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Structure-Related Changes of the Electron Spin Resonance Spectra of the Monomeric Nitrosyl Haemoglobin IV from *Chironomus thummi thummi*

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Abstract. The monomeric haemoglobin IV from *Chironomus thummi thummi* (CTT IV) is an allosteric protein characterized by pH-dependent ligand affinities (Bohr-effect). The ligand-linked proton dissociation gives rise to a $t \rightleftharpoons r$ conformational transition. Furthermore, the Bohr-effect is ligand-dependent and decreases in magnitude following the order of ligands, $O_2 > CO > NO$. Although the Bohr-effect for NO is smallest, the electron spin resonance (ESR) spectra of frozen solutions of ^{15}NO-ligated CTT IV measured as higher derivatives at 77 K reflect this pH-dependent conformation change. g Tensor and hyperfine constants coinciding with the principal directions of the g tensor have been evaluated for ^{57}Fe, ^{15}NO, $^{14}N_{\varepsilon}$-imidazole, and ^{14}N-pyrroles.

Hyperfine parameters and g values of both conformation states of this haemoglobin, i.e., of the t state at low pH with low ligand affinity and of the r state at high pH with high ligand affinity, are characteristic for a hexacoordinated nitrosyl haem complex. The change in pH leads to a variation of the Fe-N-O bond angle which is larger at high pH (r conformation) than at low pH (t conformation). Furthermore, the spin transfer from NO into iron orbitals is larger at high pH than at low pH. These results are consistent with the assumption that the interaction of proximal imidazole and iron is smaller in the r conformation than in the t conformation.

Binding of anionic detergents to nitrosyl CTT IV causes a conversion of the native (t, r) into a denatured (super-r) structure. The latter, on the basis of hyperfine and g values, apparently contains a pentacoordinated nitrosyl haem complex. Because of the extreme displacement of the proximal imidazole in the super-r structure, the Fe-N-O gouping is nearly linear and a large spin transfer from NO into iron orbitals occurs. Removal of anionic detergents from the protein leads to a full reconversion of the super-r into the native conformations.

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These structure-related changes of hyperfine constants and g tensor further support the assumption that the *trans*-effect of the proximal imidazole is an important link of allosteric interactions in haemoglobins.

Key words: Monomeric *Chironomus* haemoglobin – Nitric oxide binding – Electron spin resonance – Hyperfine constants – Allostery

Introduction

NO-ligated haemoglobins [1–17] and haem model complexes [18–20] have been extensively investigated with respect to their electron spin resonance (ESR) properties. In all cases two distinct types of ESR spectra of the nitrosyl derivatives have been observed at liquid nitrogen temperature. The type II spectrum (the spectral specification refers to references [11, 13]) exhibits a rhombic g tensor and a more or less resolved hyperfine structure coinciding with the central resonance at g_{zz}. The latter is due to the interaction of the unpaired spin with the two axial ligands of the haem iron, i.e., NO and nitrogen base in *trans*-position to NO. If the nitrogen base is not bound to the central iron atom, the still rhombic complex exhibits a type I spectrum characterized by a high-field shift of the g_{zz} resonance and a hyperfine pattern due to nitrosyl only. A displacement of the proximal imidazole, which is the nitrogen base in haemoglobins, can be achieved by a structural change of the protein moiety. This structure change can be generated by the binding of allosteric effectors [21], i.e., Bohr-protons, CO_2 and polyphosphates, or by an interaction of the haemoglobin with detergent molecules [22–25]. As a result of the interaction with detergents, in nitrosyl haemoglobins a pentacoordinated NO-haem complex with an ESR type I spectrum is observed [1].

Whereas most of the nitrosyl haemoglobins exhibit a rather poor resolution of their ESR spectra, the monomeric *Chironomus* haemoglobins, CTT I, III, and IV, show a rather good resolution of the hyperfine structure coinciding with g_{zz}, but a much less resolved hyperfine pattern in g_{yy} and nearly no resolution in g_{xx} [8, 13, 17]. From the ESR first derivative spectra it is very difficult to get accurate information about hyperfine interactions for the x and y directions and to correlate changes of the hyperfine pattern with protein-structural changes. The *trans*-effect has been proposed as a trigger of ligand-binding in allosteric haemoglobins [13]. However, the structure-induced displacement of the proximal imidazole has been described only on the basis of changes of hyperfine components observed parallel to the direction of g_{zz} [8, 13, 28]. The improved experimental and computational techniques to obtain higher derivatives of the ESR spectra and the Fourier transform-assisted analysis [26] led to a substantial resolution enhancement.

In this paper, for a nitrosyl haemoglobin a total set of hyperfine tensor components which coincide with the principal directions of the g tensor is described using resolution enhancement techniques. Subject of our investigations is the monomeric allosteric haemoglobin IV, CTT IV, from *Chironomus*

thummi thummi which exhibits the best resolution compared to others. Since CTT IV is the simplest allosteric haemoglobin (see reference [27] for a more detailed description of allosteric phenomena in CTT IV), its conformational change can be indicated by the change in hyperfine interactions. Finally, up to now it is not clear, if type I spectra in nitrosyl haemoglobins are really due to pentacoordination. High-resolution ESR techniques should allow to see even small interactions of the nitrogen base with the central iron in the socalled "pentacoordinated" NO complex of CTT IV.

Materials and Methods

Preparation of Native and ^{57}Fe-Substituted Haemoglobin IV

Haemoglobin IV (CTT IV), a monomeric component of the lymphe of insect larvae from *Chironomus thummi thummi,* has been purified and checked for chemical homogeneity as described recently [27]. The preparation of globin and the substitution of CTT IV for ^{57}Fe (isotopic enrichment was 80%) has been performed according to [13].

NO Derivatives of Haemoglobin IV

The hexa- and pentacoordinated nitrosyl compounds of CTT IV have been prepared as described in [13].

The reversible transformation of nitrosyl CTT IV from the hexa- into a pentacoordinated state has been performed according to [22, 23]: At 37° C to an alkaline solution of native NO-ligated CTT IV, which has been characterized at 77 K by an ESR type II spectrum, potassium tetradecylsulfate (TDS) has been added up to a molar ratio TDS : CTT IV = 30. Then this haemoglobin solution has been divided into two samples: (i) One sample showed, frozen to 77 K, a pure ESR type I spectrum, indicating the typical structural transformation of the nitrosyl CTT IV. (ii) The other sample was kept at 4° C for 24 h. At this temperature, potassium tetradecylsulfate precipitated and has been removed then from the haemoglobin solution by centrifugation. The supernatant then frozen to 77 K exhibited an ESR type II spectrum indicating the retransformation of the penta- into the hexacoordinated complex.

Electron Spin Resonance Spectra

X-band ESR spectra were recorded at 77 K on a spectrometer (Type ER 420, Bruker Analytische Meßtechnik, Karlsruhe, Germany). Samples of frozen haemoglobin solution were measured in quartz tubes with 3.3 mm inner diameter. The microwave power was attenuated to 6 mW. At this microwave power no saturation was observed. In the first derivative mode the amplitude of the 100 kHz field modulation was 0.02 mT. The second derivative of the ESR

spectra was obtained by using in addition to the 100 kHz field modulation a 50 kHz field modulation with an amplitude of 0.4 mT each. A 1 kHz field modulation with an amplitude of 0.8 mT was additionally applied to perform the third derivative spectra. The microwave frequency was measured with a frequency counter, the magnetic field strength with a nuclear magnetic resonance oscillator.

Q-band ESR spectra were recorded at 100 K on a spectrometer (Type E-9, Varian, Palo Alto, California) with modulation amplitudes of 0.4 mT and a frequency of 35.4 GHz. Quartz tubes with inner diameter of 1.3 mm were used. Since in Q-band experiments the magnetic field strength could not be measured with high accuracy, the g_{xx} and g_{zz} values obtained by X-band ESR have been used as internal standards to calibrate the magnetic field axis of the Q-band spectra. By this procedure g_{yy} has been determined with high accuracy.

Resolution Enhancement by the Fourier-Transform Technique

The resolution enhancement and the simulation of ESR spectra were performed by using the Fourier-transform technique described elsewhere [26]. The low-modulated experimental first derivative spectra have been used as input data to calculate the higher derivatives which exhibit much better resolution of the hyperfine lines than the experimental higher derivatives. For experimentally generated higher derivatives large modulation amplitudes are required, which broaden the hyperfine lines.

The analysis of ESR high-derivative spectra yields more accurate hyperfine constants, which in turn are used as input data for the simulation of ESR spectra. For simulation of randomly oriented ESR spectra with $S = \frac{1}{2}$ a non-quantum mechanical procedure has been used [26]. Experimental and simulated spectra were compared. Finally, the set of hyperfine data has been chosen which exhibited the best fit for all three derivatives. Simulations were performed with an apparent linewidth of 2.2 mT which has been found to be optimal for all spectra under all conditions. The apparent linewidth is due to the computing algorithm used and does not represent the natural linewidth which remains unknown.

Results

Electron Spin Resonance of the Hexacoordinated ^{15}NO Complex of Haemoglobin IV

The ESR spectrum of a frozen solution of ^{15}NO-ligated CTT IV, shown in Fig. 1, is characteristic for a low-spin complex with $S = \frac{1}{2}$ and rhombic symmetry with resonances for the three principal directions (g_{xx}, g_{yy}, g_{zz}). Principally, the electron spin interacts with the nuclear magnetic fields of the six ligands and the central iron atom if substituted for ^{57}Fe. The observed hyperfine interactions

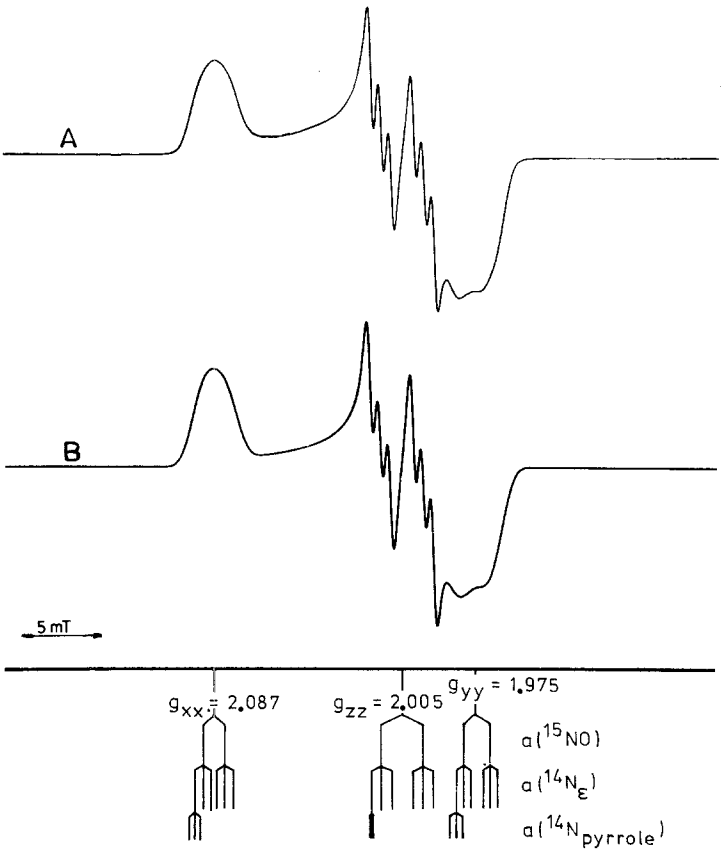

Fig. 1. Experimental (A) and simulated (B) X-band ESR first derivative spectra of the hexacoordinated ^{15}NO-ligated haemoglobin IV. Temperature: 77 K; pH 9.3

reflect nuclear magnetic fields of ^{15}NO and ^{57}Fe with $I = \frac{1}{2}$, and of the four ^{14}N-pyrroles and $^{14}N_{\varepsilon}$-imidazole with $I = 1$.

For the low-field resonance, $g_{xx} = 2.087$ has been determined according to the inflection point of the second derivative spectrum and the minimum of the third derivative (see Fig. 2). This g value, however, does not coincide with the maximum of the first derivative spectrum (see Fig. 2). Whereas the first derivative of the low-field resonance does not exhibit any hyperfine pattern, the second and third derivatives do. Since the higher derivatives clearly demonstrate three hyperfine lines and not two – the latter could be expected as a consequence of the interaction of the spin with ^{15}NO – we must assume that additionally the $^{14}N_{\varepsilon}$-imidazole interaction is reflected by the hyperfine pattern coinciding with g_{xx}. But even from the well resolved higher derivatives no direct and exact determination of any hyperfine constant is possible. Finally, the total set of hyperfine parameters coinciding with the x component of the g tensor is obtained by comparing simulated and experimental spectra in the higher derivative modes.

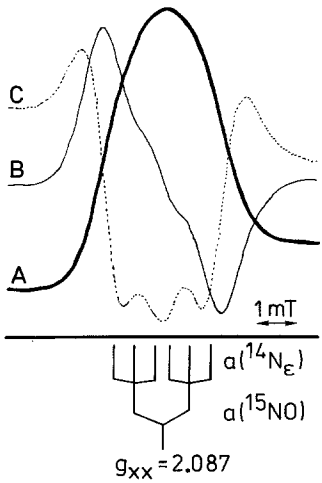

Fig. 2. Low-field resonance (g_{xx}) of the X-band ESR spectrum of the hexacoordinated ^{15}NO-ligated haemoglobin IV. Temperature: 77 K; pH 9.3: A, B, and C refer to the first, second and third derivative respectively, experimentally obtained by the modulation technique (see Methods)

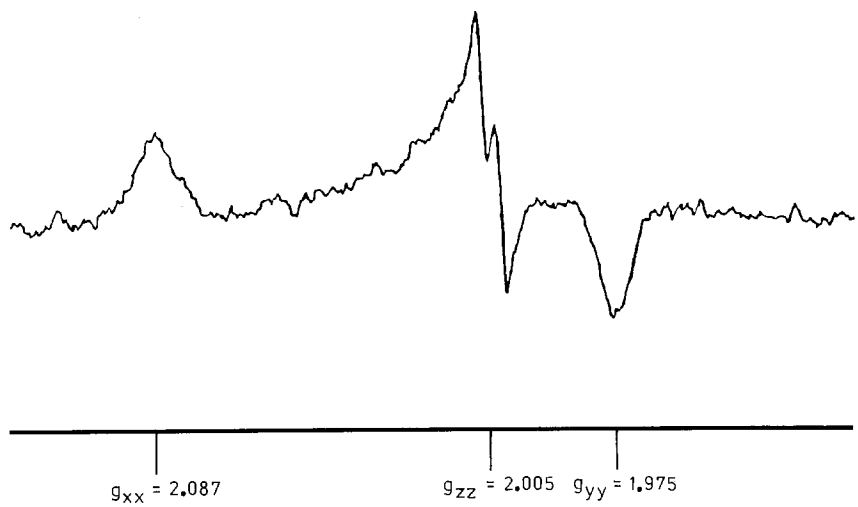

Fig. 3. Q-band ESR spectrum of the hexacoordinated ^{15}NO-ligated haemoglobin IV. Temperature: 100 K; pH 9.3

The center of the g_{zz} resonance corresponds to $g_{zz} = 2.005$. The first derivative spectrum demonstrates the hyperfine interaction of the electron spin with the ^{15}NO and $^{14}N_{\varepsilon}$-imidazole (see Fig. 1). The respective hyperfine constants can be determined from the resolved doublets and triplets repectively. The higher derivatives do not yield further information.

The high-field resonance attributed to g_{yy} exhibits a two-line hyperfine pattern (see Fig. 1). But due to the lack of further spectral information these two hyperfine lines can not be definitely attributed to a ^{15}NO doublet and thus the determination of the g_{yy} value seems to be difficult. However, $g_{yy} = 1.975$ has been determined in the minimum of the Q-band ESR first derivative spectrum (see Fig. 3). Marking the g_{yy} value obtained by the Q-band spectrum in the

Table 1. ESR parameters of hexacoordinated ^{15}NO-ligated haemoglobin IV at 77 K

Direction		g Value	$a(^{15}NO)$ (mT)	$a(^{14}N\varepsilon$-imidazole) (mT)	$a(^{14}N$-pyrrole) (mT)	$a(^{57}Fe)$ (mT)
x	△	2.087 ± 0.0003	1.51 ± 0.02	0.47 ± 0.02	0.38 ± 0.01	0.98 ± 0.02
	*		1.49 ± 0.02	0.49 ± 0.02	0.38 ± 0.01	1.03 ± 0.02
y	△	1.975 ± 0.0003	1.96 ± 0.03	0.56 ± 0.02	0.38 ± 0.01	0.65 ± 0.02
	*		1.93 ± 0.03	0.58 ± 0.02	0.38 ± 0.01	0.72 ± 0.02
z	△	2.005 ± 0.0002	2.98 ± 0.02	0.70 ± 0.01	0.01 ± 0.01	0.58 ± 0.01
	*		2.98 ± 0.02	0.70 ± 0.01	0.03 ± 0.01	0.60 ± 0.01

△ Determined for pH 5.4; * determined for pH 9.4

X-band spectrum shows that g_{yy} is exactly located between the two hyperfine lines. Therefore, we assume that this hyperfine pattern is a doublet reflecting the interaction of the electron spin with ^{15}NO. The evaluation of the hyperfine parameters, however, can be obtained only by comparing simulated with experimental spectra in the higher derivative modes.

The components of the hyperfine constants of ^{15}NO and $^{14}N_{\varepsilon}$-imidazole which coincide with the three principal directions of the g tensor are compiled in Table 1. The simulations of spectra have further shown that the ^{14}N-pyrrole nitrogen atoms contribute considerably to the hyperfine interaction of the electron spin. No fitting of the experimental spectra without assuming ^{14}N-pyrrole hyperfine constants was possible. $A(^{14}$N-pyrrole) values are small but significant for the z direction and are large and equivalent for x and y directions.

Electron Spin Resonance of the Hexacoordinated ^{57}Fe-Substituted ^{15}NO Complex of Haemoglobin IV

The replacement of the central iron atom in the ^{15}NO-ligated CTT IV for its isotope ^{57}Fe results in additional doublets which occur in all ^{15}NO hyperfine lines of the ESR spectra described already in the last chapter. The substitution for ^{57}Fe does not lead to substantial changes in the hyperfine features coinciding with g_{xx} and g_{yy} when measuring the first derivative spectrum (see Fig. 4). In z direction eight hyperfine lines instead of the expected 12 lines are resolved, so, even in this usually well resolved g_{zz} resonance, overlapping of lines occurs and the direct determination of the ^{57}Fe hyperfine constant is impossible. The second derivative spectrum demonstrates the influence of the ^{57}Fe nuclear field on the electron spin for x and y directions (see Fig. 5), but the hyperfine constants for ^{57}Fe cannot be directly obtained.

The ^{57}Fe hyperfine constants are determined by varying this parameter in the Fourier transform-assisted simulation of the ESR spectra, until exact agreement

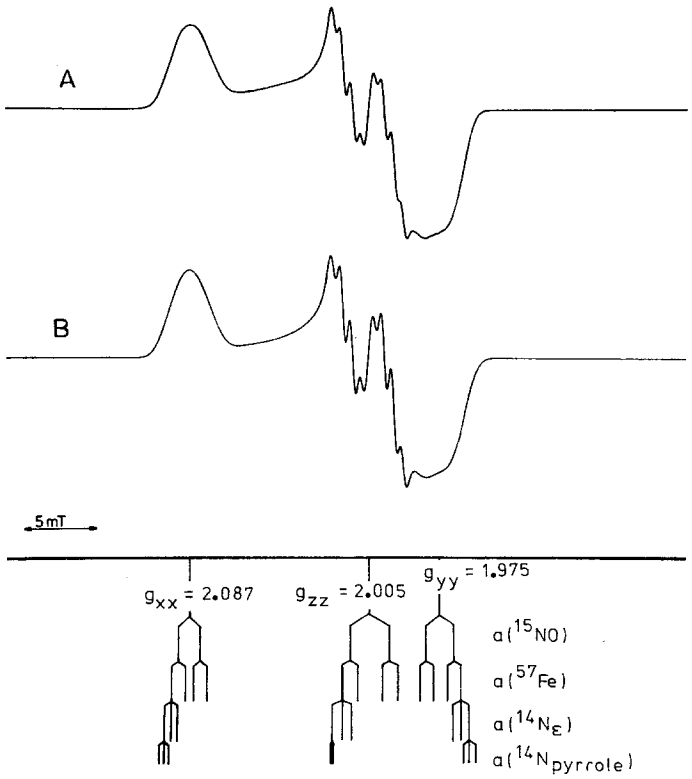

Fig. 4. Experimental (A) and simulated (B) X-band ESR first derivative spectra of the hexacoordinated ^{15}NO-ligated ^{57}Fe-reconstituted haemoglobin IV. Temperature: 77 K; pH 9.3

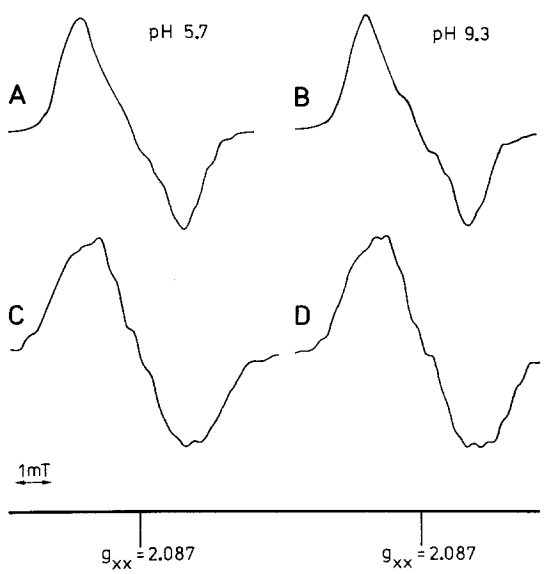

Fig. 5. Low-field resonance (g_{xx}) of the X-band ESR second derivative spectrum of the ^{15}NO-ligated haemoglobin IV. Temperature: 77 K; pH 5.7 (A and C); pH 9.3 (B and D); ^{56}Fe (A and B); ^{57}Fe (C and D)

between experimental and simulated spectra for the three derivatives occurs. During this procedure, all the other parameters taken from the evaluation of spectra of non-substituted CTT IV have been kept constant. An excellent agreement between the experimental and the computed ESR first derivative spectra is demonstrated in Fig. 4 (for parameters see Table 1).

pH-Dependence of the Hyperfine Interactions in ^{15}NO-ligated Haemoglobin IV

CTT IV which exhibits a Bohr-effect for O_2- and CO-binding [29, 31] apparently did not show any pH-dependent change of the ESR first derivative spectra of its nitrosyl complex [8, 13]. However, generating the second derivatives from the small-amplitude-modulated experimental first derivatives by computation with the Fourier-transform technique a pH-dependent hyperfine pattern can be observed for the resonances at g_{xx} (see Fig. 5) and g_{yy} and to a much less extent for the resonance at g_{zz}. These differences in the hyperfine structures are small but significant. In general, the hyperfine constants for ^{15}NO become smaller, those for $^{14}N_{\varepsilon}$-imidazole, ^{14}N-pyrrole, and ^{57}Fe larger when changing the pH from 5.4 to 9.3 (for a detailed description see Table 1). Furthermore, the increase of the ^{14}N-pyrrole hyperfine constants in the g_{zz} resonance with increasing pH is large enough to be detected for both, the native and the ^{57}Fe-reconstituted form of CTT IV. The g values remain fairly constant with variation of the pH value.

Electron Spin Resonance Spectra of the Pentacoordinated ^{15}NO-ligated Haemoglobin IV

In the presence of a 30-molar excess of sodium dodecylsulfate, the NO-ligated CTT IV is transformed into a "superrelaxed" conformation which is characterized by a long or broken iron-imidazole bond [1, 13]. The ESR spectrum of this socalled pentacoordinated complex exhibits rhombic symmetry (g_{xx}, g_{yy}, g_{zz}) (see Fig. 6). In this complex g_{yy} is shifted from high- to low-field, so that g_{zz} is now the high-field resonance. On the basis of the experimental third derivative spectra the exact values for g_{xx} and g_{yy} as well as the components of the ^{15}NO hyperfine constants which coincide with these directions of the g tensor have been determined (see Fig. 6 and Table 2).

Even more information about the hyperfine structures for x and y directions, i.e., hyperfine constants for the pyrrole nitrogens, can be obtained by the transformation of the experimental first derivative into the second derivative spectrum using the Fourier-transform technique. The computer simulation of the ESR spectrum is performed until it fits exactly to the second derivative (see Fig. 6). The ESR parameters of ^{15}NO-ligated CTT IV which is structurally perturbed by the association with detergent molecules are compiled in Table 2.

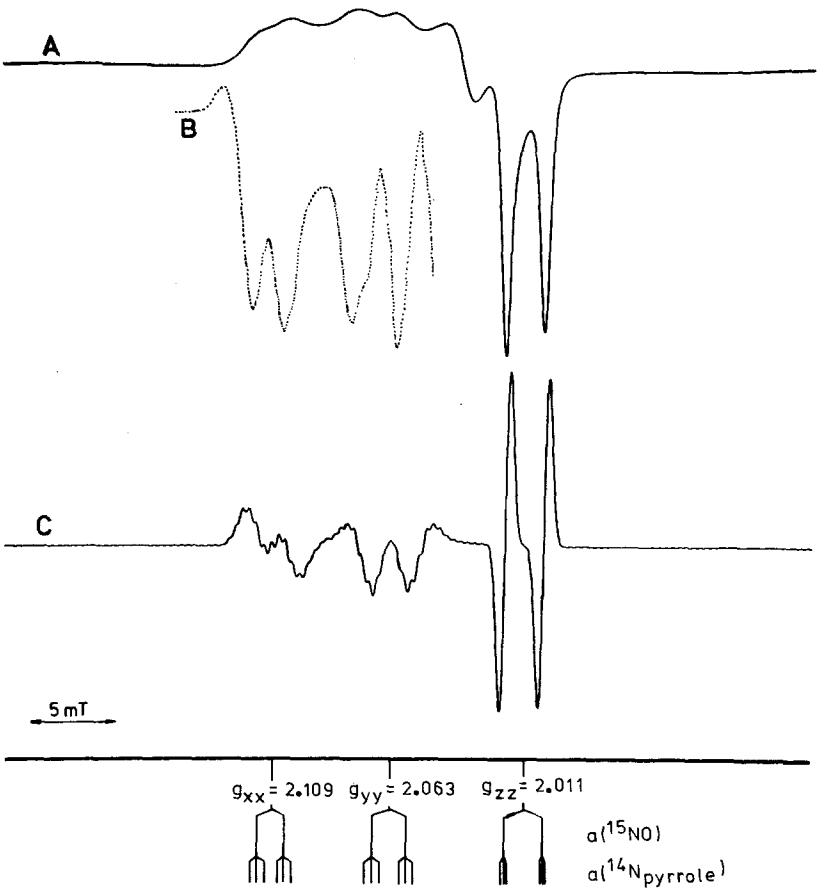

Fig. 6. Experimental (A, B) and simulated (C) X-band ESR spectra of the pentacoordinated ^{15}NO-ligated haemoglobin IV. Temperature: 77 K; pH 9.3; A, B, and C refer to the first, third and second derivative, respectively

Table 2. ESR parameters of the pentacoordinated ^{15}NO-ligated haemoglobin IV at 77 K

Direction	g Value	$a(^{15}NO)$ (mT)	$a(^{14}N$-pyrrole) (mT)	$a(^{57}Fe)$ (mT)
x	2.109 ± 0.0005	1.74 ± 0.05	0.40 ± 0.01	0.88 ± 0.1
y	2.063 ± 0.0005	2.06 ± 0.05	0.41 ± 0.01	0.88 ± 0.1
z	2.011 ± 0.0002	2.36 ± 0.03	0.12 ± 0.01	0.67 ± 0.05

The always occuring unknown resonance at $g_? = 2.04$ (see Fig. 6) [1, 11, 13, 20] is omitted in the simulation.

The substitution for ^{57}Fe results in a doublet splitting of all hyperfine lines of the ESR spectra. As it can be seen from Fig. 7 these ^{57}Fe doublets are only

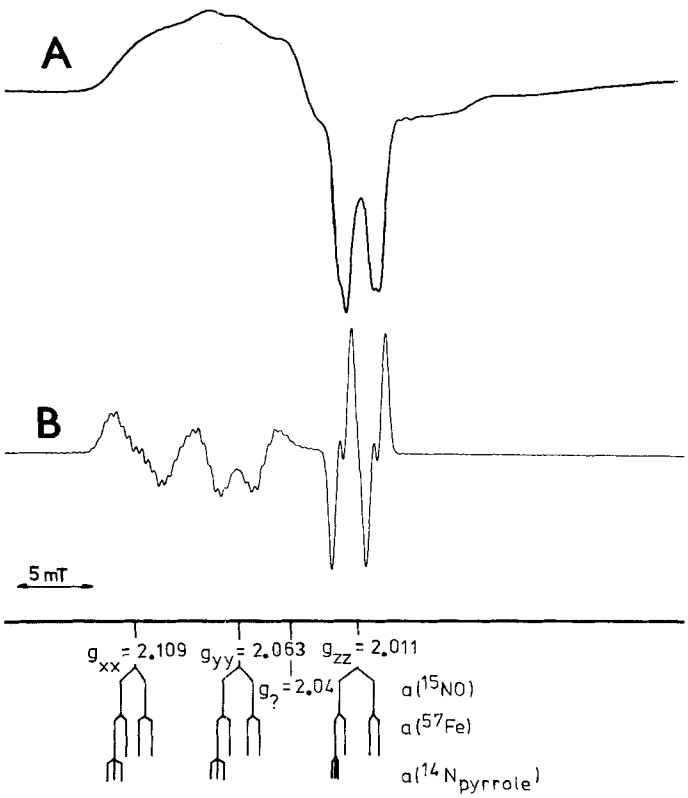

Fig. 7. Experimental (A) and simulated (B) X-band ESR spectra of the pentacoordinated ^{57}Fe-reconstituted ^{15}NO-ligated haemoglobin IV. Temperature: 77 K; pH 9.3. A and B refer to the first and second derivative, respectively

poorly resolved even in the *z* direction, whereas a complete superposition of lines occurs in *x* and *y* directions. The ^{57}Fe hyperfine constants have been determined by using the above mentioned fit procedure on the basis of a fixed data set for the ^{15}NO and ^{14}N-pyrrole hyperfine constants, which latter were obtained from the ^{56}Fe-containing form. The simulated ESR spectra of the pentacoordinated ^{57}Fe-reconstituted ^{15}NO-ligated CTT IV is shown as second derivative in Fig. 7B. It is remarkable that the hyperfine constants for ^{57}Fe and ^{14}N-pyrrole respectively determined for *x* and *y* directions are equivalent.

The structural transformation of the nitrosyl complex of CTT IV into a superrelaxed conformation is reversible. After the removal of the detergent anions from the haemoglobin solution by precipitating the potassium salt of tetradecylsulfate at 4° C, again a type II spectrum of the native nitrosyl complex occurs (see Fig. 8). Comparison of the spectra, shown in Figs. 8B and 1A, indicates that in this particular experiment only small amounts of the

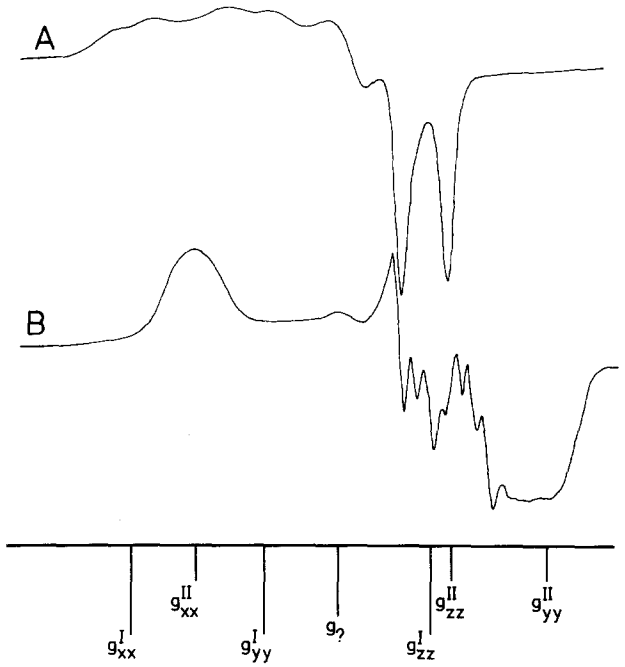

Fig. 8. Reversible structural transformation of ^{15}NO-ligated haemoglobin IV. Temperature: 77 K, pH 9.4. (A), type I spectrum (g^{I}) after binding of tetradecylsulfate; (B), type II spectrum (g^{II}) after removal of tetradecylsulfate by precipitating of potassium salt at 4° C

pentacoordinated nitrosyl complex contribute to the ESR spectrum of the reconverted native form.

Discussion

Coordination States of Iron and Binding Geometries of NO in Nitrosyl Haemoglobin IV

Single crystal ESR investigations have demonstrated that g and $A(^{15}NO)$ tensors do not coincide in nitrosyl haemoglobin and myoglobin [3, 10, 15]. However, from powder ESR spectra hyperfine tensors cannot be evaluated. The hyperfine constants compiled in Tables 1 and 2 are therefore components of the A tensors coinciding with the well-known g tensor. Nevertheless, some conclusions with regard to the binding geometries of the NO ligand in the hexa- and pentacoordinated state of CTT IV may be allowed on the basis of hyperfine data given in Tables 1 and 2 and on the basis of crystal ESR data published by Chien.

In the case of the hexacoordinated NO complex of CTT IV the hyperfine constants for ^{15}NO and $^{14}N_{\varepsilon}$-imidazole determined in the principal directions of the g tensor decrease following the order of directions: $z > y > x$, whereas those

for ^{14}N-pyrrole and ^{57}Fe increase. From this, we conclude that the principal directions of the two pairs of hyperfine tensors differ strongly. It is more likely, that the hyperfine tensors for ^{14}N-pyrrole and ^{57}Fe coincide with the g tensor.

The iron-NO bond in haemoglobin is not linear. The Fe-N-O angle varies from 105–165° [15]. The interaction of the iron d_{z2} orbital with the lone pair orbital of NO is strongest for a linear iron-NO bond and is relieved upon bending of the Fe-N-O angle. Thus we can expect that the affinity of haemoglobin to NO is smaller, if the Fe-N-O angle deviates more and more from linearity and likewise the σ donor property of NO is weakened. Furthermore, the d_{z2} orbital is destabilized by a strong σ donor in *trans*-position to NO. Therefore, shortening of the imidazole-iron bond induced by a structural transition leads to a larger inclination of the Fe-N-O angle and a weakening of the NO-iron bond.

The interaction between the π^* orbital of NO and the iron d_{xz}, d_{yz} orbitals is also destabilized with the bending of the Fe-N-O angle. Both, short imidazole-iron bond and strong bending of NO remove the degeneracy of these orbitals. Therefore, we can conclude that the two directions in the haem plane (x and y) are not any more equivalent. One of the consequences of a bent Fe-N-O bond angle is a difference in the spin densities of the iron d_{xz} and d_{yz} orbitals. The ESR spectrum of the hexacoordinated form (type II spectrum) should exhibit differences in the x and y components of the ^{15}NO hyperfine tensor. Comparison of the ^{15}NO hyperfine constants demonstrates that the hyperfine splitting coinciding with g_{yy} is 30% larger than that coinciding with g_{xx}. On the other hand, the ^{57}Fe hyperfine constant determined in g_{yy} is 30% (at high pH) – 34% (at low pH) smaller than that determined in g_{xx}. It follows, that the NO ligand must be bent towards the x direction of the haem plane. The bending seems to be larger at low pH as indicated by the pH-dependent change of the ^{57}Fe hyperfine constants.

The conversion of the hexacoordinated (type II spectrum) into a pentacoordinated NO-complex (type I spectrum) by associating anionic detergents to nitrosyl CTT IV leads to a hyperfine constant for ^{15}NO, which is only 18% larger in direction of g_{yy} than in direction of g_{xx}. The difference in hyperfine splitting is zero for the ^{57}Fe hyperfine constants coinciding with g_{yy} and g_{xx}. The structure-related change in the hyperfine constants indicates that the Fe-N-O bond angle is much larger in the pentacoordinated than in the hexacoordinated nitrosyl complex. Crystal ESR measurements on haemoglobin Kansas have confirmed that the type I spectrum has to be attributed to a species with a Fe-N-O bond angle of 167° [15]. On the basis of crystal ESR data the conversion of the hexa- into a pentacoordinated NO complex in haemoglobins is accompagnied by an increase of the iron-NO bond angle from 105° to 165°.

The difference in hyperfine constants measured in g_{xx} and g_{yy} is much smaller in protein-free NO-haem-nitrogen base model complexes than in hexacoordinated nitrosyl CTT IV, indicating less bending of the iron-NO bond (Gersonde et al. unpublished results). An example for a nearly linear iron-NO bond angle is given by nitrosyl cytochrom c oxidase which does not exhibit different ^{15}NO hyperfine constants in g_{xx} and g_{yy} for the hexacoordinated complex [26].

pH-Dependence of the Hyperfine Structure in Hexacoordinated Nitrosyl Haemoglobin IV and its Relationship with the Bohr-Effect

Chironomus haemoglobin IV is a monomeric protein which exhibits allosteric properties, i.e., pH-dependent affinities for O_2 and CO [27, 29, 30]. The long-range interaction between ligand- and proton-binding sites is called "Bohr-effect". The pH-induced interconversion between two affinity states is based on a tertiary conformation transition *t*(ense) $\rightleftharpoons$ *r*(elaxed) [28, 31–34]. It has been recently discovered by NMR [33, 34] and ESR [27] investigations, that the electronic structure of the central atom is affected by the conformational change only in the ligated state of the haemoglobin. Because of the very small off-rate constant and the extremely high affinity of NO, the Bohr-effect for NO could not be measured by equilibrium studies. However, on the basis of proton NMR titration studies of the Bohr-proton binding site in nitrosyl CTT IV it has been concluded that the Bohr-effect for NO has to be much smaller than for O_2 or CO [8]. The ligand-linked pK shift of the Bohr-proton group in CTT IV varies with different ligands and correlates with the magnitude of the Bohr-effect for these ligands in the following order: $O_2 > CO > NO$. Since nitrosyl CTT IV exhibits a small Bohr-effect, we assume that there also exists a pH-induced tertiary structure change which can be detected by ESR.

The ESR first derivative spectra of the NO-ligated CTT IV do not show any pH dependence (see also reference [8]), whereas the second derivative spectra do (see Fig. 5). A comparison of the second derivative spectra at low and high pH shows small, but significant changes of the hyperfine structures which are more distinct for the ^{57}Fe-substituted CTT IV (see Table 1). The spectra measured in the range of pH 5–10 are all of type II indicating that nitrosyl CTT IV remains hexacoordinated during the $t \rightarrow r$ transition. Binding of Bohr-protons has no effect on the coordination state of the haem iron in nitrosyl CTT IV.

The pH-induced $t \rightarrow r$ transition leads to a change of the spin distribution in the hexacoordinated nitrosyl complex. The high-pH form which corresponds to the high-affinity state shows larger $a(^{57}\text{Fe})$ values for all principal directions; the low-pH form which correlates with the low-affinity state, exhibits smaller $a(^{57}\text{Fe})$ values. Therefore, in the high-affinity state of CTT IV spin transfer from NO to iron is some what larger than in the low-affinity state. Additional evidence for an increase of the spin density on the iron atom in the high-pH form (high-affinity state) is given by the substantial increase of the ^{14}N-pyrrole hyperfine constants in g_{zz} from 0.01 to 0.03 mT. On the contrary, the ^{15}NO hyperfine constants seem to show an inverse behaviour consistent with the just mentioned correlation between affinity states and spin distribution on iron. The larger spin transfer from NO to iron in the high-affinity state of CTT IV correlates with an increase of the σ bond strength of the NO ligand and a weakening of the imidazole-iron bond in *trans*-position. The allosteric protein controls the binding of the external ligand via the proximal imidazole, i.e., *t* and *r* conformation in CTT IV differ with regard to the imidazole-iron bond strength which is only relieved in the *r* conformation. The approach of the proximal imidazole to iron

via the transition from *r* to *t* conformation leads to a back-transfer of spin density into NO orbitals and to the formation of a longer iron-NO bond.

Furthermore, at low pH the ^{57}Fe hyperfine constant is found to be 51% larger for the *x* than for the *y* direction, whereas at high pH the ^{57}Fe splitting in g_{xx} increases only by 43%. Thus in the low-affinity state the Fe-N-O bond is more bent than in the high affinity state of CTT IV.

In tetrameric haemoglobins a pH-induced change of the quaternary structure is often accompagnied by a more dramatic change of the ESR spectrum from type II to type I indicating a transition from a hexa- to a pentacoordinated nitrosyl complex [5–7, 11, 14, 16]. Nitrosyl CTT IV does not show such a pH-dependent transition from a hexa- to a pentacoordinated iron complex. Our conclusion is that tertiary structure changes in this monomeric allosteric haemoglobin are less affective on the ligand-binding site than those occuring in tetrameric haemoglobins.

Reversible Formation of a "Superrelaxed" Structure Indicated by a Pentacoordinated Nitrosyl CTT IV

Nitrosyl CTT IV can be transformed from a native into a denatured structure by a stoichiometric association of anionic detergents. This structure change is reversible and has been followed by ESR. The native structure (*t* and *r* conformation) is characterized by type II spectra, the denatured structure (superrelaxed conformation) by type I spectra. Type I spectra are typical for pentacoordinated nitrosyl haemoglobins, i.e., the proximal imidazole-iron bond is broken or extremely lengthened. A haemoglobin structure which relieves the imidazole-iron bond to such a great extent is defined as a "superrelaxed" structure and has to be distinguished from the relaxed conformation of the native structure which still contains the hexacoordinated nitrosyl haem complex. The formation of this superrelaxed structure is reversible and can be reconverted into the native conformations (*t* or *r*) as demonstrated by the ESR spectra. This means, that, after the removal of the detergent anions, the proximal imidazole takes exactly its original position.

We now want to discuss the question if the superrelaxed nitrosyl CTT IV is a pentacoordinated or a hexacoordinated complex with a very weak interaction of the proximal imidazole with the haem iron. The simulation of ESR spectra leads to a good fit of the experimental spectra also if additional hyperfine constants for $^{14}N_\varepsilon$ with < 0.33 mT, < 0.37 mT, and < 0.12 mT for the *x*, *y*, and *z* directions respectively are used. Although the simulation of ESR spectra is no proof of the validity of a proximal histidine-iron interaction, the latter cannot be excluded. The full reversibility of the structural transformation indicates that the proximal imidazole should be located very near to its original position held in the native protein and therefore could be another hint that at least nitrosyl CTT IV is a socalled "pentacoordinated" complex, i.e., with very long imidazole-iron bond. On the basis of ENDOR experiments the largest haem proton hyperfine constant which has been found is about 0.1 mT (Gersonde et al. unpublished results). Most of the proton hyperfine splitting constants are ≤ 0.03 mT.

Therefore haem protons could not be resolved in the hyperfine pattern and their contribution to the ESR spectra are part of the linewidths which were assumed to be constant for all complexes and under all conditions.

It has been already discussed that the Fe-N-O bond angle is more stretched in the superrelaxed state of CTT IV than in the native conformations.

Spin Transfer to Iron in Hexa- and Pentacoordinated Nitrosyl Haemoglobin IV

According to the Fe-N-O bond angle in hexacoordinated nitrosyl CTT IV the overlapping of NO orbitals is supposed to be much stronger with the iron d_{xz} than with the d_{yz} orbital. As already discussed before, this is shown by the hyperfine constants for ^{15}NO which is smaller in directions of g_{xx} than of g_{yy}, because the spin density is transferred from NO more into the d_{xz} than into the d_{yz} orbital. The spin transfer is reflected also by the ^{57}Fe hyperfine constants determined in the g_{xx} and g_{yy} resonances. They are nonequivalent for the hexacoordinated complex of CTT IV. The deviation from equivalence can be expressed by the ratio $a_{xx}(^{57}Fe) : a_{yy}(^{57}Fe)$, which is unity for an equal spin transfer to d_{xz} and d_{yz} in the case of a linear Fe-N-O bond and increases upon bending of the Fe-N-O bond. These ratios are 1.51, 1.43, and 1.0 for the *t*, *r*, and super-*r* conformations of nitrosyl CTT IV respectively.

The transformation of the hexacoordinated (*t*, *r* conformation) into the pentacoordinated (super-*r* conformation) of nitrosyl CTT IV is accompagnied by an increase of the spin density in the d_{z2} orbital of the iron. This can be demonstrated by comparing the hyperfine constants measured in g_{zz}. The hyperfine constant for ^{57}Fe increases by 16% upon transition from type II to type I spectra. The hyperfine constant for ^{15}NO decreases by 21%. Finally, the hyperfine constant for ^{14}N-pyrrole changes from 0.01 mT to 0.12 mT. These changes of the hyperfine constants in g_{zz} are again strong argument for the important role of the proximal histidine for triggering the ligand-induced allosteric transition and for controlling the ligand-binding by conformational equilibria.

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