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Refolding of Denatured Trichosanthin in the Presence of GroEL

Chi-Kong Lau,* Ricky N. S. Wong,† Samuel C. L. Lo,‡ and Francis Kwok†

**Department of Biochemistry, The University of Tennessee, Knoxville, Tennessee 37996-0840; †Department of Biology, Baptist University, Kowloon Tong, Hong Kong; and ‡Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, Kowloon, Hong Kong*

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The stability of trichosanthin (TCS), a 27-kDa ribosome-inactivating protein, was investigated in the presence of guanidinium chloride (GdnHCl). The process of unfolding was monitored by CD and fluorescence spectroscopy. Both methods show the presence of partially folded intermediates. Unfolding of TCS is attained in 6M GdnHCl, but the inactive species recover a good deal of its DNase activity upon dilution with buffer containing GroEL and ATP. The mechanism of recognition of unfolded TCS by GroEL was studied by fluorescence spectroscopy.

Trichosanthin is an N-glycoside that attacks the 28 S rRNA of the ribosome at a highly conversed adenine residue. (1). Recently, trichosanthin has also been shown to possess DNase activity on supercoiled DNA (2).

The primary sequence of the cDNA codes for a protein of 247 amino acids, which has been expressed in E. coli (3). The mature protein is homologous to other ribosome-inactivating proteins such as Ricin A (4). The structure of trichosanthin has been determined to 1.73 Å resolution using x-ray crystallography (5). It has an overall structural similarity to Ricin A. The role of the amino acids GLU 160 and GLU 189 in the catalytic function of trichosanthin has been assessed by site-specific mutagenesis (6).

Relatively little information has been reported on the stability of trichosanthin in the presence of denaturing agents. In an attempt to understand the mechanisms underlying the refolding of trichosanthin, we have investigated the denaturation curves obtained in the presence of guanidinium chloride using two spectroscopic techniques; i.e., circular dichroism and fluorescence spectroscopy. It is shown that trichosanthin, treated with 6 M guanidinium chloride, recovers a good deal of its DNase activity after refolding in the presence of GroEL and Mg-ATP.

EXPERIMENTAL PROCEDURE

Sample preparation for fluorescence and CD spectroscopy. Samples of trichosanthin subjected to fluorescence and circular dichroism measurements were dissolved in 10 mM Tris-HCl buffer (pH 7). For denaturation experiments, each protein sample was prepared by diluting 0.2 ml of protein stock solution (2mg/ml) to 2 ml using 10mM Tris-HCl buffer (pH 7) in the presence of various concentrations of guanidinium chloride. All measurements were carried out at 25° C after 30 min incubation in the presence of guanidinium chloride. Control experiments were used to indicate the time of incubation which was required to achieve unfolding and folding equilibrium.

Fluorescence spectroscopy. Emission spectra were recorded in a Perkin-Elmer LS-50B spectrofluorimeter. For the derivatized anthraniloyl-trichosanthin, the excitation wavelength was set at 330 nm, and for 1-anilino naphthalene-8-sulfonate, the excitation wavelength was 360 nm. Excitation and emission bandwidths were set at 2.5 nm.

Circular dichroism. CD spectra were recorded on a Jasco (J-40 A) spectropolarimeter using cells of 0.2 cm path length. The spectropolarimeter was routinely calibrated with the asymmetric compound, (+)-10-camphorsulfonic acid. Spectral data were acquired over the range 250-200 nm.
Ellipticity (θ), expressed in terms of mean residue ellipticity was calculated with the aid of the following equation:

$$[\theta] = \theta \text{ M}/10 \text{ lc}$$

where M is the mean residue mass, 1 the path length (0.2 cm), and c the concentration in g/ml.

Labelling of trichosanthin. Trichosanthin (2 mg/ml) was reacted with a 5-fold molar excess of isatoic anhydride in 0.1 M potassium phosphate, pH 7 at 37° C. Three hours after the reaction, excess isatoic anhydride was removed by dialysis against 2 liters of 10 mM Tris-HCl (pH 7). Then, the degree of labelling was determined spectrophotometrically using an extinction coefficient of 4600 M^{-1} cm^{-1} at 330 nm (10) for the anthraniloyl chromophore and an extinction coefficient of 18,000 $M^{-1}cm^{-1}$ for the protein at 280 nm.

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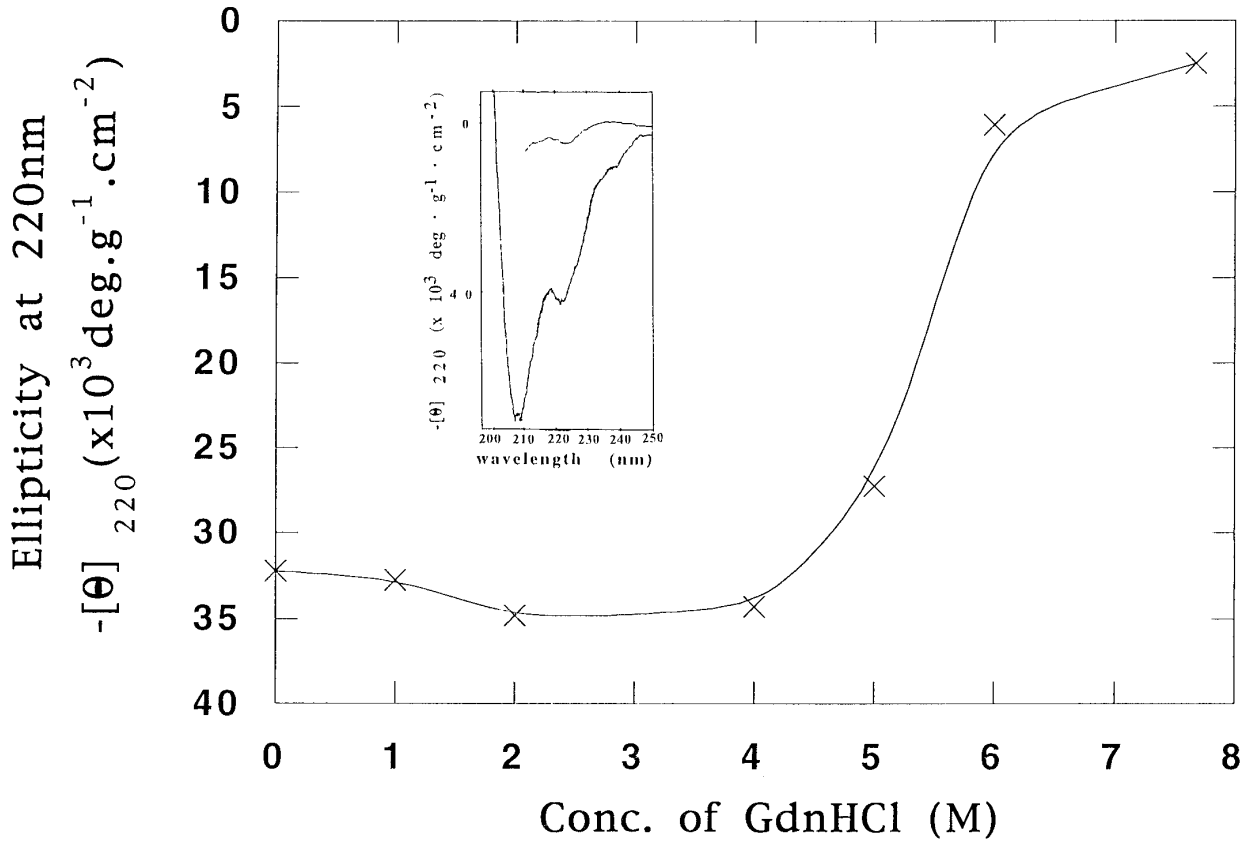

FIG. 1. Changes in circular dichroism. Changes in the mean residue ellipticity [θ] at 220 nm of trichosanthin (0.2 mg/ml) as a function of guanidinium chloride concentration in 10 mM Tris-HCl buffer (pH 7). Inset: circular dichroism spectra of the protein (0.2 mg/ml) treated with 6 M guanidinium chloride and dialyzed against 10 mM Tris-HCl buffer (pH 7). (Top). The spectrum of the native protein at the same concentration (0.2 mg/ml) is included in the figure. (Bottom).

Enzymatic assay. The endonucleolytic property of trichosanthin was assayed as previously described (2). One microgram of supercoiled DNA (p-Bluescript II) was incubated with appropriate amounts of trichosanthin in a total volume of 20 μl of 1 $\times$ OPA buffer (Pharmacia) at 37° C for 1 h. At the end of the incubation, electrophoresis of the sample was carried out under non-denaturing conditions using 1% agarose gel at 50 V until the tracking dye moved to the appropriate position. Gel were stained with ethidium bromide at a final concentration of 0.5 mg/ml.

Gels were then placed over the imaging densitometer (BIO-RAD, Model GIS-670 and the bands were quantitatively analyzed.

Purification of proteins. Recombinant trichosanthin, expressed in E. coli, purified by following the procedure as previously described (1).

The E. coli GroESL gene (7) was expressed in E. coli strain BL 21 (DE 3) cells from the plasmid provided by Dr. Frank Lorimer (Oak Ridge). The protein was purified to homogeneity by modification of a published procedure (8). After gel filtration through Sepharose-CL-GB, the peak fractions containing GroEL were identified by measuring ATPase activity using a coupled enzymatic system (9) and by SDS/PAGE.

The protein concentration was determined using an extinction coefficient at 280 nm of 29,000 $M^{-1}cm^{-1}$ for a molecular mass of 57 kDa.

RESULTS AND DISCUSSIONS

Unfolding of Trichosanthin by Guanidinium Chloride

The unfolding of trichosanthin by guanidium HCl (GdnHCl) was monitored by CD spectroscopy in the far ultraviolet region of the spectrum. All measurements were performed after incubation of the protein for 30 min at 25° C in the presence of increasing concentrations of Gdn HCl in 10 mM Tris-HCl buffer (pH 7). Control measurements showed no changes in the signal at 220 nm after exposure to the denaturant for 30 min.

As shown in Fig. 1, the signal at 220 nm is slightly decreased as the Gdn HCl concentration changes from 0 to 4 M. At Gdn HCl concentrations above 4 M, the secondary structure of trichonsanthin is disrupted and the CD signal reaches a plateau at denaturant concentrations near 6 M. The sharp transition observed between 4 and 6 M Gdn HCl is irreversible, since dialysis of the denatured protein does not restore the original ellipticity values corresponding to the unfolded protein (inset Fig. 1).

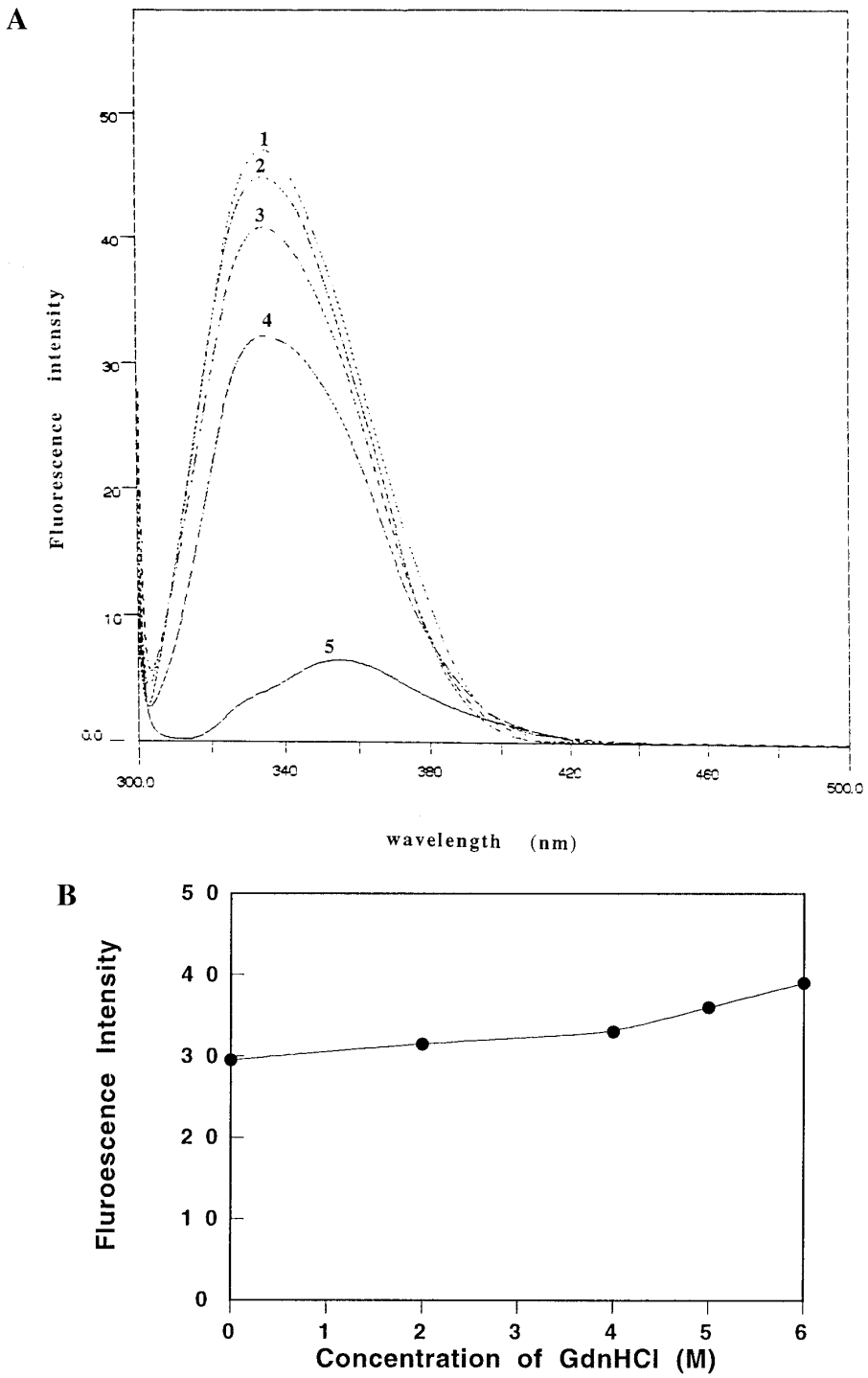

FIG. 2. Emission spectra of trichosanthin. **(A)** Samples of trichosanthin (0.2 mg/ml) were treated with guanidinium chloride for 30 min prior to recording their emission spectra. Spectra recorded in the absence (curve 3) and presence of 4 M (curve 1), 2 M (curve 2), 5 M (curve 4) and 6 M (curve 5) guanidinium chloride. Excitation wavelength 295 nm. **(B)** Fluorescence-intensity changes recorded at 520 nm (excitation 360 nm) when a sample of trichosanthin (0.2 mg/ml) in the presence of increasing concentration of guanidinium chloride was mixed with ten fold molar excess of ANS.

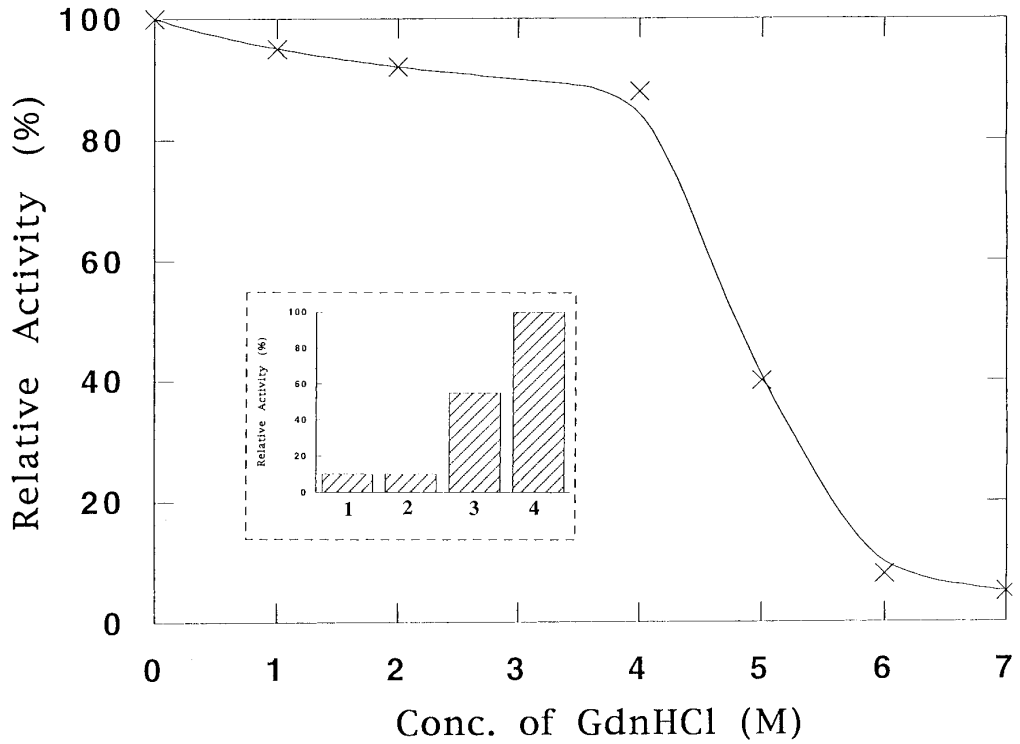

FIG. 3. Inactivation of trichosanthin by guanidinium chloride. Samples of the protein (0.5 mg/ml), exposed to the indicated concentrations of guanidinium chloride for 30 min at 25° C, were diluted and assayed for catalytic activity in the absence of denaturing agent. Inset: reactivation of trichosanthin by GroEL and MgATP. A sample of guanidinium chloride-denatured trichosanthin was dialyzed against the renaturation buffer, 10 mM Tris-HCl (pH 7) containing 10 mM KCl, 1 mM $MgCl_2$ and 1 mM dithiothreitol. The dialyzed protein (1 μM) was allowed to react with GroEL (1.4 μM) and Mg-ATP (2 mM) for 1 hour at 25° C prior to assaying endonulease activity as described in "Experimental procedures." (1. Denaturated trichosanthin; 2. Denaturated trichosanthin + GroEL; 3. Denaturated trichosanthin + GroEL + Mg-ATP; 4. Native trichosanthin).

In order to further assess the effect of Gdn HCl on the microenvironment of trp 200, the only tryptophan residue of the protein, emission spectra were recorded in the presence of increasing concentrations of Gdn HCl. Addition of the denaturant to a final concentration of 4 M, results in small enhancement of the protein fluorescence yield without any significant change in the band position centered at around 338 nm. Further increase in Gdn HCl to a final concentration of 6 M causes quenching of protein fluorescence together with a spectral shift of approximately 10 nm (Fig. 2A). The red shift in the band position could be attributed to exposure of the tryptophanyl residue to the solvent as result of disruption of α-helical structures, since the indole chromophore is positioned in an α-helix surrounding the catalytic site of trichosanthin (5). The unfolding of trichosanthin by guanidinium chloride was also followed by measuring changes in the fluorescence emission of the extrinsic probe 1-anilinonaphthalene-8-sulfonate (ANS), which is known to recognize hydrophobic clusters in proteins (11). This probe is commonly used to detect molten-globule intermediate during the unfolding of proteins (12). Fig. 2B shows the results obtained when native and Gdn HCl-treated trichosanthin were allowed to mix with ten-fold molar excess of 1-anilinonaphthalene-8-sulfonate and their emission recorded upon excitation at 360 nm. An enhancement of fluorescence yield was detected when the concentration of denaturant was increased in solutions of 10 mM Tris/HCl buffer (pH 7.5), suggesting a gradual exposure of hydrophobic residues as the protein becomes exposed to Gdn HCl. It is worthy to note that small changes in the ellipticity at 220 nm occur over Gdn HCl concentrations ranging from 0 to 4 M (Fig. 1), whereas more pronounced changes in the emission of the extrinsic probe are detected under similar experimental conditions. Thus, it seems reasonable to suggest that several intermediates, which preserve most of their secondary structure, are in equilibrium before their irreversible denaturation caused by 6 M guanidinium chloride. Therefore, the following model is proposed to explain the denaturation of trichosanthin by guanidinium chloride.

$$N \leftrightarrow (I)n \rightarrow U$$

Consistent with this interpretation of the spectro-

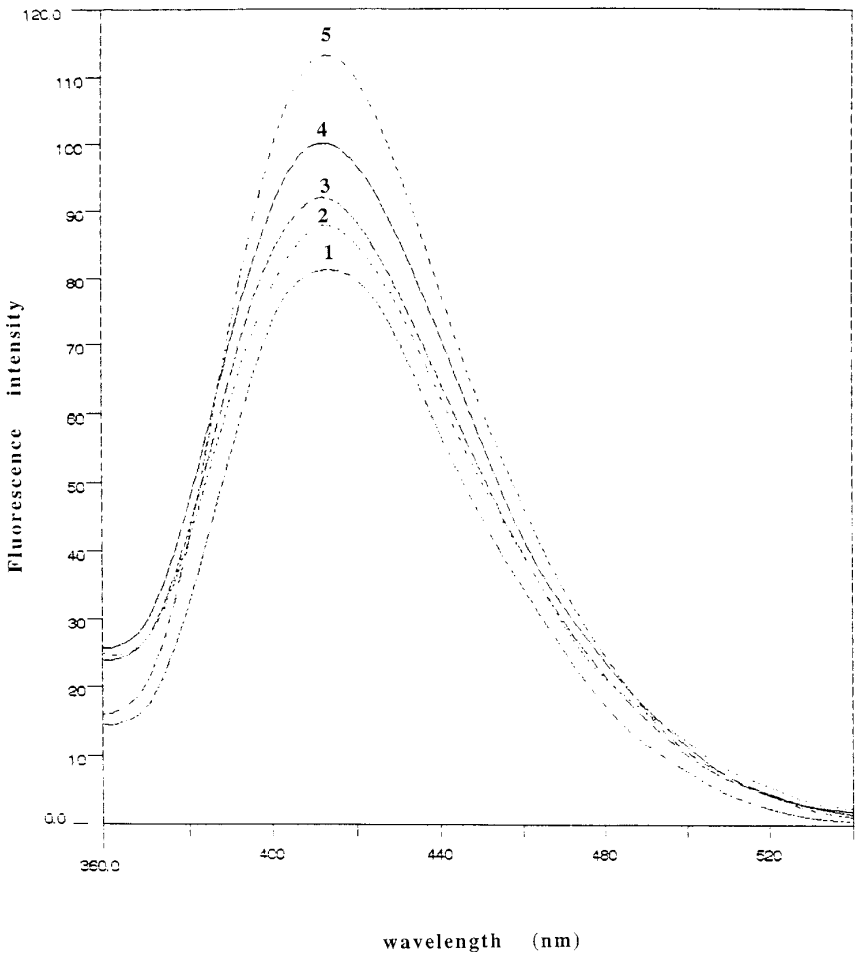

FIG. 4. Emission spectra of denatured anthraniloyl-trichosanthin in the presence of GroEL. A sample of denatured trichosanthin (1μM), dialyzed against the renaturing buffer, was mixed with GroEL (1.4 μM) and the emission spectra recorded after addition of Mg-ATP (2 mM). Emission spectra of denatured anthraniloyl-trichosanthin in the absence (curve 1) and presence of GroEL (curve 2). Emission spectra recorded 30 min (curve 3) and 60 min (curve 4) after addition of Mg-ATP. The emission spectrum of the undenatured anthraniloyl-trichosanthin in renaturation buffer containing Mg-ATP (2 mM) is given in the figure (curve 5).

scopic data, is the finding that trichosanthin exposed to 1, 2, 3 and 4 M guanidinium chloride exhibits catalytic activity following removal of the denaturing agent by dialysis against buffer. In marked contrast, trichosanthin treated with 6 M guanidinium chloride and dialyzed against buffer does not recover its catalytic activity (Fig. 3).

Reactivation of Denatured Trichosanthin

The effect of GroEL on the reactivation of partially-folded trichosanthin was investigated at pH 7 in the presence of Mg-ATP. For these experiments, trichosanthin was denatured by 6 M Gdn HCl and dialyzed against buffer at 4° C to remove the denaturing agent. Under this set of experimental conditions, the CD spectrum of the denatured protein in the far-UV region shows residual secondary structure (Fig. 1), but the protein is devoid of catalytic activity. Upon dilution of denatured trichosanthin, (1 μM) with the renaturing buffer, Tris/HCl, pH 7.5, containing GroEL (1.4 μM), MgATP (2 mM), $MgCl_2$ (2 mM), dithiothreitol (2 mM) and KCl (20 mM), followed by incubation for 1 hour at 25° C, the protein recovers a good deal of its catalytic activity as shown by the results included in Fig. 4. Denatured trichosanthin is not reactivated by GroEL in the absence of ATP, suggesting that the endogenous ATPase activity of the chaperonine plays some role in either the refolding or release of folded protein substrate.

To study the recognition of partially folded trichosanthin by GroEL, samples of trichosanthin labelled with the chromophore anthraniloyl were denatured with 6 M Gdn HCl, dialyzed against buffer, and allowed to mix with equimolar amounts of GroEL.

Derivatized anthraniloyl-trichosanthin is suitable for these experiments because its maximum of absorption at 320 nm does not interfere with the absorption properties of the chaperonine; moreover the emission properties of the anthraniloyl group can be exploited to detect very low concentrations of the tagged protein.

Fig. 4 shows the fluorescence spectra of native and denatured trichosanthin derivatized with the fluorescence probe, where it can be seen that denaturation of the protein brings about a decrease in the fluorescence yield recorded upon 320 nm excitation. In the absence of ATP, the addition of GroEL has a small effect on the fluorescence emitted by the anthraniloyl group, but addition of ATP results in a significant increase in the fluorescence detected over the wavelength range 360-550 nm. No effect of addition of GroEL and ATP on the emission of Ant-trichosanthin was detected at GroEL concentrations of 2mM.

The fluorescence enhancement observed in the presence of ATP and GroEL could be attributed to the formation of species which acquire a conformation similar to that of the native protein ,since denatured anthraniloyl-trichosanthin is also reactivated by GroEL and ATP.

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