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The Redox Potential Measurements for Heme Moieties in Variants of D-Fructose Dehydrogenase Based on Mediator-assisted Potentiometric Titration

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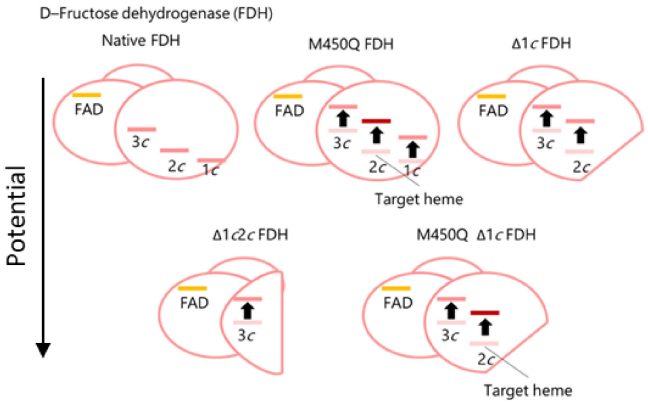
ABSTRACT

The effect of mutation on the redox potentials ($E^{\circ\prime}$) of the heme moieties in the variants of D-fructose dehydrogenase (FDH) was investigated by mediated spectroelectrochemical titrations. The replacement of the axial ligand of heme from methionine to glutamine changes the $E^{\circ\prime}$ value more negatively than that of the corresponding heme moiety in the recombinant (native) FDH (rFDH). The determined $E^{\circ\prime}$ values of non-targeted heme moieties in the variants were also shifted in a negative direction from that in rFDH. Thus, enzyme modification changes $E^{\circ\prime}$ of the heme moieties in unmodified protein regions.



Keywords : D-Fructose Dehydrogenase, Potentiometric Titration, Heme Moieties, Mutations

1. Introduction

Redox potentials ($E^{\circ\prime}$) are important physical quantities for understanding the physiological functions and intramolecular electron transfer of redox enzymes. Many attempts have been made to determine the $E^{\circ\prime}$ values of various enzymes or to shift the $E^{\circ\prime}$ values by site-directed mutations.[1–6] It has been revealed that $E^{\circ\prime}$ values depend on various factors such as water accessibility of heme,[7–9] nonpolar environments around heme moieties,[9,10] and total distribution patterns of surface charges on proteins.[11] In addition, it is known that heme redox potentials are shifted to negative direction by heme ruffling.[12,13] However, as far as redox proteins that contain several heme moieties, little has been discussed about the influence of point mutations or deletions in the enzyme structure on other unmodified regions. Therefore, determination of $E^{\circ\prime}$ is important to estimate the influence of mutations.

Here, we focused on D-fructose dehydrogenase (FDH; EC 1.1.99.11) from *Gluconobacter japonicus* NBRC 3260 as a model enzyme. This heterotrimeric membrane-bound protein contains a covalently bound flavin adenine dinucleotide (FAD) and three heme *c* moieties called heme 1*c*, heme 2*c*, and heme 3*c*, from its N-terminus.[14–17] FDH has high direct electron transfer (DET)-type bioelectrocatalytic activity. The $E^{\circ\prime}$ values of the three heme moieties in FDH were determined using the spectroelectrochemical method.[18] Several FDH variants have been constructed using protein engineering approaches. These include variants in which the axial ligand of heme 2*c* was replaced with glutamine (M450Q FDH),[19] 143 amino acid residues involving heme 1*c* were removed (Δ1*c* FDH),[20] these two mutations were introduced simultaneously (M450Q Δ1*c* FDH),[21] and 199 amino acid residues involving heme 1*c* and heme 2*c* moieties were removed (Δ1*c*2*c* FDH).[22] Although DET-type catalytic voltammograms of these variants have already been examined,[23] the $E^{\circ\prime}$ values of these variants have not yet been determined.

In this communication, the $E^{\circ\prime}$ values of these FDH variants are determined by spectroelectrochemical redox titration based on a sequential three-step one-ET model.[18] It clarifies the influence of point mutations or deletions in the enzyme structure on other unmodified regions.

2. Experimental

2.1 Materials

$Ru(NH_3)_6Cl_3$ and diaminodurol (DAD) were purchased from Sigma-Aldrich Co. (USA). Sodium dithionite and phenazine methosulfate (PMS) were obtained from Nacalai Tesque Co., Ltd. (Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Japan). All chemicals were of analytical grade and used without further purification.

Recombinant (native) FDH (rFDH), M450Q FDH, Δ1*c* FDH, M450Q Δ1*c* FDH, and Δ1*c*2*c* FDH were expressed and purified as described in previous works[14,15,19–22] and stored as a solution of pH 4.5 McIlvaine buffer containing 0.1 % Triton® X-100 and 1 mM (M: mol dm^{-3}) 2-mercaptophenol. The specific activities of rFDH,

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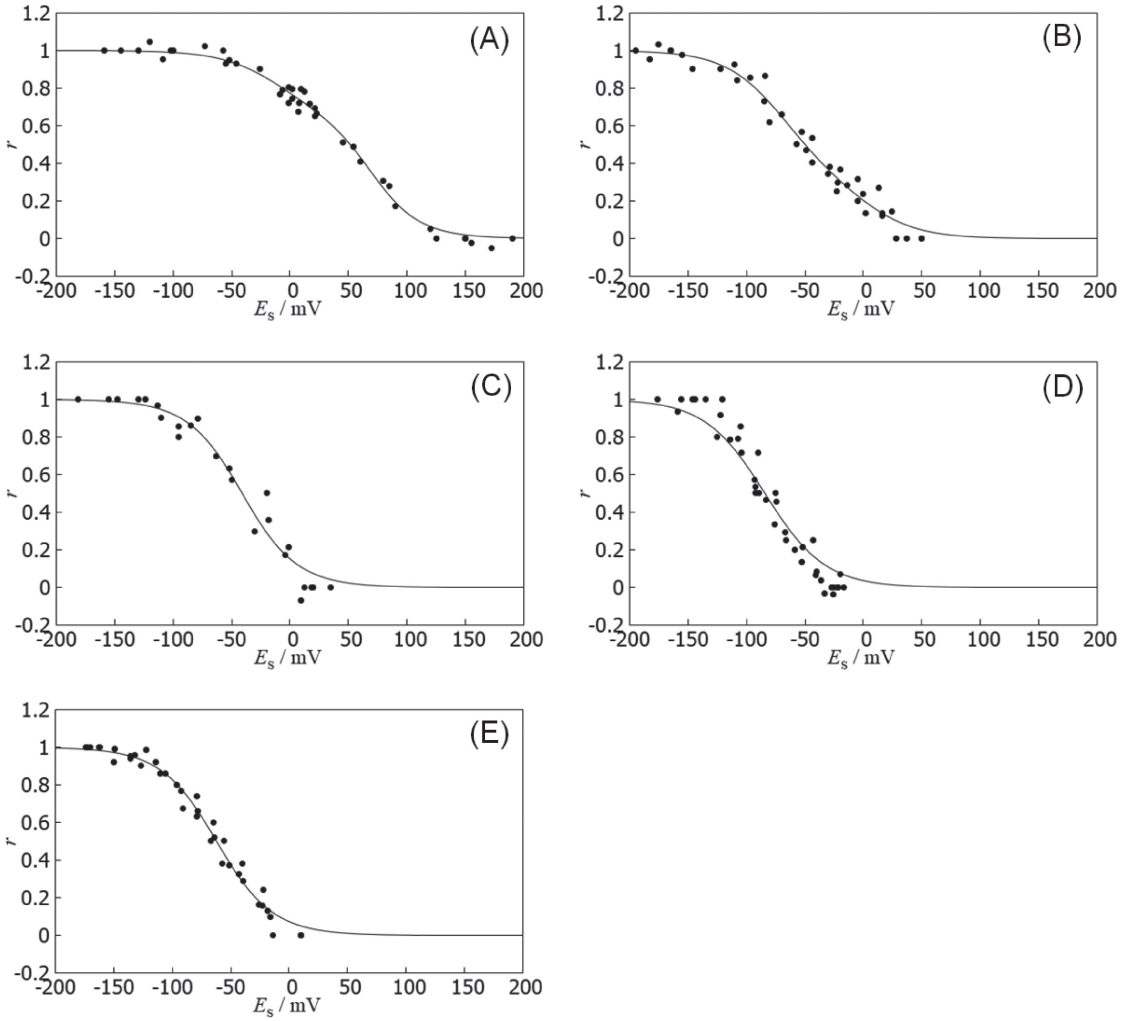

Figure 1. Solution potential dependence of the reduction ratio determined at 550 nm. The solid lines show the best fitted curves evaluated from Eqs. S5a, S5b, and S5c. Panels (A), (B), (C), (D), and (E) represent results of rFDH, M450Q FDH, Δ1*c* FDH, Δ1*c*2*c* FDH, and M450Q Δ1*c* FDH, respectively.

M450Q FDH, Δ1*c* FDH, M450Q Δ1*c* FDH, and Δ1*c*2*c* FDH in their solutions were evaluated to be 150, 70, 120, 170, and 30 U mg^{-1} (U = μmol min^{-1}), respectively.

2.2 Spectroelectrochemical measurements

Potentiometric oxidative/reductive titrations were performed at 25 °C under stirring and anaerobic conditions in a cuvette with the light-path length of 1 cm containing 2 mL of McIlvaine buffer (pH 4.5). The buffer solution contained 0.3 mM DAD, 0.3 mM PMS, 0.5 mM Na[Fe(edta)], 0.5 mM $Ru(NH_3)_6Cl_3$, and 0.01 % Triton® X-100. The concentration of each enzyme in the bulk solution was 1–7 μM. The solution potential (E_s) was changed by adding $K_3[Fe(CN)_6]$ or sodium dithionite and monitored by an electrometer (HE-106A, Hokuto Denko Co., Ltd., Japan) using a Pt and a Ag|AgCl (sat. KCl) electrodes as a sensing and reference electrodes, respectively. All potentials in this work were referred to the reference electrode. The spectra were recorded using a spectrophotometer (UV-2550, Shimadzu Co., Ltd., Japan) when E_s was sufficiently stable (potential change: $<\pm 1$ mV min^{-1}). Since FDH had no absorption at 600 nm irrespective of the oxidation state,[14] each spectrum was offset to have the absorbance at 600 nm of zero to subtract the effect of the scattered light by aggregates.

3. Results and Discussion

The E_s-dependence of absorbance at 550 nm was analyzed by a sequential three-step one-ET model[18] (details are shown in SI). Figure 1 shows the E_s-dependence of the reduction ratio (r) of each FDH. There were no differences in the results of oxidative/reductive titration at each FDH (data not shown); thus, redox equilibrium can be achieved between the enzyme and solution. The sigmoidal shapes were steeper when the number of heme moieties was deleted. Therefore, redox states in the heme moieties were changed in narrower E_s ranges according to the number of heme moieties. The inflection points on the sigmoidal curves of the variants were more negative than that of rFDH; thus, the $E^{\circ\prime}$ values of variants should be more negative than that of rFDH.

The $E^{\circ\prime}$ values were estimated by fitting Eqs. (S5) to the experimental results. Table 1 summarizes the estimated $E^{\circ\prime}$ values of the heme moieties in each FDH. In the case of rFDH, the results are generally consistent with results in the previous work,[18] except for the results on the high potential side. The difference is probably due to the poor potential buffer capacity in the positive E_s region.

Since heme 2*c* is considered as an electrode active site,[18] the catalytic voltammogram of M450Q FDH[19] supports the negative shift in $E^{\circ\prime}$ of heme 2*c*. Interestingly, the potentials of the other hemes are also shifted to the negative direction. The $E^{\circ\prime}$ values also

Table 1. Evaluated $E^{\circ\prime}$ of heme moieties in each FDH.

	$E^{\circ\prime}$/mV		
rFDH (Previous work)[18]	-10 ± 4	60 ± 8	150 ± 4
rFDH (This work)	-11 ± 4	65 ± 7	77 ± 7
M450Q FDH	-87 ± 8	-55 ± 7	2 ± 6
Δ1*c* FDH	-54 ± 8	-28 ± 7	—
Δ1*c*2*c* FDH	-84 ± 2	—	—
M450Q Δ1*c* FDH	-76 ± 2	-48 ± 3	—

The error ranges correspond to the standard-errors for the fitting.

shifted negatively at the two heme moieties in Δ1*c* FDH. In addition, Δ1*c*2*c*-deletion caused a further negative shift in the remaining heme moiety. In summary, the $E^{\circ\prime}$ values of unmodified heme moieties shift in the negative direction by mutations. In contrast, the two heme moieties in M450Q Δ1*c* FDH had almost the same $E^{\circ\prime}$ value as M450Q FDH. Thus, it may also be said that the $E^{\circ\prime}$ values are not necessarily shifted to a negative direction by mutations.

The $E^{\circ\prime}$ values of non-targeted heme moieties shifted in the negative direction for all variants used in this study except M450Q Δ1*c* FDH. We consider that some changes in the environments around heme moieties may occur, such as the charge or hydrophobicity of amino acids around them. Moreover, the degree of heme ruffling is changed by a single amino acid mutation, so the negative shift of $E^{\circ\prime}$ values of variants are possibly caused by out-of-plane distortion of heme pyrroles as a result of mutations.[12] Enzymes are complicated and steric structures made of polypeptide chains; thus, it is rational that overall enzyme structures are influenced even by point mutations or deletion of part of the enzyme.

In our previous work, the $E^{\circ\prime}$ values of the variants were evaluated from catalytic waves.[23] In the case of rFDH, the spectroelectrochemically determined $E^{\circ\prime}$ values agreed with those evaluated by analysis of the catalytic voltammogram (54 ± 1 mV). In contrast, the $E^{\circ\prime}$ values of the variants estimated in this study were significantly lower than those determined from the voltammograms (Δ1*c* FDH: 34 ± 1 mV, M450Q Δ1*c* FDH: 18 ± 1 mV, Δ1*c*2*c* FDH: 20 ± 1 mV). This disagreement demonstrates that the states of heme moieties are changed by the adsorption of the enzyme at the electrode surface. Therefore, mutation and the adsorption of enzymes will change the $E^{\circ\prime}$ values of the heme moieties in the enzyme. Unfortunately, the determination of $E^{\circ\prime}$ values in solution may not be ideal for characterization of the catalytic waves of electrode-immobilized enzymes. The strict analysis of the bioelectrocatalytic waves seems to require the $E^{\circ\prime}$ values of the electrode-active site to be determined by direct electrochemistry.

4. Conclusions

The $E^{\circ\prime}$ values of native and some variant FDH were successfully determined by potentiometric redox titration. The $E^{\circ\prime}$ values of unmodified heme moieties shift in the negative direction by replacement of the axial ligand of heme 2*c* or deletion of amino acid residues, including other heme moieties. These results revealed that mutations in redox enzymes can influence other unmodified protein regions. In contrast, the bioelectrocatalytic activities were unaffected by mutations. The susceptibility and durability of mutations may reflect in the intramolecular electron transfer pathway and the flexibility of the enzyme structures in the solution.

Supporting Information

The Supporting Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.21-00044.

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