Nonplanar porphyrins and their significance in proteins

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Nonplanar distortions of tetrapyrroles are prevalent in the hemes of hemoproteins, the pigments of photosynthetic proteins, and cofactor F_{430} of methylreductase. The nonplanarity of these porphyrin cofactors is currently believed to influence factors in the biological activity of the proteins, in part, because the porphyrin deformations are often conserved within functional classes of proteins. The occurrence, classification, and study of nonplanar porphyrins in proteins and synthetic nonplanar porphyrin analogs are reviewed.

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1 Introduction

Macrocyclic tetrapyrroles, including porphyrins, are found as cofactors in a bewildering array of proteins. Tetrapyrrole derivatives occur biologically in many enzymes as heme (iron porphyrin), in photosynthetic proteins as chlorophyll and pheophytin, and in other proteins as corrin (vitamin B_{12}) and

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corphin (methylreductase). Macrocyclic tetrapyrroles in biological systems are either metal free (pheophytins biosynthetic intermediates, catabolites) or contain iron (hemes), magnesium (chlorophylls), cobalt (vitamin B_{12}), nickel (cofactor F_{430}), and copper (pigments). Some of these porphyrin derivatives are illustrated in Fig. 1. Their biological functions range from O_2 transport (hemoglobins) and storage (myoglobins), collection and transport of light energy (antennae complexes), conversion of solar energy to chemical energy (photosynthetic reaction centers), electron transfer (cytochromes), oxygen reduction (oxidases), and a large number of other enzymatic reactions (peroxidases, catalases, cytochromes P450, methylreductases, methyltransferases, *etc.*)

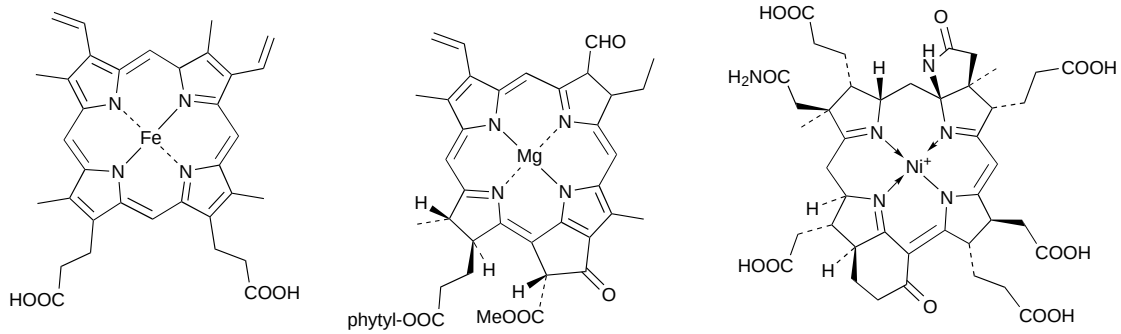
Beginning over 70 years ago, the heme proteins have been investigated intensely with the aim of determining the structural mechanisms controlling their varied biological functions. The major questions remaining concern the role of the protein in modulating the properties of the iron-porphyrin cofactor to yield its specific biological function. The immediate surroundings of the heme active site certainly have a dominant influence on function. In particular, axial coordination to the central iron atom, covalent attachment of the heme to the protein, and the nature of amino acid side chains in the immediate vicinity of the active site are undoubtedly important. More subtle influences on the structure of the active site are also sometimes observed to modify the activity of the protein. For example, the number and type of axial ligands of the iron atom, the axial ligand binding geometry, and the nature of the hydrogen bonds between the axial ligands and the protein appear to be of importance in governing the redox properties.[1] For hemoglobins, the O_2 affinity of each of the four hemes depends on whether the other hemes have O_2 bound as an Fe axial ligand. The differences in heme O_2 affinity for hemoglobin have been ascribed to subtle structural changes in axial coordination of the iron atom that are transmitted from one heme to the others through the protein's tertiary and quaternary structure.[2] In a similar manner, protein-induced conformational differences in the porphyrin itself might influence enzymatic reactivity. Early X-ray crystal structures of heme proteins led to the false impression that the porphyrin macrocycle in the proteins was planar or nearly planar, partly because the heme was constrained to a planar conformation during the refinement procedure. Later crystal structures of heme proteins and photosynthetic proteins almost invariably show nonplanar heme conformations. For the photosynthetic proteins, the nonplanar distortions observed in the crystal structures have been suggested to influence the photophysical and redox properties of chlorophyll pigments, with consequent effects on electron-transfer rates in photosynthetic reaction centers and antennae complexes.[3] Currently, attempts are underway to determine whether these nonplanar porphyrin distortions in proteins have functional significance, and if so, to determine the mechanism by which nonplanarity influences activity.

Some typical nonplanar structures of porphyrin macrocycles are shown in Fig. 2. Only the atoms of the porphyrin macrocycle and the central metal are shown, *i.e.* substituents that are present at the twelve atoms around the perimeter of the macrocycle have been omitted. The distortions illustrated are simple symmetric deformations; more complicated asymmetric distortions that combine these simple distortions are often observed, especially in the proteins.

Arriving at a detailed structural understanding of the function of tetrapyrrole-containing proteins requires a thorough knowledge of the various influences of structure on function. Toward this end, researchers often look for structural features that are conserved across functionally related proteins from many species, for example conserved residues in the amino acid sequence. These conserved structural features are most likely to influence enzymatic function and are where one normally looks for relationships between structure and function. Yet, the structure of the macrocycle has often been ignored in proposed structural mechanisms. This in spite of the fact that crystallographers have recently noted some highly nonplanar conformations in X-ray structures of proteins[4] and these distortions are often conserved (*vide infra*).

The hemoproteins provide a representative example of the occurrence of nonplanar porphyrins in proteins. It has been recognized for about 10 years that the hemes in many hemoproteins are highly distorted from planarity and that these nonplanar distortions might play a role in their biological function.[3] Further, by using a new normal-coordinate structural decomposition (NSD) procedure[5–7] for characterizing and quantifying heme distortions, our group has recently found that these distortions are often of different types for hemoproteins with different functions. Moreover, the types of distortion observed are conserved for proteins belonging to the same functional class.[4,7] Since nonplanar distortion is energetically unfavorable for hemes,[8] conservation of the heme conformation strongly suggests that the biological function of hemoproteins might be modulated by protein control over the conformation of the heme prosthetic group.

The possible importance of the nonplanar distortions of the heme is also emphasized by recent studies of model nonplanar porphyrins showing, first, that hemes are expected to be nearly planar in the absence of interactions with the protein moiety[8] and, second, that the nonplanar structure of the heme influences relevant chemical and photophysical properties (*e.g.* axial ligand affinity, redox potentials, transition dipoles and energies).[3,6,9–12] Moreover, the advent of many model nonplanar porphyrins has contributed to an improved understanding of the origin of nonplanar distortions of porphyrins. A great variety of sterically constrained nonplanar porphyrins has been synthe-



Protoheme Chlorophyll-b Cofactor F_{430}

Fig. 1 Structures of protoheme (iron protoporphyrin IX), chlorophyll *b*, and cofactor F_{430}

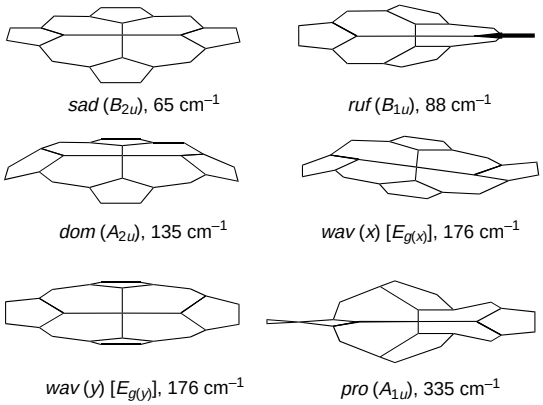


Fig. 2 Symmetric normal-coordinate deformations used to decompose the structures of hemes. (1 Å displacements are shown).

sized and their photophysical and chemical properties have been determined. These model studies indicate how the functional role of the porphyrin might be altered when the protein induces a particular distortion of the macrocycle.

Currently, a few spectroscopic techniques for distinguishing the magnitude of nonplanar distortion have been found, although, distinguishing the different types of distortion (*e.g.* doming, ruffling, saddling, illustrated in Fig. 2) proves to be more challenging. If the nonplanar conformers are not interconverting too rapidly, NMR spectroscopy can sometimes distinguish the different distortion types. Improved spectroscopic probes of porphyrin structure are needed to characterize fully the different distortions for a wide range of environments and time scales.

Molecular modeling is a recent development in the investigation of nonplanar porphyrins. Molecular mechanics calculations in particular provide insight into the possible mechanisms by which the surroundings of the porphyrin may induce various nonplanar distortions, especially when coupled with spectroscopic data and NSD analysis. Molecular mechanics force fields have been developed for porphyrins and exhaustively validated experimentally for the prediction of porphyrin conformations.[6,8,12–14] The molecular modeling studies provide information about the energetics of nonplanar distortions as well as additional structural information. The computational capabilities are essential because present spectroscopic and crystallographic methods alone are inadequate for fully distinguishing and measuring the types of distortions shown in Fig. 2 under all conditions. Molecular mechanics calculations are helpful for interpreting experimental results, but more importantly they can predict the presence of conformers that may be energetically accessible, though not populated (and thus not observed spectroscopically or in X-ray structures). These stable high-energy conformational states of the heme may also have functional significance.

The NSD method is described here briefly first, since it provides the framework appropriate for reviewing the nonplanar distortions of porphyrins that occur in the X-ray crystal structures of proteins and model porphyrins. Next, the novel properties associated with porphyrin nonplanarity are described, along with the spectroscopic methods for investigating these nonplanar structures. Subsequently, the question of how the environment of the porphyrin influences its conformation is addressed. Finally, investigations of the relationship between chemical and biological function and heme structure are reviewed.

1.1 Normal-coordinate structural decomposition of the out-of-plane distortions of porphyrins

The NSD method is simple in concept.[5,6] It relies on the fact that the distortions of the 24 macrocycle atoms from ideal square-planar geometry can be given in terms of the $3N - 6 = 66$ (N is the number of atoms) normal coordinates of the macrocycle, instead of simply giving the x, y and z displacements of each atom in the porphyrin skeleton. The normal coordinates are special linear combinations of the x, y and z displacements of each of the atoms from their equilibrium positions. Only in the normal coordinate system is the molecular vibrational energy expressed simply as a sum of energies for each of the $3N - 6$ coordinates. In addition, vibrations along the normal coordinates are what one observes in vibrational spectroscopy, *i.e.* Raman and infrared spectroscopy. The advantage of a description in terms of the normal coordinates is that the distortional energy of the macrocycle takes its simplest form in this representation. Further, because the restoring forces are smallest for displacements along these coordinates, the distortion of the porphyrin takes place primarily along only the lowest-frequency normal coordinates. In other words, the largest deformations are usually observed for the normal coordinates of lowest-frequency because they are the softest modes of distortion.

A mathematical procedure has been described[5,7] which projects out the displacements from an ideal geometry [chosen to be a planar copper(II) porphyrin macrocycle] along the normal coordinates.[5] The coordinate eigenvectors of each symmetry type are obtained from a normal coordinate calculation. Fig. 2 illustrates a 1 Å distortion along the lowest-frequency vibrational mode of each out-of-plane symmetry type. One easily identifies each of these normal deformations, *ruf*fling (B_{1u}), *sad*dling (B_{2u}), *dom*ing (A_{2u}), *wav*ing (E_g), and pyrrole *pro*pellering (A_{1u}), with various nonplanar macrocyclic conformations that commonly occur in X-ray crystal structures of symmetrically substituted porphyrins. A 1 Å distortion means that the square root of the sum of the squares of the z-displacements from the mean plane is equal to one.

For a particular porphyrin structure, the NSD computational procedure ascertains the contribution of each normal mode to the structure. Combining only the individual displacements along the lowest-frequency normal coordinates (*ruf, sad, dom, wav* and *pro*)[5,15] gives a simulated structure that typically closely matches the observed structure. In general, an exact representation of the observed conformation usually requires that the deformations along all normal coordinates be added into the simulated structure. However, in practice, an essentially exact representation of the structure requires far less than the total number of out-of-plane normal coordinates ($N - 3 = 21$). When applied to protein crystal structures (Fig. 3), the poorer resolution (compared to porphyrin crystals) usually precludes the determination of displacements for more than the lowest-frequency normal coordinates. That is, the high-frequency normal coordinate displacements are smaller than the statistical positional uncertainties in the X-ray refinements. The dotted lines in Fig. 3 show the simulated structures based on just the displacements of the lowest-frequency normal coordinates for some hemes in protein X-ray structures.

All of the hemes of the approximately 350 X-ray crystal structures of hemoproteins contained in the Protein Data Bank have been analyzed by NSD. The heme conformations have been examined with regard to structural motifs that are maintained within functional classes of proteins. In most of the proteins, the nonplanar heme structures have been found to be characteristic of the specific protein type.[4,5,9] Detailed examination of the NSD results for the X-ray structures of hemoproteins has delineated a variety of structural effects of natural amino acid sequence variation, mutation, axial ligation, and other protein differences on the conformation of the heme, thus tying the primary, secondary, and tertiary structure of the protein moiety to the conformation of the heme. In the case of the mitochondrial cytochromes *c*, a structural mechanism by which the protein produces the strong ruffling of the heme skeleton has been suggested by the NSD results.[4,5,7] NSD characterization of the hemes of other proteins will lead to other detailed mechanisms by which protein structure modulates heme conformation and function.

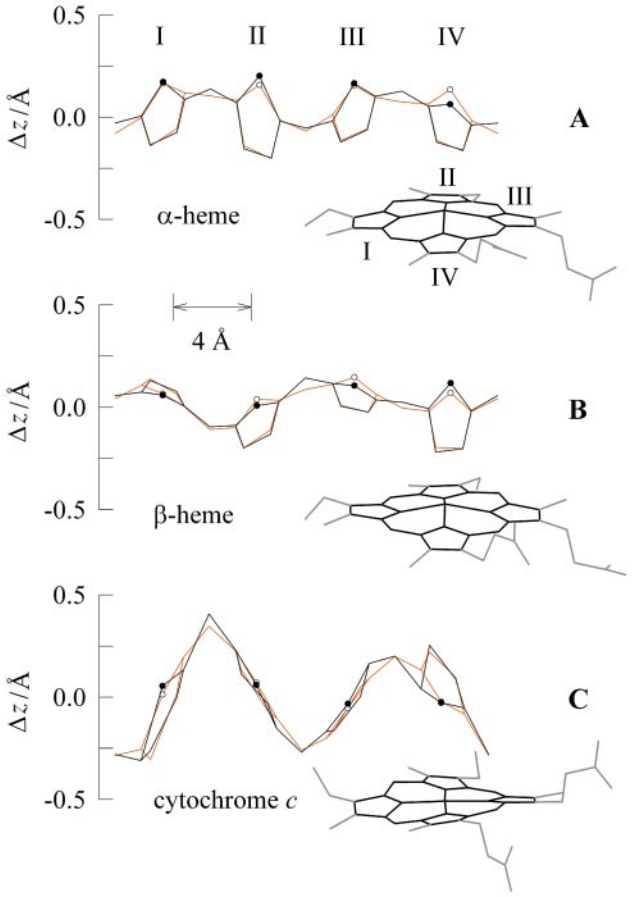


Fig. 3 Linear or 'clothesline' displays of the hemes in the α- and β-subunits of deoxyhemoglobin A and in isoenzyme-1 of cytochrome *c* isolated from baker's yeast. The dotted line represents the simulated structure obtained from a linear combination of displacements along only the lowest-frequency normal coordinates of each of the out-of-plane symmetries (see text).

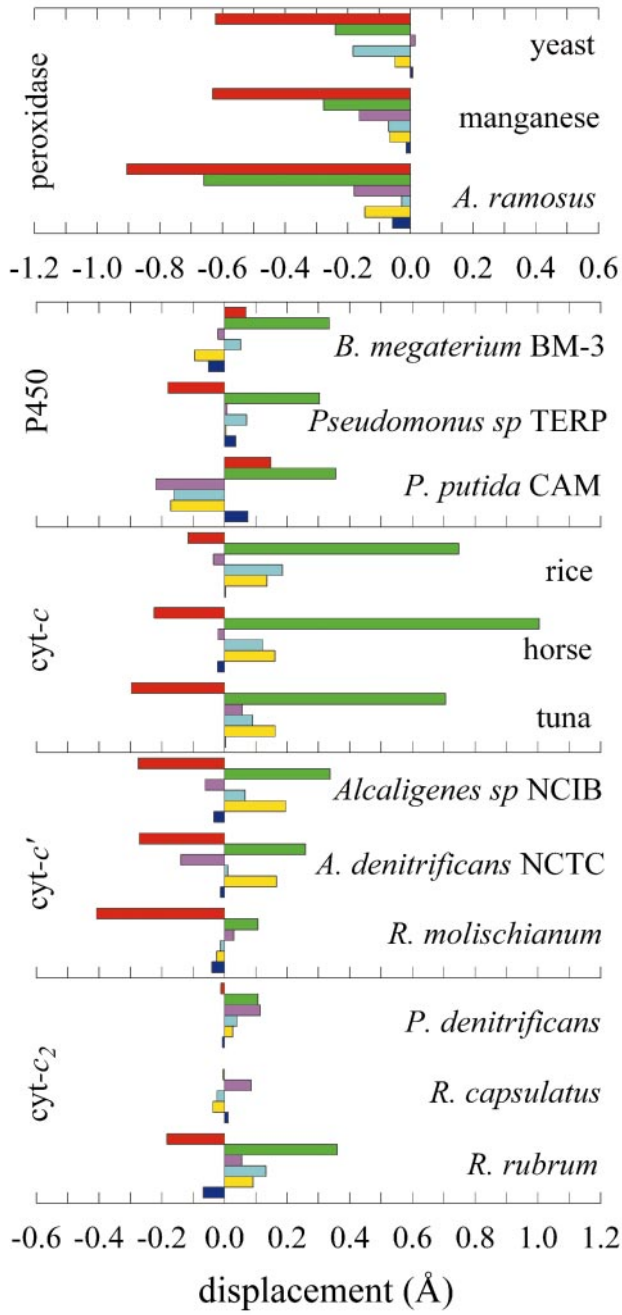


Fig. 4 Normal-coordinate Structural Decomposition results for several types of hemoproteins (peroxidase, cytochrome P450, cytochrome *c*, cytochrome *c*′ and cytochrome c_2). Hemes from three different species are shown. The color scheme for the displacements is as shown in Fig. 7 [red-*sad*, green-*ruf*, pink-*dom*, cyan-*wav*(*x*), yellow-*wav*(*y*), blue-*pro*]. For full details see ref. 7.

1.2 Occurrence and characterization of nonplanar heme conformations in proteins

Nonplanar porphyrin macrocycles are observed in many heme proteins, with the largest heme distortions observed so far in the *c*-type cytochromes and the peroxidases. Fig. 3 shows the conformations of typical hemes in X-ray crystal structures of human hemoglobin and yeast mitochondrial cytochrome *c*.[7] In Fig. 3, the *z*-displacements of the atoms relative to the mean plane of the macrocycle are shown in linear or 'clothesline' displays, with the *z*-displacements expanded to show clearly the deviations from planarity. These clothesline displays also clearly illustrate the variety and complexity of the heme distortions occurring in proteins.

The NSD method allows the nonplanar distortions to be quantified in terms of a few displacements along the most deformable normal coordinates of the square-planar (D_{4h}) symmetric porphyrin macrocycle.[5,7] Thus, the NSD procedure greatly simplifies the discription of these geometrically complex structures. A simple bar graph of the displacements (Fig. 4) clearly shows the similarities and differences in the structures of hemes from different proteins. The lengths of the bars represent the normal-coordinate displacements that best represent the X-ray structure. The great variety in the types and magnitudes of the deformations in the hemoproteins is clear from Fig. 4. (Also, see Fig. 9 of reference 5). For example, the heme of myoglobin (not shown) is predominantly domed with a significant *wav*(*y*) component.[5] In contrast, cytochrome *c* peroxidase (Fig. 4) is mainly saddled and much more distorted.

What is surprising is that in many cases these distortions are conserved for proteins of the same type but from different species. Fig. 4 shows the NSD results for the hemes of three species, for several different types of hemoproteins. Considering the approximately 0.1 Å uncertainties in the atomic positions inherent in the X-ray crystal data and thus in the displacements determined using NSD, the heme conformations for each type of protein are remarkably similar. Individual displacements along the normal deformations as large as 1.0 Å are observed. Although only three proteins are shown in Fig. 4, the conformation is generally conserved for all known crystal structures of a particular protein. For example, the hemes of all of the more than 25 peroxidases and their mutants are predominately saddled as Fig. 4 indicates. (Prostaglandin synthase, which is also a peroxidase, is an exception.)

It is clear from the NSD results that the heme conformation is conserved in many instances, and thus may be expected to play

a role in the function of these enzymes. An alternative view is that the structure of the heme may have no functional significance but simply reflects the protein's tertiary structure, which is known to be remarkably similar for proteins within a class. From this point of view, the NSD analysis of the hemes in proteins provides a useful probe of the protein's tertiary structure at the active site. That is, the heme serves as a reporter group for the structure of the protein. In either case, NSD analysis is necessary in order to further develop spectroscopic methods for precisely determining the conformations of porphyrins in hemoproteins.

1.3 Occurrence and characterization of the nonplanar structures in synthetic porphyrins

A large number of studies of model nonplanar porphyrins has given new insight into the conformational flexibility of porphyrins and the energetics of the interactions necessary to produce nonplanar distortions.[6,8–13,16] Some of these nonplanar porphyrins simulate the structures of hemes in specific proteins. Over the last decade, a variety of porphyrins with nonplanar structures has been synthesized. At least four methods have been used to induce nonplanarity in porphyrins. One method of inducing nonplanarity is by substituting sterically bulky groups at some or all of the peripheral positions of the macrocycle. Even for tetra-substituted porphyrins, large distortions from planarity are observed when the substituents are sufficiently bulky. Fig. 5 (top) shows the ruffling and doming that occurs for

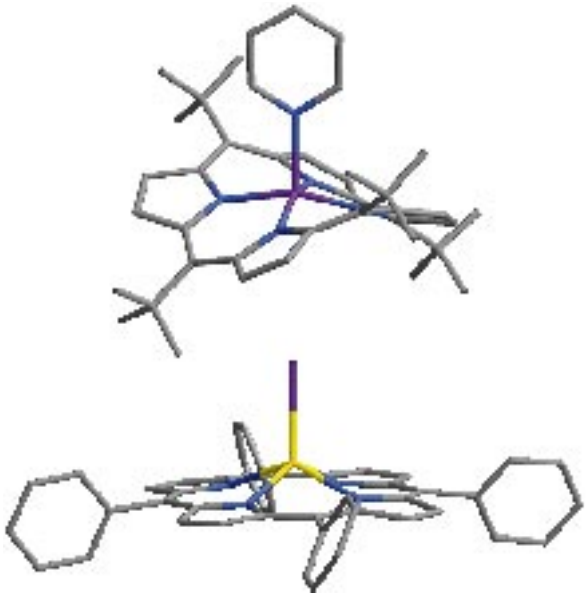


Fig. 5 Crystal structures of the mono-pyridine complex of zinc(II) tetra-*tert*-butylporphyrin and thallium(III) tetraphenylporphyrin iodide. Structure taken from the Cambridge Structural Database.

the Zn(py) complex of *meso*-tetra(*tert*-butyl)porphyrin.[16] Octa-substituted porphyrins with bulky groups at the β-pyrrole positions are also nonplanar. Nonplanarity relieves the steric strain by increasing the volume available for the substituent groups to occupy. More highly substituted porphyrins generally exhibit large distortions even for relatively small substituents. For example, dodeca-substituted porphyrins are highly sterically crowded at the periphery of the macrocycle, and, consequently, they show large deviations from planarity. Good examples are dodecaphenylporphyrin,[11,17] octaethyltetraphenylporphyrin,[12] and octabromotetraphenylporphyrin[18] shown in Fig. 6. The structures of these porphyrins are generally ruffled or saddled or a combination of these deformations.

A second method that has been used to create nonplanar porphyrins is to incorporate very small [*e.g.* Ni(II)] or very large [*e.g.* Ag(II)] metal ions into the porphyrin core. When the optimum metal–nitrogen(pyrrole) distance for a metal is at

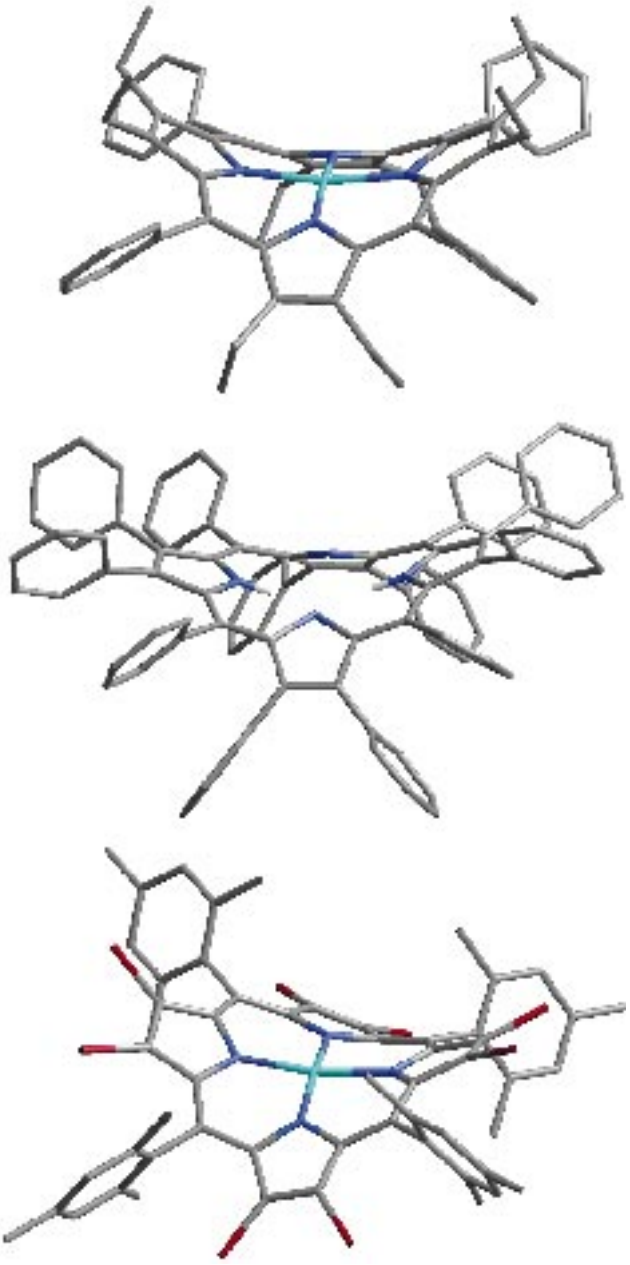


Fig. 6 X-ray crystal structures of nickel(II) octaethyltetraphenylporphyrin (OETPP), free base dodecaphenylporphyrin (DPP) and nickel(II) octabromotetramesitylporphyrin taken from the Cambridge Structural Database

variance with optimum core size of the planar porphyrin macrocycle (~2.01 Å), then the macrocycle distorts to accommodate the metal ion. The deformations can be of the in-plane [Sn(IV)] or the out-of-plane [Ni(II), Ag(II)] variety. While ruffling and saddling often are necessary to accommodate small metals, doming is often observed for large metals. The doming for large metals is illustrated by the X-ray structure of Tl(III)TPP iodide in Fig. 5.[16] Doming is usually small, as seen in Fig. 5, because of the large energy required for deformation along the *dom* normal coordinate.

A third strategy for inducing nonplanar distortion is to incorporate 'straps' or 'basket-handles' between peripheral substitution sites that are too short for a planar porphyrin. Chandrashekar, Ravikanth, and coworkers have most recently investigated and reviewed the properties of these nonplanar porphyrin models.[19]

Axial ligand–metalloporphyrin interactions are also known to induce nonplanar distortions of the porphyrin. This effect

may also play a role in the doming of the thallium porphyrin shown in Fig. 5. Safo *et al.* have used X-ray crystallography and other techniques to investigate the electronic and steric forces influencing axial ligand orientation in porphyrins.[20] These porphyrin systems serve as models for the observed eclipsed (parallel) and staggered (perpendicular) orientations of the axial histidine ligands of hemes in proteins. In these systems, the steric crowding at the periphery is small, thus, the conformation of the macrocycle is directly influenced by the weak steric interactions of the axial ligands with the macrocycle. The results obtained strongly support the suggestion that axial ligand orientation may alter the spectroscopic and redox properties of heme proteins.

Our group has investigated axial ligand orientation effects in highly substituted porphyrins for which the deformation of the macrocycle is determined by the substituents rather than by the axial ligands.[21] For the bis-ligand complex of Co(III) octaethyltetraphenylporphyrin, which has the *sad* macrocyclic conformation, the planes of the ligands are perpendicular to each other and aligned with the metal–nitrogen bonds, *i.e.* along the saddle. In contrast, for the bis-ligand complexes of Co(III) tetra(*tert*-butyl)porphyrin which has the *ruf* conformation, the planes of the ligands are perpendicular and aligned with the direction of opposite *meso* carbons and the groove formed by the *ruf* macrocycle.[21] The observed orientations of the ligands are thus those that minimize steric interactions with the macrocycle. In some cases, steric interactions between the ligand and porphyrin were sufficient to result in hindered rotation of the axial ligands.

2.1 Novel spectroscopic properties and consequences of nonplanar distortion of porphyrins

2.1.1. Photophysical properties: UV–visible absorption spectra

The most commonly observed spectroscopic consequence of porphyrin nonplanarity is a red shift in the π–π* absorption bands in the UV–visible spectrum. Shifts in the Soret or B band, typically near 400 nm, of as much as 50 nm have been observed as a result of nonplanar distortion. For example, the Soret band of NiTPP, a mixture of planar and nonplanar conformers, is at 424 nm, while highly ruffled Ni *meso*-tetraadamantylporphyrin has its Soret band at 478 nm, a 54 nm red shift. The size of the red shift is proportional to the magnitude of the distortion, albeit in a nonlinear fashion.[6] For a series of ruffled Ni tetraalkylporphyrins with increasingly bulky alkyl substituents, ruffling angles (angle between the planes of adjacent pyrroles) of up to 20° produce only small shifts; however, small increases in the ruffling angle beyond 20° begin to give large red shifts. A similar nonlinear relationship holds when the normal coordinate displacements are used to quantify the deformation. This must be the case since there is a linear relationship between ruffling angle and the size of the *ruf* deformation.[6] In addition, other types of distortions besides ruffling have a similar but not quantitatively equivalent effect on the absorption spectrum.[13,22]

The Q band, located in the red region of the absorption spectrum, red shifts to an even larger extent than the B (Soret) band. For example, NiTPP has Q_0 at 561 nm and Ni adamantylporphyrin has Q_0 at 648 nm. The red shifts are a result of a decrease in the energy separating the filled $a_{1u}(\pi)$ and $a_{2u}(\pi)$ orbitals and the empty $e_g(\pi)$ orbitals of the macrocycle.[6,13] In addition to the spectral shifts, a broadening of the absorption bands is usually observed as the porphyrin becomes more distorted.

Iron porphyrin complexes have not been investigated sufficiently. Many questions remain as to whether the spectroscopic markers of nonplanar conformation, primarily deduced for nickel porphyrin complexes, can be reliably used for iron porphyrins. For example, the oxidation and spin state of the iron atom likely influence the dependence of these absorption bands on the magnitude of distortion. Systematic studies of a series of progressively more distorted iron porphyrins are needed.

2.1.2 Non-linear optical properties

Nonlinear optical properties such as optical limiting and harmonic generation are closely connected with molecular optical spectral properties. Various molecular factors such as π-delocalization length, donor-acceptor groups, conformation, and orientation influence the nonlinear optical properties of porphyrins. Porphyrins, including some nonplanar Cu basket-handle porphyrins,[23] have been investigated as nonlinear optical materials. Tailoring the nonplanarity of porphyrins offers a method for conformational control over optical properties such as lifetimes and intersystem crossing rates which influence nonlinear optical properties. In addition, photo-conversion between stable nonplanar conformers provides another opportunity for controlling nonlinear optical properties.

2.1.3 Luminescence spectra

Ravikanth, Chandrashekar and coworkers[19] and Holten *et al.*[24] have investigated the fluorescence from nonplanar basket-handle porphyrins and sterically crowded porphyrins, respectively. They demonstrated that the quantum yields for fluorescence are reduced as a consequence of the nonplanarity. The reduced yield is a result of the decreased lifetime of the excited singlet states, which in turn is a result of increased intersystem crossing to the triplet manifold and increased non-radiative decay rates.

2.1.4 Vibrational spectra: resonance Raman and IR spectroscopy

Resonance Raman spectroscopy has proved to be one of the best probes of the conformation of the porphyrin. In fact, it was the first technique to detect,[25] and the only method currently known that can quantify, the conformational equilibrium between planar and nonplanar conformers of some metal porphyrins.[8,9,10,13,25] We now outline the major conclusions of recent resonance Raman studies of model nonplanar porphyrins.

Our group's contribution[6,8,10–13,15,25] to the development of resonance Raman spectroscopy as a means of quantifying the type and magnitude of distortion began in 1988, when we investigated a new crystalline form of NiOEP. At that time, two other crystalline forms were known—a ruffled form and a planar form. The new crystal morphology also exhibited a planar porphyrin ring. Having three crystals with known porphyrin conformations, we used single-crystal resonance Raman spectroscopy to verify that certain structure-sensitive Raman lines could be used as indicators of the nonplanar conformation of the porphyrin.[26]

A year later we found that NiOEP in solution is a roughly equal mixture of planar and nonplanar conformers,[25] identifying these solution forms with the planar and nonplanar crystalline forms investigated earlier. This finding was subsequently confirmed in a study by Czernuszewicz and coworkers.[13] We later showed this to be a general property of β-pyrrole-substituted Ni porphyrins, including biological porphyrins like protoporphyrin.[9] We also showed that the environment of Ni porphyrin influences the equilibrium between these conformers. Specifically, the formation of π–π dimers and insertion into the active site of hemoglobin and its α-subunits shift the equilibrium in favor of one conformer or the other. In particular, binding NiProtoP to the hemoglobin active site forces the macrocycle to be nearly planar. This occurs for the hemoglobin binding sites that do not have the proximal histidine coordinated to the Ni atom. This was a particularly interesting finding in that the protein was shown to exercise direct control over the macrocycle structure through only nonbonding interactions.

Subsequent to these findings, we published a series of Raman studies[6,11] of nonplanar synthetic porphyrins and the Ni-corphinoid cofactor F_{430} of methylreductase.[10] F_{430} has a highly

reduced porphyrin ligand. In the enzyme, F_{430} catalyzes methyl-coenzyme M reduction. The reduced porphyrin ring of F_{430} is thought to facilitate changes in the size and oxidation state of the Ni atom by increasing the out-of-plane flexibility of the macrocycle.[27]

The principal result of our studies with model nonplanar porphyrins has been the elucidation of the role of the substituents and their orientations in determining the macrocycle conformation. In addition, these Raman studies have also clarified the existence of stable nonplanar conformational isomers at higher energy than the ground-state conformer. With regard to the latter, these stable conformers (local minima) are sometimes thermally occupied at room temperature.[7,21] The studies of the model compounds have also provided some useful correlations between the frequencies of structure-sensitive Raman lines and the degree of nonplanarity of the macrocycle.[6,11,12]

Some of the different types of deformations in Fig. 2 were examined in more recent Raman investigations.[6,13] For example, a series of nickel *meso*-tetraalkyl-substituted porphyrins with alkyl groups of varying steric size exhibit nearly pure *ruf* deformations.[6] The magnitude of the distortion increases with the bulkiness of the substituent, so that the variations of the Raman-line frequencies and absorption-band positions were determined and correlated with molecular mechanics structural parameters and transition energies calculated using INDO/s semiempirical methods. A study of 5,15-dialkyl substituted porphyrins investigated the consequences of the addition of a *dom* deformation component to a *ruf* deformation.[13] Differing dependencies of the Raman frequencies on the magnitude of distortion are found when the *dom* and *ruf* deformations are both present compared with when only the *ruf* deformation occurs. Both calculated and X-ray structures were available for direct comparison in this study.

Because almost all substituted Ni porphyrins show ruffled conformers, we became interested in whether Ni porphyrin (NiP), with the reduced steric requirements of its hydrogen substituents, would also be nonplanar.[16] In other words is at least some steric interaction of the peripheral substituents necessary to give nonplanar conformers? An X-ray crystal structure of NiP was obtained and showed a nearly planar macrocycle. The Raman spectra taken both in solution and in the crystal show that NiP also exists in solution as only the planar species.[15]

Other resonance Raman investigations of nonplanar porphyrins looked at the influence of the size of the central metal on nonplanar structure by using resonance Raman spectroscopy.[6,8,12] These studies also served to develop suitable force-field parameters for the molecular mechanics calculations for additional metals, including Co(II), Cu(II), Zn(II) and Fe(III). The results show that large metals reduce the magnitude of the distortion for sterically crowded porphyrins. These studies also explained why small metals reduce the slope of the well-known core-size correlations for the structure-sensitive Raman lines. For porphyrins that coexist in both planar and nonplanar conformers, it was also found that increasing the metal size shifts the equilibrium in favor of the planar form.[8] The latter work also provides an estimate of the steric repulsion energy necessary to induce nonplanar distortion. It demonstrated that biological Fe porphyrins like FeProtoP should exhibit only the planar conformer in the absence of a perturbing protein environment.[8]

2.1.5 NMR spectroscopy

As the prevalence of nonplanar porphyrin conformers and their possible importance in biological systems has become evident, studies of nonplanar porphyrins have become more diverse. They now include a wide range of nonplanar porphyrin models and other spectroscopic probes such as NMR. Proton NMR studies of model nonplanar porphyrins have revealed the presence of numerous dynamic processes, including inversion of the porphyrin macrocycle, hindered rotation of aryl or alkyl substituents at the *meso* or β-pyrrole positions, and hindered rotation of axial ligands.[21] Some of these processes are unique to nonplanar porphyrins. Proton NMR studies have also been used to determine the solution structures of cobalt(II) complexes of nonplanar porphyrins and, most recently, to measure the effect of nonplanarity on the porphyrin ring current. Using a double-dipole model of ring current effects, it was shown that nonplanarity caused little if any decrease in the ring current even for extremely nonplanar *sad* or *ruf* porphyrins compared with planar porphyrins.

2.1.6 Electron paramagnetic resonance

EPR spectroscopy of copper octaethyltetraphenylporphyrin has added support to the proposal by Reed, Scheidt, and coworkers that nonplanarity controls the magnetic coupling between paramagnetic metals and the macrocycle radical cation, giving antiferromagnetic coupling with the cation for highly nonplanar porphyrins.[28] This was also verified by Ravikanth, Chandrashekar and coworkers using other techniques.[19] Time-resolved EPR measurements of the photoexcited triplet states of free base and zinc derivatives of OETPP reveal fast exchange between different conformers, suggesting fluxional excursions from the saddled ground-state conformer observed in X-ray crystal structures.[29]

2.2 Novel functional properties of nonplanar synthetic porphyrins

2.2.1 Redox potentials

The most studied influence of nonplanarity on porphyrin chemistry is its effect on redox potentials. Fajer *et al.*,[3] Ravikanth and Chandrashekar,[19] and Reddy have shown that nonplanar porphyrins are easier to oxidize and harder to reduce than planar porphyrins. This was initially shown for ZnOETPP,[3] but the same trend has now been verified for many other nonplanar porphyrins. In a particularly striking example of this effect, it was shown for a series of increasingly brominated tetraphenylporphyrins (Fe and H_2, Br_xTPP, x = 0 to 8), that the porphyrin initially becomes harder to oxidize due to the electron withdrawing ability of the added bromine substituents, but subsequently becomes easier to oxidize as the added bromines make the porphyrin more nonplanar.[30] Electron withdrawing groups appear to effect oxidations and reductions equally. Kadish and others have also shown that nonplanarity affects the site (metal or ring) of oxidation[30] and whether 2-electron *versus* 1-electron oxidation is observed.

2.2.2 Axial ligand affinity

Our group has shown that the affinity of nickel porphyrins decreases as the ruffling of the macrocycle increases if the electron-withdrawing capabilities of the substituents are held constant. Based on resonance Raman spectra, Desbois *et al.* have associated the increased macrocycle distortion in a series of strapped hemes with a decrease in O_2 ligand off-rates. The decrease does not occur for CO off-rates in the models. Thus, heme distortion provides a mechanism for differentiating CO and O_2 binding in hemoglobins and myoglobin.[31]

2.2.3 Iron spin states

Novel spin states have been suggested to result from distorted hemes. FeOETPPCl has been claimed to be in a quantum-mixed S = 5/2, 3/2 intermediate spin state[32] as have some cytochromes *c*′ and peroxidases, all of which have been noted to have saddled deformations.

2.2.4 Chirality

Some porphyrins that are not chiral when planar become chiral when nonplanar. The chirality results from an out-of-plane location of the substituents. Furthermore, inversion of the macrocycle geometry and substituent rotation result in racemization. A good example is the single-armed porphyrin derived

by mono-*meso*-substitution of metalloetioporphyrin I with a single pivaloylamino group [$(CH_3)_3CCONH$].[33] Mono-substituted etioporphyrin I, though asymmetrically substituted with alternating methyl and ethyl groups at the β-pyrrole carbons, is not chiral when planar since the mirror image can be superimposed by a C_2 rotation. However, steric interaction with the adjacent substituents moves the *meso* substituent out-of-plane making the porphyrin nonplanar and the porphyrin becomes chiral. The enantiomers can be separated by chiral chromatography, and slow racemization occurs at room temperature by flipping of the bulky arm through the macrocycle mean plane and rotating about the *meso* position. Free base porphyrins apparently racemize more rapidly than metalloporphyrins, and a metal dependence of the racemization rate is observed. A number of workers have also reported photo-induced atropisomerization of these chiral porphyrins, *i.e.* the racemization rate increases on radiation by visible light. Aida and coworkers have very recently shown that a chiral nonplanar porphyrin can sense the chirality of asymmetric carboxylic acids and retain a memory of the chirality of the acid even after the asymmetric acid has been removed.

2.2.5 Excited state lifetimes

The lifetimes and other dynamic photophysical properties of the excited states of porphyrins are altered by nonplanarity.[19,24] For example, the formation of the excited (d,d) state *via* the (π,π*) state of NiDPP (see Fig. 6) exhibits complex spectral evolution involving both cooling and conformational changes. The results are interpreted in terms of photoinduced access to multiple low-energy nonplanar conformers.

2.2.6 Basicity and metallation rate

Nonplanarity alters the basicity of the porphyrin nitrogen atoms and the rate constants for metal insertion. For example, proton dissociation constants for the mono- and di-cations of dodecaphenylporphyrin are at least 10^9 times less than those for TPP.[17] Copper insertion in the nonplanar DPP system is accelerated by a factor of 6×10^5.[17] Moreover, increased metal insertion rates have been observed for nonplanar porphyrins with substituents that decrease the basicity of the nitrogen atoms, *e.g.* metal insertion is faster by a factor of 10^2–10^3 in Br_8TPP *versus* TPP. Nonplanarity also appears to be responsible for the unusual optical spectra of H_2DPP in certain solvents, where it has been suggested that the exposed porphyrin NH protons can hydrogen bond with solvent molecules.[17]

3.1 Non-protein environmental influences on porphyrin conformation

3.1.1 π–π Aggregation and complexation

The formation of π–π dimers usually results in a flattening of the porphyrin macrocycle.[9] Presumably, the stacking of porphyrins is favored when the macrocycles are nearly planar. The effect is most clearly seen for porphyrins that exist in solution as a mixture of planar and nonplanar species. Aggregation causes the equilibrium to shift in favor of the planar conformer. Similarly, π–π complex formation has an influence on the equilibrium between planar and nonplanar conformers.

For highly nonplanar porphyrins, π–π aggregation and complex formation is apparently disfavored or brings about unusual aggregation behavior. Specifically, cobalt(II) complexes do not stack with 1,3,5-trinitrobenzene. In addition, π–π dimerization does not occur for some dodeca-substituted porphyrins, most likely because of the interference caused by the nonplanar structure and the bulky substituents surrounding the macrocycle. This leads to some unusual properties when nonplanar porphyrins are incorporated into surfactant micelles or when the porphyrin moiety of a lipoporphyrin is nonplanar.[13,34] The altered aggregation properties can lead to interesting polymers of porphyrins as in the case of zinc octaethyltetranitroporphyrin.[16]

3.1.2 Surfactant interactions

Incorporation of porphyrins into detergent micelles and films also influences the equilibrium between planar and nonplanar conformers.[34] Often the micellar environment induces a shift toward the planar conformer; however, in general the effect on porphyrin structure depends on the nature of the detergent molecules. For example, incorporation into cholate micelles induces a shift toward nonplanarity.

3.2 Protein influences on porphyrin conformation and possible roles of nonplanar conformers in protein function

Since the isolated heme group is nominally planar, the distortions evident in Figs. 3 and 4 are a consequence of the protein environment of the heme. In some cases, it is easy to see how the protein might strongly influence the macrocyclic structure. In particular, hemes that are covalently linked to amino acid residues, such as those of the cytochromes *c* and myeloperoxidase, might easily be distorted through these strong interactions with the protein. Indeed, the hemes in these proteins show some of the largest nonplanar distortions.[4,5,7] On the other hand, many proteins that lack covalent connections to the heme also show large deviations from planarity. A specific example of the latter case is the peroxidases whose deformations are characterized in Fig. 4. In fact, the crystal structures of myeloperoxidase and all of the approximately 50 peroxidases exhibit a strong saddling of the heme.

3.2.1 Mitochondrial cytochrome c

Examination of Fig. 3 shows that the most out-of-plane atom of the heme for yeast cytochrome *c* is the *meso*-carbon between pyrroles I and II. This is a characteristic feature of the nonplanar conformation of the hemes of most *c*-type cytochromes, and has led to the suggestion that the covalent attachments to the protein at the 2- and 4-positions of pyrroles I and II cause the distortion from planarity.[4] Specifically, the short protein segment between the cysteine residues could contract the distance between the thioether linkages to the heme, causing the porphyrin to buckle. In particular, we proposed[4] that the hydrogen-bonding network in this segment might act to contract this protein segment, giving rise to the nonplanar distortion. Molecular mechanics calculations show that the heme-pentapeptide unit alone does account for the *ruf*, *wav*(*x*), and *wav*(*y*) components of the observed heme distortion.

3.2.2 Cytochrome c_3

Additional support for this structural hypothesis comes from the NSD results for other cytochromes. The NSD results for the four-heme cytochromes c_3, reproduced in Fig. 7, are particularly convincing.[5,7] Hemes 2 and 4 typically have four intervening residues between the cysteines, whereas hemes 1 and 3 have only two intervening residues as for the mitochondrial cytochromes *c*. The relationship between the short segment and the heme conformation does not simply correlate with the number of residues, but depends on the detailed assembly of the amino acids in the peptide unit. Fig. 7 shows that the heme conformations for these proteins are generally conserved for different strains even though there is very little amino acid sequence identity among these proteins. In fact, excluding the eight cysteines and eight histidines bound directly to the hemes, only a handful of residues (out of more than 110) are conserved for the strains listed. The maintenance of the conformation of the heme, given so little sequence identity between the strains, suggests that only a small portion of the protein is required to generate the major part of the distortion.

The NSD results for heme 4 directly point to the short segment that includes the cysteines, the intervening residues and the adjacent histidine ligand. Notice that hemes 4 of the *baculatum* strains (Fig. 7) are distinctly different from those of the other hemes 4. The structural origin of this difference is likely the result of the differences in the number and folding of the amino acids between the cysteines for heme 4; the

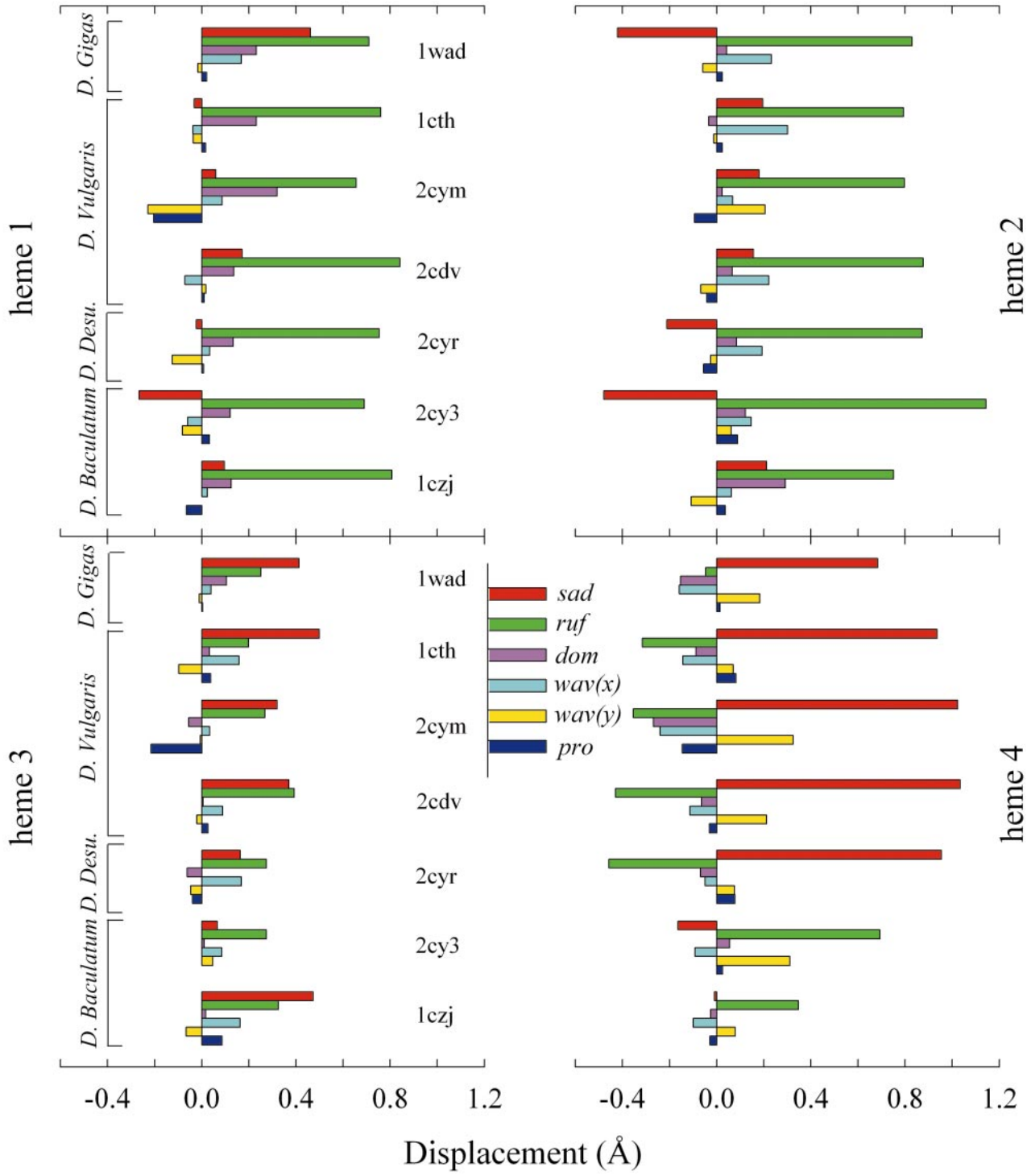


Fig. 7 NSD results for X-ray crystal structures of cytochromes c_3 from four different strains of *Desulfovibrio desulfuricans* (ATTC 27774), *Desulfovibrio vulgaris* (Miyazaki, Hildenborough), *Desulfomicrobium baculatum* (Norway 4), and *Desulfovibrio gigas*

baculatum strains have two residues, not the four residues common to the other strains.

3.2.3 Microperoxidase

Desbois *et al.*[31] have investigated Fe(III) microperoxidase-8 using resonance Raman spectroscopy. Microperoxidase is a digestion product of cytochrome *c* that leaves only a covalently attached, 8-amino acid segment of the protein. Resonance Raman spectroscopy shows that, by itself, the short protein segment induces a nonplanar distortion of the heme.

3.2.4 Cytochrome c_2

The influence of conserved amino acids on the structure of the heme of the cytochrome c_2 has been examined by Desbois *et al.*[31] They find evidence for structural heterogeneity in the macrocycle distortion for the wild type protein in both oxidation states, although higher deformability is found for the ferric state. Similar conformational flexibility was observed for microperoxidase. The distortion of the macrocycle is sensitive to mutation of some conserved residues, particularly tryptophan 67 which is hydrogen bonded to one of the heme propionates. The distortion could have an obvious influence on the redox

potential, but in the case of *R. capsulatus* cytochrome c_2 the distortion is not large (Fig. 4).

3.2.5 Cytochrome oxidase

Hildebrandt *et al*. have noted species-specific differences in the distortion of the macrocycle in the hemes *a* of cytochrome oxidases.[35] Specifically, for the oxidized heme *a* of *Paracoccus denitrificans* oxidase, Raman frequency differences are interpreted in terms of a nonplanar porphyrin structure for the *Paracoccus* enzyme but a planar conformation for the beef heart oxidase. Differences in the oxidases are also observed by EPR. The conformational changes influence the formyl substituent and its electronic coupling with the porphyrin ring. Since the hemes *a* are apparently planar in the fully reduced oxidases, a more large-scale redox-linked conformational transition is indicated for the bacterial oxidase. Flattening of the heme is the main conformational change in the bacterial enzyme upon reduction, and it occurs because of a reorganization of the protein surroundings of the heme. The protein reorganization also influences the interaction with the heme *a* formyl substituent. No significant redox-linked conformational changes can be inferred for heme a_3. The heme *a* protein transition could have significance for the function of oxidase.

3.2.6 Nickel-reconstituted hemoglobin

Resonance Raman studies of nickel-reconstituted hemoglobin and myoglobin further underline the influence of the protein on the conformation of the heme.[9] Nickel protoporphyrin in solution exists as an equilibrium mixture of planar and nonplanar conformers. Upon binding to the active site of hemoglobin, only the planar conformer is observed. This is true even for the NiProtoP molecules that are bound in the active site but not coordinated to the proximal histidine. NiProtoP is known to be in the active site because the histidine ligand is transiently acquired during 10 ns pulsed photo-excitation. These Raman results show that the heme-binding site strongly favors a planar rather than a ruffled macrocyclic conformation, more so in hemoglobin than in the isolated α-subunits. Further, since the ruffled form has lower axial ligand affinity, its prevalence in the α-subunits of hemoglobin may account for their lower affinity for proximal histidine. This macrocyclic distortion effect on axial ligand affinity is similar to the noted influence of macrocyclic distortion on the relative CO and O_2 affinities of strapped heme models.

3.2.7 Methyl-coenzyme M reductase

Methyl reductase is the terminal enzyme in methanogenesis. The enzyme has an $(\alpha\beta\gamma)_2$ protein composition and contains two molecules of coenzyme F_{430}, coenzyme M (mercaptoethane sulfonate) and coenzyme B (mercaptoheptanoyl threonine phosphate). It catalyzes the final step in the methanogenic pathway, reducing methyl-coenzyme M to methane and forming the disulfide coenzyme M–S–S–coenzyme B. The nickel atom of cofactor F_{430} is apparently coordinated to an oxygen atom of a glutamine amino acid. Coenzymes M and B are located on the other side of the Ni-corphin plane at the active site.

Cofactor F_{430} is the prosthetic group of the enzyme. Its nickel atom is reduced to Ni(I) in the catalytic cycle, which entails a large change in metal size. The reduction of the porphinoid ring increases the out-of-plane flexibility for F_{430}, permitting facile changes in the metal core size. Nonplanarity gives F_{430} the ability to accommodate large changes in core size and may also influence its axial ligand affinity.[27] Ligand affinity differences are noted for the F_{430} and its 12,13-di-epimer, and these differences may be attributable to differences in macrocycle conformation resulting from epimerization.[36] Multiple coexisting nonplanar conformers have been observed by resonance Raman scattering for model nickel hydrocorphinates related to cofactor F_{430}.[36] The Ni corphinate models also show altered photodynamics compared with Ni porphyrins, and this too has been attributed to the corphin's higher out-of-plane flexibility.

3.2.8 Photosynthetic pigments

Although this review has focused primarily on nonplanarity in heme proteins and model porphyrins, crystallographic structures of antenna and reaction center proteins have revealed nonplanar conformations for many of the photosynthetic pigments. Fajer and coworkers, in particular, have pointed out that the protein may provide a microenvironment that defines a protein scaffolding which controls the out-of-plane conformation of the bacteriochlorophylls.[3,28] Further, many experimental and theoretical studies have demonstrated that modulating the conformation can vary the optical, redox, and electron-transfer properties of the photosynthetic chromophores. Temperature-dependent conformational changes in the bacteriopheophytins of *Rhodobacter sphaeroides* reaction centers that are detected by Raman spectroscopy have been related to changes in function.[37] Undoubtedly, the entire microenvironment, including chromophore conformation, will be involved in directing the flow of energy and electrons in photosynthetic proteins.

4 Summary

New tetrapyrrole-containing proteins are being found each year, generating the continued interest in an understanding of the molecular basis of their function. Studies of synthetic nonplanar porphyrins are providing an improved understanding of the porphyrin's role in the biological function of these proteins. Conserved heme structural motifs within the proteins indicate the importance and richness of the heme's share in determining the function of these versatile prosthetic groups. In addition, nonplanar porphyrins may have practical uses in biomimetic processes and other commercial applications. Continued experimental and theoretical investigations of nonplanar porphyrins may lead to important new processes, materials, and technological applications in electronics, photonics, catalysis, and other chemical technologies.

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