Notable deuterium effect on the electron transfer rate of myoglobin

Yasuhiro Mie,*[a] Chiho Yamada,[b] Tadayuki Uno,[c] Saburo Neya,[d] Fumio Mizutani,[a] Katsuhiko Nishiyama[b] and Isao Taniguchi[b]

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The electron transfer reaction of wild-type myoglobin at an electrode was significantly facilitated in a D_2O buffer as compared with that in an H_2O buffer, with $k^{0\prime}_{H2O}/k^{0\prime}_{D2O} = 0.13$, while a minimal deuterium kinetic isotope effect on the myoglobin with modification at distal histidine (His-64) was observed.

Since electrode surfaces for protein molecules have become compatible, the electron transfer reaction of metalloproteins at electrodes has been studied in detail to understand the kinetics and thermodynamics of the biological redox process.[1,2] Myoglobin (Mb) is an oxygen storage hemoprotein and the values of its electron transfer kinetics are smaller than those of electron-transfer hemoproteins such as cytochrome *c*.[1] Significantly, autooxidation, the reaction of reduced Mb(Fe(II)) with O_2 that converts into physiologically inactive oxidized Mb(Fe(III)), is extremely slow. Slower redox kinetics of Mb is the suggested result(s) of the gated redox process caused by the dissociation and re-association of bound H_2O in the Fe(II) form[3] and a high reorganization energy.[4] Dyke *et al.*[5] and Taniguchi *et al.*[6] also reported that the breakage of the hydrogen bond network including water molecules in distal sites of the heme pocket of Mb increases its electron transfer rate due to lowered reorganization during the redox reaction. We recently found that when the active centre protoheme of native Mb was replaced with diazaheme, the sixth ligand of the heme iron changed to His-64 from H_2O, and its electron transfer rate significantly increased.[7] The hydrogen bond network including a H_2O molecule bound to heme iron, the network of which must be rearranged during electron transfer, coupled with dissociation and reassociation of the water molecule to and from the heme iron (Fe(II)), is crucial to determine the electron transfer kinetics of Mb. Therefore, a deuterium isotope effect on the kinetics should be discernible.

Although there have been several reports regarding the deuterium (isotope) effect on the ligand binding property of Mb to understand its mechanism,[8] no experiment to study the effect of deuteration on the electrochemical properties of Mb has been conducted. We electrochemically measured the wild-type (WT) Mb and K45S mutant, having a water molecule as the sixth ligand of heme iron, and the H64V mutant and His-64 chemically modified Mb with cyanogen bromide (BrCN-Mb), without a water molecule bound to heme iron, in H_2O and D_2O buffers at an In_2O_3 electrode.

WT pig Mb and its mutants were expressed in *Escherichia coli* and purified as previously described.[9] Chemically modified Mb with cyanogen bromide was prepared as described.[10] Cyclic voltammograms (CV) were obtained at an In_2O_3 electrode with a highly hydrophilic surface.[7] All voltammograms obtained in this study showed a diffusion-controlled electrode reaction. Phosphate buffers were prepared by dissolving NaH_2PO_4 in pure H_2O (MilliQ) or D_2O and the pH or pD was adjusted to 6.5 (pH) or 6.1 (pD) by adding concentrated NaOH in H_2O or D_2O, respectively. The UV-visible (700–250 nm) and CD spectra (500–200 nm) of the Mbs in the H_2O and D_2O buffers apparently did not differ.

Fig. 1 shows the cyclic voltammograms of WT Mb in the H_2O and D_2O buffers at a scan rate of 20 mV s^{-1}. The difference between the profiles of the voltammograms of the H_2O and D_2O buffers was remarkable. As regards D_2O, the peak sharpness and reversibility (electrochemical sense) were significantly improved as compared with those of H_2O. The peak separations between the anodic and cathodic peak potentials (ΔE) were 245 and 98 mV in H_2O and D_2O, respectively. The estimated heterogeneous electron transfer rate constant $k^{0\prime}$, by CV simulation using BAS DigiSim® software, was 1.3 ($\pm$0.3) $\times$ 10^{-4} cm s^{-1} in H_2O and 1.0 ($\pm$0.2) $\times$ 10^{-3} cm s^{-1} in D_2O. The electron transfer rate of Mb increased with increasing the concentration of D_2O in the buffer solution, as shown in Fig. 2. This indicates that deuterium incorporation facilitates electron transfer. The $k^{0\prime}$ value in the D_2O buffer was approximately one order of magnitude greater than that in H_2O, yielding a k_{H2O}/k_{D2O} value of 0.13. This large 'inverse' isotope (deuterium) effect was not predicted. Since the electron transfer of Mb is coupled with reorganization of the H-bonding network including a water molecule bound to a heme iron in the distal heme pocket, the isotope effect should be normal. In order to precisely understand the deuterium effect on the electron transfer of Mb, we prepared Mbs (K45S, H64V mutants and BrCN-Mb) in which the distal H-bonding network was modified, and

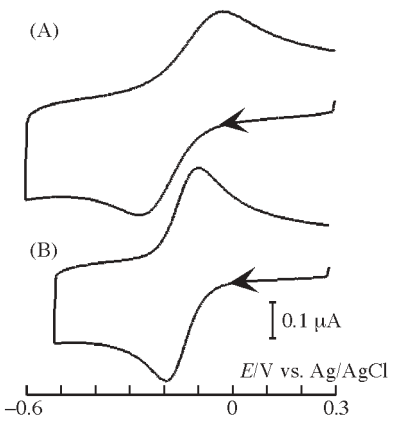

Fig. 1 Cyclic voltammograms of 40 μM WT Mb at a scan rate of 20 mV s^{-1} in 50 mM phosphate buffer: (A) H_2O and (B) D_2O.

*yasuhiro.mie@aist.go.jp

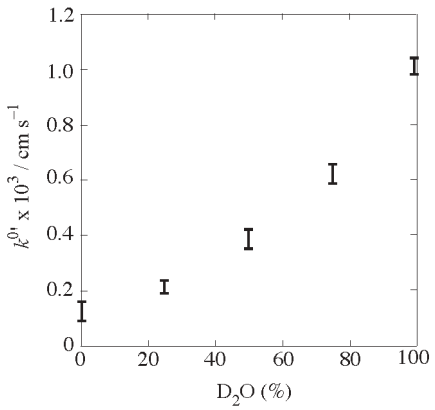

Fig. 2 The effect of increasing the concentration of D_2O in the buffer solutions on the heterogeneous electron transfer rate of WT Mb.

measured their electrochemical parameters in H_2O and D_2O buffers. The $k^{0\prime}$ values of all the Mbs estimated from their voltammograms are summarized in Table 1.

When the lysine-45 residue is replaced with a less bulky serine (K45S), the hydrogen bond between residue-45 and the histidine-64 residue *via* the water molecule is broken; however, the iron–water–His-64 network remains.[11] The $k^{0\prime}$ value obtained for the K45S mutant in H_2O was 2.2 ($\pm$0.3) $\times$ 10^{-4} cm s^{-1}, which was slightly greater (approximately double) than that of WT Mb. This might be due to the hydrogen bond network being disrupted by the mutation, which might decrease the structural reorganization during electron transfer. The $k^{0\prime}$ value of the K45S mutant in the D_2O buffer was 1.0 ($\pm$0.2) $\times$ 10^{-3} cm s^{-1}, which was five times greater than that in H_2O. Similar to WT Mb, the inverse isotope effect was observed (k_{H2O}/k_{D2O} = 0.22).

The H64V mutant has a five coordinated heme iron structure without a water molecule as the sixth ligand, and the hydrogen bond network around the hydrophobic Val-64 residue is significantly destroyed.[12] The electrode reaction of the H64V mutant was quite reversible and the estimated $k^{0\prime}$ value of 2.5 ($\pm$0.2) $\times$ 10^{-3} cm s^{-1} in H_2O was one order of magnitude greater than that of WT Mb, which closely agrees with the results of the electron transfer kinetics of sperm whale WT and H64 mutated Mbs.[6] The improved electron transfer ability of the H64V mutant is due to less reorganization being required during the redox reaction since the hydrogen bond network is lost.[5,6] Importantly, the estimated $k^{0\prime}$ value of 2.7 ($\pm$0.2) $\times$ 10^{-3} cm s^{-1} for the H64V mutant in D_2O was similar to that in H_2O. In contrast to WT and K45S mutant, the deuterium kinetic isotope effect on the electron transfer of the H64V mutant was small (k_{H2O}/k_{D2O} = 0.93).

The chemical modification of Mb by a stoichiometric amount of cyanogen bromide ejects a water molecule bound to the heme iron to yield a five coordinated heme iron structure.[10] Since cyanogen bromide specifically modifies the Nε atom in the imidazole ring of His-64 that interacts with H_2O in WT Mb,[12] the H bond network at the distal heme pocket should remain (except for the ejected H_2O molecule). The obtained $k^{0\prime}$ value of 1.9 ($\pm$0.2) $\times$ 10^{-3} cm s^{-1} in H_2O for BrCN-Mb was much greater than that of WT Mb as previously reported.[6] The $k^{0\prime}$ value in D_2O was found to be 2.0 ($\pm$0.2) $\times$ 10^{-3} cm s^{-1}, yielding k_{H2O}/k_{D2O} = 0.95. Similar to the H64V mutant, the deuterium effect on the electron transfer of BrCN-Mb was minimal.

In summary, the deuterium kinetic isotope effect for WT and K45S mutant was large, whereas that for the H64V mutant and BrCN-Mb was not. Considering the heme structural environment of each Mb, the deuterium effect occurs in Mbs with an iron–water–His-64 network in the heme pocket. Thus, the key to the deuterium effect on the electron transfer kinetics is an 'iron–water–His-64 network'. This suggests that the structural reorganization for this network during the redox process would be significantly smaller in D_2O than in H_2O. Since the inverse deuterium isotope effect discovered here is quite large, this could not be explained in terms of the change(s) in hydrogen bond coupling between H_2O and D_2O along the electron transfer pathway[13] by assuming that the electron transfers through an iron–water–His-64 unit.

Assuming that the rate of electron transfer was changed only by the term of the activation energy, ΔG^* (= $\lambda/4$ at ΔG^0 = 0, where λ is reorganization energy, and in this study ΔG^0 = 0 for each situation because the $k^{0\prime}$ values were estimated at the equilibrium potential), the change in the $k^{0\prime}$ value for each Mb in D_2O compared with that in H_2O at 25 °C, with $k^{0\prime}{}_{H2O}/k^{0\prime}{}_{D2O}$ *ca.* 0.13 for WT and 0.22 for K45S mutant, corresponded to a difference in $\Delta\Delta G^*$ (= ΔG^* (H_2O) − ΔG^* (D_2O)) of approximately 5.1 and 3.8 kJ mol^{-1} or $\Delta\lambda$ = 20 and 15 kJ mol^{-1}, respectively, according to the Marcus theory.[14] These $\Delta\lambda$ values are equivalent to one or two hydrogen bond(s). The present results might indicate that the hydrogen bond(s) between His-64 and water molecule(s) in H_2O is significantly perturbed in D_2O. This would be consistent with the fact that Mb has a larger pK value in D_2O than in H_2O for an acid–alkaline transition (a water molecule bound to the heme iron changes to $OH(D)^-$ with increasing solution pH(D)).[15] Since the hydrogen bond between the imidazole Nε atom of His-64 and water is important for the deprotonation of the water molecule to yield the alkaline form,[15] the larger pK value (*i.e.*, harder deprotonation) indicates perturbed hydrogen bonding.

In conclusion, the electron transfer of Mb was notably facilitated in D_2O as compared with H_2O. The precise structural reorganization of the iron–water–His-64 unit in D_2O during the electron transfer reaction remains unknown; however, the electrochemical results presented in this study suggest that it would differ significantly from that in H_2O.

Table 1 Comparison of heterogeneous electron transfer rate constants of Mbs in H_2O and D_2O buffers

Mb	$k^{0\prime}{}_{(H2O)}$ × 10^4/ cm s^{-1}	$k^{0\prime}{}_{(D2O)}$ × 10^4/ cm s^{-1}	Ratio $k^{0\prime}{}_{(H2O)}/k^{0\prime}{}_{(D2O)}$
WT	1.3 ± 0.3	10 ± 2	0.13
K45S	2.2 ± 0.3	10 ± 2	0.22
H64V	25 ± 2	27 ± 2	0.93
BrCN	19 ± 2	20 ± 2	0.95

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[a]*National Institute of Advanced Industrial Science and Technology, 2-17-2-1, Tsukisamu-higashi, Sapporo, 062-8517, Japan*
E-mail: yasuhiro.mie@aist.go.jp; Tel: +81-11-857-8913
[b]*Department of Applied Chemistry and Biochemistry, Kumamoto University, 860-8555, Japan*
[c]*Graduate School of Pharmaceutical Sciences, Kumamoto University, 862-0973, Japan*
[d]*Graduate School of Pharmaceutical Sciences, Chiba University, 263-8522, Japan*

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