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Purification and Characterization of Membrane-bound Quinoprotein Quinate Dehydrogenase

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Several bacterial strains carrying quinoprotein quinate dehydrogenase (QDH) were screened through acetic acid bacteria and other bacteria. Strong enzyme activity was found in the membrane fraction of *Gluconobacter melanogenus* IFO 3294, *G. oxydans* IFO 3292, *G. oxydans* IFO 3244, and some strains of *Acinetobacter calcoaceticus*. Interestingly, in the membrane fraction of *A. calcoaceticus* AC3, which is unable to produce pyrroloquinoline quinone (PQQ), fairly large amounts of apo-QDH were formed, and were converted to holo-QDH only by the addition of PQQ. It was difficult to detach PQQ from the holo-QDH by EDTA treatment, and EDTA treatment with apo-QDH prior to PQQ addition gave no significant holo-QDH. For QDH purification, *Gluconobacter* strains were not suitable due to the presence of huge amounts of quinohemoprotein alcohol dehydrogenase (ADH) in the same membrane, which was co-solubilized with QDH and disturbed purification of QDH. Purification of holo-QDH was done with *Acinetobacter* sp. SA1 instead, which contained no ADH. Apo-QDH was purified from *A. aclcoaceticus* AC3.

This is the first report dealing with QDH purification, and two different criteria of QDH purification were given. A combination of two steps using butyl-Toyopearl and hydroxyapatite columns gave a highly purified holo-QDH which was monodispersed and showed enough purity, though the specific activity did not increase as much as expected. When QDH purification was done with *A. calcoaceticus* AC3 in the absence of PQQ, purified apo-QDH appeared to be a dimer, which was converted to the monomer on addition of PQQ. Since QDH was highly hydrophobic, one-step chromatography on a DEAE-Sepharose column was tried. Purified holo-QDH of higher specific activity was obtained with a higher yield. The molecular mass of QDH was estimated to be 88 kDa. There was no characteristic absorption spectrum with the purified QDH except for a small bump around 420 nm. QDH oxidized only quinate and shikimate so far examined. The optimal QDH activity was found at pH 6–7 when assayed with artificial electron acceptors. QDH was formed in the presence or absence of quinate in the culture medium, although stronger induction was usually observed in the presence of quinate.

Key words: acetic acid bacteria; *Acinetobacter calcoaceticus*; pyrroloquinoline quinone (PQQ); quinoprotein; quinate dehydrogenase

Quinate utilization by microorganism is an important index in the systematic determinative bacteriology for *Pseudomonas* and many other aerobic bacteria.[1–4] 3-Dehydroquinate dehydratase (synonymous with 5-dehydroquinase, EC 4.2.1.10) was isolated from cell extract of *Escherichia coli*.[5] The presence of quinate 5-dehydrogenase (synonymous with NAD(P)-dependent quinate dehydrogenase, EC 1.1.1.24) and shikimate 5-dehydrogenase (synonymous with 5-dehydroshikimate reductase, EC 1.1.1.25) were shown in *Aerobacter aerogenes*,[6] and 5-dehydroshikimate reductase in *E. coli*.[7] These enzymes were also indicated from plant origins in a series of aromatic biosyntheses.[8–11] In 1967, the first and sole report on a quinate oxidizing enzyme produced by acetic acid bacteria was made by Whiting and Coggins.[12] They pointed out quinate oxidation to 5-dehydroquinate by an NAD(P)-independent quinate dehydrogenase (QDH) (EC 1.1.99.25) and D-shikimate oxidation to 3-dehydroshikimate by an enzyme associated with the particulate enzyme in the cytoplasmic membrane. van Kleef and Duine[13] indi-

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Abbreviations: ADH, quinoprotein alcohol dehydrogenase; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; GDH, quinoprotein glucose dehydrogenase; $\alpha\beta$-OG, α,β-octyl-D-glucopyranoside; β-OG, β-octyl-D-glucopyranoside; OT, β-octyl-thio-D-glucopyranoside; PMSF, phenylmethanesulfonyl fluoride; PQQ, Pyrroloquinoline quinone; QDH, quinoprotein quinate dehydrogenase; TX, Triton X-100; Z3-12, Zwittergent

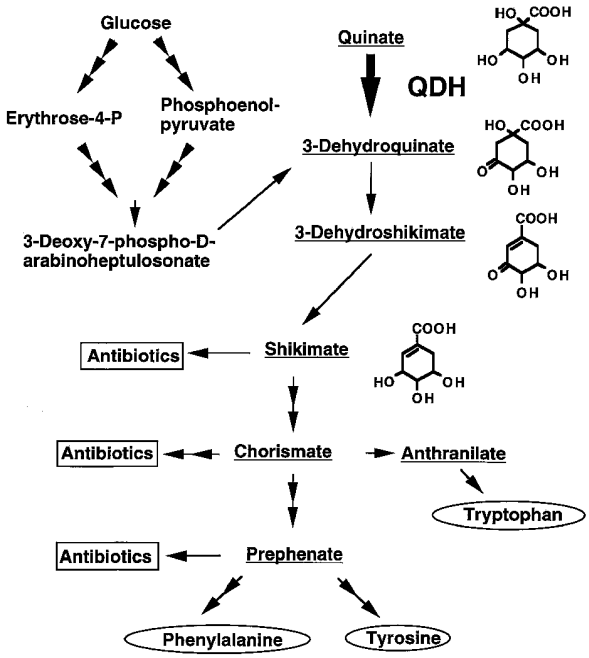

Fig. 1. Entrance to the Shikimate Pathway from Quinate.
Quinoprotein quinate dehydrogenase (QDH) is shown by a fat arrow.

cated the occurrence of QDH in the periplasm of *Acinetobacter calcoaceticus* LMD 79.41 and suggested that QDH is a quinoprotein in which pyrroloquinoline quinone (PQQ) is involved in the reaction. However QDH purification was not successful due to the hydrophobicity of the enzyme. Elsemore and Ornston did a genetic analysis of protocatechuate catabolism[14] and claimed that the consecutive action of three genes, *quiA* encoding quinate dehydrogenase, *quiB* for 3-dehydroquinate dehydratase, and *quiC* for 3-dehydroshikimate dehydratase, are required in the catabolism of quinate to protocatechuate.[15]

Quinate is oxidized to 3-dehydroquinate, which can be linked to 3-dehydroshikimate and then to shikimate in the shikimate pathway. Since shikimate is remote from D-glucose in the shikimate pathway, reports regarding microbial production of the intermediates in the pathway are scarce. However, it is a subject worth to challenge, because most of the intermediates would be useful for the salvage synthesis of many kinds of antibiotics, readily degradable herbcides and pesticides, and aromatic amino acids. If shikimate or any intermediates in the shikimate pathway become available as commodity chemicals from quinate, progress in pharmaceutical sciences and agricultural applications would be much developed (Fig. 1), although production of aromatic amino acids that are related to the shikimate pathway has been developed enzymatically using the terminal parts of the pathway. Synthesis of L-tryptophan by L-tryptophan synthase or by a reverse reaction of L-tryptophanase and L-tyrosine by L-tyrosine phenol lyase from phenol, ammonia, and pyruvate, are typical examples.[16,17] Cinchona was used for production of quinine hydrochloride, a potent anti-malarial agent, and quinate came out as a byproduct in quinine-hydrochloride manufacturing. In order to develop the intermediates in the shikimate pathway, quinate dehydrogenase, the first enzyme leading to the pathway from quinate must be characterized. Purification and characterization of QDH were done with acetic acid bacteria and other oxidative bacteria.

Materials and Methods

Chemicals. NAD, NADP, NADH, NADPH, and yeast extract were kind gifts from Oriental Yeast Co. (Tokyo, Japan). Mydol 10 and Mydol 12 were donated by Kao, Co. (Tokyo, Japan). Pyrroloquinoline quinone (PQQ) used was our own product.[18] Other chemicals used were from commercial sources of guaranteed grade unless otherwise stated.

Microorganisms and culture conditions. *Gluconobacter* strains were a kind donation from the Institute for Fermentation, Osaka (IFO). *Acinetobacter calcoaceticus* LMD 79.41 was generously given to us by J. A. Duine, Delft University of Technology. *A. calcoaceticus* AC3 (PQQ^- strain) was the same strain used in the previous study on quinoprotein glucose dehydrogenase, which cannot produce PQQ.[19] *A. calcoaceticus* SA1 and other *Acinetobacter* strains were isolated from urine, soil, fruits, and flowers in Thailand by enrichment culture on quinate. The culture medium consisted of 2 g of sodium quinate, 1 g of yeast extract, and 1 g of Polypepton in 1 liter of tap water. The pH of the medium was adjusted to 7.0. From the culture medium for *A. calcoaceticus* AC3, which produces apo-QDH, quinate was omitted. Microorganisms were grown in 100 ml of the medium in a 500-ml side-armed Erlenmeyer flask at 30°C, with shaking. The bacterial growth was measured by a Klett-Summerson phoptoelectric colorimeter with a red filter. For the cell mass preparation, a seed culture in 100 ml of the medium in a 500-ml Erlenmeyer flask was transferred to 5 liters of fresh medium in a 10-L table top fermentor (MD500-10L, Marubishi Bioengineering, Co., Tokyo, Japan) and cultivated for another 12 hr under vigorous aeration. About 20 g of wet cells was usually harvested under these culture conditions.

Preparation of membrane fraction and cytoplasmic fraction. Cell suspension was prepared by homogenizing the freshly harvested cells at a ratio of about 10 g wet cells per 10 ml of 10 mM potassium phosphate buffer (KPB), pH 7.5. A trace amount of RNase and DNase were added before cell disruption. A small volume of cell suspension, less than 40 ml,

was treated by passing twice through a French pressure cell press (SIM AMINCO, Spectronic Instruments, Inc., Rochester, NY, USA) at 1,000 kg/cm^2. A large volume of cell suspension was passed through a Rannie high pressure laboratory homogenizer (Rannie model Mini-Lab, type 8.30H, Wilmington, MA, USA) at 1,000 psi. After removal of intact cells by a conventional low speed centrifuge, the crude cell-free extract was further centrifuged at 68,000 × *g* for 90 min to isolate the membrane fraction from the cytoplasmic soluble fraction. The membrane fraction was washed by resuspending and homogenizing the precipitate in a glass homogenizer with the same buffer.

Assays of enzyme activity. QDH activity was measured with potassium ferricyanide or a combination of phenazine methosulfate (PMS) and 2,6-dichlorophenol indophenol (DCIP) as the electron acceptors. In the case of apo-QDH, the membrane suspension or enzyme solution prepared from *A. calcoaceticus* AC3 was incubated with 5 μM of PQQ and 5 mM of $CaCl_2$ for 20 min at room temperature before starting the enzyme reaction by the addition of electron acceptors. The reaction mixture contained 0.8 ml of McIlvaine buffer, pH 6.5, 5 μmol of sodium quinate, and enzyme solution, and the total volume was adjusted to 0.9 ml with distilled water. After incubation for 5 min at 25°C, the reaction was started by the addition of 10 μmol (0.1 ml) of potassium ferricyanide. The reaction was stopped by adding 0.5 ml of ferric-Dupanol reagent.[20] After this was left standing for a further 20 min, 3.5 ml of distilled water was added and the optical density of the reaction mixture at 660 nm was measured. An alternative enzyme assay with PMS-DCIP was done essentially in the same manner as that for quinoprotein glucose dehydrogenase (GDH) from aerobic bacteria.[21–23] One unit of enzyme activity was defined as the amount of enzyme catalyzing 1.0 μmol of substrate oxidation per min under these conditions. Protein content was measured by a modified Lowry method with bovine serum albumin as the standard protein.[24] The specific activity was defined as units of enzyme activity per mg of protein.

EDTA-treatment of the membrane fraction. The membrane suspension (1 ml containing 10 mg of protein in 10 mM KPB, pH 6.5) was mixed with EDTA of which the final concentration was adjusted to 20 mM. After this was left standing for 30 min in an ice bath, 30 ml of fresh buffer was added and EDTA in the membrane suspension was removed by ultracentrifugation at 90.000 × *g* for 60 min. The precipitate was resuspended by a glass homogenizer with an excess of the same fresh buffer (30 ml) to wash EDTA out from the membrane fraction, and then followed by ultracentrifugation again under the same conditions. The resulting precipitate was resuspended with a small amount of the same fresh buffer and the protein concentration was adjusted to 10 mg per ml. The final EDTA concentration in the membrane suspension was reduced to less than 0.01 mM after twice ultracentrifuging. Under the usual conditions, PQQ and Ca^{2+} were added to 5 μM and 5 mM, respectively, and incubated for 30 min at 25°C before the start of the enzyme reaction.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done on a 12.5% (w/v) slab gel by the methods described by Laemmli.[25] Before application, samples were treated with 6% (w/v) SDS and 0.1 mM dithiothreitol at 60°C for 30 min. The following calibration proteins (Bio-Rad, Hercules, CA, U.S.A.) with the indicated molecular masses were used as references: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.1 kDa), and lysozyme (14.4 kDa). Protein bands were stained with Coomassie brilliant blue.

Results and Discussion

Occurrence of QDH in aerobic bacteria

Bacterial strains from the genera of *Gluconobacter* and *Acinetobacter* were tested for QDH activity when grown on a medium containing quinate (0.2%). *A. calcoaceticus* AC3 (PQQ^- strain) was grown on a medium without quinate. A strong QDH activity was found in the cell-free extracts of *G. melanogenus* IFO 3294 with the specific acitivity of 0.40 units/mg protein, *G. oxydans* IFO 3292 with 0.5 of specific activity, and *G. oxydans* IFO 3244 with 0.7 of specific activity, as shown in Table 1. No reasonable enzyme activity was observed with the other *Gluconobacter* strains of which the specific activity was less than 0.01, so far examined. The specific activity of 0.03–0.09 was observed with the cell-free extracts from most of *Acinetobacter* strains grown on a medium without quinate. Addition of quinate to the culture medium facilitated QDH formation and the specific activity was increased by several times. With the cell-free extract of *A. calcoaceticus* AC3, no enzyme activity was observed but conversion of apo-QDH to holo-QDH was observed upon the exogenous addition of PQQ, and the specific activity increased to nearly 0.1. QDH was localized in the membrane fraction and scarce enzyme activity was found in the cytoplasmic fraction, when the cell-free extract was fractionated into two parts. A very weak enzyme activity of NAD-dependent QDH was detected at pH 9.5–11.0 with all cytoplasmic fractions of bacteria tested. However, the NAD-dependent cytosolic enzyme has no contributions to the oxidative fermentation, as similarly shown with other cases.[26] As mentioned below, purified QDH is alike to

Table 1. Screening of QDH Activity

All strains were cultivated in a medium containing 0.2% quinate for 24 hr at 30°C. Cell free extract was used as the enzyme solution and enzyme activity was measured by the ferricyanide reductase method.

Strain	QDH activity (U/mg)	Strain	QDH activity (U/mg)
Gluconobacter asaii IFO 3265	<0.01	*G. oxydans* IFO 14819	n.d.
G. albidus IFO 3253	<0.01	*G. sphaericus* IFO 12467	n.d.
G. asaii IFO 3275	<0.01	*G. suboxydans* IFO 3130	n.d.
G. asaii IFO 3276	<0.01	*G. suboxydans* IFO 3172	n.d.
G. cerinus IFO 3264	<0.01	*G. suboxydans* IFO 3290	n.d.
G. cerinus IFO 3268	<0.01	*G. suboxydans* IFO 3289	n.d.
G. cerinus IFO 3262	<0.01	*G. suboxydans* IFO 12528	n.d.
G. cerinus IFO 3270	<0.01	*G. suboxydans* var. αIFO 3254	n.d.
G. dioxyacetonicus IFO 3271	n.d.	*G. suboxydans* var. αIFO 3255	n.d.
G. dioxyacetonicus IFO 3272	n.d.	*G. suboxydans* var. αIFO 3256	n.d.
G. frateurii IFO 3251	n.d.	*G. suboxydans* var. αIFO 3257	n.d.
G. frateurii IFO 3286	n.d.	*G. suboxydans* var. αIFO 3258	n.d.
G. frateurii IFO 3264	n.d.		
G. frateurii CHM 16	n.d.	*Acinetobacter calcoaceticus* LMD 79.41	0.26
G. frateurii CHM 54	n.d.	*A. calcoaceticus* AC3*	0.08
G. gluconicus IFO 3285	n.d.	*Acinetobacter* sp. SA1**	0.09
G. industrius IFO 3260-1	n.d.	*Acinetobacter* sp. SA4**	0.03
G. industrius IFO 3260-2	n.d.	*Acinetobacter* sp. SA5**	0.04
G. industrius IFO 3261	n.d.	*Acinetobacter* sp. SA6**	0.04
G. melanogenus IFO 3294	0.39	*Acinetobacter* sp. SA7**	0.06
G. oxydans IFO 3990	n.d.	*Acinetobacter* sp. SA8**	0.01
G. oxydans IFO 3189	n.d.	*Acinetobacter* sp. SA9**	0.05
G. oxydans IFO 3287	n.d.	*Acinetobacter* sp. SA10**	0.05
G. oxydans IFO 3292	0.54	*Acinetobacter* sp. SA11**	0.09
G. oxydans ATCC 621	n.d.	*Acinetobacter* sp. SA12**	0.06
G. oxydans IFO 3244	0.66	*Acinetobacter* sp. SA13**	0.05

n.d.: not detected.

*: PQQ-mutant. QDH activity was measured by the addition of 5 μM PQQ and 5 mM $CaCl_2$ at 25°C for 30 min.

**: These strains were isolated in Thailand and cultivated in the medium containing 1.0% polypeptone.

the membrane-bound quinoprotein glucose dehydrogenase (GDH) in molecular properties and absorption spectra. However, QDH is distinct from GDH. Because GDH is very strong and thus a representative membrane-bound enzyme among *Gluconobacter* strains, and the specific activity assayed with cell free extract is 10 times higher than those of QDH.[27] Purified QDH did not oxidize glucose at all. In the cell free extracts from *Gluconobacter* strains, a high amount of ADH coexisted with QDH, suggesting there will be difficulty in isolating pure QDH while excluding ADH. After many disappointing results in QDH separation from ADH with *Gluconobacter* strains, in the following experiments for QDH characterization, *Acinetobacter* sp. SA1 and *A. calcoaceticus* AC3 were used preferentially.

Titration of apo-QDH with PQQ

A cell free extract of *A. calcoaceticus* AC3 containing apo-QDH was titrated with PQQ to guess the ultimate specific activity in the native membrane. Although the data are not shown, it gave a typical saturation curve like a plot of the initial velocity of a simple Michaelis-Menten reaction versus PQQ concentration. Purified apo-QDH must give the same results, if enzyme purification was successfully done without any partial denaturation in the purification steps. However, due to the strong hydrophobicity of QDH as mentioned below, there was some difficulty in obtaining the intact apo-QDH as it is in the native cytoplasmic membrane in spite of our best efforts. Furthermore, some part of such disrupted membrane must exist as so-called inside-out membrane vesicles, preventing the exact measurement of absolute content of QDH in the membrane. QDH must have its highest specific activity at more than 20 units/mg of protein as judged from the titration experiment, if the inside-out membrane vesicles make up 20–30% of the total membrane vesicles and the molecular mass of QDH is assumed to be 88 kDa as predicted from genetic information.[14] An apparent binding constant for PQQ to apo-QDH was roughly estimated to be 100–350 nM, though it was largely depend on the status of the membrane. The apparent binding constant was comparable to that of the membrane-bound apo-GDH.[19]

Unlike other membrane-bound polyol dehydrogenases in acetic acid bacteria, most of which are readily inhibited by chelation of Ca^{2+} with EDTA, allowing release of PQQ, holo-QDH in the membrane fraction of *Gluconobacter* and *Acinetobacter* strains was quite resistant to EDTA

treatment. More than 90% of the original QDH activity was maintained after EDTA treatment. This means that holo-QDH in the native membrane fraction may be kept stable through unusually strong binding of PQQ to QDH protein via Ca^{2+}. It was interesting to see that, in contrast to the above observations, if apo-QDH in the membrane fraction of *A. calcoaceticus* AC3 (PQQ^- strain) was treated with EDTA first, no reasonable holo-QDH was formed even though the PQQ concentration was increased in the presence of 5 mM Ca^{2+}. If EDTA treatment was done after the addition of PQQ to the mambrane fraction of *A. calcoaceticus* AC3, QDH was converted to holo-QDH and no clear QDH resolution was given. These results suggest the importance of Ca^{2+} in the holo-QDH formation.

QDH Purification I

A. calcoaceticus SA1 and *A. calcoaceticus* AC3 were used for QDH purification of holo-QDH and apo-QDH, respectively. Purification of two different forms of QDH was done under the same conditions. The membrane fraction was homogenized and the protein content adjusted to 10 mg/ml of 10 mM KPB, pH 7.0. To the membrane suspension, Mydol 10 and KCl were added to 1% (v/v) and 0.1 M, respectively. The solution was left at room temperature for 30 min to solubilize QDH. The enzyme solution was centrifuged at 20°C for 90 min. The resulting supernatant was designated as the solubilized enzyme. To the solubilized enzyme, ammonium sulfate was added to 2.5 M and the pH adjusted to 7.0 with ammonia water. The enzyme solution was adsorbed onto a butyl-Toyopearl column (1.5 × 10 cm) which had been equilibrated with the same buffer containing 2.5 M ammonium sulfate. After the column was washed with the same buffer containing ammonium sulfate, elution of QDH was done by a gradient system between 10 mM KPB, pH 7.0, containing 2.5 M ammonium sulfate and the same KPB containing 1.0% Mydol 10. As shown in Fig. 2, there was a clear difference about the eluted positions between holo-QDH and apo-QDH. Holo-QDH was eluted at the position where ammonium sulfate decreased to 1.0 M and Mydol 10 increased to 0.5%, while apo-QDH was not detached from the butyl-Toyopearl column until ammonium sulfate was finally decreased and the buffer solution was replaced with 1% Mydol 10. These results show that holo-QDH was much more hydrophilic than apo-QDH. In fact, as shown in Fig. 3, holo-QDH was eluted from a hydroxyapatite column at 250 mM KPB, pH 7.0, in the presence of 0.1% Mydol 10, whereas apo-QDH was not eluted from the column until KPB was increased to 1.0 M. The QDH fraction was dialyzed and concentrated by polyethylene glycol 6000. Due to the strong hydrophobicity of QDH, the specific activity of QDH did not increase through the purification, although

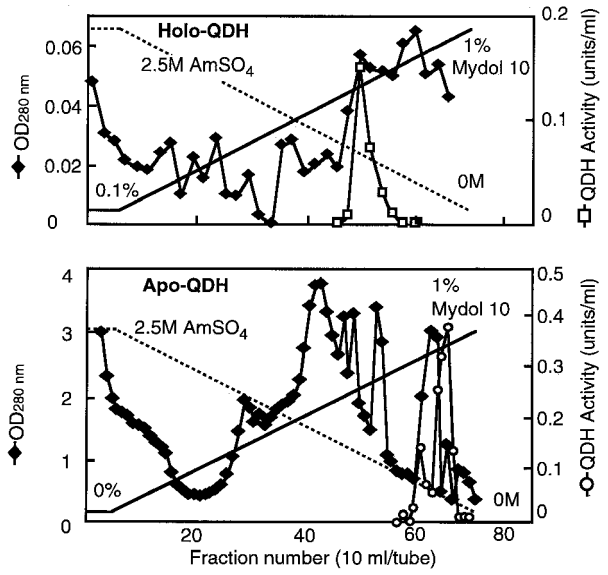

Fig. 2. Butyl-Toyopearl Chromatography of Holo-QDH (upper frame) and Apo-QDH (lower frame).

To both QDH solutions, ammonium sulfate ($AmSO_4$) was added to 2.5 M and the pH was adjusted to 7.0 with ammonia water. The enzyme solution was adsorbed onto two columns of butyl-Toyopearl (1.5 × 10 cm) which had been equilibrated with the same buffer containing 2.5 M $AmSO_4$. After the column was washed with the same buffer containing $AmSO_4$, elution of both QDHs was done by a linear gradient between 10 mM KPB, pH 7.0, containing 2.5 M $AmSO_4$ against the same KPB containing 1.0% Mydol 10. Ten-ml fractions were collected as indicated.

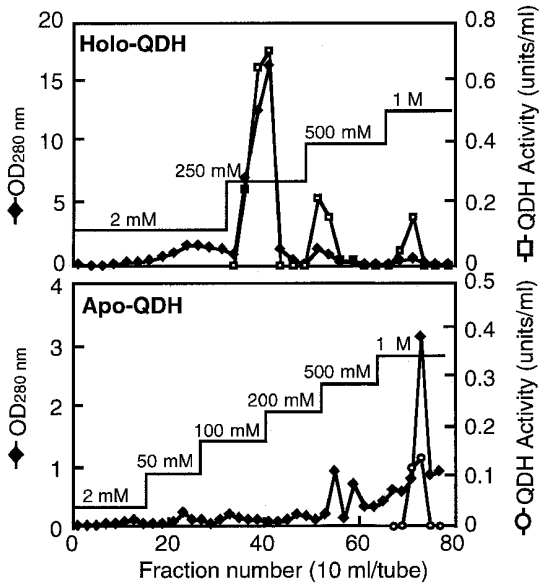

Fig. 3. Hydroxyapatite Chromatography of Holo-QDH (upper frame) and Apo-QDH (lower frame).

Both QDH solutions from the proceeding step were adsorbed onto two columns of hydroxyapatite (2 × 5 cm), which had been equilibrated with 2 mM KPB, pH 7.0, containing 0.1% Mydol 10. Elution of QDH was done stepwise by different concentrations of KPB, pH 7.0, in the presence of Mydol 10. Ten-ml fractions were collected as indicated.

Table 2. Summary of Two Criteria of Holo-QDH Purification from *Acinetobacter* sp. SA1

(Purification I)

Step	Protein	Activity	Specific activity	Recovery	Purification
	(mg)	(units)	(units/mg)	(%)	(fold)
Membrane	1,300	256	0.2	100	1.0
Solubilization	462	231	0.5	90	2.5
Butyl-Toyopearl	223	90	0.4	35	2.0
Hydroxyapatite	7	10	1.4	4	7.0

(Purification II)

Step	Protein	Activity	Specific activity	Recovery	Purification
	(mg)	(units)	(units/mg)	(%)	(fold)
Membrane	370	185	0.5	100	1.0
Washed membrane	340	206	0.6	111	1.0
Solubilization	131	275	2.1	148	4.2
DEAE-Sepharose	8	136	17.0	73	34.0
Holo-QDH formation	8	152	19.0	82	38.0
Concentrate	6	126	21.0	68	42.0

QDH itself became highly purified, as shown in the upper frame of Table 2 and SDS-PAGE in Fig. 5. The precipitate was dissolved in a small volume of KPB, pH 7.0, and passed through a Sephadex G-100 column (1.5×100 cm), which had been equilibrated with 10 mM KPB, pH 7.0, containing 0.1% Mydol 10. Holo-QDH came out at the position showing Ve/Vo of 1.75 when fractionated into 30-drop portions. On the other hand, the Ve/Vo value for apo-QDH elution was found to be 1.40. This means that apo-QDH was purified as dimer due to the interaction of a hydrophobic portion of two molecules of apo-QDH as similarly seen with ADH.[27,28] Addition of exogenous PQQ readily converted apo-QDH to the holoenzyme.

QDH Purification II

The membrane fraction of *Acinetobacter* sp. SA1 was suspended in 5 mM KPB, pH 7.5, and the protein content was adjusted to 10 mg/ml. To the suspension, various detergents were added to the final concentration of 0.1% or 1.0% followed by gentle stirring for 30 min. The following detergents were effective for QDH solubilization: β-OG, OT, Mydol 10, Mydol 12, Zwittergent, and TX. The highest QDH activity was found when the membrane fraction was treated with 1% OT. However, taking enzyme stability into consideration, Mydol 10 was finally chosen as the most effective detergent among those tested, if it was used at more than 2%. *Acinetobacter* sp. SA1 strain contains GDH in the membrane that may disturb QDH purification. Preliminary elimination of GDH from QDH was tried first. Addition of 0.2% TX and 0.3 M KCl to the membrane suspension was effective to remove GDH, while almost all of the QDH activity still existed in the washed membrane fraction. QDH was finally solubilized with 2% Mydol 10 in 5 mM KPB, pH 7.5, containing 0.1 M KCl and 1 mM PMSF. The solubilized enzyme was put on a DEAE-Sepharose column (2×20 cm) equilibrated with 50 mM Tris-HCl, pH 8.5, containing 0.3% Mydol 10 and 10% glycerol (w/v). Elution was made by a linear gradient of NaCl up to 50 mM, and QDH came out from the column at about 30 mM NaCl when the elution was monitored by dual wavelengths at 280 nm and 420 nm and QDH activity was measured. It was apparent that the fractions containing QDH activity were free from heme components monitored by the absorbance at 420 nm, as shown in Fig. 4. The active fractions from DEAE-Sepharose chromatography were pooled (shadowed area, fraction numbers from 63 to 72) and a small amount of PQQ was added to confirm holo-QDH formation before concentration of QDH in a dialyzing tube by enbedding in sucrose powder. The purification summary is shown in the lower frame of Table 2.

Properties of purified QDH

Molecular mass of QDH and absroption spectra

The molecular mass of purified QDHs from the purification I and II showed the same molecular mass of 88 kDa as shown in Fig. 5 and Fig. 6. The molecular mass estimated showed a good coincidence with 88,196 of the estimated molecular size from *quiA*, encoding the quinate dehydrogenase in *Acinetobacter*.[14] The absorption spectra of holo-QDH and apo-QDH are shown in Fig. 5. As judged from a small bump that appeared around 420 nm in both forms of QDHs was alike to that found in GDH from *G. suboxydans*,[29] through which electrons may flow to potassium ferricyanide. Different from quinoprotein GDHs from *Pseudomonas* sp.[21] and *Escherichia coli*,[22] and quinoprotein glycerol dehydrogenase from *G. industrius*,[23] most of which show a minimal enzyme activity with potassium ferricyanide, the QDH purified in this study showed a strong enzyme activity with potassium ferricyanide. The only differ-

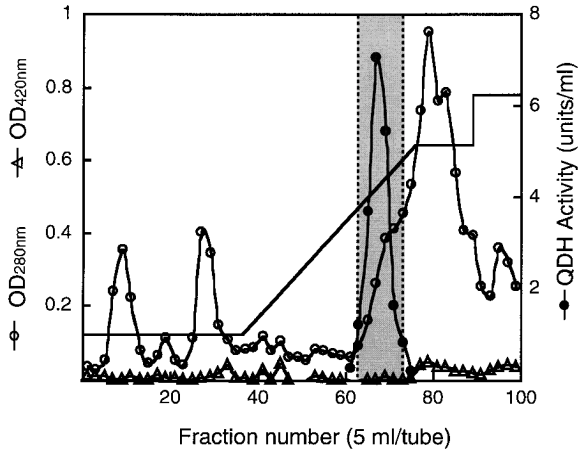

Fig. 4. DEAE-Sepharose Chromatography of Holo-QDH.
The solubilized QDH was put on a DEAE-Sepharose (2× 20 cm) column equilibrated with 50 mM Tris-HCl, pH 8.5, containing 0.3% Mydol 10 and 10% glycerol (w/v). Elution of QDH was done by a linear gradient of NaCl up to 50 mM as shown by a solid ascending line. Elution was confirmed by washing the column with 50 mM NaCl and then with 60 mM NaCl as indicated. Elution was monitored by measuring the absorbance at 280 nm and 420 nm and by assaying QDH activity.

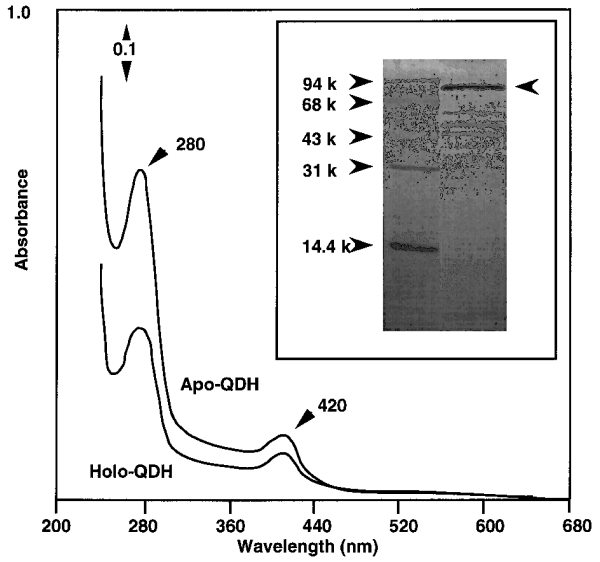

Fig. 5. Absorption Spectra and SDS-PAGE of QDHs Purified from Purification I.
Both QDHs were dissolved in 10 mM KPB, pH 7.0, containing 0.1% Mydol 10. The spectra of holo-QDH and apo-QDH were taken at the protein concentrations of 0.4 mg/ml and 0.7 mg/ml, respectively. In a frame, SDS-PAGE of only apo-QDH (5 μg loaded) is shown and an arrowhead corresponds to the protein band of QDH in the right lane.

ence among these enzymes having similar molecular masses each other comes from the presence or absence of a chromophore appeared at 420 nm. Since the purified QDH showed a negative reaction to heme staining, the existence of a heme component in the chromophore appearing at 420 nm was ruled out.

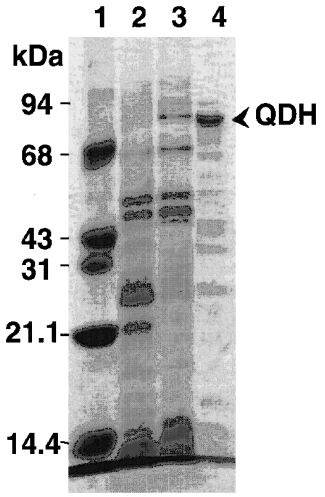

Fig. 6. SDS-PAGE of QDH Purified from Purification II.
Lane 1, markers; lane 2, membrane fraction; lane 3, solubilized QDH; lane 4, peak fraction of QDH from DEAE-Sepharose chromatography.

The function of the chromophore is unknown at this moment and is to be clarified.

Catalytic properties of purified QDH

The optimum pH in quinate oxidation was found at pH of 5.0 to 6.0 when assayed with potassium ferricyanide or PMS/DCIP as an electron acceptor. A direct quinate oxidase was observed with the intact membrane and oxygen uptake was also optimal at pH 5.0–6.0 similar to the case of assay with artificial electron acceptors. As briefly mentioned above, NAD-dependent QDH localized in the cytoplasmic soluble fraction showed the optimum pH at highly alkaline pH such as 9.5–11.0. Judging from the clear contrast in catalytic properties between membrane-bound quinoprotein QDH and NAD-dependent QDH, it is apparent that quinoprotein QDH has a function in the oxidative fermentation yielding 3-dehydroquinate from quinate. Ubiquinone-2 (Q_2) was also available for an electron acceptor as ferricyanide and PMS/DCIP, and the optimum pH of Q_2 reductase activity was found at pH 6.0. Moreover, DCIP itself was also a good electron acceptor and DCIP reductase activity was 80% of that of PMS/DCIP reductase activity. These data suggest that QDH directly coupled with the respiratory chain of the organisms, yielding bioenergy during substrate oxidation. The kinetic properties in quinate oxidation and reduction of the electron acceptor catalyzed by the purified QDH were examined. Both K_m values for quinate with potassium ferricyanide and PMS/DCIP were the same, 0.2 mM. Apparent K_m values for potassium ferricyanide and DCIP were measured to be 0.52 mM and 0.89 mM, respectively. The substrate specificity of QDH was checked with the purified QDH and the enzyme reacted only with quinate and shikimate when examined with various substrates.

The reaction rate with shikimate was 74% of that with quinate and the apparent K_m value for shikimate was measured to be 0.26 mM equally when assayed with either potassium ferricyanide or PMS/DCIP. 3-Dehydroquinate, 3-dehydroshikimate, and *myo*-inositol, of which the structures look alike to quinate or shikimate, were not available as the substrate for QDH. D-Glucose was not oxidized by QDH, although QDH looked like GDH in physicochemical and spectral properties. Furthermore, QDH was distinct from the cyclic alcohol dehydrogenase of acetic acid bacteria[30] and cyclohexanol or 1,4-cyclohexanol was not oxidized, although quinate looks like a typical cyclic alcohol.

Identification of quinate oxidation product by the membrane-bound QDH

QDH oxidized quinate and shikimate as the substrate. In order to identify the oxidation products from quinate and shikimate, these substrates were reacted with QDH under the following conditions. The reaction was done in McIlvaine buffer, pH 6.0, containing 50 mM quinate or shikimate, 160 mU of QDH, and an excess of potassium ferricyanide or PMS/DCIP. First, identification of the oxidation products was done with paper chromatography according to the method described by Yoshida and Hasegawa.[31] In this method, 3-dehydroshikimate (DHS), an oxidation product of shikimate, was detected giving a yellow spot with an *R*f of 0.54. 3-Dehydroquinate (DHQ), an oxidation product of quinate, also gave a yellow spot with a different *R*f of 0.28 (data not shown). DHQ production was detected with 3-dehydroquinate dehydratase and NADP-dependent shikimate dehydrogenase by measuring NADPH as reported previously.[32] In addition to paper chromatography, DHS production was detected enzymatically by measuring 3-dehydroshikimate reductase activity coupled with NADPH oxidation using NADP-dependent shikimate dehydrogenaese, as also reported previously.[32] Using the enzymatic method, oxidation products from quinate and shikimate catalyzed by QDH were identified to be DHQ and DHS, respectively, because of high reactivity of these products with DHQ and SKDH. These results support the earlier indications of the first report on quinate oxidation by acetic acid bacteria done by Whiting and Coggins.[12]

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