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Thermal inactivation and conformational lock studies on horse liver alcohol dehydrogenase: Structural mechanism

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ABSTRACT

Horse liver alcohol dehydrogenase (HLADH) is a two subunits metal enzyme that has two catalytic sites and two coenzyme domains for each subunit. These subunits are connected together by coenzyme domains. In this study, we investigated the number and sequences of residues that participated in interface locks of HLADH. For this purpose, the kinetics of thermal inactivation of HLADH were studied in a 50 mM pyrophosphate buffer, pH 8.8, using ethanol as a substrate and NAD^+ as a cofactor. The temperature range was between 46 °C and 55 °C and the conformational lock was developed based on the Poltorak theory and analysis of the curves was done by the conformational lock method for oligomeric enzymes. The conformational lock number of HLADH was 2 when calculated experimentally. The results were confirmed by the Ligplot program computations. Using computational method it was shown that there are two patches binding sites at the interface and they spread over two regions of each chain. In this study we also proposed a thermal denaturation mechanism for HLADH by using different techniques such as UV-Vis fluorescence and circular dichroism (CD) spectroscopy and dynamic light scattering (DLS). The subunits are dissociated and several intermediates appeared during inactivation through increasing the temperature. DLS measurement was performed to study the changes in hydrodynamic radius during thermal inactivation. The three distinct zones that were shown by DLS were also confirmed by fluorescence and CD techniques.

1. Introduction

Much interest has been devoted to the study of enzymes involved in alcohol metabolism especially mammalian liver alcohol dehydrogenase. This enzyme has been extensively studied because of its role in limiting the rate of alcohol oxidation [1] and also its availability as a commercial scale biocatalyst [2].

Horse liver alcohol dehydrogenase (HLADH; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1), is a dimer enzyme with molecular mass of 80 kDa [3]. HLADH consists of two identical subunits containing two zinc ions per subunits, one in its active site (catalytic zinc) and the other in a loop 20 Å away from the active site called conformational zinc [4,5]. Each subunit folds into two non-equal domains separated by a deep active site cleft [6]. One of them is a catalytic domain, which performs catalytic activity, and the other one is a coenzyme domain that is specialized to connect to NAD^+ coenzyme. The coenzyme domain contains the parallel strands that are called βA–βF and the helices αA–αE sequentially from the amino end. The sequence of strands from top to bottom of the sheet is C–B–A–D–E–F. The subunits are connected to each other by βF strands, the strand of each subunit run in opposite directions perpendicular to axis and are joined together by hydrogen bonds forming two strands of antiparallel β-structure [7].

Some oligomeric enzymes may contain a specific structure called "conformational lock" which is clarified as inter-subunit contact complexity that, by progressive and stepwise breaks, leads to the separation of the inactive monomers [8]. The discovery of these locks is very important in studying protein structure and folding [9]. These locks play an important role in protein stability.

The catalytic activity and stability of oligomer enzymes depend on their subunit interfaces which can be explained by two independent methods; the use of their structural data and the results of their chemical kinetics [10]. Poltorak et al. reported that in alkaline phosphate both of these results are in reasonable agreement [10,11]. In a different work on glycogen phosphorylase b, Kurganov et al. suggested that the denaturation proceeds by the dissociative mechanism of thermal denaturation by using differential scanning

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calorimetry and analytical ultracentrifugation in addition to enzymological inactivation studies [12]. Moosavi-Nejad et al. suggested a putative mechanism of lentil seedling amine oxidase denaturation based on structural data [8]. Hong et al. obtained an accepted mechanism for thermal dissociation of superoxide dismutase by studying thermal dissociation and conformational lock in addition to biochemical calculation [13]. Amani et al. showed that *Euphorbia characias* latex (ELAO) and *lentil seedlings* (LSAO) have different thermal and chemical stability, and in order to find an explanation for these differences they compared the structure of ELAO conformational lock with LSAO [14]. Alaie et al. studied the inter-subunit amino acid residues and subunits intermolecular interactions of bovine carbonic anhydrase (BCA) [15]. Sattari et al. studied the thermal inactivation and conformational lock of glucose oxidase (GOD) [16].

In the present study we investigated the number and sequences of residues that participated in interface locks of HLADH. Additionally we have reported a putative mechanism for thermal dissociation of this enzyme.

2. Methods and materials

2.1. Enzyme and chemicals

Alcohol dehydrogenase from horse liver (HLADH, EC 1.1.1.1, 1.8 U/mg) and β-nicotinamide adenine dinucleotide (NAD^+) were purchased from Boehringer Mannheim, Gmbh (Germany). Ethanol 96% was obtained from Merk Company. 8-Anilinonaphthalene-1-sulfonate (ANS) was purchased from Sigma.

2.2. Enzyme assay

The activity of HLADH was determined using NAD^+ as a cofactor and ethanol as a substrate for kinetics studies. Reduced NAD^+ (NADH) exhibits strong UV absorption at 340 nm whereas the oxidized form has practically no absorption at this wavelength. Therefore the kinetic reaction was monitored at 340 nm. The assay was performed in 0.5 ml of 50 mM pyrophosphate buffer at pH 8.8 containing 0.8 mM NAD^+, 0.05 M ethanol and 0.02 mg/ml HLADH in a Ray Leigh UV-Vis-2100 spectrophotometer.

2.3. Determination of optimum temperature

Optimum temperature (T_{opt}) is the maximum temperature at which the activity of the enzyme does not change during the incubation time [17]. First the HLADH samples at a concentration of 1.7 mg/ml were incubated at different temperatures (from 44 °C to 55 °C) in 50 mM pyrophosphate buffer at pH 8.8 for 35 min. The incubated HLADH samples were cooled rapidly to 25 °C in a water bath, and assayed for residual activity.

2.4. Thermal inactivation of HLADH

To studying the residual activity of HLADH, samples at a concentration of 1.7 mg/ml, in 50 mM pyrophosphate buffer at pH 8.8 were incubated separately between 44 °C and 55 °C. At suitable intervals, samples were removed, quickly cooled to 25 °C and assayed for activity.

2.5. Measuring the mean hydrodynamic diameter

Dynamic light scattering (DLS) is a technique that can be used to study the particle size distribution in a solution with reasonable accuracy. Hydrodynamic radius of the heated samples was measured by DLS technique using a Zetasizer Nano ZS (Brookhaven, USA) equipped with a helium/neon laser. First HLADH was filtered with 0.2 μm filters and then it was dissolved in 50 mM pyrophosphate buffer at pH 8.8 that was made of deionized water. Six different temperatures were selected in the range of 44–55 °C based on the thermal inactivation plot. Each sample was incubated for 35 min, quickly cooled to 25 °C and tested for hydrodynamic diameter measurements (0.1 mg/ml). The results are mean values of triplicates with error bars denoting SD. Dynamic light scattering technique is a useful method to study the changes in protein diameter with temperature.

2.6. Fluorescence studies

In order to study the HLADH tertiary structure changes, intrinsic fluorescence was performed. The intrinsic fluorescence of the diluted samples (0.05 mg/ml) in 50 mM pyrophosphate buffer (pH 8.8) was monitored using excitation wavelength of 280 nm and evaluated emission spectra were recorded between 290 nm and 500 nm with a Carey eclipse fluorescence spectroscopy.

For determination of accessible surface area (ASA) changes, 1-anilinonaphthalene-8-sulfonate (ANS) was used in this study as a small organic compound that works as a probe and interacts with protein hydrophobic areas in a non-covalent manner. ANS has an inverse relationship with solvent polarity and fluorescent quantum yield and therefore is a sensitive probe for identification of hydrophobic sites [18]. ANS has fluorescent intensity in 500 nm when associated with protein. In this study, protein solution (0.05 mg/ml) and ANS (0.01 mg/ml) was prepared in 0.2 ml pyrophosphate buffer pH 8.8. The emission spectra were recorded between 395 and 595 nm using an excitation wavelength of 385 nm. The bandwidth for both excitation and emission was 10.0 nm.

2.7. Circular dichroism experiments

Far-UV (190–260 nm) circular dichroism (CD) was used to measure changes in the secondary structure of HLADH (0.2 mg/ml), using JASCO J-810 Spectropolarimeter CD. The results were expressed as the molar ellipticity $[\theta]$ ($cm^2\,dmol^{-1}$) based on a mean amino acid residue weight (*MRW*) of HLADH having the average molecular weight of 80 kDa. The molar ellipticity was determined as $[\theta] = (\theta_{obs} \times MRW)/(10 \times L \times c)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in g/ml and L is the length of the light path (cm). The amount of secondary structure associated with the samples was calculated by the CDNN software.

2.8. Biochemical computation using Ligplot

LIGPLOT (v.4.4.2) is used to construct protein–protein and protein–ligand contacts map from a given PDB file [19]. The created scheme shows interactions such as hydrogen bonds presented by dashed lines between the involved atoms and hydrophobic contacts that are shown by an arc with spokes radiating toward the ligand atoms they connect. To find details about the protein surface, cavities and secondary structures, the SWISS PDB viewer 4 is used [20].

2.9. SurfRace program

SurfRace program was performed to calculate the exact ASA by the given PDB file, the molecular surface (MS) and interior cavities. The program is fast, robust and portable to practically any existing computational platform [21]. The SurfRace program was used to calculate ASA for the dimer and monomer of this enzyme.