Fluorescence Quenching of Green Fluorescent Protein during Denaturation by Guanidine

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Received November 8, 2004

Fluorescence of green fluorescent protein mutant, 2-5 GFP is observed during denaturation by guanidine. The fluorescence intensity decreases exponentially but the fluorescence lifetime does not change during denaturation. The fluorescence lifetime of the denatured protein is shorter than that of native form. As the protein structure is modified by guanidine, solvent water molecules penetrate into the protein barrel and protonate the chromophore to quench fluorescence. Most fluorescence quenchers do not affect the fluorescence of native form but accelerate the fluorescence intensity decay during denaturation. Based on the observations, a simple model is suggested for the structural change of the protein molecule during denaturation.

Key Words : GFP, Fluorescence, Denaturation, Quencher

Introduction

Green fluorescent protein (GFP) is one of rare proteins which give fluorescence in the visible region.[1,2] Since GFP was expressed[3] and the crystal structure of wild-type (wt) GFP was determined,[4] GFP has become one of the most commonly used biological markers. Various dynamic processes of gene expression and protein interactions in living cells have been visualized by using GFP.[5-7] The GFP chromophore with unique luminescent properties is embedded in the highly protected region of the barrel shape protein.[4,8,9] The chromophore is formed by spontaneous cyclization and oxidation of amino acid chain during protein folding. The fluorescence of GFP is sensitive to microscopic environments such as cellular pH. GFP gives strong fluorescence only when the chromophore is kept inside the protein barrel. Many GFP mutants have been reported and more than 20 crystal structures of GFP mutants and homologs are listed in the Protein Data Bank.[2] While the GFP mutants have very different spectroscopic characters,[1] their structural features are remarkably similar. Although GFP has been utilized in many areas, folding and unfolding of GFP have not been understood in details.

In this work, we have used the fluorescence spectroscopy to study the denaturation of GFP by guanidine and the effects of fluorescence quenchers. Guanidine is a well known denaturant which unfolds native proteins by interrupting hydrogen bondings of protein molecules.[10,11] Because the fluorescence of GFP decreases by denaturation, guanidine can be regarded as a fluorescence quencher for GFP. We also investigated the effects of known fluorescence quenchers such as Cu^{2+}, I^- and acrylamide. Fluorescence study of GFP during denaturation provides information about the structural change of the protein by guanidine.

Experimental Section

GFP has been modified genetically to improve spectroscopic features and structural stability. The GFP mutant studied in this work is called 2-5 GFP, which has four mutations of S65T, V163A, I167T and S175G.[12] The crystal structure of 2-5 GFP is not listed in the Protein Data Bank. GFP molecules with similar mutations are reported to have the well known barrel shape structure like wt GFP.[2] It can be assumed that 2-5 GFP has the same structure as wt GFP.

GFP molecules were expressed in *E. coli* and purified using Ni^{2+}-nitrotriacetic acid after breaking *E. coli* cells.[13-15] Purified protein molecules in phosphate buffer saline solution are diluted into designated solutions and the concentration of GFP is kept around a few μM by checking the absorption spectrum in the experiments. Some chemicals for denaturation and fluorescence quenching affect the pH of solution. Proper buffers such as ACES or PIPES are used to avoid the effects of pH change. The fluorescence lifetime of GFP is measured using ps TCSPC equipment. All the experiments are carried out at 25 ^oC using water bath.

Results and Discussion

Spectroscopic Change during Denaturation. Figure 1 shows the spectra of GFP. The absorption pectrum of native GFP is the same as the excitation spectrum. The absorption spectrum of Figure 1, which shows the non-fluorescent absorption band at 380 nm, is taken from partially denatured GFP by guanidine. The 380 nm band attributed to the protonated chromophore[16,17] appears when the native structure collapses by denaturants or pH variation. The shape of excitation and fluorescence spectrum does not change during denaturation. As the fluorescent absorption band

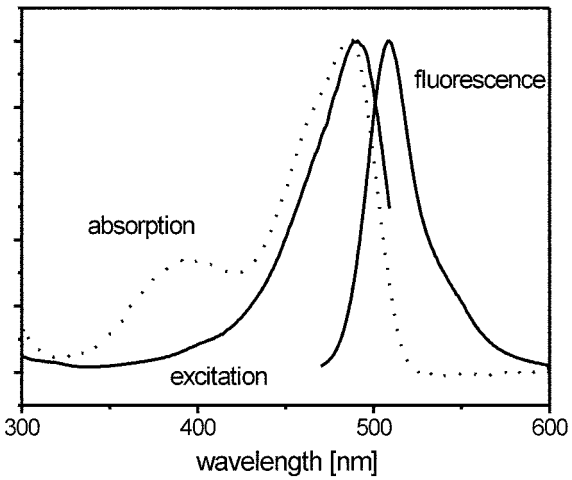

Figure 1. Absorption, fluorescence and excitation spectra of GFP. The absorption spectrum (dotted line) is taken from partially denatured GFP by guanidine.

decreases by denaturation, the fluorescence intensity decreases and the non-fluorescent band at 380 nm grows. However, the rate of the 380 nm band growth is slower than that of the 470 nm band decrease, which indicates that the non-fluorescent state is not formed directly from the fluorescent state. At the end of denaturation, the 470 nm absorption band disappears with fluorescence. The absorption spectrum change by guanidine is similar as reported for wt GFP.[17]

Temporal change of the fluorescence intensity at different guanidine concentrations is shown in Figure 2a. A single exponential decay of fluorescence intensity is observed in the investigated concentration range of guanidine. The fluorescence intensity decreases faster at higher concentration of guanidine. As the fluorescence intensity changes with time, the Stern-Volmer type analysis cannot be applied to study the fluorescence features of GFP during denaturation. Figure 2b shows the rate constant of fluorescence intensity decay at different guanidine concentrations. The solid line of Figure 2b is a fitting curve given by $k = k_a \{[Gu]-[Gu]_o\}^n$ where $[Gu]_o = 2.6 \pm 0.1$ M and $n = 4 \pm 0.1$. Only when the guanidine concentration is greater than the threshold, the fluorescence intensity begins to decrease. However, the threshold concentration of guanidine is not absolute as discussed below. Although the kinetic order is related to the molecularity of denaturation, the same kinetic order cannot be applied for all the interactions of GFP and guanidine. The kinetic order may vary under different conditions such as pH, temperature, or concentration of guanidine. Figure 2c shows the fluorescence intensity of GFP after 15 hr incubation at given guanidine concentrations and the threshold concentration of guanidine is 3.2 M. The threshold concentration determined from the rate constants of Figure 2b, corresponds to the threshold extrapolated to the time range longer than 15 hr. Change of the threshold concentration with denaturation time denotes that the denaturation of the protein is a complex process.

The fluorescence lifetime of 2-5GFP is measured as 2.8 ± 0.1 ns at native condition. The reported fluorescence lifetime of wt GFP is 3.2 ± 0.1 ns.[16,18] The deviation of the fluorescence lifetime is caused by the protein sample itself rather than by the instrument. During denaturation, the fluorescence lifetime of GFP does not change as shown in Figure 3. Just after mixing GFP in the guanidine solution, the fluorescence lifetime reduces by about 13%, thereafter, keeps constant until the fluorescence disappears. The fluorescence lifetimes are determined from 5 min accumulation of fluorescence intensity at given times after mixing. The fluorescence lifetime of GFP becomes slightly shorter at higher guanidine concentration but the dependence on the guanidine concentration is not significant in the observed

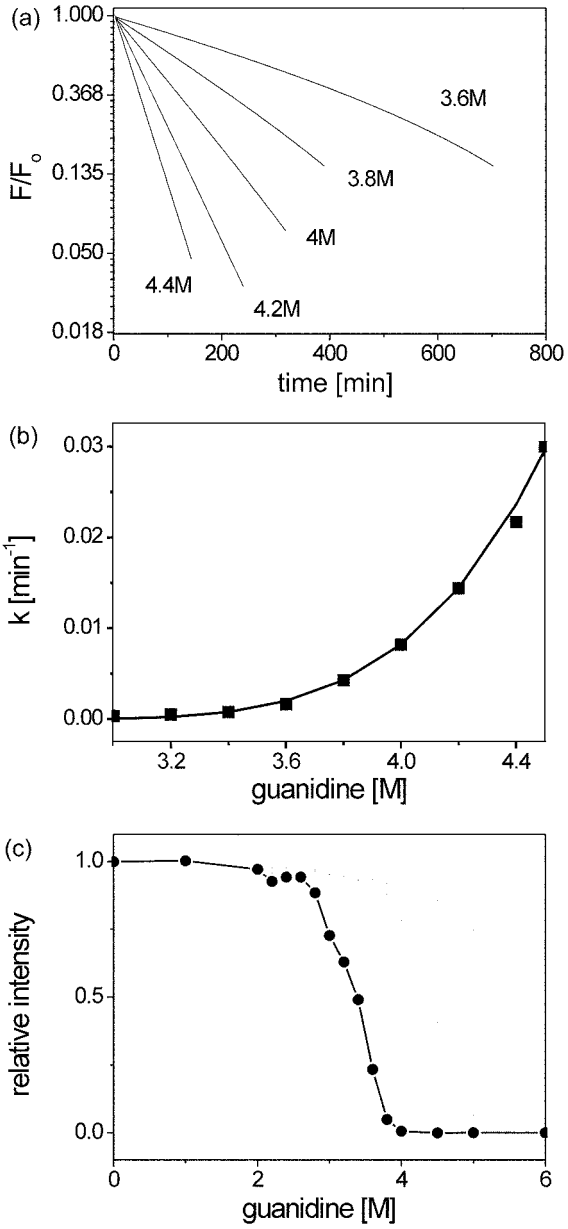

Figure 2. (a) Temporal change of GFP fluorescence intensity at different guanidine concentrations. (b) Rate constant for the fluorescence intensity decay. (c) Fluorescence intensity of GFP after 15 hr incubation in guanidine solution.

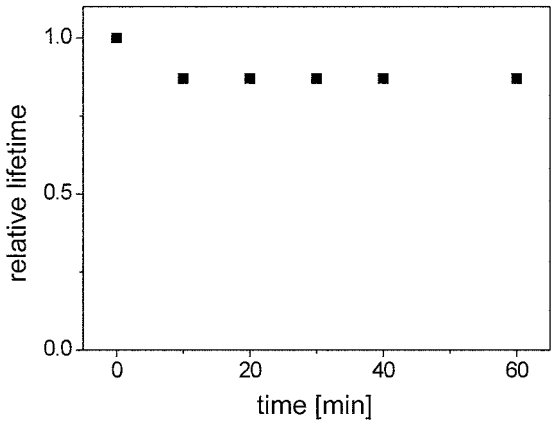

Figure 3. Fluorescence lifetime of GFP measured at different times after mixing in 4 M guanidine solution.

guanidine concentration range. The invariance of fluorescence lifetime during denaturation is not affected by the guanidine concentration. The constant fluorescence lifetime with decreasing fluorescence intensity implies that the character of the fluorescent state does not change but the number of fluorescent protein molecules decreases during denaturation.

Structural Change during Denaturation. Above results suggest a simple model for the denaturation of the GFP molecule by guanidine:

$$R_N^- \rightarrow R_D^- \rightarrow R_DH \quad (1)$$

The native GFP, R_N^-, experiences small but fast structural modification in the very early stage of denaturation. The chromophore of the modified GFP, R_D^-, has the same fluorescence spectrum as the native GFP but shorter fluorescence lifetime. The spectral shape of GFP fluorescence does not change during denaturation, which indicates that local environments around the chromophore are similar for R_N^- and R_D^-. Small difference between these states appears as different fluorescence lifetimes. The modified GFP has the shorter fluorescence lifetime than the native GFP. As the GFP molecule denatures, the chromophore converts into the non-fluorescent state, R_DH. Water molecules penetrate into the protein molecule and protonate the chromophore. The protonated chromophore does not give fluorescence. Isolated chromophore molecules do not give fluorescence in aqueous solution.[19] Fluorescence intensity decay during denaturation results from the conversion of R_D^- into R_DH. There are several protonation sites in the chromophore and the pK_a's of the protonation sites are high enough to protonate the chromophore at neutral pH.[20] It is not possible to identify the exact protonation sites during denaturation in this work.

Scheme (1) does not mean that GFP molecules denature through the two-step mechanism where the involved states can be described in details. The states of R_N^-, R_D^- and R_DH do not correspond to a single molecular state. The protein molecules of each state have common spectroscopic feature, however, the protein molecules may have different microscopic structures which are not relevant to the spectroscopic properties. The protein molecules with the same spectroscopic character can have different chemical and biological properties because of local structure differences. Conformations of amino acids far from the chromophore may not affect the fluorescence of GFP as long as the chromophore is protected from solvent molecules. As mentioned in the previous section, the growing rate of the non-fluorescent state R_DH is slower than the decay rate of the fluorescent state R_D^-. The different rates indicate that the conversion of R_D^- into R_DH is not a single step. There are numerous pathways for the transitions between the states of Scheme (1), and the energy landscape picture should be introduced for detailed understanding of the transitions.

The kinetics of denaturation by guanidine were reported for wt GFP and a mutant cycle3.[21] The single exponential decay of fluorescence intensity is observed for these two GFP's but the decay rates are different. When the guanidine concentration is 4 M, the decay rates are 3×10^{-4} min^{-1} and 6×10^{-5} min^{-1} for wt GFP and cycle3, which are slower than 8×10^{-3} min^{-1} for 2-5 GFP. The slower denaturation represents the higher structural stability in guanidine solution. The threshold concentration of guanidine for denaturation which is estimated from the long time incubation, is reported to be higher for the proteins with slower denaturation rate: 3.7 M for wt GFP and 4.3 M for cycle3. The threshold concentration for 2-5 GFP is 3.2 M.

2-5 GFP has four mutations of S65T, V163A, I167T and S175G while cycle3 has three mutations of F99S, M153T and V163A. Mutation S65T of 2-5 GFP is introduced to modify the chromophore for simple absorption spectrum and strong fluorescence.[12] When the hydrophobicity of protein reduces, the stability and folding efficiency of protein increases in aqueous solution. Mutation V163A which is common in 2-5 GFP and cycle3, is buried inside the barrel and distant from the chromophore. Alanine is less hydrophobic than valine. I167T of 2-5 GFP, replacement of isoleucine with threonine occurs near the chromophore, which is attributed to the fast modification step of Scheme (1). The OH group of threonine seems to be related to the shorter fluorescence lifetime of 2-5 GFP during denaturation. The other mutations of 2-5 GFP and cycle3 are called surface-located folding mutations, which improve the in vivo maturation of GFP at 37 °C by increasing thermal stability.[22] The surface-located folding mutations are related to the guanidine sensitivity but give different effects on the sensitivity of the mutants. According to the kinetic data of guanidine denaturation, 2-5 GFP is more sensitive to guanidine than wt GFP but cycle3 is less sensitive. Replacement with hydrophilic serine and threonine in cycle3 reduces the guanidine sensitivity but the loss of OH group of serine in 2-5 GFP increases the guanidine sensitivity.

As the fluorescence lifetimes measured during guanidine denaturation are not available for wt GFP and cycle3, it is not easy to decide whether Scheme (1) is valid for wt GFP and other mutants. The GFP molecules with only a few mutations show the denaturation kinetics with rates of

different orders of magnitude. The different denaturation kinetics suggests that Scheme (1) may be unique for the structural change of 2-5 GFP by guanidine. The shorter fluorescence lifetime during denaturation as for 2-5 GFP might not be observed for wt GFP and cycle3 which have different microscopic environments around the chromophore.

Denaturation with Fluorescence Quenchers. Folding and unfolding of proteins are greatly affected by various chemicals including ionic species.[23,24] In this work, we use three fluorescence quenchers, acrylamide, I^- and Cu^{2+} to investigate the effects of chemicals on the fluorescence of GFP. The quenchers of molecular, anionic and cationic species are chosen. As the chromophore is located inside the protein barrel, GFP gives strong fluorescence in solutions of relatively high ionic strength.[25] The protecting barrel structure of GFP prevents acrylamide and iodide ion of 1 M from quenching the fluorescence of native GFP. However, a few mM of copper ion, added as $Cu(NO_3)_2$ quenches the fluorescence of native GFP in neutral pH solution. Other nitrate salts such as sodium nitrate do not reduce the fluorescence. When Cu^{2+} ion is added, the fluorescence of native GFP decreases but the absorption spectrum does not change. Figure 4 shows the fluorescence intensity and lifetime of native GFP at different Cu^{2+} ion concentrations. Both fluorescence intensity and lifetime decrease simultaneously upon adding Cu^{2+} ion. Slow decrease of fluorescence intensity as in the guanidine solution is not observed when Cu^{2+} ion is added. Copper ions penetrate inside the barrel and quench fluorescence through direct interactions with the chromophore. As the spectrum of Cu^{2+} ion is slightly overlapped with the GFP spectrum, the concentration of Cu^{2+} ion is varied only in a limited range but the effect of Cu^{2+} ion is large enough to reveal the unique character.

In dynamic quenching of fluorescence, quenchers affect the fluorescent excited state of chromophore so that both fluorescence intensity and lifetime decrease. While acrylamide, I^- and Cu^{2+} ion are known as dynamic quenchers interacting with excited states of chromophore,[26] only Cu^{2+} ion shows the dynamic quenching effect for native GFP. In contrast to the dynamic quenching, the static quenching does not affect the fluorescence lifetime. Formation of non-fluorescent ground state complex is a main cause for the static quenching. During the GFP denaturation by guanidine, the non-fluorescent state is formed by protonation. The effect of guanidine on GFP can be regarded as static quenching.

When acrylamide, I^- or Cu^{2+} ion is added with guanidine into the GFP solution, additional decrease of fluorescence intensity is observed during denaturation. Figure 5 shows the temporal changes of absorbance and fluorescence intensity of GFP in guanidine solution with acrylamide, I^- or Cu^{2+} ion. Notice the concentrations of quenchers; [acrylamide] = 1 M, $[I^-]$ = 0.2 M and $[Cu^{2+}]$ = 1.5 mM. As in the denaturation by only guanidine, both absorbance and fluorescence decrease simultaneously during denaturation with quenchers, however, the fluorescence intensity decay deviates from the single exponential when the quenchers are added. The fluorescence lifetime of GFP is slightly shorter with quenchers but remains constant during denaturation. The effects of the quenchers on the fluorescence lifetime are similar. These results indicate that Scheme (1) is still valid for the denaturation with quenchers. There are no direct interactions between the quenchers and the chromophore to reduce both fluorescence intensity and lifetime, but the protonation of chromophore accelerates with quenchers.

Dependence on the quencher concentration represents that

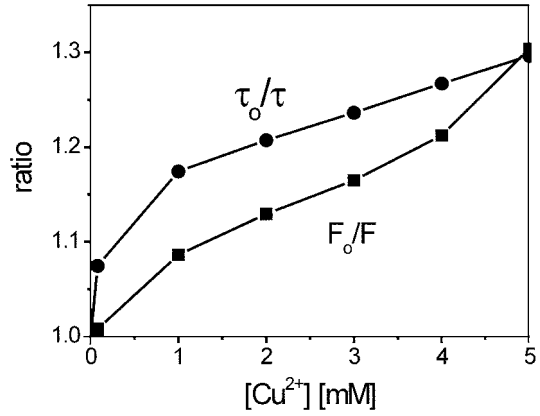

Figure 4. Fluorescence intensity and lifetime of GFP at different Cu^{2+} ion concentrations.

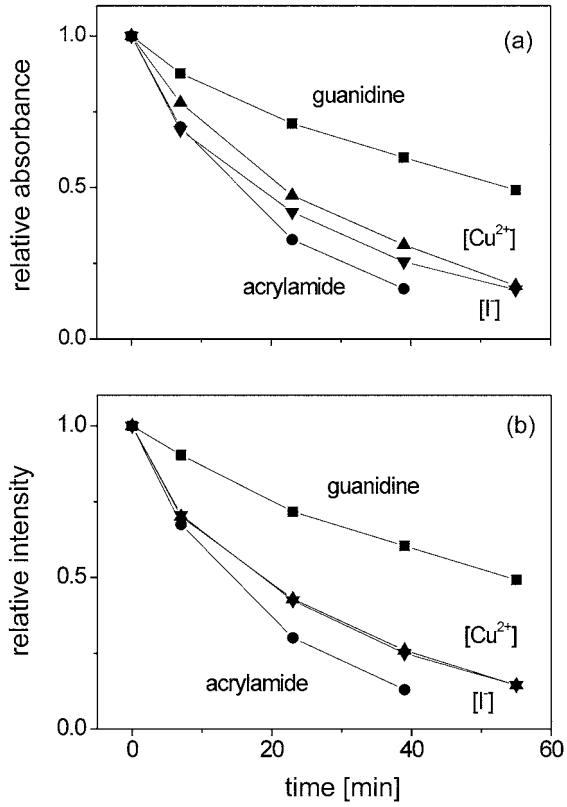

Figure 5. (a) Temporal change of absorbance of 470 nm band with guanidine and quencher. (b) Temporal change of fluorescence intensity. [guanidine] = 4 M, [acrylamide] = 1 M, $[I^-]$ = 0.2 M and $[Cu^{2+}]$ = 1.5 mM.

ionic quenchers are more effective in assisting the penetration of water molecules into the GFP barrel than neutral molecule: Cu^{2+} >> I^- > acrylamide. Hydration of ionic quenchers may play a role in the water penetration. However, the quenching efficiencies for rhodamine dye are different: I^- > Cu^{2+} >> acrylamide. Different efficiencies of quenchers for denatured GFP and rhodamine dye denote that direct interactions of quenchers and chromophore are not important for denatured GFP. The dynamic quenching effect of Cu^{2+} ion for native GFP is not significant for denatured GFP. Detailed mechanisms of assisting protonation by quenchers are not clearly understood.

Conclusion

We studied the fluorescence quenching of 2-5 GFP during denaturation by guanidine. The fluorescence intensity decays exponentially during denaturation but the fluorescence lifetime does not change. These results suggest the fast modification of protein structure followed by the protonation of chromophore. The kinetic data of fluorescence decay by guanidine denaturation shows that 2-5 GFP is more vulnerable to guanidine compared with wt GFP. The folding mutations introduced for thermal stability of 2-5 GFP seem to increase the structural sensitivity to the chemical denaturant. During denaturation, fluorescence quenchers do not affect the GFP fluorescence through direct interactions with the chromophore but assist the protonation of chromophore.

Acknowledgment. We acknowledge the support of this work by the Korea Research Foundation (2002-015-DP0212).

Refereces

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