Electron Paramagnetic Resonance Studies on Cobalt Hemoglobin, Iron–Cobalt Hybrid Hemoglobins, and Their Related Model Complexes. Characterization of Proximal Histidine Binding to Porphyrin Cobalt(II) Ion and Its Transition Associated with Subunit Interaction[†]

Toshiro Inubushi* and Takashi Yonetani

ABSTRACT: Electron paramagnetic resonance studies have been conducted on iron–cobalt hybrid deoxyhemoglobins and cobalt deoxyhemoglobin. Examination of $\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ hybrid hemoglobin at two different microwave frequencies revealed two sets of axial-symmetric electron paramagnetic resonance (EPR) signals rather than the anisotropic splitting of the $g_\perp$ signal as reported previously [Ikeda-Saito, M., Yamamoto, H., & Yonetani, T. (1977) *J. Biol. Chem. 252*, 8639–8644]. One of these two sets is a characteristically broad spectrum with $g_\perp = 2.38$ and $g_\parallel = 2.03$; the other, a sharp spectrum with $g_\perp = 2.33$ and $g_\parallel = 2.03$. The α-subunits of deoxy-CoHb also exhibited a complex spectrum with a mixture of these two different types of signals. The relative compositions of these two signals were calculated for deoxy-$\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ and the α-subunits of CoHb, which were profoundly sensitive to the pH of the medium and also to the presence of allosteric effectors. A positive correlation was noticed between the spectral characteristics and the equilibrium extent of oxygenation, showing that the broad component may be associated with the electronic state of the porphyrin metal ion in T-state hemoglobin and the sharp component with that in the R state. In contrast, the complementary hybrid $\alpha(\mathrm{Fe})_2\beta(\mathrm{Co})_2$ and the β-subunits in CoHb showed relatively narrow spectra under similar conditions, and only small changes were noted with variation of pH and addition of inositol hexaphosphate. These spectra were close to the sharp component of the α-subunits associated with the R state. Comparison of the hemoglobin EPR spectra with those of model cobaltous porphyrin complexes revealed that the restraint that is operative at the fifth ligand position of the prosthetic group in the T state is released in the R state. These ligand binding properties were discussed in relation to the regulation of oxygen binding to hemoglobin.

One of the key undertakings required to understand the regulation of oxygen binding to hemoglobin (Hb) is to disentangle the functional properties of the prosthetic group in different subunits in the two quaternary states. Substitution for the natural iron porphyrin with an artificial cobalt porphyrin has proved to be a potential tool not only in the investigation of this subject but also in the investigation of the intrinsic role of the central metal ion (Hoffman & Petering, 1970; Yonetani et al., 1974a). First, the cobalt-substituted hemoglobin (CoHb)[1] can bind to oxygen as does the parent natural Hb and, likewise, shows cooperativity in oxygen binding and heterotropic effects (Imai et al., 1977). These facts indicate that the substitution with cobalt provides a good approximation for the study of the subunit interactions of Hb. Second, Fe–Co hybrid Hb's can be prepared by a recombination of appropriate isolated chains containing Co– and Fe–porphyrins, respectively (Ikeda-Saito et al., 1977b). As a consequence, the α- and β-subunits can be specifically labeled by Co–porphyrin, and thus the function of the individual subunits can be monitored spectroscopically. Third, the most prominent advantage endowed by this substitution is that one can obtain half-liganded Hb, such as $\alpha(\mathrm{Co})_2\beta(\mathrm{Fe{\cdot}CO})_2$ and $\alpha(\mathrm{Fe{\cdot}CO})_2\beta(\mathrm{Co})_2$, with all the porphyrin metals in a 2+ valence state, since the affinity of CO toward Co subunits is negligibly small as compared with that toward Fe subunits at atmospheric pressure (Ikeda-Saito et al., 1977b). These hybrid Hb's may serve as good models for the investigation of the intermediate state of Hb in the course of oxygenation. In addition to these advantages, we can directly examine the electronic state of the Co metal ion in both oxy and deoxy states by EPR spectroscopy (Hoffman & Petering, 1970; Yonetani et al., 1947b; Ikeda-Saito et al., 1977a).

Several interesting aspects of the EPR spectra of Co-substituted Hb's were reported in the previous paper (Ikeda-Saito et al., 1977b). The most curious feature encountered was the abnormal broadening of the $g_\perp$ signal for $\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$, whose magnitude depends on pH and the presence of allosteric effectors. However, in the present work, we have reexamined this curiosity to find that the broadening is not due to the anisotropy of $g_\perp$ but due to the overlapping of two axial-symmetric signals, where one has a broad peak-to-peak width and the other has a narrow one.

Nevertheless, tremendous efforts have been contributed toward the cause of the allosteric mechanism of Co-substituted Hb's by comparison with characteristic properties of model Co–heme complexes. One of the most important conclusions drawn by a previous investigator was that the allosteric mechanism does not involve as much motion of the proximal histidyl group in CoHb as occurs in the parent Hb (Little et al., 1974). Such a conclusion had been understood mainly from the observation of only a narrow EPR signal in deoxy-

---

[†] From the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205 (T.I.), and the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 (T.Y.). *Received September 16, 1982.* This work was partly supported by a research grant from the National Heart, Lung and Blood Institute (HL-14508) and by a grant from the National Science Foundation (PCM 7922841) to T.Y. This paper is part XVIII in the series "Cobalt Myoglobins and Hemoglobins". This paper was presented in part at the 178th American Chemical Society National Meeting, Washington, D.C., 1979.

[1] Abbreviations: CoHb, cobalt hemoglobin; Fe–Co hybrid Hb, iron–cobalt hybrid hemoglobin; CoPPDME, cobaltous protoporphyrin dimethyl ester; 1-MeIm, 1-methylimidazole; 2-MeIm, 2-methylimidazole; BzIm, benzimidazole; OEP, octaethylporphyrin; TPP, *meso*-tetraphenylporphyrin; IHP, inositol hexaphosphate; EPR, electron paramagnetic resonance; P_{50}, partial oxygen pressure at half-saturation; n_{max}, maximal slope of the Hill plot; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/83/0422-1894$01.50/0

Table I: EPR Data for Fe–Co Hybrid Hemoglobins and Model Cobaltous Complexes[a]

preparations	$g_\perp$	peak-to-peak width of $g_\perp$ (mT)[b]	$g_\parallel$	$\lvert A_\parallel^{Co}\rvert$ (mT)	$\lvert a^N\rvert$ (mT)
$\alpha(Co)_2\beta(Fe)_2$ at pH 7.0	2.38	10.4	2.03	8.0	1.4
$\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ at pH 7.0	2.33	5.8	2.03	7.7	1.7
$\alpha(Co)_2\beta(Fe)_2$ at pH 9.0[c]	2.34	5.8	2.03	7.8	1.7
$\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ at pH 9.0	2.33	5.8	2.03	7.7	1.7
$\alpha(Fe)_2\beta(Co)_2$ at pH 7.0	2.34	5.6	2.03	7.7	1.7
$\alpha(Fe{\cdot}CO)_2\beta(Co)_2$ at pH 7.0	2.33	5.4	2.03	7.6	1.6
$\alpha(Fe)_2\beta(Co)_2$ at pH 9.0	2.34	5.6	2.03	7.7	1.7
$\alpha(Fe{\cdot}CO)_2\beta(Co)_2$ at pH 9.0	2.33	5.7	2.03	7.7	1.7
1-MeImCoPPDME[d]	2.33	6.5	2.03	7.7	1.7
2-MeImCoPPDME[d]	2.35	7.4	2.03	8.2	1.6
BzImCoPPDME[d]	2.36	9.3	2.03	8.5	1.6

[a] Measured by X-band EPR spectroscopy at 77 K. The error in g values is within ±0.01. [b] The width from the top to bottom of the g signal. [c] This sample gives a mixture of two different types of signals. Only the data for the sharp component are listed in this table (see the text). [d] Measured in toluene glass at 77 K.

CoHb (Hoffman & Petering, 1970; Yonetani et al., 1974b), even though model Co–porphyrin complexes showed a wide range distribution of line broadness associated with variation of the axial ligand (Little et al., 1974; Walker, 1970, 1974; Stynes et al., 1973). However, we have observed not only a narrow EPR spectrum but also a characteristically broad EPR spectrum, usually a mixture of these two, for deoxy-CoHb and deoxy Fe–Co hybrid Hb's. In addition, these two EPR spectra were found to be interconvertible with each other, with the relative composition dependent upon pH and the presence of allosteric effectors. Such observations prompted us to reinvestigate the allosteric mechanism that operates in Co-substituted Hb's. Moreover, the origin of the above spectral difference is still open for study in relation to the electronic state of the porphyrin metal ion. In this paper, we report on a comprehensive EPR study at X- and Q-bands on CoHb and Fe–Co Hb's carried out in order to explore the relationship between the electronic state of the prosthetic group and the function of the Hb molecule.

Experimental Procedures

Cobalt hemoglobin (Yonetani et al., 1974a) and iron–cobalt hybrid hemoglobins (Ikeda-Saito et al., 1977b, 1980) were prepared according to the methods reported previously. Deoxygenation of the Hb sample was achieved by an addition of solid sodium dithionite under an argon atmosphere. The half-liganded Fe–Co Hb's were prepared by flushing CO gas through the deoxygenated sample. The pH of the sample was adjusted by the addition of an appropriate amount of 1.0 M Tris, 0.1 NaOH, and 0.1 M HCl. The pH was recorded by a Model NX Sargent Welch pH meter equipped with Ingold microelectrode 6030-02.

Cobaltous protoporphyrin dimethyl ester (CoPPDME) was synthesized by a previous method (Falk, 1964). 1-Methylimidazole, 2-methylimidazole, and benzimidazole were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were purified by distillation or recrystallization. CoPPDME was dissolved into a toluene solution containing an appropriate amount of imidazole ligand, usually a 5-fold excess over CoPPDME in the EPR sample tube. The atmosphere was replaced by flushing argon gas in the EPR sample tube. The evacuation of oxygen was checked by the disappearance of the EPR signal around $g = 2.002$, which is due to the oxy CoPPDME complex.

EPR spectra were recorded at 77 K on a Varian E-109 X-band (9.17-GHz) spectrometer with 100-kHz field modulation. The spectrometer is equipped with a PDP 11/40 on-line computer system. Q-band EPR spectra (35.0 GHz) wer obtained on a Varian V-4503 spectrometer with 100-kHz field modulation coupled with a Varian E-257 temperature controller.

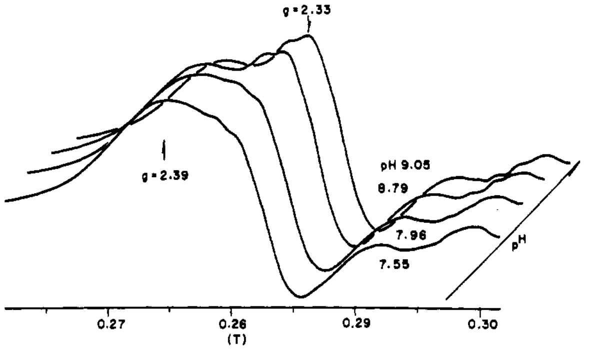

FIGURE 1: pH dependence of X-band EPR (9.17-GHz) spectra for $\alpha(Co)_2\beta(Fe)_2$ hybrid deoxyhemoglobin (77 K).

Results

General Features of EPR Spectra of Co-Substituted Hb's. The deoxy form of $\alpha(Co)_2\beta(Fe)_2$ showed two different types of EPR spectra characteristic of deoxy cobaltous porphyrin; a broad spectrum and a sharp spectrum were both exhibited. The fully deoxy-$\alpha(Co)_2\beta(Fe)_2$ at pH 7.0 exhibited mainly a broad $g_\perp$ signal resonating at $g = 2.38$, with a peak-to-peak width of 10.4 mT. It also showed a broad octet $g_\parallel$ signal centered at $g = 2.03$, with a spacing of about 8 mT due to $\lvert A_\parallel^{Co}\rvert$ hyperfine coupling. This $g_\parallel$ signal also exhibited an ambiguous triplet superhyperfine coupling with approximately 1.4-mT spacing, characteristic of the nitrogen atom of the proximal histidine ligand.

In contrast, the half-liganded $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ exhibited preferentially a narrow $g_\perp$ signal with a peak-to-peak width of 5.8 mT at $g = 2.33$, as well as a sharp $g_\parallel$ signal with 7.7 mT of $\lvert A_\parallel^{Co}\rvert$ and 1.7 mT of $\lvert a^N\rvert$ superhyperfine coupling. Accordingly, it is generally noted that the broader the $g_\perp$ and $g_\parallel$ signals, the larger the $\lvert A_\parallel^{Co}\rvert$ coupling constant and the smaller the $\lvert a^N\rvert$ superhyperfine coupling constant.

The complementary hybrid Hb, $\alpha(Fe)_2\beta(Co)_2$, and its half-liganded form $\alpha(Fe{\cdot}CO)_2\beta(Co)_2$ each showed a relatively sharp EPR spectrum resembling that of deoxy-$\alpha(Co)_2\beta(Fe{\cdot}CO)_2$. These EPR data are assembled in Table I.

pH-Dependent EPR Spectral Change for Co-Substituted Hb's. The EPR spectrum of deoxy-$\alpha(Co)_2\beta(Fe)_2$ was found to be sensitive to pH, as reported previously (Ikeda-Saito et al., 1977b). Remarkable changes in the EPR spectrum were exhibited in the $g_\perp$ region with variation of pH, as shown in Figure 1. In this figure, it should be noted that the broader portion at $g = 2.38$ decreased in its intensity with increasing

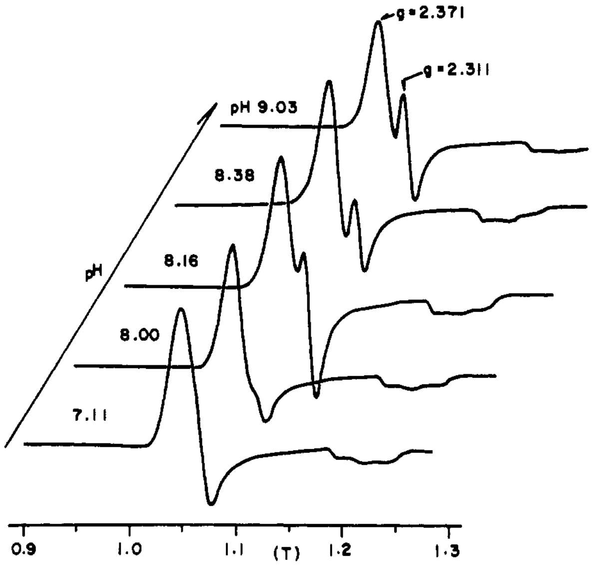

FIGURE 2: pH dependence of Q-band EPR (35-GHz) spectra for $\alpha(Co)_2\beta(Fe)_2$ hybrid deoxyhemoglobin (128 K).

pH, and concomitantly, the intensity of the sharper portion at $g = 2.33$ increased. This situation is manifested in Q-band EPR spectra as shown in Figure 2. At pH 7.0, a single $g_\perp$ signal was observed at $g = 2.371$. However, above pH 8 the $g_\perp$ signal was split into two parts with $g = 2.371$ and 2.311. This result clearly indicates that deoxy-$\alpha(Co)_2\beta(Fe)_2$ involves at least two different electronic states of the porphyrin cobalt ion.

In contrast to the case of deoxy-$\alpha(Co)_2\beta(Fe)_2$, the complement of $\alpha(Fe)_2\beta(Co)_2$ exhibited only a small change in its EPR spectrum with increasing pH from 7.0 to 9.0. This small pH perturbation can only be recognized in the $g_\perp$ signal region of the X-band spectra. Otherwise, no significant difference could be found between the spectrum at neutral pH and that at alkaline pH (spectra are not shown).

As reported previously, deoxy-CoHb showed a characteristic pH-dependent spectral change as well. The resonance positions of the two $g_\perp$ signals were explicitly determined by Q-band EPR spectroscopy. The resulting spectra are shown in Figure 3. With increasing pH, the lower field portion of the $g_\perp$ signal with a maximum at $g = 2.360$ decreased in intensity, while, simultaneously, the higher field $g_\perp$ signal at $g = 2.324$ increased. Unlike the case of deoxy-$\alpha(Co)_2\beta(Fe)_2$, shown in Figure 2, the peak height of the higher field portion is much larger than that of the lower field portion.

Allosteric Effector on Co-Substituted Hemoglobins. An addition of IHP to deoxy-CoHb and deoxy Fe–Co Hb's caused a change in their EPR spectra. The most striking change was observed in the $g_\perp$ signal region for deoxy-$\alpha(Co)_2\beta(Fe)_2$, which is illustrated in Figure 4. The lower field signal around $g = 2.38$ at pH 7.0 decreased and was slightly shifted upfield by the addition of IHP.

The IHP-induced spectra change at alkaline pH is somewhat different from that resulting around neutral pH's. At pH 8.0, the sharp component at $g = 2.33$ decreased in its intensity, and concomitantly, the broad signal at lower field, $g = 2.38$, increased on addition of IHP. Eventually, the addition of IHP caused a switch in this spectrum to the quite similar spectrum of the corresponding hybrid hemoglobin observed at pH 7.0 in the absence of IHP.

Decomposition of EPR Spectra into Two Components. In order to simulate the EPR spectra of deoxy-$\alpha(Co)_2\beta(Fe)_2$ by a linear combination of broad and sharp signal components,

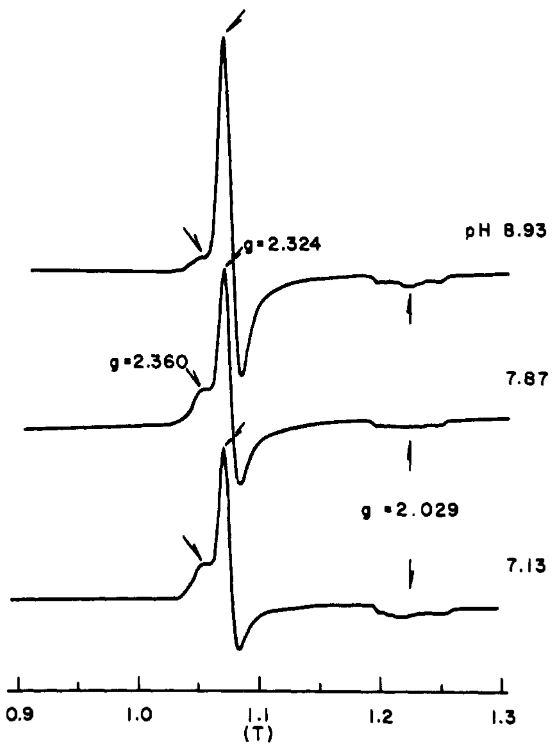

FIGURE 3: Q-band EPR spectra for deoxy-CoHb at various pH values (128 K).

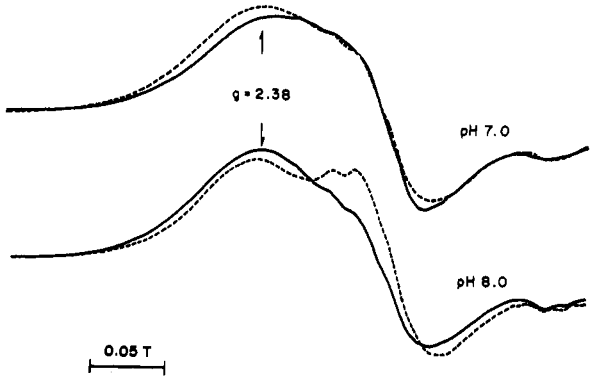

FIGURE 4: IHP effect on $g_\perp$ signal for deoxy-$\alpha(Co)_2\beta(Fe)_2$ at different pH values. Dotted lines designate the spectra of hybrid deoxyhemoglobin in the presence of 2 mM IHP, and solid lines designate the spectra of stripped hemoglobin.

we have to assume purely broad and purely sharp signals. However, at the present stage, we cannot experimentally obtain these signals because all the EPR spectra of $\alpha(Co)_2\beta(Fe)_2$ exhibited, more or less, a mixture of the two signals even at extremely low or high pH. Therefore, the broadest spectrum observed for deoxy-$\alpha(Co)_2\beta(Fe)_2$ at pH 6.5 was chosen as representative of the broad component at a close approximation. The narrowest spectrum of half-liganded $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ recorded at pH 9.0 was chosen as representative of the sharp signal. Only the $g_\perp$ signal was simulated because of its good signal to noise ratio, as well as its sensitive response to both pH variation and addition of allosteric reagents. Moreover, in order to obtain the best fit to the observed spectra, the estimated broad signals should be shifted downfield by 1 mT from the original resonance position. Generally, the resonance position of the $g_\perp$ signals was pH sensitive, although their change throughout the entire pH region was usually less than 1 mT. Therefore, the above requirement of a 1-mT shift suggests that the original position of the purely broad signal may be further shifted downfield by 1 mT more than that for

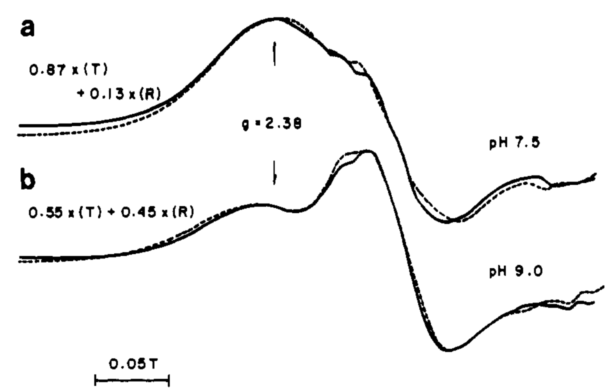

FIGURE 5: Breakdown into broad and sharp signal contribution for $\alpha(Co)_2\beta(Fe)_2$ deoxyhemoglobin. (a and b) Observed (solid line) and simulated (dotted line) spectra of $g_\perp$ signal region at different pH values. Designation of T corresponds to the broad component of the $g_\perp$ signal and that of R to the sharp component (details are described in the text).

Table II: Contribution of T State in α-Subunits for $\alpha(Co)_2\beta(Fe)_2$, Tetrameric CoHb,[a] and HbNO

preparations	pH 7.0	7.5	8.0	8.5	9.0	ref
$\alpha(Co)_2\beta(Fe)_2$, −IHP	0.90	0.87	0.75	0.65	0.55	b
$\alpha(Co)_2\beta(Fe)_2$, +IHP	0.90	0.90	0.90	0.80	0.70	b
CoHb, −IHP	0.45	0.57	0.52	0.45	0.38	b
FeHbNO, −IHP	0.07		0.0			c
CoHb, +IHP	0.58	0.63	0.63	0.52	0.52	b
FeHbNO, +IHP	0.78					c

[a] Error is estimated to be within ±0.05. The calculation procedure is described in the text. Also refer to Figures 6 and 7. [b] This paper. [c] Hille et al. (1979).

deoxy-$\alpha(Co)_2\beta(Fe)_2$ at pH 6.5.[2] The $g_\perp$ signals for the α-subunits were calculated by a trial and error method that involved changing the composition of the broad and sharp signals.

Typical examples of observed and simulated EPR spectra for deoxy-$\alpha(Co)_2\beta(Fe)_2$ at pH 7.5 and 9.0 are illustrated in Figure 5. The composition of the broad and sharp signals is designated on the left shoulder of each spectrum, and all the calculated compositions are assembled in Table II. The data in this table show that the contribution of the broad signal decreased directly with increasing pH. However, the addition of IHP caused a remarkable suppression of this decrease, indicating that the electronic state of the Co ion is forced to change due to the addition of IHP.

Tetrameric deoxy-CoHb also exhibited a complicated spectrum with a mixture of broad and sharp signals in the $g_\perp$ region. In order to calculate the composition for CoHb, the contributions of the individual α- and β-subunits to the entire spectrum should themselves be calculated. This is necessary because both subunits contribute to the observed spectra of tetrameric CoHb. The following procedure was employed to breakdown the spectrum into the individual contributions of the subunits. First, the signal of the β-subunits is assumed to involve only a sharp signal at the $g_\perp$ position. This assumption is based on the fact that the EPR spectrum of the β-subunits for deoxy-$\alpha(Fe)_2\beta(Co)_2$ showed only a sharp signal at all pH conditions. Accordingly, we can safely choose the sharp EPR signal as representative of the signal of the β-subunits in tetrameric CoHb. Second, we chose the spectrum of $\alpha(Co)_2\beta(Fe)_2$ at an interesting pH for the initial estimation of the contribution of the α-subunits, since the α-subunits are expected to involve both broad and sharp signals in the $g_\perp$ region. Then the best fit spectrum for the α-subunits was obtained by changing the combination of the broad and sharp signals as described above. In Figure 6 are demonstrated the decomposed EPR spectra of the individual α- and β-subunits in tetrameric CoHb. It is noteworthy that there is a considerable contribution of the sharp signal to the spectrum of the α-subunits even at neutral pH when compared with the data from $\alpha(Co)_2\beta(Fe)_2$. These results suggest that the substitution of Fe–porphyrin with Co–porphyrin in the β-subunits may substantially affect the electronic structure of Co–porphyrin in the α-subunits. As an example, the calculated spectrum of the α-subunits in CoHb at pH 7.5 is quite similar to the spectrum of $\alpha(Co)_2\beta(Fe)_2$ at pH 9.0. Furthermore, a breakdown of the α-subunit spectrum into the broad and sharp

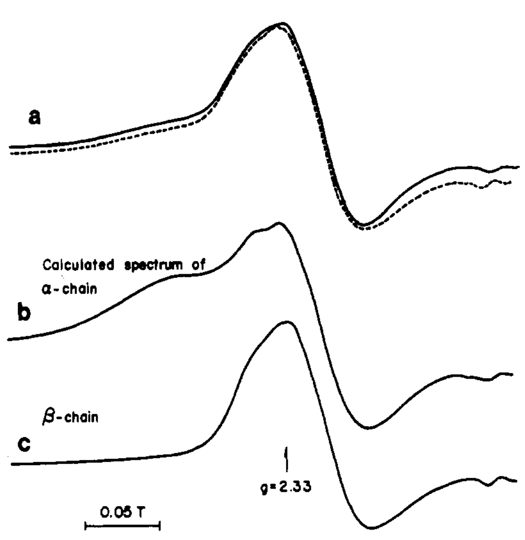

FIGURE 6: Breakdown into subunit contribution for deoxy tetrameric CoHb. (a) Observed (solid line) and simulated (dotted line) spectra of $g_\perp$ region. The simulated spectrum was calculated from the spectra given in (b) and (c).

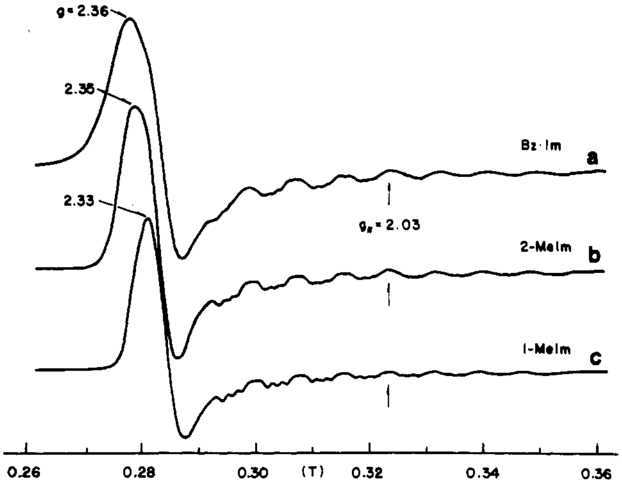

FIGURE 7: EPR spectra of pentacoordinated model complexes in toluene glass at 77 K: (a) benzimidazole complex with cobaltous protoporphyrin dimethyl ester (BzImCoPPDME); (b) 2-methylimidazole complex with cobaltous protoporphyrin dimethyl ester (2-MeImCoPPDME); (c) 1-methylimidazole complex with cobaltous protoporphyrin dimethyl ester (1-MeImCoPPDME).

---

[2] The $g_\perp$ signal for $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ was found to be shifted ca. 0.7 mT downfield by changing pH from 9.0 to 6.5. However, the signal was shifted upfield again by decreasing pH from 6.5 to 6.0 by approximately 0.5 mT. A similar situation was discerned in the $g_\perp$ signals for deoxy-$\alpha(Fe)_2\beta(Co)_2$ and $\alpha(Fe{\cdot}CO)_2\beta(Co)_2$ (T. Inubushi and T. Yonetani, unpublished results).

signal contributions was carried out according to the preceding method applied to deoxy-$\alpha(Co)_2\beta(Fe)_2$. Thus, the calculated ratios of the broad and sharp signals in the α-subunits of CoHb are compiled in Table II, where the contribution of the broad signal is described as T-state contribution. Like deoxy-$\alpha(Co)_2\beta(Fe)_2$, the contribution of the broad signal was found to be decreased monotonously with increasing pH, and the addition of IHP also resulted in an increase of the contribution while tending to force to the T state.

EPR Spectra of Model Complexes. The EPR spectra demonstrated in Figure 7 are typical pentacoordinated cobaltous protoporphyrin dimethyl esters with three different imidazole derivatives. The CoPPDME complex with 1-MeIm gives a sharp $g_\perp$ signal with an extreme at $g = 2.33$ and well-resolved hyperfine coupling in the $g_\parallel$ signal. This EPR spectrum closely resembles the typical sharp EPR spectra for deoxy-$\alpha(Fe)_2\beta(Co)_2$ and the half-liganded hybrid hemoglobins $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ and $\alpha(Fe{\cdot}CO)_2\beta(Co)_2$. With more bulky ligands, 2-MeIm and BzIm, the peak-to-peak width of the $g_\perp$ signal suffers significant broadening, and the superhyperfine coupling due to the nitrogen atom becomes more ambiguous. The triplet pattern, however, can still be discerned even in the extreme case of CoPPDME complex with BzIm, as shown in Figure 8. This indicates that BzIm still serves as the axial ligand in this complex. In this spectrum, the peak-to-peak width of the $g_\perp$ signal is comparable with that of $\alpha(Co)_2\beta(Fe)_2$ at pH 7.0, which exhibits the broadest $g_\perp$ signal of the hybrid hemoglobins. The only difference between these spectra involves the $g_\perp$ values, which are compared in Table I along with the data of the Co-substituted hemoglobins.

Discussion

Axial Ligation of Prosthetic Group in α-Subunits. According to the recent analysis (Ikeda-Saito & Yonetani, 1980) of the oxygen equilibrium for $\alpha(Co)_2\beta(Fe)_2$ in the presence and absence of CO gas, the P_{50} value of the Co subunits has been reported to be much smaller in $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ than in fully deoxy-$\alpha(Co)_2\beta(Fe)_2$. These equilibrium data indicate that the deoxy Co subunits of the half-liganded $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$ are preferentially in the high-affinity state (R state) and, eventually, those of the fully deoxy-$\alpha(Co)_2\beta(Fe)_2$ are in the low-affinity state (T state). On the other hand, the EPR spectra showed a remarkable difference between these two hybrid hemoglobins, namely, that the fully deoxy-$\alpha(Co)_2\beta(Fe)_2$ showed a characteristically broad $g_\perp$ signal while the corresponding half-liganded state showed a sharp $g_\perp$ signal. Therefore, if one considers the above relation, the broad EPR signal of the α-subunits may be responsible to the T state of Co-substituted hemoglobin and the sharp signal to the R state.[3] This correlation is also reasonably confirmed by the EPR spectral change observed by the increase of pH. As shown in Figures 1 and 2, the broad component of the $g_\perp$ signal (T state) decreases, and concomitantly, the sharp EPR signal (R state) increases. It has been reported that the oxygen affinity of the corresponding Co subunits in $\alpha(Co)_2\beta(Fe)_2$ is substantially increased by the pH change from 6.5 to 8.0 (Imai et al., 1980), indicating that the low-affinity state at pH 6.5 is partially switched to the high-affinity state.

An inspection of the EPR spectra for the model complexes illustrated in Figure 7 discloses that the peak-to-peak width of the $g_\perp$ signal increases in the order of adducts: 1-MeIm < 2-MeIm < BzIm.[4] This order can be interpreted in terms of an increase of steric hindrance in the coordination of imidazole toward the porphyrin cobaltous ion. The X-ray crystallographic data of $1,2\text{-}Me_2ImCoTPP$ show that the distance between the Co and the N atoms of the coordinated imidazole is 2.216 Å and the N–Co bond is tilted from the heme normal by about 8° (Dwyer et al., 1974). Apparently, steric hindrance forces the coordination of the imidazole ligand to be distorted against Co–porphyrin. However, crystallographic data for 1-MeImCoOEP show that the separation between the Co and the ligand N atoms is 2.150 Å and the coordinated axis is almost parallel to the heme normal (Scheidt, 1974). The structure of 1-MeImCoTPP (Little & Ibers, 1974) has also been reported to be similar to that of 1-MeImCoOEP.

The line width of the $g_\perp$ signal increased in the above order and exhibited more indistinct superhyperfine structure of $|a^N|$ coupling. Especially, the spectrum observed for BzIm-CoPPDME is close to *the broad* EPR spectra of $\alpha(Co)_2\beta(Fe)_2$. However, unfortunately, X-ray crystallographic data are not available for this model complex to discuss the structure. If one considers the bulkiness neighboring the N atom, more profound restraint is expected to be operative in BzIm-CoPPDME than in 2-MeImCoPPDME. Therefore, this spectral similarity suggests that the restraint binding of the *proximal* His to the porphyrin metal ion may be important for the T-state hemoglobin (vide infra). On the other hand, the spectral feature of the CoPPDME complex with unhindered 1-MeIm adduct is almost identical with that of the half-liganded $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$. Consequently, such an unhindered ligand binding situation may be attributable to the proximal His binding in the deoxy R state.

The studies on oxygen affinity for model cobaltous complex show positive correlation between the ligand binding situation at the metal ion and the affinity of oxygen. Co–porphyrin complex with $1,2\text{-}Me_2Im$ has been reported to discern the low affinity of oxygen binding, which is in contrast to the case of the porphyrin complex with 1-MeIm, showing high oxygen affinity (Collman et al., 1978). Generally, the greater the steric hindrance of ligand toward porphyrin metal, the lower the oxygen affinity for the corresponding cobalt porphyrin model complexes. Such a relation can be extended to the case of Co-substituted hemoglobin. The broad EPR signal of deoxy-$\alpha(Co)_2\beta(Fe)_2$, which is associated with restraint ligation of the proximal His, may be correlated to low oxygen affinity, namely, to the T state. In fact, oxygen binding data of $\alpha(Co)_2\beta(Fe)_2$ showed relatively low affinity for the $\alpha(Co)_2$ subunits (Imai et al., 1980). In contrast, the sharp EPR signal of $\alpha(Co)_2\beta(Fe{\cdot}CO)_2$, which is associated with unhindered

---

[3] Complete disappearance of the broad $g = 2.38$ signal for CoHb Ranier, which is preferentially in the R state even in the deoxy form of CoHb Rainier, also supports the above correlation (K. Imai, M. Ikeda-Saito, A. Hayashi, and T. Yonetani, unpublished results). In light of all these EPR data, we can safely correlate the sharp $g_\perp$ signal to the R state. A similar relationship has been found in the pattern of the hyperfine-shifted NMR signals of the porphyrin peripherals for the corresponding deoxy Fe–Co Hb's in solution state (Ikeda-Saito et al., 1978). In addition, careful inspection of the $g_\perp$ signals revealed a small difference in the resonance position between $\alpha(Fe)_2\beta(Co)_2$ and $\alpha(Fe{\cdot}CO)_2\beta(Co)_2$, which could only be recognized in the g shifts. They are summarized in Table I. At the present stage, however, this spectral change in the β-subunits is too small to allow a firm conclusion to be drawn about the electronic state of the Co ion when compared with that in the α-subunits. Further analysis of these EPR spectra is under way in our laboratory by S-band EPR spectrometry.

[4] EPR data have been reported for the model complexes of cobaltous porphyrin (Walker, 1970, 1974; Stynes et al., 1973). However, information about the line shape and the peak-to-peak width of the $g_\perp$ signal has not been reported. In this paper, we have reexamined the EPR spectra for typical model complexes in order to directly compare them with those of Co-substituted hemoglobins.

ligation of the proximal His, may be responsible for the high oxygen affinity, namely, the R state. In addition, the $g_\perp$ signal of the Co ion of $\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ is found to be broader than that of $\alpha(\mathrm{Fe})_2\beta(\mathrm{Co})_2$, suggesting that the oxygen affinity in the α-subunits is expected to be lower than that in the β-subunits. This prediction is in agreement with the analysis of the oxygen equilibrium curve for FeHb, in which the β-subunits show higher oxygen affinity than do the α-subunits (Imai et al., 1980).

Alkaline Bohr Effect and Allosteric Effector. An addition of IHP to deoxy-$\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ gave rise to an increase of the broad signal contribution,[5] which may be associated with the T state, as shown in Table II. In alkaline condition, the addition of IHP to a hemoglobin solution causes, at least, an increase of the T-state contribution as judged by the increase of the broad component of the $g_\perp$ signal in Figure 4. This result reasonably corresponds to the previous observation that the addition of IHP can shift the allosteric equilibrium to the T state. In contrast to the change in the alkaline solution, an addition of IHP at pH 7.0 gives a different change in the EPR spectra as shown in Figure 5a. If the hemoglobin conformation is simply switched to the T state by the addition of IHP, as expected from the spectral change in the alkaline condition, the peak at $g = 2.38$ should then be expected to increase in height. However, against this expectation, the peak height at $g = 2.38$ decreased in the presence of IHP. Probably, $|A_\perp^{\mathrm{Co}}|$ decreased somewhat without changing the resonance position of the $g_\perp$ signal. Therefore, this spectral change suggests that the addition of IHP results in a different electronic state of the porphyrin metal ion when compared with that of the deoxy quaternary T state without IHP. One of the two states in the three-state model, which has been predicted by Minton & Imai (1974), may be responsible for this electronic state of the porphyrin metal ion.

Effect of Porphyrin Metal Substitution. The composition data listed in Table II suggest that more than 50% of the sharp signal contributes to the α-subunits at pH 7. This situtation is comparable to the case of the α-subunits of deoxy-$\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ at pH 9.0, as shown in Figure 1. The smaller allosteric constant of $L = 10^3$ for CoHb (Imal et al., 1977), which is substantially smaller than that of the natural Hb, where $L = 10^9$, may be partly responsible for these ligand binding properties of the α-subunits. This increase of the sharp signal contribution is apparently caused by the substitution of Fe with Co in the β-subunits. Since the coordination of the proximal His to Co–porphyrin is expected to be weaker than that to the natural Fe–porphyrin, some of the homotropic interaction may be lost in tetrameric CoHb. Subsequently, the affinity state of the α-subunits is partially switched to the R state through the Co substitution. Such an increase of the R-state contribution was also observed by a kinetic CO binding study for the hydrid hemoglobins (Blough et al. 1980).

Electronic State of Porphyrin Metal Ion in β-Subunits. In sharp contrast to the remarkable response of the EPR spectrum of deoxy-$\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ to the allosteric transition, the complementary $\alpha(\mathrm{Fe})_2\beta(\mathrm{Co})_2$ shows a surprisingly small response to similar variations. For example, the largest change discerned in the EPR spectrum is caused by ligation of CO to the α-subunits containing Fe–porphyrin, as demonstrated previously (Table I). In addition, the half-liganded hybrid hemoglobins, such as $\alpha(\mathrm{Co})_2\beta(\mathrm{Fe{\cdot}CO})_2$ and $\alpha(\mathrm{Fe{\cdot}CO})_2\beta(\mathrm{Co})_2$, also exhibit small response to pH variation and the addition of IHP. Noteworthy are the characteristics of these insensitive EPR spectra, which closely resemble that of the model porphyrin complex with 1-MeIm adduct. This similarity suggests that these subunits have the same ligand binding situation as the unhindered 1-MeIm ligand in model complex. Therefore, judging from the electronic structure of the porphyrin metal ion, it can be suggested that the ligand binding situation in the β-subunits is not noticeably changed by the ligation in the α-subunits or by the addition of allosteric effector.

Despite the insensitivity of the EPR spectrum of $\alpha(\mathrm{Fe})_2\beta(\mathrm{Co})_2$, the analysis of the oxygen equilibrium curve shows an $n_{\max}$ value of 2.0 for the β-subunits involving Co–porphyrin (Imai et al., 1980). This fact indicates that the β-subunits in this hybrid Hb show cooperative oxygen binding. Consequently, the insensitivity in the EPR spectrum suggests that the oxygen binding to the β-subunits may not be regulated by changes of the electronic state of the porphyrin metal ion. This situation is quite different from the case of the α-subunits, which were discussed above. Conformational change of the protein in the vicinity of the heme may be responsible for this regulation rather than the change of the porphyrin electronic state itself (Perutz, 1970; Baldwin & Chothia, 1979).

Comparison with Nitrosyl-Hb Data. Recently, Hille et al. (1979) reported that EPR and visible spectra for the α-subunits of HbNO showed a hexacoordinated heme structure as well as a pentacoordinated form, where the former structure is related with the R state and the latter with the T state. They also reported the relative ratios of pentacoordinated to hexacoordinated structures in the α-subunits, which are listed in Table II and are directly compared with those of the broad to sharp components in the EPR spectra for Co-substituted Hb's.

The value of 7% for the α-subunits of stripped HbNO is apparently much lower than 45% for those of CoHb. However, in the presence of IHP, HbNO gave 78% for the ratio, which is larger than the 58% value for CoHb. Moreover, the 78% value for HbNO with IHP is smaller than the 90% for deoxy-$\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ with and without IHP. Therefore, the composition value obtained from Co-substituted Hb's seems to be independent of those of HbNO. Generally, the increase of T-state contribution for HbNO by the addition of IHP is much more drastic than that for deoxy-CoHb and deoxy Fe–Co Hb's. Such a discrepancy may be due mostly to the difference in the liganded status of these two hemoglobins; HbNO is in a fully liganded state, while CoHb is in a totally unliganded state. For liganded hemoglobins, the physicochemical properties are more or less characterized by the ligation of external ligand to the sixth coordination position. This is because the coordination of NO to the heme iron is the primary perturbation for the properties of deoxy-Hb. In this sense, HbNO is particularly unique among ferrous hemoglobin derivatives in that it responds remarkably to the addition of IHP

---

[5] In a previous paper (Ikeda-Saito et al., 1977b), we reported that the broadening of the EPR signals for deoxy-$\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ and deoxy-CoHb was interpreted in terms of the increase of the in-plane anisotropy of cobaltous ion. However, a careful inspection of the spectra reveals that the $g_\parallel$ signal, as well as the $g_\perp$ signal, is composed of two characteristic signals: a broad signal and a sharp one. This observation indicates that two axially symmetric EPR signals are overlapped with each other. In addition, X-band EPR signals on the crystal form of deoxy-$\alpha(\mathrm{Co})_2\beta(\mathrm{Fe})_2$ showed that the $g_\perp$ signal was partly resolved due to the $|A_\perp^{\mathrm{Co}}|$ coupling with a spacing of about 1.5 mT, although resolved hyperfine structure was not observed for the counterpart $\alpha(\mathrm{Fe})_2\beta(\mathrm{Co})_2$ (H. Hori and T. Yonetani, unpublished results). The S-band (3.7-GHz) EPR spectra of these hybrid hemoglobins also resolved a part of the $|A_\perp^{\mathrm{Co}}|$ coupling. However, serious overlapping of $g_\perp$ signal with $g_\parallel$ signal hampered a precise reading of the coupling constants. These data will be published elsewhere. Therefore, in this paper, the above broadening of the $g_\perp$ signal is analyzed in terms of an increase in the $|A_\perp^{\mathrm{Co}}|$ coupling constant, although they cannot be resolved at the magnetic field employed in this study.

by changing its quaternary structure from R to T states as discerned from the resulting alternations of both EPR and visible spectra. HbO_2 and HbCO, however, change their spectra only slightly with the addition of IHP (Perutz et al., 1976; Adams & Schuster, 1974).

Despite numerical disagreement, the decreasing tendency of the T-state contribution with increasing pH in the range from 7.5 to 9 and the increasing bias of the value by the addition of IHP, as well, were observed commonly for both Co-substituted Hb's and HbNO. Therefore, these phenomena can be interpreted in terms of the weakening of the bond between the porphyrin metal and the proximal histidine, as is usually interpreted for HbNO (Maxwell & Caughey, 1976).

Conclusions

Two electronic states, which can be differentiated by EPR spectroscopy, can be correlated to the quaternary states T and R for Co-substituted hemoglobins. The ligation of CO to the β-subunits of $\alpha(Co)_2\beta(Fe)_2$ gave rise to a substantial increase of the R-state contribution. Comparison with the EPR data of the model complexes suggests that the restrained coordination of the *proximal* His toward porphyrin Co ion in the T state is switched to the less restrained ligand binding condition by the ligation of CO to the β-subunit. By contrast, the EPR spectra of the deoxy β-subunits were relatively insensitive to homotropic and heterotropic effects. These observations indicate that the electronic state of the porphyrin metal ion in the β-subunits is close to that of the R state in the α-subunits even in the fully deoxy state and cannot be perturbed by the ligation in the counterpart α-subunits. Accordingly, some other part of the protein may play a crucial role in the regulation of oxygen binding in the β-subunits.

Acknowledgments

We are grateful to Dr. John K. Yandell, Joseph M. Palumbo, and Elmer L. Leininger for preparation of the manuscript, to Dr. Hiroshi Hori and Dr. Masao Ikeda-Saito for discussion, and to Dr. Edwin D. Becker for encouragement.

Registry No. 1-MeImCoPPDME, 36544-48-6; 2-MeImCoPPDME, 64188-26-7; BzImCoPPDME, 41188-40-3; IHP, 83-86-3.

References

Adams, M. L., & Schuster, T. M. (1974) *Biochem. Biophys. Res. Commun. 58*, 525–531.

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Blough, N. V., Zemel, H., Hoffman, B. M., Lee, T. C. K., & Gibson, Q. H. (1980) *J. Am. Chem. Soc. 102*, 5685–5686.

Collman, J. P., Brauman, J. I., Doxsee, K. M., Halbert, T. R., Hayes, S. E., & Suslik, K. S. (1978) *J. Am. Chem. Soc. 100*, 2761–2766.

Dwyer, P. N., Madura, P., & Scheidt, W. R. (1974) *J. Am. Chem. Soc. 96*, 4815–4819.

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, Elsevier, New York.

Hille, R., Olson, J. S., & Palmer, G. (1979) *J. Biol. Chem. 254*, 12110–12120.

Hoffman, B. M., & Petering, D. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 637–643.

Ikeda-Saito, M., & Yonetani, T. (1980) *J. Mol. Biol. 138*, 845–858.

Ikeda-Saito, M., Iizuka, T., Yamamoto, H., Kayne, F. J., & Yonetani, T. (1977a) *J. Biol. Chem. 252*, 4882–4887.

Ikeda-Saito, M., Yamamoto, H., & Yonetani, T. (1977b) *J. Biol. Chem. 252*, 8639–8644.

Ikeda-Saito, M., Inubushi, T., McDonald, G. G., & Yonetani, T. (1978) *J. Biol. Chem. 253*, 7134–7137.

Imai, K., Yonetani, T., & Ikeda-Saito, M. (1977) *J. Mol. Biol. 109*, 83–97.

Imai, K., Ikeda-Saito, M., Yonetani, T., & Yamamoto, H. (1980) *J. Mol. Biol. 138*, 635–648.

Little, R. G., & Ibers, J. A. (1974) *J. Am. Chem. Soc. 96*, 4452–4463.

Little, R. G., Hoffman, B. M., & Ibers, J. A. (1974) *Bioinorg. Chem. 3*, 207–215.

Maxwell, J. C., & Caughey, W. S. (1976) *Biochemistry 15*, 388–396.

Minton, A. P., & Imai, K. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1418–1421.

Perutz, M. F. (1970) *Nature* (*London*) *228*, 726–734.

Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A., & Simon, S. R. (1976) *Biochemistry 15*, 378–387.

Scheidt, W. R. (1974) *J. Am. Chem. Soc. 96*, 90–94.

Stynes, D. V., Stynes, H. C., Ibers, J. A., & James, B. R. (1973) *J. Am. Chem. Soc. 95*, 1142–1149.

Walker, F. A. (1970) *J. Am. Chem. Soc. 92*, 4235–4244.

Walker, F. A. (1974) *J. Magn. Reson. 15*, 201–218.

Yonetani, T., Yamamoto, H., & Iizuka, T. (1974a) *J. Biol. Chem. 249*, 2168–2714.

Yonetani, T., Yamamoto, H., & Woodrow, G. V., III (1974b) *J. Biol. Chem. 249*, 682–690.