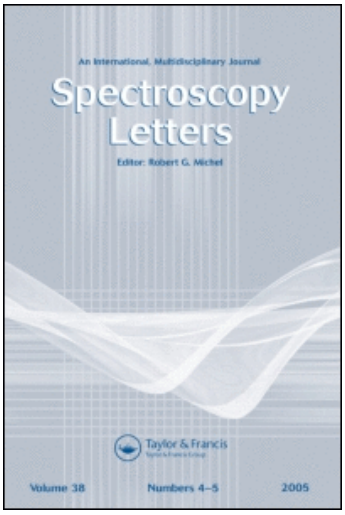

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^{1}H NMR Saturation Transfer Studies of Exogenous Ligands Binding to Metmyoglobin

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^{1}H NMR SATURATION TRANSFER STUDIES OF EXOGENOUS LIGANDS BINDING TO METMYOGLOBIN

Key Words: Metmyoglobin, 1D Saturation transfer, 2D Exchange spectrum, Assignment and kinetics

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ABSTRACT

Two-dimensional(2D) ^{1}H nuclear magnetic resonance exchange spectroscopy, 1D saturation transfer have been utilized to study the binding of imidazole(Im), 1-Methylimidazole(MeIm), 1-Ethylimidazole(EtIm) to the heme iron of metmyoglobin. Some heme peripheral proton resonances of these complexes have been first time assigned. The rates and equilibrium constants for Im, MeIm, EtIm binding to metMb are calculated from the 2D peak amplitudes. Analysis of the heme methyl shifts indicates that the orientations of the binding Im, MeIm, EtIm are very similar. The steric effects and hydrogen bonding between the distal histidine and bound ligand are important factors regulating affinity.

INTRODUCTION

Myoglobin, whose primary function in tissue is to reversibly bind molecular oxygen, has been generally studied as a prototype for exploring the relationship between protein structure and function. The iron high-spin form of myoglobin(metMb) can coordinated with a wide range of ligands, such as Fe-bound ligand H_2O and external ligands CN^-, N_3^-, Im *etc.*[1-5]. Through studying these ligands binding, it is found the amino acids in the distal pocket play an important role in modulating the kinetics of ligand binding. The Im ring of distal histidine residue (His 64) may act as a gate along the pathway for ligand entry into and escape from the distal pocket [5-8]. In order to analysis the role of the distal ligand within the protein, we use Im and subtle varied Im, such as 1-MeIm, 1-EtIm, to systematically explore the accessibility of the metal's sixth coordination position in heme proteins.

Saturation transfer(ST) experiments are very useful for relating resonances which are connected by a suitable time-scale dynamic exchange process. With the known assignments in one form, the same resonances in the other form are identified [4,9,10]. Two-dimensional chemical exchange (2D EXSY) studies can not only provide a unique tool for assigning the resonances, but also obtain accurate rate constants of chemical exchange between sites [11-13]. We present here the results from 1D ST method and 2D EXSY experiment study of Im, 1-MeIm and 1-EtIm binding to metMb. Some heme hyperfine shifted resonance assignments are first time presented. The kinetic values and equilibrium constants for the reactions have been measured, and the reasons for the different affinity are discussed.

EXPERIMENTAL SECTION

Preparation of Samples. Horse skeletal muscle Mb was obtained from Sigma Chemical Co.(lyophilized, salt-free power) and used without

further purification. 1-Methylimidazole and 1-Ethylimidazole were synthesized according to the procedure [14], the purity was checked by ^1H NMR spectrum. Solution of metMb was prepared by dissolving the Mb sample in H_2O, oxidized by adding fivefold molar excess of potassium hexacyano-ferrate(III). The solution was centrifuged to remove precipitate, ultrafiltered to remove the anions and lyophilized finally. The NMR samples of Im-metMb, MeIm-metMb and EtIm-metMb were prepared by dissolving weighed amount of lyophilized metMb in known volume of D_2O, adding D_2O solutions of [$^2H_4$]Im, MeIm and EtIm. NaOD and DCl solutions were used to adjust pH. Reported pH values are uncorrected for isotope effect.

^1H NMR Measurement. All ^1H NMR spectra were recorded on a Bruker AM-500MHz spectrometer, equipped with an Aspect 3000 computer system. Typically, 32K data points over 100KHz band width with 6.5μs 90° pulse were used for 1D NMR experiments. The carrier was centered on the residual water peak which was suppressed by presaturation. The ST spectra were recorded by applying a 120ms presaturation pulse on the desired resonances, corresponding reference spectra were collected under the same conditions but with the decoupler pulse off-resonance. On and off resonance frequencies were alternated every 32 scans. 2D exchange spectra (EXSY) were recorded using the standard 2D NOESY pulse sequence[15] with mixing time of 10ms and 512 × 2048 time-domain size. Zero-filling was performed in the F1 dimension, and the time-domain data were multiplied by phase-shifted sine bell window function in both dimensions. Chemical shifts for all the spectra were referenced to 1,4-dioxane at 3.743ppm.

The ligation of metMb can be represented by the following reaction:

$$\text{metMb} + \text{L} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{L-metMb}$$

The magnetization exchange between the species is a first-order reaction:

$$M_{metMb} \underset{k_{BA}}{\overset{k_{AB}}{\rightleftarrows}} M_{L\text{-}metMb}$$

For a spin system involving chemical exchange, the peak amplitude in 2D EXSY spectra is related to the exchange rate constant k, the relaxation rate and the mixing time τ by the expression[16]:

$$A = \exp(-R\tau_m) \qquad (3)$$

where A is the amplitude matrix whose elements are defined by:

$$A_{ij} = \frac{I_{ij}}{M_j^\circ} \qquad (4)$$

R is the dynamic matrix which contains the parameters to be determined:

$$R = \begin{vmatrix} \rho_A & -k_{BA} \\ -k_{AB} & \rho_B \end{vmatrix} \qquad (5)$$

The solution to equation(3) is given by equation (6)[11]

$$R = -\frac{\ln A}{\tau_m} = -\frac{X(\ln \Lambda) X^{-1}}{\tau_m} \qquad (6)$$

where X is the square matrix of eigenvectors of A, such that $X^{-1}AX = \lambda = \mathrm{diag}(\lambda_i)$ and $\ln\Lambda = \mathrm{diag}(\ln\lambda_i)$, with λ_i the ith eigenvalue of A. In our experiment, the equilibrium magnetization, M_i°, is evaluated by simply replacing each M_i° by p_i, the relative population of each site, from

$$k_1 = \frac{k_{AB}}{[L]}, \quad k_{-1} = k_{BA}, \quad k_{app} = \frac{k_1}{k_{-1}}$$

integration of the 1D spectrum[11]. The chemical exchange in our

experiment is between two sites, the peak amplitudes integration of the 2D exchange spectra provide the data matrix A. Thus we can calculate the reaction rate constants and equilibrium constant from a single 2D spectrum with mixing. The integral values of the 2D peaks were obtained by direct reading from the spectra using a frame. The same frame was used to estimate the average noise intergral value in order to remove the noise effects.

RESULTS AND DISCUSSION

1.Assignment of Some Hyperfine Shifted Resonances in Im-metMb.

The ST method is used in connection with the known hyperfine shifted resonance assignments for metMb[1,15] to make unambiguous assignments for Im-metMb. Four heme methyl proton signals of equine Im-metMb have been assigned previously [4]. The ^{1}H NMR spectra of native metMb, Im-metMb are illustrated in Fig.1(a)(b). The resonances of metMb and Im-metMb in the downfield can be resolved due to the large scaler interaction. The ST difference spectra obtained upon saturation selected signals in the mixture of metMb and Im-metMb are shown in Fig.2.

In the spectrum of Fig.2b, the resonance of metMb at 44.6ppm which assigned to 4α, 6α'-CH is saturated. ST is observed to two peaks at 23.5ppm and 13.2ppm. By comparing the spectrum of pure Im-metMb with that of the metMb, it appears that these two proton resonances are really from Im-metMb. The two peaks can be distinguished in fig.2c, which the peak of 6α-propionate in metMb at 56.7ppm is saturated. A large ST connectivity to the corresponding proton signal in Im-metMb at 15.8ppm is observed. Saturation the peak of 6α also leads to an NOE to peak 6α' in metMb at 44.7ppm. Compare with the difference spectrum of Fig.2b, we find the peak at 13.2ppm in Fig.2c is due to transfered NOE generated by geminal proton 6α in Im-metMb. So we assigned the peak at 13.2ppm to 6α', and the peak at

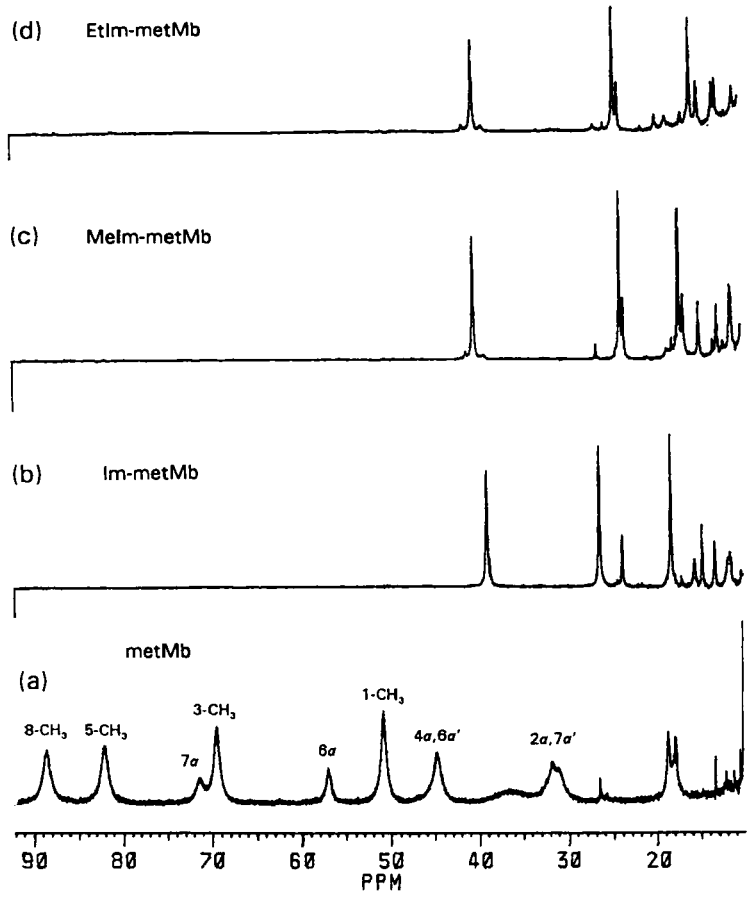

Fig.1. The downfield hyperfine shifted portions of the 500MHz ^{1}H NMR spectra of (a)metMb(C_{metMb} = 4mM); (b)Im-metMb(C_{metMb} = 5mM, C_{Im} = 0.20M); (c)1-MeIm-metMb(C_{metMb} = 5mM, C_{MeIm} = 0.23M); (d)1-EtIm-metMb(C_{metMb} = 5mM, C_{EtIm} = 0.28M) at 308K and pH7.4. The signal assignments of the heme methyl pronton resonances of metMb are indicated.

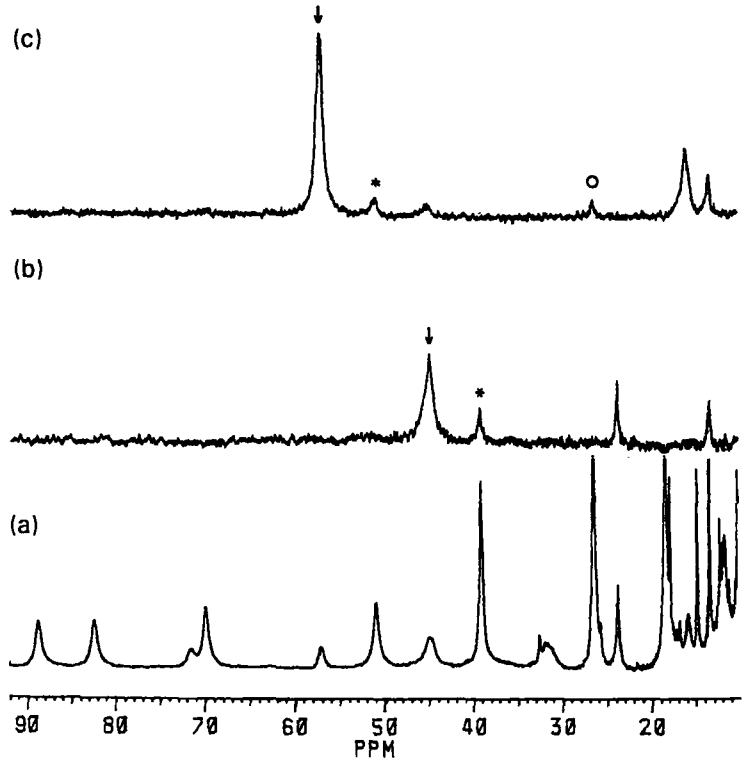

Fig.2. The downfield regions of the ^{1}H NMR spectrum and ST difference spectra of the mixture of metMb and Im-metMb(C_{metMb} = 5mM, C_{Im} = 20mM) at 308K and pH7.4. (a) Reference spectrum. The arrow indicates the peak being saturated, and the signal due to the off-resonance saturation is indicated with an asterisk. The circle denotes the ST connectivity from resonances other than that indicated with the arrow. ST difference spectrum on saturation of (b) the 4α,6α'signal of metMb; (c) the 6α signal of metMb.

23.5ppm to 4α. The 7α, 2α and 7α' signals of Im-metMb can not be located in the ST difference spectra. When the peak at 31.6ppm is saturated, only one ST peak at 7.75ppm can be clearly observed. The assigned resonances of Im-metMb at 308K and pH7.4 are collected in Table 1.

2. Assignment of Heme Peripheral Proton Resonances in MeIm-metMb and EtIm-metMb

We use 1D saturation transfer and 2D EXSY experiments to make the assignments for MeIm-metMb and EtIm-metMb. The ^{1}H NMR spectra of pure MeIm-metMb and EtIm-metMb are illustrated in Fig.1(c)(d). Some heme peripheral protons could be assigned directly by 2D EXSY experiments. A portion of the 2D exchange spectrum of the mixture of MeIm-metMb and metMb is given in Fig.3. Due to the t_1 noise caused by the enormous intensity of the diamagnetic envelope(0-10ppm), we only displayed the upper left portion of the EXSY spectrum. The cross peaks indicate the connectivities between the corresponding heme peripheral proton resonances of the two forms. With the known assignments in metMb, the corresponding resonances of heme 8, 5, 3, 1 methyl groups and 6α-CH of MeIm-metMb are unambiguously assigned. The 4α and 6α' signals of MeIm-metMb can be distinguished by 1D ST difference spectrum, shown in Fig.4(b)(c). When the 4α, 6α' signal and 6α signal in metMb are irridated respectively, transfered NOE is observed between the peaks of 6α and 6α' in MeIm-metMb. Therefore, the resonances at 23.1ppm and 11.3ppm in MeIm-metMb are assigned to 4α and 6α' protons respectively. The 1D saturation transfer experiments are also used to assigned the 7α, 7α' and 2α-CH resonances of MeIm-metMb, shown in Fig.4(d)(e). When the peak of 7α-CH in metMb at 70.4ppm is saturated, the saturation transfer to a signal at 1.95ppm is observed (Fig.4d). The signal at 3.02ppm which has been assigned to 3-CH_3 in MeIm-metMb is due to the off-resonance saturation on nearby 3-CH_3 signal in metMb. So the peak at 1.95ppm is assigned to 7α-CH in MeIm-

TABLE 1

^{1}H NMR Assignments and Chemical Shifts (ppm) for Heme Peripheral Protons of Im-metMb, 1-MeIm-metMb and 1-EtIm-metMb at 308K, pH7.4.

Assignment	Im-metMb	1-MeIm-metMb	1-EtIm-metMb
8-CH_3	18.0[a]	17.0	15.7
5-CH_3	38.6[a]	39.9	39.8
3-CH_3	7.36[a]	3.03	3.00
1-CH_3	26.0[a]	23.5	24.0
4α	23.5	23.1	23.6
6α'	13.2	11.3	12.7
6α	15.8	12.7	13.0
2α		6.76	6.55
7α'		0.51	0.52
7α		1.95	2.13

[a] Ref.4.

metMb. Irradiation on the 2α, 7α' signal of metMb at 31.3ppm results in the difference spectrum shown in Fig.4e, two peaks at 6.76, 0.51ppm are clearly observed. By comparing the transfered NOE between geminal protons 7α and 7α' of MeIm-metMb in Fig.4(d)(e), we assign the peak at 0.51ppm to 7α'.

The same assignment methods are applied to EtIm-metMb. With the 2D EXSY spectrum of the mixture of EtIm-metMb and metMb shown in Fig.5, the heme 8, 5, 3, 1-CH_3 and 6α-CH in EtIm-metMb are straight assigned. The 4α and 6α' signals can be distinguished in Fig.6(b)(c) by observing transfered NOE between 6α and 6α' signals in EtIm-metMb. The results of the ST experiments with saturation on the heme 7α and 2α, 7α' signals of metMb are given in Fig.6(d)(e). The ST connectivity provides the corresponding signal assignments in EtIm-metMb. Transfered NOE is observed between geminal protons 7α and 7α' of

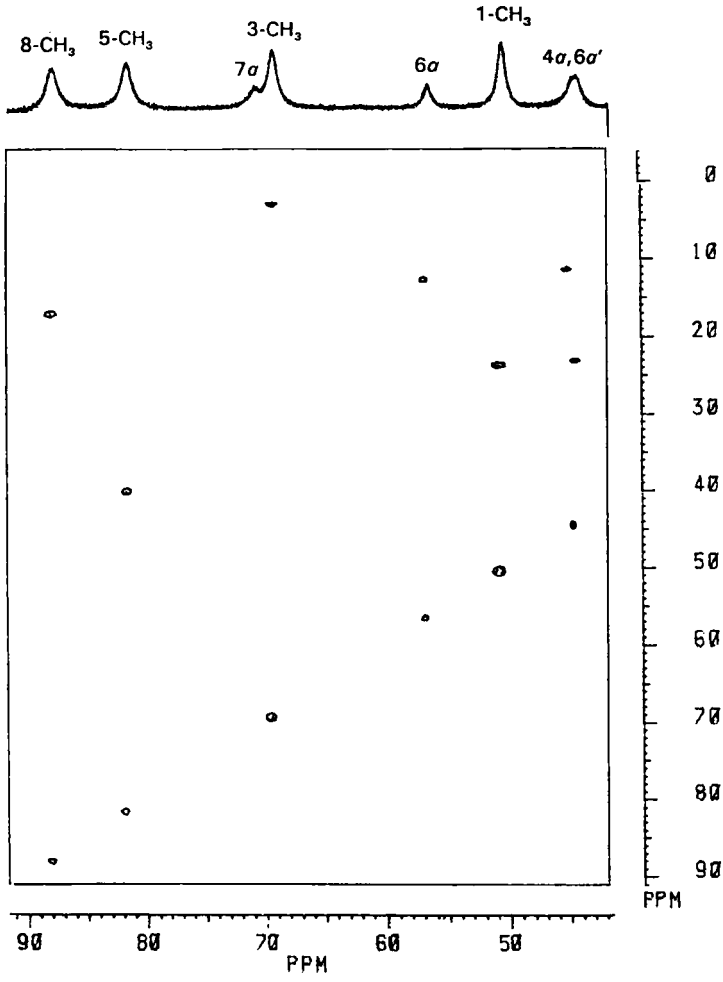

Fig.3. Downfield region of the 2D EXSY spectrum of the mixture of metMb and 1-MeIm-metMb(1:1) at 308K and pH7.4 with mixing time 10ms. 1D spectrum is shown at the top with the signal assignments in metMb are indicated.

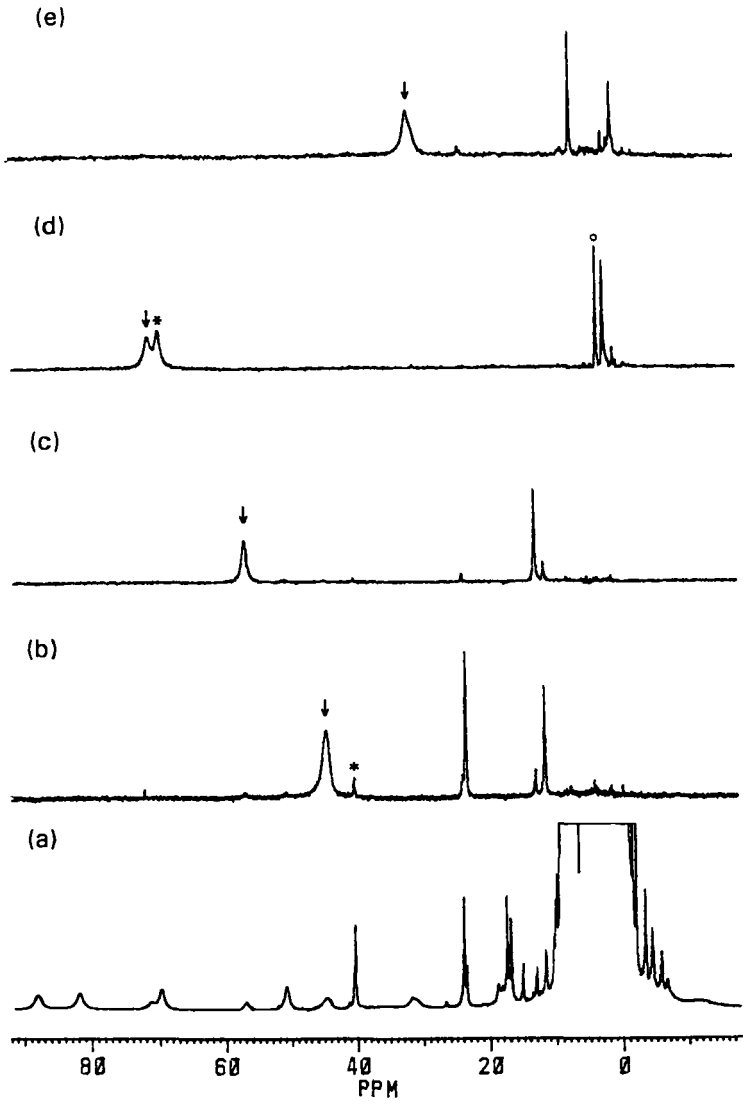

Fig.4. The ^{1}H NMR spectrum and ST difference spectra of the mixture of metMb and 1-MeIm-metMb(1:1) at 308K and pH7.4. (a) Reference spectrum; ST difference spectrum on saturation of the (b) 4α, 6α' signal of metMb; (c) 6α signal of metMb; (d) 7α signal of metMb; (e) 2α, 7α' signal of metMb.

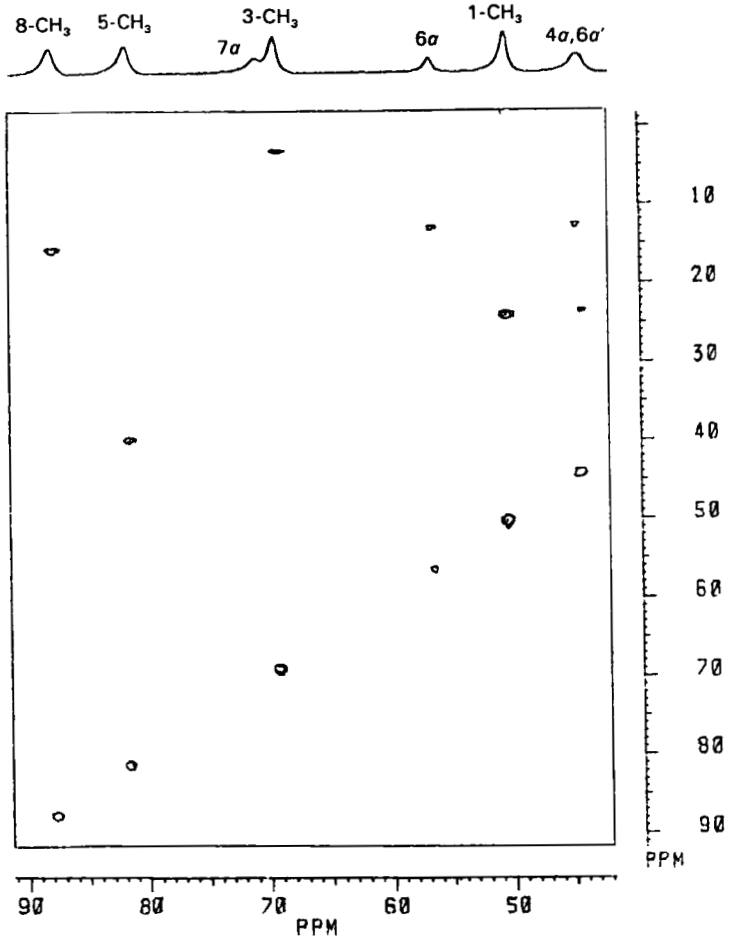

Fig.5. Downfield region of the 2D EXSY spectrum of the mixture of metMb and 1-EtIm-metMb(1.1:1) at 308K and pH7.4 with mixing time 10ms. 1D spectrum is shown at the top. The signal assignments in metMb are indicated with the 1D spectrum.

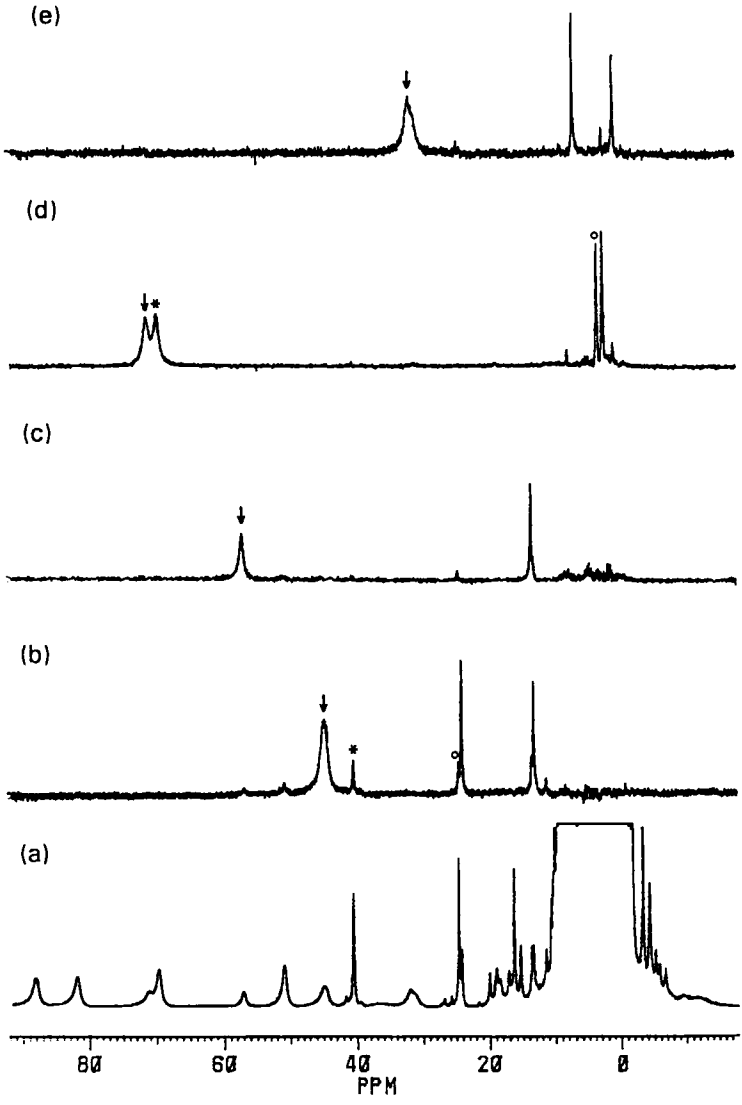

Fig.6. The ^{1}H NMR spectrum and ST difference spectra of the mixture of metMb and 1-EtIm-metMb(1.1:1) at 308K and pH7.4. (a) Reference spectrum; ST difference spectrum on saturation of the (b) 4α, 6α' signal of metMb; (c) 6α signal of metMb; (d) 7α signal of metMb; (e) 2α, 7α' signal of metMb.

EtIm-metMb. All the assigned resonances of MeIm-metMb and EtIm-metMb are listed in Table 1.

^{1}H NMR spectroscopy of heme proteins is very useful for characterizing both the protein structure and the heme electronic structure. The pattern of hyperfine-shifted resonances of the heme peripheral protons reflects the electronic asymmetry in the heme produced by the orientation of the axial ligands[17,18]. According to the X-ray crystal structure analysis of the ferric sperm whale Im-metMb complex. The overall conformation of the protein is little affected by binding of the ligand, but distinguishable local changes at the distal site in the surrounding protein residues are resulted [7]. Due to a steric repulsion with the bound bulky imidazole, the side chain of His64(E7) swings out of the distal pocket and becomes substantially exposed to the solvent. The ligated imidazole ring plane adopts an orientation that is 16° tilted with respect to the plane of the heme and forms an angle of -41° with the pyrrole N2-N4 direction [7]. According to our study, it is found the hyperfine shifts of heme methyl resonances of Im-metMb, 1-MeIm-metMb and 1-EtIm-metMb are very similar. This manifests that although the steric bulk and structure of imidazole, 1-methylimidazole and 1-ethylimidazole are different, the orientations of their ring planes are nearly the same. The hydrogen bonding between the ligated imidazole and distal histidine residue seems make little difference on the ring plane orientation compared with 1-MeIm and 1-EtIm, which have no hydrogen bondings. Swinging of the His64(E7) side-chain towards the protein surface could make room large enough for the ligand binding, the ligand may enter the heme pocket through a channel with certain orientaton.

3. Affinity of MetMb for Imidazole, 1-MeIm and 1-EtIm

In Table 2, the association and dissociation rate constants of Imidazole, 1-MeIm and 1-EtIm binding to metMb and the equilibrium constants are listed. Due to the different sizes of the ligands, imidazole

TABLE 2

Rate and Equilibrium Constants of Im, 1-MeIm and 1-EtIm Binding to MetMb at 308K, pH7.4.

	k_1 ($M^{-1}S^{-1}$)	k_{-1} (S^{-1})	K (M^{-1})
Im	2993	14.4	207
1-MeIm	831	26.2	31.7
1-EtIm	584	38.4	15.2

is less sterically hindered from entering the heme pocket and thus has far larger association rate constant than 1-MeIm and 1-EtIm. The affinities of imidazoles binding to the distal site varies in the order: Im > 1-MeIm > 1-EtIm. The affinity constant of imidazole binding is several times larger than 1-MeIm, whereas the affinity constant of 1-MeIm is only one times larger than 1-EtIm's. This means although the affinity constant increases with a decrease in the size of the ligand, the steric effect upon ligation is not the only factor regulating the stability of the Im, 1-MeIm and 1-EtIm complexes. The bound-imidazole is stabilized by the hydrogen-bonding interaction with His64(E7), in which free NH proton of the Fe bound imidazole interacts with the lone-pair electrons of the His-E7 imidazole. Hydrogen-bonding interaction plays an important role in stabilizing the exogenous ligand.

REFERENCES

1. G.N.La Mar, L.B.David, M.S.Kevin, J.Am.Chem.Soc., 1980; 102: 1822.
2. S.D.Emerson, G.N.La Mar, Biochemistry, 1990; 29: 1545.

3. L.P.Yu, G.N.La Mar, K.Rajarathnam, J.Am.Chem.Soc., 1990; 112: 9527.
4. Y.Yamamoto, Y.Inoue, T.Suzuki, Magn.Reson.in Chem.,1993;31:S8.
5. Y.Yamamoto, T.Suzuki, H.Hori, Biochim.Biophys.Acta, 1995; 1248:149.
6. J.A.Bell, Z.R.Koeszun, K.Moffat, J.Mol.Biol., 1981; 147: 325.
7. C.Lionetti, M.G.Guanziroli, F.Frigerio, P.Ascenzi, M.Bolognesi, J.Mol.Biol., 1991; 217: 409.
8. A.Brancaccio, M.Brunori, S.J.Smerdon, A.J.Wilkinson, Y.Dou, R.E.Brantley, J.Bio.Chem., 1994; 269: 13843.
9. R.M.Keller, K.Wüthrich, Biochim.Biophys.Res.Common., 1978; 83: 1132.
10. V.Thanabal, J.S.Ropp, G.N.La Mar, J.Am.Chem.Soc., 1987; 109: 265.
11. E.R.Johnston, M.J.Dellwo, J.Hendrix, J.Magn.Reson., 1986;66:399.
12. E.W.Abel, T.P.J.Coston, K.G.Orrell, V.Šik, D.Stephenson, J.Magn.Reson., 1986; 70: 34.
13. C.L.Perrin, T.Dwyer, J.Chem.Rev., 1990; 90:935.
14. A.Badische, A.G.Soda-Fabrik, Chemical Abstracts, 1966; 64: 19629a.
15. G.Bodenhausen, H.Kogler, R.R.Ernst, J.Magn.Reson., 1984; 58: 370.
16. R.R.Ernst, G.Bodenhausen, A.Wokau, Principles of Nuclear Magnetic Resonance in One and Two Dimensions,Oxford;Oxford University 1987.
17. T.G.Traylor, A.P.Berzinis, J.Am.Chem.Soc., 1980; 102: 2844.
18. Y.Yamamoto, N.Nanai, R.Chûjô and T.Suzuki, FEBS Lett. 1990; 264: 113.

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