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ORIGINAL ARTICLE

Alcohol-induced structural transitions in the acid-denatured *Bacillus licheniformis* α-amylase

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Thermal denaturation

Abstract Alcohol-induced structural changes in the acid-denatured *Bacillus licheniformis* α-amylase (BLA) at pH 2.0 were studied by far-ultra violet circular dichroism, intrinsic, three-dimensional and 8-anilino-1-naphthalene sulfonic acid (ANS) fluorescence, acrylamide quenching and thermal denaturation. All the alcohols used in this study produced partial refolding in the acid-denatured BLA as evident from the increased mean residue ellipticity at 222 nm, increased intrinsic fluorescence and decreased ANS fluorescence. The order of effectiveness of these alcohols to induce a partially folded state of BLA was found to be: 2,2,2-trifluoroethanol/*tert*-butanol > 1-propanol/2-propanol > 2-chloroethanol > ethanol > methanol. Three-dimensional fluorescence and acrylamide quenching results obtained in the presence of 5.5 M *tert*-butanol also suggested formation of a partially folded state in the acid-denatured BLA. However, 5.5 M *tert*-butanol-induced state of BLA showed a non-cooperative thermal transition. All these results suggested formation of a partially folded state of the acid-denatured BLA in the presence of these alcohols. Furthermore, their effectiveness was found to be guided by their chain length, position of methyl groups and presence of the substituents.

1. Introduction

In order to perform their biological functions, proteins fold spontaneously into three-dimensional structures (native state) from a linear polypeptide chain of amino acid residues [4]. Misfolding in the transformation to the native state of the protein has been shown to cause the development of protein folding diseases such as prion, Alzheimer and Parkinson's diseases [44,54,60]. In order to overcome this problem, studies on the mechanism of protein folding and protein stability involving

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characterization of various intermediates and denatured state of a protein become an important subject [14,38,43,50]. Many techniques have been employed to identify these states of proteins [15,20,38]. Information about how and when various non-covalent forces guide the folding process of a polypeptide chain into a functional protein can be obtained by the identification of various intermediates [22,47]. Formation of an intermediate state (a partially-folded state) can be induced by exposure of the protein to the low pH or addition of alcohols or other denaturing agents [23,25,30]. Many reports have shown retention of some native-like secondary structures in the partially folded states of proteins, obtained in the presence of fluoroalcohols [9,16,17,24,49,59]. Alcohols are known to stabilize the α-helical structure by reducing the exposure of peptide backbone in the unfolded proteins [27,48]. However, the effectiveness of an alcohol in inducing α-helical structure depends on the number of hydrocarbon groups, location of the hydroxyl groups, presence of fluorine atoms or any other constituent groups in the alcohol [17,24,59]. Although, a similar order of effectiveness has been shown among various alcohols in inducing α-helical structure in various proteins, differences have been noticed in terms of the concentration requirement to achieve the completion of the transition [17,24,59].

Bacillus licheniformis α-amylase (BLA) has been widely used in various industries in the production of maltodextrin, alcohols, baking, textile and detergent industries for the initial starch hydrolysis [2,6,33,37,52]. BLA is a highly thermostable enzyme and possesses higher thermal stability compared to *Bacillus amyloliquefaciens* α-amylase (BAA) even though both enzymes show 81% identity and 88% similarity in their primary sequence [10]. Thus, it becomes important to understand the mechanism of protein folding and structural-stability of BLA [46]. Acid denaturation studies on BLA have shown the completion of transitions at pH 2.0, while at pH 4.0, the state of this enzyme has been characterized as a molten globule state [46]. Although the effect of 2,2,2-trifluoroethanol (TFE) on the native state of BLA has been shown in an earlier study [5] a detailed study involving the effect of various alcohols on the acid-denatured state of BLA at pH 2.0 is lacking. Therefore, in the present study, we investigated the effect of various alcohols on the acid-denatured state of BLA obtained at pH 2.0, as monitored by far-ultra violet circular dichroism (far-UV CD), intrinsic fluorescence, 8-anilino-1-naphthalene sulfonic acid (ANS) fluorescence, three-dimensional (3-D) fluorescence, acrylamide quenching and thermal denaturation.

2. Materials and methods

2.1. Materials

Lyophilized *B. licheniformis* α-amylase (Catalog #A4551), 8-anilino-1-naphthalene sulfonic acid, acrylamide, *N*-acetyl-L-tryptophanamide (NATA), 2,2,2-trifluoroethanol, *tert*-butanol, 2-propanol and methanol were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. 2-Chloroethanol, ethanol and 1-propanol were procured from Merck, Darmstadt, Germany. Tris base was obtained from Amresco, Irvine, CA, USA. All other chemicals used were of analytical grade purity.

2.2. Analytical procedures

The concentrations of the stock solutions of BLA, ANS and NATA were determined spectrophotometrically on a Shimadzu double-beam spectrophotometer, model UV-2450, using molar extinction coefficients of 139,690 M^{-1} cm^{-1} at 280 nm [35], 5000 M^{-1} cm^{-1} at 350 nm [29] and 5630 M^{-1} cm^{-1} at 280 nm [1], respectively. The molar enzyme concentrations used in this manuscript were estimations based on the value of the molar extinction coefficient of BLA reported by Nazmi et al. [35].

2.3. Circular dichroism

A Jasco spectropolarimeter, model J-815 was used for circular dichroism (CD) measurements after calibrating the instrument with (+)-10-camphorsulfonic acid under constant nitrogen flow. All CD measurements were made at 25 °C using a thermostatically-controlled cell holder. The far-UV CD spectra were recorded in the wavelength range, 200–250 nm, using a protein concentration of 1.7 μM in a 1 mm path length cuvette while 3.4 μM protein concentration and 10 mm path length cuvette were used to measure the near-UV CD spectra in the wavelength range, 250–300 nm. Each spectrum was the average of three scans with a scan speed of 100 nm/min and a response time of 1 s. The results are expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ after subtracting the CD spectral contribution of the appropriate blanks, using the following equation:

$$\mathrm{MRE} = \theta \times \mathrm{MRW}/10 \times c \times l$$

where different terms *i.e.* θ, MRW, c and l refer to ellipticity in millidegrees, mean residue weight (molecular weight, 55,200 Da/number of amino acid residues, 483 [57] of the protein, protein concentration in mg/mL and the optical path length in cm, respectively. The MRE values, thus obtained were estimations based on the data of molecular weight and amino acid residues obtained from the above reference. The method of Chen et al. [8] was used to calculate the α-helical content in the protein.

2.4. Fluorescence spectroscopy

Fluorescence measurements were carried out on a Hitachi fluorescence spectrophotometer, model F-2500. The emission spectra were recorded in the wavelength range, 300–400 nm upon exciting the protein solution (0.1 μM) at 280 nm, using a quartz cuvette of 1 cm path length. Both excitation and emission slits were fixed at 10 nm each.

ANS fluorescence spectra were measured in the wavelength range, 400–600 nm upon exciting the protein sample (0.6 μM) at 380 nm. The ANS: protein molar ratio was fixed at 50:1 throughout the measurements and the fluorescence spectra were corrected by subtracting the fluorescence contribution of the appropriate blanks.

A Jasco spectrofluorometer, model FP 6500 was used for three-dimensional (3-D) fluorescence measurements. The fluorescence spectra of the protein (0.1 μM) were measured using the emission and excitation wavelength ranges as 220–500 and 220–400 nm, respectively, with 10 nm increment. The number of scanning curves was 19. The fluorescence spectra

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