INFLUENCE OF UREA ON STABILITY OF INVERTASE FROM *SACCHAROMYCES CEREVISIAE*

(INFLUÊNCIA DA URÉIA NA ESTABILIDADE DA INVERTASE DE SACCHAROMYCES CEREVISIAE)

Ubirajara COUTINHO FILHO[1]; Nilson PENHA-SILVA[2]

ABSTRACT: This paper aimed to investigate the dependence between the unfolding equilibrium of invertase by urea, according to a two-state mechanism, with the thermodynamic properties of the solvent as calculated by application of the McMillian-Mayer theory. Unfolding equilibrium of invertase was monitored by spectrophotometric titration at 280 nm with a 8 mol.L^{-1} urea solution, and compared with data for RNase A and RNase T_1. The good fitting of the equilibrium data for all proteins using a first order model is consistent with the idea that the high carbohydrate content of invertase does not affect the general mechanism of protein unfolding. However, an increase in the urea concentration produced a decrease in the Gibbs free energy of unfolding ($\Delta G°_U$) for invertase, and an increase in the $\Delta G°_U$ values for RNase A and RNase T1. Invertase also required higher water activity values (A_w) than RNAse A and RNAse T_1 for unfolding. The different behavior of invertase in relation to those enzymes may be relevant to provide additional information about the detailed mechanisms of protein folding and unfolding.

UNITERMS: invertase, McMillan-Mayer theory, protein unfolding, urea, water activity.

Abbreviations used: A_w, water activity; F, folded state of a protein; U, unfolded state of a protein; D, molar concentration of denaturant; $\Delta G°_U$, unfolding free energy, always expressed in kcal.mol^{-1}; K_{eq}, equilibrium constant.

INTRODUCTION

According to the literature (PACE et al., 1991; BOLEN; YAO, 1995; SANTORO; BOLEN, 1988), the equilibrium between the folded (**F**) and unfolded (**U**) states of a protein can be described by a two state equilibrium model,

$$\mathbf{F} \leftrightarrows \mathbf{U} \qquad \textbf{(1),}$$

in which the summation of the fractions of unfolded ($\mathbf{f_U}$) and folded ($\mathbf{f_F}$) molecules is considered equal to 1,

$$\mathbf{f_U + f_F = 1} \qquad \textbf{(2).}$$

At an intermediate point of the transition (**y**),

$$\mathbf{y = y_U . f_U + y_F . f_F} \qquad \textbf{(3),}$$

where y_F is the intermediate point of the folding curve and y_U is the intermediate point of the unfolding curve.

The fraction of unfolded molecules ($\mathbf{f_U}$) can be determined according to a expression derived from **Equation 3**,

$$\mathbf{f_U} = \frac{\mathbf{y_F - y}}{\mathbf{y_F - y_U}} \qquad (4).$$

Since the equilibrium constant, $\mathbf{K_{eq}}$, for the unfolding process is the relation between $\mathbf{f_U}$ and $\mathbf{f_N}$, it is implicit from **Equation 2** that

$$\mathbf{K_{eq}} = \frac{\mathbf{f_U}}{\mathbf{1 - f_U}} \qquad (5).$$

[1] Professor, Faculdade de Engenharia Química, Universidade Federal de Uberlândia.
[2] Professor, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia.
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From that equilibrium constant, we can obtain the unfolding free energy ($\Delta G°_U$) by the expression

$$\Delta G°_U = - RT \ln K_{eq} \quad (6).$$

With the aim to obtain more detailed information on the protein unfolding process, the present study describes the effect of urea on unfolding of invertase in comparison to RNAase A and RNAase T_1. Invertase (β-D-fructofuranosidase, EC 3.2.1.26) was here chosen due to its high carbohydrate concentration, approximately 50% w/w, which improves the solubility of folded and unfolded states of the protein without affecting its mechanism of folding (ATHÈS; COMBES, 1997; BERGGREN, 1967; LI; YANG; ZHOU, 1998; NAUMOV; DOROSHENKO, 1998).

MATERIAL AND METHODS

Material

Invertase from *Saccharomyces cerevisiae* (Sigma Chemical Company, grade VII) was obtained and used without further purification. Potassium chloride, urea, and all others chemicals here used were of analytical grade. 0.0020 $g.L^{-1}$ invertase solutions were prepared daily in 0.05 $mol.L^{-1}$ KCl at pH 7.0. Urea solutions at 8 $mol.L^{-1}$ were prepared daily in deionized water.

Invertase unfolding

The unfolding equilibrium of invertase by urea was determined according to the literature (LI; ZHOU; YANG, 1998; SCHÜLKE; SCHIMID, 1990), from absorbance measurements at 280 nm and 25 °C, in a solution containing invertase in 0.05 $mol.L^{-1}$ KCl against a blank with 0.05 $mol.L^{-1}$ KCl, before and after addition of small increments of a 8 $mol.L^{-1}$ urea solution, until the urea concentration in the measurement cell reach the limit of 4 $mol.L^{-1}$. All absorbance values were duly corrected for the protein dilution.

Equilibrium constant (K_{eq}), Gibbs free energy ($\Delta G°$) and water activity (A_w)

The equilibrium constant (K_{eq}) and the unfolding Gibbs free energy change ($\Delta G°_U$) were estimated assuming a two state model for the unfolding transition. The water activity (A_w) values for the urea solutions were obtained with basis on the McMillan-Mayer model (SIMONIN, 1999):

$$\ln(A_w) = \frac{-\Phi^{LR} x_w}{x_{urea}} \quad (7),$$

where x_{urea}, x_w and ϕ^{LR} are the molar fractions of urea and water, and the osmotic coefficient of the urea solutions, respectively.

RESULTS AND DISCUSSION

The spectrophotometric unfolding curve of invertase by urea is shown in **Figure 1**. The high carbohydrate content of invertase did not affect the general sigmoidal shape of the unfolding equilibrium.

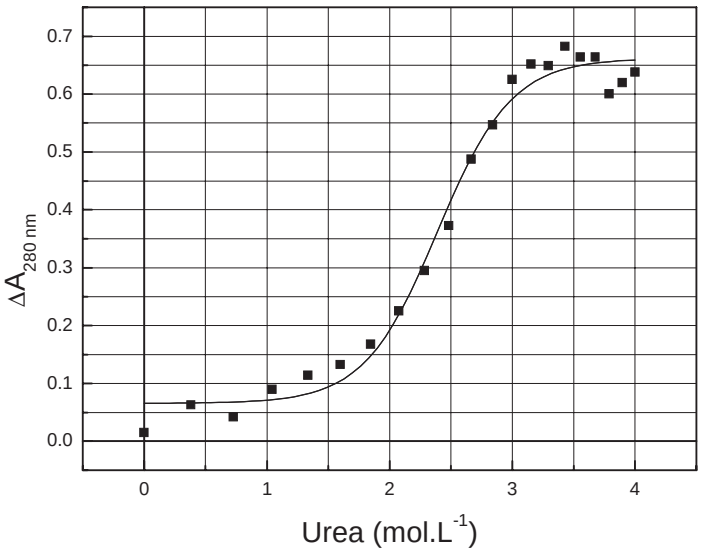

Figure 1. Dependence of absorbance at 280 nm of invertase with the urea concentration of the solution, showing the transition between the folded and unfolded states of the protein.

The increase in urea concentration is associated to a decrease in the unfolding Gibbs free energy for invertase (**Figure 2**), differently from RNAse A and RNAse T1, which presented direct relations between urea concentrations and the unfolding Gibbs free energy (BOLEN; YAO, 1995; PACE et al., 1991; PACE; SHIRLEY; THOMSON, 1990; PACE et al. 1990).

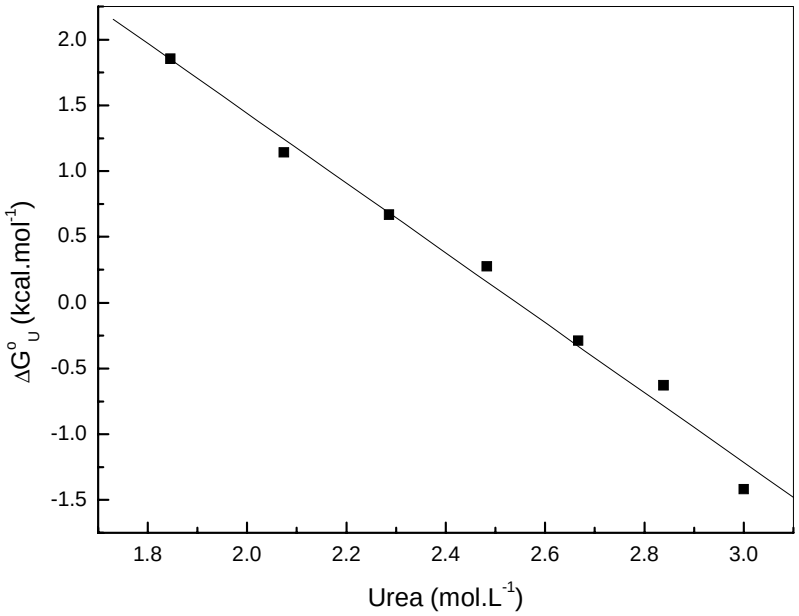

Figure 2. Dependence of the Gibbs free energy for the unfolding of invertase by urea with the concentration of urea.

As shown in Table I, the dependence of the free energy changes for unfolding of invertase by urea were linearly correlated to the water activity (A_w) values of the urea solutions. **Table I** also shows the parameters obtained from the analysis of RNAse A and RNAse T1 data, and reveals that invertase requires much higher values of A_w than RNase A and RNase T1 to stabilize the folded protein.

Table I. Linear regression parameters for the dependence of $\Delta G°_U$ with the activity of water, according the general equation $\Delta G°_U = \Delta G° (A_w) + m \, . \, A_w$

Protein	$\Delta G^o (A_w)$	m	$(A_w)_{1/2}$	r	p
RnaseA	95.3987	-101.6221	0.9388	- 0.97622	< 0.0001
RnaseT1	60.7984	-66.4469	0.9150	- 0.98896	< 0.0001
Invertase	-136.3337	142.9653	0.9536	+ 0.99402	< 0.0001

The equilibrium constant, K_{eq}, was fitted to a first order equilibrium model (**Table II**), based in the following relation:

$$\mathbf{\ln K_{eq} = \ln K_0 + \eta \, . \ln [D]} \qquad \mathbf{(8),}$$

in which $\mathbf{K_0}$ is the equilibrium constant at concentration of urea equal to 0 mol.L^{-1}, η is number of molecules of urea per aminoacid residue, and **[D]** is the concentration of urea (SCHELMAN, 2002). The dependence of $\mathbf{\ln K_{eq}}$ with **ln [D]** for invertase is not significantly different from the same dependence obtained for RNase A and RNase T_1. This observation is consistent with the fact that the carbohydrate content of invertase does not affect the mechanism of invertase unfolding. This suggests that invertase, and perhaps others glycoproteins, may contribute to overcome the lack of information about the detailed mechanisms of protein folding and unfolding.

Table II. Linear regression parameters for the dependence of $\ln K_{eq}$ with **ln [D]**, according the general equation $\ln K_{eq} = \ln K_0 + \eta \cdot \ln [D]$.

Protein	$\ln K_0$	η	r	P
RnaseA	-12.233	10.3870	0.9956	< 0.0001
RnaseT1	-14.334	9.6381	0.9948	< 0.0001
Invertase	-9.796	10.6209	0.9881	< 0.0001

CONCLUSIONS

The good fitting to a first order model presented by the equilibrium data for invertase, as also for RNase A and RNase T1, is consistent with the idea that the high carbohydrate content of invertase does not affect the general mechanism of protein unfolding. However, an increase in the urea concentration produced a decrease in the Gibbs free energy of unfolding ($\Delta G°_U$) for invertase, but an increase in the $\Delta G°_U$ values for RNase A and RNase T1. Invertase also required higher water activity values (A_w) than RNAse A and RNAse T1 to be unfolded, which may be due to its high carbohydrate content.

RESUMO: Este trabalho objetivou investigar a dependência entre o equilíbrio de desenovelamento da invertase por uréia, segundo um mecanismo de equilíbrio entre dois estados, com algumas propriedades termodinâmicas do solvente. Os valores das propriedades termodinâmicas do solvente foram calculados com aplicação da teoria de McMillian-Mayer. O equilíbrio de desenovelamento da invertase foi acompanhado por titulação espectrofotométrica em 280 nm com uma solução de uréia a 8 $mol.L^{-1}$. O bom ajuste a um modelo de primeira ordem apresentado pelos dados de equilíbrio da invertase, bem como da RNase A e da RNase T1, é consistente com a idéia de que o alto conteúdo em carboidratos da invertase não afeta o mecanismo geral de desenovelamento. Entretanto, um aumento na concentração de uréia produziu uma diminuição na energia livre de desenovelamento ($\Delta G°_U$) para a invertase, e um aumento nos valores de $\Delta G°_U$ para RNase A e RNase T_1. A invertase também exigiu maiores valores de atividade de água (A_w) do que a RNAse A e a RNAse T1 para ser desenovelada. O diferente comportamento da invertase em relação a aquelas enzimas pode se relevante para fornecer informação adicional sobre os mecanismos detalhados do enovelamento e desenovelamento de proteínas.

UNITERMOS: Invertase, Teoria de McMillan-Mayer, Desenovelamento de proteínas, Uréia, Atividade de água.

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