Thermodynamic Redox Properties Governing the Half-Reduction Characteristics of Histamine Dehydrogenase from Nocardioides simplex

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Histamine dehydrogenase from Nocardioides simplex is a homodimer and belongs to the family of iron-sulfur flavoproteins having one [4Fe-4S] cluster and one 6-S-cysteinyl FMN per monomer. In the reductive titration with histamine, two-electron reduction occurred per monomer at pH < 9, while single-electron reduction proceeded at pH > 9. The substrate-reduced histamine dehydrogenase yielded an electron paramagnetic resonance spectral signal assigned to the flavin semiquinone. The signal intensity increased with pH up to pH 9 and reached a maximum at pH > 9. These unique features are explained in terms of the redox potential of the cofactors, where the redox potential was evaluated over a pH range from 7 to 10 by using a spectroelectrochemical titration method for the flavin and cyclic voltammetry for the [4Fe-4S] cluster. The bell-type pH dependence of the enzymatic activity is also discussed in terms of the pH dependence of the centers’ redox potential.

Key words: histamine dehydrogenase; 6-S-cysteinyl flavin mononucleotide; [4Fe-4S] cluster; intramolecular electron transfer; thermodynamic redox property

Histamine is a powerful biologically-active amine that can directly cause smooth muscle motion and stimulate the heart and neurons. Histamine is produced in food by the microbial decarboxylation of histidine during spoilage and is a marker of the freshness of seafood, especially scombroid fish and fermented foods such as cheese and wine. Because an intake of fish with a high concentrations of histamine causes a potentially fatal allergic reaction, the United States Food and Drug Administration (FDA) has ruled the upper limits for the amount of histamine in tuna and related fish.

Histamine dehydrogenase (HmDH) from gram-positive actinobacterium Nocardioides simplex and gram-negative bacteria Rhizobium sp. 4-9 catalyzes the oxidative deamination of histamine to imidazole acetaldehyde and ammonia (Scheme 1). Due to its high catalytic activity and narrow substrate specificity, HmDH shows promise as a catalyst in histamine sensors for food analysis and clinical use. The determination of histamine using HmDH from N. simplex and from Rhizobium sp. 4-9 has been reported. In order to develop biosensors based on HmDH, it is important to understand the physicochemical properties of the enzyme.

HmDH is a homodimer whose subunits each have a molecular mass of about 150 kDa. HmDH is classified as an iron-sulfur flavoprotein. Each subunit has one unusual, covalently-bound flavin mononucleotide, 6-S-cysteinyl flavin mononucleotide (CFMN), and one [4Fe-4S] cluster. HmDH from N. simplex also has one adenosine diphosphate (ADP) per monomer, although the function of this ADP remains unclear.

Scheme 1.

Translated by: J. Umemoto
N (CH₃)₃ + H₂O + A → NH (CH₃)₂ + HCHO + AH₂

A: 2e⁻-electron acceptor

Scheme 2.

overexpressed the hmd gene in E. coli strain Rosetta (DE3) by using the pET26b(+) vector. Furthermore, we have purified the recombinant enzyme to homogeneity and for the first time verified complete flavinylation in a recombinant iron-sulfur flavoprotein. The results of a steady-state enzyme-kinetic analysis and chemical analysis of the cofactors have revealed that the recombinant enzyme showed enzymatic properties almost identical to those of the native enzyme from N. simplex. However, the mechanisms for the catalytic reaction, intramolecular electron transfer, and substrate recognition remain to be elucidated.

In contrast, trimethylamine dehydrogenase (TMADH) from Methylophilus methylotrophus W₁₃₁₁ catalyzes the oxidative demethylation of trimethylamine to produce dimethylamine and formaldehyde, as shown by Scheme 2. TMADH is also an iron-sulfur flavoprotein that is a homodimer and has the same cofactors as HmDH: one CFMN, one bacteria-type [4Fe-4S] cluster and one ADP per monomer. The DNA sequence of HmDH is similar to that of TMADH (40% identical). Therefore, it is possible that the mechanism for the catalytic reaction of HmDH will be similar to that of TMADH, including the intramolecular electron transfer reaction.

It has been suggested that the electron transfer sequence of the TMADH reaction may involve two-electron transfer from trimethylamine to the oxidized CFMN (CFMN₈), then by the intramolecular transfer of electrons from fully reduced CFMN (CFMN₉) to the oxidized [4Fe-4S] cluster (FeS₀₈), and finally the transfer of the reducing equivalents from the reduced [4Fe-4S] cluster (FeS₀₈) to an electron-transferring flavoprotein (ETF) as the physiological electron acceptor. ETF consists of an αβ heterodimer with a molecular mass of 62 kDa that possesses one flavin adenine dinucleotide (FAD) and one adenosine monophosphate (AMP) per monomer. FAD bound to ETF acts physiologically as a single-electron acceptor from TMADH as it cycles between the oxidized and the semiquinone states, although the two-electron reduction of FAD in ETF can be achieved electrochemically.

It has been reported that a trimethylamine-treatment of TMADH caused two-electron reduction per monomer in the absence of electron acceptors of the enzyme. TMADH can accept a total of up to three electrons per monomer; two at the CFMN moiety and one at the [4Fe-4S] cluster. Such partial reduction by the substrate appears to be due to the odd number of electrons involved in reducing the TMADH monomer, in contrast with the even number of electrons donated by the substrate.

Substrate-reduced (i.e., two-electron per monomer-reduced) TMADH is in equilibrium between two ultimate states: one state is characterized by CFMN₈ and FeS₀ (here referred to as CFMN₉–FeS₀), while the other state is characterized by the flavin semiquinone (CFMN₈) and FeS₈ (here referred to as CFMN₉–FeS₈). It has been reported that, in TMADH, the CFMN₉–FeS₀ state is more favorable below pH 7.5, whereas the CFMN₈–FeS₈ state becomes more favorable as the pH value rises above 8. Such equilibrated redox reactions should be understood in terms of the redox potentials of the cofactors. Although the redox potential of TMADH has been evaluated by micro-coulometry at pH 7, the pH dependence of the redox potential has not yet been extensively examined.

Apart from the partial two-electron reduction of the subunit, complete three-electron reduction per monomer seems to be thermodynamically feasible during substrate reduction if intermolecular electron transfer occurs between the partially reduced enzymes. In addition, single-electron reduction per monomer might also be expected with substrate reduction if intersubunit electron transfer occurs and the redox potential of [4Fe-4S] cluster is much more negative than that of CFMN. However, there is no report of such a single- or three-electron reduction state of the TMADH monomer during substrate reduction. These equilibrium properties must also be governed by the redox potentials of the cofactors within the enzyme as well as by the intra- or intermolecular electron transfer probability.

In this work, we focused our attention on the equilibrated redox properties of HmDH as one of the typical iron-sulfur flavoproteins. HmDH from N. simplex is superior to HmDH from Rhizobium sp. 4-9 and TMADH from M. methylotrophus for observing the redox properties of enzymes in this family, since recombinant HmDH from N. simplex is completely flavinylated and those from the other sources are not. Recombinant HmDH was titrated with histamine as well as with dithionite over the pH range from 7 to 10. Single-, two-, and three-electron reduction of the enzyme per monomer were achieved by selecting the reductants and pH. In order to understand the redox properties observed during the titration, the redox potential of CFMN was evaluated by using a spectroelectrochemical method over the pH range, while that of the [4Fe-4S] cluster was assessed by using cyclic voltammetry. The electron paramagnetic resonance (EPR) spectroscopic method was utilized to characterize the reduction product of recombinant HmDH. The pH profile of the catalytic property is also discussed in view of the pH dependence of the intramolecular electron transfer driving force.

Materials and Methods

Enzyme purification. Wild-type recombinant HmDH was expressed in E. coli and purified as previously
The protein concentration was determined by using the modified Lowry method with a DC Protein Assay kit (Bio-Rad, USA) with bovine serum albumin from Sigma as a standard sample. Complete flavinylation was confirmed through the determination of CFMN as described in the literature. Wild-type recombinant HmDH is simply referred to as HmDH in this paper.

**UV-vis spectroscopy.** Reductive titration was performed in a quartz cuvette with a 1-cm light path under anaerobic conditions at 30 °C, using a water-jacket cell holder and a thermostat. The quartz cuvette was sealed with a rubber septum, and a gas-tight syringe was used to inject each sample and reagent. Absorption spectra were recorded by a UV-2500PC UV-vis spectrophotometer (Shimadzu, Japan).

Histamine dihydrochloride used as the substrate was purchased from Sigma, and sodium dithionite was obtained from Nacalai Tesque (Japan). The dithionite solutions were prepared by dissolving dithionite powder in anaerobic water which had been prepared by repeated evacuation and flushing with O2-free argon. The dithionite solutions were standardized by titration against the free flavin mononucleotide, using an absorption coefficient of 12.3 mm−1 cm−1 at 448 nm.23) The HmDH solutions were prepared at concentrations of 3.8–6.0 μM in a 0.1 M Britton and Robinson buffers (containing 0.033 M sodium citrate, 0.033 M sodium phosphate and 0.033 M borate) of pH 7.0–10.0. These HmDH solutions, in a total volume of 2.5 ml, were made anaerobic by repeated evacuation and flushing with O2-free moisturized argon. The HmDH solution was then reduced anaerobically by adding histamine or dithionite. Benzyl viologen was added at a concentration of 0.5 μM as a mediator in the reductive titration with dithionite.20)

**EPR spectroscopy.** EPR spectra were recorded by a Nikkiso ES–10A electron spin resonance spectrometer (Japan), using a with glass capillary cell with an internal diameter of 1.0 mm. The magnitude of the modulation (100 kHz) was chosen to be as low as possible to optimize the resolution and signal-to-noise ratio of the observed spectrum. The microwave power was set in the range of 1–2 mW. An HmDH sample was placed in a glass capillary EPR tube just after adding excess histamine and subjected to the EPR spectroscopic analysis. The HmDH concentration was in the range of 0.4–0.5 mm in a final volume of about 25 μL. All EPR spectral measurements were performed at room temperature.

**Spectroelectrochemistry.** Mediated spectroelectrochemical titration for measuring the redox potential was performed by controlling the solution potential in a quartz cuvette with an HSV-100 potentiostat, Hokuto Denko (Japan). Absorption spectra were recorded simultaneously on a Shimadzu UV-2500PC UV-vis spectrophotometer as previously described.24) All values for potential in this paper refer to the standard hydrogen electrode (SHE).

Two osmium complexes, [Os(bipyridylamine)2Cl2]PF62− and [Os(4-imidazole carboxylic acid)2(bipyridylamine)2]PF62−, were synthesized according to the literature25) and used as mediators of the redox titration. The redox potentials of [Os(bipyridylamine)2Cl2]PF62− and [Os(4-imidazole carboxylic acid)2(bipyridylamine)2]PF62− were 47 mV and −103 mV, respectively, at pH 7. The electrolysis solution was a 0.1 M Britton and Robinson buffer containing 5–10 μM HmDH, 35 μM [Os(bipyridylamine)2Cl2](PF6)2, and 43 μM [Os(4-imidazole carboxylic acid)2(bipyridylamine)2](PF6)2 in a total volume of 1.7 ml. An antifoaming reagent (Antifoam PE-L, Wako, Japan) was added to the electrolyte solution to a final concentration of 0.3% (w/w). Preliminary experiments confirmed that the antifoaming reagent did not affect the enzymatic activity of HmDH.

The spectral changes of the electrolysis solutions were simultaneously monitored during electrolysis by the UV-vis recording spectrophotometer.24) Once the spectrum no longer changed with time, i.e., where the solution potential became identical with the electrode potential, UV-vis spectra were measured at several given potentials in the range from −203 mV to +147 mV. These experiments were performed under anaerobic conditions at 30 °C.

**Cyclic voltammetry.** Electrochemical measurements were carried out with a three-electrode system. Cyclic voltammetric measurements were performed with a CV-50W electrochemical analyzer, BAS (Japan) under anaerobic conditions. A platinum wire and Ag/AgCl-saturated KCl were used as the reference and counter electrodes, respectively. The working electrodes used were glassy carbon (GC), highly oriented edge-plane pyrolytic graphite, gold, and indium tin oxide (ITO).

The GC, highly oriented edge-plane pyrolytic graphite, and gold electrodes were polished with emery paper (No. 600), before being rinsed with and sonicated in distilled water. The ITO electrodes (5 × 5 mm, BAS) were cleaned by ultrasonication for more than 10 min in a 1% New-Vista® (an anionic surfactant, AIC Corp.) aqueous solution, this being followed by ultrasonication in Milli-Q water according to the literature.26) The electrolysis solution was a 0.1 M Britton and Robinson buffer containing 50–70 μM HmDH and 0.2 M NaCl at pH 7–10 in a total volume of 1 ml.

All anaerobic experiments by spectroscopy and electrochemistry were performed in a model A glove box, Coy Laboratory Products (USA). The concentration of O2 in the glove box was maintained at less than 10 ppm.
Results

Reductive titration with dithionite

Figure 1 illustrates the UV-vis spectral changes of HmDH during the reductive titration with dithionite at pH 8.0. Each spectrum was recorded 3 min after adding dithionite, at which time the spectrum no longer changed with time. The oxidized HmDH showed an absorption peak with a maximum at 440 nm and a shoulder at around 330 nm, which were assigned to CFMN as in the case of TMADH.21) The absorption band at around 440 nm decreased with the addition of dithionite. No isosbestic points appeared during the titration. The absorbance at 365 nm increased the beginning of titration and then decreased with the progress of titration (Fig. 1). These characteristics indicate the generation of an intermediate redox state during the reductive titration (Scheme 3).

The inset to Fig. 1 shows the titration curve monitored at 440 nm which indicates a stoichiometric reaction of three moles of dithionite per mole of HmDH. This means that HmDH took up a total of three electrons per monomer upon reduction with dithionite: two at the CFMN moiety and one at the [4Fe-4S] cluster. The fully reduced form contained CFMN_R and FeS_R.

The increase in absorbance at 365 nm at the beginning of the reductive titration was due to the generation of CFMN_S.27) With the progress of titration, the absorption due to CFMN_S was replaced with that of CFMN_R, which gave a broad absorption band at around 360 nm. Similar behavior was observed over the pH range of 7–10 in the reductive titration of HmDH with dithionite.

Reductive titration with histamine

Figure 2 illustrates the UV-vis spectral changes of HmDH in the reductive titration with histamine at pH 8.0. The pattern of the spectral changes is different from that observed in the dithionite titration just described. The absorption band at 440 nm decreased linearly with the addition of histamine, while the band at 365 nm increased. As shown in the inset to Fig. 2, HmDH reacted stoichiometrically with two molecules of histamine per enzyme at pH 8.0. This indicates that HmDH accepted two electrons per monomer stoichiometrically. Similar results have been reported for TMADH.16,19,20) The isosbestic points appeared at 338 nm and 385 nm, indicating that there was no

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Fig. 1. Reductive Titration of HmDH with Dithionite.

The concentration of HmDH was 6.0 μM in a 0.1 M Britton and Robinson buffer at pH 8.0. The inset shows the absorbance change at 440 nm as a function of the concentration ratio of dithionite against HmDH (dimer).

Fig. 2. Reductive Titration of HmDH with Histamine at pH 8.0.

The concentration of HmDH was 3.7 μM in a 0.1 M Britton and Robinson buffer at pH 8.0. The inset shows the absorbance change at 440 nm as a function of the concentration ratio of histamine against HmDH (dimer).
detectable intermediate such as single-electron (per monomer)-reduced HmDH during the titration. Similar behavior was observed in the reductive titration of HmDH with histamine over the pH range of 7–9.

Figure 3 illustrates the spectral changes of the reductive titration of HmDH with histamine at pH 10.0, at which the enzyme activity of HmDH was about 30% of the maximum at pH 9.0. HmDH was not denatured and gave very stable absorption spectra. The titration was completely different from that observed at pH < 9. Upon the addition of histamine, the absorption band at 440 nm was partially quenched and sharpened, and a strong absorption band at 365 nm and a small broad band at around 500 nm appeared. The isosbestic points were observed at 338 nm and 385 nm. The titration curve shown in the inset to Fig. 3 indicates that HmDH seems to have reacted with one mole of histamine per mole of enzyme. Similar behavior was observed at pH > 9 in the reductive titration of HmDH with histamine. The behavior was not compared with that of TMADH, since there is no report on the substrate titration of TMADH in such an alkaline pH region.

**EPR spectroscopy**

HmDH reduced with an excess of histamine yielded a strong isotropic EPR signal at \( g \approx 2 \) (Fig. 4). The total line width of the EPR spectra was about 3.6 mT. HmDH fully reduced by dithionite was EPR-silent. Therefore, it is reasonable to assign the EPR signal to CFMNs. The EPR signal intensity of the substrate-reduced HmDH increased with pH up to pH 9, as in the case of TMADH\(^{20,21}\) but it reached a maximum at pH > 9.

**Spectroelectrochemical evaluation of the redox potentials of CFMN**

In order to understand the characteristic properties of the substrate-reduced HmDH, the redox potentials of the two redox cofactors were evaluated. The electrochemical and spectroscopic properties of the two redox cofactors of HmDH were different. If CFMN were localized in the cavity of HmDH, then the CFMN cofactor would be silent in a direct electrochemical analysis. Therefore, a mediated spectroelectrochemical titration method based on separator-less one-compartment bulk electrolysis\(^{24}\) was adopted to evaluate the redox potentials of CFMN.

Figure 5 shows the background-corrected spectra of HmDH. The enzyme concentration was 5.7 \( \mu \)M in a 0.1 M Britton and Robinson buffer at pH 10.0, containing 35 \( \mu \)M [(Osbipyridyl-amine)_3Cl]_2(PF_6)_2, 43 \( \mu \)M [Os(4-imidazole carboxylic acid)_2(bipyridylamine)_2](PF_6)_2, and 0.3% (w/w) Antifoam PE-L. The absorption spectra have been equilibrated stepwise in the potential region from \(-203\) mV to \(+147\) mV. The inset shows the potential dependence of the absorbance at 440 nm during reductive (●) and oxidative titration (○). The broken lines represent the regression lines based on Eq. 3.
and reductive titrations. The absorption band at around 440 nm decreased with the shift of electrode potential toward negative, and the maximum wavelength of the absorption shifted slightly to the longer wavelength. The results indicate that an intermediate existed during the titration, which we assigned to CFMN S.

The absorption spectral change was predominantly assigned to CFMN, since the [4Fe-4S] cluster-related absorption change was negligibly small as compared with that of CFMN and was located in the UV region. 38 Considering the existence of an intermediate, the two-step single-electron transfer model was adopted for an analysis of the Nernstian response of the spectroscopic data. Nernst equations for CFMN are given by:

\[
\frac{[\text{CFMN}_0]}{[\text{CFMN}_S]} = \exp\left[F/RT(E - E'_{O/S})\right] = \eta_1 \\
\frac{[\text{CFMN}_S]}{[\text{CFMN}_R]} = \exp\left[F/RT(E - E'_{S/R})\right] = \eta_2
\]

where \(E'_{O/S}\) and \(E'_{S/R}\) are the redox potentials of the CFMN O/CFMN S and CFMN S/CFMN R redox couples, respectively. \(F, R, T\) are the Faraday constant, the gas constant, and the absolute temperature, respectively. The relationship between the absorbance \(A\) and the electrode potential \(E\) can be expressed by: 29

\[
A = \frac{[\text{CFMN}_0][\text{CFMN}_0] + \epsilon_{\text{FMNO}}[\text{CFMN}_S]}{[\text{CFMN}_R]} = \frac{\epsilon_{\text{FMNO}}}{R/\eta_1 + \eta_2} (\epsilon_{\text{FMNO}} \eta_1 \eta_2 + \epsilon_{\text{FMNS}} \eta_1 \eta_2 + \epsilon_{\text{FMNR}})
\]

where \(\epsilon_{\text{FMNO}}\), \(\epsilon_{\text{FMNS}}\), and \(\epsilon_{\text{FMNR}}\) are the absorption coefficients of the corresponding species, \([E]/(=[\text{CFMN}_0]+[\text{CFMN}_S]+[\text{CFMN}_R])\) denotes the total concentration of HmDH, and \(R/\eta_1\) is the light-path length. \(\epsilon_{\text{FMNO}}, \epsilon_{\text{FMNS}},\) and \(\epsilon_{\text{FMNR}}\) can be evaluated experimentally from the initial and final spectra in the reductive titration with dithionite (Fig. 1). The values for \(\epsilon_{\text{FMNO}}\), and the two redox potentials \(E'_{O/S}\) and \(E'_{S/R}\) were optimized as adjustable parameters by a nonlinear regression analysis according to Eq. 3. As shown by the inset to Fig. 5, the potential dependence of the absorbance changes at 440 nm was well reproduced by a set of the parameters based on the two-step electron transfer model.

Figure 6 illustrates the pH dependence of the redox potentials of the cofactors. The \(E'_{O/S}\) value, indicated by the open circles, was almost independent of pH. This means that the electron transfer was not coupled with proton transfer. Therefore, the isoalloxazine moiety in CFMN S must have had a single negative charge at the N1 atom over the pH region investigated. On the other hand, \(E'_{S/R}\) represented by the closed circles, shifted with pH in the direction of the negative potential with a slope of \(-60 \text{ mV pH}^{-1}\). This indicates single-electron transfer coupled with single-proton transfer. Therefore, the isoalloxazine moiety in CFMN R also had a single negative charge at the N1 atom.

The thermodynamic stability of the intermediate, CFMN S, was expressed by the semiquinone formation constant (or comproportionation constant, \(K_S\)) given by:

\[
K_S = \frac{[\text{CFMN}_S]^2}{[\text{CFMN}_0][\text{CFMN}_S]} = \exp\left(F(E'_{O/S} - E'_{S/R})/RT\right)
\]

Since \(E'_{S/R}\) was shifted to the negative direction linearly with increasing pH, \(K_S\) increased exponentially with pH. This means that CFMN S became predominant with increasing pH at \(E = (E'_{O/S} + E'_{S/R})/2\).

Voltammetric evaluation of the redox potentials of the [4Fe-4S] cluster

It was difficult to extract spectroscopic information about the redox reaction of the [4Fe-4S] cluster from the experimental data because the spectral change was small and appeared in the UV region, where the signal-to-noise ratio was too small to detect such a small spectral change under our experimental conditions (see Fig. 5). We next tried to get a direct electrochemical signal of the [4Fe-4S] cluster by using cyclic voltammetry. HmDH gave no detectable faradaic response with GC, highly oriented edge-plane pyrolytic graphite, or gold electrodes. However, the enzyme showed a couple of well-defined redox peaks with ITO electrodes. Figure 7 shows a cyclic voltammogram of HmDH at pH 7.0 with ITO as the working electrode. Ultrasonic pretreatment of the ITO electrodes in the presence of an anionic surfactant was essential to observe well-defined redox waves. The midpoint potential of the anodic and cathodic peaks of the redox wave was evaluated to be +36 mV at pH 7.0. Almost identical cyclic voltammograms were obtained when ITO electrodes adsorbed in the alkaline pH region were transferred to a solution at pH 7.0, and vice versa. The peak current was proportional to the scan rate (\(v\)) in the \(v\) region from 20 to
200 mV s\(^{-1}\). This indicates that HmDH was adsorbed to the ITO electrode. The total width of the half-height (\(\Delta E_{p/2}\)) was ca. 130 mV at pH 7.0. The theory of the reversible voltammogram in the ideally surface-confined case (with equivalent interaction of the reactants) indicates that \(\Delta E_{p/2}\) was 90.6/n mV at 25 \(^{\circ}\)C,\(^{30}\) \(n\) being the number of electrons involved. The experimental value for \(\Delta E_{p/2}\) is close to that predicted for the ideally reversible case of \(n = 1\). In addition, liberation of the [4Fe-4S] cluster from HmDH under acidic conditions (pH \(\approx\) 1) led to disappearance of the redox peak, although the spectrum for denatured HmDH was similar to that of the CFMN-containing peptide in HmDH.\(^{10}\) Therefore, the redox peak can be assigned to the [4Fe-4S] cluster. The discrepancy in \(\Delta E_{p/2}\) from that of the ideally reversible case is due in part to the quasi-reversible characteristics of the wave and also to non-equivalent interaction between FeS\(_0\) and FeS\(_1\).\(^{31}\) The surface concentration of adsorbed HmDH (\(\Gamma\)) at pH 7.0 was evaluated to be \(6.0 \times 10^{-11}\) mol cm\(^{-2}\) from the electricity (\(Q\)) of the peak, according to Faraday’s Law, by considering that \(n = 1\):

\[
Q = nF\alpha \Gamma
\]

The \(\Gamma\) value seems to be reasonable for monolayer adsorption of HmDH.

The open triangles in Fig. 6 show the pH dependence of the redox potential of the [4Fe-4S] cluster (\(E'_{\text{FeS}}\)), which was evaluated as the midpoint potential of the cyclic voltammogram with ITO electrodes. The \(E'_{\text{FeS}}\) value was almost independent of pH at pH < 9, but it shifted with pH above 9 in the direction of the negative potential with a slope of about \(-180\) mV pH\(^{-1}\). The open triangles in Fig. 6 show the pH dependence of the redox potential of the [4Fe-4S] cluster (\(E'_{\text{FeS}}\)), which was evaluated as the midpoint potential of the cyclic voltammogram with ITO electrodes. The \(E'_{\text{FeS}}\) value was almost independent of pH at pH < 9, but it shifted with pH above 9 in the direction of the negative potential with a slope of about \(-180\) mV pH\(^{-1}\).

**Discussion**

It has been reported that several ferredoxins gave a (quasi)reversible cyclic voltammetric response with indium oxide electrodes in the presence of suitable promoters such as polypeptides and aminosilane.\(^{32-34}\) In our case, however, it was found that ITO electrodes were very effective for communicating with the [4Fe-4S] cluster of HmDH even in the absence of a promoter. That a cleaning pretreatment of the ITO electrodes was required to get a well-defined voltammogram suggests that the hydrophilicity of the ITO electrode surface was important. This direct electrochemistry of the [4Fe-4S] cluster of HmDH is the first to be reported for the family of iron-sulfur flavoproteins.

The CFMN moiety of HmDH was silent even with the ITO electrodes. It is likely that the CFMN moiety became buried in the polypeptide shell as an insulator. The redox potentials of CFMN have been evaluated by using a spectroelectrochemical method based on mediated bulk electrolysis.\(^{24}\) The data have been successfully analyzed based on a two-step single-electron transfer model. The redox potentials of the CFMN\(_{O/S}\), CFMN\(_{O/R}\) and FeS\(_{O/R}\) couples (\(E'_{O/R}, E''_{O/R}\) and \(E'_{\text{FeS}}\)) have thus been evaluated to be +34 mV, +30 mV and +36 mV, respectively, at pH 7. In the case of TMADH, \(E'_{O/R}, E''_{S/R}\) and \(E'_{\text{FeS}}\) have been reported to be +44 mV, +36 mV and +102 mV, respectively, at pH 7.\(^{21}\) The redox potentials of CFMN in HmDH are close to those in TMADH, while \(E'_{\text{FeS}}\) of HmDH is more negative than that of TMADH. However, \(E'_{\text{FeS}}\) of HmDH (as well as TMADH) is more positive than that of most bacteria-type [4Fe-4S] clusters (\(-0.45\) V to \(-0.25\) V\(^{35-40}\)). \(E'_{\text{FeS}}\) of HmDH is almost independent of pH over the range 7–9. However, at pH > 9, \(E'_{\text{FeS}}\) sharply shifts with pH in the direction of the negative potential. There is no report on \(E'_{\text{FeS}}\) of TMADH in such an alkaline pH region. The pH dependence of \(E'_{\text{FeS}}\) suggests the deprotonation of inorganic sulfurs in the oxidized form of the [4Fe-4S] cluster in the alkaline region. Similar phenomena have been reported for *Azotobacter vinelandii* ferredoxin I variants.\(^{40}\)
HmDH showed unique redox behavior in the reductive titration. HmDH could take up three electrons per monomer upon reduction with dithionite: two at CFMN and one at the iron-sulfur center. Physiologically, single-electron transfer seems to have occurred from the reduced [4Fe-4S] cluster to the electron acceptor, although the physiological electron acceptor for HmDH is unknown. This is because ETF, which uses FAD, has a two-electron capacity but physiologically acts as a single-electron acceptor cycling between the oxidized and semiquinone state for TMADH. In the reductive titration with histamine, however, two–electron reduction per monomer occurred at pH < 9. The occurrence of partial reduction by two electrons per monomer is also verified by the detection of the isotropic EPR signal at g ≈ 2 due to CFMN₈. Similar behavior has been reported for TMADH. These results seem to be reasonable, since the substrates were two-electron donors and did not work as single-electron donors in the enzymatic reaction. This property is in contrast with that of dithionite, which can work as a single–electron donor. On the other hand, this work has revealed that single–electron reduction proceeded per monomer during the substrate titration of HmDH at pH > 9. This is the first finding of its kind for the family of iron-sulfur flavoproteins.

An important point regarding the partial reduction of the enzyme by the substrate is that this fact rules out the possibility of intermolecular electron transfer between partially reduced enzymes. Full reduction of the enzymes by the substrate could be feasible if such intermolecular electron transfer occurred under conditions in which the electron transfer from the substrate to the enzyme were thermodynamically favored, as in the case of the dithionite reduction.

Another important point to be considered is the distribution of the electrons in the partially reduced enzyme. Scheme 4 shows the redox species to be generated during the reductive titration of HmDH. The single-electron-reduced monomer has two possible redox states: one has CFMN₈ and FeSO (CFMN₈–FeSO), and the other has CFMN₀ and FeSR (CFMN₀–FeSR). The equilibrium constant (K₁) between the two states of the single-electron-reduced form can be expressed as a function of the difference between E'_FeS and E'_O/S:

\[
K_1 = \frac{[\text{CFMN}_0-\text{FeSR}]}{[\text{CFMN}_8-\text{FeSO}]} = \exp(F(E'_\text{FeS} - E'_\text{O/S})/RT)
\]

(6)

The two-electron-reduced monomer also has two possible redox states: CFMN₉–FeSO and CFMN₈–FeSR. The distribution between the two states is determined by E'_FeS and E'_S/R:

\[
K_2 = \frac{[\text{CFMN}_9-\text{FeSO}]}{[\text{CFMN}_8-\text{FeSR}]} = \exp(F(E'_\text{FeS} - E'_\text{S/R})/RT)
\]

(7)

At pH < 8.0, E'_S/R and E'_FeS are close to each other, and therefore the two-electron-reduced subunit is almost evenly distributed between the CFMN₉–FeSO and CFMN₈–FeSR states at equilibrium. E'_S/R shifts with pH in the direction of the negative potential, while E'_FeS remains almost unchanged at pH < 9. Therefore, E'_S/R becomes more negative than E'_FeS as pH increases to 9. This situation leads to an increase in the K₂ value of the two-electron-reduced state of the subunit. This property seems to be responsible for the fact that the EPR signal intensity increased with pH up to 9 for the two-electron-reduced subunit of HmDH.

At pH > 9, E'_FeS shifted sharply in the direction of the negative potential (Fig. 6). Therefore, it might be expected that the CFMN₉–FeSO state would become thermodynamically favorable for the two-electron-reduced subunit, as predicted by Eq. 7. However, the substrate titration indicates a stoichiometric single-electron reduction per subunit. Here, it might appear that the heterogeneous redox state dimer consisting of a two-electron-reduced subunit and a fully oxidized subunit could be generated. However, the spectra for histamine-reduced HmDH at pH > 9 do not share the characteristics of CFMN₀ (Fig. 3). This rules out the generation of the heterogeneous redox state dimer, suggesting that the product must be the homogeneous single-electron reduced dimer.

As expected from Eq. 6, CFMN₉–FeSO became thermodynamically favorable compared with CFMN₀–FeSR, because E'_O/S ≫ E'_FeS in the single-electron-reduced form at pH > 9. This is clearly evident from the strong EPR signal of substrate-reduced HmDH at pH > 9 (Fig. 4). As a result, the sharp absorption bands at 365 and 440 nm of the reduction product (Fig. 3) can reasonably be assigned to CFMN₈ in partially reduced HmDH.

We have to consider how a single-electron-reduced subunit was generated during the histamine titration in spite of the fact that histamine is a two-electron donor in the enzymatic reaction. One of the subunits may first be reduced by one mole of histamine to generate a
heterogeneous redox state dimer consisting of the CFMN_R–FeS_R subunit and the CFMN_O–FeS_O subunit. Under these conditions, CFMN_R–FeS_O would be the predominant form over CFMN_O–FeS_R in the two-electron-reduced subunit because \( E'_{S/R} \gg E'_{FeS} \). Therefore, single-electron transfer must have occurred from the CFMN_R–FeS_O subunit to CFMN_O–FeS_O subunit to generate a homogeneous dimer composed of the CFMN_O–FeS_O state subunits. The intersubunit electron transfer would be driven thermodynamically as evident from the fact that \( E'_{O/S} \) was much more positive than \( E'_{S/R} \) and that \( K_S \) was extremely large (Eq. 4) under these conditions.

To explain the difference in the stoichiometry of the half-reduction of HmDH by dithionite and histamine at neutral and alkaline pHs, we can refer to their absorption spectra. As already mentioned, the strong but somewhat broad absorption band at 440 nm and the shoulder at 330 nm (Fig. 1) are assigned to CFMN_O. The sharp absorption bands at 365 and 445 nm and the small broad band at around 500 nm (Fig. 3) are assigned to CFMN_R. CFMN_R also showed an absorption band at 365 nm, but the band was broad compared with that of CFMN_S (Fig. 2). The fact that there was no inflection point in the pH dependence of \( E'_{O/S} \) and \( E'_{S/R} \) (Fig. 6) means that CFMN did not have a pK_a in the pH region investigated (pH 7–10) and that the spectral properties of each redox species of CFMN did not depend on pH.

The enzymatic activity of HmDH shows a bell-type dependence on pH with a maximum at around pH 9. Such pH dependence of the enzyme activity may usually be explained in terms of acid-base equilibria of the amino acid residues located at or near the active site, as well as those of the substrate and product. In the case of redox enzymes, the redox potentials of the cofactor(s) will also depend on pH. This means that the thermodynamic driving force for electron transfer depends on pH. Therefore, in order to understand the pH dependence of the redox enzyme activity, we have to consider the pH dependence of the thermodynamic driving force for electron transfer as well as acid-base equilibria.

We suggest from this work an explanation for the pH dependence of the HmDH activity in view of the thermodynamic driving force of the intramolecular electron transfer. The reduction of the enzyme by histamine yields the CFMN_R–FeS_O state, followed by intramolecular single-electron transfer from CFMN_R to FeS_O to yield the CFMN_O–FeS_R state. Since \( E'_{S/R} \) becomes more negative than \( E'_{FeS} \) with pH < 9 (Fig. 6), the driving force for electron transfer increases with pH. This situation may be responsible for the observed increase in the enzyme reaction rate constant as pH approaches 9. The driving force for electron transfer from CFMN_S to FeS_O is almost independent of pH at pH < 9, since \( E'_{FeS} \) and \( E'_{O/S} \) are almost independent of pH in this pH range (Fig. 6). This single-electron transfer process from CFMN_S to the [4Fe-4S] cluster would probably not be the rate-determining step.

At pH > 9, the \( E'_{FeS} \) value shifts markedly with pH in the direction of negative potential and becomes more negative than \( E'_{S/R} \) (Fig. 6). Therefore, the driving force for electron transfer from CFMN_R to FeS_O decreases with increasing pH. The electron transfer from CFMN_S to FeS_O also becomes extremely unfavorable, since \( E'_{FeS} \ll E'_{O/S} \) (Fig. 6). These situations may be responsible for the observed decrease in the enzyme reaction rate constant when pH > 9.

The present work has revealed characteristic features of the equilibrated redox reaction of HmDH. The spectral change and reaction stoichiometry obtained by the titration have been well demonstrated in terms of the redox potentials. Such thermodynamic properties also seem to be responsible for the pH profile of the enzymatic reaction. In addition, these spectral assignments will become useful for following and analyzing the transient spectral changes of the HmDH reaction in stopped-flow experiments in the future.

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**References**


