) —, wild-type ferrochelatase; ·····, free protoporphyrin; — — —, protoporphyrin IX–wild-type ferrochelatase in a 0.5:1 molar ratio (see the Experimental section for experimental details). (B) —, protoporphyrin IX–wild-type ferrochelatase in a 0.5:1 molar ratio; — — —, E287Q; ·····, E287A. Spectra were recorded in buffer H, in a 1 cm cell, as described in the Experimental section.

is typical of [2Fe-2S]-containing proteins (Figure 1A and [11]). The purified E287Q and E287A mutant enzymes elute as symmetrical, single bands from a Superdex 200 gel-filtration column (results not shown), but in contrast with wild-type ferrochelatase they display very different visible absorbance spectra. The comparison of the absorption spectra of E287Q and E287A with that of the wild-type ferrochelatase with bound protoporphyrin indicates that the variants, as purified, contain protoporphyrin IX (Figure 1B). Specifically, the binding of protoporphyrin IX to wild-type ferrochelatase, in a 0.5:1 molar ratio, caused a sharpening and a 10 nm red shift of the Soret absorbance maximum. In fact, the Soret band shifted from 400 nm for the protoporphyrin IX free in solution to 413 nm when bound to wild-type ferrochelatase (Figure 1A). Although there are differences (i.e. in terms of intensity and width) in the Soret band and 300–380 nm regions of the absorption spectra of the E287Q, E287A and wild-type enzyme with bound protoporphyrin, the E287Q and E287A variants exhibited maxima at 413 nm, clearly suggesting that these enzymes have porphyrin bound within their active sites. The specificity of the

prophyrin binding was investigated previously by the analysis of the structure-sensitive lines and the vinyl vibrational mode in the resonance Raman spectra of the E287-directed variants [14].

Chemical nature of the E287A- and E287Q-bound porphyrin

To identify the chemical nature and the relative abundance of the E287-variant-bound porphyrin, the deep-red extract from the acidified acetone treatment of purified E287Q was applied directly to a reversed-phase chromatography column. Three major peaks, corresponding to fractions containing protoporphyrin IX (retention time, 24.8 min), mesoporphyrin IX (retention time, 13.5 min) and Zn-protoporphyrin IX (retention time, 11.3 min), were identified. The majority of the bound porphyrin corresponded to protoporphyrin IX, i.e. protoporphyrin IX, mesoporphyrin IX and Zn-protoporphyrin IX accounted for 78%, 14% and 8% of the total extracted porphyrins, respectively, suggesting that E287 variants could bind the protoporphyrin substrate tightly. It is worth emphasizing that resonance Raman studies of the E287Q variant have demonstrated previously that the endogenous protoporphyrin was not even displaced upon the addition of other porphyrins or metalated porphyrins (i.e. Ni-protoporphyrin or Ni-mesoporphyrin) [14]. In fact, Ni-porphyrins at concentrations sufficient to yield a 1:1 porphyrin–wild-type ferrochelatase complex failed to remove the porphyrin endogenously bound to the E287Q variant [14].

Conversion of E287Q- and E287A-bound porphyrin into Zn-protoporphyrin

The Soret absorbance maximum for E287Q- and E287A-bound protoporphyrin IX (413 nm) is distinct from those of E287Q-bound Zn-protoporphyrin IX (423 nm) and of free Zn-protoporphyrin (417 nm). Further, the mode of binding endogenous porphyrin to wild-type ferrochelatase and to the E287Q variant is also distinct, as the resonance Raman spectra of the endogenously-bound porphyrin of the wild-type enzyme and the E287Q variant differ substantially [14]. To verify whether the bound protoporphyrin could be used as a substrate in the ferrochelatase-catalysed reaction, Zn^{2+} was reacted with either the purified E287A or the purified E287Q, and the appearance of the enzyme-bound product, Zn-protoporphyrin IX, was monitored by rapid-scanning stopped-flow spectroscopy (Figures 2A and 2B). The results indicate that the protein-bound protoporphyrin IX (Soret maximum at 413 nm) was converted into protein-bound Zn-protoporphyrin IX (Soret maximum at 423 nm; Figures 2A and 2B). This is in striking contrast to the wild-type enzyme, with which the reaction of Zn^{2+} and protoporphyrin IX, under single-turnover conditions, yields free Zn-protoporphyrin IX (Figure 2C). The time course for the formation of bound Zn-protoporphyrin in the E287A-catalysed reaction could be best described by a two-exponential process with rates of 0.35 s^{-1} and 0.07 s^{-1} (Figure 2A, inset). Representative spectra from the 1500 collected spectra (350–450 nm) are displayed in Figure 2, indicating that the spectrum at 0.17 s corresponds to that of bound protoporphyrin whereas that, for instance, at 14.2 s corresponds to bound Zn-protoporphyrin. The time-course data for the pre-steady-state reaction of E287Q with Zn^{2+} were also best fit to a biphasic process (Figure 2B, inset). Thus as with E287A, binding of Zn^{2+} to E287Q and conversion into bound Zn-protoporphyrin occur in two kinetic steps, albeit with lower observed rates, of 0.05 s^{-1} and 0.01 s^{-1} , and with approx. 2-fold greater amplitudes (compare Figures 2A and 2B, insets).

All of the protoporphyrin bound to the E287-directed variants could be used as a substrate and metallated to yield a protein-

Figure 2 Pre-steady-state kinetics of (A) E287A and (B) E287Q in the forward reaction and (C) single turnover in the wild-type ferrochelatase forward reaction

(A) Time, 0–40 s. The reaction mixture contained 20 μM E287A and 200 μM Zn^{2+} . Spectra shown are for 0.17, 2.2, 4.2, 6.2, 8.2, 10.2, 12.2, 14.2 and 16.2 s. Inset: the absorbance data, collected at 423 nm ($\circ$), were fitted to a two-exponential process equation with rates of 0.35 and 0.07 s^{-1} and amplitudes of 0.003 and 0.014 absorbance units. (B) Time, 0–170 s. The reaction mixture contained 20 μM E287Q and 200 μM Zn^{2+} . Spectra shown are for 1.0, 12.5, 24.0, 36.0, 47.0, 59.0, 70, 82 and 93 s. Inset: as for (A), with rates of 0.05 and 0.01 s^{-1} and amplitudes of 0.006 and 0.036 absorbance units. (C) Time, 0–10 s. The reaction mixture contained 30 μM ferrochelatase, 100 μM Zn^{2+} and 3 μM protoporphyrin IX. Spectra shown are for 0.144, 0.256, 0.336, 0.656, 0.976, 2.416 and 6.416 s. Inset: as for (A), but collected at 417 nm ($\circ$), with rates of 2.29 and 0.43 s^{-1} . Note that absorbance maxima for free and bound Zn-protoporphyrin are at 417 and 423 nm, respectively.

bound product, Zn-protoporphyrin IX, indicating that all of the bound-porphyrin substrate was potentially accessible to be used in porphyrin metallation. Significantly, the conversion of the enzyme-bound protoporphyrin into Zn-protoporphyrin is an indication that the substrate is not only bound to the enzyme but also that the protein determinants, leading to the porphyrin macrocycle distortion, are present in the E287A and E287Q variants. The extremely low activity under turnover conditions observed in the variants in relation to the wild-type enzyme ($\approx 1\%$) is a strong indication that E287 is crucial for function. In fact, the crystal structure of the *B. subtilis* ferrochelatase–*N*-methylmesoporphyrin complex revealed that the binding and positioning of the porphyrin is stabilized by ‘ionic, and aromatic stacking and steric interactions’ between the protein side chains and the pyrrole rings, and that a small tilt of the distorted pyrrole ring A would put it in close proximity to the conserved E287 [6]. However, the extent of the non-planar distortion of the porphyrin ring varies for the wild-type- and E287Q-bound porphyrins [14], but a definite interpretation of the distinct resonance Raman spectra of porphyrin bound endogenously to the wild-type and E287 ferrochelatases has not yet been made. Consequently, the distinct modes of the macrocycle distortion and the mechanism undergone by the wild-type and E287-directed variants to accomplish the distortion are a matter for further investigation. In conclusion, whereas the murine ferrochelatase E287 residue appears not to have a direct role in porphyrin binding, it has critical roles in the release of the product and the function of the enzyme. Non-conservative substitution of this residue yielded variants with endogenously bound porphyrin substrate, which were capable, to a certain extent, of catalysis, but incapable of releasing the product of the catalytic reaction. Studies underway should offer more insight into this impaired enzymic activity and the ferrochelatase reaction mechanism.

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