Spectroelectrochemical Characterization of Quinohemoprotein Alcohol Dehydrogenase from *Gluconobacter suboxydans*

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Direct electron transfer of alcohol dehydrogenase (ADH) from *Gluconobacter suboxydans*, which contains one pyrroloquinoline quinone (PQQ) and four hemes *c*, are realized on a carbon-wool column electrode. Potential-dependent spectral change monitored with a photodiode array detector is well explained by a five-step Nernstian electron transfer model. The evaluated redox potentials are reasonable to explain the chemical reactivity of ADH with various redox reagents.

A membrane-bound quinohemoprotein ADH of acetic acid bacteria consists of three subunits I (78 kDa), II (48 kDa), and III (14 kDa) and functions as the primary dehydrogenase of the ethanol respiratory chain to transfer electrons from ethanol to ubiquinone. Subunit I contains one mol each of PQQ and heme *c* as the prosthetic groups and subunit II is a triheme cytochrome *c*, while subunit III has no prosthetic group.[1] Deeper knowledge of the redox potentials ($E^{\circ\prime}$) of the PQQ and four heme *c* groups is of particular interest for a better understanding of the intramolecular electron transfer of ADH and the ubiquinone reduction mechanisms. We (T.I. and coworkers) have shown that ADH adsorbed on several electrodes exhibits electroreflectance spectra and unmediated anodic catalytic current in the presence of ethanol, indicating the possibility of the direct electrochemistry of ADH.[2] On the other hand, continuous-flow column electrolysis is very useful in achieving rapid and quantitative bulk electrolysis and has an advantage to be easily coupled with spectroscopy.[3] The method has been successfully applied to the direct electrolysis of relatively small hemoproteins such as horse heart cytochrome *c* (Cyt *c*, 12 kDa).[4] These reports encouraged us to characterize spectroelectrochemical properties of the large molecular ADH.

Column electrolysis was performed using a Hokuto Denko HX-110 cell consisting of a carbon wool working electrode with a fibrous structure packed tightly in a Bicole® glass tube (i.d. 8 mm, length 50 mm), an Ag/AgCl (sat. KCl) reference, and a platinum coiled counter electrode. The electrode potential (E) was changed in a staircase mode with a potential width of 25 mV and a pulse interval of 3 min, unless otherwise noted. The sample solution was continuously flowed at 0.5 mL min^{-1} with a peristaltic pump. The electrolyzed solution was spectroscopically monitored using a Shimadzu SPD-M10Avp photodiode array detector in the range from 300 to 600 nm at a data acquisition interval of 2 s. All experiments were carried out under nitrogen atmosphere. ADH was isolated from *Gluconobacter suboxydans* (IFO 12528) as described in the literature.[5] Because of a large molar absorption coefficient of ADH and high sensitivity of the photodiode array detector, ADH concentrations as low as ca. 1×10^{-7} M (1 M = 1 mol dm^{-3}) suffice for the spectroscopic detection.

ADH exhibited reproducible E-dependent spectral changes at pH 7.0 (phosphate buffer, ionic strength 0.5 M with KCl) containing 0.1% of Triton X-100 and 1×10^{-7} M $K_3Fe(CN)_6$. Triton X-100 was essential to stabilize ADH and to prevent adsorption of ADH on the electrode surface. $K_3Fe(CN)_6$ was not essential for direct electrolysis, but it was found to facilitate the heterogeneous electron transfer rate. Such promoter-like effect of $K_3Fe(CN)_6$ was also observed for Cyt *c*. Figure 1 shows an absorbance (A)-E plot at 422 nm, in which A values of 30 points just before each potential pulse were averaged (standard deviation (s.d.) < 0.5%). The curve was practically independent of the direction and width of the potential step, which strongly supports the electrochemical reversibility under the present conditions.

The A-E plot is composed of three sigmoidal parts with four plateaus. Absorption spectra at the plateaus (referred as state A-

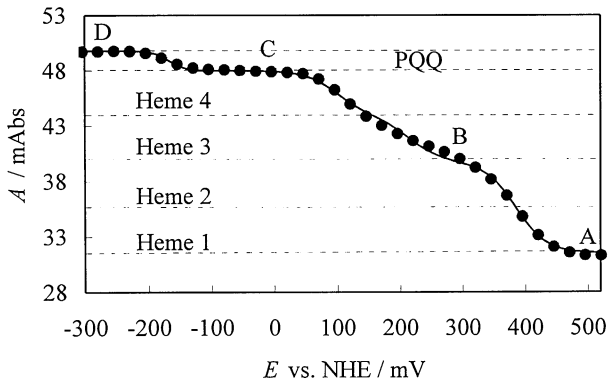

Figure 1. A-E curve of ADH in pH 7.0 phosphate buffer at 422 nm. (•): Experimental data, (—): fitting curve based on a five-step electron transfer mechanism.

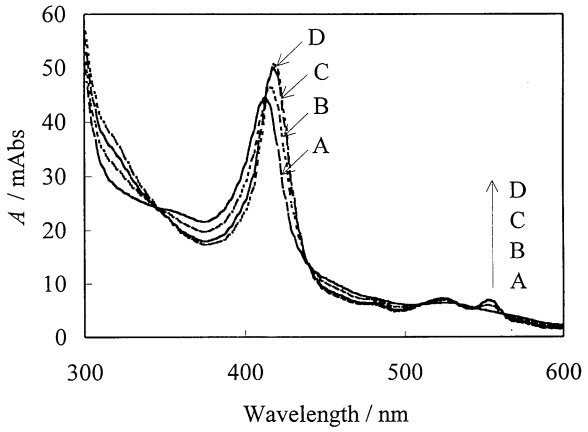

Figure 2. Absorption spectra of ADH at state A (0.5 V), B (0.3 V), C (0 V), and D (-0.3 V vs. NHE) represented in Figure 1.

D in turn from the positive to negative potentials) are depicted in Figure 2. At state A (0.5 V vs. NHE), all of the heme *c* groups are fully oxidized, as evidenced by disappearance of the α and β bands. During the potential change from state A to C (0 V), increases of the α and β bands and a red shift of the Soret band are observed. The spectral changes are reasonably assigned to the reduction of the heme *c* moieties. On the potential change from state C to D (-0.3 V), the Soret band as well as the α band increase slightly, although the maximum wavelength of the Soret band remains unchanged. Then we concluded that the four hemes *c* are completely reduced at state C and that the spectral change from state C to D is owing to the two-electron reduction of PQQ, which could affect the electronic circumstance of a heme(s) *c*, most probably that in subunit I.

Thus we tried to fit a theoretical Nernstian curve to the *A-E* data based on a four-step one-electron and one-step two-electron transfer mechanism by means of a non-linear least square method. The regression curve given by the solid line in Figure 1 reproduces the data well. Evaluated $E^{\circ\prime}$ values are: 0.401 (heme 1), 0.370 (heme 2), 0.216 (heme 3), 0.101 (heme 4), and -0.167 V (PQQ) vs. NHE at pH 7.0 (s.d. ≅ 0.004 V). The analysis also indicated that the spectral change from state A to C is assigned almost equally to the redox reactions of the four hemes, as shown by the dotted lines in Figure 1. This supports our assignment of the spectral change. Almost identical results were obtained by the analysis at other wavelengths. As the alternative, the total spectral change from state A to D might be assigned to the redox reaction of the hemes *c* alone. Although the curve fitting analysis based on a four-step one-electron transfer mechanism yielded a regression curve similar to that given in Figure 1, the absorbance change due to the redox reaction of one heme *c* moiety became quite different from each other. It seems to be difficult to accept such a situation. Then we ruled out the assignment.

For further confirmation of the evaluated $E^{\circ\prime}$ of ADH, spectral changes of ADH were monitored on addition of several redox reagents at 500-fold in the molar concentration. The reduction with $Na_2S_2O_4$ resulted in slight increase of the Soret band, while no change was observed by the addition of vitamin K_3 ($E^{\circ\prime}$ = -0.024 V at pH 7), suggesting that the isolated ADH is in state C. On the other hand, the oxidation with $K_3Fe(CN)_6$ ($E^{\circ\prime}$ = 0.461 V) and 2,3-dimethoxy-5-methyl *p*-benzoquinone ($E^{\circ\prime}$ = 0.146 V), respectively, exhibited a spectrum very similar to that of state A and B. All these results are well explained by considering the $E^{\circ\prime}$ values of ADH and the reagents.

Similar spectroelectrochemical data were obtained at pH 6.0 (McIlvaine buffer), of which analysis yielded five $E^{\circ\prime}$ as 0.343 (heme 1), 0.315 (heme 2), 0.189 (heme 3), 0.086 (heme 4), and −0.230 V (PQQ). However, at pH 5.0 (McIlvaine buffer), the *A-E* plot had only two sigmoidal parts. This would be considered as a result of the $E^{\circ\prime}$overlapping between heme 4 and PQQ. Values of $E^{\circ\prime}$ were evaluated as 0.39 (heme 1), 0.14 (heme 2), -0.08 (heme 3), and -0.14 V (heme 4 and PQQ). Although it does not seem to be simple to explain the pH dependence of $E^{\circ\prime}$, this result might be related to the complicated pH dependence of ADH activity[1] or reflect pH dependence of the interaction among the subunits. Anyway, this work has proved the usefulness of column-electrolytic spectroscopy for the determination of $E^{\circ\prime}$ values of redox proteins under suitable conditions.

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