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The compact state of reduced bovine pancreatic trypsin inhibitor is not the compact molten globule

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Reduced bovine pancreatic trypsin inhibitor (BPTI) has been shown to be in a compact state [(1988) Biochemistry 27, 8889–8893]. This leads to the proposal that this compact state may be a compact molten globule folding intermediate. Optical rotatory dispersion in the visible region failed to show the presence of pronounced secondary structures in the reduced BPTI and no binding of 8-anilino-1-naphthalenesulphonic acid to reduced BPTI could be detected. Yet, no cooperative thermal transition was detected by tyrosine fluorescence. These experiments show that reduced BPTI is not in the compact molten globule state.

Bovine pancreatic trypsin inhibitor; Molten globule state; Optical rotatory dispersion; Fluorescence; Protein folding

1. INTRODUCTION

The compact molten globule (CMG) state of globular proteins is defined as an intermediate state between the unfolded and the folded states. It is characterized by native-like secondary structures and a tertiary structure devoid of unique packing of side chains [1–3]. Methods used for detection of the CMG state in proteins include far- and near-UV circular dichroism (CD) measurements, measurements of 8-anilino-1-naphthalenesulfonic acid (ANS) binding by detection of fluorescence enhancement, and monitoring the thermal unfolding transitions [2–4]. Bovine pancreatic trypsin inhibitor (BPTI) is a small (58 residues) single domain protein. The folding pathway of BPTI has been studied by many approaches and a few intermediates were identified [5–7]. Long-range, non-radiative energy transfer measurements have shown that reduced BPTI in 0.5 M GuHCl has a compact structure [6]. This raised the question as to whether reduced BPTI is in a CMG state under these conditions. Here we report experiments designed to address this question. So far, we have found no evidence for a CMG state in BPTI.

2. MATERIALS AND METHODS

Guanidine hydrochloride (GuHCl; Aldrich), dithiothreitol (DTT; Sigma), ethylenediaminetetra-acetic acid (EDTA; BDH), glycine (Gly; Sigma), *N*-acetyl-tyrosine ethyl ester (ATEE; Sigma), and 8-anilino-1-naphthalenesulfonic acid (ANS; Sigma) were used. BPTI (Trasylol) was a kind gift of Bayer A.G. To reduce BPTI the preparation was dissolved in 50–53 mM Gly buffer, pH 7.8–8.2 containing 6 M GuHCl, 25–45 mM DTT and 1.0–1.2 mM EDTA. The solution was incubated for 4 h at room temperature and then diluted in the same buffer containing EDTA only [6]. The final concentration of DTT and GuHCl was 1.25 mM and 0.17 M, respectively, after dilution. All measurements were done with freshly diluted solution.

Absorption spectra were measured with an AVIV 17DS spectrophotometer. Absorbances at the excitation bands were 0.05–0.25 (corrected for light scattering). Fluorescence spectra were measured with an I.S.S. Greg PC photon-counting spectrofluorimeter with thermostated cell-holder and a LAUDA thermostated circulating bath. Quantum yield (q) of BPTI tyrosine fluorescence (excitation at 277 nm) was calculated relative to ATEE fluorescence (q = 0.05 [8]) using peak intensity at 304 nm according to Cowgill [9]. Quantum yields of reduced BPTI were corrected for inner filter effect (due to the oxidized DTT absorption [10]). The viscosity of the solutions (η) was assumed to be close to that of water since we used low salt, GuHCl and protein concentrations. ANS fluorescence was excited at 360 nm and emission was measured at 400–650 nm (band width 8 nm). Optical rotatory angles were measured with a Perkin-Elmer 141 spectropolarimeter with an accuracy of 0.001 degrees. We have used molecular rotation per mean residue weight (M') reduced by refractive index (n) dispersion [11]:

$$[m'] = 3M\alpha/[100(\eta_\lambda^2+2)cd]$$

where M = 12,327 Da is defined as BPTI mol. wt. divided by the number of residues, α is the optical rotatory angle in degrees, c = 0.0009 g/ml is the protein concentration, d = 10.0 cm is the optical pathlength. Refractive index dispersion was determined by Cauchy's dispersion formula with n-values for water at 20°C [12].

3. RESULTS

The far-UV CD of BPTI was shown to contain a large contribution from the aromatic side chains [13–15] which might mask contributions due to changes in the main chain conformation. Therefore the method used here for estimation of changes of the secondary structure content of BPTI was the optical rotatory dispersion

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(ORD) in the visible region [11], which is convenient for comparative studies. The secondary structure changes could be estimated using the Moffitt-Yang equation:

$$[m'] = a_0 \Lambda + b_0 \Lambda^2$$

where a_0 and b_0 are parameters which reflect α-helix and β-structure contents and $\Lambda = \lambda_0^2/(\lambda^2-\lambda_0^2)$, $\lambda_0 = 212$ nm [11,16].

Fig. 1 shows, first, that the experimental data are in good agreement with the Moffitt-Yang equation and, second, that a_0 and b_0 values are very different for native and reduced BPTI: $a_0 = -640 \pm 12$ and $b_0 = -153 \pm 53$ for native and $a_0 = -446 \pm 38$ and $b_0 = -9 \pm 38$ degr · cm^2 · decimol^{-1} for reduced BPTI. Many reduced proteins have b_0 values close to zero, corresponding to a statistical coil state [17]. The Moffitt-Yang plots are in fact the same for reduced BPTI in 0.17, 1 and 6 M GuHCl. 6 M GuHCl is known to have a strong effect on secondary structures [17]. The negligible effect of increasing GuHCl concentrations on the ORD parameters indicates the absence of secondary structures in reduced BPTI. No α-helices and β-sheets in reduced BPTI have been qualitatively shown by the CD technique [13].

Fig. 2 shows the temperature dependence of the thermal (solvent) quenching of tyrosine fluorescence in reduced and native BPTI. The quantum yield of tyrosine fluorescence in reduced BPTI at 20°C (0.048) is 2.2-fold higher than that of the native state (0.022), and is very close to that observed for ATEE. It is possible that the quenching of tyrosine fluorescence in the native state is caused by a disulphide bond and/or carbonyl quenching, as shown for other proteins [9]. In the reduced state, even partial disorder near tyrosine residues might relieve the quenching. At high temperatures, 96–98°C, both the reduced and native BPTI fluorescence reach the same quantum yield, indicating a similar environment of the phenolic chromophore, probably fully solvated.

A decrease of $1/q$ values is observed at the temperature range 85–96°C with a clear, sharp cooperative transition. This change probably reflects a cooperative thermal conformational transition in native BPTI, like that of ribonuclease A [18]. A probable candidate tyrosine residue which may probe this transition is Tyr-23. This residue is buried, as shown by its resistance to iodination [19] or nitration and its higher pK_a value [20]. Such buried phenol chromophores are not fluorescent in many proteins [9], and solvation of Tyr-23 by the thermal unfolding can add its contribution to the quantum yield at the high temperatures and counter-balance the incremental thermal quenching. A reduction of tyrosine fluorescence appears above 85°C following full exposure of the buried tyrosine residue. A thermal transition in native BPTI in this temperature range has been shown using the Cotton effect [21] and calorimetric

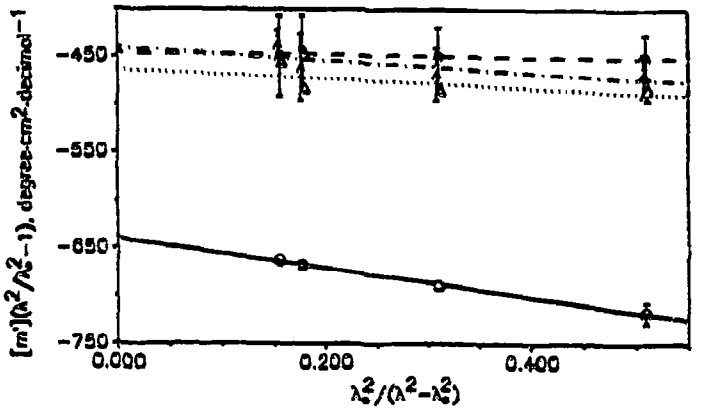

Fig. 1. Moffitt-Yang plot of the mean residue weight molar rotation of native and reduced bovine pancreatic trypsin inhibitor (BPTI). Open circles (solid line) indicate native BPTI. Filled circles (dashed line), filled triangles (dashed-dotted line) and open triangles (dotted line) indicate fully reduced BPTI in 0.17, 1 and 6 M GuHCl, respectively (DTT and EDTA concentrations are 1.25 and 1 mM; pH 7.8).

measurements [22]. An additional weak conformational transition can be deduced from the slope of $1/q$ in the range 12–25°C.

The same experiment done with reduced BPTI does not show any cooperative transition over the temperature range from 9 to 98°C. A plot of $1/q$ vs. T/η (where T is the absolute temperature) shows two temperature ranges in which $1/q$ vs. T/η is linear with different slopes ($\eta/Tq = 0.046$ at 20–82°C and 0.094 centipoise/K at 88–98°C). Similar types of results were obtained earlier with tyrosine fluorescence of cobra cytotoxin [23]. A linear $1/q$ vs. T/η plot indicates a homogeneity of all the chromophores with regard to their quantum yield [24]. Thus in reduced BPTI there are two temperature-controlled states which have different tyrosine fluorescence properties but, unlike native BPTI, there is no cooperative thermal conformational transition of reduced BPTI denaturation.

We have measured the fluorescence of ANS mixtures with native and reduced BPTI as it had been used by others [4,25]. Under all conditions used here, the quantum yield and the wavelength of the emission maximum of ANS was not changed relative to the results obtained for ANS in the same solvents in the absence of BPTI. We have scanned the ANS fluorescence with reduced and native BPTI over a GuHCl range of 0.5–6 M and over a pH range of 0.8–8.2, but could not detect any enhancement due to binding.

4. DISCUSSION

The main features which were used to characterize the CMG state of globular proteins were: compact structure, dense interior, loose packing of side chains, no cooperative thermal melting transition, and high content of the native secondary structures [2]. Reduced

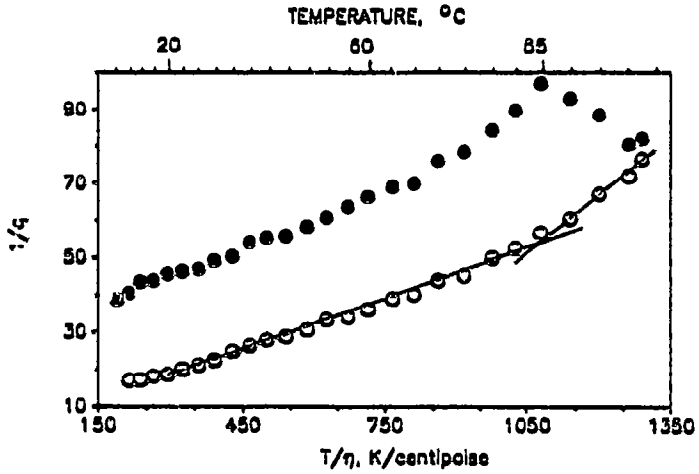

Fig. 2. Temperature quenching of tyrosine fluorescence (inversed quantum yield $1/q$) of native (filled circles) and reduced (open circles) bovine pancreatic trypsin inhibitor. Native BPTI was in 53 mM Gly-buffer, pH 7.6, plus 1 mM EDTA. Reduced BPTI was in the same buffer containing 0.17 M GuHCl and 1.25 mM DTT.

BPTI has some of these features but does not show a major feature, i.e. the presence of a high content of native secondary structures. It has been shown to be compact by energy transfer measurements [6,26], and no thermal cooperative transition was observed in the experiments reported here. 65% of the residues in native BPTI are included in secondary structures (40% in antiparallel β-sheet (residues 15–37), 25% in helices (residues 2–7 and 48–56) [27]) but our present experiments show no indication of any pronounced native-like secondary structures in the compact reduced state.

The lack of ANS binding (no spectral shift and no 100-fold intensity increase [28]) indicates the lack of a loose hydrophobic core which can accommodate the dye. Yet it may be that the small size of the hydrophobic core of BPTI leaves very few potential binding sites.

The present results and others [6,26] show that reduced BPTI has a compact structure which is temperature dependent, without cooperative transition, and without most of the native secondary structure content. That means that it does not fall under the category of the CMG-type intermediate. CMG intermediates can often be trapped using moderate concentrations of GuHCl, high salt concentrations or low pH [1–3]. The present experiment shows that even at mild conditions of neutral pH and low GuHCl concentrations, reduced BPTI contains a large fraction of unfolded chains. It is possible that reduced BPTI is in a compact state without specific packing of native-like seondary structure elements which form later in the folding pathway. Single or double disulphide intermediates [7] may be intermediate states closer to the definition of a CMG. Fully reduced BPTI may be similar to what has been termed compact denatured protein [29] but without considerable secondary structure. Other methods are currently being used to probe the kinetics of formation of the two major secondary structures in refolding BPTI in order to resolve the current question and identify the intermediate state which forms the native-like secondary structures.

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