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Effect of chaotropic agents on reversible unfolding of a soybean (*Glycine max*) seed acid phosphatase

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Abstract

In this work we examined the effect of urea and guanidinium chloride on the structural stability of a single isoform of soybean seed acid phosphatase, based on the intensity of tryptophan fluorescence as a function of denaturant concentration. The free energy of unfolding, ΔG_u, was calculated at 25 °C as a function of the concentrations of both chaotropic agents; the conformational stability, ΔG (H_2O), was determined to be 2.48 kcal mol^{-1}. Center of mass, determined from analysis of fluorescence data, was used as a parameter to assess conformational changes. Our results indicate that complete enzyme inactivation occurred before full enzyme unfolding in both cases, and suggest that there are differences between the conformational flexibility of the active-site and that of the macromolecule as a whole.

Keywords: *Glycine max*; Leguminosae; Acid phosphatase; Unfolding; Chaotropic agents

1. Introduction

Acid phosphatases or orthophosphoric monoester phosphohydrolases [EC 3.1.3.2.] are a group of enzymes that catalyze the hydrolysis of a wide range of orthophosphate monoesters, and usually exhibit pH optima below 6.0. These enzymes are widely distributed in nature and have been studied in numerous organisms and tissues (Vincent et al., 1992). Plant acid phosphatases have been reported in several tissues, including seeds (Biswas and Cundiff, 1991; Ferreira et al., 1998a; Granjeiro et al., 1999), roots (Panara et al., 1990; Penheiter et al., 1997), leaves (Staswick et al., 1994), tubers (Gellatly et al., 1994) and bulbs (Guo and Pesacreta, 1997).

Many roles have been ascribed to acid phosphatases in plants, including participation in signal transduction (Plaxton, 1996), regulation of metabolism by protein dephosphorylation (Duff et al., 1994; Tsou, 1993) and the release of inorganic phosphate from organic phosphate in the environment. However, the metabolic function of these enzymes is not yet well understood, partially because of the occurrence of multiple isoforms. We have previously observed that the four soybean seed acid phosphatase isoforms could play important roles in plant metabolism acting on key glycolytic intermediates, specially phosphoenolpyruvate (Ferreira et al., 1999), and that one of the isoforms might be regulated by endogenous lectins (Aoyama et al., 2001). Shinano et al. (2001) have recently purified a tetrameric phosphatase from *Allium cepa* with high specificity for phosphoenolpyruvate.

Studies of protein and peptide unfolding may reveal information on the properties of partially folded intermediate states which could be relevant to understanding the pathway of folding (Plaxton, 1996; Dobson and Fersht, 1996; Levitt et al., 1997; Radford, 2000).

The unfolding and refolding studies by chemical denaturants have been reported for of a great number of proteins, such as human α_1-antitrysin (Kwon and Yu, 1997), staphylococcal nuclease R (Tian et al., 1998), chicken liver fatty acid synthase (Wu et al., 2001), horse heart cytochrome *c* (Bhuyan, 2002), and β-lactoglobulin (Owusu-Apenten, 2002). However, little work has been done on the stability and unfolding of acid

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phosphatases. Some authors have observed that the homodimeric human prostatic acid phosphatase displayed a highest relative quantum yield of the fluorescence in the native form, and a lowest in the denatured one (Ostrowski et al., 1993), and the occurrence of an inactive intermediate during refolding of the denatured enzyme (Kuciel et al., 1996). Chen et al. (1997) have observed more conformational flexibility at the active site of *Pinaeus penicillatus* acid phosphatase than in the enzyme molecule as a whole. In relation to plant acid phosphatase, Cashikar and Rao (1996) demonstrated that phosphate not only stabilized the red kidney bean enzyme to denaturation but also altered the unfolding pathway. All these studies of protein denaturation by urea and GdmCl have been performed through fluorescence spectroscopy and circular dichroism measurements. Bonafé et al. (1998) have studied tobacco virus disassembly by high pressure in combination with urea following the red shift of the center of mass of fluorescence spectra.

In a previous work, we demonstrated that soybean seed acid phosphatase was unusually stable in the presence of *p*-nitrophenylphosphate (Ferreira et al., 1998b). This enzyme contains carbohydrate and iron in its structure which may contribute to protein stability. In this work, we examined the reversible unfolding of soybean seed acid phosphatase obtained in the presence of chaotropic agents by fluorescence spectroscopy and evaluated the center of mass as a parameter of the enzyme stability in the presence of these compounds.

2. Results and discussion

The stability of proteins, especially enzymes, has long been a practical concern because this is usually the factor that most limits their usefulness (Pace, 1986). Moreover, the accurate determination of conformational stability is very important for understanding the physical interactions that stabilize the structure–function relationships of proteins.

2.1. Reversibility of the denaturation of soybean seed acid phosphatase

Since we are dealing with thermodynamic measurements, it is essential that the unfolding reaction has reached equilibrium before measurements are made and the unfolding reaction is reversible. In order to confirm these requirements, a purified acid phosphatase isoform (API) was incubated in 100 mM acetate buffer (pH 5.0), in the absence and presence of urea and GdmCl, at 25 °C, for 30 and 24 h, respectively, and fluorescence spectra were periodically taken. The reversibility of unfolding was tested by diluting the chaotropic agent 10-, 100- and 1000-fold (Fig. 1). Denaturation by urea and GdmCl was reversible, although renaturation with the latter required a greater dilution. The reversibility of unfolding was also confirmed by fluorescence measurements (not shown).

2.2. Fluorescence of soybean seed acid phosphatase denatured with urea and GdmCl

Fluorescence is a useful technique for studying the structure and dynamics of proteins (Pace, 1986). In the intrinsic fluorescence of proteins the environment surrounding tryptophan residues exhibits a red-shift of emission maximum as consequence of changes from nonpolar to polar. The conformational changes of a protein by denaturation exposes further these groups inducing increases in transfer rate and maximum emission.

Fluorescence spectra were obtained at 25 °C with varying concentrations of chaotropic agents and there were important differences between the effects of urea and GdmCl as demonstrated in Fig. 2 (only selected spectra are shown for illustration purposes). The intensity of emission increased only slightly up to 4 M urea (Fig. 2a) and then sharply decreased at 330 nm between 4 M and 8 M urea, with a red-shift in the maximum to 349 nm at 8 M. Changes in fluorescence intensity were larger when GdmCl was used, with a red-shift in the maximum to 355 nm at 6 M (Fig. 2b). The red-shift observed is due to energy transfer from the aromatic rings of tyrosine residues to the tryptophan residues, and to solvent exposition of buried tryptophan side chains of the denatured protein (Chen et al., 1997). When no tryptophan residues are present, such as with the two short N-terminal fragments of staphylococcal nuclease R (Tian et al., 1998), changes in intrinsic fluorescence for tyrosine can be used to indicate protein unfolding and refolding.

2.3. Denaturation curves for soybean seed acid phosphatase in the presence of urea and GdmCl

Denaturation curves are useful to probe the relationship between chemical structure and protein stability, providing information as to whether a small change in the chemical structure can affect the mechanism of protein denaturation.

The denatured fraction (F_u) was calculated as a function of the wavelengths of maximum intensity characteristic of the folded and unfolded states, λ_f and λ_u respectively, and the maximum wavelength (λ) observed for each concentration of denaturant (Fig. 3). Thus,

$$F_u = (\lambda_f - \lambda)/(\lambda_f - \lambda_u) \quad (1)$$

A two-state mechanism was assumed in both cases and only folded and unfolded conformations were considered to be present in significant concentrations (Pace,

1986). Since $F_u + F_f = 1$, where F_f is the folded fraction, the equilibrium constant can be considered as $K = F_u/F_f$, and the Gibb's free energy change resulting for the unfolding process, ΔG_u, can be calculated as a function of denaturant concentration at 25 °C, as shown in Fig. 3 (inset) for both chaotropic agents:

$$\Delta G_u = -RT \ln K \quad (2)$$

where R is the gas constant (1.987 cal mol^{-1} K^{-1}) and T is the absolute temperature.

Linear extrapolation to zero concentration for the denaturant gives the value for the conformational stability of the enzyme, ΔG (H_2O). Although GdmCl was a much more potent denaturant agent than urea, the ΔG (H_2O) values obtained for both chaotropic agents were in agreement and were equal to 2.48 kcal mol^{-1}. This value is in agreement with reported values of the energy of stabilization, in the range of 1–4 kcal mol^{-1} (Reilly et al., 1994). Other ΔG (H_2O) values have been reported for other proteins such as recombinant human FK, 5.9 kcal mol^{-1} (Egan et al., 1993) and ribonuclease T1, 7.9 kcal mol^{-1} (Pace and Laurents, 1989) through denaturation by urea, and myoglobin, 9.7 kcal mol^{-1} (Pace and Laurents, 1989) and red kidney acid phosphatase, 5.2 kcal mol^{-1} (Cashikar and Rao, 1996), through denaturation by guanidinium chloride.

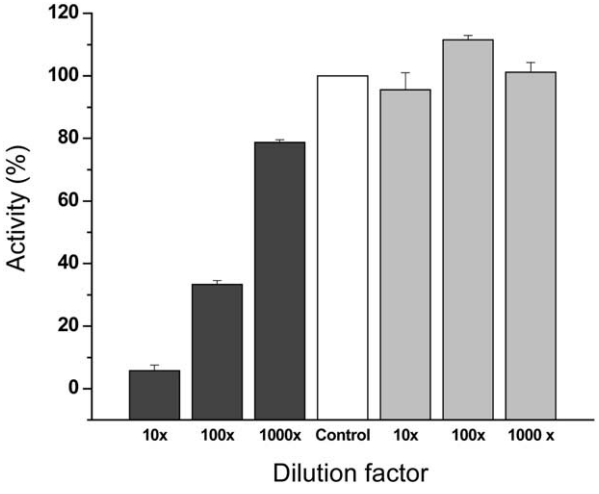

Fig. 1. Reversibility of the denaturation of soybean seed acid phosphatase by GdmCl and urea. GdmCl (light gray columns) and urea (dark gray columns) were diluted 10-, 100- and 1000-fold and the enzymatic activities were determined as described in Section 4. The white column represents 100% activity (control). The experiment was performed in triplicate and bars represent the standard deviations.

2.4. Activity of soybean seed acid phosphatase as a function of urea and GdmCl concentrations

Relevant information about macromolecule conformational flexibility can be obtained by considering the center of mass (ν_p). In this case, the fluorescence spectra were calculated by specifying the center of mass for each concentration of both chaotropic agents (Bonafé et al., 1998):

$$\nu_p = \sum_i \nu_i E_i / \sum_i E_i \quad (3)$$

where E_i stands for the fluorescence emitted at wave number ν_i, with determinations carried out over a range of appreciable values of E. Fig. 4 shows the relationship between the change in the spectral center of mass and the loss of enzyme activity. There were changes in the catalytic performance of the enzyme for both chaotropic agents, even before a noticeable conformational change could be detected during unfolding (Chen et al., 1996). This behavior was more pronounced for unfolding

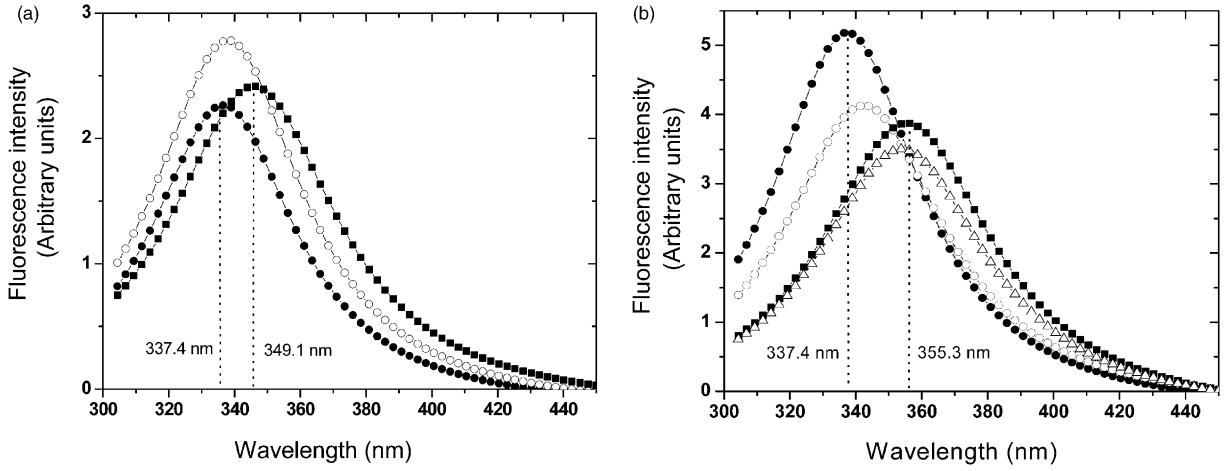

Fig. 2. Fluorescence emission spectra of soybean seed acid phosphatase denaturated with urea and GdmCl. Fluorescence emission spectra were determined for urea (a): 4.0 M (□), 8 M (■) and in the absence of denaturant (●), and for GdmCl (b): 2.2 M (○), 4.4 M (△), 6.0 M (■) and in the absence of denaturant (●). The enzyme was dissolved in 0.1 M acetate buffer at pH 5.0 containing the chaotropic agent at the desired concentration. The solutions were incubated until complete equilibrium was reached before fluorescence measurements were done using an excitation wavelength of 293 nm.

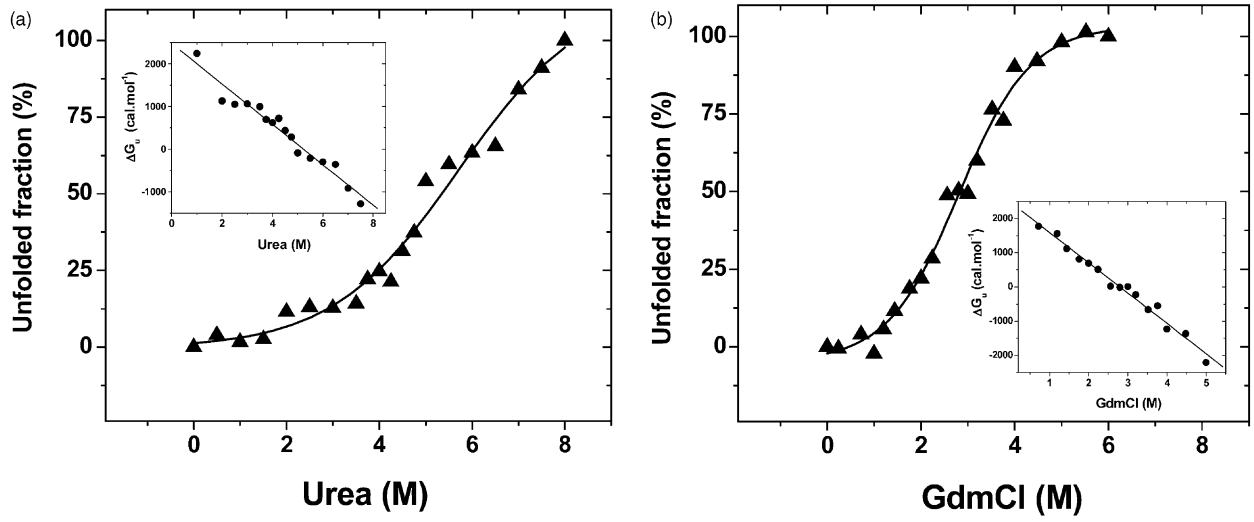

Fig. 3. Denaturation curves for soybean seed acid phosphatase in the presence of urea and GdmCl. All points were obtained in duplicate and the standard deviations were smaller than the symbols used. Inset: the linear dependence of Gibb's free energy on the concentration of chaotropic agent.

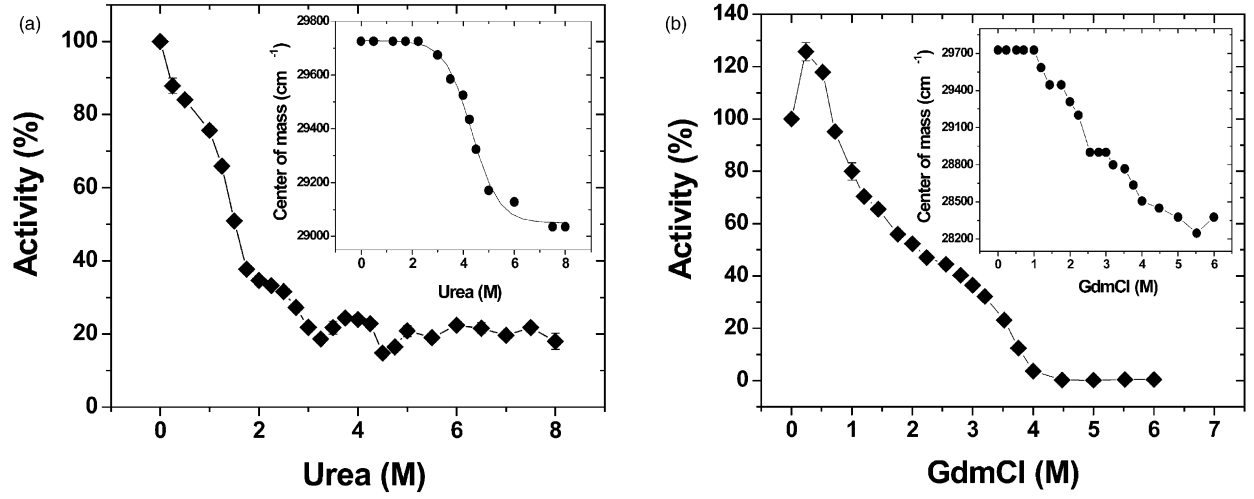

Fig. 4. Activity of soybean seed acid phosphatase as a function of urea and GdmCl concentrations. Activity measurements were done in triplicate as indicated in Experimental and their standard deviations are shown as bars (most are smaller than the symbols). Inset: effect of urea and GdmCl on the center of mass of emission fluorescence spectra.

induced by urea than by GdmCl. Our results suggest that the active site of the enzyme may be displaying a great conformational flexibility, probably because the enzyme active site is formed by relatively weak molecular interactions.

A similar behavior was observed for *Penaeus penicillatus* acid phosphatase. Chen et al. (1997) studying the conformational changes of this enzyme during denaturation by GdmCl showed that the extent of unfolding, measured by several different methods, closely coincided with each other. Moreover, their results suggested that the active sites of acid phosphatase displayed more conformational flexibility than the enzyme molecule as a whole. This same finding had already been reported by Tsou (1993) after comparison of unfolding and inactivation of several enzymes in the presence of urea and GdmCl, suggesting that actives sites were usually situated in a limited region of the enzyme molecule more fragile to denaturants.

3. Conclusions

The pathways of protein folding can occur through two mechanisms, one involving secondary structure formed before tertiary structure and the other involving general hydrophobic collapse of the protein leading to formation of secondary structure (Fersht, 1995). Many of the difficulties associated with identifying protein folding pathways can be overcome if folding is coupled

to disulphide bond formation (Creighton, 1995). It is worthwhile mentioning that sweet potato, soybean, red kidney bean and *Arabdopsis thaliana* purple acid phosphatases are closely related to mammalian purple acid phosphatases as predicted through the similarities of their secondary structures (Schenk et al., 2000). In contrast to castor bean seed acid phosphatase (Granjeiro et al., 2003), the soybean seed acid phosphatase is not a sulfhydryl-dependent enzyme. Conclusively, secondary structure elements can be involved in the pathways of soybean seed acid phosphatase folding.

Our results suggest that the center of mass, used to assess conformational changes in soybean seed acid phosphatase, enable quantitative evaluation conformational flexibility of this macromolecule during denaturation by chaotropic agents.

4. Experimental

4.1. Materials

Ultrapure urea and guanidinium chloride (GdmCl) were purchased from Pharmacia Biotech and *p*-nitrophenylphosphate (*p*NPP) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). AP1 isoform of acid phosphatase was purified (903 fold) to apparent homogeneity from soybean seeds with a specific activity of 822 nkat mg^{-1} of protein, according to Ferreira et al. (1998a). All the other reagents used were of analytical grade.

4.2. Enzymatic assays

The reaction mixture (final volume 1 ml), contained 100 mM sodium acetate buffer (pH 5.0), 5 mM *p*NPP and enzyme. After 10 min incubation at 37 °C, the reaction was stopped by addition of 1 M NaOH (1 ml). Acid phosphatase activity was measured at 405 nm by monitoring the *p*-nitrophenol released (Granjeiro et al., 2003).

4.3. Fluorescence measurements

Stock solutions of urea and GdmCl (10 and 8 M, respectively) in 100 mM acetate buffer (pH 5.0) were prepared as described by Pace (1986). The final protein concentration was about 10 μg/ml. Equilibrium unfolding, as a function of urea and GdmCl concentrations, was monitored by fluorescence spectroscopy on a Hitachi 2000 spectrofluorimeter, at 25 °C. The excitation wavelength was 293 nm and the emission intensity was monitored at wavelengths in the range of 300–450 nm. All samples were allowed to reach complete equilibrium at the appropriate final urea and GdmCl concentrations for 30 and 24 h, respectively, before spectra were obtained.

4.4. Renaturation of enzyme

Renaturation of the acid phosphatase was tested from the maxima concentrations of each denaturant (6 M for urea and 8 M for GdmCl) using 10-, 100- and 1000-fold dilutions of the denatured enzyme in 0.1 M acetate buffer (pH 5.0) followed by fluorescence and activity measurements.

4.5. Data analysis

Fluorescence data were converted to center of mass wave number for each spectrum, as described by Bonafé et al. (1998). The apparent free energy difference between native and unfolded species, ΔG_u, was assumed to linearly depend on the denaturant concentration, $\Delta G_u = \Delta G\ (H_2O) - m$ [denaturant], where $\Delta G\ (H_2O)$ is the free energy difference in the absence of denaturant (conformational stability) and m is the angular coefficient, which describes the sensitivity of the unfolding transition to denaturant (Pace, 1986).

All activity assays were done in triplicate and fluorescence measurements were done in duplicate.

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