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Nitric oxide interaction with insect nitrophorins and thoughts on the electron configuration of the $\{FeNO\}^6$ complex

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Abstract

The nitrophorins are NO-carrying heme proteins that are found in the saliva of two species of blood-sucking insects, the kissing bug (*Rhodnius prolixus*) and the bedbug (*Cimex lectularius*). In both insects the NO is bound to the ferric form of the protein, which gives rise to K_ds in the micromolar to nanomolar range, and thus upon injection of the saliva into the tissues of the victim the NO can dissociate to cause vasodilation and inhibition of platelet aggregation. The structures of the proteins from each of these insects are unique, and each has a large component of β-sheet structure, which is unusual for heme proteins. While the *Rhodnius* nitrophorins increase the effectiveness of their NO–heme proteins by also binding histamine, secreted by the victim in response to the bite, to the heme, the *Cimex* nitrophorin does not bind histamine but rather binds two molecules of NO reversibly, one to the heme and the other to the cysteine thiolate which serves as the heme ligand in the absence of NO. This requires homolytic cleavage of the Fe–S–Cys bond, which produces an EPR-active Fe(II)–NO complex having the $\{FeNO\}^7$ electron configuration. For the *Rhodnius* nitrophorins, the heme of the $\{FeNO\}^6$ stable NO complex could have the limiting electron configurations Fe(III)–NO$^{\cdot}$ or Fe(II)–NO$^+$. While vibrational spectroscopy suggests the latter and Mössbauer spectroscopy cannot differentiate between a purely diamagnetic Fe(II) center and a strongly antiferromagnetically coupled Fe(III)–NO$^{\cdot}$ center, the strong ruffling of the heme (with alternate *meso*-carbons shifted significantly above and below the mean plane of the porphyrin, and concomitant shifts of the β-pyrrole carbons above and below the mean plane of the porphyrin ring, to produce a very nonplanar porphyrin macrocycle) may suggest at least an important contribution of the latter. The strong ruffling would help to stabilize the $(d_{xz}, d_{yz})^4(d_{xy})^1$ electron configuration of low-spin Fe(III) (but *not* low-spin Fe(II)), and the d_{xy} orbital does not have correct symmetry for overlap with the half-filled π* orbital of NO. This Fe(III)–NO$^{\cdot}$ electron configuration would facilitate reversible dissociation of NO.

Keywords: Nitrophorin; Nitric oxide; Vasodilation; $\{FeNO\}^6$; Antiferromagnetic coupling; Ruffled heme; d_{xy} orbital; $a_{2u}(\pi)$ porphyrin orbital

1. Background

Nitric oxide has been shown to serve as an autacoid and neurotransmitter in vertebrates, as well as a toxic defense substance for eliminating invading organisms [1–7]. The 1998 Nobel Prize in Medicine was awarded to Ignarro, Furchgott and Murad, whose papers are referenced herein, for their work in showing how NO, a gaseous molecule produced by many cells, interacts with various tissues. NO is produced by various forms of the enzyme NO synthase (NOS), which was originally thought to include a fusion of the heme enzyme cytochrome P450 with its own reductase [1–18]. This complex enzyme contains FAD, FMN and NADPH binding sites as well as heme, and a cofactor not present in cytochromes P450, tetrahydrobiopterin, as well as a non-heme iron. In addition, the enzyme either includes a calmodulin-like subunit that is activated by binding Ca^{2+} or, as in neuronal NOS, is activated by association

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with Ca^{2+}-bound calmodulin [1–19]. The crystal structure of the domain that contains the heme and tetrahydrobiopterin centers has been reported, and although the heme is bound axially to a cysteine, as in all cytochromes P450, the protein folding pattern is quite different; P450s are largely α-helical [20], while the NOS heme/tetrahydrobiopterin domain has a number of β-sheets as well as α-helices in a structure known as a curved α–β domain [21]. Thus, the heme/biopterin domain of NOS and the heme domains of cytochromes P450 probably achieved similar catalytic activities through convergent evolution [21].

To aid in defense against microbes, immune cells such as neutrophils and activated macrophages produce large amounts of NO [22,23], which helps to kill phagocytosed bacteria and parasites [24]. Activated macrophages may induce production of NO synthase, which then produces NO. Detailed studies of inhibition of these enzymes by L-arginine analogs [25–27], and of subunit organization and conditions for dimer dissociation [28,29] have been reported. Production of large amounts of NO can result in metabolic dysfunction of cells in the surrounding tissues leading to several autoimmune diseases, including insulin-dependent diabetes mellitus [30–33], allergic encephalomyelitis [34], and possibly multiple sclerosis [34]. Inducible NOS also may be stimulated by pro-inflammatory cytokines [35,36], possibly leading to myocardial depression following cardiopulmonary bypass. The excess NO is believed to complex with the iron–sulfur centers in proteins such as aconitase, which leads to inactivation of the tricarboxylic acid pathway cycle [37] of invading bacteria and parasites. The induction of NO synthase by interferon-γ also appears to inhibit viral replication [38].

As a physiological regulatory molecule, NO is produced by a variety of tissues, such as endothelium and neural tissue; hence, NO is believed to be synonymous with the endothelial-derived relaxing factor (EDRF) that was described by Furchgott in the early 1980s [39]. It acts by activating the soluble form of the heme protein guanylyl cyclase [40]. It has been proposed that the binding of NO to the heme moiety of guanylyl cyclase triggers dissociation of the proximal histidine, causing a conformational change in the enzyme that lead to its activation [41–45]. Increased cyclic 2′,3′-guanosine monophosphate (cGMP) then activates phosphorylation events that change the state of the target tissue. In smooth muscle, relaxation occurs, with effects including lowered blood pressure [4–7] and mediation of penile erection [46,47]. In platelets, inhibition of aggregation occurs [40]. A role for NO in mediating neurotransmitter release in cerebral cortex has also been found [48], and it has been suggested that NO, in conjunction with light, may be involved in resetting the human circadian clock [49–51].

It is interesting to note that both the synthesis and many of the physiological actions of NO involve heme proteins. Indeed, the accepted NO synthase model proposes that the arginine binding site resides in the distal heme pocket [6], and the action of NO in guanylyl cyclase also requires NO binding to heme [6]. Additionally, because NO is a very reactive substance, it is believed that in biological systems NO reacts with superoxide anions, including those produced by autoxidation of hemoglobin, to produce nitrite and nitrate, or reacts with hemoglobin to form complexes that are very stable to dissociation, and can decay to methemoglobin and nitrite. Cellular NO synthesis has been found to cause the loss and degradation of certain enzyme-bound hemes, and to impede the action of the ferrochelatase enzyme involved in heme synthesis [52]. The interaction of nitrite with myoglobin during the curing of meat has been shown to produce a red pigment that has identical optical spectral characteristics to those of the mononitrosyl derivative of heme [53], and treatment of metmyoglobin with high concentrations of nitrite at pH <7, as in improper curing of meat, produces a green pigment known as nitrimyoglobin [54], in which the 2-vinyl group of the heme has been nitrated in the β-*trans* position. Thus, biochemically speaking, the synthesis, signaling, and destruction of NO all involve heme proteins.

2. Strategies used by blood-sucking insects to insure that they obtain a sufficient meal

There are approximately 15,000 species of blood-sucking insects in the world, each of which has various components in its saliva that aid the insect in obtaining a sufficient blood meal. These substances help to cause vasodilation and to counteract blood coagulation, platelet aggregation, and/or swelling and the beginning of the immune response in the victim [55,56]. They include a wide range of anticoagulants, anti-platelet aggregation compounds, substances that cause vasodilation, and/or create antihistaminic agents, and multiple substances are typically found in the saliva of a given insect, hence providing redundancy in aiding it to feed successfully. The presence of these substances in the saliva allows blood-sucking arthropods to minimize the time taken for obtaining a blood meal [57,58]. Because the evolution of blood feeding crosses a number of classes of arthropods, it is believed that these organisms developed a large variety of anti-hemostatic compounds via convergent evolution [59].

Within the class *Insecta* and order *Hemiptera*, the blood sucking bug *Rhodnius prolixus*, a member of the Kissing Bug family that is native to the Amazon river basin but is found in all temperate areas of South, Central and even southern North America, has a group of salivary nitrovasodilators [60]. These nitrovasodilators

are composed of unique heme proteins that act as storage and delivery systems for NO [61,62]. This new class of heme proteins has accordingly been named nitrophorins (nitro = NO, phorin = carrier), and abbreviated NP [63]; there are a total of four nitrophorins in the adult *R. prolixus* insect, denoted NP1–NP4, or in recent papers rNP1-rNP4, to differentiate them from the *Cimex* nitrophorin, cNP (see below). The nitrophorins provide a means of stabilizing NO for long periods of time in the insect salivary glands, and then releasing it upon dilution and increase in pH, when the saliva is injected into a victim. NO can dissociate from the carrier proteins by dilution at neutral or alkaline pH, but less readily at pH 5.0, the approximate pH of the insect's saliva. Indeed, the titration curve indicates an ionizable group with a pK_a of 6.5 [61,64]. In the notation of Feltham and Enemark [65], these are $\{FeNO\}^6$ centers, which are expected to be EPR-silent. In fact, the appearance of the high-spin Fe(III) EPR (electron paramagnetic resonance) signal when argon was blown over a sample of homogenized *R. prolixus* salivary glands, and its disappearance when NO was blown over the same sample (Fig. 1) [61], provided the initial clue as to how NO could be released upon dilution of the protein, for Fe(II)NO centers, having the $\{FeNO\}^7$ electron configurations, have dissociation constants in the pico- to femtomolar range [41] and would thus not release NO upon dilution by a factor of ~100 or so.

We have also shown that these proteins provide an additional means of assuring the insect a sufficient blood meal by binding histamine [66,67], which is produced by the victim in response to the wound, and thereby at least slowing the defense mechanisms of the victim, as diagrammed in Fig. 2. The histamine complex dissociation constant of rNP1 is 19 nM at pH 7.0 [67] and 11 nM at pH 8.0 [64], while the NO complex dissociation constant is 950 nM, about 50 times larger, at pH 7.0 [68]; the K_d at pH 8.0 is 850 nM [64], a factor of 80 times larger. For rNP4 and rNP2 the histamine K_d is 10 and 9 nM at pH 8.0 and 25 °C, while the NO K_d is 540 and 20 nm, respectively (NP3 has K_ds for both ligands that are too small to measure at 25 °C) [64]. Hence, histamine can displace NO from the Fe(III) form of the nitrophorins, and the tight binding of histamine provides yet another means of insuring a successful meal for the insect. The structural basis for this tight binding is discussed below. rNP2 is also able to interfere with the activation of Factor Xa in the blood coagulation cascade [69,70], and thus possesses a third antihemostatic function.

The binding of histamine to the nitrophorins, mentioned above, which leads to the insect's not being detected for long periods of time during feeding, is clearly a contributing factor in the potential infection of the victim with the protozoan *Trypanosoma cruzi*, which is carried by *Rhodnius prolixus*, because the trypanosome is left at the site of the bite in the feces of

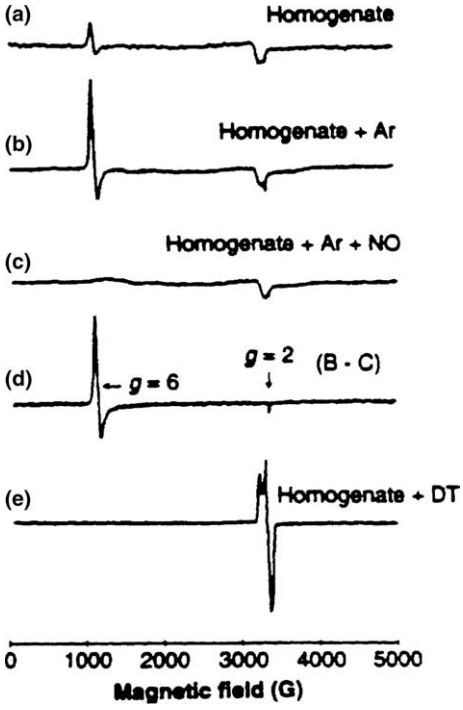

Fig. 1. EPR spectra of 100 pairs of *Rhodnius* salivary glands obtained in 125 μL of PBS (a) before argon equilibration; (b) after equilibration in an argon atmosphere for 4 h, and (c) after equilibration of (b) with NO for 2 min. (d) is the difference spectrum, that is, (b–c). (e) is the homogenate as in (a) treated with dithionite (DT) to reduce Fe(III) to Fe(II). All spectra are on the same scale, except (e), which is shown reduced 3× in amplitude. The spectra were recorded on a Bruker ESP-300E X-band spectrometer equipped with a helium cryostat (Oxford Instruments). Conditions were as follows: Power attenuation, 30 dB; modulation frequency, 100 kHz; modulation amplitude, 3.2 G; receiver gain, 1.25×10^5; resolution 1024 points; time constant, 82 ms; and sweep width, 5000 G. Reprinted from [67] with permission from the American Association for the Advancement of Science.

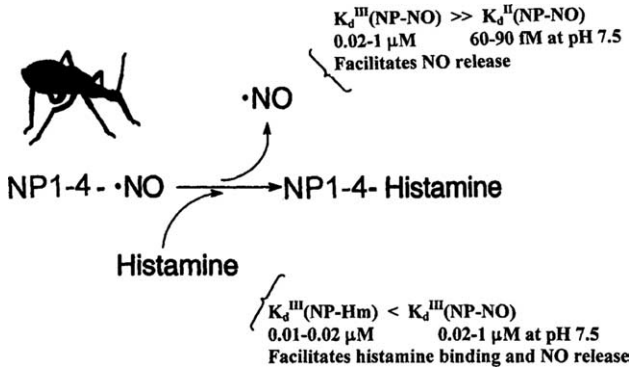

Fig. 2. Role of the *R. prolixus* salivary nitrosyl heme proteins NP1–4 in feeding by the insect. The NP–NO complex is stored in the insect saliva and is delivered to the victim's tissue while the insect probes for a blood vessel. Dilution, binding of histamine and an increase in pH facilitate the release of NO. Histamine is released by the victim in response to tissue damage and the detection of foreign antigens. Histamine binding to the nitrophorins aids in the transmission of *T. cruzi,* the causative agent of Chagas' disease, to the victim, by preventing detection of the insect for a period of time (see text). Modified from [67] with permission from Nature.

the insect after feeding [71]. Later, secretion by the victim of more histamine than can be bound by the nitrophorins injected by the insect causes itching and the beginning of the immune response, which may lead the victim to scratch the bite, thus possibly introducing the trypanosome into the blood stream. *T. cruzi* causes Chagas' disease, a debilitating disease that leads to weakening of the heart and gut muscles on a very slow timescale, and in some cases to death, after periods of tens of years [72–75]. Recent studies have also shown that the mitochondrial kinoplast DNA (kDNA) of *T. cruzi* can be transferred to and integrated into the mitochondrial genome of higher animals, where it can disrupt protein-coding sequences and thus lead to autoimmune responses in a percentage of Chagas disease patients [76]. Both the insect and the trypanosome are found in tropical areas of the New World, and now as far north as southern Texas and Arizona [74,77]. This, combined with migration of large numbers of Latin Americans, some of whom may be infected with Chagas' disease, now puts the U.S. blood supply at risk, for while the blood supplies in Latin America are screened with antibodies to *T. cruzi*, no such tests are currently used in the U.S. country [74]. There is only one drug approved for use against *T. cruzi* in the U.S. (Nifurtimox, a nitrofuran), while a second drug (Benznidazole, a nitroimidazole) is also available in Latin American countries [72,73]. Neither is very effective. Much [76,78–80,78,81–86], though certainly not all [87–92], of the research currently being carried out on *T. cruzi* and Chagas' disease is being done in Latin America, where so many people are infected with the disease.

3. Protein sequences and structures of the nitrophorins from *Rhodnius prolixus*

Following the discovery of the salivary nitrophorins in the early 1990s [60,61], further work in Dr. Ribeiro's laboratory led to the purification of four salivary heme proteins from *R. prolixus*, the predominant one, NP1, being 50% of the total heme protein content and 25% of the total gland protein [63]. The amino terminal sequence for all four heme proteins and partial sequences from proteolytic digests of the two most abundant proteins were obtained [63]. A cDNA library was also produced from *R. prolixus* salivary glands and the gene for the most abundant nitrophorin, NP1, was cloned [63] and expressed [68]. While the work in our laboratory first concentrated on NP1, the genes for NP2, NP3 and NP4 have also been cloned, sequenced, and found to be similar in protein sequence to NP1 [67]. In fact, the four nitrophorins fall into two pairs of nearly homologous protein sequences: NP1 and NP4 as one pair, and NP2 and NP3 as the other, as shown in Fig. 3. NP1 and NP4 are 91% identical in sequence, while NP2 and NP3 have 78% sequence identity. Overall, the four proteins have only 34% sequence identity. In line with the two pairs of similar protein sequences,

Protein Sequences of *Rhodnius prolixus* Nitrophorins

```
     1           __α1__       _βA__ |--A-B loop--| ___βB___
NP1  KCTKNALAQT GFNKDKYFNG DVWYVTDYLD LEPDDVPKRY CAALAAGTAS
NP4  ACTKNAIAQT GFNKDDYFNG DVWYVTDYLD LEPDDVPKRY CAALAAGTAS
NP2  DCSTNISPKQ GLDKAKYFSG .KWYVTHFLD KDP.QVTDQY CSSFTPRESD
NP3  DCSTNISPKK GLDKAKYFSG .TWYVTHYLD KDP.QVTDFY CSSFTPKESG

    51 __βC_____       _____βD_______      ___βE_____
NP1  GKLKEALYHY DPKTQDTFYD VSELQEESPG .KYTANFKKV EKNGNVKVDV
NP4  GKLKEALYHY DPKTQDTFYD VSELQVESLG .KYTANFKKV DKNGNVKVAV
NP2  GTVKEALYHY NANKKTSFYN IGEGKLESSG LQYTAKYKTV DKKKAVLKEA
NP3  GTVKEALYHF NSKKKTSFYN IGEGKLGSLG VQYTAKYNTV DKKRKEIEPA

   101  ___βF_____      _____βG___          __βH__          _____
NP1  TSGNYYTFTV MYADDSSALI HTCLHKGNKD LGDLYAVLNR NKDTNAGDKV
NP4  TAGNYYTFTV MYADDSSALI HTCLHKGNKD LGDLYAVLNR NKDAAAGDKV
NP2  DEKNSYTLTV LEADDSSALV HICLREGSKD LGDLYTVLTH QKDAEPSAKV
NP3  DPKDSYTLTV LEADDSSALV HICLREGPKD LGDLYTVLSH QKTGEPSATV

   151 α2__                          ___α3___
NP1  KGAVTAASLK FSDFISTKDN KCEYDNVSLK SLLTK
NP4  KSAVSAATLE FSKFISTKEN NCAYDNDSLK SLLTK
NP2  KSAVTQAGLQ LSQFVGTKDL GCQYD.DQFT SL~~~
NP3  KNPVAQAGLK LNDFVDTKTL SCTYD.DQFT SM~~~
```

Fig. 3. Sequences of the four nitrophorins from *R. prolixus*, arranged to show sequence identities within the pairs. Helices and beta sheet strands are labeled α and β, respectively. Definition of the secondary structure is based on the structure of NP4.

the rates of NO release also fall into two groups, with NP1 and NP4 having much larger K_ds than NP2 and NP3 [64]. More recently, additional nitrophorin genes have been found to be expressed in earlier life stages of *R. prolixus* [93,94], and we are currently investigating several of these.

Data base searches showed little similarity between the sequences of NP1–NP4 and other proteins [63]. The presence of the non-covalently bound protoheme, the size of the protein, and the interaction of a histidine with the heme (indicated by the pH dependence of NO binding and the EPR spectral behavior of salivary homogenates [61]) first suggested a possible relationship to hemoglobins or cytochromes *b*. Alignment of the sequences of NP1–NP4 with hemoglobin sequences from the insect *Chironomus* [95], the annelids *Lumbricus* and *Tylorrhynchus* [96,97], the mollusc *Glycera* [98], the parasitic nematode *Ascaris* [99], human β chain hemoglobin, leghemoglobin, and vertebrate cytochromes [100] indicated an overall sequence similarity of only 38–45%, with no pronounced regions of sequence identity. No other higher overall sequence similarities could be found with other proteins, and thus we initially assumed that NP1–NP4 might be found to have globin folds. However, determination of the three-dimensional structures by Montfort and co-workers of various ligand complexes of recombinant NP1 [67], NP4 [101–103], and NP2 [104] by X-ray crystallography show that the protein fold of the nitrophorins belongs to a diverse class of proteins called lipocalins [105,106], a family of relatively small secreted proteins that typically bind small, principally hydrophobic molecules such as pheromones [107–109], retinol [110,111], prostaglandins [110,112], retinoic acid [113], and biliverdin [110,114–118], although a recently discovered lipocalin binds two molecules of histamine [119,120], another binds ADP and other adenine nucleotides [121], another binds a Fe(III)-bound tris-catecholate siderophore [122], one is an enzyme [123], one, C8γ, is a subunit of the eighth component of complement that interact to form the cytolytic membrane attack complex, MAC [124], and two, lactoglobulin and α_1-microglobulin, play important, but as yet incompletely understood roles in the processing of fatty acids in milk [125,126] and free hemoglobin and heme in the circulatory system [127–129], respectively. Preliminary evidence suggests that α_1-microglobulin degrades hemin in the bloodstream and in tissues [129]. The lipocalins have little sequence homology and are almost entirely β-sheet proteins that are folded into eight-stranded β-barrels that are closed at one end and bind the small molecule within the other end of the barrel [105,130–132]. *R. prolixus* also utilizes lipocalins that do not bind heme for several other antihemostatic purposes [121,133]. The ribbon structure of the cyanide complex and the histamine-bound heme pocket of NP1 [67] are shown in Fig. 4. Our first structure of an NO complex, NP1–NO, had a highly bent Fe–N–O unit, with angles of 123° and 135° for molecules I and II in the unit cell [134], values consistent with a Fe(II)–NO (Enemark/Feltham notation $\{FeNO\}^7$ [65]) center, which is not the stable oxidation state of the protein in solution, as shown by the EPR spectra of Fig. 1. At the time of the determination of that structure, we suspected that the Fe–NO center might have been photoreduced by the X-rays used for structure determination. Thus, when the first structure of NP4–NO was determined, the X-ray data were collected at low temperatures [102]. For one of two resolved conformations of the NP4–NO complex, the Fe–N–O unit appeared to be linear (174–178°), as shown in Fig. 5; however, in the same structure there were molecules that had a strongly bent Fe–N–O unit, with an angle of 138–144° [102]. With further study, Dr. Montfort and his co-workers confirmed that the nitrophorin–NO complexes are readily photoreduced by the X-rays used for single crystal X-ray diffraction [135]. In order to prevent photoreduction it was found necessary to collect the X-ray diffraction data rapidly, preferably using synchrotron radiation, and at very low temperatures, as well as to use short wavelength X-rays [135], and as the techniques were improved, the Fe–N–O unit has become more and more linear [103]. As shown in a close-up view, Fig. 6, the NP4–NO unit is still somewhat bent, with a Fe–N–O angle of 156° [103], and it is now believed that the previous resolution of two very different angles [102] was due to partial reduction of the FeNO complex to $\{FeNO\}^7$ [103]. However, whether the 156° angle may also contain some contribution from photoreduction is not known.

4. Source of NO in the insect saliva: a salivary gland NO synthase from *R. prolixus*

Ribeiro and co-workers [136] have shown that the salivary gland homogenates of *Rhodnius* contain NO synthase activity, which is activated by tetrahydrobiopterin, Ca^{2+}, calmodulin, FAD, and requires NADPH (but not NADH) to convert arginine to citrulline and NO. Furthermore, similarly to vertebrate enzymes, NO synthase activity coelutes with a diaphorase activity (a two-electron transfer from NADPH to tetrazolium dyes) in a molecular sieving column. Taken together, the data show that the activity is thus similar to the vertebrate soluble constitutive NOS enzyme [136]. The salivary cDNA from *Rhodnius* salivary NOS was cloned and expressed, and shown to have homology to the vertebrate constitutive enzymes, including binding sites for flavins, NADPH and calmodulin [137]. Ultrastructural localization of the associated diaphorase activity of the NO synthase indicates that it is present within cellular vacuoles, similarly to the vertebrate neural NOS enzyme [138], with which it has highest sequence homology [139].

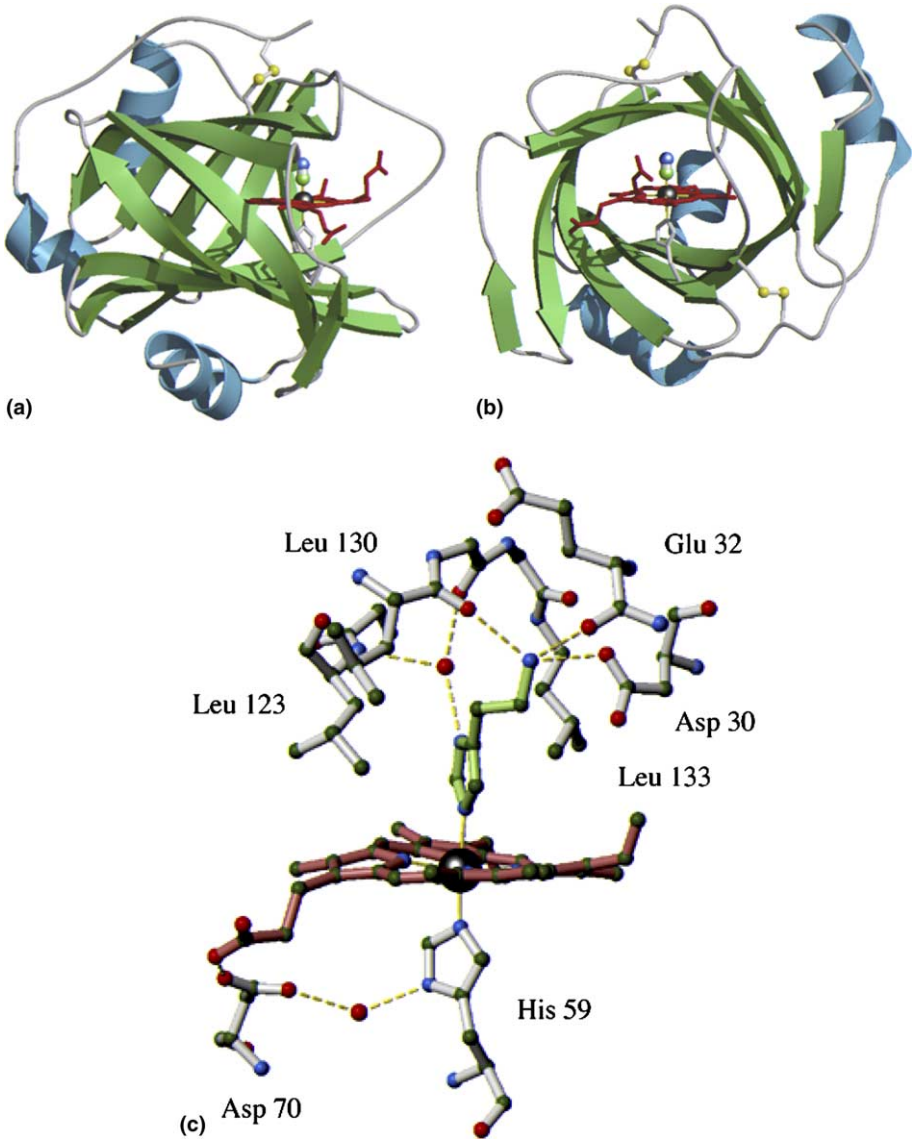

Fig. 4. (a,b) Ribbon and ball-and-stick diagram of the NP1-CN^- structure. Heme is colored red, iron black, His-59 gray, C and N green and blue, respectively. CN^- and iron are shown in a space-filling representation. The strands of the β-barrel are shown in green, α-helices in blue, and loops that connect the individual α and β structural elements are shown in light grey. Disulfide bonds linking Cys-2 to Cys-122 and Cys-41 to Cys-171 are shown with yellow sulfurs. The view in (b) is rotated approximately 90° about the vertical axis from the view (a) on the left. The mobile loops A–B and G–H discussed in the text are above and next to the heme in this view. (c) Histamine binding to NP1. Heme is shown with red bonds, histamine with green bonds, and protein with gray bonds. Hydrogen bonds are shown as dashed yellow lines, nitrogen–Fe bonds as solid yellow lines. Oxygens are colored red, nitrogens blue, carbons green and iron black. Shown are hydrogen bonds between histamine and Asp-30 (2.7 Å), Glu-32 (3.1 Å), Leu-130 (2.7 Å), and an ordered water molecule (2.8 Å, red), which further hydrogen bonds to Thr-121 (3.2 Å), Leu-123 (3.0 Å) and Gly-131 (2.7 Å). Van der Waals contacts are made to Leu-123 (3.7 Å), Leu-130 (4.2 Å), and Leu-133 (3.7 Å). Also shown are hydrogen bonds between an ordered water molecule (red) and residues His-59 (2.7 Å) and Asp-70 (2.6 Å), and between Asp-70 and a heme propionate (2.5 Å). The other propionate has been omitted for clarity. Reprinted from [67] with permission from Nature.

5. A nitrophorin from another insect

We have shown that the bedbug, *Cimex lectularius*, also has a salivary nitrophorin that shows similar, yet unique, pH-dependent reversible NO binding behavior, and optical and EPR spectral properties [140]. The size (~30 kD), amino acid sequence [140], and even the heme ligand of the *Cimex* nitrophorin (Cys thiolate [141]), cNP, are all quite different from those of the *Rhodnius* nitrophorins. The three-dimensional structure of this protein has now been solved by the Montfort group [142], and the heme is indeed bound to a cysteinate ligand, one of two Cys in the protein. The structure, shown in Fig. 7, is that of a β-sandwich with the heme on the outside of the sandwich, and covered by only one helix, which contains the Cys ligand. A closeup of the heme and its cysteine ligand, showing the hydrophobic nature of the protein side chains surrounding the distal pocket

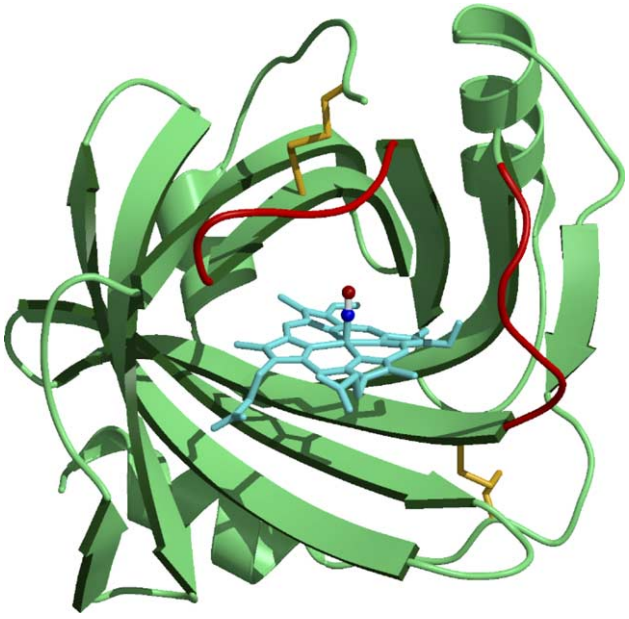

Fig. 5. Ribbon drawing of the NP4–NO structure. Except for the loops that move on NO binding (loop A–B, residues 31–37, and loop G–H, residues 125–132), which are colored red, the rest of the protein backbone (α-helices, β strands, and other loops) are colored green, the heme cyan (stick representation), the disulfide bonds yellow, and the linear NO orientation in blue and red (ball-and-stick representation), respectively. Reprinted from [102] with permission from Nature.

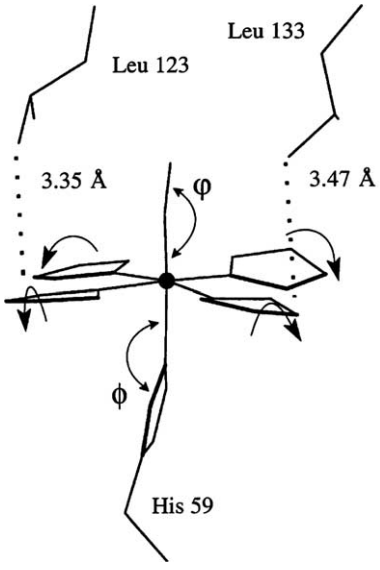

Fig. 6. Near-linearity of the Fe–N–O unit of NP4–NO, and interaction of the two distal leucines with the highly ruffled heme of this complex. The methyl groups of both leucines are within van der Waals contact of the heme π system. Reprinted from [103] with permission of the American Chemical Society.

is shown in Fig. 7(c). The second Cys is inside the β-sandwich and is isolated from solvent and other potential reactive protein side chains. The heme is thus fairly exposed on the surface of the protein. Despite this exposure, neither histamine nor imidazole form stable complexes with the heme at ambient temperatures [143], probably because of the tight packing of the distal side of the heme against the β-sandwich (Fig. 7(c)). The only sequence homology found for this protein is with an exonuclease [144] and with some inositol polyphosphate binding proteins and phosphatase enzymes [145,146]. The structure of one of the latter has been reported, and its protein backbone overlays very well with that of the *Cimex* nitrophorin [147], as shown in Fig. 8. This *Cimex* nitrophorin gene has been expressed, and an improved purification method for the protein has been developed [141]. The fact that *Rhodnius* and *Cimex* come from different families of *Hemiptera* suggests that occurrence of salivary nitrovasodilators in blood-sucking insects may be fairly widespread, and may serve as a common strategy for such insects, to assure them a sufficient blood meal. The fact that other members of each family do not have salivary nitrophorins suggests that these proteins developed in the particular species in which they are found via convergent evolution.

A fascinating finding during the structural investigations of this protein is that when crystals were soaked in an argon-saturated solution for 1 h, followed by moving them to a similar solution saturated with NO for 20 min and then flash-freezing them in liquid nitrogen, the diffraction data obtained for such crystals revealed a structure having NO bound to the heme in the distal pocket, as expected, but also with a modification to the proximal cysteine that is consistent with formation of a *S*-nitrosyl (SNO) conjugate, as shown in Fig. 9(b), as compared to the heme-bound cysteine present in the absence of NO, Fig. 9(a). We have investigated the protein in the absence of NO by EPR spectroscopy and found that it indeed has similar EPR spectra to those of cytochromes P450 in both the high-spin Fe(III) state [148], as shown in Fig. 9(c), and the low-spin Fe(III) complex present as a minor species in Fig. 9(c), or formed by addition of imidazole at the low temperatures used for EPR spectroscopy [149], indicating the presence of a cysteinate proximal ligand to the heme [141].

The very bent Fe–N–O bond, 119°, shown in Fig. 9(b) is typical of a $\{FeNO\}^7$ center [65], and indeed, an EPR spectrum is observed for this complex in frozen solution, as shown in Fig. 9(d). The shape of the EPR signal is indicative of a Fe(II)–NO complex that has no sixth ligand [150], which is consistent with the electron density map of the complex shown in Fig. 9(b). Thus, formation of the Cys–SNO moiety is accomplished by homolytic cleavage of the Fe–S bond to produce Fe(II)–NO and a thiyl radical, which can readily react with a second molecule of NO, as diagrammed in Fig. 10. Both steps of the reaction of NO with cNP shown in the Fig. 10 are reversible at pH 5.6, and the spectral changes that occur at each step exhibit excellent isosbestic behavior [142].

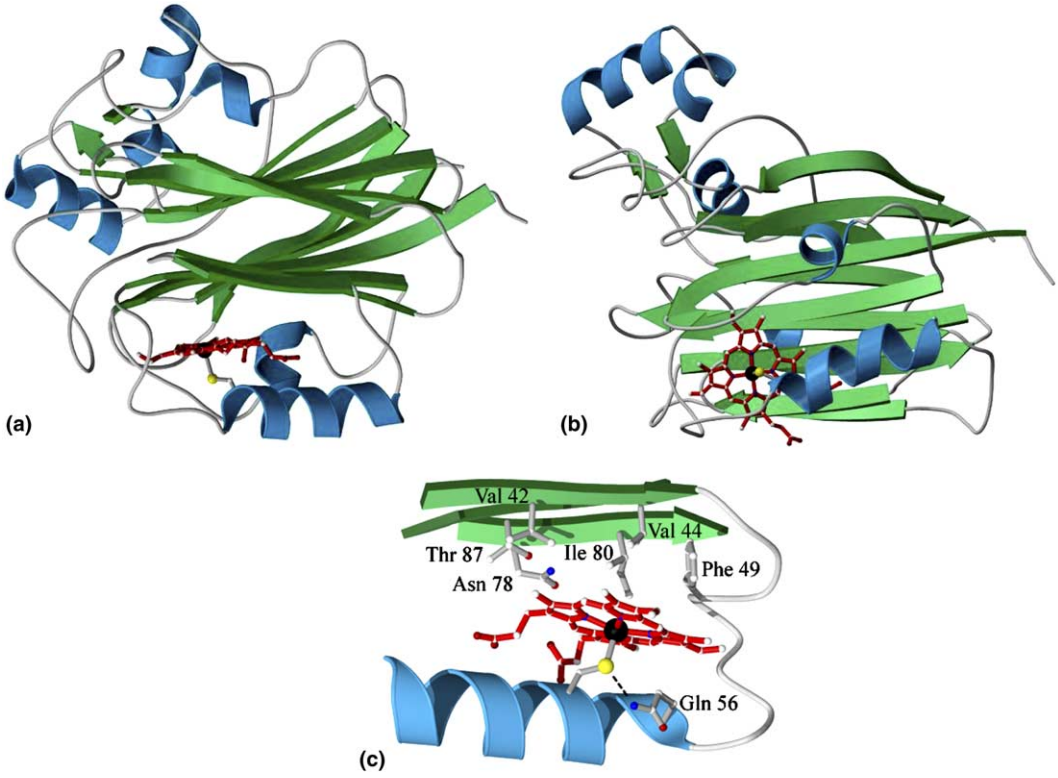

Fig. 7. (a,b) Two views of the structure of cNP, showing the thiolate coordination of the heme and the β-sandwich structure of the protein; β-strands are colored green and shown as arrows with the C-terminal at the head of the arrow, α-helices blue, loops grey, the heme red, iron black and the cysteine sulfur yellow. (c) Closeup of the surroundings of the heme and cysteine thiolate as viewed from the opposite side of the protein as shown in (a), showing the hydrophobic nature of the NO binding site; colors are the same as in (a) and (b), except that oxygen and nitrogen atoms of the side chains are shown as red and blue balls, respectively. Reprinted from [142] with permission from Proc. Natl. Acad. Sci. USA.

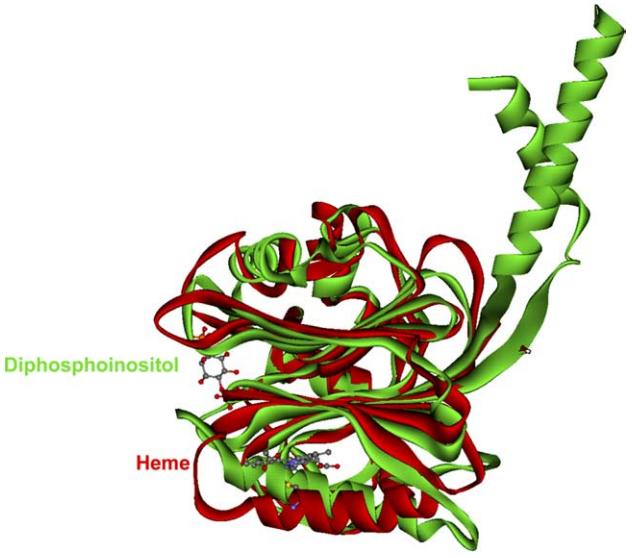

Fig. 8. Overlay of the protein backbone structures of cNP and IPP5P [142] showing the similarity in protein fold. The protein backbone of cNP is shown in red and that of IPP5P in green; the visible atoms of heme, the proximal cysteine and diphosphoinositol are shown as balls: grey = carbon, red = oxygen, blue = nitrogen, yellow = sulfur.

Cys–SNO formation from S^- and NO requires a one-electron oxidation, which is readily facilitated by metal centers. In cNP this is accomplished through reversible reduction of the heme iron [142]. In this mechanism, NO first binds to the ferric heme, giving rise to a complex that remains vulnerable to undesirable side reactions. Although not yet observed crystallographically, the loss of EPR signal and absorption spectral changes confirm the existence of the Fe(III)–NO complex [142]. A second NO molecule binds at the Cys-60 thiolate, leading to reduction of the heme iron and formation of a neutral SNO conjugate. NO release, in response to lowered NO concentrations, involves simple reversal of these steps: decomposition of Cys–SNO is induced by Fe(II) [151], and release of NO from the now-ferric heme readily takes place, more so at higher pH.

These data represent the first observation of reversible Cys–SNO conjugation in a protein [142]. It is possible that Cys–SNO formation at metal centers may be common. For example, inhibition of the ferrous state of inducible NOS (iNOS) by NO is known to occur, but apparently does not involve release of proximal Cys-415; however, if a hydrogen bond to the proximal cysteine is removed through mutation in endothelial NOS (eNOS), a five-coordinate Fe(II)–NO complex is formed [152], much like that of cNP. Whether SNO formation also occurs in this case, or in any of the many proteins with cysteines linked to redox active metal centers, is not known, but this would be difficult to discover

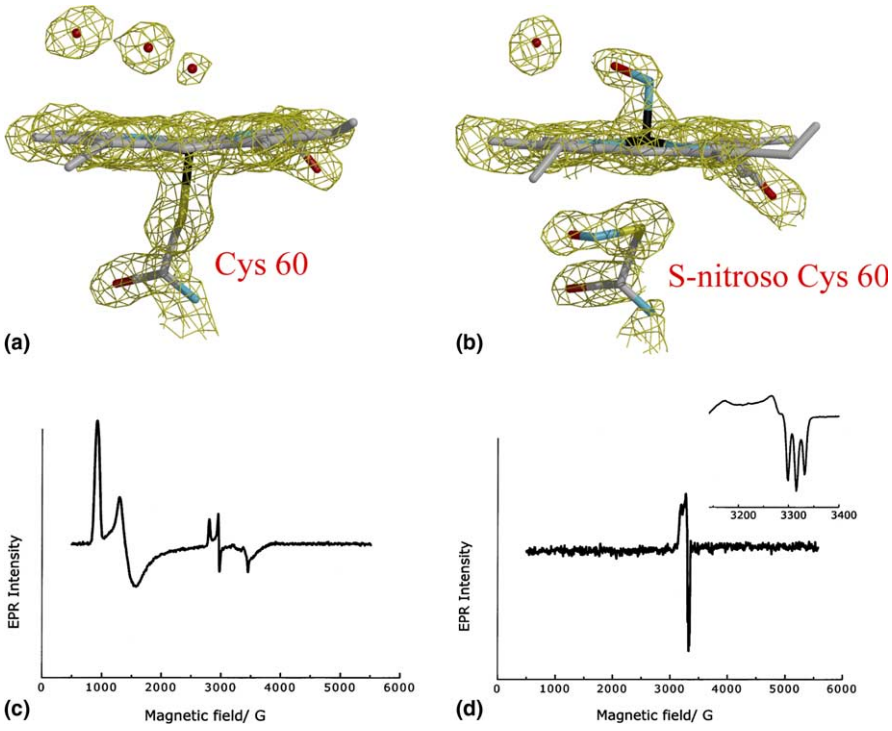

Fig. 9. (a,b) Electron density maps of the heme in the absence (a) and presence (b) of NO. Electron density is shown as gold wires, the heme and cysteine 60 are represented as sticks with carbons = grey, nitrogens = blue, oxygens = red, iron = black, and sulfur = yellow. Note that in the presence of NO there is no electron density between the cysteine sulfur and the heme iron, but that there is electron density to the side of the cysteine sulfur that is consistent with formation of a thionitrosyl (Cys–SNO). (c,d) EPR spectra in the absence (c) and presence (d) of NO. Reprinted from reference [142] with permission from Proc. Natl. Acad. Sci. USA.

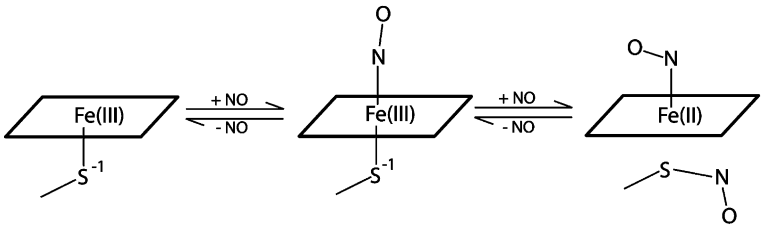

Fig. 10. The reaction of NO with cNP first forms an EPR-silent Fe(III)–NO complex with the Fe–N–O bond expected to be linear; homolytic cleavage of the Fe–S bond and reaction of the thiyl radical with the second NO forms the Cys–SNO, and at the same time the iron–NO moiety has become a Fe(II)–NO complex, which is EPR active. Reprinted from reference [142] with permission from Proc. Natl. Acad. Sci. USA.

without freeze-trapping in the crystal, since the typically employed spectroscopic signals do not readily detect sulfur adducts.

6. Nitric oxide reactivity with heme centers

Nitric oxide is unique among diatomic molecules in being able to interact with both iron(II) and iron(III) heme proteins [41]. In the notation of Feltham and Enemark [65], these are $\{FeNO\}^7$ and $\{FeNO\}^6$ centers, respectively. NO reacts rapidly according to second-order kinetics with the heme center in hemoglobins [153–156], myoglobins [157,158], catalase [158] and less rapidly with cytochrome *c* [158] in both their ferrous and ferric forms. The association rate constants are of the order of 10^7–10^8 M^{-1} s^{-1} for ferrous Hb [153–155] and Mb and 10^3–10^4 M^{-1} s^{-1} for most species of ferric Hb [157] and Mb [157,158] (except elephant metMb, 10^7 M^{-1} s^{-1} [157], for which the ferric form has no coordinated water). The dissociation rates are vastly different for the two oxidation states: for Fe(II), dissociation rate constants are of the order of 4×10^{-4} s^{-1} [154], leading to very large equilibrium constants for NO binding ($K_{eq} = k_f/k_r = 10^{11}$–$10^{12}$ M^{-1} [41]); for Fe(III), dissociation rate constants range from 0.65 to 40 s^{-1} [157], leading to relatively small binding constants for NO ($K_{eq} = k_f/k_r = 10^3$–10^5 M^{-1}) [41,157,158]. And unlike the binding of CO or O_2, the equilibrium constant for binding NO to *unligated* ferrous hemes is 10^3–10^4 *larger*

than when the heme carries a ligand (histidine or, presumably, cysteinate) [41,159]. This has led to the hypothesis that *the loss of protein axial ligand* upon binding NO to a ferrous heme protein could begin the activation of soluble guanylyl cyclase (sGC) [41]. Guanylyl cyclase itself has also been shown to have the largest known NO dissociation rate from the Fe(II) form of the protein, $k_{obs} = 6 \times 10^{-4}$ s^{-1}, leading to a predicted half-life of the NO complex of about 2 min at 37 °C [159]. This value may have implications for the mechanism of regulation of the activity of guanylyl cyclase, since this enzyme releases NO relatively quickly. Resonance Raman spectroscopy has lent strength to this hypothesis [43]. Marletta and co-workers have recently shown that guanylyl cyclase is activated by reaction of NO with the heme of sGC in a two-step reaction, the first of which has a very large rate constant ($k_{on} > 1.4 \times 10^{8}$ M^{-1} s^{-1}) and is NO-concentration dependent. Formation of this Fe(II)–NO complex weakens the proximal histidine-Fe bond, which is then followed by a second NO-concentration-dependent reaction with a rate constant $k_{6c-5c} = 2.4 \times 10^{5}$ M^{-1} s^{-1}, where the second NO is believed to bind to the proximal side of the heme after the histidine has dissociated [160,161]. A similar reaction path is followed by cytochromes *c*′ [162–165], whose main function is now believed to be NO scavenging [166,167].

Spectroscopic techniques that have been utilized to characterize nitrosylheme proteins include magnetic circular dichroism (MCD) [168,169], infrared (IR) [134,170–174], resonance Raman (RR) [43,175–180], Mössbauer [181–185], and EPR spectroscopies [61,134,150,186–200]. MCD has been used to characterize the protein-provided axial ligand bound to both Fe(II) and Fe(III) forms of various heme proteins, using the NO, CO, phosphine, and other adducts to develop MCD spectra that are unique for the fifth (protein-provided) ligand [168,169]. MCD information particularly definitive for the paramagnetic forms of heme proteins is obtained at very low temperatures (4.2 K) in the near-IR region of the optical spectrum [201,202]. IR spectroscopy has been used to characterize the N–O stretching frequencies of ferrous (1675 and 1618–1635 cm^{-1} for five- and six-coordinate model hemes, respectively [171], 1615–1617 cm^{-1} for HbA [170,172] and 1611 cm^{-1} for NP1 [134]) and ferric (1925 cm^{-1} for HbA [172,174] 1917 and 1904 cm^{-1} for NP1 [134] and 1865–1910 cm^{-1} for related heme-based Fe(III)NO centers [174]), while RR spectroscopy has been used to characterize not only this stretch [175,176], but also the low-energy Fe–NO stretch for ferrous (522–527 cm^{-1} for model hemes [179] and 551–554 cm^{-1} for HbA [175]) and ferric (601–603 cm^{-1} for models [179], 595 cm^{-1} for HbA [176] and 591 cm^{-1} for NP1 [203]), as well as porphyrin ring vibrations. It should be noted with respect to the N–O stretching frequencies that those of the nitrophorin–NO complexes (and other ferric–NO complexes, 1865–1917 cm^{-1}) are similar to those of ferrous–CO stretching frequencies (1936, 1960 cm^{-1}) [134], which lends support to the suggestion that the electron configuration is Fe(II)–NO^{+}.

7. Redox chemistry of NO–heme systems and the nitrophorins of *Rhodnius prolixus*

Since NO binds to both Fe(III) and Fe(II) hemes, the redox chemistry that links these two oxidation states of the nitrosylheme complexes is of major importance to the functioning of these proteins. Ferric nitrosylhemoglobin undergoes rapid autoreduction to the ferrous protein under 1 atm NO gas; the autoreduction process is pseudo-first-order in Fe(III)NO, the reaction rate is enhanced by medium-intensity broad-band optical illumination, and excess NO is clearly the reductant [204]. Recent studies have shown that the rate law contains not only NO, due to the pre-equilibrium between metHb and NO, but also hydroxide ion; attack of hydroxide on coordinated NO^{+} (Fe(III)NO ↔ Fe(II)NO^{+}) is the rate determining step in the autoreduction reaction [205]. This rate determining step is followed by rapid dissociation of HNO_2 and rapid binding of a second NO to the Fe(II) formed by hydroxide attack on coordinated NO^{+} [205]. Methemoglobin–NO autoreduces more than ten times faster than metmyoglobin–NO ($k_1' = 9.67 \times 10^{-4}$ s^{-1} and 0.64×10^{-4} s^{-1}, respectively), and good isosbestic points are not observed [204]. However, upon "recycling" the protein, the pseudo-first-order rate constant decreases markedly ($k_1' = 0.18 \times 10^{-4}$ s^{-1}) and slightly different isosbestic points are observed, suggesting that some chemical modification of the protein may be involved. The authors point out that both lysine and histidine could be nitrosylated [204]. Presumably, free cysteine residues could also be nitrosylated, and Stamler and co-workers [206,207] have shown evidence for a physiological role in lowering blood pressure for the nitrosylation of the Cys β93 thiols of hemoglobin.

Electrochemical investigations of several synthetic nitrosylheme complexes have been reported [208–211]. Fe(II)NO complexes can be reversibly oxidized by one electron to Fe(III)NO in the case of octaethylporphyrin (OEP) and tetraphenylporphyrin (TPP) complexes. Based upon vibrational spectroscopic data mentioned above, Fe(III)NO heme complexes are commonly believed to have the electron configuration Fe(II)–NO^{+}, and from this standpoint it is not surprising that they readily act as electrophilic nitrosating agents [212–222]. In aqueous solution, nitrosation can occur at –S, –N, –O, and –C in organic molecules [212,213]. With particular relevance to biological systems, primary amines are readily deaminated and secondary and tertiary amines are readily N-nitrosated by NO^{+} [3]. Nitrosation of

the N atoms of DNA bases can lead to carcinogenesis [219,221]. Thiols have a particularly high propensity for nitrosation under physiological conditions [206,219–222], but this requires 1-electron oxidation of the thiol (or NO), as discussed above for the case of cNP. Inhibition of the catalytic activity of alcohol dehydrogenase by NO is believed to be associated with S-nitrosylation of the zinc-bound cysteine, followed by release of zinc [223].

Our investigations of the electrochemistry of NP1 and NP1–NO [134] as well as the other three nitrophorins [64,224] have pointed out striking differences from the results obtained for metmyoglobin and its NO complex [64,134,204,205] and model hemes [208–210]. We have measured and reported the reduction potentials of NP1-4 in the absence of ligand and in the presence of NO [64,134], histamine [64,224], imidazole [224] and 4-iodopyrazole [224]. We find that in marked contrast to methemoglobin and metmyoglobin [158,204,205], the nitrosylheme protein of *Rhodnius prolixus* autoreduces slowly only after prolonged treatment with gaseous NO [61,102]. The reduction potentials of the four nitrophorins are about 300 mV more negative than that of metMb: The metMb reduction potential ranges from +28 to 0 mV at pH 5.5–7.5, while those of the nitrophorins range from −274 to −303 mV (NP1), −259 to −278 mV (NP4), −287 to −310 mV (NP2), and −321 to −335 mV (NP3) vs. the standard hydrogen electrode (SHE) over the pH range 5.5–7.5 [64,134]. The negative shifts in potential relative to metMb are consistent *both* with the sluggishness of autoreduction of NP1–NO by excess NO *and* with the presence of several buried potentially negatively charged residues in the heme pocket of NP1 [68,134]. Such negative charges have previously been shown to stabilize the Fe(III) state of mutant Mbs, thus shifting the reduction potential in the negative direction [225]. However, the very large difference in the rate of autoreduction of metMbNO (seconds) [204,205] and NP1-NO (hours) is likely mainly a result of the difficulty of OH^- attack on the $Fe(II)NO^+$ form of the protein [205] in the presence of the potential negative charges in and near the heme pocket [67,101–103,134].

The electrochemistry of MbNO and all four NP–NO complexes has also been investigated [64,134]. We find that reversible Fe(III)NO–Fe(II)NO reduction is not observed for myoglobin because of rapid dissociation of NO from the Fe(III) state of MbNO in the absence of excess NO [134]. In contrast, NP1 shows this reversible reduction at +154 mV vs. SHE at pH 5.5, and at +127 mV at pH 7.5, although NO also readily dissociates from the Fe(III) form at the latter pH [134]. NP2–4 exhibit similar redox behavior in the presence of NO [64]. From the shift in reduction potential upon complexation with NO and the K_d for Fe(III)NO for each of the nitrophorins, we have calculated the K_d for Fe(II)NO from the Nernst equation. We find that K_d = 8 fM at pH 5.5 and 50 fM at pH 7.5, or the NO *binding* constants at the two pH values are 1.3×10^{14} and 2×10^{13} M^{-1}, respectively for the Fe(II)NO complex [64,224]. These NO binding constants are larger than for most other heme proteins [41,153–158]. Such large binding constants for the Fe(II)NO state makes it all the more important that these nitrophorins are set up so as to have potentially negatively charged residues nearby and a very ruffled heme (with alternate *meso*-carbons shifted strongly above and below the mean plane of the porphyrin, and concomitant shifts of the β-pyrrole carbons above and below the mean plane of the porphyrin ring, to produce a very nonplanar porphyrin macrocycle), to remain in the Fe(III) state, in order that NO can be released upon dilution, to do its jobs of vasodilation and preventing platelet aggregation (Fig. 2); if the Fe(II)NO oxidation state were the stable form, NO would not dissociate upon ~100-fold dilution and pH change.

We have prepared mutants of NP2 to examine the effect of various residues on the reduction potentials of these proteins. We have created the D1A mutant of NP2 in order that the methionine-0 would be processed off as it is in NP4, and have shown that the D1A mutant has a reduction potential that is 15 mV more negative than the wild-type protein at pH 7.5, although it is 33 mV more positive at pH 5.5 [226]; we have utilized the D1A mutant in creation of the other mutants mentioned below, except where noted. Based upon the structures of the nitrophorins, we expected that the carboxyl groups of the A-B loop plus the buried glutamic acid (E53 of NP2) would have the greatest effect on the reduction potential at pH 7.5, which is close to the pH of most tissues. This expectation is partially borne out, in that mutation of E53 to glutamine causes a 9 mV positive shift in the reduction potential of NP2 in the absence of added ligand, and a 75 mV positive shift when NO is bound [226], and the A-B loop mutants D29A and D36A both have more positive reduction potentials in the absence (+12, +18 mV, respectively) and presence (+22, +28 mV, respectively) of NO, but the D31A mutant has a more *negative* potential in the absence of NO (−41 mV), yet a somewhat more positive potential when bound to NO (+12 mV)! The reason for these odd findings is rooted in the changes in binding constant of NO to each of the oxidation states of iron, since the shift in reduction potential measures only the ratio of the equilibrium constants for NO binding:

$$E_{NO} - E^\circ = -(2.303RT/nF)\log_{10}(K_{eq}^{III}/K_{eq}^{II})$$

where E_{NO} is the reduction potential of the NO complex and E° is the reduction potential of the nitrophorin in the absence of added ligand, R is the gas constant, T is the absolute temperature, n is the number of electrons involved in the redox reaction (one in this case), F is the Faraday constant, and the K_{eq} are the equilibrium constants for binding of NO to each oxidation state of iron.

The values of K_{eq}^{III} can be measured by optical spectroscopic methods, and hence the values of K_{eq}^{II} can be calculated from the potential shifts, E_{NO}–$E°$. The results show that the values of K_{eq}^{III} are very similar for D1A, D1A,D29A, D1A,D31A and D1A,D36A, but that K_{eq}^{II} is a factor of ~4 larger for D1A,D31A than for the others; likewise, D1A,E53Q has a value of K_{eq}^{III} that is smaller by a factor of ~4 smaller than D1A itself, presumably because the Fe(III) complex is destabilized by loss of the negative charge of the E53 carboxylate at pH 7.5, while the value of K_{eq}^{II} is almost a factor of 4 *larger* for the former than the latter, making the overall ratio of equilibrium constants larger by nearly a factor of 16 for this mutant [226]. Other more drastic changes including replacement of the entire A-B loop of wild-type NP2 with the corresponding loop residues of NP4 produces a +68 mV shift in potential in the absence of NO, and a +80 mV shift when NO is bound, the single K30A and D33+ mutants yield a +18 and −68 mV shift in the absence of NO, respectively, and a +61 and +38 mV shift when NO is bound, respectively; measurement of NO binding constants for these mutants is in progress. Thus, the observed potential shifts of the various mutants cannot be understood without separately measuring the equilibrium constants for ligand binding. Nevertheless, the sum of all of the differences in potential shifts combined, in the absence and presence of NO at pH 7.5 (+10, +53, +10, +66, and +43 mV = +182 mV), account for almost 2/3 of the ~300 mV more negative potential of the *Rhodnius* nitrophorins as compared to met-myoglobin [64,134,224].

The heme group in all of the wild-type (WT) nitrophorins is ruffled [67,71,101–104], and is even more ruffled for the NO complexes [101–103,227]. The structure of NP4–NO indicates that close contacts of the two distal pocket leucines (L123 and L133) probably contributes significantly to this ruffling (Fig. 6), and may help to stabilize the Fe(III)NO state [103], and recent structures of NP2–NO also show the close contacts of the two distal leucines (L122 and L132 in the case of NP2); the NP2 heme is even more ruffled than the NP4 heme [227]. Hence, we have investigated the role of the distal leucines of NP2 on the reduction potential of the protein [228]. For wild-type and L → V distal pocket mutants of NP2 and their NO and imidazole complexes we find that the change in reduction potential when NO binds to the mutant proteins is more positive than that of the wild-type protein, a 90 mV more positive shift for the L132V single and the L122,132V double mutants when NO binds at pH 7.5 than for the wild-type protein [228], thus indicating stabilization of the Fe(II)–NO state in the mutants at this pH this 90 mV difference represents the majority of the remaining ~120 mV more negative shift of the reduction potential of the nitrophorin heme as compared to that of met-myoglobin [64,134,224].

At lower pH values, the double L → V mutant in the absence of NO behaves uniquely, in that it shows strong pH dependence of its reduction potential (−363, −337, and −223 mV vs. SHE at pH 7.5, 6.5 and 5.5, respectively), suggesting a 2-proton dependence on the reduction potential between pH 5.5 and 6.5 [228]. None of the other nitrophorins that we have investigated behave in this manner, but we suspected at the time of publication of this work that the mutation of one or two leucines to valines might create enough additional space in the distal pocket for several additional water molecules to be present. These water molecules could have acid dissociation constants in the pH range 5.5–6.5 when in hydrogen-bonding contact with the water molecule bound to the heme iron [228]. Indeed, the structure of this mutant NP2 protein, L122,132V, has been solved, and there are four water molecules in the cavity created by shortening the side chains of the two leucines in the distal pocket to valines [227].

However, independent of the low pH behavior of the L132V single and L122,132V double mutants in the absence of NO, the more positive shift in reduction potential upon binding NO to the mutant proteins is consistent with the expectation that the smaller valine residues allow the heme to regain some planarity as compared to the significant ruffling observed in the wild-type protein, and this is observed [227], as discussed further below. It thus appears, as postulated by us recently [103,228], that ruffling stabilizes the Fe(III)–NO state, which is required for facile NO dissociation.

8. Possible role of heme ruffling in stabilizing the {FeNO}^{6} center of the nitrophorin–NO complexes

The hemes of all *Rhodnius* nitrophorin complexes are somewhat ruffled, but those of NP4–NO [103] and NP2–NO [227], NP2–H_2O [227], and NP4–CN^- are highly ruffled, much more ruffled than the NP4-NH_3 complex [101]. Furthermore, the resolutions of these structures are higher than those of structures of NP1 [67] and the first structure of NP2 [104], thus making it possible to measure accurately the deviations of the 25 atoms of the heme core from the mean plane, a measure that has frequently been used for model heme complexes [229]. More recent structures of a number of NP2 ligand compexes have been obtained to high resolution [227], and show that in all cases the NP2 complexes are more ruffled than the corresponding NP4 complexes (Table 1). Although these structures are highly ruffled, the most highly nonplanar hemes are found in the heme–NO and oxygen binding (H–NOX) domain of the O_2-binding protein of the obligate anaerobe *Thermoanaerobacter tengcongensis*, where the hemes in two crystalline forms have RMS deviations of 0.33, 0.44, 0.45 and 0.46 Å, which normal coordinate structural decomposi-

tion shows to be composed of close to equal contributions from saddled and ruffled conformations [230]. This protein domain has a sequence very similar to that of human soluble guanylyl cyclase (sGC) and is believed to have a structure similar to that of sGC [231].

Formal core diagrams of the nitrophorin complexes of NP4 [101,102] and NP2 [227] have been constructed, and root-mean-square deviations (RMSD) from the mean plane, in units of 0.01 Å, are listed in Table 1, where they are compared to the values obtained for relevant model heme complexes [232–248]. In analyzing these data, several factors must be kept in mind: (1) Among synthetic hemes, it is known that *meso*-substituted metalloporphyrinates are in general more ruffled than β-pyrrole-only-substituted metalloporphyrinates [229] and (2) Fe(III) porphyrinates are usually much more ruffled than Fe(II) porphyrinates [229,236, 242,243]. From consideration of the data of Table 1, it appears that RMSD values of less than 0.10 Å may be considered essentially planar, while as RMSD values increase above 0.10 Å, a moderately to significantly ruffled heme is indicated. Of the reported structures of six-coordinate Fe(III)–NO synthetic porphyrinates [232], only the complex in which the sixth ligand is indazole is ruffled to an extent similar to that of NP4–NO and NP2–NO. No comment is made about any difference in crystal packing of this complex as compared to the others [232], but it is possible that the larger ligand, with a six-membered ring fused to the five-membered pyrazole ring, takes up enough space in the crystal so as to contribute to this ruffling. Neglecting this complex, all other $[(OEP)Fe(L)(NO)]^+$ complexes have RMSD values less than or close to 0.10 Å, while NP4–NO has a value nearly twice that, 0.189 Å, and the cyanide complex has a larger yet RMSD (0.214 Å). As mentioned above, all complexes of NP2 are more ruffled than the corresponding NP4 complexes, with the NP2–NO complex having a RMSD (0.285 Å) [221], which approaches three times that of the model hemes of the same coordination. Although all of the Fe(III)–NO complexes may be classified according to the notation of Feltham and Enemark as $\{FeNO\}^6$ complexes [65], we choose in this discussion and in Table 1 to classify them according to their metal d-electron count, as d^5 systems, for reasons that will soon become apparent. In this classification, we can compare the –NO and $–CN^-$ complexes directly, and can also compare them to Fe(III) bis-ligand complexes of other types.

There are two possible electron configurations of low-spin iron(III) porphyrinates, the more commonly observed $(d_{xy})^2(d_{xz}, d_{yz})^3$, or d_π, electron configuration, which is usually observed for the elecron-transferring ferricytochromes *a*, *b*, *c*, *f* and *o* [243–247], and the "novel" $(d_{xz}, d_{yz})^4(d_{xy})^1$, or d_{xy}, electron configuration, which is often observed in model hemes having axial ligands that are good π-acceptors, such as isocyanides [235,236,248], low-basicity pyridines [234], and under certain conditions, cyanide ions [249–252]. The d_{xy} ground state is also believed to exist for some reduced hemes in biological systems, including heme *d* and d_1 and siroheme, but this appears to depend upon the nature of the axial ligands [253–255]. A highly ruffled heme macrocycle is one of the hallmarks of d_{xy} ground state complexes, although many d_π heme centers are also ruffled. Rousseau and co-workers [256,257] have noted that the NOS heme is also quite ruffled, and that this may indicate a major role for the d_{xy} electron configuration in that heme protein.

Probably the most relevant comparison of porphyrin ring ruffling that can be made, among model heme complexes, is to the bis-(*tert*-butylisocyanide) complex of (OEP)Fe(III). This complex is highly ruffled, with a RMSD of 0.232 Å [236], while the Fe(II) analog, although highly saddled, has RMSD *meso*-carbon displacements of only 0.01 Å [237], and thus has no ruffling component. *Meso*-only substituted metalloporphyrins having planar six-membered ring axial ligands in perpendicular planes, as is the case for all of the (TMP)Fe(III) bis-pyridine complexes, are highly ruffled [233,245], whereas the corresponding Fe(II) complexes, all of which have the axial pyridine ligands in parallel planes [242], are quite planar; Fe(III) porphyrinates having axial ligands in parallel planes, such as $[(TMP)Fe(1\text{-}MeIm)_2]^+$ and $[(OEP)Fe(4\text{-}NMe_2Py)_2]^+$, are also quite planar [233]. Iron(III) dodecasubstituted porphyrin complexes such as the octaalkyltetraphenylporphyrins, which have both *meso*- and β-pyrrole substituents, are highly saddled, irrespective of axial ligand(s) or coordination number, but often have a ruffling component as well [246,247,258]. However, the bis- *t*-butylisocyanide complex of octamethyltetraphenylporphyrinatoiron(III) is purely saddled in two crystalline forms [248], showing that a low-spin Fe(III) complex with an electron configuration of $(d_{xz}, d_{yz})^4(d_{xy})^1$ can exist without ruffling of the porphyrinate ring.

Thus, apart from model hemes having planar axial ligands in nearly perpendicular planes lying over the *meso* positions, most of the cases for which the porphyrinate ring is highly ruffled are those for which the iron(III) center has the electron configuration $(d_{xz}, d_{yz})^4(d_{xy})^1$, *i.e.,* the bis-isocyanide complexes of both (OEP)Fe(III) [236] and various (TPP)Fe(III) derivatives [235,236], and the bis-4-cyanopyridine complex of (TPP)Fe(III) [234], as well as the bis-cyanide complexes of *meso*-alkyl-substituted hemins [244,249–251] and other modified porphyrin ring systems [252]. Complexes of this electron configuration have EPR g-values close to 2.0 [234–236,244], as well as unique NMR, Mössbauer and MCD spectra [244], that readily differentiate them from the more typical low-spin Fe(III) complexes that have the electron configuration $(d_{xy})^2(d_{xz}, d_{yz})^3$, such as the vast majority of the cytochromes *a*, *b*, *c*, *f* and *o*

Table 1
RMSD from 25-atom mean plane for various Fe(III) and Fe(II) heme complexes

Complex	Fe electron configuration	RMSD, 0.01 Å	Type of distortion	Reference
Fe(III)–L complexes				
NP4–NH_3	d^5	13.8	Ruffled	[101]
NP4–NO	d^5	18.9	Ruffled	[103]
NP4–CN^-	d^5	21.4	Ruffled	[103]
NP4–*t*-BuNC[a]	d^5	14.5	Ruffled	[266]
NP4–Histamine	d^5	13.1	Ruffled	[103]
NP4–ImH[a]	d^5	11.8	~Ruffled	[224]
NP4–4IPzH[a]	d^5	8.8	~Planar(Ruffled)	[224]
NP2–H_2O	d^5	25.0	Ruffled	[228]
NP2–NO	d^5	28.5	Ruffled	[228]
L122,132V NP2–H_2O	d^5	20.8	Ruffled	[228]
L122,132V NP2–NO	d^5	24.6	Ruffled	[228]
[(OEP)Fe(1-MeIm)(NO)]ClO_4[a]	d^5	10.0	Ruffled	[233]
[(OEP)Fe(PzH)(NO)]ClO_4[a]	d^5	9.5	~Ruffled	[233]
[(OEP)Fe(Iz)(NO)]ClO_4[a]	d^5	20.9[b]	Ruffled	[233]
[(OEP)Fe(Prz)(NO)]ClO_4[a]	d^5	10.5	Ruffled	[233]
[(OEP)Fe(PzH)(NO)]ClO_4[a]	d^5	8.0	Ruffled	[233]
{[(OEP)Fe(NO)]$_2$(Prz)}ClO[a]	d^5	4.9	~Planar(Saddled)	[233]
[(TMP)Fe(1-MeIm)$_2$]ClO_4[a]	d^5	1.8	Planar	[234]
[(TMP)Fe(1-MeIm)$_2$]ClO_4[a]	d^5	5.4	~Planar	[234]
[(OEP)Fe(4-NMe_2Py)$_2$]ClO_4[a]	d^5	6.0	~Planar	[234]
[(TMP)Fe(4-NMe_2Py)$_2$]ClO_4[a]	d^5	31.7	Ruffled[c]	[234]
[(TMP)Fe(4-CNPy)$_2$]ClO_4[a]	d^5	24.4	Ruffled[c]	[246]
[(TPP)Fe(4-CNPy)$_2$]ClO_4[a]	d^5	35.6	Ruffled[c]	[235]
[(TPP)Fe(*t*-BuNC)$_2$]ClO_4[a]	d^5	36.8	Ruffled[c]	[237]
[(p-TTP)Fe(2,6-XylylNC)$_2$]ClO_4[a]	d^5	33.8	Ruffled[c]	[236]
[(OEP)Fe(*t*-BuNC)$_2$]ClO_4[a]	d^5	23.2	Ruffled	[237]
[(OETPP)Fe(1-MeIm)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 3)	Saddled	[248]
perp-[(OMTPP)Fe(1-MeIm)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 10)	Saddled	[248]
paral-[(OMTPP)Fe(1-MeIm)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 1)	Saddled	[248]
[(TC_6TPP)Fe(1-MeIm)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 26)	Saddled	[248]
[(OETPP)Fe(4-NMe_2Py)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 28)	Saddled	[247]
[(OMTPP)Fe(4-NMe_2Py)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 35)	Saddled	[248]
[(OMTPP)Fe(4-NMe_2Py)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 7)	Saddled	[248]
[(OMTPP)Fe(*t*-BuNC)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 3)	Saddled	[249]
[(OMTPP)Fe(*t*-BuNC)$_2$]ClO_4[a]	d^5	(mean *meso* displ. = 6)	Saddled	[249]
Fe(II)–L complexes				
[(OEP)Fe(*t*-BuNC)$_2$][a]	d^6 (mean *meso* displ. = 1)	42.1	Saddled	[238]
[(TPP)Fe(Py)(CO)][a]	d^6	5	~Planar	[239]
[(C_2-capTPP)Fe(1-MeIm)(CO)][a]	d^6	10	~Ruffled/Planar[c,d]	[240]
[(OC_3O-capTPP)Fe(1-MeIm)(CO)][a]	d^6	8	~Planar[c,d]	[241]
[(OC_3O-capTPP)Fe(1,2-Me_2Im)(CO)][a]	d^6	7	~Planar[c,d]	[241]
[(10-strapTPP)Fe(1-MeIm)(CO)][a]	d^6	(mean *meso* displ. = 15.5)	Ruffled[c,e]	[242]
[(8-strapTPP)Fe(1-MeIm)(CO)][a]	d^6	(mean *meso* displ. = 29)	Ruffled[c,e]	[242]
[(6-strapTPP)Fe(1-MeIm)(CO)][a]	d^6	(mean *meso* displ. = 44)	Ruffled[c,e]	[242]
[(TMP)Fe(4-CNPy)$_2$][a]	d^6	2.5	Planar	[243]
[(TMP)Fe(3-CNPy)$_2$][a]	d^6	2.6	Planar	[243]
[(TMP)Fe(4-MePy)$_2$][a]	d^6	3.8	Planar	[243]

[a] Ligand abbreviations: *t*-BuNC = *t*-butylisocyanide, ImH = imidazole, 4IPzH = 4-iodopyrazole, 1-MeIm = 1 methylimidazole, PzH = pyrazole, Iz = indazole, Prz = pyrazine, 4-NMe_2Py = 4-dimethylaminopyridine, 4-CNPy = 4-cyanopyridine, 2,6-XylylNC = 2,6-xylylisocyanide, Py = pyridine, 1,2-Me_2Im = 1,2-dimethylimidazole, 3-CNPy = 3-cyanopyridine, 4-MePy = 4-methylpyridine.
[b] Fused six-membered ligand ring may cause crystal packing effects that induce ruffling.
[c] TPPs are always more ruffled.
[d] Superstructure may cause ruffling.
[e] Strap definitely causes ruffling.

[243,244]. It has been suggested [234–236,244] that the reason for the strong departure of the porphyrin ring conformation from planarity in the bis-isocyanide and related complexes of Fe(III) porphyrins is *electronic*, because the d_{xy} unpaired electron does not have proper symmetry to engage in porphyrin → Feπ donation un-

less the porphyrin ring ruffles so that the nitrogen p_z orbitals are twisted away from the normal to the mean plane, as shown in Fig. 11, and thus have a component in the *xy* plane that has proper symmetry to overlap with the d_{xy} orbital. This type of overlap allows significant spin delocalization via π donation from the $a_{2u}(\pi)$ orbital of the porphyrin ring to the hole in the d_{xy} orbital, and gives rise to very large contact shifts at the *meso* positions and very small contact shifts at the β-pyrrole positions of the porphyrin ring [234–236,244], thus yielding unique NMR and EPR spectra for all metalloporphyrins with $(d_{xz}, d_{yz})^4(d_{xy})^1$ electron configurations. Since the nitrophorin–NO complexes are even-electron systems, no EPR signal is observed, and it is thus not possible to determine what the electron configuration of the metal actually is. *If* it were $(d_{xz}, d_{yz})^4(d_{xy})^1$, then the unpaired electron of NO would be in an orbital that is orthogonal to the metal d_{xy} orbital, and there could therefore be no direct bonding interaction between the two unpaired electrons. Thus, the NO unpaired electron would be isolated on the NO unit in the ruffled heme complex, which would allow facile bending of the Fe–NO bond, possibly contributing to the nonlinearity of the NP4–NO unit in the latest structure (Fig. 6) [103]. The Fe(d_{xy}) and NO$^{\cdot}$ unpaired electrons would therefore, because of their proximity in space, simply have to be magnetically coupled, either antiferromagnetically (antiparallel spins) or ferromagnetically (parallel spins). Mössbauer spectroscopic investigations show that for both model ferriheme–NO complexes that undoubtedly have quite planar porphyrin cores in frozen solution [259] and the highly ruffled NP2–NO and NP4–NO complexes, also in frozen solution [260], the spectra are those of fully electron-paired "diamagnetic" species, and whether those electrons are truly paired, as in a Fe(II)–NO^+ electron configuration, or strongly antiferromagnetically coupled, as in a Fe(III)(d_{xy})–NO$^{\cdot}$ configuration, cannot be differentiated from the Mössbauer data. If the latter were the case, then this antiferromagnetically coupled configuration of Fe(III)–NO would allow more facile departure of NO upon dilution of the protein into the tissues of the victim than would the valence tautomer, Fe(II)–NO^+, which has long been believed to be the likely electron configuration of the $\{FeNO\}^6$ centers, based on infrared spectroscopic data [134], as mentioned above. However, both the original review of Feltham and Enemark [65], as well as a recent chapter by Westcott and Enemark [261], emphasize the fact that the $\{FeNO\}^n$ triatomic fragment is highly covalent, and thus assigning electrons to the metal and NO may be meaningless, except for those die-hard scientists such as the author who want to know the exact orbital occupation and possible coupling mechanism involved.

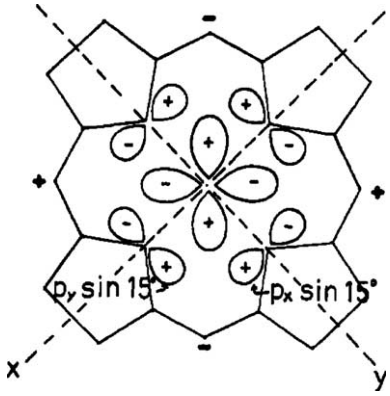

Fig. 11. Possible interactions of the d_{xy} metal orbital with the porphyrin nitrogens in the case of strong S_4 ruffling. The remaining nitrogen pπ projections have the proper symmetry to allow delocalization via the $3a_{2u}(\pi)$ porphyrin orbital. Reprinted from [234] with permission of the American Chemical Society.

DFT calculations on the $\{FeNO\}^6$ nitrophorin centers do not allow "visualization" of the possibility of the existence of the antiferromagnetically coupled Fe(III)–NO$^{\cdot}$ electron configuration, because the even number of electrons and apparent diamagnetism of the system leads to the calculations assuming complete pairing of the electrons. In all calculations, whether for the ruffled porphyrin core observed for the NP4–NO complex, an optimized planar core, or an optimized planar core that is "relaxed" to allow ruffling, the $3a_{2u}(\pi)$ porphyrin orbital is the HOMO, but it contains no significant amount of metal character in any of these cases [260]. However, the larger Mössbauer quadrupole splitting observed for NP2–NO (1.84 mm s^{-1}) [260] as compared to that of NP4–NO (1.61 mm s^{-1}) [260] and a model heme complex, $[OEPFe^{III}(NO)(1\text{-MeIm})]^+$ (1.61 mm s^{-1}) [259] can only be calculated if it is assumed that the heme is very ruffled [260].

Since the NP4–NH_3 complex, which has the open conformation for the loops surrounding the distal heme pocket, is also somewhat ruffled (RMSD = 0.138 Å, Table 1), it is tempting to speculate that the leucines pointing toward the heme in the distal pocket predispose all nitrophorins to be ruffled, thus stabilizing the $(d_{xz}, d_{yz})^4(d_{xy})^1$ ground state for Fe(III). However, both the NP4-NH_3 and the NP4-CN^- complexes have totally normal "large g_{max}" EPR signals at $g = 3.41$ and 3.28, respectively [262], indicating that both of these complexes have $(d_{xy})^2(d_{xz}, d_{yz})^3$ ground states, yet the cyanide complex is more ruffled and the NH_3 complex is much less ruffled than the NO complex, and both have open loop conformations. But these facts do not tell us about the NP4–NO or NP2–NO complex electron configuration. The NP4–histamine complex, which also has the open loop conformation, is significantly ruffled, considering the fact that the dihedral angle between the ligand planes of the proximal histidine and histamine imidazoles is approximately 29°, which is much closer to parallel than to perpendicular orientation of the axial ligands, and its EPR spectrum is that of the normal

rhombic type ($g = 2.92$, 2.24, 1.52) [263]. Thus, one might have expected this complex to have a nearly planar heme, as do those of $[(TMP)Fe(1\text{-}MeIm)_2]^+$ and $[(OEP)Fe(4\text{-}NMe_2Py)_2]^+$ [233]. The imidazole complex, which has the closed loop conformation, is among the least ruffled complexes of NP4, and the 4-iodopyrazole complex, which has a half-closed loop conformation, has the very least ruffled ring conformation among all NP4 complexes (Table 1). Perhaps the most enigmatic NP4-L complex is that where L = *tert*-butylisocyanide, which shows an RMSD (0.145 Å) [264] not much different from the NH_3, ImH and histamine complexes, in spite of the large ruffling of the bis-*t*-BuNC complexes of OEPFe(III) and TPPFe(III) [236]. However, in the protein-bound complex, the axial *t*-butylisocyanide ligand binds very far off the heme normal [264], and it is clear that the binding of this ligand to the heme of NP4 is highly hindered. Thus no major conclusions can be made with regard to the much smaller RMSD of this complex than expected. Nevertheless, the NMR and EPR spectra of this complex are both consistent with a $(d_{xz}, d_{yz})^4(d_{xy})^1$ electron configuration [264].

For NP2-L complexes, as mentioned above, all are more ruffled than the NP4-L counterparts (Table 1). NP2-H_2O, for example, is more ruffled (RMSD = 0.250 Å) than NP4–NO, and NP2–NO is yet more ruffled (RMSD = 0.285 Å); both L122,132V mutant complexes are less ruffled (0.208 and 0.246 Å, respectively) than the wild type protein counterparts [227]. Clearly, the ruffled nature of the heme of NP2 and NP4 exists for all ligand complexes, and mutating the two leucines that are within van der Waals contact of the heme to valines causes only a modest decrease in ruffling [227].

It has been said that nonplanar porphyrins are easier to oxidize and more difficult to reduce than planar porphyrins, but the data on which this statement is based involves only (β-bromo)$_{1-8}$tetraphenylporphyrin complexes of Fe(III)Cl [265–267] and Co(II) [268]. While the proper controls appear to have been done to separate the inductive effects of the halogens from the steric crowding effects, leaving still a large residual difference between predicted and observed oxidation potentials [265] that is consistent with the conclusion stated above, the type of nonplanarity of the porphyrinate complexes having 5–8 bromo substituents on the β-pyrrole positions is *saddled* rather than *ruffled*. Unfortunately, it is the *ruffled* conformation which would be more appropriate for comparison to the current work on NP2–NO and NP4–NO, and only one recent study of the reduction potentials of ruffled vs. planar hemes has been carried out that has been able to factor out the effects of heme substituents [274]. In that study, the reduction potentials of imidazole or N-methylimidazole complexes of model ferrihemes with varying documented tendencies to have ruffled, planar or saddled porphyrinate ring conformations were compared to those of the corresponding 2-methylimidazole complexes. The rationale for that study was that while the imidazole or N-methylimidazole ligands could bind to all of the iron porphyrins studied in parallel planes in both oxidation states, thus encouraging a planar ring conformation [233,242], the 2-methylimidazole ligands would be required to bind in perpendicular planes in both oxidation states, which would encourage ruffling of the porphyrin ring [269–271,274]. It was found, as summarized in Table 2, that all iron(III) porphyrinates that could readily ruffle when the hindered 2-methyimidazole ligands were bound, including three $[(2,6\text{-}X_2)_4TPPFeL_2]^{+/0}$ complexes (X = Cl, Br or CH_3) [271], have half-wave potentials ($E_{1/2}$) for the Fe(III)/Fe(II) redox step that are more negative when the ligands L are 2-methylimidazole than when they are imidazole or N-methylimidazole. Since $\Delta G = -nFE$, a negative shift in potential indicates a stabilization of the oxidized form. The same was found to be true of the iron complex of tetra-isopropylporphyrin [274], which is known to be very ruffled [272,273]. How-

Table 2
Reduction potentials for iron porphyrinates with imidazole ligands[a]

Porphyrin	Core type	Ligand		
		ImH[b] $E_{1/2}(Fe^{III}/Fe^{II})$ (mV)	2-MeImH[b] $E_{1/2}(Fe^{III}/Fe^{II})$ (mV)	"2-MeImH-ImH" $E_{1/2}(Fe^{III}/Fe^{II})$ (mV)
FeOETPP	Saddled	−430[c]	−400[c]	+30[c]
FeOMTPP	Saddled	−396[c]	−290[c]	+106[c]
$FeTC_6TPP$	Saddled(Ruffled)	−387[c]	−300[c]	+87[c]
FeTiPrP	Ruffled or Planar	−368[c]	−417[c]	−59[c]
		−343[e]		−74[c,e]
FeTMP	Ruffled or Planar	−169[c]	−212[d]	−43[c]
		−130[d,e]		−82[d]
$Fe(2,6\text{-}Cl_2)TPP$	Ruffled or Planar	−3[d,e]	−115[d]	−118[d]
$Fe(2,6\text{-}Br_2)TPP$	Ruffled or Planar	−40[d,e]	−131[d]	−91[d]

[a] Measured in dimethylformamide. Potentials tabulated vs. SCE, mV.
[b] ImH = imidazole, 2-MeImH = 2-methylimidazole.
[c] Reference [274].
[d] Reference [271].
[e] 1-MeIm instead of ImH.

ever, for three iron octaalkyltetraphenylporphyrin complexes, where the dominant nonplanar distortion is saddled rather than ruffled, it was found that the 2-methylimidazole complexes have more positive reduction potentials for the Fe(III)/(II) couple than do the imidazole complexes [274], thus indicating that for porphyrins that cannot adopt ruffled ring conformations, the binding of axial ligands to Fe(II) in perpendicular planes is not so unfavorable as it is for those that readily adopt ruffled ring conformations.

Thus, in all cases, the oxidized state of *ruffled* heme complexes is favored over those of planar heme complexes, but by different amounts, depending on the nature of the substituents. This is probably because Fe(II) does not appear to favor a ruffled heme core [242–244]. Beyond this correlation, however, studies of additional carefully-designed model heme systems, specifically designed to test the hypothesis of the effect of heme ruffling on stabilization of the oxidized (ferric) state, should be carried out. Nevertheless, it appears possible that provision of a ruffled heme at least aids in maintaining the iron in the ferric state and preventing autoreduction of the Fe(III)NO complex. Steric contacts with protein residues force the heme to be ruffled, thus favoring a ferric state for the iron in all ligand complexes, most importantly the NO complex. The closure of the mobile loops that form a hydrophobic pocket around the NO create a tightly constrained and desolvated NO binding site [102], which may further discourage reduction, since water can assist in this reaction [205].

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