Non-Equivalence and Inverse Allosteric Response of the α and β Chains in Haemoglobins. An Electron Spin Resonance Study of NO-Ligated Hb Kansas

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Nitrosyl Haemoglobin Kansas, Non-Equivalence and Inverse Allosteric Response, Electron Spin Resonance

The electron spin resonance (ESR) spectra of ^{15}NO- and ^{14}NO-ligated Hb Kansas have been measured at 77 K in the range of pH 5 to 10. At low pH the ESR spectrum is the composite of a type I and a type II spectrum which changes to another composite of a type I and type II spectrum at high pH. For the definition of type I and type II spectra and the correlation of these types with two tertiary conformation states see Overkamp *et al.*, Z. Naturforsch. **31 c**, 524 [1976]. Both, the type I and the type II spectra observed at low and high pH respectively are different with regard to g-tensors and hyperfine-splitting constants. Therefore at intermediate pH values the ESR spectra of NO-Hb Kansas are the composites of four spectral components.

The assignments of the four spectral components to the α and the β chains are arrived at from the comparison of the ESR spectra of the $\alpha_2^{Mmet}\beta_2^{NO}$ and of the $\alpha_2^{MNO}\beta_2^{NO}$ species of Hb M Iwate. α and β chains are both characterized by a pH-dependent spectral transition from a type I to a type II spectrum. The chains are non-equivalent with regard to both the type I and the type II spectra. The type I spectra assigned to the α and the β chains are characterized by $g_{zz}^{*}=2.009_5$ with a hyperfine splitting of a_{zz}^{*} (^{15}NO) = 2.36 mT and $g_{zz}=2.008_5$ with a hyperfine splitting of a_{zz} (^{15}NO) = 2.41 mT respectively. The type II spectra assigned to the α and the β chains are characterized by $g_{zz}^{*\prime}=2.005$ and a hyperfine splitting of $a_{zz}^{*\prime}$ (^{15}NO) = 3.07 mT and $g_{zz}^{\prime}=2.005$ and a hyperfine splitting of $a_{zz}^{\prime}$ (^{15}NO) = 3.31 mT.

The change of the hyperfine splitting at g_{zz} during the transition from type I to type II corresponds to an increase of the spin density at the NO by about 25% in both types of chains. Comparison of type I spectra of the NO-ligated α and β chains respectively demonstrates that the spin density at the NO is larger in the β chains than in the α chains.

The spectral types are correlated with functional states defined by the kinetics of NO-binding.

Binding of inositol hexaphosphate has no influence on the ESR spectra in the whole range of pH as it is expected if NO-Hb Kansas is in the quaternary T structure.

Introduction

Recently studies of the NO-binding of haemoglobins gained an increasing interest because these experiments shed light upon three aspects of haemoglobin function:

i. The allostery of haemoglobins depends on the electronic structure of the ligand bound at the 6th position of the haem iron [1, 2]. NO being a strong σ donor as well as a strong π acceptor ligand stabilizes the tertiary conformation of *monomeric haemoglobins* in a conformation which is ESR-spectroscopically characterized by a type II spectrum [1, 3–9]. A detailed description and definition of type I and II spectra of NO-haemoglobins is given in references [3] and [8], respectively.

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i.i. The NO-ligated *isolated α and β chains* of Hb A are ESR-spectroscopically and functionally non-equivalent [4, 6, 8]. Both types of subunits change their spectra from type II to I with pH. The isolated α chains show this spectral transition at low pH ($<$ pH 6.5), the isolated β chains, however, at high pH ($>$ pH 8) [6, 8].

i.i.i. The NO-ligated *α- and β-chains involved in the quaternary structure* of haemoglobin respond differently to tertiary structure changes of their partner chains [6]. This allosteric response of the subunits seems to be modified by the binding of the allosteric effectors 2,3-bisphosphoglycerate [5] and inositol hexaphosphate [10], and depends on the respective quaternary structure state of haemoglobin [8, 11].

The NO-haemoglobins with a total spin of $S=1/2$ show ESR spectra being sensitive indicators of conformation states of the protein molecule. These conformation states are correlated to two types of ESR

spectra indicating different probability densities of the unpaired spin at the axial ligands and at the central metal ion [3]: The type I spectrum of ^{14}NO-haemoglobin with a typical 3-line hyperfine structure at $g_{zz} = 2.010$ ($a_{zz}(^{14}NO) = 1.66$ mT, $a_{zz}(^{57}Fe) = 0.48$ mT) corresponds to a relatively small spin density at the NO, a relatively large spin density at the iron and a very small or zero spin density at the Nε of imidazole; the type II spectrum of ^{14}NO-haemoglobin with a typical 9-line superhyperfine structure at $g_{zz} = 2.005$ ($a_{zz}(^{14}NO) = 2.19$ mT, $a_{zz}(^{57}Fe) = 0.38$ mT, $a_{zz}(^{14}N\varepsilon) = 0.72$ mT) demonstrates an increase of the spin density at the NO by 25% and a decrease of the spin density at the ^{57}Fe by 26%. The superhyperfine splitting originating from the ^{14}Nε at the 5th position of the iron increases and a further triplet appears in the three hyperfine lines of NO. This change of the spin density induced by the conformational transition elucidates most obviously how bond length and binding-geometry of the NO in haemoglobin are controlled via the trans-effect by the 5th ligand, which is the Nε of imidazole of His F8.

The spectral transition from a type II to a type I spectrum (inflection point at pH 5.35) described for Hb A has been correlated with the acid Bohr effect [5]. Inositol hexaphosphate [10] and 2,3-bisphosphoglycerate [5] bound to the central cavity of Hb A shift this spectral transition to higher pH values. The spectral change from a type II to a type I spectrum can also be induced by detergent binding [3, 12].

A comparison with pentacoordinated nitrosyl iron compounds [13, 14] lead to the conclusion that the type I spectrum could correspond to a broken or stretched imidazole-iron bond and a small NO-iron distance [3, 14]. The type II spectrum, however, reflects a smaller imidazole-iron distance and a larger NO-iron distance compared with the type I spectrum.

This paper makes a contribution to the third aspect of haemoglobin function mentioned above describing the influence of pH and inositol hexaphosphate-binding on the NO-ligated Hb Kansas. Hb Kansas is a mutant haemoglobin characterized by a replacement of Asn G4(102) by Thr in the primary structure of the β chains [15]. This abnormal haemoglobin shows a low oxygen affinity and a reduced cooperativity [15–17]. Fully O_2- or CO-ligated Hb Kansas which is in the quarternary R structure is switched to the quaternary T structure by binding of inositol hexaphosphate [18]. Stripped (polyphosphate-free) Hb Kansas can be already transformed to the quaternary T structure by ligation with NO [19]. If stripped NO-ligated Hb Kansas exists in the quaternary T state, one would expect no influence of the IHP-binding on the ESR spectrum. On the other hand a pH-dependent transition of the ESR spectrum would indicate a tertiary structure change of the subunits being involved in the quaternary T structure. Furthermore, a comparison of the spectra of the NO-ligated forms of Hb Kansas and of Hb M Iwate allow an assignment of the ESR signals to the α- and β-chains. With the assigned spectra the intrinsic allostery of the chains of Hb Kansas can be described. Hb M Iwate with a replacement of His F8 by Tyr is a mutant haemoglobin being stabilized in the quaternary T structure even in the ligated form. Contrary to Hb Kansas Hb M Iwate normally provides only the β chains for the binding of NO, whereas the α-chains are hexacoordinated and in the met-form [8, 20–22].

Materials and Methods

Preparation of haemoglobin

Haemoglobin Kansas prepared according to [15] was a gift of Dr. S. Ogawa. The CO-ligated haemoglobin was stripped from organic phosphates by gel filtration on a Sephadex G25 column equilibrated with a 0.01 M Tris/HCl buffer pH 8.5 containing 0.10 M NaCl. Then the haemoglobin solution was dialyzed against 0.10 M NaCl and concentrated by vacuum dialysis. The concentration of this stock solution was 148 mg/ml Hb. The material was stored at 4 °C under CO gas.

NO Derivatives

The nitrosyl Hb Kansas was prepared at room temperature under oxygen-free nitrogen gas. A volume of 0.05 ml stock solution of the CO-ligated haemoglobin was mixed with 0.2 M buffer up to a final volume of 0.2 ml. Citrate/NaOH, bis-Tris/HCl, Tris/HCl and glycine/NaOH were used as buffers in the range from pH 5 to 10. Then successively 6 mg sodium ascorbate and 1 mg sodium nitrite were added. After a reaction time of 10 min the pH was measured with a glas electrode (type pH 406 M3, Ingold, Frankfurt a. Main) and a pH-Meter (type PHM 63, Radiometer, Copenhagen). The solution was transferred to a quartz tube with 3.2 mm diameter, quickly frozen in liquid nitrogen and sealed up. $Na^{14}NO_2$ (analytical grade) was a product of Merck

(Darmstadt), $Na^{15}NO_2$ with an isotopic enrichment of 95% was perchused from Prochem (London).

For reaction of inositol hexaphosphate with CO-ligated Hb Kansas the respective buffer contained a 6 molar excess of the polyphosphate. Thus NO-ligation was performed after having switched the Hb Kansas to the quaternary T structure. Sodium inositol hexaphosphate was a product of Sigma (St. Louis).

Electron spin resonance spectra

The ESR measurements were carried out at 77 K with an X-band spectrometer (type BER 420, Bruker-Physik, Karlsruhe). Quartz tubes with an inner diameter of 3.2 mm were filled with 0.2 ml solution of NO-haemoglobin. The amplitude of the 100 kHz field modulation was 0.05 mT. The microwave power was attenuated to 8 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. For measuring the pH-dependence of the signal amplitude of the hyperfine lines at g_{zz} the setting of the signal gain, the Q-factor of the cavity and the above mentioned parameters of the instrument were proved to be constant by measuring the signal amplitude of a reference sample after each run. The second derivative of the spectrum was recorded by use of the 100 kHz and the 1 kHz field modulation unit. The modulation amplitudes were in both cases 0.1 mT.

Calculation of proton dissociation curves

Model proton dissociation curves were calculated with a computer (type TEK 31, Tektronix) with digital plotter.

Results

Electron spin resonance spectra of nitrosyl haemoglobin Kansas

A cursory inspection of the ESR spectra of NO-ligated Hb Kansas measured at 77 K demonstrates rhombic symmetry of the heam iron ($g_{xx} \neq g_{yy} \neq g_{zz}$). The exact determination of the g tensors and the analysis of the influence of the pH on the g-value anisotropy manifest that the ESR spectra are essentially the composites of at least two anisotropic spectra. Hyperfine structures and g tensors characterize these overlapping individual spectra as type I and type II spectra respectively.

It shall be demonstrated later that the type I and the type II spectra can be attributed to both the α and the β chains of Hb Kansas; furthermore, it is shown that the type I as well as the type II spectra of both the α and the β chains differ significantly with regard to the g-tensors and to the hyperfine splitting constants. Therefore under special conditions the maximum of four individual spectra (type I and II of the α chains and type I and II of the β chains) contribute to the total spectrum of the NO-Hb Kansas. This complex ESR spectrum originating from the superposition of two or four individual spectra shows a characteristic pH-dependent change of the intensity of the signals, of the g tensors, and of the hyperfine splitting constants. Raising the pH from 5 to 10 a pair of spectra (type I and II) changes to another pair of spectra (type I and II) differing in the g-tensors and hyperfine splitting constants.

The analysis of the complex spectrum for describing the spectral types and assigning the hyperfine splitting constants to one of the axial ligands is facilitated by comparing the spectra of the ^{15}NO and ^{14}NO derivatives. The attribution of the resonance lines to the particular spectra components is possible if one compares spectra obtained at different pH values. The assignments of the two types of spectra to the α and the β chains respectively were made possible by comparison with the ESR spectra of the nitrosyl haemoglobin M Iwate [8]. Typical ESR spectra measured at pH 5.1, pH 7.1 and pH 9.8 defining characteristic conformation states of Hb Kansas are described in more detail.

Fig. 1 shows the ESR spectrum of ^{15}NO-ligated Hb Kansas at pH 5.1. The dominant feature of this spectrum is a type I spectrum which overlaps a type II spectrum. This type I spectrum of Hb Kansas at pH 5.1 is characterized by the g-tensors: $g^*_{xx} = 2.105$, $g^*_{yy} = 2.063$ and $g^*_{zz} = 2.009_5$. In each of the three resonances a 2-line hyperfine structure appears which has to be attributed to the interaction of the unpaired spin with the ^{15}N nucleus of the NO ligand. The hyperfine splitting constants are: $a^*_{xx} = 1.87$ mT, $a^*_{yy} = 2.36$ mT and $a^*_{zz} = 2.36$ mT (see Table I). Thus at pH 5.1 a considerable anisotropic term contributes to the hyperfine splitting of the type I spectrum. The type II spectrum at pH 5.1 can be identified by the 2-line hyperfine structure at $g'_{zz} = 2.005$. Although the g'_{zz} value cannot exactly be determined from the complex spectrum and was taken from the literature [3, 8], the hyperfine splitting constant $a'_{zz}(^{15}NO) = 3.31$ mT and the superhyperfine splitting $a'_{zz}(^{14}N_\varepsilon) = 0.60 \pm 0.03$ mT attributed to the $^{14}N_\varepsilon$ of imidazole were measured

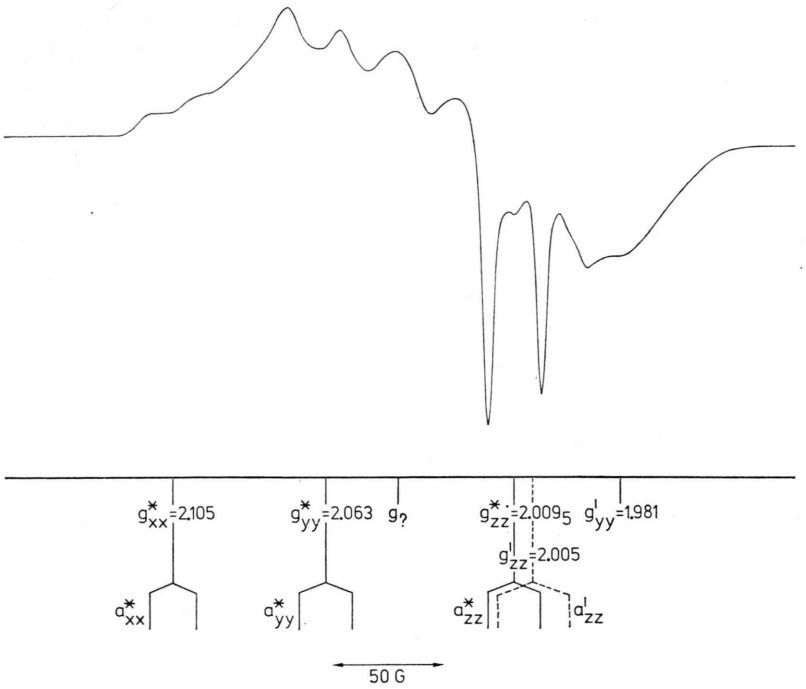

Fig. 1. ESR spectrum of ^{15}NO-ligated Hb Kansas at pH 5.1; temperature: 77 K.

with high accuracy from the second derivative spectrum and correspond to the respective values found for type II spectra [3, 8]. $g'_{yy} = 1.981$ is identical to that described for a type II spectrum of monomeric haemoglobins[3]. g'_{xx} totally superimposed by the type I spectrum cannot be determined.

The ESR spectrum of the ^{15}NO-Hb Kansas at pH 9.8 again is the composite of a type I ($g_{xx} = 2.09$, $g_{yy} = 2.057$ and $g_{zz} = 2.008_5$) and a type II spectrum ($g^{*\prime}_{yy} = 1.981$ and $g^{*\prime}_{zz} = 2.005$) (see Fig. 2). Comparison with the spectrum obtained at pH 5.1 demonstrates slight but significant changes of the g-tensors and the hyperfine splitting constants of both types of spectra. The type I spectrum is characterized by a decrease of $g_{yy} = 2.057$ ($< g^*_{yy}$) and of $g_{zz} = 2.008_5$ ($< g^*_{zz}$). Furthermore, the hyperfine splitting at g_{yy} is smaller ($a_{yy} = 2.09$ mT) and that at g_{zz} is larger ($a_{zz} = 2.41$ mT) than the respective type I parameter at pH 5.1. In the type II spectrum a decrease of the hyperfine splitting at $g^{*\prime}_{zz}$ is observed ($a^{*\prime}_{zz} = 3.07$ mT) if one increases the pH from 5 to 10.

Table I. ESR parameter of NO-Hb Kansas at 77 K.

Complex	^{15}NO—^{56}Fe—^{14}Nε							
pH	5.1		9.8		7.1			
type of ESR spectrum	I(g^*)	II(g')	I(g)	II($g^{*\prime}$)	I(g^*)	I(g)	II($g^{*\prime}$)	II(g')
subunit	α	β	β	α	α	β	α	β
g_{xx}	2.105	n.r.	2.09	n.r.	2.105	2.09		n.r.
hyperfine lines	2	n.r.	n.r.	n.r.	n.r.	n.r.		n.r.
hyperfine splitting [mT]	1.87	n.r.	n.r.	n.r.	n.r.	n.r.		n.r.
g_{yy}	2.063	1.981	2.057	1.981	2.063	2.057		1.981
hyperfine lines	2	n.r.	2	n.r.	2	2		n.r.
hyperfine splitting [mT]	2.36	n.r.	2.09	n.r.	2.47	2.09		n.r.
g_{zz}	2.009_5	2.005 [a]	2.008_5	2.005 [a]		2.008_9		2.005 [a]
hyperfine lines	2	2	2	2		2		2
hyperfine splitting [mT]	2.36	3.31	2.41	3.07		2.39 [c]		3.28 [b]

[a] g-tensor taken from [3, 8]. [b] $a^{*\prime}_{zz} < 3.28$ mT $< a'_{zz}$. [c] $a^*_{zz} < 2.39$ mT $< a_{zz}$. n.r., Not resolved.

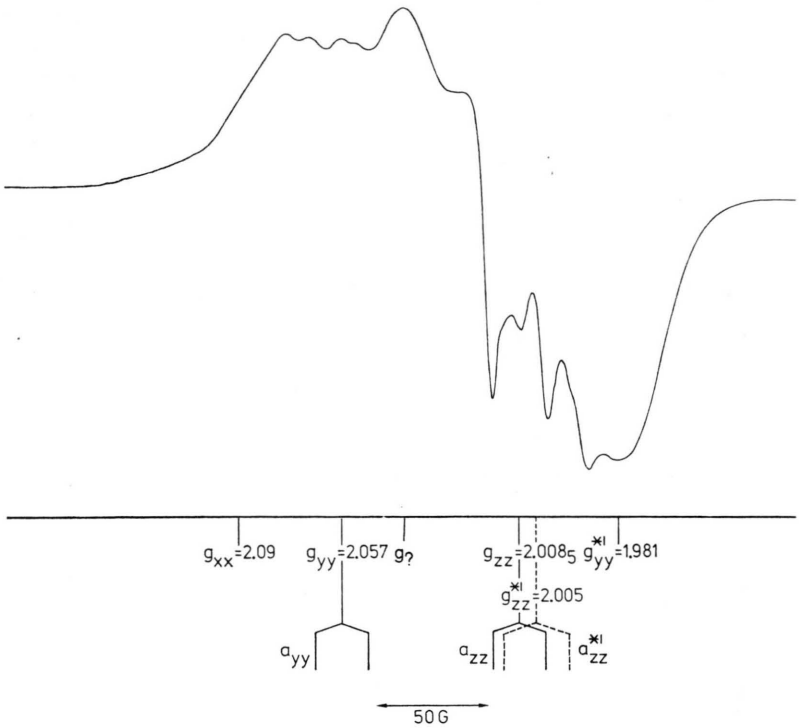

Fig. 2. ESR spectrum of ^{15}NO-ligated Hb Kansas at pH 9.8; temperature: 77 K.

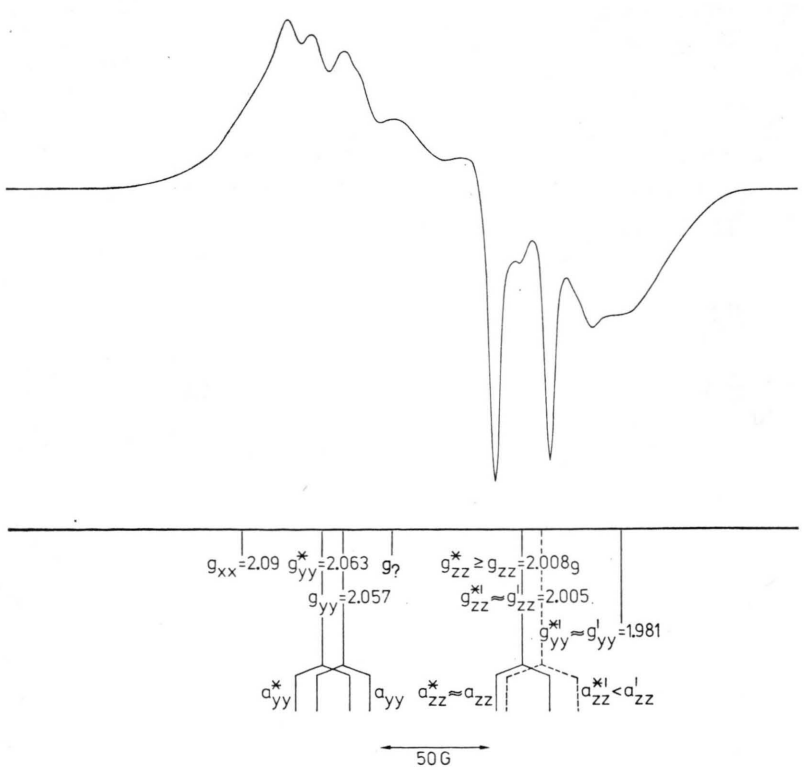

Fig. 3. ESR spectrum of ^{15}NO-ligated Hb Kansas at pH 7.1; temperature: 77 K.

These differences at pH 5.1 and 9.8 lead to the conclusion that the spectral transitions are linked to the dissociation of protons. Thus the ESR spectra measured at values between pH 5.1 and 9.8 are the composites of four spectral components. This is shown in Fig. 3. At pH 7.1 the ESR spectrum of ^{15}NO-Hb Kansas exhibits *two* type I spectra defined by g^* and g values respectively (see Table I). The g-tensors of these type I spectra for the x- and y-direction are identical to those described for the spectra at pH 5.1 and 9.8. The g-tensors of the type I spectra for the z-direction cannot be resolved, thus only *one* g-tensor $g^*_{zz} > 2.008_9 > g_{zz}$ and *one* hyperfine splitting $a^*_{zz} < 2.39\,\mathrm{mT} < a_{zz}$ is observed. The pH-dependent variation of the g_{zz} tensor and of the hyperfine splitting demonstrates that the g_{zz} values and hyperfine splitting constants measured at intermediate pH are the composites of $g^*_{zz} = 2.009_5$ (at pH 5.1) and $g_{zz} = 2.008_5$ (at pH 9.8) and of $a^*_{zz} = 3.36\,\mathrm{mT}$ and $a_{zz} = 2.41\,\mathrm{mT}$, respectively. The *two* type II spectra defined by $g^{*\prime}$ and $g^{\prime}$ are not resolved in the spectrum at pH 7.1, but their existence can be proven by the hyperfine splitting $a^{\prime}_{zz} > 3.28\,\mathrm{mT} > a^{*\prime}_{zz}$ which continuously changes with pH. The decrease of the splitting constant with increasing pH indicates that this parameter of the type II spectrum is the composite of $a^{\prime}_{zz} = 3.31\,\mathrm{mT}$ (at pH 5.1) and $a^{*\prime}_{zz} = 3.07\,\mathrm{mT}$ (at pH 9.8).

The exchange of ^{15}NO by ^{14}NO in NO-Hb Kansas leads to a replacement of the doublet hyperfine structures by triplet hyperfine structures (see Fig. 4). The g-tensors determined in the ^{14}NO-Hb Kansas spectrum at pH 6.3 are identical to those determined in the respective ^{15}NO-Hb spectrum. Furthermore, the hyperfine splitting constants of the ^{14}NO-Hb spectrum are identical to those calculated on the basis of magnetogyric ratios from the ^{15}NO-Hb spectrum. This identity of the g-tensors and hyperfine splitting constants makes evident that the assignments for the resonances to the spectral components (two type I and two type II components) made in the ^{15}NO-Hb spectra are correct.

The ESR signal observed at $g = 2.04$ is to be found in all spectra of NO-Hb Kansas. Furthermore, this resonance occurs in other NO-ligated haemoglobins [3, 5, 8, 12, 14, 28]. The amplitude and the position of this signal does not change with pH. Therefore we assume, that it can not be attributed to one of the above described spectral components. The origin of this signal is up to now unknown.

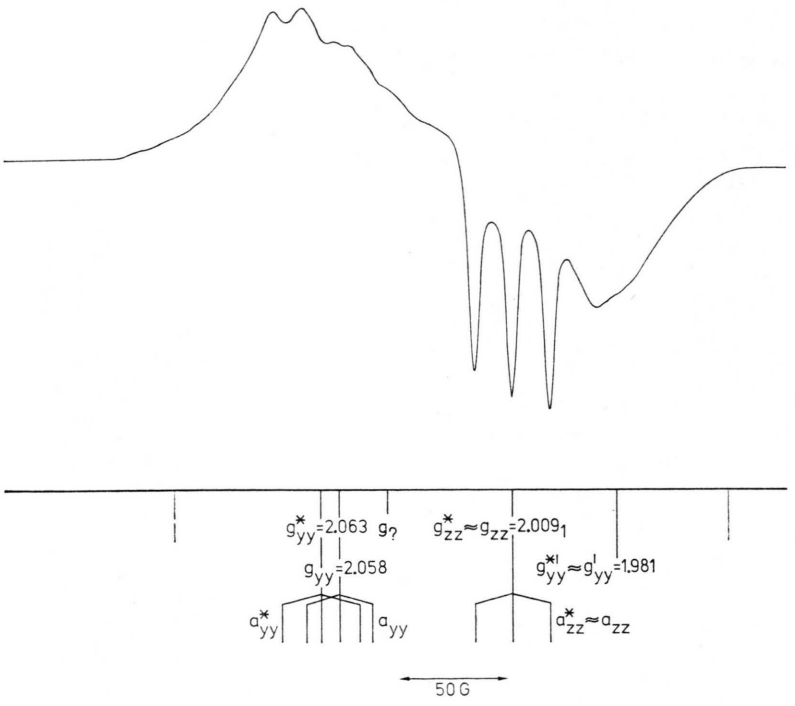

Fig. 4. ESR spectrum of ^{14}NO-ligated Hb Kansas at pH 6.3; temperature 77 K.

Assignment for the spectral types to the subunits of NO-Hb Kansas

As described in Table I two components of the ESR spectra of NO-Hb Kansas can be attributed to the α chains and two further components to the β chains. These assignments can be arrived at from the comparison of the ESR spectra of two different ligation states of Hb M Iwate. The spectra of the $\alpha_2^{\mathrm{Mmet}}\beta_2^{\mathrm{NO}}$ and the $\alpha_2^{\mathrm{MNO}}\beta_2^{\mathrm{NO}}$ species are published in Fig. 5 and Fig. 6 of reference [8] respectively. The second derivatives of the ESR spectra of Hb M Iwate, studied in the course of this work, exhibit a higher resolution especially in the resonance region of g_{zz} (unpublished results).

Under mild conditions of reduction the haem iron of the α chains remains oxidized and then Hb M Iwate binds NO only at the haem iron of the β chains. Thus the ESR spectrum of the $\alpha_2^{\mathrm{Mmet}}\beta_2^{\mathrm{NO}}$ species is exclusively that of the β chains in a tetrameric structure. Furthermore, this mutant haemoglobin, as Hb Kansas, is stabilized in the quaternary T structure by ligation with NO. Therefore it has to be supposed that the ESR spectra of the NO-ligated β chains in Hb M Iwate and Hb Kansas are spectroscopically equivalent. The study of the ESR of the $\alpha_2^{\mathrm{Mmet}}\beta_2^{\mathrm{NO}}$ species enables us to identify those spectral components in Hb Kansas which have to be attributed to the β chains.

The second derivative spectra are now demonstrating that the NO-ligated β subunits of Hb M Iwate undergo a pH-dependent tertiary conformation change reflected by a spectral transition from a type II ($g'_{xx}=2.066$, $g'_{yy}=1.983$, and $g'_{zz}=2.005$) to a type I spectrum ($g_{zz}=2.008_3$) with increasing pH. This pH-dependent tertiary conformation change of the β chains also occurs in Hb Kansas and is reflected by the identical spectral transition from a type II (g') to a type I (g) spectrum. Not only the g-tensors but also the hyperfine splitting constants are identical for the β chains in Hb M Iwate and Hb Kansas. At $g'_{zz}=2.005$ a hyperfine splitting of a'_{zz} (^{15}NO) = 3.34 mT and a superhyperfine splitting of a'_{zz} (^{14}Nε) = 0.62 mT are observed. At $g_{zz}=2.008_3$ the hyperfine splitting amounts to $a_{zz}=2.45$ mT.

With the help of Hb M Iwate one further spectral component of NO-Hb Kansas can be assigned to the α chains. At low pH ($<$pH 6) Hb M Iwate can be totally reduced. Then this mutant haemoglobin binds additional NO at the haem iron of the α chains forming the $\alpha_2^{\mathrm{MNO}}\beta_2^{\mathrm{NO}}$ species. The ESR properties of this species, however, can only be studied at low pH where a type I spectrum ($g^*_{xx}=2.109$, $g^*_{yy}=2.062$ and $g^{*|}_{zz}=2.009_1$) different from that found at pH 9 overlaps the type II spectrum of the β chains. Therefore this type I spectrum has to be attributed to the α chains. Again, g-tensors and hyperfine splitting constants are identical with those found for α chains in Hb Kansas at low pH. The NO-ligated α chains of Hb Kansas undergo a pH-dependent tertiary conformation change reflected by the transition from a type I to a type II spectrum with increasing pH. Although the ESR spectrum of the NO-ligated α chains of Hb M Iwate cannot be studied at high pH we must assume that the fourth spectral component of NO-Hb Kansas being of type II ($g^{*\prime}$) represents the conformation state of the α chains at high pH.

Influence of pH on the electron spin resonance of nitrosyl haemoglobin Kansas

The ESR properties of NO-Hb Kansas are dependent on pH. Both the type I and the type II spectral conponents are involved in this process. The pH-effect is reflected by the change of the signal amplitude of the 2-line hyperfine structure at g_{zz}, by a shift of the g_{zz} tensor and by a variation of the hyperfine splitting at g_{zz}. These results demonstrate that both types of chains undergo a tertiary conformation change with pH.

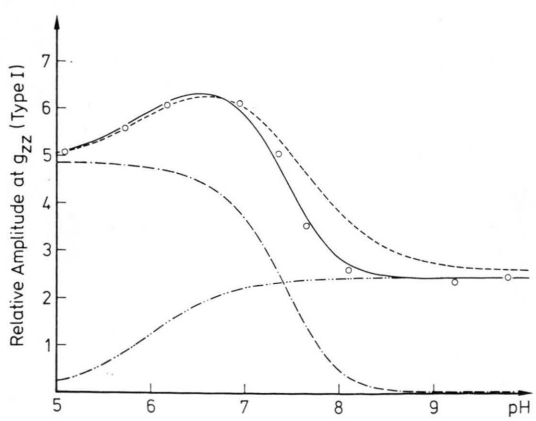

Fig. 5. pH-dependence of the type I spectrum of NO-Hb Kansas. Calculated dissociation curves with $pK_{\alpha 1,2}=7.6$ and $pK_\beta=6.0$: —··—, β subunit with one NO-linked proton; ——·——, α subunit with two NO-linked protons; ———, composite of the dissociation curves of β and α subunits with one and two NO-linked protons respectively; — — —, composite of the dissociation curves of β and α subunits with one NO-linked proton respectively.

In Fig. 5 the signal amplitude of one hyperfine line at $g_{zz} \approx 2.009$ (type 1 component) is plotted *versus* pH. This plot results in a biphasic proton dissociation curve with a maximum intensity at pH 6.6 and inflection points at pH 5.65 and 7.50. The calculation of the dissociation curve for the ligand-linked proton-binding sites of NO-Hb Kansas is based on the following properties:

1. Hb Kansas is stabilized in the quaternary T structure by NO-ligation [19].
2. The α and the β chains are non-equivalent with regard to both types of spectral components.
3. The α and the β subunits respond inversely to a change of pH: With increasing pH α subunits change their conformation from r (type I component) to t (type II component), β subunits, however, from t (type II component) to r (type I component).

Furthermore, it is assumed in this model that the NO-binding to the α chains leads to a dissociation of two cooperative protons, and the NO-binding to the β chains to a dissociation of one proton. The NO-linked proton-binding site of the α chains is independent of the NO-linked proton-binding site of the β chains. Then the dissociation curve can be expressed by the following equation:

$$A = \frac{[H^+]^2}{[H^+]^2 + [H^+]\cdot K_{\alpha_1} + K_{\alpha_1}\cdot K_{\alpha_2}} + \frac{K_\beta}{K_\beta + [H^+]}. \quad (1)$$

A represents the amplitude of the hyperfine line at g_{zz}, $K_{\alpha_{1,2}}$ and K_β are the proton dissociation constants of the α and β chains respectively. The best fit on the experimental points is obtained with $pK_{\alpha_{1,2}} = 7.6$ and $pK_\beta = 6.0$ (see Fig. 5). For comparison a dissociation curve was calculated on the assumption that α chains dissociate only one proton upon NO-binding. As shown in Fig. 5 no fit on the experimental points could be obtained with this assumption. The pH-dependence of NO-Hb Kansas supports the conclusion that the α and the β chains involved in a quaternary T structure are not only non-equivalent with regard to the NO-binding site, but also non-equivalent with regard to the number of conformation-linked proton-binding sites and inverse with regard to the allosteric response.

The inverse allosteric response of the subunits in NO-Hb Kansas can be demonstrated by the pH-dependence of the g-tensors and hyperfine splitting constants in the z-direction (parallel to the haem normal).

In the type I spectrum g_{zz} changes continuously from $g^*_{zz} = 2.009_5$ at pH 5.1 to $g_{zz} = 2.008_5$ at pH 9.8, *i. e.* an increasing pH corresponds to a spectral change from the type I component of the α chains to the type I component of the β chains. The same pH-dependence is reflected by the hyperfine splitting constant which increases from $a^*_{zz}(^{15}NO) = 2.36$ mT at pH 5.1 to $a_{zz}(^{15}NO) = 2.41$ mT at pH 9.8.

In the type II spectrum the hyperfine splitting in the z-direction changes continuously from $a'_{zz}(^{15}NO) = 3.31$ mT at pH 5.1 to $a^{*\prime}_{zz}(^{15}NO) = 3.07$ mT at pH 9.8, *i. e.* an increasing pH corresponds to a spectral change from the type II component of the β chains to the type II component of the α chains.

Influence of inositol hexaphosphate on the electron spin resonance of nitrosyl Hb Kansas

In the presence of a 6 molar excess of inositol hexaphosphate no change in the ESR spectrum of the NO-Hb Kansas is observed. The intensity of the resonance lines, the magnitude of the hyperfine splitting constants, and the position of the g-tensors are constant. The change of pH reflects only the above described spectral variations, but no additional effect upon inositol hexaphosphate-binding occurs.

Discussion

Inverse allosteric response of the subunits of Hb Kansas

The α chains of the NO-ligated Hb Kansas can exist in two spectroscopically defined conformation states termed "relaxed" (r) and "tense" (t) conformation respectively. From the consistency of the model calculation with the experiment we may conclude that these conformation states are in the state of equilibrium ($r \rightleftharpoons t$) which is controlled by an allosteric site being the binding site of two cooperative protons. The r state is represented by the protonated form (low pH species of the α chains), the t state is represented by the deprotonated form (high pH species of the α chains). The pK value of this allosteric site was found to be $pK_{\alpha_{1,2}} = 7.6$ for both NO-linked protons, *i. e.* at neutral pH 76% of the NO-ligated α chains are in the tertiary r state.

The β chains of the NO-ligated Hb Kansas also can exist in two spectroscopically defined conformation states specified by type II and I spectra. Contrary to the α chains the $r \rightleftharpoons t$ equilibrium is con-

trolled by an allosteric site which is the binding site of only one proton. The t state is represented by the protonated form (low pH species of the β chains), the r state by the deprotonated form (high pH species of the β chains). The pK-value of this allosteric site was found to be $pK_\beta = 6.0$. Thus at neutral pH 92% of the NO-ligated β chains are in the tertiary r state whereas 24% of the NO-ligated α chains are in the tertiary t state.

The r conformation is ESR-spectroscopically characterized by the type I spectrum, the t conformation by the type II spectrum. The type I spectrum has been attributed to an NO complex with a relatively small NO-iron distance and a stretched or broken imidazole-iron bond [3]. The type II spectrum has been assigned to an NO complex with a relatively large NO-iron distance, but a relatively small imidazole-iron distance [3]. Since 1972 when we detected this correlation of the two types of ESR spectra with the two conformation states of NO-haemoglobin characterized by a variation of binding distances of both axial ligands [5, 1, 8, 3], this idea gained more and more support by three fundamental results:

i. Kon and Kataoka [23] demonstrated in their pioneer work that NO-haem model compounds tend to exhibit type I spectra if a weak base is occupying the 5th coordination site of the iron. NO-haem model compounds are showing type II spectra if a strong base is bound at the 5th position of the iron. Later this observation was confirmed by Henry *et al.* [24] who demonstrated, that electron supplying substituents of *p*-substituted pyridines lead to a type II spectrum, electron withdrawing substituents to a type I spectrum.

i.i. Pentacoordinated nitrosyl haem complexes are exclusively characterized by type I spectra [13, 14].

i.i.i. Finally, the simultaneous observation of the change of the spin density at the NO, the Nε of imidazole and the iron in ^{57}Fe-substituted monomeric haemoglobins during the transition from the t to the r conformation has not only proved the above mentioned correlation between σ donor strength of the base and type of ESR spectrum but also the reciprocal binding behaviour of the axial ligands known as the trans-effect of these ligands [3]. Furthermore, the ESR data and the trans-effect idea of triggering the binding distance and binding geometry of the NO are consistent with a qualitative MO scheme described in reference [8].

The question arises: How can one correlate the spectral data and the complex structure of the nitrosyl compounds with functional states of the subunits of haemoglobin? Strictly speaking, this correlation is only allowed for the NO-binding states and not for CO or O_2 affinity states because of the different electronic structures of the latter ligands. No equilibrium data for the NO-binding are available. Therefore kinetic data have to be used to describe the functional states of NO-Hb. Henry and Cassoly [25] have found that the α chains at pH 6.5 are the preferentially binding sites for NO in deoxy Hb A. The apparent association rate constant of NO is larger for the α than for the β chains. On the other hand in fully ligated Hb A the β chains preferentially dissociate the NO ligand. Henry and Cassoly attributed their kinetic data to particular chains assuming that NO-ligated α chains are characterized exclusively by a type I spectrum, NO-ligated β chains, however, exclusively by a type II spectrum. In case of NO-Hb Kansas at pH 6.5 92% of the α-chains and 77% of the β chains are of the spectral type I whereas 8% of the α chains and 23% of the β chains are of the spectral type II (see Fig. 5). No exclusive attribution of the spectral types to α or β chains can be made. This result allows the conclusion that functional states and spectral types can be correlated af follows: A large association rate and a small dissociation rate for NO correspond to a type I spectrum indicating a relatively strong σ bond at the 6th position of the iron and a relatively weak or broken σ bond at the 5th position. A smaller association rate and a larger dissociation rate for NO, however, correspond to a type II spectrum indicating a relatively weak σ bond at the 6th and a relatively strong σ bond at the 5th position.

Moore and Gibson [26] have found that the rate of dissociation of NO from partially liganded Hb A (quaternary T structure) is substantially faster than that from the fully liganded Hb A (quaternary R structure). It should be stated at this point again, that the ESR spectra of NO-ligated Hb A are reflecting only the tertiary conformation states of the subunits which may be modified by the particular quaternary structure. The resolution of the available ESR spectra of tetrameric haemoglobins is not sufficient for demonstrating the quaternary structure effects. Therefore the correlation of the type I and type II spectrum with quaternary T and R struc-

tures of NO-haemoglobins as it is found in the recent literature [19, 27] has been considered as an oversimplification without any spectroscopic meaning.

Finally the conclusion may be allowed, that the α chains of NO-Hb Kansas change from a high affinity state (r conformation, protonated form, type I spectrum) to a low affinity state (t conformation, deprotonated form, type II spectrum) by increasing the pH from 5 to 10. The β chains of NO-Hb Kansas change from a low affinity state (t conformation, protonated form, type II spectrum) to a high affinity state (r conformation, deprotonated form, type I spectrum) with increasing pH. Thus the spectral non-equivalence of the chains strongly depends on the respective pH. Both types of subunits behave inversely with regard to the pH-induced conformation change.

Non-equivalence of the subunits reflected by the binding-distance and the binding-geometry of the NO

The comparison of the type I spectra of α (at low pH) and β chains (at high pH) respectively demonstrates remarkable differences. The g_{zz} tensor of the β chains is smaller than that of the α chains ($g_{zz} < g^*_{zz}$) indicating a smaller spin orbit coupling and a larger spin density at the NO. This larger spin density at NO in the β chains is also reflected by a 2% larger hyperfine splitting ($a_{zz}(^{15}NO) > a^*_{zz}(^{15}NO)$). Thus the NO-iron distance should be larger in case of the NO-ligated β chains compared with that of the NO-ligated α chains. The g_{yy} tensor of the β chains is shifted to high field compared with that of the α chains ($g_{yy} < g^*_{yy}$) indicating an increase of the back-donation from the d_{yz} orbitals of the iron to the π system of the proximally bound imidazole. The anisotropy of the *g*-tensors and the anisotropic term of the hyperfine splitting are different for α and β chains. From this we assume a different binding-geometry of the NO in both types of subunits. With crystals of ^{15}NO-Hb Kansas Chien has determined at pH 6.6 by ESR the Fe – N – O bond angles of 167° and 105°. The bond angle of 167° is characteristic for the type I spectrum, the bond angle of 105° for the type II spectrum (personal communication). This correspondence of bond angles with the two types of spectra allows the conclusion that in the case of a relatively short imidazole-iron distance (type II spectrum) the Fe – N – O bond angle decreases and in the case of a relatively large imidazole-iron distance (type I spectrum) this bond angle increases. Furthermore, the comparison of the hyperfine splitting constants of the type I spectra of α and β chains respectively leads to the conclusion that the Fe – N – O bond angle in the β chains should approach 167°, but in any case it has to be smaller than that observed in the α chains.

The comparison of the type II spectra of α chains (at high pH) and β chains (at low pH) reflects the non-equivalence of the chains again. The *g* tensors could not be determined exactly enough. The hyperfine splitting a'_{zz} of the β chains is larger than that of the α chains ($a^{*\prime}_{zz}$). This indicates a decrease of the spin density at NO by 7% and a smaller NO-iron distance in the t state of the α chains compared to that in the t state of the β chains. The resolution of the spectra did not allow the determination of further ESR parameters.

The pH-dependent conformation change of both types of subunits leads to a change of the spin density at the NO by about 25% in both the α and the β chains.

The amplitudes of the hyperfine lines at g_{zz} (β chains) have been found to be smaller by a factor of 2 than those at g^*_{zz} (α chains). This decrease is caused by a greater line width in case of the NO-ligated β chains. Approaching imidazole nearer to iron the type I spectrum changes more and more to a spectrum with type II characteristics exhibiting a superhyperfine structure from $^{14}N\varepsilon$ (imidazole). The line broadening of the hyperfine lines at g_{zz} indicates therefore a small superhyperfine contribution from the Nε. We must therefore assume that the imidazole-iron bond is not broken in the β chains when going to type I, but may be broken in the α chains at low pH. Spectral changes in NO-Hb are dominated by those of the α chains because their hyperfine lines have smallest line widths. This led to the erroneous conclusion that the α chains are mainly involved in structural changes induced by pH and polyphosphates, but the β chains are not [27].

Finally it should be pointed out that the non-equivalence of the chains and their inverse response on allosteric effectors may persist in case of CO- or O_2-ligated haemoglobins. Because NO is a stronger σ donor than CO and O_2 the possible rupture of the imidazole-iron bond in the α chains may only occur if NO is bound.

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[1] E.Trittelvitz, H. Sick, K. Gersonde, and H. Rüterjans, Eur. J. Biochem. **35,** 122–125 [1973].
[2] H. Sick and K. Gersonde, Eur. J. Biochem. **45,** 313–320 [1974].
[3] M. Overkamp, H. Twilfer, and K. Gersonde, Z. Naturforsch. **31 c,** 524–533 [1976].
[4] T. Shiga, K.-J. Hwang, and I. Tyuma, Biochemistry **8,** 378–383 [1969].
[5] E. Trittelvitz, H. Sick, and K. Gersonde, Eur. J. Biochem. **31,** 578–584 [1972].
[6] Y. Henry and R. Banerjee, J. Mol. Biol. **73,** 469–482 [1973].
[7] L. C. Dickinson and J. C. W. Chien, Biochem. Biophys. Res. Commun. **59,** 1292–1297 [1974].
[8] E. Trittelvitz, K. Gersonde, and K. H. Winterhalter, Eur. J. Biochem. **51,** 33–42 [1975].
[9] M. Tamura, K. Kobayashi, and K. Hayashi, Biochem. Biophys. Res. Commun. **70,** 265–270 [1976].
[10] H. Rein, O. Ristau, and W. Scheler, FEBS Letters **24,** 24–26 [1972].
[11] W. E. Antholine, A. G. Mauk, H. M. Swartz, and F. Taketa, FEBS Letters **36,** 199–202 [1973].
[12] H. Kon, J. Biol. Chem. **243,** 4350–4357 [1968].
[13] B. B. Wayland and L. W. Olson, J. Amer. Chem. Soc. **96,** 6037–6041 [1974].
[14] H. Kon, Biochim. Biophys. Acta **379,** 103–113 [1975].
[15] J. Bonaventura and A. Riggs, J. Biol. Chem. **243,** 980–991 [1968].
[16] Q. H. Gibson, A. Riggs, and T. Imamura, J. Biol. Chem. **248,** 5976–5986 [1973].
[17] J. J. Hopfield, S. Ogawa, and R. G. Shulman, Biochem. Biophys. Res. Commun. **49,** 1480–1484 [1972].
[18] S. Ogawa, A. Mayer, and R. G. Shulman, Biochem. Biophys. Res. Commun. **49,** 1485–1491 [1972].
[19] J. M. Salhany, S. Ogawa, and R. G. Shulman, Biochemistry **14,** 2180–2190 [1975].
[20] J. Greer, J. Mol. Biol. **59,** 107–126 [1971].
[21] K. Gersonde, M. Overkamp, H. Sick, E. Trittelvitz, and W. Junge, Eur. J. Biochem. **39,** 403–412 [1973].
[22] A. Mayer, S. Ogawa, R. G. Shulman, and K. Gersonde, J. Mol. Biol. **81,** 187–197 [1973].
[23] H. Kon and N. Kataoka, Biochemistry **8,** 4757–4762 [1969].
[24] Y. Henry, J. Peisach, and W. E. Blumberg, Biophys. J. **15,** 286 a [1975].
[25] Y. Henry and R. Cassoly, Biochem. Biophys. Res. Commun. **51,** 659–665 [1973].
[26] E. G. Moore and Q. H. Gibson, J. Biol. Chem. **251,** 2788–2794 [1976].
[27] M. F. Perutz, J. V. Kilmartin, K. Nagai, A. Szabo, and S. R. Simon, Biochemistry **15,** 378–387 [1976].
[28] T. Yonetani, H. Yamamoto, J. E. Erman, J. S. Leigh, jr., and G. H. Reed, J. Biol. Chem. **247,** 2447–2455 [1972].