

# 'Molten-globule' state accumulates in carbonic anhydrase folding

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Kinetics of folding and unfolding of bovine carbonic anhydrase B were monitored by circular dichroism, viscometry and esterase activity. It was shown that kinetic intermediate states accumulating in folding process reveal a native-like compactness and secondary structure but have a symmetrized average environment of aromatic side groups and no esterase activity. These properties allow one to consider these intermediate states as the 'molten-globule' state of a protein molecule previously described by us for several equilibrium forms of bovine and human  $\alpha$ -lactalbumins and bovine carbonic anhydrase B.

*Carbonic anhydrase B      Protein folding      Molten-globule state of protein molecules*  
*Folding intermediate*

## 1. INTRODUCTION

Recently [1–3] we have described a new equilibrium state of a protein molecule, 'compact globular state with fluctuating tertiary structure', using the examples of bovine and human  $\alpha$ -lactalbumins. Both proteins can be transformed into this state under the influence of various agents: acid pH, moderate concentrations of Gu-HCl, heating, etc. A protein molecule in this state is nearly as compact as native protein and has a native-like secondary structure, but at the same time it has a symmetrical average environment of aromatic and other side groups, no cooperative temperature melting and fast deuterium exchange. A model of this 'intermediate' (between the native and unfolded states) state has been proposed [1–4] as a compact globule with pronounced fluctuations of its three-dimensional structure (first of all with large fluctuations of its side groups). According to

this model the increase of fluctuations is due to a slight increase of the molecular volume leading to a sharp decrease of Van der Waals intramolecular interactions [4].

Quite recently we have found that a similar intermediate state also exists in bovine carbonic anhydrase B at pH  $\sim$ 3.6 [3,5]. CA B at pH 3.6 has a compact structure with a far UV CD spectrum even more pronounced than in the native state but with a near UV CD spectrum similar to the completely unfolded state [6]. We have shown [5] that CA B at pH  $\sim$ 3.6 has a native-like secondary structure (evidenced from its infrared spectrum) and that the main part of its molecule has fast deuterium exchange and does not melt cooperatively on heating. Therefore all properties of the main part of its molecule at pH  $\sim$ 3.6 correspond to the properties of the intermediate state described earlier [1–3] for  $\alpha$ -lactalbumins. It has also been shown that CA B has a similar state at  $\sim$ 2 M Gu-HCl as it has far and near UV spectra similar to those at pH 3.6 [7,8], it is compact [9] and does not melt cooperatively on heating (unpublished).

A similar intermediate state has also been found for ribonuclease A [10] and for cytochrome *c* [11]. Wada proposed for this state the term 'molten globule' [11]. The existence of a similar inter-

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*Abbreviations:* CA B, carbonic anhydrase B;  $\alpha$ -LA,  $\alpha$ -lactalbumin; Gu-HCl, guanidine hydrochloride; CD, circular dichroism; UV, ultraviolet; states of CA B – N (native), I (intermediate) and U (unfolded)

mediate or molten globule state for such different proteins as  $\alpha$ -lactalbumins, carbonic anhydrase, ribonuclease and cytochrome *c* suggests that this state is not a rare exception and may be of general importance for protein physics.

Carbonic anhydrase folds much slower than  $\alpha$ -lactalbumins, ribonuclease and cytochrome *c* (see e.g. [12]) which facilitates the investigation of the possible role of this intermediate state in its folding pathway. The kinetics of CA B refolding and unfolding have been studied by several authors [6,9,13–18]. Complex (multi-phase) kinetics of both processes have been shown [6,15–18] which, taken by itself, does not prove the existence of kinetic intermediates as CA B has 20 prolines and therefore may have several unfolded forms differing in proline isomerization [12]. However it has been shown [17] that the secondary structure of CA B (monitored by the far UV CD spectra) is restored in the course of folding much faster than the tertiary one (monitored by  $[\theta]_{270}$ ) which is good evidence for the existence of intermediate(s) with secondary structure but without a specific tertiary one. It has also been shown [9] that the esterase activity of CA B is restored slowly in the course of protein folding and that the rates of this recovery are similar at the transitions both from the unfolded state (5 M Gu-HCl) and from the intermediate one (2 M Gu-HCl).

We show here that the state which accumulates during carbonic anhydrase folding has not only a native-like secondary structure but also a native-like compactness, i.e. it is similar to the intermediate state which exists in this protein at pH 3.6 and at  $\sim$ 2 M Gu-HCl. We also compare the kinetics of all 6 transitions between 3 different states of carbonic anhydrase (native, intermediate and unfolded).

## 2. MATERIALS AND METHODS

Bovine CA B was obtained from Serva Feinbiochemica and purified on a column with cellulose DE-52 (Whatman) as in [19] with small modifications. The homogeneity of the proteins was checked by disc-electrophoresis in polyacrylamide gels with and without SDS.

Protein concentrations were determined using extinction coefficients  $E_{1\text{ cm}, 280\text{ nm}}^{1\%} = 18.3$  [6]. Gu-HCl concentrations were determined from the

refractive index at 589 nm [20].

Measurements of transitions from the intermediate state (at 1.97 M Gu-HCl) to the other states are hampered by the slow (half-time  $\sim$ 2 h) transitions of CA B at  $\sim$ 2 M Gu-HCl to an irreversibly denatured state [9]. Although in our experiments this effect was substantially smaller than in [9], to exclude it completely we transferred CA B to 1.97 M Gu-HCl from 5.45 M Gu-HCl 5–10 min before the transition from 1.97 M Gu-HCl to the other states.

Time courses of molar ellipticities  $[\theta]_{222}$  and  $[\theta]_{270}$  have been monitored by dichrograph (Marks III-S, Jobin Ivon) directly during the transition of CA B from the initial to final state in the spectrophotometric cell. The protein concentration in CD measurements was usually  $\sim$ 1 mg/ml and the path length of the cell was 1 cm for the near UV and 0.5 mm for the far UV experiments.

Specific viscosity  $\eta_{sp}$  was measured with a capillary viscometer (Viscomatic) with an automated solution pump which permits monitoring of the time course of  $\eta_{sp}$  for the same protein sample by the change of its flow time through the capillary. The viscometer was thermostated to within 0.01°C. CA B concentration in viscosity measurements was  $\sim$ 3 mg/ml, the duration of each measurement being  $\sim$ 100 s.

Esterase activity was determined as in [19] by the increase of absorbance at 348 nm due to hydrolysis of *p*-nitrophenylacetate (*p*-NPA) corrected for the spontaneous hydrolysis in the absence of the enzyme. Aliquots (0.1 ml) of the renaturing protein ( $\sim$ 1 mg/ml) were placed into the cell containing 2 ml of the reactive mixture with 1 mM *p*-NPA. The duration of each measurement was  $\sim$ 2 min. As Gu-HCl is an inhibitor of CA B the activity of the renaturing protein at 0.97 M Gu-HCl was compared with the activity of native CA B under the same conditions.

The temperature was 19–20°C for all experiments. All kinetics were studied at neutral pH values.

## 3. RESULTS

### 3.1. Kinetics of renaturation

#### 3.1.1. U $\rightarrow$ N transition

Refolding kinetics were studied by diluting CA B solution in 5.45 M Gu-HCl (where molecules are

completely unfolded [6]) to 0.97 M Gu-HCl (where the protein is in the native state [6]). Fig.1 presents the data plotted as the 'fraction of nativity'

$$f_N = (X - X_D)/(X_N - X_D)$$

where  $X$  is the value for the studied parameter at a given time and  $X_N$  and  $X_D$  are the values for the native and denatured states, respectively.

Fig.1 shows that the values of reduced viscosity (reflecting the compactness of molecules) as well as the values of  $[\theta]_{222}$  (reflecting their secondary structure as it has been demonstrated [17] that these values represent well the changes of the whole far UV CD spectrum of CA B during its refolding) are restored by ~85% during the dead time of our experiments (~2 min) thus approaching the values for native CA B. However the values of  $[\theta]_{270}$  (reflecting the specific tertiary structure) and the esterase activity change by only 10–15% during the dead time and continue to change afterwards with a half-time ~25 min. This means that in the course of transition of CA B from the unfolded to the native state the intermediate state(s) accumulates which is native-like by its compactness and secondary structure but unfolded-like by its aromatic group environment and enzymatic activity.

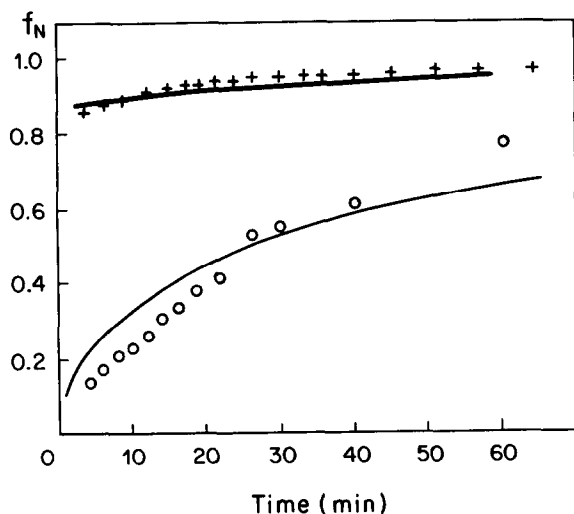


Fig.1. Time dependences of fraction of nativity  $f_N$  for CA B refolding from the completely unfolded state (U  $\rightarrow$  N transition from 5.45 M to 0.97 M Gu-HCl) monitored by ellipticities at 270 nm (—) and 222 nm (—), esterase activity (o) and reduced viscosity (+).

### 3.1.2. I $\rightarrow$ N transitions

Fig.2 represents the refolding kinetics at the I  $\rightarrow$  N transition of CA B from the molten-globule state (at 1.97 M Gu-HCl) to the native one monitored by the parameters which change during this transition ( $[\theta]_{270}$  and esterase activity). The kinetics of the U  $\rightarrow$  N transition (monitored by the same parameters) are reproduced from fig.1 for comparison. One can see that kinetics of I  $\rightarrow$  N and U  $\rightarrow$  N transitions are very similar having a half-time ~25–30 min.

## 3.2. Kinetics of unfolding

### 3.2.1. N $\rightarrow$ U and I $\rightarrow$ U transitions

The kinetics of the N  $\rightarrow$  U (0.97 M  $\rightarrow$  5.45 M Gu-HCl) transition have been monitored by  $[\theta]_{222}$ ,  $[\theta]_{270}$  and esterase activity and those of the I  $\rightarrow$  U (1.97 M  $\rightarrow$  5.45 M Gu-HCl) transition by  $[\theta]_{222}$ . All values approach those for the unfolded protein during the dead time of our experiments ( $\leq 2$  min). Thus the unfolding of CA B from both the native and the intermediate states is much faster than its folding.

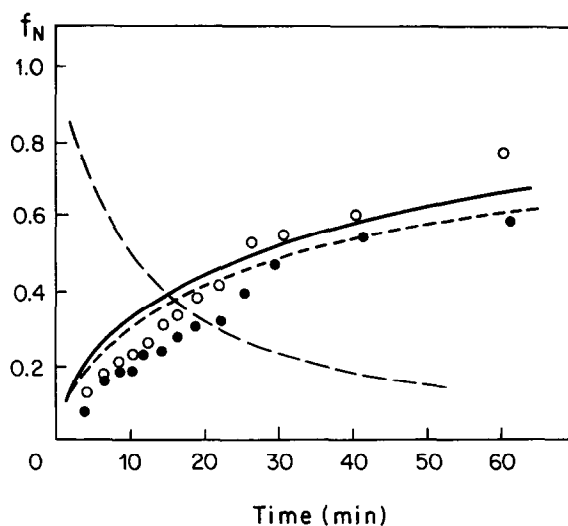


Fig.2. Time dependence of fraction of nativity  $f_N$  in the course of CA B refolding from the molten-globule state (I  $\rightarrow$  N transition from 1.97 M to 0.97 M Gu-HCl) monitored by ellipticity at 270 nm (----) and esterase activity (o). Time dependences of these values at U  $\rightarrow$  N transition (— and o, respectively) are reproduced from fig.1 for comparison. Curve (—) shows the kinetics of the N  $\rightarrow$  I (0.97 M  $\rightarrow$  1.97 M Gu-HCl) transition.

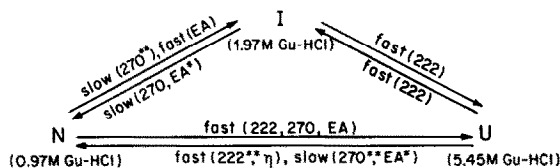


Fig.3. Scheme of kinetics of transitions of carbonic anhydrase B between the native (N), intermediate or molten globule (I) and unfolded (U) states. 222, 270,  $\eta$  and EA indicate that the kinetics have been monitored by  $[\theta]_{222}$ ,  $[\theta]_{270}$ , reduced viscosity and esterase activity, respectively. \* and \*\* indicate that similar measurements have been performed earlier in [9] (\*) and in [17] (\*\*).

### 3.3. Kinetics of transitions into the molten-globule state

#### 3.3.1. U $\rightarrow$ I transition

The kinetics of the U  $\rightarrow$  I (5.45 M  $\rightarrow$  1.97 M Gu-HCl) transition have been monitored by  $[\theta]_{222}$ . All changes take place during the dead time of our experiments ( $\leq 2$  min) similar to the fast stage of the U  $\rightarrow$  N transition (see above).

#### 3.3.2. N $\rightarrow$ I transition

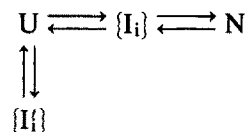
The kinetics of the N  $\rightarrow$  I (0.97 M  $\rightarrow$  1.97 M Gu-HCl) transition have been monitored by  $[\theta]_{270}$  and esterase activity. The esterase activity vanishes during the dead time of our experiments ( $\leq 2$  min), however  $[\theta]_{270}$  changes slowly with a half-time  $\sim 10$  min (see fig.2) which confirms the data in [17]. We cannot exclude, however, the possibility that these slow changes are due to the reversible aggregation during the time course of the N  $\rightarrow$  I transition.

Fig.3 summarizes our data on the kinetics of the transitions between 3 states of carbonic anhydrase B. One must remember that each kinetics has been monitored under the condition corresponding to the final stage of the transition and therefore only the kinetics of the transition to the same final state can be compared directly.

## 4. DISCUSSION

Analyzing their data on kinetics of CAB refolding and unfolding, authors in [17] came to the conclusion that these processes can be described by

the equation



where  $\{I_i\}$  denotes the intermediate states in the folding pathway and  $\{I\}$  the abortive states. We have shown here that esterase activity recovers almost simultaneously with the specific tertiary structure of CA B and that its specific viscosity recovers (as its secondary structure [17]) much faster, mainly during the dead time of our experiments ( $\leq 2$  min). It follows that in the time course of CA B refolding the state(s) accumulates which has a native-like secondary structure and a native-like compactness but has no specific tertiary structure and no enzymatic activity. Thus this state(s) is similar to the intermediate or molten-globule state which is thermodynamically stable for CA B at pH  $\sim 3.6$  and  $\sim 2$  M Gu-HCl. As the specific viscosity of the unfolded CA B is an order of magnitude greater than that of the compact one, this means that all states accumulating during the U  $\rightarrow$  N transition (both intermediate and abortive ones) are compact and therefore belong to the intermediate or molten-globule type. This suggests that this type of state may play a significant role in protein self-organization.

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