NMR analysis. Uncorrected percentage yields (Table I) are determined by cutting and weighing five copies of each spectrum and combining this information with the cis/trans ratio determined by GC. Since the small amount (5.7%) of **1b** in starting material increases to ~20% by the end of the reaction, the "uncorrected" data are corrected to zero time and 100% **1a** as starting material, to reflect participation of this "wrong" isomer in the dimerization. Note that in calculating the rate constants for e_1 and t_1 processes, the percentage values must first be divided by two, the statistical degeneracy of two equivalent rotations.

Product distribution within each series, erythro or threo, is predicted quite accurately (Table I) by a simple statistical model, the basic assumption behind which is total independence of α,β, and α',β' bond rotations. This assumption is antithetical to the theory of orbital symmetry control in concerted reactions. If x is the probability of observable rotation (and $1 - x$ the probability of no rotation) about one of the two identical bonds, the statistical distribution of products from zero, one, and two rotations is $(1 - x)^2{:}2x(1 - x){:}x^2$, respectively. Best values of x are 0.38 at 209.5 and 0.41 at 246.4 °C in the erythro series, 0.34 and 0.36 in the threo series, respectively (in a hypothetical freely rotating diradical $x = 0.50$). The higher values of x at 246.4 °C correspond to more rotation, consistent with an activation energy barrier. The slightly lower values of x in the threo series and formation of trans products in amounts slightly higher than predicted indicate a kinetic preference for trans. The origin of the slight favoring of erythro processes is unclear to us.

Cycloreversion of the cyclobutane **2d** has been examined in the gas phase from 238.8 to 290 °C. At 257 °C, where cleavage is approximately twice as fast as geometrical isomerization, acrylonitrile recovered at early points comprises 60.2% **1c** (Scheme II), corresponding to an excess of the s + s process. Excess retention of configuration is consistent with most other studies of cyclobutane pyrolysis.[3] Simple reversal of those dimerization processes ($e_0 + e_2 + t_1$) leading to cis products, when corrected to 257 °C, would predict 61.3% retention.

Our findings are inconsistent with a stereorandom process and unsupportive of even a vestige of concerted $[{}_{\pi}2_s + {}_{\pi}2_a]$ reaction. They are compatible with a statistically controlled process in which nonrotation is favored over rotation by factors of 1.44–1.94. If the results were to be discussed in the terms of the widely current diradical model,[2] these factors would correspond to ratios of rates of closure to rotation of 0.88–1.88. Yet unresolved is the question of whether hypothetical intermediary diradicals have antiperiplanar conformations and fates then determined by relative rates of cleavage, rotation about the α,β and α',β' bonds, and rotation about the β,β' bond to continuous diradicals.

Acknowledgments. We thank the National Science Foundation and the John Parker Bequest for their support of this work.

References and Notes

(1) (a) H. Fujimoto and T. Sugiyama, *J. Am. Chem. Soc.*, **99**, 15–22 (1977); (b) G. A. Segal, *J. Am. Chem. Soc.*, **96**, 7892–7898 (1974); (c) J. S. Wright and L. Salem, *Ibid.*, **94**, 322–329 (1972); (d) R. Hoffmann, S. Swaminathan, B. G. Odell, and R. Gleiter, *Ibid.*, **92**, 7091–7097 (1970); R. B. Woodward and R. Hoffmann, *Angew. Chem., Int. Ed. Engl.*, **8**, 781–853 (1969).
(2) (a) J. E. Baldwin, "Pericyclic Reactions", Vol. II, A. P. Marchand and R. E. Lehr, Ed., Academic Press, New York, N.Y., 1977, pp 273–302; (b) G. Scacchi, C. Richard, and M. H. Back, *Int. J. Chem. Kinet.*, **9**, 513–524 (1977); (c) G. Scacchi and M. H. Back, *Ibid.*, **9**, 525–534 (1977); (d) P. D. Bartlett and J. B. Mallet, *J. Am. Chem. Soc.*, **98**, 143–153 (1976), and references cited therein; (e) P. D. Bartlett, K. Hummel, S. P. Elliott, and R. A. Minns, *Ibid.*, **94**, 2898–2899 (1972); (f) P. D. Bartlett, G. M. Cohen, S. P. Elliott, K. Hummel, R. A. Minns, C. M. Sharts and J. Y. Fukunaga, *Ibid.*, **94**, 2899–2902 (1972); (g) A. Padwa, W. Koehn, J. Masaracchia, C. L. Osborn, and D. J. Trecker, *Ibid.*, **93**, 3633–3645 (1971).
(3) H. R. Gerberich and W. D. Walters, *J. Am. Chem. Soc.*, **83**, 3955–3939, 4884–4888 (1961); (b) R. Srinivasan and J. N. C. Hsu, *J. Chem. Soc., Chem. Commun.*, 1213–1214 (1972); (c) A. T. Cocks and H. M. Frey, *Chem. Commun.*, 458–459 (1969); (d) see, however, J. E. Baldwin and P. W. Ford, *J. Am. Chem. Soc.*, **91**, 7192 (1969).
(4) (a) E. C. Coyner and W. S. Hillman, *J. Am. Chem. Soc.*, **71**, 324–326 (1949); (b) Y. N. Shuraeva and V. Y. Katsobashvili, *Zh. Org. Khim.*, **11**, 923–926 (1975).
(5) R. E. Davis, M. R. Willcott III, R. E. Lenkinski, W. von E. Doering, and L. Birladeanu, *J. Am. Chem. Soc.*, **95**, 6846–6848 (1973).
(6) C. N. Banwell and N. Sheppard, *Mol. Phys.*, **3**, 351 (1960).
(7) We gratefully acknowledge the invaluable help of Dr. B. W. Bangerter, who developed the hardware and techniques for deuterium decoupling in these spectra.
(8) W. Hartman, *Chem. Ber.*, **102**, 3974–3984 (1969).
(9) W. von E. Doering and K. Sachdev, *J. Am. Chem. Soc.*, **96**, 1168–1187 (1974).

W. von E. Doering,* Catherine A. Guyton
Department of Chemistry, Harvard University
Cambridge, Massachusetts 02138
Received December 21, 1977

A Deuterium-Labeling Method for the Assignment of Histidine Nuclear Magnetic Resonance Peaks of Proteins

Sir:

A series of extensive NMR studies by Jardetzky and coworkers has demonstrated that ^{1}H NMR peaks of histidines give invaluable information about the structure of proteins in solution once each individual resonance can be assigned to a particular histidine residue in the amino acid sequence.[1] Differential deuterium exchange has been used in combination with enzymatic modification for the assignment of the histidine C-2 H peaks of ribonuclease A.[2-4] Markley and Kato[5] have developed a differential deuterium exchange technique, and used it for the assignment of the C-2 H peaks of the two histidine residues in soybean trypsin inhibitor. To determine the level of exchange at histidines in definite residue positions, they used cyanogen bromide to cleave the differentially deuterated protein into two large fragments each of which contains a single histidine residue. With large peptide fragments such as used in this method, however, the histidine C-2 H proton signals are very broad, and the C-4 H proton signals which would be used as an appropriate standard of intensity measurements are obscured by the envelope of a large number of aromatic proton signals; moreover, resonances from slowly exchangeable hydrogens may exist in the same spectral region, making the intensity measurements less reliable. Therefore, it appears that a more general method is needed, particularly to deal with larger proteins.

It has been shown that a tritium-labeling method, which is a combination of Matsuo and Narita's method[6] involving differential tritium exchange at the C-2 H position of histidines and ^{1}H NMR of differentially deuterated proteins, can be a general method for the assignment of the histidine NMR peaks.[7,8] This communication reports a deuterium-labeling method which is a modification of the tritium-labeling method. In this modification, tritium is replaced by deuterium for the analysis of the level of exchange at histidines in definite residue positions. Thus, the use of tritium becomes unnecessary, and at the same time any ambiguity which may result from the isotope effect in the tritium-labeling method is cleared away. In the deuterium-labeling method, differentially deuterated proteins are cleaved by trypsin into smaller peptides each containing a single histidine residue, which are separated in a conventional way using paper electrophoresis and chromatography, and finally extracted from the filter paper. Smaller histidine peptides thus obtained give sharp and well-resolved C-2 as well as C-4 H proton NMR peaks. Therefore, even in the case of large proteins such as used in the present experiment, ^{1}H NMR can be used very effectively to determine ac-

0002-7863/78/1500-3230$01.00/0

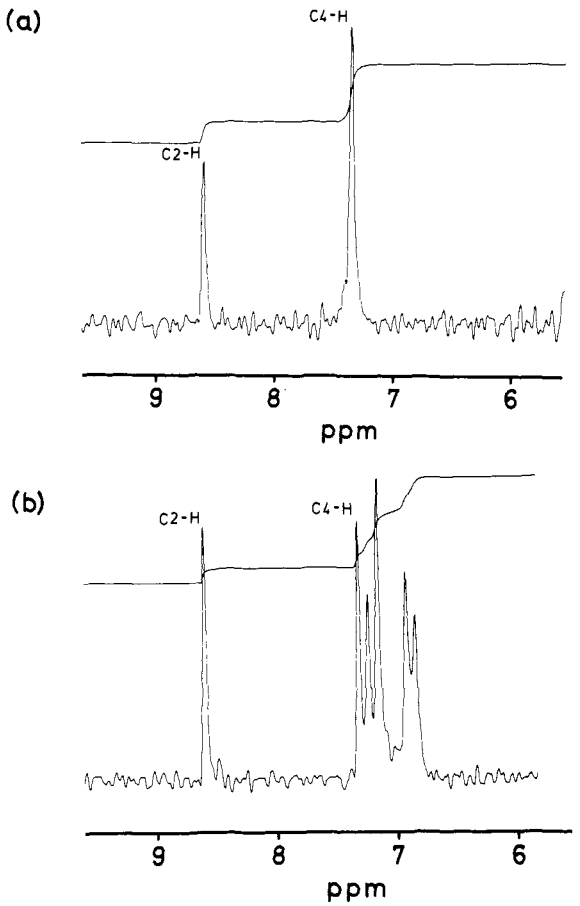


Figure 1. 100-MHz ^{1}H NMR spectra of (a) peptide I and (b) peptide II derived from protein Ak (40 mg) incubated for 14 h in D_2O at 37 °C and pH 8.5. Integrated intensity is also given in the figure. A trace amount of EDTA was added to each sample solution. 1000 transients were accumulated for each spectrum. The probe temperature was 29 °C. Chemical shifts are in parts per million from external DSS (5% in D_2O).

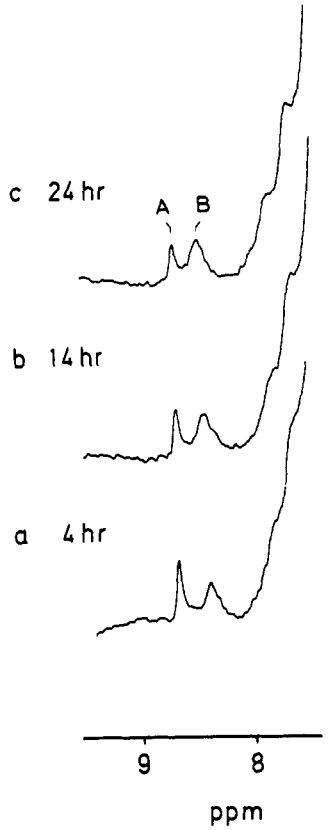


Figure 2. 100-MHz ^{1}H NMR spectra of the C-2 H protons of Bence Jones dimer Ak incubated for (a) 4, (b) 14, and (c) 24 h in D_2O at 37 °C and pH 8.5. In each experiment, 5 mg of incubated protein Ak was dissolved in 0.15 mL of D_2O at pH (a) 4.3, (b) 4.2, (c) 4.2. 5000 transients were accumulated for each spectrum. The probe temperature was 29 °C. Chemical shifts are in parts per million from external DSS (5% in D_2O).

curately the level of exchange at histidines using the C-4 H proton signal of each peptide as the standard of intensity measurements.[9]

λ-type Bence Jones dimer designated Ak contains two histidine residues (His^{189} and His^{198}) in the constant domain of each of the light chain; no histidine exists in the variable domain of this protein.[10] The native Ak protein gives between 7.5 and 8.7 ppm (from external DSS) two peaks, A and B, which titrate with pH; pK_a values for the A and B peaks are 7.5 and 4.9 (μ = 0.2, 29 °C), respectively. Protein Ak (40 mg) was incubated for 14 h in 2.5 mL of D_2O at pH 8.5 and 37 °C.[11] The differentially deuterated protein Ak was oxidized by performic acid and digested by trypsin in 1% ammonium bicarbonate solution (pH 8.5) for 2 h at 37 °C. Two histidine peptides, peptide I (Ser-His^{189}-Arg) and peptide II (Ser-Tyr-Ser-Cys-Gln-Val-Thr-His^{198}-Glu-Gly-Ser-Thr-Val-Glu-Lys), were separated by high voltage paper electrophoresis (pH 3.7), followed by paper chromatography (butanol/acetic acid/pyridine/water, 10:15:3:12), and extracted from the filter paper. Except during tryptic digestion, the peptides I and II were always kept in acidic conditions to minimize the possibility of back-exchange of the deuterium label.[12] ^{1}H NMR spectra of the peptides thus obtained are given in Figure 1.[13] After 14 h, the C-2 H peak of His^{189} loses 60% of its area, whereas that of His^{198} retains more than 95% of its area. The native Ak protein was treated under the identical condition as used to obtain the deuterated histidine peptides. As shown in Figure 2,[13] peak A loses its intensity quite rapidly with a half-time of <20 h; virtually no change in intensity is observed in peak B. On the basis of these experimental findings, peaks A and B can unambiguously be assigned to His^{189} and His^{198}, respectively.[14]

As in the case of the tritium-labeling method, the method described in the present communication can be applied to proteins having more than three histidine residues. Obviously, a necessary condition for this purpose is the existence of a significant difference in the rate of deuterium exchange at different histidine residues. A proper choice of the pH at which proteins are incubated for differential exchange is one of the key factors.

Acknowledgment. We thank Dr. K. Kangawa for his helpful discussion.

References and Notes

(1) G. C. K. Roberts and O. Jardetzky, *Adv. Protein Chem.*, **24**, 447–545 (1970).
(2) D. H. Meadows, O. Jardetzky, R. M. Epand, H. H. Ruterjans, and H. A. Scheraga, *Proc. Natl. Acad. Sci. U.S.A.*, **60**, 766–772 (1968).
(3) J. H. Bradbury and B. E. Chapman, *Biochem. Biophys. Res. Commun.*, **49**, 891–897 (1972).
(4) J. L. Markley, *Acc. Chem. Res.*, **8**, 70–80 (1975).
(5) J. L. Markley and I. Kato, *Biochemistry*, **14**, 3234–3237 (1975).
(6) H. Matsuo, M. Ohe, F. Sakiyama, and K. Narita, *J. Biochem. (Tokyo)*, **72**, 1057–1060 (1972); M. Ohe, H. Matsuo, F. Sakiyama, and K. Narita, *ibid.*, **75**, 1197–1200 (1974).
(7) Y. Arata, S. Kimura, H. Matsuo, and K. Narita, *Biochem. Biophys. Res. Commun.*, **73**, 133–140 (1976).
(8) J. L. Markley, *Biochemistry*, **14**, 3546–3554 (1975).
(9) It has been well established that no exchange occurs at the C-4 H position under the present experimental condition.
(10) Protein Ak was precipitated with ammonium sulfate from the urine of a patient with multiple myeloma and purified on a DEAE cellulose column followed by gel filtration on a Sephadex G-100 column. The numbering system used is based on protein Sh (M. Wikler, K. Titani, T. Shinoda, and F. W. Putnam, *J. Biol. Chem.*, **242**, 1668–1670 (1967)).
(11) All pH values reported in this communication are uncorrected meter readings of D_2O solutions made with electrodes standardized using H_2O buffers.

(12) On the basis of extensive experiments using several proteins, Kangawa (Ph.D. Thesis, Osaka University, 1977) has shown that, under the conditions used in the present experiments, the extent of back-exchange in finally separated deuterated histidine peptides is not >3%.
(13) ^{1}H NMR spectra were recorded on a JEOL PS-100 spectrometer operating at 100 MHz in the correlation mode (Y. Arata and H. Ozawa, *J. Magn. Reson.*, 21, 67–76 (1976)).
(14) The His^{198} peak is much broader in line width than the His^{189} peak. In addition, His^{198} has an unusually low pK_a of 4.9. Significance of the results obtained here concerning Bence Jones dimer Ak will be discussed elsewhere.

Y. Arata*
Department of Chemistry, The University of Tokyo
Hongo, Tokyo, Japan

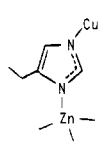
A. Shimizu
The Central Laboratory for Clinical Investigations
Osaka University Hospital
Osaka University School of Medicine, Osaka, Japan

H. Matsuo
Department of Pharmacology
Osaka University School of Medicine, Osaka, Japan
Received December 14, 1977

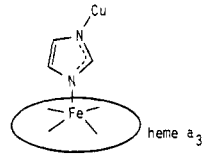
Imidazolate Anion Bridged Metalloporphyrins of Relevance to a Model for Cytochrome Oxidase

Sir:

There is wide interest in the active site structure and function of metalloproteins with magnetically interacting metal sites[1] and in probing these systems by the model complex approach.[2] We have been particularly intrigued with the metal/imidazolate/metal situation exemplified by the histidine bridged Zn^{2+}/Cu^{2+} couple **1** of bovine superoxide dismutase[3] and



1



2

suggested for the ESR inactive Cu^{2+}/heme a_3 couple **2** of cytochrome oxidase.[4] The nature of Cu^{2+}/Cu^{2+} magnetic coupling across an imidazolate bridge (Im) has been investigated by ESR in copper-substituted superoxide dismutase derivatives[5] and by magnetic susceptibility measurements on synthetic analogues.[6] Herein we report synthetic, structural, and magnetic data for some interesting, new imidazolate-bridged metalloporphyrins. Our preliminary conclusions for such systems are that (a) metal-metal antiferromagnetic coupling through an imidazolate bridge is weak, (b) the magnitude of the coupling is greatest when the orbitals containing the unpaired electrons have mutual σ symmetry, and (c) it seems unlikely that an imidazolate bridge can account for the very large magnetic coupling observed in the Cu/heme a_3 site of cytochrome oxidase.

Stoichiometric treatment of $Mn(OClO_3)(TPP)$[7] with $(Bu_4N)(Im)$ in dry THF gives an insoluble dark green precipitate of polymeric $[Mn(Im)(TPP)\cdot THF]_n$, **3**.[8] Growing single crystals of a polymer is a rarely successful endeavor but by extremely careful layering of methanolic solutions of $NaOCH_3$/imidazole/$Mn(OCH_3(TPP)$ we were able to isolate solvate-free single crystals of $[Mn(Im)(TPP)]_n$, **4**. The x-ray crystal structure[9] reveals layers of parallel polymeric chains with alternate layers having their quasi-linear chains approximately orthogonal to each other. The molecular structure

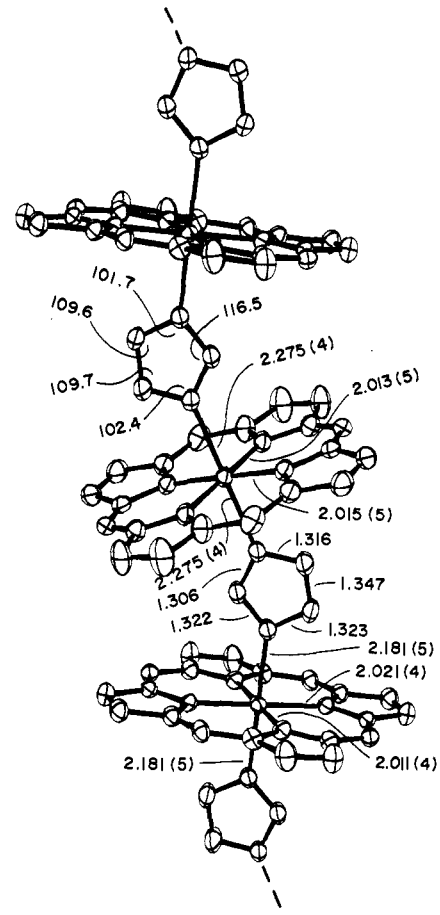


Figure 1. An ORTEP plot of three units of the $[Mn(Im)(TPP)]_n$ polymer, **4**. Each Mn(III) atom is located at an inversion center; the lowest two units (with required C_i symmetry) are the crystallographically unique portion of the polymer. For the sake of clarity, peripheral phenyl groups are omitted. Bond distances in the coordination group and bond distances and angles for the imidazolate anion are entered on the diagram. The dihedral angle between adjacent porphinato planes is 28.5°; the dihedral angles between (Im) and the porphinato planes are 86.3 and 80.4°. The intrachain Mn···Mn distance is 6.54 Å; interchain distances are >11 Å.

(Figure 1) shows the expected six-coordinate manganese stereochemistry but is unusual in that a long-long/short-short alternation of axial Mn-N_{Im} bond lengths occurs. A most reasonable explanation[10] lies in a high-spin/low-spin alternation of the d^4 Mn(III) atoms. The longer pair at 2.275 (4) Å is close to that of the known high-spin monomer $[Mn(1\text{-}MeIm)_2(TPP)]ClO_4$, **5**, where Mn-$N_{Im}$ is 2.308 (3).[11] There are no suitable low-spin manganese(III) porphyrin complexes for comparison with the shorter Mn-N_{Im} pair at 2.181 (5) Å, but the shortening of ~0.1 Å is typical of a high to low spin-state change where an electron is transferred from an antibonding to a nonbonding d orbital.[12] Consistent with these spin-state assignments, the room temperature magnetic moment of the polymer **3** has a value expected of an $S = 2/S = 1$ mixture (found, $\mu_{eff} = 4.2$; calcd spin only, $\mu_s = 4.02\ \mu_B$). The monomer **5** has $\mu_{eff} = 5.3\ \mu_B$, typical of a pure $S = 2$ species.

To look for possible antiferromagnetic coupling in the polymer **3** magnetic susceptibilities were measured over the range 4.2–300 K.[13] The essential linearity of the Curie plot ($1/\chi_M$ versus T in Figure 2a) down to 10 K shows that **3** also behaves like an uncoupled $S = 1/S = 2$ system. The small but real curvature toward 0 K at very low temperatures is suggestive of very weak antiferromagnetic coupling and/or zero-field splitting. Application of theory to a mixed-spin linear chain is nontrivial, but that[14] for an $S = 1/S = 2$ dimer places an upper limit on the exchange coupling constant, $-J \leq 8$

0002-7863/78/1500-3232$01.00/0