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Evidence for a membrane-bound pyrroloquinoline quinone-linked glucose dehydrogenase in *Acetobacter diazotrophicus*

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Abstract *Acetobacter diazotrophicus* possesses a pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH). The enzyme seemingly belongs to the type II PQQ-GDH enzymes and, at least under the culture conditions tested, the organism synthesizes enough PQQ to saturate the apo-enzyme. The synthesis of this enzyme is stimulated when the organism is grown under N_2-fixing conditions. It is proposed that this enzyme may play an important role in providing extra energy in N_2-fixing cells.

Introduction

Acetobacter diazotrophicus, a recently described N_2-fixing species, colonizes roots and stems of sugar cane and seemingly plays an important role in providing nitrogen to this crop through biological N_2 fixation (Cavalcante and Döbereiner 1988; Cojho et al. 1993). When cultivated in a defined minimal medium this bacterium oxidizes glucose extracellularly to gluconic acid as a necessary step for the initiation of exponential growth (Stephan et al. 1991).

The production of gluconic acid from glucose by bacterial cultures was reported as long ago as 1880 (Boutroux 1880). Since then, extracellular oxidation of glucose to gluconic acid has been observed in a wide variety of organisms, such as *Pseudomonas fluorescens* (Wood and Schwerdt 1953), *Aerobacter aerogenes* (Dalby and Blackwood 1955) and *Bacterium anitratum* (*Acinetobacter calcoaceticus*) (Hauge 1964). In 1964 Hauge demonstrated that the glucose dehydrogenase from *A. calcoaceticus* contained an unknown cofactor. In 1979 Duine et al. established that this cofactor was pyrroloquinoline quinone (PQQ). Later it was reported that glucose dehydrogenases (GDH) of the above-mentioned organisms and several others, were quinoproteins (Duine 1989) proteins containing PQQ as the prosthetic group). Surprisingly, it was also found that several species, such as *Escherichia coli* (Hommes et al. 1984) and *Acinetobacter lwoffii* (van Schie et al. 1987) could not synthesize PQQ, but only possessed the capacity to synthesize the glucose dehydrogenase apo-enzyme. Reconstitution of the holo-enzyme in vivo was possible by adding small amounts of PQQ to the culture medium.

Among the N_2-fixing organisms, *Klebsiella pneumoniae* possesses the holo-enzyme (Hommes et al. 1985) [although the strain that was used in studies on GDH did not possess nitrogenase activity (O. M. Neijssel, personal communication)], whereas *Rhizobium* and *Azotobacter* species were reported to possess only the apo-enzyme (van Schie et al. 1987). However, we and others were unable to confirm the presence of an apo-GDH in *Azotobacter vinelandii* (unpublished results, and J. L. Snoep, personal communication).

It has been proposed that the major physiological function of the PQQ-GDH is to generate extra energy in the addition to the usual energy-yielding pathways (Hommes et al. 1985). Because N_2 fixation is a process that imposes a heavy energy burden on the cells, it could be that this ancillary energy-yielding pathway plays a crucial role in some N_2-fixing organisms. It was therefore of interest to study whether the extracellular oxidation of glucose observed in cultures of *A. diazotrophicus* was catalysed by a PQQ-linked glucose dehydrogenase, and whether the synthesis of this enzyme was increased when the organism was grown under N_2-fixing conditions.

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Materials and methods

Organisms and culture methods

Acetobacter diazotrophicus strain PAL 3 (kindly provided by Dr. J. Döbereiner, CNPBS/EMBRAPA, Rio de Janeiro, Brasil) was maintained on agar slopes on a potato medium (Stephan et al. 1991). N_2-fixing cultures were obtained using the defined minimal medium (LGIM) described by Stephan et al. (1991) with 0.132 g l^{-1} $(NH_4)_2SO_4$. When the organism was grown under non-N_2-fixing conditions the $(NH_4)_2SO_4$ concentration was raised to 1.32 g l^{-1}.

Escherichia coli strain HB 101 was maintained on and grown in Luria broth medium.

Both organisms were grown in batch culture (shaken flasks) at 30°C. Cells of *E. coli* HB 101 were harvested from an overnight culture for the preparation of a membrane fraction.

Incubations with washed cell suspensions

The incubations with washed suspensions of cells were carried out in shaken flasks at 30°C. *A. diazotrophicus* cells were centrifuged and washed twice in TRIS/HCl buffer (pH 7.0). Cells (1.0 g l^{-1} equivalent dry weight) were incubated in the same buffer and glucose was added to a final concentration of 5.0 g l^{-1}. Samples taken at 1 h intervals during the first 4 h (after glucose addition) were centrifuged and gluconate measured in the supernatants by HPLC.

Preparation of cell-free extracts and membrane fractions

About 2 l culture of *A. diazotrophicus* (at mid-exponential growth phase) or 1 l *E. coli* culture was centrifuged and washed twice by resuspension in and sedimentation from 10 mM Na_2HPO_4 buffer (pH 6.0) supplemented with 5 mM $MgCl_2$. The pellet was resuspended in about 10 ml of the same buffer and disrupted by passing the frozen suspension (− 20°C) three times through an X25 X-Press (AB Biox, Sweden). The disrupted cell suspension was centrifuged for 20 min at 12 000 *g* to remove intact cells and cell debris, and the resulting supernatant used as the cell-free extract. Cell-free extracts of both *A. diazotrophicus* and *E. coli* were separated into crude soluble and membrane fractions by centrifugation at 120 000 *g* for 90 min. Centrifugation was repeated and soluble and membrane fractions obtained from the crude counterparts.

Enzyme assay

Glucose dehydrogenase (GDH) activity was measured spectrophotometrically using Wurster's blue as the electron acceptor essentially as described by Hommes et al. (1985). The assay was carried out at 30°C, using NaCN (1.5 mM final concentration) to block activity of the electron-transport chain. The molar absorption coefficient of Wurster's blue at 612 nm was taken to be 12 000 $M^{-1} cm^{-1}$. Specific GDH activities were expressed as nmol Wurster's blue reduced min^{-1} mg $protein^{-1}$. When enzyme activity was measured in the presence of added PQQ, the mixture was preincubated for 15 min at 30°C with 100 µM PQQ in 10 mM Na_2HPO_4/5 mM $MgCl_2$ prior to the addition of glucose.

In order to demonstrate the presence of PQQ in the GDH preparations of *A. diazotrophicus* a denatured extract of both cell-free extracts and membrane fractions was obtained. A 3-ml sample of these preparations was kept at 100°C for 10 min, either with or without the addition of 0.2% sodium dodecyl sulphate. The resulting suspensions were filtered through an ultrafiltration micropartition system (nominal molecular mass 10 000 kDa) (MSI, CFP 010K 3SM9). The filtered supernatants were used as a potential source of PQQ in the assay of GDH activity with the membrane fraction of *E. coli* HB 101.

Other analytical procedures

Protein was assayed by the biuret method using bovine serum albumin as standard (Gornall et al. 1949). Glucose was measured with glucose oxidase, using a diagnostic test kit. Gluconate was determined by HPLC with an ULTRASIL AX organic acid column (Beckman Instruments Inc.) using 0.01 M KH_2PO_4/5% methanol (pH 3.35) as eluent, with an M 156 refractive index detector (Beckman Instruments Inc.) and an SP 4270 Integrator (Spectra Physics, San Jose, USA), at room temperature.

Chemicals

All chemicals used were of the best analytical grade commercially available. Wurster's blue was prepared according to Michaelis and Granick (1943). PQQ was purchased from Fluka Chemie AG (Switzerland).

Results

When growing in the LGIM culture medium *A. diazotrophicus* PAL 3 consumed 90% of the glucose during the first 10 h of culture, producing concomitantly an equivalent amount of gluconic acid, without a significant increase in biomass. The culture pH dropped, during the same period, from 5.75 to 2.5. The exponential growth phase began when almost 50%–60% of glucose was converted into gluconic acid (Fig. 1). These results are in agreement with those reported by Stephan et al. (1991). No modification in the above growth characteristics was observed when the culture

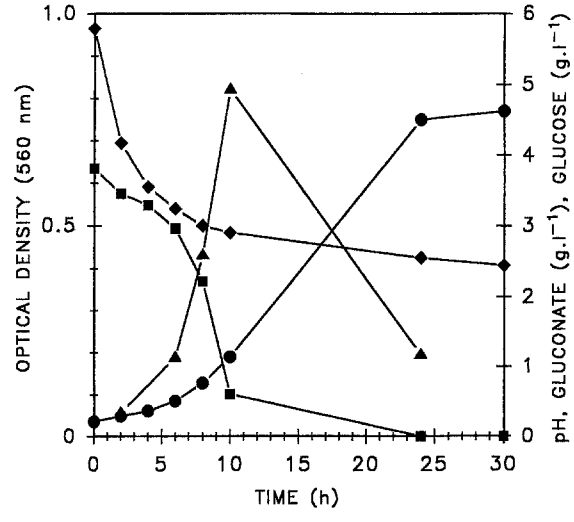

Fig. 1 Glucose (■) and gluconate (▲) concentrations, and pH evolution (◆) during growth (●) of *Acetobacter diazotrophicus* (strain PAL 3) in LGIM culture medium (Stephan et al. 1991)

medium was supplemented with either PQQ (1 μM) or calcium gluconate (2.5 $g l^{-1}$). No growth was observed in LGIM medium without $(NH_4)_2SO_4$; supplementation of this latter medium with PQQ or calcium gluconate did not restore growth.

Washed suspensions of *A. diazotrophicus*, prepared from samples taken after 10 h and 30 h of culture, showed a level of in vivo GDH activity, independent of the culture age, of around 3.3–3.5 mM gluconate h^{-1} (g dry weight cells)$^{-1}$. Addition of PQQ (1 μM) to the incubation mixture did not affect the rate of gluconate production.

A cell-free extract of this organism was prepared and GDH activity was measured using Wurster's blue (the one-electron acceptor generally used for detecting PQQ-linked dehydrogenases). As can be seen in Table 1 such extracts showed, as in the production of gluconate by washed cell suspensions, reduction of Wurster's blue at a rate that was independent of the presence of PQQ in the incubation mixture. Dialysis of cell-free extracts at pH 8.5 against an EDTA (5.0 mM)-containing buffer (50 mM TRIS/HCl) did not lead to a significant decrease in GDH activity whereas dialysis at pH 6.0 against EDTA (10 mM Na_2HPO_4) gave an extract with a reduced enzyme level.

Soluble and membrane fractions of *A. diazotrophicus* were prepared, after cell disruption, by repeated ultracentrifugation. As shown in Table 1 no GDH activity could be detected in the soluble fraction. With the membrane fraction, again the presence of PQQ in the incubation mixture did not modify the rate of dye reduction. In this case dialysis (against the same EDTA-containing buffers as used for the cell-free extract) of the membrane fraction at pH 8.5 did not modify significantly the rate of Wurster's blue reduction, but dialysis at pH 6.0 led to a lower activity. This latter activity, however, could not be restored to the original level by addition of PQQ.

To establish definitively whether PQQ is the cofactor of the GDH from *A. diazotrophicus* a biological assay was performed. Denatured cell-free extract and membrane fraction of *A. diazotropicus* were filtered and the supernatants used to reconstitute a quinoprotein apo-enzyme in a Mg^{2+}-containing buffer. On the basis of the observation that *E. coli* cells contain only apo-GDH (Hommes et al. 1984), a membrane preparation of this organism was obtained and used as the source of the apo-enzyme in the assay. GDH activity of the membrane fraction of *E. coli*, and also of a cell-free extract of this organism (data not shown), could be reconstituted either by preincubation with PQQ or with the filtered supernatants of the denatured GDH preparations from *A. diazotrophicus* (Table 2). Moreover, GDH activity of *E. coli* membranes could also be reconstituted with filtered extracellular fluids from *A. diazotrophicus* cultures.

Finally the activities of GDH in cells of *A. diazotrophicus* growth either under N_2-fixing conditions or under non-N_2-fixing were compared. It was found that N_2-fixing cells contained higher levels of this enzyme than cells grown in the presence of excess $(NH_4)_2SO_4$ (Table 1).

Table 1 Glucose dehydrogenase (*GDH*) activities in cell-free extract (*CFE*) and membrane preparations (*MF*) of *A. diazotrophicus* (strain PAL 3). *WB* Wurster's blue dye, *PQQ* pyrroloquinoline, *CFE-N* cell-free extract prepared from cells grown under non-N2-fixing conditions, *dial. EDTA pH: 8.5 or 6.0* the corresponding preparation was dialysed against 50 mM TRIS/HCl (PH: 8.5) buffer with 5.0 mM EDTA or 10 mM phosphate buffer (pH: 6.0) with 5.0 mM EDTA at 4°C for 36–40 h, *CFE and MF dial. buffer Pi pH: 6.0* the preparations were dialysed against 10 mM phosphate buffer (without EDTA), under the same conditions indicated for the EDTA treatments, as controls. Data are the average of five experiments for CFE and three for MF. Standard deviation, 20% absolute

Sample	GDH activity (nmol WB min^{-1} mg $protein^{-1}$)	
	− PQQ	+ PQQ[a]
CFE	40	41
CFE-N	12	10
CFE (dial. buffer Pi pH: 6.0)	35	37
CFE (dial. EDTA pH: 8.5)	27	27
CFE (dial. EDTA pH: 6.0)	19	22
MF	50	—
MF (dial. buffer Pi pH: 6.0)	46	44
MF (dial. EDTA pH: 8.5)	35	30
MF (dial. EDTA pH: 6.0)	14	19
Soluble fraction	0	0

[a] Preincubated in 10 mM phosphate/5 mM $MgCl_2$ with 100 μM PQQ

Table 2 Glucose dehydrogenase activity of *E. coli* (strain HB 101) membrane fraction preincubated with PQQ and different denatured extracts from *A. diazotrophicus* (strain PAL 3). Preincubations were performed at 30°C for 15 min in 10 mM phosphate/5 mM $MgCl_2$ buffer (pH: 7.0) with the addition of PQQ (100 μM) or the indicated extracts. Denatured CFE (1) and MF (1) were obtained by keeping the corresponding extract from *A. diazotrophicus* at 100°C for 10 min. Denatured CFE (2) was obtained by incubating the CFE for 10 min at 100°C in the presence of SDS (0.2%). Data are the average of three experiments. Standard deviation, 10% absolute

E. coli (HB 101) membrane fraction preincubated with:	GDH activity (nM WB min^{-1} mg $protein^{-1}$)
No addition	0
PQQ	8.5
50 μl denatured CFE (1)	1.4
200 μl denatured CFE (1)	2.1
50 μl denatured CFE (2)	1.6
200 μl denatured MF (1)	5.2
200 μl extracellular fluid[a]	0.7

[a] Filtered extracellular fluid from a culture of *A. diazotrophicus* in LGIM medium (Stephan et al. 1991)

Discussion

In glucose-containing media oxidation of glucose to gluconate by *A. diazotrophicus* is essential for the start of exponential growth (Stephan et al. 1991) (Fig. 1). Periplasmic oxidation of glucose has been observed in a wide variety of bacterial species and, in most cases, a quinoprotein GDH was found to be involved in this reaction (Duine 1989). Therefore it was reasonable to speculate that this was also the case for *A. diazotrophicus*. From the reconstitution of activity of *E. coli* apo-GDH, using either denatured cell-free extract or membrane fraction from *A. diazotrophicus* (Table 2), it can be concluded that GDH from this organism is a quinoprotein.

Two types of quinoprotein bacterial GDH have been described: type I enzymes having a low isoelectric point and from which GDH activity is totally lost after dialysis against EDTA-containing buffers, because of the removal of PQQ (e.g. GDH from *K. pneumoniae* and *Pseudomonas* sp.), and type II enzymes that show a high isoelectric point and from which PQQ can not be removed by dialysis against EDTA-containing buffers (e.g. GDH from *Acinetobacter calcoaceticus* and *Gluconobacter* sp.) (Dokter et al. 1986). Cell-free extract and membrane fraction from *A. diazotrophicus* did not show a significant loss of GDH activity after dialysis against EDTA-containing buffer at pH 8.5 and the decrease in enzyme activity observed after dialysis at pH 6.0 could not be restored by preincubation with PQQ (Table 1). From these results, and from the observation that GDH from this organism showed its maximum activity in vitro at a pH value around 8.0 (data not shown), this enzyme seems to belong to the type II PQQ-linked GDH. Whereas in *A. calcoaceticus*, an organism possessing a type II GDH, a soluble form of GDH in addition to the membrane-bound GDH was found (Duine et al. 1982), in *A. diazotrophicus* only the membrane-bound GDH could be detected (Table 1). With regard to the influence of pH on the *A. diazotrophicus* GDH activity there is a paradox: while Stephan et al. (1991) observed growth of this organism at pH values as low as 3.0, no GDH activity could be detected in vitro at pH values lower than 4.0 (data not shown). Therefore, there are at least two possibilities, either (a) the effect of external pH under in vivo conditions is different from that under in vitro conditions, or (b) at low pH values the direct glucose oxidative pathway is not expressed in *A. diazotrophicus*.

Finally, the regulation of the synthesis of this enzyme is in agreement with the proposal that it serves as an ancillary energy-generating system (Neijssel et al. 1989), because N_2-fixing cultures contained around three-times higher enzyme levels than those grown under non-N_2-fixing conditions (Table 1). It is therefore possible that this enzyme plays an important role in the generation of metabolic energy in N_2-fixing cells.

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