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Influence of the oligomeric state of yeast hexokinase isozymes on inactivation and unfolding by urea

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Abstract

The effect of the association–dissociation equilibrium on the urea-induced inactivation and unfolding of the yeast hexokinase isoforms, PI and PII, showed that these enzymes are more stable as dimers. For the monomeric PII, the inactivation and unfolding processes occurred in parallel. However, inactivation precedes the unfolding of monomeric PI or dimeric PI and PII. The unfolding transitions are biphasic for PI indicating stable intermediates, whereas for the PII isoform the unfolding occurs in a single step. Our data suggest that although PI and PII present a 78% identity in their amino acid sequences, they probably have distinct inactivation and unfolding by urea behavior.

Keywords: Yeast hexokinase; Unfolding; Protein stability; Fluorescence; Urea

Abbreviations: Cm_i, concentration of urea at the midpoint of the inactivation transition; Cm_u, concentration of urea at the midpoint of the unfolding transition; f_U, fraction of unfolded protein; G6PD, glucose-6-phosphate dehydrogenase

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1. Introduction

The phosphorylation of hexoses in yeast is catalyzed by two hexokinases, PI and PII and also by a specific glucokinase. Hexokinases PI and PII are homodimers of 52 kDa per subunit [1–4], which can be dissociated into monomers by increasing the pH [1–4] or the ionic strength [3,4].

Both hexokinase isozymes are responsible for triggering catabolite repression in *Saccharomyces cerevisiae*, where the expression of a large number of genes is repressed in response to the carbon source [5–7]. However, each hexokinase responds in a different way to the carbon source for catabolite repression [8]. In addition, it was recently shown that the hexokinase PII can be found both in the cytosol and in the nucleus of the yeast [9]. The nuclear localization of this enzyme seems to be implicated in the regulation of gene expression [10–12].

At neutral pH, hexokinases PI and PII are known to exist as dimers, and the evidence that dissociation occurs at pH 8.0 and above suggests that ionizable groups are involved in this process. The fact that increasing ionic strength causes dissociation of the dimer indicates the electrostatic nature of the association between subunits. The binding of glucose has a weak effect on the dissociation of the dimer [3,4], which is increased when Mg–ADP is present with glucose [2]. Neither D-glucose-6-phosphate nor D-galactose induces dissociation of the hexokinase PII [2].

The N-terminus of the protein is essential for the self-association of the enzyme [13]. When yeast hexokinases PI and PII are converted into the proteolytically modified short-form (SI and SII) which lacks an 11-residue peptide from the N-terminus, the protein is essentially monomeric, even at low pH or low ionic strength [13].

The equilibrium association–dissociation of the yeast hexokinases seems to play an important role in the regulatory properties of the enzymes. In particular, the effect of substrates on that equilibrium can be very important in physiological conditions, since the monomer and the dimer have different affinities for glucose [3,4]. Kraakman et al. [14] recently showed the importance of the monomer–dimer equilibrium of the hexokinase PII on establishing the catabolite repression by correlating it with the phosphorylation of Ser-15, a chemical modification that shifts the equilibrium to the monomeric form [15].

The differences found with the hexokinase isoforms in vivo are very intriguing when considering the great structural similarity of these isozymes, as found by crystallographic studies [16–18] and also by the great identity (78%) and homology (90%) in their amino acid sequences.

Here we show that, despite of their structural similarities, these isozymes do present a different behavior in the inactivation and unfolding by urea. Both isozymes are more stable as dimers but stable unfolding intermediates were only detected for the hexokinase PI, while for the PII isoform the unfolding transitions followed a single step.

2. Experimental procedures

2.1. Assay for hexokinase activity

The activity of 0.2 U/ml of yeast hexokinases PI (type C-301, Sigma) and PII (type C-302, Sigma) was determined according to Guerra and Bianconi [19], with increasing concentrations of urea. The concentration of glucose-6-phosphate dehydrogenase (G6PD) was increased to 32 U/ml and was sufficiently high enough to overcome any effects on enzyme activity caused by the addition of urea. The buffers used were Mes–Tris, pH 6.5, or Mops–Tris, pH 8.5.

2.2. Preparation of proteolytically modified hexokinase (S-forms)

The short-form of the yeast hexokinases was prepared by incubation of the enzyme (17 μg ml^{-1}) with trypsin (0.02%, w/w) at 30°C in 50 mM Mops–Tris, pH 8.5 containing 100 mM glucose. After 1 h, PMSF (1 mM) was added and the sample was transferred onto ice for 30 min before elution from a Sephadex G-25 column by the

centrifuged–column procedure (see below). A control experiment was done by adding Milli-Q water instead of trypsin to the samples.

2.3. Centrifuged–column procedure

The oligomeric state of the yeast hexokinase isoforms (PI and PII) and the modified short-forms (SI and SII) was determined by the centrifuged–column procedure [20]. Sephadex G-50 columns were packed in a 1-ml plastic syringe, filled with the gel previously swelled in a solution consisting of a 50-mM buffer (Mes–Tris, pH 6.5 or Mops–Tris buffer, pH 8.5). The gel was packed by a series of centrifugations (2000 r.p.m./2 min) at 4°C, until reaching the 0.9-ml mark in the syringe. Samples (100 μl) were eluted in one single centrifugation and collected in a test tube. The presence of protein in the eluate was determined by measuring the enzyme activity as described before. At pH 8.5, where the hexokinases PI and PII isoforms are in the monomeric state, the proteins are totally retained in the column and no enzyme activity is detected in the eluate. The same occurs with the short-forms, which are unable to form dimers at both pH values. However, when the enzymes are in a dimeric state (PI and PII at pH 6.5) over 95% of the protein is recovered in the eluate, as detected by the enzyme activity. This procedure was used as a routine control of the oligomeric state of the PI and PII isozymes since their N-terminus can be cleaved in the ammonium sulfate stock solution. Likewise, in order to check if the preparation of the short-forms was successful, this procedure was also applied. The centrifuged–column procedure is much faster than HPLC experiments which also require a higher protein concentration in the samples.

2.4. Fluorescence

The hexokinase PI and PII steady-state intrinsic fluorescence spectra were obtained in a Hitachi F-3010 spectrofluorimeter from 310 to 400 nm, with excitation at 295 nm, with 5-nm bandpass for both excitation and emission. The proteins were prepared in a 50-mM buffer containing 10 mM glucose and 5 mM $MgCl_2$, with increasing concentrations of urea. The background fluorescence due to the buffer and urea was subtracted from the obtained spectra with the proteins. The protein concentrations used were 1 and 10 μM. At lower protein concentrations, the equilibrium was reached in a few minutes. However, the spectra with 10 μM protein were recorded after a 36-h incubation with urea at 4°C. The results were essentially the same in both conditions.

The center of mass of the fluorescence spectra ($\lambda_{[D]}$) was calculated at each urea concentration according to Eq. (1):

$$\lambda_{[D]} = \Sigma\lambda I_\lambda / \Sigma I_\lambda \quad (1)$$

where λ is the emission wavelength and I_λ is the fluorescence intensity at wavelength λ.

The extent of unfolding at each concentration of urea was calculated by Eq. (2):

$$f_U = (\lambda_{[D]} - \lambda_0)/\Delta\lambda \quad (2)$$

where f_U is the fraction of the unfolded protein, λ_0 is the center of mass of the native protein in the absence of urea and $\Delta\lambda$ is the total change in the spectral center of mass upon complete unfolding.

3. Results and discussion

The effect of urea on the inactivation and unfolding of the yeast hexokinase PI and PII isozymes was studied at pH 6.5 and 8.5 were only dimers and monomers, respectively, are found [3,4]. The proteolytically modified S-forms, however, are essentially monomeric in both pH values [13]. In order to check the oligomeric state of the proteins, a routine control was done by the centrifuged–column procedure as described in the experimental section.

Fig. 1 shows the inactivation of the enzymes by urea. For both PI (Fig. 1a) and PII (Fig. 1b), it was observed that the monomer was more susceptible to inactivation than the dimer. The inactivation transition curves for the dimeric forms of the isozymes are displaced to higher urea concentra-

Table 1
Midpoint of the inactivation (Cm_i) and unfolding (Cm_u) transitions by urea of the yeast hexokinase PI and PII

Enzyme	Oligomeric state[a]	Cm_i (M)	Cm_u (M)
PI	Monomer	1.7 ± 0.08	3.1; 4.5[b]
	dimer	2.5 ± 0.09	4.2; 5.3[b]
PII	Monomer	2.1 ± 0.04	2.3
	dimer	2.9 ± 0.05	4.3

[a]The monomeric and dimeric forms were studied at pH 8.5 and pH 6.5, respectively and the dimeric ones at pH 6.5 as discussed in the text. The oligomeric state of the enzymes was checked by using the centrifuged column procedure described in Section 2.
[b]The values correspond to the first and second midpoints of the biphasic unfolding transitions of the PI isozyme.

tions (Fig. 1a,b). The concentration of urea at the midpoint of the inactivation transition (Cm_i, Table 1) was calculated by the curve fitting of the experimental data, according to a sigmoidal curve. On the other hand, with the respective monomeric S-forms the inactivation transitions were very similar at both pH values (Fig. 1c,d). In addition, the Cm_i values for SI and SII (Table 2) were comparable to those obtained with the monomeric PI and PII (Table 1). Since the S-forms are monomeric at any pH, these results show that the differences in Cm_i observed with the intact PI and PII were not due to the changes in pH but, in fact, to the oligomeric state of the proteins. Upon dilution of the samples, at least 75% of the original activity was recovered at once, indicating that the inactivation was reversible.

In the unfolding induced by urea, we also found that the dimers are more stable than the monomers. The unfolding process was studied with the intact proteins by fluorescence as described in the experimental section, and the midpoints of the unfolding transitions (Cm_u) were calculated from the curves shown in Fig. 2. For the yeast hexokinase PI, the transition curves were biphasic for both monomer and dimer (Fig. 2a) indicating the presence of stable intermediates during unfolding. However, the transition curves for the unfolding of hexokinase PII followed a single step at both pH values (Fig. 2b). Only with the hexokinase PII at pH 8.5, where monomers are found, the inactivation and unfolding processes induced by urea occurred in parallel (Table 1). However, for monomeric PI or dimeric PI and PII, the inactivation by urea precedes the loss of protein structure. This kind of behavior was already observed for other enzymes and it is probably due to a local perturbation at the catalytic domain by the chaotropic agent without affecting the entire structure [21,22].

Table 2
The effect of pH on the midpoint of the inactivation transition (Cm_i) by urea of the monomeric S-forms of yeast hexokinase

S-form	pH	Cm_i (M)
SI	6.5	1.4 ± 0.04
	8.5	1.4 ± 0.10
SII	6.5	2.2 ± 0.08
	8.5	2.1 ± 0.06

The short-forms of the yeast hexokinase, SI and SII were prepared by incubation of the monomeric PI and PII, respectively, with trypsin as described in Section 2. The S-forms are monomeric at both pH 6.5 and 8.5.

One interesting feature was observed when the midpoints of inactivation (Cm_i) and unfolding (Cm_u) transitions of both isozymes by urea where compared (Table 1). In the first case, the hexokinase PII seems to be more stable than PI, since the inactivation curves of the PII are shifted to higher concentrations of urea. However, PII undergoes structural changes with lower urea concentrations than PI, especially in the monomeric form (Table 1).

An attempt to use circular dichroism (CD) to

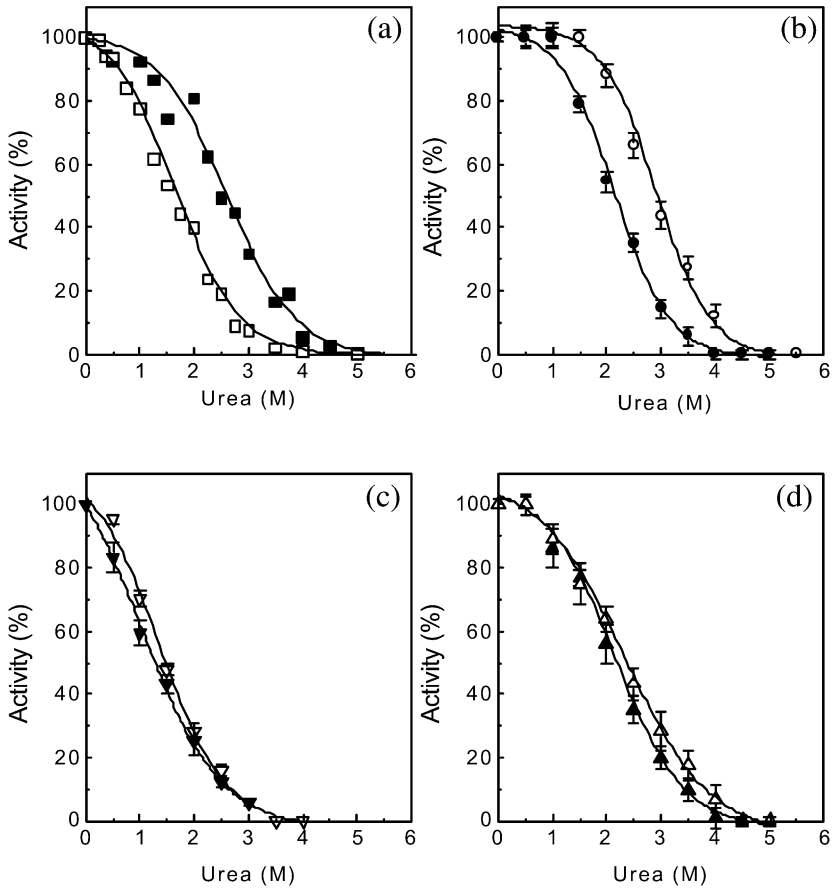

Fig. 1. Inactivation of yeast hexokinase isoforms by urea. The activity of the intact hexokinase PI (a), PII (b) and of the short-forms SI (c), and SII (d) was determined at 25°C with 10 mM glucose, 5 mM $MgCl_2$, and 1 mM ATP in 50 mM Mes–Tris, pH 6.5 (open symbols) or 50 mM Mops–Tris, pH 8.5 (solid symbols), in the presence of increasing concentrations of urea. The initial rate of reaction was normalized in order to compare both pH values, considering as 100% the rate measured in the absence of urea. The values are means ± S.D., for $n = 5$.

study the unfolding process by urea failed since higher protein concentrations, than that used in the fluorescence experiments, was required due to the noise level observed in the CD spectra with urea. However, at high protein concentration both hexokinase PI and PII had a tendency to form large aggregates with a molecular mass at approximately 560 kDa at pH 8.5 as observed by HPLC (data not shown). Therefore, it was not possible to find a condition were monomers were found giving a CD signal for a reliable data collection at 222 nm to study the unfolding process.

4. Conclusions

The study of the equilibrium unfolding of proteins provides information for the understanding of different features that influence the conformational stability, as well as the mechanisms that are involved in the folding of globular proteins. The conformational stability of a protein is a consequence of several non-covalent interactions within the molecule, where many microstates can be found. These microstates have similar folding patterns but distinct thermodynamic properties due

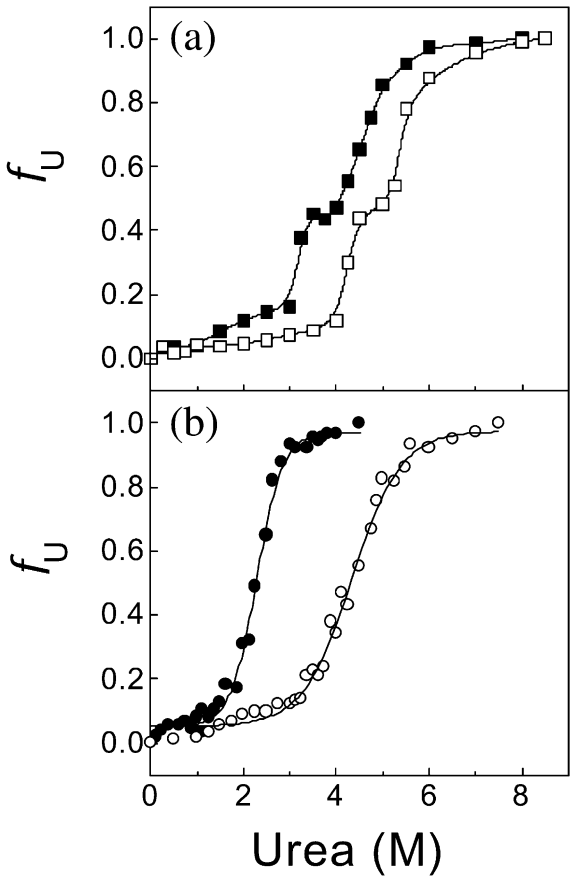

Fig. 2. The extent of the unfolding of yeast hexokinase isozymes by urea. The fraction of protein unfolded (f_U) as a function of urea for the monomeric (solid circles) and dimeric (open circles) forms of the yeast hexokinase PI (a) and PII (b). The value of f_U was calculated from Eq. (2) (Section 2) by the changes in the center of mass of the fluorescence spectra of the proteins caused by urea. The protein concentration was 10 μM and the spectra were recorded after a 36-h incubation with the denaturant at 4°C. The experimental data for hexokinase PI were fitted resulting in two sigmoidal curves and for PII, one sigmoid curve.

to the differences in the extent of intramolecular non-covalent contacts or exposure to the solvent, among other features [23–25]. The unfolded state of a protein has a random conformation with a high degree of solvent exposure and flexibility of the side chains and the backbone [23–25].

The unfolding of many small globular proteins is a highly cooperative reversible process where the population of an intermediate is negligible. Therefore, equilibrium unfolding transition for these proteins can be described as a two-state model, where the transition between the native (N) and unfolded (U) states occurs in one single equilibrium step, described by $N = U$ [14]. However, this behavior is not usual for oligomeric or multidomain proteins where the presence of stable, partially folded, intermediates can be detected [26–31]. Generally, the unfolding transition curves are biphasic when stable intermediates are formed. This biphasic behavior of the transitions is also associated with the dissociation of oligomeric proteins [26–31].

Crystallographic studies showed a great similarity in the structures of the yeast hexokinase isozymes [16–18]. Each subunit of the yeast hexokinases consists of two domains separated by a deep cleft. Binding of glucose induces a great conformational change in the protein, partly closing the cleft between the two domains [32]. A 78% identity, with approximately 90% homology, is found by comparing the amino acid sequence of both enzymes [33,34]. Nevertheless, the binding of glucose is strongly cooperative in the dimeric PI [3,4] in contrast with the dimeric PII where both sites are equivalent and binding is non-cooperative [4,35]. They both present four Trp residues per subunit at the same position in the amino acid sequence (W51, W109, W219 and W414), two of them located in the major lobe and the other two in the minor lobe.

Despite the great structural similarity between the yeast hexokinase PI and PII isozymes, our data suggest that they have distinct behavior on the inactivation and unfolding by urea. While the hexokinase PI is more susceptible to the inactivation than the PII isoform, the latter undergoes structural changes with lower concentrations of the chaotropic agent. Furthermore, the unfolding transition of the hexokinase PI is biphasic for both the monomeric, or in the dimeric forms, indicates the presence of stable intermediates. However, stable intermediates were not detected in the unfolding of the PII isoform by urea, which followed a two step transition either as monomers or dimers. This distinct behavior of the yeast

hexokinase isozymes towards urea may or may not represent distinct mechanisms of unfolding.

Somehow, the small structural differences may account for the different behavior of these two very similar proteins towards urea. The particularities of each isozyme, such as the differences in the affinity for glucose [3,4,35], or in the response to catabolite repression [5–12] as well as the different stability of these isozymes observed here, may be some of the many reasons why there are two very similar proteins with the same metabolic function in yeast.

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