Cytochrome *b*-562 from *Acinetobacter calcoaceticus* L.M.D. 79.41

Its characteristics and role as electron acceptor for quinoprotein glucose dehydrogenase

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A soluble cytochrome *b* was purified from *Acinetobacter calcoaceticus* L.M.D. 79.41. On the basis of the α -band maximum of a reduced preparation, measured at 25 °C, it is designated as cytochrome *b*-562. This cytochrome is a basic monomeric protein (pI 10.2; M_r 18000), containing one protohaem group per molecule. The reduced form, at 25 °C, showed absorption bands at 428, 532 and 562 nm. At 77 K the α -band shifted to 560 nm (with a shoulder at 558 nm). The reduced cytochrome did not react with CO. Cytochrome *b*-562 is most probably (loosely) attached to the outside of the cytoplasmic membrane, since substantial amounts of it, equimolar to quinoprotein glucose dehydrogenase (GDH), were present in the culture medium when cells were grown in the presence of low concentrations of Triton X-100. The midpoint potential at pH 7.0 was found to be +170 mV, a value that was lowered to +145 mV by the presence of GDH. Since the GDH was shown to have a midpoint potential of +50 mV, cytochrome *b*-562 could function as the natural primary electron acceptor. Arguments to substantiate this view and to propose a role of ubiquinone-9 as electron acceptor for cytochrome *b*-562 are presented.

INTRODUCTION

The physiological function of microbial incomplete oxidation is still poorly understood. In particular, the point whether the reactions provide useful energy for the organism has been a matter of debate for several years. Recently, however, the question has been definitely answered in the case of *Acinetobacter calcoaceticus*. A large number of strains of this bacterium convert glucose into gluconolactone without showing growth on either of these compounds. The oxidation step is mediated by a NAD(P)-independent glucose dehydrogenase, which is clearly involved in energy metabolism, since it can lead to the generation of a protonmotive force that can drive solute transport [1–3], to ATP accumulation in the cells [2,3] and to an increase of the molar growth yield [4,5]. The increase in yield was, however, unexpectedly high [6], suggesting that the auxiliary energy source (glucose) is able to improve the efficiency of energy generation from the carbon and energy source (acetate). In order to shed light on this phenomenon, it should be known at which level reduction equivalents derived from the incomplete oxidation step enter the respiratory chain. This prompted a study of the dehydrogenase and its electron acceptors at the molecular level.

The glucose dehydrogenase (GDH) from *A. calcoaceticus* contains pyrroloquinoline quinone (PQQ) as a cofactor, and is therefore designated as 'quinoprotein glucose dehydrogenase' (EC 1.1.99.17) [7]. It appears to be widespread among bacteria [8,9], and is most probably situated in the periplasm since the activity was found in the spent culture medium of *A. calcoaceticus* after growth in the presence of low concentrations of

Triton X-100 [10]. The enzyme has been purified and characterized [11,12], but, since further knowledge of the redox properties could be helpful in the indication of the site of interaction with the electron-transfer chain, determination of its midpoint potential was attempted.

Much controversy exists on the nature of the primary electron acceptor of GDH. Hauge [13,14] reported that a soluble cytochrome *b* was present in the early stages of isolation of GDH from *Bacterium anitratum* (nowadays known as *A. calcoaceticus*). This cytochrome could be reduced by glucose in the presence of GDH, suggesting that it is the primary electron acceptor of this dehydrogenase. No evidence for this was obtained in a recent study by Beardmore-Gray & Anthony [15]. The authors concluded that glucose, succinate and NADH are all oxidized by way of the same *b*-type cytochromes and that GDH donates its electrons to ubiquinone-9. In view of these contradicting reports, it was decided to purify and characterize the soluble cytochrome *b* and to study its capability as electron acceptor for GDH.

EXPERIMENTAL

Culture conditions

Acetate-limited chemostat cultures of *A. calcoaceticus* L.M.D. 79.41 and mutants (13.37) and (N12), derived from this strain and unable to oxidize glucose (obtained from Dr. N. Goossen), were provided by Dr. B. J. van Schie (see ref. [16]). After centrifugation and washing, the cells were stored at –20 °C. *A. calcoaceticus* was also grown overnight at 30 °C in batch cultures (50 ml) on the same mineral medium, supplemented with 0.2% (v/v) ethanol. For production of GDH and cytochrome *b*-562

Abbreviations used: GDH, glucose dehydrogenase; PES, phenazine ethosulphate; PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione); Q-0 and Q-9, ubiquinone-0 and ubiquinone-9 respectively; Wurster's Blue is the free radical of *NNN*'*N*'-tetramethyl-*p*-phenylenediamine.

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in the culture medium, growth was performed on heptadecane in the presence of Triton X-100, as described previously [10].

Chemicals

All chemicals were obtained from commercial sources and were of reagent grade.

Purification of cytochrome *b*-562

Cell-free extract (260 ml) was prepared from cells (130 g wet wt.) as described previously [7] and applied to a CM-Sepharose column (4.4 cm × 5 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. The column was washed with the same buffer (5 column volumes). The cytochrome was eluted with 0.2 M-potassium phosphate buffer, pH 7.0. The fractions having significant absorbance at 418 nm were pooled. After concentration by pressure filtration on a Pellicon membrane (Millipore, type PTGC 047.10), the concentrate was diluted 20-fold with water and the preparation was applied to a CM-Sepharose column (1 cm × 5 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. Cytochrome *b*-562 was eluted with a linear gradient of 0–0.2 M-NaCl in 10 mM-potassium phosphate buffer, pH 7.0 (total volume of the gradient 150 ml). The fractions having significant absorbance at 418 nm were pooled and concentration was performed as described above. The concentrate was applied to a gel-filtration column [Fractogel TSK HW-50(S), 2 cm × 60 cm] and the chromatography was performed in 50 mM-potassium phosphate buffer, pH 7.0. Finally, h.p.l.c. gel filtration was carried out on a Serva Si-300-polyol column (0.5 cm × 9.1 cm) in 0.1 M-potassium phosphate buffer, pH 6.5, at a flow rate of 1 ml/min. The eluate was monitored with a Hewlett-Packard HP1040 A photodiode-array detector. GDH was purified as described previously [11].

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out in gel slabs of 7.7% polyacrylamide, cross-linked with 0.2% bisacrylamide, in 0.02 M-potassium phosphate buffer, pH 7.0, with the anode above the application side of the gel. Electrophoresis under denaturing conditions was performed on polyacrylamide gradient gels (Pharmacia, PAA 4/30) in the presence of SDS, with the use of markers from the high- M_r and low- M_r electrophoresis calibration kits as a reference (method described by Pharmacia [17]). Cytochrome staining was done with tetramethylbenzidine in accordance with Thomas *et al.* [18] and protein staining with Coomassie Blue R [19].

M_r determinations

The M_r of the native cytochrome *b*-562 was determined by the method of Andrews [20] by gel filtration on a Sephadex G-100 column (1 cm × 55 cm) in 50 mM-potassium phosphate buffer, pH 7.0, containing 0.1 M-NaCl, at a flow rate of 7.8 ml/h. Proteins used for calibration were horse heart cytochrome *c*, chymotrypsin, carbonic anhydrase and ovalbumin. The M_r of the SDS-denatured cytochrome was determined by electrophoresis as described above.

Isoelectric point

Isoelectric focusing was performed in a Phast-System apparatus (Pharmacia) with the use of markers from the broad-pI kit (Pharmacia) [21].

Potentiometric titrations

The cytochrome *b*-562 was titrated in 0.1 M-Hepes, pH 7.0, with the use of a mediator cocktail and the equipment described by van Wielink *et al.* [22]. Reductive titrations were performed by stepwise addition of an anaerobic solution of $\text{Na}_2\text{S}_2\text{O}_4$ to the cytochrome *b*-562 as isolated. In oxidative titrations the mixture was first reduced by the addition of an adequate amount of $\text{Na}_2\text{S}_2\text{O}_4$ before stepwise addition of an anaerobic solution of $\text{K}_3\text{Fe}(\text{CN})_6$. GDH was titrated in 0.1 M-Hepes, pH 7.0, with 20 μM -phenazine ethosulphate (PES), 25 μM -duroquinone and 25 μM -trimethylhydroquinone as mediators. Reductive titrations were performed by stepwise addition of anaerobic solutions of glucose to the enzyme as isolated. In oxidative titrations the mixture was first reduced by an appropriate amount of glucose.

Protein determinations

During purification, protein concentrations were determined by the method of Bradford [23], with bovine serum albumin (Serva, product no. 11920) as a standard. The concentrations of purified proteins were derived from their specific absorption coefficients (a) at 280 nm. For the purified cytochrome *b*-562 the a value was obtained from measurements at 205 and 280 nm by using the chromatographic procedure described by van Iersel *et al.* [24].

Cytochrome analysis

The cytochrome *b*-562 concentration was also calculated from absorption difference spectra, measured with a Hewlett-Packard model HP8450 UV/VIS spectrophotometer, by using the wavelength pair 562 nm–576 nm and the absorption coefficient calculated from haemochrome spectra (see below). Spectra at 77 K were recorded with a DW-2a spectrophotometer (American Instruments Co.) equipped with a low-temperature accessory (JA-9603, American Instruments Co.). Pyridine haemochrome spectra were measured in aqueous alkaline pyridine solutions and the haem content was determined by the method of Fuhrhop & Smith [25]. Absorption spectra of cells, membrane vesicles and cell-free extract were recorded with a Beckman UV5260 spectrophotometer at room temperature. The absorption coefficient and wavelength pair used to determine total haem *b* content were those used by Ensley & Finnerty [26].

Electron transfer from GDH to cytochrome *b*-562 *in vitro*

The ability of cytochrome *b*-562 to function as electron acceptor for GDH *in vitro* was determined by measuring the rate of increase of the absorbance difference between 562 and 576 nm. The experiments were performed in a quartz cuvette containing 0.1 M-Tris/HCl buffer, pH 7.0, cytochrome *b* (10 μM), GDH (10 μM) and glucose (10 mM) (total volume 3 ml). After cytochrome *b*-562 and GDH were mixed, the solution was made anaerobic by gassing with argon (< 3 p.p.m. O_2) for 30 min. The reaction was started by adding an anaerobic glucose solution. Experiments were also performed in which phospholipids (0.25 mg/ml) and/or ubiquinone-0 (Q-0) or ubiquinone-9 (Q-9) (300 μM) were added [the latter

compounds dissolved in ethanol, resulting in a final concentration of 5% (v/v) ethanol].

GDH assays

Activities of quinoprotein GDH with Wurster's Blue as electron acceptor were determined as described previously [11]. To test the natural quinone in this organism (Q-9; see Whittaker [27] and Makula *et al.* [28]) as electron acceptor, the method described by Matsushita *et al.* [29] was used. The assay mixture (3 ml) consisted of GDH (45 nM), Q-9 or Q-0 (60 μ M), glucose (10 mM), 0.1 M-potassium phosphate buffer, pH 7.0, and 5% (v/v) ethanol. The rate of absorbance change of 275 nm was measured in a stirred cuvette. Experiments were also performed in which the effect of cytochrome *b*-562 (100 nM) was tested on this system.

O₂ uptake rates

The O₂ consumption of whole cells, membrane vesicles and reconstituted proteoliposomes was assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) at 30 °C in 0.1 M-potassium phosphate buffer, pH 7.0, containing 10 mM-MgSO₄. Substrates tested were succinate (10 mM), ethanol (20 mM), glucose (20 mM) and duroquinol (300 μ M).

Membrane vesicles

Preparations of right-side-out membrane vesicles of *A. calcoaceticus* and the mutant 13.37 were made according to the method of Stinnett *et al.* [30], as modified by van Schie *et al.* [3]. Removal of GDH from the vesicles by incubation with Triton X-100 was carried out as described previously [31].

Phospholipids

Phospholipids were extracted from *A. calcoaceticus* cells by the method described by Viitanen *et al.* [32].

Proteoliposomes

Q-9 was mixed with phospholipids as described by Matsushita & Kaback [33]. Proteoliposomes were prepared by the detergent dilution method [34]. Sonicated *A. calcoaceticus* phospholipids in 50 mM-potassium phosphate buffer, pH 7.5, containing Q-9 (50 mg of lipids and 500 nmol of Q-9) were mixed with cytochrome *o* oxidase (0.6 mg) [prepared by a method devised by P. Dokter, J. E. van Wielink, A. Geerlof, L. F. Oltmann, A. H. Stouthamer & J. A. Duine (unpublished work)], GDH (0.2 mg), cytochrome *b*-562 (0.2 mg), octyl glucoside (final concn. 1.25%, w/v) and 50 mM-potassium phosphate buffer, pH 7.5 (final vol. 2 ml). The mixture was incubated on ice for 20 min and then diluted with 75 ml of 50 mM-potassium phosphate buffer, pH 7.5, having a temperature of 25 °C. The proteoliposomes formed were collected by centrifugation at 110 000 *g* and resuspended in 50 mM-potassium phosphate buffer, pH 7.5, containing 5 mM-MgSO₄. The presence of GDH was checked by assaying the rate of reduction of Wurster's Blue and that of cytochrome *o* oxidase by measuring the rate of reduction of duroquinol. The incorporation of Q-9 could be monitored from the change in colour of the liposomes from transparent white to light pink. The presence of cytochrome *b*-562 in the proteoliposomes was not checked.

RESULTS

Purification

Results of the purification procedure for the soluble cytochrome *b*-562 are presented in Table 1. Indications for the presence of other soluble cytochromes *b* during the purification steps were not found. The final preparation appeared to be homogeneous, as revealed by electrophoresis since a single band was observed after protein staining as well as after cytochrome staining.

Purification started with a cell-free extract containing a total amount of 1050 nmol of haem *b* and 8750 mg of protein. Assuming a quantitative recovery in the CM-Sepharose chromatography step, 3.3% of the haem *b* in the extract originates from the soluble cytochrome *b*-562, the rest originates from membrane-bound cytochromes *b*.

M_r determinations

The M_r of the native cytochrome *b*-562, determined by gel filtration, was 17 500 ($\pm$ 900). The M_r of the denatured cytochrome *b*-562 was 17 800 ($\pm$ 500), so therefore this cytochrome is a monomeric protein.

Isoelectric point

The pI was found to be 10.2, indicating that cytochrome *b*-562 is a basic protein, a property in accordance with its chromatographic behaviour.

Potentiometric titrations

From a reductive redox titration, the midpoint potential at pH 7.0 of cytochrome *b*-562 was calculated to be +170 mV. In the presence of quinoprotein GDH the midpoint potential of cytochrome *b*-562 was found to be +145 mV (Fig. 1). The midpoint potential of GDH itself was +50 mV (Fig. 2). With oxidative redox titrations essentially the same midpoint potentials were found.

Absorption spectra

The absorption spectrum of the cytochrome as it is isolated (Fig. 3) shows maxima at 418 and 360 nm. After

Table 1. Purification of cytochrome *b*-562

The quantity of protein in the final preparation was derived from the specific absorption coefficient (*a*) at 280 nm. For the quantity of cytochrome *b*-562 (Cyt. *b*-562), the *a* value was obtained from measurements at 205 and 280 nm in accordance with van Iersel *et al.* [24].

Step	Protein (mg)	Cyt. <i>b</i> -562 (nmol)	Cyt. <i>b</i> -562/protein (nmol/mg)	Yield (%)
CM-Sepharose (0.2 M buffer eluate)	160	35	0.22	100
CM-Sepharose (NaCl gradient)	9	24	2.6	70
Gel filtration (Fractogel)	1.1	9.6	8.7	27
Gel filtration (h.p.l.c.)	0.14	7.7	55	22

Fig. 1. Potentiometric titrations of cytochrome *b*-562

Titration of cytochrome *b*-562 (10 μ M) were performed in the absence (O) and in the presence (●) of GDH (10 μ M). The percentage reduction was calculated from the peak areas of the α -bands in the 540–580 nm region. The continuous line represents the best fit for the cytochrome *b*-562 in the presence of GDH (giving $n = 1$, n being the number of electrons involved in the oxidation reduction reaction of the cytochrome, and $E'_0 = +145$ mV). The broken line represents the best fit for cytochrome *b*-562 (giving $n = 1$ and $E'_0 = +170$ mV) in the absence of GDH. Only data for the reductive titrations are shown.

Fig. 2. Potentiometric titration of GDH

The percentage reduction of GDH (10 μ M) in 0.1 M-Hepes buffer, pH 7.0, containing 20 μ M-PES, 25 μ M-duroquinone and 25 μ M-trimethylhydroquinone as mediators was calculated from the absorbance at 338 nm. The line represents the best fit ($n = 1$, $E'_0 = +50$ mV). Data are given for the oxidative titration.

reduction with a few grains of NaBH_4 , maxima at 428, 530 and 562 nm were observed (Fig. 3). At 77 K, the α -band shifted to 560 nm and a shoulder was observed at 558 nm. Reduced cytochrome *b*-562 did not react with CO, as deduced from absorption difference spectra.

Fig. 3. Absorption spectra of cytochrome *b*-562

The absorption spectra of cytochrome *b*-562 (2.6 μ M) were measured in 0.1 M-potassium phosphate buffer, pH 6.5, before (—) and after (---) the addition of a few grains of NaBH_4 .

Haem identification and quantification

The absorption maxima at 418, 526 and 557 nm, observed in the pyridine haemochrome spectrum, are indicative of a protohaem. From these experiments, a quantity of 55 nmol of protohaem per mg of protein was calculated (the protein concentration was derived from the a value at 280 nm, which was estimated to be 13 litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$). Given the M_r of 18000, 0.96 protohaem group per cytochrome molecule was present. Therefore, assuming that protohaem is not removed during purification, cytochrome *b*-562 contains one protohaem group per molecule. Calculation of the molar absorption coefficient of cytochrome *b*-562 at the wavelength pair 562 nm–576 nm, by using the haem concentration derived from the pyridine haemochrome spectra, gave a value of 23 000 M $^{-1}$ $\cdot$ cm $^{-1}$.

Localization

Similarly to GDH, cytochrome *b*-562 appeared in the culture medium when *A. calcoaceticus* was grown in a mineral medium supplemented with 0.5% heptadecane and a low concentration (0.005%) of Triton X-100. Results described previously [10] indicated that significant amounts of periplasmic enzymes, but insignificant amounts of cytoplasmic enzymes, occur in the culture medium under such conditions. The identity and amount of cytochrome *b*-562 were determined after the first purification step with CM-Sephadex. No membrane-bound cytochromes *b* were detected in the culture medium.

Table 2 shows that the ratios of GDH to cytochrome *b*-562, present in the culture medium of several cultures of *A. calcoaceticus* and the mutant, are approximately 1:1. This could indicate that GDH and cytochrome *b*-562 occur in equimolar amounts in the periplasm. It is also clear that the inability of the mutant to oxidize glucose is not due to the absence of GDH or of cytochrome *b*-562 or to an aberrant ratio of these two components.

Reduction of cytochrome *b*-562 *in vitro*

Ferricytochrome *b*-562 (10 μM) was reducible with glucose in the presence of GDH (10 μM), though the activity (20 nmol of cytochrome *b*-562 reduced/min per mg of GDH) was rather low compared with the activity of GDH measured with Wurster's Blue as electron acceptor (40 μmol of Wurster's Blue reduced/min per mg of GDH). Anaerobic conditions were essential for activity determination since the ferrocyclochrome was auto-oxidizable. Addition of Q-0, Q-9 or phospholipids (0.25 mg/l) did not result in a change of the reduction rate of ferricytochrome *b*-562.

Enzyme activity with Q-0 and Q-9

GDH was not able to reduce Q-0 or Q-9 *in vitro* with glucose as substrate. Also, addition of cytochrome *b*-562 did not lead to reduction of ubiquinone. Ethanol at a concentration of 5% (v/v) did not inhibit the GDH activity, as assayed with Wurster's Blue, or inhibit the GDH-mediated reduction of cytochrome *b*-562.

Influence of O_2 concentrations on respiration rates

The respiration rate of cells grown in batch culture on ethanol did not depend on the O_2 concentration when ethanol was used as a substrate (Fig. 4). On the other hand, with glucose as a substrate the respiration rate

Table 2. Ratios of GDH to cytochrome *b*-562 in the culture medium

The amount of GDH was calculated by assuming a specific enzyme activity of 635 μmol of glucose/min per mg of protein [11] and an M_r of 94000 [11]. The amount of cytochrome *b*-562 (Cyt. *b*-562) in the CM-Sepharose eluate was calculated by using the absorption coefficient 23000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at the wavelength pair 562 nm–576 nm.

Strain (L.M.D.)	GDH (nmol)	Cyt. <i>b</i> -562 (nmol)	GDH/Cyt. <i>b</i> -562 ratio
13.37	14.1	14.1	1.0
N-12	15.2	12.2	1.2
79.41	9.1	10.6	0.9
79.41	13.5	16.2	0.8
79.41	10.1	9	1.1

Table 3. Respiration rates of whole cells and membrane vesicles

Cells of the wild-type strain and the mutant 13.37 originated from acetate-limited continuous cultures, grown at a dilution rate of 0.15 h^{-1} at 30 $^\circ\text{C}$.

Sample	Substrate ...	Respiration rate (nmol of O_2 /min per mg of protein)		
		Glucose (20 mM)	Glucose (20 mM) + PES (0.3 mM)	Succinate (10 mM)
Cells (strain)				
79.41		580	610	210
13.37		0	500	230
Vesicles (strain)				
79.41		1900	2300	90
13.37		0	1200	100

diminished substantially at lower O_2 concentrations (nearly 4-fold lower at 22.5 μM - O_2 compared with the value at 225 μM - O_2).

Comparison of oxidative capacities of wild-type and mutant

Cells and membrane vesicles of the wild-type organism were able to oxidize glucose as well as succinate (Table 3). The presence of the artificial electron acceptor PES did not lead to significant changes in the rates. Reduction of the *b*-type cytochromes was apparent from the absorption spectra of cells or membrane vesicles. Addition of purified GDH, cytochrome *b*-562 or Q-9 to membrane vesicles did not lead to an enhancement of O_2 consumption rates. Treatment with Triton X-100 resulted in vesicles that were completely unable to oxidize glucose, as described elsewhere [31]. After centrifugation and resuspension of the membrane

Fig. 4. Recorder tracings of O_2 consumption of whole cells

The initial air-saturated reaction mixture contained 0.1 M-potassium phosphate buffer, pH 7.0, 10 mM- MgSO_4 and whole cells (0.5 mg wet wt./ml, 75 μg of protein/ml). Glucose (20 mM) or ethanol (20 mM) was added at the time indicated by arrows.

Table 4. Comparison of soluble cytochromes *b*-562 from different bacteria

Abbreviations: (s), shoulder; N.D., not determined.

Property	<i>A. calcoaceticus</i> (present work)	<i>B. anitratum</i> [13,14]	<i>E. coli</i> [35,36]
$\lambda_{\max.}$ at 25 °C (oxidized preparation)	418 and 360 nm	N.D.	418 and 360(s) nm
$\lambda_{\max.}$ at 25 °C (reduced preparation)	562, 532 and 428 nm	562, 532 and 428 nm	562, 532 and 428 nm
$\lambda_{\max.}$ at 77 K (reduced preparation)	560 and 558(s) nm	N.D.	558 nm
M_r	18000	N.D.	12000
E'_0	+170 mV	+120–140 mV	+113 mV [35] +180 mV [36]
pI	10.2	N.D.	7–8

vesicles, activity could be restored to 25 % of the original value by addition of GDH plus cytochrome *b*-562.

Mutant 13.37 readily oxidized succinate but not glucose (Table 3). Not unexpectedly, addition of succinate to cells or membrane vesicles led to reduction of *b*-type cytochromes, but glucose did not have the same effect. Active GDH was present in this mutant, since addition of PES resulted in significant respiration rates with glucose. Removal of GDH from the vesicles with Triton X-100 and subsequent addition of wild-type GDH plus cytochrome *b*-562 did not induce activity. Most probably the GDH and cytochrome *b*-562 of mutant 13.37 are identical with those of the wild-type, as they showed the same behaviour on chromatography and electrophoresis (results not shown). The behaviour of mutant 13.37 is not unique, since 20 other mutants unable to oxidize glucose showed the same properties.

Glucose oxidation activity of proteoliposomes

The yield of phospholipid extraction (300 mg of 50 g wet wt. of *A. calcoaceticus* cells) was comparable with the yield obtained with *Escherichia coli* cells [32]. Proteoliposomes made with these phospholipids and containing GDH, cytochrome *b*-562, Q-9 and cytochrome *o*-type oxidase were unable to oxidize glucose. Incorporation of the components in the proteoliposomes seemed adequate, since 12 % of the added GDH and 15 % of the added cytochrome *o* were found in the preparations.

DISCUSSION

Cytochrome *b*-562 was purified to homogeneity. It appears to be a soluble cytochrome *b*, since detergents were not necessary to keep it solubilized once it was detached from the membranes. It is a basic (pI 10.2) monomeric protein (M_r 18000) containing one protohaem group per molecule. Although the soluble cytochrome *b* of *Bacterium anitratum* has only been partly characterized [13], most of its properties are very similar to those of cytochrome *b*-562 (Table 4). It is highly probable, therefore, that the cytochrome *b* described by Hauge [13] is identical with cytochrome *b*-562. As is also apparent from Table 4, the cytochrome *b*-562 from *E. coli* [35,36] is very similar to that from *A. calcoaceticus*.

GDH showed a midpoint potential of +50 mV at pH 7.0, a value comparable with that for the couple PQQ/PQQH₂ (+90 mV) [37]. The reduced GDH was

able to transfer electrons to ferricytochrome *b*-562, albeit at a low rate, but was unable to transfer them to Q-0, Q-9 or membrane-bound cytochromes *b* (results not shown). Attempts to enhance the rate by adding Q-0, Q-9 or phospholipids failed. A similar situation exists for the quinoprotein methanol dehydrogenase, where cytochrome *c_L* is a very poor electron acceptor in the assay carried out *in vitro*. Recently it was discovered that appreciable stimulation could be achieved by addition of an O₂-sensitive factor [38]. Therefore searches for the presence of such a factor in *A. calcoaceticus* might be worthwhile. Besides the possibility of electron transfer between reduced GDH and cytochrome *b*-562, another indication of the acceptor role of cytochrome *b*-562 is found in the fact that both components occur in the periplasm in a 1:1 ratio and most probably interact with each other, since the midpoint potential of cytochrome *b*-562 was significantly lowered in the presence of GDH.

Respiration rates of whole cells with glucose as a substrate were strongly dependent on the dissolved O₂ concentration but the same is not true for ethanol (ethanol oxidation in this organism proceeds via NAD- and NADP-dependent alcohol dehydrogenase [39,40], so that ethanol oxidation is in fact NAD(P)H oxidation), pointing to the possibility that the dehydrogenases, reduced by glucose and ethanol, transfer the electrons to different electron acceptors. A clear indication of such a possibility is apparent from the comparison of respiration capacities of wild-type and mutant strains: succinate and ethanol were oxidized at normal rates by the mutant, but glucose was not oxidized at all. Not unexpectedly, succinate and ethanol induced reduction of cytochromes *b* in cells and vesicles of the mutant whereas glucose did not. Since the mutant has normal concentrations of GDH and cytochrome *b*-562, which were active in assays and showed normal behaviour on chromatography and electrophoresis, and as addition of wild-type GDH to GDH-depleted vesicles from the mutant did not result in measurable activity, an O₂-sensitive factor (discussed above) might be present in the organism. This interpretation of the results can also explain why active proteoliposomes were not obtained, since adequate functioning was not achieved owing to the (partial) absence of this unknown factor.

The cofactor PQQ has a high redox potential compared with other coenzymes and cofactors. Analogously, quinoprotein dehydrogenases could have much higher midpoint potentials than other types of dehydrogenases.

Scheme 1. Electron transport from NADH and glucose to O_2 in *A. calcoaceticus*

Methanol dehydrogenase is indeed coupled to the respiratory chain at the level of cytochrome *c* [41], methylamine dehydrogenase has a midpoint potential of +100 mV [42], and its electron acceptor, the blue copper protein amicyanin, has a midpoint potential of between +260 and +294 mV [43]. Both dehydrogenases are situated in the periplasm, together with their natural electron acceptor proteins [44]. The results presented here show that GDH from *A. calcoaceticus* is no exception. The midpoint potential is high (+50 mV) and, as outlined above, the periplasm-located high redox-potential cytochrome *b*-562 is the most likely candidate for the primary electron acceptor for GDH in this organism.

Although the experiments carried out *in vitro* with Q-9 in this work gave no indication of a role as electron acceptor for either GDH or cytochrome *b*-562, Beardmore-Gray & Anthony [15] reported reduction of Q-9 in whole cells on addition of glucose. The latter report and the role of cytochrome *b*-562 as electron acceptor for GDH, taken together, suggest that Q-9 could function between cytochrome *b*-562 and the membrane-bound cytochromes *b* from the oxidase complex(es) (Scheme 1). However, the couple Q-9/Q-9H₂ has a redox potential (+90 mV) substantially lower than that of cytochrome *b*-562. Several explanations can be put forward to circumvent this difficulty. One of these is that electron transfer from glucose is only possible if the Q-9 pool is largely in the oxidized form. In other words, glucose oxidation occurs at high O_2 concentrations but scarcely at all at low O_2 concentrations, whereas the oxidation of other substrates, for which the electrons are donated at a lower level to the respiratory chain, is less sensitive to the O_2 concentration. This is precisely what is observed for the substrates glucose and ethanol (Fig. 4).

A quite opposite view of the role of soluble GDH and cytochrome *b*, as studied here, has been given by Beardmore-Gray & Anthony [15]. They stated [15] that they could find no evidence for the periplasmic location of soluble GDH and interaction of it with cytochrome *b*. However, it is clear from their paper that these authors missed the cytochrome *b*-562 that is reported here, since the pI of the cytochrome *b* was given as 4.5 and rapid loss of haem occurred so that its M_r could not be determined. On the basis of this it was reported that 'no evidence was obtained to support a previous suggestion that the soluble form of the dehydrogenase and the soluble cytochrome associated with it are involved in the

oxidation of glucose'. Although this statement is strictly invalid as it is contradicted by the findings of the present work, it is nevertheless worthwhile considering. In that view *A. calcoaceticus* contains, besides a soluble GDH, a membrane-bound GDH transferring its electrons to Q-9, just as has been reported for glucose oxidation via GDH in *Escherichia coli* [45] and *Pseudomonas fluorescens* [46]. The existence of two different types of GDH has already been proposed [11], and the occurrence of both in *A. calcoaceticus* cannot be excluded. It should be realized, however, that the implication of this hypothesis is that the role of soluble GDH and cytochrome *b*-562 remain unexplained.

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