Modulation of inhibition of ferrochelatase by *N*-methylprotoporphyrin

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Protoporphyrin IX ferrochelatase catalyses the terminal step of the haem-biosynthetic pathway by inserting ferrous iron into protoporphyrin IX. NMPP (*N*-methylprotoporphyrin), a transition-state analogue and potent inhibitor of ferrochelatase, is commonly used to induce haem deficiency in mammalian cell cultures. To create ferrochelatase variants with different extents of tolerance towards NMPP and to understand further the mechanism of ferrochelatase inhibition by NMPP, we isolated variants with increased NMPP resistance, bearing mutations in an active-site loop (murine ferrochelatase residues 248–257), which was previously shown to mediate a protein conformational change triggered by porphyrin binding. The kinetic mechanisms of inhibition of two variants, in which Pro²⁵⁵ was replaced with either arginine (P255R) or glycine (P255G), were investigated and compared with that of wild-type ferrochelatase. While the binding affinity of the P255X variants for NMPP decreased by one order of magnitude in relation to that of wild-type enzyme,

the inhibition constant increased by approximately two orders of magnitude (K_i^{app} values of 1 μM and 2.3 μM for P255R and P255G respectively, as against 3 nM for wild-type ferrochelatase). Nonetheless, the drastically reduced inhibition of the variants by NMPP was not paralleled with a decrease in specificity constant ($k_{\text{cat}}/K_{\text{m,protoporphyrin IX}}$) and/or catalytic activity (k_{cat}). Further, although NMPP binding to either wild-type ferrochelatase or P255R occurred via a similar two-step kinetic mechanism, the forward and reverse rate constants associated with the second and rate-limiting step were comparable for the two enzymes. Collectively, these results suggest that Pro²⁵⁵ has a crucial role in maintaining an appropriate protein conformation and modulating the selectivity and/or regiospecificity of ferrochelatase.

Key words: ferrochelatase, haem biosynthesis, *N*-methylprotoporphyrin, porphyrin, tetrapyrrole.

INTRODUCTION

Protoporphyrin IX ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1; hereafter called simply ‘ferrochelatase’), the terminal enzyme of the haem-biosynthetic pathway, catalyses the insertion of ferrous iron into protoporphyrin IX to yield protohaem [1,2]. Distortion of the porphyrin macrocycle has long been recognized to be a critical step in porphyrin metallation [3,4]. Porphyrin distortion heightens metal chelation by endowing porphyrin with an appropriate configuration for metal-ion complexation. In this configuration, the lone-pair orbitals of the pyrrole nitrogen atoms are exposed to the incoming metal ion [3].

Pursuing both experimental and theoretical studies, investigators have demonstrated that an out-of-plane distortion occurs on porphyrin binding to ferrochelatase and that this induced distortion is energetically favourable [5–9]. Further, the tilting of one of the pyrrole rings lowers the energy of metal insertion by about 50 kJ·mol^{−1} [9]. In particular, the crystal structure of the complex between ferrochelatase and *N*-methylmesoporphyrin revealed a largely saddled–ruffled structure, with a tilt angle in relation to the mean porphyrin plane of approx. 36° for pyrrole ring A and approx. 5° for the other three pyrrole rings [7]. While most of the distortion certainly results from the *N*-methyl substitution, the high selectivity and affinity of ferrochelatase for this porphyrin conformation [7] suggest that it is the favoured distorted structure adopted by the protein-bound porphyrin substrate and corroborates the notion that *N*-methylporphyrins are transition-state analogues for porphyrin metallation [10]. In fact, antibodies elicited to non-planar *N*-methylporphyrins catalyse metal-ion incorporation into porphyrin [5,10–12]. Significantly, the mode and extent of the ferrochelatase-induced distortion of the porphyrin substrate were recently proposed to enhance the reaction

rate by decreasing the activation energy of the reaction and control the metal-ion selectivity by modulating which bivalent metal ion is incorporated into the porphyrin ring [1,13].

The importance of a conserved active-site loop motif (Figure 1) in the interaction between ferrochelatase and protoporphyrin was previously assessed using a combination of random mutagenesis and steady-state kinetic analysis [14]. While multiple functional substitutions were tolerated within the 10-amino-acid-loop motif, the positions occupied by Gln²⁴⁸, Ser²⁴⁹, Gly²⁵², Trp²⁵⁶ and Leu²⁵⁷ exhibited high informational content, as permissible substitutions were limited and only observed in multiply substituted variants [14]. Strikingly, resonance-Raman-spectroscopy studies indicated that the extent of a specific non-planar porphyrin deformation contributed to the catalytic efficiency of ferrochelatase and loop variants. When compared with the wild-type enzyme, the reduced non-planar porphyrin deformation (saddling) associated with the variants correlated with their decreased catalytic efficiencies towards porphyrin [15]. Indeed, one variant with a catalytic efficiency one order of magnitude lower than that of wild-type enzyme produced less than 30% of the wild-type saddling deformation [15]. The loop residues also appeared to orient, specifically, the porphyrin vinyl substituents and, probably, the entire porphyrin macrocycle [15].

NMPP (*N*-methylprotoporphyrin), a potent inhibitor of ferrochelatase [$K_i \sim 10$ nM [16,17]], resembles a strained substrate, and it is precisely this non-planarity of the porphyrin ring that has been generally accepted to confer the inhibitory properties of the alkylated porphyrin. Recently, the characterization of ferrochelatase variants with mutations in the porphyrin-interacting loop led us to suggest that specific conserved loop residues are directly involved in the saddling of the porphyrin substrate [15]. Similarly, we hypothesized that if loop mutations affect

Abbreviations used: COX, cytochrome *c* oxidase; LB, Luria–Bertani; NMPP, *N*-methylprotoporphyrin; PDB, Protein Data Bank.

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where K_d is the dissociation constant, F is the measured fluorescence, F_0 is the fluorescence in the absence of NMPP, ΔF is the total change in fluorescence, E_t is total protein concentration and L is the total NMPP concentration.

Transient kinetic analysis

The kinetic steps involved in NMPP binding to ferrochelatase were assessed using stopped-flow absorption-spectroscopic analysis by monitoring the enzymatic reaction in the presence of various concentrations of the inhibitor NMPP. The reactions were performed using a rapid scanning stopped-flow spectrophotometer (model RSM-1000; Olis Inc., Bogart, GA, U.S.A.), equipped with a stopped-flow mixer and an observation chamber with an optical pathlength of 4 mm. The dead time of the instrument is ~ 2 ms. Scan spectra spanning the wavelength range 347–574 nm were collected at a rate of 1000 scan/s. The syringes containing the reaction components and the stopped-flow cell were maintained at 30°C using an external water bath. The concentrations of reagents loaded into each syringe were two-fold greater than the final concentrations in the observation chamber. Enzymatic activity was monitored by following the increase in absorbance at 420 nm for zinc-protoporphyrin production using Zn^{2+} and protoporphyrin as substrates.

The enzyme and substrate stock solutions were diluted in 100 mM Tris/acetate, pH 8.0, containing 0.5% Tween-80. The stock solutions for substrates and inhibitor were prepared as follows. A 3 mM zinc acetate solution was made by dissolving zinc acetate in distilled deionized water. A 200 μ M protoporphyrin solution was prepared by dissolving protoporphyrin IX in 50 mM NH_4OH containing 0.5% Tween-80. A 2 mM NMPP solution was prepared by dissolving NMPP in 100 mM NH_4OH containing 0.5% Tween-80. Typically, a 2 ml solution that was 1 μ M in ferrochelatase and 10 μ M in protoporphyrin was preincubated on ice for 30 min. The porphyrin/enzyme solution was titrated with NMPP. The mixture was subsequently transferred to one of the stopped-flow syringes. The other stopped-flow syringe was loaded with a reaction buffer solution of volume about 2 ml that was 30 μ M with respect to zinc acetate. The enzymatic reaction was initiated by mixing the contents from both syringes, and reaction progression was monitored by following the increase in absorbance at 420 nm. The data were analysed by fitting the observed absorbance values to eqn (2):

$$A = A_0 + v_s t + \frac{v_0 - v_s}{k} (1 - e^{-kt}) \quad (2)$$

where A is the observed absorbance, A_0 is the initial absorbance, v_s is the steady-state velocity, v_0 is the initial velocity and k is the pseudo-first-order rate constant for inhibitor binding. Eqn (2) describes the progress of enzymatic reactions in the presence of tight-binding competitive inhibitors [20].

Measurement of ligand-binding-pocket size

Structural models for wild-type murine ferrochelatase and variants P255R and P255G were generated using the amino acid sequence and co-ordinates for human ferrochelatase [PDB (Protein Data Bank) accession code 1HRK] as the template. Sequence alignments, molecular modeling, energy minimization and model analysis using PROCHECK were performed on the Geno3D servers at the IBCP (Institute of Biology and Chemistry of Proteins, UMR 5086, CNRS, Université de Lyon, Lyon, France) [21]. Dimensions of the porphyrin-binding pocket in the monomeric models of wild-type ferrochelatase and variants were calculated on the CASTp server [22]. Values for the area and volume

of Connolly's surface of the active-site cavity were determined using the CAST program [22].

RESULTS AND DISCUSSION

NMPP has long been known to be a potent inhibitor of mammalian ferrochelatase, which competes with the porphyrin substrate for binding to the enzyme [16,17]. Since alkylation of a pyrrole nitrogen atom introduces non-planarity into the porphyrin macrocycle [23], the inhibitory properties of NMPP are thought to arise from its structural resemblance to the distorted porphyrin intermediate in the ferrochelatase-catalysed reaction [2]. In addition, the analysis of the X-ray crystal structural of *Bacillus subtilis* ferrochelatase complexed with *N*-methylmesoporphyrin indicated that the active-site loop residues are in close proximity to the porphyrin macrocycle [7]. This observation led us to hypothesize that different mutations in the loop motif should modulate binding of *N*-alkylated porphyrins and consequently control their potency as inhibitors of ferrochelatase. In order to test this hypothesis, selected loop variants were examined with respect to the kinetics of inhibition by NMPP. Also, ferrochelatase variants with different extents of tolerance towards NMPP could be instrumental in cell-culture systems designed to model haem deficiency, as the use of NMPP to inhibit ferrochelatase and haem biosynthesis is generally accompanied by cellular toxicity.

Isolation of ferrochelatase loop variants with increased resistance to NMPP

To isolate *E. coli* Δvis transformants harbouring loop variants more tolerant to NMPP than wild-type ferrochelatase, 33 variants isolated from a library of 214 functional ferrochelatase clones [14] were plated on LB/ampicillin medium containing 0.2, 2 or 20 μ M NMPP. *E. coli* Δvis cells can only grow when haemin is added to the medium or when the cells are transformed with an active ferrochelatase expression plasmid [14,18]. Transformants harbouring a subset of the functional loop variants, including K250N, V251L, P255R, P255G, the triply mutated variant K250M/V251L/W256Y and the quadruply mutated variant Q248P/S249G/K250P/G252W were grown on LB/ampicillin plates with increasing concentrations of NMPP. Whereas Δvis cells expressing variants P255R and P255G could form colonies onto medium containing more than 20 μ M NMPP, Δvis cells transformed with either wild-type ferrochelatase or the other variants could not. On the basis of this observation, the P255 variants were selected for further characterization. [Note: this study was not intended to provide a comprehensive screen to cover the full spectrum of the loop mutations that can affect interactions with the inhibitor; however, it revealed variants which behaved differently from wild-type enzyme and therefore provided support to the hypothesis that mutation(s) in the loop should modulate inhibitor binding.]

Equilibrium binding of NMPP to ferrochelatase

Tryptophan is a major contributor to intrinsic protein fluorescence, and thus quenching of tryptophan fluorescence is frequently used for detection of changes in the local environment of the fluorophore upon ligand binding [24]. In wild-type murine mature ferrochelatase, five tryptophan residues (two of them being in the active site) contributed to the intrinsic protein fluorescence, which exhibited an excitation maximum at ~ 280 nm ($\lambda_{\text{emission}} = 331$ nm) and an emission maximum at ~ 330 nm ($\lambda_{\text{excitation}} = 283$ nm) (Figure 2A). Intrinsic wild-type fluorescence, as monitored at 331 nm upon excitation at 283 nm, was linearly dependent on protein concentration (Figure 2B). Although the P255 variants exhibited

Figure 2 Intrinsic fluorescence of ferrochelatase

(A) Fluorescence excitation and emission spectra of wild-type murine ferrochelatase are shown for 0.9 μM enzyme in 10 mM Tris/acetate buffer containing 0.05% Tween-80. The emission spectrum exhibits a maximum at 331 nm ($\lambda_{\text{excitation}} = 283$ nm), while the excitation spectrum displays a maximum at 283 nm ($\lambda_{\text{emission}} = 331$ nm). (B) The intrinsic protein fluorescence is proportional to enzyme concentration. Fluorescence of wild-type murine ferrochelatase was measured at 331 nm ($\lambda_{\text{excitation}} = 283$ nm).

decreased fluorescence intensities, the same linear relationship between intrinsic protein fluorescence and protein concentration was observed (results not shown). The decrease in intrinsic fluorescence intensity suggests that the mutations induced changes in protein conformation, which agrees with a previous proposal based on the analysis of structural models for the wild-type enzyme and variants [14].

The equilibrium constant for NMPP binding to ferrochelatase could be determined from the change in intrinsic protein fluorescence upon ligand addition. Because incubation of ferrochelatase with NMPP resulted in a decrease in intrinsic protein fluorescence, the dissociation constant of NMPP for either ferrochelatase or the P255X variants was estimated by titration of the enzyme with NMPP and quantification of the quenching effect. Whereas the value for the dissociation constant (K_d) of NMPP in respect of wild-type ferrochelatase was 9 ± 5 nM, values for the variants increased by one order of magnitude with a K_d of 0.16 ± 0.07 μM for P255R and K_d of 0.30 ± 0.10 μM for P255G (Figure 3). In fact, the order of magnitude of the affinity of wild-type ferrochelatase towards NMPP corroborates the results of previous studies on inhibition of bovine ferrochelatase

Figure 3 Fluorescence titration of NMPP binding to either wild-type ferrochelatase or P255X variants

Aliquots of NMPP were added to a solution of enzyme in 10 mM Tris/acetate buffer, pH 8, containing 0.05% Tween-80 and incubated on ice for 1 h. The intrinsic protein fluorescence was monitored at 331 nm ($\lambda_{\text{excitation}} = 283$ nm). The K_d value for NMPP was determined by fitting the data to eqn (1). The enzymes used in each set were: (A) wild-type ferrochelatase (40 nM); (B) P255R (250 nM); (C) P255G (120 nM).

by NMPP [16]. However, the stability of the ferrochelatase–NMPP complex was clearly diminished by the substitutions of loop residue Pro²⁵⁵. The lower affinity of the mutated enzymes for NMPP could have resulted from altered positioning of NMPP in the porphyrin-binding site and, consequently, a weakened interaction between ligand and protein. Binding of *N*-methylmesoporphyrin to *B. subtilis* ferrochelatase promotes a conformational change with widening of the binding cleft [7]. A loop covering residues 221–233 (i.e., murine residues 248–257) and the N-terminal loop flanked by helices 1 and

2 appear to be the major participants in the adopted 'open' conformation; thus mutations introduced in the Pro²⁵⁵ position most likely disrupt the interaction between NMPP and the protein. Alternatively, the loop might have a role in molecular recognition. Of the eight possible isomers of NMPP with different non-planar distortions of the macrocycle, only one specific isomer was complexed with wild-type *B. subtilis* ferrochelatase [7]. The methyl group of the selected isomer was attached to pyrrole ring A and positioned above the mean plane of the macrocycle [7]. Since the present study was performed with commercially available NMPP, which is a racemic mixture of the eight isomers, the possibility that mutation of Pro²⁵⁵ alters the geometry of the binding site and the selectivity of the enzyme towards the NMPP isomer cannot presently be ruled out.

Kinetics of NMPP inhibition

For some enzymes, such as thrombin [25] and pyruvate dehydrogenase [26], a two-step binding mechanism has been invoked to characterize the enzymatic inhibition by certain substrate analogues. The high affinity of the enzyme towards the inhibitor has been related to the slow second kinetic step corresponding to the rearrangement of the initial weak enzyme–inhibitor complex into a more stable enzyme–inhibitor complex. To evaluate the kinetic pathway for inhibition of ferrochelatase and variants by NMPP, the kinetic steps involved in NMPP binding to the enzymes were investigated by monitoring the enzymatic reaction with Zn²⁺ and protoporphyrin as substrates using rapid scanning stopped-flow absorbance spectroscopy. Although Fe²⁺ is the physiological substrate, we opted to use Zn²⁺, given that ferrochelatase can incorporate Zn²⁺ into porphyrin and thus we could eliminate the technical constraints of maintaining the iron substrate in the reduced state and the requirement of strict anaerobicity. Figure 4(A) illustrates representative spectra from the 3000 spectra (350–450 nm) collected for the P255R-catalysed reaction in the absence of NMPP. The clear isosbestic point (at 412 nm) indicated inter-conversion between the two absorbing species, protoporphyrin and zinc-protoporphyrin (Figure 4A), and thus the progress of the reaction could be monitored by following the formation of zinc-protoporphyrin. The time course for the first 3 s of the reaction records a pre-steady-state burst of zinc-protoporphyrin production, followed by steady-state turnover (Figure 4B). The presence of a burst of zinc-protoporphyrin formation implies that a step occurring after catalysis, which we assign to product release, is at least partially rate-limiting.

In order to obtain the pseudo-first-order rate constants for NMPP binding, the kinetic traces for zinc-protoporphyrin formation in the presence of various concentrations of inhibitor were fitted to eqn (1) (see the Experimental section). The hyperbolic nature of the pseudo-first-order rate constant dependence on the inhibitor concentration suggests that NMPP binds to wild-type ferrochelatase (Figure 5A) and P255R variant (Figure 5B) via a two-step pathway with a kinetically significant intermediate (Scheme 1). The first step is assumed to involve the formation of an initial complex ([FC–NMPP]₁), which subsequently is rearranged into a more stable complex ([FC–NMPP]₂). Hence the second step of the kinetic pathway is likely to be associated with a slow conformational change of the inhibitor–protein complex. The data were fitted to eqn (3) for a two-step process:

$$k = k_{-2} + \frac{k_2 I_0}{I_0 + K_i \left[1 + \frac{S_0}{K_m} \right]} \quad (3)$$

Figure 4 Transient kinetic analysis of the ferrochelatase-catalysed reaction

Purified variant P255R (2 μ M) was pre-incubated with protoporphyrin (10 μ M) in 100 mM Tris/acetate buffer, pH 8, containing 0.5% Tween-80. The solution was mixed with zinc acetate (15 μ M) to initiate the enzymatic reaction. **(A)** Selected pre-steady-state spectra from the 3000 spectra collected during the reaction. Spectra were collected at 96, 144, 192, 240 and 288 ms from the start of the reaction. **(B)** Time course of the reaction at 420 nm, which records zinc-protoporphyrin formation. The collected data (open circles) were fitted to eqn (2) to obtain the rate constant k .

where k is the pseudo-first-order rate constant for the approach to the steady-state phase, I_0 is the inhibitor concentration, S_0 is the initial substrate concentration, $K_i/[1 + (S_0/K_m)]$ is the apparent inhibition constant, k_i^{app} , and k_2 and k_{-2} are the second step forward and reverse rate constants respectively. The best fit of the points for the reaction with the wild-type enzyme (Figure 5A) gave values of $k_2 = 6.4 \text{ s}^{-1}$, $k_{-2} = 24.5 \text{ s}^{-1}$ and $K_i^{\text{app}} = 3 \text{ nM}$, whereas the fitting of the data for the reaction with the P255R variant returned values of $k_2 = 8.3 \text{ s}^{-1}$, $k_{-2} = 19.3 \text{ s}^{-1}$ and $K_i^{\text{app}} = 1 \text{ } \mu\text{M}$. A two kinetic step process for binding of NMPP to either wild-type ferrochelatase or P255R (i.e. a fast initial step for NMPP binding to the enzyme, followed by a slow step, possibly related to a conformational rearrangement of the protein–inhibitor complex) is consistent with the porphyrin-triggered conformational change of the active-site pocket deduced from the analysis of the crystal structure of *B. subtilis* ferrochelatase with bound *N*-methylmesoporphyrin in comparison with that of the unliganded protein [7]. Although the loop residues [248–257 (murine numbering); 221–231 (yeast numbering)] are involved in the

Figure 5 Concentration-dependence of the rate constants of NMPP binding to either ferrochelatase or P255X variants

Enzyme (0.5 μM) was allowed to react with protoporphyrin (5 μM) and zinc acetate (15 μM) in the presence of various concentrations of NMPP. The pseudo-first-order rate constant, k , was obtained from analysis of the time course (see the Experimental section and Figure 4) and plotted against the NMPP concentration. The enzymes used in each set were: (A) wild-type ferrochelatase; (B) variant P255R; (C) variant P255G.

porphyrin-induced wild-type protein conformational change [7], the values for the forward and reverse rate constants of the second step (k_2 and k_{-2}), which is rate-limiting in the two-step binding pathway, were comparable between the P255R and wild-type enzymes. Therefore the diminished inhibition of variant

Scheme 2 One-step kinetic pathway for inhibition of P255G by NMPP

P255R by NMPP ($K_i^{\text{app}} = 1 \mu\text{M}$ versus $K_i^{\text{app}} = 3 \text{ nM}$ for wild-type ferrochelatase) cannot be accounted for by differences in these rates.

In contrast with wild-type ferrochelatase and the P255R variant, the linear dependence of the pseudo-first-order rate constant for NMPP binding to P255G on the inhibitor concentration (Figure 5C) suggests that NMPP binds to the P255G variant via a single one-step process (Scheme 2). The pseudo-first-order rate constant dependence on NMPP concentration is best described by eqn (4):

$$k = k_{-1} + \frac{k_1 I_0}{1 + \frac{S_0}{K_m}} \quad (4)$$

where $k_1/[1 + (S_0/K_m)]$ and k_{-1} are the forward and reverse rate constants respectively. By fitting the data displayed in Figure 5(C) to eqn (4), the forward and reverse rate constants were determined to be $8.2 \mu\text{M}^{-1} \text{ s}^{-1}$ and 18.7 s^{-1} respectively, whereas resolution of $(k_{-1}/k_1)/[1 + (S_0/K_m)]$ yielded a value for K_i^{app} of $2.3 \mu\text{M}$. Although NMPP binding to P255G occurred via a one-step reaction, the values for the rate constants associated with this step (k_1 and k_{-1}) were similar to those determined for the rate-limiting step of the kinetic pathway for inhibition of ferrochelatase and P255R by NMPP. This suggests that the rate of NMPP binding was unlikely to contribute to its lessened inhibitory effect on P255G.

While the binding affinity of the P255 variants for NMPP was one order of magnitude lower than that of the wild-type protein (K_d values of $0.16 \mu\text{M}$ and $0.30 \mu\text{M}$ versus 9 nM), the inhibition constant increased by approximately two orders of magnitude (K_i^{app} values of $1 \mu\text{M}$ and $2.3 \mu\text{M}$ versus 3 nM). These results suggest the P255 residue is involved in conferring specificity towards the recognition of *N*-alkylated porphyrins. Indeed, the loop residues were previously proposed to function in specifically orienting the porphyrin vinyl substituents and probably the entire porphyrin macrocycle [15].

Analysis of resonance Raman spectra revealed an enhancement of the vinyl stretching band $\nu_{\text{C}=\text{C}_b}$ of Ni-protoporphyrin upon binding to either ferrochelatase or loop variants. Additionally, changes in the vinyl-related lines of haemin bound to the P255R and P255G variants were also observed when compared with those of wild-type ferrochelatase-bound haemin. In particular, a downshift of the core-size marker ν_2 for haemin bound to P255R and P255G indicated that the porphyrin ring was even more expanded than when haemin was bound to wild-type ferrochelatase [15]. These results again implicate a mechanism of recognition and interaction between the loop residues, including P255, and the porphyrin ring.

Size measurement of the active-site pocket

In order to compare the size of the active site in the P255 variants with that of wild-type ferrochelatase, the volume and Connolly's

Scheme 1 Two-step kinetic pathway for inhibition of ferrochelatase or P255R by NMPP

surface area of the porphyrin-binding pocket in the structural models were estimated using the program CAST [22]. The active site of wild-type ferrochelatase was found to occupy a surface area of 2138 Å² (1 Å = 0.1 nm) and a volume of 3015 Å³, whereas variant P255R had a smaller surface area of 1340 Å² and a decreased volume of 1932 Å³, and variant P255G had a surface area of only 1048 Å² and a volume of 1604 Å³. Thus the binding-pocket size was significantly decreased as a result of the P255 mutations. Variant P255R retained ~70 % of the volume and surface area relative to the wild-type protein, whereas P255G only kept ~50 % of the size of the wild-type porphyrin-binding pocket. Previous structural modelling of variants with mutated loop residues suggested that loop mutations affect the active-site architecture [14]. On the basis of the measurements of the porphyrin-binding pocket and previous structural modelling of loop variants, the Pro²⁵⁵ residue appears to have a crucial role in supporting an open conformation of the active site in wild-type ferrochelatase. By contrast, the P255 variants are possibly impaired to widen the active-site cleft, leading to a restricted NMPP entry and decreased binding affinity. This possibility is consistent with the function of proline as a conformational switch, as demonstrated in a variety of proteins such as interleukin-2, tyrosine kinase and Hsp70 (heat-shock protein 70) chaperones [27,28]. On the other hand, the regiospecificity of porphyrin binding is critical to ferrochelatase function [17,29] and any variation of the steric characteristics of the active site of the enzyme will most certainly affect the binding affinity for NMPP. In 1981, Ortiz de Montellano et al. [17] demonstrated that the potency of *N*-alkylprotoporphyrin structures to inhibit ferrochelatase depends on the specific location of the *N*-alkyl group on the porphyrin ring. Isomers with the *N*-alkyl group on pyrrole rings A or B were significantly much stronger inhibitors (i.e. 30–100 times) than those with the *N*-alkyl group on pyrrole rings C or D. In fact, the authors reported that “the identity of the nitrogen alkylated in the protoporphyrin IX is a major determinant of the inhibitory activity”. How stereoselective inhibition of ferrochelatase by *N*-alkylated porphyrin is achieved was further investigated to prove that, within the ring A-substituted (N_A) and the ring B-substituted (N_B) regioisomers and enantiomers of *N*-ethylprotoporphyrin, the N_A regioisomer and the epi-N_B isomer were the more potent inhibitors of ferrochelatase [29]. These results led these investigators to conclude that the greater inhibitory activity of these two isomers probably reflected their macrocycle non-planar distortions being more on a par with that undergone by the porphyrin substrate during ferrochelatase-catalysed porphyrin metallation. In other words, the N_A and the epi-N_B isomers were stronger inhibitors because they were more effective as transition-state analogues of the ferrochelatase-catalysed reaction [29]. Clearly the regiospecificity of ferrochelatase for *N*-alkylated porphyrins, including NMPP, mirrors defined interactions between porphyrin and protein. Mutation of P255 in murine ferrochelatase could have disrupted this interaction and/or altered the stereospecificity of the enzyme towards NMPP.

Despite the marked differences with respect to NMPP binding, the variants exhibited steady-state kinetic properties similar to those of the wild-type enzyme. Whereas wild-type ferrochelatase had a k_{cat} of $4.1 \pm 0.3 \text{ min}^{-1}$, the catalytic activity was slightly increased in the variants, with values of k_{cat} of $7.8 \pm 0.8 \text{ min}^{-1}$ for P255R [14] and $5.9 \pm 1.4 \text{ min}^{-1}$ for P255G. The $K_{\text{i,protoporphyrin IX}}$ value for the wild-type enzyme was $1.4 \pm 0.2 \mu\text{M}$, and it was slightly greater in the variants, with values of $2.65 \pm 0.44 \mu\text{M}$ for P255R [14] and $2.51 \pm 0.80 \mu\text{M}$ for P255G. Thus the specificity constant for protoporphyrin IX, i.e., $k_{\text{cat}}/K_{\text{i,protoporphyrin IX}}$, was $2.92 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$ for wild-type ferrochelatase, $2.94 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$ for P255R and $2.35 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$ for P255G. These results

imply that specificity towards the physiological protoporphyrin IX substrate is distinct from the observed selectivity towards NMPP. One possible explanation for these findings is that the mechanism of interaction between the enzyme and the protoporphyrin substrate differs from that for inhibitor binding. It is conceivable that the active site of ferrochelatase is optimized for interaction with the protoporphyrin substrate and retains a certain amount of plasticity towards this end. Possibly this facilitates the induction of porphyrin deformation in the protein matrix and, in so doing, ensures that the catalytic reaction is able to proceed. In contrast, the constraints of the active site towards NMPP are presumably more stringent than towards the protoporphyrin substrate, and any alterations of the active site affect the selectivity towards NMPP. Another possible explanation, and not necessarily mutually exclusive from the former, is that mutations introduced in the active site (e.g. at the Pro²⁵⁵ position) cause an alteration of the regiospecificity of ferrochelatase, such that the mutated active site can accommodate either a regioisomer or a combination of regioisomers of NMPP different from those of the wild-type active site. It is worth noting that: (1) the commercially available NMPP used in the present study consists of a racemic mixture of the eight potential isomers and (2) only one NMPP isomer was identified in the crystal structure of the *B. subtilis* ferrochelatase. The final answer to the question of whether P255R and P255G exhibit different stereospecificity and bind a different NMPP stereoisomer awaits the determination of the crystal structures of the complexes between NMPP and P255R (or P255G).

Finally, haem deficiency has been recently reported to be associated with various disorders, such as Alzheimer's disease, frataxin-deficiency-mediated diseases and oxidative damage resulted from hyperoxia [30–32]. In these studies, NMPP treatment of cultured cells has provided a convenient model system to induce haem deficiency [30,31]. Typically, NMPP-treated cells exhibit impaired enzymatic activity and assembly of the mitochondrial electron transport complex IV, i.e. COX (cytochrome *c* oxidase) [30,31,33]. COX has been proposed to have a cytoprotective role by removing reactive oxygen species and its deficiency renders cells more susceptible to oxidative stress [30,31]. Thus the ferrochelatase variants with improved tolerance towards NMPP could be potentially used in cell assay systems to study physiological responses to haem deficiency. It should be possible to determine whether the observed NMPP-induced cytotoxicity could be alleviated by expressing constitutively either of the P255 variants; these variants would confer resistance to NMPP inhibition and thereby keep haem synthesis uninterrupted.

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