The Separation, Purification, and Characterization of Ethanolamine Kinase and Choline Kinase from Rat Liver†

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ABSTRACT: Ethanolamine kinase activity from rat liver was separated by DEAE-cellulose chromatography into two distinct enzymes, ethanolamine kinase I and ethanolamine kinase II. Ethanolamine kinase I had a molecular weight of 36,000, while ethanolamine kinase II had a molecular weight of 160,000. Each had distinctly different K_m's for ethanolamine and ATP. Ethanolamine kinase II was strongly inhibited by choline (K_i = 0.03 mM) while ethanolamine kinase I was not inhibited by choline. The choline inhibition was competitive with ATP. The fraction that contained ethanolamine kinase II activity also had choline kinase activity. Ethanolamine kinase I had no choline kinase activity. Ethanolamine kinase II and choline kinase were not separated by the purification procedures. However, evidence is presented which suggests that the choline kinase and ethanolamine kinase II do not use a common active site. The results suggest that the rate of ethanolamine phosphorylation could be regulated by both the level of choline and ATP.

Ethanolamine kinase and choline kinase (ATP:choline phosphotransferase, EC 2.7.1.32) catalyze the phosphorylation by ATP of ethanolamine and choline, respectively. Choline kinase was demonstrated initially in yeast and in acetone powders from liver, brain, kidney, and intestinal mucosa (Wittenberg and Kornberg, 1953). The properties of the partially purified yeast enzyme were described in some detail. Choline kinase has subsequently been described in plant tissue (Ramasarma and Wetter, 1957; Setty and Krishnani, 1972), in the mollusc *Helix lactea* (Liang *et al.*, 1970), in brain and nerve tissue (Haubrich, 1973; Berry *et al.*, 1958; McCaman, 1962; McCaman and Cook, 1966), and in Ehrlich ascites cells (Sung and Johnstone, 1967). Most investigations did not involve extensive purification or detailed analysis of the enzymatic properties. Studies on ethanolamine kinase are very limited. Wittenberg and Kornberg reported that ethanolamine was phosphorylated by the choline kinase preparation from yeast. However, the activity with ethanolamine was much less than with choline. Crude preparations from the mollusc *Helix lactea* catalyze the phosphorylation of both ethanolamine and choline (Liang *et al.*, 1970). Sung and Johnstone (1967) were the first to make any clear attempt to distinguish between choline kinase activity and ethanolamine kinase activity. They presented evidence that the two reactions may be catalyzed by separate enzymes although physical separation of the enzymes was not obtained. We have reported previously the activity and properties of ethanolamine kinase in the liver from adult and fetal rats (Weinhold and Rethy, 1972). We found that choline was a strong inhibitor of ethanolamine kinase and, interestingly, the inhibition was competitive with ATP. These observations lead to speculations about the regulation and interrelationships of choline kinase and ethanolamine kinase and to a more thorough investigation of the identity of these enzymes. In this paper we present evidence for: (1) a specific ethanolamine kinase which has no choline kinase activity and is not inhibited by choline, and (2) a separate fraction which has choline kinase activity and an ethanolamine kinase activity that is inhibited by choline.

Materials and Methods

Female rats, 180–250 g, were obtained from Holtzman Co., Madison, Wis. [1,2-^{14}C]Ethanolamine and [CH_3-^{14}C]choline were purchased from New England Nuclear Corp. The [1,2-^{14}C]ethanolamine was purified by applying the material to a small column (0.5 × 4 cm) of AG 50-H^+, 100–200 mesh ion exchange resin. The column was washed with 20 ml of water and the [1,2-^{14}C]ethanolamine eluted with 1.0 N HCl. The HCl eluent was evaporated and the [1,2-^{14}C]ethanolamine was dissolved in water and stored at −20°. This procedure removed material which produced a high blank in the assay. ATP, ethanolamine-HCl, choline chloride, and dithiothreitol were purchased from Sigma. All ion exchange resins were obtained from Bio-Rad. DEAE-cellulose (DE 32) and carboxymethyl-cellulose (CM 52) were purchased from Whatman Co. Sephadex 200 and protein standards for molecular weight estimations were purchased from Pharmacia Co.

Determination of Enzyme Activities. Ethanolamine kinase activity was determined by a modification of the method of Sung and Johnstone (1967). Incubations contained, unless specified otherwise, 60 mM glycylglycine (pH 8.5), 3.0 mM ATP, 3.0 mM $MgCl_2$, 0.2 M KCl, 0.5 mM [1,2-^{14}C]ethanolamine (2 Ci/mol), and enzyme in a final volume of 0.1 ml. Incubations were performed in 1.0 ml of polyethylene centrifuge tubes. The reaction was started by the addition of enzyme and incubated at 37°. After the desired time (usually 15 min), the reaction was stopped by placing the reaction tube in a boiling water bath for 2 min. Blank reactions were placed in the boiling water bath immediately after the addition of the enzyme. The ethanolamine phosphate was separated from ethanolamine by applying a 50 μl sample to a small column of AG 50-H^+ X8, 100–200 mesh. The columns were prepared using the 1.0-ml plastic pipet tip for an Eppendorf micropipet. A No. 3 cotton pellet (Richmond Dental Cotton Co.) was packed

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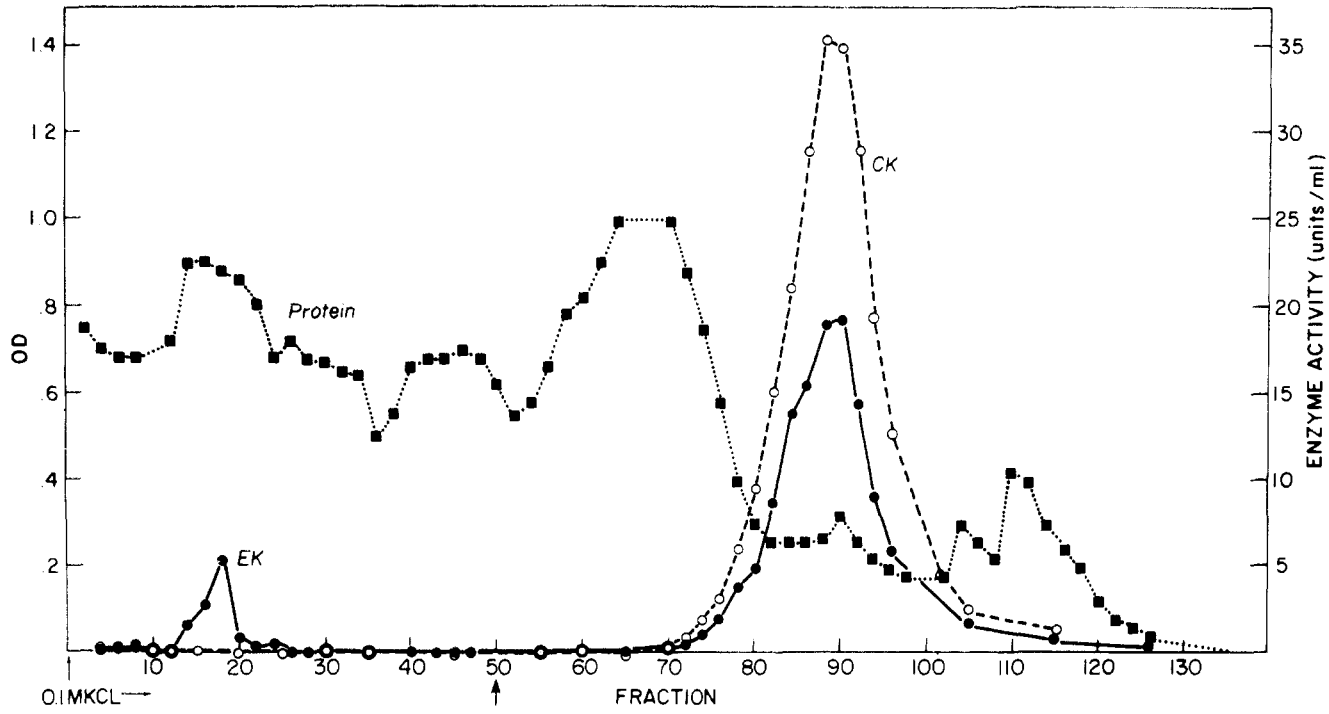

FIGURE 1: The separation of ethanolamine kinase (●) and choline kinase (○) by DEAE-cellulose chromatography. One unit of activity represents the production of 1 nmol of product/min. The arrow at fraction 50 indicates the beginning of the gradient elution.

tightly into the tip and a slurry of resin added until a volume of 0.1 ml was obtained. The column was placed through a plastic stopper which was placed onto a polyethylene liquid scintillation vial. The sample was added to the moist resin column and the vial with the column was placed into a No. 320 centrifuge holder for an International Centrifuge. The sample was centrifuged for 2 min at about 1000 rpm. The columns were washed twice with 1.5 ml of water. Each wash was accomplished by centrifuging at 1000 rpm for 3 min. The radioactive ethanolamine phosphate was completely washed from the resin while the radioactive ethanolamine was retained by the resin. The water eluent in the scintillation vial was prepared for counting by the addition of 10 ml of aquasol (New England Nuclear Corp.).

Choline kinase activity was determined with an incubation mixture that contained, unless specified otherwise, 0.1 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, 0.25 mM [Me-^{14}C]choline (0.7 Ci/mol), and enzyme in a final volume of 1.0 ml. The reaction was started by the addition of enzyme and incubated at 37°. After the desired incubation time (usually 20 min), the reaction was stopped by placing the reaction tube into a boiling water bath for 2 min. Blanks were done by stopping the reaction immediately after the addition of enzyme. A 0.5-ml portion of the reaction mixture was applied to a column (0.5 cm × 4 cm) of AG-1.0H$^-$-X8, 100–200 mesh ion exchange resin. The column was washed with 10 ml of water and the choline phosphate was eluted into scintillation vials with 0.5 ml of 1 N NaOH followed by 1.5 ml of 0.1 N NaOH. Aquasol was added to the NaOH eluent and radioactivity was determined in a liquid scintillation spectrometer.

The KCl was included in the ethanolamine kinase assay because preliminary experiments indicated that the addition of 0.2 M KCl increased the activity by about 20%. Choline kinase activity was not affected by KCl. The inclusion of dithiothreitol in the assay mixtures had no affect upon the velocity of the reactions. However, the presence of dithiothreitol during the preparation improved the yield of activity. Since dithiothreitol was present in the final enzyme preparations, both assay mixtures contained dithiothreitol, usually at a concentration of 0.2 mM.

Protein was determined with the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Results

Separation and Purification of Ethanolamine Kinase and Choline Kinase. Rat liver was homogenized with a Potter-Elvehjem homogenizer in 4 volumes (4 ml/g) of solution that contained 0.25 M sucrose, 1.0 mM EDTA, and 2.0 mM dithiothreitol. The homogenate was centrifuged at 100,000*g* for 60 min in a Spinco Model L2.65 centrifuge. The 100,000*g* × 60 supernatant contained essentially all of the ethanolamine kinase and choline kinase activity. The pH of the supernatant was adjusted to 5 with 1.0 M acetic acid and allowed to stand at 4° for 30 min. The precipitate was removed by centrifugation at 20,000*g* for 20 min and discarded. The pH of the supernatant was adjusted to 6 with 1.0 M KOH. Moist carboxymethyl-cellulose (20 g/50 ml of supernatant), previously equilibrated with 0.02 M potassium phosphate (pH 6.0) and 2.0 mM dithiothreitol, was added to the supernatant. This mixture was stirred for 30 min at 4°, poured into a column, and allowed to drain. The column was washed with 2 volumes of 0.02 M potassium phosphate (pH 6.0) and 2.0 mM dithiothreitol and the eluent and washes were combined. The pH of the combined eluents was adjusted to 7.0 and pumped onto a column of DEAE-cellulose (2.5 cm × 40 cm). The DEAE-cellulose had previously been equilibrated with 0.02 M potassium phosphate (pH 7.0), 2.0 mM dithiothreitol, and 0.02% sodium azide. A flow rate of about 60 ml/hr was maintained. The column was eluted with 500 ml of 0.1 M KCl in 0.02 M potassium phosphate (pH 7.0), 2.0 mM dithiothreitol, and 0.02% sodium azide followed by a 1000-ml linear gradient

TABLE I: Purification of Ethanolamine Kinase (EK) and Choline Kinase (CK).

Step	Protein	Choline Kinase Specific Activity (nmol min^{-1} mg^{-1})	Choline Kinase Yield, %	Ethanolamine Kinase II[a] Specific Activity (nmol min^{-1} mg^{-1})	Ethanolamine Kinase II[a] Yield, %	EK/CK
100,000*g* supernatant	3510	2.5	100	2.3	100	0.90
pH 5.0 supernatant	1998	4.1	90	3.3	82	0.82
CM-cellulose effluent	884	6.9	68	6.3	69	0.91
DEAE-cellulose	41.4	123	57	52.8	27	0.43
Sephadex 200	10.3	169	20	70.5	9	0.42
				Ethanolamine Kinase I[a]		
DEAE-cellulose	79.7			1.56	1.3	
Sephadex 200	8.5			2.50	0.64	

[a] Ethanolamine kinase II refers to the ethanolamine kinase activity that is associated with choline kinase activity while ethanolamine kinase I is the activity separated from CK by DEAE-cellulose chromatography.

from 0.1 M KCl to 0.4 M KCl. Fractions of 10.0 ml were collected and assayed for ethanolamine kinase and choline kinase activities. A typical DEAE-cellulose column separation is shown in Figure 1. A peak of ethanolamine kinase activity is eluted between 100 and 200 ml of 0.1 M KCl. There is no detectable choline kinase activity associated with this fraction. The choline kinase activity is eluted at about 0.2 M KCl in the gradient. This fraction also contains ethanolamine kinase activity. The bulk of the protein is eluted prior to the choline kinase-ethanolamine kinase peak. The fractions included in the separate peaks were combined and designated ethanolamine kinase and choline kinase-ethanolamine kinase. The ethanolamine kinase activity associated with the choline kinase will subsequently be called ethanolamine kinase II and the separate ethanolamine kinase, ethanolamine kinase I. The ethanolamine kinase and choline kinase-ethanolamine kinase preparations were dialyzed against 0.02 M potassium phosphate (pH 7.0) and 2.0 mM dithiothreitol. The dialyzed preparations were concentrated by absorption of the protein onto small columns of DEAE-cellulose followed by elution of the protein with 0.4 M KCl in 0.02 M potassium phosphate (pH 7.0) and 2.0 mM dithiothreitol buffer. The ethanolamine kinase and choline kinase-ethanolamine kinase preparations were purified further by Sephadex 200 chromatography. A column (2.5 cm × 60 cm) of Sephadex 200 was equilibrated and eluted with 0.02 M potassium phosphate (pH 7.0) and 2.0 mM dithiothreitol. The fractions that contained enzyme activity were combined and concentrated by absorption and elution from small DEAE-cellulose columns. All steps were routinely performed at 4°. The resulting ethanolamine kinase and choline kinase-ethanolamine kinase preparation were used in the subsequent characterization experiments. A summary of the purification steps and the results from a typical preparation is shown in Table I. The choline kinase was purified about 68-fold with a yield of 20%. The total ethanolamine kinase was recovered in two fractions; 9% of the total was recovered associated with choline kinase and 0.6% was recovered as a separate enzyme. The yield and purification of ethanolamine kinase and choline kinase were similar through the CM-cellulose step. A considerable amount of ethanolamine kinase activity was lost during DEAE-cellulose chromatography. If we assume that the ratio of ethanolamine kinase II and choline kinase in the final preparation (*i.e.*, 0.42) is also applicable to the previous steps, about 44% of the total ethanolamine kinase activity in the CM-cellulose effluent is associated with ethanolamine kinase II and 56% is associated with ethanolamine kinase I. Apparently, ethanolamine kinase I activity was recovered in low yield from the DEAE-cellulose column. All attempts to further purify the ethanolamine kinase I were unsuccessful due to loss of activity during subsequent proce-

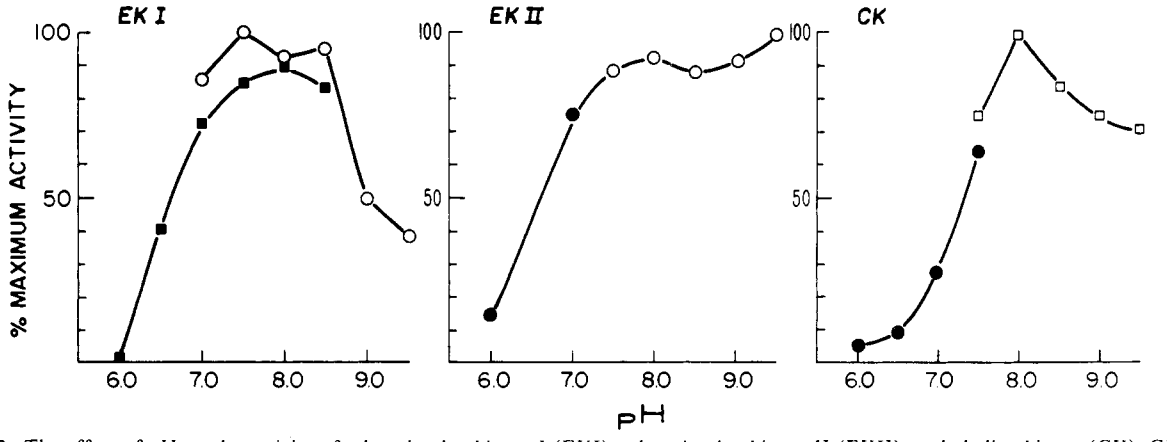

FIGURE 2: The effect of pH on the activity of ethanolamine kinase I (EKI), ethanolamine kinase II (EKII), and choline kinase (CK). Glycylglycine (○), potassium phosphate (●), Tris (□), and Tris-maleate (■) buffers were used at a final concentration of 100 mM for choline kinase and 60 mM for ethanolamine kinase. Other assay conditions were exactly as described in the Materials and Methods section.

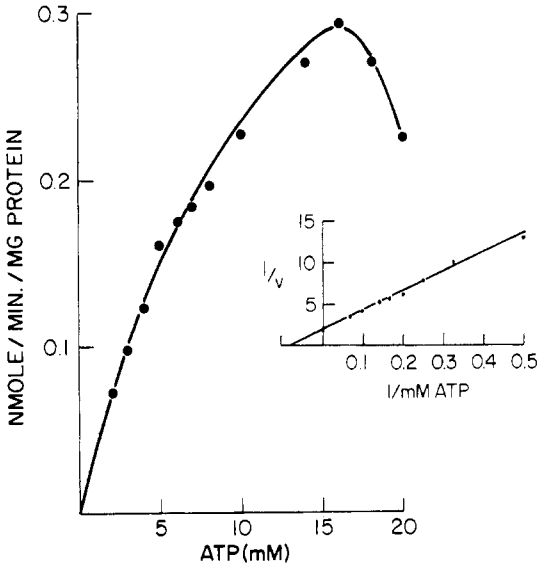

FIGURE 3: The effect of MgATP concentration on the velocity of ethanolamine kinase I. The Mg^{2+} concentration was maintained at double the ATP concentration. The other reaction conditions were the same as described in the Materials and Methods section.

dures. Examination of the preparation by disc gel electrophoresis indicated that both preparations contained a major protein and several other proteins in lesser amounts.

Comparison of the Properties of Ethanolamine Kinase I, Ethanolamine Kinase II, and Choline Kinase. MOLECULAR WEIGHTS. The molecular weights of ethanolamine kinase I and choline kinase-ethanolamine kinase were estimated by Sephadex 200 chromatography. A Sephadex 200 column (2.5 cm × 40 cm) was equilibrated with 0.02 M potassium phosphate (pH 7.0) and 2.0 mM dithiothreitol. The column was standardized with chymotrypsinogen, ribonuclease, ovalbumin, and aldolase. The plot of K_{av} *vs.* log MW for each standard protein and for ethanolamine kinase and choline kinase-ethanolamine kinase was linear. The estimated molecular weight for ethanolamine kinase I was 36,000 and for choline kinase-ethanolamine kinase was 166,000.

STABILITY OF ENZYMES. The relative stabilities of ethanolamine kinase II and choline kinase were investigated in some detail since these activities were not separated during the purification process. Preparations of ethanolamine kinase-choline kinase after the DEAE-cellulose chromatography step and after the final Sephadex 200 step were stored in 1.0-ml aliquots at either −20 or 4°. Both preparations were stored in 0.02 M phosphate buffer (pH 7.0), 2.0 mM dithiothreitol at a protein concentration of 1.0 mg/ml. The activities of ethanolamine kinase and choline kinase in both the DEAE preparation and the Sephadex 200 preparation decreased by 40% after 2 days of storage at −20°. The activity dropped by 75% after 6 days at −20° and was completely gone after 12 days. The ratio of choline kinase to ethanolamine kinase II remained constant during storage. The activity was relatively stable when stored at 4°. Ethanolamine kinase II and choline kinase activity in the DEAE preparation and in the Sephadex 200 preparation dropped only 20% during 20 days of storage at 4°. Again, the ratio of choline kinase to ethanolamine kinase II remained constant throughout the storage.

The stability of ethanolamine kinase I was not investigated as thoroughly. The enzyme was routinely stored at 4° and about 50% of the activity was lost after a week of storage.

EFFECT OF pH ON THE REACTIONS. Ethanolamine kinase I had maximal activity between pH of 7.5 and 8.5 and the activity declined significantly at pH 9.0 (Figure 2). The ethanolamine kinase II activity also approached maximal activity at pH 8.0 but, in contrast to ethanolamine kinase I, the activity remained maximal through pH 9.5. Choline kinase activity had a sharper maximum at pH 8.0.

Kinetics of the Reactions. ETHANOLAMINE KINASE I. The requirements for ATP and Mg^{2+} were investigated in some detail. When the Mg^{2+} concentration was held at 3.0 mM and the ATP concentration was varied from 1 to 6 mM, maximal enzyme activity occurred when the ATP concentration was equal to the Mg^{2+} concentration. ATP inhibited the activity at concentration above the Mg^{2+} concentration (67% inhibition at 6.0 mM ATP). In a second experiment the ATP concentration was maintained at 5.0 mM and the

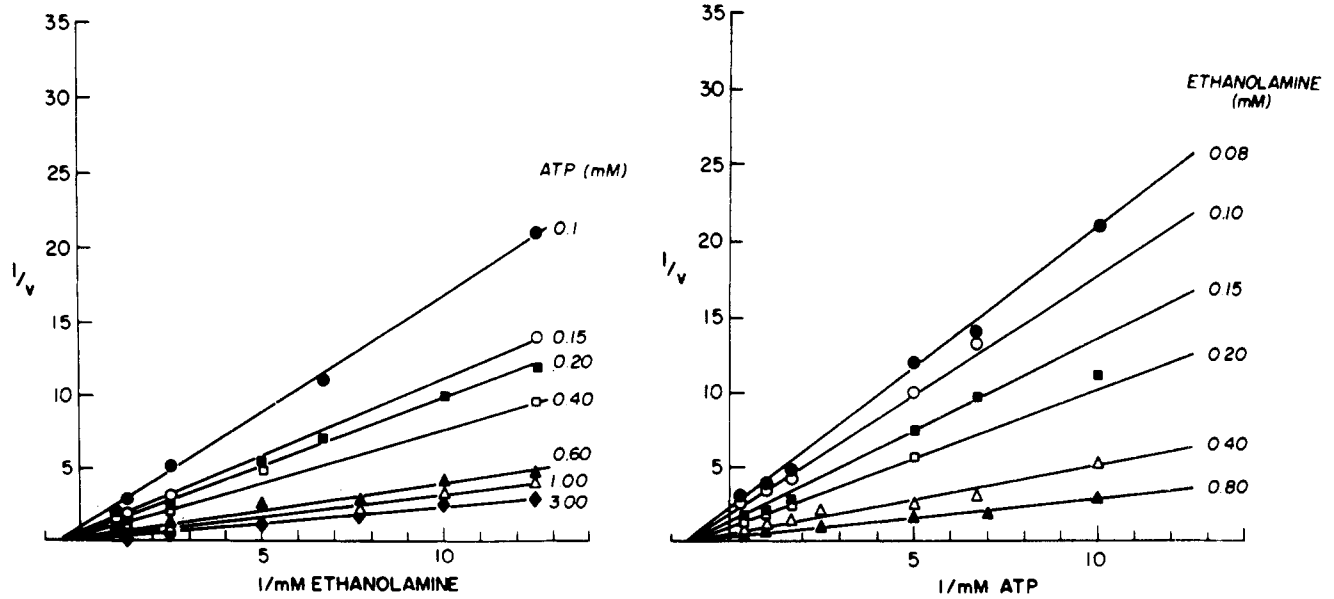

FIGURE 4: The double-reciprocal plots of ethanolamine kinase II. The reactions were run with Mg^{2+} concentrations at 5.0 mM greater than the ATP concentrations.

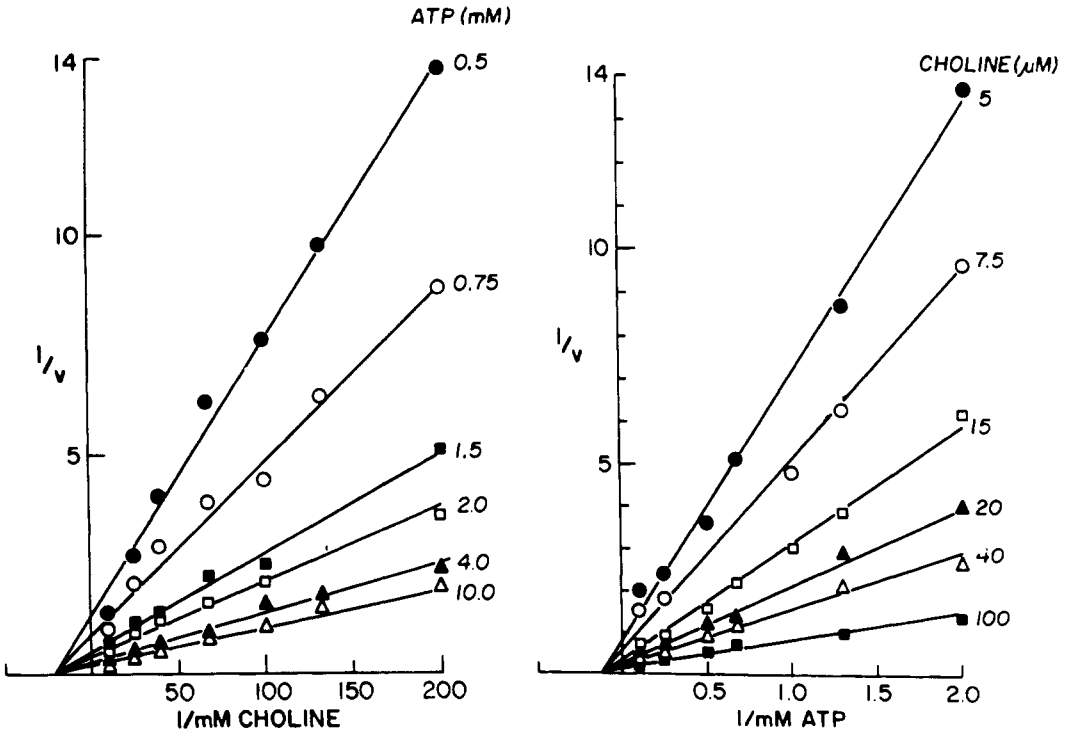

FIGURE 5: The double-reciprocal plots of choline kinase activity. Mg^{2+} concentrations were equal to the ATP concentrations.

Mg^{2+} concentration was varied. Maximal activity was obtained when the Mg^{2+} concentration was double the ATP concentration. Mg^{2+} concentrations of three times the ATP concentration produced a slight inhibition. Thus, ethanolamine kinase I apparently uses Mg-ATP as substrate but also has an additional requirement for Mg^{2+}. The velocity of the reaction increases with increasing concentrations of Mg-ATP until a concentration of 16 mM is reached (Figure 3). Significant inhibition occurs at Mg-ATP concentration above 16 mM. The reciprocal plot of the velocity *vs.* Mg-ATP was linear when the Mg-ATP concentrations were below 14 mM. The apparent K_m for Mg-ATP was 14 mM and the apparent V_m was 0.5 mmol min^{-1}/mg of $protein^{-1}$.

The dependency of the reaction velocity on ethanolamine concentration was determined at ATP and Mg^{2+} concentration of 10 and 15 mM, respectively. The double-reciprocal plot of the data produced a straight line and an apparent K_m of 0.4 mM for ethanolamine was calculated.

The ethanolamine kinase I activity was not inhibited by choline. Choline concentrations as high as 1.0 mM were used with no inhibitory affect. The ATP concentration used in these experiments was 3.0 mM, well below the apparent K_m for ATP. The ethanolamine concentration of 0.5 mM was only slightly above the apparent K_m for ethanolamine.

CHOLINE KINASE-ETHANOLAMINE KINASE II. Ethanolamine kinase II also required MgATP as the substrate, as is the case for most kinase reactions. However, maximal activity was observed when the Mg^{2+} concentration was 5 mM in excess of the ATP concentration. Enzyme activity decreased when the ATP concentration exceeded the Mg^{2+} concentration (50% inhibition at 3 mM excess ATP).

The Mg^{2+} and ATP relationships for choline kinase activity were somewhat different from those for ethanolamine kinase II. Maximal activity was obtained when both Mg^{2+} and ATP concentrations were at 10 mM. The activity at 10 mM ATP and 15 mM Mg^{2+} was 20% lower than that obtained with 10 mM ATP and 10 mM Mg^{2+}. An inhibition of activity also occurred when the ATP concentration was greater than the Mg^{2+} concentration (20% inhibition at 2 mM excess ATP). There was no apparent inhibition by Mg ATP.

The kinetic parameters of choline kinase activity and ethanolamine kinase activity were determined by the double-reciprocal plot method for two substrate reactions (Florini and Vestling, 1957). The double-reciprocal plots for ethanolamine kinase II (Figure 4) and for choline kinase (Figure 5) both intersect at a common point on the X axis. This meets the diagnostic criteria for a random ordered reaction mechanism (Dixon and Webb, 1964). The K_m values for ATP, ethanolamine, and choline were determined from the secondary plots of the reciprocals of the apparent V_m from Figures 4 and 5 *vs.* the reciprocals of the corresponding substrate concentrations. Linear plots were obtained in all cases. The K_m and V_m values thus obtained are shown in Table II along with the apparent K_m values for ethanolamine kinase I. The various K_m values for the different enzyme activities and substrates all differ. Ethanolamine kinase I has a very high K_m for ATP and a relatively

TABLE II: Comparison of the Kinetic Constants for Ethanolamine Kinase I (EKI), Ethanolamine Kinase II (EKII), and Choline Kinase (CK).

Term	EKI	EKII	CK
K_m (ATP)	14.3 mM	0.5 mM	3.7 mM
K_m (ethanolamine)	0.4 mM	1.7 mM	
K_m (choline)			0.03 mM
V_m[a]	33	463	138

[a] nmol min^{-1} mg of $protein^{-1}$.

low K_m for ethanolamine. Ethanolamine kinase II, however, has a relatively low K_m for ATP but a high K_m for ethanolamine. Choline kinase activity has a very low K_m for choline but a high K_m for ATP. A comparison of the V_m for ethanolamine kinase I and ethanolamine kinase II is meaningless since there are differences in the extent of purification and stability. However, the comparison of V_m for ethanolamine kinase II and choline kinase are useful since both activities are associated with the same preparation and appear to have similar stabilities. Thus, ethanolamine kinase II under maximal conditions is almost four times more active than choline kinase.

The ethanolamine kinase II was rather strongly inhibited by choline. The velocity of the reaction was determined with various concentrations of ATP and with either no choline in the reaction or with choline concentrations of 0.03 and 0.10 mM. The double-reciprocal plots of these data were linear and all intersected at the same point on the Y axis, as would be expected for choline inhibition that was competitive with ATP. The K_i for choline was calculated to be 0.03 mM. In similar experiments the choline inhibition was found to be noncompetitive with ethanolamine. These results with the purified preparation are similar to results obtained previously (Weinhold and Rethy, 1972) with ethanolamine kinase activity in the 100,000g supernatant fraction of rat liver.

Ethanolamine at relatively high concentrations inhibited choline kinase activity. This inhibition appeared to be competitive with choline and also competitive with ATP. The K_i for ethanolamine when calculated from experiments with choline competition was 4.5 mM. The K_i when calculated from experiments with ATP competition was 9.8 mM. A clear understanding of these relationships will require additional investigation.

Discussion

Since the initial investigations by Wittenberg and Kornberg (1953) on partially purified choline kinase from yeast, several reports have appeared concerning choline kinase activity from a variety of sources. The enzyme activity was extensively purified from rabbit brain (Haubrich, 1973) and from the filaments of *Cuscuta reflexa* (Setty and Krishnow, 1972). Ethanolamine kinase, on the other hand, has not been investigated in any detail. Several investigators have studied the ability of choline kinase preparation to phosphorylate ethanolamine and have observed either much lower rates (Wittenberg and Kornberg, 1953; Haubreck, 1973; Lang, *et al.*, 1970; McCaman, 1962) or no activity at all (Ramasarma and Wetter, 1957). In all instances the phosphorylation of ethanolamine was studied under essentially the same conditions as employed for choline kinase activity. A definitive investigation of the properties of both choline kinase and ethanolamine kinase was reported by Sung and Johnstone, (1967). They reported indirect evidence that the two activities in Ehrlich ascites cells involved separate enzymes. However, no physical separation of the activities was reported. Our results show that there are at least two ethanolamine kinase activities in rat liver, one of which can be clearly separated from the choline kinase activity. Furthermore, the properties of the two ethanolamine kinases are distinctly different from each other. Their molecular weights are quite different. Even though both have maximal activity at pH 8.0, ethanolamine kinase I activity remains maximal out to pH 9.5 while ethanolamine kinase II activity drops considerably at pH 9.0. The K_m values for both ATP and ethanolamine are significantly different for the two ethanolamine kinase activities. However, the most striking difference is the ability of choline to inhibit ethanolamine kinase II activity while having no inhibitory affect upon ethanolamine kinase I activity.

Ethanolamine kinase II and choline kinase purified together. The ratio of activities remained constant through the DEAE chromatography and several Sephadex 200 chromatographic steps. Both activities had the same stability properties. These characteristics are consistent with the activities being associated with the same protein. However, the preparations were not homogeneous and no definitive conclusion can thus be made. Although the kinetic properties of the two reactions do not distinguish whether one protein or separate proteins are involved, the data do suggest that there are separate binding sites for choline and ethanolamine. The choline inhibition of ethanolamine kinase is not competitive with ethanolamine as would be expected if both bases bound to the same site. The inhibition of choline kinase by ethanolamine, however, is apparently competitive with choline, suggesting that if the activities are associated with the same protein the binding of one of the bases influences the subsequent binding of the other base. If, on the other hand, the activities are associated with separate proteins, the binding site for ethanolamine on ethanolamine kinase has a stricter specificity than the binding site for choline on choline kinase. In either case, the binding site for ATP apparently interacts in some fashion with the base binding sites.

The implications of the three activities in terms of the possible regulation of phospholipid synthesis are of major importance. Ethanolamine kinase II activity is affected by the relative concentrations of choline and ATP. The choline concentrations in the liver have been reported by Sundler *et al.* (1972) to be around 0.3 mM. The ethanolamine kinase II activity would be strongly inhibited at this concentration of choline. However, with increased concentration of ATP this inhibition could be decreased resulting in an increase in ethanolamine kinase activity. If, on the other hand, choline concentrations were to decrease, for example, as a result of choline deficiency, the choline inhibition of ethanolamine kinase would also decrease and ethanolamine kinase activity would increase. This could cause an increase in the formation of phosphatidylethanolamine, a required intermediate in the synthesis of choline *via* the methylation of phosphatidylethanolamine to phosphatidylcholine (Bremer *et al.*, 1960). Ethanolamine kinase I is apparently unaffected by choline and would continue to form phosphorylethanolamine if the ATP concentration was sufficient. Thus the relative activities of the two very similar pathways, *i.e.*, the incorporation of ethanolamine into phosphatidylethanolamine and the incorporation of choline into phosphatidylcholine, may be responsive to each other through choline levels and to the total cellular metabolism through relative ATP levels.

References

Berry, J. F., McPherson, C. F., and Rossiter, R. J. (1958), *J. Neurochem. 3*, 65.

Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N.Y., Academic Press.

Florini, J. R., and Vestling, C. S. (1957), *Biochim. Biophys. Acta 25*, 575.

Haubrich, D. R. (1973), *J. Neurochem. 21,* 315.
Liang, C., Segura, M., and Strickland, K. P. (1970), *Can. J. Biochem. 48,* 580.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
McCaman, R. E. (1962), *J. Biol. Chem. 237,* 672.
McCaman, R. E., and Cook, K. (1966), *J. Biol. Chem. 241,* 3390.
Ramasarma, J., and Wetter, L. R. (1957), *Can. J. Biochem. Physiol. 35,* 853.
Sung, C., and Johnstone, R. M. (1967), *Biochem. J. 105,* 497.
Weinhold, P. A., and Rethy, V. B. (1972), *Biochim. Biophys. Acta 276,* 143.
Wittenberg, J., and Kornberg, A. (1953), *J. Biol. Chem. 202,* 431.

Choline Acetyltransferase and Acetylcholinesterase: Evidence for Essential Histidine Residues†

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ABSTRACT: Choline acetyltransferase (EC 2.3.1.6) catalyzes the biosynthesis of acetylcholine according to the following chemical equation: acetyl coenzyme A + choline ⇌ acetylcholine + coenzyme A. Ethoxyformic anhydride inactivates the enzyme prepared from bovine brain. Acetyl coenzyme A and coenzyme A, but not choline or acetylcholine, substantially protect against inactivation. The enzyme is reactivated by hydroxylamine treatment. The apparent pK_a of the reactive group is about 6.5. Ethoxyformic anhydride also inactivates, and hydroxylamine reactivates, the partially purified electric eel acetylcholinesterase (EC 3.1.1.7). High concentrations of acetylcholine substantially protect against inactivation. The apparent pK_a of the reactive group is about 6.1. Inhibition by ethoxyformylation which is reversed by hydroxylamine treatment provides evidence that histidine plays a role in the choline acetyltransferase and acetylcholinesterase reactions.

Acetylcholine is an established neurotransmitter at the vertebrate neuromuscular junction and a probable, but not proven, transmitter in the vertebrate central nervous system (*cf.* Iverson, 1970). Choline acetyltransferase (EC 2.3.1.6) catalyzes the following reversible reaction: acetyl coenzyme A + choline ⇌ acetylcholine + coenzyme A. Several studies support the notion of an essential enzymic sulfhydryl group. For example, thiol reagents inhibit choline acetyltransferase from squid head ganglia (Reisberg, 1954), primate placenta (Schuberth, 1966), torpedo (Morris, 1967), and mammalian brain (Potter *et al.,* 1968; Chao and Wolfgram, 1973; Roskoski, 1974a). Experiments with the bovine brain transferase suggest that an active site -SH reacts with acetyl coenzyme A to form an acetyl-thioenzyme intermediate (Roskoski, 1973, 1974a). This alleged thio ester intermediate, isolated by Sephadex gel filtration, further reacts with choline to form acetylcholine.

Thio ester intermediates are also associated with the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and papain (EC 3.4.4.10) reactions (Harris *et al.,* 1963; Lowe and Williams, 1965). Crystallographic structural analysis of the lobster muscle dehydrogenase (Buehner *et al.,* 1973, 1974) and papain (Drenth *et al.,* 1968) reveal a histidine residue in close proximity to the active site -SH. The interaction of the imidazole with the sulfhydryl and with the substrates may be important in the catalytic mechanism of these enzymes. These findings prompted a study of the effects of bromoacetyl coenzyme A and bromoacetylcholine, possible alkylating reagents, on choline acetyltransferase. These studies failed to implicate histidine; instead, the enzyme was inhibited by bromoacetylation of the active site sulfhydryl (Roskoski, 1974b).

In the present studies ethoxyformic anhydride inactivation and reversal by hydroxylamine treatment implicate histidine in the choline acetyltransferase and acetylcholinesterase (EC 3.1.1.7) enzyme reactions. Furthermore, both enzymes are inhibited by *N*-acetylimidazole and their activity spontaneously returns to control values within 1 hr. These results are consistent with the hypothesis that choline acetyltransferase and acetylcholinesterase are inhibited by chemical modification of enzymic histidine residues.

Experimental Section

Materials. Ethoxyformic anhydride and *N*-acetylimidazole were purchased from Sigma Chemical Co. Electric eel acetylcholinesterase (1058 units mg^{-1}) was a product of Worthington Biochemical Corp. Acetylthiocholine chloride was purchased from Pfaltz and Bauer, Inc. Decamethonium bromide was purchased from K and K Laboratories, Inc., and indoxyphenyl acetate, Calbiochem.

Methodology for Chemical Modification. For reaction with choline acetyltransferase, ethoxyformic anhydride, dissolved in absolute ethanol, was added to give the specified concentration of inhibitor. Ethoxyformic anhydride and *N*-acetylimidazole were dissolved in acetonitrile for the other experiments. These solutions were prepared immediately before use. A 1-μl solution of inhibitor was added to 100 μl of enzyme solution to initiate the reaction unless specified otherwise. Solvent alone was added to the control samples.

Acetylcholinesterase Assay. The spectrophotometric

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