Function of Multiple Heme c Moieties in Intramolecular Electron Transport and Ubiquinone Reduction in the Quinohemoprotein Alcohol Dehydrogenase-Cytochrome c Complex of Gluconobacter suboxydans*

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Alcohol dehydrogenase (ADH) of acetic acid bacteria functions as the primary dehydrogenase of the ethanol oxidase respiratory chain, where it donates electrons to ubiquinone. ADH is a membrane-bound quinohemoprotein-cytochrome c complex which consists of subunits I (78 kDa), II (48 kDa), and III (14 kDa) and contains several hemes c as well as pyrroloquinoline quinone as prosthetic groups. To understand the role of the heme c moieties in the intramolecular electron transport and the ubiquinone reduction, the ADH complex of Gluconobacter suboxydans was separated into a subunit I/III complex and subunit II, then reconstituted into the complex. The subunit I/III complex, probably subunit I, contained 1 mol each of pyrroloquinoline quinone and heme c and exhibited significant ferricyanide reductase, but no Q1 reductase activities. Subunit II was a triheme cytochrome c with no enzyme activity, but it enabled the subunit I/III complex to reproduce the Q1 and ferricyanide reductase activities. Hybrid ADH consisting of the subunit I/III complex of G. suboxydans ADH and subunit II of Acetobacter aceti ADH was constructed and it had showed a significant Q1 reductase activity, indicating that subunit II has a ubiquinone-binding site. Inactive ADH from G. suboxydans exhibiting only 10% of the Q1 and ferricyanide reductase activities of the active enzyme has been isolated separately from active ADH (Matsushita, K., Yakushi, T., Takaki, Y., Toyama, H., and Adachi, O (1995) J. Bacteriol. 177, 6552–6559). Using these active and inactive ADHs and also isolated subunit I/III complex, we performed kinetic studies which suggested that ADH contains four ferricyanide-reacting sites, one of which was detected in subunit I and the others in subunit II. One of the three ferricyanide-reacting sites in subunit II was defective in inactive ADH. The ferricyanide-reacting site remained inactive even after alkaline treatment of inactive ADH and also after reconstituting the subunit I/III complex from the subunits, in contrast to the restoration of Q1 reductase activity and the other ferricyanide reductase activities. Thus, the data suggested that the heme c in subunit I and two of the three heme c moieties in subunit II are involved in the intramolecular electron transport of ADH into ubiquinone, where one of the two heme c sites may work at or close to, the ubiquinone-reacting site and another between that and the heme c site in subunit I. The remaining heme c moiety in subunit II may have a function other than the electron transfer from ethanol to ubiquinone in ADH.

Alcohol dehydrogenase (ADH)1 of acetic acid bacteria, consisting of the genera Acetobacter and Gluconobacter, catalyzes the first step of acetic acid production, oxidation of ethanol to acetaldehyde. ADH is a quinohemoprotein-cytochrome c complex bound to the periplasmic side of the cytoplasmic membrane and functions as the primary dehydrogenase in the ethanol oxidase respiratory chain, where ADH oxidizes ethanol by transferring electrons to ubiquinone embedded in the membrane phospholipids. The resulting ubiquinol is oxidized by terminal ubiquinol oxidase, cytochrome o or a3 (1). ADH has been purified from five strains and it consists of subunits I, II, and III (1–4), except for one ADH purified from Acetobacter polyoxogenes which consists only of subunits I and II (5). ADH contains pyrroloquinoline quinone (PQQ) (6) and several heme c moieties in subunits I and II (1). The genes encoding subunits I and II have been cloned and sequenced from several sources including Acetobacter aceti (7, 8), A. polyoxogenes (9), and A. pasteurianus (10). Takeda et al. (11) have also cloned the gene encoding the CO-binding cytochrome c from Gluconobacter suboxydans, which is identical to subunit II of ADH. These genetic data suggest that subunit I is a typical secretory protein with a cleavable signal sequence which has significant homology to the putative PQQ-binding motif found in the methanol dehydrogenase α subunit, and a heme c binding motif, and that subunit II is also a secretory protein with three heme c binding motives.

Coupled with ethanol oxidation, ADH reduces phenazine methosulfate, dichlorophenolindophenol, or ferricyanide as an artificial electron acceptor in vitro (12). Since ferricyanide reacts with heme components having a high redox potential, the heme c sites in the ADH complex should reduce ferricyanide. Furthermore, ADH reacts with several ubiquinone homologues and also with native ubiquinone in proteoliposomes (1). To couple with the reduction of ubiquinone, an electron from ethanol must be transferred inside the ADH complex, where PQQ...

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1 The abbreviations used are: ADH, alcohol dehydrogenase; KPB, potassium phosphate buffer; PAGE, polyacrylamide gel electrophoresis; PQQ, pyrroloquinoline quinone; PVD, polyvinylidene difluoride microporous membrane; Q, ubiquinone; CAPS, 3-(cyclohexylaminomethyl)propane-sulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
and several heme c moieties may be involved in the electron transfer and thus in the reduction of ubiquinone. Furthermore, ADH is involved in the CN-insensitive by-pass oxidase system of the G. suboxydans respiratory chain (13, 14) and may mediate electron transfer from another primary dehydrogenase, glucose dehydrogenase, to ferricyanide (15). Thus, ADH appears to have several additional functions in vivo, besides the oxidation of ethanol to acetaldehyde.

To understand why there are so many prosthetic groups and how the intramolecular electron is transported in the ADH complex, we separated and reconstituted individual subunits from the ADH complex. In addition, during the course of the investigation, inactive ADH was isolated from G. suboxydans (16), which has at least 10 times lower activity, although there are no differences in the subunit composition or prosthetic groups. Thus, we also studied the kinetic properties of active and inactive ADH and the reactivation of inactive ADH. The results indicated that subunit I of ADH is a quinohemoprotein which contains one molecule each of PQQ and heme c, that subunit II contains three heme c moieties which are responsible for ubiquinone reduction and that the four heme c sites of ADH are separately involved in the various ferricyanide reductase activities of the ADH complex. Furthermore, based on the results obtained in this study, the intramolecular electron transport of the ADH complex to ubiquinone is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**

Monoclonal antibodies against the subunit I of ADH of G. suboxydans were prepared as described (17). Ubiquinone homologs (Q) were supplied by Elizai Co., Tokyo, DEAE- or CM-Toyopearl, which was used as a medium performance ion-exchanger, was from Tosoh Co. (Tokyo). Phenyl-Sepharose and Amphotrine (pH 3.5–10.0 for IEF) were purchased from Pharmacia LKB. POQ was from Wako Chemical Co. (Osaka). An immunoblotting kit and prestained marker proteins were obtained from Bio-Rad. The polyvinylidene difluoride microporous membrane (PVDF) was obtained from Millipore. High performance liquid chromatography marker proteins and pi marker proteins were supplied by Oriental Yeast Co. Ltd. (Osaka). All other materials were of reagent grade and obtained from commercial sources.

**Bacterial Strains, Plasmids, and Growth Conditions**

The bacterial strains and plasmids used in this study are listed in Table I. These organisms were cultivated at 30 °C with rotary shaking (200 rpm). Acetic acid bacteria were maintained on agar slants containing 1.5% agar, 0.5% CaCO3, and potato medium (18). Cells maintained on the agar slant were inoculated into 5 ml of the respective medium in a 500-ml Erlenmeyer flask, which was then shaken for 16–24 h. For large scale cultivation, 100 ml of the culture was transferred into 1.5 liters of the same medium in a 3-liter Erlenmeyer flask and when necessary, further transferred into 20 liters of the same medium in a 50-liter jar fermentor.

**Preparation of A. pasteurianus and P. aeruginosa Strains Harboring the Plasmid Containing adh Gene**

Plasmids, pAA025 (21) and pRK2013 (22), and A. pasteurianus NP2503 (23) (Table I) were supplied by Dr. Masao Fukuda (Department of Bioengineering, Nagoya University of Technology). E. coli HB101 was transformed with the plasmids by a standard CaCl2 procedure (23). The transformants were screened on a LB plate containing tetracycline or kanamycin. The plasmid pAA025 was transferred from E. coli to A. pasteurianus NP2503 or P. aeruginosa IFO 3445 by the triparental mating method using pRK2013 as a helper plasmid (7). The resulting transconjugants were plated on plates of glycerol medium containing 1% acetic acid or of the minimal medium supplemented with glutconate, respectively, both of which contained tetracycline. The transconjugated strains were termed A. pasteurianus 2503C or P. aeruginosa 3445A. These strains were cultivated in glycerol medium or the minimal medium supplemented with 0.5% ethanol, respectively, and both contained tetracycline.

**Preparation of the Membrane Fraction**

Cells were harvested by centrifugation at 9,000 × g for 10 min, and washed twice with 50 ml KPB (pH 6.0). The washed cells were suspended at about 1 g of wet cells per 5 ml of 50 ml KPB (pH 6.0), and passed twice through a French press (American Instrument Co.) at 16,000 psi. After centrifugation at 9,000 × g for 10 min to remove intact cells, the supernatant were ultracentrifuged at 86,000 × g for 90 min to obtain the membrane fraction.

**Purification of ADH, Subunit I/III Complex, and Subunit II from G. suboxydans**

ADH was purified essentially as described (1, 2) with some modifications as follows. The membrane fraction was suspended in 10 ml KPB (pH 6.0) at a protein concentration of 20 mg/ml, and Triton X-100 was added to the suspension at a final concentration of 1.0% (w/v). After an incubation at 4 °C for 60 min, solubilized ADH was recovered by ultracentrifugation and dialyzed against 5 ml KPB (pH 6.0) containing 0.1% Triton X-100. The dialyze was applied to a DEAE-Toyopearl column (about 5 mg of protein per 1 ml of bed volume) equilibrated with the same buffer. ADH was eluted with a linear gradient consisting of 5-bed volumes each of 5 and 50 ml KPB (pH 6.0), both of which
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Enzyme Assays

Ferricyanide reductase activity of ADH was measured colorimetrically using potassium ferricyanide as an electron acceptor as described (12, 16). Ferricyanide reductase activity was also measured spectrophotometrically in the reaction mixture (1 ml) containing buffer, potassium ferricyanide, and enzyme solution. The reaction was started by adding 10 mM ethanol and the absorbance at 417 nm was followed. Enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate per min, calculated from a millimolar extinction coefficient of potassium ferricyanide of 1.0 mM−1 cm−1. Q1 reductase activity of ADH was measured spectrophotometrically by following the decrease of absorbance at 275 nm at 25°C in a reaction mixture (1 ml) consisting of appropriate amounts of enzyme, 10 mM ethanol, 50 μM Q1, and Mcl-vaine buffer (pH 4.5), as described (1). One unit of these activities was defined as the amount of enzyme oxidizing 1 μmol of ethanol per min.

Analytical Procedures

Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% acrylamide slab gels. The standard marker proteins were a mixture of phosphorylase b (92 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and lysozyme (14 kDa) or prestained molecular weight markers (low molecular weight range, Bio-Rad) for protein staining or heme staining and immunoblotting, respectively. The gel was stained for protein or heme using 0.1% Coomassie Brilliant Blue R-250 or heme-catalyzed peroxidase activity (25), respectively. Immunoblotting was performed as described (26) after the samples treated with 2.0% SDS were applied to SDS-PAGE and resolved as described above. Isoelectrofocusing was performed on 7% (w/v) polyacrylamide gels containing 5.0% (w/v) Ampholine (pH 3.5-10.0) as described (26).

Analysis of N-terminal Sequence—Purified ADH was applied to SDS-PAGE using glycine-free buffers 200 mM Tris-HCl (pH 8.5) and 100 mM Tris-Tricine containing 0.1% SDS (pH 8.25) as the anode and cathode buffers, respectively. The proteins in the gel were transferred electrophoretically onto a PVDF membrane in 10 mM CAPS-NaOH buffer (pH 11.0) containing 10% methanol for 9 h at 25 to 50 mA. The transferred membrane was stained with Coomassie Brilliant Blue R-250 and destained with 50% methanol. The visible bands were excised and applied to a peptide sequence analyzer (Shimadzu).

Measurement of POQ Content—Purified enzyme solution was mixed with 9 volumes of methanol. After incubation at 25°C for 30 min, this solution was centrifuged at 3,000 g for 10 min. The supernatant was evaporated under vacuum and used as POQ extract. The POQ content was measured enzymatically using the membrane fraction containing apo-glucose dehydrogenase from E. coli K12 strain as described (27).

Heme c Contents—Heme was measured from dithionite-reduced minus ferricyanide-oxidized difference spectrum of its pyridine heme-chrome with a dual-wavelength spectrophotometer. The pyridine hemochrome was prepared by mixing the sample with 1.5 mM NaF, and with heme solutions of 20% (w/v) pyridine and 0.2 mM NaOH. The heme content was calculated by a millimolar extinction coefficient of 24.3 (549–535 nm).

Protein Content—The protein content was determined by the modified Lowry method (28). Bovine serum albumin was used as the standard protein.

RESULTS

Isolation of Subunit I/III Complex and Subunit II of ADH from G. suboxydans—ADH purified by DEAE-Toyopearl column was dialyzed against pH 5 buffer, then applied to a CM-Toyopearl column to separate three cytochrome c fractions (Fig. 1). The first cytochrome fraction that eluted at 40 mM buffer had CO-binding ability, according to the CO-reduced minus reduced difference spectrum (data not shown), but no ADH activity. The second fraction that eluted at 70–80 mM buffer showed a relatively high ADH activity. The third fraction that eluted at 200 mM buffer had lower ADH activity. As shown in SDS-PAGE (Fig. 2), these fractions consisted of a single peptide of 48 kDa, three bands of 78, 48, and 14 kDa, and two peptides of 78 and 14 kDa. Thus, although the principle of this separation was unclear, the ADH complex was separated into three fractions in the CM-Toyopearl column chromatography. The second fraction was the same as the reported native ADH.
complex (2), and the first and third fractions seemed to correspond to subunit II and subunit I/III complex of ADH, respectively.

CO-binding cytochrome c$_{553}$ has been purified from G. suboxydans where the cytochrome can be solubilized from the membrane with 0.2% Triton X-100 and separated from ADH by CM-cellulose column chromatography (24). Since the first cytochrome c fraction exhibited CO-binding ability and almost the same molecular weight and heme c contents as the cytochrome (see below), it seems to be similar to the CO-binding cytochrome c$_{553}$. Therefore, the immunocross-reactivity of the first cytochrome c fraction with the antibody raised against the CO-binding cytochrome c$_{553}$ was examined by immunoblotting (Fig. 2). The antibody cross-reacted at the same intensity with the cytochrome of the first fraction and also with the second subunit in ADH complex of the second fraction but not with the third fraction. Immunoblotting confirmed that the third fraction contained the subunit I present in the ADH complex (Fig. 2). Thus, it was shown that the ADH complex can be separated into subunit II, which is identical to the CO-binding cytochrome c$_{553}$, ADH complex, and subunit I/III complex by CM-Toyopearl column chromatography. The pl values of the ADH complex, subunit I/III complex, and subunits I and II were also determined by isoelectrofocusing to be 5.1, 5.3 (5.5 in the apo-form), 6.4, and 4.7, respectively.

Characterization of Subunit I/III Complex and Subunit II of G. suboxydans ADH—The contents of the prosthetic groups, POQ and heme c, in ADH complex, subunit I/III complex, and subunit II were determined (Table II), and their contents were estimated based on their relative molecular masses of 140, 92, and 48 kDa with the ADH complex, subunit I/III complex, and subunit II, respectively. The ADH and subunit I/III complexes contained about 0.6 mol of POQ per mol. Heme c was present at 3.5, 0.74 and 2.5 mol/mol of the ADH complex, subunit I/III complex, and subunit II, respectively. In addition to subunit II, as shown by heme-stained SDS-PAGE (Fig. 2), the heme c moiety was detected in subunit I but not in subunit III. The absorption spectra of these fractions are shown in Fig. 3. The ADH and subunit I/III complexes were completely reduced whereas subunit II was oxidized. ADH complex exhibited the same absorption spectrum as the purified ADH (2), having \(\alpha\), \(\beta\), and \(\gamma\) peaks of 553, 522, and 417 nm. The absorption spectra of subunit II was similar to that of CO-binding cytochrome c$_{553}$ (24), which exhibited \(\alpha\), \(\beta\), and \(\gamma\) peaks of 553, 522, and 418 nm, respectively, in the reduced state and \(\alpha\) \(\gamma\) peak of 410 nm in the oxidized form. The subunit I/III complex exhibited absorption peaks at 551, 522, and 416 nm.

Although the N-terminal amino acids of subunits I and II were blocked by some modifications and thus could not be determined, the N-terminal amino acid sequence of subunit III was determined without deblocking, to be Gln-Asp-Gln-Leu-Gly-Ala-Pro-Val-Gly.

Reconstitution of ADH Activity from the Separated Subunits—The first fraction, subunit II, did not exhibit any ADH activity, while the third fraction, subunit I/III complex, showed a relatively weak ADH activity of around 100 units/mg at pH 5.0. In contrast to the ADH complex acting at a broad pH range from acidic to neutral pH, the subunit I/III complex exhibited ADH activity only at acidic pH (Fig. 4). Notably, the subunit I/III complex showed no Q$_{1}$ reductase activity although it had ferricyanide reductase activity (Table II).

ADH complex was reconstituted from the isolated subunits by mixing subunit I/III complex and subunit II in 10 mM KPB (pH 6.0) containing 0.1% Triton X-100 and incubating it at 25°C for 20 min. ADH activities, ferricyanide reductase activities at pH 5.0 and pH 7.0, and Q$_{1}$ reductase activity at pH 5.0, of subunit I/III complex were titrated with subunit II, in which the enzyme activities were measured following holoenzyme formation with both POQ and Ca$^{2+}$. As the added subunit II was increased, ferricyanide reductase activity increased slightly at pH 5.0 and drastically at pH 7.0 and most importantly, ubiquinone reductase activity was recovered to almost the same level as that of the native ADH complex (Fig. 5). In the reconstitution experiments, the enzyme activity of the reconstituted ADH seemed to reflect that of the subunit I/III complex used. Since subunit I/III complex was so unstable that the activity was difficult to maintain constantly during storage, the activity of the reconstituted ADH varied largely among experiments even if the holoenzyme was formed with POQ (see Fig. 4 and Fig. 5). Nonetheless, the ratio between Q$_{1}$ reductase activity and ferricyanide reductase activity at pH 7.0 of the reconstituted enzyme was constant through the study. When the molar ratio was calculated based on the heme contents of the subunits where subunit I/III complex and subunit II were estimated to contain 1 and 3 mol of heme c, respectively, the activities were saturated with 0.5–10 mol of subunit II per mol of subunit I/III complex. In high performance liquid chromatography gel filtration (data not shown), the reconstituted enzyme was eluted at the same position as the native ADH. This was faster than subunit I/III complex, suggesting that subunit II binds with subunit I/III complex at an equimolar ratio to form the ADH complex. Considering that the reconstituted enzyme consisted of a one to one ratio of both subunits, it seems that the reconstituted activity can also be saturated at a ratio of roughly 1 mol of subunit II per 1 mol of subunit I/III complex. One specific ferricyanide reductase activity of the native ADH, which functions at acidic to neutral pH regions, was not functional in the reconstituted ADH (Fig. 4). This also shows the pH profiles of the ferricyanide reductase activities of the reconstituted ADH.

Construction of Hybrid ADH from Subunit I/III Complex of
G. suboxydans ADH and Subunit II of A. aceti ADH—The affinity for $Q_1$ between ADH from G. suboxydans and that from A. aceti IFO 3284 largely differs (1). Therefore, if a hybrid ADH can be prepared from the subunits of both strains, the subunit containing $Q_1$ site could be identified. Since plasmid pAA025 encodes the genes for subunits I and II, but not subunit III, of ADH of A. aceti K6033 (21), transformants harboring this plasmid may produce whole ADH complex or part of the subunits.

**TABLE II**
ADH activities and the prosthetic groups of ADH complex, subunit I/III complex, and subunit II purified from G. suboxydans

| ADH and subunits | ADH activity (units/mg) | Prosthetic group (nmol/mg) |
|------------------|-------------------------|---------------------------|
|                  | Ferricyanide at pH 5.0  | Ferricyanide at pH 7.0    | $Q_1$ at pH 5.0 | PQQ | Heme c |
| ADH complex      | 277                     | 282                       | 68.1           | 4.07 | 24.9   |
| Subunit I/III complex | 115                  | ND*                               | <0.30          | 5.78 | 8.07   |
| Subunit II       | ND                      | ND                        | <0.05          | <0.40 | 52.0 |

*ND, not detected.

**Fig. 2.** Protein and heme staining as well as immunoblotting of G. suboxydans ADH, the subunit II, and the subunit I/III complex in SDS-PAGE. ADHs were heated in SDS sample buffer with dithiothreitol (for protein staining and for immunoblotting) or without dithiothreitol (for heme staining) for 30 min at 60 °C, then applied to a SDS gel containing 12.5% acrylamide. The gels were stained for protein and heme, and also immunoblotted as described under "Experimental Procedures." Protein staining: 12, 4, and 8 μg of protein were applied on the lanes for ADH (lane 1), subunit II (lane 2), and subunit I/III complex (lane 3), respectively. M shows protein staining markers as described under "Experimental Procedures." Hemestaining: lanes 1, 2, and 3 contained 140, 100, and 40 pmol of heme c of ADH, subunit II, and subunit I/III complex, respectively. Lane M contained pre-stained markers. Immunoblotting with anti-CO-binding cytochrome $c_553$ (A) and with anti-subunit I (B); 0.38, 0.19, and 0.59 μg of protein of subunit I/III complex, subunit II, and ADH were applied to lanes 1, 2, and 3, respectively, in both A and B. Prestained markers are in lane M.

**Fig. 3.** Absorption spectra of subunit II, subunit I/III complex, and ADH purified from G. suboxydans. Triton X-100 included in ADH and subunit II was depleted as described (1). First, each spectrum (broken lines) was taken with subunit II (0.3 mg/ml), subunit I/III complex (0.58 mg/ml), and ADH (0.24 mg/ml), then taken again after adding a few grains of borohydride (solid lines).

G. suboxydans ADH and Subunit II of A. aceti ADH—The affinity for $Q_1$ between ADH from G. suboxydans and that from A. aceti IFO 3284 largely differs (1). Therefore, if a hybrid ADH can be prepared from the subunits of both strains, the subunit containing $Q_1$ site could be identified. Since plasmid pAA025 encodes the genes for subunits I and II, but not subunit III, of ADH of A. aceti K6033 (21), transformants harboring this plasmid may produce whole ADH complex or part of the subunits.
An inactive ADH has been detected and purified, and thus may be useful for the purpose described above. As shown in Fig. 6, when this plasmid was transconjugated into the ADH-deficient strain, A. pasteurianus NP2503, the transconjugant A. pasteurianus 2503C, produced whole ADH complex, probably because the mutant strain retains the ability to produce subunit I/III, but not subunits I and II. On the other hand, when the transconjugant, P. aeruginosa 3445A, was prepared with the same plasmid, the strain produced only subunit II of A. acetii ADH. Although the reason for this is not yet clear, the host strain, P. aeruginosa, may not have any genes for the ADH of acetic acid bacteria and thus subunit I of ADH encoded in the plasmid might not be produced properly without subunit III, which is not present in the plasmid. Thus, the ADH complex and subunit II of A. acetii K6033 were purified from the membranes of these transconjugants, A. pasteurianus 2503C and P. aeruginosa 3445A, respectively.

To construct a hybrid ADH, we attempted to reconstitute ADH from subunit I/III complex of G. suboxydans ADH with subunit II of A. acetii ADH, and the kinetics for Q1 reductase activity were compared with those of whole ADH complexes of A. acetii K6033 and G. suboxydans. When the subunit I/III complex was titrated with the subunit II, ADH activity was gradually increased but not saturated, even when excess subunit II was added to the subunit I/III complex (data not shown). This implies that affinity of the interaction between subunit I/III complex and subunit II from different origins is not so high. Importantly, however, Q1 reductase activity could also be reproduced in the “hybrid ADH” as well as the ferricyanide reductase activities at pH 5.0 and 7.0. Thus, kinetics of Q1 reductase activity can be compared between native complex and hybrid ADH complex (Table III). Affinity for Q1 of ADH from G. suboxydans was high (Km 32–40 μM) while that of native ADH from A. acetii was relatively low (Km 204 μM). The Km value for Q1 of the hybrid ADH (205 μM) was comparable to that of A. acetii native ADH. Thus, the results suggested that the ubiquinone-binding site of ADH is present in subunit II of ADH.

Kinetic Characterization in the Subunits of Active and Inactive ADHs—An inactive ADH has been detected and purified, separate from the active (native) enzyme, in the membranes of the cells grown on acidic media, and it has enzyme activities that are 10 times lower than those of active ADH (16). Like active ADH as described above, inactive ADH was also partially dissociated into the subunit I/III complex and subunit II. Although the subunit I/III complex from inactive ADH exhibited less ferricyanide reductase activity, it was re-activated by holoenzyme formation with PQQ and Ca2+ to the level with the subunits obtained from active ADH. Thus, the Km values for electron acceptors, ferricyanide and Q1, were determined and compared with active and inactive ADHs, and also with the subunit I/III complex derived from inactive ADH (Table IV). When ferricyanide reductase activity was measured at pH 5.0 and 7.0, active ADH exhibited two significantly distinct Km values (0.40 and 4.5 mM) at pH 7.0, whereas Km values at pH 5.0 and 7.0 were 0.47 and 4.5 mM. Two of these Km values (0.40 and 0.47 mM) seemed to be identical. On the other hand, inactive ADH exhibited only the low value (0.09 mM) at pH 5.0 and only the high value (more than 2 mM) at pH 7.0, in which the saturation curve became sigmoidal against ferricyanide concentrations so that Vmax could not be obtained. Furthermore, although subunit I/III complex exhibits ferricyanide reductase activity only at pH 5, the Km value for ferricyanide was also the same as that of ADH complex. In addition to these activities, an additional Km value for ferricyanide was detected with ADH complex at pH 3.5, in which the Km value with active ADH was below 20 μM. The value is so high that a real Km value could not be
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Table IV

| Ferricyanide | pH 3.5, II₂ | pH 5.0 | pH 7.0 |
|--------------|------------|--------|--------|
|               | Kₘ (mM)    | Vₘₐₓ  | Kₘ (mM) | Vₘₐₓ  | Kₘ (mM) | Vₘₐₓ  |
| Active ADH    | <0.020     | NE     | 0.090   | 225    | 0.404   | 543    | 0.47   | 4.47   | 725    | 0.032   | 71     |
| Inactive ADH  | ND         | 0.093  | 13.7    | nd     | nd      | >2.0   | nd     | nd     | 0.040   | 7.10   | nd     |
| Subunit I/III complex | nd      | 0.095  | 38.2    | nd     | nd      | nd     | nd     | nd     | nd     | nd     | nd     |

a. NE, not estimated.
b. ND, not determined.
c. nd, not detected.

determined from the usual steady state kinetics. Although the Kₘ value at pH 3.5 was not determined with inactive ADH, the enzyme also had this high affinity site that reacted with ferricyanide, judging from the pH profile (see Fig. 7). On the other hand, both active and inactive ADHs were measured with active (- Δ) and inactive (C) ADHs and the alkali-treated inactive ADH (●), as described under “Experimental Procedures.” Right panel, ferricyanide (ferri at pH 5 and 7) and Q₁ reductase activities were also measured with active and inactive ADHs and the alkali-treated inactive ADH (alkali inactive), as described under “Experimental Procedures.”

Electron flow of ADH from ethanol to ubiquinone was reproduced by reconstituting subunit I/III complex with subunit II, indicating that subunit I/III complex, probably subunit I, is responsible for the dehydrogenation of ethanol. By sequence homology with the methanol dehydrogenase of methylotrophs (29, 30) and the alcohol dehydrogenase of Comamonas testosteroni, as well as by the presence of a heme c-binding motif in their amino acid sequences, subunit I of ADH complex should have PQ and heme c as the prosthetic group. Actually, this study showed that subunit I/III complex contained 1 mol each of PQ and heme c and functioned as the dehydrogenase. Thus, subunit I can be classified as a quinohemoprotein ADH termed type II ADH (31), which includes ADHs from C. testosteroni (32), Pseudomonas putida (ADHs IIB and IIG; 26), and Rho-dopseudomonas acidophila (33), as well as polyvinyl alcohol dehydrogenase from Pseudomonas sp. VM15C, all of which have 1 mol each of PQ and heme c and a relative molecular mass of around 70 kDa.

Subunit II of ADH was shown to be identical to cytochrome C₅₅₃ isolated from the membranes of G. suboxydans (24), which had been thought to contain 2 mol of heme. However, the amino acid sequence of the cytochrome c deduced from the DNA sequence has suggested that there are three heme c-binding motives (11). The heme determination of the purified subunit II or ADH in this study actually showed that subunit II contained three heme c moieties. This notion has also been confirmed by redox titration with subunit II, which shows the cytochrome c behaving as three one-electron carriers. Thus it can be concluded that the ADH complex contains a total of four heme c moieties, one in subunit I and three in subunit II.

Data obtained using active and inactive ADHs and the isolated subunit I/III complex in this study indicate that these four heme c moieties in the ADH complex can be distinguished by their kinetic differences with ferricyanide, since four specific ferricyanide-reacting sites were detected. The first site functions with high affinity at acidic pH, the second with low affinity at neutral pH, the third with extremely high affinity at acidic pH, and the fourth with middle affinity over a range of pH. Since the first ferricyanide-reacting site (high affinity at acidic pH) was detected even in subunit I/III complex (Fig. 4 and Table IV), it may be located at the heme c site in subunit I and termed heme c site I (see Fig. 4). Thus, the other three ferricyanide-reacting sites should locate at or near one of the three heme c moieties in subunit II, in which the second, third, and fourth ferricyanide-reacting sites are tentatively termed.

DISCUSSION

ADH of acetic acid bacteria is a highly sophisticated enzyme complex composed of subunits I (78 kDa), II (48 kDa), and III (14 kDa). In this study, from the ADH of G. suboxydans, subunit I was isolated as a complex with subunit III, and subunit II was isolated as a free form. The subunit I/III complex exhibited ferricyanide reductase activity only at acidic pH but not Q₁ reductase activity, whereas subunit II had no activity. The
heme c sites II₁, II₂, and II₃, respectively (see Fig. 4).

Inactive ADH and also the reconstituted ADH complex may lack one of the ferricyanide-reacting sites, namely the fourth site with middle affinity working at broad pH regions, the II₃ site. One of the heme c moieties in inactive ADH remains oxidized and is not reduced with ethanol, although the individual subunits seemingly remain intact (16). Inactive ADH can be activated by alkali treatment, where, despite the Q₁ reductase activity being almost completely recovered, the fourth ferricyanide-reacting site, II₃, remained unrecovered. This is consistent with the notion that the oxidized heme c moiety of inactive ADH remains oxidized after exposure to alkali (16). Thus, these data suggested that the ubiquinone reductase activity of ADH can function properly irrespective of whether the fourth ferricyanide site works or not. Therefore, the heme c site II₃ would not be functioning in the pathway of electron transport from ethanol to ubiquinone within the ADH complex. Thus other heme c moieties (I, II₁, and II₂) should function for intra- and inter-subunit electron transport within subunits I or II. Furthermore, this study showed that inactive ADH, except for missing one ferricyanide-reacting site, kept the same Kₘ values for ferricyanide and also for Q₁ as active ADH. Although inactive ADH has an electron transfer rate of only 10% of active ADH (16), the electrons from ethanol at the PQQ site in subunit I should be effectively extracted in inactive ADH and thus even in subunit III/I complex alone, like active ADH. Thus, we speculate that in inactive ADH, an improper interaction between subunit II and subunit III/I complex impairs efficient inter-subunit electron transport in the ADH complex.

This study also showed that Q₁ reductase activity can be reproduced by reconstituting subunit II to the subunit III/I complex and furthermore, its kinetics for Q₁ in a hybrid reconstituted ADH complex reflected the feature of the original ADH from which subunit II was derived. These results indicated that the ubiquinone-reacting site of ADH is located in subunit II. The ubiquinone site would be very close to either the second or third ferricyanide-reacting sites (II₁ or II₂ site) since three heme c sites (I, II₁, and II₂) may be involved in the electron transport to ubiquinone in ADH as described above and sites II₁ and II₂ are present in subunit II. It cannot be determined at this moment, which should be the actual site or close to the ubiquinone-reacting site, because we could not obtain any evidence indicating a relationship between the ubiquinone-reacting site and the ferricyanide-reacting sites in the ADH of G. suboxydans. Thus, to understand whether the II₁ or the II₂ site is related to the ubiquinone-reacting site, we are searching for some specific inhibitors of Q₁ reductase activity and also the ferricyanide reductase activity of G. suboxydans ADH.

Thus, we speculate that electrons extracted from ethanol at the PQQ site may be transferred via heme c site I to either heme c site II₁ or II₂ in subunit II, then to the ubiquinone site, which may also be at or near either of heme c sites II₁ or II₂. If so, the physiological function of the heme c site II₃ in subunit II remains to be elucidated. The respiratory chain of G. suboxydans branches at the site of ubiquinone, with CN-sensitive terminal oxidase and -insensitive by-pass oxidase, of which the former is cytochrome o (19) and the latter may be constituted at least partly with subunit II of ADH (13, 14, 34) which may connect the quinone pool to the by-pass oxidase (15). We found that ADH can oxidize ubiquinol and the ubiquinol-ferricyanide oxidoreductase activity works at somewhere other than the ubiquinone-reacting site, but which has similar affinity to ferricyanide as the II₃ site. Thus, the II₃ site may be involved in the electron transport from ubiquinol to the CN-insensitive by-pass oxidase independent of the intramolecular electron transport from ethanol to ubiquinone.

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