

Cloning, sequence analysis, and purification of choline oxidase from *Arthrobacter globiformis*: a bacterial enzyme involved in osmotic stress tolerance[☆]

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Received 24 September 2003, and in revised form 14 October 2003

Abstract

Choline oxidase catalyzes the four-electron oxidation of choline to glycine betaine, one of a limited number of compounds that accumulate to high levels in the cytoplasm of cells to prevent dehydration and plasmolysis in adverse hyperosmotic environments. In the present study, the highly GC rich *codA* gene encoding for choline oxidase was cloned from genomic DNA of *Arthrobacter globiformis* strain ATCC 8010 and expressed to high yields in *Escherichia coli* strain Rosetta(DE3)pLysS. The resulting enzyme was purified to high levels in a single chromatographic step using DEAE-Sepharose, as shown by SDS-PAGE analysis. Denaturation and mass spectroscopic analyses showed that the covalent linkage between the FAD cofactor and the protein is preserved in recombinant choline oxidase, consistent with protein flavinylation being a self-catalytic process. The enzyme was shown to be a homodimer of 120,000 Da by size-exclusion chromatography and to be active with both choline and betaine aldehyde as substrate. Sequencing analysis indicated that the nucleotide sequence of *codA* originally reported in GenBank contains seven flaws, resulting in a translated protein with a significantly altered amino acid sequence between position 298 and 410.

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Keywords: Choline oxidase; FAD; Flavin; Glycine betaine; Choline; Osmoprotection; GMC oxidoreductases; Self-flavinylation

Choline oxidase (E.C. 1.1.3.17) catalyzes the four-electron oxidation of choline to glycine betaine (*N,N,N*-trimethylglycine; betaine) via betaine aldehyde as intermediate. Molecular oxygen acts as primary electron acceptor in the reaction (Scheme 1) [1]. The enzyme is of importance because glycine betaine is one of a limited number of compatible solutes that accumulate to high levels in the cytoplasm of cells to prevent dehydration and plasmolysis in adverse hyperosmotic environments [2–6]. Regulation of intracellular osmolality to hyperosmotic environments is also intimately connected to a number of physiological responses, such as increased heat and cold tolerance, as well as regulation of the

internal pH and ionic strength [7–12]. Hyperosmolarity is often encountered at human infection sites [13], where choline and its precursors are very abundant [14–16], and is a major environmental signal controlling the expression of genes associated with cellular invasion and virulence in a number of human pathogens [17–24]. For these reasons, the study of choline oxidase is of considerable interest both for genetically engineering water and osmotic stress resistance in economically relevant crop plants lacking efficient glycine betaine biosynthetic systems [10,11,25–29], and for the potential development of therapeutic agents that inhibit the biosynthesis of glycine betaine thereby making bacteria more susceptible to conventional treatments.

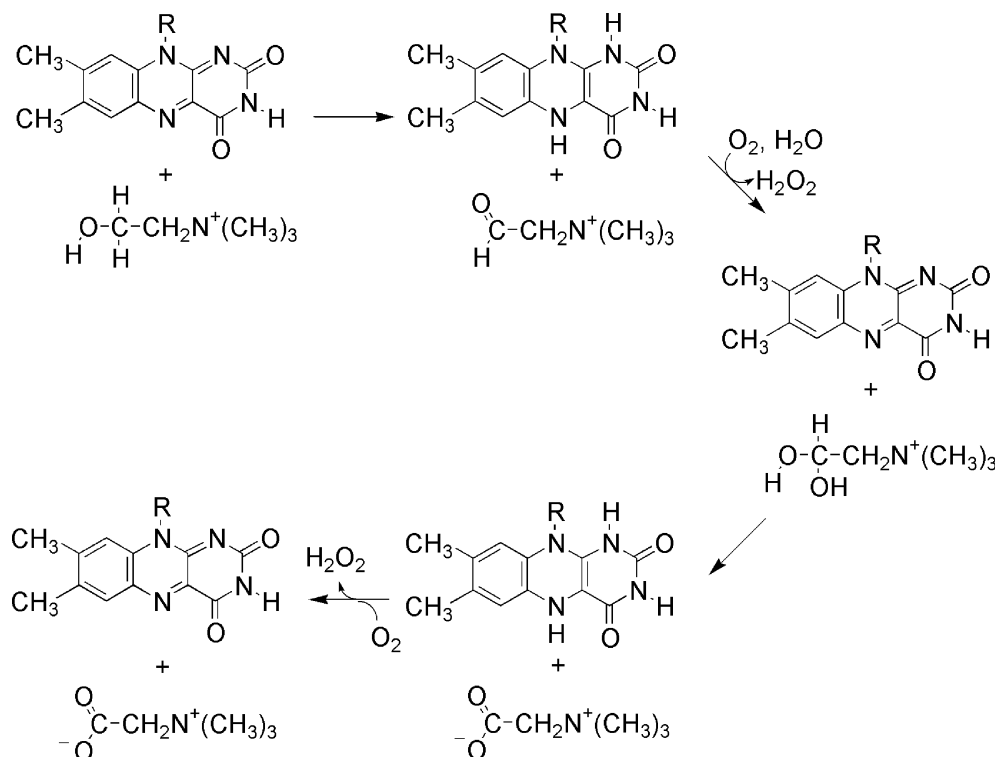
Choline oxidase has been purified from *Cylindrocarpum didymum* M-1 [30], *Alcaligenes* sp. [31], and *Arthrobacter globiformis* [1]. Based on amino acid sequence comparisons, the enzyme can be grouped in the GMC oxidoreductase enzyme superfamily [32], which

[☆] Data deposition: the sequence reported in this paper has been deposited in the GenBank database (Accession No. AY304485).

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Scheme 1.

comprises enzymes like glucose oxidase, cholesterol oxidase, or cellobiose dehydrogenase, that utilize FAD as cofactor for catalysis and non-activated primary alcohols as substrate. Despite a wealth of studies on the biotechnological applications of the enzyme, minimal biochemical and mechanistic investigations of choline oxidase have been reported to date. In this respect, our group has recently reported the analyses of the steady state kinetic isotope effects on the oxidation of choline to betaine aldehyde catalyzed by choline oxidase from *A. globiformis* [33,34]. One of the most critical impediments to future studies is the obtainment of large quantities of choline oxidase for detailed biophysical, mechanistic, and structural investigations. In the present study, we have cloned from *A. globiformis* genomic DNA the *codA* gene encoding for choline oxidase and heterologously expressed it in *Escherichia coli*. The resulting recombinant enzyme has been purified to homogeneity in a single chromatographic step and characterized for its flavin content, catalytic activity, and oligomerization state.

Materials and methods

Materials

Escherichia coli strain JM109 harboring plasmid pGAH/*codA* was a kind gift from Dr. Murata, National

Institute for Basic Biology, Okazaki, Japan [27,28]. A freeze-dried culture of *A. globiformis* (ATCC 8010) was obtained from American Type Culture Collection. Restriction endonucleases *Nde*I and *Eco*RI, calf intestinal alkaline phosphatase, T4 DNA ligase, *Taq* DNA polymerase, deoxynucleotide triphosphates, and bovine serum albumin were purchased from Promega. *Pfu* DNA polymerase was obtained from Stratagene or Roche Molecular Biomedicals. Luria–Bertani agar, Luria–Bertani broth, chloramphenicol, tetracycline, kanamycin, isopropyl-β-D-thiogalactopyranoside (IPTG),² lysozyme, phenylmethylsulfonyl fluoride (PMSF), and betaine aldehyde bromide were obtained from Sigma-Aldrich. Carbenicillin, ampicillin, choline chloride, and electrophoresis-grade agar were purchased from ICN Biomedicals. Nutrient Broth and Nutrient Agar #3 were from Difco. All other reagents were of the highest purity commercially available. Oligonucleotides were custom synthesized on a Beckman Oligo model 1000 M by the DNA Core Facility of the Biology Department of Georgia State University. *A. globiformis* genomic DNA was purified using the DNeasy midi-kit from Qiagen. Plasmids and products derived from primer extension reaction and PCR were purified by using mini-kits from Qiagen. *E. coli* strains Novablue (Novagen) and XL1-Blue (Stratagene) were used during cloning procedures,

² Abbreviations used: IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

whereas strain Rosetta(DE3)pLysS (Novagen) was used for protein expression. *E. coli* strain Novablue cells harboring plasmid pET/*codA1* or pET/*codA2*, strain XL1-Blue cells harboring plasmid pET/*codA3*, and strain Rosetta(DE3)pLysS cells harboring plasmid pET/*codA1* or pET/*codA3* were stored at -80°C as 7% DMSO 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.

Sub-cloning of codA into the expression vector pET20b(+)

E. coli strain JM109 harboring plasmid pGAH/*codA* was grown on Luria–Bertani agar medium containing kanamycin at a final concentration of $15\text{ }\mu\text{g/ml}$ for 16 h at 37°C . Single colonies were used to inoculate 2 ml of Luria–Bertani broth medium containing kanamycin ($15\text{ }\mu\text{g/ml}$) and the resulting liquid cultures were grown at 37°C for 7 h. After harvesting the cells by centrifugation at $14,000g$ for 10 min, the plasmid vector was isolated by using a QIAquick Spin Miniprep kit (Qiagen) following the manufacturer's protocol. The isolated plasmid DNA was then used for primer extension reaction of the *codA* gene encoding for choline oxidase by using the oligonucleotide primers Chox-for A and Chox-rev containing *NdeI* and *EcoRI* restriction endonuclease sites designed to anneal to the 5' and 3' ends of the gene, respectively (Table 1). The *NdeI* and *EcoRI* restriction sites introduced at the 5' ends of the sense and antisense primers allowed cloning of *codA* into the corresponding sites of pET20b(+). Primer extension reaction was performed in the presence of DMSO at a final concentration of 2% for 1 min at 95°C , followed by 31 three-step cycles of 0.5 min at 95°C , 1 min at 55°C , and 4.5 min at 68°C , with a final 5-min step at 68°C , in a total volume of $50\text{ }\mu\text{l}$ by using $\sim 15\text{ ng}$ template DNA, 2.5 U *Pfu* DNA polymerase, and the manufacturer's suggested protocol. The resulting amplicons were purified by agarose gel electrophoresis using the QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol.

The pET20b(+) plasmid vector was isolated from an *E. coli* strain XL1-Blue grown for 16 h at 37°C in 4 ml of Luria–Bertani broth containing ampicillin at a final concentration of $50\text{ }\mu\text{g/ml}$ by using the QIAprep Spin Miniprep kit from Qiagen, following the manufacturer's instructions.

Both the amplified *codA* and pET20b(+) vector were digested for 3 h at 37°C with 30 U *NdeI* and 36 U *EcoRI* in a total volume of $60\text{ }\mu\text{l}$ of 10 mg/ml bovine serum albumin, 0.15 mM sodium chloride, 6 mM magnesium chloride, 1 mM dithiothreitol, and 6 mM Tris–Cl at pH 7.9, followed by purification of the DNA by agarose gel electrophoresis using the QIAquick Gel Extraction kit. To ensure minimal self-ligation of the plasmid, pET20b(+) was further treated with 3 U calf intestine alkaline phosphatase for 3 h at 37°C , followed by purification of the DNA by gel electrophoresis extraction. The *codA* gene (160 ng) was then ligated into the pET20b(+) (120 ng) plasmid in the presence of 2% DMSO by incubation for 16 h at 4°C with 3 U T4 DNA ligase in a total volume of $60\text{ }\mu\text{l}$, and $5\text{ }\mu\text{l}$ of the ligation reaction mixture was used to transform directly $100\text{ }\mu\text{l}$ of *E. coli* strain XL1-Blue competent cells by using the electroporation method. The resulting transformant colonies obtained by plating on Luria–Bertani agar plates containing ampicillin (or carbenicillin) at a final concentration of $50\text{ }\mu\text{g/ml}$ were screened for the presence of *codA* by colony primer extension amplification with 2.5 U of *Pfu* DNA polymerase in the presence of 2% DMSO using the protocol described above. Sense and antisense oligonucleotide primers designed to bind to the DNA regions of pET flanking the inserted gene were used in the screening (Table 1). The correct construct, pET/*codA*, was sequenced in both directions by using the oligonucleotide primers shown in Table 1, and *E. coli* strain Rosetta(DE3)pLysS competent cells were then transformed with plasmid pET/*codA3* following the procedures described above.

Cloning of codA from A. globiformis genome into the expression vector pET20b(+)

Arthrobacter globiformis strain ATCC 8010 was grown on Difco #3 Nutrient Agar medium for 48 h at 26°C . Single colonies were used to inoculate eight 5 ml of

Table 1
Oligonucleotide primers used for primer extension amplification and PCR of *codA*

Primer	Nucleotide sequence	Purpose
Chox-for A	CGGCAAGGAGAACCATATGCACATCGACAACATCTG	Cloning
Chox-rev	CCCCGGAATTCGCCGCTCCCGCTTAGG	Cloning
PET-for A	CACTATAGGGAGACCACAACG	Sequencing
PET-rev A	GCTTATGCTAGTTATTGCTCAGC	Sequencing
Choxag 3	CAATGAAGTCGTGCTCTCC	Sequencing
Choxag 5	CGAAGTTCAACACC	Sequencing
Choxag 7	CGATGCAGGAGTTGTGG	Sequencing

Endonuclease restriction sites for *NdeI* and *EcoRI* enzymes are underlined.

Difco #3 Nutrient Broth and the resulting liquid cultures were grown overnight at 26 °C. The cells were harvested by centrifugation at 14,000g for 10 min and the genomic DNA was isolated using the DNeasy Midi-kit (Qiagen) according to manufacturer's instructions. The isolated genomic DNA was then used for PCR of the *codA* gene with the oligonucleotide primers described above. PCR was carried out with an Eppendorf Mastercycler in the presence of 4% DMSO for 1 min at 95 °C, followed by 31 cycles of 1 min at 95 °C, 1 min at 50 °C, 2 min at 72 °C, and a final 5-min step at 72 °C in a total volume of 50 µl by using ~200 ng of template DNA, 5 U *Taq* DNA polymerase, 3 mM magnesium chloride, and the polymerase manufacturer's suggested protocol. The resulting amplicons were purified by agarose gel electrophoresis using the QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol.

Endonuclease digestion of both the amplified *codA* and pET20b(+) with *Nde*I and *Eco*RI, dephosphorylation of the plasmid with calf intestine alkaline phosphatase, and ligation were carried out as described above, and 1 µl of the ligation reaction mixture was used directly to transform by electroporation 100 µl of *E. coli* strain Novablue competent cells. The resulting transformant colonies obtained by plating on Luria–Bertani agar plates containing 50 µg/ml ampicillin and 12.5 µg/ml tetracycline were screened for the presence of *codA* by colony PCR. PCR was performed with 5 U *Taq* DNA polymerase in the presence of 2% DMSO for 1 min at 95 °C followed by 31 three-step cycles of 1 min at 95 °C, 2 min at 55 °C, 2 min at 72 °C, and a 5-min final step at 72 °C, using sense and antisense oligonucleotide primers designed to bind to DNA regions of pET20b(+) flanking the inserted gene. The correct constructs, pET/*codA*1 and pET/*codA*2, were sequenced in both directions as described above. *E. coli* strain Rosetta(DE3)pLysS competent cells were transformed with plasmid pET/*codA*1 for protein expression.

Expression of choline oxidase in E. coli

Permanent frozen stocks of *E. coli* cells Rosetta(DE3)pLysS harboring plasmid pET/*codA*1 were used to inoculate 50 ml of Luria–Bertani broth medium containing ampicillin (or carbenicillin) and chloramphenicol at final concentrations of 50 and 34 µg/ml, respectively, at 37 °C. After 9 h, 1 ml of the starter culture was used to inoculate 3 × 1.5 liters of the same liquid culture medium at 30 °C. When the culture reached an optical density at 600 nm between 0.8 and 1.4, typically after 16 h, IPTG was added to a final concentration of 50 µM and the temperature of the culture was lowered between 21 and 25 °C. Cells were harvested by centrifugation at 20,000g for 20 min at 4 °C and stored at –20 °C. Typically, ~8 g of wet cell paste was obtained from 1.5 liters of cell culture.

Purification of recombinant choline oxidase

Unless otherwise stated, all the purification steps were carried out at 4 °C. The cell paste, typically 25 g, was suspended in 6 volumes of a solution of 0.1 mM PMSF, 0.2 mg/ml lysozyme, 1 mM EDTA, and 50 mM potassium phosphate at pH 7 and allowed to incubate with stirring for 30 min on ice. The resulting slurry was passed three times through an SLM Aminco French pressure cell at 20,000 lb/in.² and then centrifuged at 20,000g for 20 min. The supernatant was collected, incubated with stirring for 30 min on ice with 20 µg/ml RNase and 50 µg/ml DNase in the presence of 10 mM magnesium chloride, and centrifuged as described above. The soluble fraction was brought to 30% ammonium sulfate saturation, incubated for 30 min on ice, and separated from the insoluble fraction by centrifugation as described above. Choline oxidase was then collected in the pellet fraction by treatment with ammonium sulfate at a final saturation of 65%, followed by centrifugation as described above. The resulting pellet was suspended in 25 ml of 200 mM Tris–Cl, pH 8, and dialyzed against three 1-liter changes of the same buffer over 20 h. After dialysis, precipitated proteins were removed by centrifugation at 18,700g for 20 min and the resulting supernatant was loaded onto a DEAE–Sephacrose Fast Flow column (3 × 28 cm) connected to an Äktaprime Amersham–Pharmacia Biotech system equilibrated with 200 mM Tris–Cl at pH 8. The column was eluted with 2 volumes of the same buffer, followed by a linear gradient from 0 to 0.5 M NaCl developed over 5 volumes at a flow rate of 2 ml/min. The fractions with the highest purity as judged by enzymatic activity and UV–visible absorbance spectroscopy were pooled together and concentrated with the addition of 65% ammonium sulfate saturation followed by centrifugation. The resulting pellet was resuspended in 10 ml of 200 mM Tris–Cl, pH 8, and dialyzed against three 250-ml changes of the same buffer over 20 h. After removal of the precipitated protein by centrifugation, the enzyme stored at –20 °C was stable for at least six months.

Enzyme assays

The concentration of choline oxidase was determined with the method of Bradford [35], by using the Bio-Rad protein assay kit with bovine serum albumin as the standard. The oxidized flavin content per enzyme active site was determined as described in [36]. Enzyme activity was measured with the method of the initial rates [37] in air-saturated 50 mM potassium phosphate at pH 7 by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument) thermostated at 25 °C. The reactions were started by the addition of choline oxidase to a 1 ml reaction mixture, with the final concentration

of enzyme in the 0.1–0.5 μM range; the concentration of choline or betaine aldehyde was between 0.02 and 10 mM. UV–visible absorbance spectra were recorded using an Agilent Technologies diode-array spectrophotometer Model HP 8453 equipped with a thermostated water bath. Fluorescence emission spectra were recorded with a Shimadzu Spectrofluorometer Model RF-5301 PC thermostated at 15 °C.

Methods

The molecular mass of the enzyme was determined by MALDI-TOF mass spectrometry on a Micromass ToFSpec 2E at the Mass Spectrometry Laboratory of the Georgia Institute of Technology, Atlanta. Samples were prepared for MALDI-TOF analysis by gel filtration using a Sephadex G-25 column (PD-10, Amersham-Pharmacia Biotech) equilibrated with 5 mM Tris-Cl, pH 8. MALDI-TOF spectra were acquired in the positive ion mode using sinapinic acid as the matrix, with an acceleration voltage of –20 kV and a pulse voltage of –16 kV. Cytochrome *c* [12,400], myoglobin [17,000], trypsinogen [24,000], and bovine serum albumin [66,000] were used as standards for mass calibration. The molecular weight of the enzyme under non-denaturing conditions was determined by size exclusion chromatography through a Sephacryl S-400 column (1 $\times$ 50 cm) connected to an Äktaprime Amersham-Pharmacia Biotech system equilibrated with 300 mM KCl in 20 mM potassium phosphate, pH 7, at a flow rate of 0.5 ml/min. The following proteins were used as standards: horse heart cytochrome *c* [12,400], bovine serum albumin [66,000], yeast alcohol dehydrogenase [150,000], sweet potato β -amylase [200,000], and horse spleen apoferritin [443,000]. SDS-PAGE according to the method of Laemmli [38] and PAGE under non-denaturing conditions were performed in 12% polyacrylamide slab gels, with visualization of the proteins by staining with Coomassie brilliant blue G-250.

For denaturation experiments, choline oxidase was incubated at 40 °C for 1 h in the presence of urea at a final concentration of 4 M in 100 mM Tris-Cl, pH 8, before recording the UV–visible absorbance spectrum. The flavin content per monomer of enzyme was calculated from the ratio of the concentration of flavin in the denatured enzyme using a $\epsilon_{450\text{nm}}$ value of 11.3 mM⁻¹ cm⁻¹ [39] to the concentration of protein determined using the Bradford assay [35]. To establish whether the flavin is covalently bound to recombinant choline oxidase, the enzyme was incubated with trichloroacetic acid at a final concentration of 10% for 5 min at 100 °C, followed by removal of denatured protein by centrifugation at 14,000g for 10 min before measuring the UV–visible absorbance spectrum of the resulting supernatant.

Data analysis

Kinetic data were fit with the KaleidaGraph software (Adalbeck Software, Reading, PA). Apparent kinetic parameters in atmospheric oxygen were determined by fitting initial reaction rates at different substrate concentrations to the Michaelis–Menten equation (Eq. (1)), where K_a represents the Michaelis constant for choline (or betaine aldehyde) (*A*) and k_{cat} is the turnover number of the enzyme (*e*).

$$\frac{v}{e} = \frac{k_{\text{cat}}A}{K_a + A} \quad (1)$$

Results

Cloning of *codA*

The *codA* gene from *A. globiformis* encoding for choline oxidase was previously cloned in the binary vector plasmid pGAH for in vivo experiments aimed at conferring tolerance to salt stress in cyanobacteria and plants [10,11,25–28,40,41]. In this study, *codA* was amplified from pGAH/*codA* by primer extension reaction by using *Pfu* DNA polymerase in the presence of 2% DMSO. Addition of DMSO was required to ensure complete denaturation of the highly GC rich DNA template in the primer extension reaction. Directional cloning of *codA* into pET20b(+) to construct plasmid pET/*codA3* was achieved by inserting *NdeI* and *EcoRI* endonuclease restriction sites at the 5' and 3' terminal ends of the gene, respectively. Plasmid pET/*codA3* was then used to transform *E. coli* competent cells strain XL1-Blue. Successful construction of plasmid pET/*codA3* was confirmed by nucleotide sequence analysis. However, seven differences were found in the nucleotide sequence of *codA* with respect to the sequence of the gene deposited in GenBank (Accession No. X84895). Of the observed discrepancies, three accounted for missing nucleotides at positions 891, 1117, and 1232, resulting in a translated sequence of 113 amino acid residues in the central portion of the protein being different from the published sequence (GenBank Accession No. S52489). Surprisingly, no internal stop codons were created by the three single-point deletions, resulting in a translated protein of 546 amino acid residues. Two other differences were observed at positions 764 and 1496, which resulted in the substitution of an arginine with a histidine residue and of a glycine with an alanine residue, respectively. Finally, two silent nucleotide substitutions that resulted in no change at the amino acid level were observed at positions 832 and 846. The nucleotide sequence of *codA* was found to contain the same seven discrepancies when the gene was sequenced directly from pGAH/*codA*,

suggesting that the differences did not originate in the sub-cloning procedure.

To verify further that the nucleotide differences were not introduced in the sub-cloning process from pGAH/*codA*, genomic DNA from *A. globiformis* strain ATCC 8010 was used as template to amplify the *codA* gene by using PCR with *Taq* DNA polymerase. The rationale for this strategy was that possible misincorporations of nucleotides occurring in the gene amplification procedure most likely would occur at different positions in the nucleotide sequence of the gene by using different DNA templates, DNA polymerases, and PCR protocols. As a further control, *codA* was cloned from genomic DNA in two independent experiments to produce the constructs pET/*codA1* and pET/*codA2*. The nucleotide sequence of the *codA* gene cloned from genomic DNA was the same as that determined using pGAH/*codA*.

Heterologous expression of *codA* in *E. coli*

Heterologous expression of soluble choline oxidase from pET/*codA1* was achieved within 5 h of induction with 50 μ M IPTG at 25 °C in *E. coli* strain

Rosetta(DE3)pLysS, which supplies tRNAs for codons that are rarely used in *E. coli*. The choice of such a bacterial host strain for protein expression was dictated by the observation that, due to its high GC content, *codA* contains 27 of such rare codons. Recombinant choline oxidase was purified to homogeneity, as judged by SDS-PAGE analysis (Fig. 1), using 30–65% ammonium sulfate saturation followed by anion exchange chromatography onto a DEAE-Sephacrose Fast Flow column equilibrated in 200 mM Tris-Cl at pH 8. Typically, between 150 and 200 mg of pure choline oxidase could be obtained from 4.5 liters of liquid Luria-Bertani culture medium (Table 2).

Physical properties of recombinant choline oxidase

The molecular weight of recombinant choline oxidase was determined by MALDI-TOF mass spectrometry. Two peaks with m/z^+ values of 30307.5 and 60618.3 were observed using the positive ion mode, corresponding to the doubly and singly protonated species of the protein, respectively (Fig. 2). A molecular weight of $60,614 \pm 5$ was calculated from the mass spectroscopic

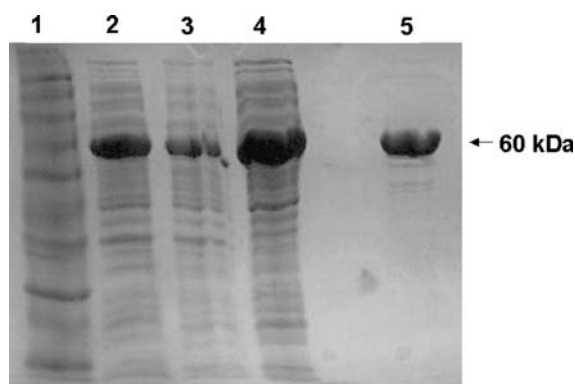


Fig. 1. Purification of recombinant *A. globiformis* choline oxidase heterologously expressed in *E. coli*. Lane 1, marker proteins; lane 2, cell-free extract of *E. coli* strain Rosetta(DE3)pLysS harboring plasmid pET/*codA1* induced with 0.05 mM IPTG for 5 h at 22 °C; lane 3, sample treated with 20 μ g/ml RNase and 50 μ g/ml DNase in the presence of 10 mM $MgCl_2$; lane 4, sample treated with 30 and 65% ammonium sulfate saturation; and lane 5, purified choline oxidase in 200 mM Tris-Cl, pH 8, after DEAE-Sephacrose column chromatography. The molecular mass of purified choline oxidase is indicated.

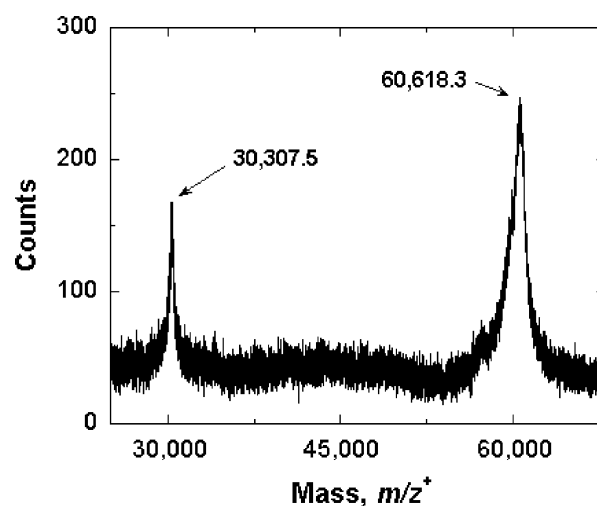


Fig. 2. MALDI-TOF mass spectrometric analysis of recombinant choline oxidase. Choline oxidase at a concentration of 46 μ M was prepared as described in Materials and methods. Peaks with m/z^+ values of 60618.3 and 30307.5 represent the singly and doubly protonated species of the protein.

Table 2

Purification of recombinant *A. globiformis* choline oxidase heterologously expressed in *E. coli*

Step	Total protein (mg)	Total activity ^a (μ mol O_2 min ⁻¹)	Specific activity (μ mol O_2 min ⁻¹ mg ⁻¹)	Yield (%)
Cell-free extract	1830	1080	0.59	100
Nuclease treated	1500	1060	0.71	98
30–65% Saturation of $(NH_4)_2SO_4$	1240	1040	0.84	96
DEAE-Sephacrose FF	185	940	5.3	87

^a Enzymatic activity was measured with 10 mM choline as substrate in air-saturated 50 mM potassium phosphate, pH 7.0, and 25 °C, by monitoring the rate of oxygen consumption with a Clark-type oxygen electrode.

data, in agreement with the expected value of 60,612 calculated from the amino acid composition of the protein with one linked FAD moiety [30,31,52].

The oligomerization state of choline oxidase was determined by size exclusion chromatography onto a Sephacryl S-400 column under non-denaturing conditions. The results indicated that at concentrations of 10 or 100 μM the enzyme has apparent molecular weights of 117,000 and 122,000, respectively (data not shown), indicating that the enzyme exists in solution as a dimer. Non-denaturing PAGE conditions of the enzyme at concentrations in the range between 2 and 48 μM yielded single bands with similar relative migration, suggesting that the oligomerization state of choline oxidase is not dependent on the concentration of the enzyme (data not shown).

Flavin content of recombinant choline oxidase

The UV–visible absorbance spectrum of the enzyme as purified is shown in Fig. 3. Absorbance maxima were observed at 272, 373, and 454 nm, as expected for a flavin-containing enzyme but with relative intensities of the peaks in the near UV and visible regions of the spectrum, suggesting that the bound flavin was present as a mixture of oxidized and anionic semiquinone states³ [42]. Significant variability in the relative intensities of the absorbance maxima in the near UV and visible regions of the spectrum was observed among different preparations of enzyme, with A_{454}/A_{373} ratios ranging from 0.4 to 0.7 (data not shown). Upon denaturation of the enzyme by treatment with urea, a decrease in the 373 nm peak with concomitant increase in the absorbance in the 450 nm region was observed (Fig. 3), consistent with oxidation of the semiquinone to form fully oxidized FAD in the denatured enzyme. This enzyme was devoid of enzymatic activity using 10 mM choline as substrate at pH 7, suggesting that the treatment with urea completely denatured the enzyme. No significant changes in the UV–visible absorbance spectrum of the denatured enzyme were seen after dialysis with decreasing amounts of urea, consistent with FAD being covalently bound to the enzyme (data not shown). Also, the UV–visible spectrum of a soluble fraction prepared upon treatment of choline oxidase with 10% trichloroacetic acid followed by centrifugation to remove the denatured protein was devoid of absorbance (Fig. 3), further consistent with FAD being covalently bound to the protein. A stoichiometry of 0.88 ± 0.12 FAD/

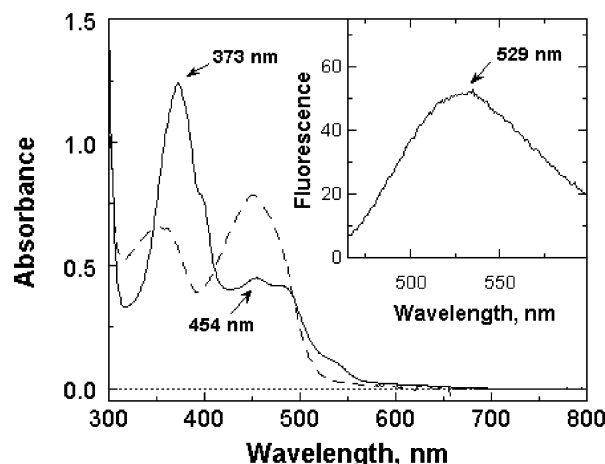


Fig. 3. UV–visible absorbance spectrum of recombinant choline oxidase. Solid line, choline oxidase as purified at a concentration of 112 μM in 200 mM Tris–Cl, pH 8; dashed line, after treatment with 4 M urea at 40 °C for 40 min; and dotted line, soluble fraction after treatment of choline oxidase with 10% trichloroacetic acid for 5 min at 100 °C and centrifugation to remove denatured protein. Inset, fluorescence emission spectrum of choline oxidase as purified at a concentration of 28 μM in 200 mM Tris–Cl, pH 8, and 15 °C. The excitation wavelength was at 454 nm.

monomer of enzyme could be calculated from the results of six independent experiments. The visible fluorescence emission spectrum of purified choline oxidase showed a maximum in the 525–530 nm region (with λ_{ex} at 454 nm), with about 6% the intensity of an equimolar solution of free FAD (Fig. 3).

Kinetic properties of recombinant choline oxidase

The steady state kinetic parameters for choline and betaine aldehyde as substrate for the enzyme as purified were determined in air-saturated 50 mM potassium phosphate at pH 7 and 25 °C. Apparent k_{cat} values of 13.4 ± 0.5 and $11.6 \pm 0.3 \text{ s}^{-1}$ per active site oxidized flavin and K_{m} values of 0.6 ± 0.1 and $2.3 \pm 0.2 \text{ mM}$ were determined with choline and betaine aldehyde, respectively. While the K_{m} values for recombinant choline oxidase compared well with the values of 0.6 and 2.4 mM previously reported for choline and betaine aldehyde as substrates for the native enzyme from *A. globiformis*, the k_{cat} values were between 3- and 6-fold larger, suggesting a higher purity of our enzyme preparation as compared to that of the commercially available enzyme [33].

Discussion

Our group recently reported studies on the steady state kinetic mechanism, pH, and substrate kinetic isotope effects on choline oxidase from *A. globiformis*

³ A spectroscopic investigation of recombinant choline oxidase by using UV–visible and fluorescence spectroscopy, electron spin resonance, and circular dichroism is consistent with the presence of an anionic semiquinone flavin in the enzyme as purified [36]. Steady state kinetic data on the enzyme as purified suggest that the semiquinone flavin of choline oxidase is not catalytically relevant [36].

[33,34]. Those studies define a framework for advanced biophysical and mechanistic studies on choline oxidase, for which large amounts of enzyme are required. To date, the enzyme from *A. globiformis* is the only choline oxidase for which the nucleotide sequence of the full gene has been reported [27]. In the present study, we have cloned and heterologously expressed in *E. coli* the gene encoding for choline oxidase from *A. globiformis* genomic DNA, and purified to homogeneity the resulting protein.

Purified choline oxidase expressed in *E. coli* contains covalently bound FAD, as indicated by the mass spectroscopic analysis of the purified enzyme. Thus, the covalent linkage between His-87 and the 8 α position of the flavin ring is preserved in the recombinant enzyme [52], consistent with flavinylation of the protein being a self-catalytic process. In this respect, self-flavinylation of histidyl, tyrosyl, and cysteinyl residues to the 8 α position of the flavin ring was previously proposed to occur through initial flavin tautomerization to yield an electrophilic iminoquinone methide, which is stabilized by the presence of a positive charge located near the N(1)–C(2)=O(2) position of the flavin [43–46]. Consistent with such a proposed mechanism for protein flavinylation, the presence of a weak positive charge in proximity of the flavin N(1)–C(2)=O(2) locus of choline oxidase is suggested by both stabilization of the anionic flavin semiquinone and formation of a complex with sulfite [36,47].

Under non-denaturing conditions, choline oxidase is a dimer of identical subunits with each subunit containing FAD in a 1:1 stoichiometry per monomer of protein. The oligomerization state of the recombinant enzyme determined in this study does not agree with earlier results on native choline oxidase from *A. globiformis* and *Alcaligenes* sp., showing masses of 83,000 and 72,000 Da, respectively [1,31]. In this respect, lower native masses were reported for cholesterol oxidase from *Schizophyllum commune* [48] and *Streptomyces hygroscopicus* [49], for which interactions of the enzyme with the matrix of the resins used for the size exclusion chromatography experiments were proposed. In contrast, a dimeric oligomerization state was determined in previous studies for the enzyme from *Cylindrocarpus didymum* M-1 [30].

Choline oxidase does not contain dissociable flavins, as suggested by acid treatment of the enzyme followed by centrifugation to remove the precipitated protein. In this respect, the enzyme is different from sarcosine oxidase, a well-characterized enzyme containing both 8 α -N(3)-histidyl-FMN and non-covalently bound FAD [50,51]. The mechanistic implication of choline oxidase containing only one type of flavin, 8 α -N(1)-histidyl-FAD [52], is that a possible catalytic mechanism in which one flavin in the dimeric protein is responsible for choline oxidation while the second flavin carries out the

oxidation of betaine aldehyde seems unlikely.⁴ These observations are consistent with oxidation of choline to betaine aldehyde and of the aldehyde intermediate to glycine betaine occurring at the same enzyme active site in two subsequent catalytic steps (Scheme 1).

Our studies show that the *A. globiformis* genomic sequence originally reported in GenBank for the gene encoding for choline oxidase contains seven flaws, resulting in an amino acid sequence of the translated protein that is significantly different from the previously reported one. Mass spectrometric analysis of the resulting enzyme expressed in *E. coli* confirmed the validity of the sequencing results.⁵ Furthermore, the protein homology between choline oxidase and other members of the GMC oxidoreductase superfamily increased significantly when the newly determined amino acid sequence was used instead of the previously determined one (Fig. 4). For example, the identities and similarities between choline oxidase and choline dehydrogenase increased from 28 to 34% and from 57 to 63%, respectively. The errors in the nucleotide sequence of the *codA* gene previously reported using the pGAH/*codA* construct likely arose from the high GC content of the *codA* gene, which could affect the fidelity of DNA polymerase in the sequencing reaction.

In summary, choline oxidase was cloned from genomic DNA of *A. globiformis* and heterologously expressed to high yields in *E. coli*. The resulting recombinant enzyme was highly purified and showed to be a dimer of identical subunits containing covalently bound FAD. To our knowledge, this study represents the first instance in which recombinant choline oxidase has been overexpressed and obtained in pure form. The availability of large quantities of recombinant choline oxidase will prove an invaluable tool for detailed mechanistic and structural studies aimed at a better understanding of four-electron oxidations of unactivated alcohols and of the mechanism of CH bond cleavage of alcohols and aldehydes catalyzed by the same flavin-linked active site. Finally, the determination of the correct nucleotide sequence of the gene encoding for choline oxidase will provide the basis for performing site-directed mutagenesis studies in this enzyme of biotechnological and medical interest.

⁴ The presence of a single type of flavin in choline oxidase also suggests that a catalytic mechanism in which one flavin in the dimeric enzyme dehydrogenates the substrate whereas a second flavin is responsible for oxygen reduction is also unlikely.

⁵ The mass of the enzyme with one linked FAD moiety is 60,612 Da when calculated by using the newly determined amino acid sequence and 59,648 Da by using the previously reported amino acid sequence. Therefore, the experimentally determined mass of 60,614 Da for the enzyme as purified is in good agreement with the sequencing results.

CHO	-----	-----	-----MH	IDNIENLSDR	EF DY I V G GG	SA G AAV A RL	32
CHD	-----	-----	-----	-----MSQAT	EF DY I I G AG	SA G NVL A TRL	25
AO	-----	-----	-----	-----MAIPE	EF DV I V C GG	ST G CVI A GR	25
GO	MQTLVLSSLV	VSLAAALPHY	IRSNGLIASL	LTDPKDVSGR	TV DY I I A GG	LT G LTT A RL	60
CHO	S-EDPAVS V A	L VE A G P DDR	GVPEVLQLDR	WMELLES--	YDWD Y PIEPQ	-ENGNSFMRH	87
CHD	T-EDSD V S V L	L LE A G G PDY	RFDFRTQMPA	ALAYPLQCKR	YNWA F ETDPE	PHMDNRRMEC	83
AO	ANVDEN L K V L	L IE N G E NNL	NNPWVYLPGI	YPRNMRLLSK	TATFYNSRPS	KHLNGRRRAIV	84
GO	T-ENPN I S V L	V IE S G S YESD	RGPIIEDLNA	YGDIFGSS--	VDHAYETVEL	-ATNNQTALI	116
CHO	AR A K V M G G S	SH N SC I AFWA	PRE D L D E W EA	KYGATG W NAE	AAW P L Y KRL E	TN-----	139
CHD	GR G K G L G G S	LI N MC Y IRG	NALD Y D H WAK	QPGLEE W DYL	SCL P YFKK S E	T-----	134
AO	PQ A NT L G G G S	SI N FM Y TRA	SASD Y D D WES	----EG W TTD	ELL P L M KK F E	TY-----	132
GO	RS G NG L G G G S	LV N GT W TRP	HKAQ V D S WET	VFGNEG W NWD	NVAAY S LQA E	RARAPNAKQI	176
CHO	-EDAG P DA P H	H G DS G PVHLM	NVPP---KD P	TGVA L L D ACE	QA G I P RAKFN	TGTTVVN- G A	194
CHD	-RDIG F ND Y H	G G DGPVSVTT	PKAG---NN P	LYRT F IE A GK	QA G Y P ETEDV	NGYQ E -- G F	188
AO	-QRPC N NRDV	H G FDG P IKVS	FGNY---TY P	QCQD F L R ACE	TQ G I P YVDDL	EDLKTSH G A E	188
GO	AAGHY F NASC	H G VNGTVHAG	PRDTGDD S P	IVK A L M S A VE	DR G V P TKKDF	CGCD P H-- G V	234
CHO	N F Q I NRRA D	GT R SSSS V S Y	I H PIVE-QE N	F T L L T GLRAR	Q L V F DADRR C	T-GVD I VD S A	252
CHD	GP M DRFVTPK	GR R ASTAR G Y	LD T AKQ-RS N	L T I E T R AVTD	V I E F E GKR--	--AV G V R Y E Q	243
AO	Q W L K WINR D F	GR R SDTA H A F	IHSTMRN K E N	L F L M TNTKVD	K V I E DGRA V	A-VRT V PS K P	247
GO	SM F PNTLHED	QV R SDAARE W	LLPNYQ-RE N	LQ V L T GQYVG	K V L S QNGT T	PRAGV V E F GT	293
CHO	FGHT---H R L	T A RNE V L S T	G AID T P K L L M	L S G I G P AAH L	AEH G I E V L VD	S P G V G E H L Q D	309
CHD	KGQP---K Q A	R A RRE V L L CG	G AIAS P Q I L Q	R S G V G N PEW L	KEL G I P V V H E	L P G V EN L Q D	300
AO	IGDSK V S R T F	K A R K Q I V V SC	G T V S S P M V L Q	R S G I G E PS K L	RAA G V K P I VE	L P G V GR N F Q D	307
GO	HKGN--T H NV	Y A K HE V L L AA	G SA V S P T I L E	Y S G I G M K S I L L	E P L G I D T V VD	L P -V G L N L Q D	350
CHO	H P EGV V Q F E A	KQPMVAESTQ	WW-----	E I G-----	-----	--IFTPTED G	342
CHD	H L BM Y I Q Y E C	KEPISLYPAL	KWYNQ---P	K I GAEWL F KG	TG V GAS N Q F E	SCGFIRSR D D	356
AO	H F CY F V P Y R I	KQDSSE F DA F	VS G DK E AQ S	A F DQWYAT G A	G P LATNG I E A	GVKIRPT E A E	367
GO	Q T TAT V RS R I	TSAGAGQ G Q A	AWFAT F N-- E	T F G----- D	Y SEKA H ELL N	TKLEQW A E E A	402
CHO	LDRPDLMMHY	GSVPFDM N T L	RH---GYPT T	ENG F SLTP N V	THAR-----	-----S R G T	387
CHD	EEWPNLQY H F	LPIAIS Y N G K	-----SA V Q	AHG F QAHV G S	MRSE-----	-----S R G R	398
AO	LATADKAF Q Q	GWSY F E N K P	DKPLMHYS V I	SG F F G DH T RL	PPGKYMT M F H	FLEYP F S R G W	427
GO	VARGGFHN T T	ALL I Q Y E N Y R	DWIVNHN V A Y	SEL F LDTAG V	AS F DVWDLL P	-----F D R G Y	457
CHO	V R L R SR D FRD	KP-MVDPR Y F	TDPEGHDM R V	MVAGIRK A R E	IAAQ P AMAE W	TGRELS P G V E	446
CHD	I R L T SK D PHA	AP-SILFN Y M	AKEK--DW E E	FRDAIRL T R E	IIAQ P AFDR Y	RGREIS P G P D	455
AO	L H I S SD D PYA	AP-DFDPG F M	NDDR--DM W P	MVWAFKK S R E	TARRME C F A G	EPTAF H P HY K	484
GO	V H I L DK D PYL	HHFAYDPQ Y F	LNEQ----LA	RNISN-----	---SGAMQ T Y	FAGEIL P G D N	505
CHO	AQT-----	-----	-----	-----	-----	-----	449
CHD	VQS-----	-----	-----	-----	-----	-----	458
AO	VDSPARALEQ	SAEDTKKVAG	PLHLTANLYH	GSWSTPIGEA	DKHDPNHVTS	SHINVYSKDI	544
GO	LAYD-----	-----	-----	-----	-----A	D-----	511
CHO	-----DEEL	QDYIRK T HNT	V Y H P V G T V R M	GAV-----ED	EMS P L D PE L R	V K G V T GL R V A	498
CHD	-----DEEL	D N F V KQHA E T	A Y H P C G S C R M	G-----EG	DMAV T D A Q G R	V H G L E GL R V V	505
AO	QYTKED E A I	E NY I KEHA E T	T W H CL G T N S M	APREG N K N A P	EGGV L D P RL N	V H G V K GL K V A	604
GO	-----LSAW	T EY I PYH F RP	N Y H D V G T C S M	MP-----KE	MGSV V D N AA R	V Y G V R GL R V I	559
CHO	D A S V M P E H V T	V NPNIT V MM I	G ER C AD L I R S	ARAG E TT T AD	AE L SA A LA--	-----	546
CHD	D A S L F P V I P T	G NLN A PT I M L	A E K I A D R I R G	REPLPRASVD	YYVANG A PA K	QAS-----	558
AO	D L S V C P D N V G	C NT F ST A L T I	G E K A A V L VA E	DLG Y SG S E L D	MEV P Q H KL K T	YEQ T GA A R Y	663
GO	D G S I P P T OMS	S H V MT V F Y AM	A L K I S DA I L E	DYAS M Q----	-----	-----	595

Fig. 4. Alignment of choline oxidase with selected members of the GMC oxidoreductase enzyme superfamily. CHO, choline oxidase from *A. globiformis* (GenBank Accession No. AAP68832); CHD, choline dehydrogenase from *Halomonas elongata* (GenBank Accession No. CAB77176); AO, alcohol oxidase from *Candida bodinii* (GenBank Accession No. JC1117); and GO, glucose oxidase from *Aspergillus niger* (GenBank Accession No. AF234246.2).

Acknowledgments

The authors thank Dr. Norio Murata at the National Institute for Basic Biology, Okazaki, Japan, for the kind gift of *E. coli* strain JM109 cells harboring the plasmid pGAH/codA; Ms. Ping Yang at the DNA Core Facility of the Biology Department of Georgia State University for preparation of the oligonucleotide primers and for DNA sequencing; and Dr. David Bostwick at the Georgia Institute of Technology for the MALDI-TOF

mass spectrometric analyses. This work was supported in part by Grant PRF #37351-G4 from the American Chemical Society (G.G.), a Research Initiation Grant, and a Quality Improvement Fund from Georgia State University (G.G.).

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