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Reduced Bovine Pancreatic Trypsin Inhibitor Has a Compact Structure†

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ABSTRACT: The conformation of reduced bovine pancreatic trypsin inhibitor (R-BPTI) under reducing conditions was monitored by measurements of nonradiative excitation energy-transfer efficiencies (E) between a donor probe attached to the N-terminal Arg^1 residue and an acceptor attached to one of the lysine residues (15, 26, 41, or 46) [Amir, D., & Haas, E. (1987) *Biochemistry 26*, 2162–2175]. High-excitation energy-transfer efficiencies that approach those found in the native state were obtained for the reduced labeled BPTI derivatives in 0.5 M guanidine hydrochloride (Gdn·HCl) and 4 mM DTT. Unlike the dependence expected for a random coil chain, E does not decrease as a function of the number of residues between the labeled sites. The efficiency of energy transfer between probes attached to residues 1 and 15 in the reduced state is *higher* than that found for the same pair of sites in the native state or reduced unfolded (in 6 M Gdn·HCl) state. This segment also shows high dynamic flexibility. These results indicate that the overall structure of reduced BPTI under folding (but still reducing) conditions shows a high population of conformers with interprobe distances similar to those of the native state. Reduced BPTI seems to be in a molten globule state characterized by a flexible, compact structure, which probably reorganizes into the native structure when the folding is allowed to proceed under oxidizing conditions.

Kinetic pathways of folding via intermediate states were proposed for the mechanisms by which proteins fold from unfolded states to their unique three-dimensional structures (Matheson & Scheraga, 1978; Creighton, 1978; Kim & Baldwin, 1982; Goldberg, 1985). Intermediate states, which restrict the conformational space available for the unfolded state, can direct the pathway of folding and accelerate the transition to the native state. Unraveling the structural characteristics of the initial states in the folding transition should help decipher the mechanism of folding and the specific amino acid sequence messages that code for the pathway.

Creighton (1978) has studied the pathway of folding of BPTI and showed that the first disulfide bonds are already formed in a nonrandom distribution; the 30–51 disulfide pair is dominant.

Ptitsyn and co-workers (Dolgikh et al., 1981) have studied the conformation of α-lactalbumin unfolded by acid and by temperature elevation and found evidence for a compact globule state with nativelike secondary structures and with slowly fluctuating tertiary structure. Ohgushi and Wada (1983) made similar observations and coined the term "molten globule state" to describe this conformation. Both groups suggested that the molten globule state is a common type of an early intermediate in the folding pathway of globular proteins.

In the present study we are interested in the structure of early folding intermediates of BPTI[1] prior to formation of

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disulfide bonds. We have developed methods based on selective labeling and nonradiative excitation energy-transfer measurements (Förster, 1948; Stryer & Haugland, 1967; Steinberg, 1971; Haas et al., 1975; Haas, 1986). Using this approach, we have detected local structures in reduced unfolded BPTI (Amir & Haas, 1988) and RNase A (Haas et al., 1988). This approach is particularly useful for investigating the structure of folding intermediates since it allows for determination of localized distributions of intramolecular distances between pairs of selected sites in the molecule and their Brownian fluctuations (Haas et al., 1978a; Haas & Steinberg, 1984). In the present study we use four BPTI derivatives which enable us to obtain information on the conformation of subdomain structures of BPTI spanning the entire molecule.

We report here results of steady-state measurements of excitation energy transfer in four labeled BPTI derivatives in the reduced state. High intramolecular excitation transfer efficiencies were found which indicate that, in the early steps in the folding pathway, BPTI has a compact average conformation similar to that expected for a molten globule state (Dolgikh et al., 1981).

Materials and Methods

Materials. Ultrapure guanidine hydrochloride (lot GB1146) and ultrapure urea (lot GB1138) were obtained from Schwarz/Mann (Spring Valley, NY) and used as provided. Oxidized glutathione (lot 107c-0119) was from Fluka (Buchs, Switzerland). Other chemicals were from the same sources as reported earlier (Amir & Haas, 1987).

Labeled BPTI Derivatives. The preparation and the characterization of the protein derivatives are reported elsewhere (Amir & Haas, 1987). In the present study we used four BPTI derivatives specifically labeled by two probes, a donor and an acceptor. The donor, (2-methoxy-1-naphthyl)methyl (MNA), was attached to the terminal primary amino group by reductive alkylation with the corresponding aldehyde. The acceptor, [7-(dimethylamino)-coumarin-4-yl]acetyl (DA-coum), was attached to the ϵ-NH_2 group of one of the lysine residues (15, 26, 41, or 46) by acylation with the *N*-hydroxysuccinimide ester. These are designated (1–*n*)BPTI and R(1–*n*)BPTI (*n* = 15, 26, 41, and 46) in the native and reduced states, respectively. Two compounds, a BPTI derivative labeled by a DA-coum at a single amino group (DA-coum-BPTI) and a lysine labeled by both donor and acceptor (MD-Lys) (Amir & Haas, 1987), served as reference compounds for zero-transfer and high-transfer efficiency, respectively.

A mixture of BPTI derivatives labeled by two or more MNA residues, $(MNA)_2$-BPTI, was prepared by reductive alkylation (Amir et al., 1986). A second mixture, consisting of multi-acceptor-labeled BPTI derivatives, $(DA\text{-}coum)_2$-BPTI, was also prepared by acylation with DA-coumarin *N*-hydroxysuccinimide ester (Amir & Haas, 1987). These were used for determination of intermolecular excitation energy transfer after reduction by DTT.

Fluorescence Measurements. Fluorescence spectra were recorded on a Perkin-Elmer (Norwalk, CT) Model MPF-44 spectrofluorometer using sample cells and a Dewar as described elsewhere (Amir & Haas, 1987). Acceptor emission polarizations *P* were measured on an ISS (Champaign, IL) Greg-PC spectrofluorometer equipped with two Glan-Thompson polarizers. The excitation wavelength was 380 nm and the emission wavelength was 480 nm, both with a bandwidth of 4 nm. The donor is not excited under this setting, and depolarization due to excitation transfer is excluded.

Fast-mixing experiments were performed by injection of unfolded BPTI derivatives in 6 M Gdn·HCl or 6 M guanidinium isothiocyanate into a 0.5 M final concentration of the corresponding guanidinium salt in 50 mM bicine, pH 7.5, 4 mM DTT, and 1 mM EDTA, using a constant fraction syringe (Hamilton). Acceptor emission was recorded by using the fast kinetics mode of the Greg-PC with an excitation wavelength of 320 nm (which is absorbed by the donor) and an emission wavelength of 480 nm (acceptor emission) (bandwidths of 4 nm). The deadtime of this measurement was about 100 ms.

Excitation energy-transfer efficiencies *E* were computed from acceptor excitation spectra (Stryer & Haugland, 1967). All spectra were normalized to the same height at the acceptor maximum (382 nm, in which the donor has no absorption). The difference excitation spectra of the test sample and the zero-transfer reference protein (DA-coum-BPTI) were then integrated over the wavelength range 285–330 nm. Similarly, an integrated difference excitation spectrum corresponding to $E = 97\%$ was calculated by using the excitation spectra of MD-Lys and DA-coum-BPTI. The transfer efficiency *E* was taken as the ratio of the integrated difference spectrum of the test sample to the integrated difference spectrum of the high ($E = 97\%$) transfer efficiency reference compound (MD-Lys). Since the samples and the reference compounds were measured under the same experimental conditions (solvent, temperature, optical setup, etc.), a major part of experimental uncertainty was eliminated.

Unfolding and Refolding of BPTI Derivatives. Samples of labeled BPTI and the reference compounds MD-Lys, DA-coum-BPTI, $(MNA)_2$-BPTI, and $(DA\text{-}coum)_2$-BPTI were dissolved in 6 M Gdn·HCl in 50 mM bicine buffer, pH 8.5, containing 12–24 mM DTT and 1 mM EDTA. The solutions were left for 4 h at room temperature for complete unfolding. The samples were then diluted 12-fold into a 50 mM bicine buffer, pH 7.5, containing a final concentration of 4 mM DTT and 1 mM EDTA. Final concentrations of the protein derivatives and the reference compounds were between 0.2 and 0.4 μM. Fluorescence excitation spectra were recorded within 30 min following dilution and remeasured 3 h later. No time-dependent changes were found in the excitation spectra.

Electrophoresis of Carboxymethylated BPTI Derivatives. Electrophoresis was carried out at room temperature in 15% polyacrylamide gels with the discontinuous buffer system according to the method of Creighton (Creighton & Goldenberg, 1984). Carboxymethylation of reduced BPTI and derivatives was done as described by Creighton (1974) with the following modifications: the DTT was not removed from the reduced sample and iodoacetic acid was added to a final concentration of 0.25 M, followed after 3 min by acidification by acetic acid and gel filtration on a Sephadex G-25 column in 0.1 M acetic acid.

Assay of Inhibitory Activity. The activity of the inhibitor was assayed by its stoichiometric inhibition of trypsin (Kassel, 1970).

Results

The four derivatives were reduced and unfolded in 6 M Gdn·HCl and 12–24 mM DTT. Upon dilution into refolding

[1] Abbreviations: MNA, (2-methoxy-1-naphthyl)methyl; DA-coum, [7-(dimethylamino)coumarin-4-yl]acetyl; Gdn·HCl, guanidine hydrochloride; BPTI, bovine pancreatic trypsin inhibitor; R-BPTI, reduced BPTI; (1–*n*)BPTI, N^{α}-MNA-Arg[1]-N^{ϵ}-DA-coum-Lys-*n*; R(1–*n*)BPTI, reduced (1–*n*)BPTI; DTT, dithiothreitol; $(MNA)_2$-BPTI, BPTI labeled by two MNA groups; DA-coum-BPTI, BPTI labeled by a single DA-coum group; $(DA\text{-}coum)_2$-BPTI, BPTI labeled by two DA-coum groups; *E*, transfer efficiency (percent); MD-Lys, N^{α}-MNA-N^{ϵ}-DA-coum-Lys; GSSG, oxidized glutathione.

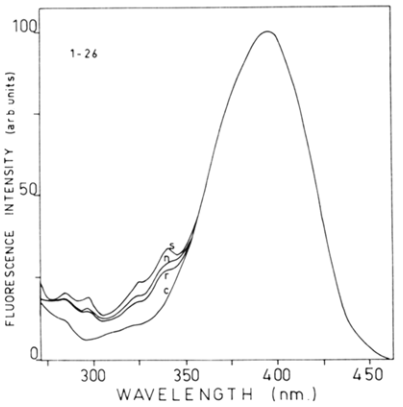

FIGURE 1: Acceptor excitation spectrum of (1–26)BPTI in 0.05 M bicine buffer, pH 7.5, the native state (n), and in 0.05 M bicine buffer, pH 7.5, 0.5 M Gdn·HCl, 2 mM DTT, and 1 mM EDTA, the reduced state (r), at room temperature. The emission wavelength is 480 nm with 10-nm bandwidth. A 2-nm bandwidth is set for the excitation wavelength. Curve c traces the excitation spectrum of DA-coum-BPTI (a reference curve for zero transfer). Curve s traces the excitation spectrum of MD-Lys, characterized by constant transfer efficiency; $E = 97\%$. Both curves s and c are recorded under the same conditions as trace n.

buffer (0.5 M Gdn·HCl in 50 mM bicine, pH 7.5, 4 mM DTT, and 1 mM EDTA), large changes in excitation transfer efficiencies were observed. The excitation spectra of R(1–26)BPTI in the refolding/reducing buffer and the native (1–26)BPTI are shown in Figure 1.

Excitation spectra obtained with the derivative R(1–15)-BPTI show a different phenomenon. The transfer efficiency between the probes attached to the ends of the 15-residue N-terminal segment of BPTI in the reduced state is considerably *higher* than in the native state.

To evaluate the contribution of chain dynamics to the enhanced excitation energy transfer, we repeated the refolding experiments with 50% glycerol as a cosolvent in the buffer. Excitation spectra were recorded for the derivatives at room temperature and at −30 °C, where conformational dynamics are slowed down by the viscous drag of the supercooled cosolvent mixture. The excitation transfer efficiencies E calculated from the excitation spectra are presented in Figure 2.

The histogram shows that excitation transfer efficiencies observed in the reduced state under folding conditions were *higher* than those found for each derivative in the reduced state, unfolded by 6 M Gdn·HCl. With the exception of R(1–15)BPTI, the reduced state under folding conditions yielded excitation energy-transfer efficiencies that were *similar to those obtained in the native state*. In R(1–15)BPTI E was *higher than that found in both the native and the unfolded states* of this derivative.

Several experiments were carried out to ascertain that the changes in E were not due to any formation of disulfide bonds. The rates of changes in E upon dilution were measured by fast mixing. The major changes in E took place within less than 100 ms after dilution, much faster than disulfide bond formation (Creighton, 1978). Furthermore, each sample was measured twice: 30 min after dilution and 3 h later. No changes in the values of E were observed, which would have been the case if oxidation took place.

Direct proof for the absence of disulfide bonds in the reduced labeled BPTI derivatives, under the conditions we used for refolding, was obtained by the method described by Creighton (1974). A mixture of double and triple MNA-labeled BPTI derivatives (MNA_2-BPTI) was reduced by 10 mM DTT in

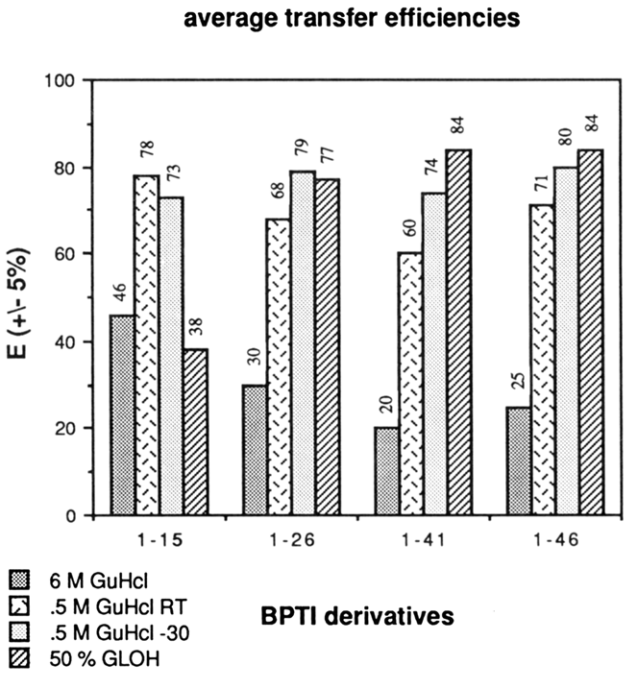

FIGURE 2: Average steady-state transfer efficiencies in (1–n)BPTI (n = 15, 26, 41, 46) in the native, reduced unfolded, and reduced states. The conformational state and solvent in each column are as follows: first column (from left), 6.0 M Gdn·HCl in 28% glycerol in 0.05 M bicine buffer, pH 7.5, 10 mM DTT, and 1 mM EDTA at −30 °C; second column, 0.5 M Gdn·HCl in 50% glycerol in 0.05 M bicine buffer, pH 7.5, 4 mM DTT, and 1 mM EDTA at −30 °C; third column, 0.5 M Gdn·HCl in 0.05 M bicine buffer, pH 7.5, 4 mM DTT, and 1 mM EDTA at room temperature; fourth column, 0.05 M bicine buffer, pH 7.5, in 50% glycerol at −30 °C. The percent transfer efficiencies calculated for each derivative and state are given on top of the corresponding column.

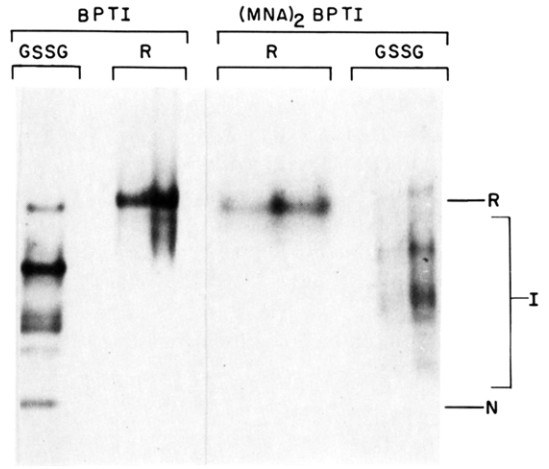

FIGURE 3: Polyacrylamide gel electrophoresis of reduced double-labeled BPTI. Samples of R($MNA)_2$-BPTI and R-BPTI reduced or partially oxidized by oxidized glutathione (GSSG) were loaded. (Each sample was loaded in a pair of lanes at two concentrations. BPTI is a basic protein; the native protein migrates faster toward the cathode at the bottom of the gel.) R, reduced protein; N, native state; I, intermediates with at least one disulfide.

6 M Gdn·HCl, 0.1 M Tris, pH 8.7, and 1 mM EDTA. After 4 h the reduced protein was diluted into the refolding buffer containing 1 mM DTT (final concentration) and left at room temperature for 15 h; then the resultant solution was divided in half. One aliquot was carboxymethylated directly with excess iodoacetic acid. To the second aliquot, which served as an "oxidized" control, were added oxidized glutathione (GSSG, final concentration 0.15 mM) and after 5 min an excess of iodoacetic acid. Analysis by gel electrophoresis

(Figure 3) revealed in the first sample only the bands corresponding to the fully reduced carboxymethylated protein. In the sample in which disulfide bond formation was initiated with oxidized glutathione, a mixture of disulfide intermediates was revealed as expected according to Creighton (1978), indicating that when disulfide bonds are formed, they are readily observable by this method. In this experiment we used derivatives labeled by the donor only (due to scarcity of the donor- and acceptor-labeled samples). The ratio of dye to protein was in excess of 2, the DTT concentration was 4-fold lower than the concentration used in the spectral measurements, and the analysis was done only after 15 h (in air). Therefore, it is clear that the probability for oxidation in the samples used in the fluorescence experiments is negligible. We also tested the samples for inhibitory activity: all samples used here were inactive when kept in the above reducing buffers.

In order to rule out any contribution of intermolecular energy transfer in microaggregates, we measured transfer efficiencies in mixtures of $R(NMA)_2$-BPTI (donor only) and $R(DA\text{-}coum)_2$-BPTI (acceptor only), at a ratio of up to 10:1, in 0.5 M Gdn·HCl, 0.05 M Tris buffer, pH 7.8, 2 mM DTT, and 1 mM EDTA, over the total protein concentration range from 0.5 to 10 μM. The minimal concentration of reduced BPTI derivatives that showed detectable intermolecular excitation energy transfer under the above conditions was 2.5 μM. Hence, submicromolar concentrations of the reduced BPTI derivatives (0.2–0.4 μM) were used.

The emission spectra of the acceptor in all the derivatives were measured in three states: native, reduced, and reoxidized (by glutathione). Only very small variations in emission maximum and bandwidth were observed despite the fact that the emission spectrum of DA-coumarin is environmentally sensitive (Haas, unpublished results).

In order to compare the extent of rotational freedom of the probes in the native and reduced states under folding conditions, the polarizations P of the light emitted by the acceptor were measured for all the derivatives in both states. All the derivatives in both the native and reduced states gave a very similar value, $P = 0.18 \pm 0.02$. The polarization values are relatively high, but considering the short lifetime of the acceptor (attached to BPTI) in aqueous solutions (1.3 ± 0.3 ns), this gives a rotational relaxation time of about 1 ns. This value is an order of magnitude lower than the rotational correlation time of native BPTI (Kasprzak & Weber, 1982). This indicates that the probes are free to rotate faster than the rotation of the whole molecule and are not trapped in a rigid hydrophobic core.

Discussion

The results presented in this study lead to the following conclusions as to the structure of reduced BPTI: (a) Even without formation of disulfide bonds, the average distances between residues separated by long segments of the backbone approach those found in the native state; this indicates that the reduced molecule has a compact conformation. (b) The efficiency of excitation energy transfer E is not correlated with the number of residues between the labeled sites; this indicates that in the reduced state BPTI *is not in a random coil conformation.* (c) The value of E observed for the pair of probes attached to residues 1 and 46 is higher than that obtained for the shorter segment 1–41. In the reduced state the N-terminal segment of BPTI thus seems to be close to the C-terminal segment as in the native state. (d) The distance between residues 1 and 15, the longest dimension of the protein in the native state according to the crystal structure (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984), is considerably shorter in the reduced state. This segment also shows high dynamic flexibility. Reduced BPTI thus seems to exist under folding conditions in what has been characterized as a molten globule state (Dolgikh et al., 1981).

Few control experiments show that the observed changes in E were not caused by other spectroscopic effects. The uncertainty in the extent of the orientational averaging and hence in κ^2, the orientation factor, has been discussed by many authors (Stryer, 1978; Jones, 1970; Dale et al., 1979; Haas et al., 1978b). We have shown that for the probes used here and their interactions with labeled native BPTI these uncertainties are not larger than the experimental error (Amir & Haas, 1987, 1988). The polarization and emission spectra indicate that in the reduced state these uncertainties were not increased. The donor quantum yield and the refractive index were determined for all solvent mixtures employed here. Their combined effect on R_0 (the Förster constant, which constitutes the distance scale for the analysis of the experiments) accounts for less than ca. 8% change in E upon dilution of the Gdn·HCl.

The probes used in the present study are hydrophobic and could conceivably add extra interactions, thereby causing the compact conformations of the protein. The emission spectra and the polarizations of the probes were similar in the native and reduced states, suggesting that the immediate environment of the probes is independent of the conformation of the protein. These experiments show that the probes were probably not trapped inside a hydrophobic core of the reduced protein.

The reduced state of BPTI has been studied by several methods [immunochemistry (Creighton et al., 1978), CD (Kosen et al., 1981, 1983), chemical activity of cystein side chains (Creighton, 1975), and hydrodynamic methods (Creighton, 1974)]. The common conclusion was that R-BPTI has a disordered conformation (Creighton, 1978). The transfer efficiencies measured here probe local structures and can reveal conformational characteristics not directly available by the above methods. It seems that Creighton's conclusions are compatible with our results within the framework of the compact overall structure, which has fluctuating partially ordered local structures. It should be kept in mind that all of the experiments of Kosen (1981) and Creighton (1978) utilized carboxymethylated or carboxamidomethylated R-BPTI derivatives. The blocking groups may have changed the local structures, as has been observed for lysozyme by Lee and Attasi (1973).

One consequence of the above conclusion is that the latter phases of the folding pathway are a sequence of rearrangements and formation of specific interactions taking place within a condensed volume element. This is probably an effective means of accelerating the rate of the folding as can also be found in Levitt's simulation studies (Levitt, personal communication).

The temperature and viscosity dependence of E in the N-terminal segment (residues 1–15) shows a higher (relative to other segments) rate of internal diffusion between the segment's ends (Figure 2; the effect of temperature in the presence of glycerol). The high flexibility of the 1–15 segment may contribute to the formation of the nonnative intermediate disulfide bonds, 5–14 and 5–38 (Creighton, 1978), and may be instrumental for the folding pathway of BPTI.

Further experiments, in particular, measurements of the fluorescence decay of the probes combined with the global analysis (Haas & Beechem, 1988) and new derivatives (currently in preparation), will enable us to establish the present conclusions and reduce the current uncertainty in the interpretation of the observed quantities.

The results presented here show that our experiments can yield significant structural parameters of the early folding intermediates both at the level of overall structure and the level of local secondary structure. It is possible that a first step in the folding transition is a fast collapse into a compact semi-ordered ensemble of conformations. These are rearranged by short-range thermal motions into the specific nativelike structures that are sequentially stabilized and fixed by the formation of the disulfide bonds at later stages of the folding pathway (Creighton, 1978).

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