Conformation-Controlled Trans-Effect of the Proximal Histidine in Haemoglobins. An Electron Spin Resonance Study of Monomeric Nitrosyl-^{57}Fe-Haemoglobins

Matthias Overkamp, Hans Twilfer, and Klaus Gersonde

Abteilung Physiologische Chemie der Rheinisch-Westfälischen Technischen Hochschule Aachen

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Nitrosyl ^{57}Fe-Haemoglobin, Conformation-Controlled Trans-Effect, Electron Spin Resonance Study

A monomeric allosteric haemoglobin from *Chironomus thummi thummi* was reconstituted with ^{57}Fe-haem. This reconstituted haemoglobin was found to be identical to the non-reconstituted material with regard to the O_2-binding properties and the visible spectra. The 270 MHz proton magnetic resonance of the bis(cyano)-^{57}Fe-haemin shows that the reconstituted haem is identical with the non-reconstituted haem. Furthermore it has been proved by proton magnetic resonance that in *Chironomus* haemoglobins the prosthetic group is proto-haem IX.

The ESR spectrum of the native nitrosyl haemoglobin demonstrates rhombic symmetry of the haem iron ($g_{xx}=2.086$, $g_{yy}=1.981$, $g_{zz}=2.005$) and hyperfine structures at g_{yy} ($a_{N\varepsilon}=1.35$ mT) and at g_{zz} ($a^{15}_{NO}=3.05$ mT, $a^{14}_{NO}=2.19$ mT, $a_{N\varepsilon}=0.715$ mT, $a^{57}_{Fe}=0.38$ mT). The spectrum is independent of pH and can be classified as a type II spectrum following the classification of ref. 2. NO-binding obviously stabilizes the tertiary structure of this haemoglobin in a "tense" conformation with a relatively strong σ bond of the 5th ligand (Nε of imidazole) and a relatively weak σ bond of the 6th ligand (NO).

Reaction of this haemoglobin with anionic, cationic and non-ionic detergents, respectively, leads to a transformation of the NO-ligated form into a "relaxed" conformation with a stretched or broken σ bond of the 5th ligand (Nε of imidazole) and a strong σ bond of the 6th ligand (NO). The ESR spectrum of this modified NO-haemoglobin shows again a rhombic symmetry of the haem iron ($g_{xx}=2.10$, $g_{yy}=2.06$, $g_{zz}=2.010$), but dramatically changes in the g tensors (low field shift), hyperfine structures and hyperfine splitting constants ($a^{15}_{NO}=2.32$ mT, $a^{14}_{NO}=1.66$ mT, $a^{57}_{Fe}=0.48$ mT). The hyperfine splitting is isotropic.

Transition from the "tense" conformation to the "relaxed" conformation corresponds with an increase of the spin density at the iron atom by 26% and a decrease of the spin density at the NO ligand by 25%. The spin density at the Nε of imidazole strongly decreases in the "relaxed" conformation, so that a hyperfine splitting of this ligand is not any more resolved. These results demonstrate the trans-effect of the proximal imidazole which in haemoglobins controls the binding properties of the external ligand in trans-position.

1. Introduction

The ligation of haem proteins with NO leads to the formation of paramagnetic complexes characterized by an odd electron spin ($S=1/2$). The binding geometry and the binding distance of the nitric oxide in these NO-haem-base complexes are controlled by the σ donor and the π acceptor properties of the nitrogen base in trans-position [1–3]. This influence of the nitrogen base on the axial ligand in trans-position is called the "trans-effect" which is involved in the trigger mechanism of allosteric haemoglobins [4, 5]. Therefore it is small wonder that in haemoglobins the 5th coordination site of the haem iron is exclusively occupied by imidazole of the invariant histidine F8 [5].

The σ donor properties of the heterocyclic nitrogen base can be modified by three ways:

i) in model compounds by the substitution of the base by electron withdrawing or electron supplying groups [1],
ii) in allosteric haem proteins by the modification of the binding distance between the imidazole and the haem iron induced by structural constraints [2],
iii) and in both types of complexes by the dissociation of a proton at N-1 of the proximal imidazole [3].

The electron spin resonance spectra of NO-haem-base complexes in model compounds [1, 6, 7] and in proteins [2, 8–21] have been extensively investigated.

Requests for reprints should be sent to Prof. Dr. Klaus Gersonde, Abteilung Physiologische Chemie der Rheinisch-Westfälischen Technischen Hochschule Aachen, Melatener Str. 213, *D-5100 Aachen*.

At the temperature of liquid nitrogen two types of electron spin resonance spectra of the nitrosyl derivatives of haemoglobins have been observed differing in the g-tensors and in the hyperfine splitting at g_{xx} and g_{zz}. The *type I spectrum* is characterized by 3-line hyperfine structures at $g_{zz} = 2.009$ and $g_{xx} = 2.064$ with an identical splitting constant of 1.8 mT [2, 9, 15, 16]. The *type II spectrum* shows a 3-line hyperfine structure only at $g_{zz} = 2.005$ with a splitting constant of 2.3 mT [2, 9, 13, 15, 18, 21]. In some haemoglobins a higher resolution leads to a *type II spectrum* demonstrating a 9-line superhyperfine structure at $g_{zz} = 2.005$, *i. e.* a further splitting of the 3-line hyperfine structure (2.3 mT) into triplets with a splitting constant of 0.6 mT occurs [2, 13, 18, 21]. The hyperfine structures at g_{zz} reflect the interaction of the unpaired electron with the nitrogen nuclei directed along the haem normal: The 3-line hyperfine structure of the type I spectrum corresponds to an interaction only with the nitrogen nucleus of NO; the 9-line superhyperfine structure of the type II spectrum demonstrates an interaction with the nitrogen nuclei of both axial ligands, *i. e.* NO and imidazole [2, 18].

A pH-induced transition from the 3-line hyperfine structure to the 9-line superhyperfine structure was observed in normal Hb A [15], Hb Zürich [2], isolated α [2, 19] and isolated β chains [2], but not in the monomeric *Chironomus* haemoglobins [18], myoglobin [13, 15, 21] and in Hb M Iwate [2]. In the presence of polyphosphates the inflection point of this pH dependent transition is shifted in case of Hb A from pH 5.35 to higher values [15, 16].

Thus the electron spin resonance spectra of nitrosyl complexes of haemoglobins have been considered to be indicators of conformational states [2, 15, 16, 18, 19]. The explanation of the two types of ESR spectra of NO-haemoglobins in terms of different Fe – Nε bonds and Fe – NO bonds respectively should have taken into account the three possibilities of modifying the σ donor properties of the nitrogen base as mentioned above and theoretical treatments of the ESR spectrum with the MO theory (a qualitative MO scheme explaining the conformational transition is given in Fig. 11 of reference 2).

In this paper the electronic structures of NO-haem-base complexes and their relations to particular protein conformations of haemoglobin are investigated in more detail by substituting the ^{14}NO by ^{15}NO with a nuclear spin $I = 1/2$ and by replacing ^{56}Fe by ^{57}Fe with $I = 1/2$. In the case of the monomeric *Chironomus* haemoglobins, which show no pH-effect on the ESR spectrum of the NO derivatives, the conformation transition is forced by the reaction with detergents. The change of the spin densities at the central iron atom and at the axial nitrogen ligands, respectively, can be demonstrated for the two types of ESR spectra. Furthermore, the two types of ESR spectra can be correlated to different conformations of the monomeric haemoglobins defined by differences in the binding distances of NO and imidazole respectively. The results obviously show that the trans-effect of the proximal imidazole plays an important part in a control mechanism which modifies the binding properties of the 6th ligand in haemoglobins.

2. Materials and Methods

Preparation of haemoglobins

The monomeric haemoglobins I and IV from *Chironomus thummi thummi* were purified as described elsewhere [22]. The salt-free materials were lyophilized and stored at -30 °C.

Preparation of globin

Lyophilized haemoglobin was dissolved in aqua destillata and was oxidized by addition of an 1.5 M

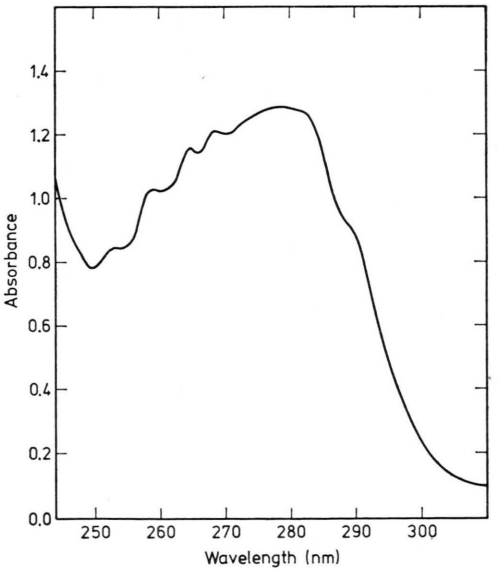

Fig. 1. UV spectrum of globin of *Chironomus* haemoglobin IV. Buffer: 0.01 M phosphate pH 7.5; temperature: 4 °C.

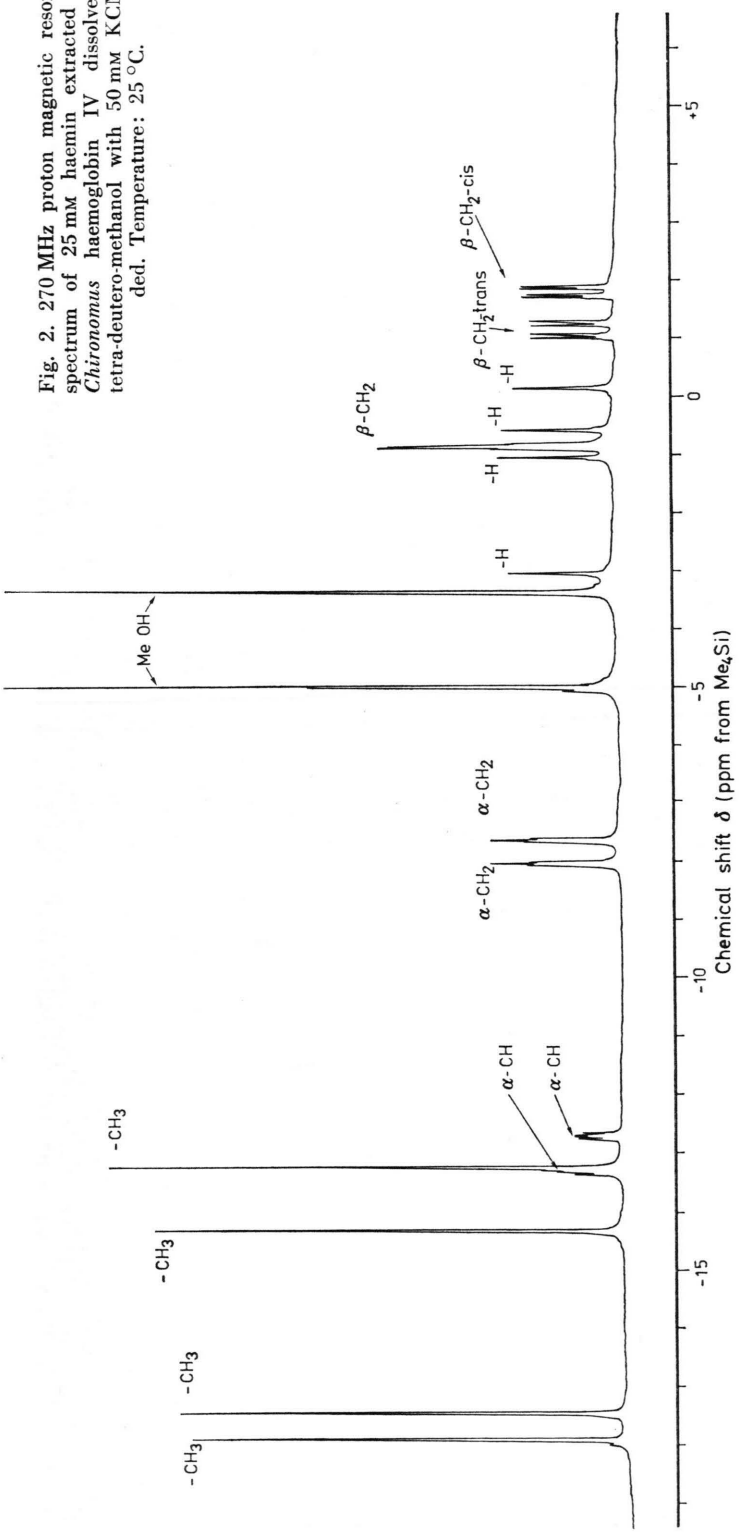

Fig. 2. 270 MHz proton magnetic resonance spectrum of 25 mM haemin extracted from *Chironomus* haemoglobin IV dissolved in tetra-deutero-methanol with 50 mM KCN added. Temperature: 25 °C.

excess of $K_3[Fe(CN)_6]$. Salts were removed by gel filtration on a Sephadex G 25 column [2.5 × 50 cm] equilibrated with aqua destillata. The globin preparation was carried out according to [23]. The procedure started with a 1% solution of haemoglobin. Finally, the globin was dialyzed against 0.01 M phosphate buffer pH 7.5. Precipitated material was discarded. The UV absorption spectrum of the globin solution was recorded at 4 °C and is shown in Fig. 1. The globin concentration was determined according to [24] using a molar absorption coefficient $\varepsilon = 11200$ [$M^{-1} \cdot cm^{-1}$] at the absorption maximum of 280 nm.

Preparation of ^{57}Fe-haemin

Haemin was isolated from bovine haemoglobin, the monomeric *Chironomus* haemoglobins III, and IV, and from mixtures of dimeric *Chironomus* haemoglobins following the procedure described by Labbe and Nishida [25]. The purified haemins were proved by thin layer chromatography [26] and by proton magnetic resonance of the paramagnetic bis(cyano)-proto-haemin [27, 28] dissolved in tetra-deutero-methanol (see Fig. 2). The haemins of bovine and *Chironomus* were found to be proto-haemin IX.

The removal of iron from the haemin followed the description of Morrell and Stewart [29]. The proto-porphyrin IX was dissolved in ether. Copro-, deutero- and mesoporphyrin were extracted with 0.54% (w/v) HCl [30]. Protoporphyrin IX was separated from haemin by extraction with 5% (w/v) HCl. Crystallization of protoporphyrin IX was performed after extraction with chloroform by evaporation of chloroform after addition of 1 ml pyridine.

^{57}Fe metal of 80% isotopic enrichment was converted to crystallized $^{57}Fe(NH_4)_2(SO_4)_2$ [31]. For insertion of ^{57}Fe the following procedure was carried out: Under anaerobic conditions 125 mg protoporphyrin IX were dissolved in 8 ml pyridine at 80 °C. Then 400 ml glacial acetic acid and 8 ml 1.2% aqueous $^{57}Fe(NH_4)_2(SO_4)_2$ solution were added. After 1 hour reaction at 80 °C under a stream of N_2 the haem was oxidized at room temperature by passing air through the solution for 20 min. Haemin and the non-reacted porphyrin were taken to ether and then the porphyrin was removed by extraction with 25% (w/v) HCl. HCl was removed from the ether by washing with H_2O. Then haemin was precipitated by evaporation of the ether and washed successively with small amounts of 50% (w/v) glacial acetic acid, H_2O, ethanol and ether. The yield was 50%.

The reconstituted haemin was found to be identical with haemin extracted from *Chironomus*

haemoglobins proved by thin layer chromatography and proton magnetic resonance of the bis(cyano)-proto-haemin complex in tetradeutero methanol.

Reconstitution of Chironomus haemoglobin IV

Reconstitution of haemoglobin IV was performed according to [32] on a Sephadex G 25 column [2.5 × 54 cm].

NO Derivatives

The NO complexes of the haemoglobins were prepared under anaerobic conditions according to [15]. An amount of 30 mg lyophilized haemoglobin was dissolved in 0.3 ml 0.2 M buffer containing 30 mg/ml ascorbic acid; citrate-phosphate pH 5.5 and Tris-HCl pH 9.0 were used as buffers. Then 3 mg sodium nitrite were added to the haemoglobin solution. After a reaction time of 10 min the precipitated material was removed by centrifugation, the final pH was measured, and the sample quickly frozen in liquid nitrogen.

The buffers contained 0.28 M sodium dodecyl sulfate, laurylpyridinium chloride and Tween 20, respectively, if NO ligation of the structurally transformed haemoglobins was required.

Electron spin resonance spectra

ESR measurements of the NO-haemoglobins were performed at 77 K with an X-band spectrometer (Type BER 420, Bruker-Physik, Karlsruhe). Quartz tubes with an inner diameter of 3.3 mm were filled with 0.3 ml solution of NO-haemoglobin. The amplitude of the 100 kHz field modulation was 0.02 mT. The microwave power was attenuated to 6 mW; saturation phenomena did not appear. The microwave-frequency was measured with a frequency counter, the magnetic field strength with a nuclear magnetic resonance oscillator.

Proton magnetic resonance spectra

PMR spectra of the bis(cyano)-proto-haemin were recorded at 25 °C with a 270 MHz spectrometer (Type WH 270, Bruker-Physik, Karlsruhe) operating in the Fourier Transform mode. A number of 100 transitions were collected using a bandwidth of 7 kHz, 16 K data points, and 10 μsec 90° pulses. Chemical shifts are reported in ppm, referenced against internal tetramethylsilane (Me_4Si).

Ultraviolet spectra

UV spectra of globin were measured at 4 °C with an UV-visible spectrophotometer (Type 118, Cary Instruments, California).

3. Results

ESR spectra of the NO derivatives of haemoglobin I

The monomeric haemoglobin I of *Chironomus thummi thummi* is a myoglobin-like haemoglobin which shows no Bohr effect for O_2 and CO binding [33, 34]. The ESR spectra of the NO derivatives of this haemoglobin measured at pH 6 and pH 9 were found to be identical.

Fig. 3 demonstrates the spectra of the ^{15}NO and ^{14}NO-ligated form, respectively. The spectra show nearly rhombic symmetry of the ligand field with $g_{xx} + g_{yy} \approx g_{zz}$ (see Table I).

Table I. ESR parameter of native and modified NO-haemoglobins I and IV from *Chironomus th. thummi.*

Haemoglobin	Hb I				Hb IV			
complex	$^{14}NO-^{56}Fe-^{14}N\varepsilon$		$^{15}NO-^{56}Fe-^{14}N\varepsilon$		$^{14}NO-^{57}Fe-^{14}N\varepsilon$		$^{15}NO-^{57}Fe-^{14}N\varepsilon$	
state	Native tense	Modified relaxed	Native tense	Modified relaxed	Native tense	Modified relaxed	Native tense	Modified relaxed
g_{xx}	2.077	2.10	2.077	2.10	2.086	2.10	2.086	2.10
Hyperfine lines	0	broadened	0	2	0	broadened	0	broadened
Hyperfine splitting [mT]	—	n.d.	—	2.3	—	n.d.	—	n.d.
g_{yy}	1.981	2.06	1.981	2.06	1.981	2.06	1.981	2.06
Hyperfine lines	3	3	3	2	n.d.	3	3	2
Hyperfine splitting [mT]	1.40	1.7	1.40	2.3	n.d.	1.7	1.35	2.3
g_{zz}	2.005	2.010	2.005	2.010	2.005	2.010	2.005	2.010
Hyperfine lines	9	3	6	2	10	6	8	4
Hyperfine splitting [mT]								
a_{NO}	2.19	1.66	3.05	2.32	2.19	1.66	3.05	2.32
$a_{N\varepsilon}$	0.715	—	0.715	—	0.715	—	0.715	—
a_{Fe}	—	—	—	—	0.38	0.48	0.38	0.48

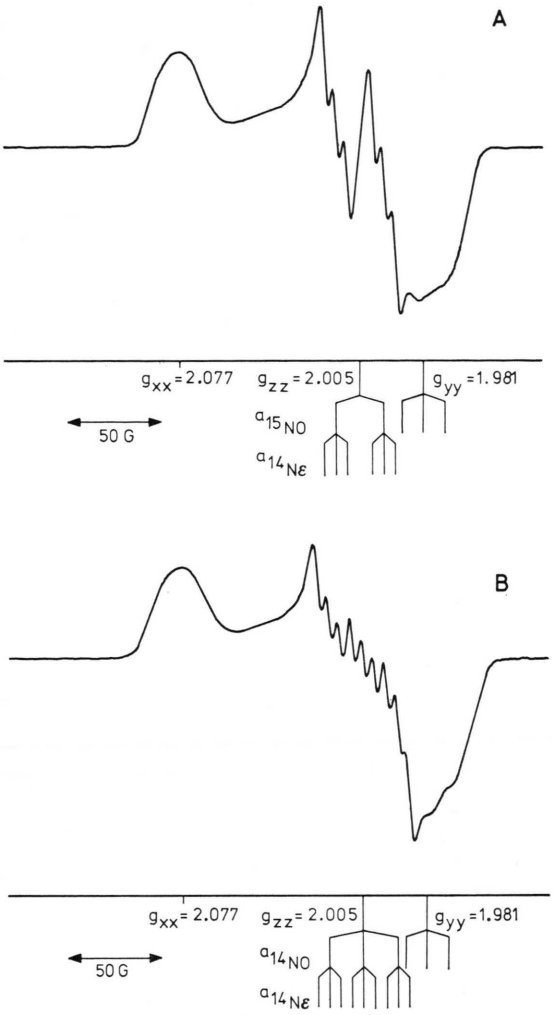

Fig. 3. ESR spectra of NO-ligated haemoglobin I. (A), $^{15}NO-{}^{56}Fe-{}^{14}N\varepsilon$ complex at pH 6.3; (B), $^{14}NO-{}^{56}Fe-{}^{14}N\varepsilon$ complex at pH 8.4; temperature: 77 K.

In the low field signal at $g_{xx}=2.077$ no hyperfine structure appears, the g value is determined at the maximum of this resonance.

The high field signal centered at $g_{yy}=1.981$ shows three lines of a hyperfine structure with a splitting of 1.4 mT; the g value of this resonance is defined by the position of the central hyperfine line. Replacement of ^{14}NO by ^{15}NO, *i. e.* a change of the nuclear spin and of the magnetogyric ratio of the 6th ligand from $I=1$ to $I=1/2$ and from $\gamma_N=1.934$ to $\gamma_N=-2.712$ respectively, causes a broadening of the hyperfine lines at g_{yy} but does not destroy the 3-line hyperfine structure. This indicates that the hyperfine structure arises from a non-exchangeable nitrogen ligand, *i. e.* from the $^{14}N\varepsilon$ of the imidazole at the 5th position. The hyperfine coupling attributed to ^{15}NO at the 6th position must be much smaller in this direction and causes only a broadening of the three hyperfine lines.

The most interesting feature is the hyperfine structure centered at $g_{zz}=2.005$. In case of the ^{15}NO-ligated form (see Fig. 3 A) a 2-line hyperfine structure with a splitting constant of 3.05 mT is observed. Each line of this doublet is further split into a triplet with a splitting of 0.715 mT. The 2-line hyperfine structure indicates an interaction of the unpaired electron with the ^{15}N nucleus of the 6th ligand (NO), the 3-line superhyperfine structure originates from the interaction with the ^{14}N nucleus of the 5th ligand (proximal imidazole). After replacement of ^{15}NO by ^{14}NO a 9-line structure at g_{zz} appears (see Fig. 3 B). Analysis of this structure leads to a 3-line hyperfine structure with a splitting constant of 2.19 mT and a superhyperfine structure with a splitting constant of 0.715 mT. The large splitting has to be attributed to ^{14}NO and is identical to that calculated from the splitting constant of ^{15}NO and the magnetogyric ratio.

ESR spectra of the NO derivatives of ^{57}Fe-reconstituted haemoglobin IV

The monomeric haemoglobin IV of *Chironomus thummi thummi* is characterized by an O_2 and CO Bohr effect[35, 34]. The ^{57}Fe-reconstituted haemoglobin IV has been found to be identical with the non-reconstituted material with regard to the O_2 binding properties. The ESR spectra of the NO derivatives of the non-reconstituted[18] and the reconstituted haemoglobin IV are independent of pH.

Fig. 4 demonstrates the spectra of the ^{15}NO-ligated and the ^{14}NO-ligated form of the ^{57}Fe-reconstituted haemoglobin IV. The spectra again reflect rhombic symmetry of the ligand field of the haem iron.

The low field signal at $g_{xx}=2.086$ is shifted to lower field relative to that observed in haemoglobin I (*cf.* Fig. 3). No hyperfine structure occurs.

The high field signal centered at $g_{yy}=1.981$ shows the already mentioned 3-line hyperfine structure for the $^{15}NO-{}^{57}Fe-{}^{14}N\varepsilon$ complex. A splitting of 1.35 ± 0.05 mT was found. In the case of the $^{14}NO-{}^{57}Fe-{}^{14}N\varepsilon$ complex this structure is totally destroyed by a line-broadening. This broadening effect seems to become more obvious by the ^{57}Fe then

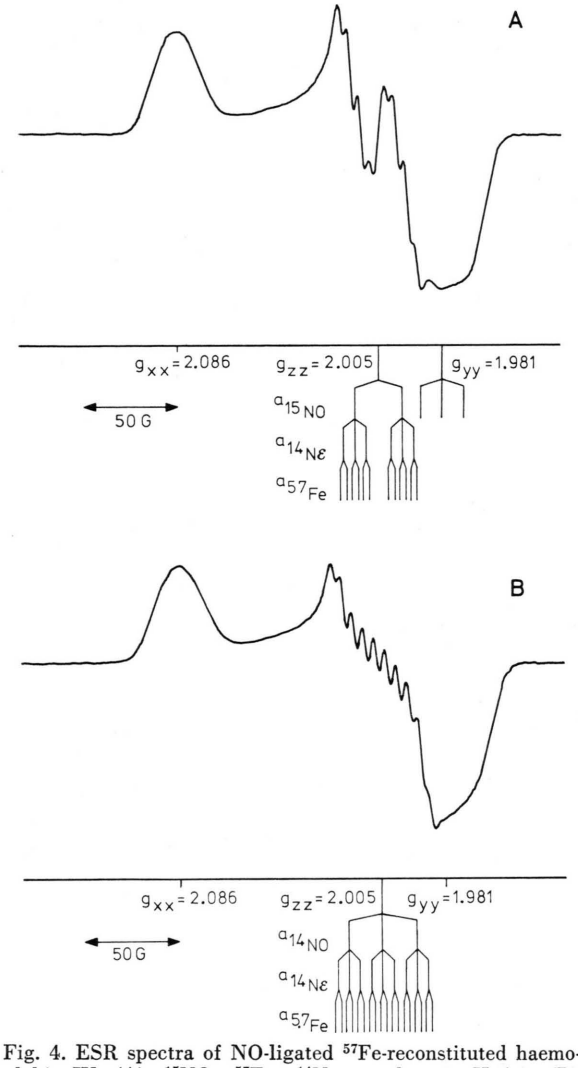

Fig. 4. ESR spectra of NO-ligated ^{57}Fe-reconstituted haemoglobin IV. (A), $^{15}NO-{}^{57}Fe-{}^{14}N\varepsilon$ complex at pH 5.4; (B) $^{14}NO-{}^{57}Fe-{}^{14}N\varepsilon$ complex at pH 9.1; temperature: 77 K.

by the ^{15}NO substitution and therefore one may argue that the hyperfine splitting of the NO is smaller than that of the central ^{57}Fe.

At $g_{zz} = 2.005$ the ESR spectrum is characterized by the additional hyperfine interaction with the nuclear spin of ^{57}Fe. In the z-direction a totally resolved spectrum should show an 18-line structure in case of the $^{14}NO-{}^{57}Fe-{}^{14}N\varepsilon$ complex and a 12-line structure in case of the $^{15}NO-{}^{57}Fe-{}^{14}N\varepsilon$ complex. Fig. 4, however, shows a 10-line and an 8-line structure respectively. This reduction of the number of lines is due to the overlapping of the broad hyperfine lines (see Fig. 4). In case of the $^{15}NO-{}^{57}Fe-{}^{14}N\varepsilon$ complex the doublet due to the interaction of the unpaired electron with the ^{15}NO is clearly seen. Each line of the doublet is split further into four lines. The splitting of the outer lines a_0 is the sum of the ^{57}Fe splitting a_{Fe} and an unknown splitting quantity a_x (see formula (1)), the splitting of the inner lines a_i is the sum of the ^{57}Fe splitting and twice of the unknown splitting quantity (see formula (2)).

$$a_0 = a_{Fe} + a_x \,. \tag{1}$$

$$a_i = a_{Fe} + 2\,a_x = a_{N\varepsilon} \,. \tag{2}$$

Thus the ^{57}Fe splitting can be calculated using equations (1) and (2). Formula (2) further shows that a_i must be identical to $a_{N\varepsilon}$ which is determined by analysis of the spectra of the $^{15}NO-{}^{56}Fe-{}^{14}N\varepsilon$

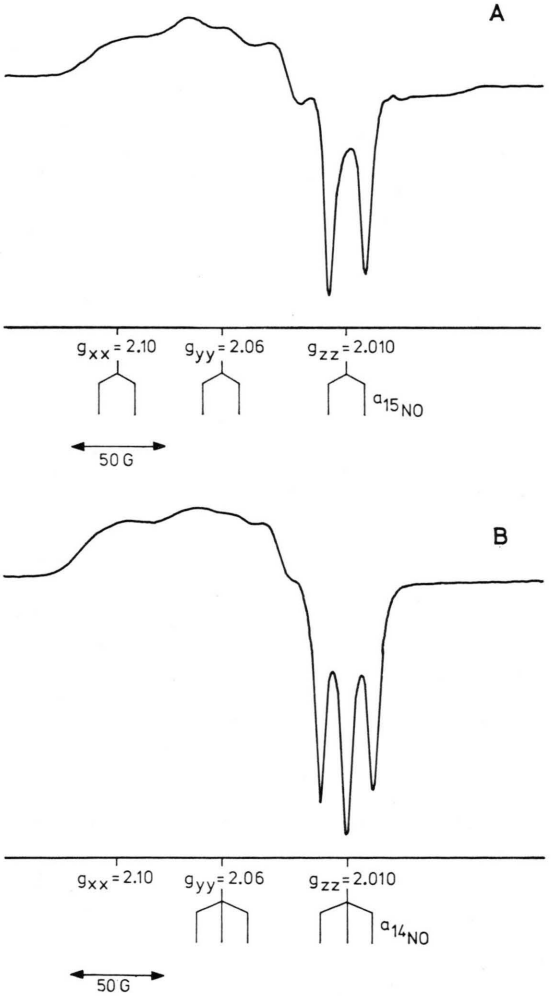

Fig. 5. ESR spectra of NO-ligated haemoglobin I modified by binding of sodium dodecylsulfate. (A) $^{15}NO-{}^{56}Fe-{}^{14}N\varepsilon$ complex at pH 9.0; (B) $^{14}NO-{}^{56}Fe-{}^{14}N\varepsilon$ complex at pH 8.6; temperature: 77 K.

complex. The analysis of the spectra of Fig. 4 leads to an iron hyperfine splitting constant of $a_{Fe} = 0.382 \pm 0.03$ mT. Spectrum simulation with the splitting constants, compiled in Table I, leads to an exact agreement with the experiment.

ESR spectra of the NO derivatives of haemoglobin I and IV modified by detergent-binding

In the presence of a 30 molar excess of sodium dodecyl sulfate or laurylpyridinium chloride and a higher molar excess of Tween 20 the NO-haemoglobins are transformed into a "relaxed" conformation state [9, 36–38]. This change of the protein structure is reflected by the ESR spectra of the nitrosyl complexes (see Fig. 5). The spectra are characterized by a typical low field shift of the g values (see Table I): $g_{xx} = 2.10$, $g_{yy} = 2.06$, and $g_{zz} = 2.010$. Again, a rhombic symmetry of the ligand field is observed, but contrary to the native haemoglobins in the modified haemoglobins g_{yy} approximates g_{xx}.

The resonances at g_{xx}, g_{yy}, and g_{zz} show the same hyperfine structures and the same hyperfine splitting constants. In the case of ligation with ^{15}NO 2-line structures with a splitting constant of 2.32 mT are observed (see Fig. 5 A), in the case of ligation with ^{14}NO 3-line structures with a splitting constant of 1.66 mT occur (see Fig. 5 B). Substitution of the central iron for ^{57}Fe leads to a further splitting of the hyperfine lines at g_{zz} into doublets with a splitting constant of 0.48 mT (see Fig. 6).

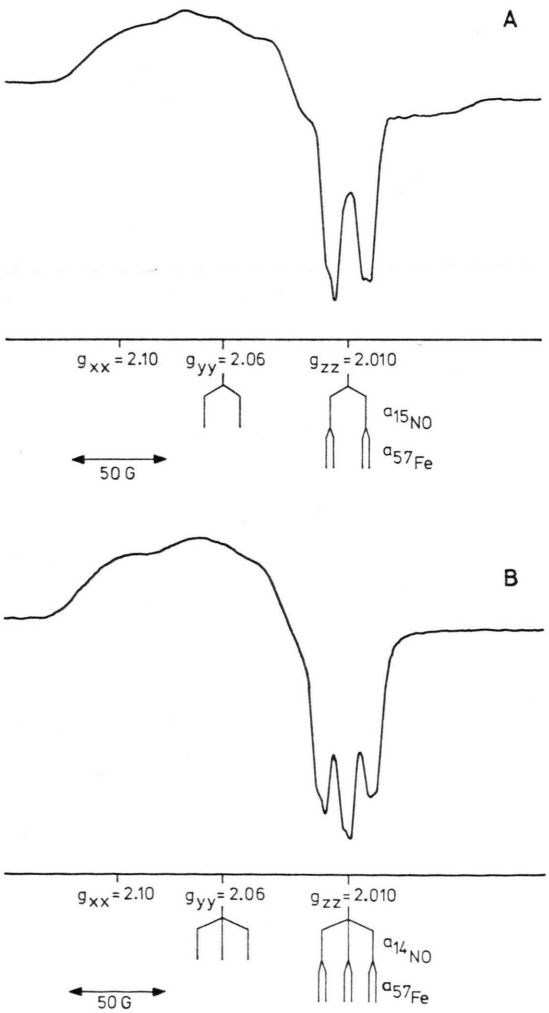

Fig. 6. ESR spectra of NO-ligated ^{57}Fe-reconstituted haemoglobin IV modified by binding of sodium dodecylsulfate. (A), $^{15}NO-^{57}Fe-^{14}N\varepsilon$ complex at pH 9.0; (B), $^{14}NO-^{57}Fe-^{14}N\varepsilon$ complex at pH 8.4; temperature: 77 K.

4. Discussion

The "trans-effect" of the histidine F8 involved in the trigger mechanism of ligand binding in haemoglobins

In 1961 Rufus Lumry proposed the rack mechanism to be a possible trigger of the ligand binding in haemoglobins [39]. This rack mechanism in haemoglobins predicted that the electronic properties of the central iron could be altered under the control of the protein structure by changing the binding geometry and/or the binding distance of the proximal imidazole [40].

This change of the orbital symmetry of the iron necessarily modifies the binding geometry and binding distance of the ligand in *trans*-position which may be further influenced by "*cis*-effects", *i. e.* direct interactions of the 6th ligand with amino acid residues at the distal site of the haem. Later this hypothesis attained more spectroscopic evidence when electron spin resonance experiments demonstrated that the conformation states of the subunits of haemoglobins can be defined by the anisotropy of the g-values and by the hyperfine structures which originate from the interaction of the unpaired spin with the nuclei of the axial ligands [4, 41]. Although these earlier electron spin resonance experiments gave information only for the binding properties of one of the axial ligands, the 6th or the 5th ligand respectively, one could conclude that the two axial ligands in haemoglobins interact in a closely balanced way. A strong delocalization of the spin

to the proximal ligand corresponds to a weak delocalization of the spin to the distal ligand and vice versa. This spin delocalization is controlled by the respective conformation *via* the proximal imidazole.

The NO-haemoglobins are excellent models for studying the "*trans*-effect". The ESR spectra of the native and the modified NO haemoglobins of *Chironomus* can be classified as type II and type I spectra respectively. Both types of spectra differ remarkably in the anisotropy, the g-values, and the hyperfine splitting constants (see Table I). In a previous paper these two types of spectra were interpreted in terms of two kinds of NO-haem-base complexes, differing in the relative iron-NO and the iron-imidazole distances [2]. The type II spectrum was attributed to a complex with a strong σ bond of the imidazole at the 5th position and a weaker σ bond of the NO in *trans*-position. Thus the type II spectrum indicates a relatively short iron-imidazole distance and a larger iron-NO distance. The type I spectrum, however, demonstrates a strong σ bond of the NO at the 6th position, but a weaker σ bond of the imidazole at the 5th position. Therefore, in a complex with a type I spectrum a short iron-NO distance correlates with a large iron-imidazole distance. The spectral transition from a type II to a type I spectrum observed in isolated α chains and tetrameric haemoglobins at low pH or in the presence of polyphosphates [2, 15, 16] offered the opportunity to discuss the "*trans*-effect" of the proximal imidazole on the basis of binding properties of both axial ligands in one complex. The additional determination of the hyperfine splitting constants of the central iron in these two types of NO complexes leads now to a much clearer picture of the change of the spin density during the conformation transition and proves thus excellently the "*trans*-effect" mechanism in haemoglobins.

The comparison of the two types of ESR spectra demonstrates that the transition from the native (type II spectrum) to the modified NO-haemoglobin (type I spectrum) is characterized by a decrease of the $^{15/14}$NO hyperfine splitting by 25% and an increase of the ^{57}Fe hyperfine splitting by 26%, whereas the ^{14}Nε hyperfine splitting becomes so small, that it cannot be resolved in the type I spectrum. This change of the hyperfine splitting constants corresponds to a change of the spin density and confirms our earlier interpretation, that a conformation-controlled weakening or break of the σ bond at the 5th position causes a strengthening of the σ bond at the 6th position [2].

From the type I spectrum itself it cannot be finally decided between two possibilities, namely a stretched σ bond or a broken σ bond at the 5th position. Pentacoordinated NO-haem model compounds showed ESR spectra which correlate closely to type I spectra of NO-haemoglobins [7, 42].

Other spectral features are also consistent with the *trans*-effect picture: the low field shift of the g_{zz} tensor in the type I spectrum indicates an increase of the spin-orbit coupling and corresponds to an increase of the probability of the unpaired electron in the d_{z^2} orbital. The type II spectrum describes a strong interaction of the d_{yz} orbitals with the π orbitals of the proximal imidazole. The g_{yy} tensor is shifted to high field and shows a 3-line hyperfine structure which cannot be removed by the isotopic exchange of NO. The hyperfine coupling constant of 1.4 mT demonstrates strong back donation of the iron to the imidazole. In the modified NO haemoglobins the g_{yy} tensor shifts to low field and leads to a hyperfine structure at g_{yy} which has to be attributed exclusively to the NO ligand. In this NO-complex the hyperfine coupling is nearly identical in the three directions and has to be assumed more or less isotropic.

Correlation of the types of ESR spectra with affinity states of haemoglobins

The observation of the conformation dependent spectral transition from the type II to the type I spectrum has motivated Salhany and coworkers to correlate these types of spectra with CO or O_2 affinity states of haemoglobin [43]. Some examples may illustrate the difficulties of such a simple correlation: Hb M Iwate is a mutant haemoglobin which is stabilized at pH 7 in the quaternary T structure with low O_2 and CO affinity [44]. Ligation with O_2 and CO induces no quaternary transition to the high affinity state (R-structure) [45]. The ESR spectrum of the $\alpha_2^{Mmet}\beta_2^{NO}$ species of this haemoglobin can be classified as a type II spectrum without pH-dependent transition to a type I spectrum. From the Hb M Iwate experiments we can conclude that the type II spectrum correlates to a low affinity state of this mutant.

The monomeric *Chironomus* haemoglobins III and IV exhibit an allosteric transition from a low affinity to a high affinity state in case of O_2 and CO binding [34, 35]. Myoglobin and *Chironomus* haemo-

globin I, however, show no Bohr effect for O_2 and CO [33]. Although these four monomeric haemoglobins differ with regard to their CO affinity states, the NO derivatives are characterized by type II spectra and no pH dependent transition from the type II to a type I spectrum occurs. A spectral transition of these monomeric haemoglobins can be induced by the association of anionic, cationic, and non-ionic detergent molecules respectively. The ionic detergent molecules are bound to the protein in a stoichiometric manner and penetrate with their hydrophobic tails into the hydrophobic core of the protein, thus stretching or breaking the iron-imidazole bond [38]. The monomeric haemoglobins demonstrate the difficulty in correlating CO affinity states to the type of ESR spectra of nitrosyl compounds. In the case of *Chironomus* haemoglobin III it was shown that O_2, CO and NO differ remarkably with regard to their influence on the allosteric properties of this haemoglobin [18, 22, 35]. NO with its strong σ donor properties stabilizes this haemoglobin in the low affinity conformation. This result and the identity of the ESR spectra of NO ligated Hb M Iwate and the monomeric haemoglobins seem to prove that NO transforms the tertiary conformation of these haemoglobins into one state which is the t conformation with low affinity. Therefore the conformational transition of haemoglobins occuring above pH 6.5 associated with the alkaline Bohr effect can not be observed in the case of NO haemoglobins.

Hb A and isolated α-chains show the spectral transition from the type II to the type I spectrum at low pH (inflection point pH 5.35) [2, 15]. Isolated β-chains, however, demonstrate the type I spectra at high pH ($>$pH 8) [2]. The type I spectrum described for Hb A and isolated α-chain can be possibly correlated to the high affinity state of the acid Bohr effect, that of the isolated β-chains to the high affinity state of the alkaline Bohr effect.

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