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pH Dependence of Oxy and Deoxy Cobalt-Substituted Leghemoglobin from Soybean

An Electron Spin Resonance Study

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Abstract. In leghemoglobin a, which is the major hemoglobin component in soybean root nodules, the haem iron has been replaced by cobalt. The electron spin resonance (ESR) of frozen solutions of the cobalt-substituted leghemoglobin has been studied at 77 K in the deoxy and oxy forms respectively. Both ligation states exhibit rhombic g tensors. The hyperfine constants of ^{59}Co, ^{14}N-imidazole (residue of the proximal histidine) and ^{14}N-pyrroles are determined for the three principal directions of the g tensor. Both, the oxy and the deoxy state exhibit pH-dependent changes of the hyperfine structures. For oxy cobalt leghemoglobin a quantitative analysis of the pH titration and of the ESR parameters of the low and high-pH forms respectively are performed. The interconversion of the low and the high-pH forms is controlled by a proton-dissociating group with pK = 6.4 which is most probably the distal histidine. g tensors and hyperfine constants are compared with those described for oxy cobalt myoglobin crystal spectra [34] allowing assignments of the low and high-pH species of leghemoglobin to stereoelectronic structures with non-equivalent and equivalent dioxygen atoms respectively. Hydrogen-bonding of the distal histidine with dioxygen favours the structure with equivalent oxygen atoms. The pH dependence of the deoxy form is interpreted as interaction of the proximal imidazole with the central cobalt atom.

Key words: Cobalt-substituted leghemoglobin – Electron spin resonance – Hyperfine constants – pH dependence

Introduction

Leghemoglobin is a plant heme protein which reversibly binds dioxygen with a very high affinity [1]. It is an important functional part of the nitrogen-fixing system of the legume root nodules. Leghemoglobins isolated from different legumes [2–4] have molecular weights ranging from 15,000–20,000 Dalton. All of them exhibit O_2-binding properties resembling myoglobin. The crystal

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structure of lupin leghemoglobin is similar to that of myoglobin [5]. The amino acid sequence of leghemoglobins of broadbean [6], kidney bean [7], and soybean [8, 9] extensively coincide with those of myoglobin and hemoglobin γ-chains.

The major hemoglobin component of the nitrogen-fixing soybean root nodules is the monomeric leghemoglobin a [11] with a molecular weight of 15,775 [8]. This hemoglobin contains a total of two histidines. One imidazole, which is the residue of the proximal histidine, is covalently bound to the heme iron, the other imidazole, which is the residue of the distal histidine, does not bind to the iron [10], but is close to the external ligand e.g., dioxygen.

Optical and ESR spectra of several ligated leghemoglobin a complexes, both in the ferrous and in the ferric state, have been extensively investigated [10–15]. The deoxy ($S = 2$) as well as the oxy ($S = 0$) state of leghemoglobin a are ESR-silent. The replacement of the central heme iron by cobalt leads to deoxy and oxy hemoglobins with $S = {}^1/_2$. The nuclear spins of the cobalt ($I = 7/2$) and of the nitrogen atoms ($I = 1$) of imidazole and pyrroles respectively are the origin of specific hyperfine interactions with the electron spin. ESR spectra of cobalt hemoglobins have been described for human hemoglobin [16–19], for myoglobins of aplysia [20] and sperm whale [18, 20–22] and for monomeric hemoglobins of glycera [21], Chironomus thummi thummi [23] and legume nodules [24].

We report the ESR spectra of cobalt leghemoglobin a from soybean in the oxy and deoxy state respectively. The total number of hyperfine constants, i.e., of the central cobalt and of the ligand atoms respectively, is determined for both ligation states of this hemoglobin and assignments to the principal axis of symmetry are discussed. The pH dependence of the hyperfine structure of the oxy form can be quantitatively described in terms of a transition between two spectral species which is controlled by one proton-dissociating group. A possible functional role of the distal histidine which influences the spin transfer to oxygen via a direct interaction with the dioxygen (socalled *cis*-effect) is discussed. The smaller changes in the hyperfine structure with pH, observed in the pentacoordinated deoxy form, may relfect the possible control of the electronic structure by the proximal histidine (socalled *trans*-effect.)

Materials and Methods

Leghemoglobin was purified in the ferric form free of exogenous ligands as described previously [25]. Apoleghemoglobin was obtained by removing the heme with the methylethyl ketone method [26]. Cobaltic protoporphyrin IX was prepared and specified according to [27]. Reconstitution of leghemoglobin and final purification of the deoxy form were performed according to [27].

The cobalt leghemoglobin was dissolved in 0.2 M *bis*-Tris and Tris buffers, respectively. The protein concentration was ca. 50 mg/ml. The deoxy form was prepared under nitrogen atmosphere by adding sodium dithionite (p. a. grade, Merck, Darmstadt). The oxy form was obtained by bubbling pure oxygen gas through the hemoglobin solution for 5 min. After the formation of the respective

complexes, the solutions were immediately frozen at 77 K in quartz ESR tubes of 3.0 mm inner diameter.

The first derivatives of the ESR spectra were recorded at 77 K with an X-band spectrometer (Type BER 420, Bruker-Physik, Karlsruhe). The amplitude of the 100 kHz field modulation was 0.08 mT. The microwave power was attenuated to 8 mW, so that saturation phenomena did not occur. The microwave frequency was measured with a frequency counter, the magnetic field strength with a nuclear magnetic resonance oscillator. The second derivatives of the ESR spectra were obtained by using two 50 kHz field modulations with an amplitude of 0.25 mT.

The pH titrations of the cobalt leghemoglobin derivatives were performed by mixing equal amounts of a stock solution of salt-free cobalt hemoglobin and buffer solutions which were adjusted to the desired pH value before mixing. The second derivatives of the ESR spectra were measured under identical conditions of the spectrometer and with constant spin concentrations of the sample but variable pH values. The change in the amplitudes of the resolved cobalt hyperfine lines, characteristic for the acid and the alkaline species respectively, was determined. The experimental titration curves are not complete, as below pH 5 denaturation phenomena occurred. The amplitude-versus-pH plot was fitted by a least squares procedure to theoretical dissociation curves yielding pK values and Hill-parameters.

The determination of the hyperfine constants is based on the analysis of the experimental first derivative spectra. These first derivatives were transformed by the Fourier transform technique to higher derivatives [28] and then analyzed for hyperfine constants and g tensors. With these ESR parameters simulations using a non-quantummechanical procedure for systems with $S = \frac{1}{2}$ and with average linewidths of 0.22 mT were performed [28]. The simulated spectra were compared with the experimental spectra to obtain the best data set of ESR parameters. The calculation procedures were carried out on the computer (Type Cyber 175, Control Data) of the Rechenzentrum der RWTH Aachen.

Results

ESR Spectra of Deoxy Cobalt Leghemoglobin a

The deoxygenated state of the cobalt-substituted leghemoglobin a exhibits a first derivative ESR spectrum with an axial symmetry (see Fig. 1). The inflection point of the large low-field signal yields a g value of $g_\perp = 2.308$. Although weak shoulders can be observed in the low-field and in the high-field part of the signal respectively, no hyperfine structure seems to be resolved. The high-field signal is centered at $g_\parallel = 2.03$ and consists of eight resonances, which are separated by 8.02 mT, mainly due to the nuclear magnetic field of the ^{59}Co atom. The four low-field ^{59}Co hyperfine resonances show clearly resolved triplets. The triplet hyperfine constant of 1.78 mT is assigned to the $^{14}N_\varepsilon$-nucleus of the proximal imidazole. The four high field hyperfine resonances become more and more broadened at higher magnetic field and are poorly resolved. No further

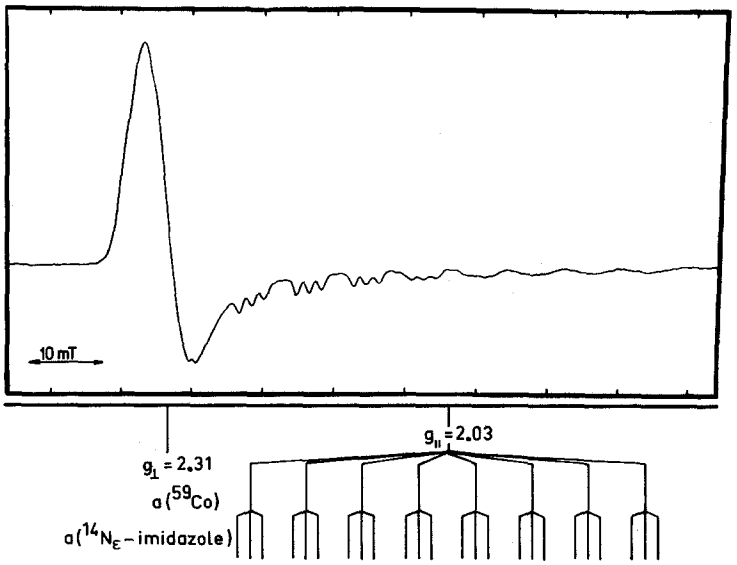

Fig. 1. ESR first derivative spectrum of the cobalt-substituted deoxy leghemoglobin a. pH = 6.2; temperature: 77 K

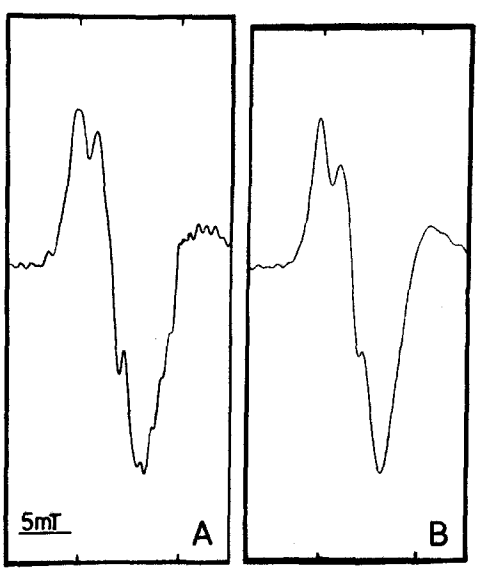

Fig. 2. ESR second derivative spectrum of the cobalt-substituted deoxy leghemoglobin a for $g_{\perp}$. The experimental first derivative is transformed to the second derivative by Fourier transform technique. **A** pH 5.8; **B** pH 8.6; temperature: 77 K

information may be drawn from this simple analysis of the experimental first derivative spectrum, which, beyond this, seems to be pH-insensitive.

However, the Fourier transform-assisted spectrum analysis allows a deeper insight into the resonance system. Considering the $g_{\perp}$, signal, the two already mentioned shoulders in the experimental first derivative of this signal give rise to a clearly resolved hyperfine structure when the second derivative is generated

Table 1. ESR parameters of deoxy cobalt leghemoglobin a at 77 K and pH 5.8

g Tensors		$a(^{59}Co)$ [mT]	$a(^{14}N_\varepsilon$-imidazole) [mT]	$a(^{14}N$-pyrrole) [mT]
$g_{\perp 1}$	2.32 ± 0.01	0.45 ± 0.03	0.54 ± 0.03	0.41 ± 0.01
$g_{\perp 2}$	2.29 ± 0.01	0.45 ± 0.03	0.54 ± 0.03	0.41 ± 0.01
$g_\parallel$	2.03 ± 0.01	7.95 ± 0.01	1.70 ± 0.01	0.05 ± 0.01

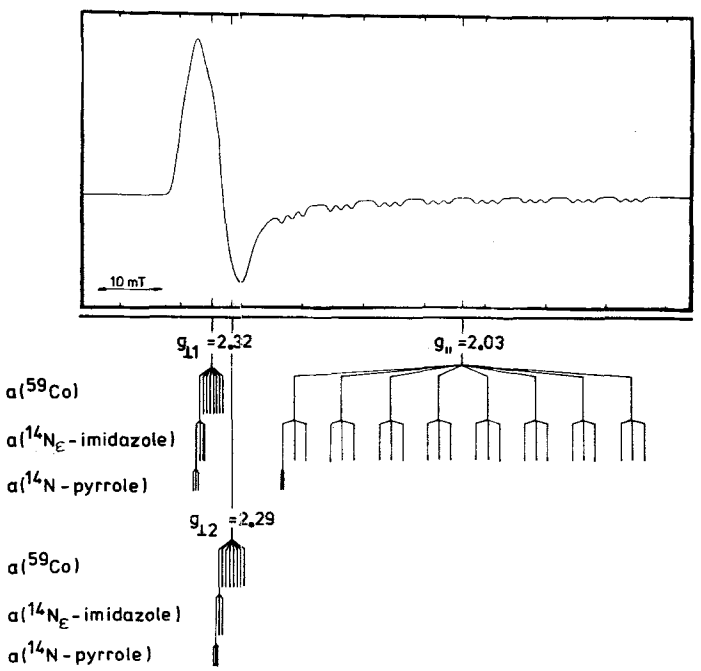

Fig. 3. Simulated ESR first derivative spectrum of the cobalt-substituted deoxy leghemoglobin a at pH 5.8

with the Fourier transform technique (see Fig. 2). Now, a small but significant and definite pH dependence of the hyperfine structure in $g_\perp$ can be seen.

Furthermore, the re-analysis of the $g_\perp$ value performed on the basis of the second derivative spectrum results in the splitting into $g_{\perp 1}$ and $g_{\perp 2}$ indicating slight rhombic distortion of the axial symmetry. $g_{\perp 1}$ and $g_{\perp 2}$ respectively as well as the hyperfine constants of the ^{59}Co center, of the $^{14}N_\varepsilon$-imidazole and the ^{14}N-pyrroles were found by simulation of the ESR spectra and by comparison with the experimental first and second derivatives at pH 5.8 (see Table 1). In the first approximation the hyperfine constants seem to be equivalent in $g_{\perp 1}$ and $g_{\perp 2}$. By this simulation additional information from the hyperfine lines in $g_\parallel$ can be obtained, namely the pyrrole nitrogen hyperfine constants (see Table 1). The simulated first derivative spectrum of the deoxy cobalt leghemoglobin a is shown in Fig. 3.

ESR Spectra of Oxy Cobalt Leghemoglobin a at High pH

The ESR first derivative spectrum of oxy cobalt leghemoglobin a at pH = 8.6 is shown in Fig. 4. The spectrum demonstrates rhombic symmetry with $g_1 = 2.078$, $g_2 = 2.005$, and g_3, the latter of which superimposing g_2. In the central resonance with g_2 five of the eight Co hyperfine lines are clearly resolved yielding the exact position of g_2 and the Co hyperfine constant in this direction. Besides the also well resolved Co hyperfine lines in $g_1 = 2.078$, no further information can be drawn from the conventional analysis of the experimental ESR spectrum. In order to analyze the hyperfine structures in g_3 we make use of the substantial resolution enhancement which is achieved by the generation of the third derivative spectrum from the measured first derivative by the Fourier transform technique (see Fig. 5). In this third derivative the eight Co hyperfine lines of g_2 are clearly to be seen and they are marked by an arrow. The remainder consists of five different lines of equal distance (marked with asterisks) which can now be declaired as Co hyperfine lines of $g_3 = 1.988$.

The thorough investigation of the third derivative yields even more information. The Co hyperfine lines in g_2 and g_3 show relatively small linewidths without further splitting. From this it follows, that the hyperfine constant of the proximal imidazole nitrogen must be much smaller, than that of cobalt. On the other hand, the Co hyperfine lines in g_1 exhibit superhyperfine structures indicating larger ^{14}N-imidazole hyperfine constants in this direction. Inspection and analysis of the enlarged section of the g_1 region shown in Fig. 6 enables us to determine directly the ^{14}N-imidazole splitting constant. Beyond that, this Fourier transform-generated third derivative gives evidence for the coupling of the electron spin with the nuclei of the pyrrole nitrogens. The ratio of intensities

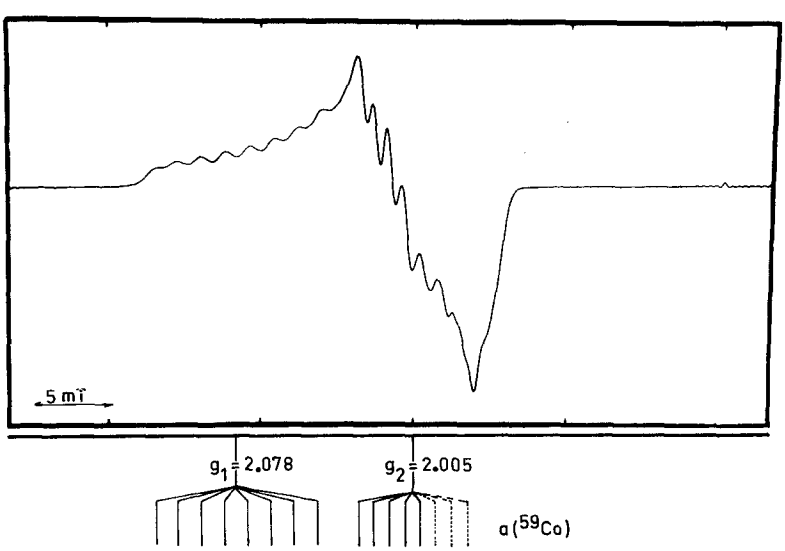

Fig. 4. ESR first derivative spectrum the cobalt-substituted oxy leghemoglobin a. pH 8.6; temperature: 77 K

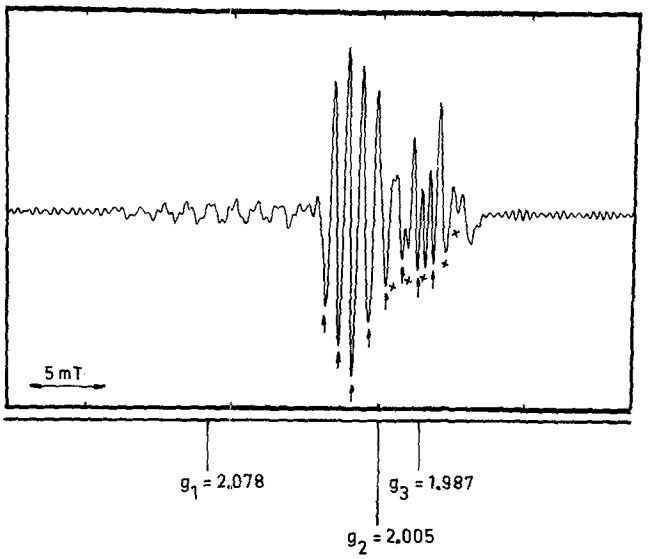

Fig. 5. ESR third derivative spectrum of the cobalt-substituted oxy leghemoglobin a. pH = 8.6; temperature: 77 K; the experimental first derivative is transformed to the third derivative by Fourier transform technique

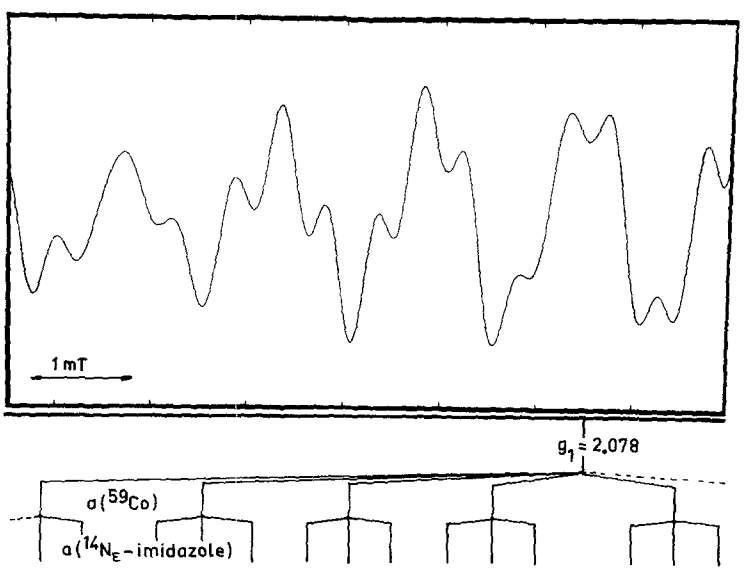

Fig. 6. ESR third derivative spectrum of the cobalt-substituted oxy leghemoglobin a at pH = 8.6; Expanded low-field (g_1) part of the spectrum which is also shown in Fig. 5

in each triplet of the Co hyperfine lines with a strengthening of the central lines can be explained only, if there exists another hyperfine coupling, superimposed on each of the ^{14}N-imidazole triplet lines. We attribute this additional coupling to the nuclear field of the pyrrole nitrogen atoms with $I = 1$. The corresponding hyperfine constant should be approximately one half of the ^{14}N-imidazole splitting, i.e., 0.2 mT. The Fourier transform-assisted spectrum analysis yields the g tensor, the ^{59}Co hyperfine tensor and the hyperfine constants for ^{14}N-imidazole and ^{14}N-pyrrole in g_1. Using these constants as input data for the computer simulation to fit the experimental spectrum, the complete data set including the lacking hyperfine constants for ^{14}N-imidazole and ^{14}N-pyrrole in g_2 and g_3 is obtained (see Table 2 and Fig. 7).

ESR Spectra of Oxy Cobalt Leghemoglobin a at Low pH

Varying the pH from the alkaline to the acid range, drastic changes in the first derivative ESR spectra of the oxy cobalt leghemoglobin a occur (see Fig. 8). The resolution of the cobalt hyperfine structure in g_2 seems to be completely lost at pH values below 6.4. But with the higher derivatives one finds $g_2 = 2.005$ and a cobalt hyperfine constant at g_2 which is not different from that at high pH. Thus, the less resolution of the Co hyperfine structure in g_2 must be due to an increase of the ^{14}N-imidazole and/or of the ^{14}N-pyrrole coupling. The low-field resonance

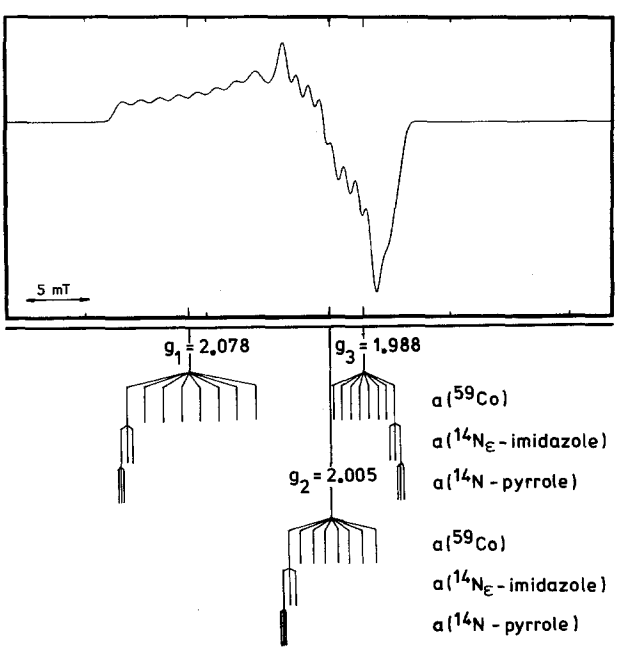

Fig. 7. Simulated ESR first derivative spectrum of the cobalt-substituted oxy leghemoglobin a at pH = 8.6

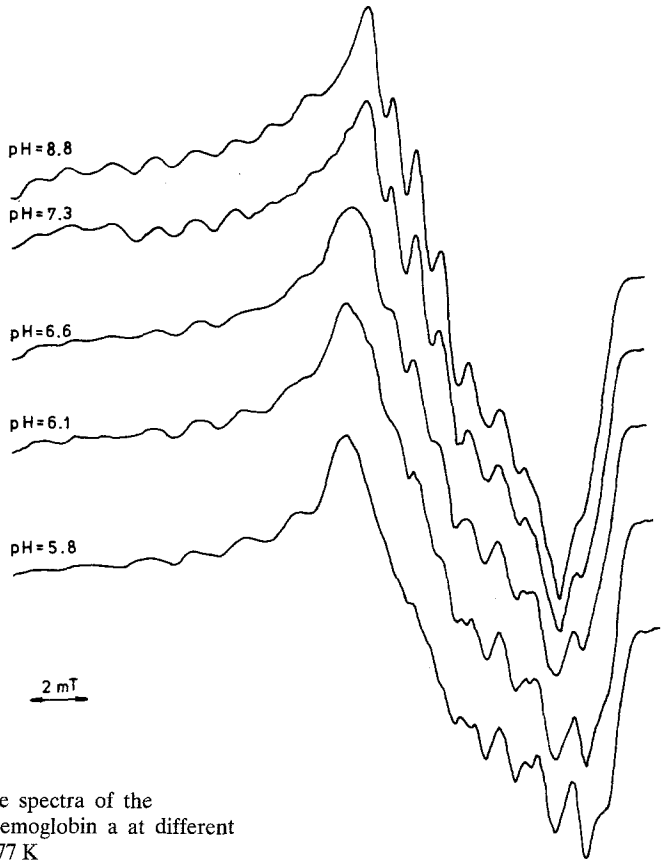

Fig. 8. ESR first derivative spectra of the cobalt-substituted oxy leghemoglobin a at different pH values. Temperature: 77 K

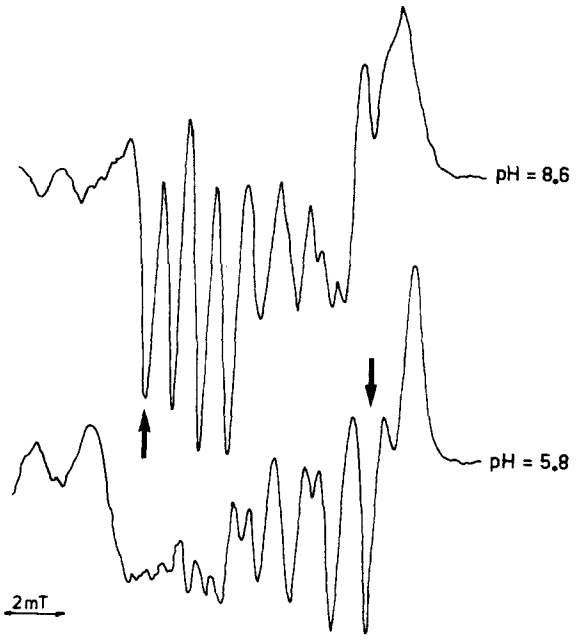

Fig. 9. ESR second derivative spectra of the cobalt-substituted oxy leghemoglobin a at low and high pH. Temperature: 77 K. Arrows indicate cobalt hyperfine lines of the acid and alkaline species, respectively

Table 2. ESR parameters of oxy cobalt leghemoglobin a at 77 K

pH	g Tensor		$a(^{59}Co)$ [mT]	$a(^{14}N$-imidazole) [mT]	$a(^{14}N$-pyrrole) [mT]
8.6	g_1 2.078 ± 0.001	xx	1.55 ± 0.05	0.44 ± 0.05	0.20 ± 0.02
	g_3 1.988 ± 0.002	yy	0.74 ± 0.05	0.49 ± 0.10	0.24 ± 0.02
	g_2 2.005 ± 0.001	zz	0.97 ± 0.05	0.24 ± 0.05	0.03 ± 0.01
5.8	g_1 2.092 ± 0.001	xx	2.10 ± 0.05	0.65 ± 0.05	0.24 ± 0.05
	g_3 1.989 ± 0.002	yy	0.96 ± 0.05	0.40 ± 0.05	0.14 ± 0.05
	g_2 2.006 ± 0.001	zz	0.98 ± 0.05	0.59 ± 0.05	0.34 ± 0.05

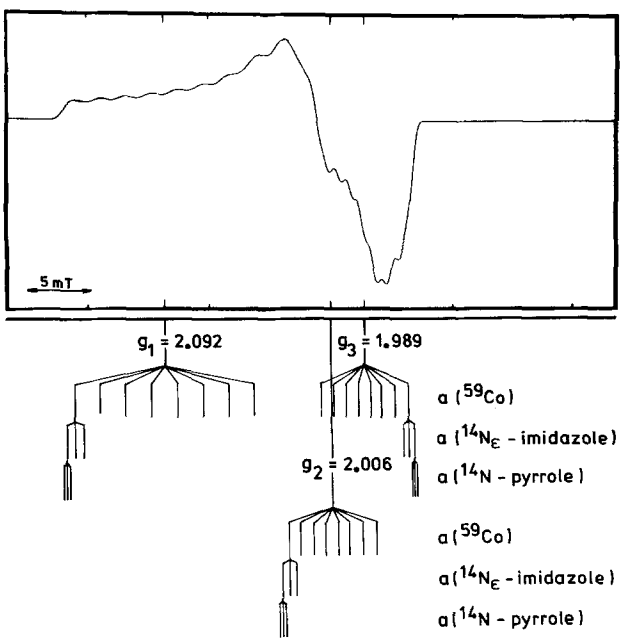

Fig. 10. Simulated ESR first derivative spectrum of the cobalt-substituted oxy leghemoglobin a at pH = 5.8

at g_1 is shifted further down-field by lowering the pH. In this direction the cobalt hyperfine constant increases, which in turn is accompanied by a line-broadening of the eight hyperfine lines. It follows that here too, the imidazole and/or the pyrrole coupling must have been increased. The most significant pH-dependent change takes place in the high-field resonance g_3 which is determined on the basis of the third derivative spectrum. At alkaline pH in the high field part of this g_3 resonance two separated lines of the cobalt hyperfine structure can be observed in the experimental second derivative (see Fig. 9). Since these two cobalt lines are not resolved in the first derivative because of overlapping by other lines, we conclude, that the ^{14}N-imidazole hyperfine constant should be approximately one half of the cobalt hyperfine constant, i.e., about 0.5 mT. The

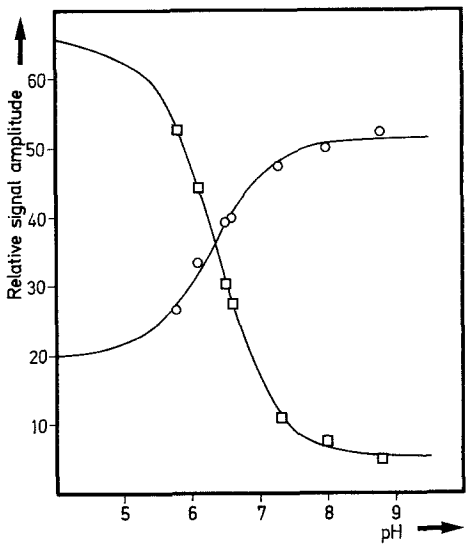

Fig. 11. pH dependent spectral transition of cobalt-substituted oxy leghemoglobin. ○, alkaline species; □, acid species. pK = 6.4; $n \approx 1$

computer-simulated spectrum which most perfectly fits to the experimental ESR spectrum yields the data set listed in Table 2 (see also 10).

pH Titration of Oxy Cobalt Leghemoglobin a

The pH-dependent change of the ESR spectra of the cobalt-substituted oxy leghemoglobin a is demonstrated in Fig. 8. The transition from the acid to the alkaline species is quantitatively measured by the use of the second derivative spectra (see Fig. 9). The species, which is stable at alkaline pH, is characterized by the four sharp cobalt hyperfine lines of g_2, whereas the acid species shows at least two distinct sharp lines in g_3. By measuring the intensity of one of the resolved lines characteristic for each species (marked with arrows) while varying the pH, the titration curves shown in Fig. 11 are obtained. Both titration curves show identical inflection points at pH = 6.4. Furthermore, Hill plots exhibit identical Hill coefficients for both titration curves indicating that only one titrating group is involved in this process. The inverse titration behaviour of the two spectral species is evidence that this proton-dissociating group controls the transition between the two hyperfine structures of one oxy leghemoglobin molecule.

Discussion

Two Stereoelectronic Structures of Oxy Cobalt Leghemoglobin a

Deoxy cobalt leghemoglobin a is a pentacoordinated cobalt(II) low spin complex with $S = {}^1/_2$. The unpaired spin is localized in a d_{z^2} *orbital. The* g values are close

to the free spin value because spin-orbit coupling is extremely small in the d_{z2} *orbital.* g and a tensors obtained at 77 K from frozen solution spectra of leghemoglobin are closely corresponding to those determined in crystals of deoxy cobalt myoglobin [22, 23]. Whereas deoxy myoglobin seems to exhibit a nearly axial g tensor [22, 23], leghemoglobin is characterized by a significant rhombic distortion. Despite this difference we assume for leghemoglobin that, as in myoglobin, g and a tensors share the same principal axes with the z-axis parallel to the heme normal. Thus the deoxy forms of both monomeric hemoglobins seem to be very similar with regard to their electronic structures.

If for example in leghemoglobin dioxygen approaches the 6th coordination site of the central cobalt atom, overlapping of the d_{z2} orbital with two π^* orbitals of O_2 will result in three molecular orbitals: ψ_1 (bonding); ψ_2 (non-bonding); and ψ_3 (anti-bonding). Spin pairing in ψ_1 gives rise to the formation of σ bonding. One unpaired spin remains in ψ_2 which is preferentially a dioxygen orbital [29, 30]. ESR experiments on $^{17}O_2$-ligated cobalt complexes have confirmed the strongly delocalized spin densities on the dioxygen [31, 32]. The large unpaired spin densities on dioxygen have its origin from the fact that dioxygen has two unpaired electrons before ligation and retains most of its second electron unpaired after bonding to the Co center [33]. Recently, Dickinson and Chien [34] demonstrated by an ESR crystallographic study that also in the ^{17}O-enriched oxy cobalt myoglobin the unpaired spin density is largest on the dioxygen. It further has been shown by these authors that at 77 K oxy cobalt myoglobin forms two distinct ESR spectral species, I and II, which do not differ in their g tensors but differ largely with regard to the Co hyperfine tensors [22]. The conclusion was, that the spin transfer from the dioxygen to the cobalt is larger in the species I indicated by 40% increase in the Co hyperfine constants [22]. In oxy leghemoglobin we find also two spectral species which because of their pH behaviour can be assigned as low-pH and high-pH forms respectively. The comparison of the g and ^{59}Co hyperfine constants obtained from myoglobin crystal and leghemoglobin powder spectra respectively shows a good correlation between species I and the low-pH form and species II and the high-pH form respectively. Crystals of myoglobin were analyzed in the range of pH 6–6.8. The similar proportion of the two spectral species found in myoglobin and leghemoglobin at this pH supports the suggestion that these stereoelectronic structures of the oxy form are also existent in leghemoglobin. The spectral species of oxy myoglobin differ in the geometries of the Co-O-O bonds [22, 34]: the ozonoid-type structure [35] corresponds to inequivalent oxygen atoms and the olefin-type (π-bonded) structure [36] to equivalent oxygen atoms. This must hold true also for the two spectral species of leghemoglobin: i.e., the high-pH form can then be characterized by a bond angle of about 120° which is in good agreement with the X-ray results obtained with myoglobin [37, 38] and by two inequivalent oxygen atoms; on the other hand the low-pH form exhibits equivalent oxygen atoms. These distinct binding geometries are also reflected by differences in the spin transfer between the dioxygen molecule and the cobalt atom. The high-pH form shows a greater spin density on the oxygen atom which is reflected by smaller hyperfine constants for ^{59}Co, ^{14}N-imidazole, and

^{14}N-pyrrole (see Table 2). The low-pH form with its strong back-donation of the spin density to the central cobalt atom exhibits an increase of the before mentioned hyperfine constants. Transition from high to low-pH form is accompagnied by a down-field shift of g_1 (see Table 2). This is taken to mean that the unpaired spin is populated in the d_{xz} and d_{yz} orbitals. The direction of the g_1 value coincides with the *x*direction of the ^{59}Co hyperfine tensor. The cobalt and nitrogen hyperfine constants in *x*direction increase also during this transition. Both phenomena support the pH-dependent transition between an ozonoid and a π-bonded structure in the oxy cobalt leghemoglobin.

Interaction Between Distal Histidine and Dioxygen in Oxy Cobalt Leghemoglobin a

The two stereoelectronic structures of oxy cobalt leghemoglobin can be reversibly transformed into each other by changing the pH. This transition is controlled by one proton-dissociating group with a pK value of 6.4. The pK value strongly indicates that this group is the distal histidine which is the only one histidine in leghemoglobin besides the covalently bound proximal histidine. This conclusion is also confirmed by NMR studies on the C-2 proton resonance of the distal histidine in ligated ferrous leghemoglobins [39]. The protonated form of the distal histidine which correlates with the low-pH spectral form can interact with the dioxygen via formation of a hydrogen bond. Therefore, we must conclude that hydrogen-bonding stabilizes the olefin-type binding geometry in myoglobin and leghemoglobin, respectively. However, the deprotonated distal histidine without interaction with dioxygen, preferentially leads to the formation of the ozonoid-type bonding of dioxygen. Rise in temperature would cause the same effect. The *cis*-effect of the distal histidine, which is present in most of the hemoglobins and myoglobins, contributes considerably to the binding behaviour of dioxygen at low pH.

It should be mentioned that the pK of the stereoelectronic transition in leghemoglobin correlates with the physiological pH of the legume nodules [40]. Therefore, the physiological function of leghomoglobin may be linked to the titration behaviour the distal histidine and the pH-dependent transition between the stereoelectronic structures of the O_2-ligated forms.

Possible Influence of the Proximal Histidine on the Electronic State of Deoxy Cobalt Leghemoglobin a

Two pH-dependent spectral species were also described for deoxy cobalt leghemoglobin, though exact hyperfine constans for both forms could not be determined. The changes in hyperfine constants are very small, but significant. Because of the difficulties in analyzing the ESR spectra of the deoxy forms no dissociation curve is shown and no exact pK value can be obtained from these spectral data. The direct interaction of the distal histidine with the penta-coordinated cobalt complex is unlikely. Therefore we assume a long-range

interaction of another proton-titrating group via the proximal imidazole. Again, leghemoglobin shows an interesting similarity to myoglobin, where pH-dependent changes (pK = 5.7) in the chemical shifts of the NH proximal imidazole and heme methyl protons respectively indicate a conformation change in deoxy myoglobin, which is not controlled by the distal histidine [41].

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