Cloning, Expression, and Purification of Choline Dehydrogenase from the Moderate Halophile *Halomonas elongata*

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Choline dehydrogenase (EC 1.1.99.1) catalyzes the four-electron oxidation of choline to glycine-betaine via a betaine-aldehyde intermediate. Such a reaction is of considerable interest for biotechnological applications in that transgenic plants engineered with bacterial glycine-betaine-synthesizing enzymes have been shown to have enhanced tolerance towards various environmental stresses, such as hypersalinity, freezing, and high temperatures. To date, choline dehydrogenase has been poorly characterized in its biochemical and kinetic properties, mainly because its purification has been hampered by instability of the enzyme in vitro. In the present report, we cloned and expressed in *Escherichia coli* the *betA* gene from the moderate halophile *Halomonas elongata* which codes for a hypothetical choline dehydrogenase. The recombinant enzyme was purified to more than 70% homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by treatment with 30 to 50% saturation of ammonium sulfate followed by column chromatography using DEAE-Sepharose. The purified enzyme showed similar substrate specificities with either choline or betaine-aldehyde as the substrate, as indicated by the apparent V/K values (where V is the maximal velocity and K is the Michaelis constant) of 0.9 and 0.6 μmol of O₂ min⁻¹ mg⁻¹ mM⁻¹ at pH 7 and 25°C, respectively. With 1 mM phenazine methosulfate as the primary electron acceptor, the apparent V_max values for choline and betaine-aldehyde were 10.9 and 5.7 μmol of O₂ min⁻¹ mg⁻¹, respectively. These V_max values decreased four- to sevenfold when molecular oxygen was used as the electron acceptor. Altogether, the kinetic data are consistent with the conclusion that *H. elongata betA* codes for a choline dehydrogenase that can also act as an oxidase when electron acceptors other than molecular oxygen are not available.

Availability of water is the most important limiting factor affecting the survival and growth of both beneficial and pathogenic microorganisms. Since microorganisms lack systems for active water transport, environmental changes in osmolality are compensated by either transport or synthesis of compatible solutes, a group of inert organic solutes that can be accumulated in the intracellular milieu to very high levels without affecting the metabolic function of the cell (for a recent review, see reference 7). Among the compatible solutes that have been identified to date, glycine-betaine is the most widely used in bacteria, such as *Corynebacterium glutamicum* (52, 256) and animals (29, 35), identified to date, glycine-betaine is the most widely used in bacteria, such as *Arthrobacter globiformis* and *Arthrobacter pascens* (19, 37) and in the fungus *Cylindrocarpon didymum* (28, 47). Choline monoxygenase (EC 1.14.15.7), in combination with betaine-aldehyde dehydrogenase (EC 1.2.1.8), has been identified in a limited number of higher plants, such as spinach (36), sugar beet, and amaranth (38). A membrane-associated choline dehydrogenase (EC 1.1.99.1) coupled to betaine-aldehyde dehydrogenase has been described in marine invertebrates, such as oysters (31, 32), and in bacteria, such as *Escherichia coli* (27) and *Pseudomonas aeruginosa* (39, 45). In *E. coli*, the biosynthetic pathway for the production of glycine-betaine from choline has been well characterized at the genetic level, where it has been shown that four genes encoding choline dehydrogenase (*betA*), betaine-aldehyde dehydrogenase (*betB*), a putative regulator (*betF*), and a choline transporter (*betT*) are clustered in the bet operon (3). Based on nucleotide sequence alignment, similar Bet pathways have been recently proposed for *Sinorhizobium melloti* (34) and the moderate halophile *Halomonas elongata* (9). Since *H. elongata* is capable of thriving in hypersaline environments with NaCl concentrations as high as 4 M (46), its Bet pathway represents an excellent model for studying the molecular basis of osmoprotection.

Bacterial glycine-betaine-synthesizing enzymes recently have become a major target in the creation of stress-resistant transgenic plants with the goal of genetically engineering stress tolerance in crop plants of economic interest (for a recent review, see reference 40). Model studies of *Synechococcus* sp. PCC 7942 or *Arabidopsis thaliana* transformed with the gene from *A. globiformis* that encodes choline oxidase, *codA*, have demonstrated an improved tolerance to salt stress (11) or high and freezing temperatures (2, 16, 40). Enhanced tolerance to low temperatures or high-salt conditions has been also observed during germination of transgenic seeds of *A. thaliana* transformed with *codA* (1, 17). Similarly, improved resistance towards salinity and low temperature has been shown in trans-
genic tobacco expressing the two *E. coli* *beta* and *betB* (18), demonstrating that choline dehydrogenase and/or betaine-aldehyde dehydrogenase are also of considerable interest for practical applications. Despite these significant biotechnological advances, the biochemical and kinetic characterization of choline dehydrogenase has lagged behind and only minimal biochemical studies on choline dehydrogenase purified from rat liver mitochondria (44) and a *Pseudomonas* strain (39) have been reported to date.

Choline dehydrogenase catalyzes the four-electron oxidation of choline to glycine-betaine via a betaine-aldehyde intermediate (44) (Fig. 1) and shows an absolute requirement for an electron acceptor other than molecular oxygen for catalysis (39, 44). Although, based on primary sequence alignment, the enzyme has been grouped in the glucose-methanol-choline flavin-dependent oxidoreductase superfamily (10), no clear biochemical evidence has been shown indicating the presence of flavin adenine dinucleotide (FAD) as a cofactor. In contrast, pyrroloquinoline quinone (PQQ) has been proposed to be bound to *Pseudomonas* choline dehydrogenase (39).

As a first step towards a biochemical and mechanistic characterization of choline dehydrogenase aimed at a better understanding of the properties of the enzyme, we cloned and expressed the *beta* gene from *H. elongata* and partially purified and characterized the resulting enzyme by using steady-state kinetics with choline or betaine-aldehyde as the substrate. The data presented in this study indicate that in *H. elongata* the *beta* gene codes for a choline dehydrogenase, which is also able to utilize molecular oxygen in catalysis in the absence of other electron acceptors.

**MATERIALS AND METHODS**

**Materials.** Plasmid pDC4 containing the *bet* operon from *H. elongata* was a kind gift of Joaquín J. Nieto, University of Seville, Spain. Restriction endonucleases NcoI and BamHI, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from Promega. *Pfu* DNA polymerase was obtained from Stratagene or from Roche Molecular Biomedicals. Luria-Bertani agar, Luria-Bertani broth, and the ligation mixture was used directly to transform *E. coli* strain OrigamiB(DE3)pLysS cells harboring plasmid pETCHDhe or pETCHDhe-S2A and strain OrigamiB(DE3)pLysS cells harboring plasmid pETCHDhe were stored at −80°C as 7% dimethyl sulfoxide suspensions. DNA sequencing was carried out with an Applied Biosystems Big Dye kit on an Applied Biosystems model ABI 377 DNA sequencer by the DNA Core Facility of the Biology Department of Georgia State University.

**Cloning of *beta* into expression vector pET23d(+)**. *E. coli* strain MKH13 harboring plasmid pDC4 was grown on Luria-Bertani agar medium containing 50 μg of chloramphenicol/ml for 16 h at 37°C. Single colonies were used to inoculate 5 ml of Luria-Bertani broth containing 50 μg of chloramphenicol/ml, and the resulting liquid cultures were grown for 16 h at 37°C. The cells were harvested by centrifugation at 14,000 × g for 10 min, and the plasmid pDC4 was isolated by using a QIAquick Spin mini-prep kit (Qiagen) according to the manufacturer’s instructions.

The resulting plasmid pDC4 was sequenced in both directions by using oligonucleotide primers designed to bind to DNA regions of pET23d(+) (40) and the ligation mixture was used directly to transform *E. coli* strain XL-1 Blue competent cells by using the heat shock method of Inoue et al. (20). The resulting plasmid was sequenced containing plasmid pDC4 was isolated by using oligonucleotide primers designed to bind to the DNA regions of PET flasking the inserted gene.

**Expression of choline dehydrogenase in *E. coli***. A single colony of *E. coli* strain OrigamiB(DE3)pLysS harboring plasmid pMETCHDhe was used to inoculate 50 ml of Luria-Bertani broth containing 50 μg of ampicillin/ml, 34 μg of chloram-

**FIG. 1.** Oxidation of choline to glycine-betaine catalyzed by choline dehydrogenase.
phenicol/ml, and 12 μg of tetracycline/ml at 37°C. After 16 h, 18 ml of the starter culture was used to inoculate 1.5 liters of Luria-Bertani broth containing 50 μg of ampicillin/ml at 37°C. When the culture's optical density at 600 nm reached 0.8, IPTG was added to a final concentration of 0.5 mM and the temperature of the culture was lowered to 25°C. After 4 h, cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C and stored at −20°C. Typically, 1.5 liters of culture yielded approximately 5 g of cell wet paste.

**Purification of choline dehydrogenase.** The purification of choline dehydrogenase was carried out at 4°C as follows. The cell paste, typically 5 g, was suspended in 4 volumes of a solution containing 1 mM EDTA, 100 mM NaCl, 0.1 mM PMSF, 0.2 mg of lysozyme/ml, and 50 mM Tris-Cl (pH 8.0) and allowed to incubate with stirring for 30 min on ice. The resulting slurry was passed through an SLM Amino French pressure cell two times at 20,000 lb/in² and then centrifuged at 5,000 × g for 20 min. The supernatant was brought to 30% ammonium sulfate saturation, incubated with stirring on ice for 30 min, and then centrifuged at 12,500 × g for 20 min. The supernatant was brought to 50% ammonium sulfate saturation and treated as described above. The resulting pellet was suspended in 5 ml of 1 mM EDTA-20 mM potassium phosphate (pH 7.0) and dialyzed against three 250-ml 1-h changes of the same buffer. After dialysis, precipitated proteins were removed by centrifugation at 12,500 × g for 20 min and the resulting supernatant was loaded onto a Hi-Prep 16/10 DEAE Fast Flow column connected to an Äktaprime Amersharm Pharmacia Biotech system equilibrated with 1 mM EDTA-20 mM potassium phosphate (pH 7.0). The column was eluted with 100 ml of the same buffer followed by a linear gradient from 0 to 1 M NaCl developed over 200 ml at a flow rate of 4 ml/min. The fractions with the highest purity as judged by enzymatic activity, UV-visible absorbance, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were pooled together and concentrated with the addition of 50% ammonium sulfate saturation followed by centrifugation. The resulting pellet was suspended in 2 M sorbitol-20% glycerol-20 mM potassium phosphate (pH 7.0) and dialyzed overnight against three 250-ml 1-h changes of the same buffer. After removal of precipitated proteins by centrifugation, the enzyme was stored at −20°C.

**Assays.** The concentration of choline dehydrogenase was determined by the method of Bradford (6) by using the Bio-Rad protein assay kit with bovine serum albumin as the standard. The oxidase activity of the enzyme was measured with 10 mM choline as the substrate in air-saturated 50 mM potassium phosphate, pH 7.0, 20 mM potassium phosphate (pH 7.0). System equilibrated with 1 mM EDTA was suspended in 2 M sorbitol-20% glycerol-20 mM potassium phosphate (pH 7.0) and dialyzed overnight against three 250-ml 1-h changes of the same buffer. After removal of precipitated proteins by centrifugation, the enzyme was stored at −20°C.

**RESULTS**

**Cloning of betA from H. elongata.** The bet operon of *H. elongata* containing the genes for choline uptake (*betI*) and glycine-betaine biosynthesis (*betBA*) was recently cloned in plasmid pDC4 by Canovas et al. (9). Plasmid pDC4 was used to amplify betA by primer extension reaction with *Pfu* DNA polymerase. Directional cloning of betA into plasmid pET23d(+) was accomplished by engineering NcoI and BamHI endonuclease sites at the 5' and 3' ends of betA, respectively. The resulting construct, pETCHDhe-S2A, was then used to transform *E. coli* strain XL1-Blue competent cells by using the heat shock method of Inoue et al. (20). Of the 125 transformant colonies obtained by plating on Luria-Bertani agar plates containing 50 μg of ampicillin/ml, 12 were screened for the presence of betA by colony primer extension reaction with *Pfu* DNA polymerase. One-third of the screened colonies were found to contain betA, as shown by the presence of an ~1,700-bp band when the primer extension reaction mixture was analyzed by agarose gel electrophoresis. Successful construction of plasmid pETCHDhe-S2A was confirmed by nucleotide sequence analysis.

In the cloning procedure, a single nucleotide substitution (T → G) was introduced at position 4 of betA as a result of the use of an NcoI endonuclease restriction site at the 5' end of the gene. The choice of an NcoI site was dictated by the observation that this site contains an ATG start codon and is not present at any internal portion of the gene. The single nucleotide substitution was removed from pETCHDhe-S2A by using the method of Kunkel (25), and the sequence of the resulting plasmid pETCHDhe was found to be correct.

**Characterization of choline dehydrogenase expressed in *E. coli.*** Recombinant choline dehydrogenase was produced in large quantities, greater than 5% of the total cell proteins, by using *E. coli* strain OrigamiI(DE3)pLysS cells, which provide an oxidizing environment in the cytosol. The choice of such a host cell strain was suggested by preliminary experiments on the expression of recombinant choline dehydrogenase from *Sinorhizobium meliloti* (Bethany Russell and Giovanni Gadda, unpublished results). Soluble and active enzyme was obtained by incubating the cell cultures in the presence of 0.8 mM IPTG at 25°C for 4 h after the optical density at 600 nm reached 0.8 (Fig. 2). Cell extracts of host cells which did not harbor the pETCHDhe plasmid did not show any protein band with the mass expected for choline dehydrogenase (Fig. 2) and were devoid of enzymatic activity, indicating that no choline-oxidizing activities other than the overexpressed enzyme were present in the cell extracts. Initial attempts to purify the enzyme expressed in *E. coli* by using octyl-, phenyl-, carboxymethyl-, or Q-Sepharose columns were unsuccessful as a result of the significant instability of the enzyme in the presence of high salt concentrations and the fact that choline dehydrogenase did not bind to these matrices (data not shown). Initial attempts to use streptomycin sulfate to precipitate nucleic acids from the cell extract led to the observation that a substantial fraction of the enzymatic activity partitioned in the insoluble fraction (data not shown) and could not be rescued in solution. In contrast, most of the contaminant proteins could be removed from the cell extract by binding to a Hi-Prep 16/10 DEAE Fast Flow column after treatment of the cell extract with 30 to 50% saturation of ammonium sulfate and dialysis (Table 1). The resulting partially purified enzyme could then be stabilized in solution in the presence of 2 M sorbitol and 20% glycerol, at pH 7.0, and was found to be more than 70% homogeneous by SDS-PAGE (Fig. 2). The UV-visible absorbance spectrum of the purified active enzyme did not show significant absorbance above 300 nm; in contrast, a peak centered at ~256 nm was observed in the UV region of the spectrum, suggesting that nucleic acids might copurify with the enzyme (data not shown).

Based on the deduced amino acid sequence of betA and in vivo experiments with [methyl-14C]choline on *E. coli*-harboring plasmids containing an *E. coli* choline transporter and betA from *H. elongata*, it has been previously proposed that betA from *H. elongata* encodes choline dehydrogenase (9). Since
choline dehydrogenase is capable of using either choline or betaine-aldehyde as a substrate (27, 44), we determined the apparent steady-state kinetic parameters for the partially purified enzyme with these substrates in air-saturated 50 mM potassium phosphate (pH 7.0 and 25°C). Initial rates of reaction were measured by monitoring the rate of oxygen consumption in the presence of 1 mM phenazine methosulfate as the primary electron acceptor with various concentrations of choline or betaine-aldehyde as the substrate for the recombinant enzyme. As shown in Fig. 3, the enzyme was active on both substrates. During the course of this study, it was noticed that when phenazine methosulfate was not added in the assay reaction mixture the enzyme was still able to oxidize choline (Fig. 4). Consequently, the apparent steady-state kinetic parameters for either choline or betaine-aldehyde were also determined with molecular oxygen as the primary electron acceptor. As shown in Fig. 3, the apparent $V_{max}$ values for choline and betaine-aldehyde in the absence of phenazine methosulfate decreased seven- and fourfold, respectively. In contrast, no significant changes were seen in the $V/K$ values. The values of the kinetic parameters determined in this study are summarized in Table 2.

The effect of exogenous FAD or PQQ on the activity of purified choline dehydrogenase was determined by measuring the initial rates of reaction with choline as the substrate and 1 mM phenazine methosulfate as the primary electron acceptor in the presence or absence of 1 mM FAD or PQQ in the assay reaction mixture. With 10 mM choline, the specific activity of the enzyme was 11.8 μmol of O$_2$ min$^{-1}$ mg$^{-1}$ in the absence of exogenous cofactors, 12.2 μmol of O$_2$ min$^{-1}$ mg$^{-1}$ in the presence of PQQ, and 11.5 μmol of O$_2$ min$^{-1}$ mg$^{-1}$ in the presence of FAD. When the concentration of choline was lowered to 5 mM, the specific activity was 8 μmol of O$_2$ min$^{-1}$ mg$^{-1}$ in the absence of exogenous cofactors, 7.4 μmol of O$_2$ 

![FIG. 2. Purification of recombinant choline dehydrogenase from H. elongata. Lanes: 1, marker proteins (E. coli β-galactosidase [116 kDa], rabbit muscle phosphorylase b [97 kDa], rabbit muscle fructose-6-phosphate kinase [84 kDa], bovine serum albumin [66 kDa], bovine liver glutamic dehydrogenase [55 kDa], chicken egg ovalbumin [45 kDa], rabbit muscle glyceraldehydes-3-phosphate dehydrogenase [36 kDa], bovine erythrocytes carbonic anhydrase [29 kDa], bovine pancreas trypsinogen [24 kDa], and soybean trypsin inhibitor [20 kDa]); 2, cell extract of E. coli strain OrigamiB(DE3)pLysS; 3, cell extract of E. coli strain OrigamiB(DE3)pLysS harboring plasmid pETCHDhe induced with 0.8 mM IPTG; 4, partially purified choline dehydrogenase.](image)

![FIG. 3. Steady-state kinetic parameters of choline dehydrogenase with choline (A) or betaine-aldehyde (B) as the substrate. Initial rates of reaction were determined with choline or betaine-aldehyde as the substrate in the presence (○) or absence (○) of 1 mM phenazine methosulfate in air-saturated 50 mM potassium phosphate (pH 7.0 and 25°C).](image)

**TABLE 1. Purification of recombinant H. elongata choline dehydrogenase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total units (μmol of O$_2$/min)</th>
<th>Total protein (mg)</th>
<th>Sp act (μmol of O$_2$/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>47</td>
<td>67</td>
<td>0.7</td>
</tr>
<tr>
<td>30–50% saturation of (NH$_4$)$_2$SO$_4$</td>
<td>26</td>
<td>30</td>
<td>0.9</td>
</tr>
<tr>
<td>Hi-Prep 16/10 DEAE</td>
<td>20</td>
<td>11</td>
<td>1.8</td>
</tr>
</tbody>
</table>

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FIG. 4. Dehydrogenase and oxidase catalytic activity of choline dehydrogenase. Shown is the time course of oxygen consumption during catalysis of choline dehydrogenase with 10 mM choline as the substrate in air-saturated 50 mM potassium phosphate (pH 7.0 and 25°C) and with 1 mM phenazine methosulfate (solid line) or 0.28 mM molecular oxygen (dashed line) as the final electron acceptor.

Based on nucleotide sequence analysis, it was previously proposed that in the moderate halophilic bacterium *H. elongata* the *betA* gene codes for choline dehydrogenase (9). As a first step towards the biochemical and mechanistic characterization of choline dehydrogenase, we cloned and expressed the *H. elongata betA* gene in *E. coli* cells under control of the T7 promoter, partially purified the resulting protein, and carried out a preliminary kinetic characterization of the solubilized enzyme. Our initial attempts to purify recombinant choline dehydrogenase expressed in *E. coli* cells by using a number of different matrixes for column chromatography were not successful as a result of the instability of the enzyme in high salt conditions. Keep in mind that the enzyme did not bind to the matrixes used. Previous attempts to purify choline dehydrogenase from other cellular sources, such as *Pseudomonas* (39) or rat liver mitochondria (44), also have been hampered by the difficulty of keeping the enzyme stable and active after extraction from its cellular source. In the present study, partial purification of stable and active *H. elongata* choline dehydrogenase was achieved by using column chromatography with a DEAE-Sepharose matrix after treating the cell extract with 30 to 50% saturation of ammonium sulfate.

The purified enzyme expressed from *betA* is a choline dehydrogenase. Clear evidence supporting such a conclusion comes from steady-state kinetic studies showing that the enzyme is capable of using either choline or betaine-aldehyde as a substrate in the presence of phenazine methosulfate as the primary electron acceptor (Fig. 3). These results are consistent with previously reported data showing that the product of the enzymatic oxidation of choline, glycine-betaine, can be formed in vivo when *E. coli* cells containing a choline transporter and *betA* from *H. elongata* are incubated with [methyl-14C]choline (9). The ability of the enzyme to catalyze both oxidative steps in the conversion of choline to glycine-betaine has previously been observed in choline dehydrogenase from *E. coli* (42) and rat liver mitochondria (44) and in choline oxidase from *Arthrobacter globiformis* (19).

In the absence of other primary electron acceptors, choline dehydrogenase can utilize molecular oxygen for catalysis (Fig. 4), indicating that the enzyme is also able to act as an oxidase. However, when oxygen is the electron acceptor, the *V*max values with betaine-aldehyde or choline as the substrate decrease four- to sevenfold with respect to the values seen with phenazine methosulfate, indicating that an electron acceptor other than oxygen is preferred by the enzyme. Previous studies on the enzyme from rat liver mitochondria suggested that coenzyme Q might be the primary electron acceptor for the enzyme in vivo (44). The observation that the enzyme from *H. elongata* can act either as a dehydrogenase or an oxidase depending on the availability of electron acceptors makes this enzyme different from choline dehydrogenase from rat liver mitochondria, *E. coli*, or *Pseudomonas*, for which an absolute requirement for an electron acceptor other than molecular oxygen has been shown (27, 39, 44).

The substrate specificity of choline oxidase is not affected to a great extent upon substitution of an alcohol for an aldehyde substrate, as indicated by the apparent *V*/*K* values for choline and betaine-aldehyde (Table 2). Since aldehydes are partly hydrated in aqueous solution to yield a gem-diol of the formula R-CH(OH)2 (4), it is likely that the hydrated form of betaine-aldehyde is the actual substrate for choline dehydrogenase. If this is the case, one would not expect significant differences in the enzyme specificity upon substituting an alcohol-containing substrate, such as choline, for a gem-diol-containing substrate, such as betaine-aldehyde. The alternative explanation that the hydroxyl group of choline and the carbonyl group of betaine-aldehyde play only a minor role in defining the substrate spec-

### TABLE 2. Apparent steady-state kinetic parameters for choline dehydrogenase from *H. elongata*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Electron acceptor</th>
<th><em>V</em>max (μmol of O₂/min/mg)</th>
<th><em>K</em>m (mM)</th>
<th><em>V</em>/<em>K</em> (μmol of O₂/min/mg/mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline</td>
<td>O₂</td>
<td>1.5 ± 0.1</td>
<td>2.2 ± 0.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Phenazine methosulfate⁵</td>
<td>10.9 ± 0.5</td>
<td>11.6 ± 1.4</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Betaine-aldehyde</td>
<td>O₂</td>
<td>1.4 ± 0.04</td>
<td>3.0 ± 0.3</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Phenazine methosulfate</td>
<td>5.7 ± 0.3</td>
<td>9.6 ± 1.2</td>
<td>0.6 ± 0.04</td>
</tr>
</tbody>
</table>

⁵ Assays were performed in air-saturated 50 mM potassium phosphate (pH 7.0) at 25°C, and the reaction was monitored using a Clark-type oxygen electrode.

⁵ The concentration of phenazine methosulfate was 1 mM.
ificity of the enzyme appears less likely in that both these groups are intimately involved in catalysis.

As pointed out by Landfald and Strom, a glycine box of the type G-X-G-X-G is found in the N-terminal region of choline dehydrogenase from *E. coli* (27), suggesting that the enzyme might use FAD as a cofactor for catalysis. Such a sequence is also present in the enzyme from *H. elongata*, which shares 74% identity with the *E. coli* enzyme in the deduced amino acid sequence (9). However, no direct biochemical evidence has been previously reported indicating that a flavin cofactor is actually bound or required for catalysis with these enzymes. The active choline dehydrogenase purified in our study does not show any significant absorbance in the 300- to 500-nm region of the UV-visible spectrum, which would be expected for a flavin-containing enzyme with the cofactor bound in the oxidized state. Furthermore, as in the case of choline dehydrogenase from rat liver and *E. coli* (27, 44), addition of exogenous FAD to the assay reaction mixture did not yield an increase in the enzymatic activity of *H. elongata* choline dehydrogenase, indicating no requirement for soluble FAD for catalysis. Although the data presented in this study do not rule out with absolute certainty an involvement of FAD in catalysis, it is possible that a different and as-yet-unidentified cofactor is required by *H. elongata* choline dehydrogenase. In this respect, it has previously been shown that the enzyme from a *Pseudomonas* strain, which also contains a G-X-G-X-G putative FAD-binding site, does not contain or require FAD for catalysis (39). In that case, a positive result was obtained when the enzyme was analyzed for the presence of POQ. However, the activity of the choline dehydrogenase purified in our study did not change when exogenous POQ was added to the assay reaction mixture, indicating that soluble POQ is also not required for catalysis by the enzyme.

In conclusion, the data reported in the present study are consistent with the enzyme encoded by betA in *H. elongata* being a choline dehydrogenase that can also act, although with a lower catalytic efficiency, as an oxidase. To our knowledge, this study represents the first instance in which a choline dehydrogenase from a bacterial source has been cloned, expressed in soluble form, and partially purified. The availability of recombinant enzyme will prove to be a useful tool for the study of the biochemical and kinetic properties of choline dehydrogenase that is aimed at achieving a better understanding of the molecular basis of osmoprotection in bacteria. This, in turn, has great potential for biotechnological applications aimed at genetically engineering stress tolerance in crop plants of economic interest.

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**REFERENCES**


