Noncooperative Temperature Melting of a Globular Protein without Specific Tertiary Structure: Acid Form of Bovine Carbonic Anhydrase B

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Synopsis

Heat denaturation of native globular proteins is a cooperative process usually connected with the melting of the main part of their regular secondary structure. In this paper, a noncooperative temperature-induced melting of the regular secondary structure in the carbonic anhydrase B at pH 2.6 in heavy water is observed by ir spectroscopy. The molecules of carbonic anhydrase B in an acid medium, unlike the native ones, do not have a specific tertiary structure. Nevertheless, the β-structure content is about the same in both of these states. A temperature-induced noncooperative melting process takes place from 10 to 67°C with a decrease of the antiparallel β-form content by about one third. The remaining part of the β-form melts with a more intensive heat absorption, with a maximum at 87°C. The whole melting process is practically reversible. We assume that the observed noncooperative process displays a general property of a new type of structural state of the globular protein—the "molten globule state."

INTRODUCTION

The study of protein conformations intermediate between the native and completely unfolded states is important for understanding the problem of protein folding. Carbonic anhydrase B is an appropriate protein for studying intermediate forms.[1-8] According to x-ray data, this protein contains approximately 40% of the β-form and 10% of α-helical structure.[9] The protein has a molecular weight equal to 29 kD, and the molecule has one Zn^{2+} ion and no disulfide bridges.

In this paper, the temperature-induced melting of the acid form of bovine carbonic anhydrase B in D_2O solution at pH* 2.6 has been examined by the methods of ir spectroscopy and scanning microcalorimetry. Under these conditions, the protein molecule exists in a state intermediate between the native and the fully unfolded ones.[2,7] In this state, the carbonic anhydrase molecule is rather compact,[2] with a nativelike secondary structure[7]; nevertheless, the environment of the aromatic side chains is symmetrical.[2,7] Under these conditions, the

(pH* is a direct reading of pH meter in D_2O solutions.)

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Zn^{2+} ion is not imbedded in the protein molecule (Dolgikh and Smolyaninova, unpublished results).

In the native globular protein, the temperature-induced melting of the main part of the secondary structure is cooperative and takes place simultaneously with considerable heat absorption. In this paper, we describe the noncooperative temperature-induced melting of the regular secondary structure of carbonic anhydrase B, the process embracing the disruption of about a third of the protein β-structure.

MATERIALS AND METHODS

Bovine carbonic anhydrase B was obtained from Serva feinbiochemica and purified on DE-52-cellulose (Whatman) according to the procedure of Armstrong et al.[10] with minor modifications. Sample homogeneity was checked by electrophoresis with sodium dodecylsulfate and without it. All measurements were carried out in D_2O solutions with $0.02M$ KCl. The protein solutions were prepared by dissolving lyophilized protein in heavy water followed by adjustment of the solution pH* to 2.6 with $0.4M$ DCl. Dialysis was then carried out against the appropriate buffer for 15 h at room temperature to improve the isotopic quality of the solution. Concentrations were determined by an EPS-3T spectrophotometer (Japan) using the value, $E^{1 \text{ cm}, 1\%}_{280 \text{ nm}} = 18.3^1$ at pH 7.5. For solutions at pH 2.6, we determined this value to be equal to 17.3.

The ir absorption spectra were obtained with the Perkin-Elmer M180 double-beam spectrophotometer connected with an EC 1010 computer (Hungary). Protein spectra were recorded with thermostated demountable CaF_2 cells of 80- and 120-μm fixed thickness at 0.05–1.0% concentration. The temperature accuracy was $\pm$ 0.5°C, and the heating rate was 0.5 deg/min. The technique used for obtaining quantitative ir spectra of proteins in heavy water has been described earlier.[11,12] The difference spectra were obtained by subtraction of the appropriate spectra with the computer. Decomposition of the ir amide I band into symmetric curves was performed with CK-1 curve resolver.[13] Microcalorimetric measurements were carried out on a DASM-4M microcalorimeter.[14] The heating rate was 1 deg/min and the protein concentration was $\leq$ 0.3%. The equilibrium centrifugation experiments were performed with a MOM 3170 ultracentrifuge (Hungary). The initial protein concentration in a cell was ~0.4 mg/mL.

RESULTS

The ir spectra of bovine carbonic anhydrase B in heavy water solution are shown in Fig. 1 at different temperatures. At 10°C, the amide I band has a maximum at 1642 cm^{-1} and the presence of a significant amount of β-structure in this protein seems to be obvious.[11] At higher temperatures, the intensity of the amide I band decreases and the
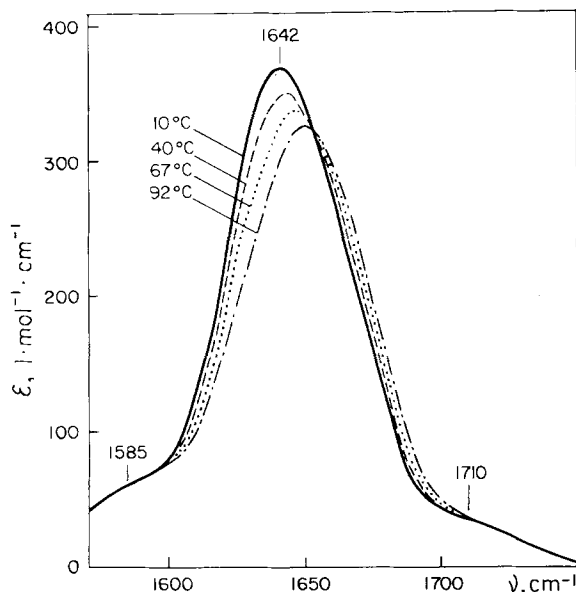
Fig. 1.   The ir spectra of bovine carbonic anhydrase B in heavy water at different temperatures, 20 mM KCl–DCl, pH 2.6, concentration 0.05–1.0%

frequency of the maximum becomes slightly higher. The shoulders with the maxima near 1585 and 1710 cm^{-1}, are due to side-chain absorption.[15]

The temperature-induced melting of the secondary structure is clearly reflected in the difference ir spectra (Fig. 2). Here, the band near 1630 cm^{-1} and the shoulder near 1685 cm^{-1} pertain to the antiparallel β-form, and the band at 1665 cm^{-1} seems to be assigned to the α-helical or unordered form. The curves at 10, 40, and 67°C intersect at the one isobestic point, which is evidence of the two-state transition; the intersection point of the curve at 82°C is moved slightly to the lower frequency. From the difference curves and the spectra in Fig. 1, it is clear that the content of regular β-form decreases with temperature elevation.

The continuous intensity change of the amide I band in the 10–92°C temperature region is shown in Fig. 3(a). This curve has been recorded at 1630 cm^{-1}, as the maximum change of intensity is near this frequency (see Fig. 2). In the temperature region of 10–67°C, the intensity changes linearly. At higher temperatures (67–92°C), the intensity falls more sharply and in a nonlinear way. The observed effect does not depend on the concentration in the investigated range (0.05–1.0%).

The calorimetric curve of carbonic anhydrase temperature melting is shown in Fig. 3(b). This curve has a practically linear part in the 10–67°C region and a peak with a maximum at 87°C. From a compar-
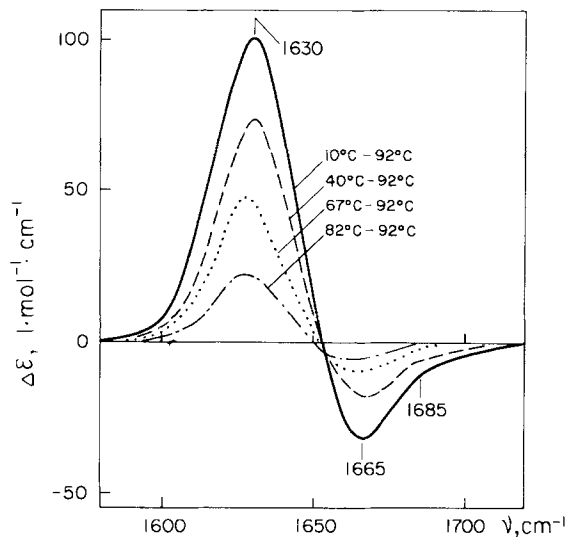
Fig. 2. Difference ir spectra calculated from the data of Fig. 1 by subtraction of the 92°C spectrum from the spectra at all other temperatures.
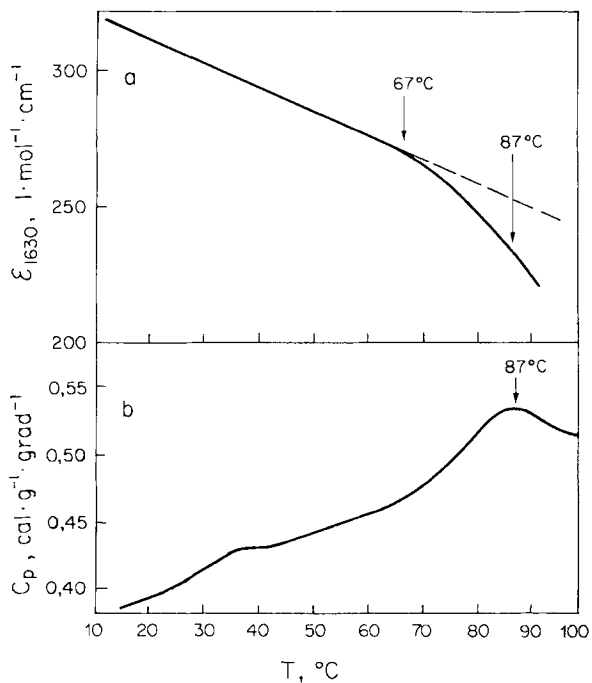

Fig. 3. (a) Temperature dependence of ir intensity at 1630 cm^{-1} for bovine carbonic anhydrase B. Solution parameters are the same as in Fig. 1; heating rate, 0.5°C/min. (b) Temperature dependence of partial specific heat capacity for bovine anhydrase B. Solution parameters are the same as in Fig. 1; heating rate, 1°C/min.

ison of spectrophotometric and calorimetric curves, it is seen that the linear change of the secondary structure is not accompanied by remarkable cooperative heat absorption, while the nonlinear part of spectroscopic curve corresponds to the cooperative heat absorption (the peak of the heat capacity at 87°C). It should be noted that the enthalpy of conformational transformation with a maximum of 87°C is very low and is equal to ~3 J/g (~0.7 cal/g), which is about an order of magnitude less than the melting enthalpy of native globular proteins at this temperature.[16]

We carried out a quantitative analysis of the ir spectra of carbonic anhydrase at different temperatures (Fig. 4). This analysis was made for spectra at 10, 67, and 92°C, using an approach described previously.[7] First, we subtracted the amino acid side-chain absorption from the experimental spectra.[15] The resulting spectrum, which corresponds to the absorption of peptide groups, is shown in Fig. 4. Then, we decomposed the amide I band into several components, using the following assumptions: (1) The contours of the components must be symmetrical; (2) the extinction coefficients of the components (belonging to different structures) do not depend on temperature; (3) the frequency, the band width, and the shape of the contour of the amide I component for the unordered structure are similar to the mean parameters determined for various proteins in the unordered state[17] and are the same as those determined earlier[7] for the native carbonic anhydrase. The first assumption follows from the symmetry of amide I bands for polypeptides in different conformations.[11,12] The second assumption follows from the fact that the contour of the amide I band for the *native* protein (at pH* 7.5 and 10.9) is practically unchanged with temperature to ~60°C (Brazhnikov, Chirgadze, and Dolgikh, unpublished data). Therefore, the change of this contour for the acid form of the same protein (at pH* 2.6) can be totally attributed to the change of its structure. The third assumption follows from the conservation of amide I parameters for different proteins in the unordered state.[17]

Using these assumptions, the amide I band resolves into two large components at all temperatures, with the maxima at ~1630 and 1653 cm^{-1} and into two small ones with the maxima at 1673 and 1685 cm^{-1} (see Fig. 4). The components with the maxima at ~1630 and 1685 cm^{-1} belong to the antiparallel β-structure, and the components with the maxima at 1653 and 1673 cm^{-1} to the unordered structure.[7,11] It is important that the amide I band can be decomposed with high accuracy into components with almost the same frequencies, bandwidths and band shapes of the contours at different temperatures. In addition, decomposition of the amide I band at 92°C gives a small narrow component with a maximum at 1617 cm^{-1} that can be attributed to the presence of ~3% of the aggregated β-form.[18] (This form remains in the cooled solution after heating to 92°C.) No other components attributable to α-helices or β-turns appear in the resolved spectra. In
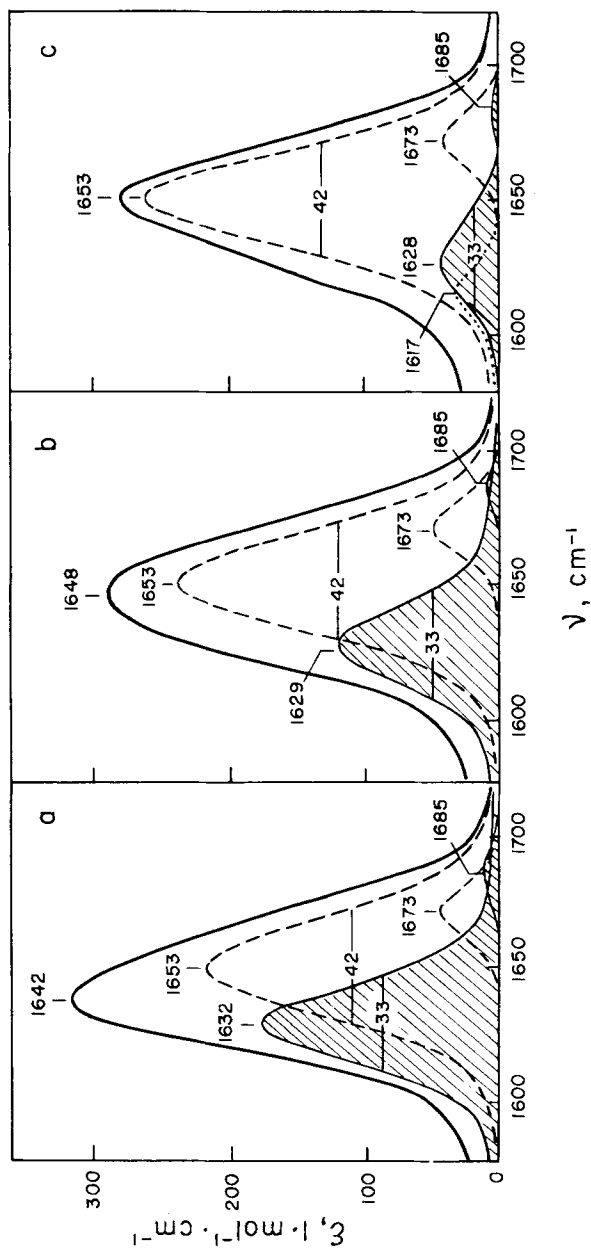
Fig. 4.   Graphic analysis of the amide I band for determining bovine carbonic anhydrase B secondary structure at different temperatures: (a) 10°C, (b) 67°C, and (c) 92°C. Experimental spectra were corrected for the absorption of side groups of amino acid residues, according to Ref. 15. The β-form bands with maxima of $\sim$1630 and 1685 cm^{-1} are hatched; bands at 1653 and 1673 cm^{-1} pertain to the unordered form.

all cases, the difference between the experimental band contour and that obtained by summarizing the individual components was small, in the range of 1–2%.

Using the integral intensities of the components of the antiparallel β-structure and of the unordered structure, we estimated the content of the antiparallel β-form in carbonic anhydrase as 33% at 10°C, 21% at 67°C, and 8% at 92°C. During the melting process, the antiparallel β-form is transformed into the unordered one. In a wide temperature interval (10–67°C), the antiparallel β-structure of the acid form of carbonic anhydrase melts noncooperatively. The effective melting enthalpy estimated from the decrease of the β-content in this temperature region is only ∼2 kcal/mol, which is two orders of magnitude smaller than the melting enthalpies of native carbonic anhydrase at 40–65°C.[16] Therefore, we conclude that the high melting enthalpies of native carbonic anhydrase (as well as of other native globular proteins) are not due to the melting of its β-structure. The rest of the antiparallel β-structure of the acid form of carbonic anhydrase melts with a more intensive heat absorption, which is reflected by a heat capacity peak at 87°C (see Fig. 3).

DISCUSSION

We recently found that bovine and human α-lactalbumin molecules can be transformed into similar forms having properties that are an unusual mixture of the properties of the native and unfolded proteins.[19–21] These forms can be obtained at acid pH, high temperatures, or moderate Gu-HCl concentrations. In these forms, the protein molecule has the nativelike compactness and the secondary structure content, but at the same time, it has a symmetrical averaged environment of side groups, a high rate of hydrogen exchange, and noncooperative melting on heating. On the basis of these facts, we assigned[19–21] all these forms to the novel state of a protein molecule (intermediate between native and fully unfolded ones), later called the "molten-globule state."[22] According to our model, the protein molecule in this state differs from the native one mainly by a significant increase in the thermal fluctuations of its structure. The amplitude of these fluctuations increases as a result of the small increase in the molecular volume, leading to a remarkable weakening of van der Waals intramolecular interactions.[23] At the same time, the other intramolecular interactions (backbone–backbone hydrogen bonds, hydrophobic forces) remain largely unchanged.

Bovine carbonic anhydrase at pH* 3.6 has a nativelike compactness and β-structure content, but the main part of the molecule has a high hydrogen exchange rate and does not melt cooperatively on heating.[2,7] Accordingly, we assume that the main part of the carbonic anhydrase molecule at pH* 3.6 occurs in the "molten-globule state," and a rather

small part of the molecule seems to retain the native structure, which has a slow hydrogen exhange rate and melts cooperatively on heating.[7]

Bovine carbonic anhdrase contains about 40% β-structure. Therefore, we have studied the structural transitions by the ir spectroscopy method, which is especially sensitive to the presence of β-structure. Heating carbonic anhydrase at pH* 3.6 leads to the aggregation effects evidenced by the appearance of the 1617-cm^{-1} band. These effects diminish at lower pH. That is why we carried out the present investigation at pH 2.6. According to Wong and Hamlin,[2] the intrinsic viscosity of carbonic anhydrase B at pH 3.6–2.0 increases at room temperature from 4.1 to 8.1 cm^3 g^{-1}, while for the native and the fully unfolded protein, these values are equal to 3.7 and 29 cm^3 g^{-1}, respectively. Therefore, carbonic anhydrase at pH 2.6 may be somewhat less compact than at pH* 3.6. But at this pH, the volume of the protein molecule is much smaller than in the fully unfolded state and the protein retains all the other features of the "molten-globule state."

To get an idea of the aggregation of carbonic anhydrase at pH* 2.6, we determined the molecular weight of the protein by the centrifuge equilibrium method. In the concentration range of ~0.02 to ~0.3%, the observed values are equal to 30–45 kD, depending on the concentration. As the actual molecular weight is equal to 29 kD, it suggests limited molecular association, at least at relatively high concentrations (~0.3%). However, this aggregation does not influence the secondary structure, as the ir spectrum does not depend on concentration in the range from 0.05 to 1.0%.

The noncooperative melting of secondary (α-helical) structure in the "molten-globule state" was observed earlier for the acid forms of bovine[24] and human α-lactalbumins (Venyaminov and Bychkova, unpublished) by CD in the far-uv region. Our present data for the melting of the acid form of carbonic anhydrase B, together with the data for α-lactalbumins, suggests that noncooperative temperature-induced melting of the secondary structure may be more or less general for the "molten-globule state." At temperatures higher than 67°C, the remaining part of the β-form melts more intensively, and this melting is accompanied by a small peak of heat capacity. It is possible in this case to assume that the melting cooperativity also is connected not with the melting of the β-structure *per se*, but with the disruption of the structure of the part of the protein molecule that is both stabilized by the backbone hydrogen bonds and by the other interactions—including, firstly, the van der Waals forces.

Note that the β-structure content of bovine carbonic anhydrase in the native form is similar to that of the "molten-globule state."[7] However, the half-width of the main β-structural component (with the maximum at ~1630 cm^{-1}) of the amide I band for the native state is equal to ~25 cm^{-1},[7] while that for the "molten-globule state" is 33 cm^{-1} (see Fig. 4). The broadening of the β-structural component of the amide band can be connected with some disordering of the regular β-

structure.[25,26] Thus, the noncooperative temperature-induced melting of the β-structure of carbonic anhydrase B described in this paper is related to the melting of the β-structure, which is less ordered than that in the native protein.

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