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ORIGINAL PAPER

# Guanidine hydrochloride and urea-induced unfolding of *Brugia malayi* hexokinase

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**Abstract** Guanidine hydrochloride and urea-induced unfolding of *B. malayi* hexokinase (BmHk), a tetrameric protein, was examined in detail by using various optical spectroscopic techniques, enzymatic activity measurements, and size-exclusion chromatography. The equilibrium unfolding of BmHk by guanidine hydrochloride (GdmCl) and urea proceeded through stabilization of several unique oligomeric intermediates. In the presence of low concentrations of GdmCl, stabilization of an enzymatically active folded dimer of BmHk was observed. However an enzymatically inactive dimer of BmHk was observed for urea-treated BmHk. This is the first report of an enzymatically active dimer of hexokinase from any human filarial parasite. Furthermore, although complete recovery of the native enzyme was observed on refolding of BmHk samples denatured by use of low concentrations of GdmCl or urea, no recovery of the native enzyme was observed for BmHk samples denatured by use of high concentrations of GdmCl or urea.

**Keywords** Guanidine hydrochloride · Urea denaturation · Multimeric protein · Dimer · BmHk

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**Abbreviations**

| | |
|---|---|
| BmHk | *Brugia malayi* hexokinase |
| GdmCl | Guanidine hydrochloride |
| $C_m$ | Concentration of denaturant for which 50% denaturation of protein is observed |
| $\lambda_{max}$ | Wavelength maximum |
| $\theta$ | Mean residual ellipticity |

## Introduction

The folding of protein monomers and their assembly into functional oligomers is a fundamental step in the biosynthesis of multimeric proteins. Multimeric proteins are an attractive system for investigating spontaneous self-assembly of protein structures and for examining regulatory interactions between subunits. Several studies clearly show that quaternary structure plays a fundamental role in the stabilization of native protein forms (Mei et al. 1997; Janicke and Lillie 2000; Gokhale et al. 1999). Understanding the folding/unfolding and self-assembly processes of such macromolecules remains a major problem in protein chemistry. Inter and intra-subunit interactions in oligomeric and monomeric proteins are more complex than those of smaller monomeric proteins and involve many local and global processes that occur by sequential and/or concerted mechanisms (Janicke 1987; Seckler and Janicke 1992). Hence elucidation of the hierarchy of events occurring during the denaturation of an oligomeric protein is an important means if delineating such a process.

Hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1.) is the key regulatory enzyme of the glycolytic pathway catalyzing transfer of a phosphoryl group from ATP to glucose to form glucose-6-phosphate and ADP with

the release of a proton (Phillips et al. 1981). Hexokinases can be distinguished on the basis of their molecular weight and sensitivity to inhibition by the product G6P. In mammals, three isozymes, hexokinases I, II, and III, having molecular weights of approximately 100 kDa and with marked sensitivity to inhibition by G6P have been identified (Mochizuki 1981; Tielens et al. 1994).

The tertiary structures of the hexokinases from human Hk I (100 kDa) (Aleshin et al. 1998a, b, c, 2000), Hk IV (50 kDa) (Kamata et al. 2004), *Schistosoma mansonii* Hk (50 kDa) (Mulichak et al. 1998), *S. cerevisiae* Hk P I (54 kDa) (Bennet and Steiz 1980), and P II (54 kDa) (Anderson et al. 1978; Kuser et al. 2004) are known. All 50–54 kDa Hks are similar in primary, secondary, and tertiary structure and in the substrate binding site (Kamata et al. 2004; Mulichak et al. 1998; Bennet and Steiz 1980; Anderson et al. 1978; Kuser et al. 2004; Steiz et al. 1976). The tertiary structures of these Hks consist of two domains (large and small domains), between which substrate (glucose and ATP)-binding sites are formed. The 100 kDa Hks consist of N and C terminal halves (Aleshin et al. 1998a, b, c, 2000; Cardenas et al. 1998). The two halves of the 100 kDa Hks are mutually similar to those of 50–54 kDa Hks in primary, secondary, and tertiary structure and in the substrate-binding site (Kamata et al. 2004; Mulichak et al. 1998; Bennet and Steiz 1980; Anderson et al. 1978; Kuser et al. 2004; Steiz et al. 1976; Cardenas et al. 1998). On the basis of the similarity of the N and C terminal halves it has been suggested that the gene encoding 100 kDa Hk evolved from a gene coding for 50 kDa glucose kinases in group Hk, by duplication and fusion of the gene (Kamata et al. 2004; Mulichak et al. 1998; Bennet and Steiz 1980; Anderson et al. 1978; Kuser et al. 2004; Steiz et al. 1976; Cardenas et al. 1998).

We have recently reported the cloning and characterization of *Brugia malayi* hexokinase (BmHk) (Singh et al. 2008). This recombinant BmHk is a tetramer with a subunit molecular mass of $\sim$72 kDa and 59% homology with *C. elegans* hexokinase. In the era of proteomics, it is becoming increasingly important to understand the structure–function relationships of proteins by using various biophysical tools. Despite the importance of hexokinase in parasite metabolism, few biophysical studies have been conducted on this enzyme (Kumar et al. 2004). With the objective of gaining insight into the structural organization of BmHk, we have carried out a detailed characterization of the structural and functional changes associated with the GdmCl and urea-induced unfolding of BmHk. Various optical spectroscopic techniques, for example fluorescence and CD, were used to study the changes in the tertiary and secondary structures of the protein during denaturant-induced unfolding. Changes in the molecular dimensions of the protein were studied by size-exclusion chromatography. Significantly different pathways of BmHk unfolding were observed with the two denaturants, with GdmCl leading to stabilization of an enzymatically active dimer during unfolding of the protein whereas urea-induced unfolding led to stabilization of an enzymatically inactive dimer.

## Materials and methods

### Materials

Guanidine hydrochloride, urea, imidazole, SDS, acrylamide, bis-acrylamide, TEMED, ammonium persulfate, glutaraldehyde, chloramphenicol, and ampicillin were purchased from Sigma–Aldrich Chemical, USA. Molecular weight markers for SDS–PAGE were purchased from MBI Fermentas, Maryland, USA. Ni–NTA agarose matrix was procured from Qiagen, Germany. Standard molecular weight markers for size-exclusion chromatography (e.g. albumin, aldolase, catalase, and ferritin) and the Superdex 200HR column were purchased from GE health care, Singapore. All solutions were prepared by using Millipore water.

### Over-expression and purification of BmHk

The expression and purification of recombinant BmHk was carried out as described previously (Singh et al. 2008). BmHk was overexpressed in *E. coli* strain Rosetta (DE3) harboring the plasmid pTriEx-BmHk. Cells were grown in Luria–Bertani (LB) broth with 40 μg/ml chloramphenicol and 100 μg/ml ampicillin and induced with 0.5 mM isopropyl-$\beta$-thiogalactopyranoside (IPTG). Cultures were then grown at 20°C for 14–16 h before harvesting. Cells were harvested by centrifugation at 8,000$\times g$ for 10 min at 4°C. Protein was purified by $Ni^{2+}$–nitrilotriacetic acid resin ($Ni^{2+}$–NTA) affinity chromatography. The homogeneity of the purified protein was evaluated by SDS–PAGE.

### Guanidine hydrochloride and urea denaturation of BmHk

BmHk (3 μM) was dissolved in 50 mM sodium phosphate buffer (pH 7) in the presence and absence of increasing concentrations of guanidine hydrochloride (GdmCl) or urea and incubated for 4 h at 4°C before the measurements were made.

### Assay for enzyme activity

Hexokinase activity was estimated spectrophotometrically by the method of Armstrong et al. (1996) at 340 nm in

a system coupled with glucose-6-phosphate dehydrogenase (G6PDH). The reaction mixture contained 100 mM Tris–HCl (pH 7.6), 10 mM ATP, 0.04 mM glucose, 0.25 mM NADP, 25 mM $MgCl_2$, 10–20 μg hexokinase, and 1 unit of G6PDH. For studies using increasing concentrations of GdmCl or urea the enzyme was incubated with assay buffer containing desired concentrations of the denaturants.

### Fluorescence spectroscopy

Fluorescence spectra were recorded with a Varian fluorescence spectrophotometer in a 5 mm path-length quartz cell. The protein concentration was 3 μM for all experiments, and the measurements were carried out at 25°C. For monitoring tryptophan fluorescence, the excitation wavelength of 290 nm was used and the spectra were recorded between 300 and 450 nm.

### Circular dichroism measurements

CD measurements were made with a Jasco J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as the mean residual ellipticity ($\theta$), which is defined as $(\theta) = 100 \times \theta_{obs}/(lc)$, where $\theta_{obs}$ is the observed ellipticity in degrees, $c$ is the concentration in mol residue per liter, and $l$ is the length of the light path in centimeters. The CD spectra were measured at an enzyme concentration of 3 μM with a 1 mm cell at 25°C. The values obtained were normalized by subtracting the baseline recorded for buffer having the same concentration of denaturants under similar conditions.

### Size-exclusion chromatography

Gel filtration experiments were carried out on a Superdex 200 HR 10/300 column (manufacturer's exclusion limit 600 kDa for proteins) on AKTA FPLC (Amersham Pharmacia Biotech, Sweden). The column was equilibrated and run with 50 mM phosphate buffer (pH 7) containing the desired GdmCl or urea concentration at 25°C with a flow rate of 0.3 ml/min.

### Cross-linking using glutaraldehyde

Glutaraldehyde cross-linking studies were carried out as described previously (Joshi et al. 2008). To native and GdmCl/urea-treated BmHk (0.2 mg/ml) was added an aliquot of 25% (*v*/*v*) glutaraldehyde to achieve a final concentration of 1% glutaraldehyde and, finally, the cross-linked samples were analyzed by 8% SDS–PAGE.

## Results

We have studied the effect of GdmCl and urea-induced changes on the structural and functional properties of BmHk. Time-dependent changes in the structural properties and enzymatic activity of BmHk in 0.5 and 1 M GdmCl and 0.5, 2, and 4 M urea were monitored to standardize the incubation time required to achieve equilibrium under these conditions. Under all the conditions studied, the changes occurred within maximum of 4 h, with no further alterations in the values obtained up to 12 h (data not shown). These observations suggest that a minimum time of 4 h is sufficient to achieve equilibrium under any of the denaturing conditions studied.

### Changes in molecular properties of BmHk associated with GdmCl-induced unfolding

Enzyme activity can be regarded as the most sensitive probe with which to study the changes in enzyme conformation during various treatments, because it reflects subtle readjustments at the active site, enabling very small conformational variations of an enzyme structure to be detected. The effect of increasing concentrations of GdmCl on the enzymatic activity of BmHk is summarized in Fig. 1a. A biphasic curve corresponding to two independent transitions centered at about 0.3 and 0.8 M GdmCl, respectively was observed. About 55% loss of enzymatic activity was found to be associated with the first transition and a complete loss of activity was observed above 1.5 M GdmCl.

Far-UV CD studies of GdmCl-induced unfolding of BmHk were carried out to study the effect of GdmCl on the secondary structure of the protein. In the far-UV region, the CD spectra of BmHk showed the presence of substantial α-helical conformation (Singh et al. 2008). The effect of increasing GdmCl concentration on the elipticity of BmHk at 222 nm is illustrated in Fig. 1b. At low concentrations of GdmCl, i.e. up to 0.3 M, no significant change in elipticity of native BmHk was observed. For GdmCl concentrations between 0.4 and 1 M, a large decrease in elipticity at 222 nm was observed, indicating significant disruption of secondary structure of the enzyme under these conditions. However, even at 1.5 M GdmCl, significant secondary structure (~8% of the native enzyme) was retained by the enzyme. Gradual disruption of the secondary structure was observed on increasing the GdmCl concentration above 1.5 M, when almost complete loss of secondary structure was observed, indicating complete unfolding of enzyme at this concentration.

Fluorescence spectra provide a sensitive means of characterizing proteins and their conformation. The spectrum is determined chiefly by the polarity of the

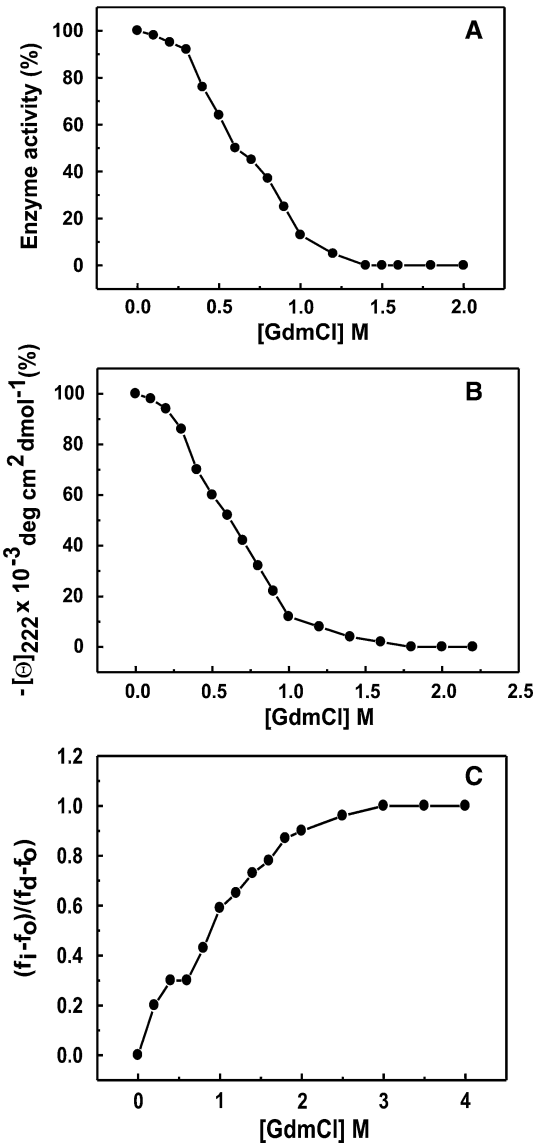


**Fig. 1** GdmCl-induced denaturation of BmHk. **a** Change in enzymatic activity of BmHk with increasing concentrations of GdmCl. The enzyme was incubated with the desired concentration of GdmCl for 4 h at 4°C and the activity was measured by the method described in "Materials and methods". The data are represented as percentages with enzymatic activity observed for BmHk in the absence of GdmCl taken as 100%. **b** GdmCl-induced changes in the secondary structure of BmHk, as monitored by following changes in ellipticity at 222 nm obtained from the far-UV CD curve of BmHk, with increasing concentrations of GdmCl. The data are represented as percentage of ellipticity at 222 nm, taking the value observed for native BmHk as 100%. **c** Plot of the fractional change in the wavelength of maximum fluorescence emission of BmHk, $(f_i - f_o)/(f_d - f_o)$, versus GdmCl concentration; $f_i$ is wavelength for a particular sample, $f_o$ is the wavelength in the absence of GdmCl, and $f_d$ is the wavelength at GdmCl concentrations >2.5 M

environment of the tryptophan and tyrosine residues and by their specific interactions (Hayashi and Nakamura 1981). In order to study the effect of denaturant on the structural properties of BmHk, tryptophan fluorescence was studied in the presence of increasing concentrations of GdmCl. If a significant difference exists between the fluorescence emission wavelength maxima of native and denatured proteins, correlation between GdmCl concentration and the fractional changes in wavelength at which the fluorescence emission is maximum provides useful information on the stabilization of intermediates during protein unfolding (Parr and Hammes 1975). For BmHk, the value of the emission wavelength maximum in the absence of GdmCl ($f_o$) and at GdmCl concentrations >2.5 M GdmCl ($f_d$) were 335 and 354 nm, respectively. Two well-separated transitions were observed for BmHk when the fractional change in the wavelength at which the tryptophan fluorescence emission spectrum of BmHk is maximum was plotted against the concentration of GdmCl. The first transition was very sharp and occurred between 0 and 0.4 M GdmCl. A plateau region existed from 0.4 to 0.6 M GdmCl and was followed by second transition that was essentially complete at 2.5 M GdmCl (Fig. 1c). For BmHk incubated with 2.5 M GdmCl tryptophan emission $\lambda_{max}$ of 354 nm was observed. Normally exposed tryptophan in the unfolded protein shows emission $\lambda_{max}$ between 340 and 356 nm (Lakowicz 1999), indicating that incubation of BmHk with a higher concentration of GdmCl results in significant unfolding of the protein molecule.

The gradual change in enzymatic activity of BmHk with increasing concentrations of GdmCl is indicative of a GdmCl-induced incremental change in BmHk structure. Further support for GdmCl-induced multiphasic unfolding of BmHk comes from the observation of two transitions in tryptophan fluorescence studies, which are indicative of stabilization of an intermediate during the GdmCl-induced unfolding of BmHk. Furthermore, it is observed that the denaturation profile, as measured by enzymatic activity, ellipticity measurements at 222 nm, and the first transition of tryptophan corresponds to the first unfolding of BmHk, whereas that of the second transition (main transition) of the tryptophan fluorescence corresponds to the final phase of unfolding of the protein.

## Changes in molecular properties of BmHk associated with urea-induced unfolding

Although urea and GdmCl are believed to have a similar mode of action (Pace 1986), GdmCl is a monovalent salt that has both ionic and chaotropic effects (Monera et al. 1994; Kohn et al. 1995a) whereas urea has only chaotropic effects. Thus, urea is an ideal control agent for distinguishing between the ionic and chaotropic effects of GdmCl.

The effect of increasing concentrations of urea on the enzymatic activity of BmHk is illustrated in Fig. 2a.

No significant change in the enzymatic activity of the native enzyme was observed up to 1 M urea. On increasing the concentration of urea from 1 to 4 M, a sharp decrease in enzymatic activity was observed, and at concentrations greater than 4 M complete loss of enzymatic activity was observed.

The effect of increasing urea concentration on BmHk secondary structure was studied by monitoring changes in ellipticities at 222 nm as determined by far-UV CD measurements under these conditions. When urea concentration was increased to 1.5 M, a slight decrease (approx. 15%) in elipticity at 222 nm was observed. However, on increasing the concentration from 1.5 to 4 M, a large decrease in elipticity at 222 nm was observed (Fig. 2b), indicating significant dissociation of secondary structure.

Although all the probes used for studying the urea-induced unfolding of BmHk as reported above showed the presence of single transition associated with this process, the different curves were not superimposable, that is, the slopes of these transitions were significantly different. This can be seen clearly from the plot of fraction folded versus urea concentration (Fig. 2c).

The stabilization of an intermediate during urea-induced unfolding of BmHk was demonstrated conclusively by the tryptophan fluorescence studies at increasing urea concentrations. Figure 2c shows the fractional change in the wavelength at which the tryptophan fluorescence emission spectrum of BmHk is maximum plotted against urea concentration. A well separated transition was observed between 0.5 and 1.5 M urea concentration, followed by another transition between 2 and 4 M urea. These observations indicate that an intermediate is stabilized at low concentrations of urea and the subunit configuration can be studied by size-exclusion chromatography.

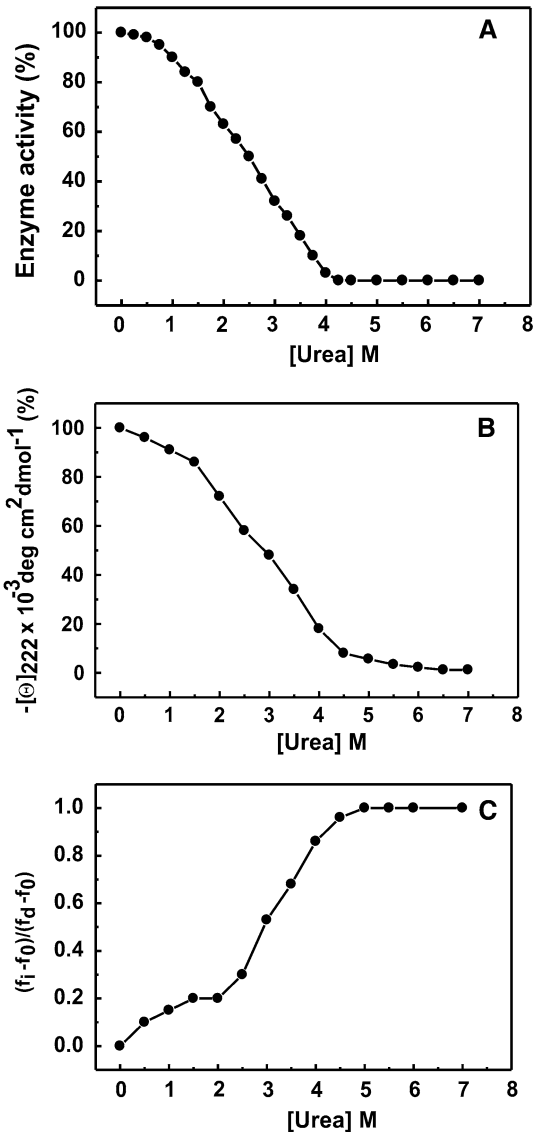


**Fig. 2** Urea-induced denaturation of BmHk. **a** Change in enzymatic activity of BmHk with increasing concentrations of urea. The value corresponding to native BmHk was taken as 100%. **b** Urea-induced changes in the secondary structure of BmHk as monitored by following changes in the far-UV CD curve for BmHk in the presence of increasing concentrations of urea. **c** Plot of the fractional change in the wavelength of maximum fluorescence emission of BmHk, $(f_i - f_o)/(f_d - f_o)$, versus urea concentration; $f_i$ is the wavelength for a particular sample, $f_o$ is the wavelength in the absence of urea, and $f_d$ is the wavelength at urea concentrations >3 M

## Size-exclusion chromatography (SEC)

To study the effect of GdmCl and urea-induced structural changes on the quaternary structure of BmHk, the effect of increasing concentrations of GdmCl or urea on the molecular dimensions of BmHk was monitored by size-exclusion chromatography experiments on a Superdex 200 HR column (Amersham Pharmacia Biotech, Sweden).

Figure 3a summarizes the results of gel-permeation experiments on BmHk in the absence and presence of increasing concentrations of GdmCl at 25°C on the Superdex 200 HR 10/300 column. For native BmHk, a single peak centered at 12.8 ml was observed; this is slightly greater than the retention volume of 13 ml observed for a molecular mass of 232 kDa, and less than the retention volume of 12.07 ml observed for a molecular mass of 420 kDa in this column under similar conditions. This result suggests that under native conditions BmHk is tetrameric. For the 0.4 M GdmCl-treated enzyme a single peak with retention volume of 13.4 ml was observed, indicating a large decrease in the molecular size of the enzyme under these conditions. This retention volume was slightly higher than the retention volume of 13.36 ml observed for aldolase ($M_r$ 158 kDa). with 1 M GdmCl-treated BmHk two peaks centered at 8 ml and 14 ml (designated A and B), respectively were observed. Peak A appeared at a dramatically reduced retention volume of

8 ml, which is the void volume of the column, indicating greatly increased hydrodynamic radii for the protein under these conditions (i.e., an aggregated species). Peak B, observed at enhanced retention volume of 14 ml, was found to be close to the retention volume of 14.5 ml observed for the molecular mass marker of 66 kDa (BSA) on this column. The monomer of BmHk has a molecular mass of 72 kDa, indicating that the peak B corresponds to the monomeric species of BmHk. For 1.5 M GdmCl, only one peak with retention volume of 7.8 ml was observed. This highly reduced retention volume indicates the presence of aggregated species under these conditions. These aggregates are possibly attributable to unstable GdmCl-stabilized unfolded BmHk.

Figure 3b summarizes the results of gel permeation experiments on BmHk, on the Superdex 200 HR 10/300 column, at 25°C, in the absence and presence of increasing concentrations of urea. For native and 1 M urea-treated BmHk, a single peak centered at 12.8 ml was observed, indicating no change in the molecular dimensions of the enzyme in 1 M urea. For 2 M urea-denatured BmHk, two peaks centered at 12.1 ml and 13 ml were observed (designated as peaks A and B). Peak A appeared at a retention volume of 12.1 ml, which is close to 12.07 ml observed for a protein with molecular mass of 420 kDa, indicating the presence of aggregated species under these conditions. Peak B, on the other hand, had an increased retention volume, 13 ml, close to the 12.8 ml observed for tetramer. The enzyme activity observed in the species eluted as peak B may be because of remaining tetramer species. For 3 M urea-treated BmHk, a single peak at a retention volume of 13.2 ml was obtained. The slight reduction in retention volume compared with 13.4 ml, observed for dimers (as in GdmCl-induced unfolding) indicated the stabilization of the dimer under these conditions. However, for 4 M urea-denatured BmHk a single peak at a retention volume of 8 ml was observed. This highly reduced retention volume indicates the presence of aggregated species under these conditions.

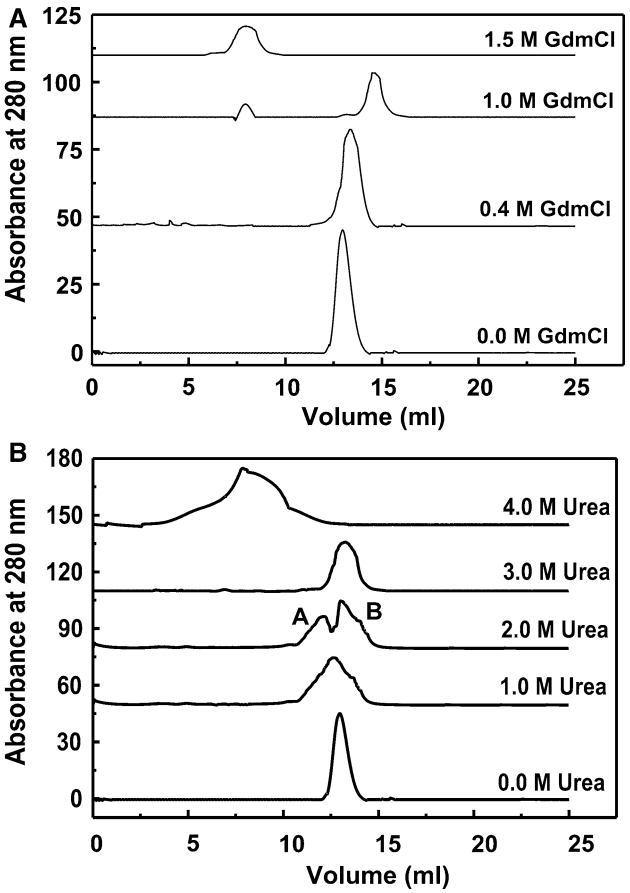


**Fig. 3** Size-exclusion chromatographic profile on Superdex 200 HR column. **a** and **b** represent the chromatographic profiles of native BmHk on a Superdex 200 HR 10/300 column on AKTA FPLC (Amersham Pharmacia Biotech, Sweden) at different GdmCl and urea concentrations, respectively. All the curves have been displaced along the *Y*-axis for display purposes

The results obtained in this study indicate that the dimer obtained with GdmCl was enzymatically active whereas the dimer obtained with urea was enzymatically inactive. Furthermore, the tetramer obtained from the 1 M urea-treated sample had less enzymatic activity than the native enzyme.

## Glutaraldehyde cross-linking studies

Results from glutaraldehyde cross-linking studies carried out to study the effect of GdmCl and urea on the subunit configuration of BmHk are summarized as in Figs. 4a, b. For native and 1 M urea-treated BmHk, a single protein species corresponding to the tetramer was observed. However, for the 0.4 M GdmCl-treated enzyme, only one species, corresponding to the dimer, was observed. For 2 M urea-treated BmHk, a protein band corresponding mainly to the dimer with a faint band corresponding to the tetramer were observed, indicating the presence of two association states under these conditions. These observations indicate that treatment of BmHk with up to 1 M urea does not bring about any change in the subunit conformation of the protein; treatment of BmHk with 0.4 M GdmCl or 3 M urea, however, resulted in dissociation of native tetramer in to dimer of the enzyme. The results from glutaraldehyde cross-linking studies agree well with those from size-exclusion chromatography.

## Reversibility of GdmCl and urea-stabilized oligomers of BmHk

Studies were performed to study the reversibility of GdmCl and urea-denatured BmHk refolding. The BmHk was

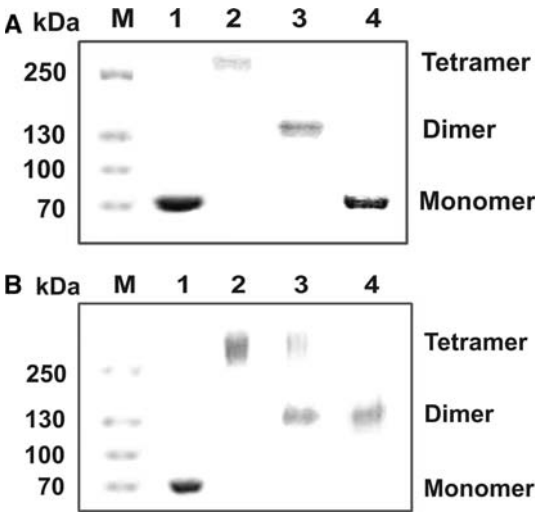


**Fig. 4** SDS–PAGE profiles of glutaraldehyde cross-linked GdmCl-treated and urea-treated BmHk samples. **a** *Lane M* represents marker, *lanes 1–4* represent native BmHk, native glutaraldehyde cross-linked BmHk, glutaraldehyde cross-linked 0.4 M GdmCl-treated BmHk, and glutaraldehyde cross-linked 1 M GdmCl-treated BmHk, respectively. **b** *Lane M* represents marker, *lanes 1–4* represent native BmHk, glutaraldehyde cross-linked 1 M urea-treated BmHk, glutaraldehyde cross-linked 2 M urea-treated BmHk, and glutaraldehyde cross-linked 3 M urea-treated BmHk, respectively. Experimental details are given in the "Materials and methods" section

incubated with increasing concentrations of GdmCl or urea for 4 h at 4°C. To perform refolding studies, these samples were then diluted 20-fold with 0.1 M phosphate buffer (pH 7) and stirred continuously for almost 4 h at 4°C. The extent of renaturation of hexokinase was judged by the recovery of enzymatic activity. For BmHk samples treated with up to 0.4 M GdmCl or 1 M urea, complete refolding was observed (data not shown). However, for BmHk samples denatured with 1 M GdmCl or 4 M urea, no recovery of enzymatic activity was observed on refolding. These observations demonstrate that complete refolding to native BmHk can be achieved only after denaturation of BmHk with low concentrations of denaturant, whereas denaturation at high concentration is irreversible, resulting in inability to recover the native protein on refolding.

## Discussion

Guanidine hydrochloride (GdmCl) and urea are the most common species used for protein denaturation. The conformational stability of proteins can be measured by equilibrium unfolding studies using GdmCl and urea solutions. The unfolding of BmHk in urea and GdmCl suggests different pathways and mechanisms for the two denaturants. The equilibrium-induced unfolding of BmHk in GdmCl and urea proceeds by stabilization of several unique oligomeric

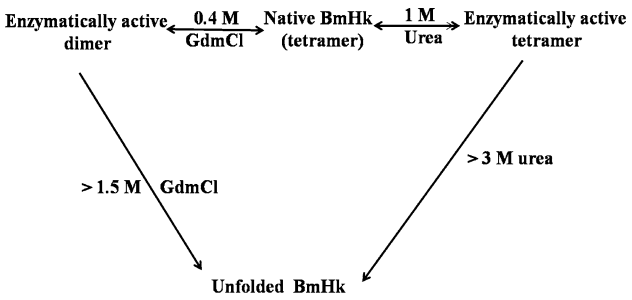


**Fig. 5** Schematic representation of urea and GdmCl-induced structural and functional changes in BmHk

intermediates. The formation of dimers during unfolding of the tetramer is a general phenomenon. For many enzymes, the formation of a dimer of dimers is the common unfolding intermediate, which is followed by unfolding of the dimer into monomers (Misra and Bhakuni 2003; Venkatesha et al. 1998). The unfolding of BmHk by low concentrations of GdmCl resulted in stabilization of an enzymatically active dimer, whereas urea-induced unfolding of BmHk leads to the stabilization of an enzymatically inactive dimer. Complete refolding of the native enzyme was observed on refolding of the folded dimer and tetramer, but no recovery of native enzyme was observed on refolding of partially unfolded dimer. Based on these results, we can suggest the following scheme for the unfolding of BmHk (Fig. 5).

The exact molecular mechanism(s) of the denaturing action of GdmCl and urea has (have) not been clearly defined (Schellman 2002; Makhatadze and Privalov 1992). It has been presumed that both urea and GdmCl molecules unfold proteins by solubilizing the non-polar parts of the protein molecule along with the peptide backbone CONH groups and the polar groups in the side chains of the proteins (Nandi and Robinson 1984). According to this mechanism, the unfolding of BmHk should follow the same path with both denaturants. However significant differences in the unfolding pathway of BmHk were observed for GdmCl and urea. This prompted us to look for other possible differences between the two denaturants which would explain their different effects on unfolding process.

GdmCl is an electrolyte and is, therefore, expected to ionize into $Gdm^+$ and $Cl^-$ in aqueous solution. Structurally, urea and $Gdm^+$ are very similar; however, urea is a neutral (uncharged) molecule whereas the guanidium ion has a positive charge delocalized over the planar structure. At high concentrations, GdmCl is a denaturant because binding of $Gdm^+$ ions to the protein predominates and pushes the equilibrium towards the unfolded state; this results in denaturation of the protein. However, at low concentrations, $Gdm^+$ ion can by preferentially adsorbed on to the protein surface because of interactions with the negatively charged amino acid side chains present in

protein molecule. This would lead to perturbations and/or weakening of the optimized electrostatic interactions present in the native conformation of protein and, as a result, stabilization of intermediates can be observed under these conditions.

In GdmCl-induced denaturation of BmHk, complete loss of enzymatic activity was observed at GdmCl concentrations above 1.5 M. Far UV studies on GdmCl-induced unfolding of BmHk indicated the complete unfolding of the enzyme at GdmCl concentrations above 1.5 M. The tryptophan fluorescence experiments indicated two well separated transitions. The folded conformation of a protein can be perturbed in various ways, for example by addition of urea or guanidinium chloride (GdmCl). In contrast the absorbance of Tyr and Trp, at 287 nm and 291 nm respectively, was increased substantially when denaturants were added, even in the absence of structural transitions (Schmid 1997). The optical density of tryptophan standard was increased at 291 nm, reflecting a slight red-shift of the spectrum, which originates from the change in refractive index (i.e. the polarity) of the solvent with the concentrations of GdmCl or urea. Consequently, even in the absence of structural changes, protein absorbance is expected to increase with denaturant concentration because of effects on those chromophores in the folded protein that are already exposed to the solvent. Hence these effects might be responsible for the observed 30% retention of the tertiary structure in 1.5 M GdmCl.

In proteins that contain tryptophan (Trp) residues both shifts in wavelength and change in intensity are generally observed upon unfolding. Unfolding by GdmCl results in a strong decrease in Trp fluorescence and a concomitant red-shift of the maximum to about 354 nm. Aromatic contributions can invalidate secondary structure estimates for proteins with a low helix content and a large proportion of aromatic residues (Perczel et al. 1992). For urea-induced denaturation of BmHk, enzymatically inactive dimer was observed. The urea-stabilized tetramer had less enzymatic activity than the native enzyme.

The studies presented in this paper demonstrate that cation binding to native BmHk leads to dissociation of the native tetramer resulting in stabilization of an enzymatically active dimer, whereas partial unfolding leads to stabilization of an enzymatically inactive, partially unfolded dimer. The studies also demonstrate that GdmCl and urea-induced unfolding of BmHk follow different pathways.

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## References

Aleshin AE, Zheng C, Bourenkov GP, Bartunik HD, Fromm HJ, Honzatko RB (1998a) The mechanism of regulation of hexokinase: new insights from the crystal structure of recombinant human brain hexokinase complexed with glucose and glucose-6-phosphate. Structure 6:39–50

Aleshin AE, Fromm HJ, Honzatko RB (1998b) Multiple crystal forms of hexokinase I: new insights regarding conformational dynamics, subunit interactions, and membrane association. FEBS Lett 434:42–46

Aleshin AE, Zheng C, Bartunik HD, Fromm HJ, Honzantko RB (1998c) Regulation of hexokinase I: crystal structure of recombinant human brain hexokinase complexed with glucose and glucose-6-phosphate. J Mol Biol 282:345–357

Aleshin AE, Kirby C, Liu X, Bourenkov GP, Bartunik HD, Fromm HJ, Honzatko RB (2000) Crystal structures of mutant monomeric hexokinase I reveal multiple ADP binding sites and conformational changes relevant to allosteric regulation. J Mol Biol 296:1001–1015

Anderson CM, Stenkamp RE, McDonald RC, Steiz TA (1978) Sequencing a protein by X-ray crystallography II. Refinement of yeast hexokinase B co-ordinates and sequence at 2.1 Å resolution. J Mol Biol 123:15–34

Armstrong RL, Wilson JE, Shoemaker CB (1996) Purification and characterization of the hexokinase from *Schistosoma mansoni*, expressed in *Escherichia coli*. Protein Expr Purif 8:374–380

Bennet WS, Steiz TA (1980) Structure of a complex between yeast hexokinase A and glucose I. Structure determination and refinement at 3.5 Å resolution. J Mol Biol 140:183–209

Cardenas ML, Cornish-Bowden A, Ureta T (1998) Evolution and regulatory role of hexokinases. Biochim Biophys Acta 1401:242–264

Gokhale RS, Ray SS, Balram H, Balram P (1999) Unfolding of *P. falciparum* triose phosphate isomerase in urea and guanidinium hydrochloride: evidence for novel disulphide exchange reaction in a covalently cross-linked mutant. Biochemistry 38:423–431

Hayashi S, Nakamura S (1981) Multiple forms of glucose oxidase with different carbohydrate compositions. Biochim Biophys Acta 657:40–51

Janicke R (1987) Stability and folding of domain proteins. Prog Biophys Mol Biol 49:117–237

Janicke R, Lillie H (2000) Folding and association of oligomeric and multimeric proteins. Adv Protein Chem 53:329–401

Joshi S, Singh AR, Kumar A, Siddiqui I, Misra PC, Saxena JK (2008) Molecular cloning and characterization of *Plasmodium falciparum* transketolase. Mol Biochem Parasitol 160:32–41

Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y (2004) Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. Structure 12:429–438

Kohn WD, Kay CM, Hodges RS (1995) Protein destabilization by electrostatic repulsions in the two stranded alpha helical coiled/coil leucine zipper. Protein Sci 4:237–250

Kumar DP, Tiwari A, Bhatt R (2004) Effect of pH on the stability and structure of yeast hexokinase A. J Biol Chem 279:32093–32099

Kuser PR, Krauchenco S, Antunes OA, Polikarpov I (2004) The high resolution crystal structure of yeast hexokinase PII with the correct primary sequence provides new insights into the mechanism of action. J Biol Chem 275:20814–20821

Lakowicz JR (1999) Principles of fluorescence spectroscopy, 2nd edn. Kluwer Academics/Plenum Publishers, New York

Makhatadze GI, Privalov PL (1992) Protein interactions with urea and guanidium chloride: a calorimetric study. J Mol Biol 226:491–505

Mei G, Venere A, Burgonza M, Vecchini P, Rosato N, Argo F (1997) Role of quaternary structure in the stability of dimeric proteins: the case of ascorbate oxidase. Biochemistry 36:10917–10922

Misra SK, Bhakuni V (2003) Unique holoenzyme dimers of the tetrameric enzyme *E. coli* methylenetetrahydrofolate reductase: characterization of structural features associated with modulation of the enzymes function. Biochemistry 42:3921–3928

Mochizuki Y (1981) Evolutionary aspects of lower deuterostomian hexokinases. Comp Biochem Physiol 70B:745–751

Monera OD, Kay CM, Hodges RS (1994) Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. Protein Sci 3:1984–1991

Mulichak AM, Wilson JE, Padmanabhan K, Garavito RM (1998) The structure of mammalian hexokinase I. Nat Struct Biol 5:555–560

Nandi PK, Robinson DR (1984) Effects of urea and guanidine hydrochloride on peptide and non-polar groups. Biochemistry 23:6661–6668

Pace CN (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves. Methods Enzymol 131: 267–280

Parr GR, Hammes GG (1975) Subunit dissociation and unfolding of rabbit muscle phosphofructokinase. Biochemistry 14:1600–1605

Perczel A, Park K, Fasman GD (1992) Deconvolution of the circular dichroism spectra of protein, the circular dichroism spectra of the antiparallel beta-sheet in proteins. Proteins Struct Funct Genet 13:57

Phillips D, Blake CCF, Watson HC (eds) (1981) The enzymes of glycolysis: structure, activity and evolution. Philos Trans R Soc Lond B Biol Sci 293:1–214

Schellman JA (2002) Fifty years of solvent denaturation. Biophys Chem 96:91–101

Schmid FX (1997) Optical spectroscopy to characterize protein conformation and conformational changes. In: Creighton TE (ed) Protein structure: a practical approach. Oxford University Press, Oxford, pp 261–297

Seckler R, Janicke R (1992) Protein folding and protein refolding. FASEB J 6:2545–2552

Singh AR, Joshi S, Arya R, Kayastha AM, Srivatava KK, Tripathi LM, Saxena JK (2008) Molecular cloning and characterization of *Brugia malayi* hexokinase. Parasitol Int 57:354–361

Steiz TA, Fletterick RJ, Anderson WF, Anderson Ch M (1976) High resolution X ray structure of yeast hexokinase, an allosteric protein exhibiting a non-symmetric arrangement of subunits. J Mol Biol 104:197–222

Tielens AG, Heuvel JM, Mazijk HJ, Wilson JE, Shoemaker CB (1994) The 50-kDa glucose 6-phosphate-sensitive hexokinase of *Schistosoma mansoni*. J Biol Chem 269:24736–24741

Venkatesha B, Udgaonkar JB, Rao NA, Savithri HS (1998) Reversible unfolding of sheep liver tetrameric serine hydroxymethyl transferase. Biochim Biophys Acta 1384:141–152