aromatic diimine complexes.[10,11] The compound Fe(GMI)$_3^{2+}$ can be oxidized by strong oxidizing agents (*e.g.*, Ce(IV)).

We now present some evidence for the reaction between $HO_2\cdot$ radicals and Fe(GMI)$_3^{2+}$. In Figure 1 the changes in concentration of hydrogen peroxide (Figure 1a), complex (Figure 1b), and evolution of oxygen (Figure 1c) with time are shown. The rate of decomposition of hydrogen peroxide decreases as the concentration of complex increases, and Fe(III) decreases. When all the colored species are destroyed, the decomposition of hydrogen peroxide catalyzed by iron(III) is observed. The rate of oxygen evolution decreases as the concentration of complex increases. This slower rate of oxygen evolution can be easily explained in the light of the mechanism proposed by Barb, *et al.*,[12] for the decomposition of hydrogen peroxide by Fe(III) ions.

$$\text{Fe(III)} + H_2O_2 \longrightarrow \text{Fe(II)} + HO_2\cdot + H^+ \quad (1)$$

$$\text{Fe(II)} + H_2O_2 \longrightarrow \text{Fe(III)} + HO\cdot + HO^+ \quad (2)$$

$$HO\cdot + H_2O_2 \longrightarrow HO_2\cdot + H_2O \quad (3)$$

$$HO_2\cdot + \text{Fe(III)} \longrightarrow H^+ + \text{Fe(II)} + O_2 \quad (4)$$

$$HO_2\cdot + \text{Fe(II)} \longrightarrow HO_2^- + \text{Fe(III)} \quad (5)$$

By this mechanism, the reaction between $HO_2\cdot$ radicals and Fe(III) is responsible for the oxygen evolution (eq 4). To slow the rate of oxygen evolution, the complex must compete with Fe(III) for $HO_2\cdot$ radicals. Thus the complex is oxidized to Fe(GMI)$_3^{3+}$, regenerating hydrogen peroxide (*cf.* eq 5) and acting as an $HO_2\cdot$ radical trap. The ferric complex so formed can undergo an internal redox reaction[9] producing a radical at the ligand. The complex–radicals[13] which are generated may react with $HO_2\cdot$ or $HO\cdot$ radicals to form the labile ligand-oxidized species.

As the formal electrode potential of the couple $HO_2\cdot|H_2O_2$ is approximately 1.5 V,[14] it is reasonable to assume that the oxidation of Fe(GMI)$_3^{2+}$ is by $HO_2\cdot$ radicals. This reaction must be very fast since the rate of electron exchange in analogous systems, *e.g.*, between Fe(phen)$_3^{2+}$ and Fe(phen)$_3^{3+}$ is very high[15] ($3 \times 10^8\ M^{-1}\ \text{sec}^{-1}$) (*cf.* Figure 1b).

Attempts to oxidize Fe(GMI)$_3^{2+}$ with $HO\cdot$ radicals gave a very low yield of the labile ligand-oxidized complexes. However, these experiments are not completely analogous since the acid concentration is much higher (0.5 *M* H_2SO_4) than that of the iron(III) experiments (pH 2.4). These radicals were generated by treating hydrogen peroxide with excess iron(II)[16] (in the presence of complex).

The rate laws deduced, including a reaction between the complex and $HO\cdot$ radicals and the reactions of the Barb, *et al.*, mechanism,[12] seem not to be compatible with our preliminary kinetic data. This observation indicates that a simple reaction between the complex and $HO\cdot$ radicals is not likely to be in the main reaction path.

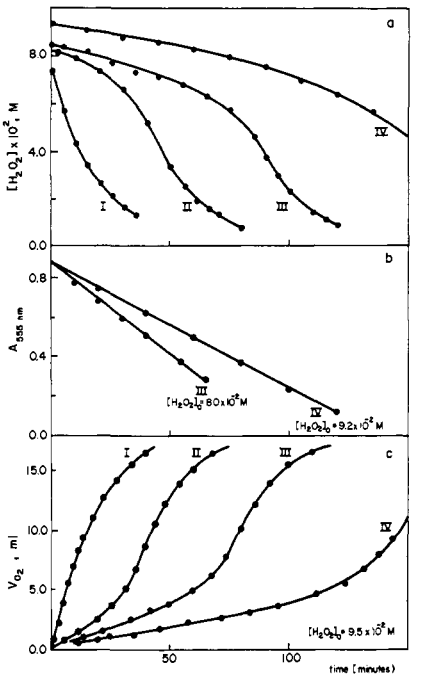

Figure 1. Plots of (a) hydrogen peroxide concentration *vs.* time, (b) absorbance at 555 nm (absorption maximum of Fe(GMI)$_3^{2+}$, $\epsilon_{555} = 8.8 \times 10^3$) *vs.* time, and (c) volume of oxygen evolved *vs.* time. All data refer to reactions at 35.0° and pH 2.4 (H_2SO_4). Concentration of iron(III) is the same in experiments I, II, and III ($4.2 \times 10^{-3}\ M$), and $2.1 \times 10^{-3}\ M$ in experiments IV. Concentration of Fe(GMI)$_3^{2+}$ is the same in experiments III and IV ($1.0 \times 10^{-4}\ M$) and $5.0 \times 10^{-5}\ M$ in experiment II. Experiments I contain no Fe(GMI)$_3^{2+}$.

The kinetics of this reaction are being carefully measured. The effect of Fe(GMI)$_3^{2+}$ and of other diimine complexes on other catalysts of the hydrogen peroxide decomposition is under investigation.

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(10) W. W. Brandt, F. P. Dwyer, and E. C. Gyarfas, *Chem. Rev.*, **54**, 959 (1954).
(11) P. Krumholz, *Struct. Bonding (Berlin)*, **9**, 139 (1971).
(12) W. G. Barb, J. H. Baxendale, P. George, and K. R. Hargrave, *Trans. Faraday Soc.*, **47**, 591 (1951).
(13) The presence of these complex–radicals was detected by the polymerization of acrylonitrile added to the reaction mixture. The red polymer obtained contains molecules of the complex chemically attached to it.
(14) P. B. Sigler and B. J. Masters, *J. Amer. Chem. Soc.*, **79**, 6353 (1957); P. S. Rao and E. Rayon, *Biochem. Biophys. Res. Commun.*, **51**, 468 (1973).
(15) I. Ruff and M. Zimonyi, *Electrochim. Acta*, **18**, 515 (1973).
(16) W. G. Barb, J. H. Baxendale, P. George, and K. R. Hargrave, *Trans. Faraday Soc.*, **47**, 462 (1951).

Nuclear Magnetic Resonance Studies of Hemoproteins. Unusual Temperature Dependence of Hyperfine Shifts and Spin Equilibrium in Ferric Myoglobin and Hemoglobin Derivatives[1]

Sir:

Nuclear magnetic resonance (nmr) spectroscopy of paramagnetic macromolecules has recently developed into a powerful tool for investigating structure and structure–function relationships in metalloproteins such

(1) A part of our systematic investigation on nuclear magnetic resonance studies of hemoproteins and hemoenzymes.

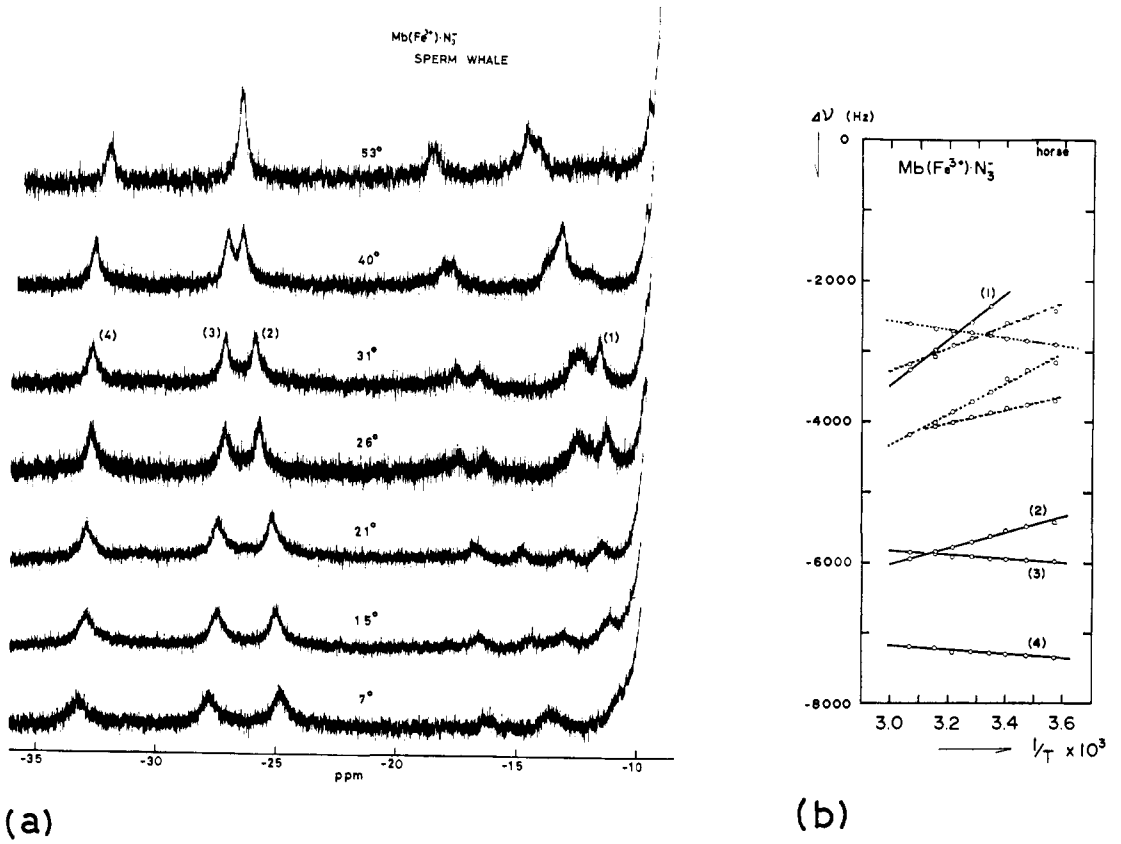

Figure 1. (a) Hyperfine shifted proton resonances (at 220 MHz) of heme side chain at various temperatures for sperm whale azidoferrimyoglobin (Mb·N_3^-). (b) Plots of the shifts *vs.* $1/T$ for horse Mb·N_3^-.

as iron–heme proteins[2] and iron–sulfur proteins.[3] It has been demonstrated that hyperfine shifted resonances are sensitive probes for elucidating the environment of the metal ion, for example, in detecting cooperativity in hemoglobins,[4] elucidating the tertiary structure of myoglobin, and characterizing the ligand binding and configuration around the heme iron.[2,5] This is the advantage of studying such metal-containing proteins.

Most of the nmr studies on ferric hemoproteins so far reported have been performed with the cyanide complexes of myoglobin(Mb),[2] hemoglobin(Hb),[2,4] and cytochrome c[3] and b_5[6] with purely low spin state. However, some of the ferric proteins such as azide, imidazole, and hydroxide complexes of ferric myoglobin and hemoglobin, which have been found to exhibit a temperature-dependent spin equilibrium between high spin ($S = {}^5/_2$) and low spin ($S = {}^1/_2$) states by magnetic susceptibility measurement,[7,8] have been open to the nmr investigation.[9]

We wish to report here one of the initial aspects of systematic investigation by the 220-MHz nmr[10] of the thermal mixing of high and low spin states in azide, imidazole, and deuterioxide complexes of sperm whale and horse heart myoglobin and human hemoglobin.[11]

In Figures 1 and 2 are exemplified the temperature dependence of hyperfine-shifted resonances of the heme side chain in azide and imidazole complexes of sperm whale and horse ferrimyoglobins (Mb·N_3^- and Mb·Im), respectively. Basic features of nmr signals for these examples are similar to those of cyanide complexes which have been well established.[2] However, hyperfine-shifts are larger and the signal lines are broader than those of Mb·CN^-. All the ring methyl signals are observed at high temperatures for both complexes except for sperm whale Mb·Im where the ring methyl signal (1) was not detected even at 60°. The most striking feature of Figures 1 and 2 is the abnormal temperature dependences of the higher field two methyl signals designated (1) and (2) in both complexes, show-

(2) K. Wuthrich, *Struct. Bonding (Berlin)*, **8**, 53 (1970).
(3) C. C. McDonald and W. D. Phillips, *Biochemistry*, **12**, 3170 (1973).
(4) S. Ogawa and R. G. Schulman, *J. Mol. Biol.*, **70**, 315 (1972); S. Ogawa, R. G. Schulman, M. Fujiwara, and T. Yamane, *ibid.*, **70**, 301 (1972).
(5) K. Wuthrich, "Probes of Structure and Function of Macromolecules and Membranes," Vol. 2, B. Chance, T. Yonetani, and A. S. Mildvan, Ed. Academic Press, New York, N. Y., 1971.
(6) R. Keller, O. Groudinsky, and K. Wuthrich, *Biochim. Biophys. Acta*, **328**, 233 (1973).
(7) J. Beetlestone and P. George, *Biochemistry*, **3**, 707 (1964).
(8) (a) T. Iizuka and M. Kotani, *Biochim. Biophys. Acta*, **181**, 275 (1969); (b) *ibid.*, **194**, 351 (1969).
(9) Features of nmr spectra of azidoferrimyoglobin have been quite briefly mentioned by Wuthrich.[2] The nmr study of spin equilibrium has been reported for the heme–pyridine–water system by Degani and Fiat (H. A. Degani and D. Fiat, *J. Amer. Chem. Soc.*, **93**, 4281 (1971)), and for the heme–pyridine–methanol system by Hill and Morallee (H. A. O. Hill and K. G. Morallee, *ibid.*, **94**, 731 (1972). However, spin-state interconversions in these iron(III) hemin–pyridine systems have been shown to be related to a ligand substitution process on iron(III); see J. K. Beattie and R. J. West, *ibid.*, **96**, 1933 (1974), and G. N. LaMar and F. A. Walker, *ibid.*, **94**, 8607 (1972).
(10) Proton nmr spectra were obtained on a Varian HR-220 at 220 MHz (Department of Hydrocarbon Chemistry, Kyoto University) equipped with a variable temperature unit and a Varian C-1024 time averaging computer. Chemical shifts are expressed in parts per million from internal sodium 2,2,3,3-tetradeuterio-3-(trimethylsillyl)propionate.
(11) For the proton nmr measurements, approximately 7 mM solutions of the lyophilized ferric proteins in 0.01 M deuterated phosphate buffer (pD 7.0) were prepared. The ratios of external ligands to heme concentrations were 10:1 and 50:1 for azide and imidazole complexes, respectively. Solutions of the deuterioxide complex of myoglobin were obtained at pD 10.2 by the addition of small amount of 4 M glycine–NaOD buffer, pD 11.7.

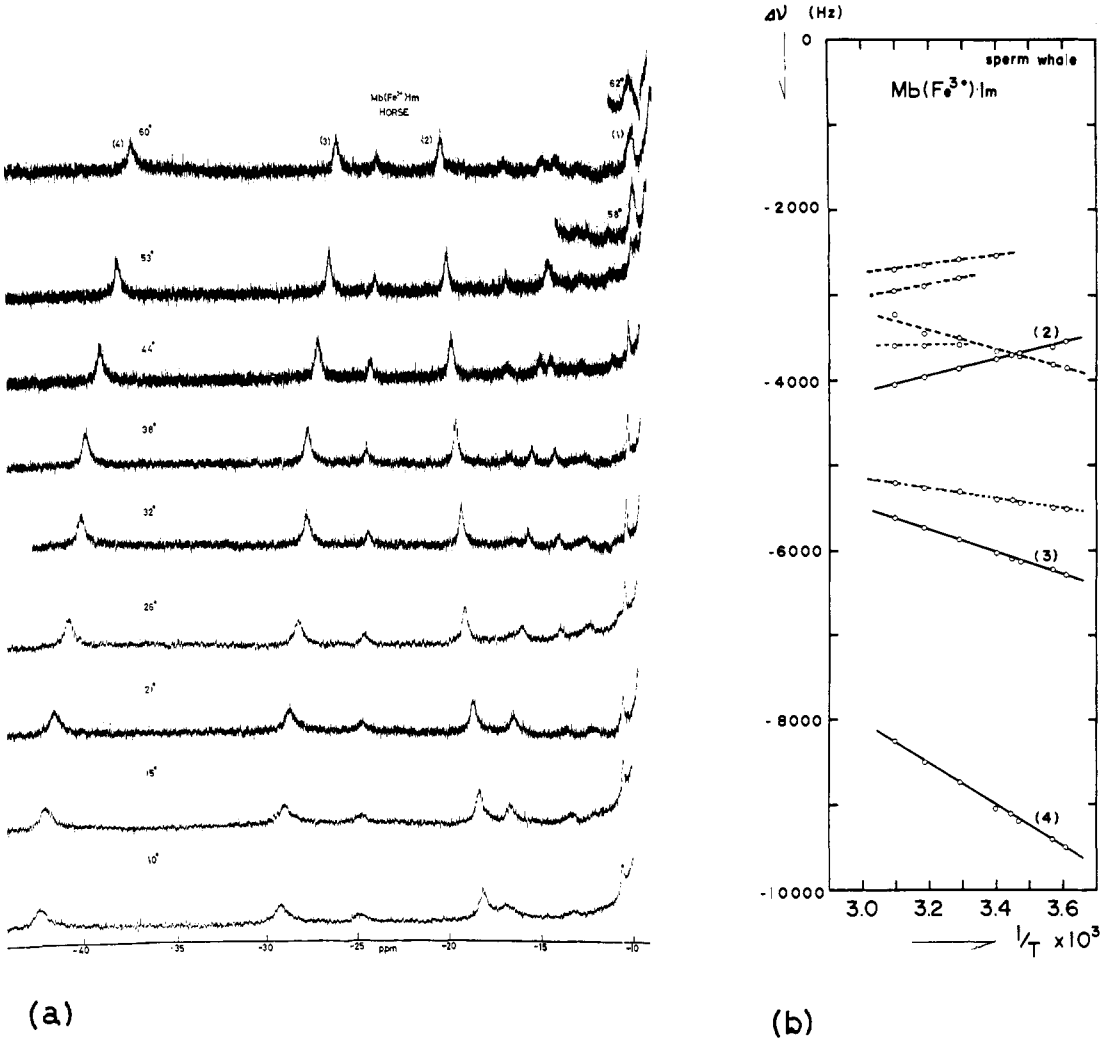

Figure 2. (a) Hyperfine shifted proton resonances (at 220 MHz) at various temperatures for imidazole complex of horse ferrimyoglobin. (b) Plots of the shifts *vs.* $1/T$ for sperm whale Mb·Im.

ing drastic deviations from Curie's law. Some of the hyperfine-shifted signals, other than the ring methyl groups which can most probably be asssigned to the protons of vinyl heme substituent and of the imidazole ring of the axial histidine, exhibit similar abnormal behavior (see the dotted lines in Figures 1b and 2b). Similar unusual temperature dependences were also found for all of the hyperfine-shifted signals of myoglobin deuterioxide (Mb·OD^-, see Figure 3).[12]

These observations are consistent[7,8] with the mixing of a high spin state ($S = 5/2$) as the temperature increases,[13] the rate of exchange[13a] between the two spin states being very much greater than the frequency shift difference of the protons in the two environments, so that a single resonance is observed for each set of protons. The methyl resonances of the high spin complex of ferrimyoglobin (Mb·D_2O, $S = 5/2$) exhibit quite a large downfield shift between −30 and −90 ppm, as has been reported by Kurland, *et al.*[14] Therefore, the mixing of a high spin state when the temperature is raised could lead to downfield shift of the proton signals in Mb·N_3^-, Mb·Im, and Mb·OD^- in proportion to the state of the mixture[13] and the frequency shift difference of the two spin states. The observation that two of the four methyl signals, (1) and (2), in Mb·N_3^- and Mb·Im show non-Curie's law behavior may lead us to expect quite a large difference for these methyl protons in purely high and low spin states; the methyl resonance (1) in Mb·N_3^- and Mb·Im would appear at an appreciably high and a very low field in purely low and high spin states, respectively. Mixing of a high spin state with a low spin state could therefore cause a down-

(12) Since the resonance lines were very much broadened (line width, *ca.* 700 Hz) due to a large high spin content (about 70%),[7] signal accumulation of more than 300 transients was performed to improve the signal-to-noise ratio. Positions of these signals were found to be highly sensitive to the pD values, which is considered to result from a slight amount of residual acidic form (Mb·D_2O, $S = 5/2$). The high spin complex (acidic form) causes a large downfield hyperfine shift.[14] Therefore, a very small amount of residual acidic form would shift the signals downfield for the alkaline form (Mb·OD^-) adjusted to pD 10.2. Details of the nmr spectra of Mb·OD^- (horse and sperm whale) will be published shortly (T. Iizuka and I. Morishima, *Biochim. Biophys. Acta*, in press).

(13) The low spin content varies from 0.86 to 0.70 and from 0.94 to 0.48 for sperm whale Mb·N_3^- and Mb·Im, respectively, when the temperature is raised from 10 to 60° (see ref 8).

(13a) Note Added in Proof. Recently Beattie and West have reported that the rate of change of spin state in ferric myoglobin hydroxide is greater than 2×10^5 sec^{-1}, obtained by temperature-jump relaxation method (J. K. Beattie and R. J. West, *J. Amer. Chem. Soc.*, **96**, 1933 (1974)).

(14) R. J. Kurland, D. G. Davis, and C. Ho, *J. Amer. Chem. Soc.*, **90** 2700 (1968).

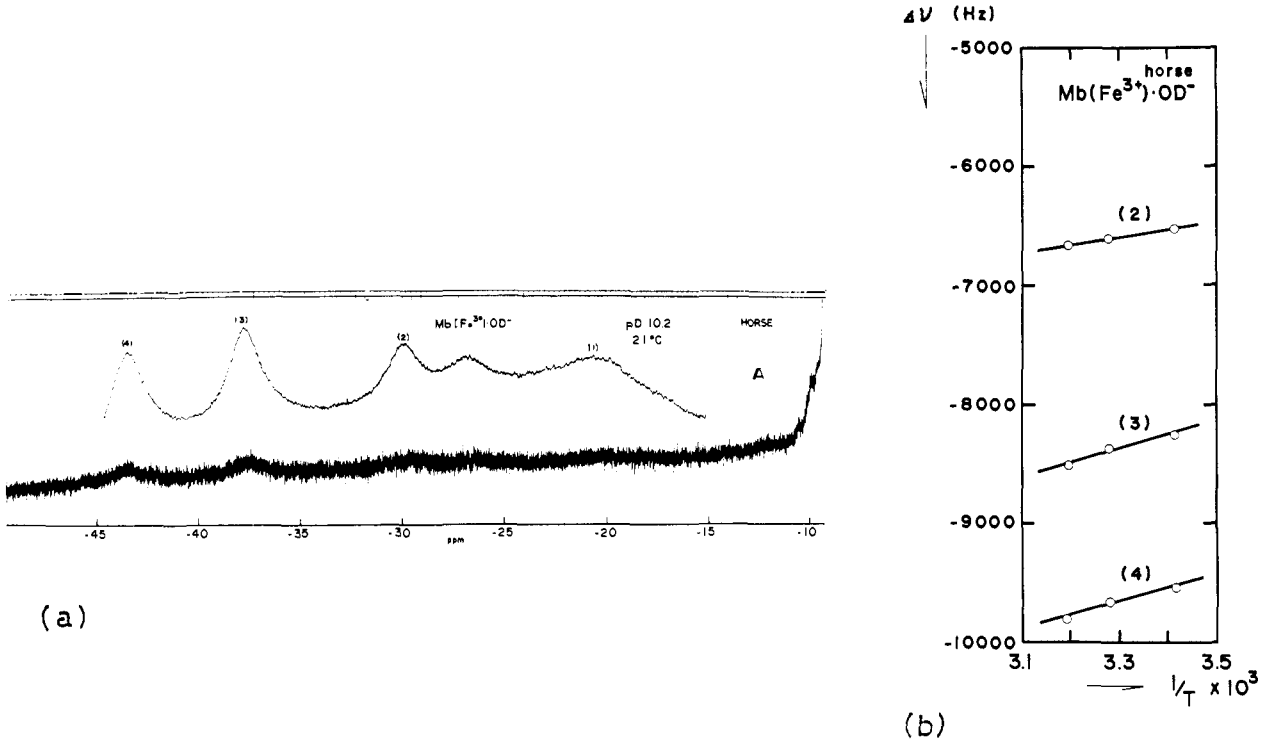

Figure 3. (a) Hyperfine shifted proton resonances (at 220 MHz) of a deuterioxide complex of horse ferrimyoglobin (Mb·OD^-) at pD 10.2 and 21°. The S/N ratio was improved by accumulation of 300 transients (upper trace). The assignment of these signals is not established. (b) Plots of the shifts *vs.* $1/T$ for horse Mb·OD^-.

field shift with raising of the temperature, overcoming the upfield shift resulting from normal Curie's law behavior. These two opposite temperature-dependent shifts appear to be compatible for the methyl signals (3) and (4) in Mb·N_3^- (see Figure 1). This may be also the case for the methin protons (dotted lines in Figures 1 and 2).

It should be noted that the resonance position of hyperfine-shifted signals are quite different between azide and imidazole complexes even at 33° where the low spin content is equal (79%)[8,13] for both complexes. This clearly shows that there is a substantial effect of different axial ligand on the hyperfine shifts of the heme group in purely low spin or high spin states. Temperature dependence of low and high spin contents for sperm whale Mb·N_3^- and Mb·Im obtained by magnetic susceptibility measurement[7,8,13] and linear plots of hyperfine shifts *vs.* $1/T$ allowed us to obtain the limiting values of the shifts for purely low and high spin states in Mb·N_3^- and Mb·Im,[15] which supports the above qualitative interpretation. This implies that the resonance positions in Mb·CN^- ($S = {}^1/_2$) and Mb·D_2O ($S = {}^5/_2$) should not be used as the reference for interpreting the hyperfine shifts resulting from the mixed spin states.[15]

It is also of interest to take a look at the temperature dependence of the ring methyl signals of azide and imidazole complexes of human hemoglobin. The methyl signals in Hb·N_3^- at about −15 and at −25 ppm, which exhibit doubling due to the environmental difference of heme between α and β subunits of hemoglobin,[16] obey Curie's law. The methyl signal at −15 ppm, however, shows a non-Curie's law shift and is split into two peaks at temperatures higher than 25°. This is also due to mixing of a high spin state when the temperature is raised, which has been evidenced by magnetic susceptibility measurement[8b] to be the case for Mb·N_3^-. All of the hyperfine-shifted signals of the imidazole complex (Hb·Im) showed normal temperature-dependent shift, corresponding to the finding by magnetic susceptibllity measurement[8b] that Hb·Im is in a purely low spin state.

From the above results and discussion it could be concluded that proton nmr serves as a very sensitive probe for studying spin equilibrium between low and high spin states in hemoproteins. More detailed discussions on each complex of ferric myoglobin and hemoglobin will appear separately elsewhere.[17]

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(15) The hyperfine shift for the complex with the mixed spin state is given by the equation, $\Delta\nu = \alpha\Delta\nu^{LS} + (1 - \alpha)\Delta\nu^{HS}$, where α is the low spin content, $\Delta\nu$ is the observed shift, and $\Delta\nu^{LS}$ and $\Delta\nu^{HS}$ are the limiting shifts in purely low and high spin states, respectively. The α and $\Delta\nu$ values depend on temperature. The use of the α value at any temperature obtained by magnetic susceptibility measurement[7,8,13] and the linear plots of $\Delta\nu$ *vs.* $1/T$ allowed us to obtain the $\Delta\nu^{LS}$ and $\Delta\nu^{HS}$ values for Mb·N_3^- and Mb·Im. The $\Delta\nu^{HS}$ values for Mb·N_3^- are −11,850 Hz(methyl(1)), −12,180(2), −9220(3), and −11,040(4) at 33°. The corresponding values of $\Delta\nu^{LS}$ are −240, −3940, −5160, and −6050, Hz, respectively. The $\Delta\nu^{HS}$ for sperm whale Mb·Im are −5740 Hz (methyl(2)), −6100(3), and −8610(4) at 33°. The corresponding values of $\Delta\nu^{LS}$ are −3690, −5820, and −8750 Hz, respectively. Details of determination of the $\Delta\nu^{HS}$ and $\Delta\nu^{LS}$ values for the mixed spin state will be presented in a separate paper (T. Iizuka and I. Morishima, *Biochim. Biophys. Acta*, in press).

(16) D. G. Davis, S. Charache, and C. Ho, *Proc. Nat. Acad. Sci. U. S.*, 63, 1403 (1969).

(17) I. Morishima and T. Iizuka, to be submitted for publication.

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Poly(U)-Directed Transamidation between Adenosine 5′-Phosphorimidazolide and 5′-Phosphoadenosine 2′(3′)-Glycine Ester

Sir:

Many derivatives of adenosine form organized triple-helical structures with polyuridylic acid (poly(U)) in which one column of molecules of the adenosine derivative is held in position by two chains of poly(U).[1] We have shown that adenosine 5′-phosphorimidazolide (ImpA) forms such a structure at 0°, and then undergoes template-directed polymerization to form short 2′–5′-linked oligoadenylic acids.[2] More recently we have confirmed that the 2′(3′)-glycyl ester of adenosine 5′-phosphate (pA-Gly) forms a similar helix melting at 17.5°[3] and have discovered a novel template-directed reaction between ImpA and pA-Gly.

We prepared two mixtures (pH 7.0), each containing 0.0125 *M* ImpA-**8**-^{14}C (specific activity 0.8 mCi/mmol), 0.0125 *M* pA-Gly, 0.075 *M* $MgCl_2$, and 0.20 *M* NaCl. Mixture 1 contained in addition 0.05 *M* poly(U), while mixture 2 served as a template-free control. The mixtures were held at 0° and their pH's maintained at 7.0 by titration with NaOH. Aliquots were withdrawn at various times for analysis. Each aliquot was subjected to electrophoresis in a 0.03 *M* potassium phosphate buffer (pH 7.1) and to chromatography in system I, 1-propanol–concentrated ammonia–water (55:10:35, v/v), and system II, 95% ethanol–1 *M* ammonium acetate (pH 7.5) (7:3, v/v).

In reaction 2 we observed a slow disappearance of ImpA, and the appearance of two new radioactive compounds which we readily identified as adenylic acid (pA) and P_1P_2-diadenosine 5′-pyrophosphate (AppA).[4] In reaction 1 we noted a much more rapid disappearance of ImpA and the appearance of a major new radioactive product (compound I) which failed to move from the origin on chromatography in system II, and had an electrophoretic mobility of 0.85 on electrophoresis at pH 7.1 (adenosine, 0.0; pA, 1.0). In the ammonia-containing system I, a mixture of radioactive adenylyl-(5′ → *N*)-glycine (Gly-N-pA) and adenylyl-(5′ → αN)-glycinamide ($NH_2C(=O)CH_2NHpA$) was formed in a total amount that roughly corresponded to the yield of compound I determined in the other systems.

These observations clearly point to the structure pA-2′(3′)-Gly-N-pA for compound I. This was confirmed by showing that it yielded equimolar amounts of pA and Gly-N-pA when treated with aqueous alkali (pH 12, 20°, 4.5 hr). The hydrolysis products pA and Gly-N-pA were identified by paper chromatography in system II and in a mixture of isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2), as well as by electrophoresis at pH 7.1. Furthermore, compound I could be prepared by the action of a water-soluble carbodiimide (1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride) on an aqueous solution containing equimolar quantities of pA and Gly-N-pA, with or without poly(U), but preferably in the presence of imidazole buffer (see below).

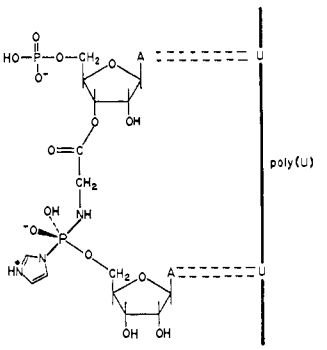
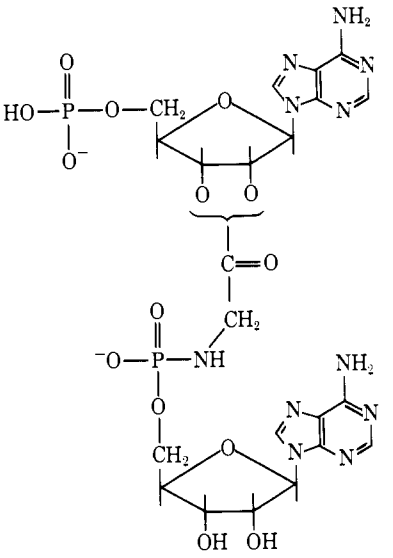


Figure 1. Proposed transition state for the template-directed transamidation reaction. The broken lines joining A and U represent Watson–Crick hydrogen bonds. A second poly(U) chain has been omitted in order to simplify the diagram.



Our quantitative studies showed that after 2 hr at 0° the yield of compound I was 55.8% in reaction 1 and 1.5% in reaction 2. After 24 hr the yields were 79.1 and 5.2%, respectively. These results demonstrate that the incorporation of pA-Gly and ImpA in an organized helix with poly(U) brings the NH_2 group of glycine sufficiently close to the phosphate residue of ImpA to permit a relatively rapid transamidation reaction at 0° (Figure 1). In our experiments the template increased the rate of reaction by a factor of at least 35. This ratio has no particular significance—it would be larger in more dilute solutions and smaller in more concentrated solutions.

The carbodiimide-induced condensation of Gly-N-

(1) F. B. Howard, J. Frazier, M. F. Singer, and H. T. Miles, *J. Mol. Biol.*, **16**, 415 (1966).
(2) B. J. Weimann, R. Lohrmann, L. E. Orgel, H. Schneider-Bernloehr, and J. E. Sulston, *Science*, **161**, 387 (1968).
(3) To be submitted for publication.
(4) J. Sulston, R. Lohrmann, L. E. Orgel, and H. T. Miles, *Proc. Nat. Acad. Sci. U. S.*, **59**, 726 (1968).