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Linear Electric Field Effect in Electron Paramagnetic Resonance for Two Bisimidazole–Heme Complexes, Model Compounds for B and H Hemichromes of Hemoglobin and for Cytochrome b_5†

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ABSTRACT: Bisimidazole-ferric heme is considered to be the structure at the heme site of cytochrome b_5 and two different low spin ferric hemochromes spontaneously formed from ferric hemoglobin. The addition of strong base to bisimidazole-ferric heme in organic solvents alters the optical and magnetic properties of this compound. With the use of the linear electric field effect in the electron paramagnetic resonance, we demonstrate that addition of base does not lead to the exchange of hydroxide anion for ligated imidazole and that the bisimidazole structure is retained. Analysis of optical titrations indicates that 2 equiv of base react reversibly with bisimidazole-ferric heme. It is suggested that the two hemichromes formed from hemoglobin differ in structure from one another by the state of protonation of N-1 in the bound imidazoles.

The slow, spontaneous denaturation of high-spin ferric hemoglobin A, as well as the more rapid denaturation of ferric β or ferric α chains (Rachmilewitz et al., 1971), lead to the formation of a variety of low-spin ferric heme compounds collectively called "hemichrome" (Peisach and Blumberg, 1971; Peisach et al., 1972). One of these, designated "H hemichrome" (Blumberg and Peisach, 1971), can be reversibly renatured to functional hemoglobin (Rachmilewitz et al., 1971) and is thought to represent the ligation of histidine imidazole E7 to heme (Rachmilewitz, 1969) displacing water which is normally bound to the iron in the ferric protein. With time, H hemichrome is converted to a second product, termed "B hemichrome," which does not renature to functional hemoglobin.

An attempt has been made to identify the chemical nature of the axial ligands for both of these hemichromes observed in denatured hemoglobin using the EPR[1] technique (Blumberg and Peisach, 1971). The g values and the crystal field parameters for B hemichrome (Table I) were found to be similar to those observed for cytochrome b_5 (Bois-Poltoratsky and Ehrenberg, 1967; Ikeda et al., 1974) and for the model compound bisimidazole-ferric heme (Peisach et al., 1973). X-ray crystallographic analysis shows that the latter two compounds have the same heme ligand structure (Mathews et al., 1971). On the other hand, the g values of H hemichrome were close to those obtained for the compound which is formed by adding strong base to bisimidazole-ferric heme (Peisach et al., 1973) or cytochrome b_5 (Bois-Poltoratsky and Ehrenberg, 1967; Ikeda et al., 1974).

Recently, Peisach et al. (1973) proposed that imidazole bound to heme can exist in two forms which are stable in nonaqueous medium. In one form, the N-1 nitrogen atom is protonated and in the other, not. It was suggested, on the basis of EPR of model compounds, that B hemichrome contains protonated imidazole ligands, and H hemichrome, deprotonated imidazole ligands, deprotonation being brought about in the model compound by the addition of strong base (Peisach et al., 1973). It remained, however, to demonstrate that the changes observed in the model compound did not involve the removal of an imidazole ligand and, in particular, that they did not correspond to the substitution of hydroxide anion. Moreover, it was necessary to determine experimentally the number of equivalents of base required to react with bisimidazole-ferric heme in order to effect the transformation from the ligand structure of B hemichrome to that of H hemichrome. According to the above assumption, this should be one or two, depending on the number of imidazole ligands undergoing deprotonation.

The considerable difference between the g values for hemoglobin or myoglobin hydroxide (Table I) (Gurd et al., 1967), where the ligands are assumed to be imidazole and hydroxide anion, and the g values of the other heme centers discussed above already affords some evidence against the

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[1] Abbreviations used: EPR, electron paramagnetic resonance; LEFE, linear electric field effect; g_{max}, the low-field end g value of the EPR spectrum; g_{min}, the high-field end g value of the EPR spectrum.

TABLE I: Magnetic Parameters of Low-Spin Ferric Heme Complexes.

Compound	g values			Reference
B hemichrome	2.95	2.26	1.47	Peisach and Blumberg (1971)
Cytochrome b_5	3.03	2.23	1.43	Bois-Poltoratsky and Ehrenberg (1967)
Bisimidazole-heme, in imidazole	3.02	2.24	1.51	Peisach et al. (1973)
H hemichrome	2.80	2.26	1.67	Peisach and Blumberg (1971)
Bisimidazole heme, + OH^- (nonaqueous)	2.78	2.26	1.72	Peisach et al. (1973)
Cytochrome b_5, pH 12	2.76	2.28	1.68	Bois-Poltoratsky and Ehrenberg (1967)
Ferric myoglobin hydroxide	2.55	2.17	1.85	Gurd et al. (1967)

possibility that the complex formed in the presence of strong base has a hydroxide ligand. In this present study, we demonstrate from optical titrations that, in dimethyl sulfoxide, 2 equiv of base react with the bisimidazole-heme complex, suggesting that the two hemichrome structures discussed above differ from each other by two protons. Yet further evidence has been obtained by making a series of measurements of the linear electric field effect in electron paramagnetic resonance (Mims and Peisach, 1976) for bisimidazole-ferric heme complexes prepared with and without strong base, and for the compound, myoglobin hydroxide. The resulting curves obtained for the two bisimidazole preparations show a clear resemblance to one another and are markedly different from the curves obtained with myoglobin hydroxide, once more suggesting that, in the presence of base, a bisimidazole structure is retained which differs magnetically from the parent compound.

Materials and Methods

In order to determine the overall stoichiometry of the reaction of bisimidazole-ferric heme with base, optical titrations were performed. Hemin chloride was dissolved in dry dimethyl sulfoxide (Burdick and Jackson Laboratories, Muskegan, Mich.). Excess imidazole was added as a concentrated solution in a small volume of dimethyl sulfoxide. For a typical titration, the final concentration of heme was 100 μM, while that of imidazole ranged from 30 to 180 mM. Small volumes of tetra-*n*-butylammonium hydroxide (purchased as a 1 M methanol solution from Southwestern Analytical Chemicals, Austin, Texas) diluted in dimethyl sulfoxide were titrated with a 5-μL Hamilton syringe into the heme solution contained in a Teflon-stoppered 1-cm light-path cuvette, and the optical spectrum was recorded on a Cary 14R spectrophotometer between 500 and 650 nm. The Teflon stopper had two holes large enough only to accommodate the syringe needle and too small to permit any significant evaporation. If the reaction is carried out in the presence of a large excess of imidazole and with minimal amounts of water, essentially two spectral species are observed in the titration, the initial bisimidazole-heme complex and the product derived from the reaction with base (Table II). The use of dry pure solvents for this reaction cannot be overemphasized. At any point in the titration, the addition of water caused significant spectral changes and large losses from isosbesticity. The overall reaction may be written:

$$S + n(OH^-) \rightleftarrows P + H_2O \quad (1)$$

where S is the bisimidazole-heme complex, P is the product of the reaction, and n the number of equivalents of base employed. The equilibrium constant, K, is given by:

$$K = \frac{[P][H_2O]}{[S][OH^-]^n} \quad (2)$$

Since F, the fractional population of unchanged bisimidazole-heme, is a function of [S], [OH^-], n, and K, one can determine n and K by fitting the optical data to the equation:

$$Z = AF + B(1 - F) \quad (3)$$

where Z is the optical density at any concentration of base, A is the optical density at the beginning of the titration, and B the optical density at the end of the titration. In order to test the reversibility of this reaction with base, tetrabutylammonium hydroxide (final concentration 830 μM) was added to bisimidazole-heme complex prepared as described above but with 90 mM imidazole. Small volume increments of acetic acid in dimethyl sulfoxide were added and the spectrum was recorded after each addition.

In another experiment aimed at finding out whether any deprotonation of the imidazole ligands occurred without the addition of base, hemin chloride (1 mM) was dissolved in aqueous base and the pH was carefully adjusted to 9.0 with HCl. An aqueous solution of imidazole (10 mM) that had also been adjusted to pH 9.0, a pH too low to completely deprotonate the imidazole, was added in small increments to the hemin solution and the pH was followed on a Corning Model 52 pH meter.

LEFE experiments were performed at 4.2 K by the electron spin echo method (Mims, 1964, 1972) at X-band using an apparatus designed to generate electric fields (E) up to 70 kV/cm which could be applied to the sample under study at any angle in relation to the magnetic field. The sample cavity as well as the apparatus used for these studies are described elsewhere (Mims, 1974). The shift parameters, S, were deduced from the electron spin echo amplitude in the manner described previously (Peisach and Mims, 1973; Mims and

TABLE II: Spectral Maxima and Configuration Interaction for Low-Spin Ferric Heme Compounds.[a]

Compound	λ_{max} (nm)	B (cm^{-1})	Q (cm^{-1})	$B - Q$ (cm^{-1})
Bisimidazole-heme in dimethyl sulfoxide	414, 535, 562	24 155	17 794	6361
Bisimidazole heme + *tert*-butylammonium hydroxide in dimethyl sulfoxide	423.5, 548, 577	23 613	17 331	6282
Myoglobin hydroxide	413, 542, 583	24 213	17 182	7060

[a] The absorption maxima (λ_{max}) are given for the Soret and visible regions. The terms B and Q as first defined by Platt (1956) refer to the absorptions of the Soret and α peak expressed in energy terms. $B - Q$, the configuration interaction, is a measure of the difference in energy of the first two optically excited states of the porphyrin π-electron system which for iron porphyrins have an accidental degeneracy.

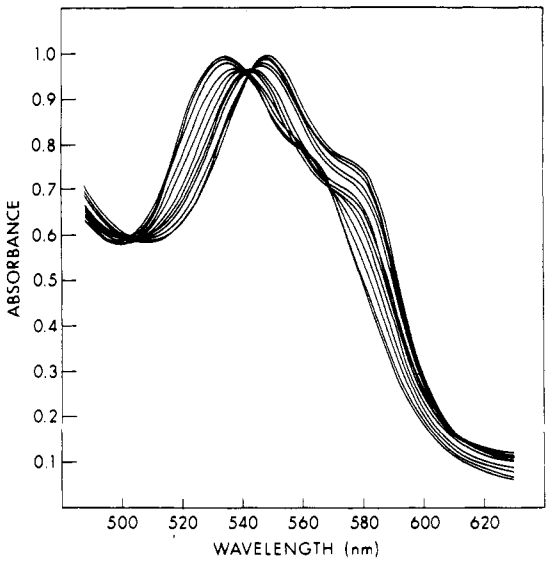

FIGURE 1: Titrations of bisimidazole-ferric heme with tetra-*n*-butylammonium hydroxide. The titration was performed by adding microliter quantities of 20 mM base to 100 μM heme in dimethyl sulfoxide to which a saturating concentration (90 mM) of imidazole was added in a total volume of 3.0 mL. The first eight additions of base were 5 μL each, the next three were 10 μL each, and the last addition was 5 μL of 80 mM base.

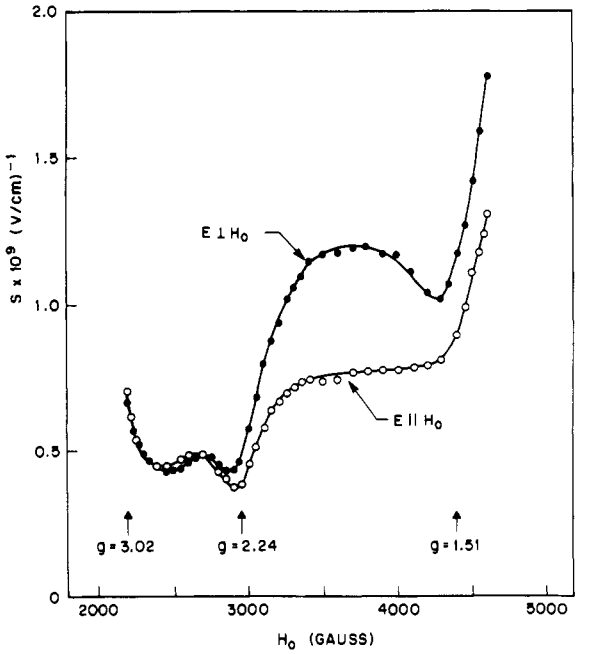

FIGURE 2: Linear electric field effect in EPR for an amorphous sample of bisimidazole heme in $CHCl_3$-Me_2SO. The shift parameter, S, is plotted as a function of the magnetic field H both with the electric field E aligned parallel to H ($E \parallel H_0$) and perpendicular to H ($E \perp H_0$). The principal g values as determined from the EPR are indicated beneath the vertical arrows.

Peisach, 1974). In all experiments, the concentration of heme was from 1 to 5 mM.

For LEFE experiments, bisimidazole-ferric heme was prepared by suspending hemin chloride (Eastman) in $CHCl_3$. After the addition of solid imidazole, the heme dissolved in solution, and a large excess of ligand (200-fold) was added in order to drive the reaction toward completion. Dimethyl sulfoxide (20% v/v) was added. In another experiment, the same compound was prepared in melted imidazole. To prepare the model compound for H hemichrome, solid KOH was added to a melt of hemin chloride in imidazole (Peisach et al., 1973).

Myoglobin hydroxide, prepared from sperm whale myoglobin, was purified according to the method of Hugli and Gurd (1970). The protein was oxidized by passage over $K_3Fe(CN)_6$ layered on a Sephadex G-25 column and concentrated by ultrafiltration, and the hydroxide form was prepared by raising the pH to 10.4 with dilute NaOH.

For the compounds studied using LEFE, the magnetic purity of the samples was verified by taking X-band EPR spectra at 1.4 K and by checking that the electron spin echo signal appeared within a range of magnetic fields corresponding to the relevant g values.

Results and Discussion

When base is added to low-spin bisimidazole-ferric heme in organic solvent, there is a gradual shift of the optical spectrum to the red (Figure 1) so that the new species that is formed also has the optical characteristics of low-spin ferric heme and yet differs markedly from myoglobin hydroxide (Table II) (Smith and Williams, 1970). In myoglobin hydroxide, the ligands to the heme are imidazole and hydroxide anion. The new spectral species that is formed has a smaller $B - Q$ level spacing, a measure of the configuration interaction (Platt, 1956, Table II), than does the bisimidazole-ferric heme precursor. For myoglobin hydroxide (Smith and Williams, 1970), the $B - Q$ spacing measured between the α band and the Soret is increased. This suggests that the addition of base to bisimidazole-ferric heme leads to the formation of a complex having a ligand composition different from that of myoglobin hydroxide.

The LEFE results for bisimidazole heme in $CHCl_3$-Me_2SO and for the same compound prepared in the presence of KOH are shown in Figures 2 and 3 respectively.[2] It will be noted that the two sets of curves (where the magnetic field is aligned parallel to or perpendicular to the electric field) are similar in form, although the magnitudes differ somewhat. A similar study was also made for bisimidazole-ferric heme prepared in dimethyl sulfoxide and for the product obtained from the addition of imidazole to ferric myoglobin. In the former case the results were essentially the same as those observed for the $CHCl_3$ preparation, and in the latter case the curves had the same general form but with small differences in the actual magnitudes. The curves obtained for myoglobin hydroxide (Figure 4) were, however, entirely different in form, thus demonstrating that the optical and EPR changes which occur when bisimidazole-ferric heme complexes are prepared in the presence of strong base under nonaqueous conditions are not due to the exchange of a ligated imidazole by a hydroxide group and strongly suggest that both imidazole ligands are retained.

The optical changes that occur when increments of base are added to the bisimidazole-ferric heme complex (Figure 1) can be analyzed in order to determine the stoichiometry of the overall reaction. By analyzing the results of the optical titration carried out at three different wavelengths (Figure 5), we find that the parameter n in eq 2 has in each case the value 2.0,

[2] In spite of the fact that the axial ligands of bisimidazole heme have the same chemical composition, the paramagnetic site of the complex is noncentrosymmetric. From x-ray crystallographic studies (Collins et al., 1972), it has been shown that the iron imidazole distances in ferric tetraphenylporphine are not the same for both axial ligands and, furthermore, that the planes of the two imidazoles are rotated with respect to one another about the axis in question.

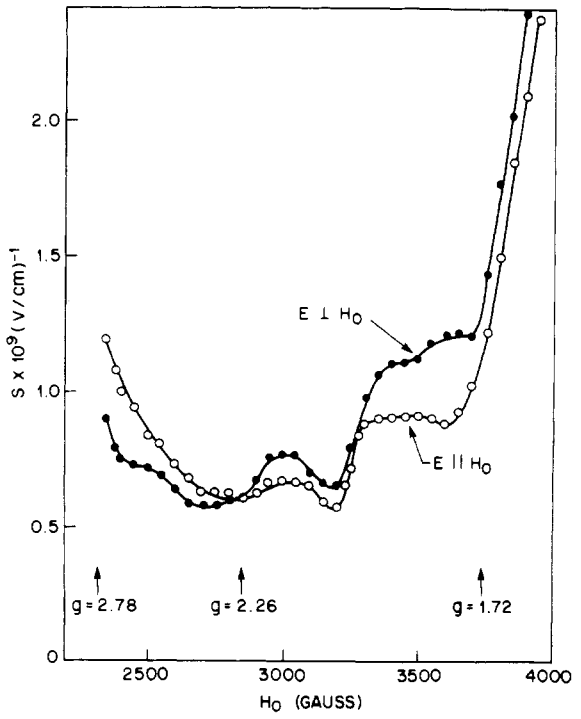

FIGURE 3: Linear electric field effect in EPR for bisimidazole-heme prepared in the presence of tetra-*n*-butylammonium hydroxide. Results are presented as in Figure 2.

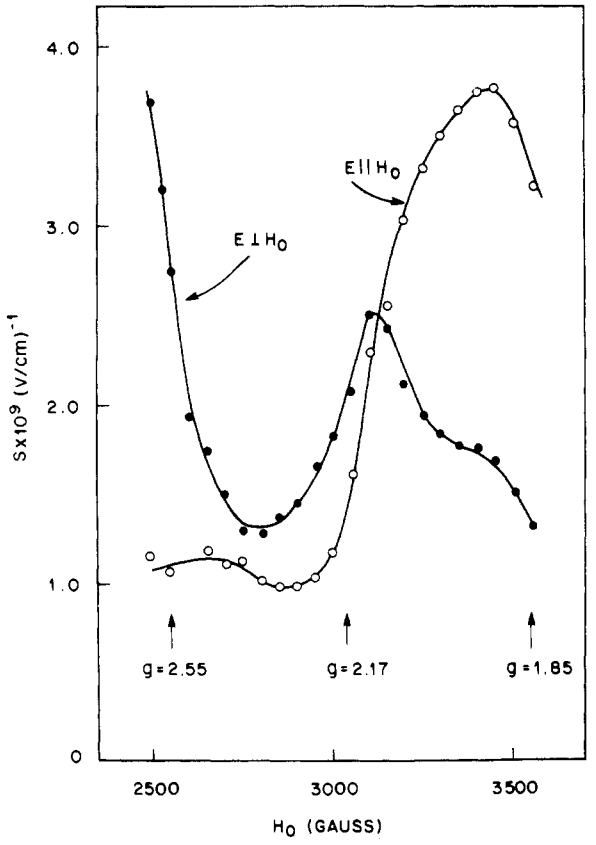

FIGURE 4: Linear electric field effect in the EPR for sperm whale myoglobin hydroxide. Results are presented as in Figure 2.

showing that two hydroxides participate in the reaction where bisimidazole-ferric heme is converted to a structure which differs from the parent compound by two protons. Also, the addition of protons from acetic acid to the newly formed

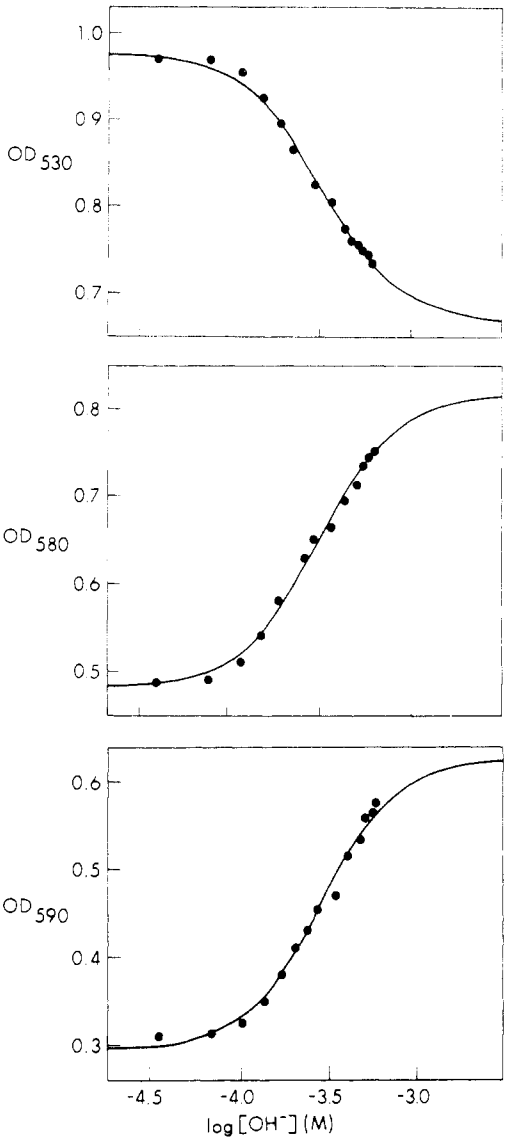

FIGURE 5: Least-squares fit of the optical density of bisimidazole-heme titrated with tetra-*n*-butylammonium hydroxide to a function described in the text relating optical density at 530, 580, and 590 nm to the equilibrium constant, the concentrations of base and heme, and the stoichiometric equivalents of base. The heme concentration was 100 μM, while that of imidazole was 90 mM. The same experiments were performed with imidazole concentrations varying from 30 to 180 mM, yielding identical results.

complex restores the optical properties of the original one. The value of *n* for the back titration varies from 1.8 to 2.0. In a subsidiary experiment it was found that no protons are released during the formation of the bisimidazole complex from heme and imidazole in an aqueous medium. One is therefore led to conclude that the complex formed without base has retained both protons and that the addition of base causes the removal of a single proton from each of the bound imidazoles.

In summary we should like to comment on what we believe to be the significance of these results and on their possible relationship with certain other heme protein observations. From the similarity of *g* values, we assume that the normal pH form of bisimidazole-heme and the form obtained in the presence of strong base are models for B and H hemichromes, respectively. Furthermore, from the results obtained here we infer that B hemichrome contains two neutral imidazole ligands each protonated at N-1, whereas in H hemichrome both imidazole ligands are deprotonated.[3] Since H hemichrome can

be readily reconstituted to functional hemoglobin, while B hemichrome cannot (Rachmilewitz et al., 1971), it is possible to speculate that, within the native structure, the same forms of the imidazole are maintained, at least when the protein is fully ligated.

It should be pointed out here that the structural change of the bisimidazole-ferric heme model compound that is described in this work is one that takes place in nonaqueous medium. The addition of water radically alters the optical spectrum and produces a species with EPR strongly resembling that of myoglobin hydroxide.[4] It is therefore not unreasonable to suggest that the deprotonation of bisimidazole-ferric heme is a reaction that can occur within a protein structure where the heme and ligands are physically shielded from water. The lack of isosbesticity in the titration shown in Figure 1, especially near 565 nm, may in fact be indicative of interaction with water, either produced by the reaction of bisimidazole heme with base or introduced as a contaminant in the reagents employed in our study. Another interpretation is that a single proton can be removed from bisimidazole-ferric heme producing an intermediate species. It should be pointed out, however, that the differences in crystal field parameters between B and H hemichrome of hemoglobin and cytochrome b_5 at neutral and elevated pH (Blumberg and Peisach, 1971) are nearly equal to the difference in crystal field parameters for bisimidazole heme in the absence and presence of excess base (Peisach et al., 1973), suggesting that the deprotonated forms of all three are the same. In any case, it should be noted that the acid dissociation constant of imidazole in aqueous medium (Yagil, 1967) is not the same as imidazole when ligated to heme in a water-free solution. Furthermore, metal ligation to imidazole can alter the pK over a range of about 4 pH units (Sundberg and Martin, 1974).

The demonstration of the existence of two different forms of imidazole ligand in heme compounds is also of interest in connection with certain EPR studies of mixed state hemoglobins. These studies indicate that, when the paramagnetic heme of α chains is ligated either to H_2O (Peisach et al., 1971) or to NO (Henry and Banerjee, 1973), their EPR spectra can be made to change reversibly when O_2 is bound to the β chains. As EPR is a site probe of structure in the immediate vicinity of paramagnetic centers, these studies suggest alteration of heme ligand structure of the α chains dependent upon the state of ligation of the β chains. It would seem possible, then, that these EPR observations indicate alternate states of protonation of the proximal imidazole belonging to the heme of the α chains (Peisach, 1975), the change being in some way brought about by the oxygenation of the β chains. In a likewise manner, the difference in the EPR of fully ligated tetrameric nitrosylhemoglobin $(\alpha_{NO}\beta_{NO})_2$ which is dependent upon whether the molecule is in a high- or low-affinity form has also been interpreted in terms of alternate structure of the proximal imidazole ligand of the heme (Chevion et al., 1977). Taking the argument that, if the proximal ligand can assume alternate structures in nitrosyl hemoglobin, then for the B and H hemichromes of hemoglobin it is suggested that the proximal ligand can also assume different forms depending upon the structural integrity of the molecule as a whole. It would be of interest to speculate that the chemical nature of imidazole is such that many of the functional properties of metalloproteins in general are implicated with alternate structures of this rather unique heterocyclic compound widespread in biology.

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[3] It would, of course, have been interesting to extend the LEFE measurements to H hemichromes of hemoglobin A or to H hemichrome of its constituent chains but attempts of this kind have not been made because of the difficulty of preparing these species in pure form (Peisach et al., 1972).

[4] J. Peisach and W. E. Blumberg, unpublished observations.