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Reconstitution of the Ethanol Oxidase Respiratory Chain in Membranes of Quinoprotein Alcohol Dehydrogenase-Deficient *Gluconobacter suboxydans* subsp. α Strains

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The ethanol oxidase respiratory chain of *Gluconobacter suboxydans* was characterized by using *G. suboxydans* subsp. α, a variant species of *G. suboxydans* incapable of oxidizing ethanol. The membranes of *G. suboxydans* subsp. α exhibited neither alcohol dehydrogenase, ethanol oxidase, nor glucose-ferricyanide oxidoreductase activity. Furthermore, the respiratory chain of the organism exhibited an extremely diminished amount of cytochrome *c* and an increased sensitivity of the respiratory activity for cyanide or azide when compared with *G. suboxydans*. The first-subunit quinohemoprotein and the second-subunit cytochrome *c* of alcohol dehydrogenase complex in the membranes of *G. suboxydans* subsp. α were shown to be reduced and deficient, respectively, by using heme-staining and immunoblotting methods. Ethanol oxidase activity, lacking in *G. suboxydans* subsp. α, was entirely restored by reconstituting alcohol dehydrogenase purified from *G. suboxydans* to the membranes of *G. suboxydans* subsp. α; this also led to restoration of the cyanide or azide insensitivity and the glucose-ferricyanide oxidoreductase activity in the respiratory chain without affecting other respiratory activities such as glucose and sorbitol oxidases. Ethanol oxidase activity was also reconstituted with only the second-subunit cytochrome *c* of the enzyme complex. The results indicate that the second-subunit cytochrome *c* of the alcohol dehydrogenase complex is essential in ethanol oxidase respiratory chain and may be involved in the cyanide- or azide-insensitive respiratory chain bypass of *G. suboxydans*.

Acetic acid bacteria, which belong to two genera, *Acetobacter* and *Gluconobacter*, of the family *Acetobacteraceae*, are well known as vinegar producers. These bacteria are able to oxidize ethanol to acetic acid by two sequential catalytic reactions of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase, which are localized in the cytoplasmic membrane and are both known to be quinoproteins having pyrroloquinoline quinone as the prosthetic group (3). These quinoprotein dehydrogenases have been shown to function by linking to the respiratory chain, which transfers electrons to the final electron acceptor, oxygen, and generates a bioenergy for the growth.

Gluconobacter strains have a highly active respiratory chain, which oxidizes several sugars and sugar alcohols besides ethanol. The respiratory chain of *Gluconobacter suboxydans* has been shown to consist of a large amount of cytochrome *c*, ubiquinone, and cytochrome *o* terminal ubiquinol oxidase and also to have a cyanide-insensitive bypass (4, 5, 10). Such characteristics of the respiratory chain, i.e., high cytochrome *c* content and cyanide insensitivity, have been shown to fluctuate with the extracellular pH of the culture. The fluctuation may be caused mainly by the change of ADH components (9). ADH of *G. suboxydans* has been shown to consist of three polypeptides, of about 80, 50, and 14 kDa (2). Also, the components of ADH have recently been characterized; the first subunit is a quinohemoprotein dehydrogenase containing both pyrroloquinoline quinone and heme *c* as the prosthetic groups, and the second subunit is a diheme cytochrome *c* able to react with CO by being separated from the ADH (unpublished results). There are subspecies of *Gluconobacter* strains, named *G. suboxydans* subsp. α, which are characterized by an inability to oxidize ethanol despite having the ability to oxidize other sugars and sugar alcohols. Therefore, a knowledge of the respiratory chain in these strains seems to be useful for understanding that of all *Gluconobacter* strains.

In this communication, we show that *G. suboxydans* subsp. α lacks the second-subunit cytochrome *c* of ADH and has a greatly diminished amount of cytochrome *c* and an increased sensitivity for cyanide or azide in the respiratory activity, which is contrary to the characteristics observed in the respiratory chain of typical *Gluconobacter* strains. Furthermore, we show that ethanol oxidase activity and other respiratory characteristics deficient in *G. suboxydans* subsp. α can be restored by introducing ADH or its second subunit to the membranes of the strain.

MATERIALS AND METHODS

Materials. Ubiquinone-2 (Q_2) was kindly supplied by Eizai Co., and ubiquinol-2 (Q_2H_2) was prepared by the method of Rieske (12). Octyl-β-D-glucopyranoside (octylglucoside) was purchased from Calbiochem. The immunoblotting kit was purchased from Bio-Rad. ADH (2) and the second-subunit cytochrome *c* (11; unpublished data) were purified from the membranes of *G. suboxydans* in the presence of Triton X-100 which was depleted as follows. For ADH, the purified

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TABLE 1. Enzyme activities of ethanol, glucose, and sorbitol oxidase respiratory chains and cytochrome content in the membranes from *Gluconobacter* species

Strain	Oxidase activity[a]				Dehydrogenase activity[b]						Cytochrome content[c]	
	Ethanol	Glucose	Sorbitol	Q_2H_2	Alcohol		Glucose		Sorbitol		Cytochrome *c*	Cytochrome *o*
					PMS	FC	PMS	FC	PMS	FC		
G. suboxydans												
IFO 12528	1.16	0.78	0.39	2.91	4.17	2.52	2.41	1.01	0.45	1.40	1.42	0.27
G. oxydans												
IFO 3287	1.56	1.17	0.29	4.66	5.12	3.19	3.05	1.59	0.44	0.57	1.56	0.30
G. suboxydans subsp. α												
IFO 3254	0.00	0.77	0.55	4.47	0.03	0.01	1.27	0.07	1.85	2.16	0.18	0.23
IFO 3255	0.00	0.46	ND[d]	3.87	0.02	0.00	0.66	0.05	ND	ND	0.15	0.21
IFO 3256	0.00	0.52	ND	3.96	0.01	0.01	0.82	0.01	ND	ND	0.19	0.24
IFO 3257	0.00	0.42	0.49	3.56	0.01	0.00	0.69	0.00	1.28	1.23	0.16	0.23
IFO 3258	0.01	0.86	ND	7.18	0.04	0.04	1.27	0.08	ND	ND	0.27	0.40

[a] Oxidase activities are expressed as micromoles of substrate oxidized per minute per milligram of protein.
[b] Dehydrogenase activity was measured with PMS-DCIP (PMS) or ferricyanide (FC) as the electron acceptor and is expressed as micromoles of substrate oxidized per minute per milligram of protein.
[c] Cytochrome contents are expressed as nanomoles per milligram of protein.
[d] ND, Not determined.

enzyme dialyzed against 10 mM K phosphate buffer (pH 6.0) containing 0.1% Triton X-100 was adsorbed on a minimal volume of hydroxyapatite column equilibrated with 10 mM K phosphate buffer (pH 6.0). The column was washed with the same buffer containing 1% octylglucoside until no Triton X-100 was detected by A_{275} measurement and then washed with 3 volumes of the same buffer without the detergent. Thereafter, ADH was eluted with 150 mM K phosphate buffer (pH 6.0). Triton X-100 included in the purified second-subunit cytochrome *c* was also depleted by almost the same method as for ADH, except that a DEAE-cellulose column was used instead of hydroxyapatite and 250 mM phosphate buffer (pH 6.0) was used instead of 150 mM as the eluting buffer. Monoclonal antibody specific for the first subunit of ADH was prepared as described previously (14); preparation of polyclonal antibody raised against the second subunit of ADH will be published elsewhere. All other materials were of reagent grade and were obtained from commercial sources.

Bacterial strains, growth conditions and preparation of membranes. Seven *Gluconobacter* strains (Table 1) were used in this experiment. *G. suboxydans* subsp. α strains used are ones isolated in 1958 by Kondo and Ameyama (7), and *G. suboxydans* and *G. oxydans* are used as the typical *Gluconobacter* species (1). The organisms were grown up to the late logarithmic phase in sugar-rich medium (4). Cells were harvested by centrifugation and then washed twice with distilled water. The washed cells were resuspended in 10 mM K phosphate buffer (pH 6.0) and passed through a French pressure cell at 16,000 lb/in^2. The membranes were prepared as described previously (4) and resuspended in 50 mM K phosphate buffer (pH 6.5).

Reconstitution of ADH or the second-subunit cytochrome *c* to the membranes prepared from *G. suboxydans* subsp. α. Membranes prepared from *G. suboxydans* subsp. α IFO 3254 were resuspended in 10 mM K phosphate buffer (pH 6.0) at a protein concentration of 10 mg/ml. The membrane suspension was mixed with an ADH or the second-subunit cytochrome *c*, in which the detergent was depleted as described above, and filled up with 10 mM K phosphate buffer (pH 6.0) to 3 volumes of the membrane suspension. The mixture was incubated on ice for 30 min and then rapidly frozen in liquid nitrogen. After being thawed and sonicating twice for 5 s, the mixture was centrifuged at 80,000 rpm (Hitachi RP100AT rotor) for 60 min. The resultant precipitate was suspended in 10 mM K phosphate buffer (pH 6.0) for use as the reconstituted membranes.

Enzyme assays. Oxidase activity for ethanol, glucose, or sorbitol was measured polarographically with a Clark-type oxygen electrode in 50 mM K phosphate buffer (pH 6.0) for ethanol and glucose oxidases or in 50 mM citrate phosphate buffer (pH 4.5) for sorbitol oxidase. Q_2H_2 oxidase activity was measured spectrophotometrically (10). Alcohol, glucose, or sorbitol dehydrogenase activity was measured spectrophotometrically with phenazine methosulfate (PMS) and 2,6-dichlorophenol indophenol (DCIP) as electron acceptors (8) in 50 mM K phosphate buffer (pH 6.5). Dehydrogenase activities were also measured colorimetrically with ferricyanide as an electron acceptor (2) in 50 mM K phosphate buffer (pH 6.5) for ADH and glucose dehydrogenase or in 50 mM citrate phosphate buffer (pH 4.5) for sorbitol dehydrogenase. These dehydrogenase activities were measured in the presence of 8 mM azide to repress the reoxidation of the electron acceptors by a terminal oxidase.

Immunoblotting. Membranes were treated with 2% sodium dodecyl sulfate (SDS), SDS-polyacrylamide gel electrophoresis was performed with 12.5% polyacrylamide gels, and then protein bands were transferred onto a nitrocellulose sheet electrophoretically for 12 h at 50 mA (16). After blocking with 3% gelatin and washing, the sheet was incubated with the appropriate antibody solution for 2 h. After washing, the sheet was incubated with peroxidase-conjugated protein A and stained for peroxidase activity as specified by the supplier.

Other analytical procedures. The cytochrome *c* or *o* content was measured from reduced-minus-oxidized and CO-reduced-minus-reduced difference spectra, respectively, as described previously (4, 10). Heme staining of SDS-polyacrylamide gels was performed with a heme-catalyzed peroxidase activity (15). The protein content was determined by the modified Lowry method (6).

RESULTS

Characterization of the respiratory chain of *G. suboxydans* subsp. α strains. The membrane-bound respiratory chain of

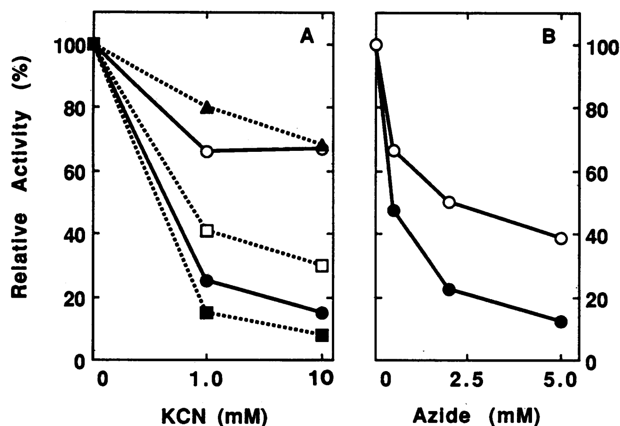

FIG. 1. Effect of cyanide on glucose oxidase activity (A) and effect of azide on sorbitol oxidase activity (B) of the membranes from *Gluconobacter* strains. Glucose oxidase activity was measured with the membranes of *G. suboxydans* (○), *G. oxydans* (▲), and three *G. suboxydans* subsp. α strains, IFO 3254 (●), IFO 3255 (■), and IFO 3258 (□); sorbitol oxidase activity was measured with the membranes of *G. suboxydans* (○) and *G. suboxydans* subsp. α IFO 3254 (●). The activities are expressed as the percent remaining activity relative to the activity without cyanide or azide.

G. suboxydans has several sugar-oxidizing systems for ethanol, acetaldehyde, glucose, sorbitol, glycerol, etc., besides an NADH oxidase system. To examine the respiratory activity of *G. suboxydans* subsp. α, we measured ethanol, glucose and sorbitol oxidase activities in comparison with those of the typical *Gluconobacter* strains, as well as Q_2H_2 oxidase, an activity for cytochrome *o* terminal oxidase (10). As shown in Table 1, it is clear that ethanol oxidase activity is deficient only in *G. suboxydans* subsp. α strains. In addition, ADH and glucose and sorbitol dehydrogenase activities measured with PMS-DCIP or ferricyanide as the electron acceptors were compared between *G. suboxydans* subsp. α strains and other *Gluconobacter* strains (Table 1). As shown in Table 1, no ADH activity was detected with either electron acceptors in any *G. suboxydans* subsp. α strains, whereas other *Gluconobacter* strains exhibited an intense ADH activity. Interestingly, glucose dehydrogenase activity measured with ferricyanide was also extremely low in *G. suboxydans* subsp. α strains, but the activity measured with PMS-DCIP was not much different from that in other *Gluconobacter* strains.

Furthermore, the respiratory chain of *G. suboxydans* subsp. α strains exhibited a critical difference in cyanide or azide sensitivity from the chain of other *Gluconobacter* strains, which is relatively insensitive to cyanide (4). As shown in Fig. 1, the glucose oxidase activity of the membranes from *G. suboxydans* subsp. α strains was much more sensitive to cyanide than was the activity of *G. suboxydans* or *G. oxydans* membranes. Also, sorbitol oxidase activity was much more sensitive against azide, which was used instead of cyanide not active at acidic pH, in *G. suboxydans* subsp. α than in *G. suboxydans*.

It has been shown that only an α absorption peak at 552 nm is seen in the reduced-minus-oxidized difference spectrum of the membranes of *G. suboxydans*, since the cytochrome *c* content is extremely high compared with cytochrome *o*, the only *b*-type cytochrome detected in the respiratory chain (4, 5), whereas the membranes of *G. suboxydans* subsp. α showed double α absorption peaks at 552 and 560 nm with an almost equivalent amount (data not shown). Thus, as summarized in Table 1, the cytochrome *c* content in the membranes from *G. suboxydans* subsp. α strains was considerably lower than that in the membranes from *G. suboxydans* or *G. oxydans*, whereas almost the same level of cytochrome *o* was detected in all *Gluconobacter* strains.

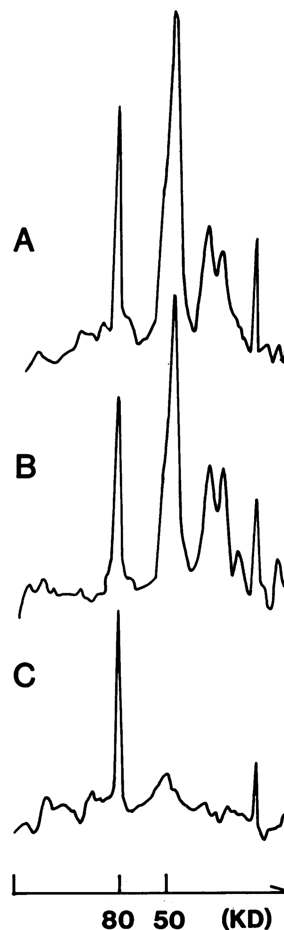

FIG. 2. Densitometric traces of heme-staining patterns in an SDS-polyacrylamide gel of the membranes from *Gluconobacter* species. The membranes prepared from *G. suboxydans* (A), *G. oxydans* (B), and *G. suboxydans* subsp. α IFO 3254 (C) were treated with 2% SDS and then applied to a 12.5% gel with protein contents of 15, 15, and 45 μg, respectively. The gels were stained by the heme staining method as described in Materials and Methods.

Difference of the cytochrome component between *G. suboxydans* subsp. α and other *Gluconobacter* strains. To examine the type of cytochrome *c* that is present at reduced levels in *G. suboxydans* subsp. α, we performed heme staining of an SDS-polyacrylamide gel with the membranes from both types of strains. As shown in Fig. 2, two major heme-staining bands, corresponding to about 80 and 50 kDa, and some minor bands are seen in the membranes from both *G. suboxydans* and *G. oxydans*. In the membranes from *G. suboxydans* subsp. α, however, the second band (50 kDa) was almost completely diminished and the first band (80 kDa) was decreased by about one-third. Since the hemoproteins of 80 and 50 kDa seemed to correspond to the first and second subunits of ADH, both of which are known to contain heme *c* (2), an immunoblotting analysis was per-

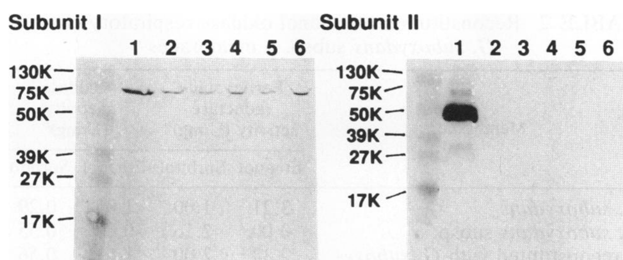

FIG. 3. Immunoblotting analysis of the membranes from several *Gluconobacter* strains with an antibody for the first (left) or second (right) subunit of ADH. Each membrane (40 μg of protein) was treated with 2% SDS, subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted. Lanes: 1, *G. suboxydans*; 2, *G. suboxydans* subsp. α IFO 3254; 3, *G. suboxydans* subsp. α IFO 3255; 4, *G. suboxydans* subsp. α IFO 3256; 5, *G. suboxydans* subsp. α IFO 3257; 6, *G. suboxydans* subsp. α IFO 3258. The left-hand lane shows prestained marker proteins (Bio-Rad).

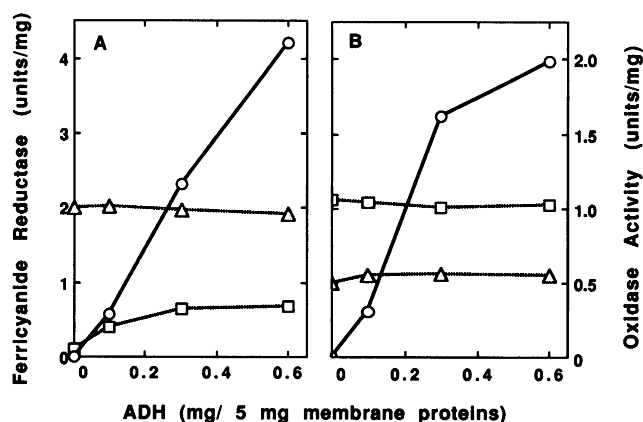

FIG. 4. Restoration of ADH to the membranes of *G. suboxydans* subsp. α. ADH purified from *G. suboxydans* was reconstituted at different concentrations with the membranes of *G. suboxydans* subsp. α as described in Materials and Methods. Ferricyanide reductase (A) and oxidase (B) activities for ethanol (○), glucose (□), and sorbitol (△) were measured as described in Materials and Methods with the reconstituted membranes.

formed by using antibodies prepared against the subunits of ADH (Fig. 3). As shown in the figure, a decreased amount of the first subunit of ADH was observed in the membranes of *G. suboxydans* subsp. α strains when compared with that in the membranes of *G. suboxydans*. On the other hand, no membranes of *G. suboxydans* subsp. α strains contained the antigen that cross-reacts with an antibody raised against the second-subunit cytochrome *c* of ADH, whereas the membrane of *G. suboxydans* did.

Restoration of ADH to the membranes of *G. suboxydans* subsp. α. As described above, data obtained in this study suggest that the membranes of *G. suboxydans* subsp. α lose the ability to oxidize ethanol and possibly other functions as a result of a defect of ADH or its component. To demonstrate the idea, therefore, we tried to reintroduce ADH into the membranes prepared from ADH-deficient strain *G. suboxydans* subsp. α IFO 3254 by several different methods as follows. (i) The membranes and ADH containing 1% octylglucoside were incubated in the presence of a final concentration of 0.67% octylglucoside and then dialyzed against buffer without any detergent. (ii) The membranes and ADH containing no detergent were incubated without any detergent and then dialyzed against buffer. (iii) After incubation, the same mixture as in method (ii) was frozen in liquid nitrogen, thawed, and sonicated. Finally, any samples in any methods were spun down to obtain the membranes binding ADH. Although all the methods had the capability of binding ADH to the membranes and to restore ethanol oxidase activity, the methods including dialysis, such as (i) and (ii), caused some inactivation of dehydrogenases, which tends to repress the restoration of ethanol oxidase activity and to decrease other oxidase activity as the control. Thus, the freeze-thaw-sonication method described in method (iii) was selected to restore ADH to the membranes of *G. suboxydans* subsp. α.

When the detergent-free ADH was restored to the membranes of *G. suboxydans* subsp. α by the method selected above (Fig. 4A), ADH was able to bind almost completely to the membranes within the range tested. As the amount of ADH bound increased, the ethanol oxidase activity increased while the glucose and sorbitol oxidase activities were unchanged (Fig. 4B). Interestingly, glucose dehydrogenase activity, when measured with ferricyanide but not with PMS-DCIP as the electron acceptor, was also restored by reintroducing ADH to the membranes of *G. suboxydans* subsp. α (Fig. 4A). Furthermore, the respiratory chains of the glucose and sorbitol oxidase systems showed a decreased sensitivity against azide in the membranes reconstituted with ADH when compared with the native membranes of *G. suboxydans* subsp. α, which are sensitive to azide or cyanide (data not shown). The insensitivity to azide of sorbitol oxidase activity was further examined with the membranes reconstituted with different amounts of ADH. As shown in Fig. 5, such an azide or cyanide insensitivity seems to be dependent on the amount of ADH bound to the membranes.

Since the membranes of *G. suboxydans* subsp. α contain smaller but significant amounts of the first subunit of ADH (Fig. 3), reconstitution of the ethanol oxidase respiratory chain was also examined by using the second-subunit cytochrome *c* of ADH (Fig. 6). As shown in the figure, ADH activity was also reconstituted with only the second subunit

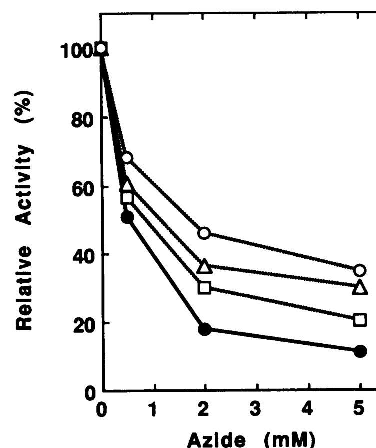

FIG. 5. Effect of azide on sorbitol oxidase activity in *G. suboxydans* subsp. α membranes reconstituted with the different amounts of ADH. The native membranes (●) (no ADH activity) and the reconstituted membranes with different ADH activities of 1.4 (□), 4.2 (△), and 7.0 (○) U/mg were used for this experiment.

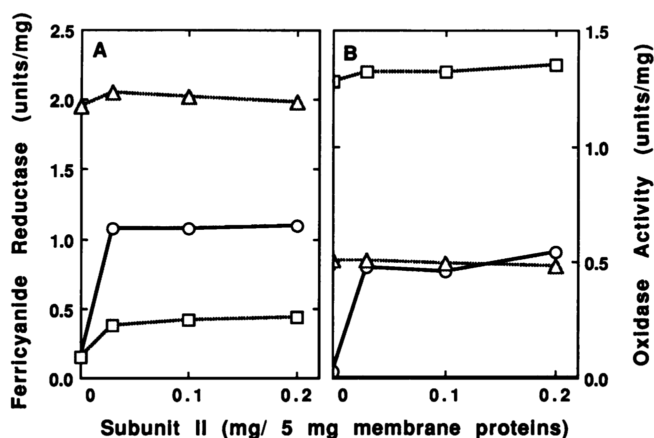

FIG. 6. Restoration of the second-subunit cytochrome *c* of ADH to the membranes of *G. suboxydans* subsp. α. ADH second subunit purified from *G. suboxydans* was reconstituted at different concentrations with the membranes of *G. suboxydans* subsp. α as described in Materials and Methods. Ferricyanide reductase (A) and oxidase (B) activities for ethanol (○), glucose (□), and sorbitol (△) were measured as described in Materials and Methods with the reconstituted membranes.

TABLE 2. Reconstitution of ethanol oxidase respiratory chain in *G. suboxydans* subsp. α membranes

Membrane[a]	Ferricyanide reductase activity (U/mg)[b]		Oxidase activity (U/mg)[b]	
	Ethanol	Sorbitol	Ethanol	Sorbitol
G. suboxydans	3.21	1.00	1.05	0.29
G. suboxydans subsp. α	0.00	2.16	0.00	0.55
reconstituted with *G. suboxydans* ADH	2.32	2.00	1.61	0.56
reconstituted with subunit II	0.74	2.48	0.31	0.53
reconstituted with *A. aceti* ADH	2.05	2.10	1.59	0.53

[a] The membranes of *G. suboxydans* subsp. α (5 mg of protein) were reconstituted, as described in Materials and Methods, with ADH (0.6 mg of protein) purified from *G. suboxydans*, with the second-subunit cytochrome *c* (0.2 mg of protein) purified from *G. suboxydans* (subunit II), and with ADH (0.6 mg of protein) purified from *A. aceti*.

[b] Ferricyanide reductase and oxidase activities for ethanol and sorbitol were measured with the native membranes of *G. suboxydans* and *G. suboxydans* subsp. α and the reconstituted membranes of *G. suboxydans* subsp. α as described.

of ADH, which is probably due to complex formation of the added second subunit with the first subunit present in the membranes. In this case, however, added ADH activity was saturated at a relatively low level, probably because of the limited amount of the first subunit present in the membranes (see Fig. 3). The restoration of the second subunit to the membranes also restored ethanol oxidase and glucose-ferricyanide oxidoreductase activities to a lesser degree compared with that of the ADH complex. However, the azide or cyanide insensitivity in the oxidase activity was not clearly increased by the reconstitution with the second subunit (data not shown). Because *Acetobacter aceti*, an acetic acid bacterium that is different taxonomically from *Gluconobacter*, has the same kind of ADH but a cyanide-sensitive respiratory chain, the reconstitution was performed with *A. aceti* ADH as a control. When ADH of *A. aceti* was reintroduced into the membranes of *G. suboxydans* subsp. α, the reconstituted membranes exhibited a fairly high ethanol oxidase activity (Table 2) but showed no increase of insensitivity against azide or cyanide (data not shown).

DISCUSSION

The present study has shown that all strains of *G. suboxydans* subsp. α have a characteristic common feature in the respiratory chain, which is summarized as follows. (i) ADH and ethanol oxidase activities are extremely low or deficient, (ii) the cytochrome *c* content is greatly reduced, (iii) the respiratory activity is sensitive to azide or cyanide, and (iv) the first and second subunits of ADH are reduced and deficient, respectively. These characteristics are totally different from those of other *Gluconobacter* species. Since ADH contains three heme *c* moieties per mol and is present in relatively large amounts in the membranes (2), lack of ADH in the membranes is expected to result in a decrease in the cytochrome *c* content as well as a decrease in ethanol oxidase activity in the membranes of *G. suboxydans* subsp. α. Furthermore, since cyanide insensitivity of the respiratory activity is known to be diminished by removing cytochromes *c* or ADH from the membranes of *G. suboxydans* (4), it is reasonable that the respiratory chain of ADH-deficient *G. suboxydans* subsp. α is sensitive to azide or cyanide. In addition, it is not surprising that *G. suboxydans* subsp. α exhibits little glucose-ferricyanide oxidoreductase activity, since the activity has been shown to be diminished by removing ADH from the membranes of *G. suboxydans* and to be restored by adding ADH to the depleted membranes (13). Therefore, almost all aspects of the features of the *G. suboxydans* subsp. α respiratory chain could be explained by the lack of ADH in the membranes; this has been strongly supported by the present experiments, which show that both ethanol oxidase and glucose-ferricyanide oxidoreductase activities can be restored by the restoration of *G. suboxydans* ADH to the membranes of *G. suboxydans* subsp. α.

Another finding in this study is that ethanol oxidase respiratory chain can be reconstituted in the membranes of *G. suboxydans* subsp. α with the second-subunit cytochrome *c* of ADH as well as with the ADH complex. Since *G. suboxydans* subsp. α membranes contain less of the first-subunit dehydrogenase component of ADH, although the amount they contain is still significant (Fig. 3), it is reasonable to conclude that the second-subunit cytochrome *c* is essential for the ethanol oxidase respiratory chain as well as for ADH activity.

It has been shown here that an azide- or cyanide-insensitive respiratory bypass can also be restored in the respiratory chain of *G. suboxydans* subsp. α, which is cyanide sensitive, by binding of ADH purified from *G. suboxydans* to the membranes. This may be intensified by the findings that ADH purified from *A. aceti* was unable to increase the azide insensitivity of the respiratory activity, despite being able to restore an intensive ethanol oxidase activity to *G. suboxydans* subsp. α membranes. The respiratory chain of *A. aceti* has been shown to be sensitive to cyanide (unpublished data); this is totally different from the situation for *G. suboxydans*. Furthermore, the second-subunit cytochromes *c* of ADH between *A. aceti* and *G. suboxydans* are distinct from each other, as shown by immuno-cross-reactivity and by heme *c* content between both subunits (unpublished data). Thus, it is reasonable that ADH of *A. aceti* does not have any ability to make such an azide-insensitive bypass.

The membranes in which the second subunit of *G. suboxydans* ADH was restored could not exhibit any clear azide insensitivity of the respiratory chain. This is probably because the restored ADH activity is too low to exhibit the azide insensitivity, since an azide or cyanide insensitivity may be largely dependent on the level of ADH activity (Fig. 6). In *G. suboxydans*, the amount of the second-subunit cytochrome *c* has been shown to be increased, concomitant with the increase of cyanide-insensitivity, by lowering of the extracellular pH (9). The data presented here, taken together with the previous findings (4, 9), have suggested that the second-subunit cytochrome *c* of ADH complex may be involved in the pathway of the azide- or cyanide-insensitive respiratory chain bypass of *Gluconobacter* species, although the exact components and the character of the terminal oxidase involved in the respiratory bypass of the organisms remain unclear.

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REFERENCES

1. **Adachi, O., K. Tayama, E. Shinagawa, K. Matsushita, and M. Ameyama.** 1978. Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans*. Agric. Biol. Chem. **42**:2045–2056.
2. **Ameyama, M., and O. Adachi.** 1982. Alcohol dehydrogenase from acetic acid bacteria, membrane-bound. Methods Enzymol. **89**:450–457.
3. **Ameyama, M., K. Matsushita, Y. Ohno, E. Shinagawa, and O. Adachi.** 1981. Existence of a novel prosthetic group, PQQ, in membrane-bound, electron transport chain-linked, primary dehydrogenases of oxidative bacteria. FEBS Lett. **130**:179–183.
4. **Ameyama, M., K. Matsushita, E. Shinagawa, and O. Adachi.** 1987. Sugar-oxidizing respiratory chain of *Gluconobacter suboxydans*. Evidence for a branched respiratory chain and characterization of respiratory chain-linked cytochromes. Agric. Biol. Chem. **51**:2943–2950.
5. **Daniel, R. M.** 1970. The electron transport system of *Acetobacter suboxydans* with particular reference to cytochrome *o*. Biochim. Biophys. Acta **216**:328–341.
6. **Dulley, J. R., and P. A. Grieve.** 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal. Biochem. **64**:136–141.
7. **Kondo, K., and M. Ameyama.** 1958. Carbohydrate metabolism by *Acetobacter* species. I. Oxidative activity for various carbohydrates. Bull. Agric. Chem. Soc. Jpn. **22**:369–372.
8. **Matsushita, K., and M. Ameyama.** 1982. D-Glucose dehydrogenase from *Pseudomonas fluorescens*, membrane-bound. Methods Enzymol. **89**:149–154.
9. **Matsushita, K., Y. Nagatani, E. Shinagawa, O. Adachi, and M. Ameyama.** 1989. Effect of extracellular pH on the respiratory chain and energetics of *Gluconobacter suboxydans*. Agric. Biol. Chem. **53**:2895–2902.
10. **Matsushita, K., E. Shinagawa, O. Adachi, and M. Ameyama.** 1987. Purification, characterization and reconstitution of cytochrome *o*-type oxidase from *Gluconobacter suboxydans*. Biochim. Biophys. Acta **894**:304–312.
11. **Matsushita, K., K. Tayama, E. Shinagawa, O. Adachi, and M. Ameyama.** 1981. Purification and characterization of CO-binding cytochrome *c*-553 from the membrane of *Gluconobacter suboxydans*. FEMS Microbiol. Lett. **10**:267–270.
12. **Rieske, J. S.** 1967. Preparation and properties of reduced coenzyme Q-cytochrome *c* reductase (complex III of the respiratory chain). Methods Enzymol. **10**:239–245.
13. **Shinagawa, E., K. Matsushita, O. Adachi, and M. Ameyama.** 1990. Evidence for electron transfer between quinoproteins D-glucose dehydrogenase and alcohol dehydrogenase in *Gluconobacter suboxydans*. J. Biochem. **107**:863–867.
14. **Shinagawa, E., K. Matsushita, T. Inoue, O. Adachi, and M. Ameyama.** 1989. Monoclonal antibody recognizing the quinoprotein subunit of alcohol dehydrogenase complex from *Gluconobacter* species. Agric. Biol. Chem. **53**:2011–2012.
15. **Thomas, P. E., D. Ryan, and W. Levin.** 1976. An improved staining procedure for the detection of the peroxidase activity of cytochrome *P*-450 on sodium dodecyl sulfate polyacrylamide gels. Anal. Biochem. **75**:168–176.
16. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.