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Electron Spin Resonance Spectra of Penta- and Hexacoordinated Nitrosyl Iron Protoporphyrin IX Complexes

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Abstract. Electron spin resonance (ESR) spectra of frozen aqueous solutions of NO · haem · base complexes and NO · haem intercalated into dodecyl sulfate micelles have been measured at 77 K and analyzed for the hyperfine components of ^{15}NO, ^{14}N-base, ^{14}N-pyrroles and ^{57}Fe which coincide with the principal directions of the g tensor. The influence of the basicity of the nitrogen base on the spin distribution and geometry of the Fe-N-O grouping has been demonstrated by replacing imidazole for pyridine and by comparing the ESR spectra with those obtained for the monomeric insect haemoglobin CTT IV.

The comparison of the hyperfine parameters described for the so-called pentacoordinated nitrosyl complex of CTT IV with those of the NO · haem intercalated into detergent micelles has furnished evidence that the ESR spectrum of this conformation state of haemoglobin has to be definitely assigned to a pentacoordinated nitrosyl complex.

The a_{zz} values increase with the following orders: CTT IV (2.98 mT) < imidazole complex (3.04 mT) < pyridine complex (3.15 mT) for ^{15}NO, and pyridine complex (0.59 mT) < imidazole complex (0.67 mT) < CTT IV (0.70 mT) for the ^{14}N-base. This result is in conformity with an increase of the σ donor and the π acceptor strengths of the nitrogen base in trans-position to ^{15}NO. The a_{yy} and a_{xx} components of ^{15}NO and the ^{14}N-base are strongly nonequivalent in the nitrosyl haemoglobin CTT IV, and less nonequivalent in the NO · haem · pyridine complex, indicating bending of the Fe-N-O grouping. The hyperfine components of the axial ligands coinciding with the x and y component of the g tensor are nearly equal for the NO · haem · imidazole complex.

Key words: Electron spin resonance – Nitrosyl-haem complexes – Coordination state – Hyperfine constants

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Introduction

On the basis of electron spin resonance (ESR) spectra two coordination states of the nitrosyl haem complexes can be differentiated [1–6]. The ESR spectra of the "pentacoordinated" and the hexacoordinated nitrosyl haemoglobins have been assigned as type I and type II spectra respectively (for a more detailed description see references [3, 4, 14]). By changing the protein structure of haemoglobins these two coordination states of the nitrosyl haem group can be reversibly and mutually interconverted [4, 5, 7–13]. Furthermore, for each coordination state particular structures and particular conformations of the nitrosyl haemoglobin are reflected by typical hyperfine structures of the ESR spectra [14]. The binding geometries and the bond distances of the axial ligands of the haem iron, i.e., of NO and of proximal imidazole, depend on the nature of the haem protein (special haem environment) and are modified by conformational transitions ($t \rightarrow r$ tertiary structure and $T \rightarrow R$ quaternary structure changes). In haem proteins the NO-binding properties are influenced by the basicity and the binding geometry of the proximal imidazole in *trans*-position as well as by direct interactions of the NO with protein side groups [3–5, 7, 14, 15].

Simple haem complexes, i.e., without any protein environment but with different degrees of basicity at the 5th coordination site, should be excellent model systems to investigate the trans-effect of the axial ligands. Such simple model compounds have already been investigated [1, 2, 6, 16–18]. However, because of the insufficient resolution of the ESR spectra, hyperfine parameters for these nitrosyl complexes could not be evaluated with high accuracy and therefore, only qualitative assumptions on the spin distribution and the geometry of the Fe-N-O grouping could be made.

In this paper we report on the ESR investigations of three NO-haem model complexes, i.e., NO-haem-imidazole, NO-haem-pyridine and NO-haem. ESR frozen solution spectra have been analyzed for hyperfine constants of the iron and its six ligands. Furthermore, the ESR parameters of the nitrosyl-haem-imidazole complex which is free of protein-structural constraints has been compared with those recently described for a monomeric nitrosyl haemoglobin [14], which contains no distal histidine but which nevertheless may exert constraints on the NO-ligand via non-polar interactions produced by side groups of the protein pocket. The analysis of the hyperfine constants has been facilitated by the substantial resolution enhancement with the Fourier-transform technique [19]. This procedure has been used to transform low-amplitude modulated experimental first derivative spectra into the higher derivatives which exhibit much better resolution of the hyperfine pattern than the experimental higher derivatives and to simulate ESR spectra for comparison with experimental spectra.

A more exact knowledge of the complete set of hyperfine components which latter coincide with the three principal directions of the g tensor, could be helpful in future for quantum-mechanical calculations on nitrosyl haem complexes and nitrosyl haemoglobins. The results obtained with pure NO-haem model complexes may lead to a better understanding of the mechanism of protein-ligand interactions in allosteric haemoglobins.

Materials and Methods

Protohemin Chloride

Protohemin chloride in crystalline form prepared from bovine haemoglobin has been purchased from Sigma Chemie (München). The material checked for purity by thin-layer chromatography [20] and by NMR spectroscopy of the bis(cyano)-protohaemin [4] has been used without further purification.

^{57}Fe-Protohemin Chloride

The preparation of ^{57}Fe-protohemin chloride has been performed under exactly anaerobic conditions as already described [4]. Special care has been taken to avoid an admixture of grease to the final product. The purity has been checked as described in the chapter "Protohemin Chloride". Protoporphyrin IX disodium salt has been purchased from Sigma Chemie (München).

Mohr's salt enriched with ^{57}Fe has been prepared from $^{57}Fe_2O_3$ (isotopic enrichment of 80%). The reduction of $^{57}Fe_2O_3$ to metallic iron has been performed by annealing for 10 h at 1000° C under a stream of hydrogen gas in a quartz tube. The completion of the reduction process has been controlled by weighing. Then 100 mg ^{57}Fe (metal) has been dissolved in 1.8 ml 1 M H_2SO_4 at 90° C by reflux boiling for about 40 h. To this solution 237 mg $(NH_4)_2SO_4$, dissolved in 0.5 ml H_2O, were added. In a desiccator the ^{57}Fe-enriched Mohr's salt crystallized while water has been absorbed by P_2O_5. The exact amount of constitutional water has been obtained if P_2O_5 was replaced by H_2O for 12 h and then by Mohr's salt for 3 days. The yield of pure ^{57}Fe-enriched Mohr's salt was about 50% of the used ^{57}Fe metal.

NO-Haem-Base Complexes

The nitrogen bases, coordinated to the NO-haem complex, were imidazole and pyridine respectively. Both substances were products of Merck (Darmstadt, Germany). Hemin chloride (final concentration: 2.5 mM) has been dissolved in aqueous NaOH solution at pH 10. To this hemin chloride solution the base was added until the final base concentration was 3 M. Then under anaerobic conditions 3 mg $Na_2S_2O_4$ (Merck, Darmstadt) and after that 15 mg $Na^{15}NO_2$ with 95% isotopic enrichment (Amersham-Buchler, Braunschweig), per ml of the haem-base solution were added. No precipitation occured during this procedure. The solution of the NO-haem-base complex immediately has been anaerobically filled into quartz tubes and frozen in liquid nitrogen.

NO-Haem Complexes

An alkaline aqueous hemin chloride solution (final concentration: 5.0 mM) has been reduced by adding 3 mg/ml $Na_2S_2O_4$. Then an equal volume of an aqueous solution containing 60 mg/ml sodium dodecyl sulfate (Henkel, Düsseldorf) and

30 mg/ml $Na^{15}NO_2$ has been added under anaerobic conditions. Under these conditions the NO-haem complex is intercalated as a monomer into micelles of sodium dodecyl sulfate [22]. The formation of dimers of the NO-haem complex has been followed by ESR measurements which indicated line-broadening. Immediately after mixing of the above mentioned solutions the samples have been filled into quartz tubes and frozen in liquid nitrogen.

Electron Spin Resonance Spectroscopy

The ESR spectra were recorded at 77 K with an X-band spectrometer (Type ER 420, Bruker Analytische Meßtechnik, Karlsruhe, Germany). The conditions of the instrumental setting and the techniques used have been already described elsewhere [14]. Quartz tubes of 3.3 mm inner diameter and anaerobically sealed have been used.

Resolution Enhancement and Simulation of Electron Spin Resonance Spectra

The resolution enhancement of ESR spectra has been carried out by transforming experimental low-amplitude modulated first derivative spectra into higher derivatives by means of the Fourier-transform technique [19]. The higher derivatives of the spectra gained by this computational technique, exhibited oscillations and noise components of the base line. In order to distinguish these noise components from ESR hyperfine patterns with small splittings and low intensities of the lines, two procedures have been performed:
i. A change of the truncation (smoothing) function led to changes in the shape but not in the magnetic field position of these hyperfine lines. On the other hand, the noise lines showed shifting in position and changing of their linewidth.
ii. When applying a rectangular function (i.e., no smoothing) the number of Fourier-transform components, which are used for the inverse transformation, can be varied. If, by applying this process, peaks do not shift within the limit of resolution, we can assume that these peaks represent a real ESR signal.

The simulation of ESR powder spectra is based on a non-quantum mechanical procedure [19]. The linewidths have been assumed to be equal for all principal directions of the g tensor because of the relatively small differences in the g values. By comparison of calculated and experimental spectra the linewidth of 0.265 mT has been determined for the z direction which already shows a well-resolved hyperfine pattern in the first derivative spectrum. Nevertheless, this linewidth does not represent the natural linewidth, because unresolved hyperfine structures, i.e., from haem protons, can contribute to the linewidth. We refined the computational algorithm described and used earlier [14, 19, 21], so that the appearent linewidth appears now to be close to the natural linewidth. It has to be stated further that the appearent linewidths used in both computational algorithms had no influence on the hyperfine parameters.

Results

Electron Spin Resonance of ^{15}NO · Haem · Base Complexes

At high pH (pH > 9.5) stable NO · haem · base complexes are formed. The ESR spectra of these complexes, measured at 77 K, are characteristic for a spin system with S = 1/2 and for rhombic symmetry (Figs. 1 and 2). These spectra can be classified as typical type II demonstrating, in addition to the doublet hyperfine splitting of the axially bound ^{15}NO, a triplet hyperfine splitting of the ^{14}N-base the latter being the axial ligand in trans-position to NO. Therefore, the ESR spectra furnish evidence of the hexacoordinated state of these nitrosyl haem complexes.

When imidazole at the 5th coordination site of the haem iron is replaced by pyridine the ESR spectrum remaines type II but changes with regard to the g tensor as well as to the hyperfine constants (Figs. 1 and 2). The three principal g tensor components, g_{xx}, g_{yy}, g_{zz} and the hyperfine components assigned to ^{15}NO and the ^{14}N-base which both coincide with g_{zz} have been determined from the

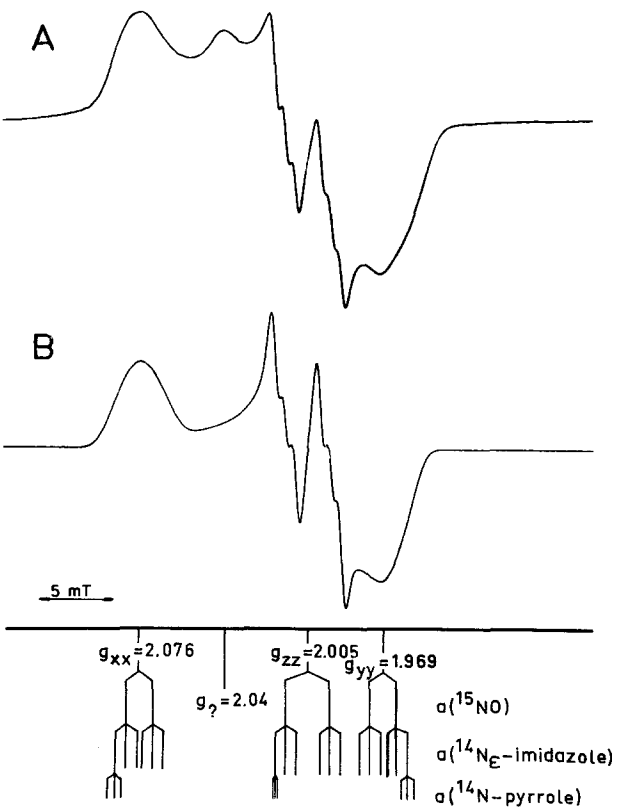


Fig. 1. ESR experimental **(A)** and simulated **(B)** first derivative spectra of the ^{15}NO · haem · imidazole complex. Solvent: H_2O/NaOH pH 10; concentrations: 2.0 mM haem, 3 M imidazole; temperature: 77 K. The simulation has been performed without the $g_?$ = 2.04-species

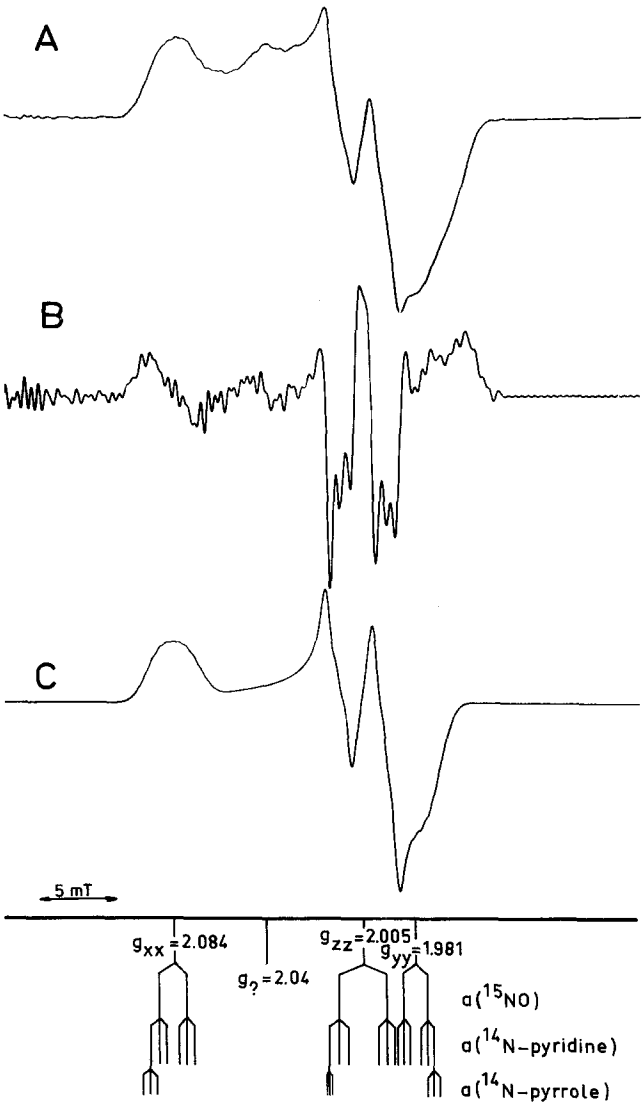


Fig. 2. ESR experimental **(A)** and simulated **(C)** first and resolution-enhanced **(B)** second derivative spectra of the ^{15}NO · haem · pyridine complex. Solvent: H_2O/NaOH pH 10; concentrations: 2.0 mM haem, 3.1 M pyridine; temperature: 77 K. The simulation has been performed without the $g_?$ = 2.04-species

first derivative spectrum (Table 1). Furthermore, an additional signal with $g_?$ = 2.04 can be observed (Figs. 1 and 2).

The hyperfine components coinciding with the g_{xx} and g_{yy} principal directions have been obtained by comparing the simulated spectra with the resolution-enhanced experimental spectra (Fig. 2). Furthermore, hyperfine constants for four equivalent ^{14}N-pyrroles have been evaluated by fitting simulated and experimental spectra. The hyperfine data are compiled in Table 1.

Table 1. ESR parameters of the $^{15}NO \cdot$ Haem $\cdot$ Base complexes at 77 K

Direction		g value	a (^{15}NO) [mT]	a (^{14}N-base) [mT]	a (^{14}N-pyrole) [mT]
x	Δ	2.076 ± 0.001	1.79 ± 0.08	0.75 ± 0.05	0.46 ± 0.03
	✲	2.084 ± 0.001	1.84 ± 0.08	0.49 ± 0.05	0.48 ± 0.03
y	Δ	1.969 ± 0.002	1.84 ± 0.08	0.76 ± 0.05	0.41 ± 0.03
	✲	1.981 ± 0.003	1.62 ± 0.08	0.44 ± 0.05	0.43 ± 0.04
z	Δ	2.005 ± 0.0001	3.04 ± 0.02	0.67 ± 0.02	0.08 ± 0.02
	✲	2.005 ± 0.0001	3.15 ± 0.02	0.59 ± 0.02	0.10 ± 0.02

The bases at the 5th coordination site of the haem iron are Δ imidazole and ✲ pyridine

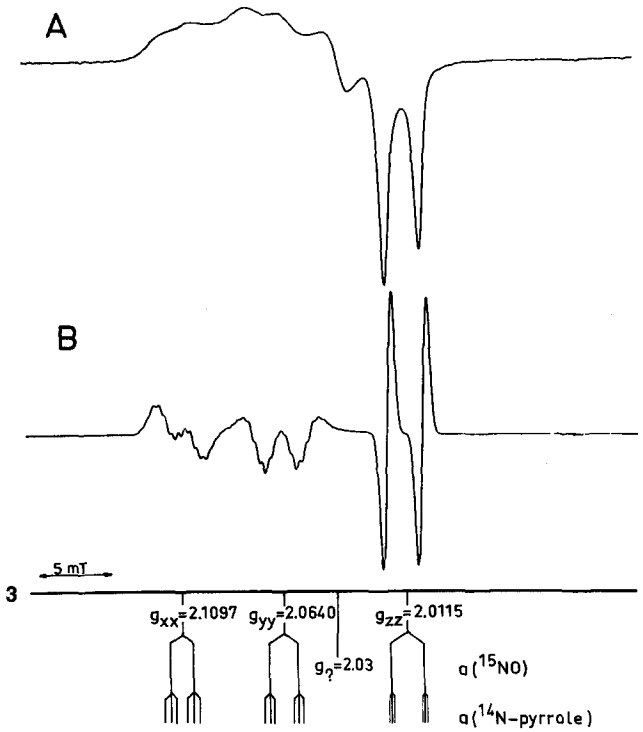
Electron Spin Resonance of $^{15}NO \cdot$ Haem Complexes intercalated into Detergent Micelles

In an aqueous alkaline solution of anionic detergents NO · haem complexes are intercalated into micelles formed by the detergent molecules. In this solution no nitrogen base is present. Therefore, we have to expect a pentacoordinated nitrosyl haem complex. Although the haem concentration is relatively high, the detergent micelles prevent the NO · haem complexes from dimerization. The polymerization leads to a line-broadening via dipolar magnetic interaction of the paramagnetic centres.

At 77 K the frozen solution spectra of the NO · haem complexes exhibit rhombic symmetry. No triplet hyperfine splitting of the axial nitrogen base is observed (Figs. 3 and 4). Therefore, these ESR spectra are of type I and are indicative for "pentacoordinated" nitrosyl complexes. The principal g tensor components g_{xx}, g_{yy} and g_{zz} as well as the components of the ^{15}NO hyperfine splitting constants which coincide with the g tensor components are determined directly from the experimental first derivative spectrum and the resolution-enhanced high-derivative spectra (Table 2). The respective components of the ^{14}N-pyrrole hyperfine constants have been determined by fitting simulated spectra to resolution-enhanced experimental spectra.

The replacement of the natural iron in NO · haem by ^{57}Fe leads to a splitting of all resonances into doublets due to the nuclear spin I = 1/2 for ^{57}Fe. This ^{57}Fe splitting is obviously demonstrated by the first derivative spectra for the nitrosyl doublet lines coinciding with g_{zz} with a splitting constant of a_{zz} (^{57}Fe) = 0.67 mT (Fig. 4). However, the resolution of the hyperfine pattern coinciding with g_{xx} and g_{yy} does not allow a direct evaluation of the splitting constants for ^{57}Fe and ^{14}N-pyrroles. For the simulation of the ESR spectra the hyperfine constants, already determined for the natural NO · haem complex, have been used. Comparison of simulated and experimental spectra yields equivalent hyperfine constants of a (^{57}Fe) = 0.88 mT and a (^{14}N-pyrrole) = 0.40 mT for both principal directions x and y of the g tensor (Table 2). Furthermore, the four pyrrole nitrogens have been assumed to be equivalent.

Again, the signal at $g_? = 2.04$ which does not seem to belong to the nitrosyl · haem complex investigated here has not been considered in the simulations.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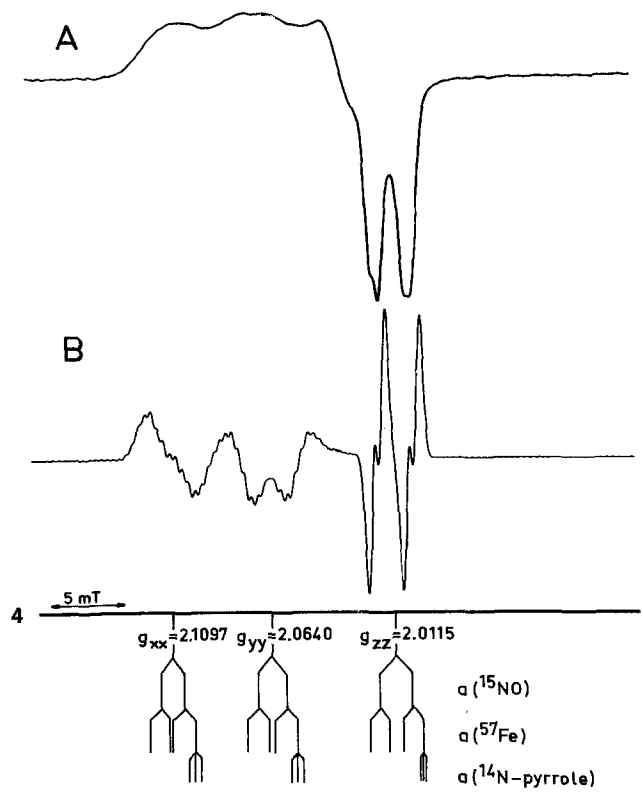
g_{zz}=2.0115
a(^{15}NO)
a(^{14}N-pyrrole)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g_{xx}=2.1097
g_{yy}=2.0640
g_{zz}=2.0115
a(^{15}NO)
a(^{57}Fe)
a(^{14}N-pyrrole)

Table 2. ESR parameters of $^{15}NO \cdot$ Haem complex, intercalated into dodecylsulfate micelles, at 77 K

Direction	g value	a (^{15}NO) [mT]	a (^{14}N-base) [mT]	a (^{14}N-pyrole) [mT]
x	2.1097 ± 0.0003	1.70 ± 0.05	0.88 ± 0.05	0.40 ± 0.02
y	2.0640 ± 0.0005	2.05 ± 0.05	0.88 ± 0.05	0.41 ± 0.02
z	2.0115 ± 0.0002	2.38 ± 0.02	0.67 ± 0.02	0.08 ± 0.02

Discussion

Coordination States of Nitrosyl · Haem Complexes in Protein-Free Solutions and in the Monomeric Insect Haemoglobin IV

The ESR investigations of nitrosyl haem in nitrogen base-free aqueous solutions and of nitrosyl · haem · base complexes allow a nondisprovable assignment of the two spectral types, I and II, to the penta- and hexacoordinated state respectively. On the other hand, the well-resolved ESR type I spectra of the monomeric nitrosyl CTT IV did not allow a final decision, whether the type I spectra observed in this haemoglobin are indicative for a pentacoordinated state of the haem iron or for a hexacoordinated state with a very weak imidazole-iron interaction [4, 7, 14]. Therefore, a comparison of the hyperfine constants evaluated for the NO · haem model complex with those determined for the nitrosyl CTT IV should give clear evidence for one of the two possibilities of proximal imidazole-interaction in haemoglobins. On the basis of the hyperfine parameters determined for ^{15}NO-, ^{57}Fe and ^{14}N-pyrroles and the principal g values (compare Table 2 of reference [14]) we now can state, that the type I spectra of both nitrosyl complexes are identical, and therefore, nitrosyl CTT IV, which is structurally modified by detergent association, has to be pentacoordinated.

Geometry of the Fe-N-O Grouping of the Hexacoordinated Haem Group in Protein-Free Solutions and in the Monomeric Insect Haemoglobin IV

The replacement of imidazole for pyridine in the hexacoordinated nitrosyl haem complex reveals differences in the hyperfine interactions which reflect the influence of the basicity on the geometry of the Fe-N-O grouping. Pyridine with

Fig. 3. ESR experimental first **(A)** and simulated second **(B)** derivative spectra of the $^{15}NO \cdot$ haem complex intercalated into detergent micelles. Solvent: H_2O/NaOH pH 10; concentrations: 2.5 mM haem, 0.1 M sodium dodecylsulfate; temperature: 77 K. The simulation has been performed without the $g_?$ = 2.04-species

Fig. 4. ESR experimental first **(A)** and simulated second **(B)** derivative spectra of the $^{15}NO \cdot {}^{57}Fe$-haem complex intercalated into detergent micelles. Solvent: H_2O/NaOH pH 10; concentrations: 2.5 mM haem, 0.1 M sodium dodecylsulfate; temperature: 77 K. The simulation has been performed without the $g_?$ = 2.04-species

its less basicity is not as strongly bound to the haem iron as imidazole [23]. Consequently, when pyridine is replacing imidazole, we find a significant decrease of $a_{zz}(^{14}N\text{-base})$. However, pyridine is also a weaker π acceptor than imidazole, so that a ligand in *trans*-position, i.e., NO, is more weakly bound, as indicated by an increase of $a_{zz}(^{15}NO)$. The imidazole of the proximal histidine F8 plays an important role as a trigger for the allosteric O_2-binding in haemoglobins [24]. Therefore, the proximal histidine is one of the few invariant amino acids in haemoglobins.

The σ donor and π acceptor strengths of the proximal imidazole may be modified by the protein structure [24]. In the native nitrosyl CTT IV two conformations correspond to two ligand affinity states with different imidazole-haem interactions [14, 21]. When comparing the σ donor strengths of the nitrogen bases of the three nitrosyl-haem-base configurations mentioned above, the hyperfine component a_{zz} follows the order:

i. CTT IV (2.98) < imidazole (3.04) < pyridine (3.15) for $a_{zz}(^{15}NO)$ and

ii. pyridine (0.59) < imidazole (0.67) < CTT IV (0.70) for $a_{zz}(^{14}N\text{-base})$.

The hyperfine splitting constants in mT are given in brackets. The spin distribution from the n orbital of NO into the d_{z^2} orbital is different for all three haem complexes and is smallest for the pyridine complex and largest for the CTT IV. This correlates with an increased spin-orbit coupling in CTT IV, which is smaller in the protein-free imidazole complex as indicated by the low-field shift of g_{xx} from 2.078 (protein-free imidazole complex) to 2.087 in CTT IV. Therefore, one can conclude that the structural constraints exerted by the protein environment leads to a closer imidazole-iron distance with an increased back-donation from the iron to the imidazole or to a distortion of the imidazole-haem grouping in CTT IV.

The inequivalence of the hyperfine constants measured in g_{xx} and g_{yy} for ^{15}NO and the ^{14}N-base, respectively, is an indicator for the decrease in symmetry due to the bending of the axial ligands. The inquivalence of the in-plane a tensor components, $(a_{xx}-a_{yy})$, for both, ^{15}NO and ^{14}N-base, decreases in the following order: CTT IV > pyridine > imidazole. Thus, the protein-free NO · haem · imidazole complex seems to be more symmetric with a nearly linear Fe-N-O grouping and the normal of the imidazole ring is parallel to the x or y in-plane axis. On the contrary, CTT IV shows the largest inequivalence of a_{xx} and a_{yy} indicating a more pronounced deviation of the Fe-N-O grouping from linearity and an imidazole normal not coinciding with the in-plane axis. In the NO · haem · pyridine complex the deviation of the Fe-N-O grouping from linearity should be relatively small.

Both, strong iron-base interaction and strong bending of NO, remove the degeneracy of the d_{xz} and d_{yz} orbitals. This results in the above mentioned inequivalence of the x and y components of the ^{15}NO hyperfine tensor. Whereas in CTT IV the ^{15}NO hyperfine constant coinciding with g_{yy} is 30% larger than that coinciding with g_{xx}, in the case of the NO · haem · pyridine complex $a_{yy}(^{15}NO)$ is about 10% smaller than $a_{xx}(^{15}NO)$. In the NO · haem · imidazole complex both hyperfine constants are practically equivalent. It follows, that the NO ligand must be bent to the x direction of the haem plane in the case of CTT IV, but to the y direction in the case of the pyridine complex. The bending of NO

in the pyridine complex should be similar to that expected for the pentacoordinated NO · haem complex where $a_{yy}(^{15}NO)$ is 17% larger than $a_{xx}(^{15}NO)$. In cytochrome c oxidase with a hexacoordinated NO · haem · imidazole grouping, the a_{xx} and a_{yy} values for ^{15}NO are identical [19].

Principally, in haemoglobins the trans-effect of the nitrogen base on the nitrosyl ligand is much more distinct than in the protein-free imidazole complex. This is reflected by the high spin density on the $^{14}N_{\varepsilon}$-imidazole as well as the strong bending of the Fe-N-O grouping. A specific influence of the protein moiety on the nitrosyl ligand in haemoglobins originates not only from the proximal imidazole (*trans*-effect) but also from direct interactions with amino acid side groups of the nearest environment (*cis*-effect). Conformation changes in CTT IV can alter the binding geometries of both axial ligands, i.e., the proximal imidazole and NO. In the tetrameric haemoglobin Kansas the nonequivalence of α and β chains is reflected by a large difference in the bending of the Fe-N-O grouping which varies between 105° and 167° [25]. Furthermore, the spin density on ^{15}NO is higher in the model compounds than in CTT IV. CTT IV has no distal imidazole and therefore, polar interactions with the nitrosyl ligand are impossible. Thus, in this particular haemoglobin any influence on the bending of the Fe-N-O grouping is the result of hydrophobic interactions with isoleucin E11 which is in closest vicinity to the haem iron.

Even the model complexes gain further evidence that the main influence on ligand binding in haemoglobins is controlled by the proximal imidazole. The geometry of the iron-imidazole grouping and the iron-imidazole bond can be modified by conformation changes in haemoglobins.

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