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The PQQ-alcohol dehydrogenase of *Gluconacetobacter diazotrophicus*

Saúl Gómez-Manzo [a], Martha Contreras-Zentella [a], Alejandra González-Valdez [a], Martha Sosa-Torres [b], Roberto Arreguín-Espinoza [c], Edgardo Escamilla-Marván [a,*]

[a] *Departamento de Bioquímica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, A. P. 70242, México, D.F. 04510, México*
[b] *División de Estudios de Posgrado, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, México D.F. 04510, México*
[c] *Departamento de Bioquímica, Instituto de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, México D.F. 04510, México*

Abstract

The oxidation of ethanol to acetic acid is the most characteristic process in acetic acid bacteria.*Gluconacetobacter diazotrophicus* is rather unique among the acetic acid bacteria as it carries out nitrogen fixation and is a true endophyte, originally isolated from sugar cane. Aside its peculiar life style, *Ga. diazotrophicus*, possesses a constitutive membrane-bound oxidase system for ethanol. The Alcohol dehydrogenase complex (ADH) of *Ga. diazotrophicus* was purified to homogeneity from the membrane fraction. It-exhibited two subunits with molecular masses of 71.4 kDa and 43.5 kDa. A positive peroxidase reaction confirmed the presence of cytochrome *c* in both subunits. Pyrroloquinoline quinone (PQQ) of ADH was identified by UV–visible light and fluorescence spectroscopy. The enzyme was purified in its full reduced state; potassium ferricyanide induced its oxidation. Ethanol or acetaldehyde restored the full reduced state. The enzyme showed an isoelectric point (pI) of 6.1 and its optimal pH was 6.0. Both ethanol and acetaldehyde were oxidized at almost the same rate, thus suggesting that the ADH complex of *Ga. diazotrophicus* could be kinetically competent to catalyze, at least in vitro, the double oxidation of ethanol to acetic acid.

Keywords: *G. diazotrophicus*; Alcohol dehydrogenase; PQQ; Cytochrome *c*; Respiratory system

1. Introduction

Fermentation industries producing vinegar, ascorbic acid, dihydroxyacetone, sorbose and other products of high commercial value, have exploited the tremendous metabolic power of acetic acid bacteria to oxidize a wide range of sugars, alcohols and aldehydes (Adachi et al., 2003). Such oxidation reactions are termed "oxidative fermentations", since they involve incomplete oxidation of substrates accompanied by accumulation of huge quantities of the oxidation products in the growth medium (Matsushita et al., 1994). In addition to the standard membrane respiratory complexes found in other aerobic bacteria, acetic acid bacteria possess a large and diverse set of membrane-bound dehydrogenases localized in the periplasmic space; these enzymes deliver electrons to the respiratory chain (Matsushita et al., 1994). Almost all primary membrane dehydrogenases involved in the alcohol- and sugar-oxidizing systems of acetic acid bacteria have their substrate site oriented to the periplasmic space and are either quinoproteins, which contain PQQ, or flavoproteins containing FAD. Some quinoproteins also contain, type-*c* cytochromes, therefore they are named quinohemoproteins (Goodwin and Anthony, 1998).

The oxidation of ethanol to acetic acid is the most characteristic process in acetic acid bacteria and is catalyzed by two membrane-bound enzymes complexes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). These enzymes are usually recognized as quinohemoproteins since they have PQQ and cytochromes *c* as prosthetic groups. Quinohemoprotein ADHs have been solubilized by detergents and purified to homogeneity from *Acetobacter* spp. (Adachi et al., 1978a; Adachi et al., 1978b;Tayama et al., 1989; Frébortová et al., 1997), *Gluconobacter* spp. (Ameyama and Adachi, 1982; Matsushita et al., 1995; Matsushita et al., 1996) and *Gluconacetobacter* ssp. (Trcek et al., 2006). So far, all the purified enzymes are heteroligomers constituted by a 72–80 kDa protein (S-I) having a single moiety of each, PQQ and cytochrome *c*; a

* Corresponding author. Tel.: +52 55 56225627; fax: +52 55 56225630.
E-mail address: eescami@ifc.unam.mx (E. Escamilla-Marván).

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44–48 kDa protein (S-II) containing three cytochromes *c*. In some cases, an additional 8–16 kDa protein (S-III) with unknown function has been reported (Kondo and Horinuochi, 1997).

Gluconacetobacter diazotrophicus is rather unique among the acetic acid bacteria because it carries out nitrogen fixation and is a true endophyte originally isolated from sugar cane (Alvarez and Martínez-Drets, 1995; Cavalcante and Döbereiner, 1988; Gillis et al., 1989; Jiménez-Salgado et al., 1997). Its presence in soils as free living bacteria has not been reported. In addition to its peculiar life style, *Ga. diazotrophicus* possesses a constitutive periplasmic oxidizing system for ethanol and acetaldehyde that is upregulated during N_2-dependent growth (Flores-Encarnación et al., 1999; Lee et al., 2004). In the present communication we describe the purification and kinetic properties of the membrane-bound ADH from *Ga. diazotrophicus*. The substrate specificity of the purified ADH was rather unique since both ethanol and acetaldehyde were oxidized at almost equal rates.

2. Materials and methods

2.1. Chemicals

Ethanol, $CaCl_2$, $MgCl_2$, K_2HPO_4, KH_2PO_4, phenylmethylsulfonyl fluoride, NaCl, Triton X-100, potassium ferricyanide, phenazine methosulphate, 2,6 dichlorophenolindophenol, glucose, Coomassie brilliant blue R, nitroblue tetrazolium, dimethylsulfoxide, sodium dithionite, ammonium persulfate, bovine serum albumin, acetaldehyde, HA-Ultrogel and Sephacryl-200 were obtained from Sigma-Aldrich Corporation, St. Louis Missouri, USA. QAE-Toyopearl was obtained from SUPELCO-Sigma-Aldrich Corporation, St. Louis Missouri, USA. Natriumlauryl-sulfate (SDS), acrylamide and bis-acrylamide were obtained from Bio-Rad Laboratories, Inc., Hercules, California, USA. Pyridine was obtained from MERCK & Co., Inc, Germany.

2.2. Strain, culture methods and preparation of membranes

Ga. diazotrophicus PAL5 (ATCC 49037) was grown at 30 °C in a sugar-rich medium (SRM) consisted of: 5 g K_2HPO_4, 4 g KH_2PO_4, 0.2 g $MgCl_2$, 0.02 g $CaCl_2$, 0.01 g $FeCl_3$, 5 g sodium citrate, 0.2642 g $(NH_4)_2SO_4$, 0.002 g Na_2MoO_2, and 50 g sucrose in 1 l of water (pH adjusted to 5.5) and in the same medium but supplemented with 0.75% ethanol (SREM); in a 60 l working-volume Bioflow 5000 fermentor (New Brunswick Scientific, NJ, USA) stirred at 350 rpm and sparged with 60 l air min^{-1}. The fermentor was seeded with 2 l of an active culture. Cells were harvested in the early stationary phase (48 h) and washed twice with cold 50 mM potassium phosphate buffer (pH 6.0) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The cell suspension was supplemented with phenylmethylsulfonyl fluoride (15 μg ml^{-1}) and disrupted in a Dyno-mill (WAB Maschinen-Fabrik, Basel, Switzerland). The membranes were prepared as described by Escamilla, Ramirez, Del Arenal, Zarzosa and Linares (1987). Membranes were stored under liquid nitrogen without appreciable lost of enzymatic respiratory activities.

2.3. Purification of the ADH complex

Cell membranes (10 mg protein ml^{-1}) were suspended in a 10 mM potassium phosphate buffer, pH 6.0 (KP buffer). Triton X-100 was added to a final concentration of 0.5% (v/v) and incubated at 4 °C for 2 h under gently stirring. The mixture was thereafter centrifuged at 144,000 ×g for 60 min and the supernatant (4 mg protein ml^{-1}) containing the ethanol dehydrogenase activity was applied to a QAE-Toyopearl column (3 × 18 cm) previously equilibrated with 0.01 M KP buffer containing 0.1% Triton X-100. Non-retained protein was washed away with 3 bed-volumes of the same buffer. ADH was eluted with a 0 to 0.25 M NaCl linear gradient in equilibration buffer. Fractions containing ethanol dehydrogenase activity were pooled and dialyzed overnight against 20 volumes of KP buffer containing 0.1% Triton X-100. The dialyzed ADH fraction was applied to a HA-Ultrogel column (3 × 15 cm) previously equilibrated with KP buffer containing 0.1% Triton X-100. Non-retained protein was washed as above and ADH was eluted with a 0 to 0.25 M potassium phosphate linear gradient containing 0.1% Triton X-100 (pH 6.0). The active fractions were collected and 10-fold concentrated in an Amicon YM30 MW filtration system (Amicon Corporation, Danvers, Mass, USA). The concentrated fractions were applied to a Sephacryl-200 column (64 × 5 cm), previously equilibrated with five volumes of KP buffer containing 0.1% Triton X-100. The active fractions were pooled, concentrated as above and stored at 4 °C for further analysis.

2.4. Enzymatic activity assays

Dehydrogenase activities associated to membranes and purified fractions were determined by spectrophotometry using potassium ferricyanide as the electron acceptor according to the standard method described by Matsushita et al. (1995), or with phenazine methosulphate (PMS) plus 2,6-dichlorophenolindophenol (DCPIP) as electron acceptors (Adachi et al., 1978a). A pH of 6.0 was routinely used. Ethanol, acetaldehyde, glucose and other substrates tested were used at a concentration of 20 mM. In the case of glucose dehydrogenase activity, the method with PMS plus DCPIP was used. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1.0 μmol of substrate per minute under the conditions indicated.

2.5. Analytical procedures

2.5.1. Electrophoresis

The purified ADH of *Ga. diazotrophicus* was analysed by SDS-PAGE in 16 × 14 cm slab gels with 10% polyacrylamide and 5% stacking gel by the method of Goodhew, Brown andPettigrew (1986). For native PAGE, the same system was used, except that 7.5% polyacrylamide resolving gel was used and the detergent SDS was replaced by 0.1%Triton X-100.

Native gels were stained for protein with 0.05 % Coomassie brilliant blue R-250 or alternatively, for enzyme activity, in a medium containing 2 mM PMS, 0.34 mM nitroblue tetrazolium (NTB) and 100 mM of either, ethanol or acetaldehyde (Adachi et al., 1978a). The heme-catalyzed peroxidase staining assay described by Thomas, Ryan and Levin (1976) was used to detect cytochrome *c* bands in SDS-gels.

The isoelectric point (pI) of the purified ADH was determined with the Phast system and gels with a pH range of 3.4 to 9.0 (Amersham Biosciences).

2.5.2. Identification of PQQ

The prosthetic group PQQ of ADH was spectroscopically identified from its absorption spectrum at 25 °C. The purified enzyme solution was mixed with 9 volumes of methanol. After incubation at 25 °C for 30 min, the extract was centrifuged at 3000 ×g for 10 min. The supernatant was concentrated by evaporation and used as the PQQ-fraction extracted. Fluorescence emission at 25 °C was scanned from 290 to 460 nm at an excitation wavelength of 370 nm for PQQ (Matsushita et al., 1995).

2.5.3. Determination of cytochromes and hemes

The purified enzyme was suspended in KP buffer containing 50% (v/v) dimethylsulfoxide and examined with an OLIS-SLM DW 2000 spectrophotometer, using cuvettes with a 2-mm light path. Samples were reduced with 20 mM substrate or a few grains of sodium dithionite, whereas references were oxidized with a few grains of ammonium persulfate. The cytochrome *c* (heme C) content of the purified ADH complex was calculated from the dithionite-reduced minus persulfate-oxidized spectrum of its pyridine hemechrome, which was prepared by mixing the sample with 20% pyridine and 0.2 M NaOH final concentrations. An absorption coefficient of 19.1 mM^{-1} cm^{-1} (550–540 nm) was used (Escamilla et al., 1987; Matsushita et al., 1992).

2.6. Other methods

Protein concentrations were determined according to Dulley and Grieve (1975), using bovine serum albumin as standard.

3. Results and discussion

3.1. Expression of ADH and detergent solubilization

We previously showed (Flores-Encarnación et al., 1999), that *Ga. diazotrophicus* express higher levels of periplasmic dehydrogenase activities during growth in batch culture under nitrogen-fixing conditions in SRM medium. Here, we optimized the conditions for the expression levels of ADH. A five fold increase in the ethanol-ferricyanide reductase activity (i.e. 10.8 units/mg membrane protein) was obtained by replacing sucrose with 0.75% ethanol as sole carbon source in the SRM medium; however ethanol was a poor carbon source. On the other hand, a significant up-regulation of ADH (i.e. 7.6 units/mg membrane protein) without compromising growth yield was obtained when ethanol was added as second carbon source (SREM). Under the culture conditions tested, the expression of ALDH paralleled the expression of ADH, without affecting the expression of glucose dehydrogenase (GDH). Thus, the SREM medium was routinely used.

ADH and ALDH complexes of *Ga. diazotrophicus* were conveniently released from membranes by 0.5% Triton X-100. Higher detergent concentrations (i.e. 1.2%) were required for the solubilization of GDH.

3.2. Chromatographic separation of ADH from ALDH

The protein released from membranes (2.5 g of protein) by 0.5%Triton X-100 was applied to QAE-Toyopearl column (Fig. 1) as described under Materials and methods. The protein that eluted by the 0 to 0.25 M NaCl linear gradient was distributed in two main peaks (Fig. 1A). The first peak eluted at 0.06 M NaCl contained the ethanol-ferricyanide reductase activity; the same fractions were also catalytically active with acetaldehyde. A second peak eluted at 0.11 M NaCl exhibited activity with

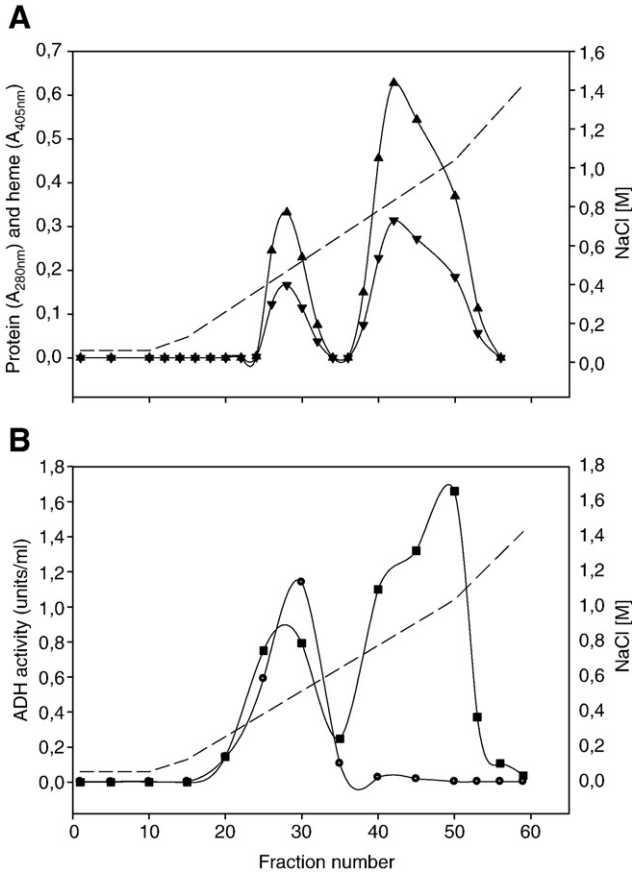

Fig. 1. Separation of ADH and ALDH complexes by anionic exchange chromatography (QAE-Toyopearl) column. Protein (2.5 g protein) solubilized in 0.5% Triton X-100 was applied to a QAE-Toyopearl column (3×18 cm) previously equilibrated with 0.01 M potassium phosphate buffer (pH 6) containing 0.1% Triton X-100. The enzyme was eluted with a linear gradient of NaCl (0 to 0.25 M). (A) Protein (A_{280},▲) and heme (A_{405},▼) elution profiles. (B) Elution profiles for the ferricyanide reductase activities with ethanol (●) and acetaldehyde (■). Broken line in both panels shows the profile of the NaCl molar gradient used.

Table 1
Purification summary of membrane-bound ADH from *Ga. diazotrophicus*

Purification steps	Total (mg)	Specific activity (units/mg)[a]	Total activity (units)	Yield (%)
Membrane	2515	6	15090	100
Solubilized	546	15	8190	54
QAE-Toyopearl	228	21	4788	31
DEAE-Toyopearl	62	54	3348	22
Ha-Ultrogel	15	180	2700	18
Sephacryl 200	9	258	2250	15

[a] The ferricyanide reductase activity was determined under the standard conditions described in Materials and methods.

acetaldehyde and showed no activity with ethanol (Fig. 1B). Both peaks were orange coloured and had strong absorption at 405 nm. Further purification steps (Table 1) rendered a homogeneous ADH complex that was active on both, ethanol and acetaldehyde (see below).

3.3. Molecular characterization

Native PAGE analysis of the purified ADH complex showed a homogeneous protein band with a Mr of 115 kDa. In the zymographic activity assay, the band was equally stained by ethanol (Fig. 2A) or acetaldehyde (not shown), as alternative electron donors. Under denaturing conditions in SDS-PAGE (Fig. 2B), the purified ADH was dissociated into two bands with molecular weights of 71.4 kDa and 43.5 kDa, as determined by mass spectroscopy (unpublished results); they were equally stained by Coomassie blue R 250. Both protein bands gave positive reaction with the heme-peroxidase staining assay; however, the stain was stronger with the 43.5 kDa band, suggesting that it had a larger content of cytochrome *c*. The ADH complex contains 4 mol of heme *c* per mol of enzyme complex as determined from the pyridine hemechrome spectrum (not shown). Thus, the ADH complex of *Ga. diazotrophicus* belongs to the group of membrane-ADHs that have two subunits. Interestingly, all the membrane-bound ADHs so far purified from *Gluconacetobacter* species (Table 2 and references therein) are hetorodimers formed by 71–72 kDa and 43–45 kDa subunits (SI and SII, respectively). In contrast, among the few cases so far reported of *Acetobacter* and *Gluconobacter* species besides the catalytic core SI–SII, and additional 8–16 kDa subunit (SIII) with unknown function has been described (Table 2 and references therein). Thus, we suggest that the SI–SII heterodimeric structure found repetitively in *Gluconacetobacter* species could be a distinctive feature of the genus; in fact the SI–SII heterodimeric structure seems to be the minimal catalytic unit of membrane-bound ADHs of acetic acid bacteria. In this regard, it is recalled that there are genetic and biochemical evidence that show that the smallest subunit of the three-subunit ADH from *G. suboxydans* is not essential for activity (Kondo et al., 1997).

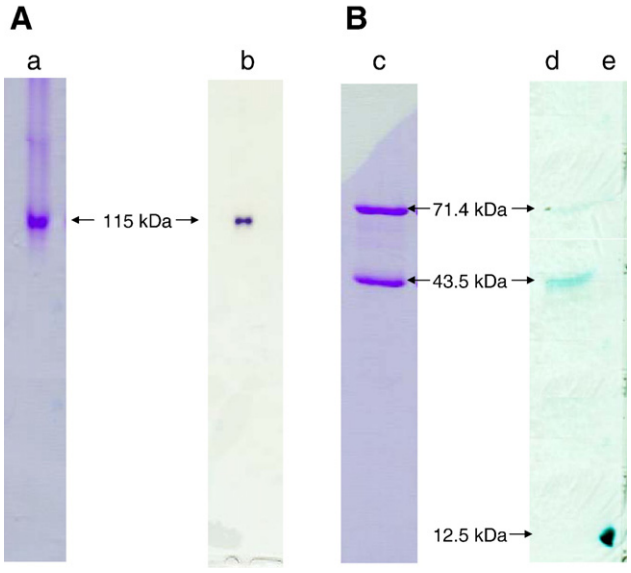

Fig. 2. (A) Polyacrylamide gel electrophoresis (PAGE) analysis of the purified ADH complex (100 μg protein) stained with, (a) Coomassie brilliant blue R 250 and, (b) ethanol dehydrogenase activity stained by zymography with Phenazine methasulfate and nitro blue tetrazolium. (B). SDS-PAGE analysis of the ADH-subunits stained by (c) Coomassie brilliant blue R 250 and (d) peroxidase activity associated to heme-*c* stained by zymography. (e) Horse cytochrome *c* (12.5 kDa; 10 μg) was included as positive control of the peroxidase stain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Spectroscopic properties

The ultraviolet/visible absorption spectrum of ADH complex "as prepared" (Fig. 3A) showed that the enzyme remained essentially reduced during its purification; indeed, incubation with ethanol or dithionite did not increase significantly the initial reduction levels (not shown). This suggested the presence of an unidentified reducing agent during its purification and that the enzyme is poorly auto-oxidizable. Similar results have been reported for other ADHs and it has been claimed that these are due to contaminating trace amounts of alcohols in the chemicals used during purification (Adachi et al, 1978a; Toyama et al., 2004). In our case, it must be borne in mind that the detergent used during purification (Triton X-100) possesses an ethoxyethanol residue that may be a potential substrate for ADH. The ultraviolet/visible absorption spectrum of the purified enzyme showed (Fig. 3A), the typical spectral signature of authentic

Table 2
Molecular structure of membrane-bound ADHs purified from acetic acid bacteria

Organism	Molecular mass subunit (kDA)			Reference/source
	I	II	II	
A. aceti	72	50	15	Matsushita et al. (1992)
A. methanolicus	80	54	8	Frébortová et al. (1997)
A. pasteurianus	74	44	16	Kondo et al. (1997) Trcek et al. (2006)
G. suboxydans	78	46	14	Matsushita et al. (1995)
Ga. europaeus	72	45	–	Trcek et al. (2006)
Ga. intermedius	72	45	–	Trcek et al. (2006)
Ga. polyoxogenes	72	44	–	Tayama et al. (1989)
Ga. xylinus	71	44	–	Unpublished results
Ga. diazotrophicus	71.4	43.5	–	This work

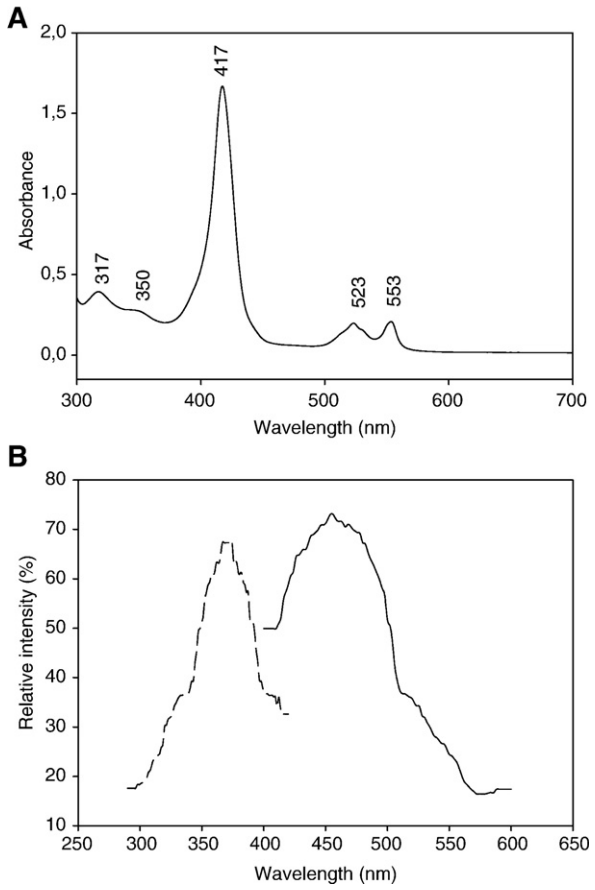

Fig. 3. Spectroscopic properties of the purified ALDH-complex of *Ga. diazotrophicus*. An enzyme preparation (0.5 mg of protein) with a specific activity of 256 units/mg protein was used for both panels. (A) Ultraviolet/visible absorption spectrum and (B) excitation/emission fluorescence spectra. The UV/visible difference spectrum was generated by adding sodium dithionite and ammonium persulfate to sample and reference cuvettes, respectively. For the fluorescence spectra, the same enzyme preparation was treated with methanol as described in Materials and methods and the methanol extract was used to generate the excitation spectrum (broken trace) and emission spectrum (solid trace).

PQQ (peaks at 317 and 350 nm) and maxima of reduced cytochrome *c* (peaks at 417, 523 and 553 nm). The existence of PQQ was further confirmed by fluorescence spectroscopy of the methanol-fraction extracted from purified ADH complex (Fig. 3B). Its excitation at 370 nm produced the characteristic emission signal at 480 nm of the authentic PQQ (Duine et al., 1987; Duine and Jongejan, 1989).

3.5. Kinetic characterization

The substrate specificity of ADHs of acetic acid bacteria (membrane-bound ADHs-III), is relatively restricted when compared with the soluble-type (ADHs-I and ADHs-II) quinoprotein alcohol dehydrogenases (Goodwin et al); generally, the ADH-III enzymes oxidize primary alcohols (C_2–C_6) but they do not oxidize methanol, secondary alcohols or aldehydes (Goodwin et al., 1998; Matsushita et al., 1992, 1994). However, the ADH of *Ga. polyoxogenes* exhibited some activity with formaldehyde and acetaldehyde (Tayama et al., 1989). Since our data (Fig. 1) suggested that the ADH of *Ga. diazotrophicus* could be exceptionally active with acetaldehyde, we studied the substrate specificity of the purified enzyme (Table 3). Similarly to other membrane-bound ADHs, the enzyme of *Ga. diazotrophicus* oxidizes primary alcohols (C_2–C_4); interestingly, allyl alcohol was the best substrate, followed by *n*-butanol, ethanol and *n*-propanol. Isopropanol was oxidized poorly and methanol was not a substrate. On the other hand, formaldehyde, propionaldehyde and acetaldehyde were oxidized with high efficiency. Indeed, acetaldehyde was almost as good substrate as ethanol, i.e. yielding 91% relative activity (Table 3). The Michaelis constants (K_m) of our purified ADH complex for ethanol and acetaldehyde were 4.6×10^{-4} M and 2.9×10^{-3} M, respectively (unpublished results).The substrate specificity of purified ADHs of acetic acid bacteria, has been tested in few case only. The ADHs of *A. aceti* (Ameyama et al., 1982) and *G. suboxidans* (Adachi et al, 1978a,b; Ameyama et al, 1982), could not oxidize aldehydes at all. On the other hand, the ADH of *Ga. polyoxogenes* showed 60% and 18% of relative activity on formaldehyde and acetaldehyde respectively, as compared with ethanol (Tayama et al., 1989). Thus, the ADH complex of *Ga. diazotrophicus* seems to have a distinctive substrate specificity that recalls the catalytic properties of soluble type (ADH-I and ADH-II) quinoprotein alcohol dehydrogenases (Goodwin et al, 1998).

3.6. Optimal pH, isoelectric point and thermostability of purified ADH

Ferricyanide reductase activity of the purified ADH showed a symmetrical and sharp response to pH; maximal activity was detected at pH 6.0 with either ethanol (Fig. 4A) or acetaldehyde (not shown) as electron donors. The same optimal pH was obtained when PMS plus DCPIP were used as electron acceptors (not shown). These results indicate that the catalytic

Table 3
Substrate specifity of purified membrane-bound DH of *Ga. diaxotrophicus*

Substrates	Relative activity (%)[a]
Methanol	9
Ethanol	95
n-Propanol	90
n-Butanol	98
Allyl alcohol	100
Isopropanol	18
Gycerol	0
Sorbitol	0
Acetone	0
Formaldehyde	4
Acetaldehyde	87
Glutaraldehyde	25
Propionaldehyde	42
Benzaldehyde	0
Glucose	0

[a] An enzyme preparation with a specific activity (ethanol as substrate) of 256 units/mg was used. The ferricyanide reductase activity was measured under the standard conditions described in Materials and methods. Substrates were used at final concentration of 20 mM. Enzyme reaction was carried out in the presence of 0.1% Triton X-100.

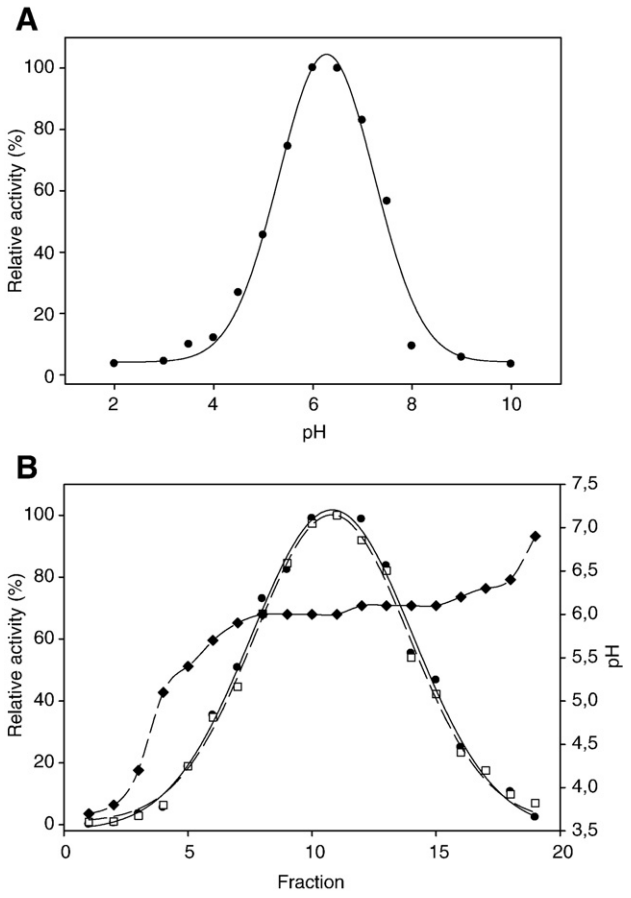

Fig. 4. pH dependent properties of the purified ADH complex (A) pH profile for the ethanol-ferricyanide reductase activity. The activity was measured in Mcllvaine buffer at pH 3.0 to 9.0 (B) the isoelectric focusing of the purified ADH-complex. (pI), for the (●) ethanol and (□) acetaldehyde ferricyanide reductase activities associated to the purified ADH-complex, was determined in the BioRad Phast system in the presence of a (♦) 3.4 to 9 pH gradient of polyampholites, using 0.10 mg of purified ADH with a specific activity of 256 units mg protein^{-1}.

response to pH fully depends on the properties of the enzyme complex, regardless of the substrate and the electron acceptors used. Contrasting with our results, the kinetic response of previously purified membrane-bound ADHs towards pH is rather complex and depends on the bacterial source and electron acceptors used. The three-subunit ADH of *G. suboxydans* showed ferricyanide reductase in a broad pH range from acidic to neutral (Matsushita et al., 1996) whereas, the three-subunit ADH of *A. methanolicus* exhibited a more complex response with different optimal pH values that depend on the electron acceptor used (Frébortova et al., 1998). The three-subunit ADH of *A. aceti* (Adachi et al., 1978a), exhibited a ferricyanide reductase activity with a single and sharp optimum at pH 4.0. Finally for the two-subunit ADH of *Ga. polyoxogenes* (Tayama et al., 1989), the optimum pH was in the range of pH 5.0 to 6.0 for the ferricyanide reductase activity. Membrane-bound ADHs of acetic acid bacteria posses multiple redox centres, which are potentially reactive sites from which electrons can be withdrawn by redox-dye electron acceptors. Therefore, it is it likely that the complex response to pH obtained for some ADHs with different electron acceptors, reflects the properties of particular redox centres in the enzyme. In fact, Matsushita et al. (1996) have decomposed the broad pH profile of ferricyanide reductase of *Ga. suboxydans* in four theoretical pH-profiles that corresponds to the same number of cytochrome *c* centres in the enzyme. Accordingly, the single and sharp optimum pH obtained for the ferricyanide reductase activity (Fig 4), suggests that only one cytochrome *c* centre, among the four present in the ADH of *Ga. diazotrophicus*, reacts with ferricyanide. This conclusion might be extended to the ADHs of *Ga. polyoxogenes* (Tayama et al., 1989) and *A. aceti* (Adachi et al., 1978a).

An isoelectric point (pI) of 6.1 was calculated for the purified ADH complex of *Ga. diazotrophicus* (Fig. 4B), as determined in the Phast system in the presence of polyampholites with a pH gradient of 3.4 to 9.0. The pI profile and relative ethanol-ferricyanide and acetaldehyde-ferricyanide reductase activities were almost identical. The pI determined for our ADH complex was less acidic than that (i.e. pI = 5.1) reported for the ADH of *G. suboxydans* (Matsushita et al., 1996).

The thermal stability of the purified ADH complex was tested. After incubation at 40 °C for increasing periods (i.e. 0 to 20 min), the ferricyanide reductase activities with ethanol or acetaldehyde, as alternative substrates, were determined at 30 °C (not shown). The inactivation profile revealed that the *Ga. diazotrophicus* ADH complex is fairly thermosensible. The ferricyanide reductase activities with ethanol or acetaldehyde decline in parallel with first order kinetics; after 15 min of incubation, 80% of the activity was lost.

3.7. Intramolecular electron transfer

Electrons removed from substrate by ADH complex are initially transferred to the PQQ centre and further tunnelled across four cytochromes *c*, (one in subunit I, *c*I and three in

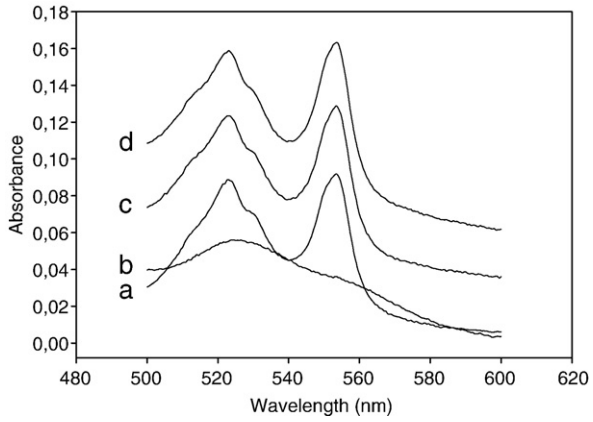

Fig. 5. Redox properties of the purified ADH-complex of *Ga. diazotrophicus*. An enzyme preparation containing 100 μg protein, with a specific activity of 256 units, was used. (a) Spectrum of the ADH complex "as prepared". (b) Spectrum of oxidized ADH-complex, obtained after titration of the enzyme with small amounts of potassium ferricyanide. (c) Spectrum of the ethanol-reduced enzyme generated by addition of 20 mM ethanol to the enzyme previously oxidized by ferricyanide. (d) Spectrum of the "fully reduced enzyme" obtained after addition of sodium dithionite to the enzyme previously reduced by ethanol. Note that dithionite did not increase the reduction levels previously evoked by ethanol.

subunit II, cII$_1$, cII$_2$ and cII$_3$), to the membrane ubiquinone (UQ_{9-10}). Matsushita et al., (1995) have detected and characterized a second type of ADH (named "inactive ADH") in *G. suboxydans*. Compared to the normal "active ADH", the purified "inactive ADH" was 10-fold less active and 37% less reduced as compared to the "active ADH". Ethanol did not increase the reduction level of the purified "inactive ADH". Thus, it was concluded that one of four cytochromes *c* of ADH is not functional in the "inactive ADH". Here, the redox properties of the ADH complex of *Ga. diazotrophicus* were examined (Fig. 5). As noted (Fig. 3a), the purified enzyme "as prepared" was in its reduced state (Fig. 5, trace a) and thus, its reduction level could not be increased by the addition of ethanol (not shown). Addition of small amounts of potassium ferricyanide induced full oxidation (Fig. 5, trace b). The ferricyanide-oxidized enzyme reversed to its original reduction state after a few seconds of incubation with ethanol (Fig. 5, trace c), or acetaldehyde (not shown). The reduction level produced by substrates was not increased by the further addition of dithionite (Fig. 5, trace d). These data indicate that the four cytochrome *c* sites in the purified enzyme are fully reducible by substrate and catalytically active.

4. Conclusions

The ADH and ALDH complexes of *Ga. diazotrophicus* were separated by column chromatography. The ADH complex was further purified to homogeneity. Its molecular characterization revealed that the enzyme is a heterodimer formed by a 71.4 kDa subunit I and a 43.5 kDa SII. The ADH complex contained one moiety of PQQ and four cytochromes *c*, as revealed by spectroscopic analysis of the methanol extract and pyridine hemochromes derived from the purified enzyme, respectively. So far, The SI–SII heterodimeric structure has been found in all the ADHs purified from *Gluconacetobacter* species; therefore, we conclude that the heterodimeric structure of ADHs could be a distinctive feature of the *Gluconacetobacter* genus that in contrast with the heterotrimeric structure found in membrane-bound ADHs, so far purified, from *Acetobacter* and *Gluconobacter* species.

The purified ADH complex showed an isoelectric point (pI) of 6.1 and an optimal pH of 6.0 for the ferricyanide and PMS-DCPIP reductase activities. The purified enzyme preferentially oxidized aliphatic alcohols with a straight carbon chain, except for methanol. Aldehydes, including formaldehyde, were also oxidizable substrates. Noteworthy, ethanol and acetaldehyde were oxidized at almost equal rates. Whether the catalytic properties of ADH from *Ga. diazotrophicus* endow this enzyme with the potential capacity to produce acetic acid from ethanol without the necessity of ALDH warrants further investigation.

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