chemical constraints, imposed by the Pro and Aib residues, make **1** a useful, conformationally rigid model for the study of the spectroscopic properties[33] of peptide disulfides.

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Supplementary Material Available: Tables of structure factor amplitudes, thermal parameters of non-hydrogen atoms, and positional parameters of hydrogen atoms (26 pages). Ordering information is given on any current masthead page.

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^{14}N and ^{1}H ENDOR of Nitrosylhemoglobin

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Abstract: ^{1}H and ^{14}N ENDOR spectra were obtained from NO derivatives of human hemoglobin, its separate α and β subunits, myoglobin, and hemin dimethyl ester. ^{14}N couplings of approximately 17 MHz are found for N_ϵ of the proximal histidine (F8), showing substantial anisotropy. Small but systematic differences exist in the N_ϵ couplings of the different compounds. Of the proton interactions, one is assigned to a meso proton ($\sim$0.4 MHz) and another one to a methyl proton of the valine (E11) on the distal side of the hemepocket ($\sim$3.8 MHz maximum coupling). One H-D exchangeable proton coupling ($\sim$1.9 MHz) is attributed to the N_{ϵ_2} proton of the distal histidine (E7). Distinct differences between α and β chains are observed upon r-t transitions. The β-chain couplings show no discernible change between coupling in both states, indicating that none of the amino acids (F8, E7, E11) are involved. For the α chains, both the N_ϵ-histidine and the distal side interactions (E7) are lost upon a transition from r to t. The quaternary R and T states of HbNO reflect the behavior of the subunits with slight but significant differences in the coupling size of several interactions, showing the influence of the quaternary state on the subunits.

Extensive electron spin resonance (ESR) studies on NO-ligated hemoglobins (HbNO)[1] and their derivatives both in single crystal form and as frozen solutions have yielded detailed information on the stereochemical and electronic structure of the nitrosyl heme group.[2-6] Since HbNO undergoes a quaternary R-T transition effected by a change in pH and by addition of inositol hexaphosphate (IHP), it has been considered a useful model for the elucidation of the allosteric behavior of hemoglobin upon oxygenation.[7,8]

The quaternary R and T states in HbNO are manifest by two distinctly different ESR spectra, both being of rhombic symmetry with respect to the iron ion. The R state corresponds to a hexacoordinated heme iron in which, in the frozen solution powder spectra used typically, the ligand NO and the proximal histidine (His F8) appear to contribute to a hyperfine structure via ^{14}N interactions for at least one g turning point, usually denoted g_z. The spectrum observed in the T state is characterized by a high-field shift of the g_z resonance that again carries a well-expressed ^{14}NO hyperfine substructure. An interaction with the His (F8) nitrogen cannot be resolved.[5,9,10]

Isolated NO-ligated subunits of Hb exhibit different ESR spectra that, in addition, react differently with respect to changes of pH. Whereas the spectra of the β chains, within the limits of the ESR resolution, appear to experience no change between basic and acidic conditions,[11,12] there is a drastic effect for the α chains, which exhibit features comparable to those of the quaternary R and T state spectra, respectively, when going from alkaline to acid pH.[11,12,13] Since the spectra of the tetramer HbNO were shown to be a linear superposition of the subunit spectra for both the R and the T state,[14] the conclusion of these and several related findings with hybrids[11,15] and natural hemoglobin mutants[9,10,16,17] is that the α chains undergo a distinct tertiary structure change well sensed at the heme upon an R-T transition. The β chains,

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(1) Abbreviations used: hemoglobin, Hb; myoglobin, Mb; hemin dimethyl ester, hemin DME; nitrosyl derivatives are written with NO used as suffix; inositol hexaphosphate, IHP; *p*-hydroxymercury benzoate, pMB; electron spin resonance, ESR; electron-nuclear double resonance, ENDOR.

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on the other hand, while lacking a pronounced tertiary conformational change, are believed to play a more important role in triggering the quaternary transition.[11,18]

With the exception of some monomeric hemoglobins,[19] the spectral resolution in ESR for both powder-type and crystalline samples of tetramers is too low to allow for the evaluation of the complete hyperfine tensor of the ^{14}N–His (F8) interaction in the hexocoordinated systems. Also, for the T-state spectra a decision whether the N_ϵ bond to iron is still intact but stretched to reduce the hyperfine interaction or is broken to yield a pentacoordinated iron complex cannot be made. Furthermore, the question of a participation of the distal histidine (His E7) in the stereochemistry of NO binding to iron that is considered effective in oxyhemoglobins[20-22] cannot be tackled by ESR. As was shown by Feher and co-workers for aquomet-hemoglobin and -myoglobin,[23,24] the resolution enhancement of electron–nuclear double-resonance (ENDOR) spectroscopy is suitable for obtaining a more detailed picture of the immediate heme-iron environment. This report presents, to our knowledge, the first ENDOR data on ^{14}N and ^{1}H couplings from NO-ligated tetrameric HbA, its isolated α and β chains, myoglobin (Mb), and a model compound, hemin dimethyl ester (hemin DME). The frequency range studied involves the ^{14}N–histidine (His F8) and the weakly coupled proton region. The experimental conditions were chosen in order to gain an understanding of the inequivalence of the Hb subunits with respect to the quaternary R–T transition in terms of structural differences in the close vicinity of the heme groups.

Experimental Section

Apparatus. ESR spectra were taken on a Bruker ER 420 instrument (X-band, ~9.4 GHz) equipped with an NMR gaussmeter and a frequency counter (Systron Donner). The ENDOR apparatus consists of a Wavetek 3000 synthesizer, the frequency of which is swept computer controlled by means of a Dietz 621/8 computer. The 10-kHz FM-modulated output is fed into two 10-W broad-band amplifiers (Amplifier Research). A power coupler (ENI) combines the output of the amplifiers, which passes a current-control device (Bruker) before reaching the ENDOR coil, which is an eight-turn Helmholtz arrangement placed inside a double-walled quartz finger which is part of a He-gas flow cryostat (Thor).[25] The current-proportional output of the control box is fed into a power-regulator system based on a voltage-variable Attenuator (Anzac AT 101) that controls the input power of the amplifiers. A parallel signal is used to monitor the waveform of the amplifier output with an oscilloscope to avoid overtones and nonlinearities. The ENDOR spectra are stored in the computer as first derivatives taken either in single-scan- or multi-scan-averaging modes. Data acquisition (1K data points) was typically performed at 10 K by using microwave powers between 3 and 12 mW, an rf sweep rate of 0.5 MHz/s in multi-scan-average mode, and an FM modulation amplitude of 500 kHz. The assignment of the ^{14}N hyperfine lines was checked by calculating the line positions using the MAGNSPEC program.[26] The corresponding stick spectra obtained are included in the spectra shown.

Reagents. All common salts used were pA grade from Merck. D_2O (99.75%) and 2-mercaptoethanol were from Merck. Inositol hexaphosphate (IHP) (type V) and p-hydroxymercury benzoate (pMB) (sodium salt) as well as Trizma and Bistris buffers were from Sigma. Myoglobin was Sigma type II sperm whale metmyoglobin. Freshly drawn human blood was obtained from the Red Cross (Regensburg).

Table I. Sample Conditions of NO Derivatives Investigated

sample	concn in heme, mM	buffer	pH
HbNO	8.1	50 mM Tris	8.5
HbNO (+3 molar excess IHP)	6.9	0.1 M phosphate	6.0
MbNO	3.8	0.1 M phosphate	7.5
αNO	4.7	0.1 M phosphate	7.4
αNO	4.7	0.1 M phosphate	6.0
βNO	2.0	50 mM Tris	8.5
βNO	2.0	0.1 M phosphate	6.0
HbNO (D_2O)	6.5	50 mM Tris	8.5
βNO (D_2O)	2.0	50 mM Tris	8.5
hemin DME-NO	4.0	chloroform	

Sample Preparation. Human hemoglobin was prepared from red blood cells by the method of Drabkin.[27] The hemoglobin was stripped of phosphates by chromatography on Sephadex G-25 equilibrated with 50 mM Bistris buffer, pH 7.4. Separation of the subunits by reaction with pMB and chromatography on DEAE-52 and CMC-52 (Whatman) was performed according to the literature.[28,29] Separated chains were freed of pMB by addition of 2-mercaptoethanol followed by chromatography on Sephadex G-25. The purity of the chains was controlled by isoelectric focussing (IEF) and by ESR spectroscopy subsequent to X irradiation of the oxygenated chains.[30,31] Typically, a purity of 85–90% was obtained. Met-Mb was reduced by sodium dithionite under an N_2 atmosphere in a 0.1 M phosphate buffer at pH 7.5. Excess dithionite was removed on a Sephadex G-25 column.

All samples were concentrated to their final concentration in heme by means of an Amicon ultrafiltration cell using P-10 membranes. NO ligation was performed by adding 20 cm^3 of NO gas to 2–5 mL of sample that was previously deoxygenated by several cycles of pumping and N_2 flushing in 50-mL vessels. After reaction of NO for 4 h at 4 °C, 0.3–0.5 mL of sample solution was withdrawn and frozen at 77 K. Concentrations, buffer conditions, and pH of samples studied are listed in Table I.

Results and Discussion

^{14}N–His (F8) Couplings. All interactions concerned being much smaller than the electron Zeeman term, the part of the spin hamiltonian $\mathcal{H}_{EN}$ relevant to the ^{14}N transitions in ENDOR can be written approximately as

$$\mathcal{H}_{EN} = \vec{S}\mathbf{A}\vec{I} + \vec{I}\mathbf{P}\vec{I} + g_n\beta_n\vec{H}\vec{I} \quad (1)$$

in which $\vec{S}$ and $\vec{I}$ are the operators of electron and nuclear spins, respectively, **A** and **P** the hyperfine and quadrupolar interaction tensors, $\vec{H}$ the magnetic field operator, and g_n and β_n the nuclear g factor and magneton, respectively. The resulting ENDOR resonances in first order on a frequency scale follow as

$$\nu_{EN} = |a_i/2 \pm p_i \pm \nu_n| \quad (2)$$

in which a_i and p_i are the elements of tensors **A** and **P** for a canonical orientation i. With $\nu_n \simeq 1$ MHz for a nitrogen nucleus in a field of about 3300 G and values of a_i of about 16–20 MHz as expected, e.g., from approximate estimates taken from the ESR spectra, eq 2 should yield a quartet of ENDOR lines centered around $a_i/2$ for which the lines are arranged in pairs separated by $2\nu_n$, the distance of the centers of the pairs giving the quadrupolar coupling $2p_i$. In a polycrystalline system of rhombic g-tensor symmetry, two canonical orientations for the ^{14}N–His (F8) couplings should be found around the extreme g-turning points of the ESR spectrum. Depending on the magnitude of a_i and p_i, the quartet may collapse into a doublet ($p_i \simeq 0$).

All samples studied comply with these expectations and exhibit, moreover, qualitatively the same behavior of resonance lines at all g turning points that we denote, for the sake of clarity, g_1, g_2, and g_3 starting from the low-field side of the ESR spectrum. For

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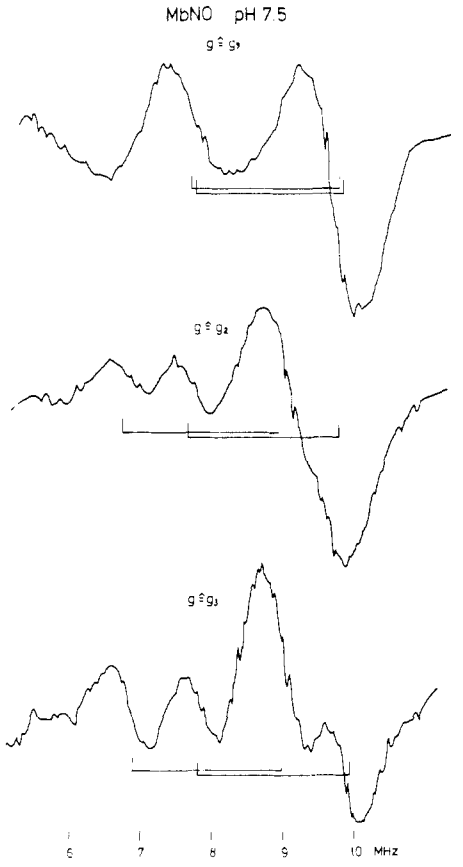

Figure 1. ^{14}N ENDOR on nitrosylmyoglobin at the three g turning points (g_1, g_2, g_3). Each spectrum took 13 min of signal averaging. Magnetic field and g values together with splitting parameter are given in Table II. Experimental conditions are given in the Experimental Section. Stick diagrams indicate calculated line positions.

the subunit spectra, there is an unambiguous correspondence between this and the usual nomenclaure (e.g., $g_1 = g_x$, $g_2 = g_z$, and $g_3 = g_y$ in a spectrum of a hexacoordinated α and β chain as well as $g_2 = g_y$ and $g_3 = g_z$ in a "pentacoordinated" α chain). This correlation, however, does not apply to the tetrameric molecule (HbNO + IHP) as a superposition of subunit spectra being in different coordination states.[14]

The ENDOR response typical for all samples at the three g turning points is depicted in Figure 1 for NO-ligated myoglobin (MbNO) as example. Along g_1 (top spectrum), a doublet is observed, indicating a small quadrupolar interaction term in this direction. When the response along g_3 is monitored (Figure 1, bottom), all samples display a well-resolved quartet that must be ascribed to a large quadrupolar interaction in this direction giving rise to a sufficient spacing of the nuclear Zeeman line pairs. Along g_2, the low-frequency lines are typically well resolved whereas the high-frequency partners merge to one broader line. Only HbNO yields a doublet in this direction, too, a behavior discussed below. The stick diagrams beneath each spectrum give the line positions arrived at by simulation using the spin hamiltonian of eq 1. All three types of spectra are well reproduced. For g_2, the sticks give the centers of line positions arrived at by the powder type simulations for the plane unfolded by $|\vec{g}| = g_2$.

The assignment of these lines to those of the ^{14}N–His (F8) couplings derives from the fact that neither a pentacoordinated model compound (NO-hemin dimethyl ester) nor the α chain in the t state display any ENDOR resonance in the frequency range 6–11 MHz. Also for MbNO or the r state α chain, the resolution of the ESR spectrum on g_2 is good enough to ascertain that the ^{14}N–His (F8) interaction observed in ESR matches the data derived from the ENDOR analysis.

Although the qualitative behavior of all samples studied is roughly identical, the magnitude of the ^{14}N–His (F8) interactions differs distinctly. Figure 2 shows the g_3 responses for a representative selection of samples studied, together with the stick diagrams obtained from stimulation. The corresponding set of hyperfine and quadrupolar couplings together with the values obtained on the other g turning points are listed in Table II, together with those of the other specimen.

Table II. Nitrogen ENDOR of NO Derivatives of Hb, α and β Chains, and Mb

compd and conditions	ENDOR frequencies, MHz	derived couplings, MHz
HbNO, pH 8.5		
$g_1 = 2.07$	7.92 ± 0.05	$\|a_1\| = 17.82 \pm 0.05$
$H = 3.15$ kG	9.91 ± 0.05	$\|p_1\| = 0.18 \pm 0.07^b$
$2\nu_N = 1.93$ MHz		
$g_2 = 2.006$	7.90 ± 0.05	$\|a_2\| = 17.74 \pm 0.05$
$H = 3.25$ kG	9.85 ± 0.05	$\|p_2\| = 0.18 \pm 0.07^b$
$2\nu_N = 2.00$ MHz		
$g_3 = 1.98$	6.83 ± 0.05	$\|a_3\| = 16.82 \pm 0.05$
$H = 3.30$ kG	7.87 ± 0.05	$\|p_3\| = 0.36 \pm 0.05$
$2\nu_N = 2.03$ MHz	9.22 ± 0.05	
	9.72 ± 0.05	
HbNO + IHP		
$g_1 = 2.11$	8.00 ± 0.05	$\|a_1\| = 17.90 \pm 0.05$
$H = 3.09$ kG	9.90 ± 0.05	$\|p_1\| \simeq 0.0^b$
$2\nu_N = 1.90$ MHz		
$g_2 = 2.06$	7.20 ± 0.08	$\|a_2\| = 17.56 \pm 0.06$
$H = 3.15$ kG	8.07 ± 0.05	$\|p_2\| = 0.43 \pm 0.06$
$2\nu_N = 1.93$ MHz	9.92 ± 0.05	
$g_3 = 2.003$	7.00 ± 0.08	$\|a_3\| = 17.24 \pm 0.06$
$H = 3.26$ kG	7.97 ± 0.05	$\|p_3\| = 0.40 \pm 0.06$
$2\nu_N = 2.00$ MHz	9.20 ± 0.05	
	9.80 ± 0.05	
αNO pH 7.4		
$g_1 = 2.08$	7.95 ± 0.04	$\|a_1\| = 17.92 \pm 0.05$
$H = 3.27$ kG	9.97 ± 0.04	$\|p_1\| \simeq 0.08^b$
$2\nu_N = 2.01$ MHz		
$g_2 = 2.014$	6.93 ± 0.04	$\|a_2\| = 16.82 \pm 0.05$
$H = 3.37$ kG	7.90 ± 0.04	$\|p_2\| = 0.42 \pm 0.05$
$2\nu_N = 2.07$ MHz	9.05 ± 0.04	
	9.75 ± 0.04	
$g_3 = 1.98$	6.83 ± 0.04	$\|a_3\| = 16.76 \pm 0.05$
$H = 3.39$ kG	7.90 ± 0.04	$\|p_3\| = 0.50 \pm 0.05$
$2\nu_N = 2.08$ MHz	8.95 ± 0.04	
	9.85 ± 0.04	
βNO		
$g_1 = 2.071$	8.05 ± 0.06	$\|a_1\| = 18.14 \pm 0.08$
$H = 3.28$ kG	10.10 ± 0.06	$\|p_1\| \simeq 0.08^b$
$2\nu_N = 2.01$ MHz		
$g_2 = 2.011$	7.22 ± 0.04	$\|a_2\| = 17.34 \pm 0.05$
$H = 3.39$ kG	8.10 ± 0.04	$\|p_2\| = 0.35 \pm 0.05$
$2\nu_N = 2.08$ MHz	9.70 ± 0.04	
$g_3 = 1.987$	7.20 ± 0.03	$\|a_3\| = 17.44 \pm 0.04$
$H = 3.41$ kG	8.25 ± 0.03	$\|p_3\| = 0.43 \pm 0.05$
$2\nu_N = 2.09$ MHz	9.45 ± 0.05	
	10.05 ± 0.05	
MbNO		
$g_1 = 2.072$	7.90 ± 0.05	$\|a_1\| = 17.7 \pm 0.05$
$H = 3.29$ kG	9.80 ± 0.05	$\|p_1\| \simeq 0.0^b$
$2\nu_N = 2.02$ MHz		
$g_2 = 2.006$	6.90 ± 0.04	$\|a_2\| = 16.62 \pm 0.05$
$H = 3.39$ kG	7.80 ± 0.04	$\|p_2\| = 0.46 \pm 0.05$
$2\nu_N = 2.08$ MHz	9.27 ± 0.05	
$g_3 = 1.985$	6.85 ± 0.03	$\|a_3\| = 16.82 \pm 0.04$
$H = 3.42$ kG	7.87 ± 0.03	$\|p_3\| = 0.46 \pm 0.04$
$2\nu_N = 2.10$ MHz	9.05 ± 0.03	
	9.87 ± 0.03	

[a] As the values for β chains at high and low pH are identical, they are only listed once as "βNO". [b] Estimated from line width and SPUR P = 0.

Comparing first the isotropic couplings of the monomers MbNO (17.05 MHz) αNO at pH 7.4 (17.17 MHz) and βNO (17.64 MHz), one observes a definite tendency toward larger interactions in this order, indicating an increasing bond strength between the iron and the N_ϵ nitrogen. Especially the relatively large difference of 0.47 MHz between α and β chains (both in the r state) indicates

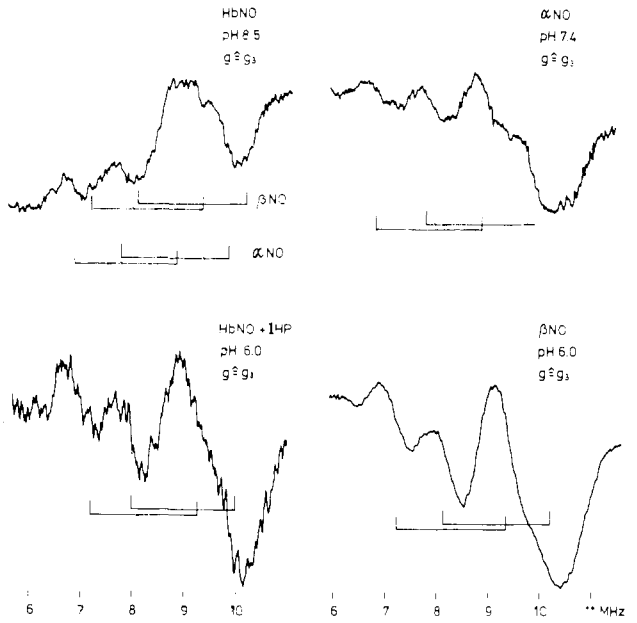

Figure 2. ^{14}N ENDOR on NO derivatives of Hb (in the absence and presence of IHP; left side) and of α and β chains (right side). All spectra are taken along g_3. Magnetic field and g values together with splitting parameters are given in Table II; experimental conditions are described in the Experimental Section. Signal averaging was as follows: 4 min for HbNO + IHP and βNO; 7 min for HbNO; 13 min for αNO.

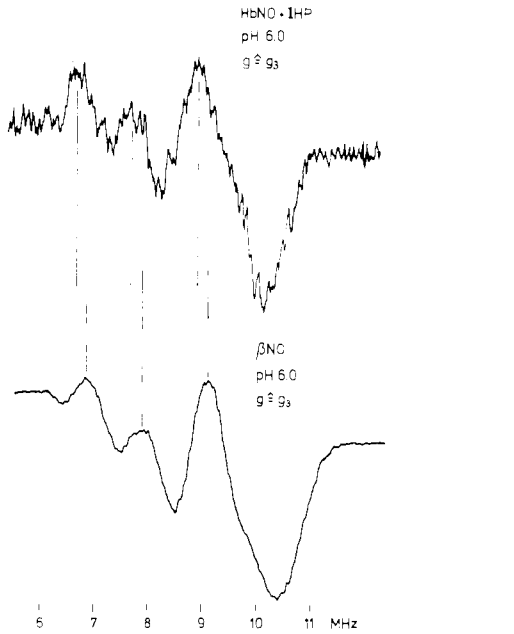

Figure 3. ^{14}N ENDOR on HbNO + IHP and βNO along g_3 under equivalent pH and buffer condition. Note the lines drawn from the maxima of the first derivative signals showing a distinct difference between the two spectra. Signal averaging was as for spectra in Figure 2.

a structural inequivalence of the subunits. It becomes more pronounced when considering the t-state spectra. Whereas, within the experimental limits of ENDOR resolution, the couplings of the β chains remain identical at both acid and alkaline pH, the α chains lose the N_ϵ interaction to an extent that the corresponding ENDOR lines, if present, would be masked by the many resonances (pyrrole- and matrix-^{14}N couplings) present in the 1-3-MHz region. This observation gives direct support to previous hypotheses claiming that the transition from r to t states of the α chains goes along with the loss of the N_ϵ-iron whereas no change occurs for the β chains.[12,13,18,32]

It is interesting to note that the ^{14}N-His (F8) intraction in MbNO is smaller than that of both the α and the β chains. A decrease in coupling strength could already have been assumed on the basis of different iron spin densities between MbNO and HbNO estimated from single crystal work.[2-4] Interestingly, the difference in coupling strength between MbNO and the β chain is larger than for the α chain, somewhat contrasting the usual assumptions about the close similarity of β chains and Mb.[33,34]

Turning now to the tetramer in the T state, HbNO + IHP, we obtain an isotropic coupling of 17.56 MHz. Although this value is slightly smaller than that found for the isolated β chains, the spectrum of HbNO + IHP is assigned to contain the β-chain N_ϵ interaction. This assignment can be made since previous ESR studies have produced evidence for the α chains to reveal very similar spectral changes both isolated and in the tetrameric molecule for the tertiary r and t states,[11] a change that the above ENDOR data proved to be connected with a loss of the ^{14}N-His (F8) interaction. Therefore, a contribution of the α chains to the spectrum of HbNO + IHP in the frequency region 6-11 MHz can be safely excluded. The small, but reproducible, differences in the N_ϵ interaction between the isolated β chains and those in the tetrameric molecule are found on each of the three g turning points. Figure 3 shows two spectra obtained along g_3 for comparison. These differences, which amount to about 0.24 MHz, are ascribed to a slightly altered conformation of the heme environment of the β chains when embedded in the tetramer HbNO.

Somewhat surprisingly, the isotropic coupling obtained from the R-state tetramer HbNO is 17.48 MHz, less than the value for HbNO + IHP. Bearing in mind, however, that both the α and β subunits should contribute to the signals of HbNO and assuming their couplings to equal the values obtained from the isolated chains, one can interpret the relatively broad ENDOR spectrum of HbNO satisfactorily as a superposition of the corresponding subunit spectra as depicted in the spectrum of Figure 2 (top left) by the stick diagrams. The large line width of about 1 MHz for the HbNO lines are compared to roughly 0.6 MHz in the isolated subunits and MbNO also can be used to explain the doublet appearence of HbNO ENDOR lines observed along g_2 mentioned above.

It should be noted that the interpretation of the HbNO spectrum in terms of those of the isolated α and β chains can only be approximate. As was shown above, there is a slight but distinct difference in N_ϵ couplings of isolated β chains and those coming from the β subunits in the tetramer HbNO + IHP. A similar situation could hold for the α chain in the case of R-state tetramers, too. In order to clarify the exact coupling of both subunits within the tetramer, work on hybrids is under way in this laboratory.

^{1}H Couplings. In all samples studied, several different ENDOR lines are observed in the "weakly coupled" proton region (10-18 MHz in a field of ca. 3300 G), all of which are symmetrically disposed about ν_H ($\sim$14 MHz), the free-proton frequency, as expected from the resonance condition

$$\nu_{EN} = |\nu_H \pm a_j/2| \quad (3)$$

There is a significant variation in the number of line pairs observed at different g turning points. Deuteration experiments on two specimens bring about a loss of only one coupling, identical for both, indicating that probably in all samples there is one interaction due to an exchangeable proton.

Table III lists all couplings observed. It is noted that, for all samples studied, monitoring on g_1 brings about the smallest number of line pairs. An interaction of about 1.4 MHz is displayed by all samples ranging from 1.35 MHz (HbNO, βNO (D_2O)) to 1.5 MHz (αNO (pH 7.4)). An additional coupling of about 2.4 MHz is found for αNO (pH 7.4), MbNO, and HbNO. Only the latter specimen also shows a very small interaction of 0.38-0.45 MHz (H_2O and D_2O samples, respectively). Figure 4 shows the corresponding spectra for HbNO at pH 8.5 (top left) and βNO

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Table III. Proton ENDOR of NO Derivatives of Hb, α and β Chains, and Mb

sample[a]	derived couplings,[b] MHz, at g turning points g_1	g_2	g_3
HbNO, pH 8.5	0.38 (AA′)	0.35 (AA′)	0.35 (AA′)
	1.35 (BB′)	1.30 (BB′)	1.30 (BB′)
	2.40 (EE′)	3.80 (DD′)	1.95 (CC′)
			3.40 (DD′)
HbNO + IHP	1.40 (BB′)	0.40 (AA′)	0.30 (AA′)
		1.35 (BB′)	1.30 (BB′)
		5.20 (FF′)	1.90 (CC′)
		3.80 (DD′)	3.85 (DD′)
βNO, pH 8.5	1.45 (BB′)	0.35 (AA′)	0.35 (AA′)
		1.30 (BB′)	1.35 (BB′)
		3.90 (DD′)	2.00 (CC′)
			3.60 (DD′)
βNO, pH 6.0	1.40 (BB′)	0.35 (AA′)	0.35 (AA′)
		1.40 (BB′)	1.40 (BB′)
		3.90 (DD′)	2.00 (CC′)
			3.50 (DD′)
αNO, pH 6.0	1.40 (BB′)	1.20 (BB′)	1.25 (BB′)
		2.70 (GG′)	2.65 (GG′)
		5.20 (FF′)	
αNO, pH 7.4	1.50 (BB′)	0.35 (AA′)	0.35 (AA′)
	2.40 (EE′)	1.50 (BB′)	1.25 (BB′)
		3.75 (DD′)	1.85 (CC′)
			3.65 (DD′)
MbNO, pH 7.5	1.40 (BB′)	1.40 (BB′)	1.80 (CC′)
	2.35 (EE′)	3.65 (DD′)	3.55 (DD′)
		5.25 (FF′)	
βNO, pH 8.5, D_2O	1.35 (BB′)	0.42 (AA′)	0.38 (AA′)
		1.30 (BB′)	1.20 (BB′)
		4.00 (DD′)	3.55 (DD′)
HbNO, pH 8.5, D_2O	0.45 (AA′)	0.45 (AA′)	0.50 (AA′)
	1.35 (BB′)	1.25 (BB′)	1.25 (BB′)
	2.10 (EE′)	3.80 (DD′)	3.50 (DD′)

[a] Magnetic field and g values as given in Table II. [b] Typical error margins are ± 0.05 MHz.

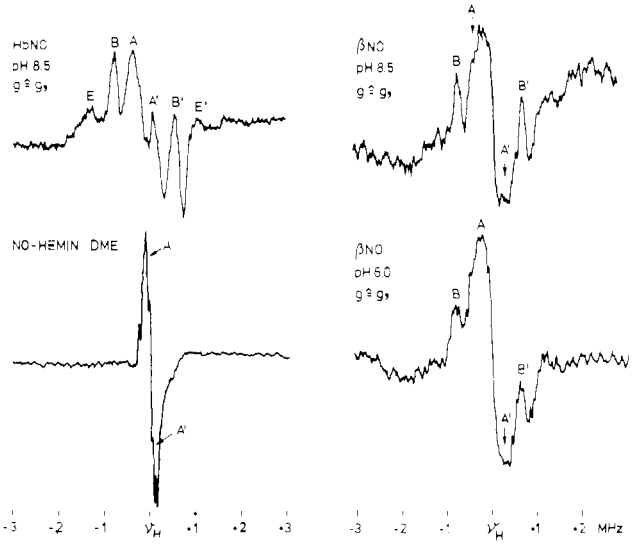

Figure 4. ^{1}H ENDOR on NO derivatives of Hb, hemin DME, and β chains at high and low pH. Assignment of the couplings is made in the text. All spectra are taken along g_1. Signal averaging was as follows: 1 min for HbNO, hemin DME, and βNO (pH 8.5); 5 min for βNO (pH 6.0).

at the same pH (top right) and at pH 6.0 (bottom right) together with the spectrum of NO-hemin dimethyl ester (bottom left) obtained from an aprotic glass. This latter spectrum sets the range for any protons in the porphyrin plane, their interaction being apparently not more than about 0.3–0.4 MHz. Therefore, the line pair denoted AA′, well visible in HbNO but somewhat obscured in βNO, is assigned to protons in the heme plane, specifically to the meso protons. Comparison with Table III, e.g.,

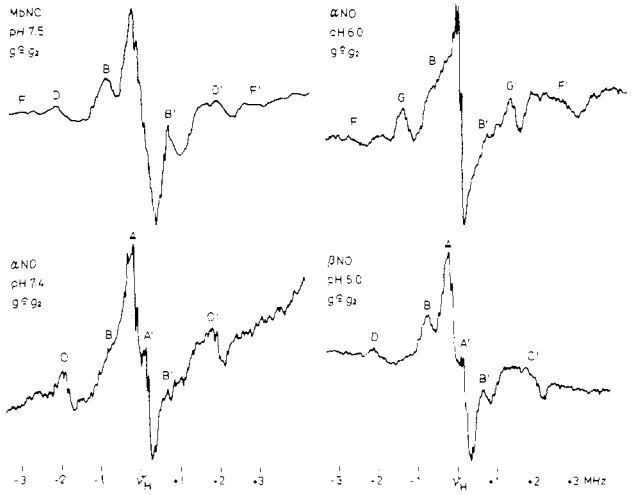

Figure 5. ^{1}H ENDOR on NO derivatives of Mb, α (at pH 7.4 and 6.0) and β (at pH 6.0). Assignment of the couplings is made in the text. All spectra are taken along g_2. Signal averaging was 4 min for βNO (pH 6.0) and αNO (pH 6.0), and 8 min for MbNO and αNO (pH 7.4).

for HbNO both in the H_2O and D_2O case, shows that the pair AA′ is not exchangeable upon deuteration and is also isotropic or nearly so. Mulks et al.[35] find a value of 0.8 MHz for the meso proton interaction in $Hb^{+}H_2O$. If we correct for the change in iron spin density (about 44%[4]), the value of about 0.4 MHz found in HbNO appears reasonable. The only two substances for which, on none of the g turning points (cf. Table III), the meso-proton interaction AA′ is not found are αNO (pH 6.0) and MbNO. This may be due to both a slight reduction in coupling strength and overlap with other lines in the center.

Apart from the pair AA′, HbNO in Figure 4 displays two other pairs, BB′ and EE′. The former group is also displayed by the isolated β chains along g_1 at both alkaline and acid pH, the latter conditions yielding but a slight decrease in coupling strength (1.4 MHz vs. 1.45 MHz at pH 8.5).

Figure 5 shows some of the spectra obtained along g_2. According to Table III, typically three different proton couplings are observed along this direction, their values being about 0.4 MHz (AA′), 1.2–1.4 MHz (BB′), and 3.65–4.0 MHz (DD′). MbNO (spectrum top left), while lacking pair AA′, clearly shows both BB′ and DD′ together with a small but reproducible signal FF′ that has a rather large interaction (5.25 MHz). This latter group is also found in αNO (pH 6.0) together with only badly expressed BB′ lines. The pair DD′ in this substance is either very small in intensity or absent. Instead, a new line group GG′ (2.7 MHz) is displayed (spectrum top right). Both FF′ and GG′ are either lost or have experienced a significant intensity loss when the pH is increased to 7.4 in αNO (bottom left). Instead, the groups AA′ and DD′ are well discernible, the expression of the pair BB′ remaining somewhat weak. The spectrum of the β chains are, as was found for g_1 above, again unaffected by a change in pH. Both at acid (spectrum bottom right) and alkaline pH (cf. Table III), the β chains display the groups AA′, BB′, and DD′. Except for the shape of the BB′ pair, the overall appearance of αNO (pH 7.4) and βNO seems comparable. The tertiary structure switch from r to t for the α chains involves the loss of groups AA′ and DD′ and the appearance of groups GG′ and FF′. MbNO carries a nonsystematic mixing of all line groups, thus resembling neither the α nor the β chains.

Another line group, labeled CC′, is observed on all samples except αNO in the t state when the ENDOR response is monitored along g_3. Its assignment is aided by the fact that it is exchangeable in both HbNO and βNO at pH 8.5 (by repeated redissolving of the samples in D_2O instead of H_2O) as shown in Figure 6. An exchangeable proton of this coupling strength (about 1.9 MHz) should be in relatively close contact to either the ligand or the

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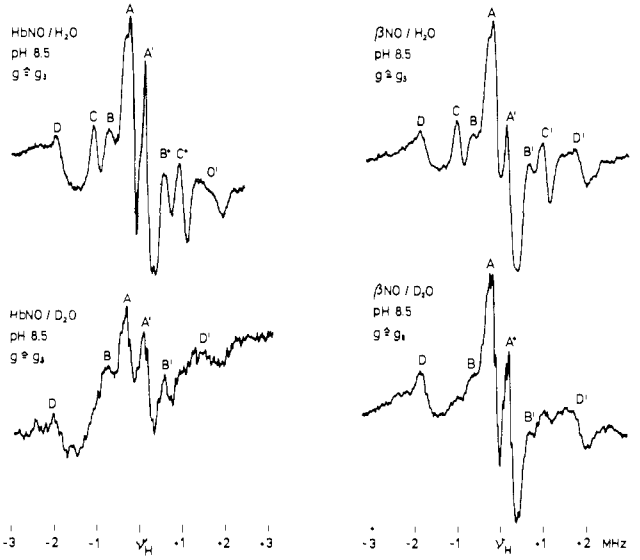

Figure 6. ^{1}H ENDOR on HbNO and βNO along g_3 under equivalent buffer and pH conditions dissolved in H_2O (upper spectra) and in D_2O (lower spectra). For the D_2O cases the disappearance of the coupling CC′ is observed for both samples. Signal averaging was 1 min for the H_2O cases and 8 min for the D_2O cases.

iron bonded to a nitrogen. Unlike the situation in Hb^+H_2O,[35] the proton on the δ nitrogen of the proximal histidine can be ruled out in accounting for this interaction since dipole calculations yield couplings of at most 0.5 MHz due to the reduced iron spin density in HbNO. The other candidate is the proton bound to the nitrogen N_{ϵ_2} of the distal histidine. Figure 7 shows the distal heme environment in two projections containing the heme normal. Using the atomic coordinates given by Watson[36] and the position of the NO ligand at low temperature as well as the g tensor element direction cosines from the work of Hori et al.,[37] we calculate a distance between H (N_{ϵ_2}–H) and O (NO) of 1.56 Å and an angle φ between the direction of g_3 and the vector connecting H and O of 53.9°. Using the conventional point-dipole interaction formula

$$a_H = (c/r^3)\rho(3\cos^2\varphi - 1) \qquad (4)$$

with $c = g_n\beta_n g_e\beta_e$ and ρ the spin density of the atom concerned (ρ_0 of NO $\simeq$ 10%[2]), the angle φ obtained from eq 4 with a_H = 1.9 MHz and r = 1.56 Å is 36°. The agreement appears very satisfactory bearing in mind the sensitivity of (4) to the distance that had to be calculated from standard bond lengths and angles since the H-atom coordinates are not available. It is interesting to note that these approximate calculations yield an angle N–H···O of 135° in comparison to 157° found for MbO_2 by neutron diffraction.[22]

The range of proton ENDOR lines observed along g_3 for HbNO and its isolated subunits prepared from H_2O is depicted in Figure 8. In their tertiary r state, both α and β chains display, as was observed also for g_2, nearly identical spectra except for slight coupling differences. All groups found in one subunit, AA′, BB′, CC′, and DD′, are present in the other and also in HbNO in the quaternary R state (left-hand column). A switch to the tertiary t state is sensed, as on g_2, mainly by the α chains and is expressed by the collapse of AA′ and the loss of DD′ and CC′ combined with the appearance of pair GG′. Allowing for slight differences in coupling strength, the spectrum of HbNO + IHP is identical with that of the β chains at low pH except for a buildup of intensity around ν_H that should come from the α-chain contributions. The pair GG′ of the α chains seems to be buried under the β-chain groups DD′ and CC′. This is different from the situation along g_2 for which the α-chain contribution in the spectrum of the T-state

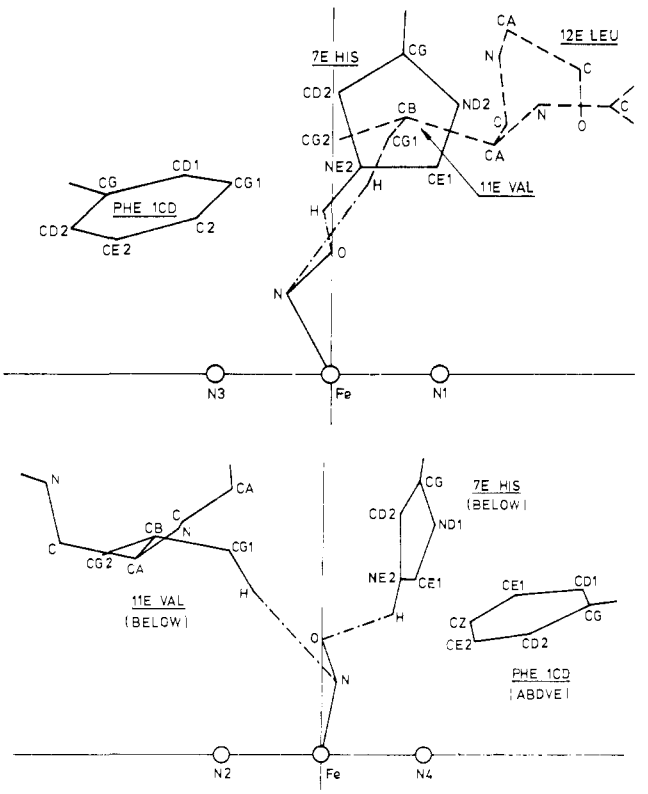

Figure 7. (a) Top, sections of the E helix and other amino acid side chains of myoglobin in projection on a plane perpendicular to the heme, intersecting it along a line containing the nitrogen atoms of pyrroles 1 and 3 and the iron atom. Sections of the E helix shown in dotted lines lie below the plane, other sections above it (after ref 34). Lines (·····) draw connections between atoms relevant to proton coupling assignments. (b) Bottom, a projection of aminoacid side chains of myoglobin in a plane perpendicular to the heme plane, intersecting it along a line containing pyrrole nitrogens 2 and 4 and the iron atom (after ref 34).

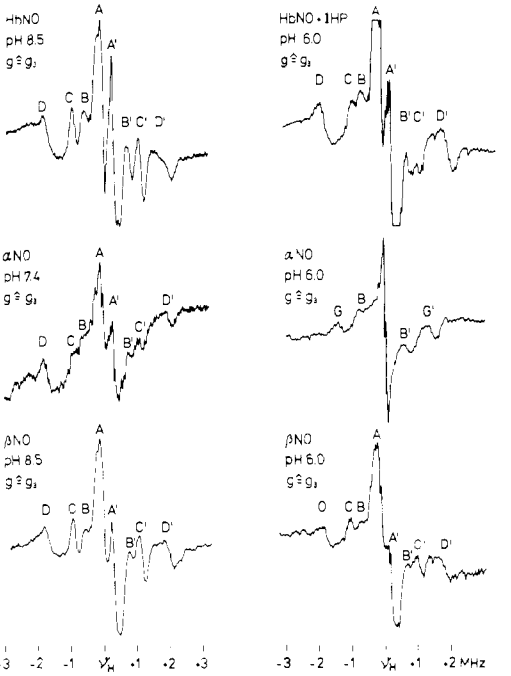

Figure 8. ^{1}H ENDOR on NO ligated tetrameric Hb and its separate chains at high (left column) and low (right column) pH. All spectra are taken along g_3. Signal averaging was as follows: 1 min for HbNO and βNO (pH 8.5); 14 min for αNO (pH 7.4); 4 min for HbNO + IHP, αNO (pH 6.0), and βNO (pH 6.0).

tetramer was distinctly visible from the appearance of the pair FF′ in the outer wings.

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Since all samples except αNO (pH 6.0) display the CC′ pair along g_3, it is tempting to extrapolate from the HbNO and βNO findings in D_2O that this group should be exchangeable in the other specimen too. Provided its correct assignment to the proton of the distal histidine N_{ϵ_2} nitrogen, some interesting implications pertain. First one notes that this coupling is strongest for the β chains (2.0 MHz) and smallest for αNO (pH 7.4) and MbNO (1.85 and 1.8 MHz, respectively), indicating a difference in coordination strength of the ligand by the distal histidine (E7). Moreover, this coordination is apparently lost in the α chains upon a tertiary transition to the t state, whereas the interaction remains in the β chains, providing for a substantial structural difference between the two. These findings may be connected to ESR work on NO-ligated mutant hemoglobins, which shows that the quaternary T structure is stabilized in the absence of the distal histidine in the α chains (Hb_{Boston} and $Hb_{Opossum}$) whereas the lack of His E7 in the β chains ($Hb_{Saskatoon}$) appears to have little influence on the quaternary state.[17,38,39]

It is difficult to arrive at a definite structural assignment for the other proton ENDOR lines, which all are from nonexchangeable hydrogens. Single-crystal studies that should clarify some of the assignments are being performed for MbNO presently. For example, the other two dipolar components of the pair CC′ on g_1 and g_2 could not be safely traced in HbNO and βNO by comparison between H_2O and D_2O at high pH, although some indications were found in the HbNO spectrum pointing toward a value of about 1 MHz or less. Also, the conspicuous appearance of pair BB′ (~1.4 MHz) for nearly all samples on all g turning points suggests the existence of a proton with a fairly large isotropic contribution that we cannot so far rationalize. Looking for feasible candidates for dipolar interactions of the observed size, we obtain a good fit for the pair DD′ along g_3 with one of the methyl protons C_{γ_1} of valine E11 (cf. Figure 7). Assuming that one of the protons points directly toward the nitrogen of the NO ligand, a distance of 2.1 Å can be calculated. Estimating the angle φ between the direction of g_3 and the vector (C_{γ_1})-H···N(NO) yields $\varphi = 75°$. With about 60% spin density, ρ, on N of NO[4,16] one then obtains from eq 4 $a_H = 3.8$ MHz, which is in surprising agreement with the typical values 3.4-3.85 MHz obtained. A survey of the nearest protons of both the proximal and the distal histidines in relation with either iron or the ligand atoms yields no satisfying alternative coincidence.

The assignment of the pair DD′ on g_3 to a valine proton, though tentative at this stage, derives support from the crystallographic data from which an interaction of valine E11 with the heme has been concluded.[36] The β chains parallel to the situation for the distal histidine show no significant difference in coupling strength of pair DD′ between the tertiary r and t states. The α chains appear to lose the interaction at low pH. Correcting, however, for the spin density change on the ligand NO between the two tertiary states it appears possible that the pair GG′ on g_3 (2.65 MHz) in αNO (pH 6.0) could be the corollary of the DD′ pair found for α chains in their tertiary r state.

If we adopt the position that the lines BB′ on g_1 and g_2 are the other dipolar elements of the DD′ pair on g_3, one still has to explain the occurrence of pairs of similar magnitude on g_2 and g_3. Taking the group DD′ on g_2 as a maximum dipole interaction, we find no agreement for it with any of the possible candidates in the heme pocket. The same holds the pair FF′ on g_2, which has a rather large (~5.2 MHz) coupling that we are unable to account for. It is interesting to note that this pair is found only in samples containing the α chains in the t state (αNO (pH 6.0) and HbNO + IHP) and in MbNO. The assignment of these groups has to await further studies.

Conclusions

ENDOR measurements on ^{14}N-His (F8) and ^1H couplings of NO-ligated hemoglobin and its derivatives give clear evidence for structural changes occurring in the heme environment of only the α chains on either a tertiary r-t transition of the isolated subunit or upon a quaternary change of the tetrameric Hb molecule. These involve the loss of the coupling of the proximal histidine (His F8) and most probably of the distal histidine (E7) N_{ϵ_2}. Although the large amount of proton lines observed in all samples needs further investigations on hybrids and Mb single crystals to fully elucidate the heme environment stereochemistry, the gamut of spectral variations obtained in this study allows the conclusion that the control of the structural state, both tertiary and quaternary, by the globin part is distinctly different in the heme environment of α and β chains.

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Registry No. $HisF_8$, 71-00-1; nitrosylhemin DME, 58357-23-6; nitrosylhemoglobin A, 52228-24-7; L-valine, 72-18-4.

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Communications to the Editor

Preparation of a Mixture of Nucleoside Triphosphates from Yeast RNA: Use in Enzymatic Synthesis Requiring Nucleoside Triphosphate Regeneration and Conversion to Nucleoside Diphosphate Sugars[1]

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This paper describes a practical procedure for converting yeast RNA into a mixture of ATP, UTP, GTP, and CTP (Scheme I). This mixture can be used as a source of nucleoside triphosphates for the synthesis of nucleoside diphosphate sugars. These latter substances are required in most enzyme-catalyzed syntheses of oligo- and polysaccharides.[3] In addition, since many ATP-utilizing enzymes (especially phosphotransferases) will also accept GTP, UTP, and CTP,[4] the mixture of nucleoside triphosphates serves

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(2) National Science Foundation Predoctoral Fellow.
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