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# 'All-or-none' mechanism of the molten globule unfolding

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The Gdm-HCl-induced unfolding of bovine carbonic anhydrase B and *S. aureus* β-lactamase was studied at 4°C by a variety of methods. With the use of FPLC it has been shown that within the transition from the molten globule to the unfolded state the distribution function of molecular dimensions is *bimodal*. This means that equilibrium intermediates between the molten globule and the unfolded states are absent, i.e. the molten globule unfolding follows the 'all-or-none' mechanism.

Protein folding; Molten globule; Size exclusion chromatography; Carbonic anhydrase B; β-Lactamase

## 1. INTRODUCTION

The molten globule is a state of protein molecules which, in many cases, is thermodynamically stable under mild denaturing conditions. It is almost as compact as the native state (**N**), has a pronounced secondary structure and differs from **N** mainly by the absence of tight packing of side chains in the protein core and by a substantial increase of fluctuations [1–4]. The molten globule state (**MG**) accumulates during the renaturation of globular proteins from the fully unfolded state (**U**) [1–8] and therefore may play a universal role in protein folding [7]. It has been also suggested [9] and shown experimentally that the molten globule is trapped by Gro-EL chaperons ([10] and unpublished data of G.V. Semisotnov) and is involved into protein insertion into the membrane [11]. All this determines the interest in the structural and thermodynamic properties of **MG**.

It has been shown [1,12,13] that the transition between **N** and **MG** is the 'all-or-none' one, just as in other types of protein denaturation [14]. However, the question remained as to whether **MG** is separated from **U** by another all-or-none transition, or if it is no more than a limiting case of the 'squeezed' coil and which can gradually 'swell' into a full **U**.

Here we show that at least Gdm-HCl-induced unfolding of the molten globule is an all-or-none transition. This means that a protein molecule can be in three discrete states: **N**, **MG** and **U**.

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*Abbreviations:* N, native state; MG, molten globule state; U, unfolded state of protein molecules; BCAB, bovine carbonic anhydrase B; Gdm-HCl, guanidinium hydrochloride; ANS, 1-anilino-naphthalene-8-sulphonate; CD, circular dichroism; UV, ultraviolet.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Bovine carbonic anhydrase B (BCAB) was purified from bovine blood erythrocytes by N.V. Kotova (Institute of Protein Research) according to [15] with minor modifications. β-Lactamase was purified from *Staphylococcus aureus* (strain PC1) by T. Picard (University of Newcastle-upon Tyne).

### 2.2. *Preparation of protein solutions*

The proteins were incubated at 4°C for 40 h in 100 mM sodium phosphate buffer, pH 6.8, containing the desired Gdm-HCl concentration (plus 0.01 mg/ml ANS in the case of fluorometric experiments). Protein concentrations for FPLC measurements were about 0.01 mg/ml, for fluorometric measurements 0.001 mg/ml, for activity measurements 0.1 mg/ml, for activity check just after chromatography 1.0 mg/ml and for circular dichroism (CD) measurements 0.8 mg/ml.

### 2.3. *Equipment*

Size-exclusion chromatography (SEC-FPLC) experiments were carried out in a cold room, using a Superose-12 column and FPLC equipment (Pharmacia, The Netherlands). CD measurements were made with a Jasco-600 spectropolarimeter (Japan). Enzymatic activity measurements were made using a Specord M40 spectrophotometer (Germany). Fluorescent measurements were made with a Aminco (SPF-1000CS) corrected spectrofluorimeter (USA). All these instruments were equipped with a temperature-controlled holder.

### 2.4. *Experimental procedures*

Size exclusion chromatography (SEC-FPLC) measurements were made by loading a protein stock solution (0.01 mg/ml) with the desired Gdm-HCl concentrations on to a column, equilibrated by the same buffer with the same Gdm-HCl concentration. The flow rate was 20 ml/h. The elution profiles were obtained with the use of a 2158 Uvicord SD (LKB) equipped with a 226 nm filter.

Esterase activity of BCAB was measured by the rate of increase of absorption at 348 nm, which is the measure of *p*-nitrophenylacetate cleavage [15]. The reaction was initiated by adding 20 liters of protein stock solution to the 2,000 liters of reactive mixture containing *p*-

nitrophenylacetate (~0.18 mg/ml). For the check of activity in samples just after chromatography the protein stock solution (1 mg/ml) was loaded as above, the elution peak, which corresponds to the compact protein molecules, was collected and 40 l of this sample was added to 4000 liters of the reactive mixture (see above).

Enzymatic activity of β-lactamase was measured by the rate of decrease of absorption at 235 nm, which is the measure of benzylpenicillin cleavage [16]. The reaction was initiated by adding 20 liters of protein stock solution to the 2,000 l of reactive mixture containing benzylpenicillin (~0.1 mg/ml).

The ANS binding was registered by the characteristic increase of the ANS fluorescence intensity at 480 nm [17] in the presence of BCAB ([BCAB]/[ANS] = 1/50).

## 3. RESULTS AND DISCUSSION

The most direct approach to establish an all-or-none transition is to show that molecules can be only in one of the two states in the transition region, i.e. that their distribution function is *bimodal*. If these two states substantially differ by their volumes, and if the exchange between these states is slower than a characteristic time of chromatography (which is about 10–20 min), the bimodal distribution can be demonstrated by the highly efficient size exclusion chromatography [18,19].

The curves of urea- or Gdm-HCl-induced denaturation (**N** ⇆ **U** transition) of proteins on a column (monitored either by relative areas of these two peaks or, for fast exchange, by the position of an average peak) coincide with the curves monitored by far UV CD in solution [18,19] and our unpublished data). We have shown that this is true also for **MG** ⇆ **U** transition of proteins which denaturate through **MG** (unpublished). Moreover it was shown that Gdm-HCl-induced **N** ⇆ **MG** transition in BCAB (monitored by esterase activity) coincides on a column and in solution (see below, Fig. 1B). Thus, a column does not shift the equilibrium between **N**, **MG** and **U** and therefore can be used for a quantitative study of protein denaturation.

The time of protein renaturation from **U** (i.e. of

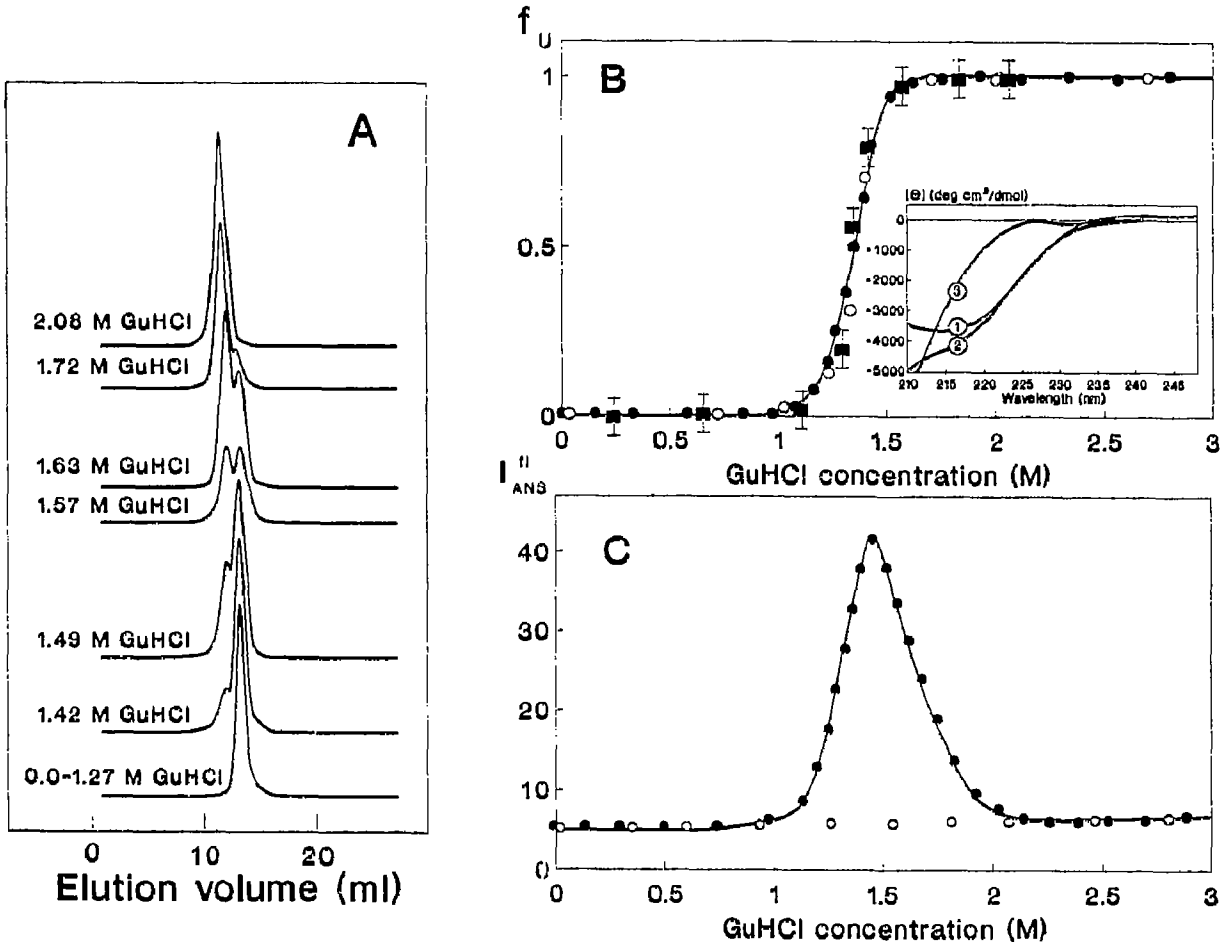


Fig. 1. Gdm-HCl-induced equilibrium unfolding of bovine carbonic anhydrase B at 4°C. (A) Elution profiles of size exclusion chromatography (FPLC). Gdm-HCl concentrations are shown above the curves. (B) Decrease of near UV CD (molar ellipticity at 270 nm) (○) in solution and of esterase activity (●) in solution as well as just after the size exclusion chromatography (■). $f_U = (X - X_D)/(X_N - X_D)$, where $X_N$ and $X_D$ are the values of the measured parameters for denatured and native states, respectively. The insert presents the far UV CD spectra at 0, 1.45 and 3.43 M Gdm-HCl (curves 1, 2 and 3, respectively). (C) Changes of fluorescence intensity of the hydrophobic probe ANS, at 480 nm in the presence (●) and in the absence (○) of BCAB.

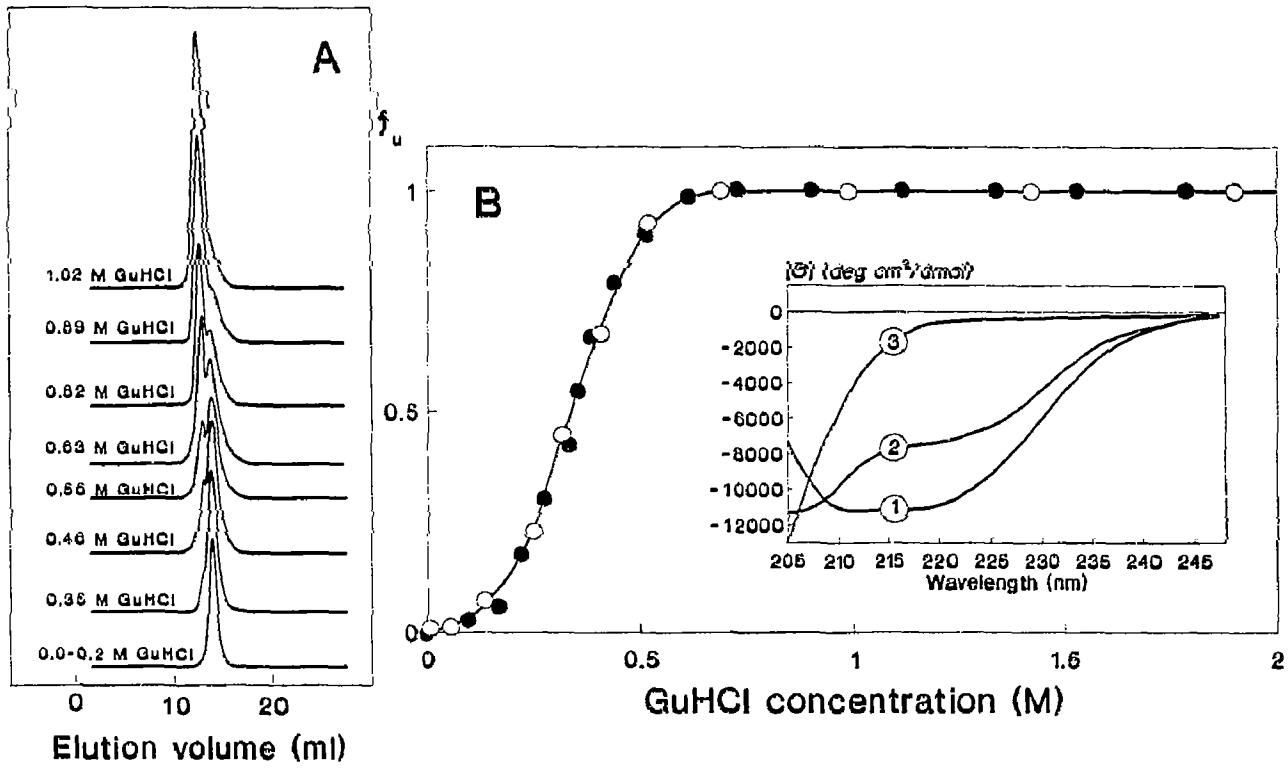


Fig. 2. Gdm-HCl-induced equilibrium unfolding of *S. aureus* β-lactamase at 4°C, monitored by (A) size exclusion chromatography (FPLC). (B) Decrease of molar ellipticity at 270 nm (○) and of enzymatic activity (●). The insert presents the far UV CD spectra at 0, 0.63 and 4.83 M Gdm-HCl (curves 1, 2 and 3, respectively).

U → N transition) can be as large as ~ 40 min at 23°C [1,2], which suggests the possibility of observing a bimodal equilibrium size distribution even at room temperature. This distribution has been actually observed for equilibrium urea-induced unfolding of myoglobin [18] and of bovine serum albumin [19] at 25°C.

The U → MG transition usually takes place in a few seconds [4,7]. However, this transition takes much more time for some proteins in cool Gdm-HCl solutions. For example, U → MG transition takes ~ 40–50 min in BCAB at 1.5 M Gdm-HCl and in *S. aureus* β-lactamase at 0.6 M Gdm-HCl (data not shown). Therefore one can expect that at least for these two proteins U ⇌ MG exchange can be slow enough to permit separate observations of MG and U by FLPC.

Figs. 1A and 2A show elution profiles for Gdm-HCl-induced unfolding of these proteins at 4°C. At moderate concentrations of Gdm-HCl (up to ~ 1.3 M for BCAB and ~ 0.2 M for β-lactamase) a single elution peak is observed the position of which virtually coincides with that of the native protein. At a higher Gdm-HCl concentration a second peak appears which corresponds to a *more expanded* state of protein molecules. The intensity of this peak increases with Gdm-HCl concentration at the expense of the intensity of the 'old' peak and finally (at ~ 1.7 M Gdm-HCl for BCAB and ~ 1.0 M for β-lactamase) only non-compact molecules remain. Thus, the Gdm-HCl-induced unfolding of BCAB and β-lactamase is an 'all-or-none' transition accompanied by a substantial increase of molecular dimensions.

At Gdm-HCl-induced unfolding of BCAB [20], β-lactamase [21] and many other proteins [4], activity and near UV CD change at smaller Gdm-HCl concentrations than far UV CD. This means the existence of two transitions: the first being interpreted [1] as the *denaturation* of a protein (i.e. N → MG transition), while the second is interpreted as the further *unfolding* of a protein (i.e. MG → U transition).

The all-or-none transition observed by size exclusion chromatography is certainly a MG → U rather than a N → MG transition. In fact, Figs. 1B and 2B show that the denaturation, monitored by activity and near UV CD, occurs mainly between 1.0 and 1.5 M Gdm-HCl for BCAB, and between 0.1 and 0.6 M Gdm-HCl for β-lactamase. On the other hand, the all-or-none transition monitored by FPLC occurs between 1.3 and 1.8 M Gdm-HCl for BCAB and between 0.35 and 0.9 M Gdm-HCl for β-lactamase. Thus, although these transitions overlap partially (especially in β-lactamase), they are resolved clearly enough to exclude the possibility that the all-or-none transition monitored by FPLC may be N → MG transition.

Fig. 1B shows also that activity of BCAB collected from an elution peak of FPLC corresponding to compact protein molecules follows the same Gdm-HCl dependence as that measured without a column. Such a

coincidence shows that also on a column the **N** ⇄ **MG** transition occurs mainly before the appearance of the second elution peak.

Thus, we can conclude that the all-or-none Gdm-HCl-induced transition monitored by FPLC *is not* the transition between N and the denatured states. Rather it is the transition between two denatured state, a compact and an expanded one. The following experiments show that this intermediate compact state fulfills all criteria for the typical molten globule [1–4]:

(i) The main elution peak for the intermediate states of BCAB (Fig. 1A) and β-lactamase (Fig. 2A) practically coincides with that of **N**. Thus, these intermediates are nearly as compact as **N**.

(ii) Far UV CD spectra of these intermediates for BCAB (Fig. 1B) and for β-lactamase (Fig. 2B) are very pronounced, which suggests that these intermediates have a pronounced secondary structure.

(iii) Near UV CD spectra of BCAB and β-lactamase (not shown) almost completely vanish in the intermediate states, which shows the absence of an asymmetrical rigid environment of aromatic side groups.

(iv) The fluorescence intensity of the hydrophobic probe (ANS) in the presence of BCAB (Fig. 1C) has a sharp maximum at 1.45 M Gdm-HCl. This shows that BCAB strongly binds ANS under these conditions, which is a specific test for **MG** [4,6,7,17].

It follows that the intermediate states of BCAB and β-lactamase at moderate Gdm-HCl concentrations correspond to **MG**, and therefore the observed all-or-none transitions (Figs. 1A and 2A) are those between **MG** and a more expanded state.

Figs. 1A and 2A show also that the elution peaks of expanded molecules shift to smaller elution volumes with the increase of Gdm-HCl concentrations, suggesting the further increase of molecular dimensions.

Thus, all-or-none transitions occur not only between **N** and **U** [14] and Native **MG** [12,13], but also between **MG** and essentially **U**. This suggests that globular proteins can exist in at least three discrete states: **N**, **MG** and **U**. These three states of globular proteins resemble, to some extent, the three states of low molecular weight compounds, the crystal, the liquid and the gas. Thus, the molten globule is not like a squeezed coil, rather it is the real third state of protein molecules (in addition to **N** and **U** described previously).

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