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Effect of pH on Thermal- and Chemical-Induced Denaturation of GFP

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Abstract

Green fluorescent protein (GFP) is an unusually stable autofluorescent protein that is increasingly being exploited for many applications. In this report, we have used fluorescence spectroscopy to study the effect of pH on the denaturation of GFP with sodium dodecyl sulfate (SDS), urea, and heat. Surprisingly, SDS (up to 0.5%) did not have any significant effect on the fluorescence of GFP at pH 7.5 or 8.5 buffers; however, at pH 6.5, the protein lost all fluorescence within 1 min of incubation. Similarly, incubation of GFP with 8 *M* urea at 50°C resulted in time dependent denaturation of GFP, but only in pH 6.5 buffer. At higher pH values (pH 7.5 and pH 8.5), the GFP was quite stable in 8 *M* urea at 50°C, showing only a slight decrease in fluorescence. Heat denaturation of GFP was found to be pH dependent as well, with the denaturation being fastest at pH 6.5 as compared to pH 7.5 or pH 8.5. Like the denaturation studies, renaturation of heat-denatured GFP was most efficient at pH 8.5, followed by pH 7.5, and then pH 6.5. These results suggests that GFP undergoes a structural/stability shift between pH 6.5 and pH 7.5, with the GFP structure at pH 6.5 being very sensitive to denaturation by SDS, urea, and heat.

Index Entries: *E. coli*; GFP; SDS; urea; thermal denaturation; renaturation.

Introduction

The green fluorescent protein (GFP) is an autofluorescent protein that was first identified and isolated from the jellyfish *Aequorea Victoria.* GFP is a compact protein of 238 amino acids consisting of a fluorophore composed of three posttranslationally modified amino acids (–Ser^{65}–Tyr^{66}–Gly^{67}). Owing to its autofluorescence both in vitro and in vivo, as well as its remarkable stability, it is widely used for numerous cell biology and molecular biology applications *(1–5)*. Mutant forms of this protein that

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allow efficient in vivo folding, high levels of expression in *Escherichia coli*, and increased and shifted fluorescence are increasingly used as fusion proteins for protein engineering, expression studies, as well as biotechnology applications *(6–8)*. For example, Waldo et al. have reported on a technique to screen for properly folded recombinant proteins in *E. coli*, using a GFP fusion tag *(9)*. Recently, GFP variants have been used in (nondestructive) fluorescence resonance energy transfer assays to study protein–protein interactions in living cells *(10)*.

One of the interesting properties of GFP is its unusual stability to heat, pH, proteases, and denaturants, which is probably because of the tight and compact "β-can" structure of the GFP molecule *(11,12)*. GFP has been shown to be resistant to denaturation by 8 *M* urea, 6 *M* guanidine hydrochloride, and even 1% sodium dodecyl sulfate (SDS) *(13,14)*. Another interesting feature of GFP is that its fluorescence has been found to be pH dependent *(15)*. Although, a great deal of research has been published on the stability of GFP in the presence of various denaturants, the combined effect of pH on these denaturants has not been carried out.

In this study, we were interested in studying the stability/denaturation of GFP by various kinds of denaturants, and to examine the combined effect of pH on GFP denaturation. As expected, we found that 0.5% SDS did not have any significant effect on the fluorescence of GFP at pH 7.5 or 8.5 buffers; however, at pH 6.5, the protein surprisingly lost all fluorescence within 1 min of incubation. Similarly, incubation of GFP with 8 *M* urea at 50°C resulted in time-dependent denaturation of GFP, but only when the pH was 6.5. As expected, at higher pH values (pH 7.5 and pH 8.5), the GFP was quite stable in 8 *M* urea at 50°C. Heat denaturation study of GFP also showed a pH dependency, with the denaturation being fastest at pH 6.5 as compared to pH 7.5 or pH 8.5. Furthermore, like the denaturation studies, renaturation of heat-denatured GFP was found to be most efficient at pH 8.5, followed by pH 7.5, and then pH 6.5. These results strongly suggests that GFP undergoes a structural/stability shift between pH 6.5 and pH 7.5, such that the GFP at pH 6.5 assumes a structure that is very sensitive to denaturation by SDS, urea, and heat.

Materials and Methods

Cloning of GFP

Plasmid pQBI T7-GFP (Qbiogene) was used as a template to PCR amplify the *GFP* gene. The *GFP* gene had the following mutations to allow for enhanced fluorescence and solubility in *E. coli*: Phe64 → Leu, Ser65 → Cys, Ile167 → Thr. Before the PCR amplification, the internal *NdeI* in the GFP gene (in pQBI T7-GFP) was eliminated using site-directed mutagenesis (^{229}CA**T**ATG^{234} → ^{229}CA**C**ATG^{234}) using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). For PCR amplification of GFP, the primers were designed such that the resultant PCR product had an *NdeI* restriction site at the 5' end, and a *BamHI* site at the 3' end. This PCR product

was digested with *NdeI* and *BamHI* after which it was ligated into pET-N-AT *(16)* vector which had been digested with *NdeI* and *BamHI* as well. DNA sequencing was used to confirm the sequence of resultant GFP-construct, which had an N-terminal His_6 tag.

Purification of GFP

The His_6-tagged GFP was purified as described previously *(16,17)*. Briefly, bacterial pellet was lysed by two cycles of freeze–thaw in 50 m*M* Tris, pH 8.0, and containing 0.1 mg/mL lysozyme. The lysates were cleared by centrifugation (30 min at 25,000*g*) and applied in a batch-mode to a Ni^{2+}-NTA resin (Sigma). After allowing 30 min (at room temperature) for binding, the resin/lysate mixture was briefly centrifuged, washed extensively with 50 m*M* Tris buffer containing 500 m*M* NaCl, and then with successive washes of the same buffer containing 5 and 10 m*M* imidazole. Bound GFP was eluted with 50 m*M* Tris, pH 8.0 containing 250 m*M* imidazole, and dialyzed into 50 m*M* Tris, pH 7.5 buffer.

Fluorescence Analysis

Fluorescence spectra of GFP were determined using the Cary Eclipse Fluorescence Spectrophotometer using a quartz fluorescence cell in 3 mL of 50 m*M* Tris buffers. For the chemical denaturation studies, GFP was diluted in the Tris buffer with the specified pH and then SDS or urea was then added to this solution. For the heat denaturation studies, the GFP solution was incubated in a waterbath at the specified temperature, and fluorescence spectra were recorded at various time intervals.

Results and Discussion

Figure 1 shows the excitation and emission spectra of the recombinantly expressed and purified GFP. Although the protein had an N-terminal His_6-tag, it had the same fluorescence characteristics as the wild-type, untagged protein.

Because the fluorescence of GFP is linked to its properly folded structure, it is therefore possible to use the fluorescence of GFP as an indication of its properly folded structure *(9)*. Hence, many groups have used GFP fluorescence to track the stability of GFP to various denaturants and have shown that GFP is extremely stable to many proteases, heat treatment, 8 *M* urea, and SDS *(11–14)*. In fact, the finding that GFP does not denature in SDS and retains its fluorescence has been used to develop a novel GFP-display SDS-polyacrylamide gel electrophoresis (PAGE) technique *(14)*. When we tested SDS to denature GFP, we also found that GFP retained its fluorescence in the presence 0.5% SDS, but only when the protein was in pH 8.5 or pH 7.5 buffer. Remarkably, at pH 6.5, the GFP lost all fluorescence in a time-dependent fashion in 1 min (Fig. 2). It is well known that fluorescence of GFP is pH dependent, a property which has been cleverly exploited to use GFP as a noninvasive intracellular pH indicator *(15)*. As expected,

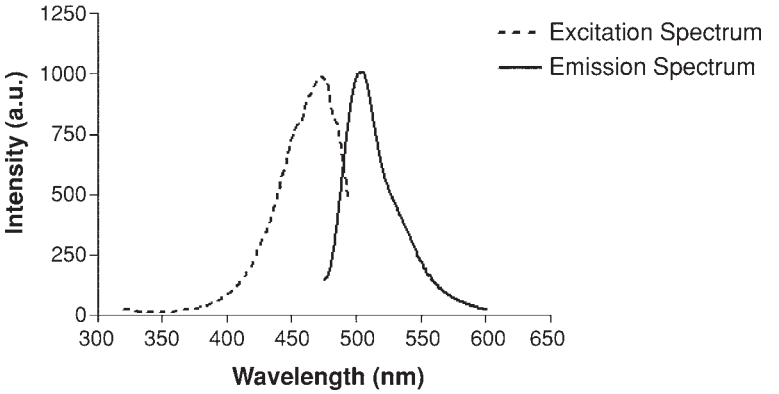

Fig. 1. Excitation and emission spectra of His_6-tagged-GFP. The excitation spectrum was obtained with the emission set at 500 nm and the emission spectrum was obtained with the excitation set at 474 nm. The spectra were recorded by using Ni^{2+}-NTA-purified GFP in 3 mL 50 m*M* Tris buffer pH 8.5.

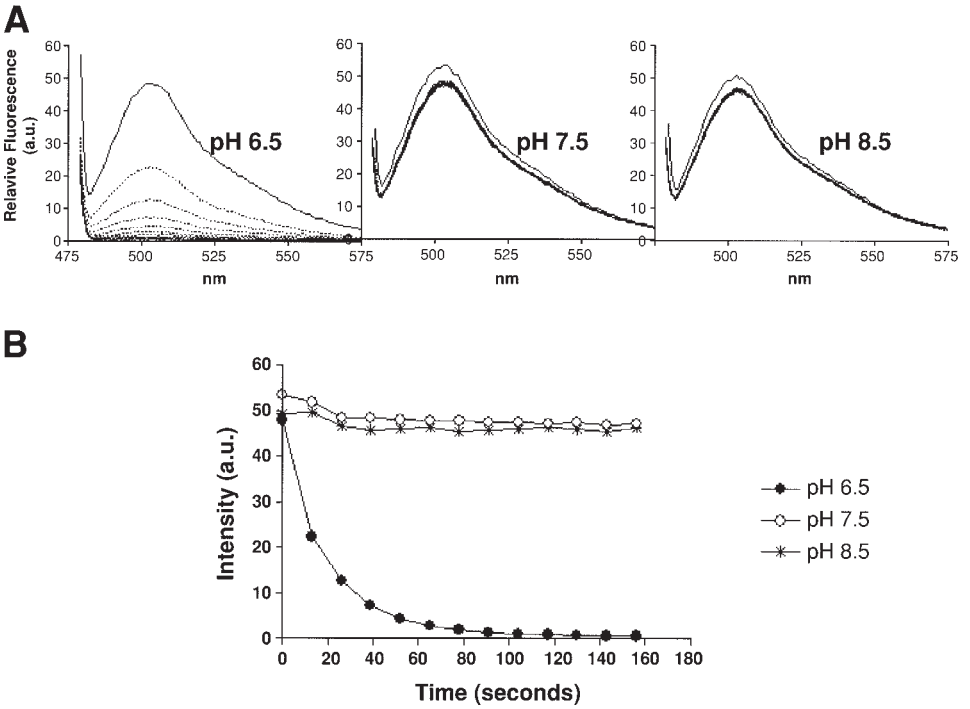

Fig. 2. **(A)** Denaturation of GFP by SDS in various pH buffers. SDS (0.5% final concentration) was added to 100 n*M* GFP in various pH buffers and the fluorescence scans were collected at 12-s intervals. Solid line is the fluorescence scan with out any SDS. **(B)** Summary graph of GFP fluorescence (506 nm) vs time in the presence of SDS in different pH values.

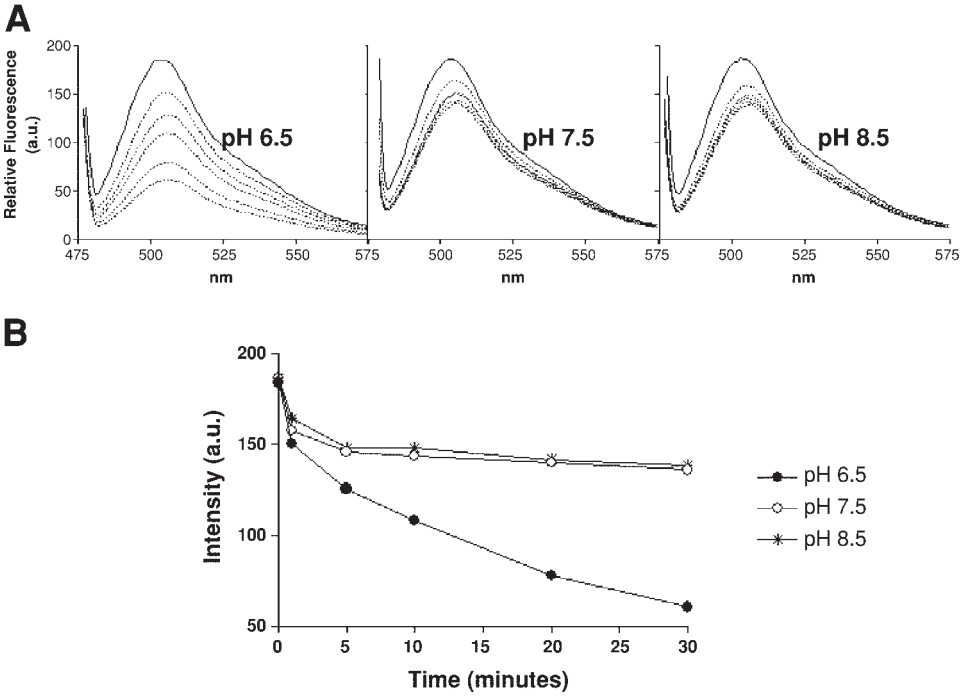

Fig. 3. **(A)** Denaturation of GFP by urea at 50°C in various pH buffers. Urea (6 *M* final concentration) was added to 300 n*M* GFP in various pH buffers at 50°C and the fluorescence scans were collected at 1, 5, 10, 20, and 30 min intervals. Solid line is the fluorescence scan with out any urea. **(B)** Summary graph of GFP fluorescence (506 nm) vs time in the presence of urea in different pH values.

in the absence of any SDS, the fluorescence of GFP in pH 6.5 buffer was slightly less than that observed for pH 7.5. However, as soon as SDS (as little as 0.05%) was added to the pH 6.5 buffer, a time-dependent loss of GFP fluorescence is observed (Fig. 2).

A similar effect of pH on GFP denaturation was observed when we tested the effect of 8 *M* urea at 50°C on GFP fluorescence. GFP was found to be quite stable to 8 *M* urea at 50°C in pH 7.5 and pH 8.5 buffers; however, like SDS, 6 *M* urea caused a time-dependent loss of GFP fluorescence in pH 6.5 buffer at 50°C. In pH 6.5 buffer containing 6 *M* urea, GFP lost more than 75% of its fluorescence in 30 min (Fig. 3). Again, this was unexpected and has not been reported earlier. A similar, but much slower, effect was observed when the experiment was carried out at 25°C (data not shown).

We were interested in following this phenomenon further and tested the effect of heat as a denaturant on GFP fluorescence. When heated at 70°C, GFP in pH 7.5 and pH 8.5 buffers lost about half of its fluorescence in 5 min, and then showed no further decrease with additional incubation at 70°C (up to 25 additional minutes). However, at pH 6.5, GFP lost all of its fluorescence in a time-dependent manner in 30 min (Fig. 4). Around the time we were carrying out these studies, a report by Penna et al. was published that

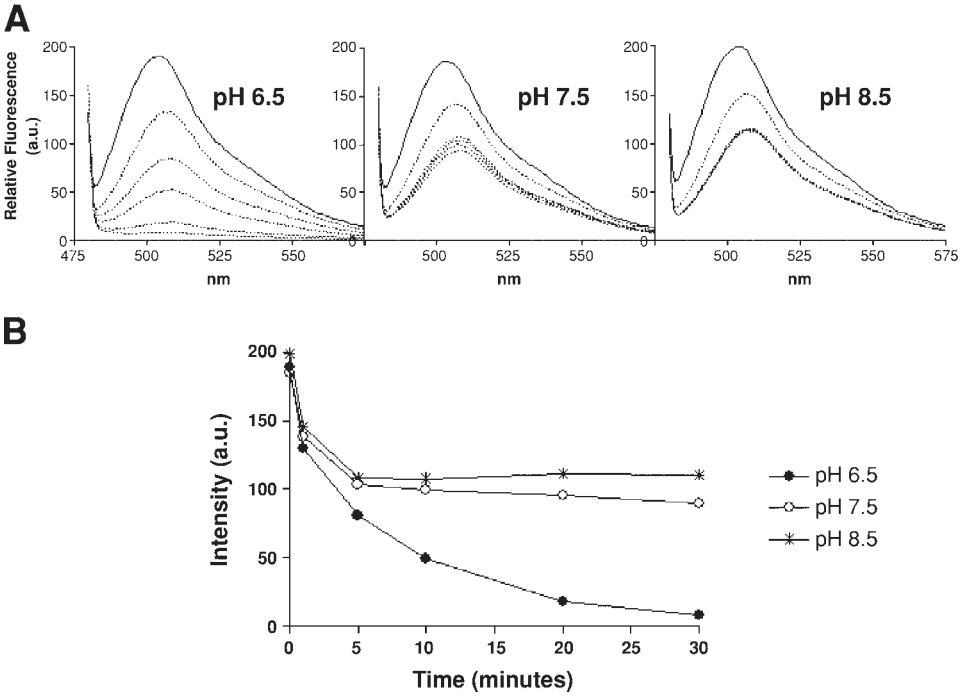

Fig. 4. **(A)** Denaturation of GFP by heating at 70°C in various pH buffers. 300 n*M* GFP in various pH buffers was heated and maintained at 70°C and the fluorescence scans were collected at 1, 5, 10, 20, and 30 min intervals. Solid line is the fluorescence scan of the unheated protein. **(B)** Summary graph of GFP fluorescence (506 nm) vs time when heated in different pH buffer solutions.

essentially showed the same pH dependence on heat denaturation of GFP *(12)*. In fact, these authors used three different buffers (including phosphate whose pKa is not as sensitive to pH changes as Tris) to test the stability of GFP at different temperature, and saw the same results with all three buffers.

Because heat denatured GFP has been reported to be renaturable *(18)*, we wanted to test if GFP renaturation was also pH dependent. As can be seen in Fig. 5, it appears that, like denaturation, renaturation of thermally denatured GFP is also pH dependent, with the renaturation being most rapid and complete at pH 8.5 followed by pH 7.5. No significant renaturation was observed at pH 6.5 buffer (Fig. 5).

Taken together, the data presented here sheds new light on the structural stability of GFP at different pH values. It is tempting to hypothesize that GFP undergoes a slight structural/conformational shift between pH 6.5 and pH 7.5. This shift is perhaps not significant enough to cause a big change in GFP fluorescence, but it is speculated that it renders the protein susceptible to denaturation by both chemical as well as thermal

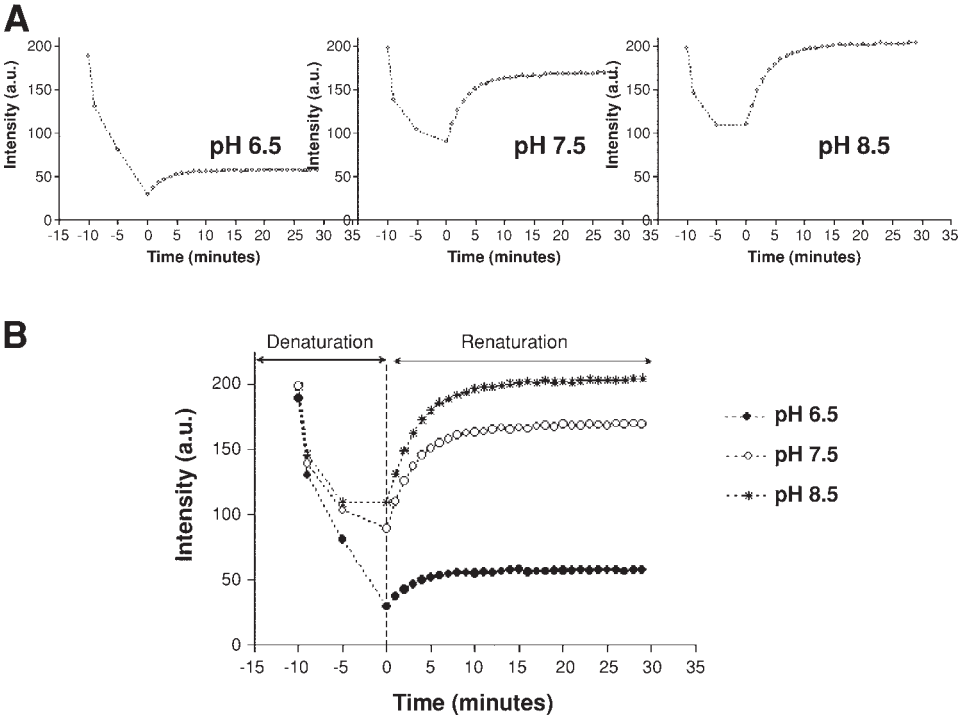

Fig. 5. **(A)** Renaturation of heat-denatured GFP in different pH buffers. Thermally denatured GFP (10 min at 70°C) in different pH buffers were allowed to cool at room temperature with fluorescence scans being collected every minute. **(B)** Summary graph of GFP fluorescence (506 nm) vs time, showing thermal denaturation and renaturation in different pH values.

means. More studies along these lines, including structural analyses, are needed to test this hypothesis.

Conclusion

In summary, we have shown that GFP stability and denaturation by various chaotropic agents, as well as heat, is highly dependent on the pH of the solution. GFP is very sensitive to denaturation by heat, SDS, or urea at pH value around 6.5. This was also seen in protein renaturation studies where heat-denatured GFP fails to renature effectively in pH 6.5 buffer. These observations indicate that GFP probably undergoes a structural shift between pH 6.5 and 7.5 such that, although at both pH values the protein retains its fluorescence properties, GFP becomes very sensitive to denaturation at the lower pH.

Acknowledgments

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