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Kinetics of the thermal inactivation and aggregate formation of rabbit muscle pyruvate kinase in the presence of trehalose

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ABSTRACT

In a previous study we found that 30–40% dimethylsulfoxide induces the active conformation of rabbit muscle pyruvate kinase. Because dimethylsulfoxide is known to perturb structure and function of many proteins, we have explored the effect of trehalose on the kinetics of thermal inactivation and stability of pyruvate kinase; this is because trehalose, in contrast to dimethyl sulfoxide, is totally excluded from the hydration shell of proteins. The results show that 600 mM trehalose inhibits the activity of pyruvate kinase by about 20% at 25 °C, however, trehalose protects pyruvate kinase from thermal inactivation at 60 °C, increases the Tm_{app} of unfolding by 7.2 °C, induces a more compact state, and stabilizes its tetrameric structure. The inactivation process is irreversible due to the formation of protein aggregates. Trehalose diminishes the rate of formation of intermediates with propensity to aggregate, but does not affect the extent of aggregation. Remarkably, trehalose affects the aggregation process by inducing aggregates with amyloid-like characteristics.

Introduction

The effects of cosolvents on the catalytic properties and conformation of pyruvate kinase from several sources have been reported. For example, poly(ethylene glycol) increases the binding of substrates to the catalytic site of liver pyruvate kinase [1] and promotes stabilization of the quaternary conformation of plant cytosolic pyruvate kinase [2]. In the same token, we explored the effect of several cosolvents that decrease the activity of water on the catalytic properties of rabbit muscle pyruvate kinase [3–10]; in our previous work, we found that in the absence of K^+, dimethylsulfoxide induces the formation of the active conformation of the enzyme, whereas in media with 100% water, it is known [11] that the enzyme acquires its active or tightly closed conformation only when K^+, PEP analogs and Mg^{2+}-ATP are bound to the active site [12,13]. Dimethylsulfoxide is known to perturb the structure and function of proteins [14–17], and it has been shown that in aqueous solutions, it undergoes two competing reactions: it associates with water at low cosolvent concentrations, whereas at high concentrations, dimethylsulfoxide binds to the nonpolar surface regions of proteins [18]. Since trehalose is a well known stabilizing solvent of biological macromolecules [19,20], we have studied the impact of this solvent on the activity and stability of muscle pyruvate kinase, as well as the kinetics of its thermal inactivation process. The reason for studying the effect of trehalose, is that this particular cosolvent, in contrast to dimethyl sulfoxide, is totally excluded from the hydration shell of proteins [21].

Rabbit muscle pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) is a complex multidomain homotetrameric enzyme of 237 kDa that catalyzes the transfer of the phosphoryl group of phosphoenolpyruvate to ADP yielding pyruvate and ATP. During catalysis, pyruvate kinase undergoes extensive conformational changes [12,13,22]. Since the enzyme is formed by four identical subunits, it provides a good experimental system to study if trehalose affects the association constant between its constituent monomers. In this regard it is noted that although the effect of trehalose has been explored in many different enzymes, its effect of the association constant between monomers in oligomeric proteins has not been studied in detail.

Our data show that 600 mM trehalose inhibits the catalytic rate of pyruvate kinase by 20% at 25 °C and that it exerts a strong protective effect of pyruvate kinase against thermal inactivation at 60 °C, it increases the Tm_{app} by 7.2 °C. furthermore, we observed that trehalose stabilizes the tetrameric structure of the protein. As evidenced by the accessibility of an external probe to sulfhydryl groups, the effects of trehalose are very likely related to the formation of a more compact structure. Finally, we found that the

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Abbrevations used: CD, circular dichroism; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); Gdn-HCl, guanidine hydrochloride; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; ThT, thioflavin-T.

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thermal inactivation process is irreversible due to the formation of protein aggregates with and without trehalose. However, trehalose diminished the rate of formation of intermediates with propensity to aggregate, although it does not affect the extent of aggregation. In this regard, it is noted that the aggregates formed in the presence of trehalose are amyloid-like, and clearly different from those formed in aqueous media.

2. Materials and methods

2.1. Reagents

Rabbit muscle pyruvate kinase and hog muscle lactate dehydrogenase were obtained as ammonium sulfate suspensions from Roche Diagnostics. Phosphoenolpyruvate (PEP) (cyclohexylammonium salt), ADP sodium salt, NADH disodium salt, $(CH_3)_4NCl$ salt, thioflavin-T (ThT), dithiothreitol, trehalose, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and all the other analytical grade reagents were from Sigma–Aldrich Co.

Prior to the experiments, the enzyme was desalted. The $(NH_4)_2SO_4$ suspensions of pyruvate kinase and lactate dehydrogenase (100 μl aliquots) were centrifuged and the pellets dissolved in 90 μl of 50 mM Tris–HCl, pH 7.6. The solutions were centrifuged twice through Sephadex G-25 hyperfine (Sigma) insulin columns by centrifugation [23]. Protein concentrations were determined by measuring the absorbance at 280 nm using the extinction coefficients 0.54 mL mg^{-1} cm^{-1} for pyruvate kinase [24] and 1.45 mL mg^{-1} cm^{-1} for lactate dehydrogenase [25]. Before using trehalose, a 50 mL of 1.3 M trehalose was precipitated in 1 L of 90% ethanol (an overnight incubation at 4 °C) and thereafter decanted; the procedure was repeated. The precipitated trehalose was dried at 60 °C and used [26].

2.2. Assay of pyruvate kinase activity

The formation of pyruvate was measured in a coupled system with lactate dehydrogenase and NADH [27] at 25 °C. One milliliter reaction mixture contained 25 mM Tris–HCl, pH 7.6, 90 mM KCl, 1 mM PEP, 3 mM ADP, 3 mM $MgCl_2$, 0.2 mM NADH and 10 μg of lactate dehydrogenase. Activity was initiated by introducing pyruvate kinase. The decreased in absorbance at 340 nm after 5 min of reaction was used for the calculation of activity, which was linear with time until NADH became limiting.

2.3. Assay of pyruvate kinase thermal inactivation

Microfuge tubes containing 1 mL of 25 mM Tris–HCl, pH 7.6, 100 mM $(CH_3)_4NCl$, 10 mM dithiothreitol, and the concentrations of pyruvate kinase indicated were placed in a temperature-controlled water bath at 60 °C (Lauda Brinkmann Ecoline RE106). The pH of the buffer was adjusted at 60 °C. Where indicated trehalose was included at a concentration of 600 mM. Pyruvate kinase was added 10 min after the mixtures were pre-equilibrated at 60 °C. Aliquots that had 0.1 μg pyruvate kinase were withdrawn at different time intervals and residual activity was determined as described.

2.4. Thermal inactivation analysis

The kinetic mechanism of pyruvate kinase thermal inactivation was determined as described by Henley and Sadana [28]. The residual activities were plotted against incubation time, and the simplest mechanism that fitted the data was obtained by analyzing the inactivation kinetics with the lineal sequential model:

$$\mathrm{E} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} \mathrm{E1} \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} \mathrm{E2} \underset{k_{-n}}{\overset{k_n}{\leftrightarrows}} \ldots \mathrm{E}n$$

where E is the native enzyme form; E1, E2 and E*n* are alternate enzyme forms that are also active

The general mathematical model for the activity is:

$$A_R = A_1\, e^{-\lambda 1 t} + A_2\, e^{-\lambda 2 t} + \cdots + A_n\, e^{-\lambda n t}$$

where A_R is the residual activity at time (t) related to each rate process, A_i is the apparent amplitude of each phase and λ_i is the apparent rate constant for each phase. A_i and λ_i contain information on the real rate constants of the system. Therefore the overall change in a given measured property, such as activity, can be followed in terms of the contribution from each species. The description of the kinetic mechanism was performed first by defining the number of inactivation phases and attempting to fit the residual activity (A_R) to the corresponding equation using the program Origin version 7.0. In all the experimental conditions studied, the best fit of the data was to the monoexponential decay model $A_R = A_0\, e^{-\lambda t}$, where A_R is the residual activity at time t, A_0 is the activity at 0 time and λ is the apparent rate constant.

2.5. Assay of reactivation of pyruvate kinase

For the experiments, pyruvate kinase (2.5, 5, 40, 100 or 200 μg/mL) was inactivated at 60 °C to the desired levels, and then, the enzyme was transferred to an ice bath for 1 min, and thereafter to a water bath at 25 °C. At different times, aliquots were withdrawn for assay of activity.

2.6. Gel electrophoresis of pyruvate kinase

Samples derived from the reactivation assays carried out at 40 and 200 μg/mL were loaded on 4–20% gradient native gels. Gels were pre-run for 1 h at 100 V; after the samples were loaded they were run for 12 h. After electrophoresis, gels were washed with bi-distilled water and then transferred into 15 mL of 50 mM Tris–HCl, pH 7.6, and shaken gently for 10 min. The gels were then transferred to a 15 mL mixture containing 25 mM Tris–HCl, pH 7.6, 90 mM KCl, 0.5 mM PEP, 1 mM ADP, 1 mM $MgCl_2$, 0.2 mM NADH and 50 μg/mL lactate dehydrogenase and exposed to UV light for 10–30 min, or until a dark band appeared on a fluorescent background; the latter reflected pyruvate kinase activity of the samples [29]. After activity was determined, the gels were exhaustively washed with bi-distilled water and stained with either Silver stain plus kit or Coomassie brilliant blue R-250 followed by destaining with 10% (v/v) glacial acetic acid and 10% (v/v) ethanol.

2.7. Circular dichroism (CD) experiments

CD spectra were recorded in the far-UV range on a JASCO J-715 spectropolarimeter with a thermostatied Peltier-controlled cell holder. A 1 mm quartz cell was used. The scan temperature experiments were conducted from 25 to 90 °C and back to 25 °C at a rate of 1 °C/min; the CD signal at 222 nm was followed. The concentration of pyruvate kinase was 200 μg/mL. Spectral scans were also run from 200 to 260 nm at intervals of 1 nm and a time constant of 2 s. These spectra were recorded before heating (25 °C), at the higher scan temperature (90 °C) and after cooling back to 25 °C. The spectra of blanks were subtracted from those that contained the protein. CD is expressed as Θ (10^{-3} deg).

2.8. Fluorescence of ThT in aggregated pyruvate kinase

Thioflavin-T (ThT) is known to form highly fluorescent complexes with amyloid-like fibrils [30–32]; ThT does not interact with