Kaptein, R. (1978) *Nuclear Magnetic Resonance Spectroscopy in Molecular Biology* (Pullman, B., Ed.) pp 211-219, Reidel, Dordrecht, Holland.
Lenkinski, R. E., Agresti, D. G., Chen, D. M., & Glickson, J. D. (1978) *Biochemistry 17*, 1463-1468.
Lenkinski, R. E., Dallas, J. L., & Glickson, J. D. (1979) *J. Am. Chem. Soc. 101*, 3071-3077.
Mallion, R. B. (1971) *J. Chem. Soc. B*, 681-686.
Mallion, R. B. (1978) *Nuclear Magnetic Resonance Spectroscopy in Molecular Biology* (Pullman, B., Ed.) pp 183-191, Reidel, Dordrecht, Holland.
McDonald, C. C., & Phillips, W. D. (1969) *J. Am. Chem. Soc. 91*, 1513-1521.
Pauling, L. (1936) *J. Chem. Phys. 4*, 673-677.
Perkins, S. J. (1977) D. Phil. Thesis, University of Oxford.
Perkins, S. J. (1979) *J. Magn. Reson.* (in press).
Perkins, S. J., & Wüthrich, K. (1979) *Biochim. Biophys. Acta 576*, 409-423.
Perkins, S. J., Johnson, L. N., Phillips, D. C., & Dwek, R. A. (1977a) *FEBS Lett. 82*, 17-22.
Perkins, S. J., Dower, S. K., Gettins, P., Wain-Hobson, S., & Dwek, R. A. (1977b) *Biochem. J. 165*, 223-225.
Perkins, S. J., Johnson, L. N., Machin, P. A., & Phillips, D. C. (1978) *Biochem. J. 173*, 607-616.
Perkins, S. J., Johnson, L. N., Machin, P. A., & Phillips, D. C. (1979) *Biochem. J. 181*, 21-36.
Pople, J. A. (1956) *J. Chem. Phys. 24*, 1111-1111.
Roberts, G. C. K., & Jardetsky, O. (1970) *Adv. Protein Chem. 24*, 447-545.
Shindo, H., Cohen, J. S., & Rupley, J. A. (1977) *Biochemistry 16*, 3879-3882.
Shrake, A., & Rupley, J. A. (1973) *J. Mol. Biol. 79*, 351-371.
Snyder, G. H., Rowan, R., Karplus, S., & Sykes, B. D. (1975) *Biochemistry 14*, 3765-3777.
Sternberg, M. J. E., Grace, D. E. P., & Phillips, D. C. (1979) *J. Mol. Biol. 130*, 231-253.
Sternlicht, H., & Wilson, D. (1967) *Biochemistry 6*, 2881-2892.
Wagner, G., & Wüthrich, K. (1975) *J. Magn. Reson. 20*, 435-445.
Weast, R. C., Ed. (1971) *Handbook of Chemistry and Physics*, 51st ed., p F-154, Chemical Rubber Co., Cleveland, OH.
Williams, R. J. P. (1976) The Merck Sharp & Dohme Research Laboratories Scientific Lecture, Merck Sharp & Dohme, Rahway, NJ.

Proton Magnetic Resonance Study of *p*-Mercuribenzoate Binding and Structural Changes in Methemoglobin†

Saburo Neya and Isao Morishima*

ABSTRACT: Interaction of human adult methemoglobin with *p*-mercuribenzoate (*p*MB) was examined at 21 °C by monitoring the hyperfine-shifted proton nuclear magnetic resonance (NMR) spectra of several high- and low-spin derivatives. The NMR spectra show that the heme methyl proton resonances from the β subunits in methemoglobin were selectively affected by the binding of *p*MB regardless of whether the heme iron was saturated with high-spin or low-spin ligand. This observation suggests that the binding of *p*MB to methemoglobin induces a localized tertiary structural change around the β heme, leaving the α heme unaffected. The structural change of the β subunit was correlated with an increase in the high-spin character of the β heme iron. A model study of the azide-methemoglobin complex suggested that the increase of the high-spin character of the β heme iron is due to a conformational change of the proximal histidine which weakens the interaction between the heme iron and the proximal base. A similar and more pronounced spectral change due to binding of *p*MB was observed for the isolated β subunit. The NMR spectral change in the isolated β subunit also suggests that the binding of *p*MB to methemoglobin induces a localized conformational change within the β subunit.

The binding of *p*-mercuribenzoate, *p*MB,[1] to hemoglobin has been examined by many investigators either to determine sulfhydryl reactivity as a measure of quaternary conformation (Gibson, 1973) or to study the functional property of the *p*MB-bound hemoglobin and subunits (Antonini & Brunori, 1971). The spectral properties of the native and *p*MB-bound hemoglobin have been compared in several ways using, for example, visible absorption (Banerjee et al., 1969), circular dichroism (Geraci & Li, 1969), and ^{13}C NMR (Dill et al., 1978). The function of the *p*MB-bound hemoglobin was discussed on the basis of the spectroscopic results. However, the *p*MB-induced spectral changes were not intensively interpreted in terms of structure.

Recently Olson (1976a) reported that the visible absorption change induced by *p*MB is similar to that induced by an allosteric effector, inositol hexaphosphate. Since the change of visible absorption due to the binding of inositol hexaphosphate was proposed to induce a localized tertiary structural change in the β subunits of methemoglobin (Hensley et al., 1978; Edelstein & Gibson, 1975; Olson, 1976b), he speculated that the *p*MB-induced absorption change may reflect a localized structural alteration in the β subunits (Olson, 1976a). However, others (Perutz et al., 1974) have suggested that inositol hexaphosphate induces the quaternary conformational transition of methemoglobin from the relaxed to the tensed state. Thus, the structural basis for the visible absorption

† From the Department of Hydrocarbon Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606, Japan. *Received May 17, 1979.* This work was supported by research grants from the Ministry of Education, Japan, and from the Toray Research Foundation for Science and Technology.

[1] Abbreviations used: *p*MB, *p*-mercuribenzoate; NMR, nuclear magnetic resonance; ppm, parts per million; Me_4Si, tetramethylsilane; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; β(SH), the β subunit with free cysteines; β(*p*MB), the β subunit with *p*MB-bound cysteines.

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change in methemoglobin induced by the binding of *p*MB has not been definitely resolved.

In order to provide further details on the reaction of methemoglobin with *p*MB and to relate the spectral response to the structural changes around the heme, we examined the effect of *p*MB binding on the methemoglobin structure by proton NMR spectroscopy.

Materials and Methods

Human adult blood was obtained from a blood bank and oxyhemoglobin was prepared as described previously (Morishima et al., 1978). Methemoglobin was prepared by adding a threefold molar excess of potassium ferricyanide to stripped oxyhemoglobin and then stirring for 40 min at room temperature. The methemoglobin was dialyzed against several changes of 10 mM phosphate buffer, pH 6.0, for 18 h at 4 °C and was adsorbed on a CM-cellulose column (Whatman, CM 52) equilibrated with the same buffer. The CM-cellulose column was washed with the same buffer to remove residual ferri- or ferrocyanide. Solvent exchange was accomplished by elution with 0.1 M deuterated phosphate buffer, pD 7.0.

The *p*MB-bound hemoglobin was prepared by adding a 0.5 heme equiv of *p*MB (Sigma Chemical Co.) to (carbon monoxy)hemoglobin. Under this condition *p*MB is known to be attached only to β93-cysteine (Olson, 1976a; Olson & Gibson, 1971). The *N*-ethylmaleimide- and iodoacetamide- (Nakari Chemicals Ltd., Kyoto) bound (carbon monoxy)hemoglobins were prepared according to Riggs (1961) and Taylor et al. (1966), respectively. These were converted to methemoglobin by adding a sixfold molar excess of potassium ferricyanide and were treated in the same way as native methemoglobin. The visible absorption spectra of the derivatives of the modified methemoglobin were almost the same as those of the corresponding native methemoglobin.

The isolated *p*MB-bound subunits were prepared according to Kilmartin et al. (1973) in carbon monoxy form. The cysteine residues were regenerated by treatment with 2-mercaptoethanol (Geraci et al., 1969). The isolated ferric subunits were prepared according to Banerjee & Cassoly (1969) after exchanging H_2O with D_2O by repeated dilution with D_2O and concentration through a membrane cone (Amicone, CF 25).

Protohemin chloride was purchased from Sigma (type I) and deuterated dimethyl sulfoxide was available from Merck. Other chemicals were reagent grade and were used without purification.

The visible absorption spectra were recorded on a Shimadzu UV-200 spectrometer. Proton NMR spectra were recorded on a Varian HR-220 spectrometer with a Nicolet TT-100 unit in a pulse Fourier transform mode at 220 MHz and 21 °C. Quadrature phase detection was used with a pulse width of 30 μs for a 75° pulse. The spectra were obtained by 4K points transform of 40-kHz spectral width after about 10000–60000 pulses. Chemical shifts in parts per million, ppm, were referred to the proton resonance of internal 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, or tetramethylsilane, Me_4Si, with the positive sign to lower field resonances. The chemical shift was accurate to ±0.2 ppm for high-spin complexes and to ±0.1 ppm for low-spin complexes.

The pD value was the direct reading of a pH meter, Radiometer Model 28, with microcombination electrode, Toko Kagaku Ltd. (Tokyo), Model CE-103.

Results

Ferric Low-Spin Complexes. Figure 1 shows the hyperfine-shifted proton resonances of cyanide-methemoglobin and its isolated α and β subunits ranging from 10 to 30 ppm downfield from DSS in deuterated 0.1 M phosphate buffer at 21 °C. The resonances at 22.5 ppm (from the α and β subunits), 16.8 ppm (from the α subunit), and 15.6 ppm (from the β subunit) have been assigned to the heme methyl protons (Ogawa et al., 1972). The NMR spectra of the isolated α and β subunits with and without *p*MB are compared in parts b and c of Figure 1. The α subunit spectra with and without *p*MB are similar to each other, indicating that the modification of α104-cysteine affects the α heme only slightly. In contrast, the β subunit spectra with and without *p*MB are considerably different from each other. The resonance at 21.5 ppm broadened and the ring current shifted resonance at −4.0 ppm split into a triplet due to the binding of *p*MB. These observations are in good agreement with the result by Ogawa et al. (1972).

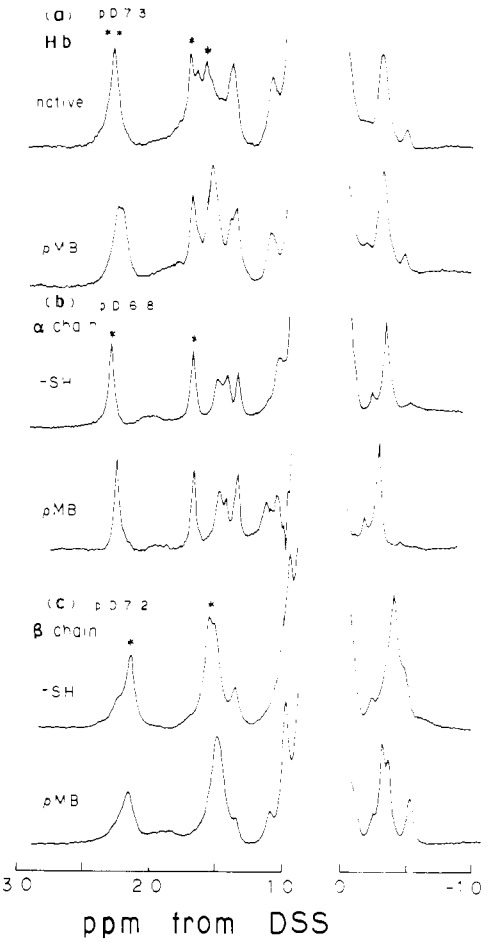

FIGURE 1: Proton NMR spectra of the cyanide complexes of methemoglobin and of the isolated α and β subunits with and without *p*MB in 0.1 M phosphate at 21 °C. Heme concentration was about 2 mM.

Since cyanide ion is known to stabilize the polypeptide structure of hemoproteins against deformation by acid, heat (Steinhardt et al., 1963), guanidine hydrochloride (McLendon & Sandberg, 1978), and pressure (Ogunmola et al., 1977), a conformational change by *p*MB binding is also thought not to be clearly resolved for the cyanide complexes. Therefore, we examined the binding of *p*MB for another low-spin complex, azide-methemoglobin, to investigate the effect more clearly. The bottom spectrum in Figure 2A shows the resonances of azide-methemoglobin. Peaks at 28.2, 22.2, and 14.5 ppm and 26.9, 21.3, and 14.0 ppm have been assigned to the heme methyl proton resonances of the α and β subunits, respectively (Davis et al., 1969; Morishima et al., 1978). When

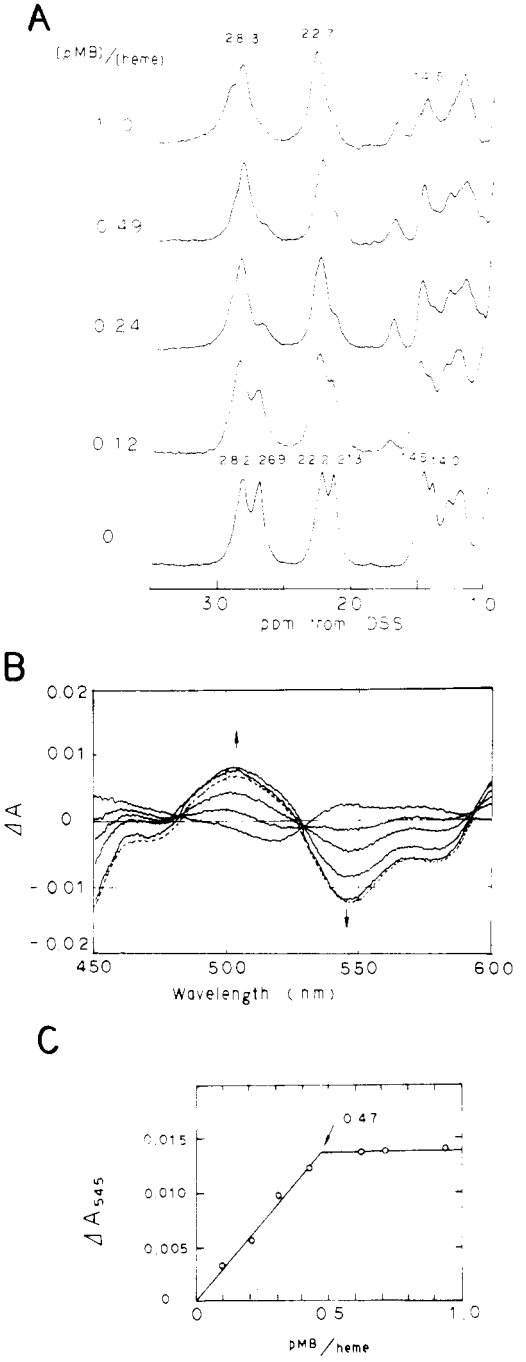

FIGURE 2: Binding of *p*MB to azide-methemoglobin. (A) Proton NMR spectral change of azide-methemoglobin upon reaction with *p*MB in 0.1 M phosphate at pD 7.1 and 21 °C. Only the β heme methyl resonances are affected. (B) Visible absorption change of azide-methemoglobin, 87 μM in heme, after reaction with *p*MB in 0.1 M phosphate at pH 7.0 and 21 °C. The dotted curve shows the difference spectrum when more than 1 equiv of *p*MB per heme was added. (C) Visible absorption change of azide-methemoglobin on the reaction with *p*MB. A stoichiometry of 0.5 equiv of *p*MB per heme was obtained.

small increments of *p*MB were added to azide-methemoglobin, the intensity of the β heme methyl resonances apparently decreased while that of the α heme methyl resonances was unaffected. The *p*MB titration was also followed by visible absorption as shown in Figure 2B. The absorption difference at 545 nm in Figure 2C indicates the binding of 0.5 equiv of *p*MB per heme, suggesting that only β93-cysteine is modified. This stoichiometry indicates that the proton NMR spectral change is the primary response of the binding of *p*MB to β93-cysteine in tetrameric hemoglobin.

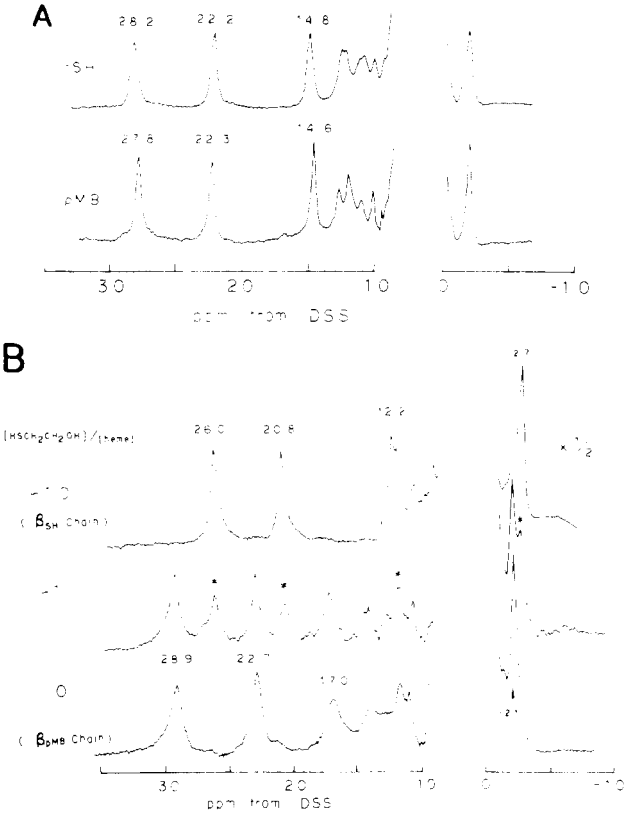

FIGURE 3: (A) A comparison of the NMR spectra of azide complexes of α(SH) (upper) and α(*p*MB) (lower) chains in 0.1 M phosphate at pD 7.0 and 21 °C. (B) Proton NMR spectral change of the isolated β(*p*MB) chain after titration with 2-mercaptoethanol in 0.1 M phosphate at pD 6.7 and 21 °C.

The effect of the *p*MB binding on the NMR spectrum is more clearly observed in the subunit spectra in Figure 3. The spectra of azide α subunit with and without *p*MB are similar to each other. In contrast, when azide β(*p*MB) subunit was titrated with 2-mercaptoethanol, a marked spectral change was observed. Upon regeneration of free cysteines, the β heme methyl resonances at 28.9, 22.7, and 17.0 ppm disappeared with concomitant appearance of a new set of heme methyl resonances at 26.0, 20.8, and 12.1 ppm. These changes suggest primarily a structural alteration related to the regeneration of β93-cysteine, because β112-cysteine located far from the heme would not affect the β heme as in the case for α104-cysteine to the α heme.

Analysis of the Heme Methyl Shifts of the Model Complexes. For interpretation of the spectral change for azide-methemoglobin and azide β subunit (Figures 2A and 3B) in terms of structure of the heme vicinity, the proton resonances of the model complexes, protohemin(1-methylimidazole)N_3^- and protohemin(2-methylimidazole)N_3^-, were analyzed. However, ligand exchange was so rapid on the NMR time scale that direct observation of the hyperfine shifts of the mixed-ligand complexes was not possible. The heme methyl shifts of the model complexes instead were determined by the analysis of the results of the 1- and 2-methylimidazole titration to azide-protohemin. The following equilibrium scheme was assumed:

$$\text{hemin}\cdot N_3^- + \text{Im} \overset{K}{\rightleftharpoons} \text{hemin}\cdot N_3^-\cdot\text{Im} \qquad (1)$$

where Im represents 1- or 2-methylimidazole. The formation constant K of the mixed-ligand complex is defined as

$$K = \frac{[\text{hemin}\cdot\text{Im}\cdot N_3^-]}{[\text{hemin}\cdot N_3^-][\text{Im}]}$$

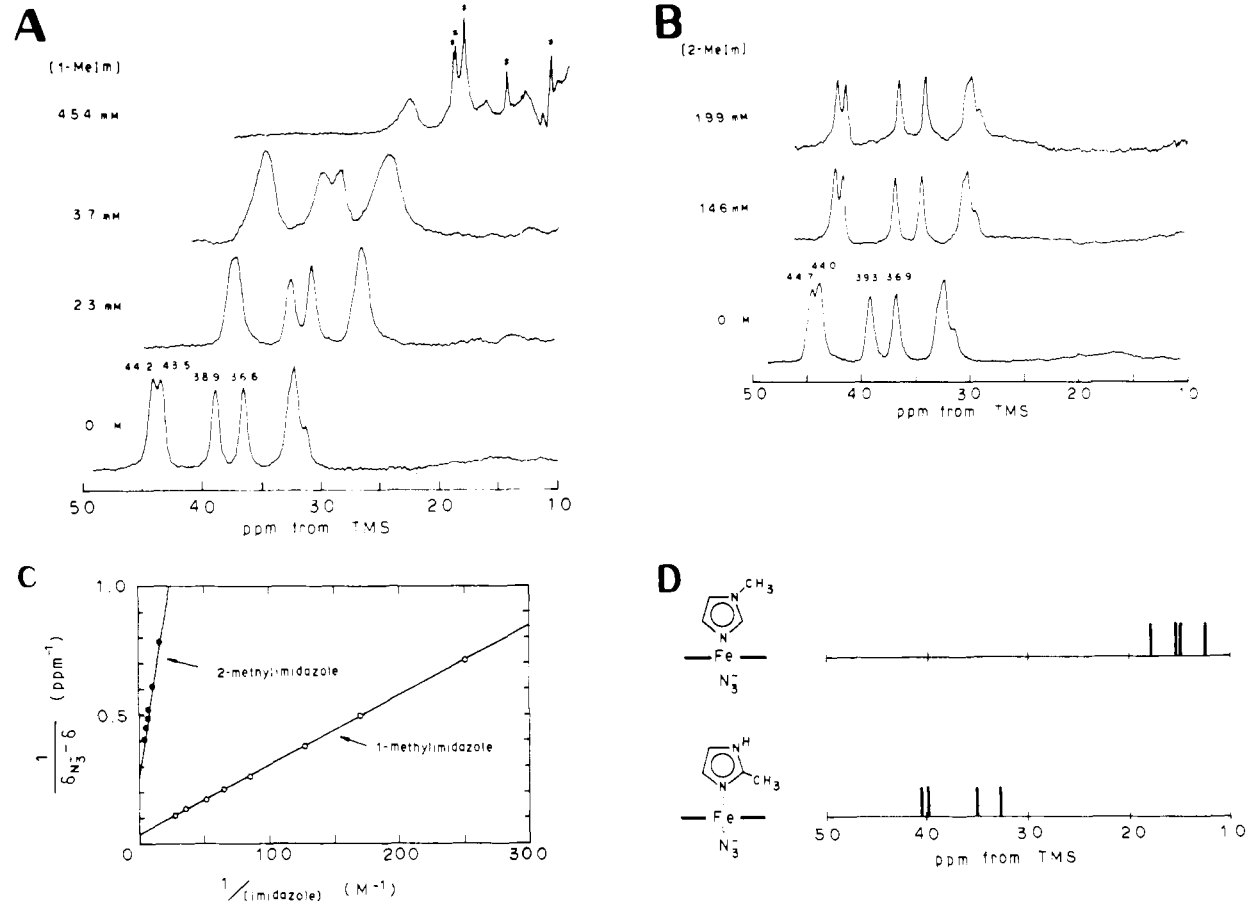

FIGURE 4: (A) Proton NMR spectral change of *mono*azide·protohemin, 7 mM, upon addition of 1-methylimidazole in deuterated dimethyl sulfoxide containing 0.8 M NaN_3 at 21 °C. The four downfield resonances are those from heme methyl protons. (B) Titration of 2-methylimidazole to *mono*azide·protohemin, 6 mM, under the same condition as described in (A). (C) Relation between the chemical-shift difference and 1-methylimidazole concentration for the heme methyl resonance at 44.2 ppm in (A) (○). Corresponding result of the 2-methylimidazole titration (●). Ordinate, reciprocal of chemical-shift difference in ppm^{-1}; abscissa, reciprocal of imidazole concentration in M^{-1}. Under the same condition as described in (A). (D) Calculated heme methyl shifts for the mixed-ligand complexes from analysis of the lines in (C). Upper, protohemin(1-methylimidazole)N_3^-; lower, protohemin(2-methylimidazole)N_3^-.

Under the fast ligand exchange condition, the observed chemical shift δ consists of the contributions from azide·hemin and the mixed-ligand complex and is expressed as a weighted average

$$\delta = \alpha\delta_m + (1 - \alpha)\delta_{N_3^-}$$

where δ_m and $\delta_{N_3^-}$ represent the chemical shifts of the resonances of the mixed-ligand complex and azide·hemin, respectively, and α is the fraction of mixed-ligand complex. The observed chemical shift δ is related to K by eq 2:

$$\frac{1}{\delta_{N_3^-} - \delta} = \frac{1}{K(\delta_{N_3^-} - \delta_m)}\frac{1}{[\mathrm{Im}]} + \frac{1}{\delta_{N_3^-} - \delta_m} \qquad (2)$$

When the concentration of imidazole is much larger than that of hemin, plots of $1/(\delta_{N_3^-} - \delta)$ against $1/[\mathrm{Im}]$ should give a straight line, from which K and δ_m can be determined.

The result of the 1-methylimidazole titration to azide·hemin in deuterated dimethyl sulfoxide at 21 °C is shown in Figure 4A. The four heme methyl resonances of *mono*azide·hemin,[2] which were assigned from their integrated intensities and line shapes, shifted upfield with line broadening upon addition of 1-methylimidazole. Further addition of 1-methylimidazole led to the formation of a new species, bis(1-methylimidazole)·protohemin, as evidenced by the appearance of the characteristic resonances designated by the asterisks in the upper spectrum of Figure 4A. A plot of $1/(\delta_{N_3^-} - \delta)$ against $1/[\mathrm{Im}]$ for the lowest field heme methyl resonance in Figure 4A is shown in Figure 4C, together with the corresponding result of the titration of 2-methylimidazole (Figure 4B). The reasonably linear relationship supports the equilibrium scheme of eq 1. From the analysis of the lines in Figure 4C and of those for other heme methyl resonances according to eq 2, the formation constant K of the mixed-ligand complexes from azide·hemin was calculated to be 15.7 ± 2.7 M^{-1} and 8.6 ± 0.7 M^{-1} for hemin(1-methylimidazole)N_3^- and hemin(2-methylimidazole)N_3^-, respectively, at 21 °C in deuterated dimethyl sulfoxide. The calculated heme methyl shifts of the two mixed-ligand complexes are illustrated in Figure 4D. The mean positions of the four heme methyl resonances, 37.2 and 15.7 ppm, for hemin(2-methylimidazole)N_3^- and hemin(1-methylimidazole)N_3^-, respectively, are closely similar to those of the pure high- and low-spin states of azide·methemoglobin estimated from the analysis of thermal spin equilibrium: 40.6 (high spin) and 17.9 (low spin) ppm for the α subunit and 32.7 (high spin) and 18.2 (low spin) ppm for the β subunit in the intact tetramer at 21 °C (Morishima et al., 1978).

The heme methyl shifts of the mixed-ligand complexes are different from each other by about 20 ppm. Since the binding of imidazole to the heme iron in hemin(2-methylimidazole)N_3^-

[2] Titration of protohemin chloride with sodium azide in dimethyl sulfoxide, followed by visible absorption spectroscopy at 21 °C, revealed that only monoazide·protohemin is formed. The formation constant of azide·hemin was 3.74 × 10^3 M^{-1}. Four heme methyl resonances in Figure 4A were thus assigned to those from *mono*azide·protohemin.

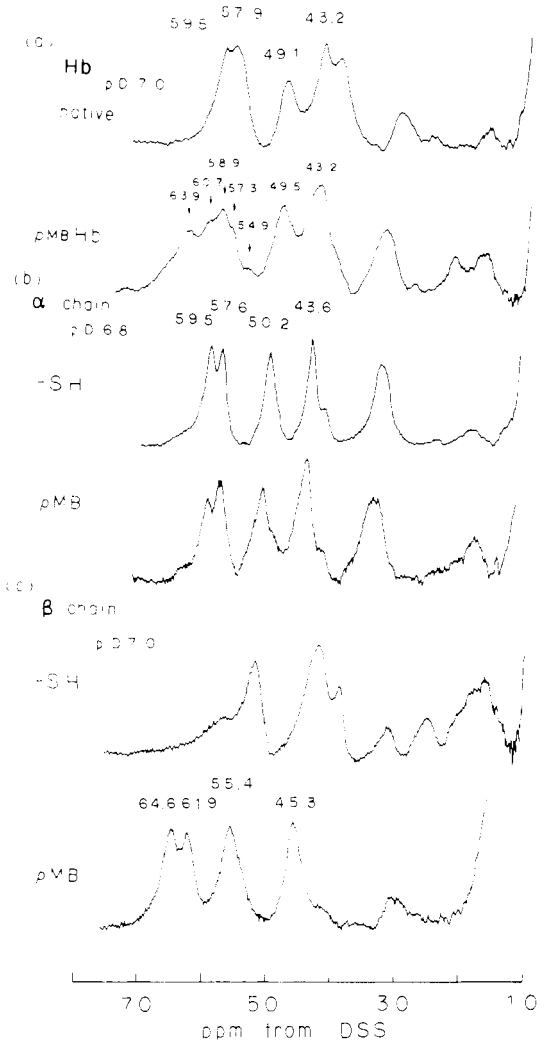

FIGURE 5: Spectral comparison of the formate complexes of methemoglobin and of the isolated subunits with and without *p*MB. (a) Methemoglobin in 0.1 M phosphate, (b) the isolated α subunit in 1 M glycine, and (c) the isolated β subunit in 1 M glycine, all at 21 °C.

is expected to be much weaker due to the steric hindrance of the 2-methyl group of imidazole, this result indicates that weakening of the iron–imidazole interaction induces a downfield shift of the heme methyl resonance.

Ferric High-Spin Complexes. For the ferric high-spin complexes of methemoglobin with fluoride, water, and formate, the NMR spectral change by *p*MB binding was also examined to obtain further insight into the *p*MB-induced structural perturbations. Figure 5 shows the hyperfine-shifted proton resonances of formate-methemoglobin and the isolated subunits with and without *p*MB. The four resonances of formate-methemoglobin at 59.5, 57.9, 49.1, and 43.2 ppm have been assigned to the heme methyl protons (Neya & Morishima, 1979). These resonances do not exhibit such $\alpha\beta$ heme methyl doublings as observed in the azide-hemoglobin spectrum in Figure 2A, probably because the line width of the heme methyl resonances from the α and β hemes in formate-methemoglobin is so large that the $\alpha\beta$ difference is not clearly resolved. Figure 5a shows that the binding of *p*MB to formate-methemoglobin caused a marked spectral change. From the spectral comparison between the isolated α(SH) and β(*p*MB) subunits in Figure 5, the resonances at 63.9, 60.7, and 59.4 ppm in the spectrum of *p*MB-bound formate-methemoglobin, $\alpha(SH)_2\beta$-$(pMB)_2$, were assigned to the β-subunit resonances. This observation indicates that the spectral change is solely associated with the β subunits of high-spin methemoglobin and is in good agreement with the result for the ferric low-spin complexes. A similar result was also obtained for the predominantly high-spin complex nitrite-methemoglobin, as shown in Figure 6a. The resonance at 46.8 ppm in the spectrum of *p*MB-bound nitrite-methemoglobin was assigned to the β subunit based on the comparison of the subunit spectra (see Figure 8a). However, fluoromethemoglobin and aquomethemoglobin exhibited less marked spectral changes upon binding with *p*MB, as shown in parts b and c of Figure 6. The apparent insensitivity of these two derivatives to *p*MB binding is presumably either because the line width of the resonance is so large that the spectral change is not clearly reflected on

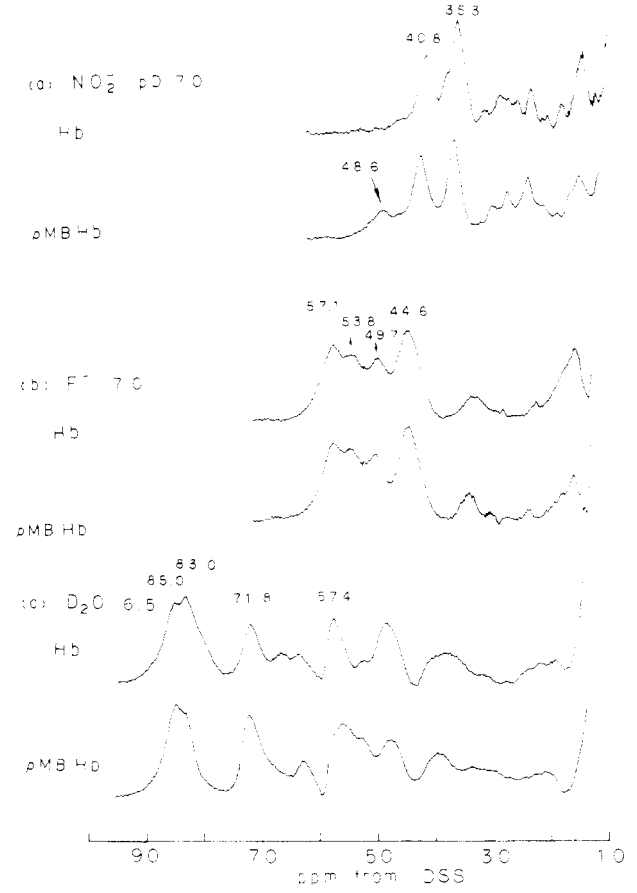

FIGURE 6: Proton NMR spectra of native and *p*MB-bound methemoglobin complexes in 0.1 M phosphate at 21 °C. (a) Nitrite complex; (b) fluoride complex; (c) aquomethemoglobin.

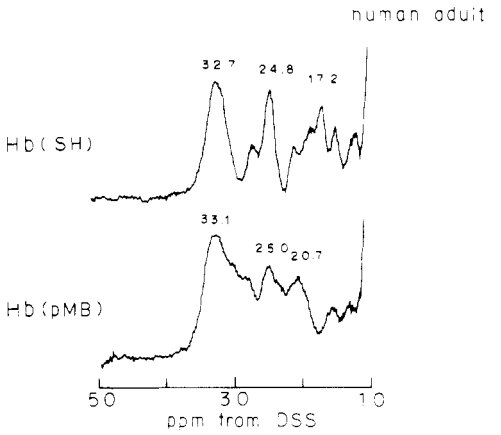

FIGURE 7: Proton NMR spectra of alkaline methemoglobin with and without *p*MB in 0.1 M NaCl at pD 10.1 and 21 °C.

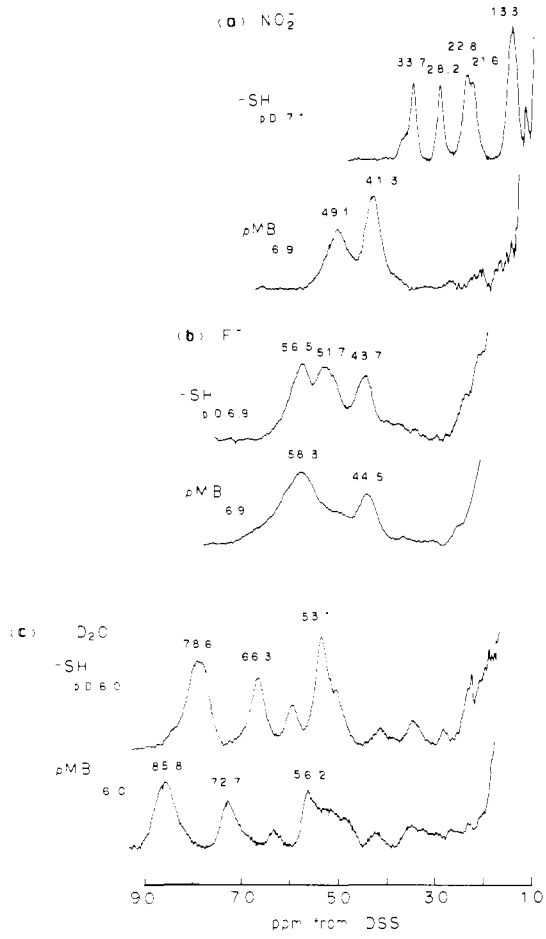

FIGURE 8: NMR spectral comparison of the isolated β subunit with and without *p*MB. (a) Nitrite complex, (b) fluoride complex, and (c) aquomet β chain; all in 1 M glycine at 21 °C. The resonances at 78.6 and 85.8 ppm in (c) are composed of two heme methyl proton signals.

the NMR spectra or because the NMR spectral responses are intrinsically small. The effect of *p*MB binding on the NMR spectrum for alkaline methemoglobin was insignificant, as presented in Figure 7.

Binding of pMB to the Isolated β(SH) Subunits. Since a large spectral change was observed by *p*MB binding of azide β subunit, which is in predominantly low-spin state, the *p*MB-induced effect was also examined for the isolated β subunit in the ferric high-spin state. Figures 5c and 8 compare the NMR spectra of the isolated β(SH) and β(*p*MB) subunits for several high-spin complexes. The four heme methyl resonances of aquomet β(SH) subunit, for example, shifted from 78.6, 78.6, 66.3, and 53.1 ppm to 85.8, 85.8, 72.7, and 56.2 ppm upon *p*MB binding. All the heme methyl resonances of other high-spin complexes also exhibited the *p*MB-induced downfield shift, indicating an increase of the high-spin character of the β heme iron. These observations suggest that the *p*MB-induced NMR spectral change of methemoglobin primarily reflects the structural alteration at the proximal side within the β subunits.

Effects of Other Sulfhydryl Reagents. Some other reagents also selectively react with β93-cysteine under specialized modification conditions (Taylor et al., 1966; Riggs, 1961; Guidotti & Konigsberg, 1964). The spectra of methemoglobin treated with *N*-ethylmaleimide and iodoacetamide are presented in Figure 9 for the low-spin (azide) and high-spin (formate) complexes, together with those of native and *p*MB-bound methemoglobins for comparison. Figure 9A

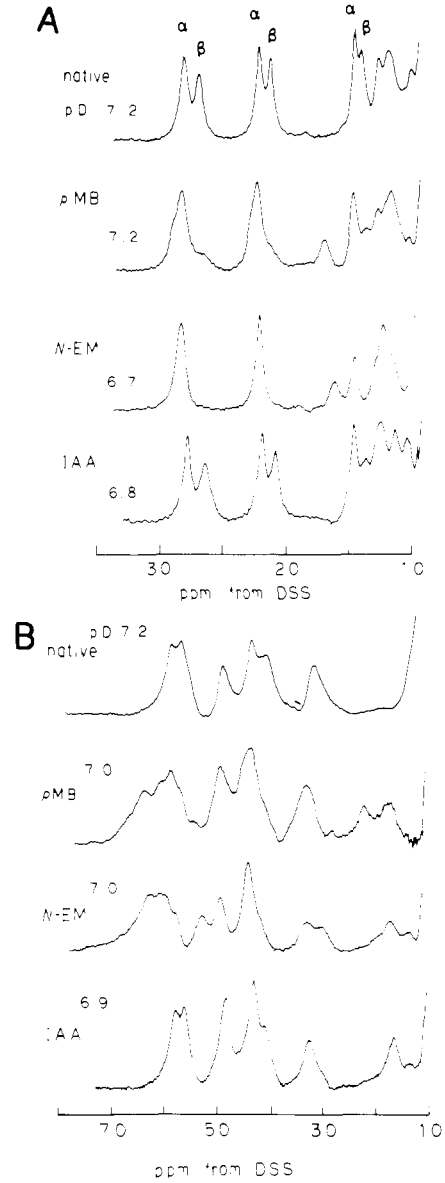

FIGURE 9: Effect of other sulfhydryl reagents bound to β93-cysteine on the NMR spectra. (A) Azide-methemoglobin and (B) formate-methemoglobin, both in 0.1 M phosphate at 21 °C. From top to bottom: native and *p*MB-, *N*-ethylmaleimide-, and iodoacetamide-treated methemoglobins.

shows that only the β heme methyl resonances of azide-methemoglobin are shifted downfield and that the $\alpha\beta$ heme methyl doublings around 21 and 28 ppm apparently disappeared upon binding with *N*-ethylmaleimide. The iodoacetamide treatment shifted the β heme methyl resonances of azide-methemoglobin to higher field by 0.1–0.4 ppm, accompanied with diminution of signal intensity. The effect of *N*-ethylmaleimide on the formate-methemoglobin spectrum was similar to that of *p*MB. It is thus likely that the spectral alteration is not characteristic of the presence of mercurial or bulky aromatic groups in *p*MB.

Discussion

p-Mercuribenzoate is known to induce changes in the visible absorption of methemoglobin derivatives similar to that induced by an allosteric effector, inositol hexaphosphate (Olson, 1976a). Upon the binding of *p*MB to methemoglobin derivatives, the optical density around 500 and 620 nm increases and that around 550 and 580 nm decreases. These visible absorption changes due to the binding of *p*MB indicated an increase of high-spin fraction in ferric heme iron on the basis

of a quantitative correlation between visible absorption bands and spin-state change for ferric hemoproteins (Smith & Williams, 1968). However, the visible spectral change of methemoglobin has not been definitely interpreted in terms of structure.

Since β93-cysteine lies near the $\alpha_1\beta_2$ subunit interface, it is reasonable to asssume that the binding of *p*MB would affect the conformation of the α subunit through the residue at the αβ subunit interface. This is what occurs in oxy- and deoxyhemoglobin with β93-cysteine bound with various N-substituted maleimides (Moffat, 1971). According to the X-ray analysis by Moffat (1971), the modification of β93-cysteine led to a tertiary structural change confined to the αβ subunit interface formed by the C helix of the α subunit and the FG corner of the β subunit 25 Å distant from β93-cysteine. The X-ray results on oxyhemoglobin also showed that the environments of the heme groups are almost entirely unaltered by treatment with the maleimides, suggesting that the major effect of the chemical modification is the introduction of the structural change at the αβ interface rather than at the direct heme vicinity. Imai (1973) reported a similar result. From observation of the ultraviolet difference and differential spectra of oxy- and deoxyhemoglobin treated with iodoacetamide and *N*-ethylmaleimide, he found that the conformation of an aromatic chromophore, probably β37-tryptophan at the $\alpha_1\beta_2$ interface, is changed by the chemical modifications. This observation was also interpreted to indicate a structural change at the subunit interface. Although the above observations suggest that the chemical modification of β93-cysteine affects the quaternary structure, they do not necessarily mean that the structural alteration is only in the quaternary level.

The present NMR results show that the structure around the β subunit is significantly altered by the binding of *p*MB, while that of the α subunit is unaffected in the tetramer and that the same is true of the isolated α subunits. It is therefore likely that, even if the quaternary structure of methemoglobin does change,[3] the environment of the α heme does not change as a consequence and that the *p*MB-induced NMR spectral change primarily reflects the localized tertiary change around the β heme. The NMR results suggest that the visible absorption change of methemoglobin derivatives upon *p*MB binding [Figure 5 of Olson (1976a)] is associated with the localized tertiary structural change around the β heme. Several previous observations suggested that the *p*MB binding might perturb only the β subunits in methemoglobin, but the present NMR data give the first direct demonstration for the preferential tertiary structural alteration in the β subunits.

The effect of the β93-cysteine modification on the methemoglobin structure is similar to the effect of hemoglobin treated with another sulfhydryl reagent, as shown in Figure 9. The β heme methyl resonances of azide- and formate-methemoglobins are significantly shifted downfield by treatment with *N*-ethylmaleimide. This finding is consistent with an increase in the high-spin fraction of the heme iron as proposed by Olson (1976a) from a study of the visible absorption spectra, because NMR formulation of the paramagnetic shift predicts the downfield shift of the resonance with increasing high-spin character of the heme iron (Dwek, 1973). Olson (1976a) noted that iodoacetamide binding to aquomethemoglobin produced a visible difference spectrum which is the inverse of those produced by *p*MB and *N*-ethylmaleimide treatments. Careful examination of the spectrum of azide-methemoglobin (Figure 9) shows that the β heme methyl resonances of native methemoglobin at 26.9, 22.1, and 13.8 ppm shifted to 26.4, 20.9, and 13.6 ppm upon iodoacetamide treatment. These small upfield shifts suggest an increase in the low-spin fraction of the heme iron and are in agreement with Olson's results for aquomethemoglobin.

A clue to the nature of the structural origin of the *p*MB-induced increase in the high-spin fraction of the heme iron may be given by a spectral comparison of the model complexes, protohemin(1- or 2-methylimidazole)N_3^-. Figure 4 shows that substitution of 1-methylimidazole with 2-methylimidazole in the mixed-ligand complex shifted the heme methyl resonances to lower field by about 20 ppm. The difference in the p*K* values of 1-methylimidazole (7.25) and 2-methylimidazole (7.86) (Hofmann, 1953) appears too small to account for such a large shift. The lower field shift of the heme methyl resonance in the 2-methylimidazole complex is rather due to a conformational difference such as tilting of imidazole ring[4] and/or lengthening of the bond between imidazole and heme iron. Thus, the observed downfield shift of the β heme methyl resonances due to binding of *p*MB to the azide complexes of methemoglobin and the isolated β subunit is expected to reflect the weakening of the interaction between the proximal β92-histidine and the heme iron.

Binding of pMB to the Isolated High-Spin β Subunit. Banerjee et al. (1969) reported from a visible absorption study that the high-spin fractions of the isolated aquomet β(SH) and β(*p*MB) chains are 0.65 and 0.95 at 1 °C in 1 M glycine, respectively. They showed that the visible spectral anomaly of the isolated β(SH) subunit disappeared either upon binding of *p*MB or upon asssociation with α(SH) subunit. They interpreted these results to indicate that the αβ subunit association affects the β heme electronic state similarly to the modification of β93-cysteine in the isolated β(SH) subunit.

The present NMR observations show that the spectral pattern of the isolated β(*p*MB) subunit in the ferric high-spin state is quite similar to that of the corresponding methemoglobin derivative, as shown in Figures 5, 6, and 8. However, the heme methyl resonances of the isolated β(*p*MB) subunit uniformly shifted to lower field compared with those of the corresponding methemoglobin derivative. The uniform downfield shifts suggest that the effects of the β93-cysteine modification and of the association of the αβ subunits on the β heme electronic state are not the same. Binding of *p*MB to the β(SH) subunit increases the high-spin fraction of the β heme iron to a greater extent than does the αβ subunit association. This is in contrast to the interpretation by Banerjee et al. (1969).

Contribution from the Distal Side on the Spectral Change of the Isolated β Subunit. Inspection of the isolated β subunit spectra with and without *p*MB in Figures 1c, 3B, 5c, and 8 shows that the extent of the spectral change due to binding of *p*MB is different among the derivatives and is the smallest for the isolated fluoride β subunit. Spectral changes which are apparently dependent on the size of the external ligands

[3] From the dimer formation constant of $K = 1.25 \times 10^{-4}$ M for *p*MB-bound methemoglobin reported by Olson (1976b), the fraction of the dissociated dimer, which is thought to be in oxyhemoglobin-like conformation, was calculated to be 0.16 under the NMR experimental condition of [tetramer] ≃ 1 mM.

[4] Gelin & Karplus (1977) and Warshel (1977) suggested that the change in the interaction between the tilted proximal histidine and pyrrole nitrogen is important to induce the quaternary transition of hemoglobin. It is thus likely that binding of *p*MB to methemoglobin induces a similar change in the tilt of the heme iron–histidine bond relative to the overall heme plane. This may explain the selective increase in the dissociation rate of water molecule from the β subunits of *p*MB-bound methemoglobin, which in turn is reflected by the increase in the rate of the azide binding to the β subunits [Table III of Olson (1976b)].

may be associated with a steric effect from the distal side. Dill et al. (1978) reported such an effect for hemoglobin complexed with alkyl isocyanide. They reported that the ^{13}C NMR chemical shifts of the iron-bound ethyl isocyanide are 173.17 and 171.57 ppm for the isolated β(SH) and β(pMB) chains, respectively. The difference in these ^{13}C chemical shifts was ascribed to the change of the contact between ethyl isocyanide and the G helix region of the subunits. In view of their observation, it is likely that some structural perturbation from the distal side upon the ligand–iron interaction is in part responsible for the proton NMR spectral difference of the isolated β(SH) and β(pMB) subunits.

In summary, the present NMR results show that the binding of pMB to β93-cysteine in methemoglobin induces a localized tertiary structural change in the β subunits accompanied by an increase in the high-spin fraction of the β heme iron. The study on the model porphyrin complex of azide-methemoglobin suggests that the increase in the high-spin character of the β heme iron is due to a conformational change of β93-cysteine to weaken the interaction between the proximal base and the β heme iron.

Acknowledgments

We thank Dr. T. Yonezawa for his continuing interest and encouragement.

References

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands*, Chapter 11, North-Holland Publishing Co., London.

Banerjee, R., & Cassoly, R. (1969) *J. Mol. Biol. 42*, 337–349.

Banerjee, R., Alpert, Y., Laterrier, F., & Williams, R. J. P. (1969) *Biochemistry 8*, 2862–2867.

Davis, D. G., Charache, S., & Ho, C. (1969) *Proc. Natl. Acad. Sci. U.S.A. 60*, 1403–1409.

Dill, K., Satterlee, J. D., & Richards, J. H. (1978) *Biochemistry 17*, 4291–4298.

Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry. Application to Enzyme Systems*, Chapter 3, Clarendon Press, Oxford.

Edelstein, S. J., & Gibson, Q. H. (1975) *J. Biol. Chem. 250*, 961–965.

Gelin, B. R., & Karplus, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 801–805.

Geraci, G., & Li, T.-K. (1969) *Biochemistry 8*, 1848–1854.

Geraci, G., Parkhurst, L. J., & Gibson, Q. H. (1969) *J. Biol. Chem. 244*, 4668–4676.

Gibson, Q. H. (1973) *J. Biol. Chem. 248*, 1281–1284.

Guidotti, G., & Konigsberg, W. H. (1964) *J. Biol. Chem. 239*, 1474–1484.

Hensley, P., Edelstein, S. J., Wharton, D. C., & Gibson, Q. H. (1975) *J. Biol. Chem. 250*, 952–950.

Hofmann, K. (1953) *Imidazoles and Its Derivatives*, Part I, Interscience, New York.

Imai, K. (1973) *Biochemistry 12*, 128–134.

Kilmartin, J. V., Fogg, J., Luzzana, M., & Rossi-Bernardi, L. (1973) *J. Biol. Chem. 248*, 7039–7043.

McLendon, G., & Sandberg, K. (1978) *J. Biol. Chem. 253*, 3913–3917.

Moffat, J. K. (1971) *J. Mol. Biol. 58*, 79–88.

Morishima, I., Neya, S., Inubushi, T., Yonezawa, T., & Iizuka, T. (1978) *Biochim. Biophys. Acta 524*, 307–316.

Neya, S., & Morishima, I. (1979) *J. Biol. Chem. 254*, 9107–9112.

Ogawa, S., Shulman, R. G., & Yamane, T. (1972) *J. Mol. Biol. 70*, 291–300.

Ogunmolar, G. R., Zipp, A. D., Chan, F., & Kautzmann, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1–4.

Olson, J. S. (1976a) *J. Biol. Chem. 251*, 441–446.

Olson, J. S. (1976b) *J. Biol. Chem. 251*, 447–458.

Olson, J. S., & Gibson, Q. H. (1971) *J. Biol. Chem. 246*, 5241–5253.

Perutz, M. F., Heidner, E. J., Ladner, J. E., Beetlestone, J. C., Ho, C., & Slade, E. F. (1974) *Biochemistry 13*, 2187–2200.

Riggs, A. (1961) *J. Biol. Chem. 236*, 1948–1954.

Smith, D. W., & Williams, R. J. P. (1968) *Biochem. J. 110*, 297–301.

Steinhardt, J., Ona-Pascual, R., Beychok, S., & Ho, C. (1963) *Biochemistry 2*, 256–266.

Taylor, J. F., Antonini, E., Brunori, M., & Wyman, J. (1966) *J. Biol. Chem. 241*, 241–248.

Warshel, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1789–1793.