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Thermochimica Acta 326 (1999) 127–131

thermochimica acta

Kinetics denaturation of yeast alcohol dehydrogenase and the effect of temperature and trehalose. An isothermal microcalorimetry study

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Received 1 August 1998; accepted 29 October 1998

Abstract

The decrease in biological activity of alcohol dehydrogenase (YADH), due to its spontaneous denaturation, is accompanied by heat exchange of the enzyme solution. The spontaneous denaturation of YADH in 50 mM phosphate buffer, pH 7.8, at two temperatures of 27° and 37°C has been investigated by monitoring the heat exchange and residual activity of an enzyme solution with respect to time. The kinetics of denaturation was found to obey a first order law, depending on the concentration of YADH. The rate constants of denaturation were found to be 0.073 and 0.126 h^{-1} at two temperatures of 27° and 37°C, respectively. These values decreased to 0.021 and 0.034 h^{-1}, respectively, in the presence of 1 M trehalose. Moreover, thermal denaturation study of YADH showed that trehalose leads to thermal stability enhancement.

Keywords: Enzyme denaturation; Enzyme inactivation; Isothermal microcalorimetry; Protein stability; Yeast alcohol dehydrogenase

1. Introduction

Alcohol dehydrogenase occurs in a variety of organisms, including different types of bacteria [1–3], yeasts [1,4], fungi [5], plants [6], insects [7], fishes [4,8], amphibians [4], birds [9] and mammals [4,10,11]. The yeast alcohol dehydrogenase (YADH) is the first pyridine nucleotide-dependent dehydrogenase to be crystallized [4]. The YADH molecule is composed of four identical subunits, each presumably containing 88 amino acid residues [12], two zinc atoms [13], and one NAD(H) coenzyme with narrow specificity for primary alcohols [14]. The molecular mass of the tetrameric enzyme is 150 kDa [15,16]. Alcohol dehydrogenases in general are temperature labile. YADH is somewhat unstable even at 25°C [17].

In most studies on the thermal inactivation of enzymes, the reaction rate constants and thermodynamic parameters are determined based on the assumption that the inactivation follows first-order kinetics [18–20]. However, in general, large deviations from first-order kinetics are observed in the log(residual enzyme activity)–time curve (i.e., enzyme activity remaining vs. heating time at a constant temperature) [20–24]. These deviations could be due to the formation of enzyme groups with differing heat stabilities, the presence of stable/labile isoenzymes [20,21], or due to a series-type enzyme inactivation kinetics [23–25]. Nonlinear enzyme activity–time relationships have recently been observed for various enzymes: horseradish peroxidase [20], *Bacil-*

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0040-6031/99/$ – see front matter
PII: S0040-6031(98)00588-7

lus licheniformis α-amylase [21], acid phosphatases [23] and alcohol dehydrogenase from baker's yeast [26]. A method to determine the enzyme inactivation rate constants from the nonlinear activity–time curves has been proposed [26]. This method has been applied at temperatures above 50°C for alcohol dehydrogenase from baker's yeast, showing first-order kinetics.

Recently, isothermal titration calorimetry (ITC) has provided a large amount of excellent data on protein stability and studies on ligand–protein interaction [27–32]. The sensitivity and reliability of ITC are so high that, under suitable conditions, it is capable of detecting a reaction with a small enthalpy change. One aspect of product quality that is important for proteins is stability, since their integrity and biological activity is known to be affected by time and temperature. The decrease of biological activity of a protein affected by time under fixed conditions, so-called time inactivation (or time denaturation), is accompanied by heat exchange. Accordingly, the isothermal calorimetric method can be used to study the time stability of proteins, drugs and other compounds [33–35].

The purpose of this paper is to propose a method for the analysis of kinetic data obtained by isothermal calorimetry. The described procedure is of general validity and can be applied to any decay process. The method was applied to spontaneous denaturation data obtained for YADH. By measuring the rate constant of spontaneous denaturation of YADH at two temperatures, activation energy related to the time denaturation process is calculated. Since sugars are commonly used as stabilizers for biological systems [36,37], experiments were carried out both in the absence and presence of trehalose and the kinetic parameters were determined using the proposed method.

2. Materials and methods

2.1. Materials

Yeast alcohol dehydrogenase and NAD^+ (99% pure) were obtained from Sigma. Trehalose and ethanol were purchased from Merck. All other materials and reagents were of analytical grade. Solutions were prepared in 50 mM phosphate buffer, pH 7.8, using double-distilled water.

2.2. Methods

2.2.1. Enzyme activity studies

Standard activity tests were performed in the presence of 200 mM ethanol and 1.65 M NAD^+ [38,39]. All measurements were triplicates. The production of NADH was monitored by the increase in absorbance at 340 nm. One unit is defined as the quantity of enzyme necessary to produce 1 μmol NADH/min.

2.2.2. Microcalorimetric stability studies

The isothermal microcalorimetry used in these studies was 2277 Thermal Activity Monitor, TAM (Thermometric, Sweden). It was equipped with four channels, allowing for the rate of heat output. Each channel of the calorimeter was independently electronically calibrated with a precision of ±0.2%. Each channel is a twin heat-conduction calorimeter, where the heat-flow sensor is a semiconductor thermopile (multijunction thermocouple plates) positioned between the vessel made from stainless steel. The insertion vessel was also made from stainless steel. The rate of heat output from the sample cell with respect to the reference cell, displayed digitally, was recorded with an accuracy of 0.1 μW by a 'DIGITAM 3' computer program. The sample vessel contained 2 ml enzyme (0.75 mg/ml) in buffer alone or buffer containing trehalose at 1.00 M concentration. The reference vessel contained all components described for the sample vessel except the enzyme was excluded. The microcalorimetric experiments were performed at two fixed temperatures of 27° and 37°C. The microcalorimeter was frequently calibrated electrically during the course of the study.

2.2.3. Temperature-scanning spectroscopy

Absorbance profiles, which describe the thermal denaturation of YADH, were obtained from Gilford model 2400-S spectrophotometer fitted with a temperature programmer which controls the speed of temperature change in melting experiments. The cuvette holder can accommodate four samples; however, two of them were used; one acts as a reference buffer solution and the other for experimental determination. The enzyme concentration was 0.33 mg cm^{-3}. The chart recorder read the temperature, reference line (from the reference cuvette) and the absorbance change at 280 nm from the sample cuvette.

3. Results and discussion

The heat-exchange curves due to the spontaneous denaturation of YADH in phosphate buffer solution, pH 7.8 at two temperatures of 27° and 37°C are shown in Fig. 1. These curves were obtained in the absence (Fig. 1(a)) and presence (Fig. 1(b)) of trehalose at 1 M concentration. The heat exchange (q) was reached a maximal value (q_{max}), which was related to the maximum concentration of denatured enzyme. The values

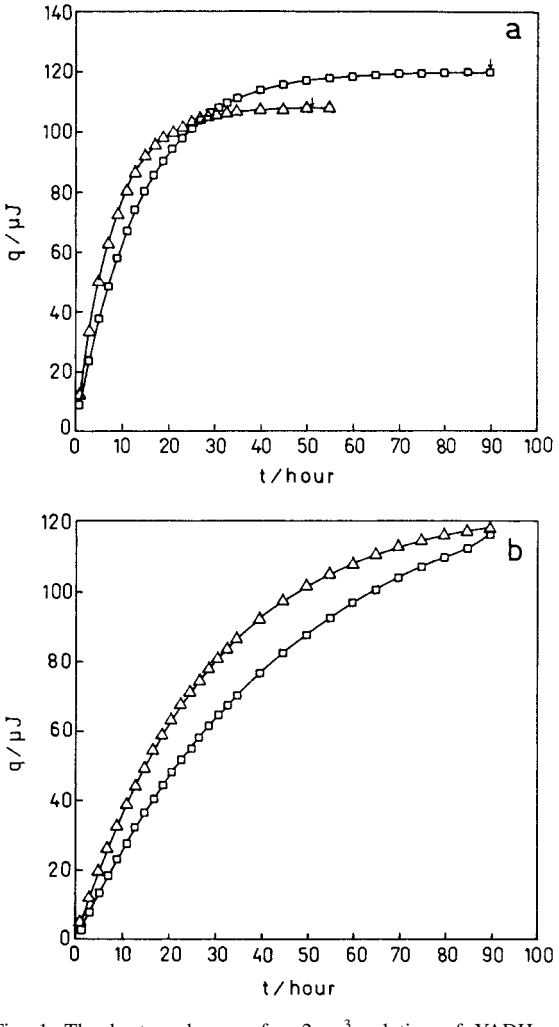

Fig. 1. The heat exchange of a 2 cm^3 solution of YADH, at 0.75 mg cm^{-3} concentration, in 50 mM phosphate buffer, pH 7.8, at two temperatures of 27°C (□) and 37°C (△) with respect to time: (a) in the absence, and (b) in the presence of 1 M trehalose. Arrows show time for zero activity of the enzyme.

of q_{max} are 120 and 108 μJ at two temperatures of 27° and 37°C, respectively, in the absence of trehalose (see Fig. 1(a)). These values for q_{max} are obtained at 90 and 50 h at two temperatures of 27° and 37°C, respectively. The enzyme loses its activity completely after these times. However, values for q_{max} can not be directly obtained from the data presented in Fig. 1(b), since inactivation of the enzyme in the presence of trehalose requires longer incubation times. Nevertheless, the conversion of the enzyme from native (N) to a denatured form (D) results in heat exchange and a saturation curve. Suppose fractions of the denatured and the native forms are α and $1-\alpha$, respectively, at time 't'. It is assumed that the transition N→D follows first-order kinetics, with the rate constant of k. With these in mind, the kinetics of denaturation can be expressed as:

$$\frac{d\alpha}{dt} = k(1-\alpha) \tag{1}$$

Integrating from Eq. (1) yields:

$$\int_0^{\alpha} \frac{d\alpha}{1-\alpha} = k\int_0^{t} dt$$

or

$$\ln(1-\alpha) = -kt \tag{2}$$

Replacing α by q/q_{max} yields:

$$\ln\left(1-\frac{q}{q_{max}}\right) = -kt \tag{3}$$

Therefore, plots of $\ln(1-q/q_{max})$ versus t were linear, and the denaturation rate constant was obtained from the slope of this linear plot.

Fig. 2 shows plots of $\ln(1-q/q_{max})$ versus t. These linear plots are consisted with first-order kinetics for YADH denaturation. Moreover, the rate constants are 0.073 and 0.126 h^{-1} at two temperatures of 27° and 37°C, respectively, obtained from Fig. 2(a). These values decrease to 0.021 and 0.034 h^{-1} at two temperatures of 27° and 37°C, respectively, in the presence of 1 M trehalose (obtained from Fig. 2(b)). Therefore, this sugar is a suitable stabilizer for YADH. The values of 135 and 124 μJ were considered as q_{max} at two temperatures of 27° and 37°C, respectively, in the presence of trehalose. These values were obtained

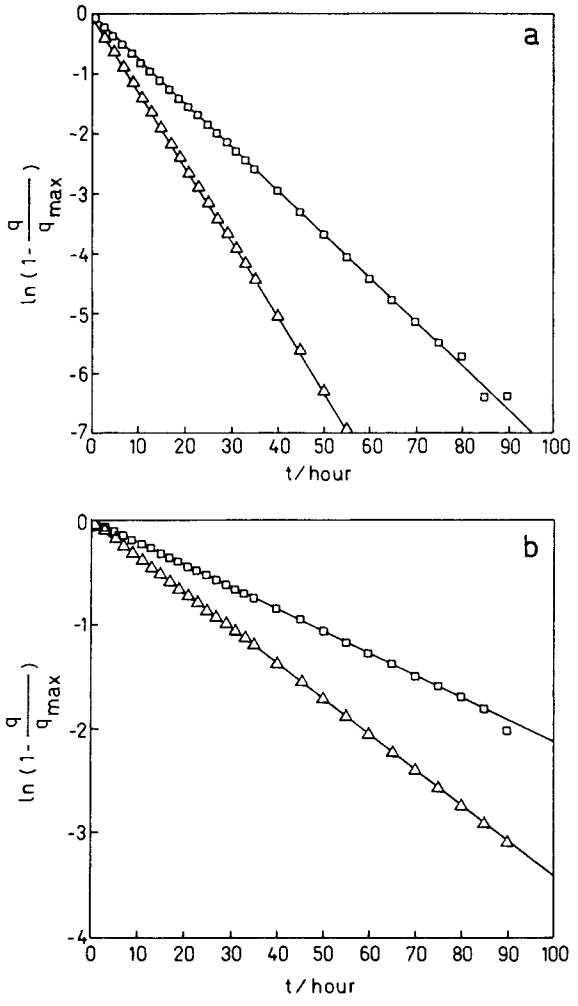

Fig. 2. The plot of ln $[1-(q/q_{max})]$ versus time using data from Fig. 1, at two temperatures of 27°C (□) and 37°C (△): (a) in the absence, and (b) in the presence of 1 M trehalose.

using experimental data which provided the best linear plots (Fig. 2(b)).

The activation energy (E_a) for the denaturation process can be calculated using the Arrhenius equation [40]

$$\ln\frac{k_2}{k_1} = -\frac{E_a}{R}\left(\frac{1}{T_2}-\frac{1}{T_1}\right) \quad (4)$$

where k_1 and k_2 are the denaturation rate constants at two temperatures of T_1 and T_2, respectively, and R is the universal gas constant. The values of the activation

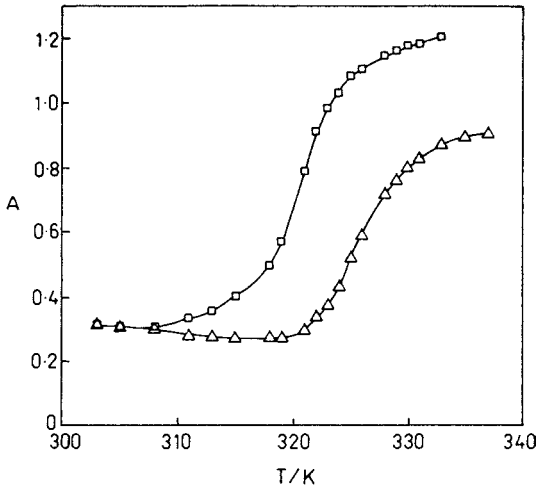

Fig. 3. Variation of absorbance of YADH at 280 nm in 50 mM phosphate buffer, pH 7.8, with temperature (K) measured using a Gilford spectrophotometer: (□) in the absence of trehalose, and (△) in the presence of trehalose, at 1 M concentration.

energies are obtained 42.20 and 37.26 kJ mol^{-1} in the absence and the presence of trehalose, respectively.

Fig. 3 shows the effect of trehalose on thermal stability of YADH. Enzyme denaturation of the enzyme occurred at a higher temperature in the presence of this sugar. The transition temperature of the enzyme was found to increase 5°C in the presence of trehalose at 1 M concentration.

4. Conclusion

A rapid method has been developed to estimate the kinetic parameters of enzyme exhibiting spontaneous denaturation behavior. The applicability of the method has been shown for denaturation microcalorimetric data on YADH. The method may not only be found useful for enzyme kinetic studies, but also be applied in a wide variety of physical, chemical, and biological processes.

Acknowledgements

The financial support from the Research Council of the University of Tehran is gratefully acknowledged.

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