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Biophysical Chemistry 125 (2007) 254–259

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Two-state irreversible thermal denaturation of *Euphorbia characias* latex amine oxidase

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Received 11 May 2006; received in revised form 7 August 2006; accepted 17 August 2006
Available online 23 August 2006

Abstract

Thermal denaturation of *Euphorbia* latex amine oxidase (ELAO) has been studied by enzymatic activity, circular dichroism and differential scanning calorimetry. Thermal denaturation of ELAO is shown to be an irreversible process. Checking the validity of two-state it really describes satisfactorily the thermal denaturation of ELAO. Based on this model we obtain the activation energy, parameter T^* (the absolute temperature at which the rate constant of denaturation is equal to 1 min^{-1}), and total enthalpy of ELAO denaturation. HPLC experiments show that the thermal denatured enzyme conserves its dimeric state. The $N_2 \xrightarrow{k} D_2$ model for thermal denaturation of ELAO is proposed: where N_2 and D_2 are the native and denatured dimer, respectively.

Keywords: Copper containing amine oxidase; Differential scanning calorimetry; Kinetic models; Irreversible denaturation

1. Introduction

Cu/TPQ amine oxidases (AOs; E.C. 1.4.3.6) are a group of heterogeneous dimeric enzymes, each subunit containing one Cu (II) and one 2,4,5-trihydroxyphenyalanine quinone (TPQ) as cofactors. TPQ is formed from a posttranslational self-processing of a tyrosine residue [1] in the amino acid sequence. These enzymes catalyze the oxidative deamination of primary amines to the corresponding aldehydes, hydrogen peroxide, and ammonia.

AOs are widely distributed in nature, occurring in plants, microorganisms, and mammals [2]. Although the functional role of Cu/TPQ AOs has not been clearly determined, it has been shown that plasma level of amine oxidases varies in diabetes [3], heart failure [4], patients suffering from serious burns and solid tumors, pregnancy and age [5]. In microorganisms these enzymes have nutritional role using primary amines as a sole source of nitrogen or carbon. In plants AOs can play a role in regulating intercellular polyamine levels, morphogenesis [6], and mobilization of seed reserves [7]. The level of plant AOs changes upon auxin treatment [8], light stress [9], germination [10], anoxic and thermal stress [11], salt stress [12], and mechanical injury [13].

In spite of intensive physiological and pharmaceutical studies on Cu/TPQ AOs, there are very few literature surveys on the thermodynamics of this group of enzymes. Moosavi-Nejad et al. [14] reported that thermal denaturation of lentil seedling amine oxidase (LSAO) showed two main reversible peaks, the first broad while the second one relatively sharp. They also deconvoluted the second peak to three subpeaks and supposed that subpeaks belonged to three hypothetical structure domains for each subunit of LSAO.

Giartosio et al. [15] analyzed thermal denaturation of bovine serum oxidase (BSAO) by differential scanning calorimetry (DSC). These authors showed that the DSC profile of BSAO had three distinct peaks. The thermogram of BSAO was deconvoluted

Abbreviations: BSAO, bovine serum amine oxidase; DSC, Differential scanning calorimetry; ELAO, *Euphorbia latex* amine oxidase; PSAO, pea seedlings amine oxidase; TPQ, 2,4,5, trihydroxyphenylalanine quinone.

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doi:10.1016/j.bpc.2006.08.006

to four two-state independent transition albeit they did not mention whether this process was reversible or not. In a successive study the same authors deconvoluted the thermogram to five two-state transitions [16] explaining discrepancies rising from some protein impurity. Moreover they reported that transitions 1 and 5 showed reversibility.

The aim of the present work is to study the thermal denaturation of amine oxidase from *Euphorbia characias* latex (ELAO) by different methods and to propose a model of thermal denaturation of the protein.

2. Material and methods

2.1. Protein purification

ELAO was purified by the procedure described in [17]. Only protein of the highest quality was utilized on the basis of 2.0± 0.1 titratable TPQ/dimer.

An ε_{278} of 3.78×10^5 $M^{-1}cm^{-1}$ was used to determine the protein concentration [18].

2.2. Enzyme assay

ELAO activity was measured by the oxidation of guaiacol in the presence of hydrogen peroxide and horseradish peroxidase. The reaction mixture contained 100 mM potassium phosphate buffer, pH 7.0, 13 mM putrescine as a substrate, 0.1 U/ml horseradish peroxidase and 0.5 mM guaiacol in a final volume of 1 ml. The increase in absorbance at 470 nm was recorded using a Cary 100 UV–visible Spectrophotometer (Varian, Australia) at 27 °C. For studying the thermal denaturation of ELAO in terms of enzymatic activity, the *Euphorbia* enzyme (8×10^{-9} M) in 100 mM phosphate buffer, pH 7.0, was incubated for 20 min at desired temperature and then cooled to 27 °C to measure the activity. Reported activity values are the activities relative to native enzyme at 27 °C and are the mean of at least three different measurements.

2.3. Circular dichroism experiments

CD spectral measurements in far-UV regions were made on Aviv model 215 (USA) CD–spectropolarimeter equipped with a water-bath circulating system using 0.1-cm path length cuvettes. Protein concentration was typically 0.25 mg/ml.

2.4. Determination of aggregation state

ELAO was denatured by heating up to 90 °C and then cooled to 27 °C. For detection of aggregation, absorbance of native and denatured ELAO was recorded using Cary 100 UV–visible Spectrophotometer in the range 200–500 nm at 27 °C.

2.5. Calorimetric study

Calorimetric studies were carried out by a Scal-1 differential scanning microcalorimeter (Russia) equipped with 0.355 ml capillary glass cells. All DSC experiments were done under 2 atm pressure. The concentrations of ELAO were 0.5–4.0 mg/ml. The experiments were performed at scan rates 0.125, 0.25, 0.475, and 1 °C/min.

2.6. Data analyses

Irreversible protein denaturation is thought to have at least two steps:

a) reversible unfolding of the native protein,
b) irreversible alteration of the unfolded protein to final state that is unable to fold back to native state.

This model is known as a Lumry–Eyring model and can be shown as:

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k} F \qquad \text{(SchemeI)}$$

where N, U, and F indicate native, unfolded, and final state of the protein; $\underline{k}_1$, $\underline{k}_{-1}$, and $\underline{k}$ are the rate constants for reversible denaturation, renaturation, and irreversible denaturation stage, respectively. If $\underline{k}>\underline{k}_{-1}$, most of the molecules are converted to the final state and the concentration of U is very low; thus, the equilibrium between U and N cannot be established. In this case the rate-limiting step is unfolding and the formation of F is determined by first order rate constant, $\underline{k}$:

$$N \xrightarrow{k} F \qquad \text{(SchemeII)}$$

There is another case in which the Lumry–Eyring model can be diminished to two-state model if $\underline{K}=\underline{k}_1/\underline{k}_{-1}<1$ and in addition $\underline{k}_{-1}>\underline{k}$. The amount of U is very low and the rate of formation of F can be determined by an apparent first order rate constant equal to $\underline{K}\underline{k}$. Thus, two-state irreversible model is a limiting case of the Lumry–Eyring model.

Sanchez-Ruiz et al. [19] proposed different methods for estimation of the energy of activation relative for two-state model:

A) The first order rate constant k at given temperature T is:

$$k = vC_p^{ex}/(Q_t - Q) \qquad (1)$$

where v is the scanning rate, C_p^{ex} is the excess heat capacity, Q_t is the enthalpy of denaturation and Q is the heat absorbance up to temperature T.

The rate constant obeys the Arrhenius equation:

$$k = \exp\left[\frac{-E_a}{R}\left(\frac{1}{T^*}-\frac{1}{T}\right)\right] \qquad (2)$$

where $\underline{E}_a$, $\underline{R}$, $\underline{T}$ and $\underline{T}^*$ are activation energy, gas universal constant, absolute temperature and temperature in which $\underline{k}=1\ min^{-1}$, respectively.