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Published in:
Journal of Biological Inorganic Chemistry

DOI:
10.1007/s00775-002-0413-8

2003

Document Version:
Peer reviewed version (aka post-print)

The importance of porphyrin distortions for the ferrochelatase reaction

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2017-04-01

Non-standard abbreviations: MMP, N-methylmesoporphyrin.

Ferrochelatase is the terminal enzyme in haem biosynthesis, i.e. the enzyme that inserts a ferrous ion into the porphyrin ring. Suggested reaction mechanisms for this enzyme involve a distortion of the porphyrin ring when it is bound to the enzyme. We have examined the energetics of such distortions using a various theoretical calculations. With the density functional B3LYP method we calculate how much energy it costs to tilt one of the pyrrole rings out of the porphyrin plane for an isolated porphyrin molecule without or with a divalent metal ion in the centre of the ring. A tilt of 30° costs 65–130 kJ/mole for most metal ions, but only ~48 kJ/mole for free-base (neutral) porphine. This indicates that once the metal is inserted, the porphyrin becomes stiffer and more flat, and therefore binds with lower affinity to a site designed to bind a distorted porphyrin. This would facilitate the release of the product from ferrochelatase. This proposal is strengthened by the fact that the only tested metal ion with a lower distortion energy than free-base porphyrin (Cd^{2+}) is an inhibitor of ferrochelatase. Moreover, it costs even less energy to tilt a doubly deprotonated $porphine^{2-}$ molecule. This suggests that the protein may lower the acid constant of the pyrrole nitrogen atoms by deforming the porphyrin molecule. We have also estimated the structure of the protoporphyrin IX substrate bound to ferrochelatase using combined quantum chemical and molecular mechanics calculations. The result shows that the protein may distort the porphyrin by ~20 kJ/mole, leading to a distinctly non-planar structure. All four pyrrole rings are tilted out of the porphyrin mean plane (1–16°) but most towards the putative binding site of the metal ion. The predicted tilt is considerably smaller than that observed in the crystal structure of a porphyrin inhibitor.

Key words: Ferrochelatase, density functional calculation, QM/MM calculation, porphyrin distortion, potential-of-mean-force calculation.

Introduction

Haem is a ubiquitous cofactor in biological systems, and it is present in numerous important proteins such as haemoglobin, cytochromes, oxidases, peroxidases, and catalases. Biosynthesis of haem consists typically of eight enzymatic steps [1]. The final step, insertion of a ferrous ion into the protoporphyrin IX ring, is catalysed by the enzyme ferrochelatase (E.C. 4.99.1.1). Similar chelatases insert magnesium and cobalt into the precursors of chlorophyll and coenzyme B_{12} [2–4]. Chelatases from various types of organisms have in general little sequence homology. However, crystal structures of *Bacillus subtilis*, yeast, and human ferrochelatases, as well as anaerobic cobalt chelatase from *Salmonella typhimyrium* show that they share a similar three-dimensional structure with conserved amino-acid residues around the active site [3,5,6]. On the other hand, the crystal structure of yeast sirohaem chelatase (Met8p) shows no resemblance to the other structures [7].

The crystal structure of ferrochelatase from *B. subtilis* in complex with a potent porphyrin inhibitor, N-methylmesoporhyrin (MMP) has recently been determined [8]. The structure shows that the porphyrin molecule binds to the enzyme in a hydrophobic pocket with the propionate side chains reaching out to the surface. The binding of MMP is stabilised by ionic, aromatic stacking, and steric interactions of protein side chains with the porphyrin.

The most widely accepted reaction mechanism of ferrochelatase involves binding of the porphyrin and the metal ion to the enzyme, deformation of the porphyrin to expose the nitrogen lone-pair orbitals towards the metal, deprotonation of the porphyrin, and metal-ligand exchange with the release of the haem product [9]. The methyl group bound to the nitrogen atom of the *A* pyrrole ring of MMP is too large to fit into the central cavity (see Figure 1). Therefore, this pyrrole ring is tilted ~30° out of the porphyrin plane [10]. In the protein, this tilt is increased to 36° [8]. It is believed that ferrochelatase enforces a distortion of protoporphyrin IX similar to the tilt in MMP [8, 9]. MMP is therefore assumed to be a transition-state analogue of the enzyme [11]. In fact, antibodies as well as catalytic DNA and RNA raised against MMP have ferrochelatase activity, although with a lower rate than the native enzyme [12–14]. Distortions of the bound porphyrin have also been observed by resonance Raman spectroscopy on ferrochelatase and catalytic antibodies [15–17].

Ferrochelatases from different sources have similar catalytic properties [10]. In vitro, they insert several divalent metal ions in addition to Fe^{2+} into the porphyrin ring, e.g. Zn^{2+} [11]. Cu^{2+} is inserted

by ferrochelatase from *B. subtilis*, but not by other ferrochelatases [18]. On the other hand, Co^{2+} is inserted by most known ferrochelatases, but not by that from *B. subtilis*. Mg^{2+} is not inserted by any known ferrochelatase. Some metal ions are even known to be inhibitors, e.g. Mn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} [11].

A conserved histidine residue, His-183 in *B. subtilis* ferrochelatase, has been identified as a putative binding site for the iron ion [6,16,17,19,20], even if some authors argue that the metal ion binds instead to a tyrosine residue (Tyr-165 in human ferrochelatase corresponding to His-88 in *B. subtilis* ferrochelatase) on the opposite side of the porphyrin ring [5,21]. In a recent crystal structure, a Zn^{2+} ion is found to bind to His-183 [22]. The distorted tetrahedral binding geometry of the zinc ion is completed by bonds to Glu-264 and two water molecules. The water molecules are stabilised by hydrogen bonds to Tyr-13 and Ser-222, respectively. His-183 is located on the same side as the methyl group of MMP in its complex with ferrochelatase, which further supports the suggestion that MMP is a transition-state analogue.

The proposed reaction mechanism of ferrochelatase with its initial distortion of the porphyrin ring is attractive. However, there is no evidence that the suggested distortions are energetically feasible. In this paper, we therefore use theoretical methods to study the energetics of porphyrin distortions both of the porphyrin ring and of the surrounding enzyme. In addition, we try to predict the structure of the true substrate, protoporphyrin IX, in the enzyme. Besides energetics and structures, the results give some clues about the metal specificity of the protein and how the haem product is released from the protein.

Methods

Quantum chemical calculations

Quantum chemical geometry optimisations were performed with the density functional B3LYP method (unrestricted formalism for open-shell systems), as implemented in the Turbomole software [23,24]. Hybrid density functional methods have been shown to give excellent geometries for first-row transition metal complexes [25–27], and the B3LYP method seems to be one of the most reliable density functional methods in general terms [28]. In particular, density functional methods have been used successfully to study geometric, energetic, and spectroscopic aspects of porphyrin distortions [29–32] The basis sets used are described in Table 1. Only the pure 5 *d* and 7 *f*-type

functions are used. Calibrations have shown that geometries obtained with this approach do not change much when the basis set is increased [33,34].

The models studied include the full porphyrin ring without any side chains (i.e. porphine, Por), either in the neutral free-base form ($PorH_2$, with two of the pyrrole nitrogen atoms protonated), in the doubly deprotonated form (Por^{2-}), or with a divalent metal ion (Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}) in the centre. In one case, a five-coordinate complex was also studied, with an axial imidazole (Im) ligand as a model of the putative histidine ligand in the protein (FePorIm). Calculations on MMP with and without side chains were also performed.

The full geometry of all models was optimised (i.e. no constraints were imposed to model steric restrictions of the protein) until the change in energy between two iterations was below 10^{-6} Hartree (2.6 J/mole) and the norm of the internal gradients was below 10^{-3} a.u. (0.053 pm or 0.057°). All Fe^{2+} porphyrins were studied in the high-spin quintet states. Cd^{2+}, Mg^{2+} and Zn^{2+} porphines were closed-shell singlets, Cu^{2+} porphine a doublet, Mn^{2+} porphine either a doublet or a quartet, whereas Ni^{2+} and Co^{2+} porphines were assumed to be in the low-spin states, because these states had a lower energy than the alternative spin states, calculated with the same method. Test calculations showed that the various spin states give rise to similar potential energy surfaces for the studied ring tilt (see below).

The energy of tilting the *A* pyrrole ring out of the porphyrin plane was studied by constraining the C1B–CHB–C4A–C3A torsion angle (see Figure 1 for the definition of the atom names) and optimising the rest of the structure. These calculations were performed in C_s symmetry, imposing a reflection plane through atoms NA and NC (implying a constraint also of the C4D–CHA–C1A–C2A torsion). In the other calculations no symmetry restraints were imposed. The reported tilt is the angle between the planes defined by NB–NC–ND and NA–C2A–C3A. The calculations were run on IBM SP2 and SGI Origin 2000 or Octane workstations.

Free energy perturbation calculations

The energy cost of tilting the porphyrin in the protein (i.e. how much the protein favours or disfavours a tilt of the porphyrin ring) was estimated by calculating the potential of mean force of the tilt in the ferrochelatase:MMP complex. These calculations were based on the crystal structure of the this complex [8], structure 1c1h in the Brookhaven protein databank. Hydrogen atoms were

added to the protein assuming the normal protonation status at pH 7.0 for the Asp, Glu, Lys, and Arg residues. After a detailed study of the surroundings and possible hydrogen bond networks, it was decided that His-116 is protonated on the ND1 atom, His-88 is protonated on the NE2 atom, whereas the other histidine residues are protonated on both nitrogen atoms (and thus positively charged).

The protein was solvated in a sphere of water molecules with a radius of 30 Å, centred on the CCA atom of MMP (2729 water molecules). The full system was then equilibrated using the sander routine of the Amber 5 software [35] until the potential energy was stable, i.e. 300 ps, cf. Figure 2. Protein atoms outside a radius of 26 Å from CCA in MMP were kept fixed at the crystal position in all calculations. No bond-length constraints were applied in any of the calculations.

The potential-of-mean-force calculations were performed by the gibbs routine in the Amber package [35]. In these calculations, the dihedral angles C1B–CHB–C4A–NA and C4D–CHA–C1A–NA were driven from the equilibrium value, ±21.7°, down to 0° and back again or up to ±45° and back again. These double calculations illustrate the hysteresis in the calculations and provide a lower limit of the error in the calculations. The change in free energy was calculated with the thermodynamic integration method using the dynamically modified windows technique [36]. Simulation parameters are gathered in Table 2.

The force field for MMP (available as supplementary material) was obtained from a B3LYP/6-31G* frequency calculation (with Gaussian98 [37]) of the whole molecule, including the side chains, using the method of Seminario [38]. Charges where estimated from the same calculation using the standard two-stage RESP method [39] in the Amber software [35]. The electrostatic potential points were selected using the Merz–Kollman scheme in Gaussian98 [37], but with a high density of about 1000 points per atom. We used the default Van der Waals parameters of the Amber force field, but the r^* parameter for the three HCA hydrogen atoms was reduced by 0.38 Å. This gives a minimised structure in vacuum that is closely similar to the optimised quantum chemical structure. In particular, the porphyrin tilt angle is the same as in the quantum structure.

The octahedral $Mg(H_2O)_6^{2+}$ ion, also present in the ferrochelatase structure, was described by a simple non-bonded potential, using standard Amber Van der Waals parameters for water and Mg^{2+} (1.17 Å and 0.10 kcal/mole) [40]. The charges of this complex were estimated with the RESP method in the same way as for the MMP molecule (Mg: 1.7663, O: –1.0146, H: 0.5268 *e*).

ComQum calculations

Finally, combined quantum mechanical and molecular mechanical (QM/MM) calculations where performed to estimate the structure of the native porphyrin substrate in the protein. These calculations were based on the MMP:ferrochelatase complex described above, but with MMP replaced by protoporphyrin IX. The calculations were started from the crystal structure with only the hydrogen atoms and the solvation water molecules equilibrated by a simulated annealing calculation.

These calculations were performed with the ComQum software [41,42], which is a combination of Turbomole [23,24] and Amber [35]. The porphine core (the porphyrin ring without side chains) was treated by quantum chemistry, using the B3LYP method and the 6-31G* basis set, as described above. The side chains of the porphyrin, the protein, and the surrounding solvent sphere were treated with molecular mechanics.

In the quantum chemical calculations, porphine was represented by a wave function, whereas all the other atoms were represented by an array of point charges, one for each atom, taken from the Amber libraries. Thereby, the polarisation of the quantum chemical system by the surroundings is included in a self-consistent manner. In the classical force and energy calculations, all atoms were represented by the Amber force field, but without any electrostatic interactions (which are already treated by quantum mechanics). Special action is taken for the porphyrin side chains, for which there is a bond between the classical and quantum chemical systems [41]. The quantum chemical system is truncated by hydrogen atoms, the positions of which are linearly related to the corresponding carbon atoms in the full system.

The total energy is calculated as

$$E_{tot} = E_{QC} + E_{MM123} - E_{MM1} \quad (1)$$

Here, E_{QC} is the quantum chemical energy of the quantum system truncated by hydrogen atoms, including all the electrostatic interactions. E_{MM1} is the classical energy of the quantum system, still truncated by hydrogen atoms, but without any electrostatic interactions. Finally, E_{MM123} is the classical energy of all atoms with normal porphyrin side-chain atoms and no electrostatics. The philosophy behind this energy is that the total energy should involve as much quantum chemistry as

possible and that terms from the porphyrin side-chain truncations shall cancel out. This approach is similar to the one used in the Oniom method [43]. The calculated forces are the gradient of this energy, but owing to the different atoms for the porphyrin side chains, the gradients have to be corrected using the chain rule.

Calculations were performed both with the protein fixed at the crystal positions and with protein atoms within 15 Å of the porphyrin ring allowed to relax by a molecular mechanics minimisation in each step of the ComQum optimisation. In these calculations, all atoms are represented by standard Amber parameters. Electrostatic interactions are included, using standard charges for the surroundings, but for the quantum system, we used quantum chemical Mulliken charges fitted to Merz–Kollman electrostatic potential charges [44], as described before [42].

The applied convergence criteria were the same as in the quantum chemical calculations. However, for calculations where the protein was allowed to relax, looser convergence criteria were used (260 J/mole and 0.53 pm), and these calculations were followed by an optimisation using a fixed enzyme and the normal convergence criteria. The strain energy of the porphyrin in the protein (ΔE_1) is calculated as the difference in energy in vacuum (i.e. without point charges) of the quantum system optimised in the protein and in vacuum. It should be noted that this energy may contain terms that are not normally considered as strain [42].

Results and Discussion

Geometry of the MMP molecule

Biologically active porphyrins have several side groups attached on the periphery of the porphyrin skeleton. The full porphyrin molecule with all these side groups is on the verge of what can be studied by accurate quantum chemical methods. Therefore, the side chains are usually ignored in quantum chemical calculations. In order to test this approximation, we first optimised the geometry of N-methylmesoporphyrin (MMP) both with and without side chains. As can be seen from the overlay of the two resulting structures in Figure 3, the geometry of the two models is very similar; the rms. difference between all the porphine atoms is only 3 pm. Similarly, the average absolute difference of all bond lengths, angles, and dihedral angles in the porphyrin ring are 0.5 pm, 0.4°, and 0.5°, respectively, and the maximum difference is 1.9 pm, 1.5°, and 2.3°, respectively.

In both structures, the *B*, *C*, and *D* pyrrole rings are in the same plane, whereas the *A* ring is tilted 30° out of the porphyrin plane. The NA–CCA bond is tilted even more, 60–61° from the porphyrin plane. This shows that it is reasonable to ignore the side chains in calculations of porphyrins, and we have applied this approximation in all other quantum chemical calculations in this article.

In Figure 4 we compare the quantum chemical structure of MMP with that observed in the crystal structure of ferrochelatase [8]. The general geometry of the two structures is quite similar with a rms. deviation of 20 pm for the heavy atoms in the porphine ring. However, the geometry of the side chains differs appreciably more (rms. deviation 96 pm). This is quite natural, because the theoretical structure is optimised in vacuum where the side chains can only interact with themselves and with the porphyrin ring. In the experimental structure, on the other hand, the side chains interact with the surrounding protein.

Interestingly, there are also significant structural differences in the overall structure of the pyrrole rings. In particular, the *B*, *C*, and *D* pyrrole rings of the quantum chemical structure are completely planar, whereas the experimental structure is strongly distorted. Some of the distortions of the porphyrin ring in the experimental structure are artefacts caused by errors in the force field used in the crystallographic refinement of the structure, but the general ruffling of the structure seems to be real and caused of the protein [38]. Moreover, the tilt angle differs; it is 6° larger in the crystal structure (36°) than in the theoretical structures. Thus, the protein seems to tilt the *A* pyrrole ring even further out of the porphyrin plane. Similarly, the NA–CCA bond in the crystal structure is tilted more than the *A* ring, about 70°.

How much energy is required to tilt various porphyrins?

As was discussed in the introduction, the most widely accepted reaction mechanism of ferrochelatase suggests that the protein deforms the porphyrin substrate in a manner similar to the tilt observed in MMP, in order to expose the lone-pair orbitals of the pyrrole nitrogens to the iron ion. As a first test of this mechanism, we studied the energetics of such a deformation for isolated porphyrin molecules in vacuum, without or with a number of different metal ions in the centre of the ring. The results are collected in Figure 5. All porphines except the MMP model and to a smaller extent also the five-coordinate FePorIm model are completely planar in their optimum geometries. MMP has its minimal energy at a tilt angle of 30° as was discussed in the previous section. The

protonation status of MMP is not fully clear: N-methyl coproporphyrin is protonated and has a positive charge of the porphyrin core at neutral pH [45], whereas N-methyl tetraphenylporphine is unprotonated and neutral [46]. Therefore, we tilted both a neutral and a monocationic MMP molecule. As can be seen in Figure 5, the two models give very similar tilt energies.

For the neutral free-base porphine molecule without any metal, it costs 46–50 kJ/mole to tilt the A pyrrole ring 30° out of the porphyrin plane. The lower energy is obtained when the tilted ring is protonated, whereas the tilt energy is slightly higher when the tilted ring is not protonated (the porphine molecule is symmetric, so it cannot be decided which is the *A* pyrrole ring).

When a Fe^{2+} ion is inserted into the porphyrin ring, the tilt energy increases by ~30 %, i.e. to 67 kJ/mole for a tilt angle of 30°. A five-coordinate iron porphine with an imidazole group (FePorIm) as a model of the putative iron ligand His-183 in ferrochelatase is slightly more easily distorted. Thus, these calculations indicate that it is energetically feasible for the protein to deform the metal-free porphyrin so that the nitrogen lone pairs become exposed, but probably not so much as is observed in the crystal structure of the ferrochelatase:MMP complex. Moreover, once the metal ion is inserted, the energy required to deform the porphyrin increases. This could provide a mechanism for the release of the product. If ferrochelatase is designed to deform the porphyrin substrate, i.e. to bind a tilted porphyrin ring stronger than a plane one, then the stiffer metal-porphyrin would become more planar in the enzyme and fit the binding site worse. Thus, its affinity to the binding site would be reduced, which could trigger its release.

A fully deprotonated porphine (i.e. with a double negative charge) requires much less energy to be deformed, about 20 kJ/mole for a tilt of 30°. The deprotonated porphyrin is probably an intermediate in the metal insertion mechanism. It is formed after the neutral (and doubly protonated) porphyrin is bound to the enzyme and before the iron ion is inserted into the porphyrin ring. This putative intermediate would then have an even stronger affinity to the binding site of ferrochelatase and would thus be stabilised. This means that the distortion of the porphyrin ring in the protein will lower the pK_a of the pyrrole groups, thereby facilitating insertion of the metal ion.

We have also studied the energy required to tilt other metal porphyrins, involving both inhibitor and substrate metals ions. Interestingly, the Mg^{2+} ion gives almost exactly the same energy curve as Fe^{2+}. In plants, Mg is inserted into protoporphyrin IX by a enzyme called magnesium chelatase during the synthesis of chlorophyll [2]. Our results may indicate that the release mechanism of

magnesium chelatase is similar to that of ferrochelatase. However, as mentioned earlier, Mg^{2+} is not inserted by ferrochelatase. The reason for this is probably that the metal binding site near the porphyrin, involving His-183, Glu-284, and two water molecules in a tetrahedral fashion [22], is a poor site for a magnesium ion, especially as there is a strong magnesium site only ~0.7 nm from that site.

Zn^{2+}, Co^{2+} and Cu^{2+} are experimentally shown to be inserted into porphyrin by at least some ferrochelatases [18]. The porphine derivatives of all these metal ions require higher energy to be tilted than Fe porphine (cf. Figure 5). Thus, our suggested release mechanism would work also for these metals.

Cd^{2+} and Mn^{2+} are known to be strong inhibitors of ferrochelatase [11]. Figure 5 shows that Cd^{2+} porphine requires much less energy to be tilted than the metal porphyrins of all the inserted ions (26 kJ/mole for a tilt of 30°). In particular, the required energy is less than for the neutral metal-free porphine. This means that Cd porphine would have higher affinity to the binding site than the metal-free porphyrin and would therefore not be released. This might be the reason why Cd^{2+} is an inhibitor. However there must be another explanation for the inhibitory effect of Mn^{2+}, because the energy curve of Mn porphine is almost identical to that of Cu porphine, although Cu^{2+} is a substrate of ferrochelatase.

Potential-of-mean-force calculations

In the previous section, we estimated the energy cost to tilt the isolated porphyrin. In this section, we want to do the opposite, i.e. to estimate what tilt angle ferrochelatase prefers for a bound porphyrin and with how large a force it may tilt a bound porphyrin. Therefore, we have performed classical free energy perturbations of MMP bound to ferrochelatase.

There is no force field available for MMP, so we had to construct it (as described in the methods section; parameters are included as supplementary material). We ensured that the MMP molecule optimised with this force field in vacuum has a tilt of 30° (by adapting the Van der Waals parameters of the HCA atoms), i.e. the same value as in quantum chemical geometry optimisations.

When the structure of MMP was optimised in the protein with this force field, the tilt angle increased somewhat, to 33°, which is 3° less than in the crystal structure. However, there are appreciable dynamics in this tilt angle; During the last 50 ps of the equilibration of the

MMP:ferrochelatase complex, the tilt angle varied between 27–40° with an average of 32°. This reflects the shallow potential of this angle, as can be seen in Figure 5.

From the equilibrated structure, we started a set of potential-of-mean-force calculations, where we either increased or decreased the tilt angle of MMP. It is not possible to constrain the tilt angle (defined as the angle between the NB–NC–ND and NA–C2A–C3A); instead we constrained the C1B–CHB–C4A–NA and C4D–CHA–C1A–NA dihedral angles. The results were reinterpreted in terms of the tilt angle by sampling a large number of coordinates along the perturbation and then calculating the best correlation between the constrained dihedral and the tilt angle by linear regression.

The potential of mean force for the tilt angle of MMP in ferrochelatase is shown in Figure 6. The perturbation runs from 0° to 45° in the constrained dihedrals, which correspond approximately to a tilt angle of 23° and 39°. It can be seen that the energy curve is fairly symmetric around the equilibrium value of 32° (tilt angle). The potential raises to ~40 kJ/mole at both endpoints of the perturbation, with an uncertainty of less than 3 kJ/mole.

Unfortunately, these curves cannot directly be interpreted as the tilt potential in the protein, because the internal distortion energy of the MMP molecule is included in these energies. In order to estimate this internal energy, we performed the same perturbations for an isolated MMP molecule both in vacuum and in a sphere of water molecules of the same size as for the protein (a radius of 3 nm). The result of the vacuum calculation is also included in Figure 6 (that in water gave very similar results, but with a larger uncertainty). The curves show that the majority of the potential of mean force in the protein arises from the MMP molecule itself. Only ~3 kJ/mole of the potential at the end points can be attributed to the enzyme. This confirms our previous results that a protein can only exert quite modest strain forces [39].

A model of the ferrochelatase complex with protoporphyrin IX

The crystal structure of the MMP:ferrochelatase complex is very interesting, because it shows the binding mode of a porphyrin in the enzyme. However, it is not clear how relevant these results with the artificial porphyrin inhibitor MMP are for the native protoporphyrin IX substrate. Unfortunately, it has not been possible to study the ferrochelatase:protoporphyrin IX by crystallography. Therefore, we have instead tried to predict the structure of this complex by a

combined quantum chemical and molecular mechanics geometry optimisation, starting from the structure of the ferrochelatase:MMP complex.

Neutral protoporphyrin IX (the dominant form at neutral and alkaline pH [45]) is protonated on two of the four pyrrole nitrogen atoms. Calculations have shown that it is energetically favourable to have the two protons on opposite nitrogens (a porphine with the two protons on neighbouring atoms has 30 kJ/mole higher energy at the B3LYP/6-31G* level), but there are two possible choices of protonated atoms, NA and NC, or NB and ND. Therefore, we tested both possible protonation states.

The structure of protoporphyrin IX bound to ferrochelatase is shown in Figure 7. The two calculations with different protonation status of the porphyrin ring gave very similar results; the rms. deviation of the porphine atoms in the two calculations is only 8 pm. In particular, the porphyrin ring of both structures are deformed by the protein. In the structure where NA and NC are protonated, the *A* ring is tilted 15° out of the plane of the porphyrin in the same direction as in the ferrochelatase:MMP complex. However, the other three rings are also tilted: The *C* ring is slightly tilted in the same direction, about 4°, whereas the B and D rings are tilted in the opposite direction, 2° and 9°, respectively. In the structure with the opposite protonation, the deformation is similar, but the tilt angles differ slightly; they are 13, 1, 9, and 10° for the *A*, *B*, *C*, and *D* rings, respectively.

Together, this alternating tilting of the pyrrole rings gives the porphyrin ring a saddled structure. Such a distortion has been suggested to facilitate the insertion of the metal into the ring [9]. It is also notable that the largest tilt is seen for the *A* pyrrole ring, which is tilted so that the nitrogen lone-pair orbitals are directed towards the side where His-183 is situated. As mentioned above, His-183 is the suggested binding site for the iron ion [6,16,17,19,20], so such a tilt would expose the nitrogen lone pairs towards the metal ion.

The two structures of protoporphyrin IX are much less distorted than that of MMP bound to ferrochelatase (cf. Figure 4) and the distortion is also smoother. This may at first be somewhat unexpected and disappointing, since it would make the insertion of the metal harder. Yet, it is fully expected; as will be shown quantitatively in the next section. MMP is tilted already in vacuum and is therefore distorted only 6° by the protein. However, protoporphyrin IX prefers a planar structure and is distorted 15° by the protein. The protein cannot distort it more, because it is also distorted by the porphyrin itself and an equilibrium is attained when the distortion force of the protein equals

that of the porphyrin. Moreover, spectroscopic studies have indicated that the porphyrin is only slightly distorted when bound to the chelatase [47].

The strain energy (ΔE_1) in the porphyrin induced by the protein is 20 kJ/mole for both protonation states. This is larger than the energy in Figure 5 for a tilt of 15° in neutral porphine (~12 kJ/mole), because the distortion of the porphyrin in the protein involves more changes besides the tilt angle of the *A* ring. Such a strain energy is smaller than what has been found for other proteins investigated with the same method, e.g. alcohol dehydrogenase, blue copper proteins, and iron-sulphur clusters, 30–110 kJ/mole, except for a few rubredoxin sites, which had a similar strain energy [41,42,48–52]. The reason for this lower strain energy is probably that the porphine ring is neutral and does not make any strong electrostatic interactions with the surrounding enzyme, in variance to the metal sites, studied in the other proteins.

Rationalisation of the results using a harmonic model

In the previous sections, we have used various theoretical methods to obtain information about the energy cost of tilting the porphyrin ring in different compounds. We have used these results qualitatively to propose a mechanism for product release and to discuss the binding of various porphyrins to ferrochelatase. In this section, we will put these suggestions on a firmer, quantitative, ground and rationalise some other results using a simple harmonic model for the strain in the protein and in the porphyrin. Such a model has been used before in a similar context [48,53].

For simplicity, we assume that the only significant distortion in the porphyrin is a tilt of the *A* pyrrole ring out of the plane of the other pyrrole rings. In Figure 5 we see that for all porphyrins except MMP, the energy cost of such a tilt can with a good approximation be described by a harmonic function

$$E_{por} = k_{por}\, x^2 \qquad (2)$$

where x is the tilt angle and k_{por} is a force constant that varies between 0.018 kJ/mole/degree^2 for porphine^{2-} to 0.11 kJ/mole/degree^2 for Co^{2+} porphine. For MMP, the function is slightly more complicated:

$$E_{MMP} = 0.087\,(x - 30)^2 \qquad (3)$$

because, the optimum value of the tilt angle is 30° rather than 0°.

Next, we assume that the strain energy of the protein follows a similar equation:

$$E_{Fc} = k_{Fc} (x - x_{Fc})^2 \quad (4)$$

However, for the protein, we have less information, because the result in Figure 6 refers to the complex between ferrochelatase and MMP, and not to the isolated enzyme, as the porphyrin results in Figure 5. Therefore, we cannot take x_{Fc} to be the tilt angle in this equilibrated structure or in the crystal structure. We will soon see how we can estimate the force constant and the ideal tilt angle of the protein by combining various calculations.

A bound porphyrin will distort if the ideal tilt angles of the protein and the porphyrin differ. If this is the case, a strain force will build up in the protein as well as in the porphyrin as they are both distorted, and an equilibrium will arise when these two forces are equal (but with opposite signs, i.e. when the sum of their forces vanishes). In this simple model, the relative size of the resulting distortion in the two molecules will be determined by the quotient of their respective force constants [53]. Therefore, we need to calculate the strain forces from the strain energies in Eqns. (2–4). The force is simply the negative of the first derivative of the strain energy, i.e. a linear function of the distortions:

$$F_{por} = -k_{por} x \quad (5)$$

$$F_{MMP} = -0.087 (x - 30) \quad (6)$$

$$F_{Fc} = -k_{Fc} (x - x_{Fc}) \quad (7)$$

Once we have estimated the constants for the protein, we can rationalise and interpret our results within this simple model.

From Figure 6, we saw that only 3 kJ/mole of the potential of mean force could be attributed to the protein, whereas the rest of the energy came from the porphyrin ring. This energy arose from a distortion of ~8° in both directions. Inserting these results in Eqn. (4), we can estimate the force constant for ferrochelatase: $k_{Fc} = 0.04$ kJ/mole/degree.

Next, we estimate x_{Fc} from the tilt angle observed in the MMP:ferrochelatase complex (36°), by solving the equation $F_{MMP} + F_{Fc} = 0$ for $x = 36$. This gives $\alpha_{Fc} = 49°$. Thus, we see that if the protein should be able to increase the tilt angle for MMP (as is observed in the crystal structure), it must prefer a tilt angle considerably larger than that found in the ferrochelatase:MMP complex. We also see that because the force constant of the protein is lower than that of the porphyrin, the protein is more distorted (13°) than the porphyrin (6°). The same applies to the strain energies; the strain

energy of the protein (Eqn. 4), 7 kJ/mole, is larger than that of MMP (Eqn. 3), 3 kJ/mole. The total strain energy is quite modest, 10 kJ/mole. This is in reasonable accordance with the strain energy of MMP estimated in the crystal structure with quantum chemical methods, 4–6 kJ/mole [54].

For protoporphyrin IX, things are different, because it has a much smaller ideal tilt angle (0°). Solving the equation $F_{por} + F_{Fc} = 0$. using $k_{por} = 0.056$ kJ/mole/degree^2 (according to Figure 5), we find that equilibrium is attained at a tilt angle of 20°. This tilt angle is slightly larger than that obtained in the ComQum calculations because in the latter calculations, there are more distortions besides the tilt of the *A* ring. In fact, all four pyrrole rings are slightly tilted, but the tilt is most pronounced for the *A* and *D* rings. This is probably because the strain energy must be kept low – the protein cannot induce very much strain into the bound group. As can be seen in Figure 5, the strain energy increases approximately quadratically with the tilt angle. Therefore, it is energetically favourable to induce several small distortions rather than one large. Similar general distortions of the whole porphyrin ring along the low-energy modes [55] are also seen for MMP in ferrochelatase [8].

Moreover, the tilt angle is appreciably less than for MMP in the protein (36°). This is perhaps a bit unexpected, but it is a direct consequence of the fact that the two porphyrins have widely different ideal tilt angles (0° and 30°) and that there must be distortions in the protein as well as in the porphyrin. Obviously, it is much harder to tilt a planar porphyrin than one that is already strongly tilted. Thus, the *distortion* of free-base porphine is almost three times larger than for MMP (20° compared to 6°), and the distortion of the protein is even larger, 29° (13° in the complex with MMP). Consequently, the strain energy is also larger, 23 kJ/mole in the porphyrin and 33 kJ/mole in the protein. Note that the first figure is similar to the estimate from the ComQum calculations.

A similar reasoning applies for the suggested release mechanism. For neutral porphine (and therefore probably also for the protoporphyrin IX substrate), the strain energy is 23 kJ/mole in the porphyrin and 33 kJ/mole in the protein (Eqns. 2 and 4), i.e. 56 kJ/mole in total. However, for iron porphine (as a model of the haem product), the force constant is 0.074 kJ/mole/degree^2, giving an equilibrium angle of 17° and a strain energy of 22 + 41 = 63 kJ/mole. This 7-kJ/mole difference in strain energy corresponds to a decrease in the binding constant by a factor of 40, which would facilitate the release of the product, as we suggest.

On the other hand, Cd porphine has a force constant of only 0.029 kJ/mole/degree^2, giving an equilibrium angle of 28° and a strain energy of 23 + 17 = 40 kJ/mole. This is 16 kJ/mole less than for the substrate, which indicates that Cd porphine would bind 600 times stronger to the protein than the substrate.

Similarly, porphine^{2-} (with a force constant of 0.018 kJ/mole/degree^2) would prefer an equilibrium tilt of 34° and therefore a strain energy of 21 + 9 = 30 kJ/mole. Consequently, the ferrochelatase:porphyrin complex would gain 26 kJ/mole in strain energy if the porphyrin is doubly deprotonated (or equivalently, it would bind the doubly deprotonated form of the porphyrin with a 30 000 times larger binding constant). This corresponds to a decrease in the pK_a of the porphyrin by 4.5 units.

In conclusion, we see sizeable effects of the porphyrin distortions for the thermodynamic properties of the protein. However, it should be noted that the main effect of porphyrin distortions are not necessarily seen for the ground state of the substrate:protein complex, but rather at the transition state for insertion of the metal ion into the porphyrin ring, for which the distortion of the porphyrin ring is probably larger. Here, we expect the largest effect of the protein's preferential binding of a distorted (tilted) porphyrin.

Concluding remarks

In this paper we have with several different theoretical methods studied the energetics of porphyrin deformations in free porphyrins, ferrochelatase, and in the complex of porphyrins and ferrochelatase. The results give quite a complete picture of such deformations in the reaction mechanism of ferrochelatase. From the ComQum calculations of the protoporphyrin IX:ferrochelatase complex, we see that the protein can induce appreciable distortions in the porphyrin molecule. In energy terms, the distortion is quite modest, about 20 kJ/mole. This is in accordance with our and other group's results on other proteins, indicating that a protein can only induce quite small strain energies onto a bound group, on the order of 10 kJ/mole [48,55,56], especially as ComQum typically overestimates the strain energy [55].

By quantum chemical calculations of the energetic cost of tilting various porphyrins, we have gained information about how easily distorted the porphyrins are. These results have then been combined by similar calculations of the cost of distorting the surrounding protein. Due to the size of

the protein, these calculations have to be done with classical force field methods, which are less accurate, but on the other hand let us estimate free energies. Moreover, since the distortion coordinate of the protein is not well-defined without the porphyrin, these calculations have to include a bound porphyrin. Yet, the calculations can be combined with those of the isolated porphyrins to obtain an approximate picture of the stiffness of the isolated protein against the relevant distortions.

Finally, we have combined the two methods together with a simple harmonic model of both the porphyrin and the protein to rationalise all results and show that they give several clues about the importance of porphyrin distortions for the reaction mechanism of ferrochelatase. We have seen that the distortions have at least two favourable functions in addition to expose the pyrrole nitrogen lone-pair orbitals against the metal ion. First, the distortion can be predicted to decrease the pK_a of the two protonated pyrrole rings in the neutral porphyrin. This is most important for the catalytic reaction, because these two protons have to be removed before the metal can bind to the porphyrin and the deprotonation of these groups and the transport of the protons out to the solution is one of the major problems of the protein. Second, the deformation automatically seems to provide a ingenious release mechanism for the product; metal porphyrins are in general harder to distort than the neutral free-base porphyrin substrate. Therefore, the strain energy will be higher for these products, and their affinity to the protein will therefore be lower. This suggestion also explains why some metal ions, e.g. Cd^{2+}, inhibit the enzyme.

Altogether, these results show how a combination of theoretical methods can be used to obtain geometric and energetic information about the reaction mechanism of ferrochelatase. Energetic information is invaluable in this context, because it can directly be converted to binding, acid, and rate constants. Together, our results show that the proposed distortion mechanism is energetically feasible and suggest what deformations are reasonable (viz. smaller than what can be expected from the structure of the inhibitor MMP).

Acknowledgements

This investigation has been supported by grants from the Swedish Research Council (VR). It has also been supported by computer resources of the Swedish Council for Planning and Coordination of Research (FRN), Parallelldatorcentrum (PDC) at the Royal Institute of Technology, Stockholm,

the High Performance Computing Centre North at the University of Umeå, and Lunarc at Lund University. We thank Mats Hansson, David Lecerof, and Salam Al-Karadaghi for fruitful discussions and John A. Shelnutt for help with normal-coordinate structural decomposition of the porphyrins.

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Table 1. Basis sets used in the calculations

Element	Basis set	Enhanced with functions (exponents)		
		p	*d*	*f*
Mn	DZP (62111111/33111/311) [57]	0.12765, 0.04028	0.1125	2.25
Fe	DZP (62111111/33111/311) [57]	0.134915, 0.041843	0.1244	1.339
Co	DZP (62111111/33111/311) [57]	0.141308, 0.043402	0.1357	1.62
Cu	DZP (62111111/33111/311) [57]	0.13	0.096	0.34
Zn	DZP (62111111/33111/311) [57]	0.162	0.132	0.39
Cd	ANO 18*s*13*p*6*d* [58], uncontracted	0.1173	–	0.2328
other	6-31G* [59]	–	–	–

Table 2. Simulation parameters used in the molecular dynamics simulation and the free energy perturbations. Words in brackets are the corresponding Amber parameter names.

Temperature (temp0)	300K
Time step (dt)	0.5 fs
Interval between the non-bonded pair list updates (nsnb)	25 fs
Cut-off distances for non-bonded interactions (cut2nd)	2.0 nm[a]
Time constant for temperature bath coupling (tautp)	0.2 ps
Dielectric constant (dielec; idiel=1)	1.0
Scale factor for 1, 4 electrostatic interactions (scee)	1.2
Number of equilibration steps in the perturbations (nstmeq)	100
Number of collection steps in the equilibration (nstmul)	100
Target energy change per window (amxmov)	0.1
Maximum energy change per window (amxrst)	0.5
Initial window size (almdl0)	0.001

[a] Non-bonded interactions between 1.0 (cut) and 2.0 nm were updated only once every 25 fs.

Figure legends

Figure 1. The structure of MMP, showing the atomic names used in this paper. Protoporphyrin IX has the same atoms, except CCA, but it has two vinyl side chains instead of the ethyl side chains in MMP.

Figure 2. The potential energy of the ferrochelatase:MMP complex in a molecular dynamics simulations as a function of time. The potential-of-mean-force calculations were started at the end of this simulation.

Figure 3. The structure of MMP with side chains overlaid with the structure without side chains (thicker lines). Both structures were optimised by the B3LYP method in vacuum.

Figure 4. A comparison of the optimised structure of MMP (thick lines) and the crystal structure of the MMP:ferrochelatase complex (thin lines).

Figure 5. The energy of various porphyrin models, with or without a central metal ion, as a function of the pyrrole A ring tilt angle.

Figure 6. The potential of mean force for the tilt angle of MMP in ferrochelatase and in vacuum. For each perturbation, two curves are shown (one perturbation forth and one back), in order to give an estimate of the uncertainty in the calculation.

Figure 7. The structure of protoporphyrin IX optimised in ferrochelatase with ComQum. The porphyrin is protonated on the NA (left) and NC atoms. Only the quantum atoms are shown. The protein was allowed to relax in the calculation.

Supplementary material

Table S1. Amber atom types and charges of the atoms in MMP.

Atom	Type	Charge	Atom	Type	Charge
CHA	CD	-0.1542	CHC	CD	-0.1931
HHA	HC	0.1157	HHC	HC	0.1522
C1A	CI	-0.0470	C1C	CI	-0.1421
NA	nm	-0.0089	NC	NA	0.0482
CCA	ct	-0.2514	HNC	H	0.0441
HCA1	hc	0.0988	C2C	CO	0.2654
HCA2	hc	0.0988	CMC	CT	-0.2583
HCA3	hc	0.0988	HMC1	HC	0.0668
C2A	CO	0.1928	HMC2	HC	0.0668
CMA	CT	-0.4083	HMC3	HC	0.0668
HMA1	HC	0.1079	C3C	CO	-0.2227
HMA2	HC	0.1079	CAC	CT	0.0075
HMA3	HC	0.1079	HAC1	HC	0.0159
C3A	CO	-0.2171	HAC2	HC	0.0159
CAA	CT	0.1660	CBC	CT	0.0239
HAA1	HC	-0.0030	HBC1	HC	-0.0381
HAA2	HC	-0.0030	HBC2	HC	-0.0381
CBA	CT	-0.3128	CGC	C	0.6555
HBA1	HC	0.0681	O1C	O2	-0.6981
HBA2	HC	0.0681	O2C	O2	-0.6981
HBA3	HC	0.0681	C4C	CI	0.1245
C4A	CI	0.0997	CHD	CD	-0.3419
CHB	CD	-0.2205	HHD	HC	0.2196
HHB	HC	0.1543	C1D	CI	0.2977
C1B	CI	-0.0549	ND	NB	-0.3025
NB	NB	-0.2466	C2D	CO	-0.2836
C2B	CO	0.2758	CAD	CT	0.0174
CMB	CT	-0.3998	HAD1	HC	0.0072
HMB1	HC	0.0939	HAD2	HC	0.0072
HMB2	HC	0.0939	CBD	CT	0.0633
HMB3	HC	0.0939	HBD1	HC	-0.0536
C3B	CO	-0.3485	HBD2	HC	-0.0536
CAB	CT	0.2064	CGD	C	0.6607
HAB1	HC	-0.0254	O1D	O2	-0.7033
HAB2	HC	-0.0254	O2D	O2	-0.7033
CBB	CT	-0.2282	C3D	CO	0.2247
HBB1	HC	0.0488	CMD	CT	-0.2546
HBB2	HC	0.0488	HMD1	HC	0.0608
HBB3	HC	0.0488	HMD2	HC	0.0608
C4B	CI	0.2158	HMD3	HC	0.0608
			C4D	CI	0.0273

Table S2. Non-standard Amber bond parameters for MMP.

Bond	force constant (kcal/mole/Å^2)	equilibrium bond (Å)
ct–nm	213.57	1.47
ct–hc	337.86	1.09
CT–HC	317.39	1.10
CD–CI	324.40	1.40
CD–HC	347.94	1.09
nm–CI	279.72	1.39
CI–CO	230.92	1.46
CO–CO	399.51	1.38
NB–CI	312.92	1.36
NA–CI	312.86	1.38
CO–CT	229.78	1.50

Table S3. Non-standard Amber angle parameters for MMP.

Angle	force constant (kcal/mole/rad^2)	equilibrium angle (degree)
nm–ct–hc	65.82	109.20
ct–nm–CI	141.10	121.29
CI–CD–CI	139.26	128.37
CI–CD–HC	63.28	115.68
CD–CI–nm	137.02	125.83
CD–CI–CO	142.96	125.43
CD–CI–NB	138.19	124.14
CD–CI–NA	158.70	126.16
CI–nm–CI	154.13	109.11
nm–CI–CO	124.67	107.57
CI–CO–CO	134.14	106.70
CI–NB–CI	154.15	105.93
NB–CI–CO	123.09	111.29
CI–NA–CI	158.19	110.04
CI–NA–H	70.93	124.94
NA–CI–CO	145.16	107.42
CI–CO–CT	124.94	125.35
CO–CO–CT	125.83	127.89
CO–CT–CT	109.58	114.25
CO–CT–HC	66.47	110.63
hc–ct–hc	47.19	109.75

Table S4. Non-standard Amber dihedral parameters for MMP. In all cases the parameter *idiv* = 1.

Dihedral	force constant (kcal/mole)	phase	period
CI–CD–CI–NA	4.82	180	2
CI–CD–CI–CO	6.23	180	2
CI–CD–CI–NB	5.80	180	2
HC–CD–CI–NA	3.07	180	2
HC–CD–CI–CO	4.12	180	2
CD–CI–NA–CI	5.47	180	2
CD–CI–NA–H	1.50	180	2
CD–CI–CO–CO	6.35	180	2
CD–CI–CO–CT	6.39	180	2
HC–CD–CI–NB	4.01	180	2
CD–CI–NB–CI	13.88	180	2
CI–CD–CI–nm	7.52	180	2
HC–CD–CI–nm	4.97	180	2
CD–CI–nm–CI	6.07	180	2
CD–CI–nm–ct	6.71	180	2
CI–NA–CI–CO	7.62	180	2
H –NA–CI–CO	1.77	180	2
NA–CI–CO–CO	7.24	180	2
NA–CI–CO–CT	7.43	180	2
CI–CO–CO–CI	9.12	180	2
CI–CO–CO–CT	8.09	180	2
CI–CO–CT–CT	2.59	180	3
CI–CO–CT–HC	1.61	180	3
CT–CO–CO–CT	7.33	180	2
CO–CO–CT–CT	2.45	0	3
CO–CO–CT–HC	1.39	0	3
CO–CT–CT–C	2.83	180	3
CO–CT–CT–HC	1.19	180	3
HC–CT–CT–C	1.42	180	3
HC–CT–CT–HC	0.75	180	3
CT–CT–C –O2	0.00	180	3
HC–CT–C –O2	0.00	180	3
CI–NB–CI–CO	17.27	180	2
NB–CI–CO–CO	7.55	180	2
NB–CI–CO–CT	7.69	180	2
CI–nm–CI–CO	9.73	180	2
ct–nm–CI–CO	11.06	180	2
CI–nm–ct–hc	1.90	0	3
nm–CI–CO–CO	10.17	180	2
nm–CI–CO–CT	9.02	180	2

Table S5. Non-standard Amber non-bonded parameters for MMP.

Atom	radius (Å)	well depth (kcal/mole)
ct	1.908	0.1094
nm	1.824	0.1700
hc	1.100	0.0157