D-Glucose Dehydrogenase of *Gluconobacter suboxydans*: Solubilization, Purification and Characterization[†]

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D-Glucose dehydrogenase was purified to homogeneity from the membrane of *Gluconobacter suboxydans* IFO 12528. The enzyme was solubilized from the membrane with Triton X-100 and fractionated with SE-cellulose and hydroxyapatite columns. Other species of membrane-bound dehydrogenases which were cosolubilized with D-glucose dehydrogenase were eliminated by treating the enzyme at fairly acidic pH in the initial step of enzyme purification. The purified enzyme was homogeneous in analytical ultracentrifugation and sucrose density gradient centrifugation. The enzyme had an apparent sedimentation constant of 4.2 S and showed a molecular weight of 87,000 by urea-sodium dodecylsulfate gel electrophoresis. The spectral studies with the purified enzyme preparation showed the participation of pyrroloquinoline quinone, a new prosthetic group, in the enzyme. Phenazine methosulfate was found to be the best electron acceptor in D-glucose oxidation with the enzyme. The optimum pH of D-glucose oxidation was at pH 3.0 with potassium ferricyanide, and pH 6.0 with phenazine methosulfate, 2,6-dichlorophenolindophenol and Wurster's blue as electron acceptor. The substrate specificity of the enzyme seemed to be restricted to D-glucose, and other sugars were not oxidized except for maltose which was oxidized at a low rate. The hydrophobicity of the enzyme was discussed from the standpoint of a typical integral protein of the bacterial membrane.

Membrane-bound D-glucose dehydrogenase is of a particular importance as the key enzyme in the oxidative metabolism of D-glucose in acetic acid bacteria and other oxidative bacteria. Such groups of bacteria have a direct oxidation system of D-glucose. The system is constructed of D-glucose and D-gluconate dehydrogenases, which are localized in cytoplasmic membrane^{1,2)} and linked to an electron transport chain contained in it.³⁾ Some properties of D-glucose dehydrogenase in membrane of *Pseudomonas fluorescens* and *Gluconobacter suboxydans* have been briefly reported by Imanaga *et al.*^{4,5)} In the previous paper,⁶⁾ we also reported the complete purification of the

enzyme from *Pseudomonas* sp. and described some molecular properties of the enzyme. Of many kinds of membrane-bound dehydrogenases involved in carbohydrate metabolism of oxidative bacteria including acetic acid bacteria, only D-glucose dehydrogenase was solubilized and purified as a single peptide having a molecular weight of about 90,000, whereas the other species of enzymes were purified as an enzyme-cytochrome complex around 150,000 daltons of molecular weight.⁷⁾

Recently, Duine *et al.*^{8,9)} reported that the prosthetic group of D-glucose dehydrogenase from *Acinetobacter calcoaceticus* was like that of methanol dehydrogenase from methylotrophs. The prosthetic group has been identified to be pyrroloquinoline quinone (PQQ) as a new prosthetic group¹⁰⁾ and the name "quinoproteins" has been proposed for this distinct class of dehydrogenase. We have also showed that the prosthetic group of D-

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glucose dehydrogenase of *P. aeruginosa* is PQQ.¹¹⁾

In this paper, we attempted to solubilize and purify D-glucose dehydrogenase from the membrane of *G. suboxydans*. With a highly purified enzyme preparation, catalytic and physicochemical properties of the enzyme were examined. Some preliminary information with respect to the prosthetic group was also presented.

MATERIALS AND METHODS

Chemicals. Yeast extract was generously supplied by the Oriental Yeast Ind. Co. Sodium D-gluconate for culture medium was kindly donated by the Fujisawa Pharmaceutical Ind. Co. Hydroxyapatite was prepared according to Tiselius *et al.*¹²⁾ Potato extract was prepared as described by Ameyama and Kondo.¹³⁾ All other chemicals used in this study were commercial products of analytical grade.

Microorganisms. Bacterial strains of genus *Gluconobacter*, listed in Table I, were generously donated by the Institute for Fermentation, Osaka (IFO).

Medium and cultivation. The medium and cultivation of bacteria were the same as described in the previous paper.¹⁴⁾

Preparation of cell homogenate, cell-free extract and membrane-fraction. Cells were harvested by centrifugation at $12,000 \times g$ for 20 min and washed twice with ice-cold water. The cell paste was suspended in distilled water and passed twice through a French pressure cell press, Aminco Co., at 20,000 psi. The resulting pressed material was designated as cell homogenate. Cell homogenate was spun down in a preparative ultracentrifuge at $68,000 \times g$ for 120 min. The resulting supernatant was collected by decanting and used for enzyme source of NADP-dependent D-glucose dehydrogenase as previously reported.¹⁵⁾ The sedimented cell debris was used as membrane fraction.

Enzyme assay. Enzyme activity was assayed by the method of Wood *et al.*¹⁶⁾ which used ferricyanide as an electron acceptor. The rate of reduction of ferricyanide to ferrocyanide gave a quantitative amount of D-glucose oxidized. The reaction mixture contained $10 \mu\text{mol}$ of potassium ferricyanide, 0.7 ml of McIlvaine buffer, pH 3.0, $100 \mu\text{mol}$ of D-glucose and enzyme solution in a total volume of 1.0 ml. The reaction was carried out at 25°C by addition of ferricyanide solution, and stopped by adding 0.5 ml of the ferric-Dupanol reagent. Then, 3.5 ml of water was added to the reaction mixture and well mixed. The

resulting stabilized Prussian blue color was measured by a spectrophotometer at 660 nm after standing for an additional 20 min at 25°C. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of $1 \mu\text{mol}$ of D-glucose per min under these assay conditions and 4.0 absorbance units corresponded to $1 \mu\text{mol}$ of D-glucose oxidized. Enzyme assay with ferricyanide was mainly used for routine assay due to its simplicity. Alternative enzyme assay was used with 2,6-dichlorophenolindophenol under the same conditions as described previously.⁶⁾

Protein determination. Protein content was determined according to the method by Lowry *et al.*,¹⁷⁾ using a bovine serum albumin as the standard. A modified method described by Dulley and Grieve¹⁸⁾ was also employed, instead, for the samples which contained detergent.

Analytical ultracentrifugation. Estimation of sedimentation velocity and examination of the purity of enzyme preparations were carried out with a Hitachi model 282 analytical ultracentrifuge operated at 59,780 rpm with a schlieren optics.¹⁹⁾ The temperature was maintained at 20°C throughout the measurements.

Sucrose density gradient centrifugation. Sucrose gradients were prepared with the following composition: 1 ml of 21%, 1 ml of 17%, 1 ml of 13%, 1 ml of 9%, and 1 ml of 5% (wt/wt) sucrose, each prepared in 20-fold diluted McIlvaine buffer, pH 7.0, containing 1% Triton X-100 and 1 mM 2-mercaptoethanol. The gradients were left to stand at 4°C for about 20 hr before use. The sample of purified enzyme (0.1 ml) was layered on the top of the gradients and centrifuged at 36,000 rpm for 16 hr in a Hitachi RPS 40A rotor at 4°C. After fractionation for 0.14 ml each, the enzyme activity and protein content were assayed as described above.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of urea and sodium dodecylsulfate (SDS) was performed by the method of Uemura and Mizushima.²⁰⁾ The enzyme was precipitated with 40% ethanol to remove excess Triton X-100. The pellet was suspended in 1% SDS and 1% 2-mercaptoethanol and heated in a boiling water bath for 5 min. Urea was added to the heated sample to about 8 M, and the enzyme was subjected to gel electrophoresis. The molecular weight of the enzyme was estimated from a calibration curve prepared with bovine serum albumin, ovalbumin, trypsin and lysozyme as the standard.

Isoelectric focusing. A 110 ml column (LKB) was employed to determine the pI value of the purified D-glucose dehydrogenase through pH 3.5 to 10.0 prepared by ampholite and sucrose density gradient (0~25%). About 15 units of the purified enzyme was applied to the column and electrofocusing was made at 300 V for 40 hr at

0°C.

Extraction and analysis of prosthetic group. The purified enzyme (1 mg) was mixed with 9 volume of methanol and the precipitate was eliminated by centrifugation at $10,000 \times g$ for 20 min. The supernatant was evaporated under reduced pressure to 0.14 ml. To the concentrated supernatant solution, 0.86 ml of 0.01 M potassium phosphate, pH 6.0, containing 5 mM $MgCl_2$ was added. One ml of chloroform was further added to the extract and well mixed. The solution was centrifuged at $1,000 \times g$ for 10 min, and the upper layer was analyzed as the prosthetic group of the enzyme. Absorption spectrum in the ultraviolet region and fluorescence spectrum were measured at 25°C with a Hitachi spectrophotometer 557 and Aminco-Bowman spectrofluorometer J4-8962, respectively.

RESULTS AND DISCUSSION

Distribution of membrane-bound D-glucose dehydrogenase in genus Gluconobacter

Distribution of membrane-bound D-glucose

dehydrogenase was surveyed with the cell homogenate of genus *Gluconobacter* as shown in Table I. High enzyme activity occurred in the cell homogenate of *G. oxydans* IFO 3287, *G. suboxydans* IFO 12528 and *G. rubiginosus* IFO 3244. In most strains of *G. cerinus*, a fair amount of the enzyme activity was observed. It was interesting that all strains of *G. suboxydans* var. α had a poor enzyme activity as a species trait in spite of good cell yield on the same medium. Finally, the strain of *G. suboxydans* IFO 12528 was selected for the further study of the purification and characterization of the enzyme. This is the same strain that was used for the purification and characterization of membrane-bound alcohol dehydrogenase¹⁴⁾ and aldehyde dehydrogenase.²¹⁾ Some of the other cytoplasmic enzymes in the intracellular metabolism of carbohydrate were also investigated by this

TABLE I. DISTRIBUTION OF MEMBRANE-BOUND D-GLUCOSE DEHYDROGENASE IN GLUCONOBACTERS

Each strain of gluconobacter was inoculated into three shaking flasks containing 100 ml each of the medium and incubated at 30°C for 24 hr under reciprocal shaking. Enzyme activity was assayed with cell homogenate under the standard assay conditions using potassium ferricyanide at pH 3.0. Results are expressed as total enzyme activity and the values in parentheses are specific activity (units/mg protein).

Strain	GDH ^a	Strain	GDH ^a
<i>G. liquefaciens</i> IFO 12388	11 (0.07)	<i>G. gluconicus</i> IFO 3286	192 (1.10)
<i>G. melanogenus</i> IFO 12257	64 (0.49)	<i>G. roseus</i> IFO 3990	123 (1.60)
<i>G. melanogenus</i> IFO 12258	51 (0.30)	<i>G. albidus</i> IFO 3250	59 (0.51)
<i>G. melanogenus</i> IFO 3293	179 (0.81)	<i>G. albidus</i> IFO 3251	78 (0.57)
<i>G. melanogenus</i> IFO 3294	181 (1.00)	<i>G. albidus</i> IFO 3253	155 (1.00)
<i>G. sphaericus</i> IFO 12467	51 (0.46)	<i>G. suboxydans</i> IFO 3130	62 (0.51)
<i>G. oxydans</i> IFO 3189	15 (0.14)	<i>G. suboxydans</i> IFO 3172	112 (0.89)
<i>G. oxydans</i> IFO 3287	266 (1.70)	<i>G. suboxydans</i> IFO 3289	56 (0.30)
<i>G. dioxyceticus</i> IFO 3271	155 (0.85)	<i>G. suboxydans</i> IFO 3290	73 (0.45)
<i>G. dioxyceticus</i> IFO 3272	184 (0.92)	<i>G. suboxydans</i> IFO 3291	53 (0.32)
<i>G. dioxyceticus</i> IFO 3274	212 (0.91)	<i>G. suboxydans</i> IFO 12528	294 (1.70)
<i>G. cerinus</i> IFO 3262	88 (0.54)	<i>G. suboxydans</i> var. α IFO 3254	tr. ^b
<i>G. cerinus</i> IFO 3263	150 (1.10)	<i>G. suboxydans</i> var. α IFO 3255	tr.
<i>G. cerinus</i> IFO 3264	194 (1.30)	<i>G. suboxydans</i> var. α IFO 3256	tr.
<i>G. cerinus</i> IFO 3265	144 (1.00)	<i>G. suboxydans</i> var. α IFO 3257	tr.
<i>G. cerinus</i> IFO 3266	47 (0.46)	<i>G. suboxydans</i> var. α IFO 3258	tr.
<i>G. cerinus</i> IFO 3267	191 (1.20)	<i>G. capsulatus</i> IFO 3462	167 (1.50)
<i>G. cerinus</i> IFO 3268	228 (1.60)	<i>G. industrius</i> IFO 3260	27 (0.19)
<i>G. cerinus</i> IFO 3269	250 (1.30)	<i>G. nonoxygluconicus</i> IFO 3275	180 (0.93)
<i>G. cerinus</i> IFO 3270	230 (1.60)	<i>G. nonoxygluconicus</i> IFO 3276	195 (0.83)
<i>G. gluconicus</i> IFO 3171	74 (0.52)	<i>G. rubiginosus</i> IFO 3244	263 (1.30)
<i>G. gluconicus</i> IFO 3285	158 (1.10)		

^a D-Glucose dehydrogenase is abbreviated as GDH.

^b Enzyme activity was less than 1.0 unit.

strain.^{14,22)} The highest enzyme formation was observed at the late exponential phase when grown on a medium containing D-glucose, glycerol and sodium D-gluconate as the carbon source. The late exponential phase was attained at about 30 hr by flask culture on a rotary shaker and at about 24 hr by jar fermentor (25 liters/50-liter tank, 30 liters air/min and 500 rpm agitation). The bacterial cells at the late exponential phase were harvested and used for further experiments mentioned below.

Solubilization of D-glucose dehydrogenase from the membrane and elimination of alcohol dehydrogenase

To the membrane fraction prepared from 300 g of wet cells, McIlvaine buffer, pH 2.5, containing 1 mM 2-mercaptoethanol was added and adjusted the protein concentration to about 20 mg/ml. To the membrane suspension, Triton X-100 was added to 1% and the suspension was gently stirred for 3 hr at 0°C. The incubation mixture was spun down at $12,000 \times g$ for 30 min and the resulting supernatant was collected (1,400 ml). The enzyme solution was put in a dialyzing tube and embedded in Polyethyleneglycol 6,000 and concentrated to about 150 ml. The dialyzing bag including the concentrated enzyme was dialyzed against 3 liters of distilled water containing 0.5% Triton X-100 and 1 mM 2-mercaptoethanol and salt concentration in the enzyme solution was reduced to about one-twentieth during dialysis. During the solubilization of the enzyme and subsequent treatments, membrane-bound alcohol dehydrogenase which was abundant in the cell membrane of the organism and was cosolubilized with D-glucose dehydrogenase, was inactivated and disappeared from the enzyme solution. It can be seen from Table II, where the effects of pH on enzyme solubilization and solubility of D-glucose dehydrogenase and alcohol dehydrogenase are shown, that alcohol dehydrogenase was partially solubilized at pH 2.5 and the solubilized enzyme was rapidly inactivated due to acidity. On the other

TABLE II. EFFECT OF pH ON SOLUBILIZATION OF D-GLUCOSE DEHYDROGENASE AND ELIMINATION OF ALCOHOL DEHYDROGENASE

Enzyme solubilization was performed at pH 2.5 and 6.0 in the presence of 1% Triton X-100. Incubation mixture was stirred for 3 hr at 0°C and centrifuged at $68,000 \times g$ for 60 min. The precipitated membrane residue was suspended in 0.1% Triton X-100. D-Glucose dehydrogenase and alcohol dehydrogenase were assayed with both the supernatant and the residual membrane. The assay of D-glucose dehydrogenase was carried out at pH 3.0 with ferricyanide. The assay of alcohol dehydrogenase was carried out at pH 6.0 with ferricyanide as previously reported.¹⁴⁾

pH	GDH ^a in		ADH ^b in	
	Sup.	Ppt.	Sup.	Ppt.
	(units)		(units)	
2.5	38	20	12	130
6.0	26	22	270	5

^a D-Glucose dehydrogenase.

^b Alcohol dehydrogenase.

hand, the solubilized D-glucose dehydrogenase was stable at pH 2.5, although about 50~60% of the total enzyme activity was solubilized. Elimination of alcohol dehydrogenase from the solubilized enzyme solution was advantageous for further purification of D-glucose dehydrogenase.

Purification by SE-cellulose and hydroxyapatite column chromatographies

The dialyzed enzyme solution was applied to a SE-cellulose column (3 × 25 cm), which had been equilibrated with 20-fold diluted McIlvaine buffer, pH 2.5, containing 0.5% Triton X-100 and 1 mM 2-mercaptoethanol. Nonadsorbable proteins were washed out with the same buffer thoroughly. The enzyme activity was eluted with the same buffer supplemented with KCl to 0.1 M. The enzyme fraction (260 ml) when put in a dialyzing tube was concentrated to about 30 ml by Polyethyleneglycol 6,000 in the same fashion as mentioned above. The concentrated enzyme was dialyzed overnight against 3 liters of 20-fold diluted McIlvaine buffer, pH 2.5, contain-

TABLE III. SUMMARY OF PURIFICATION OF D-GLUCOSE DEHYDROGENASE FROM MEMBRANE OF *Gluconobacter suboxydans* IFO 12528

Step	Total protein (mg)	Total activity (units) ^a	Specific activity (units/mg)	Yield (%)
Membrane fraction	28,500	42,750	1.5	(100)
Solubilized fraction	7,850	29,000	3.7	68
SE-Cellulose (I)	720	11,900	16.5	28
SE-Cellulose (II)	220	6,490	29.5	15
Hydroxyapatite	120	4,200	35.0	10

^a Enzyme activity was assayed with potassium ferricyanide as electron acceptor at pH 3.0.

FIG. 1. Sedimentation Pattern of Purified D-Glucose Dehydrogenase from *Gluconobacter suboxydans*.

Purified enzyme was used at an enzyme concentration of 5.6 mg/ml in 20-fold diluted McIlvaine buffer, pH 7.0, containing 5% sucrose, 1 mM 2-mercaptoethanol and 1% sodium cholate. Triton X-100 which had been in excess in the purified enzyme solution was replaced with sodium cholate by hydroxyapatite before ultracentrifugation as mentioned in the text. Photographs were taken at 24, 33, 42 and 51 min after reaching 59,780 rpm. Direction of sedimentation was right to left.

ing 0.5% Triton X-100 and 1 mM 2-mercaptoethanol. Then, SE-cellulose column chromatography (1.5 × 20 cm) was repeated with the dialyzed enzyme. After the column had been washed with the buffer containing 0.03 M KCl, the enzyme was eluted by increasing the concentration of KCl by a linear gradient to 0.15 M. The enzyme fraction (400 ml) in a dialyzing bag was concentrated to about 50 ml by Polyethyleneglycol 6,000, as mentioned above, and dialyzed for 24 hr against two changes of 3 liters of 40-fold diluted McIlvaine buffer, pH 6.0, containing 0.5% Triton X-100 and 1 mM 2-mercaptoethanol. After dialysis, insoluble materials were removed by centrifugation at 12,000 × *g* for 20 min. The supernatant was applied to a hydroxyapatite column (5 × 5 cm), which had been equilibrated with the same buffer, pH 6.0. After non-adsorbable materials had been washed out with the same buffer, elution of the enzyme

was made by a linear gradient to 0.1 M (respect to phosphate). The enzyme solution (180 ml) was concentrated in a similar fashion by Polyethyleneglycol 6,000 and subsequently dialyzed against 0.1 M McIlvaine buffer, pH 6.0 (respect to phosphate), containing Triton X-100 (0.5%) and 2-mercaptoethanol (1 mM).

The summary of a typical purification of D-glucose dehydrogenase from the membrane of *G. suboxydans* is shown in Table III. As can be seen, the enzyme was purified about 25-fold with an overall yield of 10%. From these results, it can be considered that D-glucose dehydrogenase was formed in the bacterial membrane over 4% of membrane protein. It is quite reasonable for gluconate producer to form a lot of D-glucose dehydrogenase.

Properties of purified D-glucose dehydrogenase

Homogeneity. The homogeneity of the purified enzyme was estimated by an analytical

ultracentrifuge and the sedimentation pattern of the enzyme showed a symmetrical peak as shown in Fig. 1. An apparent sedimentation constant was calculated to be 4.2 S. Before ultracentrifugation, Triton X-100 in the enzyme solution was replaced with 1% sodium cholate by adsorption into a hydroxyapatite, washing with buffer solution containing no Triton X-100 and desorption with buffer containing 1% sodium cholate. Due to the hydrophobicity of the enzyme molecule, a trace of dimeric enzyme was also observed at the position of sedimentation constant of about 9.0 S. In an analytical ultracentrifugation, the presence of Triton X-100 in excess in the enzyme solution made a huge peak near the enzyme boundary and caused a complicated sedimentation pattern that was hard to divide one from another. A curved baseline of sedimentation was due to the presence of 5% sucrose and some Poly-ethyleneglycol 6,000 which contaminated the

FIG. 2. Sucrose Density Gradient Centrifugation in the Presence of Triton X-100.

Purified enzyme (about 3 mg) in 0.1 ml of 20-fold diluted McIlvaine buffer, pH 7.0, containing 1% Triton X-100 and 1 mM 2-mercaptoethanol, was layered on the top of the gradient which contained 1% Triton X-100 in the medium. Centrifugation was conducted for 16 hr at 36,000 rpm in a Hitachi RPS 40A rotor (4°C). After fractionation, D-glucose dehydrogenase and protein content were assayed by using aliquot of fractions. Dotted line means sucrose gradient. The sucrose concentration at bottom (left) and top (right) corresponded to 21% and 5%, respectively. Direction of sedimentation was right to left.

enzyme solution during enzyme concentration. In the presence of Triton X-100 over 1%, dimerization of the enzyme did not occur when analyzed in a sucrose density gradient centrifugation as shown in Fig. 2. At the same time, the profile of the sucrose density gradient centrifugation showed that the purified enzyme was composed of a single protein species and showed a constant level of specific activity around the peak fraction. Thus, the homogeneity of the purified enzyme preparation of D-glucose dehydrogenase was proved to be homogeneous when examined by means of two different analytical methods.

Spectral study. The purified D-glucose dehydrogenase showed no typical absorption spectrum (Fig. 3). Due to the excess Triton X-100 in the enzyme solution, the absorption spectrum was partly surveyed in the ultraviolet region, and it was quite similar to that

FIG. 3. Absorption Spectrum of Purified D-Glucose Dehydrogenase.

Purified enzyme with a specific activity of 35.0, assayed with potassium ferricyanide at pH 3.0, was used at a protein concentration of 5.0 mg/ml in 20-fold diluted McIlvaine buffer, pH 6.0, containing 1% Triton X-100 and 1 mM 2-mercaptoethanol.

obtained with the enzyme from *Pseudomonas* sp.⁶⁾ Unlike the other species of membrane-bound dehydrogenases which are purified as a dehydrogenase-cytochrome complex associating with *c*-type cytochrome,⁷⁾ D-glucose dehydrogenase might be purified as an only dehydrogenase protein and freed from the cytochrome component during treatment of the membrane fraction with detergent. Enzyme preparations by Imanaga *et al.*^{4,5)} from *P. fluorescens* and *G. suboxydans* also indicate the absence of the cytochrome component and suggest a pterin derivative as the prosthetic group of these enzymes.

We attempted to characterize the prosthetic group of the purified enzyme. Extraction of the prosthetic group was made in essentially the same way as described by Duine *et al.*⁹⁾ Absorption spectra and fluorescence spectra of the isolated prosthetic group are shown in Figs. 4 and 5, respectively. Although no further purification was made with the isolated sample, a characteristic absorption spectrum of oxidized form similar to that of PQQ was obtained. By reduction with sodium borohydride, an increase in absorbance around 340 nm was also observed. Excitation spectrum of the isolated prosthetic group showed two peaks at 260 nm and 370 nm and a shoulder around 325 nm with excitation at 480 nm. An emission peak was at 455 nm with excitation at 370 nm. These fluorescence spectra were also similar to those of PQQ isolated from D-glucose dehydrogenase of *Pseudomonas* sp.¹¹⁾ Though we could not estimate whether the prosthetic group of the purified enzyme was extracted completely, these results strongly indicated the participation of PQQ as the prosthetic group of D-glucose dehydrogenase from the membrane of *G. suboxydans*. Reconstitution of D-glucose dehydrogenase from an apo-enzyme and isolated PQQ has not been performed with enzyme from *G. suboxydans*, but a preliminary experiment was performed successfully with the enzyme from *Pseudomonas* sp.¹¹⁾

Molecular weight. Due to hydrophobicity of the enzyme molecule of D-glucose dehydrogenase, conventional electrophoresis by poly-

FIG. 4. Absorption Spectra of Prosthetic Group Extracted from the Purified D-Glucose Dehydrogenase.

Purified enzyme with a specific activity of 35.0, assayed with potassium ferricyanide at pH 3.0, was treated with methanol as mentioned in MATERIALS AND METHODS. The spectra were recorded in 0.01 M potassium phosphate, pH 6.0, containing 5 mM MgCl₂. Solid line, oxidized form; broken line, reduced form made by addition of sodium borohydride to the oxidized form.

FIG. 5. Fluorescence Spectra of Prosthetic Group Extracted from the Purified D-Glucose Dehydrogenase.

The same extract with that used for absorption spectra of prosthetic group of the purified enzyme (Fig. 4) was used. A, excitation spectrum with excitation at 480 nm; B, emission spectrum with excitation at 370 nm.

acrylamide gel²³⁾ or SDS-gel electrophoresis²⁴⁾ did not give a normal electrophoretic migration profile. In the case of polyacrylamide

FIG. 6. Molecular Weight Determination by Urea-SDS Gel Electrophoresis.

Purified enzyme with a specific activity of 35.0, assayed with potassium ferricyanide at pH 3.0, was used. The enzyme was precipitated with 40% ethanol to remove excess Triton X-100. The pellet was suspended in 1% SDS and 1% 2-mercaptoethanol and heated in a boiling water-bath for 5 min. Urea was added to the heated enzyme solution to about 8M, and the enzyme was subjected to gel electrophoresis. Protein band was stained with Coomassie brilliant blue and destained in 7% acetic acid. Calibration curve was tentatively prepared with bovine serum albumin (BSA), mol. wt. 68,000; ovalbumin, mol. wt. 43,000; trypsin, mol. wt. 23,300 and lysozyme, mol. wt. 14,300. Direction of electrophoresis was left to right.

gel electrophoresis, the enzyme protein aggregated in the spacer gel and did not come into the separation gel, though a marker dye, bromophenol blue, migrated to the bottom of a gel column. Similar situation occurred in SDS-gel electrophoresis. Molecular weight determination of the enzyme was performed by urea-SDS gel electrophoresis according to Uemura and Mizushima.²⁰⁾ In addition to several minor bands of polymerized enzyme, a fair amount of the enzyme still aggregated and stayed at the top of separation gel (Fig. 6). The molecular weight of the enzyme was calculated from the most predominant protein species in

FIG. 7. Isoelectrofocusing of Purified D-Glucose Dehydrogenase.

Sucrose gradient was prepared in a 110 ml column from 25 to 0% with ampholite of pH 3.5 to 10.0. About 15 units of the purified enzyme with a specific activity of 35.0, assayed with potassium ferricyanide at pH 3.0, was layered at the position of pH 5 to 6. Triton X-100 was added to 1% throughout the medium. Electrofocusing was made at 300 V for 40 hr at 0°C.

the separation column on the basis of a calibration curve tentatively prepared with the standard marker proteins, and an apparent molecular weight was estimated to be 87,000, which was comparable to that from the enzyme of *Pseudomonas* sp.⁶⁾

Isoelectric point. Figure 7 shows the profile of isoelectric focusing performed at pH 3.5 through 10.0. The purified enzyme was originally put at the position of pH 5 to 6 predicting its relative low *pI*, because *pI* values of 5 to 6 were obtained with other species of membrane-bound dehydrogenase complex. Unexpectedly, the enzyme migrated toward the alkaline region and settled at about pH 7.8 after 40 hr isoelectric focusing. In the case of membrane-bound alcohol dehydrogenase,^{14,25)} aldehyde dehydrogenase,²¹⁾ D-gluconate dehydrogenase^{26,27)}, D-fructose dehydrogenase²⁸⁾ and others, adsorption and desorption of these enzymes on DEAE- or CM-cellulose are usually conducted at a slight acidic pH (5 to 6) and it implies that the isoelectric point of these enzyme complexes is close to pH 5 to 6. The purified D-glucose dehydrogenase that was solubilized and purified free from other heterogeneous structural

TABLE IV. ELECTRON ACCEPTOR SPECIFICITY OF D-GLUCOSE DEHYDROGENASE

Purified enzyme with a specific activity of 35.0 (assayed with potassium ferricyanide at pH 3.0) was used. pH of the reaction mixture was varied with McIlvaine buffer of various pH. Enzyme assay with PMS-DCIP, DCIP or Wurster's blue was performed spectrophotometrically under essentially the same conditions as described in the previous paper.⁶⁾

Electron acceptor	Relative activity	Optimum pH
PMS-DCIP	100 ^a	6.0
DCIP	7	6.0
CoQ ₁	0	—
Ferricyanide	8	3.0
Wurster's blue	29	6.0

^a Enzyme activity with PMS-DCIP was put to 100. Maximum enzyme activity with PMS-DCIP of 435 units/mg protein was observed.

FIG. 8. pH Dependence of D-Glucose Dehydrogenase of *Gluconobacter suboxydans*.

Enzyme activity was assayed with potassium ferricyanide at various pH as indicated. Closed circles were assayed with membrane fraction and 1.2 to 1.8 unit of enzyme activity per mg protein at pH 3.0 was observed. Enzyme activity assayed with solubilized enzyme was shown by open circles and 3.2 to 4.0 unit of enzyme activity per mg protein at pH 3.0 was observed.

subunits was a basic protein in nature.

Catalytic properties of D-glucose dehydrogenase. D-Glucose dehydrogenase could be assayed *in vitro* in the presence of either one of the following dyes as an electron acceptor : potassium ferricyanide, phenazine methosulfate (PMS), 2,6-dichlorophenolindophenol (DCIP), Wurster's blue or nitroblue tetra-

TABLE V. SUBSTRATE SPECIFICITY OF D-GLUCOSE DEHYDROGENASE FROM MEMBRANE OF *Gluconobacter suboxydans* IFO 12528

Oxidation of various substrates was performed with bacterial membrane and the purified enzyme with a specific activity of 35.0 (assayed with potassium ferricyanide at pH 3.0). Enzyme activities were assayed at pH 3.0 and the reaction rate obtained with D-glucose was put to 100. Trace amount of oxidation rate less than 0.1% of that with D-glucose is symbolized as tr.

Substrate	Relative activity with	
	Membrane	Purified enzyme
D-Glucose	100	100
D-Fructose	0	0
D-Mannose	3	0
D-Galactose	tr.	0
L-Rhamnose	9	0
D-Mannitol	4	0
D-Sorbitol	5	0
L-Sorbose	0	0
D-Ribose	2	0
D-Xylose	0	0
Maltose	7	5
Lactose	0	0
Sucrose	0	0
Ethanol	7	0

zolium : whereas NAD, NADP or oxygen were inactive as an electron acceptor. As shown in Table IV, PMS-DCIP was the most effective electron acceptor tested. Potassium ferricyanide was more pH dependent and only restricted around pH 3.0 and the rate of D-glucose oxidation with ferricyanide at pH 3.0 was just 8% of that obtained with PMS-DCIP. Potassium ferricyanide, however, was available as an electron acceptor over a wide pH range when assayed with the bacterial membrane. Once the enzyme was solubilized from the membrane with detergent, the enzyme activity with potassium ferricyanide seen around neutral pH region was bleached and the enzyme activity was only detected around pH 3.0 (Fig. 8). This means that the D-glucose dehydrogenase links directly with ferricyanide but at a relatively low rate. The predominant enzyme activity observed at a neutral pH region with the membrane enzyme was structurally disrupted in enzyme solubilization with

respect to ferricyanide. The outlet of the electron to PMS-DCIP or Wurster's blue is open at neutral pH and oxidation of D-glucose proceeds smoothly. The electron acceptor specificity of the enzyme of *G. suboxydans* was similar to that obtained with the enzyme of *Pseudomonas* sp.⁶⁾

The substrate specificity of D-glucose dehydrogenase is summarized in Table V. Substrates which were oxidized by the purified enzyme were restricted to D-glucose and maltose. Several kinds of substrates were oxidized with the membrane enzyme since the other species of membrane-bound dehydrogenases were localized in the bacterial membrane as already reported.⁷⁾ It was also shown that the elimination of such membrane-bound dehydrogenases was well conducted during the process of enzyme purification by handling the enzyme at a fairly acidic pH. The apparent Michaelis constant for D-glucose was measured with potassium ferricyanide and a similar order of the Michaelis constant such as 2.8×10^{-3} M and 7.7×10^{-3} M were obtained with the membrane enzyme and the purified enzyme, respectively.

The enzyme activity of D-glucose dehydrogenase in bacterial membrane was stable and could be stored in a frozen state without any loss of enzyme activity for over a year. The membrane enzyme was also stable over a wide range of pH 2.0 through 10.0 for several days at 4°C. On the other hand, the solubilized and purified enzyme became labile against storage for long period even it was frozen unless an excess detergent was present in the enzyme preparation. Exposure to extreme acidic or alkaline pH caused inactivation of the purified enzyme in the presence of excess Triton X-100. An examination of pH stability with the purified enzyme preparation showed that the enzyme activity with potassium ferricyanide was stable at pH 3.0 and with PMS-DCIP was stable at pH 6.0.

From these results, we can conclude D-glucose dehydrogenase should have the ability to oxidize D-glucose over a wide range of pH as the key enzyme of a series of oxidative

metabolism of D-glucose in acetic acid bacteria and other oxidative bacteria. Judging from its hydrophobicity and incomplete solubility with detergent, it seemed that the enzyme was buried deeply in, or associated tightly with, the membrane. The immunochemical study showing the structural similarity between two enzyme preparations of D-glucose dehydrogenase of *Pseudomonas* sp.⁶⁾ and *G. suboxydans* will be reported on in a forthcoming paper.

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