The molten globular intermediate form in the folding pathway of human carbonic anhydrase B

M.V. Jagannadham and D. Balasubramanian*

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

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The acid-induced and guanidinium chloride-induced conformational transitions in human carbonic anhydrase B have been analyzed. A structural form was detected at pH 3, which has a higher secondary structural order than the native enzyme but little tertiary structure. The enzyme dissolved in an intermediate concentration of the denaturant guanidinium chloride (1 M at pH 7.5) also adopts a similar conformational state. This form, denoted as the intermediate form I, possesses most of the characteristics defined for the molten globular state of globular proteins and might serve as the embryonic structural intermediate during the self-organization of the protein into its functional native form.

Globular protein renaturation *Molten globular form* *Human carbonic anhydrase B* *CD spectroscopy*

1. INTRODUCTION

The delineation of the strategy that an unordered protein chain adopts to fold into its functional and native form in a short time has been a subject of considerable interest. A hierarchy of the folding process, nucleation sites and domains, embryonic structures and discrete intermediates have been suggested [1,2]. A recent suggestion is that of a molten globular form [3–8] (the term has been proposed in [7]), which forms an intermediate state in the self-organisation of globular proteins. Such a molten globular state has been reported for several globular proteins [3–8]. We report here our results on the unfolding-refolding of human carbonic anhydrase B (HCAB), induced by acid and by the denaturant guanidinium chloride (GuCl), which suggest the presence of such a molten globular intermediate state in this enzyme, which has significant secondary, but little tertiary, structural order, which is devoid of esteratic activity and which can be converted into the native and active form.

HCAB, present in human erythrocytes, is a single chain of about 260 amino acids (no disulfide bridges), having one Zn^{2+} at the active site, and catalyzing the reversible hydration of CO_2 [9]. Its activity can be assayed either by determining the hydrolysis rate of *p*-nitrophenyl acetate [10] or by the hydration reaction of CO_2 into HCO_3^- or vice versa [9]. The isoenzyme of HCAB, termed HCAC, is also present in erythrocytes; while there is only 59% homology between the sequences of HCAB and HCAC, their 3-dimensional structures are very similar [11–13]. Early spectroscopic studies on HCAB and HCAC [14,15] have suggested that stable and partly unfolded conformations of the enzymes might occur at intermediate concentrations of denaturants. Carlsson et al. [16,17] have studied in detail the unfolding and refolding of these molecules and suggested that incorrectly folded states of the molecule might occur in 1.7 M GuCl.

It is of interest in this connection to note that bovine carbonic anhydrase B (BCAB) is remarkably similar in its 3-dimensional conformation to the human B enzyme HCAB, despite the 45% variations or heterology in their amino acid sequences [11,18]. Kinetic studies on the unfolding

* To whom correspondence should be addressed

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and renaturation of BCAB [19,20] have revealed several conformational species involved in the folding pathway. That a molten globular intermediate accumulates in the folding of BCAB has been reported by Dolgikh et al. [8].

The overall conformational similarity between HCAB and its bovine analog has prompted us to investigate whether any of the intermediate structural forms observed in HCAB bear any structural resemblance to the molten globular state. We have also attempted to compare the properties of the acid-induced form and the GuCl induced forms of the enzyme HCAB.

2. MATERIALS AND METHODS

HCAB was obtained from Sigma (lot 112F-9305) and its purity and intactness confirmed by PAGE and SDS-PAGE to be satisfactory. The esteratic activity of the enzyme was determined using *p*-nitrophenyl acetate as the substrate [21]. The rate of hydrolysis of 0.4 mM substrate in 75 mM Tris-sulfate (pH 7.5) buffer at 25°C was followed by measuring the absorbance of 400 nm. When the assay solution contained GuCl, appropriate correction was made by using the blocks containing the proper concentrations of the denaturant.

Circular dichroism (CD) spectral measurements in the near-ultraviolet (UV) and far-UV regions were made using a Jasco-20 spectropolarimeter that was previously calibrated with appropriate standards. The mean residue molar ellipticity is expressed in degree·cm^2·$dmol^{-1}$. All measurements were made at 25°C. The concentration chosen for CD studies is 0.8 mg/ml of HCAB, where self-aggregation of the protein does not occur.

3. RESULTS AND DISCUSSION

3.1. *Acid-induced conformation of HCAB*

The monitoring of the far-UV (below 240 nm) and the near-UV CD bands (310–250 nm) of the enzyme offers information about its secondary and tertiary structural features respectively, such as the helical or β-structure content, and the aromatic amino acid clustering and environment [9,14,22]. Fig.1 reveals that the native structure of HCAB has about the same structural features as its bovine analog [20]. The far-UV CD spectrum is compatible with little helical order and about 35% β-

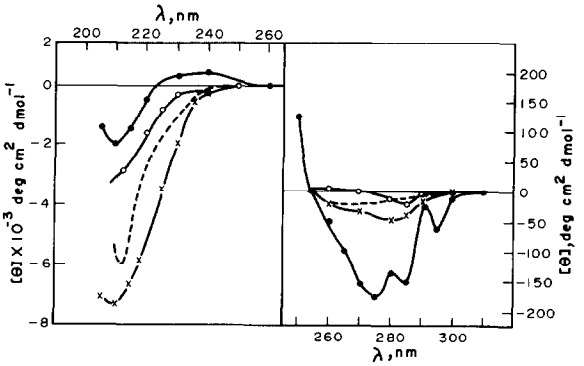


Fig.1. Far-UV (left) and near-UV (right) CD profiles of human carbonic anhydrase B. (●) Native, neutral pH; (– – –) in 1 M GuCl; (○) in 5 M GuCl; and (×) at pH 3.0.

structure while the characteristic near-UV CD bands originate from the aromatic amino acid clustering in the tertiary structure. Upon denaturation (with 5 M GuCl), the enzyme loses all its tertiary structure (fig.1, a featureless near-UV CD curve) and backbone order (monotonically decreasing far-UV CD spectrum).

Acidification of HCAB to pH 3 causes significant CD spectral changes. The intensification and slight blue-shifting of the 215 nm band suggest that the acid form has a different secondary structural order from the native form, but the near-UV CD spectrum has lost much of its features, resembling the unordered form. The reduction of ellipticity of the aromatic bands suggests a substantial loss in the tertiary structure around these structures. The molecule was found to be enzymatically inactivated at pH 3 since no esteratic activity was observed here. Efforts to renature the enzyme by restoring the pH to neutrality were unsuccessful since it led to aggregation and precipitation [20]. The solubility of HCAB at pH 3 already being low, hydrodynamic characterization of its compactness was not possible [23].

3.2. *HCAB in 1.2 M GuCl*

Fig.1 also shows the changes that occur in the CD profiles of HCAB upon addition of increasing amounts of the denaturant GuCl. Addition of as little as 0.5 M GuCl already causes a significant reduction in the tertiary structure of the molecule (near-UV CD bands) and at 1.2 M GuCl the tertiary structure is essentially disrupted. However,

the secondary structure, as monitored by far-UV CD bands, appears to be promoted even more in 1.2 M GuCl than in the native form – a situation similar to what is obtained when the enzyme is acidified to pH 3. Further increase in the denaturant concentration leads to expected loss of the secondary structure as well. Thus, there is a non-coincidence in the transition curves of the enzyme as monitored by near-UV CD and far-UV CD bands. The resemblance between the CD spectra of the enzyme in 1.2 M GuCl and at pH 3 suggests a similarity in the structure of the enzyme under both conditions. It thus appears that HCAB might adopt an intermediate conformational form I, during its denaturation and that this form possesses a pronounced secondary structure, but little or no tertiary structure. In light of the observations that such an I form exists in BCAB [8], which can be transformed into the native (N) or the unfolded (U) forms, we undertook a kinetic study of the conformational transitions in HCAB between the N, U and the putative I forms.

3.3. *Time dependence of structural transitions*

The enzyme aggregates and precipitates when one attempts to renature it from the acid-expanded form directly, just as the bovine analog does [20]. However, it is possible to take the GuCl-denatured HCAB, dilute the denaturant concentration to very low amounts (0.1–0.4 M GuCl) and reversibly renature and reactivate it. Fig.2a shows that such a U⟶N transition of HCAB is rapid with respect to the recovery of the secondary structure. The ellipticity at 222 nm of the native form is largely obtained within the dead-time of 2 min of diluting the GuCl concentration and recording the CD spectrum. However, it is slower with respect to its tertiary structure as monitored by the recovery of the native-form near-UV CD spectrum (half-time of the order of 10 min), and the esteratic activity was found to recover to 90% of its value in the native form in about 60 min. This transition may be summarized as:

$$N \underset{\text{fast (222), slow (270,EA)}}{\overset{\text{fast (222, 270, EA)}}{\rightleftharpoons}} U$$

0.1–0.4 M GuCl 5 M GuCl

where 'fast' refers to a rapid change in the property studied, i.e., within the dead-time of 2 min, 'slow' to a time scale of the order 10–20 min, 222 and 270 to the CD band intensities at 222 and 270 nm, and EA to the specific activity of the enzyme.

When the kinetics of the N⟶I form was

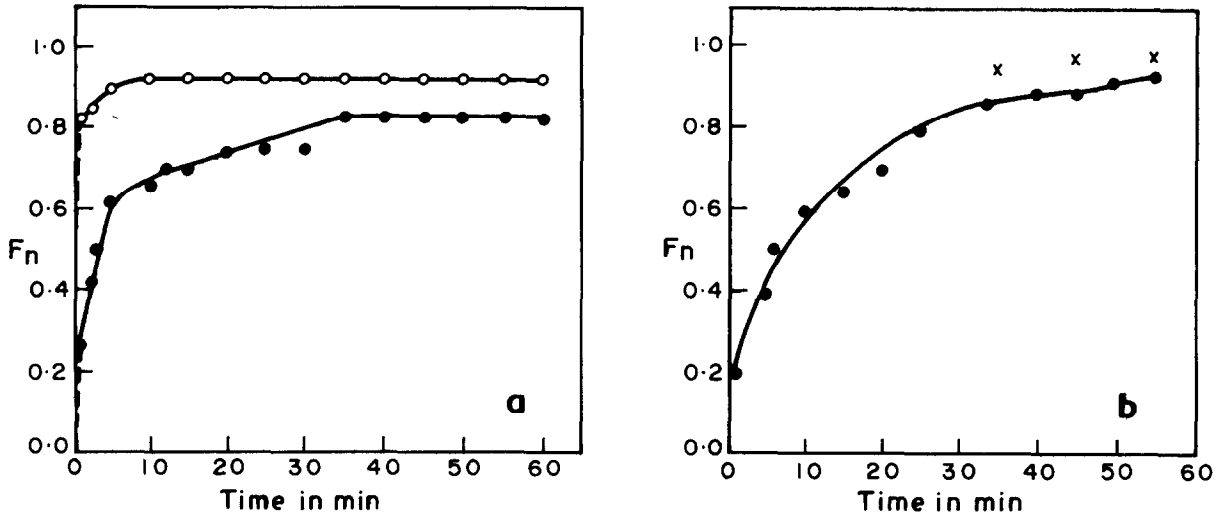
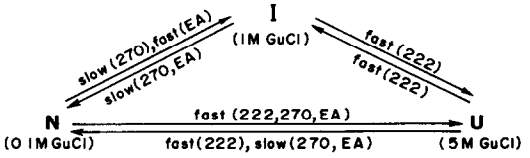


Fig.2. (a) Time dependence of the recovery of the native structure in HCAB upon renaturing the molecule from the unfolded (U) state in 5 M GuCl to the native form (N) in 0.5 M GuCl; as monitored by CD at (●) 270 nm and (○) 222 nm. (b) Time dependence of the recovery of native structure in HCAB during the transition from the intermediate (I) form in 1.2 M GuCl to the native (N) form in 0.12 M GuCl; (●) as monitored by CD at 270 nm, and (×) by the esteratic activity.

followed, it was seen that the CD spectral changes (fig.1) occurred with a half-time of the order of a few minutes, while the change from the I⟶U form occurred within 2 min. The I form has no esteratic activity. It has already been pointed out that the far-UV CD band changes (at 222 nm) are fast upon going from U to I.

Fig.2b illustrates the time profile of the transition I⟶N, as monitored by the near-UV CD spectral changes (270 nm) and the recovery of the esteratic activity. The CD bands around 270 nm are seen to recover to that of the N form with a $t_{1/2}$ of the order of 10–12 min, while the esteratic activity is recovered to that of the native state in about 40 min.

It is thus possible to summarise the structural transitions in HCAB, brought about by various concentrations of GuCl in the medium, in the form of scheme 1, identical to that proposed by Ptitsyn and co-workers [8] for BCAB:



Scheme 1. Scheme of the kinetics of the transitions in HCAB between the native (N), intermediate (I) and unfolded (U) forms.

The implication of an intermediate form, I, is consistent with the kinetic behavior of the enzyme, and also with its spectroscopic properties. Carlsson et al. [16,17] suggested that an 'incorrectly folded' form of HCAB might accumulate at medium concentrations of GuCl. Henkens et al. [24] characterized an intermediate form of the bovine enzyme at 2 M GuCl; McCoy and Wong [20] have shown that the kinetics of renaturation of the same enzyme is consistent with the presence of intermediate forms; and Ptitsyn's group has suggested that bovine carbonic anhydrase has a molten globular form in 2 M GuCl, similar to those obtained in a few other globular proteins [8]. Such a molten globule is defined as possessing a secondary structure comparable to the native form, but lacking tertiary structural features, or cooperative thermal melting. It is thought to have pronounced fluctuations in its 3-dimensional structure, due to a slight decrease in its molecular volume leading to a sharp decrease of the van der Waals interactions [5].

The I form that we have studied in HCAB appears to be such a molten globule since it shares many of its characteristics. A few more points are worthy of note: (i) The acid-induced form of HCAB might very well be a molten globular structure since it shares several properties with the form obtained at 1 M GuCl. (ii) The observations that the N⟶U transition of HCAB occurs at a lower GuCl concentration when monitored by the CD changes at 270 nm than those at 220 nm, and that the secondary structure is recovered faster than the tertiary structure during the U⟶N, U⟶I, or I⟶N transitions indicate that the secondary structure is formed earlier in the enzyme molecule. Thus, the I form appears to be a likely candidate for the 'embryonic form' with secondary structural order, that has been postulated as the intermediate form in the self-organization of a globular protein [2]. (iii) The I form of HCAB occurs at around 1 M GuCl while that of the bovine enzyme is seen around 2 M GuCl. This appears to be a reflection of the differences in their primary structure. (iv) The fact that the I form is identifiable in both HCAB and BCAB (which have only 55% homology between them) suggests that such a form might also occur in human carbonic anhydrase C, which has about 60% homology with the enzyme B, and that the entire family of carbonic anhydrases might self-organize through the I form in their folding pathway.

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