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Thermal inactivation, denaturation and aggregation of mitochondrial aspartate aminotransferase

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ABSTRACT

A comparative study of thermal denaturation and inactivation of aspartate aminotransferase from pig heart mitochondria (mAAT) has been carried out (10 mM Na phosphate buffer, pH 7.5). Analysis of the data on differential scanning calorimetry shows that thermal denaturation of mAAT follows the kinetics of irreversible reaction of the first order. The kinetics of thermal inactivation of mAAT follows the exponential law. It has been shown that the inactivation rate constant (k_{in}) is higher than the denaturation rate constant (k_{den}). The k_{in}/k_{den} ratio decreases from 28.8±0.1 to 1.30±0.09 as the temperature increases from 57.5 to 77 °C. The kinetic model explaining the discrepancy between the inactivation and denaturation rates has been proposed. The size of the protein aggregates formed at heating of mAAT at a constant rate (1 °C min^{-1}) has been characterized by dynamic light scattering.

1. Introduction

Aspartate aminotransferase (EC 2.6.1.1), a homodimeric, pyridoxal 5′-phosphate (PLP)-dependent enzyme, exists in two distinct forms in higher eukaryotes, one located in the cytosol and the other in the mitochondria [1]. The enzyme contains one molecule of PLP bound to each of the two identical active sites. A single polypeptide chain comprising each subunit folds into three distinct regions: an N-terminal extended arm that interacts with the neighboring subunit, a small domain that changes its conformation during catalysis and a large domain that contains most of the residues involved in cofactor binding [2]. The active site is adjacent to both the domain and the subunit interfaces [2]. It is formed by loops of polypeptide from the large and small domains of its own subunit and by a few residues from the large domain of the adjacent subunit. The coenzyme PLP is attached to the active site by a covalent link (Schiff base) to the side-chain of Lys258. The functionally independent active sites lie at the interface between the small and large domains on the opposite sides of the dimeric molecule [3]. Since each active site is composed of residues from both subunits, only dimeric enzyme shows catalytic activity. The monomeric form of the enzyme is catalytically inactive [4].

The dimeric cytosolic isoenzyme with molecular mass of 93.0± 2.8 kDa and dimeric mitochondrial isoenzyme with molecular mass of 91.2±2.7 kDa [5] are structurally related, with approximately 48% identity of amino acid sequence [6], but they exhibit considerable differences in stability to denaturing agents [7,8] and sensitivity to thermal denaturation [9]. The melting temperatures of the two isoenzymes at pH 7.5 as determined by differential scanning calorimetry (DSC) were 83 and 68 °C for the cytosolic and mitochondrial isoenzymes, respectively [9]. Mitochondrial aspartate aminotransferase (mAAT) is inactivated irreversibly at heating, the inactivation being a first-order process [10,11]. During heating aggregates of the enzyme are formed. Light scattering data at 360 nm have shown that the increase in turbidity is more rapid at higher temperature [10].

In the present work we have compared the rates of inactivation and denaturation of mAAT from pig heart mitochondria in the temperature interval from 50 to 77 °C. Denaturation of mAAT was studied by DSC. Inactivation and denaturation of mAAT proceed as irreversible reactions of the first order. The obtained results have shown that the active site reveals lesser stability in comparison with that of the protein globule as a whole. Thermal aggregation of mAAT has been studied by dynamic light scattering (DLS) under the regime, wherein the temperature was elevated at a rate of 1 °C min^{-1}. When comparing the increment in the light scattering intensity (I) and the portion of the denatured protein (γ_{den}), we observed that the normalized I values (I/I_{lim}; I_{lim} is the limiting value of I at $\gamma_{den}=1$) exceed the γ_{den} values.

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Abbreviations: DLS, dynamic light scattering; DSC, differential scanning calorimetry; mAAT, mitochondrial aspartate aminotransferase; PLP, pyridoxal 5′-phosphate.

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2. Materials and methods

2.1. Materials

L-aspartic acid, malate dehydrogenase (MDH), NADH and α-ketoglutaric acid were purchased from Sigma; CM-32 cellulose was from Whatman; DE-sepharose (fast flow) were from Pharmacia; Macro-Prep ceramic hydroxyapatite was from Bio-Rad; pyridoxal 5′-phosphate (PLP) and 2-mercaptoethanol were from Loba Chemie.

All solutions for the experiments were prepared using deionized water obtained with Easy-Pure II RF system (Barnstead, USA).

2.2. Purification of mAAT

The enzyme was purified according to Barra et al. [12] with several modifications. Freshly obtained pig hearts were minced and 1.5 kg of the material was homogenized in 1.8 L buffer A (10 mM K-phosphate buffer, pH 6.8, containing 10 mM glutaric acid, 5 μM PLP, 0.5 mM EDTA and 0.2 mM 2-mercaptoethanol). Insoluble material was collected by centrifugation at 2500 *g* and re-extracted with buffer A two times. The combined supernatants were dialyzed against buffer A. The dialyzed solution was clarified by centrifugation at 16900 *g* and then applied on the column (5×25 cm) of CM-cellulose equilibrated with 10 mM K-phosphate buffer, pH 6.8. The mitochondrial enzyme was eluted from the column using buffer A containing 0.25 mM NaCl. The active fractions were combined and dialyzed overnight against buffer A. The dialyzed solution was applied to a column (3.6×30 cm) of CM-cellulose equilibrated with 10 mM K-phosphate buffer, pH 6.8. The mAAT was eluted using a salt gradient with buffer A containing 10 mM NaCl (500 mL) in the mixing chamber and buffer A containing 125 mM NaCl (500 mL) in the reservoir. Flow rates of about 300 mL h^{-1} were used. The active fraction was dialyzed overnight against buffer B (10 mM K-phosphate buffer, pH 8.0, containing 1 mM α-ketoglutaric acid, 5 μM PLP and 0.2 mM 2-mercaptoethanol). Then the enzyme solution was applied on DE-sepharose column (2.5×25 cm) concatenated with the hydroxyapatite column (1.6×20 cm); both columns were equilibrated with buffer A. After washing with the same buffer columns were disconnected and mAAT was eluted from hydroxyapatite column in AKTA FPLC system under the following conditions: buffer B (10 mM K-phosphate buffer, pH 8.0, containing 1 mM α-ketoglutaric acid, 5 μM PLP and 0.2 mM 2-mercaptoaethanol), buffer C (0.5 M K-phosphate buffer, pH 8.0, containing 10 mM glutaric acid, 5 μM PLP and 0.2 mM 2-mercaptoaethanol); gradient slope was 10 column volumes of 10–50% buffer C. Fractions containing enzyme were pooled and concentrated. Application of DE-sepharose/hydroxyapatite in the final step of tandem chromatography allowed us to obtain homogeneous preparation of mAAT according to SDS-PAGE with activity not less than 229 ± 10 U mg^{-1}.

Protein concentration was determined according to Peterson [13] or from the absorbance at 280 nm using the extinction coefficient $A_{280}^{1\%}$ = 14.0 [12].

2.3. Assay of mAAT

mAAT activity was measured at 25 °C by coupling the enzyme reaction with malate dehydrogenase (MDH) and subsequent monitoring NADH oxidation at 340 nm on Cary 50 Bio UV/Visible spectrophotometer [14]. The reaction mixture (1 mL) contained 0.05 M K-phosphate buffer, pH 6.8, 20 mM α-ketoglutaric acid, 0.2 mM NADH, ~3 U ml^{-1} MDH and an appropriate amount of the sample. The reaction was started by the addition of L-aspartate to a final concentration of 10 mM. One unit of the enzyme activity was defined as the amount of the enzyme that is required for the formation of 1 μmol of the product per 1 min under the above conditions.

2.4. Calorimetric studies

Thermal denaturation of mAAT was studied by DSC. DSC experiments were performed using a DASM-4 M differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia). All measurements were carried out in 10 mM Na-phosphate, pH 7.5, with 0.5 mM EDTA. The protein solution was heated at a constant rate from 5 to 90 °C and at a constant pressure of 2.2 atm. The reversibility of the thermal transition of mAAT was tested by checking reproducibility of the calorimetric trace during the second heating of the sample immediately after cooling. The calorimetric traces of mAAT were corrected for instrumental background and possible aggregation artefacts by subtracting the scans obtained from the second heating of the samples. Calorimetric traces obtained at various scanning rates were corrected for the instrumental time-response according to Mayorga and Freire [15]. The temperature dependence of the excess heat capacity (C_p^{ex}) was further analyzed and plotted using Origin software (MicroCal Inc.).

We analyzed the temperature profiles of the excess heat capacity assuming that denaturation of mAAT proceeds as an irreversible reaction of the first order:

$$N \xrightarrow{k_{den}} D, \tag{1}$$

where N and D are the native and denatured form of the protein, respectively, and k_{den} is the denaturation rate constant.

To describe the dependence C_p^{ex} on temperature, we used the following equation system [16–18]:

$$\begin{cases} \dfrac{d\gamma_{nat}}{dT} = -\dfrac{k_{den}\gamma_{nat}}{v} \\ C_p^{ex} = Q_t \dfrac{k_{den}\gamma_{nat}}{v} \end{cases}, \tag{2}$$

where γ_{nat} is the portion of the native protein, T is the absolute temperature, v is the temperature scanning rate and Q_t is the total heat of denaturation. It is assumed that the temperature dependence of the k_{den} value (in min^{-1}) follows the Arrhenius equation:

$$k_{den} = \exp\left\{\frac{E_a^{den}}{R}\left(\frac{1}{T_1^{den}} - \frac{1}{T}\right)\right\}, \tag{3}$$

where E_a^{den} is the energy of activation, T_1^{den} is the temperature at which the rate constant k_{den} equals 1 min^{-1} and R is the gas constant.

2.5. Determination of portions of inactivated and aggregated mAAT

The enzyme was transferred into 10 mM Na-phosphate buffer, pH 7.5, with 0.5 mM EDTA by careful dialysis. Samples containing appropriate amounts of protein were incubated at a well-defined temperature in silicone-covered glass tubes. Each tube was removed at an appropriate time interval, immediately placed in the ice water bath and then centrifuged for 20 min at 20,000 *g*. Residual enzyme activity was measured at 25 °C as described above. Sample of the unheated protein served as a control. In addition to determination of the enzyme activity we measured the optical density (OD) of the supernatant at 280 nm. The portion of the aggregated protein (γ_{agg}) was calculated as $(1 - OD/OD_0)$, where OD_0 is the optical density of the initial solution.

2.6. DLS studies

For light scattering measurements a commercial instrument Photocor Complex was used (Photocor Instruments Inc., USA; www.photocor.com). A He–Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) was used as a light source. The temperature of sample cell was controlled by the proportional integral derivative (PID) temperature controller to within ± 0.1 °C. The quasi-cross