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Articles

Use of Fluorescence Energy Transfer To Characterize the Compactness of the Constant Fragment of an Immunoglobulin Light Chain in the Early Stage of Folding

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ABSTRACT: The C_L fragment of a type-κ immunoglobulin light chain in which the C-terminal cysteine residue was modified with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (C_L-AEDANS fragment) was prepared. This fragment has only one tryptophan residue at position 148. The compactness of the fragment whose intrachain disulfide bond was reduced in order for the tryptophan residue to fluoresce (reduced C_L-AEDANS fragment) was studied in the early stages of refolding from 4 M guanidine hydrochloride by fluorescence energy transfer from Trp 148 to the AEDANS group. The AEDANS group attached to the SH group of a cysteine scarcely fluoresced when excited at 295 nm. For the reduced C_L-AEDANS fragment, the fluorescence emission band of the Trp residue overlapped with the absorption band of the AEDANS group, and the fluoresence energy transfer was observed between Trp 148 and the AEDANS group in the absence of guanidine hydrochloride. In 4 M guanidine hydrochloride, the distance between the donor and the acceptor was larger, and the efficiency of the energy transfer became lower. The distance between Trp 148 and the AEDANS group for the intact protein estimated by using the energy-transfer data was in good agreement with that obtained by X-ray crystallographic analysis. By the use of fluoresence energy transfer, tryptophyl fluoresence, and circular dichroism at 218 nm, the kinetics of unfolding and refolding of the reduced fragment were studied. These three methods gave the same unfolding kinetic pattern. However, the refolding kinetics measured by fluoresence energy transfer were different from those measured by tryptophyl fluorescence and circular dichroism, the latter two giving the same kinetic pattern. In addition to the two phases observed by using tryptophyl fluoresence or circular dichroism, a very much faster phase was detected by fluoresence energy transfer. The energy-transfer efficiency reached the same level as that of the intact protein at a very early stage of refolding. Double-jump experiments also gave the same result. These findings indicate that a structure as compact as that of the native protein is formed immediately after refolding, and then the compact molecule converts slowly to the native protein by rearrangement of groups, probably involving cis–trans isomerization of the prolyl residue.

In our laboratory, unfolding and refolding of the constant fragments obtained from immunoglobulin light chains have been studied in detail (Goto & Hamaguchi, 1982a,b, 1987; Tsunenaga et al., 1987; Goto et al., 1988). However, little is known about the mechanism of folding from the fully unfolded polypeptide chain. In the present study we investigated the compactness of the constant fragment obtained from a type-κ immunoglobulin light chain immediately after refolding using fluoresence energy transfer.

The C_L fragment (type κ) is suitable for studying fluorescence energy transfer, because it contains only one tryptophan residue at position 148[1] buried in the interior of the molecule (Tsunenaga et al., 1987; Kawata et al., 1987; Kawata & Hamaguchi, 1990) and a single cysteine residue at the C terminus to which a fluorophore can be attached. We modified the SH group of the C-terminal cysteine residue with IAE-

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[1] The numbering system used in this paper is based on the type-κ light chain Ag (Putnam, 1969).

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DANS[2] and attempted to use the fluorescence energy transfer from Trp 148 to the fluorophore as a measure of the compactness of the molecule. However, Trp 148 is located very close to the intrachain disulfide bond (Lesk & Chothia, 1982), a strong fluorescence quencher, and thus scarcely any fluorescence of Trp 148 is detected (Tsunenaga et al., 1987). Therefore we reduced the intrachain disulfide bond of the C_L-AEDANS fragment to remove its quenching effect. Although reduction of the intrachain disulfide bond unfolds the C_L fragment molecule, the presence of ammonium sulfate refolds the protein molecule to its folded conformation (Goto et al., 1987, 1988).

Here we measured the change in fluorescence energy transfer from Trp 148 to the AEDANS group attached to the C-terminal cysteine residue of the reduced C_L-AEDANS fragment in the process of refolding from the denatured state in Gdn-HCl. All the experiments were done in the presence of 0.5 M ammonium sulfate. We found that the protein molecule becomes as compact as the native molecule immediately after starting the refolding reaction, by lowering the concentration of Gdn-HCl.

Materials and Methods

Materials. Bence-Jones protein Oku (type κ) was prepared from the urine of a multiple-myeloma patient by precipitation with ammonium sulfate and ion-exchange chromatography (DE-52) as described previously (Goto et al., 1979).

1,5-IAEDANS was obtained from Aldrich Chemical Co. and Gdn-HCl (specially purified grade) was from Nacalai Tesque. Other reagents were of the highest grade commercially available and were used without further purification.

Preparation of C_L and C_L-AEDANS Fragments. The C_L fragment of light-chain Oku was obtained by digestion with clostripain (EC 3.4.22.8) (Tsunenaga et al., 1987). The light chain (500 mg) was digested with clostripain in 25 mL of 0.1 M Tris-HCl, pH 7.5, containing 0.15 M KCl, 1 mM $CaCl_2$, and 5 mM dithiothreitol, for 30 min at 37 °C at a substrate-to-enzyme ratio of 400:1 (w/w). In this reaction process, the interchain disulfide bond between the C-terminal Cys residues of the Oku protein was reduced. In previous experiments (Tsunenaga et al., 1987), the digestion was stopped by addition of iodoacetamide. In the present study, we would not have been able to use the sample thus obtained, because the C-terminal cysteine residue is alkylated by this treatment and thus cannot be modified with IAEDANS. Therefore, in the present experiments, we stopped the digestion reaction by simply cooling the mixture with ice water. The reaction solution was then immediately subjected to gel filtration on a Sephadex G-75 column with 10 mM Tris-HCl, pH 8.6, at 4 °C, followed by ion-exchange chromatography (DE-52) as described previously in detail by Tsunenaga et al. (1987). Seventy milligrams of the C_L(109–214), which has a single cysteine residue at position 214, was obtained. The C_L-CAM fragment of which the terminal cysteine residue was alkylated with monoiodoacetamide was prepared as described by Tsunenaga et al. (1987).

The modification with 1,5-IAEDANS of the terminal cysteine residue of the C_L fragment was carried out as follows. The C_L fragment (35 mg) was dissolved in 5 mL of 0.1 M Tris-HCl buffer, pH 8.6, containing 2 mM dithiothreitol and left to stand for 20 min. 1,5-IAEDANS (final concentration 5 mM) was added to this solution with stirring in the dark at 25 °C. The reaction was allowed to proceed for 50 min, and then the mixture was applied to a Sephadex G-10 column (2.2 × 45 cm) equilibrated with 20 mM NH_4HCO_3. The protein fraction thus eluted was the modified C_L fragment whose C-terminal Cys 214 was blocked with an AEDANS group. Under these reaction conditions, only Cys 214 was modified with IAEDANS completely. The amino acid composition of the C_L-AEDANS fragment was within 3% of that expected, and *S*-AEDANS-cysteine was recovered as carboxymethylcysteine.

Preparation of the Reduced C_L-AEDANS Fragment. Reduction of the intrachain disulfide bond of the C_L-AEDANS fragment was carried out with 30 mM dithiothreitol in 4 M Gdn-HCl at pH 8. The reduced fragment was separated from the residual reagents on a Sephadex G-25 column equilibrated with 10 mM acetate buffer, pH 5.5, containing 0.5 M ammonium sulfate, 1 mM EDTA, and 0.1 M NaCl. Reduction of the intrachain disulfide bond of the C_L-CAM fragment was carried out in the same way.

CD Measurements. CD measurements were carried out with a Jasco spectropolarimeter, model J-500A, equipped with a data processor (DP-501). The CD instrument was calibrated with *d*-10-camphorsulfonic acid. The results were expressed as mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = (100 \times \theta_{obs})/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the residue molar concentration of the protein, and l is the length of the light path in centimeters. For calculation of c, a value of 108 was used as the mean residue molecular weight. The temperature was maintained at 25 °C with a thermostatically controlled cell holder.

Absorption and Fluorescence Measurements. Absorption spectra were measured with a Jasco-Ubest-40 spectrometer. Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, model MPF-4, equipped with a spectral corrector. The temperature was maintained at 25 °C with a thermostatically controlled cell holder.

Protein Concentration. Protein concentrations were determined spectrophotometrically. The absorption coefficient of the reduced C_L-CAM fragment was assumed to be the same as that of the intact C_L-CAM [12 100 M^{-1} cm^{-1} at 280 nm (Tsunenaga et al., 1987)]. The concentration of the C_L-AEDANS was determined by using the value of the absorption coefficient of the AEDANS group [6100 M^{-1} cm^{-1} at 337 nm (Hudson & Weber, 1973)].

Unfolding Equilibrium. All the measurements in the present studies were carried out in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 M ammonium sulfate at 25 °C. All the buffers were freshly prepared and degassed before use. Unfolding transition curves of the reduced C_L-AEDANS fragment were measured by fluoroescence at 510 nm with 295-nm light for excitation, which can be used as a measure of the fluorescence energy transfer from Trp 148 to the AEDANS group, tryptophyl fluorescence at 350 nm with 295-nm light for excitation, and CD at 218 nm. The protein concentrations were about 2 μM.

Unfolding and Refolding Kinetics. Slow unfolding and refolding kinetics were measured in terms of the fluoresence at 350 nm due to the tryptophan residue and the fluorescence at 510 nm. The excitation wavelength was set at 295 nm for

[2] Abbreviations: ANS, 1-anilinonaphthalene 8-sulfonate; CD, circular dichroism; C_L fragment, the constant fragment of type-κ immunoglobulin light chain Oku; C_L-AEDANS fragment, the constant fragment whose C-terminal Cys 214 is labeled with 1,5-IAEDANS; C_L-CAM fragment, the constant fragment whose Cys 214 is alkylated with monoiodoacetamide; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine; reduced C_L fragment, the constant fragment whose intrachain disulfide bond is reduced; Tris, tris(hydroxymethyl)aminomethane.

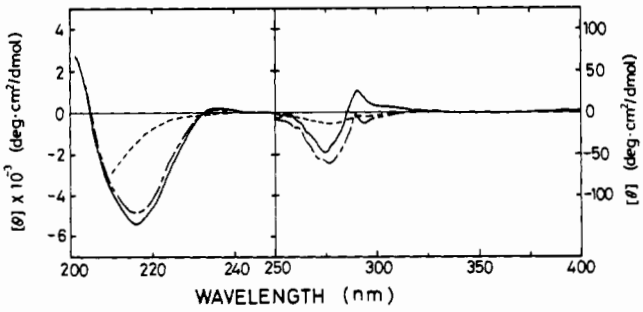


FIGURE 1: CD spectra of the C_L-AEDANS (solid line) and reduced C_L-AEDANS (dashed and dotted line) fragments in 50 mM Tris-HCl buffer containing 0.5 M ammonium sulfate at pH 7.5 and 25 °C. The dotted line indicates the CD spectrum of the reduced C_L-AEDANS fragment in 4 M Gdn-HCl containing 0.5 M ammonium sulfate at pH 7.5 and 25 °C. The protein concentrations were 0.2 mg/mL.

both measurements. A protein solution (0.1 mL) at pH 7.5 containing 0.5 M ammonium sulfate was mixed with 2.9 mL of the buffer at pH 7.5 containing 0.5 M ammonium sulfate and Gdn-HCl at a given concentration. Initial concentrations of Gdn-HCl for the unfolding and refolding measurements were 0 and 4 M, respectively.

Fast unfolding and refolding kinetics were measured on a Union Giken stopped-flow spectrophotometer, model RA-401, by use of fluorescence detection. The details of the apparatus have been given previously (Goto & Hamaguchi, 1982a). The kinetics for the reduced C_L-CAM fragment were monitored by tryptophyl fluorescence. The excitation wavelength was set at 295 nm, and the fluorescence at wavelengths longer than 330 nm obtained by a cut-off filter was measured. The kinetics for the reduced C_L-AEDANS were monitored by fluorescence observed by energy transfer. The excitation wavelength was set at 295 nm, and the fluorescence at wavelengths longer than 460 nm obtained by a cut-off filter was measured. For the unfolding reaction, the initial concentration of Gdn-HCl was 0 M, and the mixing ratio was 1:1. For the refolding reaction, the initial concentration of Gdn-HCl was 2 M, and the mixing ratio was 1:4.3. For the measurements of refolding reactions, we introduced a constriction in the flow channel from the reservoir of protein solutions to the mixer as described by Kato et al. (1981). The mixing ratio was checked by a known concentration of an acetyltryptophanamide solution. The protein concentration was 2–4 μM.

All the kinetic data were analyzed as described previously (Goto & Hamaguchi, 1982a).

pH Measurements. The pH was measured with a Radiometer PHM26c meter at 25 °C.

Results

Conformation of Reduced C_L-AEDANS Fragment. Figure 1 shows the CD spectra of the C_L-AEDANS fragment and its reduced fragment in which the intrachain disulfide bond was reduced, in 50 mM Tris-HCl buffer containing 0.5 M ammonium sulfate at pH 7.5 and 25 °C. As reported previously (Goto et al., 1987, 1988), the reduced C_L-CAM was unfolded in the absence of ammonium sulfate but had the folded conformation in the presence of 0.5 M ammonium sulfate. The reduced C_L-AEDANS fragment was also unfolded in the absence of ammonium sulfate, but as can be seen in Figure 1 the conformation of the reduced C_L-AEDANS fragment in the presence of 0.5 M ammonium sulfate was almost the same as that of the intact C_L-AEDANS fragment. Thus it was necessary to carry out all the present experiments in the presence of ammonium sulfate. The CD spectrum of the C_L-AEDANS fragment was the same as that of the C_L-CAM fragment (data not shown). The extrinsic CD bands

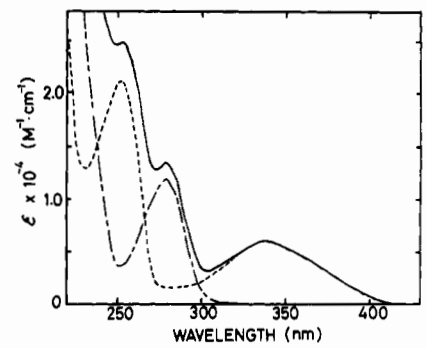


FIGURE 2: UV spectra of the reduced C_L-AEDANS (solid line) and reduced C_L-CAM (dashed and dotted line) fragments in 50 mM Tris-HCl buffer containing 0.5 M ammonium sulfate at pH 7.5 and 25 °C. The dotted line indicates the UV spectrum of *S*-AEDANS-cysteine at pH 7.5 and 25 °C.

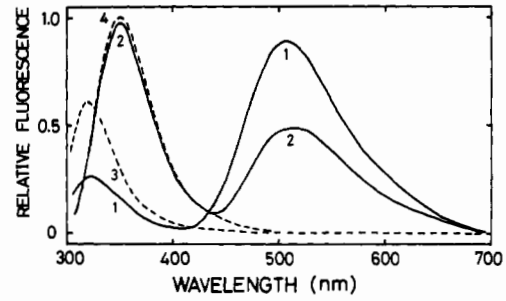


FIGURE 3: Fluorescence spectra of the reduced C_L-AEDANS (solid line) and reduced C_L-CAM (dotted line) fragments in 50 mM Tris-HCl buffer containing 0.5 M ammonium sulfate at pH 7.5 and 25 °C. (1) Reduced C_L-AEDANS fragment in 0 M Gdn-HCl; (2) reduced C_L-AEDANS fragment in 4 M Gdn-HCl; (3) reduced C_L-CAM fragment in 0 M Gdn-HCl; (4) reduced C_L-CAM fragment in 4 M Gdn-HCl. The ordinate represents the fluorescence of the reduced C_L-CAM fragment relative to that in the presence of 4 M Gdn-HCl. The protein concentrations were 2 μM. Excitation was at 295 nm. A correction was made for the fluorescence intensities due to solvent Raman scattering.

due to the incorporated AEDANS group in the C_L-AEDANS and reduced C_L-AEDANS fragments were not observed at around 350 nm. This suggests that the AEDANS group is not fixed on the protein molecule. The C-terminal region of the type-κ C_L fragment seems to be flexible because the C-terminal residues were easily removed by carboxypeptidase Y.

Fluorescence Energy Transfer from the Tryptophan Residue to the AEDANS Group in the Reduced C_L-AEDANS Fragment. Figure 2 shows the absorption spectra of *S*-AEDANS-cysteine, the reduced C_L-AEDANS fragment, and the reduced C_L-CAM fragment. As can be seen, the AEDANS group had absorption maxima at 250 and 340 nm. The latter absorption band did not overlap with that of the reduced C_L-CAM fragment.

Figure 3 shows the fluorescence spectra of the reduced C_L-CAM and reduced C_L-AEDANS fragments in the absence and presence of Gdn-HCl. Light with a 295-nm wavelength was used for excitation. The fluorescence spectrum of the reduced C_L-CAM fragment had a maximum at 310 nm in the absence of Gdn-HCl (curve 3). The blue shift of the fluorescence is due to Trp 148 being buried in the hydrophobic region of the protein molecule as reported previously by Goto et al. (1987). When the reduced C_L-CAM fragment was unfolded in 4 M Gdn-HCl, the fluorescence maximum was shifted to 350 nm, and the intensity was increased (curve 4). These emission bands overlap with the absorption band with a maximum at 340 nm for the AEDANS group at Cys 214 (see Figure 2). The fluorescence spectrum of the reduced

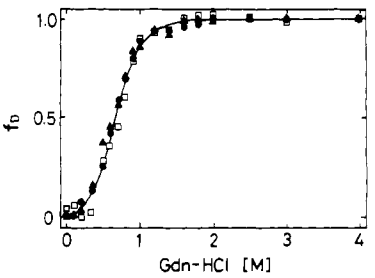


FIGURE 4: Unfolding transition by Gdn-HCl of the reduced C_L-AEDANS fragment at pH 7.5 and 25 °C. All the solvents contained 0.5 M ammonium sulfate. The ordinate represents the fraction of the unfolded protein (f_D). The unfolding transition was measured in terms of the ellipticity change at 218 nm (□), tryptophyl fluorescence change at 350 nm (●), and fluorescence energy transfer efficiency change at 510 nm (▲). The excitation wavelength in fluorescence measurements was 295 nm. The solid line indicates the theoretical curve calculated by using the equation proposed by Tanford (1970): $\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka_{\pm})$, where Δn is the difference in the number of binding sites of Gdn-HCl between the unfolded and folded states, k is the average binding constant of the sites, and $a_{\pm}$ is the mean activity of Gdn-HCl. We used 0.6 M^{-1} as the value of k [Pace and Vanderburg, (1979), see also Goto and Hamaguchi (1979)]. The solid line indicates the theoretical curve calculated by using the values of $\Delta G_D^{H_2O} = 3.0 \pm 0.3$ kcal/mol and $\Delta n = 23.6 \pm 1.5$. The concentration at the midpoint of the transition curve was 0.7 M.

C_L-AEDANS fragment had two maxima at 310 and 510 nm in the absence of Gdn-HCl (curve 1). The fluorescence intensity at 310 nm was lower than that of the reduced C_L-CAM fragment. In the presence of 4 M Gdn-HCl, the fluorescence intensity at 510 nm of the reduced C_L-AEDANS fragment was considerably lower than that in the absence of Gdn-HCl (curve 2). This fluorescence at 510 nm may be due partly to the overlap of the absorption band of the AEDANS group with the 295-nm excitation wavelength.

These findings indicate that, in the absence of Gdn-HCl, the distance between Trp 148 and the AEDANS group attached to the C-terminal cysteine residue is close in the folded conformation, that fluorescence energy transfer from the donor to the acceptor occurs effectively, and that in 4 M Gdn-HCl the distance between Trp 148 and the AEDANS group is long in the unfolded conformation, resulting in loss of energy-transfer efficiency. Since the fluorescence energy transfer efficiency depends on the inverse sixth power of the distance between the donor and acceptor (Szöllösi et al., 1987), it can be used as a measure of the compactness of the protein molecule with a high sensitivity in the process of unfolding and refolding of the reduced C_L-AEDANS fragment.

Unfolding Equilibrium of the Reduced C_L-AEDANS Fragment by Gdn-HCl. Figure 4 shows the unfolding curves obtained with Gdn-HCl of the reduced C_L-AEDANS fragment measured by ellipticity at 218 nm, tryptophyl fluorescence at 350 nm, and fluorescence at 510 nm. These three methods gave the same unfolding curve. This curve was also the same as that obtained for the reduced C_L-CAM fragment (Goto et al., 1987). The unfolding of the reduced C_L-AEDANS fragment by Gdn-HCl can thus be explained in terms of the two-state transition.

Unfolding and Refolding Kinetics of the Reduced C_L-CAM Fragment. Unfolding and refolding kinetics of the reduced C_L-CAM fragment were measured in terms of tryptophyl fluorescence. Figure 5 shows the dependence on Gdn-HCl concentration of the kinetic parameters of the fragment. The unfolding and refolding kinetics inside the transition zone were described by two exponential decay terms:

$$F(t) - F(\infty) = F_1 \exp(-\lambda_1 t) + F_2 \exp(-\lambda_2 t) \quad (1)$$

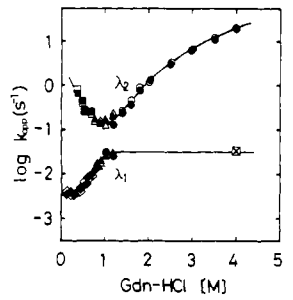


FIGURE 5: Dependence on Gdn-HCl concentration of the apparent rate constans (λ_1 and λ_2) of the fast and slow phases for the unfolding and refolding kinetics of the reduced C_L-AEDANS and reduced C_L-CAM fragments at pH 7.5 and 25 °C. The open and solid symbols indicate the values for the reduced C_L-CAM and reduced C_L-AEDANS fragments, respectively. The kinetics of the reduced C_L-CAM and reduced C_L-AEDANS fragments were measured in terms of the change in tryptophyl fluorescence and the fluorescence energy transfer efficiency, respectively. Shown are (○, ●) the unfolding kinetics obtained by stopped-flow measurements; (△, ▲) the unfolding kinetics obtained by manual mixing; (□, ■) the refolding kinetics obtained by stopped-flow measurements; (◇, ◆) the refolding kinetics obtained by manual mixing; and (cross-hatched box) the apparent rate constant of the isomerization process of U_2 to U_1 in mechanism 1 after unfolding measured by double-jump experiments with tryptophyl fluorescence detection of the reduced C_L-CAM fragment.

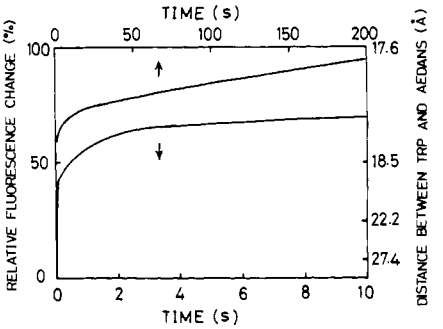


FIGURE 6: Refolding kinetics of the reduced C_L-AEDANS fragment in 0.4 M Gdn-HCl at pH 7.5 and 25 °C by stopped-flow measurement using fluorescence energy transfer detection. The distance between Trp 148 and the AEDANS group estimated by the efficiency of the fluorescence energy transfer was also shown (see text in details). Protein concentration was about 0.04 mg/mL.

where λ_1 and λ_2 are the apparent rate constants of the slow and fast phases, respectively, and F_1 and F_2 are the amplitudes of the respective phases. The total change in the fluorescence above 1.3 M Gdn-HCl was expressed by a single exponential term. The apparent rate constants of the fast phase of refolding at less than 0.37 M Gdn-HCl were not determined owing to the limitation of the mixing ratio.

Unfolding and Refolding Kinetics of the Reduced C_L-AEDANS Fragment. Figure 5 also shows the kinetic parameters for unfolding and refolding of the reduced C_L-AEDANS fragment measured in terms of the fluorescence at 510 nm at various concentrations of Gdn-HCl. As described above, the unfolding and refolding of the reduced C_L-CAM fragment at less than 1.2 M Gdn-HCl were described by two exponential terms. However, the kinetics of refolding of the reduced C_L-AEDANS fragment were described by three exponential terms, representing very fast, fast, and slow phases. The apparent refolding rate constants of the fast and slow phases were the same as those for the respective phases observed for the reduced C_L-CAM fragment, and refolding of the very fast phase was completed within the mixing dead time (~20 ms) of the stopped-flow measurement (see Figure 6).

At around 1.0 M Gdn-HCl, the values of λ_1 and λ_2 for the refolding kinetics agreed well with the respective values for

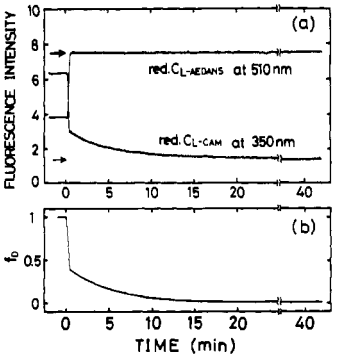


FIGURE 7: (a) Refolding kinetics by manual mixing of the reduced C_L-AEDANS and reduced C_L-CAM fragments obtained by the change in fluorescence energy transfer efficiency at 510 nm and by the change in tryptophyl fluorescence at 350 nm, respectively, in 0.13 M Gdn-HCl containing 0.5 M ammonium sulfate at pH 7.5 and 25 °C. Thick and thin arrows indicate the positions of the fluorescence intensity of the reduced C_L-AEDANS and that of reduced C_L-CAM fragments, respectively, in 0 M Gdn-HCl. (b) Refolding kinetics of the reduced C_L-AEDANS and reduced C_L-CAM fragments by the change in ellipticity at 218 nm under the same conditions as described in (a). The refolding kinetics for the two fragments were identical.

the unfolding kinetics for both fragments. The apparent rate constant of λ_2 increased sharply with decreasing concentrations of Gdn-HCl below 1 M Gdn-HCl. The apparent rate constant of λ_1 was nearly constant above 1 M Gdn-HCl.

Figure 7 shows the refolding kinetics in 0.13 M Gdn-HCl of the reduced C_L-AEDANS fragment measured in terms of the fluorescence at 510 nm and the ellipticity at 218 nm and those of the reduced C_L-CAM fragment measured in terms of the fluorescence at 350 nm and the ellipticity at 218 nm. The fluorescence intensity at 510 nm for the reduced C_L-AEDANS fragment reached the level of the value for the untreated fragment within the dead time of manual mixing (2 s). However, when the refolding reaction of the reduced C_L-AEDANS fragment was measured by using the ellipticity at 218 nm under the same conditions, a slow phase in addition to a fast phase was observed. In the refolding reaction of the reduced C_L-CAM fragment measured by fluorescence at 350 nm and ellipticity at 218 nm under the same conditions, a slow phase in addition to a fast phase was also observed. The rate constants of these slow phases were all the same. These findings indicate that while the compactness of the protein molecule measured in terms of fluorescence energy transfer was completed within about 2 s, the state of the tryptophan residue and the secondary structure of the fragment molecule were not completely recovered instantaneously. The fluorescence at 510 nm of the AEDANS group of the reduced C_L-AEDANS fragment when excited at 340 nm was not changed in the refolding process. When the refolding of the reduced C_L-CAM fragment was measured in the presence of ANS, which has similar hydrophobicity to AEDANS, no fluorescence change due to its hydrophobic interaction with the protein molecule (Stryer, 1965) was observed. These findings indicate that the AEDANS group is not caught in the interior of the reduced C_L-AEDANS molecule in the refolding process.

Double-Jump Experiment. Previously, Goto and Hamaguchi (1982b) demonstrated the existence of two forms of unfolded state for a reduced type-λ C_L-CAM fragment and showed that the unfolding and refolding kinetics can be explained on the basis of the following mechanism:

$$U_1 \rightleftharpoons U_2 \rightleftharpoons N \qquad \text{(mechanism 1)}$$

where N is native protein and U_1 and U_2 are the slow-folding and fast-folding species, respectively, of the unfolded protein.

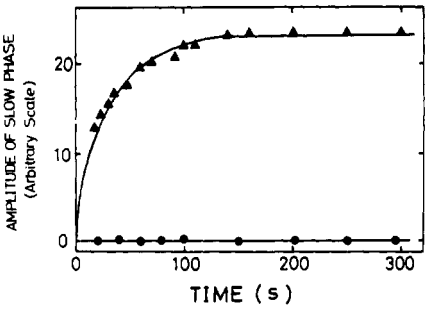


FIGURE 8: Double-jump experiments for the reduced C_L-AEDANS fragment (●) obtained by fluorescence energy transfer and for the reduced C_L-CAM (▲) fragment obtained by tryptophyl fluorescence in 0.13 M Gdn-HCl containing 0.5 M ammonium sulfate at pH 7.5 and 25 °C. Unfolding conditions included 4 M Gdn-HCl.

U_1 and U_2 are indistinguishable in terms of tryptophyl fluoresence. In order to clarify whether the existence of the two unfolded forms is also detectable by measurement of fluoresence energy transfer, we carried out double-jump experiments (Brandts et al., 1975; Nall et al., 1978) for the reduced C_L-AEDANS fragment. The results are shown in Figure 8, together with those for the reduced C_L-CAM fragment measured by tryptophyl fluoresence. The reduced C_L-CAM and reduced C_L-AEDANS fragments were first unfolded by 4 M Gdn-HCl, and then refolding was initiated with 0.13 M Gdn-HCl after various intervals under the unfolding conditions. If there is a slow equilibrium between the two forms in the unfolded state ($U_1 \rightleftharpoons U_2$), the refolding kinetics should depend on the time elapsed in the unfolded condition. We measured the amplitude of the slow phase under the refolding conditions after various times of exposure of the fragments to 4 M Gdn-HCl. For the refolding of the reduced C_L-CAM fragment measured by tryptophyl fluorescence, the dependence was expressed by one exponential decay process, with an apparent rate constant of 2.8×10^{-2} s^{-1}. For the refolding of the reduced C_L-AEDANS fragment measured by the efficiency of the fluorescence energy transfer, the slow phase was not observed at any time during the exposure to 4 M Gdn-HCl. These findings indicate that the compactness of the molecule immediately after refolding from 4 M Gdn-HCl is the same as that of the native protein at any time during exposure to 4 M Gdn-HCl, although the fraction of the species U_1 in mechanism 1 increases with the time elapsed in the unfolded condition.

Discussion

Fluorescence Energy Transfer from the Trp Residue to AEDANS in the Reduced C_L-AEDANS Fragment. The type-κ C_L fragment has only one conserved tryptophan residue at position 148. Although the fluorescence of this residue is almost completely quenched by the closely located disulfide bond (Tsunenaga et al., 1987), it does fluoresce on reduction of the disulfide bond. On reducing the intrachain disulfide bond, the type-κ C_L fragment is unfolded (Goto et al., 1987). In the presence of ammonium sulfate, however, the conformation of the reduced C_L fragment is the same as that of the intact fragment (Goto et al., 1987). These are the reasons why we used the reduced C_L-AEDANS fragment in 0.5 M ammonium sulfate to characterize the fluoresence energy transfer from Trp 148 to the AEDANS group attached to the C-terminal cysteine residue.

When excited with 295-nm light, *S*-AEDANS-cysteine fluoresced slightly at 510 nm. However, when Trp 148 of the reduced C_L-AEDANS fragment was excited with 295-nm light in the presence of 0.5 M ammonium sulfate, a fluorescence

emission band with a maximum wavelength at 510 nm was observed distinctly. This indicates that fluorescence energy transfer occurs from Trp 148 to the AEDANS group, because the fluorescence emission band at 300–450 nm due to the Trp residue overlaps with the UV absorption band of the AEDANS group and the distance between the donor and acceptor is short. In the presence of 4 M Gdn-HCl, however, the distance between the donor and acceptor is longer, and the efficiency of the fluorescence energy transfer became greatly reduced. Thus, measurement of fluorescence energy transfer is very useful for studying the change in the compactness of the protein molecule in the refolding reaction. Blond and Goldberg (1987) also used this method to study the folding of tryptophan synthase.

We attempted to estimate the distance between Trp 148 and the AEDANS group attached to Cys 214 by using the energy-transfer data obtained here and compare it with the distance obtained by X-ray crystallographic analysis. The relation between the energy-transfer efficiency, E, and the distance between donor and acceptor, R, is given by

$$R = R_0(E^{-1} - 1)^{1/6} \quad (2)$$

$$E = 1 - F_{DA}/F_D \quad (3)$$

where R_0 is the distance at which the efficiency of energy transfer between donor and acceptor is 50% (Förster, 1948), and F_{DA} and F_D are the fluorescence intensities in the presence and absence of acceptor, respectively (Fairclough & Cantor, 1978). R_0 is calculated by the equation (Stryer & Haugland, 1967)

$$R_0 = (\kappa^2 J_{DA} Q_D n^{-4})^{1/6}(9.79 \times 10^3\ \text{Å}) \quad (4)$$

where n is the refractive index of the medium between the donor and acceptor, Q_D is the quantum yield of the donor, J_{DA} is the spectral overlap integral of the donor fluorescence and acceptor absorption, and κ^2 is the orientation factor and accounts for the relative orientation of the donor emission and acceptor absorption transition dipole. The overlap integral J_{DA} is calculated by the equation (Fairclough & Cantor, 1978)

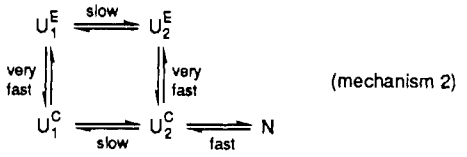
$$J_{DA} = \frac{\int_0^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda}{\int_0^\infty F_D(\lambda)\, d\lambda}(\text{cm}^3\ \text{M}^{-1}) \quad (5)$$

where $F_D(\lambda)$ and $\epsilon_A(\lambda)$ are the donor fluorescence and acceptor extinction coefficients, respectively. With the data of Figures 2 and 3, J_{DA} was calculated to be 5.50×10^{-15} cm^3 M^{-1}. Q_D was determined to be 0.047 based on the value of 0.13 for *N*-acetyl-L-tryptophanamide (Chen, 1967; Kikuchi et al., 1987). A value of 1.4 was used for the refractive index (n) of the medium. Although the AEDANS group attached to the C-terminal residue rotates freely, Trp 148 is buried in the interior of the molecule and the movement is restricted. On reduction of the disulfide bond, however, the rate of fluctuation is increased to a greater extent (Kikuchi et al., 1987). Though we could not determine the value of the orientation factor κ^2, a value of 2/3 was used in the present calculation assuming that the Trp and AEDANS groups rotate freely in a shorter time relative to the excited-state lifetime of the donor (Förster, 1948). By using these values of J_{DA}, Q_D, n, and κ^2, the R_0 value was calculated to be 18.5 Å. As the energy-transfer efficiency, E, was found to be 0.57 by using the data of Figure 3, the value of R was calculated to be 17.6 Å by eq 2.

On the basis of the X-ray structure of the F_{ab} fragment of a type-κ immunoglobulin (McPC603) (Satow et al., 1986), the distance between the C_γ atom of Trp 148 and the S_γ atom of C-terminal cysteine is found to be 20.5 Å. Thus the distance between Trp 148 and the AEDANS group estimated above agrees well with the X-ray data.

During the refolding process, the donor, the indole group of Trp 148, will be buried with a variety of orientations in the interior of the protein molecule, and thus the value of κ^2 will change. However, if we assume a constant value of 2/3 for κ^2, we can estimate the change in the distance between the donor and acceptor during the refolding process. This is shown in Figure 6. It is very interesting to note that for the refolding reaction of the reduced C_L-AEDANS fragment in 0.4 M Gdn-HCl the distance between Trp 148 and the AEDANS group decreased to about 19 Å, which is almost the same as the distance for the native protein, within 50 ms after the refolding starts.

Refolding of the Reduced C_L Fragment. For the unfolding reactions, the kinetic parameters obtained for the reduced C_L-AEDANS fragment by measurement of fluorescence energy transfer were the same as those obtained for the reduced C_L-CAM fragment by tryptophyl fluorescence measurement. For the refolding reaction of the reduced C_L-AEDANS fragment, however, the kinetic parameters obtained by the measurement of fluorescence energy transfer were different from those for the reduced C_L-CAM fragment obtained by measurement of tryptophyl fluorescence, and a very fast phase with an apparent rate constant λ_3 was detected in addition to the fast and slow phases with apparent rate constants λ_1 and λ_2, respectively, observed for the reduced C_L-CAM fragment. The very fast phase, which was completed within about 20 ms, was observed for the reduced C_L-AEDANS fragment only by use of the fluorescence energy transfer method. These findings indicate that in the refolding process a conformation as compact as the native one is first formed with an apparent rate constant λ_3, and then rearrangements of various groups in the compact molecule occur with apparent rate constants λ_1 and λ_2, and the native molecule is formed. The unfolding and refolding kinetics of the reduced C_L fragment at Gdn-HCl concentrations below the transition zone can be explained on the basis of the following mechanism:



(mechanism 2)

where N is the native protein, U_1 and U_2 are the slow-folding and fast-folding species, respectively, of unfolded protein, U^E is the extended unfolded molecule or the unfolded molecule with a large volume, and U^C is the molecule whose conformation is disordered but is nearly as compact as the native one. The appearance of the two forms of unfolded protein, U_1 and U_2, may be explained in terms of the cis–trans isomerization of the prolyl residues (Garel & Baldwin, 1975; Brandts et al., 1975), and U_1 and U_2 are indistinguishable on the basis of CD and fluorescence. U^E and U^C are distinguishable only by means of a fluorescence energy transfer measurement. The results in Figure 8 show that the cis–trans isomerization of the prolyl residue(s) can also occur in the compact states (Lang & Schmid, 1990). The rates for the reactions of $U^E \rightleftharpoons U^C$, $U_C^2 \rightleftharpoons N$, and $U_1^C \rightleftharpoons U_2^C$ are of the millisecond, second, and minute order, respectively. Although they did not directly measure the compactness of the protein molecule but measured the absorption at 292 nm, Kuwajima et al. (1989) recently proposed a similar mechanism for the folding of α-lactalbumin.

Unfolding and refolding of proteins have been so far explained in terms of the three-species mechanism (mechanism 1) (Garel & Baldwin, 1975; Brandts et al., 1975). However, this model describes only the presence of two unfolded proteins in the unfolded state and not the conformation of the protein molecule immediately after transfer from unfolding conditions to refolding conditions. The present results have further clarified the mechanism of protein unfolding and refolding. The appearance of such a compact structure in the refolding process has been suggested for a type-λ C_L fragment (Goto & Hamaguchi, 1982a), ribonuclease A (Schmid, 1981; Schmid & Blaschek, 1981; Shalongo et al., 1989), a thermolysin fragment (Dalzoppo et al., 1985), carbonic anhydrase B (Semisotnov et al., 1987), and bovine pancreatic trypsin inhibitor (Shalongo et al., 1989). This compact structure may be formed by hydrophobic interactions. Although we do not know the secondary structure of the U^C molecules precisely, extrapolation to zero time of the CD signal of the reduced C_L-AEDANS fragment for refolding (Figure 7) shows that the U^C molecules have about 50% of the secondary structure of the folded one. These characteristics of the U^C molecules are very similar to those of the molten globule (Ohgushi & Wada, 1983; Ptitsyn, 1987). Recent studies (Baum et al., 1989; Kuwajima, 1989; Baldwin, 1989; Wright et al., 1988; Fischer & Schmid, 1990; Ptitsyn et al., 1990) indicate that the molten-globule state is formed at an early stage of protein refolding.

Ptitsyn et al. (1990) used the binding of ANS to protein in support of molten-globule formation. In our present experiments, however, ANS did not bind to the reduced C_L-AEDANS fragment in the early stages of refolding. Since the number of proteins examined by use of ANS binding has been limited, the binding of ANS will not always support molten-globule formation.

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