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Kinetic studies of imidazole and its methyl derivative binding to metmyoglobin: Effects of substitute methyl on the binding affinity

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Abstract

The binding of imidazole and imidazole derivatives (4- and 2-MeIm) to the metMb was studied by ^{1}H NMR spectroscopy to elucidate the effects of different methyl substitution positions on the affinity and kinetics of binding to metMb. These exogenous ligands can form stable complexes with metMb except 2-MeIm. Kinetics and equilibrium data for the binding of imidazole and 4-MeIm to metMb have been obtained by 2D EXSY, and the reasons for the different affinity are also discussed.

Introduction

Myoglobin, as an important O_2 carrier in the body, has been the subject of intense physicochemical studies to elucidate the mechanism of control of simple exogenous ligand binding such as CO, N^{3-}, CN^- and imidazole (Springer *et al.* 1994; Antonini *et al.* 1971; La Mar *et al.* 2000; Emerson *et al.* 1990; Yamamoto *et al.* 1993). Through studying these ligands binding, it is found that the amino acids in the distal pocket play an important role in modulating the kinetics of ligand binding. The imidazole ring of the distal histidine residue (His64) may act as a gate along the pathway for ligand enter into and escape from the distal pocket. Therefore we have performed a comprehensive study of the binding reactions of imidazole derivatives including 4-MeIm and 2-MeIm to systematically explore the effects of different positions of methyl substitution in imidazole on the affinity and kinetics of binding of imidazole and its methyl derivatives to metMb. Here the kinetics values and equilibrium constants from 2D EXSY experiments of imidazole and 4-MeIm binding to metMb are presented. Based on these data a comprehensive comparison between the binding properties of the imidazole and its methyl derivatives was made.

Materials and methods

Sample preparation

Horse skeletal muscle Mb was obtained from Sigma Chemical Co. (lyophilized, salt-free power) and used without further purification. A solution of metMb was prepared by dissolving the Mb sample in H_2O, and oxidized by adding a fivefold molar excess of potassium hexacyano-ferrate (III). The solution was centrifuged to remove the precipitate, ultrafiltered to remove the anions and finally lyophilized. The NMR samples of 4-MeIm-metMb, Im-metMb and 2-MeIm-metMb were prepared by dissolving weighing amounts of lyophilized metMb in known volumes of D_2O solution of 4-MeIm, Im and 2-MeIm. The pH was adjusted to 7.0 using NaOD and DCl solutions; reported pH values are uncorrected for isotope effect.

NMR spectroscopy

All the ^{1}H NMR experiments were performed on a Brucker AM 500 spectrometer equipped with an Aspect 3000 computer system. Typically, 16K data points over 100 KHz band width were obtained for one-dimensional spectra. The carrier was centered on

the residual water peak, which was suppressed by presaturation during the relaxation delay. 2D exchange spectrum (EXSY) experiments was recorded using standard 2D NOESY pulse sequence with mixing time of 10 ms and 512 × 2048 time domain data size (Bodenhausen *et al.* 1984). 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 shift values were referenced to 1,4-dioxane at 3.743 ppm. Data processing was performed using the standard Bruker software package XWINNMR.

Results and discussion

Kinetics of 4-MeIm binding to metMb

The ^{1}H NMR spectra of native metMb, the mixture of metMb and 4-MeIm-metMb, the mixture of metMb and Im-metMb, the mixture of metMb and 2-MeIm-metMb are illustrated in Figure 1. When the concentration of 4-MeIm is 7.5 mM, about 1:1 4-MeIm-metMb complex and metMb formed in the solution, the same case is to the mixture of metMb and Im-metMb when the concentration of imidazole is 23 mM. As to 2-MeIm, only a small fraction of 2-MeIm-metMb existed in the solution even when the concentration of 2-MeIm reached 800 mM. Here we can conclude qualitatively that the binding affinity has the following order: 4MeIm > Im ≫ 2MeIm.

The assignment of the hyperfine shifts from the EXSY spectra (shown in Figure 2) is consistent with previous results (Yamamoto *et al.* 1993; Luo *et al.* 1998). The hyperfine shift pattern of the heme methyl proton resonances can be interpreted in terms of the contact shift and pseudocontact shifts. The distribution of unpaired electrons among the four pyrroles is directly perturbed by the axial ligand orbital and reflects the in-plane asymmetry of the heme electronic structure.

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

$$\text{MetMb} + \text{L} \underset{K_{-1}}{\overset{K_1}{\leftrightharpoons}} \text{L-metMb} \tag{1}$$

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

$$M_{\text{metMb}} \underset{K_{BA}}{\overset{K_{AB}}{\leftrightharpoons}} M_{\text{L-metMb}} \tag{2}$$

For the 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 by the expression (Ernst *et al.* 1987):

$$A = \exp(-R\tau_m) \tag{3}$$

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

$$A = \begin{bmatrix} I_{11}/M_1 & I_{12}/M_2 \\ I_{21}/M_1 & I_{22}/M_2 \end{bmatrix} \tag{4}$$

the quantity I_{ij} is two dimensional peak amplitudes measured and normalized. The chemical exchange in our experiment is between two sites, the peak amplitude of the 2D exchange spectra (based on the heme 8-CH_3 signal) provide the data matrix A. The integral values of the 2D EXSY peaks were obtained by direct reading from the spectra using a frame. The same frame was used to estimate the average noise integral value in order to remove the noise effects. M_i is the equilibrium magnetization values obtained from integration of another 2D EXSY spectrum without mixing and also normalized. R is the dynamic matrix which contains the parameters to be determined:

$$R = \begin{bmatrix} \rho_1 & -k_{-1'} \\ -k_{1'} & \rho_2 \end{bmatrix} \tag{5}$$

The solution to the equation (3) is given by equation (6) (Johnson *et al.* 1986):

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

R can be directly calculated in expression (5) where X is the square matrix of eigenvector matrix and $XDX^{-1} = A$.

The relationship between the magnetization exchange rate constants and the substitution reaction rate constants can be found in the equations (5) and (7). And the apparent equilibrium constant K_{app} and the equilibrium constant K of the reaction can be calculated from equation (8), where k_a is the dissociation constant of 4-MeIm. Since the concentration of 4-MeIm is known, the reaction rate constant and the equilibrium constant can be determined.

$$k_1 = k_{AB}/[\text{L}], \quad k_{-1} = k_{BA}, \tag{7}$$

$$K_{app} = k_1/k_{-1}, \quad K = K_{app}\{1 + ([\text{H}^+]/k_a)\} \tag{8}$$

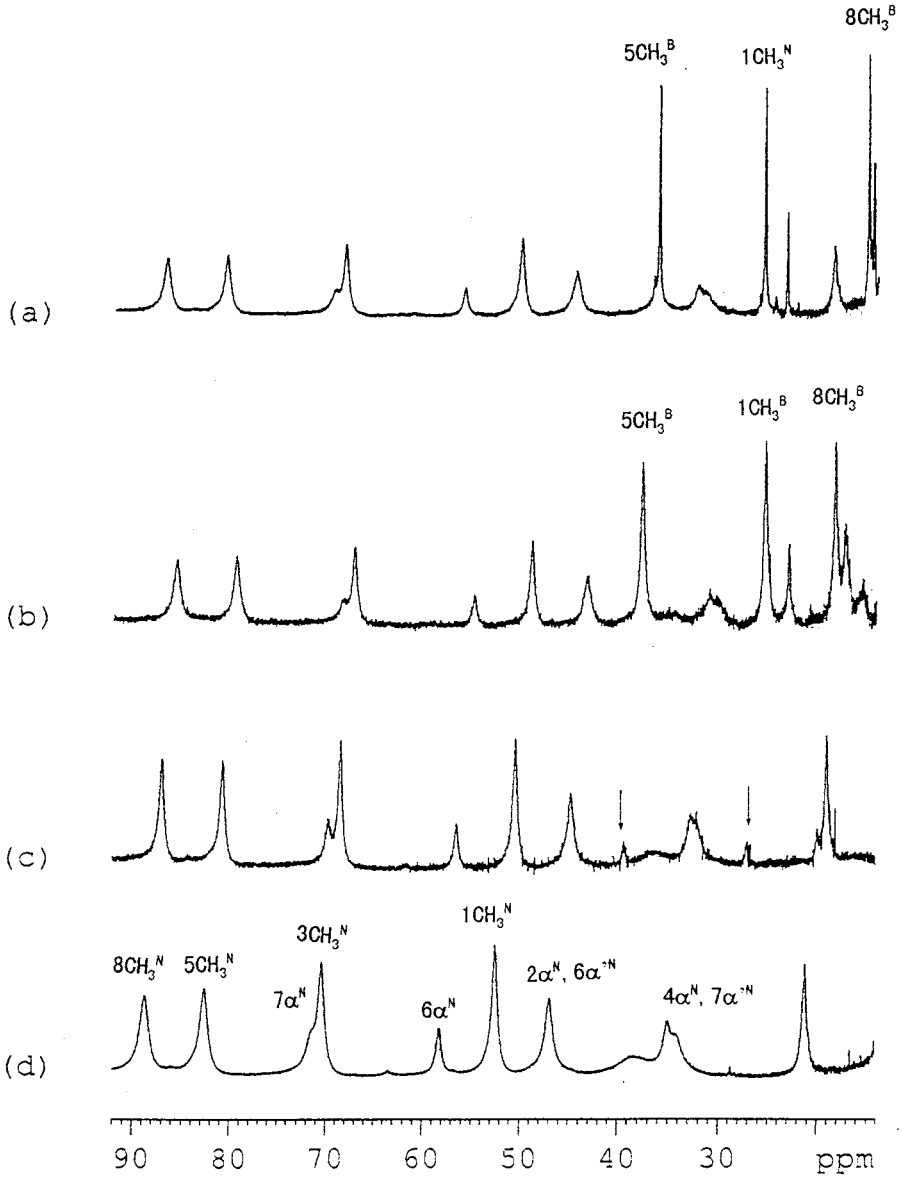

Figure 1. The comparison of the downfield hyperfine shifted portions of the 500 MHz ^{1}H NMR spectra of (a) the mixture of 4-MeIm-metMb and metMb ($C_{metMb} = 7$ mM, $C_{4-MeIm} = 7.5$ mM); (b) the mixture of Im-metMb and metMb ($C_{metMb} = 5$ mM, $C_{Im} = 23$ mM); (c) the mixture of 2-MeIm-metMb and metMb ($C_{metMb} = 5$ mM, $C_{2-MeIm} = 800$ mM); (d) metMb ($C_{metMb} = 5$ mM).

The association constant and dissociation rate constants of imidazole, 4-MeIm binding to metMb and the equilibrium constants are listed in Table 1. It is obvious that the presence of 4-MeIm significantly leads to the formation of a very stable complex of 4-MeIm-metMb, wherein its reaction rate constant k_1 is a little higher than those of the Im-metMb. In addition, we also find from Figure 1 that 2-MeIm hardly binds to metMb, it only displays very weak 2-MeIm-metMb peaks when the concentration of ligand 2-MeIm reaches 800 mM. Also it has been noted elsewhere that the binding constant of 2-MeIm-metMb is very low (Eaton *et al.* 1978). So the affinities of these ligands binding to heme Fe in the distal site of protein also conform the following order: 4-MeIm > Im $\gg$ 2-MeIm. The pk_a of Im and 4-MeIm are 6.93 and 7.37, respectively (Perrin *et al.* 1965), which means 4-MeIm can form a more stable complex with metMb than im-

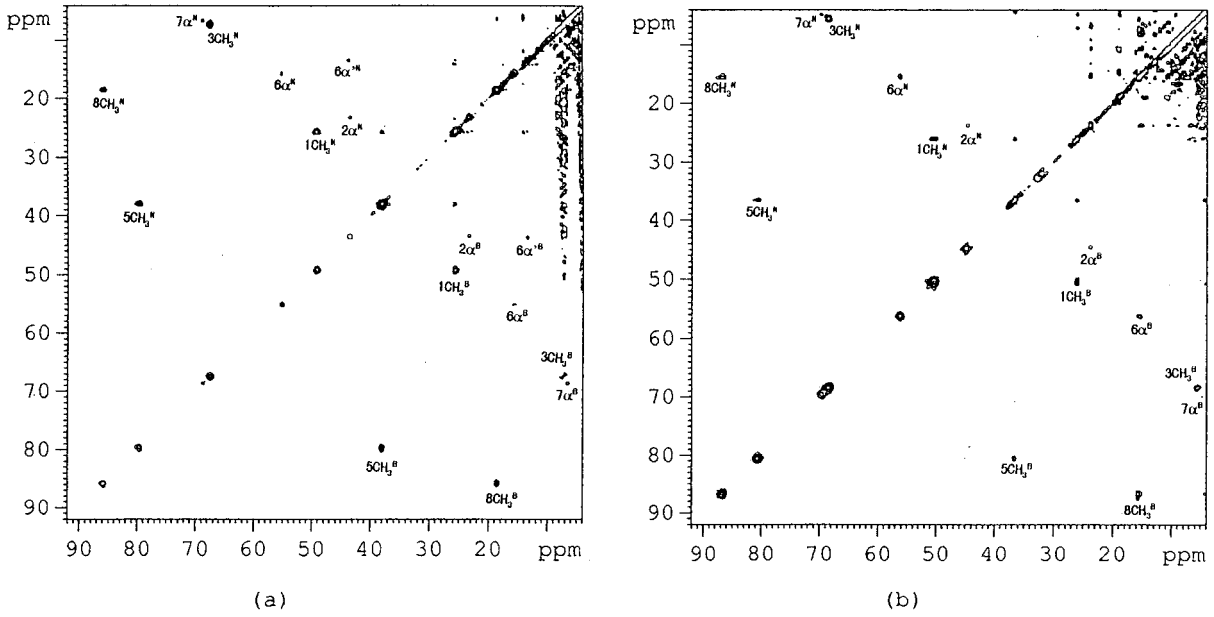

Figure 2. Downfield hyperfined-shifted region of the 2D EXSY spectrum of the mixture of (a) 4MeIm-metMb and metMb (about 1:1), (b) Im-metMb and metMb (about 1:1) at 313K and pH 7.0 with mixing time 10 ms.

idazole. As for 2-MeIm, the pk_a is 7.85, which means 2-MeIm should have stronger coordination ability than Im and 4-MeIm, but the methyl group of 2-MeIm has a strong steric hindrance for it closes with N_1H and hinders the approach of 2-MeIm to the heme and then counteracts the formation of N_1-Fe bond. So the steric hindering effect is the most important factor effecting the binding of 2-MeIm. However, the case is different for 4-MeIm. The methyl group of 4-MeIm is in the meta position of N_1H, where it has a little steric effect on the formation of N_1-Fe bond, so that the steric hindering effect is not prominent. Previous x-ray crystal structure of Im-metMb complex has indicated swinging of the His64 (E7) side-chain towards the protein surface creating an aperture large enough to fill the imidazole, and the imidazole seems to keep the His64 door in an open conformation, then forms a stabilizing hydrogen bond with distal His64 (E7) (Lionett *et al.* 1991). According to our results, it is found that the hyperfine shifts of the heme methyl resonances of Im-metMb and 4-MeIm-metMb are similar. The angle between the two heme binding ligands has been calculated for Im-metMb and 4-MeIm-metMb (Bertini *et al.* 1999), through a heuristic equation (9) that correlates the hyperfine shifts of heme peripheral protons with the orientation of two axial ligands,

$$\delta_{\mathrm{i}} = \cos\beta\left[\mathrm{A}\sin^2(\theta_{\mathrm{i}}-\phi)+\mathrm{B}\cos^2(\theta_{\mathrm{i}}+\phi)+\mathrm{C}\right] + \mathrm{D}\sin\beta \qquad (9)$$

where δ_i is the hyperfine shifts of the ith-methyl, θ_i is the angle between the Fe-ith-methyl direction and the Fe- pyrrole II nitrogen (NB) axis, β is the acute angle between the two histidine planes, ϕ is the angle between the bisector of the angle β and the Fe-NB direction, A, B, C and D are constants, determined by fitting data on similar systems and with known structure. The experimental shifts of 4-MeIm-metMb provides ϕ of 34° and β of 31°, and ϕ and β for Im-metMb are 32° and 33°, respectively. The results of the two complexes are very similar, which manifests that although the steric bulk and structure of imidazole and 4-MeIm-metMb are different, the orientations of their ring planes are nearly the same. It is reasonable that ligand 4-MeIm also has formed a hydrogen bond with His64 (E7) which facilitated to form a stable complex. Another factor is that 4-MeIm has more hydrophobic nature than imidazole which facilitates 4-MeIm binding to the heme, since the heme pocket provides a hydrophobic environment for the heme. It is also noted that the ligands enter the distal pocket through several longer hydrophobic pathways between the B, G and H helixes (Elber *et al.* 1990). These effects outweigh the steric hindrance from the methyl

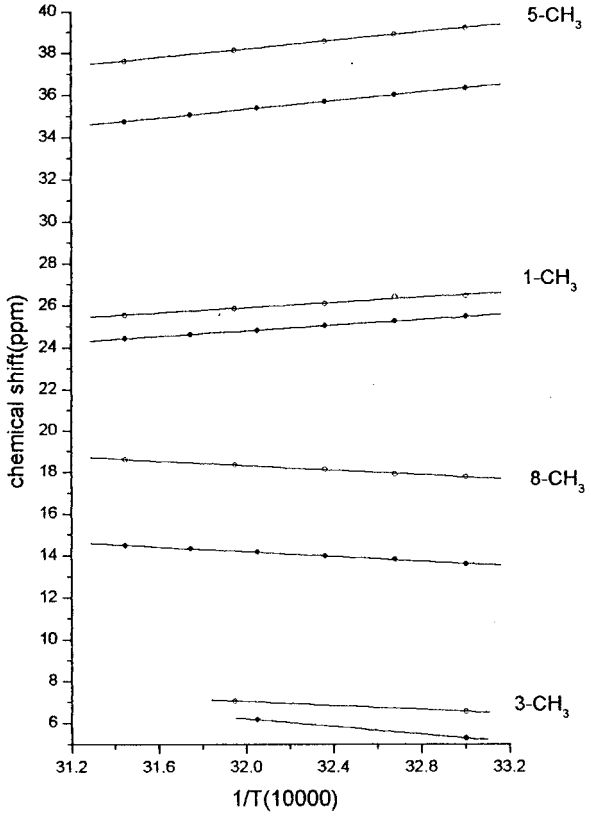

Figure 3. Curie plot for methyl resonance of 4-MeIm-metMb and Im-metMb at pH 7.0. (○ indicates Im-metMb and ● indicates 4-MeIm-metMb).

Table 1. Rate and equilibrium constants of Im, 4-MeIm binding to metMb at 313K, pH 7.0.

	k_1 ($M^{-1}S^{-1}$)	k_{-1} (S^{-1})	K_{app} (M^{-1})	K (M^{-1})
Im	805	14.3	56.3	104
4-MeIm	960	4.2	228	762

group of 4-MeIm. Thus, the steric effect upon ligation is not the only factor regulating the stability of the complex, hydrogen bonding between ND1 of His64 (E7) and bound ligand NE2 atom and hydrophobic effect are important factors regulating affinity.

Temperature dependence of methyl proton hyperfine shifts

The temperature dependence of the heme methyl resonances for Im-metMb and 4MeIm-metMb is displayed in Figure 3. Because heme 3-CH_3 chemical shift of both complexes is located in the diamagnetic envelope, it was identified from EXSY spectra recorded at different temperature. Other heme methyl chemical shifts can be obtained directly from one-dimensional spectrum at different temperature. As to both complexes, 5-CH_3 and 1-CH_3 obey the Curie law, the other methyl peaks (8-CH_3 and 3-CH_3) are abnormally according to the law of Curie, which predicts a linear downfield shift of the hyperfine shift with the raising inverse absolute temperature. In addition, both complexes present their methyl signals in the order of $5 > 1 > 8 > 3$, which is different from purely high-spin metMb whose heme methyl shift pattern is $8 > 5 > 3 > 1$. This chemical shift pattern may arise from the contribution of thermal equilibrium between the high ($s = 5/2$) and low ($s = 1/2$) states of the heme iron, reflecting different spin transfer mechanism (La Mar *et al.* 1973).

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