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Probing Local Conformational Changes during Equilibrium Unfolding of Firefly Luciferase: Fluorescence and Circular Dichroism Studies of Single Tryptophan Mutants

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Firefly luciferase is a monomeric protein composed of two globular domains. There is a wide cleft between the two domains. The N-terminal domain can be further divided into A-, B-, and C-subdomains. Previous studies showed that *in vitro* unfolding of firefly luciferase induced by guanidinium chloride can be described as a four-state equilibrium with two inactive intermediates (Herbst, R., *et al.* (1997) *J. Biol. Chem.* 272, 7099–7105). In order to monitor spectroscopically the conformational changes that occur in the different domains and subdomains during the multi-state unfolding process, we constructed a series of single-tryptophan mutants. These mutants were purified and characterized and shown to retain essentially all of the structural properties of the wild-type luciferase. Under equilibrium conditions, the unfolding of each mutant protein were studied by means of fluorescence and circular dichroism. The results show that different conformational changes occur in specific regions, suggesting a sequential unfolding process for firefly luciferase. Under 2.5 M GdmCl, whereas the N-domain unfolds partially holding half of the secondary structure content, the C-domain unfolds almost completely. In the equilibrium intermediate I_2, the secondary structure might stem mostly from the A- and B-subdomains.

Key Words: **single-Trp mutant; circular dichroism; fluorescence; unfolding; local conformational change; firefly luciferase.**

Abbreviations used: GdmCl, guanidinium chloride; ANS, 1-anilinonaphthalene-8-sulfonic acid; NBS, *n*-bromosuccinimide; CD, circular dichroism; IDA, iminodiacetic acid; Trp, tryptophan; Phe, phenylalanine; Trp^{89}, Trp^{127}, Trp^{295}, Trp^{417}, Trp^{426}, and Trp^{457}, single-tryptophan mutants of luciferase with Trp residue at position 89, 127, 295, 417, 426, and 457, respectively; WT, wild-type.

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Firefly (*Photinus pyralis*) luciferase is a monomeric enzyme of 62 kDa. In the presence of Mg^{2+}, ATP, and oxygen, the enzyme oxidizes luciferin and produces yellow-green light (1). Firefly luciferase folds into two compact domains connected by a short linker: a large N-terminal domain comprising residues 4–436 and a small C-terminal domain from residues 440–544. The N-terminal domain consists of three subdomains (2). Although the crystal structure of the enzyme has been solved, some aspects of its enzymatic mechanism still remain mysterious (2–4). The cloning of the cDNA coding for firefly luciferase has opened a wide field of applications in molecular biology in which *luc* is used as a genetic marker or reporter gene (5). Moreover, firefly luciferase is an interesting model system that has been used extensively as a substrate for chaperone-mediated folding studies (6–8).

Other investigations have been done on chaperone-independent folding of firefly luciferase (9, 10). Equilibrium unfolding results have shown that unfolding of luciferase induced by GdmCl can be described as a four-state equilibrium with two inactive intermediates (I_1 and I_2) (9). Further studies on characterizing the structural properties of the intermediate states will lead to a more detailed understanding of the folding mechanism of firefly luciferase. Since tryptophan residue has been extensively used as a sensitive intrinsic fluorescence probe of protein conformational change (11–14), we constructed six single-Trp mutants in an effort to further characterize the conformational changes that occur in the different domains and subdomains during the multi-state unfolding process.

In the six single-Trp mutants, the six tryptophan residues are located in different domains and subdomains individually (Fig. 1). Firefly luciferase contains two native Trp residues, Trp417 and Trp426, both of which are situated in the C-subdomain close to the inter-domain hinge region. Two single-Trp mutants,

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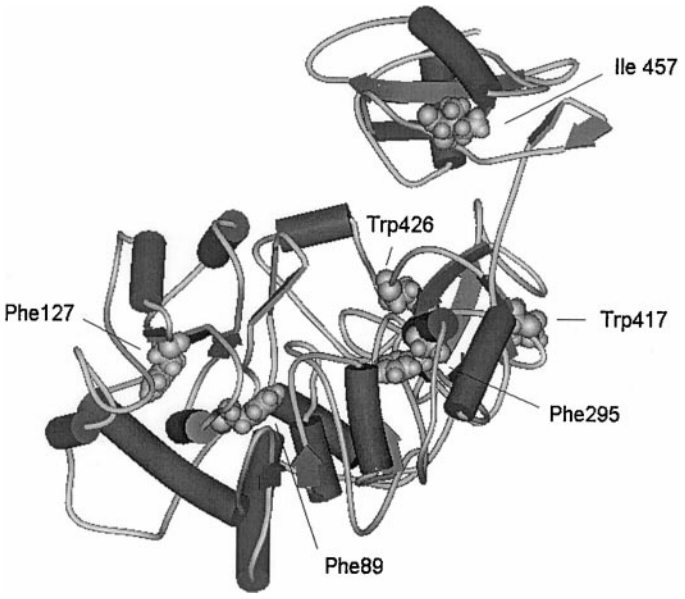

FIG. 1. Ribbon diagram of firefly luciferase (PDB code: 1lci) showing the positions of the two native tryptophan (Trp417 and Trp426 in the β-barrel C-subdomain of the N-terminal domain) and the residues mutated to tryptophan in this study (Phe89 and Phe127, β-sheet A-subdomain of the N-terminal domain; Phe295, β-sheet B-subdomain of the N-terminal domain; Ile457, the C-terminal domain. Phe89, Phe295, and Ile457 located in α-helix; Phe127 located in β-strand).

Trp^{417} and Trp^{426}, were created by replacing two native tryptophan residues individually with a phenylalanine residue. The other four single-Trp mutants, Trp^{89}, Trp^{127}, Trp^{295}, and Trp^{457}, were obtained first by replacing two native tryptophan residues together with phenylalanine residues and then replacing other hydrophobic residues individually at selected sites with a tryptophan residue. Trp89 and Trp127 locate in the hydrophobic core of the A-subdomain. Trp295 and Trp457 lie in the hydrophobic core of the B-subdomain and C-domain, respectively. The six single-Trp mutants were shown to retain essentially all of the structural properties of the WT luciferase. The unfolding transitions of these mutants have been studied by means of circular dichroism and fluorescence.

MATERIALS AND METHODS

Materials. GdmCl was purchased from USB; ANS was obtained from BDH Chemicals Ltd.; NBS was from Sigma and luciferin was from Molecular Probe. Ni-IDA sepharose was synthesized in our laboratory. Dehydroluciferin was synthesized in our laboratory. Other chemicals are of analytical grade.

Preparation, expression, and purification of luciferase variants. All site-directed mutants were constructed from a pseudo-wild-type (WT) firefly luciferase containing an N-terminal His-tag (19) by PCR procedures. It has been shown that the His-tag almost does not affect the structure, stability, or activity of firefly luciferase (data not shown; Ref. 9, 19). The Trp^{417} and Trp^{426} mutants were constructed from WT by replacing one of the native Trp codons with Phe codon alternatively. Other mutants were obtained from a Trp-free mutant (Trp417Phe/Trp426Phe) by introducing Trp codon in selected regions. The mutant genes were then cloned into a pQE plasmid and expressed in *E. coli* TG1 strain. Purification of all the mutants was performed on a Ni-IDA sepharose affinity chromatography as described previously (19). The proteins were >95% homogeneity as estimated from SDS–PAGE.

Luciferase luminescence assay. The luciferase luminescence assay was performed as described previously (19). The activities of the enzyme in GdmCl denaturation were determined in the reagent buffer containing the same concentration of the denaturant.

CD measurements. CD spectra were collected on a Jasco-715 spectropolarimeter with a scan speed of 10 nm/min and a response time of 0.25 s. For the far-UV CD, the path-length of the cuvette was 1 mm. Each spectrum was the average of at least four scans. The protein concentration was 3 μM (in 25 mM Tris, 50 mM NaCl buffer, pH 7.8), except for unfolding experiments. The ellipticity at 222 nm under different concentrations of GdmCl was recorded for the unfolding transition profile.

Fluorescence measurements. Intrinsic fluorescence measurements were performed at 20°C using an F-4010 spectrophotometer (Hitachi). The excitation wavelength was set at 295 nm, and the spectral slits were both 5 nm for excitation and emission. The emission spectra were recorded in the range of 300 to 400 nm. All spectra were corrected for background fluorescence from buffer and denaturant. The protein concentration was 3 μM in Tris buffer (100 mM Tris, 200 mM KCl, pH 7.8). The quantum yields were determined at room temperature relative to tryptophan in water (20) as described in reference (21). The Job Plot experiments of ANS binding to proteins were essentially performed as described in ref. 17. The excitation wavelength was 350 nm. After incubation at 20°C for 30 min, the ANS fluorescence intensity at 480 nm was recorded. For the binding studies of dehydroluciferin, various amounts of dehydroluciferin are titrated into a solution of luciferase and the fluorescence (440 nm emission, 350 nm excitation) were measured according to Denburg's paper (22). GdmCl-induced equilibrium unfolding was performed using different concentrations of the denaturant and recorded as the fluorescence intensity at emission maximum.

NBS modification. Excess (ca. 40 μM) NBS was added to a protein sample of 3.5 μM in Tris buffer and the reduction of the fluorescence intensity for every reaction was recorded for 30 min at 20°C. The data were processed by pseudo-first-order kinetics.

Equilibrium unfolding experiments. The equilibrium unfolding was performed on protein samples by monitoring fluorescence intensity and CD ellipticity. Protein samples were incubated with Tris buffer (100 mM Tris-Cl, pH 7.8, 200 mM KCl, 0.5 mM EDTA) at 4°C overnight (both fluorescence and CD signals reached their equilibrium within this period, data not shown) in the presence of various concentrations of GdmCl (0 ~ 6 M). The protein concentration was 50 μg/ml for CD and fluorescence measures, at which concentration the unfolding profiles of luciferase measured by both CD and fluorescence signals are correspond to those at lower concentrations (data not shown; also as described in ref. 9, 10). All of the data were normalized to relative fractions for comparison.

RESULTS AND DISCUSSION

Structures and Activities of the Single-Trp Mutant Luciferases

As presented in Table 1, the Trp^{417} and Trp^{426} mutants retain 80 and 72% of the relative activity of WT respectively, but the other four mutants retain considerably lower activity. As well known, the activity assay may be too sensitive to probe the changes of the active

TABLE 1

Spectral Properties and Relative Activities of WT Luciferase and Its Mutants

Proteins	Relative activity (%)	K_D[a] (μM)	Mean residue ellipticities		λ_{max}[b]	
			208 nm	222 nm	0 M	5 M
WT	100	2.2	−8243	−7356	334	351
Trp^{89}	0.1	36	−7952	−7210	334	351
Trp^{127}	1	14	−7802	−7189	334	351
Trp^{295}	7	3.1	−7693	−6451	334	351
Trp^{417}	80	2.0	−7994	−6964	330	351
Trp^{426}	72	2.3	−8556	−7433	334	351
Trp^{457}	27	2.4	−7369	−6238	334	351

[a] The dissociation constant of dehydroluciferin binding to proteins.
[b] Corrected fluorescence emission spectra (excited at 295 nm) in the presence and absence of 5 M GdmCl.

site geometry. The binding studies using a substrate analog (dehydroluciferin) would be more informative. As shown in Table 1, the dissociation constants (K_D) of Trp^{417}, Trp^{426}, Trp^{295}, and Trp^{457} mutants are similar to that of WT, whereas those of Trp^{89} and Trp^{127} increase by nearly 18- and 7-fold, respectively. The results indicate that the mutations at position 417, 426, 295, and 457 have only negligible effect on the geometry including active site, whereas the other two mutants present slight changes in the active site.

The far-UV CD spectra of all the mutants are similar to that of the WT luciferase (Fig. 2A), indicating that the secondary structures remain unchanged upon mutations. The mean residue ellipticities at 208 and 222 nm, which is a measure of the helicity of a protein, also show no significant changes (15, 16) (Table 1).

Fluorescence emission spectra of WT and the mutants are presented in Fig. 2B. We summed up the two fluorescence spectra of the Trp^{417} and Trp^{426} mutants, the calculated spectrum was virtually identical to that of WT, indicating that the fluorescence emission of Trp417 and Trp426 were additive. The quantum yields of the Trp^{89}, Trp^{127}, Trp^{295}, and Trp^{457} mutants were relatively low (Q = 0.10, 0.13, 0.09, and 0.07, respectively), as compared with those of Trp^{417} and Trp^{426} (Q = 0.16 and 0.18, respectively). Under native conditions, the maximum emission of WT and the mutants were at 334 nm except for Trp^{417} that displayed a blue shift to 330 nm. After denaturation in 5 M of GdmCl, all the mutants exhibited an emission maximum at a wavelength of 351 nm (Table 1).

The Job Plot experiments (17) showed that the ANS binding properties of all the mutants were also similar to that of WT (Fig. 2C; the binding ratio = 1:1), suggesting that the hydrophobic regions on the accessible surface of these mutants are the same as WT luciferase.

To test the extent of exposure of Trp residues in the protein structure, we modified the WT luciferase and mutants with NBS. The reaction curves are shown in Fig. 2D. The apparent rate constants of Trp^{417} and Trp^{426} were 0.030 and 0.008 s^{-1}, respectively. The rate constants were virtually identical to those of WT luciferase (0.029 and 0.007 s^{-1}, respectively). The other mutants, Trp^{89}, Trp^{127}, Trp^{295}, and Trp^{457}, had rate constants around 0.002 s^{-1}. These results demonstrate that the Trp residues at positions 89, 127, 295, and 457 were in a more hydrophobic environment than positions 417 and 426.

All the results above indicate that the six single-Trp mutants were shown to retain essentially all of the structural properties of WT, although mutations at positions 89 and 127 might cause slight alterations at the active site geometry. In addition, the tryptophan residues (Trp89, Trp127, Trp295, and Trp457) were individually introduced into the hydrophobic environments in different domains and subdomains as we expected.

Unfolding of the Trp^{295}, Trp^{417}, Trp^{426}, and Trp^{457} Mutants

Four mutations (Trp^{295}, Trp^{417}, Trp^{426}, and Trp^{457}) did not affect the unfolding properties of luciferase, based on CD-monitored unfolding curves (Fig. 3A). Therefore, we could assume that the same transitions are reported by the four tryptophan probes during GdmCl-induced unfolding process. The intrinsic fluorescence intensities of Trp295, Trp417, Trp426, and Trp457 gave dramatically different unfolding curves (Fig. 3B). The fluorescence unfolding curves of all the four single-Trp mutants showed multiple unfolding transitions.

Between 0–2.5 M GdmCl, all the four tryptophan probes indicated two transitions. The first transition occurred from 0 to 0.8 M GdmCl. The fluorescence intensities of Trp^{417} and Trp^{426} decreased significantly from 0 to 0.4 M GdmCl, while those of Trp^{295} and Trp^{457} increased slightly. From 0.4 to 0.8 M GdmCl, the fluorescence of Trp^{417} and Trp^{426} experienced a flat. The fluorescence of Trp^{295} and Trp^{457} reached a maximum value at 0.8–1 M GdmCl. In Herbst's paper, it reported that the equilibrium intermediate I_1 is populated

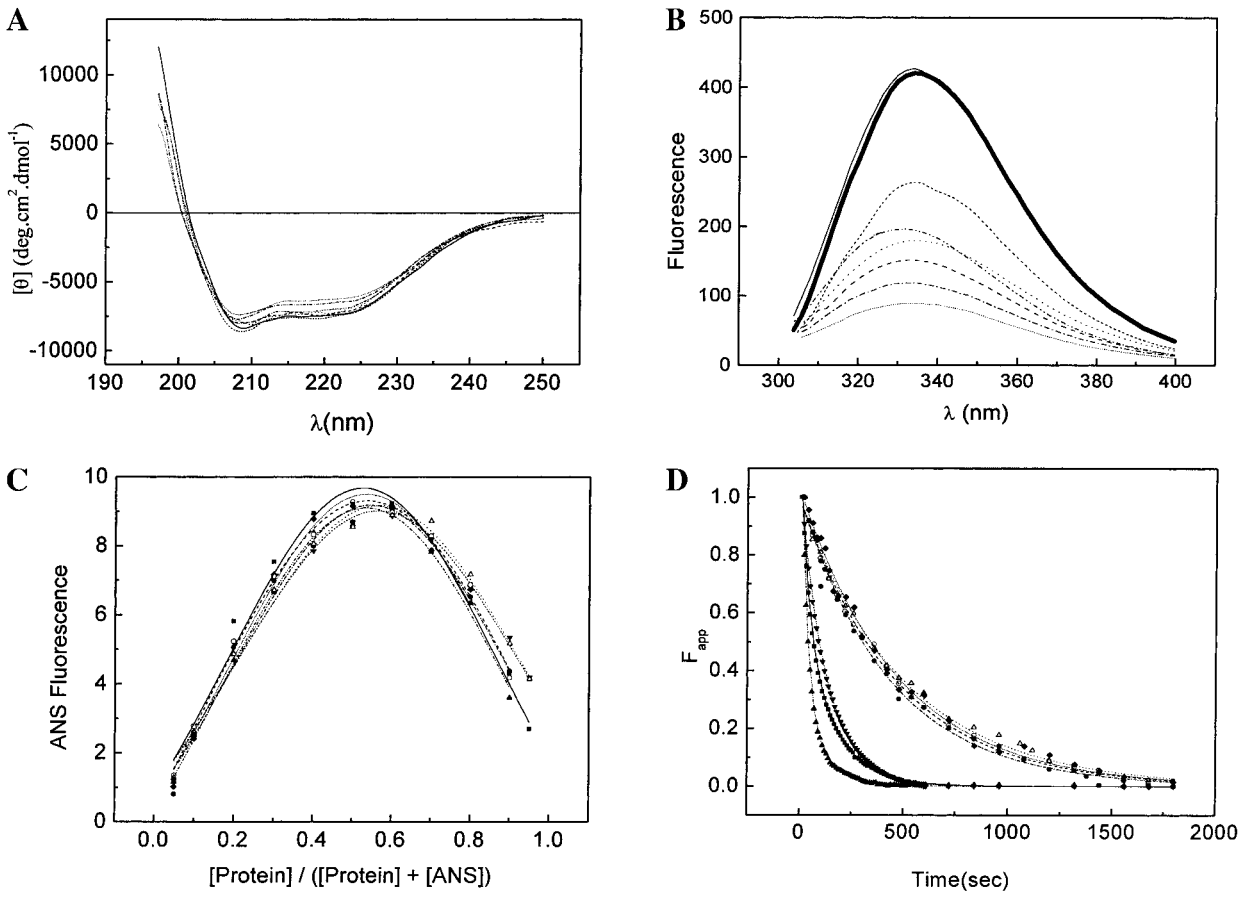

FIG. 2. Spectroscopic characterization of WT luciferse and its single-Trp mutants. (A and B) WT (—), Trp^{89} (–), Trp^{127} (. . .), Trp^{295} (-.-.-), Trp^{417} (-..-), Trp^{426} (short–), Trp^{457} (short. . .). (A) Far-UV CD spectra. (B) Fluorescence emission spectra of WT luciferase and the single-Trp mutants. Comparison of the emission spectrum of WT luciferase (thick solid line) and the calculated spectrum (thin solid line) generated by adding the emission spectra of Trp^{417}, Trp^{426} and subtracting that of Trp-free mutant. (C and D) WT (■), Trp^{89} (○), Trp^{127} (△), Trp^{295} (●), Trp^{417} (▲), Trp^{426} (▼), and Trp^{457} (◆). (C) Job Plot for ANS binding studies of WT luciferase and its mutants. (D) Time course of NBS modification of WT luciferase and the single-Trp mutants. The data were processed by pseudo-first-order kinetics.

around 0.8–1 M GdmCl. In the present study, the intermediate I_1 is characterized by the fact that the specific region in which Trp426 resides is almost completely exposed and the specific region in which Trp417 resides is partially exposed. The plateau in the fluorescence curves of the Trp^{417} and Trp^{426} implies that Trp417 and Trp426 might be partially involved in the formation of the intermediate I_1. Trp295 in B-subdomain and Trp457 in C-domain are slightly shifted to more hydrophobic regions. The ANS binding fluorescence intensity of WT luciferase and all four mutants reached a maximum around 0.8–1 M GdmCl (data not shown), indicating the extensive hydrophobic regions of the protein has been accessible in the intermediate I_1 state.

From 0.8 to 2.5 M GdmCl, the second transition occurred. The fluorescence of all four mutants decreased. Moreover, the fluorescence of Trp^{417}, Trp^{426}, and Trp^{457} all reached a minimum value at 2.5 M GdmCl, indicating Trp417, Trp426, and Trp457 were exposed completely. Interestingly, in the second transition, the fluorescence changes of Trp^{417} and Trp^{426} were consistent with that of Trp^{457}. Taking account of the fact that the tryptophan residues 417 and 426 are located close to the linker region between N- and C-domains, we imply that: (i) the local regions in which Trp417, Trp426, and Trp457 reside might be involved in some interactions in the intermediate I_1; (ii) in the second transition the local regions in which Trp417, Trp426, and Trp457 reside might unfold simultaneously to the unfolded state, with a completely unfolding of C-domain.

Between 2.5–4 M GdmCl, there was a third transition as shown by fluorescence changes of Trp^{295}, which agreed with the changes of CD. The plateau in the CD unfolding curves and the plateau in the fluorescence unfolding curve of the Trp^{295} mutant between 2.5–3 M GdmCl both indicate the existence of the equilibrium intermediate I_2, which has been reported in Herbst' paper (9). The local region in which Trp295 reside might be involved in some interactions in the intermediate I_2, but not those local regions in which Trp417,

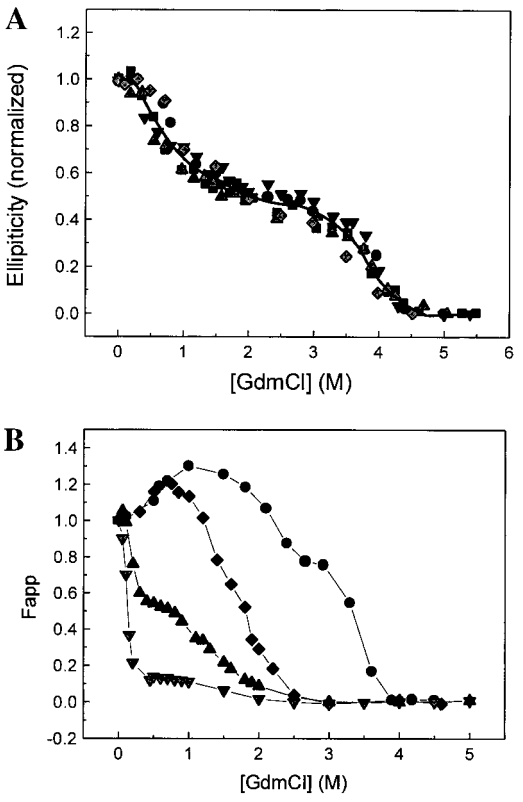

FIG. 3. Denaturation curves of WT luciferase (■), Trp^{295} (●), Trp^{417} (▲), Trp^{426} (▼), and Trp^{457} (◆). GdmCl-induced unfolding was followed by monitoring (A) CD ellipticity change at 222 nm and (B) fluorescence.

Trp426, and Trp457 reside as visualized by fluorescence. As we only selected limited sites for fluorescence reporters, it could not exclude that other parts at each subdomains participate into the transitions and intermediates as well.

The multi unfolding transitions of luciferase imply that more than two intermediates may exist. Further kinetic determination of the denaturation rate constants for each of the mutants will provide more information about the unfolding process of luciferase.

Role of Phe89 and Phe127 in the Formation of the Intermediate

There are two mutations, Trp^{89} and Trp^{127}, affecting the unfolding process. The plateau in the CD curves between 2.5–3 M GdmCl disappeared during the unfolding process of the two mutants (Fig. 4A). The CD-monitored unfolding curves of the Trp^{89} and Trp^{127} mutants were identical but were not consistent with those of WT luciferase and the other four mutants. Between 0 to 1.5 M GdmCl, the CD curves of the two mutants nearly superimpose to that of WT luciferase. It implies that the secondary structure of the intermediate I_1, which populated around 1 M GdmCl, does not change significantly in the unfolding process of the Trp^{89} and Trp^{127} mutants. The fluorescence-monitored unfolding curves of the Trp^{89} and Trp^{127} mutants were different from each other as well as the other mutants. The fluorescence of Trp^{89} decreased constantly and reached a minimum value at 4 M GdmCl. However, the fluorescence of Trp^{127} increased slightly from 0 to 0.5 M GdmCl, then decreased completely until 2.5 M GdmCl.

Phenylalanine and tryptophan are both neutral nonpolar aromatic amino acids, the substitution of Trp for Phe usually would not cause large changes in the whole protein structure and unfolding process. But the present studies indicated that a single-site conservation mutation led to a different unfolding process, in which the intermediate I_2 disappeared. It provides an additional evidence for the existence of the intermediate I_2 and suggests Phe89 and Phe127 play an important role in the formation of the intermediate I_2.

Summing up the unfolding transitions of the four mutants (Trp^{295}, Trp^{417}, Trp^{426}, and Trp^{457}) monitored by fluorescence and the role of Phe89 and Phe127, a sequential unfolding process could be proposed for firefly luciferase. Under 2.5 M GdmCl, the C-domain unfolds almost completely, whereas the N-domain unfolds partially holding half of the secondary structure content. The secondary structural content of the equi-

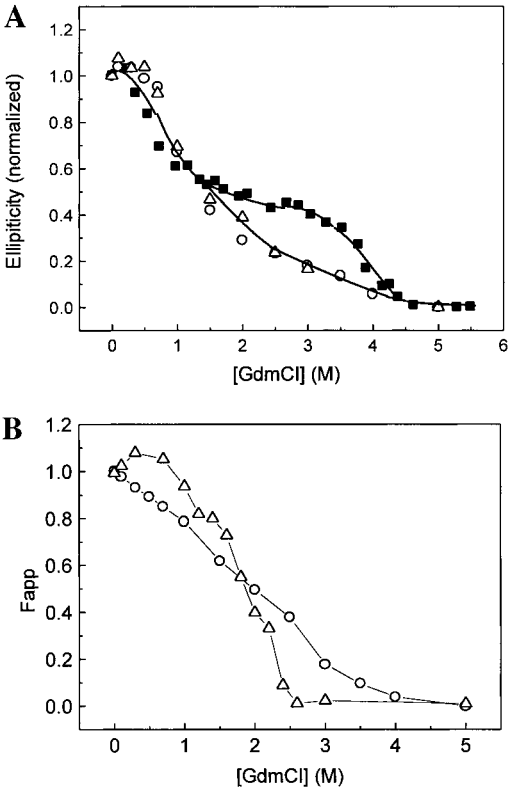

FIG. 4. Denaturation curves of WT luciferase (■), Trp^{89} (○) and Trp^{127} (△). GdmCl-induced unfolding was followed by monitoring (A) CD ellipticity change at 222 nm and (B) fluorescence.

librium intermediate I_2 might stem mostly from the A- and B- subdomains of the N-domain. The stable N-terminus is also evidenced by cotranslational folding and trypsin digest experiments (18).

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