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Spectroscopic investigation of structure-breakers and structure-makers on ornithine carbamoyltransferase

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Abstract

In this work, we investigate the effects of different cosolutes on ornithine carbamoyltransferase in order to study the role of water in stabilization processes as a function of solutes and to point out the fundamental role played by an enhancement of hydrophobic interactions.

It is well known that the cosolutes can affect the solvent properties and therefore influence the environment in which biostructures are immersed. Structure-breakers or "chaotrope" solutes do not stabilize biomolecules, whereas structure-makers or "kosmotrope" solutes are capable to create strong bonds with water, so creating good conditions for avoiding denaturation processes. The effect of different additives and temperature on ornithine carbamoyltransferase has been carried out using techniques such as the measurement of the enzymatic activity and UV–VIS spectroscopy.

Keywords: Water; Structure-breakers; Structure-makers; Enzymes; Spectroscopy

1. Introduction

New developments in the field of enzymology are emerging from many research laboratories around the world (Delcour & Verschaeve, 1987; Eerlingen & Delcour, 1995; Eerlingen, Van Haesendonck, De Paepe, & Delcour, 1994; Gruchala & Pomeranz, 1993; Marco, Gee, Cheng, Liang, & Hammock, 1993). Enzymes are used in foods and beverages to improve both processing effectiveness and quality of products. However, foods often contain naturally occurring enzymes which may cause food degradation. In this case, it can be very useful to identify ingredients which inhibit the enzymatic activity and improve the product shelf-life (Kilcast & Subramaniam, 2001). Food researchers have addressed their studies on developing products retaining their quality during shelf-life, which is determined not only by a food's chemical nature, but also the way has been processed, packaged, distributed and stored. In this context, use of trehalose, a shelf-life extending glucose disaccharide, has been considered (Patist & Zoerb, 2005).

The understanding of the basic process of stabilization is therefore fundamental; it can provide the use of better enzymes. Many additives, such as sugars, salts, polyols, organic solvents and osmolytes have been tested to increase the stability of proteins (Bellocco et al., 2005; Costa, Tzanov, Carneiro, Paar, Gubitz & Cavaco-Paulo, 2002). It has been observed that protein stabilization by cosolutes is concentration-dependent and that the stabilization becomes effective only at relatively high cosolute concentrations (Carpenter, Crowe, & Arakawa, 1990). Studies on the mechanism of interaction of these compounds with proteins have shown that, at room temperature, they are preferentially excluded from contact with the protein surface (Arakawa & Timasheff, 1982; Carpenter et al., 1990; Lee & Timasheff, 1981). Lee and Timasheff (1981) suggested that the presence of these cosolutes in a protein solution creates

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a thermodynamically unfavorable situation, since the chemical potentials of both the protein and the additive are increased. Sola-Penna et al. (Sola-Penna & Meyer-Fernandes, 1994; Sola-Penna, Vieyra, & Meyer-Fernandes, 1994; Sola-Penna, Ferreira-Pereira, Lemos, & Meyer-Fernandes, 1997) demonstrated that the effectiveness of trehalose is higher than that achieved using other sugars such as maltose, sucrose, glucose or fructose. In particular, studies performed by these authors showed that trehalose is more effective than other sugars in protecting yeast pyrophosphatase against thermal inactivation (Sola-Penna et al., 1994). It has been shown that the extraordinary effectiveness of trehalose is due to a large hydrated volume in comparison to other sugars, and consequently it is more excluded from the hydration shell. Hence, less trehalose is necessary to decrease the solvation layer of proteins and thus to stabilize and modulate enzyme activity (Sola-Penna & Meyer-Fernandes, 1998).

Solutes are generally classified on the basis of their influence on the natural hydrogen bonded network of water in biomolecules (De Xammar Oro, 2001; Wiggins, 2001). Compounds exhibiting weaker interactions with water than water itself are known as structure-breakers or "chaotropes", whereas additives having the opposite effect are known as structure-makers or "kosmotropes". This behavior is linked to the capability of increasing or decreasing, respectively, the structuring of water (De Xammar Oro, 2001; Wiggins, 2001).

In this work, we investigate the effect of different additives on ornithine carbamoyltransferase (OCTase) by activity and UV–VIS spectroscopy measurements.

2. Experimental

Ornithine carbamoyltransferase (OCT, EC 2.1.3.3.) has been isolated from the liver of the thresher shark (*Alopias vulpinus*). The enzyme (M_w 120,000) has a trimeric structure composed of identical subunits, it catalyzes the synthesis of citrulline from ornithine and carbamoyl phosphate with the release of orthophosphate during the first step in de novo biosynthesis of arginine. δ-*N*-(phosphonacetyl)-L-ornithine (δ-PALO) was synthesized and further purified as described by Hoogenraad (1978); bovine serum albumin (BSA), citrulline, L-ornithine hydrochloride, carbamoyl phosphate dilithium salt, tris-(hydroxymethyl)-aminomethane, urea and trehalose were purchased from SIGMA, St. Louis, MO; epoxy-activated Sepharose 6B was obtained from Pharmacia LKB, Uppsala, Sweden.

4 g of thresher shark (*Alopias vulpinus*) liver was homogenated with 1:4 (w/v) of Tris–HCl 50 mM at pH 7.8 with 1 mM β-mercaptoethanol. Concerning with the heat treatment, the homogenate was heated with stirring to 60 °C for 1 min in a water bath and immediately cooled in an ice bath. The amounts of the denatured protein and membrane fragments were removed by centrifugation (10,000 rpm × 10 min at 4 °C). The purified protein was stored at −20 °C.

3. Results and discussion

The OCT activity was determined in the range of temperature from 37 to 80 °C in the absence and in the presence of different additives. The OCT activity decreases as a function of temperature. The value of the midpoint of thermal inactivation T_m, defined as the temperature at which 50% of residual activity remains, of 62.9 °C with native enzyme increases in the presence of NaCl, ornithine, phosphate, sulphate and trehalose of about 2.0, 3.4, 4.3, 6.8 and 7.1 °C, respectively (see Fig. 1).

Let us analyse the opposite effect of a known denaturant as urea and a known bioprotectant as trehalose. The enzyme denaturation process follows a first order reaction without breaking-point and it is a slow sequential reaction as shown in a previous work (Bellocco et al., 2005). Citrulline enzymatically formed appears as a clear peak with a retention time of 5.57′. The peak progressively disappears by increasing the time of incubation at 66 °C. In Fig. 2 the enzyme activity in the presence of

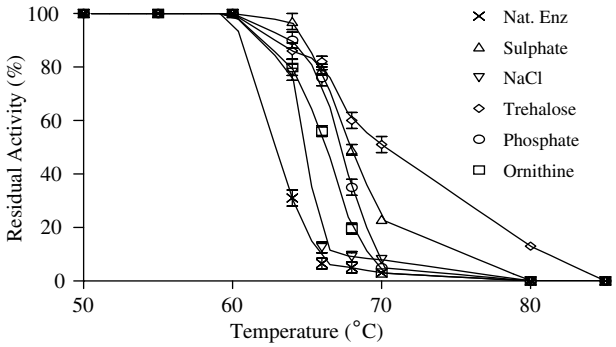

Fig. 1. Native enzyme and enzyme activity in the presence of 0.2 M ammonium sulphate, 0.5 M of trehalose, 1.0 M of NaCl, 0.05 M of ornithine and 0.025 M phosphate in the temperature range of 37–90 °C.

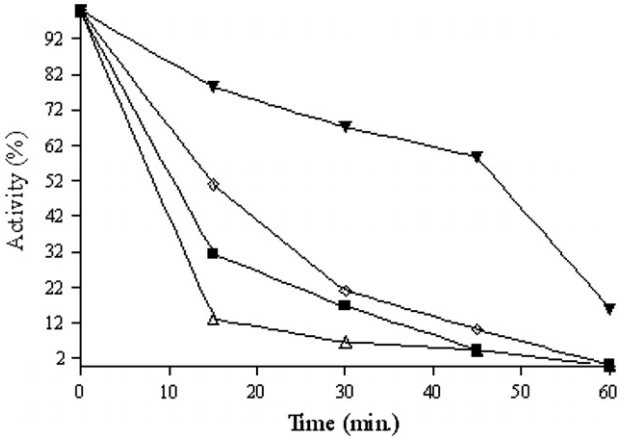

Fig. 2. OCT activity in the presence of urea, trehalose and both. Enzyme solution (0.8 mg/ml) in 50 mM Tris–HCl buffer (pH 7.8) with 1 mM β-mercaptoethanol was incubated at 66 °C in the absence (■) and in the presence of 1.0 M urea (Δ), 1.0 M trehalose (▼) and both (◇).

urea, trehalose and a combination of both the additives is shown. At 66 °C OCT activity in the presence of urea decreases by increasing the incubation time and after 15 min of incubation only 15% of original activity remains. After 30 min of incubation at 66 °C the residual activity is of about 17%, 67%, 7% and 21% in the

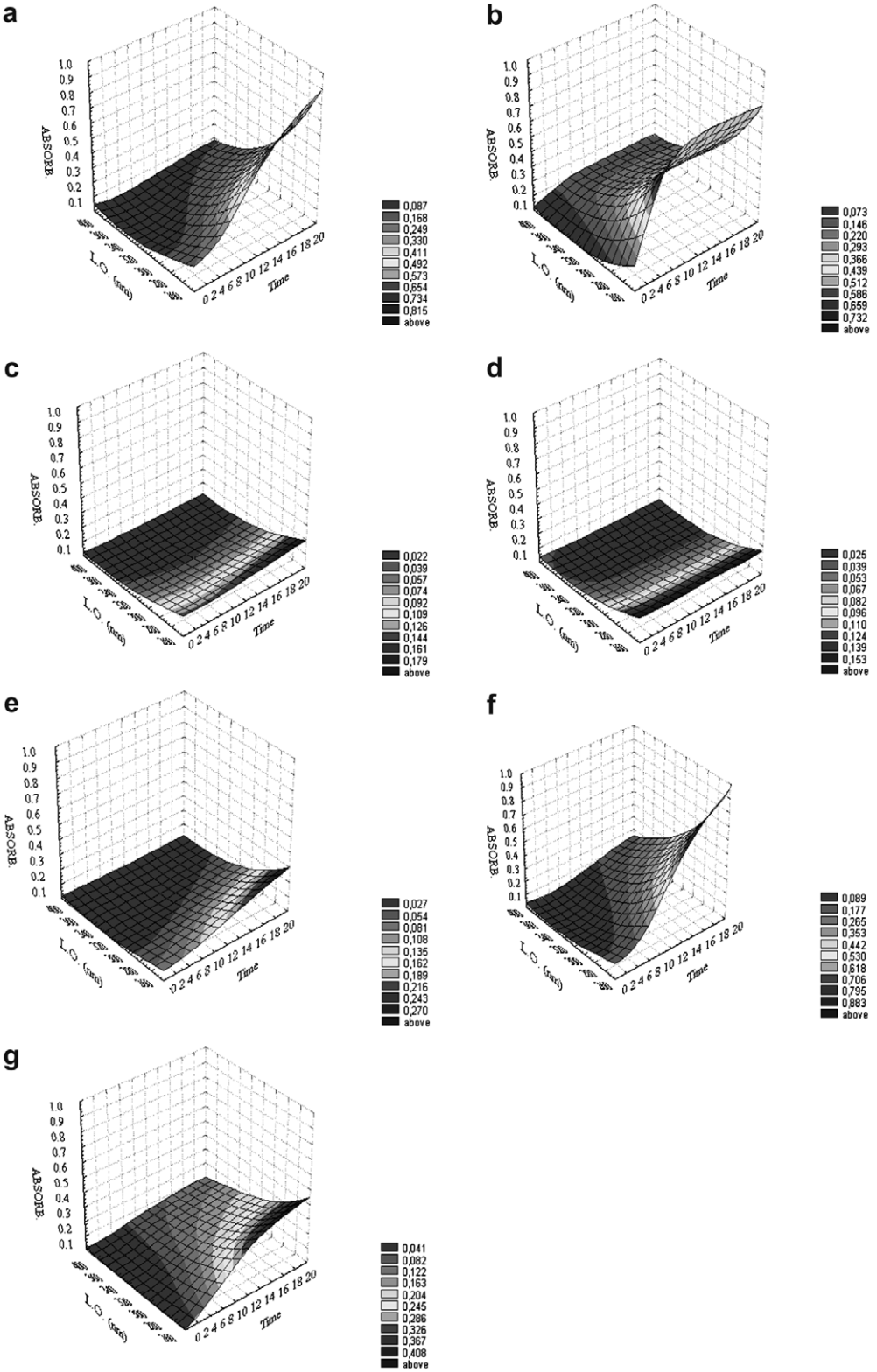

Fig. 3. 3-D UV spectra of (a) native enzyme, (b) enzyme without 2-mercaptoethanol; (c–g) enzyme with: sulphate; phosphate; ornithine; sodium chloride; trehalose, respectively.

absence and in the presence of trehalose 1.0 M, urea 1.0 M and both the additives, respectively. It can be observed that the presence of trehalose protects the enzyme against thermal inactivation and up to 58% of original activity remains after 45 min of incubation. At 66 °C trehalose mitigates also the denaturation effect induced by urea, preserving 51% of enzymatic activity after 15 min of incubation.

UV–Visible spectroscopy results, shown in Fig. 3, on the native enzyme at 60 °C in the range of 260–400 nm as a function of time point out a significant increase in absorbance, characteristic of a major exposure of interior hydrophobic group. The unfolding without a reduced additive is faster, demonstrating the importance of SH for the integrity and functionality of enzyme. The presence of phosphate (0.025 M), sulphate (0.2 M) and ornithine (0.01 M) avoids the exposure of this hydrophobic group and small changes in absorbance value were monitored, while in the presence of NaCl (1 M) and trehalose (0.5 M) the variation of absorbance is more evident.

From many experimental evidences, it emerges that trehalose shows a marked kosmotrope characteristic. This capability implies that trehalose promotes an extensive layer of structured water around its neighbourhood, which affects the tetrahedral H-bond network of pure water and provides a structure whose spatial positions and orientations are not compatible with those of ice, as pointed out by Raman, neutron diffraction and inelastic neutron scattering findings (Branca, Magazù, & Migliardo, 2002; Branca, Magazù, Maisano, & Migliardo, 1999; Magazù, Migliardo, & Ramirez-Cuesta, 2005). In addition, the water dynamics in the presence of trehalose is strongly slowed down (Magazù, Migliardo, & Telling, 2006). These experimental results have been confirmed by molecular dynamics simulation studies performed by Descamps and co-workers (Bordat, Lerbret, Demaret, Affouard, & Descamps, 2004). In particular, the distributions of Voronoi volumes, which provide useful information about the local molecular environment or the local free volume, emphasise a dilation and a distortion of the hydrogen bonded network of water from its tetrahedrality. Concerning with water dynamics, the relaxation time of water in the presence of trehalose is longer than the one of pure water, revealing that the dynamics of trehalose molecules is imposed to a larger number of water molecules.

In conclusion, OCT shows a different behavior in the presence of denaturant and stabilizing additives at room and at high temperature. In the presence of trehalose the enzyme is stabilized and also inhibits the combined denaturation effect induced by urea and high temperature. The results suggest that trehalose avoids the denaturation process through a process preserving the tertiary and secondary structures and it decreases (and in same case completely avoids) the exposure of SH-group essential for enzymatic activity maintenance.

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