Biochimica et Biophysica Acta, 936 (1988) 10–19
Elsevier

BBA 42845

Isolation and properties of oxaloacetate keto–enol-tautomerases from bovine heart mitochondria

Yuliya O. Belikova, Victor I. Burov and Andrei D. Vinogradov

Department of Biochemistry, School of Biology, Moscow State University, Moscow (U.S.S.R.)

(Received 7 March 1988)

Key words: Oxaloacetate keto–enol tautomerase; Oxaloacetate keto–enol isomerase; (Bovine heart mitochondria)

Two highly purified proteins with quite different properties capable of oxaloacetate keto–enol-tautomerase activity (oxaloacetate keto–enol-isomerase, EC 5.3.2.2) were isolated from the bovine heart mitochondrial matrix. The first protein has an apparent molecular mass of 37 kDa as determined by SDS-gel electrophoresis and Sephacryl SF-200 gel filtration. It is quite stable upon storage at 40 °C and reaches the maximal catalytic activity at pH 8.5 with a half-maximal activity at pH 7.0. The enzyme is specifically inhibited by oxalate and diethyloxaloacetate. When assayed in the enol → ketone direction at 25 °C (pH 9.0), the enzyme obeys a simple substrate saturation kinetics with K_m and V_{max} values of 45 μM and 74 units per mg of protein, respectively; the latter value corresponds to the turnover number of 2700 min^{-1}. The second protein has an apparent molecular mass of 80 kDa as determined by SDS-gel electrophoresis and Sephacryl SF-300 gel filtration. The enzyme is rapidly inactivated at 40 °C and shows a sharp pH optimum of activity at pH 9.0. The enzyme can be completely protected from thermal inactivation by oxaloacetate and dithiothreitol. The kinetic parameters of the enzyme as assayed in the enol → ketone direction at 25 °C (pH 9.0) are: K_m = 220 μM and V_{max} = 20 units per mg of protein; the latter corresponds to the turnover number of 1600 min^{-1}. The enzyme activity is specifically inhibited by maleate and pyrophosphate. About 30% of the total oxaloacetate tautomerase activity in crude mitochondrial matrix is represented by the 37 kDa enzyme and about 70% by the 80 kDa protein.

Introduction

Oxaloacetate is an essential substrate and product of many enzymatic reactions, including those located in the mitochondrial matrix. In aqueous solutions at neutral pH, oxaloacetate exists as a mixture of several species, predominantly dianions of keto and enol isomers [1–3]. The tautomeric interconversion between these two species is the subject of a general acid-base catalysis [2–4], and under certain conditions the rate of tautomerization can be much lower than the oxaloacetate turnover in the enzyme-catalyzed reactions. A search for a unique enzyme which would specifically accelerate oxaloacetate isomerization has led Annett and Kosicki [5] to the discovery of the oxaloacetate tautomerase activity (oxaloacetate keto–enol-isomerase, EC 5.3.2.2.) which is widely distributed in mammalian and avian organs, plants and bacteria [6]. The enzyme has been partially purified from porcine kidneys, and some properties of the 56-fold purified protein have been studied [5]. Quite recently it has been reported that partially purified porcine kidney enzyme catalyzes the nonstereospecific incorporation of

Correspondence: A.D. Vinogradov, Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, U.S.S.R.

the solvent protons in C3 prochiral position of keto-oxaloacetate [7].

The search for the physiological function of the enzyme was so far unsuccessful [7] apparently because all the known enzymes involved in oxaloacetate metabolism utilize (or release) the keto isomer as the substrate (product) of the enzymatic reactions.

Our interest in oxaloacetate tautomerase was dual. Firstly, the enzyme catalyzes one of the most simple chemical transformations in the one-substrate–one-product reaction. The equilibrium of the tautomerization reaction is not too far from unity, thus the reaction can easily be traced in both directions. Contrary to most of enzymatic processes, the kinetics and thermodynamics of the spontaneous reaction can be thoroughly studied under various conditions [1–5]. These features make oxaloacetate tautomerase an ideal object for a general investigation of the mechanism and control of the enzyme catalysis.

Secondly, we have recently described the malate dehydrogenase activity of succinate dehydrogenase (succinate : (acceptor) oxidoreductase, EC 1.3.99.1), the first reaction (to our knowledge) which produces the enol isomer of oxaloacetate [8,9]. An importance of the oxaloacetate tautomerization in the control of succinate dehydrogenase operating in conjunction with the other enzymes of the mitochondrial matrix thus becomes evident. In view of our long-standing interest in the operation of succinate dehydrogenase [10], we started a project on the study of a functional link between succinate dehydrogenase and oxaloacetate tautomerase. In this paper we will show that the mitochondrial matrix contains two individual proteins capable of oxaloacetate tautomerase activity. Some molecular and catalytic properties of these enzymes will be described. A preliminary account of this work has been published elsewhere [8,10].

Materials and Methods

Starting materials for enzyme purification

Bovine heart mitochondria were prepared by the conventional procedure [11] and used without further separation of heavy and light fractions. The fraction which is referred to as matrix was prepared as follows: 75 ml of mitochondrial suspension (50 mg of protein/ml) stored at −20 °C in 0.25 M sucrose were thawed at room temperature and diluted with an equal volume of a cold (0 °C) mixture containing 50 mM potassium phosphate and 2 mM EDTA (pH 8.1). The mixture was homogenized and treated by ultrasonic irradiation (MSE Soniprep 150) at a maximal output power and constant cooling twice during 1.5 min with a 2 min interval. The suspension was centrifuged for 15 min at 10000 rpm in a J-20 rotor of a Spinco Model L centrifuge. The supernatant (125 ml) was diluted 1.5-times with 50 mM potassium phosphate/0.2 mM EDTA (pH 8.1) and centrifuged for 1 h at 105000 × *g*. The clear supernatant (matrix) was collected and used for further purification. Rat liver mitochondria were prepared according to Johnson and Lardy [12] and used for subfractionation exactly as described [13].

Assay systems for oxaloacetate keto–enol-tautomerase

Since keto–enol-tautomerization is readily reversible, the enzyme activity was measured in both directions, either in coupled malate dehydrogenase assay [5], or directly recording enol absorption at 260 nm ($\varepsilon_{260}^{mM} = 11$ [1]).

(i) Coupled assay (enol → ketone). Solid oxaloacetic acid was dissolved in dry diethyl ether to give a final concentration of 20 mM. A standard spectrophotometric cuvette containing 2 ml of a mixture comprising 2 mM Tris/Cl^-, 0.2 mM EDTA, 0.2 mM NAD · H and malate dehydrogenase (EC 1.1.1.37) was placed in the cuvette compartment of a Hitachi 200-20 spectrophotometer. The pH of the mixture was adjusted so as to give the value of 9.0 after addition of oxaloacetic acid. 5 µl of an ether oxaloacetic acid solution were rapidly added with the aid of a Hamilton syringe. The initial instant decrease of absorption corresponding to the keto isomer content in a diethyl ether solution following the slow change in absorption (spontaneous tautomerization) were recorded; the proper amount of the enzyme preparation was then added, and the rate of the enzymatic ketonization was measured as the difference between the rates with and without the enzyme added.

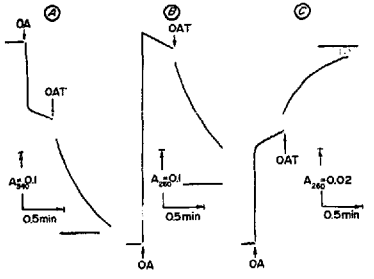

Fig. 1. The registration of the spontaneous and enzyme-catalyzed interconversion of enol and keto forms of oxaloacetate. The addition of oxaloacetate (OA) and oxaloacetate keto-enol tautomerase (oxaloacetate tautomerase, 1.1 μg/ml) are indicated by the arrows. (A) Coupled malate dehydrogenase assay (enol → ketone); (B) direct enol → ketone assay; (C) direct ketone → enol assay. The horizontal lines indicate the final absorption level reached after the reactions have been completed. See Materials and Methods section for the details.

A representative example of the assay procedure is shown in Fig. 1A.

(ii) Direct assay (enol → ketone). This was measured as described above, except that NAD · H and malate dehydrogenase were excluded. An instant increase of absorption after addition of oxaloacetic acid corresponding to the enol isomer content in diethyl ether solution was followed by its slow decrease (spontaneous ketonization) which was accelerated after addition of the enzyme preparation. A representative example of the assay is shown in Fig. 1B.

(iii) Direction assay (ketone → enol). The freshly prepared 20 mM solution of oxaloacetic acid in water was added to the mixture comprising 2 mM Tris-HCl, and 0.2 mM EDTA (pH was adjusted to give a value of 9.0 after addition of oxaloacetic acid). An instant increase in absorption corresponding to the enol isomer content in aqueous acid solutions was followed by slow spontaneous enolization which was accelerated after addition of the enzyme preparation. A representative example of the assay is shown in Fig. 1C.

It was shown that after the equilibrium has established, an addition of the enzyme preparation caused no changes in absorption either in coupled or in direct assay systems.

Assay for phenylpyruvate tautomerase (EC 5.3.2.1)

This was performed according to the published procedure [14].

Determination of the enol and keto isomers content of oxaloacetate

The relative content of enol and ketone in different solvents was measured as described above in a coupled malate dehydrogenase assay. The following values were obtained:

Solvent	Ketone (%)	Enol (%)
Dry acetone	35	65
Dry diethyl ether	33	67
Aqueous solution (pH 2.0)	93.5	6.5
Aqueous solution (pH 9.0)	87	13

Analytical methods

Amino acid analysis was performed with Hitachi 835 automatic amino acid analyzer. Protein samples were hydrolyzed in 6 M HCl at 110 °C for 24 h. Prior to hydrolysis the sample tubes were evacuated and sealed.

SDS-(10%)acrylamide gel electrophoresis was performed on slabs (12 × 12 cm) according to Laemmli [15]. Protein bands were stained with Coomassie Brilliant Blue R-250 for 30 min.

The molecular masses of native purified proteins were determined by Sephacryl SF-200 and Sephacryl SF-300 gel chromatography as described in the legend to Fig. 4.

Protein content was determined by a biuret procedure (mitochondria, submitochondrial particles, matrix protein) [16] or with Coomassie brilliant blue G-250 [17].

Hydroxyapatite was prepared according to Tiselius et al. [18].

Other methods and chemical used are described in the accompanying paper.

Results

Purification of the enzymes

All the operation procedures were carried out at 0–4 °C. Solid ammonium sulphate was slowly

added to the preparation designated as matrix (see Materials and Methods) up to 35% salt saturation. pH was controlled and maintained at 7.8–8.0 by addition of concentrated ammonium hydroxide. The mixture was stirred for 25 min and centrifuged for 15 min at 10000 rpm (J-21 rotor). The precipitate was discarded, and the supernatant was adjusted up to 70% saturation by adding solid ammonium sulphate. The sediment was collected, thoroughly suspended in 80% saturated ammonium sulphate containing 5 mM potassium phosphate, 0.2 mM EDTA and 5 mM dithioerythritol (pH 7.8) and stored overnight at 0 °C. The suspension was dialyzed against 1 litre of the same buffer containing no ammonium sulphate for 12 h with two changes of buffer, and finally for 2.5 h against 0.2 mM potassium phosphate, 0.2 mM EDTA and 1 mM dithiothreitol (pH 7.8). The cloudy material was centrifuged for 15 min at 10000 rpm (J-20 rotor) and the supernatant was further purified. At this stage the preparation had the specific activity (measured under suboptimal conditions) of 0.4 units per mg of protein.

The protein solution was applied on a column (2.5 × 5 cm) of hydroxyapatite (the total volume of the sorbent was approx. 20 ml) and the column was washed with 200 ml of the buffer used for final dialysis. The proteins were eluted stepwise with increasing concentrations of potassium phosphate buffer as shown in Fig. 2.

The fractions containing an active protein (designated as oxaloacetate tautomerase-1, Fig. 2) were collected, concentrated in a high-pressure filtration device and stored in liquid nitrogen. The protein eluted in oxaloacetate tautomerase-1 peak had the specific activity of 50 units per mg of protein and appeared in SDS-gel electrophoresis as a single protein band. Occasionally, depending on hydroxyapatite batches the oxaloacetate tautomerase-1 fraction contained some minor bands seen after SDS-gel electrophoresis. Such preparations were further purified as follows. Concentrated oxaloacetate tautomerase-1 was dialyzed against 2 mM potassium phosphate/0.2 mM EDTA (pH 7.1) and applied on a small (1 ml) column of DEAE-cellulose equilibrated with the same buffer. The elution was performed stepwise, using 2 ml of 5, 10, 20 and 40 mM potassium phosphate (pH 7.1) containing 0.2 mM EDTA. The fraction eluted with 10 mM potassium phosphate was collected and stored in liquid nitrogen. This additional purification step did not increase the specific activity of oxaloacetate tautomerase-1.

When the hydroxyapatite column was further eluted with potassium phosphate gradient as

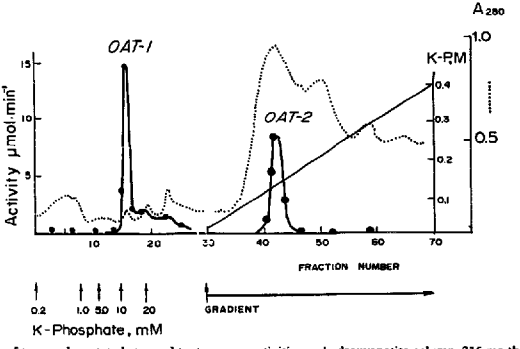

Fig. 2. The separation of two oxaloacetate keto-enol tautomerase activities on hydroxyapatite column. 215 mg the protein (35–70% of ammonium sulphate saturation) were applied and eluted as described in text. The rate of elution was 36 ml/h; the volume of each fraction was 9 ml.

shown in Fig. 2, an additional peak of activity (oxaloacetate tautomerase-2) appeared. The fractions containing an active protein were combined, concentrated in a high pressure filtration device and dialyzed against 200 ml of a buffer containing 5 mM potassium phosphate, 0.2 mM EDTA, 1 mM dithiothreitol (pH 7.2) for 3 h with two changes of the buffer. The dialyzed protein (6 mg) was applied on a small (1 ml) phosphocellulose column (0.7 × 2.4 cm) equilibrated with the buffer used for dialysis. The protein was eluted stepwise with 1.6 ml of the increasing concentrations of potassium phosphate/EDTA/dithiothreitol buffer (pH 7.2). The fraction eluted with 40 mM potassium phosphate was collected and stored in liquid nitrogen. Oxaloacetate tautomerase-2 thus prepared appeared as a single protein band after SDS-gel electrophoresis.

Composition and molecular properties of the enzymes

When subjected to SDS-gel electrophoresis both oxaloacetate tautomerase-1 and oxaloacetate tautomerase-2 purified as described above appeared as single protein bands with apparent molecular masses of 37 and 80 kDa, respectively

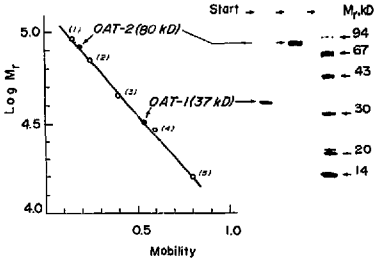

Fig. 3. The SDS-acrylamide gel electrophoresis of two purified proteins catalyzing oxaloacetate keto–enol-tautomerase reaction. The closed circles indicate the positions of two proteins on the calibration semi-log plot. The open circles (Standard Pharmacia LMW protein set, 2.5 μg of each): 1, rabbit muscle phosphorylase B (94 kDa); 2, bovine serum albumin (67 kDa); 3, egg white albumin (43 kDa); 4, bovine erythrocyte carbonic anhydrase (30 kDa); 5, soybean trypsin inhibitor (20 kDa). 3 and 6 μg of tautomerase 1 and 2, respectively, were applied on the gel.

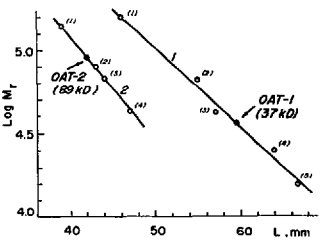

Fig. 4. Gel filtration of OAT-1 (oxaloacetate tautomerase-1) and OAT-2 (oxaloacetate tautomerase-2) on Sephacryl. The column (0.6×80 cm) was packed with Sephacryl SF-200 (line 1) or Sephacryl SF-300 (line 2) equilibrated by 0.1 M KCl, 10 mM potassium phosphate, 0.2 mM EDTA (pH 7.8) and 0.6 mg of oxaloacetate tautomerase-1 and 0.4 mg of oxaloacetate tautomerase-2 were applied and passed through the column at the flow rate of 6 ml per h. The position of oxaloacetate tautomerase-1 peak was detected by the activity measurement. The position of oxaloacetate tautomerase-2 peak and those of the standards used for calibration were detected by ultraviolet absorption measurement. Line 1: (1), aldolase (160 kDa); (2), bovine serum albumin (67 kDa); (3), egg white albumin (43 kDa); (4), chymotrypsinogen (25 kDa); (5), myoglobin (18 kDa). The filtration was performed at 22°C. Line 2: (1), lactate dehydrogenase (140 kDa); (2), creatine kinase (82 kDa); (3), bovine serum albumin (67 kDa); (4), egg white albumin (43 kDa). The filtration was performed at 4°C. L indicated on abscissa corresponds to the length of a recorder chart from the moment of a protein application to its maximum appearance.

(Fig. 3). These values are in good agreement with those estimated from the relative mobility of the proteins on Sephacryl columns (Fig. 4). A comparison of the data obtained using two methods suggests no oligomeric structure of the proteins at least in the diluted solutions. The amino acid composition of the two proteins is listed in Table I. No unusual amino acid composition is seen; both proteins are expected to be quite hydrophilic and negatively charged at neutral pH, although significant differences between oxaloacetate tautomerase-1 and oxaloacetate tautomerase-2 in the content of some amino acids (Val, Ile and Tyr) are evident.

Stability

Oxaloacetate tautomerase-1 was found to be quite stable when stored at 0°C for several days.

TABLE I

AMINO ACID COMPOSITION OF OXALOACETATE TAUTOMERASE-1 AND OXALOACETATE TAUTOMERASE-2

OAT, oxaloacetate tautomerase.

Amino acid	Mol %		Residue/protein (mol/mol)	
	OAT-1	OAT-2	OAT-1	OAT-2
Lys	5.8	6.6	17.0	41.7
His	2.1	2.9	6.1	18.2
Arg	4.3	4.4	12.8	27.8
Asp	10.4	10.9	30.8	68.7
Thr	6.5	5.9	19.2	37.2
Ser	6.4	5.4	18.9	33.9
Glu	11.3	11.0	33.4	69.5
Pro	7.9	9.3	23.4	58.6
Gly	10.4	10.0	30.8	63.4
Ala	8.6	8.7	25.4	54.7
Cys	0.7	0.9	2.1	6.0
Val	7.0	4.4	20.7	28.0
Met	0.9	1.0	2.8	6.1
Ile	2.8	4.4	8.4	27.9
Leu	9.0	8.6	26.7	54.5
Tyr	1.8	2.6	5.3	16.1
Phe	3.9	2.9	11.6	18.5

It resists freezing and thawing when dissolved in potassium phosphate buffer (pH 7.8). Oxaloacetate tautomerase-2 was much less stable. It gradually lost the activity within several days of storage at 0 °C and was rapidly inactivated at 40 °C (Fig. 5). The inactivation was completely prevented by dithiothreitol, oxaloacetate and partially prevented by cyanide. After inactivation had been completed, dithiothreitol was unable to restore the enzyme activity.

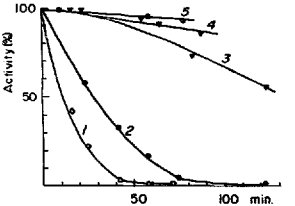

Fig. 5. Stability of oxaloacetate tautomerase-1 and oxaloacetate tautomerase-2. The proteins were incubated at 40 °C for the time intervals indicated on abscissa in 50 mM potassium phosphate, 0.2 mM EDTA (pH 7.8). The residual activities were measured in the coupled malate dehydrogenase assay. Curves 1, 2, 3 and 5, oxaloacetate tautomerase-2; curve 4, oxaloacetate tautomerase-1. 1 and 4, no other additions; 2, 2 mM neutralized potassium cyanide was added; 3, 5 mM oxaloacetate was added; 5, 5 mM dithiothreitol was added.

Catalytic properties

Both enzymes obeyed the simple Michaelis-Menten kinetics when assayed in the enol → ketone coupled malate dehydrogenase test. An inhibition of the oxaloacetate tautomerase-2 activity by the substrate was observed when the enzyme activity was measured in the direct ketone → enol assay.

TABLE II

THE KINETIC PARAMETERS OF THE OXALOACETATE KETO–ENOL-TAUTOMERASE REACTION CATALYZED BY THE PURIFIED PROTEINS

25 °C, pH 9.0, 2 mM Tris/Cl^- buffer. OAT, oxaloacetate tautomerase.

Reaction	OAT-1			OAT-2			Spontaneous reaction first-order rate constant (min^{-1})
	K_m [c] (μM)	V_{max} (μmol·min^{-1}·mg^{-1})	turnover number [d] (min^{-1})	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})	turnover number [d] (min^{-1})	
Enol → ketone [a]	45	74	2740	220	20	1600	0.24
Ketone → enol [b]	68	5.8	215	– [e]	– [e]	– [e]	0.04

[a] Determined in the coupled malate dehydrogenase assay.

[b] Determined in the direct assay.

[c] These values are calculated for the true substrate enol or ketone.

[d] Calculated assuming the presence of a single catalytic site per mole of protein.

[e] These values were not determined, since inhibition by the substrate was observed.

The kinetic parameters of the forward and reverse reactions are summarized in Table II. It should be noted that only the values determined in the coupled malate dehydrogenase assay are definitly reliable, since no conditions can be reached where 100% of either ketone or enol are present in the direct initial rate determination assay. Conceivably, both isomers can mutually compete for the enzyme active site and the reversibility of the overall reaction should also be taken into consideration. Nevertheless, some provisional values of the turnover numbers for the two proteins calculated on the basis of their molecular masses are given in Table II. A comparison of the first-order reaction rate constants for the forward and reverse spontaneous reactions with the corresponding turnover numbers gave an approximate characteristics of the catalytic efficiency of the two proteins.

The pH profiles for the activity of oxaloacetate tautomerase-1 and oxaloacetate tautomerase-2 as compared to that for spontaneous tautomerization are shown in Fig. 6. The shape of the curve for oxaloacetate tautomerase-1 suggests that the ionization of a single group with an apparent pK close to 7.0 is responsible for the activity. A bell-shaped dependence with a relatively sharp maximum at pH 9.0 was obtained for oxaloacetate tautomerase-2. As expected for general acid-base catalysis, an inverse bell-shaped curve with a minimum

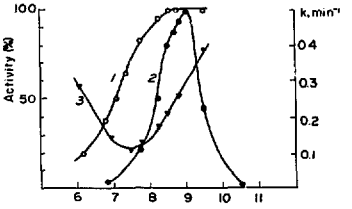

Fig. 6. pH-dependence of oxaloacetate keto–enol-tautomerase activities. The initial rates of tautomeric interconversion of 64 μM oxaloacetate were measured in the coupled malate dehydrogenase assay at different pH in 2 mM tris/Cl^- buffer containing 0.2 mM EDTA. 1, oxaloacetate tautomerase-1; 2, oxaloacetate tautomerase-2; 3, spontaneous tautomerization (first-order rate constant; right ordinate).

TABLE III

SOME INHIBITORS OF THE OXALOACETATE KETO–ENOL-TAUTOMERASES

25 °C, pH 9.0, 2 mM Tris/Cl^- buffer. The enzyme activity was assayed in the coupled malate dehydrogenase assay with 50 μM oxaloacetate. No detailed kinetics of inhibition was studied. The – denotes that 5 mM inhibitor had no detectable effect on the reaction.

Inhibitor	I_{50} (μM)	
	OAT-1	OAT-2
Maleate	–	50
Oxalate	4	–
Diethylester of oxaloacetate	4	–
Phosphoenolpyruvate	–	60
Phenylpyruvate	200	–
Pyrophosphate	–	20
Malonate	–	600

at neutral pH was observed in the case of spontaneous tautomerization.

A number of the compounds which more or less resemble the structure of the substrate were tested as the inhibitors of the enzymes (Table III). The kinetic mechanism of inhibition of the compounds listed was not studied. The clear-cut difference in the sensitivity of the two enzymes to a number of inhibitors is evident. This is especially true for maleate which is a potent inhibitor of oxaloacetate tautomerase-2, being inert to oxaloacetate tautomerase-1 and for oxalate which exhibits an opposite behavior.

Localization and relative content of oxaloacetate tautomerase-1 and oxaloacetate tautomerase-2 in mitochondria

Since no methods for the clearcut separation of different compartments of heart mitochondria are available, the intramitochondrial distribution of oxaloacetate keto–enol-tautomerase was studied, using intact rat liver mitochondria as starting material. We found that rat liver mitochondria solubilized in 0.1% Triton X-100 catalyze the oxaloacetate tautomerization reaction (enol → ketone, 25 °C, coupled malate dehydrogenase assay, 50 μM enol oxaloacetate) at a rate of 0.2 μmol/min per mg of protein. The mitochondria were fractionated and the malate dehydrogenase (EC 1.1.1.37), adenylate kinase (EC 2.7.4.3) and

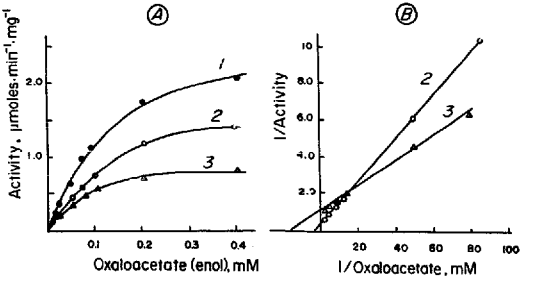

Fig. 7. The kinetics of oxaloacetate keto–enol-tautomerase reaction catalyzed by crude fraction of matrix proteins. The dialyzed 35–70% of ammonium sulphate saturation fraction was used as an enzyme. The total concentrations of oxaloacetate added from dry diethyl ether solution are indicated on abscissa. (A) Curve 1, the total reaction; curve 2, 200 μM oxalate was added; curve 3, 500 μM maleate was added. (B) 2 and 3, the linear anamorphoses of the curves 2 and 3 shown in (A), respectively.

succinate dehydrogenase (EC 1.3.99.1) activities were measured by conventional procedures as markers for matrix, intermembrane space and inner membranes, respectively. It was shown that all the oxaloacetate keto–enol-tautomerase activity was quantitatively associated with the matrix fraction.

It seemed of interest to quantitate the relative activities of oxaloacetate tautomerase-1 and oxaloacetate tautomerase-2 in the mitochondrial matrix. For this purpose the kinetics of the oxaloacetate keto–enol-tautomerase activity of the bovine heart mitochondrial matrix were studied, using the specific inhibitors, maleate (for oxaloacetate tautomerase-2) and oxalate (for oxaloacetate tautomerase-1) (see Table III). As shown in Fig. 7A (curve 1), the reaction catalyzed by the crude matrix fraction does not appear as a simple hyperbole, and at any concentration of the substrate the overall enzymatic reaction is only partly inhibited by maleate and oxalate. When the activities measured in the presence of either maleate or oxalate were presented as double-reciprocal plots, they both gave straight lines, thus indicating simple Michaelis-Menten kinetics. The K_m values for the enol determined from the plot shown in Fig. 7B are 50 and 220 μM; these values are in excellent agreement with those determined for purified oxaloacetate tautomerase-1 and oxaloacetate tautomerase-2, respectively (see Table I). Taking into account the V_{max} values of 2.0 and 0.8 μmol/min per mg of the total matrix protein determined from the double-reciprocal plots, it can be concluded that about 30% of the total tautomerase activity in the matrix are represented by oxaloacetate tautomerase-1 and about 70% by oxaloacetate tautomerase-2.

Discussion

Oxaloacetate keto–enol-tautomerase activity originally described by Annett and Kosicki [5] is widely distributed among animal tissues, plants and microorganisms. The tautomerase activity was found in all compartments of rat liver and pig kidney cells [6]. The purification procedure originally described by Annett and Kosicki for porcine kidney extract includes heat and acetone treatments, ammonium sulphate fractionation and dialysis followed by column chromatography and results in a 56-fold purified protein with the highest specific activity of about 5 units/mg [5]. This procedure has recently been modified [7] to increase the specific activity up to 11.5 units/mg. The problem of intracellular localization of the partially purified protein is still obscure, although the existence of several enzymes capable of the oxaloacetate keto–enol-tautomerase activity may

be proposed, taking into account the data on intracellular distribution of the enzyme activity [6].

In this report for the first time some properties of two well-defined SDS-electrophoretically homogeneous proteins from bovine heart mitochondrial matrix capable of the oxaloacetate tautomerase activity have been described. Both proteins are quite different from oxaloacetate tautomerase partially purified from porcine kidney extract [5]. Johnson et al. [7] have reported the molecular mass of their preparation which is evidently identical to the oxaloacetate tautomerase originally described by Annett and Kosicki [5] to be about 55 kDa as determined by gel filtration chromatography using a Sephadex G-200 column. Our values for low and high molecular mass enzymes as determined by either Sephacryl gel filtration or SDS gel electrophoresis are approx. 37 and approx. 80 kDa, respectively. The differences between the molecular masses of all the three proteins seem to be far off to be explained by different methods employed in two laboratories. Thus, it is judicious to consider the two proteins described in this report as new mitochondrial oxaloacetate keto–enol-tautomerases. All the properties of these two enzymes studied so far, namely, their molecular masses, stabilities, sensitivities to the specific inhibitors, pH optima, kinetic parameters, are quite different; therefore the 37 kDa protein cannot be considered as proteolytic product of the 80 kDa protein. Moreover, as was revealed by the kinetics of oxaloacetate tautomerism catalyzed by the crude fraction, both proteins are present in the original mitochondrial matrix.

The central question which still cannot be definitely answered is whether the proteins described in this paper are unique oxaloacetate keto–enol-tautomerases (EC 5.3.2.2) or the oxaloacetate tautomerase activities are the side or partial reactions of some others enzymes capable of specific binding of oxaloacetate. We found no oxaloacetate keto–enol-tautomerase activity in purified mitochondrial malate dehydrogenase (EC 1.1.1.37), succinate dehydrogenase (EC 1.3.99.1), glutamic acid-oxaloacetic acid transaminase (EC 2.6.1.1). These enzymes substantiate the list of those studied by Annett and Kosicki which includes citrate synthase (4.1.3.7), malate dehydrogenase, fumarase (4.2.1.2) and oxaloacetate decarboxylase (EC 4.1.1.3) from commercial sources [5]. Our preparations do not catalyze the phenylpyruvate keto–enol-tautomerase reaction described by Knox and Pitt [19]. It should be emphasized that neither of the enzymes mentioned above is composed of the 37 or 80 kDa subunits. It thus seems very likely that the proteins described herein are unique oxaloacetate keto–enol-tautomerases. Whether the last statement is correct or not, the significant oxaloacetate keto–enol-tautomerase activity of mitochondrial matrix raises the question about its physiological function. There are several enzymes in the mitochondrial matrix which utilize or produce oxaloacetate as the substrate or product; all of them utilize the keto isomer of oxaloacetate (see Refs. 5 and 7 and the references cited therein). An attempt to demonstrate enol form of oxaloacetate as a species which is transported across the inner mitochondrial membrane has not been successful [7]. Since at physiological pH in an aqueous solution an equilibrium between keto and enol forms of oxaloacetate is significantly shifted towards the keto isomer (Refs. 1, 2, this paper), the possibility that oxaloacetate tautomerase may control the concentration of the active keto isomer buffered by the inactive enol form is hardly likely. Our recent finding that succinate dehydrogenase is able to oxidize malate and produce an enol isomer of oxaloacetate [9] seems to shed some light on the possible function of oxaloacetate tautomerase. Indeed, we have shown that oxaloacetate tautomerase couples the oxidation of malate by succinate dehydrogenase with the utilization of the oxaloacetate formed in transaminase reaction, thus protecting succinate dehydrogenase from suicide inhibition [8,9]. An important role of rapid enol → ketone isomerisation of oxaloacetate in the control of the Krebs cycle operation thus becomes evident.

It may be anticipated that the enol isomer of oxaloacetate is formed as a product of malate oxidation by the membrane-bound flavin containing malate dehydrogenase which is widely distributed in microorganisms (Refs. 20–22 and the references cited therein). This proposal seems to be likely in the light of an apparent similarity between microbial membrane-bound malate dehydrogenase [20–22] and mammalian succinate dehydrogenase [10]. The essential role of oxaloace-

tate keto–enol-tautomerase in the organisms containing membrane-bound malate dehydrogenase would be to couple an oxidation of malate with further metabolism of oxaloacetate. It may be speculated that during evolution such an essential role of oxaloacetate tautomerase in mitochondria has been lost and a new function of the enzyme, namely the control of the succinate dehydrogenase activity has been developed in eukaryotic cells.

Acknowledgements

We thank Dr. A.B. Kotlyar for his stimulating discussions during this research. We are indebted to Mrs. R.L. Birnova for her helpful advice during preparation of the manuscript. We are grateful to Dr. L.A. Baratova (A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University) for having performed an amino acid analysis.

References

1 Hess, J.L. and Reed, R.E. (1972) Arch. Biochem. Biophys. 153, 226–232.
2 Banks, B.E.C. (1962) J. Chem. Soc. N 1, 63–71.
3 Kokesh, F.C. (1976) J. Org. Chem. 41, 3593–3599.
4 Cocivera, M., Kokesh, F.C., Malatesta, V. and Zinck, J.J. (1977) J. Org. Chem. 42, 4076–4080.
5 Annett, R.G. and Kosicki, G.W. (1969) J. Biol. Chem. 244, 2059–2067.
6 Wesenberg, J.C., Chaudhari, A. and Annett, R.G. (1976) Can. J. Biochem. 54, 233–237.
7 Johnson, J.D., Creighton, D.J. and Lambert, M.R. (1986) J. Biol. Chem. 261, 4535–4541.
8 Kotlyar, A.B., Belikova, Yu.O. and Vinogradov, A.D. (1986) in 4th European Bioenergetics Conference Short Reports, Prague, p. 160, Congress Edition.
9 Belikova, Yu.O., Kotlyar, A.B. and Vinogradov, A.D. (1988) Biochim. Biophys. Acta 936, 1–9.
10 Vinogradov, A.D. (1986) Biochimiya (U.S.S.R.) 51, 1944–1973 (1663–1688 in English translation).
11 Crane, F.L., Glenn, J.L. and Green, D.E. (1956) Biochim. Biophys. Acta 22, 475–493.
12 Johnson, D. and Lardy, H. (1967) Methods Enzymol. 10, 94–96.
13 Schnaitman, C. and Greenwalt, J.W. (1968) J. Cell. Biol. 38, 158–175.
14 Constantsas, N.S. and Knox, W.E. (1966) Arch. Biochem. Biophys. 117, 59–64.
15 Laemmli, U.K. (1970) Nature 227, 680–685.
16 Gornall, A.D., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–756.
17 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
18 Tiselius, A., Hjerten, S. and Levin, O. (1956) Arch. Biochem. Biophys. 65, 132–155.
19 Knox, W.E. and Pitt, B.M. (1957) J. Biol. Chem. 225, 675–688.
20 Benziman, M. and Galanter, Y. (1964) J. Bacteriol. 88, 1010–1018.
21 Reddy, T.L., Murthy, P.S. and Venkitasubramanian, T.A. (1975) Biochim. Biophys. Acta 376, 210–218.
22 Imai, T. (1978) Biochim. Biophys. Acta 523, 37–46.