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Resonance Raman Spectroscopic Examination of Ferrochelatase-induced Porphyrin Distortion

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Abstract

Ferrochelatase, the terminal enzyme of the heme biosynthetic pathway, catalyzes the insertion of ferrous iron into protoporphyrin IX to give heme. Resonance Raman spectroscopy has been instrumental in defining the distortion (mode and extent) of the porphyrin substrate, which is a critical step in the catalytic mechanism of ferrochelatase. Saddling is the predominant porphyrin out-of-plane deformation induced upon binding to ferrochelatase. Our analysis demonstrated that the intensity of the γ_{15} line, which is assigned to an out-of-plane porphyrin vibration, in resonance Raman spectra obtained for wild-type- and variant ferrochelatase-bound porphyrin, correlates with the saddling deformation undergone by the porphyrin substrate. Further analysis of the three dimensional X-ray structures of bacterial, human and yeast ferrochelatases and the type and extent of distortion of the protein-bound porphyrin substrate and inhibitors using normal structure decomposition, support the view that ferrochelatase catalysis involves binding of a distorted porphyrin substrate and releasing of a flatter, metalated porphyrin.

Keywords

Ferrochelatase; Resonance Raman; Protoporphyrin IX; Heme; Normal Structure Decomposition; Tetrapyrrole; Enzyme; X-ray Structures

INTRODUCTION

Ferrochelatase (EC 4.99.1.1, protoheme ferrolyase), catalyzes the terminal step in heme biosynthesis, corresponding to the insertion of ferrous iron into protoporphyrin IX to produce protoheme [1,2].

The catalytic mechanism of ferrochelatase (FC)¹ has been proposed to involve the stabilization of a non-planar porphyrin to present the pyrrole nitrogens to the metal ion

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substrate [1-3]. The mechanism involves, as the first step, the distortion of the tetrapyrrole porphyrin upon binding to the enzyme, in the form of a saddled structure. In Figure 1 (a), the two unprotonated nitrogen atoms of the pyrrole rings point upwards, while the two protonated nitrogens point downwards with respect to the plane of the porphyrin ring. Subsequent to the distortion of the porphyrin ring, the first metal–porphyrin bond is formed, followed by other ligand exchange steps leading to the formation of a ‘sitting-atop’ complex (ii) [3]. This is followed by the sequential deprotonation of the two pyrrole nitrogen atoms coupled with formation of the metallated porphyrin (Figure 1(b)). The saddling of the porphyrin is an out-of-plane deformation, which exposes both the protons and the lone pairs of the nitrogen atoms of the porphyrin molecule in an appropriate arrangement for metal insertion.

Although the physiologically relevant reaction catalyzed by FC is the chelation of ferrous iron (Fe(II)) into the porphyrin ring, the enzyme can chelate other divalent metals *in vitro* (e.g., Zn(II), Ni(II) and Cu(II)). Ferric iron (Fe(III)) and certain other divalent metals, e.g., Mn(II) and Hg(II) as well as N-methylprotoporphyrin, a toxic byproduct of the metabolism of some drugs, are potent inhibitors of the enzyme [4,5]. Hemin, or the oxidized product of the FC reaction (*i.e.*, Fe(III)-protoporphyrin IX) is also an inhibitor to the enzyme. While the natural porphyrin substrate of FC is protoporphyrin IX (H₂PP), a diverse range of IX isomer porphyrins, with substituents at the 2 and 4 positions of the A and B rings, can function as substrates *in vitro*. In addition, the presence of the propionate side-chains, at positions 6 and 7 on the C and D rings respectively, seems to be an essential requirement for the porphyrin to be used as a substrate. These requirements are probably related to the orientation of the porphyrin in the FC active site pocket, namely for the alignment of the central nitrogens with the substrate-iron binding site [3,4].

DISCUSSION

Resonance Raman evidence for porphyrin-substrate binding to ferrochelatase

Resonance Raman (RR) spectroscopy has been instrumental in the elucidation of the kinetic mechanism of metal chelation by FC, particularly from the point-of-view of the analysis of structural alterations undergone by the porphyrin substrate. In fact, the structure-sensitive lines in the RR spectra of porphyrins bound to the enzyme, relative to the unbound porphyrins, even in the absence of the metal ion substrate, reflect the type of distortion present in the porphyrin macrocycle [6,7]. Nickel-porphyrins are especially useful for RR spectroscopic studies, as extensive literature reports on model nonplanar nickel porphyrins are available to reliably interpret the spectral results [8-11]. Also, the presence of nickel instead of iron ensures the absence of axial metal ion coordination, thus reducing the ambiguity caused by axial ligation in interpreting the spectroscopic results [12].

Comparison of the RR spectra for the reaction of FC with sub-stoichiometric amounts of porphyrins with those of free porphyrin in the same buffer permitted us to distinguish line shifts and observe new lines with relevant structural information [13,14]. Figure 2 illustrates the low- and high-frequency regions of RR spectra for the porphyrin substrate or Ni(II)-protoporphyrin IX bound to FC (wild-type enzyme and porphyrin-binding loop variants) (H₂PP - Figure 2A; NiPP - Figure 2B) [14]. Porphyrin-binding loop variants, which are functional forms of FC harboring mutations in a loop previously predicted to interact with porphyrin [15,16], exhibit altered steady-state kinetic parameters, including catalytic efficiency towards H₂PP. These findings are consistent with disruption of the interaction

¹The abbreviations used are: FC, Ferrochelatase; H₂PP, free-base protoporphyrin IX; NiPP, nickel(II)-protoporphyrin IX; Hemin, iron(III)-protoporphyrin IX; RR, resonance Raman.

between the porphyrin substrate and FC. Crystallographic structures of porphyrin-bound FC supported the involvement of the active site loop residues in porphyrin binding [17,18]. Clear differences can be observed in the RR spectra of H₂PP bound to FC variants when compared to those of the free porphyrin in the same buffer (Figure 2A). Specifically, in the low-frequency region (Figure 2A, top spectra), a strong γ_{15} out-of-plane mode is activated upon protein binding (traces b-g vs. trace a). The RR out-of-plane vibrational mode γ_{15} , is a B_{2u} symmetry (saddling-like) mode associated with an out-of-plane porphyrin deformation [9]. RR spectra in the high-frequency region (Figure 2A, lower spectra) also exhibit distinct differences between H₂PP bound to the variants or wild-type FC and free H₂PP. Of relevance, the ν_2 line is upshifted by different degrees for the H₂PP bound to the wild-type enzyme (trace b) and all loop variants (traces c-g). This upshift is most prominent for the H₂PP bound to the triply mutated K250M/V251L/W256Y variant (trace c), in which the ν_2 line upshifts by 5 cm⁻¹ relative to the wild-type protein-bound H₂PP (trace b). This shift reflects important changes in the interaction of the vinyl group with the protein. Further, these changes are supported by the broadening and down-shifting of the $\nu_{Ca=Cb}$ vinyl stretching mode for the triply mutated variant (trace c), which is clearly not observed for the wild-type (trace b) and for the other loop variants (traces d-g). The addition of NiPP to both wild-type FC (Figure 2B, trace b) and loop variants (Figure 2B, traces c-g) results in a sharpening of the RR lines of the bound porphyrin in relation to those of NiPP in assay buffer (Figure 2B, trace a) [13,14,19]. Also, upon protein binding, the ν_2 , ν_3 , and ν_{10} structure-sensitive lines downshift, while the vinyl stretching mode ($\nu_{Ca=Cb}$) sharpens and increases in intensity (Figure 2B, lower spectra). Two porphyrin-binding loop variants, namely the triply mutated enzymes K250M/V251L/W256Y and S249A/K250Q/V251C, induce a less pronounced splitting of the ν_7 lines, broader propionate lines, and ν_8 lines that are similar to those observed for the unbound NiPP in assay buffer (Figure 2B, top spectra, traces c and f vs. trace a). These observations suggested that, in relation to the wild-type enzyme, the triply mutated variants have lower affinities for NiPP and a looser interaction with the porphyrin. When comparing the kinetic parameters for wild-type murine FC and loop variants, the triple K250M/V251L/W256Y variant exhibits a 1-order of magnitude lower catalytic efficiency towards porphyrin (*i.e.*, $k_{cat}/K_m^{H_2PP}$) than the wild-type enzyme [14]. This decrease is mainly due to a hindered porphyrin binding, as indicated by the 8-fold increase in the value of K_m for H₂PP, which contrasts with the *ca.* 30% decrease in k_{cat} [16]. Analysis of the three dimensional X-ray structure of the human FC variant, E343K, a variant with endogenously bound porphyrin (PDB code 2QD1) [20] shows that the corresponding residues in human FC are located in close vicinity to the bound substrate. For example, both the V251L and W256Y replacements may create a steric clash with the porphyrin, interfering with its proper binding, while the S249A/K250Q/V251C variant may have higher loop flexibility, and may also create a steric hindrance due to the bulky sulfur atom of the cysteine.

Porphyrin saddling distortion and ferrochelatase catalytic efficiency

The extent of substrate saddling influences the catalytic efficiency of FC towards the porphyrin substrate, H₂PP [14]. The γ_{15} line corresponds to a B_{2u} symmetry saddling-like mode, and its appearance was attributed to a saddling deformation of the porphyrin upon binding to the protein [13,21]. Figure 3 shows a direct correlation between murine FC catalytic efficiency towards porphyrin (*i.e.*, $k_{cat}/K_m^{H_2PP}$) and the degree of saddling porphyrin distortion induced by the wild-type enzyme and porphyrin-binding loop variants [14]. The degree of saddling porphyrin distortion can be determined by the integrated intensity of the γ_{15} line, normalized to the intensity of the ν_7 line for the same RR spectrum, for wild-type and mutated murine FCs incubated with H₂PP (see Figure 2A and 2B) [14].

Porphyrin binding and distortion in *Saccharomyces cerevisiae* FC and catalytic antibodies with FC activity have been also assessed using RR spectroscopy [6,22]. In both the enzyme and the antibody, porphyrin binding also activates several assigned out-of-plane vibrational modes of the macrocycle. Significantly, a trend similar to what has been observed for murine FC and its variants was also identified with the catalytic efficiencies towards porphyrin of the different catalytic antibodies, in which an approximate linear correlation between the logarithm of the catalytic efficiencies and the γ_{15}/ν_7 RR lines intensity ratio could be established [22].

Normal-coordinate Structural Decomposition (NSD) is a procedure developed by Shelnutt and co-workers [23,24] to describe a nonplanar porphyrin structure by determining the distortion from a reference planar macrocycle in terms of equivalent displacements along the normal coordinates of the reference D_{4h} -symmetric macrocycle. Briefly, the NSD program² quantifies the optimum displacements for six out-of-plane symmetry types as normal coordinates that approximately correspond to the symmetric out-of-plane deformations commonly seen in X-ray crystal structures of symmetrically substituted porphyrins, *i.e.*, the so-called ruffled, saddled, domed, x- and y-waved, and propellered structures [7]. Figure 4 represents the NSD charts obtained from the X-ray structures of N-methyl porphyrins bound to *Bacillus subtilis* FC. The analysis of the X-ray structures led Lecerof *et al.* (16) to note that the overall conformations of the non-metallated and metallated porphyrin-FC complexes are essentially similar, implying that the enzyme conformation is primarily affected by porphyrin binding rather than metallation. In figure 4, the major contributions to the distortion of N-methyl mesoporphyrin (either as free base or as a metallated Cu(II) complex) bound to FC are saddling, followed by x-waving, ruffling, and doming. Thus, assuming that the structure of the murine FC active site induces similar distortions, it is not surprising that RR data corroborate the presence of all of these deformations in the FC-bound porphyrins [13]. The preponderance of saddling distortion in these structures, becomes particularly relevant to the FC mechanism because saddling involves a B_{2u} (D_{4h} classification) out-of-plane deformation that exposes both the protons and the lone pairs of the nitrogen atoms of the porphyrin core. Saddling is then a porphyrin deformation suitable for promoting metal insertion by FC.

Amino acid residues involved in porphyrin distortion

The three-dimensional structures of several mammalian and bacterial FCs have been determined in a free form [15,17,18], in complex with inhibitors [25,26], and in complex with metal ions, porphyrin substrate and reaction products [27-29]. From the analysis of these structures it became clear that the porphyrin rings B, C and D remain relatively planar, whereas the A ring experiences an out-of-plane distortion relative to the other three. Two metal-ion binding sites have been identified, one located at the surface of the molecule, occupied by a fully hydrated Mg(II) ion, and the other located in the porphyrin-binding cleft, close to the distorted porphyrin ring A, whose nitrogen points towards His183 and Glu264 (*Bacillus subtilis* FC numbering) [30]. Molecular dynamics simulations of wild-type and variant murine FCs support that mutations of the corresponding murine FC active-site residues, His209 and Glu289 (murine FC numbering), affect hemin and, presumably, H₂PP distortion [31]. In the E289Q variant, hemin forms more contacts and interacts more strongly with the protein than in the wild-type enzyme or the H209N variant. These molecular dynamics simulation studies [31] are in good agreement with the experimental determination of tighter porphyrin binding with the E289Q and E289A variants than with the wild-type enzyme. In fact, both Glu289 variants revealed to possess H₂PP endogenously bound to the native enzyme [32]. Significantly, rapid stopped-flow experiments with these

²The NSD Program is available on-line at <http://jasheln.unm.edu/jasheln/content/nsd/NSDEngine/start.htm>

variants indicated that the endogenously bound H₂PP could be used as a porphyrin-substrate for Zn(II) chelation, but product release was hindered [32]. Normal-coordinate Structural Decomposition analysis of the endogenously bound H₂PP on an equivalent variant of human FC (E343K; PDB code 2QD1) [20] also led to the conclusion that saddling distortion was more prominent, at ca. 1 Å, while other distortion modes were at the most *ca.* 0.2 Å (Figure 5). Taken together, these observations reinforce the notion that porphyrin-substrate saddling distortion is essential for metal insertion, and that product release can only occur for a planar metallated porphyrin.

Molecular dynamics simulations also allowed to conclude that the H209N variant causes the largest distortion of the porphyrin when comparing with the wild-type enzyme or the E289Q variant, which led Szeftczyk *et al.* [31] to suggest that His209, in addition to the previously proposed roles in ferrous ion substrate binding [30,33] and proton extraction [29], has a role in the induction of the planar geometry of the product.

CONCLUSION

Resonance Raman spectroscopy has proven to be an extremely useful tool to study the enzymatic mechanism of FC by probing the distortion of the porphyrin substrate, a crucial step in the catalytic cycle of the enzyme. The intensity of the γ_{15} line in RR spectra obtained for wild-type- and variant FC-bound porphyrin correlates with the saddling deformation undergone by the porphyrin substrate. This type of out-of-plane deformation exposes both the protons and the lone pairs of the nitrogen atoms of the porphyrin core. Finally, the degree of porphyrin saddling distortion modulates the catalytic efficiency of the enzyme towards this substrate, accentuating the importance of the architecture of the active site towards binding a distorted porphyrin and releasing a flatter, metalated porphyrin.

Acknowledgments

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Figure 1. Reaction mechanism of porphyrin metalation. (a) Out-of-plane saddling deformation structure in which two pyrrole rings with unprotonated nitrogens point upwards, while the other two, protonated point downwards. (b) Steps in the mechanism for incorporation of the metal ion into the porphyrin (pyrrole rings in a darker shade), described in the text. (Adapted from [3], Copyright (2006), with permission from Elsevier).

Figure 2.

Resonance Raman spectra of H₂PP and NiPP. Low-frequency (top spectra) and high-frequency (lower spectra) regions of the resonance Raman spectra of H₂PP (A), or NiPP (B), in assay buffer (a), bound to wild-type FC (b) and to porphyrin-binding loop variants : triple mutant K250M/V251L/W256Y (c), single mutant P255R (d), single mutant P255G (e), triple mutant S249A/K250Q/V251C (f), and quadruple mutant Q248P/S249G/K250P/G252W (g). Asterisks denote buffer lines. Vibrational modes of the major structure-sensitive lines are labeled (Adapted with permission from [14]. Copyright 2006 American Chemical Society).

Figure 3.

Catalytic efficiency of murine FC towards H₂PP is directly related to the saddling porphyrin distortion induced by the enzyme. The graph is a representation of the catalytic efficiency kinetic parameters ($k_{cat}/K_m^{H_2PP}$) for H₂PP utilization by wild-type FC (WT) and porphyrin-binding loop variants (PR=P255G; SKV=S249A/K250Q/V251C; KVW=K250M/V251L/W256Y; QSKG=Q248P/S249G/K250P/G252W) vs. relative areas of the γ_{15} line in RR spectra obtained for the respective enzymes incubated with H₂PP (see spectra in Figure 2) [14].

Figure 4.

Normal-coordinate structural decomposition of the X-ray crystal structures of free-base and metallated porphyrins, bound to *B. subtilis* FC. Free-base structure, PDB code 1C1H; $H_2(N\text{-Me-MesoP})$ = N-methyl mesoporphyrin, a potent inhibitor of the enzyme; Metalated porphyrin, PDB code 1C9E; $Cu(N\text{-Me-MesoP})$ = Cu(II)-N-methyl mesoporphyrin. Hatching code: saddling, horizontal lines; ruffling, vertical lines; doming, right diagonal lines; x-waving, left diagonal lines; y-waving, vertical crossing; propellering, diagonal crossing; black is for the lowest frequency mode of each symmetry type, and grey is for the next-to-lowest frequency mode of each symmetry type. (Adapted with permission from [13], Copyright 2002 American Chemical Society).

Figure 5.

Normal-coordinate structural decomposition of the X-ray crystal structure of H₂PP, bound to human FC. The free-base porphyrin is endogenously bound to the native variant E343K of human FC, PDB code: 2QD1. The analyzed H₂PP molecule corresponds to “PP9 B 702”, one of the four H₂PP molecules bound to the FC active site present in the crystallographic unit cell. The NSD parameters for the other three H₂PP molecules are similar. Hatching code: saddling, horizontal lines; ruffling, vertical lines; doming, right diagonal lines; x-waving, left diagonal lines; y-waving, vertical crossing; propellering, diagonal crossing; black is for the lowest frequency mode of each symmetry type, and grey is for the next-to-lowest frequency mode of each symmetry type.