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A NOVEL PURIFICATION AND SOME PROPERTIES OF RAT LIVER MITOCHONDRIAL CHOLINE DEHYDROGENASE

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Summary

Choline dehydrogenase (choline:(acceptor) oxidoreductase, EC 1.1.99.1) was purified from rat liver mitochondria. An approx. 240-fold purification was achieved by chemically modified enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) through columns of DEAE-Sepharose CL-6B and choline-Sepharose 4B with C_3-spacer, and after the subsequent release of the thionitrobenzoate with dithiothreitol, through a second column of DEAE-Sepharose CL-6B in the presence of 0.1% Triton X-100.

The purified preparation gave a specific activity of 6.3 μmol of O_2 consumed/min per mg protein at 30°C with phenazine methosulfate as the primary electron acceptor. After polyacrylamide gel electrophoresis in the presence of 0.5% sodium dodecyl sulfate, the enzyme showed activity in the gel. The preparation thus purified oxidized only choline and betaine aldehyde. The K_m value for choline was 7.0 mM at an infinite concentration of phenazine methosulfate at pH 7.6 and 30°C. 2-Dimethyl aminoethanol ($K_{i,app}$ = 1.0 mM) and monoethanolamine did not work as substrates, inhibiting the enzyme competitively. The absolute requirement of any electron acceptor other than the molecular oxygen was confirmed. The K_m value for phenazine methosulfate was about 1.1 mM at infinite concentration of choline. These findings suggested that coenzyme Q served as the primary electron acceptor in vivo.

Choline dehydrogenase (choline:(acceptor) oxidoreductase, EC 1.1.99.1) found at the inner membrane of mammalian liver mitochondria has been

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

partially purified by Singer and coworkers [1,2], and identified as a flavoprotein containing non-heme iron. Barrett and Dawson [3,4] have solubilized the enzyme by Triton X-100 and have suggested that coenzyme Q is involved as the primary electron acceptor in the intact mitochondria and crude preparation so solubilized. Various techniques have been examined for the solubilization of hepatic mitochondrial choline dehydrogenase [5—7], but further purification has never been reported except with the use of a hydroxyapatite column [2]. All of the characteristics [7] reported thus far have been obtained using a relatively crude preparation and a highly purified preparation is an urgent priority in the field.

The present study purports to purify the choline dehydrogenase from rat liver and to establish some of its characteristics.

Experimental procedures

Materials

The following materials were obtained from the companies listed: Choline chloride, Tokyo Kasei Organic Chemicals (Tokyo, Japan); betaine, dimethyl glycine, sarcosine, butyryl choline chloride, phenazine methosulfate, 5,5′-dithiobis(2-nitrobenzoic acid), and dithiothreitol, Nakarai Chemicals Ltd. (Kyoto, Japan); betaine aldehyde chloride, Sigma Chemical Co. (St. Louis, MO, U.S.A.); CN-activated Sepharose 4B and DEAE-Sepharose CL-6B, Pharmacia Fine Chemicals (Uppsala, Sweden). Coenzyme Q_2 was the generous gift of Eisai Pharmaceutical Co. Ltd. (Tokyo, Japan). Choline-Sepharose 4B with C_3-spacer was synthesized in two steps; 6 g of CN-activated Sepharose 4B was coupled with *N,N*-dimethyl-1,3-propanediamine (4 mmol) in 0.1 M $NaHCO_3$ containing 0.5 M NaCl according to the method of Parikh et al. [8], washing with 10 vols. of 0.1 M $NaHCO_3$ containing 0.5 M NaCl, and then reacted with ethylene bromohydrin (20 mmol) in methanol at 70 °C for 4 h. The contents of quaternary ammonium cation in the choline-Sepharose 4B were determined by the method of Reinekate [9] after a complete water wash and drying. All other chemicals used were of the highest available commercial grade.

Rat livers were from Wistar strain killed immediately before use.

Methods

Unless otherwise noted, all assays for choline dehydrogenase were performed at 30 °C with an oxygen electrode connected to a Bioxygraph (Kyusui Kagaku Kenhyosho, Co. Ltd., Tokyo, Japan), and the assay medium was comprised of 33 mM potassium phosphate buffer (pH 7.6)/1 mM KCN/1 mM phenazine methosulfate/16.7 mM choline chloride and a suitable amount of enzyme preparation in a total volume of 3.0 ml. The reaction was started by adding the enzyme solution, and initial rate of oxygen consumption was recorded at a chart speed of 1 cm/min. The amount of soluble oxygen was calibrated according to the method of Misra and Fridovich [10].

Protein concentrations were determined by the biuret method [11] for the crude preparation, and by a modification of the method of Lowry [12] for the purified sample.

1 unit of enzyme is defined as the amount giving an oxygen consumption of

1 μmol/min under these assay conditions. Specific activity is expressed as units per mg of protein.

Results

Purification of choline dehydrogenase

Standard purification was started from approx. 150 g of liver (using 12 rats) at a time. Centrifugation was done with a usual refrigerated type of centrifuge at a maximum speed (approx. 24 000 × *g*) at 5 °C, unless otherwise noted.

Preparation of mitochondria and extraction of undesired protein by buffer. Mitochondria were prepared by grindings of diced liver in 4 vols. of 0.25 M sucrose containing 5 mM Hepes-KOH buffer (pH 7.6) with a glass-Teflon homogenizer [13], and then by centrifugation for 10 min. The resulting pellet was repeatedly treated in series as above, at least, three times. The mitochondrial pellet from the final step, was suspended in 150 ml of 60 mM glycine-NaOH buffer (pH 10), and the suspension was finely ground and then centrifuged for 30 min. The resulting pellet was resuspended in 150 ml of 0.3 M potassium phosphate buffer (pH 8.0), and the supernatant was discarded. The pellet was transferred to a beaker with 10 mM Tris-phosphate buffer (pH 8.0).

Solubilization of the enzyme. The choline dehydrogenase was solubilized by successive treatments of the mitochondrial suspension by sonication and Triton X-100. The mitochondrial suspension was disrupted by sonic treatment for 10 min, in 5 min periods (twice), at 20 KHz in an ice/water bath, to which 2% (w/v) of intact Triton X-100 was added dropwise, and mechanically stirred for 20 min. After centrifugation for 60 min, the supernatant solution was taken as solubilized enzyme preparation, which was shown not to precipitate by an ultracentrifugation at 125 000 × *g* for 60 min. Pre-chilled absolute ethanol was added to the solution to give a final 30% (v/v), which was stirred for 30 min and then centrifuged to remove the precipitate.

Preparation of SH-modified enzyme. 5,5'-Dithiobis(2-nitrobenzoic acid), dissolved in a minimal volume of 1 M Tris-phosphate buffer (pH 8.0), was added to a clear solution, the protein concentration of which was determined by the biuret method [11], so that 5,5'-dithiobis(2-nitrobenzoic acid)/protein (both total mg amounts) was 1:15. This ratio was found to give the optimum conditions on the remaining activity when assayed. Increasing the concentration was found to inhibit the activity; decreasing caused its rapid loss during subsequent purification. After dialysis against 10 mM Tris-phosphate buffer (pH 8.0) containing 0.1% Triton X-100 overnight, any precipitate was centrifuged and discarded.

Column chromatography. The SH-modified enzyme solution was applied to a DEAE-Sepharose CL-6B column (3 × 25 cm), which was preequilibrated with the same buffer as above. The column was eluted with a linear gradient of KCl established between 0 and 0.3 M in the same buffer, totalling 600 ml. The elution profile is shown in Fig. 1. Fractions of high specific activity were pooled and dialyzed against 10 mM Tris-phosphate buffer (pH 8.0) containing 0.1% Triton X-100. The dialysate * was applied to a choline-Sepharose 4B with C_3-

* The contents of thionitrobenzoate residue attached were about 4.6 nmol/mg protein, which were estimated photometrically by adding dithiothreitol.

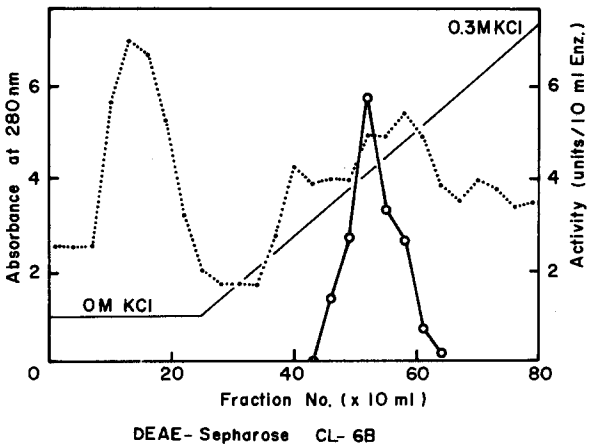


Fig. 1. DEAE-Sepharose CL-6B column chromatography of choline dehydrogenase modified by thionitrobenzoate. About 305 mg of modified preparation was applied, and fractions of high specific activity (Nos. 47—58) were pooled. Absorbance at 280 nm (●·····●), and choline dehydrogenase activity via phenazine methosulfate (○———○).

spacer (2.5 × 6 cm), which was synthesized as described in Experimental procedures and preequilibrated with 10 mM Tris-phosphate buffer (pH 8.0) containing 0.1% Triton X-100. The contents of quaternary ammonium cation [9] were approx. 0.2 mmol/g of dried mass. The column was washed with 5 mM choline chloride solution and then eluted with a linear gradient of KCl established between 0 and 0.5 M in the same buffer, totalling 600 ml. The elution profile is shown in Fig. 2. Fractions of high specific activity were pooled, and

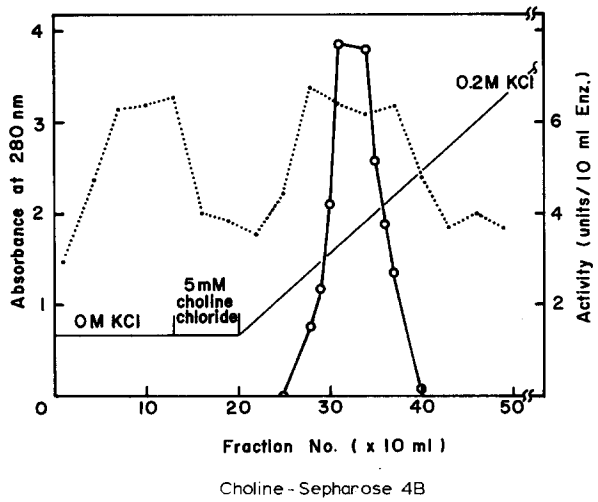
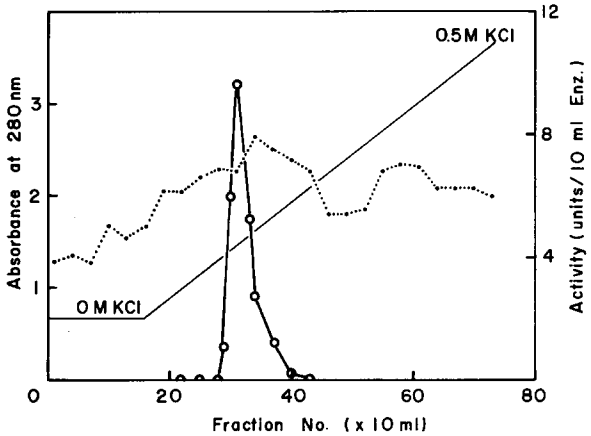


Fig. 2. Choline-Sepharose 4B (with C_3-spacer) column chromatography of choline dehydrogenase modified by thionitrobenzoate. About 33 mg of protein was applied after dialysis against 10 mM Tris-phosphate buffer (pH 8.0), containing 0.1% Triton X-100, and fractions of high specific activity (Nos. 30—35) were pooled. Absorbance at 280 nm (●·····●), and choline dehydrogenase activity via phenazine methosulfate (○———○).



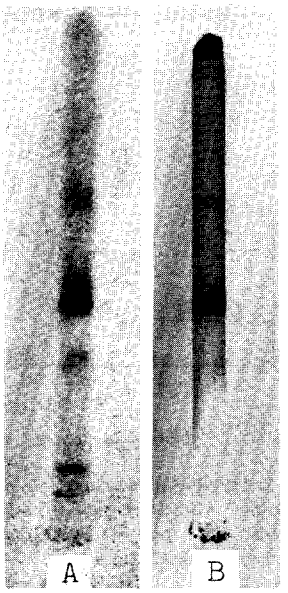


Fig. 3. Second DEAE-Sepharose CL-6B column chromatography of choline dehydrogenase freed from thionitrobenzoate by dithiothreitol. To about 60 ml of the pooled solution, 1 ml of 0.1 M dithiothreitol was added and allowed to stand for 30 min. The sample was applied after dialysis against 10 mM Tris-phosphate buffer (pH 8.0), containing 0.1% Triton X-100. Fractions of high specific activity (Nos. 30—33) were pooled. Absorbance at 280 nm (●· · · · · ·●), and choline dehydrogenase activity via phenazine methosulfate (○———○).

Fig. 4. Polyacrylamide gel electrophoresis of purified choline dehydrogenase in the presence of 0.5% sodium dodecyl sulfate. Electrophoresis was carried out with 10% acrylamide monomer at 3 mA per tube for 3 h in the cold. Protein was stained with Coomassie brilliant blue, and choline dehydrogenase activity was stained by incubating the gel in a solution containing 50 mM choline chloride/1 mM phenazine methosulfate/1 mg/ml nitroblue tetrazolium/50 mM potassium phosphate buffer (pH 8.0), at 37°C for 30 min in the dark. A. Protein band; B, activity band.

TABLE I

SUMMARY OF PURIFICATION OF CHOLINE DEHYDROGENASE FROM RAT LIVER MITOCHON DRIA

Step	Total volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg P)	Yield (%)
Mitochondria	238.0	7916	204	0.026	100
Mitochondria, impurities removed	72.2	2242	121	0.054	59.3
Solubilized preparation	69.4	1231	56	0.045	27.5
SH-Modified preparation	133.3	305	67	0.222	33.1
1st DEAE-Sapharose	118.0	33.16 *	72	2.18	35.4
Choline-Sepharose	59.0	19.94 *	45	2.26	22.1
2nd DEAE-Sepharose **	39.3	4.95 *	31	6.29	15.3

* Protein concentrations were measured using the modified method of Lowry [12].

** Sample: released thionitrobenzate group(s) by adding 1 ml of 0.1 M dithiothreitol and dialyzing exhaustively against 10 mM Tris-phosphate buffer (pH 8.0) containing 0.1% Triton X-100.

1 ml of 0.1 M dithiothreitol was added and allowed to release the bound thionitrobenzoate residues. After exhaustive dialysis against 10 mM Tris-phosphate buffer (pH 8.0), containing 0.1% Triton X-100, the sample was reapplied to a DEAE-Sepharose CL-6B column (2.5 × 25 cm). The column was eluted with a linear gradient of KCl established between 0 to 0.5 M in 10 mM Tris-phosphate buffer (pH 8.0), containing 0.1% Triton X-100, totalling 600 ml. The elution profile is shown in Fig. 3. Fractions of high specific activity were pooled and used to check the purity with polyacrylamide gel electrophoresis in the presence of 0.5% sodium dodecyl sulfate. As shown in Fig. 4, three protein bands were stained by Coomassie brilliant blue, two of them by the choline dehydrogenase activity from 30 min incubation at 37°C in the dark.

The overall purification is summarized in Table I. Pooled fractions of column chromatographies gave rather high absorbance at 280 nm, but actual protein concentration determined by the modified method of Lowry [12] was considerably lower. This should be due to the intrinsic absorbance of Triton X-100 at this wavelength. Finally, the pooled fraction gave a specific activity of 6.3 units/mg protein, and starting from the mitochondrial preparation, approx. 240-fold purification in 15% yield was achieved.

Properties

The pooled fraction from the second DEAE-Sepharose CL-6B column, giving an apparently colorless, transparent solution, was used for the characterization of the enzyme despite its content of about 0.1 M KCl.

Substrate specificity. Relative substrate specificity was examined at a concentration of 16.7 mM under the standard assay conditions. The following compounds were investigated; acetylcholine chloride, ammonium hydroxide, betaine aldehyde chloride, butyrylcholine chloride, DL-carnitine chloride, choline chloride, 2-dimethylaminoethanol, dimethyl glycine, ethanol, hydroxylamine · HCl, DL-3-hydroxybutyrate, L-malate, monoethanolamine, sarcosine, L-serine, succinate, taurine, and L-threonine. The enzyme could use only choline and betaine aldehyde. Betaine aldehyde served as the substrate, but its relative activity was very low (5.2% of choline as control). No oxygen consumption was enhanced by the addition of NAD or NADP. Other compounds having 2-hydroxyethyl function did not serve as the substrate.

To determine the K_m value for choline, the purified enzyme was reacted at various concentrations of choline and phenazine methosulfate. The results are shown in Fig. 5, in the form of Lineweaver-Burk plots. The slopes of the plots were in both cases independent of the concentrations of the invariant substrate. These results may be explained by a reaction mechanism described as a Ping-Pong-Bi-Bi type by Cleland [14], and are in good agreement with those of Barrett and Dawson [4]. However, the true Michaelis constants for choline (7.0 mM) as well as phenazine methosulfate (1.1 mM), which were determined by extrapolation of the co-substrate, were considerably higher than those of the crude solubilized enzyme [4]. The differences may be due to the temperature used (cf. 30°C vs. 38°C in Ref. 4). The K_m value for choline is in good agreement with that of Kimura and Singer [2]. As to betaine aldehyde, the $K_{m/app}$ was calculated to be 3.1 mM at a constant concentration of phenazine methosulfate (1 mM) and strong inhibition was noted in more than approx. 4 mM.

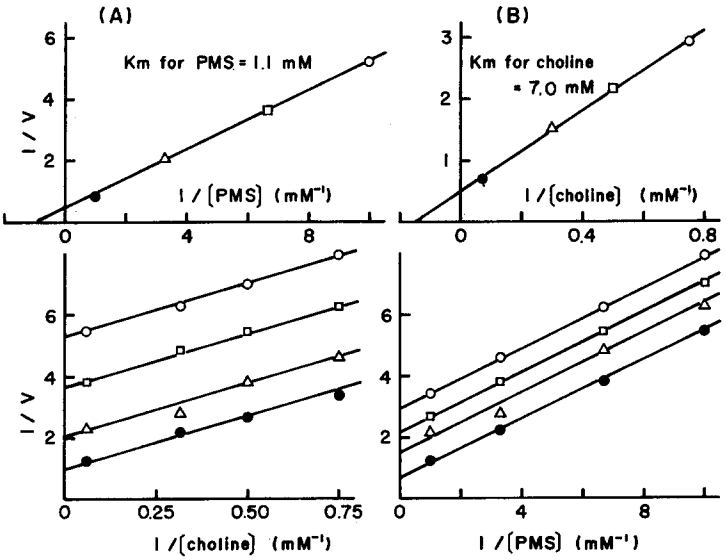


Fig. 5. Double-reciprocal plots for the determination of K_m values against choline and phenazine methosulate (PMS). About 25 μg of protein (0.08 unit) were used to assay under standard conditions except for the choline and phenazine methosulfate concentrations. (A) Phenazine methosulfate concentrations used were: ○, 0.1 mM; □, 0.15 mM; △, 0.3 mM; ●, 1.0 mM. (B) Choline concentrations used were: ○, 1.33 mM; □, 2.0 mM; △, 3.33 mM; ●, 16.7 mM.

Electron acceptor. The absolute requirement of any electron acceptor other than molecular oxygen was established in the presence or absence of KCN. Phenazine methosulfate easily accepted an electron from the reduced enzyme. When various concentrations of choline were used as the electron donor, K_m for the compound at the infinite concentration of choline was approx. 1.1 mM. Coenzyme Q has been suspected to take part in the mitochondrial oxidation of choline [3,4,15]. Instead of water-insoluble coenzyme Q_{10}, Q_2, more soluble in ethanolic medium, was tested for its feasibility as the primary electron acceptor. As shown in Fig. 6, it accepted electrons from the reduced enzyme under the N_2 gas. When the oxygen consumption was traced in the presence of coenzyme Q_2 and phenazine methosulfate as the electron acceptor under the standard assay conditions, the reaction proceeded somewhat more quickly than when in the presence of only phenazine methosulfate.

Inhibitor and activator. It should be noted here that the choline dehydrogenase activity was greatly affected by the pH of the assay medium. Thus the reaction medium to detect the inhibitor or activator for the enzyme had to be checked carefully. The following compounds, including the substrate analogs listed in the preceding section, were investigated; ATP, ADP, AMP, Ca^{2+}, Mg^{2+}, Fe^{3+}, FMN, FAD, NAD, NADP, semicarbazide · HCl and tetramethylammonium chloride. The data are summarized in Table II. Neither FMN nor NAD enhanced the activity at all, nor did Ca^{2+}, Mg^{2+}, or Fe^{3+} have any effect; 2-dimethylaminoethanol was a potent competitive inhibitor, with a $K_{i/app}$ value of about 1.0 mM. However, ethanol (16.7 mM) did not show any effect. Sarcosine, dimethylglycine and glycine, which were intermediate metabolites in

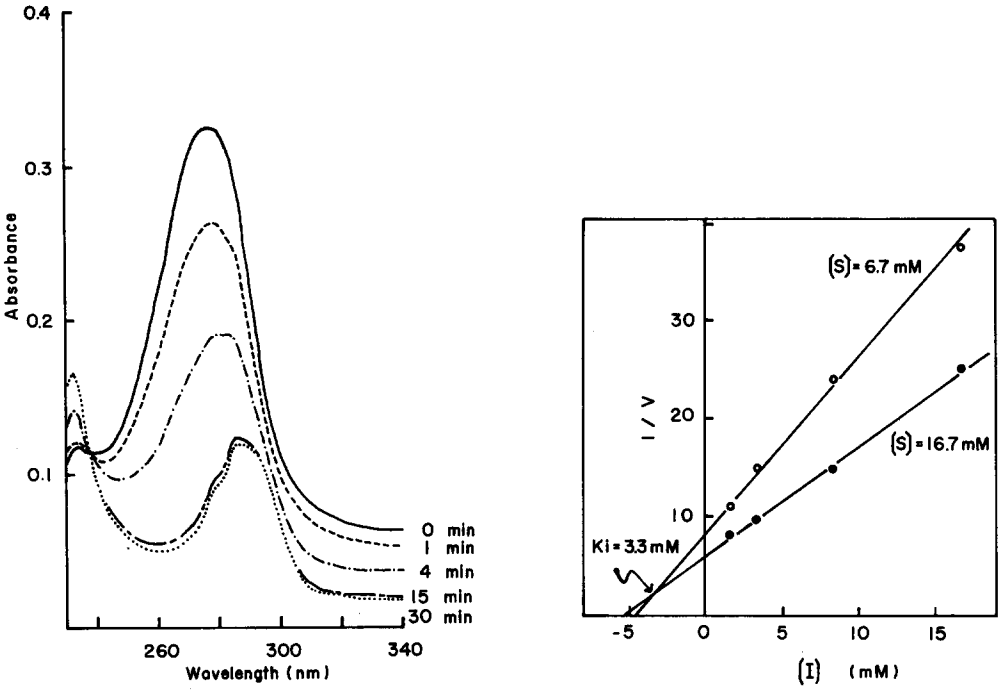


Fig. 6. Spectrophotometric trace of reduction of coenzyme Q_2 by choline chloride under N_2 gas. Thunberg-type cuvette containing 20 μM coenzyme Q_2 (as 0.5% ethanol solution), 25 μg of choline dehydrogenase (0.1 unit), 1 mM KCN, 33 mM potassium phosphate buffer (pH 7.6) in the main room and 16.7 mM choline chloride in the side arm, was evacuated and then filled with N_2 gas. Each spectrum was recorded with 100 nm/min chart speed at 25°C.

Fig. 7. Inhibitory effect of betaine aldehyde on oxidation of choline (Dixon plot). About 17 μg of choline dehydrogenase (0.1 unit) were used. S, choline chloride; I, betaine aldehyde chloride.

TABLE II

INHIBITORY EFFECT OF VARIOUS COMPOUNDS ON CHOLINE DEHYDROGENASE

Prior to testing the compound, pH of the solution was adjusted to near 7 with 1 N HCl or 1 N KOH, if necessary. Choline dehydrogenase activity (16.7 mM choline alone: 100%) was measured in the presence of 16.7 mM of the compound indicated under the standard assay conditions at 30°C.

Compound added	Relative Activity (%)
Acetylcholine	100
Betaine	92.2
Betaine aldehyde	62.9
Butyrylcholine	84.5
DL-Carnitine	100
2-Dimethylaminoethanol	31.8
Ethanol	100
3-Hydroxybutyrate	92.9
L-Malate	87.7
Monoethanolamine	71.5
Semicarbazide HCl	76.0
L-Succinate	100

the oxidative pathway of choline, failed to display any effect. As to the structural analogs of choline, DL-carnitine, and acetylcholine having trimethyl quaternary ammonium function, did not inhibit the choline oxidation. DL-3-Hydroxybutyrate (16.7 mM) acted as a weak inhibitor, as with the datum from the intact mitochondria [16]. L-Malate also served as an inhibitor.

Betaine aldehyde could be oxidized by the preparation without NAD or NADP, suggesting that the compound would compete with choline at the active site of the enzyme. In fact, betaine aldehyde, the $K_{i/app}$ value of which was estimated to be 3.3 mM as shown in Fig. 7, inhibited the oxidation of choline competitively. When semicarbazide · HCl, aldehyde-trapping reagent, was added to the reaction medium, approx. 24% of its oxidation was suppressed, suggesting that choline was oxidized to betaine via betaine aldehyde by the same protein.

Other properties. The optimum temperature was near 45° C, and an optimum pH of around 9 seemed likely. This value was rather higher than found by previous workers [2]. The enzyme was unstable even in the frozen state at −15° C for a month (remaining activity, approx. 50%), and repeated freezing and thawing decreased its activity much more quickly.

Discussion

Attempts to purify choline dehydrogenase from mammalian liver have never succeeded for the following reasons. First, when the enzyme is solubilized from the mitochondrial matrix using various methods, the activity decreased very rapidly and the enzyme contains labile SH-group(s) essential for the activity [17]; secondly, once the surfactant is omitted from the solution, the enzyme proteins are apt to associate with each other and hence to precipitate [5]; moreover, salting-out by ammonium sulfate, for instance, is hampered by the presence of surfactant; thirdly, the solubilized enzyme is strongly absorbed on cellulose derivatives so as to preclude the use of cellulose ion-exchangers [7] and the only known adsorbent is hydroxyapatite gel [2].

To overcome the above difficulties, we attempted a new approach according to which, by the positive introduction of a labelling group into the objective protein, the modified proteins could be stabilized as well as separated from each other by the force of the charged groups, when such a sample was applied to an ion-exchange column. A commercially available reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), was used to label the labile SH group(s). In fact, stabilization of the enzyme activity and reproducible chromatographies on DEAE-Sepharose CL-6B were attained. It should be noted, however, that in the effective application the introduced group could easily be released by some reactions under mild conditions.

The enzyme preparation freed from the thionitrobenzoate group(s) lost its activity easily and tended to precipitate, when the sample was dialyzed against the buffer without Triton X-100. In this connection, the earlier observations [5,6] that the choline dehydrogenase preparation solubilized by means of sodium cholate may be a dispersion of insoluble particles is probably unfounded, because several lines of evidence on integral proteins indicate that

some enzymes require the presence of phospholipids (or bile acid) to maintain the protein structure or activity [18]. In our study, the solubilization was done under mild conditions comparable to those of Williams and Sreenivasan [5]. The purified enzyme preparation migrated into the separation gel on a polyacrylamide gel electrophoresis and was sufficiently active under such conditions that it was carried out in 0.5% sodium dodecyl sulfate without any reducing agent (see the legend of Fig. 4).

Fluorescence properties of the preparation were complicated because Triton X-100 interfered with the intrinsic fluorescence from the enzyme. The existence of no dissociable prosthetic group, FAD or FMN, could be established from the enzyme assay. The enzyme may contain covalently bound flavin as in microbial choline oxidase [19,20], since after sodium dodecyl sulfate gel electrophoresis [19], its position corresponding to the activity and protein band was fluorescent in 7.5% acetic acid under ultraviolet light (unpublished observation).

The existence of succinate dehydrogenase activity was ruled out in this preparation. The enzyme purified through the above procedure showed the absolute requirement of any electron acceptor other than the molecular oxygen, and the preparation must have been free of cytochromes, since the choline oxidase activity was not affected with or without KCN.

It has long been suspected that coenzyme Q may serve as the primary electron acceptor for the reduced choline dehydrogenase, since Drabikowska and Szarkowska [15] have demonstrated such possibilities using intact mitochondria. Barrett and Dawson [3,4] have determined the K_m values for coenzymes Q_1 and Q_2 with a crude preparation solubilized by Triton X-100 or snake venom, but they have not concluded that this coenzyme takes part in the reaction. We can now conclude that the highly purified preparation utilizes coenzyme Q as the primary electron acceptor. This conclusion is corroborated by earlier observations of others.

It is of great interest that, when an excess amount of semicarbazide · HCl was added to the assay medium, oxygen consumption was suppressed thereafter by 24%. Thus, the betaine aldehyde dehydrogenase activity via phenazine methosulfate might be implicated, but this activity was not enhanced by the addition of NAD or NADP. Therefore, it can be reasonably concluded that choline dehydrogenase, like choline oxidase [21], oxidized betaine aldehyde to betaine to some extent. Drabikowska and Szarkowska [15] have observed that choline as well as betaine aldehyde reduces the endogenous ubiquinone in NAD-depleted mitochondria, suggesting that betaine aldehyde is oxidized by an enzyme without NAD. The present observations further reinforce the opinion that the choline dehydrogenase itself may well have oxidized betaine aldehyde via phenazine methosulfate.

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