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# The Membrane-Bound Quinohemoprotein Alcohol Dehydrogenase from *Gluconacetobacter diazotrophicus* PAL5 Carries a [2Fe-2S] Cluster[†]

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ABSTRACT: *Gluconacetobacter diazotrophicus* stands out among the acetic acid bacteria as it fixes dinitrogen and is a true endophyte. It has a set of constitutive enzymes to oxidize ethanol and acetaldehyde which is upregulated during $N_2$-dependent growth. The membrane-bound alcohol dehydrogenase (ADH) is a heterodimer (subunit I ≈ 72 kDa, subunit II ≈ 44 kDa) and constitutes an important component of this organism. ADH of *Ga. diazotrophicus* is a typical quinohemoprotein with one pyrroloquinoline quinone (PQQ) and four *c*-type cytochromes. For the first time, a [2Fe-2S] cluster has been identified by EPR spectroscopy in this type of enzyme. This finding is supported by quantitative chemical analysis, revealing 5.90 ± 0.15 Fe and 2.06 ± 0.10 acid-labile sulfurs per ADH heterodimer. The X-band EPR spectrum of ADH (as isolated in the presence of dioxygen, 20 K) showed three broad resonances at *g* 2.007, 1.941, and 1.920 ($g_{av}$ 1.956), as well as an intense narrow line centered at $g = 2.0034$. The latter signal, which was still detected at 100 K, was attributed to the PQQ semiquinone radical ($PQQ_{sq}$). The broad resonances observed at lower temperature were assigned to the [2Fe-2S] cluster in the one-electron reduced state. The oxidation−reduction potentials $E_m$ (pH 6.0 vs SHE) of the four *c*-type cytochromes were estimated to $E_{m1} = -64$ (±2) mV, $E_{m2} = -8$ (±2) mV, $E_{m3} = +185$ (±15) mV, and $E_{m4} = +210$ (±10) mV (spectroelectrochemistry), $E_{mFeS} = -250$ (±5) mV for the [2Fe-2S] cluster, and $E_{mPQQ} = -210$ (±5) mV for the $PQQ/PQQH_2$ couple (EPR spectroscopy). We propose a model for the membrane-bound ADH of *Ga. diazotrophicus* showing hypothetical intra- and intermolecular electron pathways. Subunit I binds the PQQ cofactor, the [2Fe-2S] cluster, and one *c*-type cytochrome. Subunit II harbors three *c*-type cytochromes, thus providing an efficient electron transfer route to quinones located in the cytoplasmic membrane.

*Gluconacetobacter diazotrophicus* is a nitrogen-fixing obligatory aerobe which ferments alcohols, aldehydes, and sugars (*1*, *2*). It is a Gram-negative bacterium and belongs to the acetic acid bacteria which consist of three groups: the *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* of the *Acetobactereaceae* family (*3*). A set of dehydrogenases is overexpressed when *Ga. diazotrophicus* grows under nitrogen-fixing conditions. Among these are the PQQ[1]-dependent enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) which are located in the cytoplasmic membrane (*4*). These enzymes are oriented toward the periplasmic space (*5*, *6*) and transfer electrons to ubiquinone $Q_{10}$. The acetic acid bacteria can oxidize ethanol to acetic acid in two consecutive reactions, using ADH and ALDH as catalysts. In view of its metabolic importance, the biochemical and structural properties of this type of ADH have been investigated in greater detail. Typically, the membrane-bound ADH of acetic acid bacteria has three subunits: SU I (72−80 kDa) which carries the PQQ cofactor involved in oxidation of the substrate, SU II (44−54 kDa) which hosts the *c*-type cytochromes to transfer electrons, and SU III (8−20 kDa) for which no function has been assigned so far (*7*−*10*). PQQ plus one heme center is usually located in SU I, while SU II harbors three *c*-type cytochromes. Note that these multicomponent enzymes exhibit a rather narrow substrate spectrum as they only will oxidize primary alcohols (C2−C6 chain length) but will not oxidize methanol and secondary alcohols.

Here we report on the identification, quantitative determination, and characterization of the cofactors of the membrane-bound ADH isolated from *Ga. diazotrophicus*. This quinohemoprotein is a heterodimer, consisting of two subunits with ≈72 and ≈44 kDa. Despite being purified in the presence of dioxygen, the cofactors of ADH remain preferentially in the reduced state as documented by UV−vis and EPR spectroscopy. In addition to PQQ and four *c*-type cytochromes, we identified a [2Fe-2S] cluster associated with ADH of *Ga. diazotrophicus* which had not been described so far for members of this class of membrane-bound alcohol dehydrogenases. A model is presented with hypothetical intra- and intermolecular pathways in the ADH heterodimer of *Ga. diazotrophicus*.

## EXPERIMENTAL PROCEDURES

*Cultivation of Bacteria and Preparation of Membranes. Ga. diazotrophicus* PAL5 (ATCC 49037) was grown at 30 °C in LGIP medium supplemented with 0.75% ethanol in a 60 L

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[1]Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; PQQ, pyrroloquinoline quinone.



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working volume Bioflow 5000 fermentor (New Brunswick Scientific; stirred at 120 rpm, aerated at 60 L of air $min^{-1}$), according to Reis et al. (*11*). Cultivation was started with 3 L of an active culture, and cells were harvested in the early stationary phase (48 h). Membranes were obtained as described earlier (*12*); they were frozen and kept in liquid nitrogen without significant loss of enzymatic activities.

*Purification of ADH*. The enzyme was solubilized from the membrane fraction with 0.5% Triton X-100 and purified as described recently (*13*). Unless mentioned, all operations were performed in 10 mM $KP_i$, pH = 6.00, containing 0.1% Triton X-100 (standard buffer). The purity of ADH was checked by SDS−PAGE (16 × 14 cm slab gels, 10% polyacrylamide (*14*)). For native PAGE, a 7.5% polyacrylamide resolving gel was used, and the detergent SDS was replaced by 0.1% Triton X-100.

Proteins were stained with 0.05% Coomassie brilliant blue R-250 (*8*) for enzyme activity; hemes were stained with the peroxidase assay (*15*). Protein was determined by a modified Lowry procedure (*16*) with bovine serum albumin as standard.

*Enzyme Activity*. Dehydrogenase activity of membranes and purified ADH was determined spectrophotometrically in standard buffer with potassium ferricyanide ($K_3[Fe(CN)_6]$) as the electron acceptor; alternatively, a mixture of phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCPIP) served as electron acceptor (*8*). The substrate ethanol was 20 mM; one unit corresponds to the amount of enzyme catalyzing the oxidation of 1.0 μmol of substrate per minute.

*Determination of PQQ*. Pyrroloquinoline quinone was extracted with 90% methanol (1.0 mg protein) as described by Matsushita et al. (*17*); quantitative analysis was performed with a Waters chromatography system equipped with a model 996 photodiode array detector and a C18 Spherisorb S5 OD52 analytical column (4.6 × 150 mm) (*12*). The column was equilibrated with a mixture of ethanol and methanol (3:2 v/v) which had been carefully degassed; this mixture was also used as the mobile phase, with a flow rate of 0.5 mL $min^{-1}$. PQQ was detected at 275 nm; PQQ from Sigma-Aldrich served as standard.

*Determination of Cytochromes and Hemes*. Samples (1 mg of ADH $mL^{-1}$) were reduced with 20 mM substrate or $Na^+$ dithionite and oxidized with $NH_4^+$ peroxydisulfate. Cytochromes *c* were determined by the pyridine−hemochrome method (*6*, *13*, *18*); in addition, hemes were analyzed according to Puustinen and Wikström (*19*) using the Waters chromatography system and a Delta- Pak HPIC18 300 Å column (2 × 150 mm). Hereby, ADH (0.9 mg) was treated with HCl−acetone, dissolved in 0.5% trifluoroacetic acid−acetonitrile, and applied to the column which had been equilibrated with 0.5% trifluoroacetic acid−25% acetonitrile in water; protoheme IX (Sigma) served as the standard.

*Metal and Acid-Labile Sulfur Analysis*. Total iron and calcium were determined by atomic absorption spectroscopy on a Perkin-Elmer 2380 instrument; for acid-labile sulfur the semimicro method of Beinert was used (*20*).

*Oxidation−Reduction Potentials*. Spectroelectrochemical titrations in the absence of dioxygen were carried out as described elsewhere (*21*, *22*). ADH, in standard buffer, was poised at different potentials in the presence of the following mediators (50 μM): anthraquinone-2-sulfonate (−225 mV), 2-hydroxy-1,4-naphthoquinone (−145 mV), menadione (0 mV), duroquinone (+50 mV), 1,4-naphthoquinone (+69 mV), phenazine methosulfate (+80 mV), 1,2-naphthoquinone (+134 mV), and 1,4-benzoquinone (+285 mV). Titrations under dinitrogen were carried out by the stepwise addition of 100 mM $K_3[Fe(CN)_6]$, or 100 mM $Na^+$ dithionite, in 50 mM $KP_i$, pH 6.0, at 25 °C, in a cell equipped with a combined Ag/AgCl-Pt electrode (Cole-Palmer) and a potentiometer (Orion 520 A+; Thermo Fisher Scientific), as described by Dutton (*21*). The electrode has been calibrated against a saturated quinhydrone solution, pH 6.0; all potentials are reported against the standard hydrogen electrode (SHE). The titration of the *c*-type cytochromes of ADH (15 mg $mL^{-1}$ in standard buffer containing 0.10% Triton X-100) was followed in the dual wavelength mode; the reduction of hemes was recorded at the α-band maximum at 553 nm with the reference wavelength set at 540 nm (OLIS-SLM DW 2000 spectrophotometer). Experimental data were fitted by Nernst curves for four single electron components ($n = 1$) with unknown redox potentials with a program kindly provided by Dr. R. Louro (Universidade Nova de Lisboa). Minimization of the sum of the squared residuals was used as a criterion for the selection of the best fitting model and gave the values for the midpoint potentials. The same procedure was applied for the EPR-monitored titrations of the [2Fe-2S] cluster and PQQ of ADH (32 mg $mL^{-1}$ in standard buffer containing 0.10% Triton X-100). Samples obtained at the various potentials were transferred to calibrated 3.0 mm (i.d.) quartz tubes, under the strict exclusion of dioxygen, and frozen in liquid nitrogen. The mediators did not interfere with the UV−vis and EPR spectra of ADH under our experimental conditions. For the potential of the [2Fe-2S] cluster, experimental data points were fitted using the Nernst equation for one single component ($n = 1$). For the potential of the PQQ cofactor, we followed the procedure developed for a semiquinone radical observed in the membrane-bound nitrate reductase of *Escherichia coli*. The variation of the normalized PQQ semiquinone ($PQQ_{sq}$) EPR signal amplitude was fitted to a theoretical curve corresponding to two successive one-electron redox processes, where $E_1$ and $E_2$ are the midpoint potentials of the $PQQ_{ox}/PQQ_{sq}$ and the $PQQ_{sq}/PQQH_2$ redox couples, respectively (*23*).

*Spectroscopy*. UV−vis spectra were recorded with the OLIS-SLM DW 2000 or the Shimadzu UV-2401 PC spectrophotometer in quartz cells (2.0 or 10.0 mm path length). EPR spectra were measured under nonsaturating conditions of microwave power on a Bruker Elexys E500 instrument at approximately 9.40 GHz (X-band) and 100 kHz modulation. Spectra were evaluated and simulated using the Bruker software, and *g*-values were calculated by measuring the magnetic field and the microwave frequency. The temperature was maintained with an Oxford liquid helium continuous flow cryostat (10−20 K) or with a liquid nitrogen insert Dewar (77 K).

## RESULTS

*Purification and Molecular Properties of ADH*. Recently, we developed an efficient procedure for the purification of active alcohol dehydrogenase (ADH) from the membrane fraction of nitrogen-fixing *Ga. diazotrophicus* (*13*). Routinely, 3−4 mg $g^{-1}$ cell of homogeneous protein (specific activity 260 (±10) units (mg of protein)$^{-1}$) could be obtained which will allow detailed spectroscopic studies and crystallization experiments. The membrane-bound ADH of *Ga. diazotrophicus* consists of two subunits, with molecular masses of ≈72 kDa (SU I) and ≈44 kDa (SU II) (SDS−PAGE), in excellent agreement with 71.4 and 43.5 kDa obtained by mass spectrometry (*13*). A positive peroxidase reaction confirmed the presence of heme centers in both subunits (Figure 1), and the organic cofactor pyrroloquinoline quinone (PQQ) of ADH was identified by UV−vis and

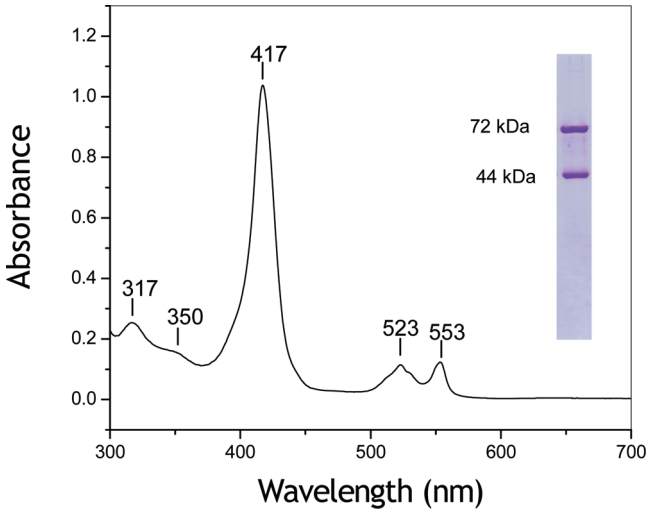


FIGURE 1: UV−visible spectrum of ADH of *Ga. diazotrophicus* (enzyme as isolated in the presence of dioxygen in standard buffer). Inset: SDS−PAGE showing the heme-stained SU I (72 kDa) and SU II (44 kDa).

fluorescence spectroscopy (*13*). Although purified in the presence of dioxygen, the heme centers of ADH were in the reduced Fe(II) state according to its UV−vis spectrum; addition of $K_3[Fe(CN)_6]$ or $(NH_4)_2S_2O_8$ caused oxidation to the Fe(III) state. Incubation with ethanol, or acetaldehyde, led to the reduction of ADH and restored the Fe(II) state of the heme centers (*13*).

In line with these observations, the EPR spectrum of ADH (enzyme as isolated in the presence of dioxygen in standard buffer) showed a narrow signal centered at $g_{iso}$ = 2.0034, which was still detectable at 100 K and was assigned to the PQQ semiquinone (Figure 2A). EPR signals, with similar *g*-values and line widths, had been reported earlier for the PQQ-dependent enzymes from *Pseudomonas aeruginosa* (*24*) and *Comamonas testosteroni* (*25*). Quantitative analysis by HPLC confirmed the presence of one PQQ (0.94 ± 0.25) per ADH heterodimer (Figure 2B). Furthermore, one calcium ion (1.10 ± 0.05) was detected by atomic absorption spectroscopy which is required for cofactor binding and stabilization of the PQQ semiquinone radical (*26*−*28*).

*Cytochromes*. The UV−vis spectrum of the ADH heterodimer (as isolated in the presence of dioxygen in standard buffer) shows intense absorption maxima at 417, 523, and 553 nm, which are characteristic for *c*-type cytochromes in the reduced Fe(II) state (Figure 1); the maxima at 317 and 350 nm result from the PQQ cofactor (*13*, *25*). Note that *b*-type cytochromes were absent according to HPLC analysis following the procedure of the Puustinen and Wikström method (*19*), whereas four *c*-type cytochromes were detected by the pyridine−hemochrome method in agreement with earlier results (*13*).

[*2Fe-2S*] *Cluster*. At 20 K, the EPR spectrum of ADH from *Ga. diazotrophicus* (enzyme as isolated in the presence of dioxygen in standard buffer) revealed a well-resolved resonance pattern with $g_{xyz}$ values at 2.007, 1.941, and 1.920 ($g_{av}$ 1.956), reminiscent of the EPR signal of an iron−sulfur protein (Figure 3). The signal intensity increased upon addition of $Na^+$ dithionite. Comparable $g_{av}$ values had been reported for the [2Fe-2S] cluster of benzene dioxygenase of *Pseudomonas putida*, aldehyde oxidoreductase of *Desulfovibrio alaskensis*, and the FhuF protein of *E. coli* (*22*, *29*−*31*). The *g* 2.0 region of the experimental EPR spectrum of ADH contains contributions of both the PQQ

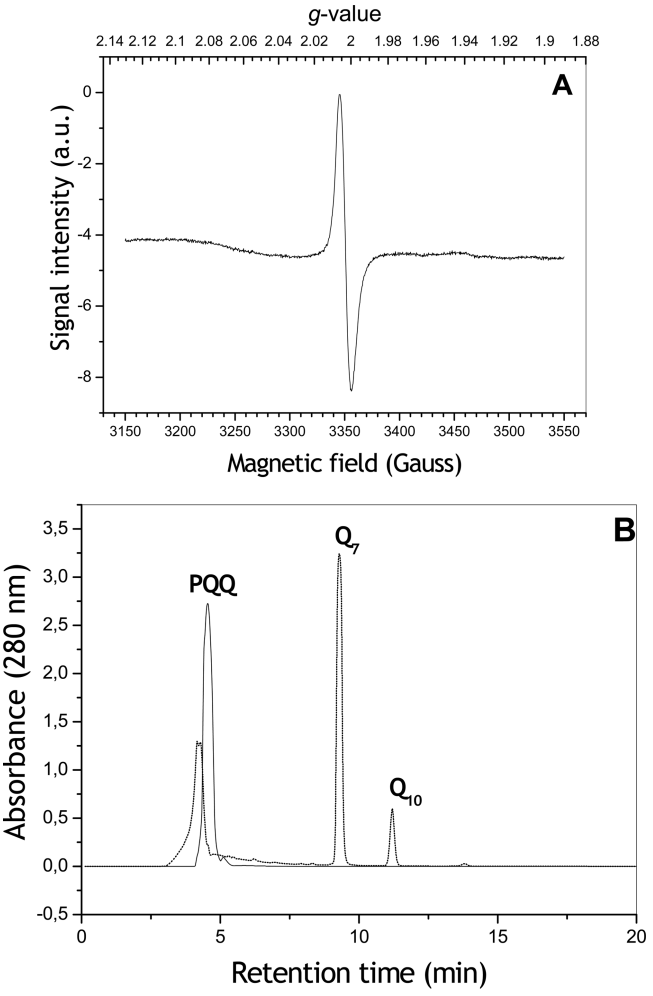


FIGURE 2: (A) X-band EPR spectrum of the PQQ semiquinone radical in ADH of *Ga. diazotrophicus* (enzyme as isolated in the presence of dioxygen in standard buffer). Conditions: microwave frequency, 9.393268 GHz; microwave power, 0.20 mW; modulation amplitude, 0.1 mT; temperature, 77 K; $g$ = 2.0034, $\Delta B_{p\text{-}p}$ = 10.5 G. (B) Identification and quantitative determination of PQQ in ADH of *Ga. diazotrophicus* as described in the Experimental Procedures section (solid line). The HPLC system was calibrated against commercially available PQQ and quinones $Q_7$ and $Q_{10}$ (dotted line, retention times 4.28, 9.28, and 11.19 min).

semiquinone radical and the [2Fe-2S] cluster as documented in the simulated spectra (Figure 3). The presence of a second type of iron center in ADH from *Ga. diazotrophicus* is supported by the quantitative determination of iron and acid-labile sulfur. The value of six (5.90 ± 0.15) Fe and two acid-labile sulfur atoms (2.06 ± 0.10) per ADH heterodimer protein agrees well with the presence of four *c*-type cytochrome centers and one [2Fe-2S] cluster.

*Midpoint Potentials and Electron Transfer*. Potentiometric titrations of the *c*-type cytochromes of ADH with $Na^+$ dithionite (reduction) and $K_3[Fe(CN)_6]$ (oxidation) were monitored by UV−vis spectroscopy following the change of the α-band maximum at 553 nm (reference wavelength set at 540 nm, dual wavelength mode). From the best fit of the data (three titrations) four potentials at $E_{m1}$ = −64 (±2) mV (31%), $E_{m2}$ = −8 (±2) mV (18%), $E_{m3}$ = +185 (±15) mV (38%), and $E_{m4}$ = +210 (±10) mV (13%) (vs SHE) could be estimated for the *c*-type cytochromes of ADH of *Ga. diazotrophicus* (Figure 4). The values given in parentheses represent the relative spectral contribution of each potential, in good agreement with previously

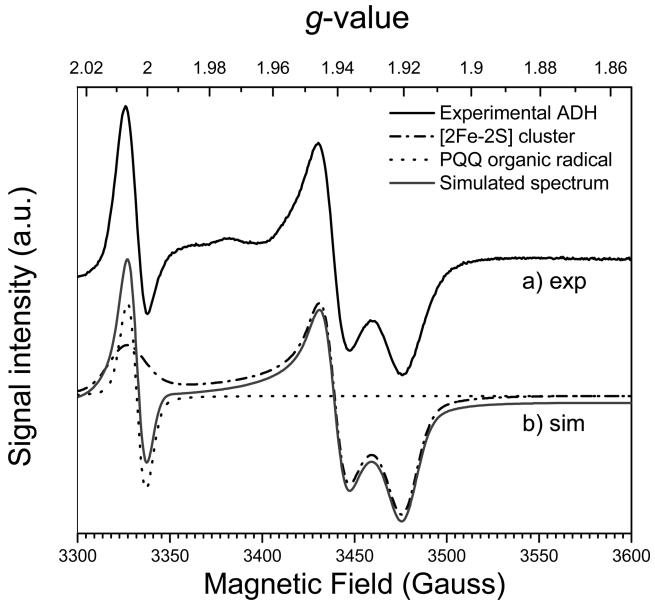


FIGURE 3: X-band EPR spectrum of the [2Fe-2S] cluster in ADH of *Ga. diazotrophicus* (enzyme as isolated, 32 mg $mL^{-1}$, in standard buffer): (a) experimental spectrum, (b) simulated spectrum, solid line; [2Fe-2S] cluster, dashed line, $g_x = 2.007$, $g_y = 1.941$, $g_z = 1.920$, $g_{av} = 1.956$; PQQ semiquinone, dotted line, $g_{iso} = 2.0034$. Conditions: microwave frequency, 9.342559 GHz; microwave power, 2.00 mW; modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; temperature, 20 K.

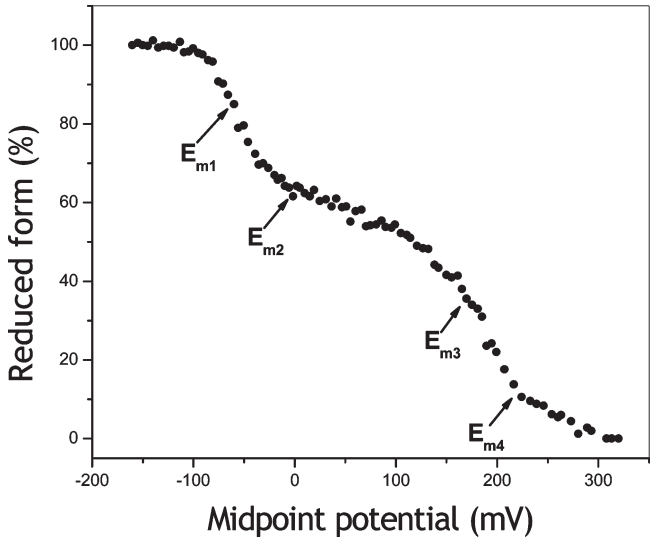


FIGURE 4: Spectroelectrochemical titration of *c*-type cytochromes in ADH of *Ga. diazotrophicus* (enzyme as isolated in the presence of dioxygen, 15 mg $mL^{-1}$, in 50 mM phosphate buffer, 0.1% Triton X-100, pH 6.0, 25 °C). Titrations were carried out by stepwise addition of 100 mM $K_3[Fe(CN)_6]$, or 100 mM $Na^+$ dithionite, under the exclusion of dioxygen, in the presence of the following mediators (50 $\mu$M): anthraquinone-2-sulfonate (−225 mV), 2-hydroxy-1,4-naphthoquinone (−145 mV), menadione (0 mV), duroquinone (+50 mV), 1,4-naphthoquinone (+69 mV), phenazine methosulfate (+80 mV), 1,2-naphthoquinone (+134 mV), and 1,4-benzoquinone (+285 mV). Data were obtained and evaluated as described in the Experimental Procedures section; from the best fit of the data (three titrations) four potentials at $E_{m1} = -64$ (±2) mV (31%), $E_{m2} = -8$ (±2) mV (18%), $E_{m3} = +185$ (±15) mV (38%), and $E_{m4} = +210$ (±10) mV (13%) (vs SHE) could be estimated.

reported redox titrations for the ADH of *Acetobacter methanolicus* (*32*). It is suggested that the cytochrome *c* center with $E_{m1} = -64 \pm 2$ mV is associated with the large subunit SU I, while the other three cytochromes *c* are located in the smaller subunit SU II, in agreement with the locations of the *c*-type cytochrome motifs CXXCH in the amino acid sequence of ADH (*33*, *34*). Dithionite-reduced ADH could be reoxidized by stoichiometric amounts of $K_3[Fe(CN)_6]$. Addition of the substrate ethanol to the ferricyanide-oxidized ADH led to rapid reduction of the enzyme and the transformation of the heme centers to the Fe(II) state. Addition of dithionite to ethanol-reduced ADH did not lead to further reduction of the enzyme. The data from these oxidation−reduction experiments show that the four *c*-type cytochrome centers are fully reducible by the substrate ethanol and can participate in electron transfer under catalytic conditions (*13*). The EPR-monitored oxidation−reduction titration (measured at 20 K, three titrations) of the [2Fe-2S] cluster in ADH of *Ga. diazotrophicus* gave an $E_{mFeS} = -250$ (±5) mV (Figure 5A). We also estimated $E_m$ of the PQQ/$PQQH_2$ couple by measuring the EPR spectrum of the PQQ semiquinone radical ($PQQ_{sq}$) in the

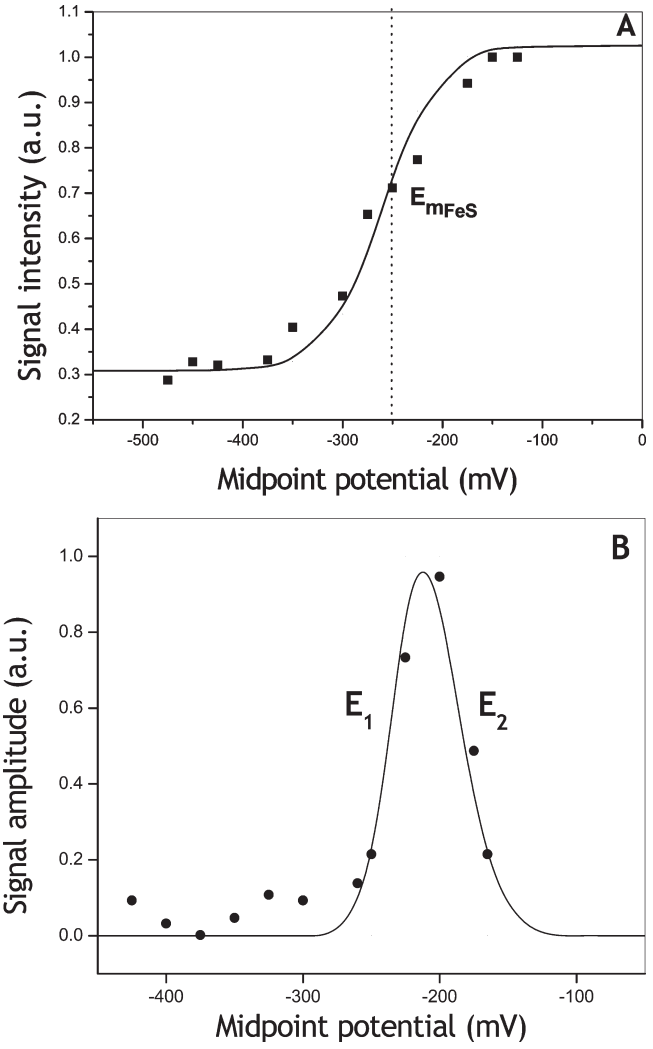


FIGURE 5: EPR-monitored potentiometric titrations of (A) the [2Fe-2S] cluster and (B) the PQQ semiquinone in ADH of *Ga. diazotrophicus* (enzyme as isolated in the presence of dioxygen, 32 mg $mL^{-1}$; experimental conditions as in Figure 4). EPR spectra were recorded at 20 K ([2Fe-2S]) or 77 K (PQQ). Data were obtained and evaluated as described in the Experimental Procedures section; from the best fit of the data (three titrations) $E_{mFeS} = -250$ (±5) mV for the [2Fe-2S] cluster and $E_{mPQQ} = -210$ (±5) mV (vs SHE) for the PQQ/$PQQH_2$ couple could be estimated.

same samples at 77 K (Figure 5B). A bell-shaped curve was obtained with two potentials, $E_1$ ($PQQ/PQQ_{sq}$) = −180 (±5) mV and $E_2$ ($PQQ_{sq}/PQQH_2$) = −240 (±5) mV, which corresponds to a value of $E_{mPQQ}$ = −210 (±5) mV (vs SHE) for the PQQ/$PQQH_2$ couple.

## DISCUSSION

Following the recently developed purification procedure by Gómez-Manzo and colleagues (*13*) pure ADH could be obtained in good yield from the membrane fraction of nitrogen-fixing *Ga. diazotrophicus*. The enzyme was active and homogeneous by criteria of SDS−PAGE, with a heterodimeric architecture consisting of a larger subunit (SU I, ≈72 kDa) and a smaller subunit (SU II, ≈ 44 kDa). So far, the heterodimeric structure has been found in all ADH enzymes isolated from *Gluconacetobacter* species, in contrast to a heterotrimeric architecture observed for the membrane-bound ADH from *Acetobacter* and *Gluconobacter* species (*13*). Although prepared in the presence of dioxygen, the metal centers of ADH were mainly in the reduced state according to UV−vis and EPR spectroscopy, a feature which is currently not fully understood. Earlier, it was suggested that reducing equivalents might come from Triton X-100 (*13*), which could not be confirmed upon replacing Triton X-100 by other detergents.

*Pyrroloquinoline Quinone*. The presence of the PQQ cofactor (0.94 ± 0.25/heterodimer) in active ADH of *Ga. diazotrophicus* could be clearly documented by chromatographic analysis and EPR spectroscopy. A large portion of the PQQ cofactor in the enzyme as isolated was in the semiquinone form ($PQQ_{sq}$), showing a strong EPR signal at $g_{iso}$ = 2.0034 (Figure 2A). Its EPR properties (*g*-value, line width, power saturation profile at 20, and 77 K) were similar to those reported for $PQQ_{sq}$ in other PQQ-dependent enzymes (*26*−*28*). Additionally, the amino acid sequence of this ADH indicates the presence of a specific binding site for the PQQ moiety in SU I (*33*, *35*−*37*). Note that the catalytic subunits of all the quino(hemo) protein ADH structures reported so far seem to have a common structural motif, a quinoprotein-specific superbarrel domain, where PQQ is deeply embedded in the interior of the superbarrel and sandwiched between the indole ring of a tryptophan residue and an eight-membered disulfide ring formed from adjacent cysteine residues (*28*, *37*). Notably, this disulfide is missing in several quinohemoproteins, such as lupanine hydroxylase from *Pseudomonas* sp. (*38*). Once we had confirmed the presence of one PQQ and one $Ca^{2+}$ in ADH of *Ga. diazotrophicus*, we estimated a value of $E_{mPQQ}$ = −210 (±5) mV for the midpoint potential of the PQQ/$PQQH_2$ redox couple by EPR spectroscopy (Figure 5B). Earlier, a value of $E_m$ = −167 mV had been obtained for the PQQ/$PQQH_2$ redox couple of the membrane-bound ADH of *Gluconobacter suboxydans* (*39*). Duine and co-workers (*40*) had reported $E_m$ = −218 mV for the PQQ/$PQQ_{sq}$ couple and $E_m$ = −242 mV for the $PQQ_{sq}/PQQH_2$ couple in methanol dehydrogenase of *Hyphomicrobium* X. In this case the PQQ cofactor had been extracted, and the $PQQ_{sq}$ radical could only be stabilized in solution at alkaline pH.

*Electron Transfer Pathways*. In addition to the four *c*-type cytochromes present in ADH of *Ga. diazotrophicus* (*13*), low-temperature EPR spectroscopy led to the identification of an iron−sulfur cluster associated with the membrane-bound enzyme. It exhibited a rhombic signal with *g*-values at 2.007, 1.941, and 1.920 (Figure 3), which we assigned to a [2Fe-2S] cluster. Its relatively high $g_{av}$ value of 1.956 can be compared to those reported for the [2Fe-2S] cluster of aldehyde oxidoreductase from *D. alaskensis*, benzene dioxygenase from *P. putida*, and the FhuF protein from *E. coli* (*22*, *29*−*31*). To our knowledge this is the first time that this type of metal center has been identified for this type of ADH.

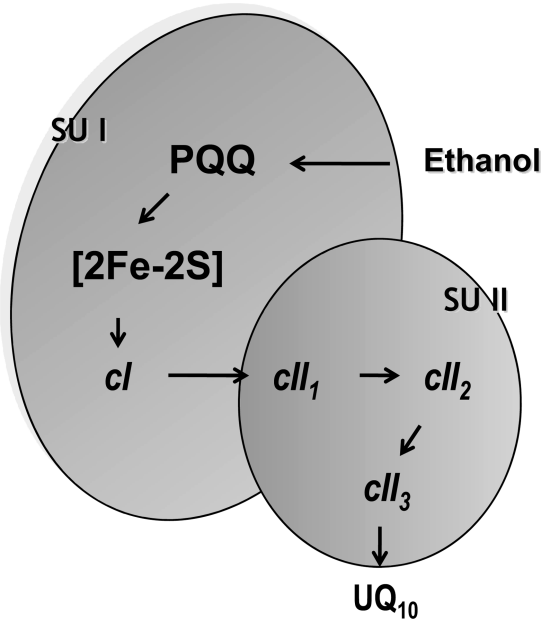


FIGURE 6: Hypothetical intra- and intermolecular electron transfer pathways in the heterodimeric membrane-bound ADH of *Ga. diazotrophicus*. PQQ, [2Fe-2S], and cytochrome *cI* are assigned to SU I and cytochromes $cII_1$, $cII_2$, and $cII_3$ to SUII, interacting with $UQ_{10}$.

It is suggested that subunit SU I, in addition to the PQQ cofactor, hosts one *c*-type cytochrome and the [2Fe-2S] cluster, whereas the residual three *c*-type cytochromes are located in subunit SU II (Figure 6). The amino acid sequence of the membrane-bound ADH from *Ga. diazotrophicus* carries 11 cysteine residues, five of them in SU I and six in SU II. In SU II, all six cysteine residues are located in three CXXCH motifs required for covalent attachment of the three *c*-type cytochromes (*33*, *34*, *37*). In SU I, two of the cysteine residues are used for the classical CXXCH motif. This leaves three cysteine residues as potential ligands for the [2Fe-2S] cluster detected by EPR spectroscopy. Two of them are part of a sequence CCDxVNRG, conserved in both type II and type III quinohemoprotein alcohol dehydrogenases, including the type III ADH of *Ga. diazotrophicus*. In the type II ADHs of *C. testosteroni* and *P. putida* these adjacent cysteines form a disulfide bond as shown by X-ray crystallography, which might be involved in electron transfer between PQQ and the *c*-type cytochrome (*41*, *42*). On the other hand, investigation of a double cysteine → alanine mutant of the quinoprotein ethanol dehydrogenase of *P. aeruginosa* showed that this characteristic disulfide in close neighborhood to the PQQ cofactor was not a prerequisite for the formation of the functionally important PQQ semiquinone (*24*). In the membrane-bound ADH of *Ga. diazotrophicus*, the two adjacent cysteines, instead of forming a disulfide, could serve as ligands for the [2Fe-2S] cluster, as reported for the [2Fe-2S] cluster of the FhuF protein in *E. coli* (*30*, *31*), or the [4Fe-4S] cluster in the assimilatory adenosine-5′-phosphosulfate reductase of *P. aeruginosa* (*43*). Usually, ferredoxin-type [2Fe-2S] clusters are bound by four cysteine residues to the protein (*44*). As there are only three cysteine residues, in addition to the two used for attachment

of one *c*-type cytochrome in SU I, we assume that the fourth ligand of the [2Fe-2S] cluster must come from another amino acid residue, such as histidine or serine. This assumption is supported by the results published recently for the outer mitochondrial membrane protein mitoNEET (*45*−*48*). In this protein, the [2Fe-2S] cluster is coordinated by three cysteines and one histidine. Along these lines, the C56S and C60S variants of the ferredoxin from *C. pasteurianum* are stable proteins with serine-coordinated [2Fe-2S] clusters (*49*−*52*).

The four *c*-type cytochromes of the ADH from *Ga. diazotrophicus* exhibit midpoint potentials $E_{m1-4}$ around −64, −8, +185 and +210 mV (vs SHE, pH 6.0), which differ from those values reported for the ADH of *A. methanolicus* (−130, +49, +188, and +188 mV, pH 7.0). In the latter case, it appears that one of the heme centers does not participate in electron transfer to ubiquinone (*32*), in contrast to ADH from *Ga. diazotrophicus* where all four *c*-type cytochromes are fully reduced by the substrate ethanol (*13*) and thus are involved in the electron transfer from PQQ to the quinone in the cytoplasmic membrane as depicted in Figure 6. In conclusion, the membrane-bound ADH from *Ga. diazotrophicus* carries three different types of redox-active centers, the PQQ cofactor, four *c*-type cytochromes, and one [2Fe-2S] cluster, which provide efficient intra- and intermolecular electron transfer pathways needed for an efficient catalyst (*53*). Note that the purified ADH from *Ga. diazotrophicus* transforms not only ethanol to acetaldehyde but also acetaldehyde to acetic acid and thus has a remarkable catalytic power (*13*).

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