D-Gluconate Dehydrogenase, 2-Keto-D-gluconate Yielding, from *Gluconobacter dioxyaceticus*: Purification and Characterization

Emiko SHINAGAWA, Kazunobu MATSUSHITA, Osao ADACHI
and Minoru AMEYAMA

Department of Agricultural Chemistry, Faculty of Agriculture,
Yamaguchi University, Yamaguchi 753, Japan

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D-Gluconate dehydrogenase catalyzing the oxidation of D-gluconate to 2-keto-D-gluconate was solubilized with Triton X-100 from the membrane of *Gluconobacter dioxyaceticus* IFO 3271 and purified to an almost homogeneous state by chromatographies on DEAE-cellulose and CM-Toyopearl in the presence of 0.1% Triton X-100. The enzyme had three subunits with molecular weights of 64,000, 45,000 and 21,000, and contained approximately 2 mol of heme per mol of the enzyme. The prosthetic group of the dehydrogenase was found to be a flavin covalently bound to the enzyme protein. The substrate specificity of the purified enzyme was very strict for D-gluconate and the apparent Michaelis constant for D-gluconate was 2.2 mM. The optimum pH and temperature of the purified enzyme were 6.0 and 40°C, respectively.

The biochemical activity of acetic acid bacteria is characterized by the high oxidative activity for many organic compounds. Of the acetic acid bacteria, particularly strains in the genus *Gluconobacter* can oxidize D-glucose and D-gluconate to ketogluconates. Such oxidative activity can be taken as one of the important characteristics of *Gluconobacter* strains. The oxidative reaction is carried out in the cytoplasmic membrane and linked with the electron transport system of the organism. To investigate the localization of the primary dehydrogenase in such an oxidative system, we prepared spheroplasts of acetic acid bacteria¹⁾ and determined the dehydrogenase activity using ferricyanide as an electron acceptor.²⁾ From the results, it was demonstrated that the primary dehydrogenases in such an oxidative system were localized on the outer surface of the cytoplasmic membrane unlike NADH, succinate or lactate dehydrogenases. This reasonably accounts for the rapid accumulation of such oxidation products. In *Pseudomonas* strains, membrane-bound D-glucose and D-gluconate dehydrogenases were also thought to be localized on the outer

surface of the cytoplasmic membrane.^{3,4)} *Gluconobacter* strains produce 2-keto-D-gluconate (2KGA) and 5-keto-D-gluconate (5KGA) at the same time but other oxidative bacteria produce only 2KGA from D-gluconate. In the previous papers, we reported the purification and characterization of D-gluconate dehydrogenase (GADH) catalyzing the oxidation of D-gluconate to 2KGA from *Pseudomonas aeruginosa*,^{5,6)} *Klebsiella pneumoniae*⁷⁾ and *Serratia marcescens*.⁸⁾ These three GADHs were very similar to each other in subunit structure and catalytic properties.

Gluconobacter strains accumulate 2KGA and 5KGA in the culture medium at the same time, and the ratio of the two ketogluconate productions varies among strains and with culture conditions as reported previously.⁹⁾ However, up to the present, it has been unclear how *Gluconobacter* strains regulate the production of 2KGA and 5KGA. It was reported that 2KGA or 5KGA was formed without NAD or NADP by the particulate fraction of *Gluconobacter* strains,^{10,11)} however, a further study on GADH catalyzing the

oxidation of D-gluconate has not been reported. Thus, it has remained unknown as to whether *Gluconobacter* strains possess two respective enzymes yielding 2KGA and 5KGA or one enzyme yielding both 2KGA and 5KGA. To resolve this question, we attempted to purify GADH catalyzing the oxidation of D-gluconate from the membrane of *Gluconobacter* strains. In this work, we selected a strain which produced mainly 2KGA, and tried to purify GADH catalyzing the oxidation of D-gluconate to 2KGA.

MATERIALS AND METHODS

Bacterial strain and cultivation. *G. dioxyaceticus* IFO 3271 was supplied by the Institute for Fermentation, Osaka (IFO), and used throughout this work. The medium consisted of 10 g of sodium D-gluconate, 3 g of D-glucose, 2 g of D-sorbitol, 2 g of yeast extract and 2 g of polypepton in 1 liter of tap water. Cultivation was carried out aerobically in a shake flask or 50-liter jar fermentor at 30°C.

Enzyme assay. D-Gluconate dehydrogenase was measured spectrophotometrically at 25°C by determining the initial reduction rate of 2,6-dichlorophenolindophenol (DCIP) at 600 nm as described in the previous paper.⁷⁾ One unit was defined as the amount of enzyme that catalyzed the oxidation of 1 μ mol of D-gluconate, or the reduction of 1 μ mol of DCIP, per minute under the standard assay conditions.

Protein determination. The protein content was determined according to the method by Lowry *et al.*¹²⁾ Crystalline bovine albumin was used as the standard. A modified method described by Dulley and Grieve¹³⁾ was also employed instead for samples which contained detergents.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out in 7.5% gel using Tris-glycine buffer (pH 8.3) as described by Davis.¹⁴⁾ Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli¹⁵⁾ using 10% separating gel. The standard proteins used for the molecular weight determination were bovine serum albumin, Mr 68,000; ovalbumin, Mr 43,000; carbonic anhydrase, Mr 30,000 and β -lactoglobulin, Mr 18,300. Protein bands were stained with Coomassie brilliant blue.

Detection of the reaction product. The reaction mixture contained 100 μ mol of sodium D-gluconate, 0.3 μ mol of phenazine methosulfate, 100 μ mol of potassium phosphate

buffer, pH 6.0, and the purified enzyme, and was incubated overnight at 30°C. The reaction product was detected by paper chromatography as described previously.¹⁶⁾ Ketoacids were identified by coloration with 2,3,5-triphenyltetrazolium chloride or *o*-phenylene-diamine·HCl as described by Gossele *et al.*¹⁷⁾

Chemicals. Sodium D-gluconate was from Fujisawa Pharmaceutical Co. and yeast extract was from Oriental Yeast Co. CM-Toyopearl was a kind gift from Toyo Soda Manufacturing Co., Ltd. All other chemicals used in this work were commercial products.

RESULTS

Solubilization and purification of D-gluconate dehydrogenase

All steps were carried out at 0 to 5°C and 0.01 M potassium phosphate buffer, pH 5.7, was used unless otherwise stated.

Step 1. Preparation of the membrane fraction. Cells were harvested at the late exponential phase and washed twice with water. The cell paste was suspended in the buffer and disrupted by passage through a French press at 20,000 psi. After centrifugation to remove intact cells, the supernatant was further centrifuged at 80,000 $\times g$ for 1 hr. The resultant precipitate was collected as the membrane fraction.

Step 2. Solubilization. About 8 g of membrane was suspended in 80 ml of a buffer containing 2% Triton X-100 and 0.1 M sodium D-gluconate. The suspension was stirred for 3 hr in an ice bath and then centrifuged at 80,000 $\times g$ for 1 hr. The precipitate was discarded and the supernatant was dialyzed overnight against two changes of 1 liter of the buffer containing 0.5% Triton X-100.

Step 3. DEAE-cellulose column chromatography. The dialysate was applied to a DEAE-cellulose column (2.5 $\times$ 10 cm) equilibrated with the buffer containing 0.1% Triton X-100 and the column was washed with the same buffer. The enzyme activity was eluted during the washing of the column.

Step 4. CM-Toyopearl column chromatography (I). The resultant enzyme solution from the preceding step was applied on a CM-Toyopearl column (1.5 $\times$ 20 cm) equili-

FIG. 1. Chromatography of D-Gluconate Dehydrogenase on CM-Toyopearl (I).

The column was equilibrated with 0.01 M potassium phosphate buffer, pH 5.7, containing 0.1% Triton X-100. After removal of nonadsorbable proteins by washing with the same buffer, elution of the enzyme was achieved with a linear gradient of sodium D-gluconate to 0.1 M (----).

TABLE I. PURIFICATION OF D-GLUCONATE DEHYDROGENASE FROM *Gluconobacter dioxyaceticus* IFO 3271

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Membrane fraction	1344	3612	2.7	100
Solubilized fraction	327	3698	11	102
Dialysate	233	3298	14	91
DEAE-cellulose	90	3248	36	90
CM-Toyopearl (I)	13	2511	193	70
CM-Toyopearl (II)	8.4	1980	237	55

brated with the buffer containing 0.1% Triton X-100. After the column was washed with the same buffer, GADH activity was eluted as a single peak with a linear gradient of sodium D-gluconate to 0.1 M as shown in Fig. 1. The fractions containing enzyme activity were pooled and concentrated with polyethylene glycol 6000. To the enzyme solution, polyethylene glycol 6000 was added to 25 g (w/v) and the mixture stirred for 1 hr. The mixture was then centrifuged at $12,000 \times g$ for 10 min and the supernatant was discarded. The precipitate was dissolved in the buffer contain-

ing 1% Triton X-100 and insoluble material was removed by centrifugation.

Step 5. CM-Toyopearl column chromatography (II). The resultant supernatant from the preceding step was applied to a CM-Toyopearl column (1.5 $\times$ 5 cm) equilibrated with the buffer containing 0.1% Triton X-100. After washing the column with the same buffer, the column was treated first with the same buffer containing 0.05 M NaCl and then with 0.2 M NaCl. The enzyme activity was eluted from the column with the buffer containing 0.1% Triton X-100 and 0.2 M

NaCl.

A summary of the enzyme purification is presented in Table I.

Properties of the purified GADH

Absorption spectrum. The purified enzyme showed a typical spectrum of a cytochrome having maxima at 418, 523 and 553 nm in the reduced form prepared by the addition of D-gluconate or dithionite (Fig. 2). The cytochrome was determined to be a *c*-type cytochrome from the pyridine hemochromogen and the content of cytochrome was calculated to be 15.2 nmol per mg of enzyme protein. This value corresponded to 1.98 mol heme per mol of the enzyme.

Electrophoretic analyses. The purity of the enzyme was examined by conventional polyacrylamide gel electrophoresis. As shown in Fig. 3, the purified enzyme migrated as a single band, indicating that the purified enzyme preparation was homogeneous. The subunit structure was analyzed by SDS-gel electrophoresis and the purified enzyme was

FIG. 2. Absorption Spectra of D-Gluconate Dehydrogenase.

The purified enzyme with a specific activity of 237 was used at a protein concentration of 0.13 mg/ml in 0.01 M potassium phosphate buffer, pH 5.7. Dotted line, oxidized form; solid line, reduced form obtained by adding sodium dithionite.

dissociated into three bands with molecular weights of 64,000, 45,000 and 21,000 (Fig. 3). The slowest moving band showed fluorescence under UV light and the second band was detected as a cytochrome by a staining method.¹⁸⁾ The sum of the molecular weights of the subunits was 130,000.

Prosthetic group. The prosthetic group of GADH was thought to be a flavin covalently bound to the enzyme protein judging from the fluorescence on SDS-polyacrylamide gel on UV irradiation. The flavin moiety could not be extracted by cold trichloroacetic acid treatment but could be released when the enzyme was digested with pronase and trypsin. The content of the covalently bound flavin thus obtained was estimated to be about 1.26 nmol per mg protein and this value corresponded to about 0.16 mol flavin per mol of the enzyme. Due to difficulties in obtaining

FIG. 3. Polyacrylamide Gel Electrophoresis of D-Gluconate Dehydrogenase.

The protein bands of both gels were stained with Coomassie brilliant blue and the direction of electrophoresis was from top to bottom. A, disc gel electrophoresis in the absence of SDS. B, slab gel electrophoresis in the presence of SDS. Bovine serum albumin (Mr 68,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 30,000) and β -lactoglobulin (Mr 18,300) were used as markers for preparation of a calibration curve.

complete proteolysis, the apparent flavin content was found to be somewhat lower than expected. As already reported, such underestimation of bound flavin was also observed at about 3 nmol per mg protein with other enzyme such as other GADH,⁵⁾ sorbitol dehydrogenase¹⁹⁾ and 2KGA dehydrogenase.²⁰⁾

Catalytic properties. Phenazine methosulfate, ferricyanide and DCIP were available as electron acceptors once the enzyme activity was solubilized from the membrane.

The substrate specificity of the enzyme was examined with various substrates including sugars, sugar alcohols and organic acids. Among these compounds, only D-gluconate was oxidized by the purified enzyme and the apparent Michaelis constant was estimated to be 2.2 mM at pH 6.0. Thus, the substrate specificity of the enzyme was found to be very strict for D-gluconate.

The optimum pH and temperature of this enzyme in D-gluconate oxidation were found to be 6.0 and 40°C, respectively.

Reaction product. After the reaction mixture containing the purified enzyme, D-gluconate and phenazine methosulfate was incubated overnight at 30°C, the reaction product was detected by paper chromatography. As a reaction product, only 2KGA was detected, 5KGA was not detected. So, the enzyme purified from *G. dioxyacetonicus* was characterized as a 2KGA yielding GADH.

DISCUSSION

In the previous papers, we reported membrane-bound GADHs from *P. aeruginosa*,^{5,6)} *K. pneumoniae*⁷⁾ and *S. marcescens*⁸⁾ which produced only 2KGA from D-gluconate during cultivation. The GADHs from these bacteria were very similar to each other in physico-chemical and catalytic properties.

This study concerned the purification and characterization of GADH from *G. dioxyacetonicus* IFO 3271. Unlike other oxidative bacteria such as *P. aeruginosa*, *K. pneumoniae* and *S. marcescens*, *Gluconobacter* strains oxidize D-gluconate and produce 2KGA and 5KGA at

the same time during cultivation. The ratio of 2KGA formation to 5KGA formation varied among the strains and also with culture conditions,⁹⁾ however, the regulatory mechanism for both ketogluconate productions has so far remained unclear. Although the existence of GADH in a *Gluconobacter* strain had been reported,^{10,11)} it was not clarified whether both ketogluconate formations were catalyzed by a single enzyme or a dual enzyme system. The GADH(s) catalyzing 2KGA and 5KGA formation was thought to be membrane-bound, since both ketogluconates accumulated so rapidly in the culture medium in large amounts. To clarify the regulatory mechanism in the two ketogluconate productions in *Gluconobacter*, we attempted to purify GADH catalyzing the oxidation of D-gluconate from a *Gluconobacter* strain. We selected *G. dioxyacetonicus* IFO 3271 which produced mainly 2KGA. GADH was solubilized from the membrane with Triton X-100 and purified to an almost homogeneous state. The enzyme never produced 5KGA under various conditions. The purified enzyme was a flavoprotein-cytochrome *c* complex and the cytochrome *c* of the purified enzyme preparation was a partially reduced form. There was a great similarity between the properties of the purified GADHs from *P. aeruginosa*, *K. pneumoniae* and *S. marcescens*, and those of that of *G. dioxyacetonicus*.

The prosthetic group of GADH was found to be a covalently bound flavin the same as in cases of other GADHs, however, the content of flavin was lower than that of other GADHs. The apparent content of flavin might be small due to incomplete digestion of the enzyme or/and the reduced form of the flavin in the purified preparation similar to the case of the cytochrome *c* component.

The apparent Michaelis constant for D-gluconate was somewhat greater than that of other GADHs. For this reason, it was thought that *Gluconobacter* strains grew generally on media containing higher concentrations of carbon source than other oxidative bacteria did. The optimum pH and temperature of the enzyme were 6.0 and 40°C, respectively, and

these optimum conditions were coincident with those for 2KGA production by resting cells as described previously.⁹⁾

It was found that GADH purified from *G. dioxycetonicus* oxidized D-gluconate to 2KGA but not to 5KGA, and was similar to other GADHs from *P. aeruginosa*,^{5,6)} *K. pneumoniae*⁷⁾ and *S. marcescens*⁸⁾ in physico-chemical and catalytic properties. Thus, *Gluconobacter* strains possess two GADHs: *i.e.* one is a 2KGA yielding GADH and the other is a 5KGA yielding GADH. At present, a study on the alternative GADH yielding 5KGA from other *Gluconobacter* strains is in progress.

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