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SEPARATION AND CHARACTERIZATION OF ACONITATE HYDRATASE ISOENZYMES FROM PIG TISSUES

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SUMMARY

Aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) activity of pig tissues is associated with two protein fractions, one present in the cytoplasm while the second is of mitochondrial origin. They differ in p*I* (pH 5.4 for the cytoplasmic and pH 7.5 for the mitochondrial enzyme) and stability, the mitochondrial being much more unstable. Chromatography on DEAE-cellulose under N_2 readily separates the aconitate hydratase isoenzymes.

INTRODUCTION

In the hands of most investigators, aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) has resisted purification beyond 24-fold, as discussed by BUCHANAN AND ANFINSEN[1], MORRISON[2], and HENSON AND CLELAND[3]. Apart from seemingly poorly controllable stability, uncertainties concerning the subcellular origin of this enzyme[4,5] emerged. The possibility could not be excluded that during various procedures of purification more than one molecular species each with different properties were extracted. It was concluded by GUARRIERA-BOBYLEVA AND BUFFA[6], that aconitate hydratase found in cell sap of liver homogenates was much less sensitive to fluorocitrate inhibition than the corresponding enzyme found in the mitochondrial fraction. Starch gel electrophoretic patterns of extracts of mouse tissues revealed two enzymatically active bands as shown by KOEN AND GOODMAN[7]. Aconitate hydratase activity in human liver was also found by SHEPHERD *et al.*[8] to be present in the soluble and particulate tissue fractions. Although the sum of these diverse experimental results suggests that aconitate hydratase activity may be associated with more than one protein, actual isolation and characterization of isoenzymes with differing molecular properties has not been achieved.

Our interest in aconitate hydratase was attracted by the apparent tissue specificity of the metabolic effect of fluorocitrate toward mitochondria isolated from various tissues[9]. One of several possible reasons for tissue specific effect of fluorocitrate could

be that various tissues contain isoenzymes of different inhibitor sensitivities. As an approach to these problems, the present paper deals with separation of aconitate hydratase isoenzymes by chromatography and isoelectric focusing of extracts prepared from pig tissues.

MATERIALS AND METHODS

Preparation of tissue extracts

Freshly obtained pig tissues were immediately chilled in cracked ice and processed within an hour. The tissues studied included heart ventricle, renal cortex and liver. All procedures were carried out at 0–4° unless stated otherwise. Initial extracts were prepared by homogenizing the tissue in citrate buffer (concentrations are shown in RESULTS) containing chloroform in the ratio 1:3:0.65 (w/v/v), respectively. Homogenization was carried out for 25 sec in a Waring blender. Crude extracts were obtained by centrifugation of homogenates at 10 000 × *g* for 15 min. Extracts prepared with 20 mM citrate were subjected to ethanol fractionation by a procedure similar to that used by MORRISON[2]. The protein fraction precipitating between 15 and 45% ethanol was collected at −10°. Alternatively tissue extracts prepared with 1 mM citrate were directly chromatographed on DEAE-cellulose.

Electrofocusing

Aconitate hydratase preparations obtained either by ethanol fractionation or by column chromatography were used for isoelectric focusing in an LKB Model 8100 apparatus. Ampholite (1%) was used in a pH range of 3–10 with a supporting linear sucrose gradient of 0–50%. The applied potential was 300 V. The enzyme preparations were added to the column at the center of the gradient. All runs were carried out for 43 h at 0–4°. The pH of each fraction collected following electrofocusing was determined at 0°.

Column chromatography and assay methods

DEAE-cellulose chromatography was carried out in dead space-free columns (Metaloglass, Inc., Boston, Mass.) of varying sizes under N_2 at 2–3°. The elution was begun with 1 mM citrate brought to pH 7.5 with Tris base at 25°, and completed by changing to 5 mM citrate also adjusted to pH 7.5 with Tris base at 25°. Fractions, containing the isoenzymes, were combined and the volume reduced either by pressure ultrafiltration (Amicon Model No. 52, Diaflo system with PM-30 membrane), or by freeze-drying.

Protein estimations were carried out by the biuret color test[10] with crystalline serum albumin (Armour & Co.) as reference standard. Enzyme activity was measured in a Gilford recording spectrophotometer in 1-ml cuvettes (1-cm light path) at 25°. The initial increase of absorbance was recorded at 240 nm with 30 mM citrate (adjusted to pH 7.5 with Tris base) as substrate. The molecular absorption coefficient for *cis*-aconitate given by HENSON AND CLELAND[3] was used in calculating the results. Maximal enzymatic activities of chromatographic or isoelectric fractions were obtained by incubation of 1 vol. of enzyme solution with 1 vol. of activating reagent (25 mM cysteine + 1.25 mM Fe^{2+}) for 15 min at 0°. After incubation, the enzyme solutions were freed from the activating reagent by passage through a Biogel P-6

column with 1 mM Tris–citrate (pH 7.5) as eluent. The activating reagent was prepared by dissolving cysteine + $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ in water at 0° under N_2 and adjusting the pH to 7.5 with Tris base.

Subcellular fractionation

Subcellular fractionation of liver homogenates (in sucrose–mannitol–albumin media) was performed by differential centrifugation according to SCHNAITMAN AND GREENWALT[11]. Mitochondria were washed twice in the preparative medium and freeze-thawed in 1 mM citrate (pH 7.5) to obtain soluble aconitate hydratase. Prior to chromatographic separation the cytosol fraction was subjected to ultrafiltration and washing through PM-30 membranes in order to replace sucrose–mannitol with 1 mM citrate.

RESULTS

The differences in the isoelectric points of aconitate hydratase isoenzymes of pig heart are illustrated in Fig. 1. The focusing Pattern A shows the presence of both isoenzymes in an alcohol fraction isolated by a procedure similar to that used by MORRISON[2]. Patterns B and C were obtained from eluates of DEAE-cellulose columns, Pattern B with 1 mM, Pattern C with 5 mM citrate. A significant difference in the stability of the isoenzymes was observed during electrofocusing. Only 2% of the original activity placed on the column was recovered from the fraction which was

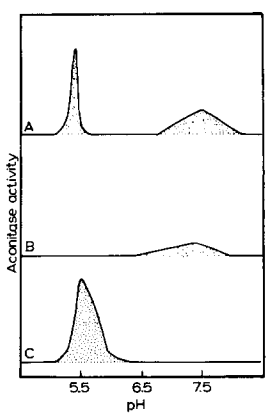

Fig. 1. Isoelectric focusing of aconitate hydratase isoenzymes from pig heart. A. Focusing pattern of enzyme preparation obtained by alcohol fractionation of a pig heart extract prepared in 20 mM Tris citrate (pH 5.4). The preparation showed a 3.5-fold increase in specific activity as compared to the crude extract. B. Focusing pattern of enzyme prepared by DEAE-cellulose chromatography. Tissue extract prepared in 1 mM Tris citrate (pH 7.5) from 400 g pig heart was placed on a 5.0 cm × 80 cm column of DEAE-cellulose. The aconitate hydratase was eluted from the column with the same buffer, and all fractions containing enzyme were combined and concentrated 50-fold using a Diaflo pressure filtration system with a PM-30 membrane. To insure maximum activity, a sample of the concentrate was activated with Fe^{2+}–cysteine prior to electrofocusing. Excess Fe^{2+} and cysteine were removed prior to the run by gel filtration. C. Focusing pattern of enzyme prepared by DEAE-cellulose chromatography. The activity represents that isoenzyme eluted from the column with 5 mM Tris citrate (pH 7.5) in the procedure initiated in B. All fractions containing this enzyme were combined, concentrated 50-fold, activated and gel filtered as had been carried out for the isoenzyme focused in B.

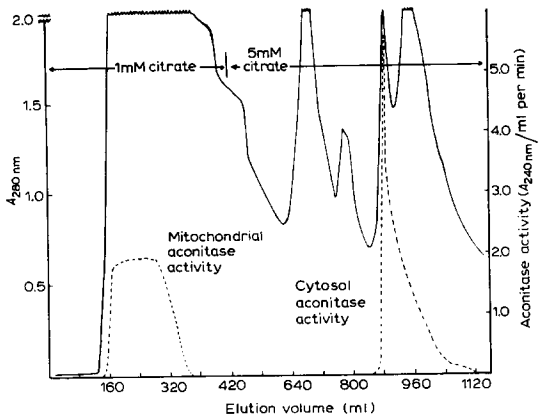

Fig. 2. The elution pattern of pig liver mitochondrial and cytosol aconitate hydratase from DEAE-cellulose. An extract of fresh liver (80 g) was prepared in 1 mM Tris citrate (pH 7.5) and chromatographed on a 2.5 cm × 48 cm column of DEAE-cellulose (Biorad Celex-D, 0.66 mequiv/g) under N_2 at 2–4°. The solid curve represents protein absorbance at 280 nm; dotted lines indicate aconitate hydratase activity.

eluted with 1 mM citrate, whereas 98% of the fraction eluted by 5 mM citrate was still present at the end of the run.

Since the DEAE-cellulose column chromatography readily separated both isoenzymes from extracts of pig heart, effectivity of this procedure was further studied for the separation of aconitate hydratase isoenzymes in other tissues. A typical run, using an extract prepared from 80 g of liver, is shown in Fig. 2. The upper interrupted curve indicates the elution pattern of proteins, while the lower two peaks represent enzymatic activities. Similar elution patterns were noted upon chromatography of extracts prepared from renal cortex and heart except that much lower quantities of the second peak were obtained than in liver. In all cases, recoveries of enzyme activity placed on the column were almost quantitative (Table I). No significant improvement

TABLE I

THE RELATIVE DISTRIBUTIONS AND SPECIFIC ACTIVITIES OF THE MITOCHONDRIAL AND CYTOSOL ACONITASES FOUND BY DEAE-CELLULOSE CHROMATOGRAPHY OF EXTRACTS OF VARIOUS TISSUES OF PIG

Specific activities are calculated on the basis of the optical test for aconitate hydratase as discussed in METHODS. The millimolar absorbance (1-cm light path) of aconitate was taken as 3.41 measured at 240 nm (*cf.* ref. 3).

Tissue	*Specific activity (μmoles aconitate/mg protein per min)*			*% of total activity in tissues*	
	Homogenate extract	*Mitochondrial eluate*	*Cytosol eluate*	*Mitochondrial eluate*	*Cytosol eluate*
Heart	0.809	1.160	0.689	94.7	2.4
Liver	0.082	0.084	1.205	49.1	49.4
Kidney cortex	0.281	0.496	1.085	75.3	24.0

in the recoveries could be obtained by activation of the eluted fractions by Fe^{2+}–cysteine (see METHODS).

The subcellular origin of the two isoenzymes separated by column chromatography from extracts of whole tissues was established by re-isolation of enzymatically active protein fractions from cytoplasm and mitochondria, obtained by differential centrifugation[11]. The active fraction isolated by chromatography of mitochondrial extracts corresponded to the aconitate hydratase of whole tissues eluted with 1 mM citrate. About 96% of enzymatic activity, applied to the column was recovered. This was in sharp contrast to the poor recovery of this enzyme after electrofocusing. Aconitate hydratase of the cytosol was eluted with 5 mM citrate, with an apparent recovery of 101%. It was of interest that cytoplasmic and mitochondrial malate dehydrogenase isoenzymes (EC 1.1.1.37), identified by kinetic analyses[12,13] appeared in the same protein peaks where cytoplasmic and mitochondrial aconitate hydratase isoenzymes were found, respectively. In separate experiments, the isoelectric point of cytoplasmic malate dehydrogenase of liver was found at pH 5.05 and the value was pH 10.0 for the mitochondrial isoenzyme.

Characteristic differences in the stability of chromatographically obtained preparations of mitochondrial and cytoplasmic aconitate hydratase isoenzymes are illustrated in Fig. 3. Storage in an atmosphere of nitrogen appreciably stabilized both enzymes. Under all conditions studied the mitochondrial enzyme was significantly less stable than was the cytoplasmic isoenzyme. The marked instability of the mitochondrial enzyme in the presence of mercaptoethanol was particularly conspicuous. Ascorbate, a frequently used reagent for the reactivation of aconitate hydratase, caused a decay in enzymatic activity for both enzymes.

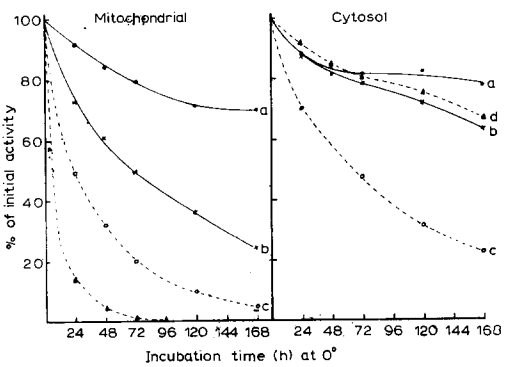

Fig. 3. The relative stabilities of mitochondrial and cytosol aconitic hydratases when stored under various conditions at 0°. The mitochondrial enzyme was a 50-fold concentrate of enzyme preparation obtained by DEAE-cellulose chromatography of heart extract. Cytosol aconitate hydratase was obtained through column chromatography of a pig kidney extract by a procedure identical to that used for pig liver (see Fig. 2). The enzyme was concentrated approx. 10-fold before use, and 5 mM citrate buffer was exchanged for 1 mM citrate buffer on a column of Biogel P-6 prior to the study. The initial enzyme activity in each test system was approx. 1.75 μmoles of aconitate produced/min per ml of enzyme preparation. All activities were assayed in 1 ml of 30 mM citrate (pH 7.5 with Tris) at 25° by following the change in absorbance at 240 nm following addition of 20 μl of enzyme preparation. a, stored in 1 mM citrate (pH 7.5 at 25 with Tris) under N_2; b, stored in the same buffer as in a but under air; c, stored in 1 mM citrate–10 mM ascorbate (pH 7.5 at 25° with Tris) under air; d, stored in 1 mM citrate–5 mM mercaptoethanol (pH 7.5 at 25° with Tris) under air.

The relative distribution of isoenzymic fractions of aconitate hydratase in various pig tissues is summarized in Table I. It is apparent that heart contains predominantly mitochondrial enzyme while the ratio of cytoplasmic to mitochondrial isoenzymes varies in liver and kidney. Although activities were determined after combining chromatographic fractions (Fig. 2), this step alone yielded an average of 15-fold increase in specific activity of the cytoplasmic enzyme of the liver (compare second and third column of Table I).

DISCUSSION

The most significant aspect of the studies reported here is that aconitate hydratase, at least in pig tissues, was found to be associated with two protein fractions of widely different p*I* (pH 5.43 and 7.50) and stability. It is apparent that available information concerning properties of this enzyme may be composed of a fortuitous combination of characteristics of both isoenzymes. Due to its greater stability, in most cases the cytoplasmic enzyme has probably been purified by previous investigators. MORRISON[2] reported that his purified enzyme retained its activity for many weeks when stored in the frozen state at pH 5.7. Our initial studies indicated lability of mitochondrial enzyme when it was stored at 0°, at pH 5.9. The cytosol enzyme seemed to retain stability at this lower pH and it was equally stable at higher pH when stored in the presence of citrate. As the result of these studies, it did not seem profitable to employ weakly acidic conditions, previously used by other workers, during initial extraction of aconitate hydratase from tissues. The chromatographic method for initial separation of isoenzymes yields protein fractions dissolved in buffers of relatively low ionic strength. This is advantageous for further purification, presently pursued, since HENSON AND CLELAND[3] reported that storage of their enzyme preparation at high salt concentrations (100 mM KCl) resulted in decay of enzymatic activity.

Different subcellular distribution of aconitate hydratase isoenzyme in the tissues studied forecast the possibility of different metabolic roles of these enzymes in various tissues.

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