$^1$H NMR study of dynamics and thermodynamics of acid–alkaline transition in ferric hemoglobin of a midge larva (Tokunagayusurika akamusi)

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Abstract

One of the components of hemoglobin from the larval hemolyph of Tokunagayusurika akamusi possesses naturally occurring substitution at the E7 helical position (Leu E7>M). Fukuda, T. Takagi, K. Shikama, Biochim. Biophys. Acta 1157 (1993) 185–191. Its oxygen affinity is almost comparable to those of mammalian myoglobins and it exhibits Bohr effect. Both acidic and alkaline forms of the ferric hemoglobin have been investigated using $^1$H NMR in order to gain insight into molecular mechanisms for relatively high oxygen affinity and Bohr effect of this protein. The NMR data indicated that the acidic form of the protein possesses pentacoordinated heme, and that the alkaline form possessing OH appears with increasing the pH value. pH titration yielded a pK value of 7.2 for the acid–alkaline transition, and this value is the lowest among the values reported so far for various myoglobins and hemoglobin. The kinetic measurements of the transition revealed that the activation energy for the dissociation of the Fe-bound OH, as well as the dissociation and association rates, decrease with increasing the pH value. These pH dependence properties are likely to be related to the Bohr effect of this protein. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Acid–alkaline transition; Monomeric hemoglobin; NMR; Paramagnetic relaxation; Paramagnetic shift; Saturation transfer

1. Introduction

Hemoglobin (Hb) from the 4th-instar larva of Tokunagayusurika akamusi, a midge (Diptera) commonly found in eutrophic lakes in Japan, is composed of at least 11 different components [1,2]. Among these components, hemoglobin V (HbV) is one of the two components of which primary structures have been determined and functional properties have been measured [1]. HbV possesses a single peptide chain
that consists of 152 amino acid residues. Its primary structure is completely different from those of mammalian Mbs, except that Phe and His occupy at the CD1 and F8 helical positions, respectively. A comparison of the sequence between HbV and the monomeric Hb components from other midge larva such as Chironomus thummi thummi [3–9] and C. thummi piger [10], on a basis of a matrix analysis, indicated the homology of less than 30% [1]. Furthermore, the mean residue ellipticity at 222 nm, obtained from the circular dichroism spectrum, indicated that HbV possesses considerably reduced helical contents, compared with mammalian myoglobins (Mbs) (Shikama et al., unpublished results). In addition, it has been suggested that Leu occupies at the E7 helical position of HbV. In spite of the lack of an amino acid residue capable of being a hydrogen-bond donor, at the E7 helical position, for stabilizing Fe-bound oxygen, HbV exhibits relatively high oxygen affinity, although they possess Val E7 27,29. X-ray measurement 43. Paramagnetic 1 H NMR saturation transfer experiment allows quantitative characterization of the kinetics of the transition, provided that the transition rate is comparable to paramagnetic relaxation rate of heme peripheral side-chain proton [32,40,41]. The acid–alkaline transition in ferric Mb (metMb) and Hb (metHb) sharply reflects characteristics of ligand binding properties of the active sites in hemoproteins [31–41]. The acidic form of metMb and metHb generally possesses H2O as Fe-bound ligand and, upon the transition to alkaline form, the bound ligand is changed to OH−, with the concomitant change of the spin state from S = 5/2 to S = 1/2. In the case of Mbs possessing His E7, in which the sixth ligation site is usually occupied by H2O, the interconversion between acidic and alkaline forms is quite fast because the protonation/deprotonation process of the His E7 imidazole bound to the Fe-bound ligand via a hydrogen bond facilitates the transition [42]. On the other hand, in the case of metMbs in which the sixth ligation site is vacant, the transition occurs in a relatively slow time scale because exogenous ligand OH− has to diffuse into/out of the heme pocket [34,37,39–41]. Recent studies revealed that the presence of an amino acid residue capable of being a proton acceptor is essential for stabilization of Fe-bound H2O [41]. Hence, H2O coordination at the sixth ligation site of heme iron in acidic form of metMb and metHb provides a diagnosis of the presence of a proton acceptor in the proximity of the binding site. Thermodynamics of the acid–alkaline transition has been analyzed using a variety of techniques such as 1H NMR [33,36], optical spectroscopy [35,43], EPR [44] and magnetic susceptibility measurement [43]. Paramagnetic 1H NMR saturation transfer experiment allows quantitative characterization of the kinetics of the transition, provided that the transition rate is comparable to paramagnetic relaxation rate of heme peripheral side-chain proton [32,40,41].

We report herein the results of 1H NMR study in dynamics and thermodynamics of the acid–alkaline transition of ferric T. akamusi Hb component V (metHbV). The present study revealed that H2O is not coordinated to heme iron of acidic form of metHbV, as in acidic form of the mollusc Mbs. The high affinity of metHbV to OH− is reflected in the value of 7.2 for the pK value of the transition, and this value is the lowest among the values reported for various metMbs and metHbs [31,34,39–41,45,46]. Furthermore, the reaction rate of the dissociation of Fe-bound OH− was found to decrease with increasing the pH value, and this result is possibly related to
alkaline Bohr effect of this protein. A unique heme active site in HbV is discussed on the basis of the analyses of the acid–alkaline transition and heme peripheral methyl proton hyperfine shifts.

2. Materials and methods

2.1. Protein preparation

Hb from the 4th-instar larva of *T. akamusi* was prepared as met form using the reported procedure [1,2]. The Hb solution was concentrated to about 1 mM, and the solvent was exchanged to 2 H₂O in an Amicon ultrafiltration cell. Sperm whale Mb was purchased from Biozyme and used without further purification. The p²H value of the sample was adjusted using 0.2 M NaO₂H or 0.2 M KH₂PO₄ in 2 H₂O solution, and the p²H value was measured using a Horiba model F-22 pH-meter with a Horiba-type 6069-10 c electrode. The isotope effect was not considered to correct the p²H value.

2.2. NMR spectroscopy

¹H NMR spectra were recorded using Bruker AC-400P FT-NMR spectrometer operating at a ¹H frequency of 400 MHz. A typical spectrum consisted of 3 k transients with 8 kbyte date points over 50 kHz band width, and a 4.6 µs 45° pulse. Solvent signal suppression, when necessary, was achieved by direct saturation during the relaxation delay. Intrinsic spin–lattice relaxation time (T¹intr) was measured using the saturation-recovery method with a selective saturation pulse. Saturation transfer experiments were carried out by selectively saturating a desired peak for a variety of time and steady-state value of the saturation transfer factor (I/I₀; I and I₀ are the signal intensities of peak A without and with the saturation of peak B which is connected to peak A by dynamic process, respectively) was achieved for the saturation time ≥ 80 ms. The spectra that resulted from the saturation transfer experiments were presented in a form of difference spectrum. The saturation transfer factor was obtained from measuring the signal intensity. The signal-to-noise of the spectra was improved by apodization, which introduced a 50-Hz line broadening. The chemical shifts are given in parts per million (ppm) relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate with the residual H²HO as internal reference.

2.3. Determination of the kinetic parameter for acid–alkaline transition via saturation transfer factor

The acid–alkaline transition in metHbV is expressed as,

\[
\text{metHbV} + \text{OH}^- \xrightarrow{k_{AB}} \text{metHbV(OH}^-) 
\]

where \( k_{AB} \) and \( k_{BA} \) are the association and dissociation rates, respectively. In the saturation transfer experiments, the pair of spectra recorded without and with the saturation of a heme methyl proton resonance of metHbV yields \( I_r \) and \( I \) for the signal intensity of the corresponding heme methyl proton resonance of metHbV(OH⁻), respectively. The saturation transfer factor \( (I/I_0) \) is related to \( k_{BA} \) and \( T_{1}^{\text{intr}} \) of the heme methyl proton resonance of metHbV(OH⁻) by the formula [47].

\[
k_{BA} = \left( T_{1}^{\text{intr}} \right)^{-1} \left( 1 - I/I_0 \right)/\left( I/I_0 \right)
\]

\( k_{AB} \) is related to equilibrium constant of the reaction, \( K \), by the following equation,

\[
K = k_{AB}/k_{BA} = \frac{[\text{metHbV(OH}^-)]/[\text{metHbV}][\text{OH}^-]}
\]

where \([\text{metHbV(OH}^-)]\), \([\text{metHbV}]\) and \([\text{OH}^-]\) are the concentration of corresponding species. Therefore, the \( k_{AB} \) value can be calculated from the \( k_{BA} \) value, \([\text{OH}^-]\) and \([\text{metHbV(OH}^-)]\)/\([\text{metHbV}]\), which is determined from the intensity of the heme methyl proton resonance arising from metHbV(OH⁻) and metHbV.

2.4. Spectrophotometric measurements

Spectrophotometric measurements were carried out using a Hitachi two-wavelength double-beam spectrophotometer (model U-3210) equipped with a thermostatically controlled cell holder. The acid–alkaline transition of metHbV was monitored at 577 nm band in 0.1 M KCl at 25°C.
3. Results

3.1. $^1$H NMR spectra of metHbV

The 400 MHz $^1$H NMR spectra of metHbV at 25°C and pH 5.10 is illustrated in trace A of Fig. 1, and is compared with that of sperm whale metMb, pH 7.10, shown in trace B. Four heme methyl proton resonances in trace A are clearly identified below 60 ppm. It has been demonstrated that heme methyl proton hyperfine shift pattern in ferric high-spin form of b-type hemoprotein is independent of the coordination structure of heme iron, as long as heme is inserted into the heme pocket with its usual orientation relative to protein moiety [39,41,49]. Consequently, the heme methyl proton resonances of metHbV are assigned to 8-, 5-, 3- and 1-Me in order of decreasing chemical shifts.

The heme methyl proton signals of metHbV are compared with those of sperm whale metMb, which possesses hexacoordinated heme with the Fe-bound water at the sixth ligation site, in Table 1. The comparison reveals some characteristic differences. First, the line width of heme methyl proton signals in TA metHbV (~ 300 Hz) is much smaller than that of sperm whale metMb (~ 600 Hz). Since the line width of these resonances is influenced by $T_{1e}$, which is primarily determined by zero-field level of the complex [50,51], relatively short $T_{1e}$ of metHbV, reflected by smaller line width of the signals, can be attributed to lower symmetric nature of the heme coordination structure. Furthermore, heme meso-proton signal of sperm whale metMb is observed at about 38 ppm. On the other hand, an extremely broad signal is observed at about $-25$ ppm in the spectrum of metHbV, and its line width strongly suggests that this signal arises from heme meso-protons of this protein. It has been demonstrated from the studies of model compounds and ferric high-spin Mb's that the meso-proton signals of hexacoordinated and pentacoordinated ferric high-spin heme complexes resonate at downfield and upfield hyperfine shifted regions of the spectra, respectively [52]. Therefore, upfield-shifted meso-proton signal in the spectrum of metHbV also supports that this protein possesses the pentacoordinated heme in its active site. In addition, the average heme methyl proton shift of metHbV is larger than that of sperm whale metMb (see Table 1). It has been reported that the average heme methyl proton shift of hexacoordinated heme is larger than that of pentacoordinated heme possibly due to the difference in zero-field splitting between the two complexes [49]. Hence, the present result is consistent with the pentacoordinated heme in metHbV. Furthermore, the spread of heme methyl proton shift of metHbV is smaller than that of sperm whale metMb, indicating that the in-plane asymmetry of the heme electronic structure in the former is smaller than that of the latter. Finally, it has been reported that the shift and the line width of the hyperfine shifted heme methyl proton resonances of ferric high-spin hemoprotein are influenced by solvent isotope composition, i.e., $H_2O/^{2}H_2O$, through hydrogen bond between the coordinated water molecule and a distal amino acid residue [53]. The fact that the resonances of metHbV are independent of the solvent isotope composition (result not shown) also supports the absence of the coordinated water molecule in this protein. Therefore, all these NMR results are consistent with the pentacoordinated heme in metHbV.

The absorption spectrum of metHbV at pH 5.5 exhibits the Soret band at 396.8 nm with the extinction coefficient of 106.5 mM$^{-1}$ cm$^{-1}$. A slight hypochromicity and a large hypsochromicity of the
Table 1  Assignment and chemical shift of heme methyl proton signals in both acidic and alkaline form of *T. akamusi* metHbV and sperm whale metMb at 25°C

<table>
<thead>
<tr>
<th></th>
<th>Acidic form</th>
<th>Alkaline form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>metHbV<em>a</em></td>
<td>metMb<em>b</em></td>
</tr>
<tr>
<td></td>
<td>metHbV(OH-)<em>c</em></td>
<td>metMb(OH-)<em>d</em></td>
</tr>
<tr>
<td>8-Me</td>
<td>90.22 (−33.06)<em>e</em></td>
<td>90.83</td>
</tr>
<tr>
<td>5-Me</td>
<td>83.15 (−31.95)<em>e</em></td>
<td>84.29</td>
</tr>
<tr>
<td>3-Me</td>
<td>80.09 (−35.12)<em>e</em></td>
<td>72.71</td>
</tr>
<tr>
<td>1-Me</td>
<td>67.47 (−33.06)<em>e</em></td>
<td>52.85</td>
</tr>
<tr>
<td>Average</td>
<td>80.23</td>
<td>75.17</td>
</tr>
<tr>
<td>Spread</td>
<td>22.75</td>
<td>11.34</td>
</tr>
</tbody>
</table>

*a* pH 5.10.

*b* pH 7.10.

*c* pH 9.95.

*d* pH 11.03.

*The numbers in parentheses are the shifts to *T*−1 → 0, obtained from the Curie plot.

*Shift difference, in ppm. 2 H2O and H2O is given in brackets; positive shifts indicate downfield bias in H2O relative to 2 H2O.  

*Average of heme methyl proton shifts.

*S* Spread of signals.

Soret band of metHbV, compared with those of mammalian metMbs possessing the usual His E7, also strongly suggested the absence of Fe-bound water in this monomeric metHb [1,54].

### 3.2. pH dependence of 1 H NMR spectra of metHbV

The pH dependence of the 400 MHz 1 H NMR spectra of metHbV is shown in A of Fig. 2. The resonances arising from alkaline form appear in the chemical shift region 15–35 ppm. The shifts of heme methyl proton signals of alkaline form are similar to those of met-hydroxyl form (metHbV(OH−)). With increasing pH, the signal intensity for the resonances of alkaline form increases at the expense of the signals of the acidic form. The fact that the two sets of the signals from the acidic and alkaline forms are separately observed in the spectra indicates that the acid–alkaline transition in this protein is slower compared with the NMR time scale. This result is in sharp contrast to the rapid acid–alkaline transition in sperm whale metMb (see B of Fig. 2). The analysis of the pH dependence of the signal intensity for the resonances arising from the two forms of metHbV yielded the p*K* value of 7.22. Thus, the p*K* values obtained from NMR and absorption spectra are essentially identical. This p*K* value is the lowest among the values reported for various ferric hemoproteins [31,34,39–41,45,46]. Since the acidic form of metHbV possesses pentacoordinated heme, its low p*K* value reflects a considerable stabilization of Fe-bound OH− with a proton-donor in this protein.

### 3.3. Heme methyl proton signal assignment of metHbV(OH−) via saturation transfer

The saturation transfer difference spectra resulted from the saturation of individual heme methyl proton signal of metHbV at 35°C, pH 7.12, are shown in Fig. 4. The traces B–E exhibit the saturation transfer connectivities between the corresponding heme methyl proton resonances in the two forms. The heme methyl proton signals of metHbV(OH−) can be assigned from the known assignments of metHbV using the connectivities. The assigned heme methyl proton signals of metHbV(OH−) are compared with those of sperm whale metMb(OH−) in Table 1 and their Curie plots, shift vs. the reciprocal of absolute temperature, are illustrated in Fig. 5. At ambient temperature, the met-hydroxyl form of hemoprotein has shown to exhibit a thermal equilibrium between the high-spin, *S* = 5/2, and low-spin, *S* = 1/2, states, due to the intermediate field strength of OH− ligand [43,55–57].
Fig. 2. pH dependence of the 400 MHz $^1$H NMR spectra of *T. akamusi* metHbV (A) and sperm whale metMb (B) in $^2$H$_2$O at 25°C. In (A), with increasing the pH value, the signals, at 15–35 ppm, arising from the alkaline form gain intensity at the expense of the signals of the acidic form, indicating that the acid–alkaline transition in this Hb is slower than the NMR time scale. A pK value of 7.2 ± 0.1 was estimated from the analysis of the signal intensity for the two forms. On the other hand, in (B), the progressive pH dependence shift of the signals shows that the transition is much faster than the NMR time scale.

The presence of the thermal spin equilibrium in this complex is manifested in the large deviation of the shift to $T^{-1} \rightarrow 0$ for the Curie plot from the diamagnetic shift of 3.1 ppm [58] (see Table 1) and the anti-Curie behavior of the plot for the average of heme methyl proton shifts. Since the shift decreases with increasing low-spin contents, the fact that the average heme methyl proton shift of metHbV(OH$^-$) is about 10 ppm smaller than that of metMb(OH$^-$) indicates the larger low-spin contents in the former protein than in the latter, although the spread of the signals are roughly the same with each other. The spin equilibrium is influenced by the strength of axial ligand field; therefore, larger low-spin contents in metHbV(OH$^-$) strongly suggests that OH$^-$ coordination to heme iron in this protein is stronger than that in metMb(OH$^-$). Furthermore, the heme methyl proton shift pattern of 5-, 8-, 1- and 3-Me, in the order of decreasing chemical shift, for metHbV(OH$^-$) is different from that for metMb(OH$^-$), i.e., 5-, 8-, 3- and 1-Me, in order of decreasing chemical shift.

The downfield hyperfine shifted regions of the 400 MHz $^1$H NMR spectra of metHbV(OH$^-$) in H$_2$O and $^2$H$_2$O are compared with each other in Fig. 6. An exchangeable proton signal is observed at about 43 ppm.
Fig. 4. Saturation transfer difference spectra of *T. akamusi* metHbV in $^2$H$_2$O, pH 7.12, at 35°C. (A) Reference spectrum. (B–E) Saturation of the heme proton signal of the acidic form exhibiting the saturation transfer to the corresponding signals of the alkaline form. The peak indicated by an arrow is saturated. The heme methyl proton signal assignments of the alkaline form are indicated in the spectra.

Fig. 5. Curie plot, the observed shift vs. reciprocal of temperature, for the individual heme methyl proton signal (open circles) and the average of all the heme methyl proton shifts (filled circles) of *T. akamusi* metHbV(OH$^-$) in $^2$H$_2$O, pH 9.95. The anti-Curie behavior for the plot of the average shift clearly demonstrates the thermal spin equilibrium in this protein.

Fig. 6. The downfield hyperfine shifted portions of the 400 MHz $^1$H NMR spectra of *T. akamusi* metHbV(OH$^-$) in H$_2$O (lower trace) and $^2$H$_2$O (upper trace), pH 8.10, at 25°C. An exchangeable proton signal is observed at about 43 ppm. In addition, there is an isotope effect on the heme methyl proton signals such that the heme methyl proton signals in the lower trace are shifted toward downfield relative to the corresponding signals in the upper trace.
ligand and an amino acid residue [16,32,53]. Although larger paramagnetic shifts in $^2\text{H}_2\text{O}$ than $\text{H}_2\text{O}$ have been reported for ferric high-spin [53] and low-spin [16,32] complexes of sperm whale Mb, upfield bias in $^2\text{H}_2\text{O}$ to $\text{H}_2\text{O}$ was observed for the present Hb.

3.4. Determination of acid–alkaline transition rate using saturation transfer experiments

The rate of the acid–alkaline transition of some Mbs have been determined using the saturation transfer method. The $T_{\text{intr}}^1$ value of 5-Me resonance of metHbV(OH$^-$) at 25°C and pH 9.95 is 2.7 ± 0.4 ms and was found to be essentially constant, within experimental error of ± 15%, in the temperature range from 20 to 35°C. The absence of the acidic form in the sample at the pH value used in the measurement ensures that the obtained $T_{\text{intr}}^1$ value is free from the acid–alkaline transition.

The substitution of both the $T_{\text{intr}}^1$ value and the saturation transfer factor, measured with the saturation of the corresponding signal in the acidic form, of 5-Me signal on metHbV(OH$^-$) into Eq. (1) yields the $k_{\text{BA}}$ value. Since the ratio between the concentration of both the acidic and alkaline forms can be calculated from their NMR signal intensities, the $k_{\text{AB}}$ value is obtained form the $k_{\text{BA}}$ value, and the equilibrium constant using Eq. (2). The $k_{\text{BA}}$ and $k_{\text{AB}}$ values for metHbV were determined at various pH and temperatures values, and the results are summarized in Table 2. Interestingly, not only the $k_{\text{AB}}$ value, but also the $k_{\text{BA}}$ value, are influenced by the pH change in a fashion that the $k_{\text{AB}}$ value increases with increasing the pH value, whereas the $k_{\text{BA}}$ value decreases. These results reflect the enhanced affinity of metHbV to OH$^-$ with increasing the pH value.

Fig. 7. Arrhenius plot of $k_{\text{BA}}$ for T. akamusi metHbV at the indicated three pH values. The activation energy ($\Delta E$) for the dissociation of Fe-bound OH$^-$ increases with increasing the pH value.

The activation energy ($\Delta E$) for the dissociation of the bound OH$^-$ ligand in metHbV is obtained from the Arrhenius plots shown in Fig. 7. As expected from the pH dependence of the $k_{\text{AB}}$ value, the obtained $\Delta E$ value increases with the pH value as summarized in Table 2. Such pH dependence of $\Delta E$ may be related to the Bohr effect of this Hb.

4. Discussion

4.1. Ligation sate of metHbV

The NMR shift data of metHbV are consistent with the absence of Fe-bound water in this protein, as

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>pH 6.64</th>
<th>pH 7.12</th>
<th>pH 7.73</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{AB}} \times 10^8 \text{mol}^{-1}\text{s}^{-1}$</td>
<td>$k_{\text{BA}} \text{s}^{-1}$</td>
<td>$k_{\text{AB}} \times 10^8 \text{mol}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>20</td>
<td>4.4 ± 0.7</td>
<td>129 ± 20</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>25</td>
<td>7.3 ± 1.1</td>
<td>213 ± 32</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>30</td>
<td>13.4 ± 2.1</td>
<td>354 ± 54</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>35</td>
<td>18.0 ± 2.7</td>
<td>478 ± 72</td>
<td>8.9 ± 1.3</td>
</tr>
<tr>
<td>$\Delta E$ (kJ mol$^{-1}$)</td>
<td>68 ± 11</td>
<td>88 ± 13</td>
<td>103 ± 12</td>
</tr>
</tbody>
</table>
described above. In addition, relatively slow acid–alkaline transition in metHbV also strongly supports the pentacoordinated heme in this Hb. Therefore, we conclude that the acidic form of metHbV possesses the pentacoordinated heme.

The absence of an amino acid residue capable of being a proton acceptor for forming a hydrogen bond in close proximity to Fe-bound $\text{H}_2\text{O}$ is common to all the metMbs and monomeric metHbs that have been shown to be pentacoordinated [39,41,49,59]. Since Leu is thought to occupy at the E7 helical position in HbV, the side-chain of this residue does not form a hydrogen bond with Fe-bound ligand. The side-chain of Arg E11 in this Hb is likely to act as a proton donor for Fe-bound $\text{OH}^-$ in the alkaline form. But its side-chain cannot be a proton acceptor for Fe-bound $\text{H}_2\text{O}$.

### 4.2. Heme electronic structure of metHbV($\text{OH}^-$)

Heme methyl proton hyperfine shift pattern of metHbV($\text{OH}^-$) is 5-, 8-, 1- and 3-Me, in the order of decreasing chemical shift. This shift pattern is completely different from that reported for sperm whale metMb($\text{OH}^-$), i.e., 8-, 5-, 3- and 1-Me, in the order of decreasing chemical shift [60]. Additionally, the value of 25.93 ppm for the average shift of the four heme methyl proton resonances of metHbV($\text{OH}^-$) is smaller by almost 10 ppm than the corresponding value of sperm whale metHbV($\text{OH}^-$), i.e., 34.16 ppm. This result indicates the presence of a significant difference in the delocalization of unpaired electron density from the heme iron to the porphyrin $\pi$-system between the two proteins. Since contact shift dominates the paramagnetic shift of these resonances, the difference in the hyperfine shift pattern between the two can be interpreted in terms of heme electronic structure. Since contact shift of heme methyl proton resonance is proportional to the quantity $S(S+1)$ [61], smaller average shift of the four heme methyl proton resonances of metHbV($\text{OH}^-$) can be attributed to smaller fraction of high-spin state in this protein, relative to that in sperm whale metHbV($\text{OH}^-$). The heme methyl proton hyperfine shift pattern of ferric low-spin Mb is modulated by the interaction of the highest $d_e$ orbital of the iron, where the unpaired electron resides, with $p_x$ orbital of the imidazole of His F8. But the shift pattern of ferric high-spin Mb is consistently 8-, 5-, 3- and 1-Me, in order of decreasing chemical shift, with significantly large magnitude of shifts. Therefore, with increasing the fraction of high-spin state in the complex, the average shift of the four heme methyl proton resonances increases, and the heme methyl proton hyperfine shift pattern tends to become similar to that in ferric high-spin form. Smaller fraction of high-spin state in metHbV($\text{OH}^-$), relative to that in sperm whale metMb($\text{OH}^-$), at ambient temperature should result in decelerating $T_2$ relaxation through the contact hyperfine interaction [62], as well as the Curie spin relaxation [63,64]. In fact, heme methyl proton signals of metHbV($\text{OH}^-$) are much narrower than those of sperm whale metMb($\text{OH}^-$).

The spin state of heme iron depends crucially on the strength of axial ligand field. Relatively large fraction of low-spin state in metHbV($\text{OH}^-$) strongly suggests that the bonding interaction between the heme iron and both His F8 imidazole and exogenous ligand in this protein is much stronger than that in sperm whale metMb($\text{OH}^-$). The strength of the iron–His F8 bond is modulated by steric interaction or the hydrogen bond of the $N_e\text{H}$ proton with a proton acceptor. The former interaction simply compresses or extends the Fe–$N_e$ bond. The latter affects the Fe–$N_e$ bond through the alteration of the imidazole basicity. Due to the lack of the knowledge about molecular structure of the active site of this protein, the effects of the steric interaction between heme iron and His F8 imidazole on the Fe–$N_e$ cannot be estimated at present. But the observation of His F8 $N_e\text{H}$ proton signal in metHbV($\text{OH}^-$) indicates that this proton is hydrogen-bonded to a proton acceptor. In addition, in the spectra of met-cyano Mb, His F8 $N_e\text{H}$ proton signal is generally observed at $\sim$ 20 ppm [14]. The observation of His F8 $N_e\text{H}$ proton signal of metHbV($\text{CN}^-$) at $\sim$ 14 ppm (unpublished result) suggests the partial deprotonation of His F8 $N_e\text{H}$ proton by a strong hydrogen bond with a proton acceptor. Furthermore, Fe-bound $\text{OH}^-$ is stabilized by a hydrogen bond with a proton donor [28,42]. The formation of this hydrogen bond enhances the localization of electron density at O atom directly bonded to the heme iron, which in turn strengthens the Fe–O bond. The increased axial ligand field strength in metHbV($\text{OH}^-$) could be partly attributed to these electronic interactions.
4.3. pK value of acid–alkaline transition in metHbV

metHbV exhibits a remarkably low pK value of 7.2 for the acid–alkaline transition. Since acidic form of metHbV possesses the pentacoordinated heme, pK value is thought to be primarily controlled by the affinity of OH\(^{-}\) to the pentacoordinated heme. Therefore, it can be concluded that there is a molecular mechanism for considerable stabilization of Fe-bound OH\(^{-}\) in this protein. Fe-bound OH\(^{-}\) demands a proton-donor to form a hydrogen bond. Considering the primary structure of HbV [1], it is conceivable that the side-chain NH proton of Arg E11 is hydrogen-bonded to the Fe-bound ligand in this protein. Similar functional properties of Arg residue in the E helix has been reported for Aplysia [25,26,28] and Dolabella [27,41] metMbs, which exhibit the pK values of 7.6 and 7.8, respectively. Cutruzzolà et al. [31] had demonstrated that the replacement of His E7 by Val in sperm whale Mb alters the pK value from 9.0 to 10.2, and that the further replacement of Thr E10 by Arg reduces the pK value to 8.8. Thus, the guanidino NH proton of the side-chain of Arg in these proteins acts as a strong proton donor for Fe-bound OH\(^{-}\).

The change of the \(k_{AB}\) and/or \(k_{BA}\) values influences the pK value. In the case of Dolabella metMb [41], its \(k_{BA}\) is one order of magnitude larger than that of metHbV, whereas the \(k_{AB}\) values are similar with each other. Therefore, the difference of 0.6 pH unit between the pK values of these proteins is attributed to the difference in the \(k_{BA}\) value. On the other hand, the difference in the pK value between Aplysia metMb [30] and metHbV arises from the fact that the \(k_{AB}\) values of Aplysia metMb is almost one order of magnitude smaller than that of metHbV, because their \(k_{BA}\) values are similar with each other. These results indicate that both \(k_{AB}\) and \(k_{BA}\) values contribute to control the pK value for the acid–alkaline transition of ferric hemoproteins that possess pentacoordinated heme in the acidic form.

4.4. Kinetics of acid–alkaline transition

As described above, the \(k_{BA}\) value of metHbV is considerably smaller than that of Dolabella metMb [40,41] at a given temperature and pH value. This result indicates that Fe-bound OH\(^{-}\) in the former protein is more strongly stabilized by the hydrogen bond with Arg E11 than that in the latter, which is stabilized by the hydrogen bond with Arg E10. Furthermore, the pH dependence of the \(k_{BA}\) value indicates that the affinity of metHbV to OH\(^{-}\) increases with increasing the pH value. The pH dependence of the OH\(^{-}\) affinity of metHbV is also clearly manifested in the activation energy (\(\Delta E\)) for the dissociation of the bound OH\(^{-}\) ligand, which also increases with increasing the pH value. This result is in a sharp contrast with those obtained for Dolabella and Mustelus Mbs [41], in which the \(\Delta E\) value are essentially independent of the pH value. Furthermore, the \(\Delta E\) values of 68–103 kJ mol\(^{-1}\) for metHbV are considerably larger than the values of 58 ± 9 and 27 ± 3 kJ mol\(^{-1}\) obtained for Dolabella and Mustelus metMbs, respectively.

The kinetic data in Table 2 demonstrate that both the \(k_{AB}\) and \(k_{BA}\) values become smaller with increasing the pH value. The difference of about three orders of magnitude in the \(k_{BA}\) value observed between Dolabella and Mustelus metMbs [41] is likely to arise from differences in intrinsic structural and dynamic properties of the active site between the two. Hence, the decrease in the \(k_{AB}\) and \(k_{BA}\) values with increasing the pH value would be attributed to the effects of pH on molecular structure and dynamic properties of the heme active site in this protein. The pH dependence of the \(k_{BA}\) and \(\Delta E\) values indicates that the stabilization of Fe-bound OH\(^{-}\) is enhanced at higher pH value. These results may be related to alkaline Bohr effect of this protein (Shikama et al., unpublished result). Since the acidic form of metHbV possesses pentacoordinated heme in its active site, the binding of OH\(^{-}\) to heme iron in alkaline form of the protein, i.e., metHbV(OH\(^{-}\)), exerts the changes in both the coordination and spin states of the iron with its oxidation state unchanged. Therefore, the acid–alkaline transition in metHbV resembles oxygenation process of the protein, although there is a difference in the oxidation state of heme iron between the two processes. Gersonde et al. [65] demonstrated that the Bohr effect of the monomeric C. thummi thummi Hb is primarily controlled by dissociation rate constant of ligand. The present results show that the enhanced affinity of metHbV to OH\(^{-}\) at higher pH stems from the decrease of the \(k_{BA}\) value with increasing the pH value. Consequently, both alkaline Bohr effect and
the enhanced affinity of metHbV to OH− at higher pH are controlled by similar molecular mechanisms. A detailed structure determination of HbV at different pH values is needed to determine such mechanisms.

References