Expression, Purification, and Characterization of Choline Kinase, Product of the CKI Gene from *Saccharomyces cerevisiae*  

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In the yeast *Saccharomyces cerevisiae*, choline kinase (ATP-choline phosphotransferase, EC 2.7.1.32) is the product of the *CKI* gene. Choline kinase catalyzes the committed step in the synthesis of phosphatidylcholine by the CDP-choline pathway. The yeast enzyme was overexpressed 106-fold in SF-9 insect cells and purified 71.2-fold to homogeneity from the cytosolic fraction by chromatography with concanavalin A, Affi-Gel Blue, and Mono Q. The N-terminal amino acid sequence of purified choline kinase matched perfectly with the deduced sequence of the *CKI* gene. The minimum subunit molecular mass (73 kDa) of purified choline kinase was in good agreement with the predicted size (66.3 kDa) of the *CKI* gene product. Native choline kinase existed in oligomeric structures of dimers, tetramers, and octomers. The amounts of the tetrameric and octomeric forms increased in the presence of the substrate ATP. Antibodies were raised against the purified enzyme and were used to identify choline kinase in insect cells and in *S. cerevisiae*. Maximum choline kinase activity was dependent on Mg2+ ions (10 mM) at pH 9.5 and at 30 °C. The equilibrium constant (0.2) for the reaction indicated that the reverse reaction was favored in vitro. The activation energy for the reaction was 6.26 kcal/mol, and the enzyme was labile above 30 °C. Choline kinase exhibited saturation kinetics with respect to choline and positive cooperative kinetics with respect to ATP (n = 1.4–2.3). Results of the kinetic experiments indicated that the enzyme catalyzes a sequential Bi Bi reaction. The V°max for the reaction was 138.7 µmol/min/mg, and the K° values for choline and ATP were 0.27 mM and 90 µM, respectively. The turnover number per choline kinase subunit was 153 s⁻¹. Ethanolamine was a poor substrate for the purified choline kinase, and it was also poor inhibitor of choline kinase activity. ADP inhibited choline kinase activity (IC50 = 0.32 mM) in a positive cooperative manner (n = 1.5), and the mechanism of inhibition with respect to ATP and choline was complex. The regulation of choline kinase activity by ATP and ADP may be physiologically relevant.

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The yeast *Saccharomyces cerevisiae* serves as a model eukaryotic cell to study the regulation of phospholipid synthesis...
The structural gene (CKI) encoding for S. cerevisiae choline kinase present in the multiple cloning site of pUC19 (41) was excised as a blunt-ended fragment using the restriction enzyme BsaA1. The CKI gene was gel-purified and A-tailed (42) with 1 unit of Taq polymerase and 200 μl deoxyribonucleotides at 72 °C for 10 min. The A-tailed DNA was then TA-cloned into the pCRII vector. A partial digest of the pCRII-CKI construct using EcoRI yielded a 1.8-kilobase pair DNA fragment containing the entire coding region of the gene. The gene was next inserted into the EcoRI site of the baculovirus vector PVLI392. The PVLI392-CKI plasmid was subsequently co-transfected with BacularGold™ Autographa (Pharmingen) into a monolayer of SF-9 cells using the CaCl₂ method. The SF-9 cells were routinely grown in TMNFH medium (43) containing 10% heat-inactivated fetal bovine serum. General procedures for the growth, maintenance, and infection of SF-9 cells followed the methods described by O’Reilly et al. (43). Routine infection of SF-9 cells for choline kinase purification used 1–2 × 10⁸ cells grown in 30 150-mm dishes. The cells were infected at a viral multiplicity of 10 and grown in TMNFH medium with 10% heat-inactivated fetal bovine serum for 69 h. The infected cells were collected by gentle trituration with medium, harvested by centrifugation, and washed twice with phosphate-buffered saline. The final cell pellet was snap-frozen over dry ice and stored at −80 °C.

**Preparation of Yeast Cell Extracts**

Cells were disrupted with glass beads with a Mini-Bead-Beater (BioSpec Products) in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Na₂EDTA, 0.3 mM sucrose, and 10 mM 2-mercaptoethanol (19). Glass beads and cell debris were removed by centrifugation at 1500 × g for 5 min. The supernatant (cell extract) was used for enzyme assays and immunoblotting of choline kinase.

**Electrophoresis and N-terminal Amino Acid Sequencing**

Native polyacrylamide gel electrophoresis (47) was performed at 5 °C using 9% slab gels. SDS-polyacrylamide gel electrophoresis (48) was performed with 9% slab gels. Proteins on polyacrylamide gels were visualized with Coomassie Blue and by the silver staining procedure (49).

**Preparation of Antibodies and Immunoblot Analysis**

Antibodies to choline kinase excised from SDS-polyacrylamide gel slices were raised in New Zealand White rabbits by standard procedures (51) at the Poono Rabbit Farm (Canadensis, PA). The IgG fraction was isolated from antisera by DEAE-Affigel Blue chromatography as described by the manufacturer. The IgG antibodies were precipitated with 50% ammonium sulfate and resuspended and dialyzed against 20 mM K₂HPO₄ (pH 8.0) buffer. Immunoblot assays were performed with anti-choline kinase antibodies as described previously (52). The density of the choline kinase bands on immunoblots was quantified by scanning densitometry. Immunoblot signals were in the linear range of detectability.

**Enzyme Assays and Protein Determination**

Choline kinase activity was measured for 10 min at 30 °C by following the formation of H-labeled phosphocholine from [methyl-3H]choline (500 cpm/nmol) as described by Porter and Kent (53). The reaction mixture contained 67 mM glycine-NaOH buffer (pH 9.5), 5 mM choline, 0.5 mM ATP, 10 mM MgSO₄, 1.3 mM dithiothreitol, and enzyme protein in a final volume of 60 μl. Radiolabeled phosphocholine was separated from the radiolabeled substrate by the precipitation of the substrate as choline reineckate (53). The amount of labeled product in the supernatant was determined by scintillation counting. The product phosphocholine was identified by thin layer chromatography on silica gel plates using the solvent system methanol, 0.5% sodium chloride, ammonia hydroxide (50:50:1) (54). The position of the labeled phosphocholine on chromatograms was determined by fluorography and compared with a standard.

**Materials**

All chemicals were reagent grade. Yeast growth medium supplies were purchased from Difco. The pCRII plasmid and the baculovirus transfer vector PVLI392 were obtained from Invitrogen. Nucleotides, choline, phosphocholine, ethanolamine, phosphoethanolamine, ammonium reinecke, phenylmethanesulfonyl fluoride, benzamide, aprotinin, leupeptin, pepstatin, and nuclease paper, and bovine serum albumin were purchased from Sigma. Radiochemicals were purchased from NEN Life Science Products. Scintillation counting supplies were purchased from National Diagnostics. Protein assay reagent, Affi-Gel Blue, DEAE-Affi-Gel Blue, molecular mass standards for SDS-polyacrylamide gel electrophoresis, electrophoresis reagents, and immunoochemical reagents were purchased from Bio-Rad. ConA-Sepharose, Mono-Q, and Superose 6 were purchased from Pharmacia Biotech Inc. Polyvinylidene difluoride paper was purchased from Millipore Corp. Silica gel G thin layer plates were purchased from Analtech.

**Methods**

Recombinant Viral Expression of the S. cerevisiae CKI Gene in Insect Cells

The structural gene (CKI) encoding for S. cerevisiae choline kinase present in the multiple cloning site of pUC19 (41) was excised as a

**FIG. 1. Pathways for the synthesis of PC in S. cerevisiae.** The pathways shown for the synthesis of PC include the relevant steps discussed under “Experimental Procedures.” The CDP-DG pathway is indicated by the boxed area. Choline kinase catalyzes reaction 1. A more comprehensive phospholipid biosynthetic pathway, which includes the steps for the synthesis of phosphatidylglycerol and cardiolipin, may be found in Ref. 5. CDP-Etn, CDP-ethylamine; CDP-Chol, CDP-choline; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidate.

**Yeast Choline Kinase**

Choline kinase was overexpressed in wild-type S. cerevisiae strain W303-1A (MATα ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100) (44) by transformation (45) with the multispecific plasmid pCK1D (41). This plasmid contains the CKI gene and directs the overexpression of choline kinase (41). Cells were grown in complex synthetic medium (46) without leucine to the exponential phase of growth (43). Routine infection of Sf-9 cells for choline kinase purification used 1–2×10⁸ cells grown in 30 150-mm dishes. The cells were infected at a multiplicity of 10 and grown in TMNFH medium with 10% heat-inactivated fetal bovine serum for 69 h. The infected cells were collected by gentle trituration with medium, harvested by centrifugation, and washed twice with phosphate-buffered saline. The final cell pellet was snap-frozen over dry ice and stored at −80 °C.

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Choline kinase on an SDS-polyacrylamide gel was transferred to polyvinylidene difluoride paper and subjected to N-terminal amino acid sequence analysis (50) using a Beckman LF-3400 sequencer.

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**Ethanolamine Kinase Activity**

Ethanolamine kinase activity was measured for 20 min at 30 °C by
following the phosphorylation of ethanolamine with \( [\gamma-^{32}\text{P}]\text{ATP} \) (100,000 cpm/nmol) (41). The reaction mixture contained 50 mM glycine-NaOH buffer (pH 9.5), 5 mM ethanolamine, 0.5 mM ATP, 10 mM MgSO_4, 1.3 mM dithiothreitol, and enzyme protein in a final volume of 25 μl. The product phosphoethanolamine was identified by thin layer chromatography on silica gel plates using the solvent system methanol, 0.6% sodium chloride, ammonium hydroxide (10:10:1). The position of the labeled phosphoethanolamine on chromatograms was determined by autoradiography and compared with a standard. The amount of the labeled phosphoethanolamine on chromatograms was determined by scintillation counting. All assays were performed in triplicate with an average S.D. of ±5%. All assays were performed with time and protein concentration. A unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min. Specific activity was defined as units/mg of protein. Protein concentration was determined by the method of Bradford (55) using bovine serum albumin as the standard.

**Analysis of Kinetic Data**

Kinetic data were analyzed according to the Michaelis-Menten and Hill models using the EZ-FIT enzyme kinetic model fitting program (56). EZ-FIT uses the Nelder-Mead Simplex and Marquardt/Nash nonlinear regression algorithms sequentially and tests for the best fit of the data among different kinetic models.

**Purification of Choline Kinase**

All steps were performed at 5 °C.

**Step 1: Preparation of Cell Extract**—The cell extract was prepared from 2.5 g (wet weight) of SF-9 cells infected with baculovirus containing the yeast CKI gene. Cells were washed once in buffer A (50 mM Tris-HCl (pH 7.5), 0.3 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin) and suspended (0.3 g, wet weight, of cells/ml) in buffer A. Cells were disrupted by sonic oscillation for seven 30-s bursts, with a 1.5-min pause between bursts. The disrupted cell suspension was then centrifuged at 1500 × g for 5 min to remove unbroken cells and cell debris.

**Step 2: Preparation of Cytosol**—The cell extract was centrifuged at 100,000 × g for 1.5 h to remove the total membrane fraction. The supernatant (cytosolic fraction) was used for the next step in the purification.

**Step 3: ConA-Sepharose Chromatography**—A ConA-Sepharose column (2 × 7 cm) was equilibrated with buffer B (50 mM Tris-HCl (pH 8.5), 10 mM MnCl_2, 10 mM 2-mercaptoethanol, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin). The cytosolic fraction was applied to the column at a flow rate of 60 ml/h. The column was then washed with 2 column volumes of buffer B. Choline kinase did not bind to the resin and emerged in the run-through and wash fractions. Fractions containing choline kinase activity were pooled and used directly for the next step in the procedure.

**Step 4: Affi-Gel Blue Chromatography**—An Affi-Gel Blue column (2 × 15 cm) was equilibrated with buffer C (50 mM glycine-NaOH (pH 9.5), 10 mM MgCl_2, 1 mM dithiothreitol, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin). The enzyme from the previous step was applied to the column at a flow rate of 60 ml/h. Choline kinase was eluted from the column in 3-ml fractions with 10 column volumes of a linear KCl gradient (0–1 M) in buffer C. The peak of choline kinase activity eluted from the column at a KCl concentration of 0.5 M. The most active fractions were pooled and the enzyme preparation was desalted by dialysis against buffer C.

**Step 5: Mono Q Chromatography**—A Mono Q column (0.5 × 5 cm) was equilibrated with buffer C. Affi-Gel Blue-purified enzyme was applied to the column at a flow rate of 24 ml/h. The column was washed with 5 column volumes of buffer C. Choline kinase activity was eluted from the column in 0.5-ml fractions with 50 column volumes of a linear KCl gradient (0–0.5 M) in buffer C. Two peaks of choline kinase activity eluted from the column. The first peak of activity eluted from the column at a KCl concentration of about 0.15 M. The second peak of activity eluted at a KCl concentration of about 0.27 M. The most active fractions of choline kinase activity from the second peak were pooled, stored at −80 °C, and used for the characterization of the enzyme.

**Superose 6 Chromatography**

Pure choline kinase in buffer D (50 mM glycine-NaOH (pH 9.5), 10 mM MgCl_2, and 1 mM dithiothreitol) in the absence and presence of 1 mM ATP was incubated for 10 min at 30 °C. The enzyme samples were then applied to a Superose 6 column (1 × 24 cm) that was equilibrated with buffer D in the absence and presence of 1 mM ATP at 5 °C. Choline kinase was eluted from the column in 0.5-ml fractions with buffer D in the absence and presence of 1 mM ATP at a flow rate of 15 ml/h. The column was calibrated with blue dextran 2000 (for the void volume), thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa).

**RESULTS**

**Expression of S. cerevisiae Choline Kinase In Insect Cells**—To facilitate the purification of yeast choline kinase, we used heterologous expression of the CKI gene in insect cells. The CKI gene was placed within the genome of baculovirus under control of the polyhedrin promoter and expressed by viral infection of SF-9 cells. Fig. 2 shows the choline kinase activity found in SF-9 cells with the CKI-containing baculovirus when compared with uninfected cells and cells infected with wild-type virus. The specific activity of choline kinase in cell extracts from insect cells expressing the CKI gene was 450–600-fold greater than the activity found in extracts from the control insect cells. The choline kinase activity from the infected insect cells was 106-fold greater than the activity found in cell extracts from wild-type S. cerevisiae and 6.4-fold greater than that found in yeast cells bearing the CKI gene on a multicopy plasmid (Fig. 2). All of the choline activity expressed in the insect cells was associated with the cytosolic fraction of the cells.

**Purification of Choline Kinase**—A summary of the purification of choline kinase is presented in Table I. The enzyme was purified from the cytosolic fraction by chromatography with ConA-Sepharose, Affi-Gel Blue (Fig. 3A), and Mono Q (Fig. 3B). During the development of the purification scheme for the enzyme, we observed a major protein band on SDS-polyacrylamide gels that migrated with a molecular mass of about 65 kDa. We determined the N-terminal amino acid sequence of this protein to be IPGTTPVIDDA. A search of the data base revealed that this protein was a serine proteinase, chitinase, which is a glycoprotein, and it was removed from the choline kinase preparation by ConA-Sepharose chromatography. The Mono Q chromatography step in the purification resulted in the isolation of two peaks of choline kinase activity (Fig. 3B). The majority of the choline kinase activity eluted from the Mono Q column under the second peak. In addition, the specific activity of the enzyme under this peak was 1.5-fold higher than the activity in the fractions under the first peak. Therefore, the
Choline kinase was purified from Sf-9 insect cells expressing the S. cerevisiae CKI gene as described under “Experimental Procedures.” The data are based on starting with 2.5 g (wet weight) of cells.

| Purification step | Total units | Protein | Specific activity | Yield | Purification |
|-------------------|-------------|---------|-------------------|-------|--------------|
|                   | µmol/min    | mg      | units/mg          | %    | -fold        |
| 1. Cell extract   | 121.2       | 67.32   | 1.8               | 100  | 1            |
| 2. Cytosol        | 120.2       | 47.70   | 2.52              | 99.2 | 1.4          |
| 3. ConA           | 110.5       | 29.23   | 3.78              | 91.2 | 2.1          |
| 4. Affi-Gel Blue  | 31.6        | 4.39    | 7.20              | 26.1 | 4            |
| 5. Mono Q         | 17.1        | 0.13    | 128.2             | 14.1 | 71.2         |

Choline kinase from the second peak was used as the source of pure enzyme. This step afforded the greatest enrichment (18-fold) in specific activity in the purification procedure. Overall, choline kinase was purified 71.2-fold over the cell extract with an activity yield of 14.1% to a final specific activity of 128.2 µmol/min/mg.

**Purity of Choline Kinase and Molecular Mass**—The purification procedure yielded essentially a homogeneous protein preparation as evidenced by polyacrylamide gel electrophoresis under non-denaturing conditions ($R_p$ of 0.39) and in the presence of SDS (Fig. 4). The SDS gel showed in Fig. 4 contained 2 µg of choline kinase and was stained with Coomassie Blue. Silver-stained SDS gels containing the purified choline kinase also showed that the enzyme preparation was homogeneous. The enzyme migrated on the SDS-polyacrylamide gel with a minimum subunit molecular mass of 73 kDa (Fig. 4). To confirm that the protein purified in the enzyme preparation was indeed choline kinase, we measured activity from native polyacrylamide gel slices. Following electrophoresis, one lane from a gel was cut into 0.5-cm slices. Each slice was minced with a razor blade in assay buffer and homogenized at 5 °C. The samples were then assayed for choline kinase activity. Choline kinase activity was found in those polyacrylamide gel slices, which were then assayed for choline kinase activity. Choline kinase activity was found in those polyacrylamide gel slices, which were then assayed for choline kinase activity.

**Immunoblotting of Choline Kinase**—Antibodies were raised against the choline kinase purified from Sf-9 insect cells. Immunoblot analysis showed that these antibodies reacted with pure choline kinase (Fig. 6). These antibodies also recognized a protein doublet in the cytosolic fraction derived from Sf-9 insect cells infected with baculovirus containing the S. cerevisiae CKI gene (Fig. 6). This immunoreactive protein migrated on SDS-polyacrylamide gels with the same mobility as the pure choline gene.
kinase protein (Fig. 6). No immunoreactive proteins were detected in the cytosolic fraction derived from uninfected Sf-9 insect cells. These antibodies also recognized a protein from wild-type S. cerevisiae cells bearing the CKI gene on a multicopy plasmid (Fig. 6). Moreover, the protein detected from S. cerevisiae migrated on SDS-polyacrylamide gels with the same mobility as the pure choline kinase isolated from the Sf-9 cells expressing the S. cerevisiae CKI gene (Fig. 6). The level of choline kinase expression in wild-type cells was too low to be detected by immunoblot analysis under the conditions used in the experiment shown in Fig. 6. Overall, the relative abundance of the protein detected in insect cells and in yeast by these antibodies correlated with the relative choline kinase activity expressed in these cells (Fig. 2).

Enzymological Properties of Choline Kinase Activity—Choline kinase activity was measured with a Tris-maleate-glycine buffer at pH values ranging from 6 to 12 (Fig. 7A). Optimum choline kinase activity was obtained at pH 9.5–12. Choline kinase activity was measured with a saturating concentration (0.5 mM) of ATP in the absence and presence of Mg$^{2+}$ ions (Fig. 7B). Activity was absolutely dependent on Mg$^{2+}$ ions, and this dependence was cooperative (Hill number of 2.8). Maximum activity was obtained at a Mg$^{2+}$ ion concentration of 10 mM (Fig. 7B). The Mg$^{2+}$ ion requirement for activity could not be substituted by Mn$^{2+}$, Co$^{2+}$, or Ca$^{2+}$ ions. Choline kinase activity was measured under standard assay conditions in the presence of various divalent cations. At a concentration of 1 mM, Hg$^{2+}$ and Cu$^{2+}$ ions totally inhibited activity. The addition of Ca$^{2+}$ ions (1–10 mM) did not affect choline kinase activity.

The equilibrium constant for the choline kinase reaction was determined using 5 mM choline and 0.5 mM ATP and allowing the reaction to come to equilibrium. At equilibrium (40 min), the concentration of ADP and phosphocholine was 0.36 mM. The equilibrium constant was calculated to be 0.2, indicating that the reverse reaction was favored in vitro. It is likely that in vivo the reaction would be driven in the forward direction by the utilization of phosphocholine by the phosphocholine cytidylyltransferase reaction.

The effect of temperature on choline kinase activity and stability was examined. Activity was measured from 5 to 50 °C (Fig. 8A). Maximum activity was observed at 30 °C. An Arrhenius plot for the enzyme was constructed by plotting the log of choline kinase versus the reciprocal of the absolute temperature. The plot was then used to calculate an activation energy.
for the reaction of 6.26 kcal/mol. The stability of the enzyme was measured at temperatures ranging from 20 to 60 °C (Fig. 8B). Choline kinase activity was unstable above 30 °C with total inactivation of the enzyme after heating for 10 min at 50 °C.

**Dependence of Choline Kinase Activity on Choline and ATP—** The dependence of choline kinase activity on choline and ATP was examined using a saturating concentration (10 mM) of Mg$^{2+}$ ions. Under these conditions, ATP in the assay system existed in the form of Mg$^{2+}$-ATP complexes (57). Choline kinase activity followed typical saturation kinetics when the choline concentration was varied at various fixed concentrations of ATP (Fig. 9A). The presence of intersecting lines in the double reciprocal plot shown in Fig. 9A was consistent with choline kinase catalyzing a sequential Bi Bi reaction (58). A replot (58) of the slopes of the data in Fig. 9A versus the reciprocal of the ATP concentration was linear and used to calculate a true $V_{\text{max}}$ value of 138.7 μmol/min/mg and a true $K_m$ value of 90 μM for ATP. Choline kinase activity did not follow typical saturation kinetics when the ATP concentration was varied at various fixed concentrations of choline (Fig. 9B). Instead, the enzyme exhibited a positive cooperative kinetic pattern (58) as demonstrated by double-reciprocal plots of the data, where the curves were not linear but were concave upward (Fig. 9B). An analysis of these data according to the Hill equation yielded Hill numbers for ATP of 1.4–2.3. Due to this positive cooperative kinetic behavior, replots of these data could not be constructed to determine a $K_m$ value for choline. Instead, we calculated a $K_m$ value for choline (0.27 mM) from the data in Fig. 9A, where the family of lines intersected on the 1/choline axis. The turnover number (molecular activity) per choline kinase subunit was calculated to be 153 s$^{-1}$ at pH 9.5 and at 30 °C.

**Effectors of Choline Kinase Activity—** We examined the effect of water-soluble phospholipid precursors, phospholipids, and nucleotides on choline kinase activity, since these compounds have been shown to regulate the activities of several phospholipid biosynthetic enzymes (1). In these experiments, we used subsaturating concentrations of choline and ATP. Thus, we could simultaneously screen compounds that were inhibitory or stimulatory to choline kinase activity. The addition of ethanolamine to the assay system resulted in a modest but dose-dependent inhibition (15%) of choline kinase activity (Fig. 10). We examined whether pure choline kinase utilized ethanolamine as a substrate. Although the enzyme catalyzed the phosphorylation of ethanolamine, this activity was only 14% of that observed when choline was used as the substrate (Fig. 10, inset). The effect of other water-soluble phospholipid precursors on choline kinase activity was also examined. Of the compounds examined, only inositol (1 mM) and CDP-choline (5 mM) affected choline kinase by inhibiting activity by 25 and 30%, respectively. The effect of the major $S$. cerevisiae phospholipids and diacylglycerol on choline kinase activity was examined. In these experiments, lipids (1–10 mol %) were added to the reaction mixture as uniform mixed micelles with Triton X-100 (59). At a final lipid concentration of 10 mol %, phosphatidylethanolamine, phosphatidylserine, phosphatidate, and diacylglycerol inhibited choline kinase activity by 30, 20, 15, and 15%, respectively.

The effects of the mono-, di-, and triphosphorylated deriva-
 effects of adenosine, guanosine, and cytidine on choline kinase activity were examined. The nucleotide concentrations used in these experiments were 0–6 mM, and the Mg²⁺ ion concentration was maintained at 10 mM. ADP inhibited choline kinase activity in a dose-dependent manner (Fig. 11). The ADP-mediated inhibition of choline kinase activity followed a cooperative (n = 1.5) kinetic pattern with an IC₅₀ value of 0.32 mM (Fig. 11, inset). The other nucleotides examined did not have a significant effect (e.g. greater than 15–20% change) on choline kinase activity.

Effect of ADP on the Kinetics of Choline Kinase Activity—The effect of ADP on the dependence of choline kinase activity on ATP was examined with subsaturating (Fig. 12A) and saturating (Fig. 12B) concentrations of choline. At the subsaturating choline concentration, ADP inhibited choline kinase activity by causing a decrease in the apparent Vₘₐₓ value (from 46 to 27 units/mg) and an increase in the apparent Kₘ value for ATP (from 80 to 180 μM). However, at the saturating choline concentration, ADP caused a decrease in the apparent Vₘₐₓ value (from 82 to 53 units/mg) but did not affect the apparent Kₘ value for ATP (38 μM). These data indicated that ADP was a mixed-type inhibitor at the subsaturating choline concentration and a noncompetitive inhibitor at the saturating choline concentration (58). At each choline concentration, ADP caused the loss of the positive cooperative kinetic behavior of the enzyme with respect to ATP (Fig. 12). The effect of ADP on the kinetics of choline kinase with respect to choline was examined with subsaturating (Fig. 12C) and saturating (Fig. 12D) concentrations of ATP. ADP inhibited choline kinase activity by causing a decrease in the apparent Vₘₐₓ values at subsaturating (from 70 to 37 units/mg) and saturating (from 136 to 48 units/mg) concentrations of ATP. ADP also caused a decrease in the apparent Kₘ values for choline at subsaturating (from 0.4 to 0.32 mM) and saturating (from 0.24 to 0.09 mM) concentrations of ATP. The enzyme exhibited saturation kinetics with respect to choline at subsaturating and saturating ATP concentrations in the absence and presence of ADP. These data indicated that ADP was an uncompetitive inhibitor of choline kinase with respect to choline (58). We were not able to calculate true Kᵢ values for ADP due to the complex kinetic behavior of the enzyme.

**DISCUSSION**

We undertook the purification of choline kinase to facilitate well defined studies on the biochemical regulation of its activity. Several attempts to purify choline kinase from *S. cerevisiae* have been unsuccessful (30, 39, 60) due to endogenous proteases in yeast (30, 40). To facilitate the purification, we used heterologous expression of the *S. cerevisiae CKI* gene encoding choline kinase in insect cells. This alleviated the problem of proteolysis in yeast and also afforded a source of enzyme that was overproduced 106-fold relative to the activity found in wild-type yeast. The five-step purification scheme reported here resulted in an apparent homogeneous preparation of choline kinase. The enzyme was purified 71.2-fold relative to the specific activity in the cell extract to a final specific activity of 128.2 μmol/min/mg. Based on the specific activity of choline kinase in *S. cerevisiae*, this level of purification would be equivalent to a 7541-fold purification relative to wild-type cells. The N-terminal amino acid sequence of the purified choline kinase
aligned perfectly with the amino acid sequence of the protein deduced from the open reading frame of the CKI gene (41). These data unequivocally identified the purified choline kinase as the product of the CKI gene.

The minimum subunit molecular mass of the purified enzyme (73 kDa) was a little larger than the predicted size (66.3 kDa) of the protein deduced from the CKI gene (41). The reason for this difference in subunit size was unclear but may be attributed to a post-translational modification of the enzyme. The modification of the enzyme could also account for the two peaks of choline kinase activity from the Mono Q column and for the doublet of protein shown in the immunoblot of insect cells expressing the CKI gene. Hosaka et al. (30) have also identified protein resembling a doublet just above the 66.2-kDa marker on an immunoblot of a yeast extract from cells that overexpress the CKI gene. Determination of whether these results were in fact due to the modification of the choline kinase by post-translational modification(s) will require additional studies.

Most of the information regarding the properties of choline kinase from yeast has been derived from studies using a relatively impure preparation of the enzyme isolated from an autolysate of brewers' yeast (39, 60). The basic enzymological properties (e.g. pH optimum, Mg$^{2+}$ ion requirement, and temperature stability) of the purified choline kinase were in general agreement with the data derived from the brewers' yeast enzyme (39, 60). However, our data on the native size of choline kinase and the kinetic properties of the enzyme differed significantly from the previous studies with the brewers' yeast enzyme. We found that the native S. cerevisiae choline kinase existed in oligomeric structures of dimers, tetramers, and octamers. On the other hand, the brewers' yeast enzyme is reported to have a native size of 67 kDa (60). We showed here that choline kinase exhibited positive cooperative kinetics with respect to ATP. The brewers' yeast enzyme has been reported to exhibit typical saturation kinetics with respect to ATP (60). In addition, the $K_m$ values determined for choline for the purified choline kinase (0.27 mM) and for the brewers' yeast enzyme (15 μM) differed significantly. These differences may be attributed to the fact that the data for choline kinase from brewers' yeast was obtained using impure enzyme, which may have also been subjected to extensive proteolysis (30, 60).

The positive cooperative kinetics exhibited by the purified choline kinase with respect to ATP is a property common to many nucleotide-dependent allosteric enzymes (58, 61). Cooperative kinetics are generally attributed to the association of enzyme subunits to form oligomeric structures (58, 61). Indeed, the relative amounts of the tetrameric and octomeric forms of the purified choline kinase increased in the presence of ATP. Thus, the yeast choline kinase behaved as an allosterically regulated enzyme. We also showed that the purified choline kinase was inhibited by ADP, the product of the reaction. This inhibition also followed positive cooperative kinetics. The regulation of choline kinase activity by ATP and ADP may be physiologically relevant. Based on the cellular concentrations of ATP and ADP and the kinetic constants for these nucleotides, an argument can be made for the regulation of choline kinase by ATP and ADP in vivo. In glucose-grown cells, the cellular concentrations of ATP (2.3 mM) and ADP (0.4 mM) are 25-fold higher than the $K_m$ value for ATP (90 μM) and 1.25-fold higher than the $IC_{50}$ value for ADP (0.32 mM), respectively (62–65). Under these growth conditions, the cellular concentration of ATP is saturating for choline kinase and its reaction would be favored. However, when cells are starved for glucose, the cellular concentration of ATP decreases 4-fold, while the cellular concentration of ADP increases 2-fold (62). This would bring the cellular ATP concentration (0.57 mM) closer to the $K_m$ value for ATP and the cellular ADP concentration (0.8 mM) 2.5-fold higher than the $IC_{50}$ value for ADP. The changes in the cellular concentrations of ATP and ADP brought about by glucose starvation could result in the inhibition of choline kinase activity and a decrease in PC synthesis. Choline kinase is the only enzyme in the pathways used for PC synthesis that utilizes ATP and ADP in its reaction (3, 5). This hypothesis is supported by the fact that glucose starvation results in a 1.46-fold decrease in total phospholipids (of which PC accounts for 30–50% (5)) and a 1.5-fold increase in triacylglycerols (65). The cellular concentrations of ATP and ADP have also been shown to play a role in the regulation of phosphorynositide synthesis in S. cerevisiae. Decreases in the cellular concentration of ATP cause decreases in the synthesis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (62, 66), and these effects have been ascribed to the regulation of membrane-associated phosphatidylinositol 4-kinase activity by ATP and ADP (64, 67).

Choline kinase plays an important role for the synthesis of PC in higher eukaryotes (68). The CDP-choline pathway is the primary route for PC synthesis in mammalian cells (69). In addition, the activation of choline kinase activity in mammalian cells is needed for the reutilization of choline in phospholipase D-mediated PC turnover signaling pathways (68). Genes encoding mammalian forms of choline kinase have been isolated and characterized (70–72), and various forms of the enzyme have been purified and characterized (73–75). The S. cerevisiae and mammalian forms of choline kinase differed with respect to structure and kinetic properties. Although the S. cerevisiae and mammalian forms of choline kinase share some amino acid sequence similarity in their C-terminal regions, they differ significantly in their N-terminal regions (40, 68). The C-terminal region of the choline kinase enzymes contains the phosphotransferase consensus sequence (76), presumed to be involved in catalytic function (40, 68). The yeast and mammalian forms of choline kinase also differed with respect to their monomeric and oligomeric structures. The subunit size of the purified yeast enzyme was 73 kDa, whereas the subunit sizes of the mammalian choline kinase enzymes range from 42 to 47 kDa (73–75). Some of the mammalian choline kinases exist as dimers (73, 75), while one form exists as a tetramer (74). The oligomeric structures of the mammalian choline kinases have not been shown to be affected by ATP as was shown here for the yeast choline kinase. Whereas the yeast choline kinase exhibited positive cooperative kinetics with respect to ATP, the mammalian forms of choline kinase exhibit saturation kinetics with respect to ATP (73–75).

There have been a number of conflicting reports as to whether choline kinase catalyzes the phosphorylation of choline and ethanolamine in various sources (40, 68). In mammalian cells, choline kinase and ethanolamine kinase activities exist in the same protein (73–75). In our studies, the purified choline kinase catalyzed the phosphorylation of ethanolamine. However, the ethanolamine kinase activity of choline kinase enzyme was only 14% of that when choline was used as the substrate. In addition, ethanolamine was a poor inhibitor of choline kinase activity. These data supported previous genetic studies showing that although the CKI gene product is responsible for essentially all of the choline kinase activity in the cell, it also exhibits some ethanolamine kinase activity (41). A null allele choline kinase cki mutant lacks detectable choline kinase activity (41); yet the cki mutant exhibits a significant amount

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*2 The purified choline kinase is phosphorylated by protein kinase A and by protein kinase C (K.-H. Kim and G. M. Carman, unpublished data).*
of ethanolamine kinase activity (41), suggesting that a distinct ethanolamine kinase enzyme exists in *S. cerevisiae* (40).

In summary, we have overexpressed the *S. cerevisiae* choline kinase in SF-9 insect cells, purified the enzyme to homogeneity, and unambiguously identified choline kinase as the product of the *CKI* gene. The purified enzyme was characterized with respect to its enzymological and kinetic properties. These studies showed that the yeast choline kinase was an allosterically regulated enzyme with respect to ATP and ADP and that this regulation may be physiologically relevant. The availability of the purified choline kinase will permit further studies on the biochemical regulation of this important enzyme, which plays a role in PC synthesis via de novo and recycling pathways in eukaryotic organisms.

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