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Role of Polyols in Thermal Inactivation of Shark Ornithine Transcarbamoylase

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Summary

The ability of activity modulators of ornithine transcarbamoylase (OCT) from the liver of the thresher shark *Alopias vulpinus* to stabilize the enzyme against thermal denaturation was investigated in the tri-buffer at pH 7.8, at temperatures ranging from 60 to 70 °C, in the presence of polyhydroxylic molecules such as glycerol and sugars. The study indicated that in the presence of 0.5 M sugars and 1.6 M glycerol in the preincubation medium the OCT activity increases. When trehalose is introduced directly in the reaction mixture in a range of concentration of 0.25-0.5 M, the activity is lower than that with maltose, glycerol and buffer alone. Kinetic data for carbamoyl phosphate and ornithine with and without maltose and glycerol are similar, whereas trehalose increases the kinetic values. Arrhenius plots show an increase of activation energy due to trehalose, whereas values obtained with maltose and glycerol are similar to the control

Key words

Elasmobranch • Ornithine transcarbamoylase • Thermostability • Sugars • Polyols

Introduction

Ornithine transcarbamoylase (OCT, EC 2.1.3.3.) catalyzes the synthesis of citrulline from ornithine and carbamoyl phosphate with the liberation of orthophosphate during the first step in the *de novo* biosynthesis of arginine. OCT has been isolated and characterized from a variety of sources: sea turtle (Bellocco *et al.* 2002a), elasmobranchs (Anderson and Casey 1984, Xiong and Anderson 1989), porcine (Koger *et al.* 1994), rat (Clarke 1976) or human (Kalousek *et al.* 1978) liver, several microorganisms (Legrain and Stalon 1976, Bates *et al.* 1985, Murata and Schacman 1996, Sanchez *et al.* 1997) and plants (Bellocco *et al.* 1993, 2002b, Slocum and Richardson 1991, Slocum *et al.* 2000). The enzyme has a trimeric structure composed of identical subunits. It catalyzes an obligatory sequential reaction with carbamoyl phosphate identified as the leading substrate (Bellocco *et al.* 2002a,b, Legrain and Stalon 1976, Wargnies *et al.* 1978, Marshall and Cohen 1972). In general, homologous enzymes isolated and characterized from a variety of sources share similar

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kinetic, molecular and structural properties. Many additives, such as sugars and glycerol at high concentrations, have been shown to stabilize proteins against disruptive elements (Wargnies *et al.* 1978). It was observed that protein stabilization by co-solutes is concentration-dependent and that the stabilization becomes effective only at relatively high (>0.3 M) co-solute concentrations (Carpenter *et al.* 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 and Timasheff 1982, Carpenter *et al.* 1990, Lee and Timasheff 1981). Lee and Timasheff (1981) suggested that the presence of these co-solutes in a protein solution creates a thermodynamically unfavorable situation, since the chemical potentials of both the protein and the additive are increased. Sola-Penna and Meyer-Fernandes (1994) and Sola-Penna *et al.* (1994, 1997) demonstrated that the efficacy of trehalose is higher than that achieved using other sugars such as maltose, sucrose, glucose or fructose. Studies made by these authors showed that trehalose is also more effective than other sugars in protecting yeast pyrophosphatase against thermal inactivation (Sola-Penna and Meyer-Fernandes 1994). Also pyrophosphatase and glucose 6-phosphate dehydrogenase were protected in a similar way against inactivation promoted by guanidinium chloride (Sola-Penna *et al.* 1997).

Recently it was shown that the extraordinary efficacy of trehalose is due to its 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 and Meyer-Fernandes 1998).

Some authors have shown that carbohydrates decrease the K_m for ortophosphate in the phosphorylation reaction catalyzed by the Ca-ATPase from sarcoplasmic reticulum (Chini *et al.* 1991) and increase the rate of ATP–P_i exchange catalyzed by membranes (Vieyra *et al.* 1989, 1991). Furthermore, it was observed previously that the extent of urea-induced denaturation of ovalbumin was reduced in the presence of sucrose, indicating a possible stabilization effect (Simpson and Kauzman 1953).

In this study we investigated the effect of sugars (trehalose, sucrose, maltose) and glycerol on the thermal stability of OCT isolated from *Alopias vulpinus* liver.

Material and Methods

Material

Livers of the thresher shark *Alopias vulpinus* were obtained from animals that had died of natural causes along the Italian coast.

δ-N-(phosphonacetyl)-L-ornithine (δ-PALO) was synthesized and further purified essentially as described by De Martines *et al.* (1981); bovine albumin, citrulline, L-ornithine hydrochloride, carbamoyl phosphate dilithium salt, N-ethylmorpholine, MES, diethanolamine, diacetylmonoxime, thiosemicarbazide and tris (hydroxymethyl) aminomethane, trehalose, sucrose, maltose and glycerol were purchased from Sigma (St. Louis, MO), epoxy-activated Sepharose 6B was obtained from Pharmacia LKB.

Enzyme purification

The enzyme was purified as described previously (Bellocco *et al.* 2002a), with small modifications.

Protein determination

Protein determination was based on the binding of Coomassie brilliant blue G-250 (Bradford 1976) using bovine serum albumin as standard.

Assay of OCT

OCT activity was measured as the rate of citrulline formation from ornithine and carbamoyl phosphate (Lusty *et al.* 1979). The reaction mixtures, in a final volume of 1.0 ml, contained the tri-buffer (diethanolamine/MES/N-ethylmorpholine 0.051 M/ 0.1 M/ 0.051 M) pH 7.8, 10 mM ornithine, 10 mM carbamoyl phosphate and an appropriate amount of ornithine transcarbamoylase. After incubation for different intervals of time at 37 °C, the reaction was terminated by the addition of 2.0 ml acid reagent (3.7 g of antipyrine and 2.5 g of ferric ammonium sulfate in 500 ml H_2O, 250 ml of concentrated H_2SO_4, and 250 ml 85 % H_3PO_4). One milliliter of 0.4 % diacetylmonoxime was added and the samples were incubated for 15 min in a boiling water bath. Citrulline concentration was determined from the absorbance at 464 nm, using appropriate standards. One unit of OCT activity was defined as the amount of the enzyme, that catalyzes the formation of 1 μmole of citrulline per min at 37 °C under standard assay conditions.

Thermal stability

The thermal stability was measured by incubating an appropriate amount of enzyme (0.4 mg/ml) in Tris-HCl buffer 50 mM (pH 7.8), 2 mM β-mercapto-ethanol in the absence or in the presence of trehalose, sucrose, maltose 0.5 M and glycerol 1.6 M at 60-64-66-68-70 °C. Aliquots (5 µl) of samples were removed at the appropriate times, diluted 50-fold, and assayed for OCT as described under experimental conditions.

Determination of OCT activation energy

The activation energy of OCT as modified by different additives was determined using an Arrhenius plot. The reactions were performed in buffer and in modified media by addition of compounds in the temperature range from 30 to 64 °C. The substrate and enzyme concentrations were 10 mM and 0.066 µM, respectively.

Table 1. Half-life of native OCT and OCT treated with different additives. OCT (0.4 mg/ml) was preincubated at different temperature and intervals of time in 50 mM Tris-HCl (pH 7.8) containing 2 mM β-mercaptoethanol and in the presence of co-solutes. After preincubation, the samples were diluted 50-fold and assayed as described in the Materials and Methods. The half-life of the enzyme was determined by plotting the logarithm of the residual activity against heating time

Additive	**Additive concentration (M)**	**Half-life (min)**				
		60 °C	**64 °C**	**66 °C**	**68 °C**	**70 °C**
(Buffer alone)		18.7	9.1	3.3	1.9	1.1
Trehalose	0.5	100.4	40	12.37	5.2	2
Glycerol	1.6	40.8	14	4.6	1.16	0.69
Maltose	0.5	50.9	28	14.7	3.6	1.2
Sucrose	0.5	32.2	22	14.6	3.15	1.0
Ornithine	0.05	84.5	30	6.47	2.3	0.6
Phosphate	0.025	100.3	50	43	9.6	1.1
Trehalose + phosphate	0.50.025	stable	1200	462	89.7	3.6
Trehalose + ornithine	0.50.05	169	120	88.8	13.8	1.2
Glycerol + phosphate	1.60.025	stable	299.8	187	22.9	2.16
Glycerol + ornithine	1.60.05	199.8	91.4	20.75	4.3	0.85
Maltose + phosphate	0.50.025	stable	360.3	161.2	23.6	5.77
Maltose + ornithine	0.50.05	88.8	68.4	41.25	5.8	1.98
Sucrose + phosphate	0.50.025	210	171.8	90.23	9.8	1.2
Sucrose + ornithine	0.50.05	180	113.6	30.1	4.1	0.6

Determination of kinetic parameters

Kinetic parameters (v_{lim} and K_m) were determined for both substrates (keeping one substrate constant at saturation and varying the concentration of the other) using a nonlinear regression method based on the Michaelis-Menten equation. The activation coefficient (%) for carbamoyl phosphate (a) and ornithine (b), for each additive, is defined as the ratio between the maximum enzyme efficiency (k_{cat}/K_a or K_b) obtained in the presence of that additive and the efficiency determined in the buffer medium.

Results

When OCT was preincubated from 60 to 70 °C its activity, measured in a standard assay medium at 37 °C, decreased as a function of preincubation time. In samples containing 400 µg/ml of enzyme extract, the half-life (min) was found to be 18.7 at 60 °C, 9.1 at 64 °C, 3.3 at 66 °C, 1.9 at 68 °C and 1.1 at 70 °C (Table 1). Figure 1 shows that the addition of ornithine, ortophosphate, sugars, or glycerol (at concentrations 0.05, 0.025, 0.5 and 1.6 M, respectively), or sugars with ornithine or ortophosphate, to the preincubation medium (60 °C) protected the enzyme against inactivation, increasing its stability at higher temperatures. Only sucrose did not affect the thermal stability of OCT, while sucrose plus ornithine or plus ortophosphate was less effective than ornithine or ortophosphate alone in protecting the enzyme. At 60 °C the presence of both

ornithine and ortophosphate plus trehalose during preincubation enhanced the OCT activity still further. Similar results were obtained with glycerol or maltose, but each of them alone had a lower effect on enzyme stability (Fig. 1). The stabilization observed in presence of glycerol could be attributed to the delicate balance between repulsion of non polar regions, attraction of the polar region of protein surface and interaction glycerol-water that could lead to an enhancement of internal protein hydrophobic interaction (Gekko and Timasheff 1981, Dreyfus *et al.* 1978). At 64 and 66 °C we obtained similar results, but at these temperatures the enzyme is stable over a shorter time.

Table 2. Kinetic parameters of OCT in the absence and presence of 0.5 M sugars and 1.6 M glycerol.

	37 °C				43 °C				53 °C			
Experimental conditions	K_a (mM)	K_b (mM)	v_{lim} ($mMmin^{-1}$) a	b	K_a (mM)	K_b (mM)	v_{lim} ($mMmin^{-1}$) a	b	K_a (mM)	K_b (mM)	v_{lim} ($mMmin^{-1}$) a	b
Enzyme alone	0.06	0.56	0.25	0.31	0.055	0.54	0.69	0.81	0.30	1.66	0.80	0.98
Trehalose	1.25	3.30	0.24	0.26	0.310	1.86	0.65	0.82	1.40	5.60	0.78	0.97
Maltose	0.10	0.40	0.21	0.27	0.065	0.46	0.57	0.82	0.51	1.98	0.77	0.93
Glycerol	0.095	0.44	0.22	0.29	0.066	0.48	0.63	0.81	0.45	1.49	0.75	0.95

Carbamoyl phosphate is denoted by "a" and L-ornithine by "b", K_a and K_b are the corresponding Michaelis constants, i.e. the concentration of additives "a" or "b" giving half-maximal velocity when the other substrate is saturating.

Table 3. Kinetic parameters of OCT in the absence and presence of 0.5 M sugars and 1.6 M glycerol.

	37 °C						43 °C						53 °C					
Experimental conditions	k_{cat} a	b	$\frac{k_{cat}}{K_a}$	$\frac{k_{cat}}{K_b}$	*A.C.* a	b	k_{cat} a	b	$\frac{k_{cat}}{K_a}$	$\frac{k_{cat}}{K_b}$	*A.C.* a	b	k_{cat} a	b	$\frac{k_{cat}}{K_a}$	$\frac{k_{cat}}{K_b}$	*A.C.* a	b
Enzymealone	4.3	5.1	72	9	100	100	12	14	218	27	100	100	11	17	36	10	100	100
Trehalose	4.1	4.4	3.3	1.3	4.6	14	11	11	35	6	16	41	13	17	9	3	25	29
Maltose	3.5	4.6	35	12	49	126	10	14	149	30	68	88	13	16	25	9	70	83
Glycerol	3.7	4.9	39	11	54	122	11	16	167	34	77	96	13	17	28	12	79	116

Carbamoyl phosphate is denoted by "a" and L-ornithine by "b"; k_{cat} is the catalytic constant and the activation coefficient (A.C. %) for each additive is defined as the ratio of the maximum enzymatic efficiency (k_{cat}/K_a or k_{cat}/K_b) obtained in the presence of that additive relative to the efficiency determined in the buffer medium. k_{cat} is expressed as $s^{-1}x10^6$ and k_{cat}/K_a or k_{cat}/K_b as $mM^{-1}\ s^{-1} \times 10^6$

At 70 °C the half-life of the enzyme with trehalose, maltose or glycerol plus ortophosphate, was found to be 3.6, 5.77 and 2.16 min, respectively, and its activity was completely lost after 7 min, whereas less than 10 % of the original activity remained after preincubation for 4 min with other co-solutes (Table 1). The enzyme activity measured during preincubation at 60 °C and in the presence of 0-0.5 M co-solute showed that at these temperatures the co-solute protected the enzyme activity in a concentration-dependent manner (data not shown). These results are in agreement with those reported by other authors (Meyer-Fernandes *et al.* 2000, Mejri *et al.* 1998, Mejri and Mathlouthi 2001). In the same way as with yeast pyrophosphatase and glutathione reductase (Sola-Penna and Meyer-Fernandes 1994, Sebollela *et al.* 2004), it was observed that the presence of trehalose in the reaction medium inhibited the enzyme activity of OCT. At 30, 37, 43, 50, 57 and 60 °C, a concentration ranging from 0.25-0.5 M of trehalose had the same effect, reducing the activity by 40, 34, 31, 26, 22 and 17 % with respect to the control value. At the same temperature, maltose at the same concentration,

glycerol at triple concentration and trehalose at 10 % concentration, did not show any effect on the activity of OCT (Fig. 2). Kinetic data of the highly purified *Alopias vulpinus* liver enzyme for carbamoyl phosphate and ornithine with and without sugars and polyols were obtained over a wide range of substrate concentrations at 37, 43 and 53 °C. The results are shown in Tables 2 and 3. The values of the kinetic constants (K_a, K_b, v_{lim}, k_{cat}) measured with or without glycerol and maltose do not show significant variation.

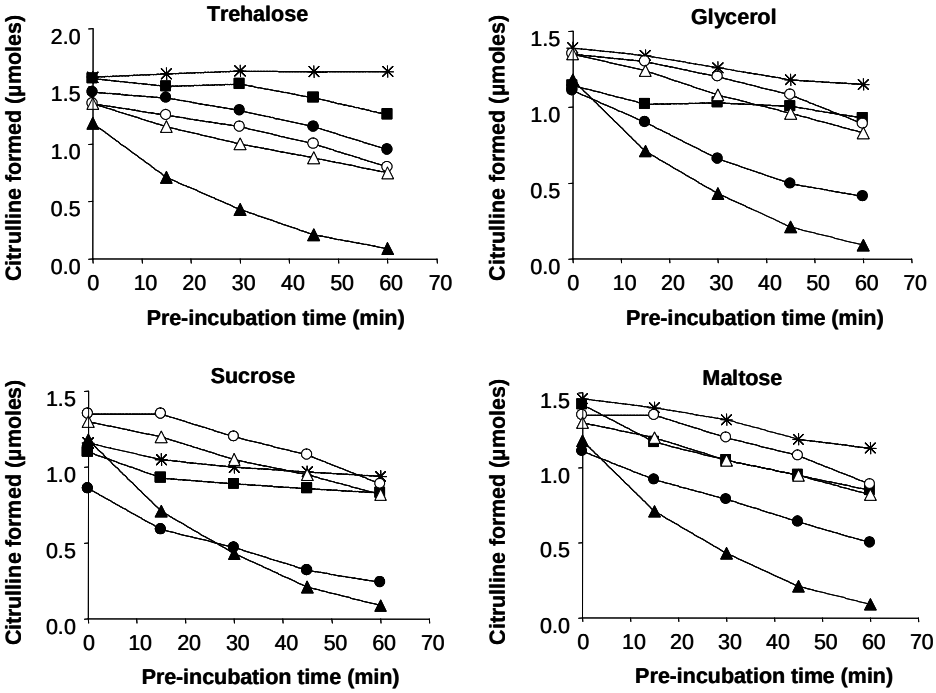

Fig. 1. The influence of sugars and glycerol on thermal stability of OCT. OCT (0.4 mg/ml) was preincubated at 60 °C for the times indicated in the absence (▲) or in the presence of 0.5 M sugars or 1.6 M glycerol (●), 0.05 M ornithine (△), 0.025 M P_i (○), or 0.05 M ornithine plus 0.5 M sugars or 1.6 M glycerol (■), 0.025 M P_i plus 0.5 M sugars or 1.6 M glycerol (✳). After preincubation, the samples were diluted 50-fold and assayed as described in the Materials and Methods.

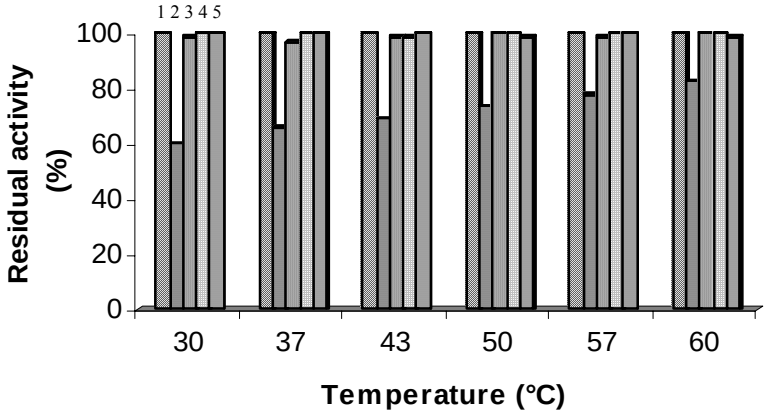

Fig. 2. Effect of additives on OCT (0.4 mg/ml) activity in a wide range of temperatures. Conditions were tri-buffer (pH 7.8), 10 mM ornithine and carbamoyl phosphate or modified media by additives. 1 - enzyme alone, 2 - 0.5 M trehalose; 3 – 0.05 M trehalose , 4 - 0.5 M maltose, 5 - 1.6 M glycerol.

Kinetic measurements reveal that K_a and K_b were affected at all temperatures by the presence of trehalose. At 37, 43 and 53 °C the K_a, values increased by factors of about 21, 6, 6, and K_b 4, 5, 4, respectively, whereas no significant changes in v_{lim} and therefore k_{cat} were observed (Tables 2 and 3). As a consequence the enzyme efficiency expressed as k_{cat}/K_a at 37, 43 and 53 °C decreased by factors of about 22, 7, 6 and k_{cat}/K_b of about

4, 4 and 3 with respect to that obtained for OCT in buffer alone.

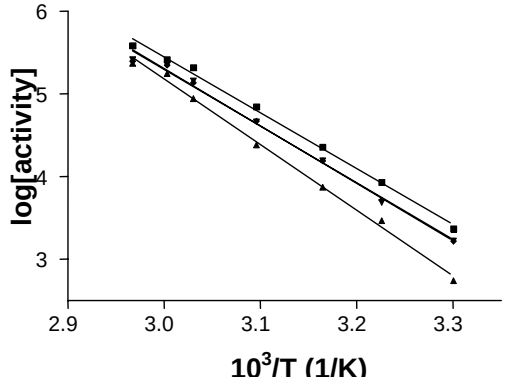

Fig. 3. Arrhenius plot for OCT. The reaction rate constant (*K*) for OCT was determined at different temperatures (30-64 °C) in the absence (■) and presence of 0.5 M trehalose (▲), 0.5 M maltose or 1.6 M glycerol (▼). Conditions were tri-buffer (pH 7.8), 10 mM ornithine and carbamoyl phosphate or sugar (0.5 M) or glycerol (1.6 M). The final concentration of the enzyme was 0.066 µM.

The activation energy (E_a) was determined by linear regression analysis of the Arrhenius plot in a temperature range of 30-64 °C (Fig. 3). The E_a values obtained by this plot were: 56.1 kJ/mol for the native enzyme, 65.3 kJ/mol in the presence of 0.5 M trehalose, 53.4 in the presence of 0.5 M maltose, and 54.0 in the presence of 1.6 M glycerol.

Discussion

The higher efficiency of several organic solutes, such as sugars or sugar alcohols in stabilizing macromolecules and modulating enzyme function has been studied (Sola-Penna and Meyer-Fernandes 1996, Miroliaei and Nemat-Gorgani 2001). It has been suggested that the ability of sugars to stabilize proteins depends on the preferential hydration of protein. This is true when the protein is dissolved in a sugar solution (Arakawa and Timasheff 1982, Carpenter *et al.* 1990).

Enzyme inhibition with trehalose may be explained in terms of its preferential hydration capacity in aqueous solution. This causes a decrease of the solvation layer of the enzyme, thereby reducing its flexibility and stabilizing it by a decreasing of its activity (Sola-Penna and Meyer-Fernandes 1998). As reported in Figure 2 the presence of trehalose, at high concentration in the mix reaction, influenced enzymatic activity in a temperature-dependent manner. Enzymatic activity inhibition induced by trehalose decrease in concomitance with temperature enhancement. This inverse proportionality between enzyme inhibition decrease and temperature increase may be attributed to the higher mobility of the enzyme which induces an increase of catalytic activity. However, this hypothesis (Sola-Penna and Meyer-Fernandes 1998, Back *et al.* 1979) does not explain why trehalose is more efficient than glycerol in inhibiting and protecting OCT, remembering that glycerol has been added at a concentration occupying the same hydrated volume. The decrease of enzyme inhibition at high temperature, observed in the presence of trehalose can be due to an increased enzyme mobility that leads to a higher catalytic activity. The considerable increase of K_a at 37 °C in the presence of trehalose can be attributed to larger hydrated volume and consequent higher viscosity of the medium.

This leads to increased steric obstruction and consequent decreased diffusion of substrate, reducing the possibility to form the enzyme-substrate complex. Recently it has been shown (Sola-Penna and Meyer-Fernandes 1998) that the specific viscosity of a trehalose solution is about 2.5-fold higher than that of a solution with the same concentration of maltose or sucrose. Trehalose and glycerol viscosities are not comparable because of the small dimension of the glycerol molecule. It appears that E_a for the native enzyme is similar to that determined in the presence of maltose and glycerol, while the presence of trehalose in the reaction medium seems to increase the energy barrier needed for the reaction to proceed and to lead to enzyme activation. It was impossible to test the effects of sucrose at high concentration, directly introduced into the reaction mixture because it interferes with the colorimetric method used for citrulline determination.

In conclusion, the results shown here are consistent with the idea that preferential hydration does not always stabilize the native structure of globular proteins (Sola-Penna *et al.* 1994, Mejri and Mathlouthi 2001, Arakawa *et al.* 1990). This study has shown that the magnitude of the stabilizing effect depends not only on the nature of the polyols and their concentration, but also on the nature of the protein. The ability to promote thermal stability or inhibition of OCT activity may be related to different physical properties of the polyols tested, in agreement with those reported by others (Duda and Stevens 1990).

References

ANDERSON PM, CASEY CA: Glutamine dependent synthesis of citrulline by isolated hepatic mitochondria from *Squalus acanthias*. *J Biol Chem* **259**: 456-462, 1984.

ARAKAWA T, TIMASHEFF SN: Stabilization of protein structure by sugars. *Biochemistry* **21**: 6536-6544, 1982.

ARAKAWA T, BHAT R, TIMASHEFF SN: Why Preferential hydration does not always stabilize the native structure of globular proteins. *Biochemistry* **29**: 1924-1931, 1990.

BACK JF, OAKENFULL D, SMITH MB: Increased thermal stability of proteins in presence of sugar. *Biochemistry* **18**: 5191-5196, 1979.

BATES M, WEISS RL, CLARKE S: Ornithine transcarbamylase from *Neurospora crassa*: purification and properties. *Arch Biochem Biophys* **239**: 172-183, 1985.

BELLOCCO E, LEUZZI U, DE GREGORIO A, LAGANÀ G, RISITANO A, DESIDERI A, CUZZOCREA G: Partial purification and properties of ornithine carbamoyltransferase from citrus *Limonum* leaves. *Biochem Mol Biol Int* **29**: 281-290, 1993.

BELLOCCO E, DI SALVO C, LAGANÀ G, LEUZZI U, TELLONE E, KOTYK A, GALTIERI A: Purification and properties of ornithine carbamoyltransferase from loggerhead turtle liver. *Physiol Res* 51: 151-158, 2002a.

BELLOCCO E, DI SALVO C, LAGANÀ G, GALTIERI A, FICARRA S, KOTYK A, LEZZI U: Ornithine carbamoyltransferase from *Spinacea oleracea*: purification and characterization. *Biol Plant* **45**: 533-538, 2002b.

BRADFORD MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing principle of protein dye binding. *Anal Biochem* **72**: 248-254, 1976.

CARPENTER JF, CROWE LH, ARAKAWA T: Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. *J Dairy Sci* **73**: 3627-3636, 1990.

CHINI EN, MEYER-FERNANDES JR, SOLA-PENNA M: Monosaccharides and disaccharides decrease the Km for phosphorylation of a membrane-bound enzyme ATPase. *Z Naturforsch C* **46**: 644-646, 1991.

CLARKE S: The polypeptides of rat liver mitochondria: identification of a 36.000 dalton polypeptide as the subunit ornithine transcarbamylase. *Biochem Biophys Res Commun* **71**: 1118-1124,1976.

DE MARTINES ML, MCINTYRE P, HOOGENRAAD NA: Rapid batch method for purifying ornithine transcarbamylase based on affinity chromatography using immobilized transition-state analog. *Biochem Int* **3**: 371-378, 1981.

DREYFUS M, VANDERBUNDER B, BUC H: Stabilization of a phosphorylase b active conformation by hydrophobic solvents. *FEBS Lett* **95**: 185-189, 1978.

DUDA CA, STEVENS ES: Trehalose conformation in aqueous solution from optical rotation. *J Am Chem Soc* **112**: 7406-7407, 1990.

GEKKO K, TIMASHEFF SN: Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* **20**: 4667-4676, 1981.

KALOUSEK F, FRANÇOIS B, ROSENBERG LE: Isolation and characterization of ornithine transcarbamoylase from normal human liver. *J Biol Chem* **253**: 3939-3944, 1978.

KOGER JB, HOWELL RG, KELLY M, JONES EE: Purification and properties of porcine liver ornithine transcarbamoylase. *Arch Biochem Biophys* **309**: 293-299, 1994.

LEE JC, TIMASHEFF SN: The stabilization of proteins by sucrose. *J Biol Chem* **256:** 7193-7201, 1981.

LEGRAIN C, STALON V: Ornithine carbamoyl transferase from *Escherichia coli* W. Purification, structure and steady-state kinetic analysis. *Eur J Biochem* **63**: 289-301, 1976.

LUSTY CJ, JILKA RL, NIETSCH EH: Ornithine transcarbamylase of rat liver, kinetic, physical and chemical properties. *J Biol Chem* **254**: 10030-10036, 1979.

MARSHALL M, COHEN PP: Ornithine transcarbamylase from *Streptococcus faecalis* and bovine liver. *J Biol Chem* **247**: 1654-1668, 1972.

MEJRI M, MATHLOUTHI M: Effect of small carbohydrates on the catalytic activity of a protease and two glycohydrolases. *Carbohydrate Polymers* **45**: 161-167, 2001.

MEJRI M, PAUTHE E, LARETTA-GARDE V, MATHLOUTHI M: Effects of polyhydroxylic additives on the activity of thermolysin. *Enzyme Microb Technol* **23**: 397-402, 1998.

MEYER-FERNANDES JR, ARRESE EL, WELLS MA: Allosteric effectors and trehalose protect larval *Manduca sexta* fat body glycogen phosphorylase B against thermal denaturation. *Insect Biochem Mol Biol* **30**: 473-478, 2000.

MIROLIAEI M, NEMAT–GORGANI M: Sugars protect native and apo yeast alcohol dehydrogenase against irreversible thermoinactivation. *Enzyme and Microbial Technology* **29**: 554-559, 2001.

MURATA LB, SCHACMAN HK: Structure similarity between ornithine and aspartate transcarbamoylase of *Escherichia coli*: characterization of the active site and evidence for an interdomain carboxy-terminal helix in ornithine transcarbamoylase. *Protein Science* **5**: 709-718, 1996.

SANCHEZ R, BAETENS M, VAN DE CASTLEELE M, ROOVERS M, LEGRAIN C, GLANSDORFF N: Ornithine carbamoylase from the extreme thermophile *Thermus thermophilus*. Analysis of the gene and characterization of the protein. *Eur J Biochem* **248**: 466-474,1997.

SEBOLLELA A, LOUZADA PR, SOLA-PENNA M, SARONE-WILLIAMS V, COELHO-SAMPAIO T, FERRIERA ST: Inhibition of yeast glutathione reductase by trehalose: possible implications in yeast survival and recovery from stress. *Intl J Biochem Cell Biol* **36**: 900-908, 2004.

SIMPSON RB, KAUZMAN W: The kinetics of protein denaturation and the behavior of the optical rotation of ovalbumin in urea solutions. *J Am Chem Soc* **75**: 5139-5139, 1953.

SLOCUM RD, NICHOLS HF, WILLIAMSON CL: Purification and characterization of *Arabidopsis* ornithine transcabamoylase (OTCase), a member of a distinct and evolutionarily-conserved group of plant OTCases. *Plant Physiol Biochem* **38**: 279-288, 2000.

SLOCUM RD, RICHARDSON DP: Purification and characterization of ornithine transcabamylase from pea (*Pisum sativum*). *Plant Physiol* **96**: 262-268, 1991.

SOLA-PENNA M, FERREIRA-PEREIRA A, LEMOS AP, MEYER-FERNANDES JR: Carbohydrate protection of enzyme structure and function against guanidinium chloride treatment depends on the nature of carbohydrate and enzyme. *Eur J Biochem* **248**: 24-29, 1997.

SOLA-PENNA M, MEYER-FERNANDES JR: Protective role of trehalose in thermal denaturation of yeast pyrophosphatase. *Z Naturforsch C* **49**: 327-330, 1994.

SOLA-PENNA M, MEYER-FERNANDES JR: Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: why is trehalose more effective than other sugars? *Arch Biochem Biophys* **360**: 10-14, 1998.

SOLA-PENNA M, MEYER-FERNANDES JR: Trehalose protects yeast pyrophosphatase against structural and functional damage induced by guanidinium chloride. *Z Naturforsch C* **51**: 160-164, 1996.

SOLA-PENNA M, VIEYRA A, MEYER-FERNANDES JR: Uncoupling by trehalose of Ca^{2+} transport and ATP hydrolysis by plasma membrane (Ca^{2+}+Mg^{2+}) ATPase of kidney tubules. *Z Naturforsch C* **49**: 141-146, 1994.

VIEYRA A, CARUSO-NEVES C, MEYER-FERNANDES JR: ATP↔^{32}Pi exchange catalysed by plasma membrane Ca^{2+}-ATPase from kidney proximal tubules. *J Biol Chem* **266**: 10324-10330, 1991.

VIEYRA A, CARUSO-NEVES C, MEYER-FERNANDES JR: Regulation of the reversal cycle of the calcium pump from kidney proximal tubules. *Methodol Surv Biochem Anal* **19**: 31-41, 1989.

WARGNIES B, LEGRAIN C, STALON V: Anabolic ornithine carbamoyltransferase of *Escherichia coli* and catabolic ornithine carbamoyltransferase of *Pseudomonas putida*. Steady-state kinetic analysis. *Eur J Biochem* **89**: 203-212, 1978.

XIONG X, ANDERSON PM: Purification and properties of ornithine carbamoyl transferase from liver of *Squalus acanthias*. *Arch Biochem Biophys* **270**: 198-207, 1989.

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