GLUCONIC DEHYDROGENASE OF *PSEUDOMONAS AERUGINOSA**

by

T. RAMAKRISHNAN** AND J. J. R. CAMPBELL

Department of Dairying, The University of British Columbia, Vancouver (Canada)

Previous work has shown that *Pseudomonas aeruginosa* ATCC 9027 oxidizes glucose by way of gluconic, 2 ketogluconic and pyruvic acids[1-3]. Dried cell preparations of the organism oxidized glucose or gluconic acid with the quantitative accumulation of 2-ketogluconate. No evidence of a phosphate requirement was found with these dried cells, and added sodium fluoride or adenosine triphosphate had no influence on the oxidations. Using *Pseudomonas fluorescens* A 312, WOOD AND SCHWERDT[4] have confirmed the conversion of glucose or gluconate to 2-ketogluconate. However, working with this same strain of *P. fluorescens* ENTNER AND STANIER[5] concluded that 2-ketogluconate was not part of the major pathway of glucose or gluconate breakdown. Moreover NARROD AND WOOD[6] have reported that extracts of *P. fluorescens* can phosphorylate gluconic acid with the formation of 6-phosphogluconate. This seems to be similar to the system in *Escherichia coli* described by COHEN[7-9]. It is possible that the mechanism available for the oxidation of gluconate may vary with the bacterial species. An analogy can be drawn with the observation by MAGASANIK *et al.*[10] that two strains of *Aerobacter aerogenes* dissimilate glycerol by different pathways, one dehydrogenating it to dihydroxyacetone while the other phosphorylates it to L-α-glycerophosphate. The present work was undertaken in an effort to clarify the picture with respect to *Pseudomonas aeruginosa* and deals with the isolation, partial purification and characterization of the gluconic acid dehydrogenase system of this organism.

MATERIALS AND METHODS

Pseudomonas aeruginosa ATCC 9027, was used throughout these studies. Cultures were grown in the liquid synthetic medium of CAMPBELL *et al.*[1] with gluconic acid substituted for glucose as the carbon source. For routine work 1 liter of medium, dispensed in 100 ml amounts in Roux flasks, was inoculated with 1.0% of a 24 hour culture grown on the gluconic acid medium at 30° C. Cells were harvested at 24 hours, washed twice with *M*/30 pH 7.0 phosphate buffer and finally suspended at a concentration of 200 mg wet weight of cells per ml in the phosphate buffer. This suspension was disintegrated by exposure to a 10 kc Raytheon sonic oscillator for 20 minutes.

Neutralized gluconic acid was used as the carbon source in the growth medium. For use in the WARBURG respirometer or the spectrophotometer, gluconolactone was diluted in a volumetric flask, brought to pH 7.6 with NaOH and made to volume. One ml of solution contained 25 μM of gluconate. Sodium adenosine triphosphate from Schwarz & Company, was dissolved in water, passed through IRC-50 resin to separate it from adenosine monophosphate and adenine and brought to pH 6.0 before use. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were used as freshly prepared solutions.

Conventional WARBURG techniques were used, and each flask contained a limiting amount of enzyme source, 1.5 ml of phosphate buffer pH 5.6, and 0.2 ml (5 μM) of substrate; total volume

* This work was carried out under a grant from the National Research Council of Canada.

** Recipient of a Fellowship from the Canada Department of Trade and Commerce, under the Colombo Plan.

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was 3.0 ml and temperature 30° C. When a hydrogen acceptor was required, 0.2 ml (750 γ) of pyocyanine solution was added. It was also possible to follow the rate of reaction spectrophotometrically by observing the rate of reduction of 2:6-dichlorophenol indophenol at 600 mμ.

In the cruder enzyme preparations, protein was estimated by the biuret method of HILLER, GRIEF AND BECKMAN[11] with slight modifications. Casein was used as the standard. Subsequent to protamine sulphate treatment protein was measured by determining the ratio of optical densities at 280 and 260 mμ[12]. Enzyme activity, as determined in the WARBURG respirometer, was calculated as the oxygen uptake at one hour of activity at maximum rate. The specific activity, defined as the microliters of oxygen uptake per hour per mg of protein, was used as an index of purity.

RESULTS

Isolation and purification of the enzyme

The sonicate resulting from the disintegration of the heavy cell suspension was centrifuged at 25,000 $\times$ *g* for 5 minutes. However, very little material was thrown down. The reddish supernatant fluid was stored at —10° C where it would retain full activity for 10–15 days. This extract contained both glucose and gluconic acid dehydrogenases and oxidized 1 μM of glucose with the uptake of 1 μM O_2 and 1 μM of gluconic acid with the uptake of $\frac{1}{2}$ μM O_2. The oxidations were complete within 30 minutes and no CO_2 was evolved.

At this stage the enzymes were not in a soluble form and did not lend themselves to purification. Attempts were, therefore, made to bring the enzymes into solution or at least to decrease the particle size by the use of surface active agents. One gram of sodium glycocholate was added to 10 ml of the sonic extract, the mixture homogenized for 1 minute in a POTTER homogenizer and finally centrifuged at 25,000 $\times$ *g* for 30 minutes. The solid material which was thrown down was resuspended in the original volume of $M/15$ phosphate buffer at pH 7.2. Neither the resuspended solid material nor the supernatant had any activity towards glucose or gluconate as measured by oxygen uptake in the WARBURG respirometer. The addition of 0.2 ml (150 γ) of a solution of 2:6-dichlorophenol indophenol or pyocyanine (750 γ) however, reactivated the supernatant presumably by completing the hydrogen transport system. The resuspended particulate matter showed no activity, even in the presence of the dye. Neither methylene blue nor potassium ferricyanide were effective as hydrogen acceptors. The former allowed a very slow reaction, while the latter apparently poisoned the system. Pyocyanine permitted the most rapid rate of oxidation as measured by oxygen uptake.

The supernatant was chilled to 5° C and solid ammonium sulphate was added slowly with constant stirring to bring the mixture to 25% of saturation. The resulting precipitate was removed by centrifugation and resuspended in the original volume of $M/15$ phosphate buffer, pH 7.2. In the presence of pyocyanine, the resuspended precipitate oxidized glucose at a good rate while its action on gluconate was very weak. The supernatant, on the other hand, oxidized gluconate at a good rate and had very weak activity towards glucose It would appear, therefore, that this step has largely separated the two enzymes.

Solid ammonium sulphate was again added to the chilled supernatant, slowly and with constant stirring, until 90% of saturation was reached. The resulting precipitate was separated by centrifuging and dissolved in the original volume of $M/30$ phosphate buffer of pH 7.0 and tested for its activity towards gluconic acid.

The 25–90 fraction was dialyzed against cold distilled water for 1 hour with constant, vigorous mechanical stirring. The dialyzed protein was treated with protamine sulphate to remove nucleoproteins by the method of LINDSTROM[13]. Sufficient protamine sulphate

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solution was added to bring the nucleic acid content of the supernatant to about 5%[12]. The precipitate was centrifuged off at 25,000 × *g* for 20 minutes and discarded. The supernatant which was clear and nearly colourless, was dialyzed against *M*/10 tris-(hydroxymethyl)aminomethane buffer pH 7.0 to precipitate any nucleoprotein which might remain dissolved due to the presence of traces of ammonium sulphate. If a precipitate formed, it too was centrifuged off and discarded.

The solution was now divided into four portions by precipitation with various degrees of saturation of ammonium sulphate, namely 0–25, –42, –59 and –90%. The activity of these various fractions (made up to the original volume) against gluconic acid was determined manometrically. The activity was found to be in the 0–25 and 25–42 fractions. Another experiment, using 0–20, –35 and –42 saturations with ammonium sulphate, resulted in the major activity being concentrated in the 20–35 fraction. This last named fraction was dissolved in the original volume of *M* 30 pH 7.0 phosphate buffer and was further purified.

This 20–35 portion was further fractionated with saturated alkaline ammonium sulphate of pH 7.5 The gluconic dehydrogenase was found to be in the 20–30 cut. Estimation of the protein content in this fraction showed that the enzyme had been purified more than 200 fold by these steps (Table I).

TABLE I

PURIFICATION OF GLUCONIC DEHYDROGENASE

Stage of purification	*Volume ml*	*Protein per ml mg*	*Activity* h/ml µl*	*Total protein mg*	*Activity h/mg protein µl*	*Total activity/h µl*
Sonic extract	10	56.00	118	560.0	2.1	1176.0
Glycocholate solution	10	29.30	960	293.0	32.8	9610.4
25–90 $(NH_4)_2SO_4$ fraction	20	6.93	444	138.0	64.0	8832.0
Protamine sulphate treated	30	2.78	300	83.4	107.8	8989.5
20–35 $(NH_4)_2SO_4$ fraction	30	2.25	300	67.5	133.3	8997.5
Alk. $(NH_4)_2SO_4$ 20–30 fraction	20	1.28	540	25.6	421.5	10790.4

* As measured by O_2 uptake in WARBURG respirometer in the presence of pyocyanine.

Stability of the enzyme

The enzyme was routinely stored at —10° C in *M*/10 tris buffer, pH 7.0. Under these conditions it was stable for several weeks. At 4° C, under the same conditions, the enzyme may be kept for 3 to 4 days without any appreciable loss of activity. When dialyzed against distilled water, there was a gradual loss of activity after 8 to 10 hours, accompanied by precipitation. Dialysis against neutral phosphate or "tris" buffers for as long as 24 hours in the cold produced no loss of activity.

Products of the reaction

The stoichiometry of the aerobic oxidation of gluconic acid catalyzed by gluconic dehydrogenase is described by the equation:

$$C_6H_{12}O_7 + \tfrac{1}{2}\,O_2 \rightarrow C_6H_{10}O_7 + H_2O$$

2-ketogluconic acid is the end-product of the reaction and no carbon dioxide is evolved. Data for this were obtained by manometric experiments with carbon dioxide estimated by the method of DIXON[12] and 2-ketogluconic acid determined by the chromatographic technique.

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For the chromatographic analysis a large Warburg cup containing ten times the usual amounts of reagents was set up side by side with the ordinary cup and the course of the reaction followed manometrically in the latter. At the end of the reaction the contents of the large cup were concentrated to one-tenth their volume by lyophilization. Chromatographic analysis of the product was carried out by the method of NORRIS AND CAMPBELL[1]. Only one spot was obtained and it corresponded to 2-ketogluconic acid.

Activity of electron acceptors

In the presence of methylene blue, 2:6-dichlorophenol indophenol or pyocyanine, gluconic dehydrogenase catalyzes the oxidation of gluconic acid. Ferricyanide appears to poison the system and brilliant cresyl blue is inactive. Reaction with methylene blue is very slow while that with indophenol cannot be used for manometric work since the dye is not reoxidized by oxygen. Reaction with pyocyanine is rapid and the rate increases with increasing concentrations of pyocyanine until an optimum concentration of 680 γ of pyocyanine per 3 ml is reached. Beyond that there is no increase in rate. Addition of cytochrome *c*, flavin monucleotide, flavin adenine dinucleotide, DPN or TPN did not increase the activity of the enzyme. Treatment with charcoal to remove any bound DPN[15] did not reduce the activity, nor was DPN or TPN reduced as measured by increase in optical density of the reaction mixture at 340 mμ.

Substrate specificity

Glucose, glucuronic acid, 2-ketogluconic acid, pyruvic acid, saccharic acid, ribonic acid, arabonic acid, fructose and mannose, were not oxidized by the enzyme system. The purified enzyme appears, therefore, to be quite specific for gluconic acid.

Effect of pH

Maximum activity of the crude extract was attained at pH 5.6 (Fig. 1), when tested in veronal buffer over the range 3.2–7.5. With the purified enzyme in the presence of pyocyanine, there was the suggestion of a second maximum at pH 4.6 but since no carbon dioxide was evolved at this pH and since the product of oxidation was still only 2-ketogluconic acid it may be presumed that the hydrogen acceptor and not the enzyme was influenced by the change in pH.

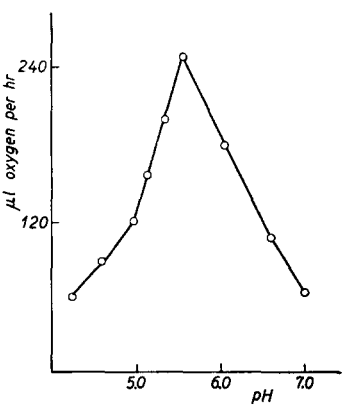

Fig. 1. Rate of oxidation of gluconate as a function of pH. Each cup contained: 0.5 ml of sonicate; 2.0 ml of Veronal buffer; 0.2 ml of gluconate (5 μM); 0.15 ml of 20% KOH; water to make up to 3.15 ml.

Other properties of the enzyme

Iodoacetate ($1 \cdot 10^{-3}$ *M*), azide ($3 \cdot 10^{-3}$ *M*), dinitrophenol ($2 \cdot 10^{-3}$ *M*), arsenite ($1 \cdot 10^{-3}$ *M*) and sodium fluoride ($2\text{–}5 \cdot 10^{-2}$ *M*) did not inhibit the action of the enzyme. The absence of fluoride inhibition indicates that phosphorylation of the substrate is not involved. Cyanide, glutathione or cysteine in concentrations of $1 \cdot 10^{-3}$ *M* increased the rate of oxidation (Fig. 2). The addition of Mg, Mn or Fe^{++} did not activate the enzyme. Similarly dialysis against versene for 40 hours, according to the procedure of RACKER *et al.*[16] did not result in decreased activity showing that a metal is not a cofactor.

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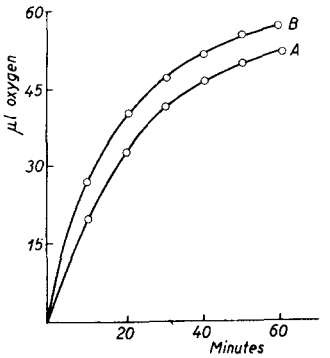

Fig. 2. Influence of reducing agents on the oxidation of gluconate. Each cup contained: 0.5 ml of purified enzyme; 1.5 ml of $M/15$ phosphate buffer pH 5.6; 0.2 ml of gluconate (5 μM); 0.3 ml of KCN (10^{-3} M) with 4 N KCN in 20% KOH in centre well; (or 0.3 ml of glutathione or cysteine 10^{-3} M); 0.2 ml of pyocyanine, A = gluconate alone; B = gluconate + KCN(or glutathione or cysteine).

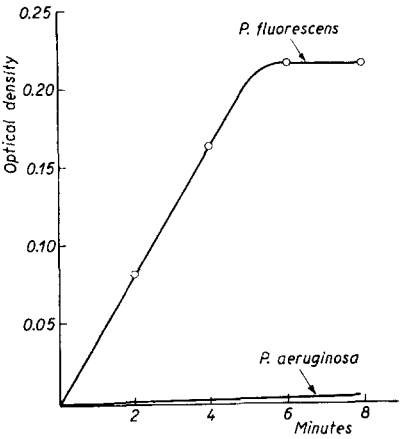

Fig. 3. Gluconate phosphorylation by *P. fluorescens* as measured by TPN reduction in the presence of excess phosphogluconate dehydrogenase. Extracts of *P. aeruginosa* 9027 gave no reduction of TPN under these conditions. The cuvette contained: 0.1 ml of sonic extract; 0.2 ml of gluconate (0.5 μM); $MgCl_2$ (0.15 μM) 0.1 ml; 0.5 ml of phosphogluconic dehydrogenase; 0.1 ml of TPN (2 mg per ml); 0.05 ml of ATP (10 μM); water to a final volume of 3.0 ml. Measurement of OD. at 340 mμ.

Phosphorylation

The enzyme was not activated by ATP or AMP and did not require inorganic phosphate for activity. Aerobic experiments at pH 8.0 and 6.0 by the method of BARKER AND LIPMANN[17] and anaerobic experiments using a mixture of 95% nitrogen and 5% carbon dioxide failed to indicate phosphorylation in sonic extracts of *P. aeruginosa*. Moreover, sonic extracts failed to reduce TPN in the presence of an excess of phosphogluconic dehydrogenase isolated from brewer's yeast by the method of HORECKER AND SMYRNIOTIS[18] (Fig. 3). In contrast to these data, it was found that by either of the last two mentioned criteria *P. fluorescens* A312 did phosphorylate gluconate.

DISCUSSION

Previous work on the metabolism of *P. aeruginosa* has indicated that this organism is unique in a number of respects and this observation has been extended by the work described above with gluconic acid dehydrogenase. The absence of inhibition by fluoride indicates that no phosphorylation is involved in the oxidation of gluconic acid by the enzyme. This is confirmed by the fact that ATP does not activate the enzyme. The absence of increased ionization as determined by the technique of BARKER AND LIPMANN[17] and the failure to reduce TPN in the presence of phosphogluconate dehydrogenase is added confirmation that no phosphorylation is involved in the oxidation of gluconic acid by extracts of this organism. The additional phosphorylated system for gluconic acid oxidation reported by WOOD AND SCHWERDT[4] to be present in *P. fluorescens* is, therefore, absent from the purified enzyme obtained from *P. aeruginosa* 9027. Pyocyanine acted as the most rapid hydrogen acceptor in the system and it is possible that this pigment functions in a similar manner in growing cells. DPN or TPN was not reduced by the

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enzyme. The system, therefore, does not seem to be linked to oxygen through the pyridine nucleotide system.

Cyanide in concentrations of 10^{-3} *M* activated the enzyme. The action of cyanide may be either in forming the cyanohydrin derivative with the product or in reducing the potential of the system. According to the first alternative, 2-ketogluconic acid, formed by oxidation of gluconic acid, may be acting as an inhibitor of the reaction, and cyanide removes it by forming the cyanohydrin derivative. However, semicarbazide did not activate the enzyme nor did increasing concentrations of 2-ketogluconic acid inhibit the activity of the enzyme. The second alternative seems to be the more acceptable one, since both glutathione and cysteine activated the enzyme to the same degree as cyanide.

The nine fold increase in total activity during purification may be due to the removal of toxic factors or of competing reactions. However, the startling increase in total activity occurred at the solublizing step and so may represent an increase in enzyme surface.

SUMMARY

1. The purification, isolation and properties of gluconic dehydrogenase from *P. aeruginosa* 9027 have been described.
2. Pyocyanine has been found to be the most active hydrogen acceptor for the enzyme.
3. The enzyme catalyzes the oxidation of gluconic acid without phosphorylation of the substrate.

RÉSUMÉ

1. La purification, l'isolement et les propriétés de la gluconique déhydrogénase de *P. aeruginosa* 9027 sont décrites.
2. La pyocyanine est, pour cet enzyme, l'accepteur d'hydrogène le plus actif.
3. L'enzyme catalyse l'oxydation de l'acide gluconique sans phosphorylation du substrat.

ZUSAMMENFASSUNG

1. Die Reinigung, Isolierung und Eigenschaften der Glukonsäuredehydrogenase aus *P. aeruginosa* 9027 wurden beschrieben.
2. Pyocyanin ist der beste Wasserstoffionenakzeptor für dieses Enzym.
3. Das Enzym bewirkt die Oxydation von Glukonsäure ohne Phosphorylierung dieses Substrates.

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Received December 4th, 1954