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Intramolecular electron transport in quinoprotein alcohol dehydrogenase of *Acetobacter methanolicus*: a redox-titration study

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Abstract

Quinohemoprotein–cytochrome *c* complex alcohol dehydrogenase (ADH) of acetic acid bacteria consists of three subunits, of which subunit I contains pyrroloquinoline quinone (PQQ) and heme *c*, and subunit II contains three heme *c* components. The PQQ and heme *c* components are believed to be involved in the intramolecular electron transfer from ethanol to ubiquinone. To study the intramolecular electron transfer in ADH of *Acetobacter methanolicus*, the redox potentials of heme *c* components were determined with ADH complex and the isolated subunits I and II of *A. methanolicus*, as well as hybrid ADH consisting of the subunit I/III complex of *Gluconobacter suboxydans* ADH and subunit II of *A. methanolicus* ADH. The redox potentials of hemes *c* in ADH complex were −130, 49, 188, and 188 mV at pH 7.0 and 24, 187, 190, and 255 mV at pH 4.5. In hybrid ADH, one of these heme *c* components was largely changed in the redox potential. Reduced ADH was fully oxidized with potassium ferricyanide, while ubiquinone oxidized the enzyme partially. The results indicate that electrons extracted from ethanol at PQQ site are transferred to ubiquinone via heme *c* in subunit I and two of the three hemes *c* in subunit II. © 1998 Elsevier Science B.V.

Keywords: Quinoprotein; Alcohol dehydrogenase; Redox titration; Heme *c*; Intramolecular electron transfer; *(Acetobacter methanolicus)*

1. Introduction

Alcohol dehydrogenase (ADH, E.C. 1.1.99.) of acetic acid bacteria is localized on the periplasmic side of cytoplasmic membrane and functions as a primary dehydrogenase in the ethanol oxidase respiratory chain [1]. It has been established that ADH donates electrons to ubiquinone embedded in membrane phospholipids and that the resulting ubiquinol is subsequently oxidized by terminal oxidase which generates an electrochemical proton gradient [2]. ADH is also able to utilize phenazine methosulfate, dichlorophenol indophenol, short chain ubiquinones and potassium ferricyanide as artificial electron acceptors. ADH of acetic acid bacteria typically consists of a dehydrogenase subunit (subunit I, 72–80 kDa), a cytochrome *c* subunit (subunit II, 44–54 kDa) and a small subunit III (8–20 kDa) [2–6]. ADH contains two different types of cofactor, pyrroloquinoline quinone (PQQ) and multiple hemes *c*. PQQ and one heme *c* are present in subunit I, while the other three hemes *c* in subunit II. Subunit

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III has been shown to be essential for the expression of active enzyme and to be detected as free form in the periplasm besides in the enzyme complex [7]. Although none of any subunits seem to have putative membrane-spanning domains, it is suggested that subunit II have membrane-binding domain since the subunit is always detected in the membrane, unlike other subunits sometimes seen in the soluble fraction [7–9]. This notion has been confirmed by the more direct evidence that the isolated subunit II but not subunit I/III complex can be bound to liposomes (unpublished observation). In *Gluconobacter suboxydans*, the four heme *c* moieties can be distinguished by their different kinetic properties with ferricyanide, since four specific ferricyanide reacting sites, one of them in subunit I (termed as site I) and the other three in subunit II (termed as site II_1, II_2, and II_3, respectively) have been detected [10]. Ubiquinone reductase activity of *Gluconobacter* ADH, that is completely diminished by dissociation into the subunits, can be reproduced by reconstituting subunit II to the subunit I/III complex and furthermore, kinetics for ubiquinone in a hybrid reconstituted ADH reflects the character of the original ADH from which subunit II is derived [10]. Thus, the ubiquinone reacting site has been shown to be present in subunit II and further suggested to be located at or close to either II_1 or II_2 site. The data have also suggested that at least the II_3 site is not involved in the reduction of ubiquinone.

Although *Acetobacter methanolicus* ADH also contains four heme *c* moieties, only three kinetically different reacting sites have been detected [11]. Kinetic study of ADH from *A. methanolicus* and of hybrid ADH constructed from subunit I/III complex of *G. suboxydans* ADH and subunit II of *A. methanolicus* ADH has suggested that ubiquinone reacting site in *A. methanolicus* ADH is not the same as either of the ferricyanide reacting sites [11]. However, no further elucidation concerning intramolecular electron transfer could be done from the kinetic data.

The aim of this work is to further analyze the intramolecular electron transfer in *A. methanolicus* ADH by focusing on the redox properties of the heme *c* components. ADH complex of *A. methanolicus* and the isolated subunits I and II, subunit I/III complex of *G. suboxydans*, and hybrid ADH constructed from this subunit I/III complex and subunit II from *A. methanolicus* were redox-titrated and redox potentials of the heme *c* moieties were determined. We also report the isolation and characterization of free subunit I of *A. methanolicus* ADH. Possible intramolecular electron transfer among the prosthetic groups, PQQ and heme *c* moieties, is discussed.

2. Materials and methods

2.1. Chemicals

Ubiquinone-1 (Q_1) was supplied by Eisai (Tokyo, Japan). 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), 2,3,5,6-tetramethyl-*p*-phenylenediamine and 2-hydroxy-1,4-naphtoquinone were obtained from Sigma. Phenazine methosulfate was from Wako (Osaka, Japan) and phenazine ethosulfate and duroquinone from Nakarai (Kyoto, Japan). All other chemicals were commercial products of guaranteed grade.

2.2. Bacterial strain, growth conditions, and preparation of soluble and membrane fractions

A. methanolicus JCM 6891 was grown aerobically to the late logarithmic phase in a glycerol medium [5]. Cells were harvested and the membrane and soluble fractions were prepared as described previously [5].

2.3. Enzyme purification

ADH and subunit II of *A. methanolicus* were purified as described previously [11]. Subunit I was purified as follows: (a) Purification from the membrane fraction. The membranes were suspended in 50 mM potassium phosphate buffer (pH 6.2) to an approximate final protein concentration of 20 mg/ml. Triton X-100 was added to a final concentration of 0.5%, the suspension was incubated on ice for 60 min, and centrifuged at $98\,000 \times g$ for 90 min. The supernatant was dialyzed against a 15-fold volume of distilled water containing 0.1% Triton X-100 and then applied onto a DEAE-Toyopearl column (1.8 cm i.d. × 25 cm) prewashed with 8 mM potassium phos-

phate buffer (pH 6.2) containing 0.1% Triton X-100. The column was washed with 78 ml of the same buffer and then with 155 ml of 25 mM potassium phosphate buffer (pH 6.2) containing 0.1% Triton X-100. The active fractions were collected, concentrated with an UP 20 ultrafilter (Advantec), and dialyzed overnight against 5 mM acetate buffer (pH 5.0) containing 0.1% Triton X-100, 1 mM calcium chloride, and 0.01 μM PQQ. The sample was then loaded onto a CM-Toyopearl column (1.6 cm i.d. × 13 cm) equilibrated with the same buffer. After washing the column with 35 ml of this buffer, the proteins were first eluted with a linear gradient from 5 to 100 mM acetate buffer (pH 5.0) containing 0.1% Triton X-100, 1 mM calcium chloride, and 0.01 μM PQQ (total volume of 100 ml), then the column was washed with another 50 ml of 100 mM acetate buffer, and the gradient was continued to 200 mM acetate buffer (total volume of 100 ml). Finally, the column was washed with 20 ml of 200 mM buffer. The orange fractions which eluted with about 200 mM buffer were collected, concentrated by ultrafiltration and used as purified subunit I.

(b) Purification from the soluble fraction. Soluble fraction was treated with Triton X-100 (final concentration 0.1%), incubated for 30 min on ice, and then dialyzed overnight against a 10-fold volume of distilled water containing 0.1% Triton X-100. The dialyzate was ultracentrifuged at 98 000 × *g* for 90 min and applied onto a DEAE-Toyopearl column (1.8 cm i.d. × 25 cm) equilibrated with 10 mM potassium phosphate buffer (pH 6.5) containing 0.1% Triton X-100. After sample loading, the column was washed with 170 ml of the same buffer. The active orange-colored fractions were collected, concentrated with an UP 20 ultrafilter, and applied onto a hydroxyapatite column (2.8 cm i.d. × 8.5 cm) prewashed with 10 mM potassium phosphate buffer (pH 6.5) containing 0.1% Triton X-100. After the column was washed with 25 ml of this buffer, the enzyme was eluted with a linear gradient from 10 to 100 mM potassium phosphate buffer (pH 6.5) containing 0.1% Triton X-100 (total volume of 340 ml). The active fraction was collected, concentrated by ultrafiltration, and dialyzed overnight against 5 mM acetate buffer (pH 5.0) containing 0.1% Triton X-100. The dialyzed sample was then applied onto a CM-Toyopearl column (1.6 cm i.d. × 13 cm) equilibrated with the same buffer. Subunit I was eluted from the column as described for the purification from the membrane fraction. The collected fraction was concentrated and used as partially purified subunit I.

2.4. Enzyme assays

Enzyme activities were measured at 25°C as described previously [11]. One unit of enzyme activity was defined as 1 μmol of substrate oxidized by the enzyme per minute. HQNO was incubated with the enzyme for 10 min prior the measurement.

2.5. Potentiometric titrations

Redox titrations were performed in an anaerobic vessel using platinum and saturated calomel electrodes (Radiometer, Copenhagen) as described previously [12]. Titrations were done in 50 mM MOPS–NaOH buffer (pH 7.0) or McIlvaine buffer (pH 4.5) at 25°C in the presence of a mixture of following electron mediators: potassium ferrocyanide, 2,3,5,6-tetramethyl-*p*-phenylene-diamine, phenazine methosulfate, phenazine ethosulfate, duroquinone and 2-hydroxy-1,4-naphtoquinone. Potassium ferrocyanide was used at 50 μM, 2-hydroxy-1,4-naphtoquinone at 10 μM, and other mediators at 20 μM. Oxygen was excluded from the vessel by continuous flushing with argon. Titrations were carried out by the stepwise additions of small amounts of anaerobic 100 mM potassium ferricyanide or 100 mM sodium dithionite in 50 mM MOPS buffer (pH 7.0) or McIlvaine buffer (pH 4.5). Reduction of hemes *c* was recorded at the α-band maximum at 553 nm in the case of subunit II, native, and hybrid ADHs and at 551 nm in the case of subunit I from *A. methanolicus* or subunit I/III complex from *G. suboxydans*, with the reference wavelength at 539 nm using a Hitachi 557 dual wavelength spectrophotometer. All potentials are relative to the standard hydrogen electrode. Experimental data were fitted by Nernst curves for 1, 3, or 4 single electron components with unknown redox potentials using Igor Pro software for Macintosh (Wave Metrics, Lake Oswego, Oregon, USA). Minimization of the sum of the squared residuals was used as a criterion for the selection of the best fitting model.

2.6. Other procedures

Protein content was determined by a modification of Lowry's method [13] with BSA as a standard protein. Absorption spectra were taken on a Hitachi U3210 spectrophotometer. Kinetic constants were calculated using the Enzyme Kinetics computer program (version 1.4, Trinity Software, Campton, NH, USA). Reconstitution of isolated subunits into hybrid ADH was performed as described previously [11]. SDS–PAGE [14] was performed on a slab gel (12.5%) in Tris–tricine running buffer. Proteins were visualized with Coomassie Brilliant Blue R-250. Heme staining was performed as heme-catalyzed peroxidase activity [15].

3. Results

3.1. Purification and characterization of subunit I

ADH complex of *G. suboxydans* was shown to partially dissociate into subunit I/III complex and subunit II on CM-Toyopearl column and this method could be used for isolating the subunits [10]. ADH of *A. methanolicus* could be also dissociated on CM-Toyopearl column, although much lesser extent. Unlike the case of *G. suboxydans* enzyme, however, only free subunit I, but not subunit I/III complex, of *A. methanolicus* ADH could be obtained on the column. Thus, only limited amount of subunit I could be purified from the membranes of *A. methanolicus* (data not shown). Since excess of subunit I or only subunit I of ADH of several acetic acid bacteria can be detected in soluble fraction under certain growth conditions [6–9], we attempted to obtain subunit I from the soluble fraction. On both DEAE-Toyopearl and hydroxyapatite columns, subunit I eluted together with ADH, but the excess of subunit I could be clearly detected by heme staining on SDS–PAGE after hydroxyapatite column. Subunit I was then separated from ADH complex on CM-Toyopearl column. By this method, partially purified subunit I was obtained (Fig. 1). To prevent loss of activity (see below), subunit I was not further purified. Subunit I was isolated as an apo-form and therefore, the activity was observed only after reconstitution of subunit I with PQQ in the presence of calcium chloride. The

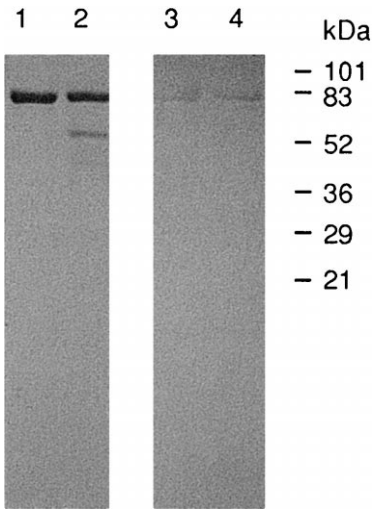

Fig. 1. SDS–PAGE of *A. methanolicus* subunit I. Lanes 1 and 3, subunit I purified from the membrane fraction (7 μg); lanes 2 and 4, subunit I purified from the soluble fraction (8.5 μg). Proteins in lanes 1 and 2 were stained with Coomassie Brilliant Blue R-250 and in lanes 3 and 4 for heme. SDS gel contained 12.5% acrylamide and Tris–tricine was used as a running buffer.

activity of subunit I was very unstable and rapidly lost. The stability was not improved by including PQQ and calcium chloride in all buffers used during the purification. Subunit I, thus, obtained showed a weak ferri-cyanide reductase activity (2–3.5 U/mg at optimum pH 4.5 depending on preparation), which is extremely low compared with ADH complex (120 U/mg at pH 4.5). The K_m value for ferricyanide was 25 μM. However, no ubiquinone (Q_1) reductase activity was detected in the isolated subunit I.

3.2. Absorption spectra

ADH complex and the subunits show absorption spectra characteristic of heme *c* (Fig. 2). The absorption maxima of α, β, and γ-bands of ADH were at 553, 522, and 417 nm, respectively. The absorption maxima of reduced subunit II were at 553, 522, and 416 nm, respectively, and of reduced subunit I at 551, 523, and 416 nm, respectively. Extinction coefficients of these peaks are summarized in Table 1.

ADH is typically isolated in its fully reduced form, which is probably due to the ubiquitous presence of the substrate, ethanol, in buffer solutions. Addition of the excess ferricyanide leads to the fully oxidized

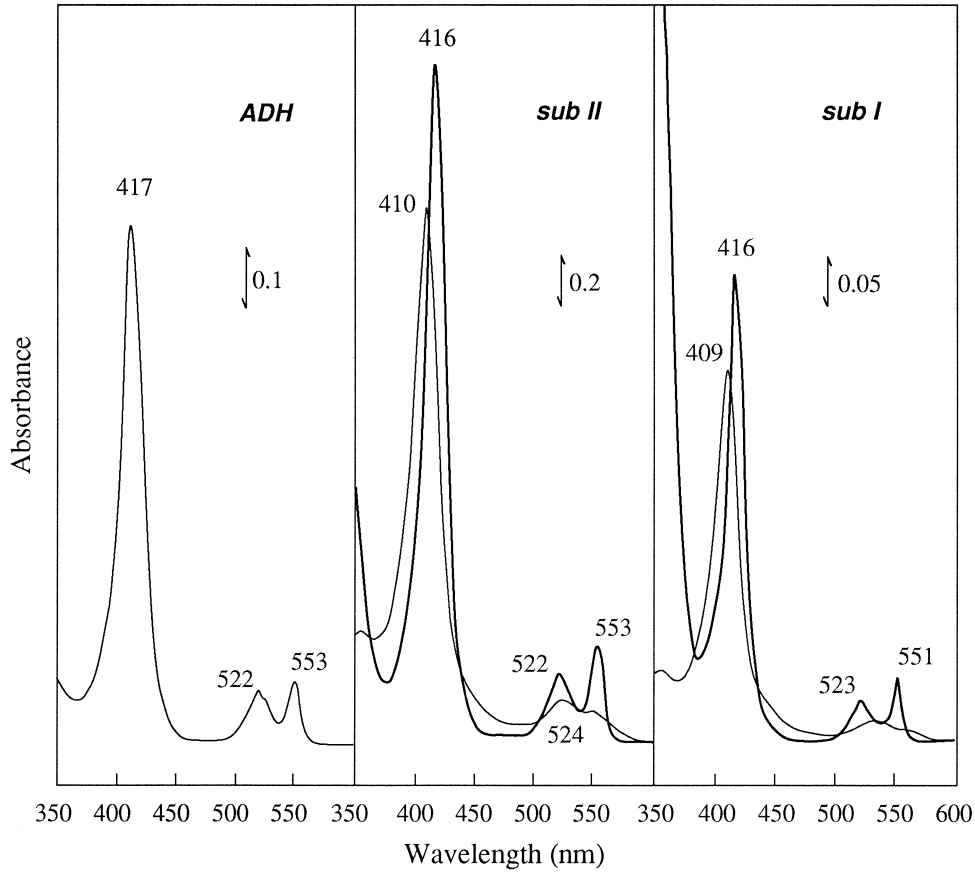

Fig. 2. Absorption spectra of ADH, subunit I and subunit II purified from *A. methanolicus*. First, each spectrum was taken with ADH (2.87 μM), subunit II (7.15 μM), and subunit I (5.93 μM) (thin line), then taken again after adding sodium dithionite (thick line). Subunit I purified from the membrane fraction was used in this experiment.

enzyme, independent of buffer pH. On the other hand, the addition of 400 μM ubiquinone-1 leads to only partial oxidation of the enzyme: at pH 4.5 about 63% of the enzyme is oxidized, while 13% is oxidized at pH 7.0 (Fig. 3). The spectra were not changed even with the higher concentration at 800 μM, which seems to be far in excess of the substrate contaminated in buffer solution. Subunit II and also subunit I are isolated in the fully oxidized state, which are reduced by the addition of sodium dithionite. However, subunit I also turns out to be an reduced form by holoenzyme formation with PQQ and calcium chloride, which is independent of the presence of substrate. Although subunit I is an oxidized form due to being isolated as the apo-form as described above, the holo-subunit I seems to be reduced by the same reason described above.

Table 1
Extinction coefficients of reduced forms of ADH, subunit I and subunit II purified from *A. methanolicus*

Wavelength (nm)	ϵ_{ADH} ($\mu M^{-1}cm^{-1}$)	ϵ_{subI} ($\mu M^{-1}cm^{-1}$)	ϵ_{subII} ($\mu M^{-1}cm^{-1}$)
553	0.064	0.009	0.055
551	–	0.011	–
523	–	0.007	–
522	0.054	–	0.040
416.2	–	0.082	0.399
416.6	0.551	–	–

3.3. Potentiometric titration

Redox titrations of hemes *c* in ADH complex, as well as in the isolated subunits I and II from *A. methanolicus*, subunit I/III complex from *G. suboxy-*

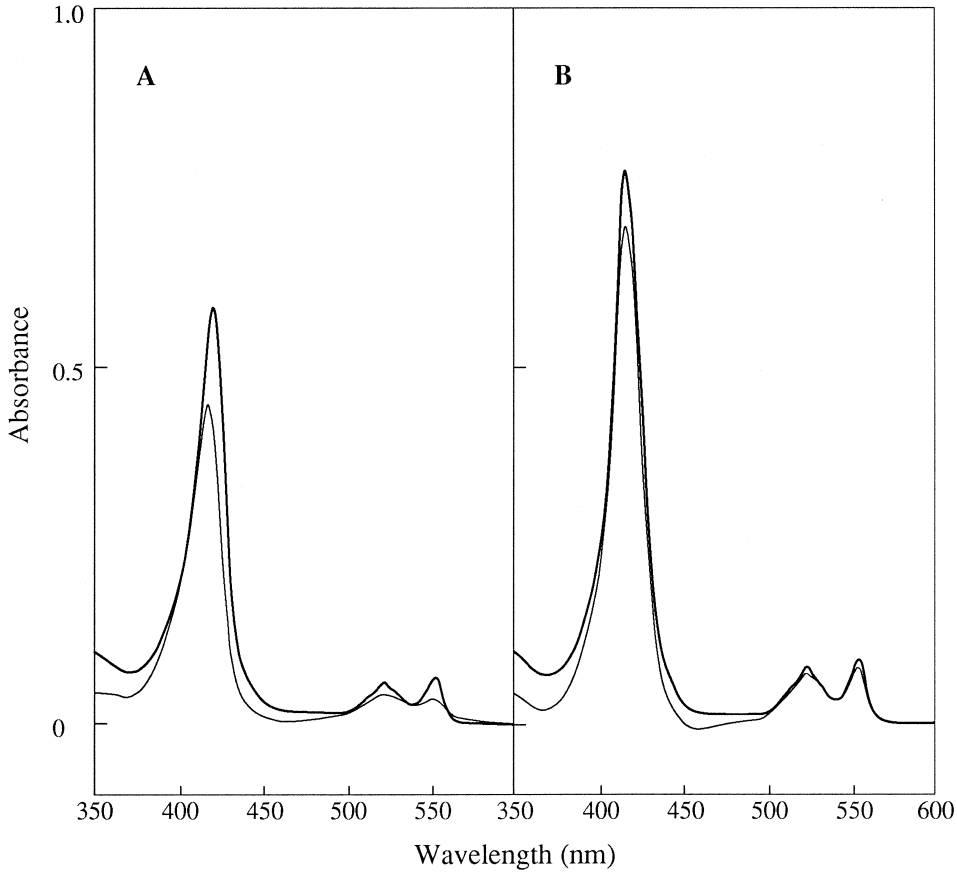

Fig. 3. Effect of ubiquinone on redox state of ADH. The absorption spectra were taken at pH 4.5 (A; 1.25 μM ADH) and 7.0 (B; 1.49 μM ADH) in the absence (thick line) and presence (thin line) of 400 μM ubiquinone-1.

dans and hybrid ADH constructed from subunit I/III complex of *G. suboxydans* and subunit II of *A. methanolicus* were carried out. The redox titration were monitored at 553 or 551 nm, and the changes in the absorbance were plotted as % of total absorbance. The experimental data were fitted with the Nernst equation for 1–4 single electron components with unknown redox potentials.

At first, redox titration of the isolated subunits was done at pH 7.0, which is not physiological pH but standard pH for the redox potential to be determined (Fig. 4). Heme *c* components in subunit II of *A. methanolicus* ADH were redox-titrated as shown in Fig. 4(A). Although the presence of only two heme *c* components was suggested according to the best fit where the component with higher redox potential contributes 39.5%, and the remaining component of lower potential does 60.5%, the data was fitted by Nernst curve for 3 components since subunit II has been shown to contain three heme *c* components [11]. Thus, the redox potentials of hemes *c* in free subunit II were determined to be 1, 41, and 168 mV with the contribution of 25.6, 34.9, and 39.5% to the absorbance change, respectively. Heme *c* component in the isolated subunit I from *A. methanolicus* ADH and the isolated subunit I/III from *G. suboxydans* ADH was also redox-titrated at pH 7.0. The titration data were fitted well by Nernst curve for single component, and then the redox potentials of these hemes *c* were determined to be −28 and −42 mV, respectively (Fig. 4 (B) and (C)).

Next, heme *c* components in *A. methanolicus* ADH complex were redox-titrated at pH 7.0, and the data were compared with the data for the isolated subunits. As shown in Fig. 5(A), the best fit of redox-titration data for ADH showed the presence of

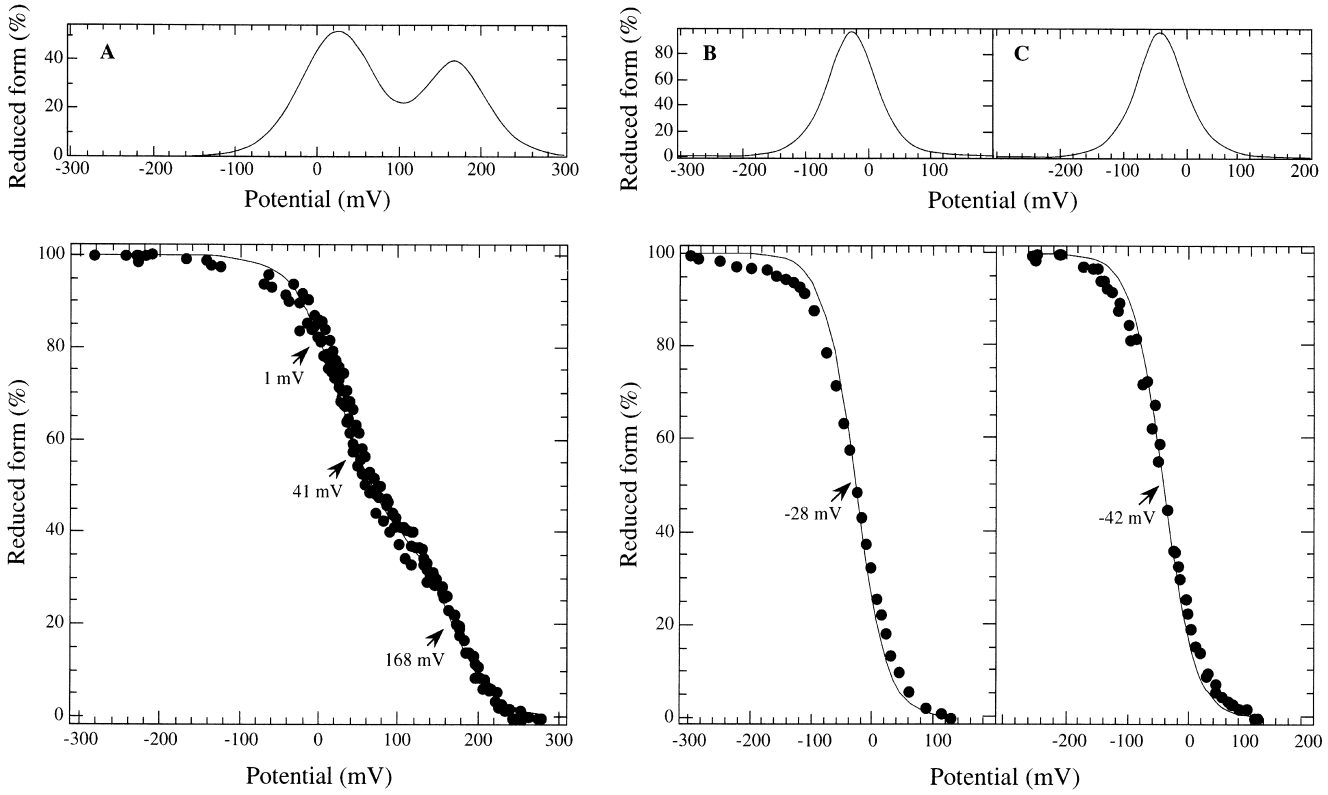

Fig. 4. Potentiometric titration of hemes *c* in *A. methanolicus* subunits I and II and *G. suboxydans* subunit I/III complex. *A. methanolicus* subunit II (panel A) and I (panel B) and *G. suboxydans* subunit I/III complex (panel C) were redox-titrated in 50 mM MOPS buffer pH 7.0 at 25°C in the presence of a mixture of following electron mediators: potassium ferrocyanide, 2,3,5,6-tetramethyl-*p*-phenylenediamine, phenazine methosulfate, phenazine ethosulfate, duroquinone, and 2-hydroxy-1,4-naphtoquinone. Potassium ferrocyanide was used at 50 μM, 2-hydroxy-1,4-naphtoquinone at 10 μM, and other mediators at 20 μM. The plots represent data from three (A) and two (B, C) independent titrations in both oxidative and reductive directions. Experimental data in panel A were fitted by Nernst curve for 3 components ($n = 1$) with unknown redox potentials and relative spectral contributions of 25.6, 34.9, and 39.5%, respectively. Experimental data in panels B and C were fitted by Nernst curve for one component ($n = 1$). The upper panels show the derivation of fitted curves.

a low-potential heme *c* component (−130 mV, 13.8%), a mid-potential heme *c* component (49 mV, 22%) and high-potential heme *c* component (188 mV, total 64.2%); the last component seems to include two different heme *c* components as shown later. Relative spectral contribution of the redox components at monitoring wavelength of 553 nm was calculated from the absorption spectra of ADH and the subunits to be 14% for subunit I and 86% for subunit II (Table 1), which is in good agreement with the results of fitting. By the spectral contribution, the heme *c* with redox potential of −130 mV (14%) is assumed to be one present in subunit I and thus, the others (86%) in subunit II. Since three heme *c* components having the lowest to highest potentials in subunit II contribute 25.6%, 34.9%, and 39.5%, respectively, of the hemes *c* in subunit II, these hemes *c* can be estimated to contribute 22%, 30%, and 34% (total 86%), respectively, of the hemes *c* in ADH complex. Thus, the mid-potential heme *c* component (49 mV, 22%) of ADH may correspond to the heme *c* in subunit II with the potential of 1 mV, and the highest potential heme *c* component (188 mV, total 64.2% contribution) of ADH would be a mixture of two different heme c components in subunit II having the potentials of 41 and 168 mV (total 64% contribution). Taken as a whole, the redox potentials of hemes *c* in ADH complex can be estimated to be −130, 49, 188, and 188 mV with contribution of each component to be 14, 22, 30, and 34%, respec-

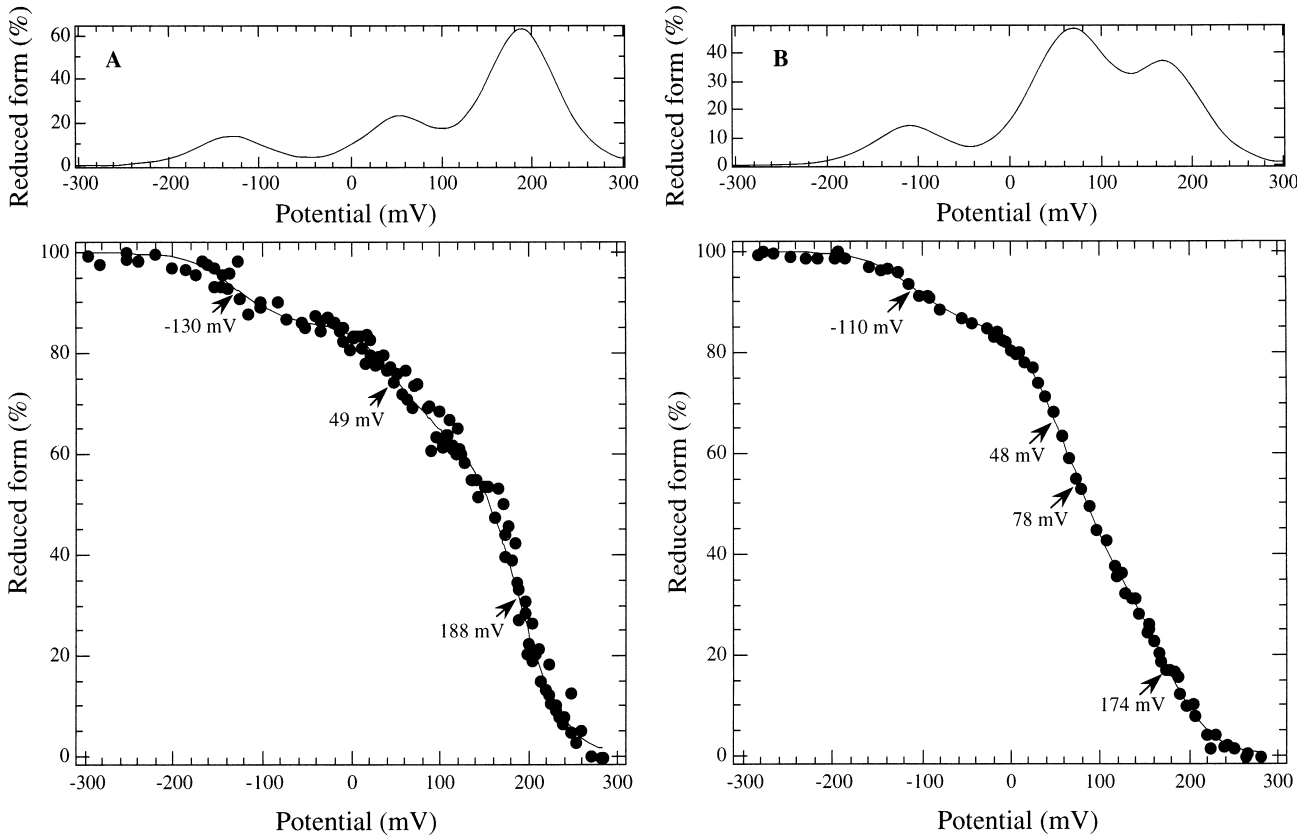

Fig. 5. Potentiometric titration of hemes *c* in native and hybrid ADH. (A) Native ADH of *A. methanolicus*. (B) Hybrid ADH. Redox titration was carried out in 50 mM MOPS buffer pH 7.0 at 25°C as described in Fig. 4. The plot represents data from two independent titrations in both oxidative and reductive directions in the case of native ADH, and one titration in both the directions in the case of hybrid ADH. Experimental data were fitted by Nernst curve for 4 components ($n = 1$) with unknown redox potentials and relative spectral contributions of 14, 22, 30, and 34%, respectively (see text for details). The upper panels show the derivation of fitted curves.

tively (Fig. 5(A)), and these heme *c* components are named as heme *c* I (−130 mV) present in subunit I, and heme *c* II_1 (49 mV), II_2 (188 mV) and II_3 (188 mV) present in subunit II. The results also clearly indicate considerable difference in the redox potentials of the hemes between the isolated subunits and

Table 2
Redox potentials of subunit I and subunit II purified from *A. methanolicus*, subunit I/III complex purified from *G. suboxydans*, native ADH purified from *A. methanolicus*, and hybrid ADH consisting of subunit I/III complex of *G. suboxydans* enzyme and subunit II of *A. methanolicus* enzyme

Heme *c* components	Redox potentials (mV) measured at pH 7.0					Redox potentials (mV) measured at pH 4.5
	A. methanolicus ADH				*G. suboxydans* ADH	*A. methanolicus*
	Subunit I	Subunit II	ADH complex	Hybrid ADH	Subunit I/III	ADH complex
I	−28	−	−130	−110	−42	24
II_1	−	1	49	48	−	187
II_2	−	41	188	78 or 174	−	190 or 255
II_3	−	168	188	174 or 78	−	255 or 190

ADH complex, which may be due to the missing interaction with the other redox components, and/or due to the conformational change of the subunits released from ADH complex (Table 2).

As we reported previously, hybrid ADH consisting of subunit I/III complex from *G. suboxydans* ADH and subunit II of *A. methanolicus* exhibits different kinetic properties from native ADH of *A. methanolicus* [11]. To see whether or not the difference reflects their redox properties, the redox potentials of heme *c* moieties in hybrid ADH were also determined at pH 7.0. As shown in Fig. 5(B), the values were estimated to be −110 (I), 48 (II_1), 78 (II_2 or II_3), and 174 mV (II_2 or II_3). From the comparison of the redox potentials between native and hybrid ADHs (Table 2), it is indicated that either of heme *c* component II_2 or II_3 has a change in the surrounding environment, which is consistent with the previous suggestion that conformational change of subunit II occurs in hybrid ADH [11].

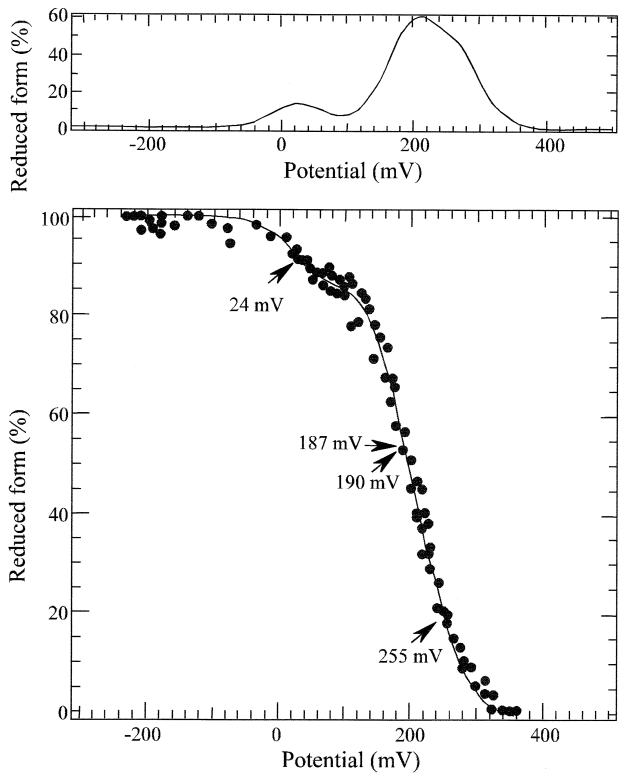

Fig. 6. Potentiometric titration of hemes *c* in native ADH measured at pH 4.5. Redox titration was carried out in 50 mM McIlvaine buffer pH 4.5 at 25°C as described in Fig. 4. The plot represents data from one titration in both oxidative and reductive directions. Experimental data were fitted by Nernst curve for 4 components ($n = 1$) with unknown redox potentials and relative spectral contributions of 14, 22, 30, and 34%, respectively. The upper panel shows the derivation of fitted curve.

As shown in the previous paper [11], ADH of *A. methanolicus* works at different pHs: ADH donates electrons to ferricyanide at both pH 4.5 and 7.0 while to Q_1 or Q_2 at pH 4.0. Thus, the redox titration of ADH complex was also determined at more physiological pH 4.5. As shown in Fig. 6, the redox potentials thus obtained with ADH were 24 (I), 187 (II_1), 190 (II_2 or II_3) and 255 mV (II_2 or II_3). Compared to the values determined at pH 7.0, the redox potential of heme *c* I shifts by 154 mV, i. e. by 60 mV per pH unit, and that of heme *c* II_1 shifts by 138 mV (Table 2). However, the potential shift of hemes *c* II_2 and II_3 could not be specified for the reason that both are electrochemically indistinguishable, and thus, one of either shifts by 67 mV in the redox potential from pH 7.0 to 4.5, while the other is pH-independent.

4. Discussion

Quinohemoprotein–cytochrome *c* complex ADH of acetic acid bacteria functions as a primary dehydrogenase in the ethanol oxidase respiratory chain, passing electrons from ethanol to ubiquinone and then to terminal ubiquinol oxidase [2]. The studies on quinohemoprotein ADH from *Comamonas testosteroni*, which is a soluble single peptide closely similar to subunit I of ADH from acetic acid bacteria, have suggested that ethanol oxidation occurs at PQQ site, after which electrons are transferred to heme *c* and possibly to the respiratory chain [16,17]. Subunit II of ADH complex has been recently shown to posses ubiquinone binding site in *Gluconobacter suboxydans* [10] and therefore, the electrons extracted from ethanol at the PQQ site in subunit I must be transferred inside the ADH complex, where several heme *c* moieties may be involved in the electron transfer and consequently in the reduction of ubiquinone. Recent kinetic study of *G. suboxydans* ADH indicates that at least one of the heme *c* sites in subunit II (termed as site II_3) is not involved in the reduction of ubiquinone [10], but no clear conclusions could be drawn in the case of *A. methanolicus* ADH [11].

In the present study, we mainly focused on analysis of redox properties of heme *c* components, as the knowledge of redox potentials is important for understanding the intramolecular electron transfer. Furthermore, subunit I from *A. methanolicus* ADH was isolated and confirmed to have a ferricyanide reacting site but not ubiquinone binding site.

Redox titration shows that the redox potentials of hemes *c* I, II_1, II_2, and II_3 in ADH complex are −130, 49, 188, and 188 mV, respectively, at pH 7.0, and 24, 187, 190, and 255 mV, respectively, at pH 4.5. Separation of ADH complex into subunits leads to significant changes in the potentials: heme *c* I attains redox potential of −28 mV, while redox potentials of hemes *c* II_1, II_2, and II_3 shift to 1, 41, and 168 mV, respectively, indicating that dissociation of ADH into the subunits induces a conformational change in the subunits and thus, a change in the hemes environment. When reconstituting subunit II with subunit I/III complex from *G. suboxydans* ADH into hybrid ADH, the heme *c* II_1 resumes its original redox potential, while redox potentials of hemes *c* II_2 and II_3 are not completely recovered: 78 and 174 mV, respectively, compared to 188 mV. Since the kinetic study has suggested that one of the ferricyanide reacting sites is not working in hybrid ADH [11], the titration data suggest that either the heme *c* II_2 or II_3 is the site not working in hybrid ADH.

So far, redox potential of heme *c* component has been determined only with quinohemoprotein ADH from *C. testosteroni* [18]. The value of 140 mV (at pH 7.5) is quite different from that of heme *c* in subunit I (−130 mV in ADH complex and −28 mV in the isolated subunit I). This difference may be of physiological importance, as the electron acceptors for the two ADHs are different. In the case of *A. methanolicus* ADH, electrons are transferred to the heme *c* in subunit II and then to ubiquinone, while the electron acceptor for *C. testosteroni* ADH seems to be a blue copper protein, azurin (K. Matsushita, unpublished data).

Absorption spectra of ADH complex in the absence and presence of ubiquinone showed that 63% of heme *c* was oxidizable by ubiquinone at pH 4.5 (Fig. 3). Considering the relative spectral contribution of each heme *c* component and one of the hemes *c* (II_3) having higher redox potential (255 mV) than ubiquinone (220 mV at pH 4.5, [19]), it is reasonable to suggest that this heme *c* II_3 is the component unaffected by ubiquinone at pH 4.5 and thus, not involved in the electron transfer to ubiquinone. As discussed above, the II_2 or II_3 site may lose its electron transfer activity to ferricyanide in hybrid ADH. Since hybrid ADH still keeps ubiquinone reductase activity [11], it can be assumed that an electron transfer from II_1 to II_2 but not from II_1 to II_3 function properly in hybrid enzyme, and thus, the heme *c* II_3 is the site defective in ferricyanide reduction. Thus, we speculate that the electrons from the ethanol are transferred to PQQ, heme *c* I site in subunit I, hemes *c* II_1 and II_2 sites in subunit II, and then to ubiquinone.

Thus, the present redox titration study of *A. methanolicus* ADH provides further experimental evidence for the intramolec-ular electron transfer pathway inside ADH complex as proposed for *G. suboxydans* ADH [10] and it thus, appears that a similar electron transfer pathway could exist in ADH of all acetic acid bacteria.

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