Biophys Struct Mech (1982) 8: 189–211

Biophysics of Structure and Mechanism

Bohr-Effect and pH-Dependence of Electron Spin Resonance Spectra of a Cobalt-Substituted Monomeric Insect Haemoglobin

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Abstract. The monomeric haemoglobin IV from *Chironomus thummi thummi* (CTT IV) exhibits an alkaline Bohr-effect and therefore it is an allosteric protein. By substitution of the haem iron for cobalt the O_2 half-saturation pressure, measured at 25° C, increases 250-fold. The Bohr-effect is not affected by the replacement of the central atom. The parameters of the Bohr-effect of cobalt CTT IV for 25° C are: inflection point of the Bohr-effect curve at pH 7.1, number of Bohr protons $-\Delta \log p_{1/2} (O_2)/\Delta pH = 0.36$ mol H^+/mol O_2 and amplitude of the Bohr-effect curve $\Delta \log p_{1/2} (O_2) = 0.84$. The substitution of protoporphyrin for mesoporphyrin causes a 10 nm blue-shift of the visible absorption maxima in both, the native and the cobalt-substituted forms of CTT IV. Furthermore, the replacement of vinyl groups by ethyl groups at position 2 and 4 of the porphyrin system leads to an increase of O_2 affinities at 25° C which follows the order: proto < meso < deutero for iron and cobalt CTT IV, respectively. Again, the Bohr-effect is not affected by the replacement of protoporphyrin for mesoporphyrin or deuteroporphyrin. The electron spin resonance (ESR) spectra of both, deoxy cobalt proto- and deoxy cobalt meso-CTT IV, are independent of pH. The stronger electron-withdrawing effect by protoporphyrin is reflected by the decrease of the cobalt hyperfine constants coinciding with $g_{\parallel} = 2.035$ and by the low-field shift of $g_{\parallel}$. The ESR spectra of oxy cobalt proto- and oxy cobalt meso-CTT IV are dependent of pH. The cobalt hyperfine constants coinciding with $g_{\parallel} = 2.078$ increase during transition from low to high pH. The pH-induced ESR spectral changes correlate with the alkaline Bohr-effect. Therefore, the two O_2 affinity states can be assigned to the low-pH and high-pH ESR spectral species. The low-pH form (low-affinity state) is characterized by a smaller, the high-pH form (high-affinity state) by a larger cobalt hyperfine constant in $g_{\parallel}$. The correlation of the cobalt hyperfine constants of the oxy forms with the O_2 affinities is discussed for several monomeric haemoglobins. The Co-O-O

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0340-1057/82/0008/0189/$ 04.60

bond angle in cobalt oxy CTT IV is characterized by an ozonoid type of binding geometry and varies little during the pH-induced conformation transition. Due to the lack of the distal histidine in CTT IV no additional interaction via hydrogen-bonding with dioxygen is possible; this is reflected by the cobalt hyperfine constants.

Key words: Monomeric *Chironomus* haemoglobin – Substitution for cobalt and mesoporphyrin – O_2 affinity – Electron spin resonance – Bohr-effect

Introduction

The two monomeric haemoglobins, CTT III and IV, which are constituents of the O_2 transport system of the larvae of the insect *Chironomus thummi thummi,* are allosteric proteins. For many reasons we believe that these haemoglobins are the simplest allosteric models. The ligand-binding is controlled by only one proton-binding site [1–7]. The pH-dependent change of the O_2 affinity of both haemoglobins follows a simple Bohr-effect curve which is characterized by inflection points at pH 7.1–7.4 [1–3, 5–7]. Therefore, both haemoglobins exhibit only an alkaline Bohr-effect, whereas no acid Bohr-effect can be observed [1–3]. The Bohr proton-binding group which forms a salt-bridge interacts with the ligand-binding site via a change of the tertiary structure [1–6] whereby the distances and the geometries of the ligands axially bound to the haem iron, i.e., the proximal imidazole and the molecular oxygen, are modified [6, 7]. Furthermore, both insect haemoglobins exhibit a lack of the distal histidine [8–10].

In principle, four effects could contribute to the change in the ligand affinity demonstrated by the Bohr-effect, or to a variation of the allosteric interaction energy indicated by different amplitudes of the Bohr-effect curves.

(i) The *trans-effect* describes the interaction of the proximal histidine, via the haem plane, with the ligand bound to the iron in trans-position. Thus, a short imidazole-iron bond length correlates with a long ligand-iron bond length and vice versa [7, 11, 12].

(ii) The *cis-effect* can be defined as a direct interaction of the external ligand with the amino acid residues in its close vicinity. A steric hindrance can cause an unusual binding-geometry [13, 14]. Such an unusual binding-geometry of the CO ligand in haemoglobins was first discovered by Huber et al. in the monomeric insect haemoglobin CTT III, where E11 Ile enforces a bond angle of 145° [13]. Haemoglobins having a distal histidine establish particular binding-geometries of the external ligands which can be stabilized by the formation of a hydrogen bond. This direct interaction of the distal histidine has been demonstrated for the binding of an azide ion [15, 16], molecular oxygen [17, 18] and fluoride [19]. In cobalt myoglobin [18] and cobalt leghaemoglobin [20] the interaction of the distal imidazole with dioxygen leads at low pH to the formation of the olefin-type bond [21], whereas at high pH the ozonoid-type bond [22] is formed.

(iii) The *porphyrin effect* is based on the change of inductive electronic and steric phenomena. The substitution of side groups in the protoporphyrin system

has an effect on the ligand affinity and influences the interaction of the haem group with its protein environment [6, 7, 23–26].
(iv) The *metal effect* on the ligand-binding can be demonstrated by substituting the iron(II) by cobalt(II). The allosteric properties are preserved when iron is replaced by cobalt [27–32]. Tetrameric cobalt-substituted haemoglobins remarkably reduce their cooperativity and their Bohr-effects [29, 30]. The substitution with cobalt in myoglobin [28], and haemoglobin [27] opened the possibility to study both the deoxy and the oxy form of haemoglobins with electron spin resonance [20, 33–37]. Both, oxy and deoxy cobalt haemoglobin, are low spin complexes with $S = 1/2$. The unpaired electron being in a d_{z^2} orbital diminishes the affinity for oxygen drastically.

The present paper describes the pH dependence of the O_2-binding equilibrium constants and the pH effect on the ESR spectra of the oxygenated monomeric insect haemoglobin IV which has been reconstituted with cobalt protoporphyrin and cobalt mesoporphyrin, respectively. We are interested to find out if the metal effect and/or the porphyrin effect influence the allostery of this protein, by comparing these results with those obtained with the native protein. Bohr-effect and amplitude of the Bohr-effect curve of CTT IV have not been changed by these substitutions, whereas the O_2 affinity increases in the following series: cobalt < iron and protoheme < mesoheme, respectively. The ESR spectra of the deoxy forms seem to be pH-independent, whereas the oxy forms show a pH-dependent hyperfine pattern. Since CTT IV has no distal histidine it will not form a hydrogen bond with dioxygen. pH effects on the ESR spectra of the oxy forms should reflect tertiary structure changes which influence the electronic structure of the cobalt atom via the proximal histidine, i.e., via the trans-effect.

Materials and Methods

Preparation of Native Haemoglobin IV

Haemoglobin IV (CTT IV) from insect larvae of *Chironomus thummi thummi* has been purified according to [2]. The collective monomeric haemoglobins have been dialyzed against 0.01 M Tris buffer pH 9.0 (starting buffer) and then adsorbed on a DEAE-Sephadex A50 column (6 × 60 cm) equilibrated with the starting buffer. The monomeric haemoglobins I, III, and IV have been eluted with a linear gradient of 5 l 0.01 M Tris pH 9.0 plus 5 l 0.01 M Tris pH 9.0 in 0.15 M NaCl. The chemical homogeneity of CTT IV has been checked by polyacrylamide gel (10%) disk-electrophoresis at pH 9.5. The R_f value was 0.60. The lyophilized salt-free protein was stored at −30° C.

Preparation of Globin

The preparation of globin was carried out as described in [11]. The haemoglobin dissolved in aqua destillata was oxidized by adding 1.5 M excess of $K_3(Fe(CN)_6)$.

Salts were immediately removed by gel-filtration on Sephadex G25. Extraction of haem was performed at −20° C under stirring. One volume of a 1% solution of met haemoglobin was added drop by drop to 20 volumes of the acetone solution being 6 mM in HCl [38]. After 30 min stirring the precipitated globin was centrifuged and washed two times with acetone. The globin was dissolved in H_2O at 4° C and then dialyzed one hour against H_2O, 12 h against 0.0015 M $NaHCO_3$ and finally 24 h against 0.01 M phosphate buffer pH 7.5. Precipitated globin was discarded. The UV absorption spectrum of the globin solution was recorded at 4° C. The globin concentration was determined by measuring the absorbance at 280 nm using a molar absorption coefficient ε = 11,200 $(M^{-1} \cdot cm^{-1})$ according to [39]. The yield of native globin was about 50%.

Preparation of Cobalt Porphyrins

Cobalt protoporphyrin IX was prepared according to [29]. Haemin was isolated from bovine haemoglobin following the procedure of Labbe and Nishida [40]. The removal of iron from the haemin was carried out as described by Morrell and Stewart [41].

The purification of the protoporphyrin was performed as published elsewhere [11]. For insertion of cobalt into protoporphyrin IX two solutions had to be prepared and heated to 80° C under anaerobic conditions: 200 mg protoporphyrin IX, dissolved in 15 ml pyridine, were added to 100 ml glacial acetic acid and 140 mg cobalt(II)-acetate-4-hydrate were dissolved in 10 ml glacial acetic acid and pyridine (10 : 1; v/v). With stirring the cobaltous acetate solution was added dropwise to the porphyrin solution. After 15 min heating at 80° C the reaction mixture cooled down to room temperature under a stream of air for 1 h and was dried in a vacuum rotatory evaporator. The dried material was dissolved in the lower phase of a mixture of pyridine, chloroform, water and iso-octane (20 : 20 : 10 : 1; v/v). The cobalt protoporphyrin was purified by chromatography on a silica gel column (2 × 30 cm) (silica gel 60, 70–230 mesh, ASTM, Merck, Darmstadt) equilibrated with the above mentioned solvent. The mobile cobalt protoporphyrin IX fraction was rechromatographed on a silica gel column (2 × 60 cm). The fractions were tested for chemical homogeneity by thin-layer chromatography. The collected fractions were dried in a vacuum rotatory evaporator and then dissolved in a minimum amount of a mixture of ethyl acetate and glacial acetic acid (6 : 1; v/v); insoluble material was separated by filtration. Then the cobalt protoporphyrin IX was precipitated from a homogeneous solution by addition of one volume n-hexane at 4° C. The material was washed with n-hexane by filtration and dried in a vacuum at room temperature. The yield of cobalt protoporphyrin IX was about 40%.

Cobalt mesoporphyrin IX was prepared according to [42]. For insertion of cobalt into mesoporphyrin IX two solutions had to be prepared and heated to 80° C under anaerobic conditions: Amounts of 200 mg mesoporphyrin IX (Carl Roth, Karlsruhe) and 400 mg cobalt(II)-acetate-4-hydrate were dissolved in 200 ml and 400 ml glacial acetic acid respectively. With stirring the mesoporphyrin solution was added to the cobaltous acetate solution. After a reaction time of 15 min the reaction mixture was cooled to room temperature. The precipitated cobalt mesoporphyrin was washed successively with glacial acetic acid, water and methanol by filtration and then dried in a vacuum at room temperature.

The purity of the cobalt porphyrins was checked by UV/visible absorption and proton nuclear magnetic resonance spectroscopy. The cobaltous porphyrin was dissolved into 0.1 N NaOH : pyridine : H_2O (3 : 10 : 17, v/v) and transformed to the cobaltic form under a stream of pure dioxygen. The optical spectra of these complexes were identical with those described by Yonetani et al. [30]. Proton magnetic resonance spectra of the bis-cyano cobaltic porphyrins were recorded with a 270 MHz Fourier spectrometer (Type WH270, Bruker Analytische Meßtechnik, Karlsruhe, FRG). The cobaltic porphyrins (50 mM) were dissolved in tetradeuteromethanol which also contained 150 mM KCN. The assignment of all heme proton resonances was possible by comparison with the spectra described for the diamagnetic zinc porphyrins dissolved in d_5-pyridine [43].

Preparation of Cobalt Haemoglobin IV

The reconstitution of cobalt haemoglobin IV from globin and cobaltous porphyrin was carried out as described by Yonetani et al. [30]. A volume of 50 ml globin solution (5 mg globin per ml 0.1 M phosphate buffer pH 7.0) was cooled in an ice bath and deoxygenated by passing humid nitrogen gas over the surface of the solution. Then 1.3 equivalents of cobalt porphyrins were dissolved in 5 ml of a pyridine : water mixture (1 : 1; v/v). This solution was also deoxygenated and then saturated with dithionite. Under unaerobic conditions and at 4° C the cobaltous porphyrin solution was added to the globin solution under gentle stirring. Immediately after the mixing, the solution was passed through a Sephadex G-25 column (5 × 25 cm) equilibrated with 0.01 M phosphate buffer pH 6.0. The solution of cobalt haemoglobin was desalted by gel-filtration and lyophilized. The dry material was stored at −30° C.

Oxygen Binding Curves

Oxygen binding curves were recorded at 25° C by means of the rapid diffusion technique [44, 45]. The samples were prepared as follows: 30 mg freeze-dried haemoglobin was dissolved in 0.2 ml 0.02 M NaCl. After centrifugation the precipitated material was discarded. For each pH value a volume of 20 µl of cobalt haemoglobin solution was mixed with 20 µl 0.4 M buffer. Glycin-NaOH, Tris-HCl, phosphate and citrate-phosphate buffers were used. The pH was measured before transferring the sample into the diffusion chamber. Changes of the absorbance occuring during the oxygenation-desoxygenation cycle were monitored in the diffusion chamber at 424 nm for cobalt proto-CTT IV and at 414 nm for cobalt meso-CTT IV.

pH Measurements

The pH of the haemoglobin solution was measured at 25° C by means of a micro glass electrode (Type 406 M3, Ingold, Frankfurt/Main, FRG) and a pH-meter (Type PHM 63, Radiometer, Copenhagen, Denmark).

UV/Visible Absorption

Optical spectra were recorded at 22° C with a spectrophotometer (Cary Model 118, Varian, Palo Alto, CA, USA).

Electron Spin Resonance

The ESR spectra were taken at 77 K with an X-band spectrometer (Type ER 420, Bruker Analytische Meßtechnik, Karlsruhe, FRG). The modulation amplitude of the 100 kHz field modulation was 0.02 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 was determined by a nuclear magnetic resonance oscillator. Second and third derivative spectra of deoxy and oxy cobalt haemoglobins were obtained by measuring the first derivative spectra with small modulation amplitudes followed by a computational transformation into the second and third derivative respectively, by means of the Fourier-transform technique as described in [46]. Samples of cobalt haemoglobin IV were prepared by dissolving 30 mg lyophilized protein in 0.25 ml 0.4 M Tris-HCl buffer pH 9.5 and 0.4 M citrate-phosphate buffer pH 5.7 respectively. After centrifugation the protein solution was filled into quartz tubes of 3.3 mm diameter. The deoxy form was obtained by adding 50 µl of a saturated $Na_2S_2O_4$ solution under nitrogen atmosphere. After a reaction time of 20 min at room temperature the sealed tube was frozen in liquid nitrogen. The oxy form was prepared at 4° C by flushing pure dioxygen over the surface of the protein solution for 30 min. The haemoglobin solution was filled into quartz tubes which were sealed with a rubber stopper and then frozen in liquid nitrogen.

Results

Visible Absorption of Cobalt Haemoglobin IV

Visible absorption spectra of deoxy and oxy cobalt haemoglobin IV are shown in Figs. 1 and 2. Deoxy cobalt proto-CTT IV exhibits a prominent absorption band at 554 nm and a marked shoulder at 520 nm. The Soret-band occurs at 403 nm. Oxy cobalt proto-CTT IV shows typical α and β bands at 570 nm and 538 nm. At 25° C and under pure dioxygen atmosphere (see also O_2-binding experiments)

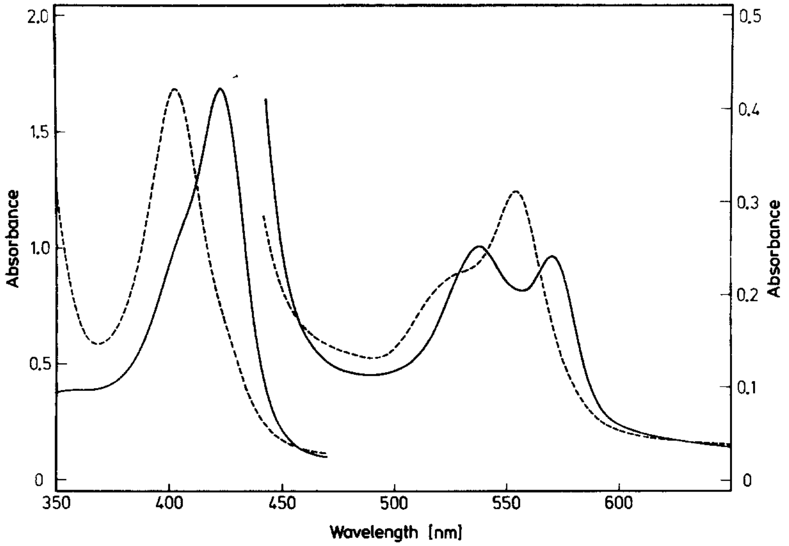

Fig. 1. Visible absorption spectra of deoxy (– – –) and oxy (———) cobalt proto-CTT IV in 0.1 M phosphate buffer pH 6.5 at 22° C

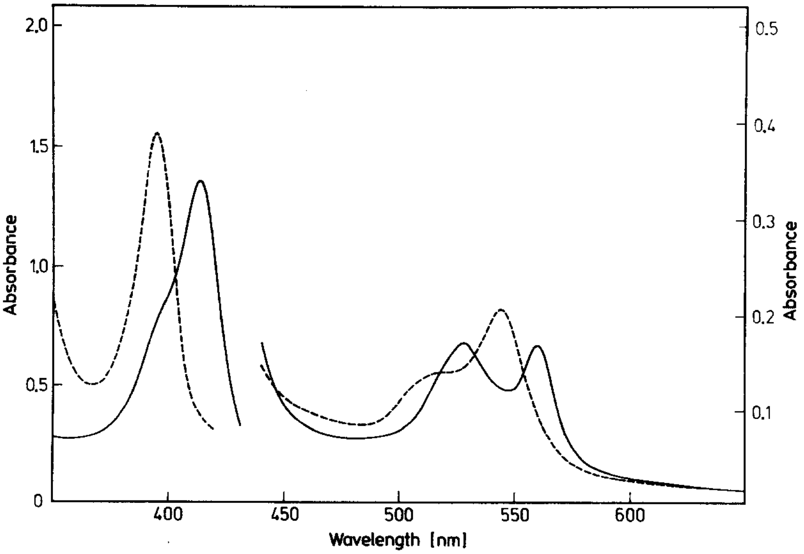

Fig. 2. Visible absorption spectra of deoxy (– – –) and oxy (———) cobalt meso-CTT IV in 0.1 M phosphate buffer pH 6.5 at 22° C

the haemoglobin is not fully oxygenated, therefore, the determination of the absorption maxima is not very exact. The shoulder at 405 nm may be also indicative for a portion of the deoxy form in the sample. As in iron haemoglobin IV the absorption bands are red-shifted by 10 nm if protoporphyrin IX is exchanged by mesoporphyrin IX. The data are compiled in Table 1 and compared with those described for human haemoglobin and sperm whale myoglobin.

Dioxygen-Binding Equilibrium Data of Cobalt Haemoglobin IV

The O_2-binding curves of cobalt-substituted CTT IV are hyperbolic as one should expect for monomeric haemoglobins. The half-saturation pressures, $p_{1/2}$ (O_2), are pH-dependent. The $\log p_{1/2}$ (O_2)-versus-pH plots are characteristic for an alkaline Bohr-effect and show inflection points at pH 7.1 for proto-CTT IV and pH 6.7 for meso CTT IV (see Fig. 3). The inflection point of the Bohr-effect curve is shifted to low pH only if iron is replaced by cobalt and protoporphyrin by mesoporphyrin. The amplitude of the Bohr-effect curves and the number of Bohr-protons released per bound O_2 have not been affected by the porphyrin substitution. Furthermore, both parameters of the Bohr-effect are identical with those determined for the respective iron haemoglobins IV (see Table 2).

After the substitution of iron for cobalt one observes an increase of $p_{1/2}$ (O_2) for proto- and meso-CTT IV, at high pH by factors of about 240 and 750 and at low pH by factors of about 280 and 630. This change in O_2 affinity is practical, i.e., within limits of ± 20%, independent of pH consistent with the invariant Bohr-effect described above.

The effect of substitution with mesoporphyrin IX on the change of O_2 affinities is more pronounced in the iron than in the cobalt-substituted haemoglobins. The $p_{1/2}$ (O_2) values increase in the iron form by a factor of about 4, whereas in the cobalt form only by a factor of about 2.

The large decrease of the O_2 affinity, characteristic for cobalt haemoglobins, led only to 80% saturation with O_2 under the conditions of the diffusion technique. Therefore, the half-saturation pressures were computed by means of an approximation procedure described elsewhere [47].

Electron Spin Resonance of Deoxy Cobalt Haemoglobin IV

The first derivative ESR spectrum of deoxy cobalt proto-CTT IV is shown in Fig. 4. This spectrum indicates rhombic symmetry with $g_{\perp 1} = 2.360$, $g_{\perp 2} = 2.322$ and $g_{\parallel} = 2.035$. The g values of deoxy meso-CTT IV differ only slightly (see Table 3). The ESR spectra are characteristic for low-spin cobalt(II) complexes with $S = 1/2$. The low-field resonances (at $g_{\perp}$) do not show resolved hyperfine structures, whereas $g_{\parallel}$ exhibits an eight-line hyperfine structure which originates from the interaction of the electron spin with the nuclear spin of the cobalt atom ($I = 7/2$). Each resonance of the eight-line hyperfine structure is further split into

Table 1. Optical absorption maxima, λ_{max} (nm), of cobalt proto- and meso-haemoglobin IV at pH 6.5 and 22° C

Ligation state	Protoporphyrin IX			Mesoporphyrin IX		
	CTT IV	Hb[a]	Mb[a]	CTT IV	Hb[a]	Mb[a]
Oxy	570 538 423	571 538 422	577 539 426	560 528 414	561 530 412	560 529 414
Deoxy	554 403	552 402	558 406	544 395	542 392	542 395

[a] For comparison, data from human haemoglobin (Hb) and sperm whale myoglobin (Mb) taken from reference [30]

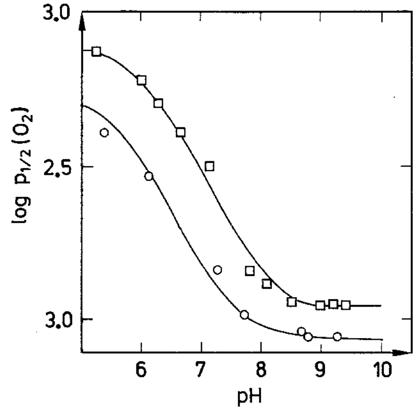

Fig. 3. Bohr-effect of cobalt haemoglobin IV at 25° C. □—□—□, proto-CTT IV: ○—○—○, meso-CTT IV

Table 2. O_2-binding equilibrium parameters of iron and cobalt haemoglobin IV at 25° C

	Iron CTT IV		Cobalt CTT IV	
	Proto-porphyrin IX[a]	Meso-porphyrin IX[b]	Proto-porphyrin IX	Meso-porphyrin IX
$p_{1/2}(O_2)_{max}$ [mm Hg]	2.75	0.79	760	500
$p_{1/2}(O_2)_{min}$ [mm Hg]	0.45	0.12	110	90
$\Delta \log p_{1/2}(O_2)$	0.79	0.82	0.84	0.75
$-[\Delta \log p_{1/2}(O_2)/\Delta pH]$ [mol H^+/mol O_2]	0.45 (7.2)	0.33 (7.3)	0.36 (7.1)	0.35 (6.7)

Numbers in brackets are the inflection points of the Bohr-effect curves, where the Bohr-proton release was determined

[a] Data taken from reference [1, 6]

[b] Data taken from reference [48]

a triplet which can be assigned to a coupling of the spin with the $^{14}N_\varepsilon$-imidazole of the proximal histidine (I = 1). The hyperfine constants of proto-CTT IV are a (^{59}Co) = 7.96 mT and a ($^{14}N_\varepsilon$) = 1.65 mT. In deoxy meso-CTT IV the cobalt splitting constant is significantly larger, whereas the N_ε splitting constant is unchanged, compared with the respective parameters of proto-CTT IV (see Table 3).

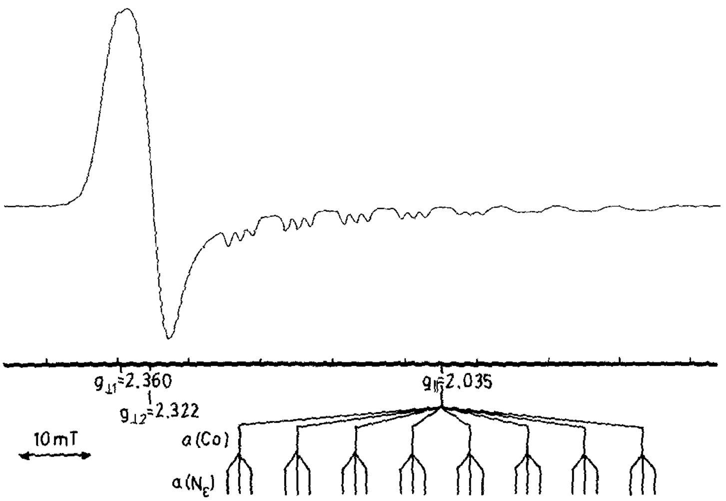

Fig. 4. ESR first derivative spectrum of cobalt-substituted deoxy proto-CTT IV. pH 5.5; temperature: 77 K

Table 3. ESR parameters of cobalt haemoglobin IV at 77 K

	Deoxy form	Oxy form	
	Low-pH/ High-pH forms	Low-pH form	High-pH form
Protoporphyrin IX			
$g_{\perp 1}$	2.360 ± 0.001	n.r.	n.r.
$g_{\perp 2}$	2.322 ± 0.001	n.r.	n.r.
$g_{\parallel}$	2.035 ± 0.001	2.077 ± 0.001	2.078 ± 0.001
$a_{\parallel}$ (^{59}Co) [mT]	7.96 ± 0.02	1.78 ± 0.01	1.81 ± 0.01
$a_{\parallel}$ ($^{14}N_\varepsilon$) [mT]	1.65 ± 0.01	n.r.	n.r.
Mesoporphyrin IX			
$g_{\perp 1}$	2.362 ± 0.001	n.r.	n.r.
$g_{\perp 2}$	2.319 ± 0.001	n.r.	n.r.
$g_{\parallel}$	2.032 ± 0.001	2.076 ± 0.001	2.078 ± 0.001
$a_{\parallel}$ (^{59}Co) [mT]	8.20 ± 0.02	1.70 ± 0.01	1.85 ± 0.01
$a_{\parallel}$ ($^{14}N_\varepsilon$) [mT]	1.66 ± 0.01	n.r.	n.r.

n.r., means not resolved

The second derivative ESR spectra of the deoxy forms measured at pH 5.7 and pH 9.5 do not show any influence of pH on the hyperfine constants in the direction parallel to the haem normal or on the spectral pattern at low-field (see Fig. 5).

Electron Spin Resonance of Oxy Cobalt Haemoglobin IV

The first derivative ESR spectrum of oxy cobalt meso-CTT IV shows rhombic symmetry (see Fig. 6). Only the low-field $g_{\parallel}$ value could be determined exactly. Exchange of mesoporphyrin IX by protoporphyrin IX does not change $g_{\parallel}$. The determination of the g values of the two other directions was not possible due to the overlapping hyperfine structures mainly originating from the cobalt atom and the N_{ε}-imidazole of the proximal histidine.

The eight-line hyperfine structure which coincides with the direction of $g_{\parallel}$ is characterized by hyperfine splitting constants of $a_{\parallel}$ (^{59}Co) = 1.85 mT (high pH) and 1.70 mT (low pH). This pH-dependent change of the cobalt splitting constant is smaller in oxy proto-CTT IV than in oxy meso-CTT IV. However, no influence on the $g_{\parallel}$ value is observed by changing the pH. Furthermore, the

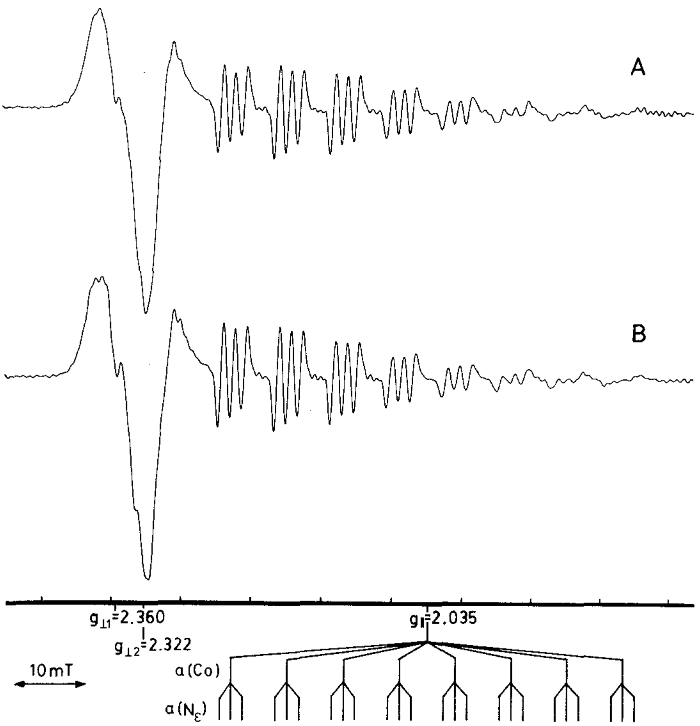

Fig. 5. A and B. ESR second derivative spectra of cobalt-substituted deoxy proto-CTT IV. pH 5.5 (**A**); pH 9.2 (**B**); temperature: 77 K; the experimental first derivative has been transformed to the second derivative by Fourier-transform technique

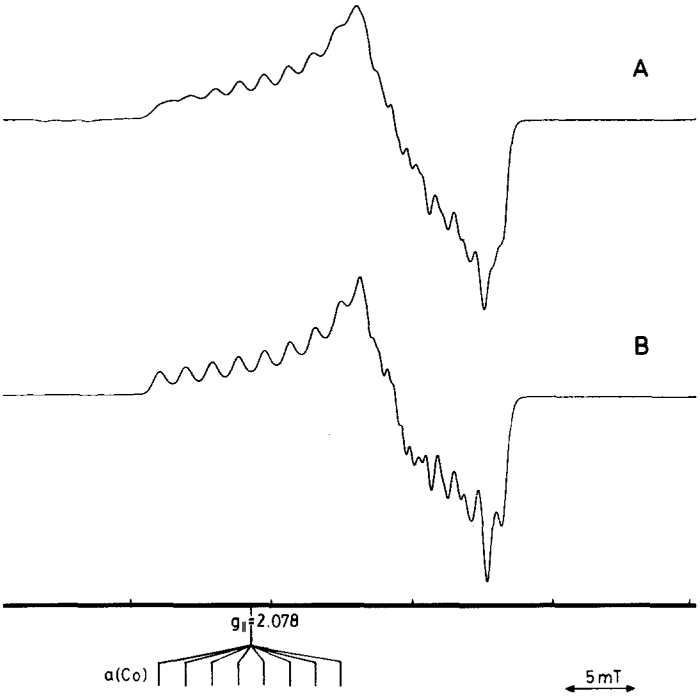

Fig. 6. A and B. ESR first derivative spectra of cobalt-substituted oxy meso-CTT IV. pH 6.1 (**A**); pH 9.6 (**B**); temperature: 77 K; g value and hyperfine splitting refer to the high-pH species

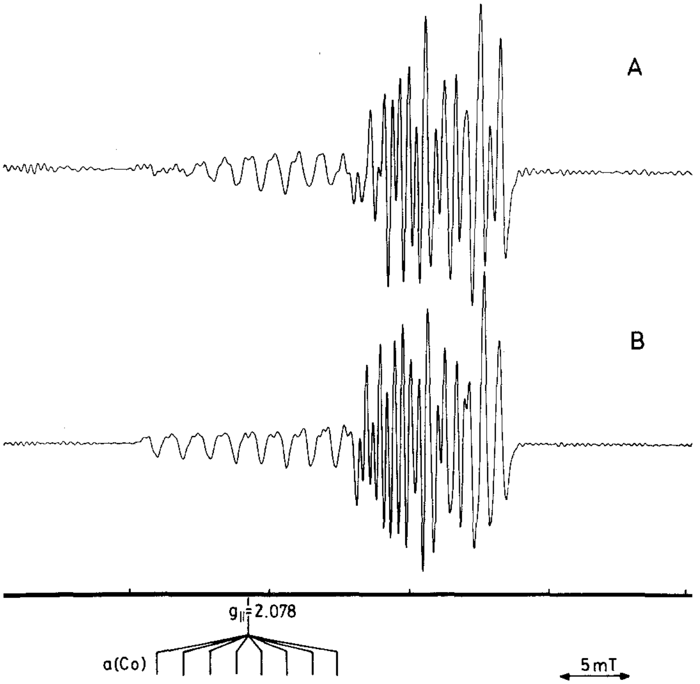

Fig. 7 A and B. ESR third derivative spectra of cobalt-substituted oxy meso-CTT IV. pH 6.1 (**A**); pH 9.6 (**B**); g value and hyperfine splitting refer to the high-pH species; the experimental first derivative has been transformed to the third derivative by Fourier-transform technique

eight-line hyperfine structure changes when replacing mesoporphyrin IX by protoporphyrin IX (see Table 3).

Comparison of the ESR spectra measured at acid and alkaline pH exhibits further spectral changes in the high-field region indicating an influence of pH also on the hyperfine pattern coinciding with the two other directions. This pH-dependent spectral change is demonstrated in the better resolved third derivative ESR spectra shown in Fig. 7. Although an exact determination of the pK value of this pH-dependent transition was not possible, the inspection of the high-field features gives evidence that this ESR spectral transition occurs with an inflection between pH 6 and 7 and therefore corresponds to the alkaline Bohr-effect. The low- and high-pH spectra correspond to the low- and high-affinity forms respectively.

Discussion

Effect of Substitution for Cobalt on the O_2 Affinity of Haemoglobin IV

The substitution with cobalt for iron in haemoglobins [30, 37, 54] and myoglobins [30, 53] is accompagnied by a dramatic decrease of the O_2 affinity. This characteristic lowering of the O_2 affinity is largest in leghaemoglobin (440-fold) (Gersonde et al., unpublished results) and CTT IV (250-fold) and is smallest in human haemoglobin A (10-fold) [30, 54] and myoglobin (50-fold) [30, 49]. On the basis of this observation CTT IV and leghaemoglobin constitute a common class and myoglobin and haemoglobin A another one.

Due to the occupancy of the d_{z^2} orbital by a single electron the central cobalt atom acts as a stronger σ donor than the iron atom and therefore, lengthening of the axial bonds must be expected [55].This fundamental behaviour is common for all cobalt-substituted haemoglobins. The strong σ donor property of cobalt(II) causes a two to three orders of magnitude larger k_{off} value for dioxygen than one can observe in the respective native haemoglobins, whereas the on-rate changes relatively little with substitution for cobalt [56, 57]. The large decrease in O_2 affinity of cobalt-substituted haemoglobin therefore originates from the increase of the off-rate constant. However, as already pointed out by Yamamoto et al. [56], the k_{off} values differ remarkably, when comparing different haemoglobins, indicating the particular influence of the protein moiety. Obviously, the apoprotein itself modifies, via specific interactions, the O_2 dissociation velocity constants and thus also the O_2 affinity.

In Table 4 for 25° C and pH 7.0 $p_{1/2}$ (O_2) values of the iron (native) and cobalt forms of three monomeric and one tetrameric haemoglobin are compiled. The O_2 half-saturation pressures increase with the following sequences,
for the iron form: leg Hb $<$ Mb $<$ CTT IV $<$ Hb A and
for the cobalt form: leg Hb $<$ Mb $<$ Hb A $<$ CTT IV.
Leghaemoglobin and myoglobin with higher O_2 affinities in both forms follow common sequences, whereas CTT IV and Hb A show an interchange. After substitution for cobalt, CTT IV exhibits a smaller O_2 affinity than Hb A. The cobalt-induced lowering-factor for the ligand affinity represented by the

Table 4. O_2-binding equilibrium parameters of iron- and cobalt-substituted leghaemoglobin (legHb), myoglobin (Mb), Chironomus haemoglobin IV (CTT IV) and human haemoglobin A (HbA) at 25° C and pH 7.0. The Hill coefficient is $n = 1$ and for Hb A given in brackets

Parameters	Central Atom	leg Hb[b]	Mb		CTT IV		HbA	
		Proto	Proto	Meso	Proto	Meso	Proto	Meso
$p_{1/2}$ (O_2) [mm Hg]	Iron	0.05	0.90**	0.54****	1.33***	0.32[b]	12.2 (2.9)[a]	2.0 (2.0)[d]
$p_{1/2}$ (O_2) [mm Hg]	Cobalt	22.2	50.0*	50.0*	309.0[c]	165.0[c]	125.0 (2.2)*	40.0 (1.2)*
F_{cobalt} = $p_{1/2}$ (O_2) cobalt/$p_{1/2}$ (O_2) iron		444.0	55.6	92.6	232.2 250 ± 20%[e]	515.6 630 ± 20%[e]	10.3	20.0
F_{meso} = F_{cobalt} (meso)/F_{cobalt} (proto)			2		2		2	

References: * [30], ** [49], *** [1], **** [52]

[a] Calculated on the basis of data published by references [50, 51]

[b] Gersonde et al., unpublished data

[c] Data of the present paper

[d] From Fig. 6 of reference [23] (conditions: 0.1 M phosphate, 22° C)

[e] Mean value calculated from data obtained at pH 5.7, 7.0, and 9.7

expression $F_{cobalt} = p_{1/2}(O_2)$ cobalt/$p_{1/2}(O_2)$ iron (see Table 4) increases with the sequence: Hb A < Mb < CTT IV < leg Hb. From the comparison of these three sequences the conclusion can be drawn that substitution for cobalt affects the k_{off} and $p_{1/2}(O_2)$ values of leg Hb and CTT IV much more than those of the two other haemoglobins.

Leghaemoglobin, myoglobin and haemoglobin A provide a distal histidine which under suitable conditions can interact with the bound dioxygen via hydrogen-bonding, whereas CTT IV does not. It is assumed that the distal histidine is responsible for the acid Bohr-effect of myoglobin [46, 50]. Although the nature of the acid Bohr-effect group in mammalian haemoglobins is yet a matter of controversy [58], the similarity of the F_{cobalt} values of Hb A and Mb support the assumption that the distal histidine in both, myoglobin and haemoglobin A, stabilizes the dioxygen-binding by reducing the off-rate. The off-rates of haemoglobins without distal histidine, i.e., *Aplysia* myoglobin [57] and *Glycera* haemoglobin [53], are about two orders of magnitude larger than that of myoglobin. Leghaemoglobin, however, although exhibiting a distal histidine, behaves on the basis of its lowering-factor of ligand affinity, F_{cobalt}, like CTT IV. Furthermore, at room temperature both, the native and the cobalt-substituted leghaemoglobin, do not show any pH dependence of the O_2 affinity, i.e., acid or alkaline Bohr effect. This result is consistent with a greater mobility of the distal histidine at room temperature which is due to the more flexible and open haem pocket in leghaemoglobin [59, 60]. However, at low temperature (at 77 K) the distal histidine of leghaemoglobin can interact with dioxygen bound to the haem group [20].

CTT IV is a lucky chance; despite of the lack of "*cis*-effects" by distal histidine, it exhibits an alkaline Bohr-effect. The Bohr-effect is substantially not changed by the metal exchange and the number of Bohr-protons released is the same in both, in the native and in the cobalt-substituted form. This result clearly demonstrates that the exchange of the central atom has no influence on the allosteric site, i.e., the Bohr-proton binding site. This is further confirmed by the invariance of the inflection points of the Bohr-effect curves, which are identical for iron and cobalt CTT IV, i.e., at pH 7.1–7.2. Furthermore, this result provides firm evidence, that a transition between two tertiary structures (*t*, *r*) or affinity states is not triggered by a spin state transition of the central metal atom and its movement relative to the haem plane as it is proposed by Perutz [61] for the quaternary structure change in Hb A. If this mechanism is effective, one should observe a larger Bohr-effect for native CTT IV than for the cobalt form. Therefore, a "*metal*-effect" as a controlling mechanism of the allostery of this haemoglobin can also be excluded.

An alternative mechanism of the allosteric control of the ligand affinity of this monomeric haemoglobin is due to the "*trans*-effect" of the proximal histidine. The proximal imidazole influences the σ donor properties of the cobalt atom by changing its bond length to the central atom either by movement of the *F* helix or by a twist. The distance between cobalt and N_ε-imidazole becomes smallest if the angle between the imidazole plane and a second plane passing through the twofold axis and one pair of the porphyrin nitrogen atoms is 45°. In this configuration the imidazole hydrogen atoms point toward the methine carbon atoms [62]. Longer bond distances result from an angle of 90°. It will be discussed later on the basis of ESR results that the "*trans*-effect" of the imidazole base influences the electronic structure of the cobalt atom solely in the ligated, i.e., in the oxygenated state, and not in the deoxy state. Thus, the two electronic states of the central atom which correlate with two tertiary conformations or two ligand affinity states exist only in the ligated form of CTT IV.

Effect of Substitution for Mesoporphyrin on the O_2 Affinity of Haemoglobin IV

The replacement of protoporphyrin by mesoporphyrin leads to an increase of O_2 affinity for both forms of CTT IV, i.e., for the iron- and the cobalt-substituted protein. The conversion of vinyl groups to ethyl groups in position 2 and 4 of the porphyrin ring is accompagnied at room temperature by an increased O_2 affinity in all haemoglobins [23–25, 30]. Ethyl groups of the mesoporphyrin are less electron attractive than vinyl groups of the protoporphyrin, resulting in an increase in the metal-oxygen bond strength. This reduction of the electron-withdrawing effect by ethyl groups is consistent with the blue-shift of the visible absorption spectra observed for CTT IV and other haemoglobins. For iron CTT IV an increase in O_2 affinity at 25° C is observed which follows the order: proto- < meso- < deutero-CTT IV. The same sequence of affinities has been described for iron and cobalt myoglobins at temperatures > 25° C, whereas at temperatures < 25° C this order changes to proto- < deutero- < meso-Mb [30]. Only the low-temperature order of O_2 affinities of myoglobin is exactly consistent with the above mentioned rule of negative inductive effects of the groups at position 2 and 4 of the porphyrin ring. On the basis of temperature dependences of the O_2 affinities of porphyrin-substituted myoglobin and haemoglobin it has been concluded that not only nucleophilicity but also size and rotational properties of the substituent groups in relation to specific interactions with the protein environment are responsible for the ligand affinity [30, 57]. Although steric effects are the primary effects which alter the intrinsic reactivities of haemoglobins [24], haem modification does not result in substantial alterations in the conformational equilibrium between two allosteric states [23–25]. Substitution for mesoporphyrin in CTT IV has no effect on the allostery of this haemoglobin, i.e., on its alkaline Bohr-effect. It has been recently demonstrated directly by ^1H NMR investigations, that one of the vinyl groups of CTT IV changes its rotational position depending on temperature and tertiary conformation [26]. The in-plane position of the vinyl group is preferred at high temperature or in the *t* state (with low O_2 affinity), whereas the out-of-plane position is possible in the *r* state (with high O_2 affinity). In the *t* state the rotational probability is little temperature-dependent, whereas in the *r* state with its more flexible protein structure the typical temperature dependence of a vinyl group with high degree of rotational freedom is shown. Although this vinyl group in CTT IV quantitatively reflects the Bohr-effect, substitution by meso- and deuteroporphyrin has no influence on the magnitude of the Bohr-effect.

Therefore, a "porphyrin effect" which controls the allostery of the protein can be excluded. Furthermore, we assume that the allosteric interaction energy is not mediated to the central atom via these side groups of the porphyrin. The above mentioned "irregular" order of O_2 affinities indicates that CTT IV produces a constraint on the porphyrin system.

The combination of a replacement for cobalt and mesoporphyrin leads to some minor alteration of the protein structure. The inflection point of the Bohr-effect curve is shifted by 0.4 units to low pH without any change in the amplitude of the Bohr-effect curve. This means that the environment of the allosteric site is changed.

F_{cobalt}, the cobalt-induced lowering-factor of the O_2 affinity is for meso-CTT IV larger by a factor of 2, than that of the proto-CTT IV (see Table 4). F_{meso} is independent of the protein structure and reflects the "pure" electronic effect of the side group substitution, i.e., the steric effects are the same in both, the iron- and cobalt-substituted, haemoglobins. This result is consistent with the observation that an exchange of the central metal atom is without any effect on the conformational equilibrium in allosteric haemoglobins. The mesoporphyrin-induced blue-shift of the visible absorption of CTT IV is another argument that the steric effects are not influenced by the exchange of the central metal atom.

pH-Independent Electron Spin Resonance Spectra of Deoxy Cobalt Haemoglobin IV

The different electron attractive effects of the substituents in position 2 and 4 of the porphyrin system can also be demonstrated by the ESR spectra at 77 K of deoxy cobalt CTT IV. Meso-CTT IV shows a smaller $g_{\parallel}$ value than proto-CTT IV. Furthermore, the ^{59}Co hyperfine constant which coincides with the z direction is larger in meso-CTT IV than in proto-CTT IV. Both observations are consistent with an increase of the unpaired spin density in the cobalt d_{z^2} orbital caused by the exchange of the vinyl by ethyl groups.

However, the hyperfine constant also coinciding with $g_{\parallel}$ and attributed to $^{14}N_{\varepsilon}$ of the proximal imidazole is not affected by the porphyrin substitution. The proximal histidine, which obviously plays the most important role for the allosteric variation of the O_2 affinity, does not change its distance to the cobalt atom or its binding geometry under the influence of the electronic effects produced by the porphyrin side groups.

The ESR feature at all and the above mentioned parameters of the z direction are invariant with pH, reflecting an invariant electronic structure of the cobalt atom. Two questions arise: Is the deoxy structure of CTT IV frozen allowing only one conformation state for oxygen-binding? Does the pH-induced conformation transition exist in the deoxy state but cannot affect the axial imidazole-cobalt bond and the electronic structure of the cobalt in the pentacoordinated state? It has been recently demonstrated by measuring hyperfine-shifted resonances of haem and proximal imidazole protons of native deoxy CTT IV that the imidazole exchangeable NH(1) proton does not show any pH dependence, whereas methyl groups at the haem periphery are sensitive to the pH-induced conformation change [7]. Therefore, we must assume that in deoxy CTT IV the conformation transition exists, but is not linked to the active site and does not induce a transition between two affinity states. This assumption is in full agreement with the on-rate constants for O_2 (Gersonde et al., unpublished data) and the temperature dependence of the quadrupole splitting of iron of deoxy CTT IV (Parak et al., unpublished data) of which both are independent of pH.

pH-Dependent Electron Spin Resonance Spectra of Oxy Cobalt Haemoglobin IV

The ESR spectra of oxy cobalt CTT IV are pH-dependent. The pH-induced transition from the low-pH to the high-pH spectral form correlates with the transition from the low-affinity to the high-affinity state of the haemoglobin as described also by the alkaline Bohr-effect. This pH dependence can be demonstrated by the hyperfine parameters of the well-resolved low-field part of spectra as well as by phenomenological comparison of the non-resolved high-field part (see Figs. 6 and 7). The transition between these two spectral forms occurs in the range of pH 6–7.

The ESR spectra are well-resolved only for one principal direction, i.e., parallel to the axial direction of the haem complex. $g_\parallel$ practically does not vary with pH and is not affected by differences in the nucleophilicity of porphyrin side groups. The eight-line hyperfine structure coinciding with $g_\parallel$ is due to the interaction of the electron spin with the cobalt nucleus ($I = 7/2$). The cobalt hyperfine constant, $a(^{59}Co)$ is smaller at low pH and becomes larger at high pH (see Tables 3 and 5). Therefore, on the basis of the above mentioned correlation, the low-affinity state of oxy cobalt CTT IV exhibits a smaller cobalt hyperfine constant than the high-affinity state indicating a pH-induced change of the spin density at the cobalt nucleus. We assume that spin transfer to dioxygen is larger for the low-pH/low-affinity than for the high-pH/high-affinity form. On the basis of ^{59}Co and ^{17}O hyperfine tensors Dickinson and Chien [29, 33] have shown for myoglobin, that dioxygen is bound to the cobalt center at 77 K in two stereoelectronic structures, i.e., in the mode of ozonoid-type and olefin-type bonding, respectively. For leghaemoglobin it has been recently demonstrated by ESR studies at 77 K that the transition between these two stereoelectronic structures is pH-induced and correlates with the protonation/deprotonation reaction of the distal histidine [20]. Both, myoglobin and leghaemoglobin, exhibit at low temperature an acid Bohr-effect (inverse to the alkaline Bohr-effect observed for CTT IV). By compiling in Table 5 the cobalt hyperfine constants coinciding with $g_\parallel$ of these three monomeric haemoglobins and by correlating them with O_2 affinity states of the acid and alkaline Bohr-effects of the respective proteins we can draw some interesting conclusions on the binding geometry of dioxygen in cobalt CTT IV. We have observed two classes of ^{59}Co hyperfine constants: $a(^{59}Co) = 2.10–2.40$ mT for the low-pH forms (high-affinity states) of myoglobin and leghaemoglobin, and $a(^{59}Co) = 1.55–1.70$ mT

Table 5. Correlation of ESR parameters with O_2 affinity states for different oxy cobalt haemoglobins

pH	ESR parameters	Haemoglobins		
		CTT IV[a]	Mb[b]	leg Hb[c]
High pH	g_1	n.d.	2.080	2.078
	g_2	n.d.	1.980	1.988
	$g_3 = g_\parallel$	2.077 (2.076)	2.008	2.005
	$a_\parallel$ (^{59}Co) (mT)	1.78 (1.70)	1.67	1.55
	$a_\parallel$ ($^{14}N_\varepsilon$) (mT)	n.d.	n.d.	0.44
	Affinity state	High-affinity	Low-affinity	Low-affinity
	Conformation state	*r*	*t*	*t*
	O_2-binding geometry	Ozonoid (more inclined)	Ozonoid	Ozonoid
	Distal imidazole interaction	No	No	No
Low pH	g_1	n.d.	2.080	2.092
	g_2	n.d.	1.980	1.989
	$g_3 = g_\parallel$	2.078 (2.078)	2.008	2.006
	$a_\parallel$ (^{59}Co) (mT)	1.81 (1.85)	2.40	2.10
	$a_\parallel$ ($^{14}N_\varepsilon$) (mT)		0.50	0.65
	Affinity state	Low-affinity	High-affinity	High-affinity
	Conformation state	*t*	r'	r'
	O_2-binding geometry	Ozonoid	Olefin	Olefin
	Distal imidazole interaction	No	Yes	Yes

[a] Data of the present paper, numbers in brackets refer to oxy cobalt meso-CTT IV

[b] Reference [33]

[c] Reference [20]

n.d., means not determined

for the high-pH forms (low-affinity states) of myoglobin and leghaemoglobin and for the low-pH form (low-affinity state) of CTT IV respectively. The large value of a(^{59}Co) is due to the interaction of dioxygen with the protonated distal histidine, the small value of a(^{59}Co) can be attributed to a lack of distal histidine interaction with dioxygen. a(^{59}Co) of the high-pH form (high-affinity state) of CTT IV is somewhat in between the above mentioned classes. Because distal histidine interaction is lacking in CTT IV the increase of a(^{59}Co) is due to the "*trans*-effect" of the proximal histidine which is controlled by the conformation transition. In both conformation states dioxygen is bound in the mode of ozonoid-type bonding, however, with a more inclined O-O-Co bond angle (< 120°) in the high-pH form (high-affinity state).

The less electron-withdrawing effect of mesoporphyrin replacing protoporphyrin in oxy cobalt CTT IV should be reflected by an increase in a(^{59}Co). This is only true for the relaxed (*r*) conformation (high-affinity state) at high pH. The transition from the *r* to tense (*t*) conformation (low-affinity state) at low pH leads to a shortening of the imidazole-cobalt bond by twisting or displacement of the base as discussed already before. Under this condition the electronic effect of the side group substitution is compensated and inverted possibly by steric effects so that the a(^{59}Co) value is now larger in oxy cobalt proto-CTT IV than in the respective meso-CTT IV. This result indirectly again gives evidence that the proximal imidazole is important for the control of the dioxygen ligand in *trans*-position. pH-induced hyperfine shifts of the NH protons of the proximal imidazole in cyanide-ligated CTT IV [6] further support the assumption that the "*trans*-effect" of the proximal imidazole has to be induced by ligand-binding. Ligand-binding makes the protein structure sensitive for the transition between functional states.

Acknowledgements. This investigation has been supported by the Deutsche Forschungsgemeinschaft.

References

1. Gersonde K, Sick H, Wollmer A, Buse G (1972) Heterotropic allosterism of monomeric haemoglobins of *Chironomus thummi thummi.* Eur J Biochem 25: 181–189
2. Sick H, Gersonde K, Thompson JC, Maurer W, Haar W, Rüterjans H (1972) The Bohr proton-binding site in a monomeric haemoglobin. A nuclear-magnetic resonance study. Eur J Biochem 29: 217–223
3. Sick H, Gersonde K (1974) Ligand-specific Bohr-effect in haemoglobins. Eur J Biochem 45: 313–320
4. Gersonde K, Noll L, Gaud HT, Gill SJ (1976) A calorimetric study of the CO Bohr-effect of monomeric haemoglobins. Eur J Biochem 62: 577–582
5. Steffens G, Buse G, Wollmer A (1977) Ligand-dependent Bohr-effect of Chironomus hemoglobins. Eur J Biochem 72: 201–206
6. La Mar GN, Overkamp M, Sick H, Gersonde K (1978) Proton nuclear magnetic resonance hyperfine shifts as indicators of tertiary structural changes accompanying the Bohr-effect in monomeric insect hemoglobins. Biochemistry 17: 352–361
7. La Mar GN, Anderson RR, Budd DL, Smith KM, Langry KC, Gersonde K, Sick H (1981) Proton nuclear magnetic resonance investigation of the nature of the solution conformational equilibria of monomeric insect deoxy hemoglobins. Biochemistry 20: 4429–4436
8. Buse G, Steffens GJ, Braunitzer G, Steer W (1979) Hämoglobin (Erythrocruorin) CTT III aus *Chironomus thummi thummi* (Diptera). Primärstruktur und Beziehung zu anderen Hämoglobinen. Hoppe-Seylers Z Physiol Chem 360: 89–97
9. Pfletschinger J, Plagens H, Braunitzer G (1980) The primary structure of the monomeric hemoglobin (Erythrocruorin) component CTT IV of *Chironomus thummi thummi (Insecta, Diptera).* Z Naturforsch 35c: 840–843
10. Steigemann W, Weber E (1979) Structure of Erythrocruorin in different ligand states refined at 1.4 Å resolution. J Mol Biol 127: 309–338
11. Overkamp M, Twilfer H, Gersonde K (1976) Conformation-controlled trans-effect of the proximal histidine in haemoglobins. An electron spin resonance study of monomeric nitrosyl-^{57}Fe-haemoglobins. Z Naturforsch 31c: 524–533
12. Twilfer H, Gersonde K (1976) Non-equivalence and inverse allosteric response of the α and β chains in haemoglobins. An electron spin resonance study of NO-ligated Hb Kansas. Z Naturforsch 31c: 664–674
13. Huber R, Epp O, Formanek H (1970) Structures of deoxy and carbonmonoxy erythrocruorin. J Mol Biol 52: 349–354
14. Heidner EJ, Ladner RC, Perutz MF (1976) Structure of horse carbonmonoxyhaemoglobin. J Mol Biol 104: 707–722

15. Stryer L, Kendrew JC, Watson HC (1964) The mode of attachment of the azide ion to sperm whale metmyoglobin. J Mol Biol 8: 96–104
16. Perutz MF, Mathews FS (1966) An X-ray study of azide methaemoglobin. J Mol Biol 21: 199–202
17. Yonetani T, Yamamoto H, Iizuka T (1974) Studies on cobalt myoglobins and hemoglobins. III. Electron spin paramagnetic resonance studies of reversible oxygenation of cobalt myoglobins and hemoglobins. J Biol Chem 249: 2168–2174
18. Dickinson C, Chien JCW (1980) Electron paramagnetic resonance crystallography of ^{17}O-enriched oxycobaltomyoglobin: Stereoelectronic structure of the cobalt dioxygen system. Proc Natl Acad Sci USA 77: 1235–1239
19. Asher SA, Adams ML, Schuster TM (1981) Resonance Raman and absorption spectroscopic detection of distal histidine-fluoride interactions in human methemoglobin fluoride and sperm whale metmyoglobin fluoride: Measurements of distal histidine ionization constants. Biochemistry 20: 3339–3346
20. Christahl M, Raap A, Gersonde K (1981) pH dependence of oxy and deoxy cobalt-substituted leghemoglobin from soybean. Biophys Struct Mech 7: 171–186
21. Griffith JS (1956) On the magnetic properties of some haemoglobin complexes. Proc R Soc (Lond) Ser A 235: 23–36
22. Pauling L (1964) Nature of the iron-oxygen bond in oxy haemoglobin. Nature 203: 182–183
23. Sugita Y, Yoneyama Y (1971) Oxygen equilibrium of hemoglobins containing unnatural hemes. Effect of modification of heme carboxyl groups and side chains at positions 2 and 4. J Biol Chem 246: 389–394
24. Seybert DW, Moffat K, Gibson QH (1976) Ligand binding properties of horse hemoglobins containing deutero- and mesoheme. J Biol Chem 251: 45–52
25. Fioretti E, Ascoli F, Brunori M (1976) Preparation of apohemoglobin trout IV and reconstitution with different hemes. Biochem Biophys Res Commun 68: 1169–1173
26. La Mar GN, Viscio DB, Gersonde K, Sick H (1978) Proton nuclear magnetic resonance study of the rotational position of oscillatory mobility of vinyl groups in allosteric monomeric insect hemoglobins. Biochemistry 17: 361–367
27. Hsu GC, Spilburg CA, Bull C, Hoffmann BM (1972) Coboglobins: Heterotropic linkage and the existence of a quaternary structure change upon oxygenation of cobalthemoglobin. Proc Natl Acad Sci USA 69: 2122–2124
28. Spilburg CA, Hoffmann BM, Petering DH (1972) Coboglobins. Influence of the apoprotein on oxygen binding to cobaltomyoglobin. J Biol Chem 247: 4219–4223
29. Dickinson LC, Chien JCW (1973) Comparative biological chemistry of cobalt hemoglobin. J Biol Chem 248: 5005–5011
30. Yonetani T, Yamamoto H, Woodrow III GV (1974) Studies on cobalt myoglobins and hemoglobins. I. Preparation and optical properties of myoglobins and hemoglobins containing cobalt proto-, meso-, and deuteroporphyrins and thermodynamic characterization of their reversible oxygenation. J Biol Chem 249: 682–690
31. Gersonde K, Twilfer H, Overkamp M (1977) Allostery of monomeric cobalt hemoglobins. 11th FEBS Meeting Copenhagen, Abstracts B2: 458
32. Gersonde K, Twilfer H, Overkamp M (1978) ESR and oxygen-binding properties of monomeric allosteric cobalt haemoglobins. VIIIth International Conference on Magnetic Resonance in Biological Systems, Nara, Abstracts E10: 106
33. Chien JCW, Dickinson LC (1972) Electron paramagnetic resonance of single crystal oxycobaltmyoglobin and deoxycobaltmyoglobin. Proc Natl Acad Sci USA 69: 2783–2787
34. Dickinson LC, Chien JCW (1973) Electron paramagnetic resonance of single crystal deoxycobalthemoglobin. Biochem Biophys Res Commun 51: 587–592
35. Yonetani T, Yamamoto H, Iizuka T (1974) Studies on cobalt myoglobins and hemoglobins. III. Electron paramagnetic resonance studies of reversible oxygenation of cobalt myoglobins and hemoglobins. J Biol Chem 249: 2168–2174
36. Gupta RK, Mildvan AS, Yonetani T, Srivastava TS (1975) EPR study of ^{17}O nuclear hyperfine interaction in cobalt-oxyhemoglobin: Conformation of bound oxygen. Biochem Biophys Res Commun 67: 1005–1012

37. Ikeda-Saito M, Yamamoto H, Yonetani T (1977) Studies on cobalt myoglobins and hemoglobins. Electron paramagnetic resonance of iron-cobalt hybrid hemoglobins and its implication for the heme-heme interaction and for the alkaline Bohr-effect. J Biol Chem 252: 8639–8644
38. Rossi-Fanelli A, Antonini E, Caputo A (1958) Studies on the structure of hemoglobin. Physicochemical properties of human globin. Biochim Biophys Acta 30: 608–615
39. Amiconi G, Antonini E, Brunori M, Formanek H, Huber R (1972) Functional properties of native and reconstituted hemoglobins from Chironomus thummi thummi. Eur J Biochem 31: 52–58
40. Labbe RF, Nishida G (1957) A new method of hemin isolation. Biochim Biophys Acta 26: 437
41. Morrell DB, Stewart M (1956) The removal of iron from haemins. Austral J Exp Biol 34: 211–218
42. Taylor JF (1940) Metalloporphyrins: Cobalt and manganese mesoporphyrins in coordination with nitrogeneous bases. J Biol Chem 135: 569–595
43. Wüthrich K (1970) Structural studies of hemes and hemoproteins by nuclear magnetic resonance spectroscopy. Structure and Bonding, vol 8. Springer, Berlin Heidelberg New York, pp 53–121
44. Sick H, Gersonde K (1969) Method of continuous registration of O_2-binding curves of hemoproteins by means of a diffusion chamber. Anal Biochem 32: 362–376
45. Sick H, Gersonde K (1972) Theory and application of the diffusion technique for measurement and analysis of O_2-binding properties of very autoxidizable hemoproteins. Anal Biochem 47: 46–56
46. Twilfer H, Gersonde K, Christahl M (1981) Resolution enhancement of EPR spectra using the Fourier transform technique. Analysis of nitrosyl cytochrome c oxidase in frozen solution. J Magn Reson 44: 470–478
47. Gersonde K, Overkamp M, Sick H, Trittelvitz E, Junge W (1973) β-Chain allostery in the frozen quaternary T-structure of haemoglobin M Iwate. Eur J Biochem 39: 403–412
48. Overkamp M (1979) Untersuchungen zur Allosterie in monomeren und dimeren Hämoglobinen der Zuckmücke *Chironomus thummi thummi (Diptera, Insecta)*. Dissertation, Aachen
49. La Mar GN, Budd DL, Sick H, Gersonde K (1978) Acid Bohr effects in myoglobin characterized by proton NMR hyperfine shifts and oxygen binding studies. Biochim Biophys Acta 537: 270–283
50. Imai K, Yonetani T (1975) pH dependence of the Adair constants of human hemoglobin. Nonuniform contribution of successive oxygen bindings to the alkaline Bohr effect. J Biol Chem 250: 2227–2231
51. Imai K, Yonetani T (1975) Thermodynamical studies of oxygen equilibrium of hemoglobin. Nonuniform heats and entropy changes for the individual oxygenation steps and enthalpy-entropy compensation. J Biol Chem 250: 7093–7098
52. Tamura M, Woodrow III GV, Yonetani T (1973) Heme-modification studies of myoglobin. II. Ligand binding characteristics of ferric and ferrous myoglobins containing unnatural hemes. Biochim Biophys Acta 317: 34–49
53. Ikeda-Saito M, Iizuka T, Yamamoto H, Kayne FJ, Yonetani T (1977) Studies on cobalt myoglobins and hemoglobins. Interaction of sperm whale myoglobin and *Glycera* hemoglobin with molecular oxygen. J Biol Chem 252: 4882–4887
54. Imai K, Yonetani T, Ikeda-Saito M (1977) Allosteric effects in cobalto haemoglobin as studied by precise oxygen equilibrium measurements. J Mol Biol 109: 83–97
55. Little GR, Ibers JA (1974) Stereochemistry of cobalt porphyrins. I. The structure and characterization of 2,3,7,8,12,13,17,18-octaaethylporphinato-bis(3-methylpyridine)cobalt(II). J Am Chem Soc 96: 4440–4446
56. Yamamoto H, Kayne FJ, Yonetani T (1974) Studies on cobalt myoglobins and hemoglobins. II. Kinetic studies of reversible oxygenation of cobalt myoglobins and hemoglobins by the temperature jump relaxation method. J Biol Chem 249: 691–698
57. Ikeda-Saito M, Brunori M, Yonetani T (1978) Oxygenation and epr spectral properties of *Aplysia* myoglobins containing cobaltous porphyrins. Biochim Biophys Acta 533: 173–180
58. Kilmartin JV, Rossi-Bernardi L (1973) Interaction of hemoglobin with hydrogen ions, carbon dioxide, and organic phosphates. Physiol Rev 53: 836–890

59. Nicola NA, Leach SJ (1977) Structural rearrangements due to ligand binding and haem replacement in myoglobin and leghaemoglobin. Eur J Biochem 78: 133–140
60. Johnson RN, Bradbury JH, Appleby C (1978) A proton magnetic resonance study of the distal histidine of soybean leghemoglobin. J Biol Chem 253: 2148–2154
61. Perutz MF (1976) Structure and mechanism of haemoglobin. Br Med Bull 32: 195–208
62. Lauher JW, Ibers JA (1974) Stereochemistry of cobaltporphyrins. II. The characterization and structure of meso-tetraphenylporphinatobis(imidazole)cobalt(III) acetate monohydrate monochloroformate, $(Co(Im)_2(TPP))\ (O\ Ac) \cdot H_2O \cdot CHCl_3$. J Am Chem Soc 96: 4447–4452

Received August 10, 1981/Accepted November 23, 1981