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ARTICLE *in* BIOCHEMISTRY · JULY 1997

Impact Factor: 3.02 · DOI: 10.1021/bi970281w · Source: PubMed

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Chromophore Formation in Green Fluorescent Protein

Brian G. Reid and Gregory C. Flynn*

Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403

Received February 6, 1997; Revised Manuscript Received April 14, 1997[⊗]

ABSTRACT: The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* forms an intrinsic chromophore through cyclization and oxidation of an internal tripeptide motif [Prasher, D. C., et al. (1992) *Gene 111*, 229−233; Cody, C. E., et al. (1993) *Biochemistry 32*, 1212−1218]. We monitored the formation of the chromophore *in vitro* using the S65T-GFP chromophore mutant. S65T-GFP recovered from inclusion bodies in *Escherichia coli* lacks the mature chromophore, suggesting that protein destined for inclusion bodies aggregated prior to productive folding. This material was used to follow the steps leading to chromophore formation. The process of chromophore formation in S65T-GFP was determined to be an ordered reaction consisting of three distinct kinetic steps. Protein folding occurs fairly slowly ($k_f = 2.44 \times 10^{-3}$ s^{-1}) and prior to any chromophore modification. Next, an intermediate step occurs that includes, but is not necessarily limited to, cyclization of the tripeptide chromophore motif ($k_c = 3.8 \times 10^{-3}$ s^{-1}). The final and slow step ($k_{ox} = 1.51 \times 10^{-4}$ s^{-1}) in chromophore formation involves oxidation of the cyclized chromophore. Since the chromophore forms *de novo* from purified denatured protein and is a first-order process, we conclude that GFP chromophore formation is an autocatalytic process.

The green fluorescent protein (GFP)[1] from the jellyfish *Aequorea victoria* is a soluble monomeric protein that displays visible fluorescent light at a peak wavelength of 508 nm when excited with ultraviolet light (Ward et al., 1980). Fluorescence is emitted from an intrinsic chromophore which arises from the covalent modification of three adjacent amino acids (Ser 65-Tyr 66-Gly 67, Figure 1) (Prasher et al., 1992; Cody et al., 1993). This chromophore is situated in the middle of a distorted α-helix that runs through the center of a β-barrel in the folded GFP structure (Ormo et al., 1996; Yang et al., 1996).

In recent years, the use of GFP as a reporter of gene expression and protein localization has increased dramatically [for a review, see Cubitt et al. (1995)]. It has been used successfully in a large and ever-growing number of species, including bacteria, fungi, plants, insects, and nematodes (Chalfie et al., 1994). Since active GFP can be observed when expressed in many divergent species, it likely requires no substrates or accessory proteins for chromophore formation. Moreover, it is this factor-independent fluorescence that makes GFP an ideal reporter protein in living cells. One potential limitation of GFP as a reporter for gene expression is its relatively slow rate of fluorescence acquisition *in vivo*, limiting its use to experiments exceeding the hour time scale. The slow rate of fluorescence acquisition is reported to be due to the oxidation step in chromophore formation (Figure 1), which has been estimated to occur with a time constant of 2−4 h *in vivo* (Heim et al., 1994).

Certain mutations in the chromophore of wild type GFP (wtGFP) have been observed to affect the rate of chromophore formation *in vivo* (Heim et al., 1995; Siemering et al., 1996) and to alter the spectral characteristics of the native fluorescent or mature protein (Heim et al., 1994). One mutant particularly useful to molecular biologists is the S65T mutant (S65T-GFP), in which a threonine is substituted for the serine at the N-terminal end of the chromophore (Heim et al., 1995). This mutation changes the spectral properties of the protein, most dramatically resolving the broad double excitation peaks at 396 and 475 nm found in wtGFP to a single one centered in the visible region. Additionally, S65T-GFP is not as labile to photobleaching as wtGFP (Cubitt et al., 1995), and is reported to gain fluorescence more rapidly *in vivo* (Heim et al., 1995). However, the fluorescence acquisition *in vivo* is still slow, on the same time scale as that of wtGFP.

Any hope of using GFP as a reporter of gene expression on shorter time scales will require GFP to acquire fluorescence more rapidly *in vivo*. To this end, it is important to have a detailed knowledge of the rates and molecular requirements of each step in folding and chromophore formation. In this paper, we present the first analysis of chromophore formation *in vitro*. Rates for each step in the production of mature chromophore were derived, and these results are discussed as they relate to mechanistic models for chromophore formation.

EXPERIMENTAL PROCEDURES

S65T-GFP Expression and Purification. The DNA coding region for a fusion protein of S65T-GFP and an N-terminal six-histidine tag was amplified from pRSETB-GFP (Heim et al., 1995) using the polymerase chain reaction (PCR) (Taq polymerase and PCR reagents from Promega). PCR primers (Genosys) contained an *XbaI* restriction site at the 5′ end of the S65T-GFP gene and a *Bsp*HI site at the 3′ end. The start primer was TCAATCTCTAGAGGAGATATACATATGCGGGGT. The end primer was TACCTCTCATGATTATTATTTGTATAGTTCATC.

* Corresponding author. Phone: 541-346-1534. Fax: 541-346-5891.

[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: GFP, green fluorescent protein; S65T-GFP, Ser → Thr chromophore mutant of GFP; drS65T-GFP, denatured and reduced S65T-GFP; ibS65T-GFP, S65T-GFP from inclusion body material; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

S0006-2960(97)00281-X CCC: $14.00

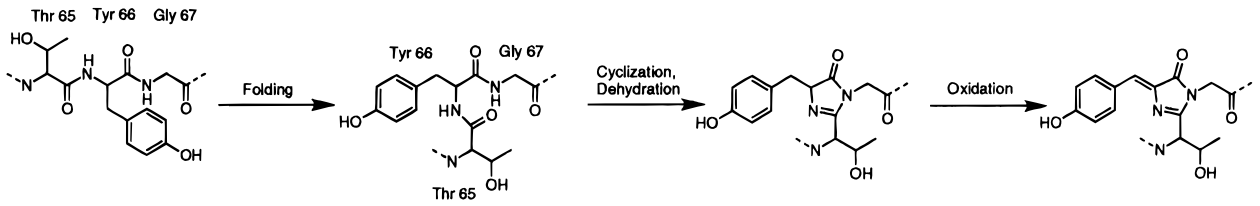

FIGURE 1: Model for chromophore formation in S65T-GFP as proposed by Heim et al. (1994). The chromophore tripeptide is represented in an extended conformation in the denatured state. Folding to the native state promotes chromophore cyclization, possibly by placing the carboxyl group of Thr 65 in close proximity to the amide of Gly 67.

The PCR product was cut with *Xba*I and *Bsp*HI and ligated into an expression vector (pQESL75) that had been linearized with *Xba*I and *Nco*I. pQEL75 is a modified version of pASK75 (Skerra, 1994). This plasmid was transformed into *Escherichia coli* strain JM109. Cells were grown at 37 °C in LB broth containing 50 μg/mL ampicillin to an OD_{600} of 0.5. Expression of S65T-GFP from the tetracycline promoter was induced by addition of anhydrotetracycline (250 μg/L, Acros).

For purification of soluble protein, S65T-GFP was expressed for 4 h at 30 °C. Cells were harvested by centrifugation at 3000*g* in a Beckmann GS-6R centrifuge at 4 °C and resuspended in lysis buffer (200 mM NaCl and 50 mM Tris at pH 8.0). Cells were lysed using a French press, and the extract was centrifuged at 27000*g* for 30 min in a Beckman JA-20 rotor. The resulting supernatant was loaded onto a 5 mL Ni^{2+}−NTA affinity column (Qiagen), and protein was purified according to the manufacturer's instructions. S65T-GFP peak fractions were determined by visual evaluation of green fluorescence, pooled, and dialyzed overnight against 100 mM NaCl and 50 mM Tris at pH 7.5.

For preparation of inclusion bodies, S65T-GFP was expressed in JM109 cells for 3 h at 37 °C. Cells were harvested and lysed, and the extract was centrifuged as above. The supernatant was discarded; the insoluble pellet was resuspended in renaturation buffer [35 mM KCl, 2 mM $MgCl_2$, 50 mM Tris (pH 7.5), and 1 mM DTT], and 20 μL aliquots were stored at −20 °C until they were needed for folding studies.

Folding and Renaturation of S65T-GFP. Denaturation solution consisted of 8 M urea and 1 mM DTT. Samples of mature S65T-GFP were heated to 95 °C in denaturation solution for 5 min and cooled to room temperature prior to renaturation. For denatured reduced S65T-GFP (drS65T-GFP), 5 mM dithionite was added to the denaturation solution in order to reduce the chromophore. S65T-GFP from inclusion bodies was solubilized in denaturation solution and centrifuged at 12000*g* for 10 min. The supernatant was used for folding reactions (ibS65T-GFP). The concentration of denatured S65T-GFP was typically around 2 mg/mL (67 μM) in denaturation solution. All renaturations and folding reactions were initiated upon 100-fold dilution from urea into renaturation buffer at room temperature

Protease Resistance of S65T-GFP. Aliquots were removed from renaturation or folding reaction mixtures at the indicated time points and incubated with trypsin (5 μg/mL, Sigma) for 5 min at 42 °C. Proteolysis was stopped by the addition of soybean trypsin inhibitor (10 μg/mL, Sigma). Samples were TCA precipitated, and then the TCA insoluble material was washed with acetone prior to resuspension in SDS−PAGE buffer. Samples were electrophoresed on 12.5% SDS−PAGE gels and Coomassie blue stained. Amounts of protease resistant S65T-GFP were determined through densitometry using the NIH image software package.

Spectroscopy. All measurements of fluorescence were done using the SLM-8000 fluorescence spectrophotometer at a 488 nm excitation wavelength, and emission was monitored at 512 nm. Absorbance spectra were determined on the Shimadzu UV2101-PC spectrophotometer.

Data Analysis. Kinetic constants were determined by two independent methods. Monophasic data were fitted to the equation $A_{(0)} = \ln[A_{(\mathrm{inf})} - A_{(t)}]$, where $A_{(\mathrm{inf})}$ is the final amount of fluorescence in a folding reaction and $A_{(t)}$ is the observed fluorescence at time *t*. The slope of the resulting fit was determined with Kaliedagraph (Abelbeck software). The constants were confirmed by fitting the raw data to a single-exponential equation (monophasic) or to a double-exponential equation (biphasic) with Mac Curve Fit (Kevin Raner software). The rate constant of cyclization (k_c) was derived through transit time analysis (Fersht, 1984) on the basis of the observable rate constants of inclusion body fluorescence acquisition (k_{ib}), the rate constant of oxidation (k_{ox}), and the folding rate (k_f).

RESULTS

The rate of GFP renaturation can be monitored directly in the fluorescence spectrophotometer upon dilution from denaturants such as 8 M urea (Bokman & Ward, 1981). When native fluorescent GFP, also referred to as mature GFP, is denatured, fluorescence is lost. Since the mature chromophore remains chemically intact in the denatured state (Ward et al., 1980), the loss of fluorescence upon denaturation is presumably due to quenching as the buried chromophore in the folded protein becomes exposed to an aqueous environment. The chromophore would not then begin emitting fluorescent light until the full tertiary structure of the protein forms around the chromophore, thus shielding it from quenching. Therefore, the rate of acquisition of fluorescence when mature denatured GFP is renatured parallels the rate of protein folding. Successful renaturation of GFP has previously been reported upon dilution from denaturants, including acid, base, 8 M urea, and 6 M guanidinium hydrochloride (Ward & Bokman, 1982; Weissman et al., 1996). Since high concentrations of urea or guanidinium alone are not sufficient to denature GFP, all the urea denaturation reaction mixtures in work were heated to 95 °C for 5 min and then brought back to room temperature prior to renaturation. Such treatment results in a complete loss of fluorescence.

A time course of fluorescence acquisition of mature S65T-GFP upon dilution from 8 M urea is shown in Figure 2. The rate of fluorescence acquisition proceeds through two distinct kinetic phases. Each phase follows first-order kinetics. The first-order rate constant for the initial phase is 2.45×10^{-2} s^{-1}, or a $t_{1/2}$ of 28 s, and the second phase has a first-order rate constant of 2.44×10^{-3} s^{-1}, or a $t_{1/2}$ of 284 s. Together, the overall rate of fluorescence acquisition for S65T-GFP is

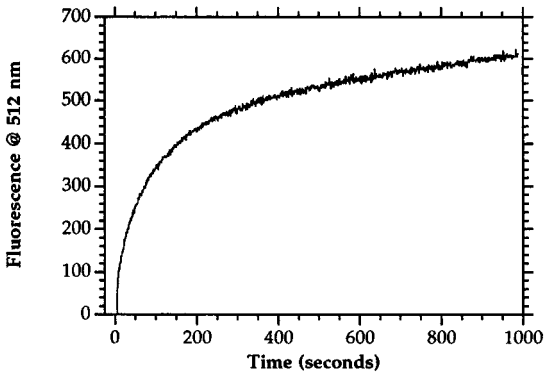

FIGURE 2: Fluorescence time course of the renaturation of S65T-GFP. Mature S65T-GFP in 8 M urea and 1 mM DTT was renatured by 100-fold dilution into renaturation buffer.

similar to that seen previously for wtGFP (Ward & Bokman, 1982).

Although our methods cannot distinguish between parallel and sequential kinetic pathways in the renaturation of S65T-GFP, a parallel pathway is more plausible. The most likely explanation for the existence of parallel pathways lies in the abundance of prolines in GFP. All but one of the nine proline residues in GFP are in the trans conformation in the native structure. We propose that the fast phase represents the folding of S65T-GFP molecules that already contain the proper distribution of proline conformers upon initiation of folding. The slow phase would then result from the slow isomerization of prolines that were in the improper conformation upon dilution from denaturant. Therefore, the existence of the two phases is likely the result of heterogeneity of the initial denatured states of urea-denatured S65T-GFP.

It was reported that a thiol-reducing agent is necessary for productive renaturation of GFP (Surpin & Ward, 1989), which probably prevents the incorrect formation of a disulfide bond. The two cysteine residues in GFP are quite far apart in native structure (Ormo et al., 1996; Yang et al., 1996), so any disulfide formation could hinder the acquisition of native structure. Therefore, 1 mM DTT was present in all denaturation solutions and renaturation buffers.

Renaturation efficiencies for S65T-GFP typically ranged from 50 to 70% as judged against a standard of mature protein. Similar inefficiency is evident when GFP folds *in vivo*, as GFP tends to form insoluble aggregates when overexpressed in *E. coli* (Heim et al., 1994). Insoluble aggregate, or inclusion body, formation is usually the result of improper protein folding in the cell (King et al., 1996). If the intramolecular reactions required for chromophore formation in GFP only occur after productive folding, and the material found in inclusion bodies had never properly folded in the cell, then the inclusion body GFP (ibGFP) chromophore tripeptide should remain unreacted. This protein could then serve as material for the study of the formation of chromophore *de novo*.

Inclusion bodies from *E. coli* expressing S65T-GFP were isolated and contained relatively pure S65T-GFP (>90% S65T-GFP by mass). In fact, S65T-GFP from inclusion bodies was of greater purity than protein prepared from soluble *E. coli* extracts by Ni^{2+}−NTA chromatography. Protein in inclusion bodies was dissolved in 8 M urea at room temperature and then was folded by dilution into renaturation buffer. Since this material likely never folded in the cell, we term this *de novo* folding. Urea-denatured

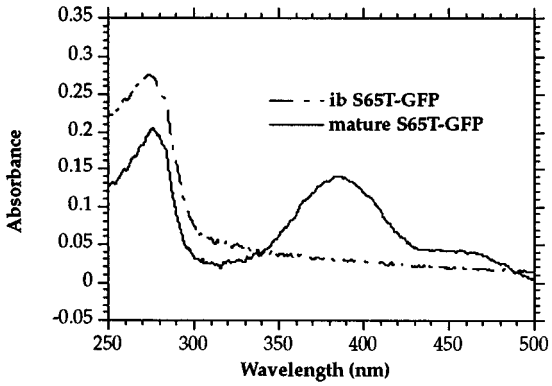

FIGURE 3: Absorbance spectra for denatured mature S65T-GFP and urea-solubilized ibS65T-GFP.

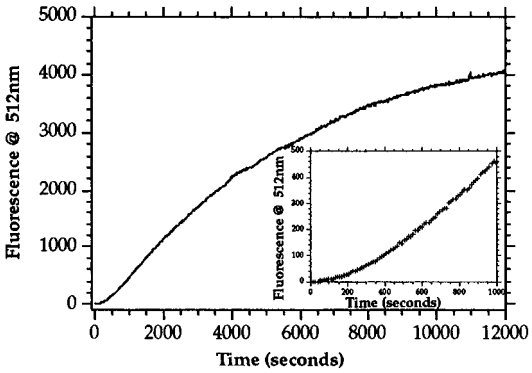

FIGURE 4: Fluorescence time course of *de novo* folding of S65T-GFP from inclusion bodies (ibS65T-GFP). Inclusion body material was solubilized in 8 M urea containing 1 mM DTT, and folding was initiated by 100-fold dilution into renaturation buffer. (Inset) The first 1000 s of the time course, showing the lag period.

inclusion bodies lacked the characteristic absorption spectrum of the GFP chromophore from 350 to 420 nm (Figure 3), indicating that the S65T-GFP aggregated *in vivo* prior to any chromophore formation. A similar absorption spectrum was recently reported for wtGFP isolated from inclusion bodies (Siemering et al., 1996). The *de novo* folding and subsequent chromophore formation proceeded with high efficiency, typically approaching 80% as measured against a standard of native S65T-GFP. Interestingly, fluorescence was acquired much more slowly for ibS65T-GFP folded *de novo* than for mature S65T-GFP refolded (Figure 4). Following an initial lag period, the acquisition of fluorescence followed first-order kinetics, with a rate constant (k_{ib}) of 1.37 $\times$ 10^{-4} s^{-1}, reflecting a $t_{1/2}$ of 84.3 min. This *in vitro* rate is similar to the rate of fluorescence acquisition seen *in vivo* (data not shown), consistent with the notion that GFP folding from inclusion bodies approximates the overall process of chromophore formation as it occurs in the cell. This also suggests that the rate of fluorescence acquisition from S65T-GFP folded *de novo* from inclusion bodies is limited by chromophore formation, not by protein folding.

It is conceivable that GFP could have multiple folding pathways depending on the nature of the starting material. For example, the material from inclusion bodies may require steps such as proline isomerizations to reach the native state, and such steps may not be necessary if mature S65T-GFP is used for denaturation−renaturation reactions. To determine whether the protein folding rate is affected by the starting material, we performed an analysis of the protease susceptibility of ibS65T-GFP over the course of a folding or renaturation reaction. Resistance to proteolysis was used as

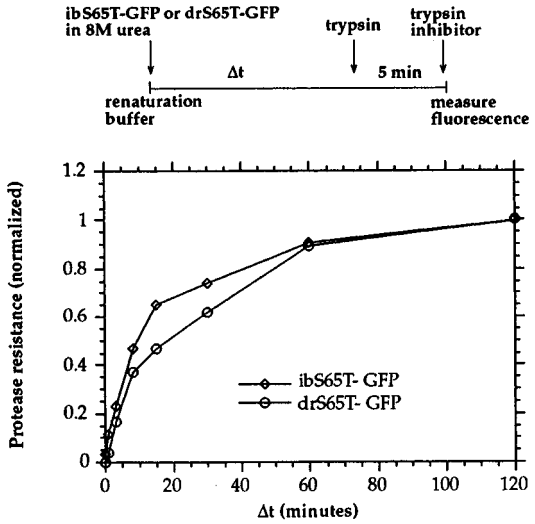

FIGURE 5: Protease susceptibility of S65T-GFP. Renaturation or *de novo* folding was initiated by 100-fold dilution from 8 M urea and 1 mM DTT. At the indicated time points (Δt), aliquots (roughly 6 μg of GFP) were removed from the folding reaction mixtures and incubated at 42 °C with 5 μg of trypsin for 5 min. Proteolysis was stopped by addition of soybean trypsin inhibitor (10 μg), and the amount of proteolytically resistant S65T-GFP was quantitated as described in Experimental Procedures. Time courses were normalized to the amount of proteolytically resistant protein at the 2 h time point of each folding reaction.

an indicator of native structure. Conditions in which non-native S65T-GFP was degraded while native S65T-GFP was resistant to proteolysis were determined experimentally. ibS65T-GFP acquired resistance to proteolysis with a half-time of around 10 min (Figure 5). This rate is on the same order as the directly observable rate of folding of mature S65T-GFP renatured from urea. These results indicate that the overall folding rates are independent of the starting material.

Chromophore formation has been proposed to proceed through a two-step process consisting of cyclization of the S-Y-G motif (T-Y-G in S65T-GFP) followed by oxidation (Heim et al., 1994). If the oxidation step is reversible, the rate of chromophore oxidation might be observed by reducing the chromophore with dithionite and then monitoring the reoxidation by reacquisition of fluorescence. The chromophore, as observed in the native structure of GFP, is buried within the core of the protein and is shielded from solvent (Ormo et al., 1996; Yang et al., 1996). In contrast to the observations of Inouye and Tsuji (1994), we found that the chromophore reduction required denaturation of the protein structure. Mature S65T-GFP was denatured in 8 M urea and reduced with 5 mM dithionite. Renaturation of the denatured reduced protein (drS65T-GFP) was initiated by dilution into buffer containing no dithionite. Since the final concentration of dithionite will be less than 50 μM in the refolding assay and the dissolved oxygen content is predicted to be greater than 250 μM (and in equilibrium with the atmosphere), residual dithionite would rapidly be lost upon initiation of refolding. The process of reoxidation of the cyclized chromophore can be initiated upon dilution from denaturant, concomitant with protein folding. The time course of fluorescence acquisition therefore represents the parallel reactions of renaturation and reoxidation (Figure 6). The observed rate of fluorescence acquisition was remarkably similar to that seen for *de novo* folding (Figure 4), with a rate constant (k_{ox}) of 1.51×10^{-4} s^{-1}, as compared to

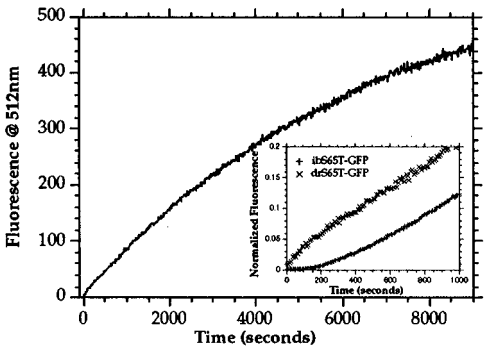

FIGURE 6: Fluorescence time course of the renaturation of drS65T-GFP. Mature fluorescent S65T-GFP was denatured by heating at 95 °C for 5 min in 8 M urea containing 1 mM DTT and 5 mM dithionite. Renaturation was initiated by 100-fold dilution into renaturation buffer. (Inset) Overlay of the first 1000 s of the normalized fluorescence time courses of ibS65T-GFP and drS65T-GFP.

1.37×10^{-4} s^{-1} for *de novo* folding. In this time course, reoxidation is the slow step in chromophore formation. drS65T-GFP was tested for protease susceptibility through a renaturation time course as before and displayed protease resistance kinetics similar to those of ibS65T-GFP (Figure 5). The fact that reoxidation and not a novel folding pathway are responsible for the slow rate of fluorescence acquisition in the renaturation of drS65T-GFP was shown as follows. Renaturation can be inhibited by diluting S65T-GFP in 6 M urea during the renaturation process. Native GFP is very stable in 6 M urea, but non-native GFP cannot fold to the native state under these conditions. Chromophore oxidation can still occur in a protein that had already folded and cyclized prior to dilution in 6 M urea, but the fraction of protein that had not yet folded upon dilution in 6 M urea will not become fluorescent. Renaturations of mature denatured S65T-GFP and of drS65T-GFP were initiated upon dilution from 8 M urea. At a number of time points during the renaturation reactions, aliquots were removed and diluted from renaturation buffer into 6 M urea. Samples were then incubated for 3 h in order to ensure that chromophore maturation had gone to completion prior to measurement of fluorescence. In other words, this protein folding assay is independent of the original oxidative state of the chromophore. Figure 7 shows time courses of renaturation of mature S65T-GFP and drS65T-GFP. The renaturation rates are remarkably similar, indicating that the reduction of the chromophore in the denatured state has little or no effect on the folding pathway of S65T-GFP. Therefore, the slow rate of fluorescence acquisition in the renaturation of drS65T-GFP is due solely to the slow reoxidation of the chromophore.

The rate of the intermediate step in chromophore formation (k_c) was determined through transit time analysis (Fersht, 1984). The method of transit time analysis is valid for determination of rates in sequential reactions. When ibS65T-GFP is folded *de novo*, the pathway leading to mature fluorescent protein is indeed an ordered sequential reaction composed of three steps: folding, cyclization, and oxidation. Therefore, the reciprocal rate constant for ibS65T-GFP fluorescence acquisition ($1/k_{ib}$) is equal to the sum of the reciprocal rate constants for each step in chromophore formation ($1/k_f + 1/k_c + 1/k_{ox}$). All of these rates have been determined independently in this paper except for k_c. We should note that only the refolding of denatured mature S65T-GFP (k_{fmat}=2.44 $\times$ 10^{-3} s^{-1}) and that of drS65T-GFP

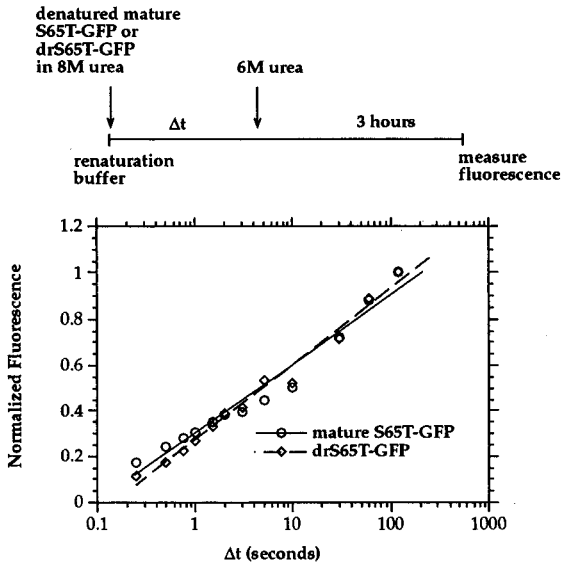

FIGURE 7: Time courses of renaturation of mature S65T-GFP and drS65T-GFP. Renaturations of denatured mature S65T-GFP and of drS65T-GFP were initiated by 100-fold dilution into renaturation buffer. At the indicated time points ($\triangle t$), aliquots were removed and further renaturation was inhibited by dilution in 6 M urea. Samples were incubated for 3 h in 6 M urea prior to measurement of fluorescence.

($k_{fdr} = 2.7 \times 10^{-3}$ s^{-1}) were measured accurately. However, protease sensitivity experiments (Figure 5) suggest that the folding rate for the ibS65T-GFP (k_{fib}) is similar to the refolding rate for denatured mature GFP. Therefore, it seems that the protein folding rates are similar if not identical for GFP regardless of the chromophore state. In the transit time analysis, we used the folding rate constant k_{fmat} instead of the less accurate k_{fib}. Solving the equation above for k_c yields a first-order rate constant of 3.8×10^{-3} s^{-1}, reflecting a $t_{1/2}$ of 180 s. The kinetic phase described by k_c would likely include cyclization of the chromophore prior to oxidation and contributes little to the overall rate of chromophore formation.

DISCUSSION

Since the S65T-GFP obtained from inclusion bodies had never reached its native conformation, which is necessary for chromophore formation, the Thr-Tyr-Gly tripeptide was unmodified. Upon dilution from urea of solubilized ibS65T-GFP, green fluorescence was slowly obtained, demonstrating conclusively that chromophore formation is an intramolecular reaction independent of added cofactors.

We have described three kinetic steps leading to the formation of active fluorescent S65T-GFP (Figure 8). The first step is folding of the protein into the native conformation. The rate of this step was monitored directly in the renaturation of mature S65T-GFP (step 1 in Figure 8). The fact that chromophore maturation is a sequential process is shown by the lag seen during *de novo* folding of ibS65T-GFP (Figure 4, inset). This lag occurs only when GFP is folded *de novo* and not when drS65T-GFP is renatured from urea (Figure 6, inset). drS65T-GFP exhibits no lag in a time course of fluorescence acquisition even though protein folding must occur prior to fluorescence, because the renaturation and reoxidation of drS65T-GFP are not sequential reactions. In this case, renaturation and reoxidation can occur in parallel since the cyclized chromophore is subject to oxidation whether or not the protein is native. Since oxidation is the slower step of the two, the observed overall rate of fluorescence acquisition of drS65T-GFP is a direct measurement of the rate of oxidation (k_{ox}) of the cyclized chromophore.

An alternative explanation for the lag seen in *de novo* folding is that it could be due to proline isomerizations that occur only when inclusion body material is used. The observation that both S65T-GFP from inclusion bodies and drS65T-GFP acquire resistance to proteolysis with similar kinetics argues against a role for proline isomerization, however. Since such isomerization would probably have a major effect on the rate of acquisition of native structure, we would expect to see a longer period of protease susceptibility in S65T-GFP folded *de novo* if the wrong proline conformers were present in high abundance.

The folding process is subject to off-pathway reactions both *in vitro* and *in vivo*. For renaturation *in vitro*, off-pathway reactions likely account for the relatively low efficiency of renaturation. *In vivo*, nonproductive folding often results in inclusion body formation in *E. coli*. J. King's laboratory has concluded that inclusion bodies form through aggregation of folding intermediates in the production of phage P22 tailspike and coat proteins (King et al., 1996). Temperature sensitive folding mutants of both the tailspike and coat proteins form inclusion bodies during infection of

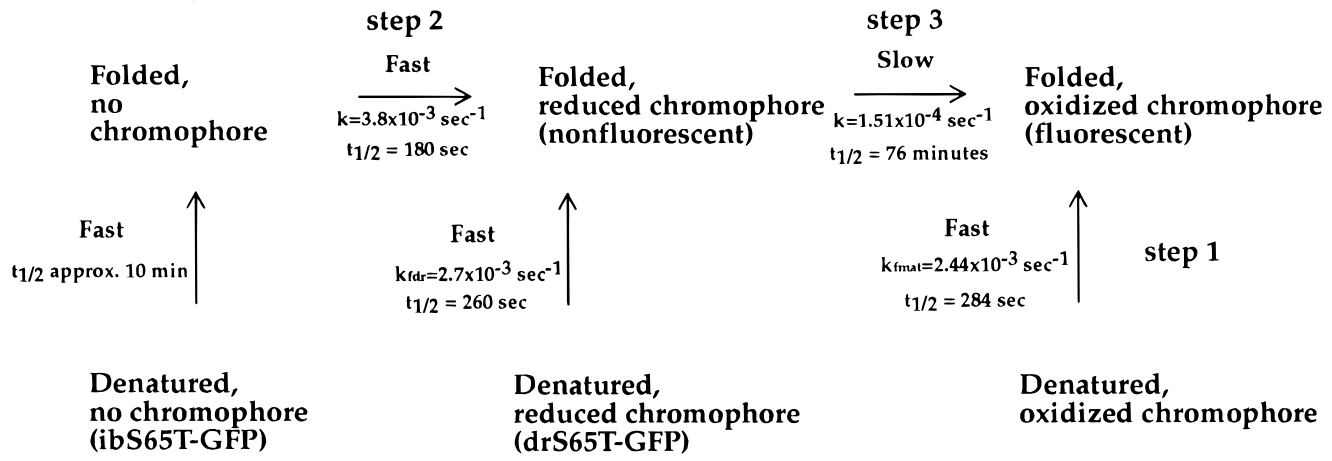

FIGURE 8: Kinetic pathway of chromophore formation in S65T-GFP. Individual steps are described in the Discussion. The only directly observable folding rate is that of the mature protein (k_{fmat}). The fact that the denatured proteins fold with the same rate is supported by the kinetics of acquisition of resistance to proteolysis (ibS65T-GFP, Figure 5) and by inhibition of renaturation followed by chromophore maturation (drS65T-GFP, Figure 7).

Salmonella typhimurium grown at the nonpermissive temperature. Using arguments based on the stability of the native P22 tailspike protein, they reasoned that protein destined for inclusion bodies never forms the native state. This argument was supported through the generation of mutations in the tailspike protein that destabilize assembly intermediates without affecting the stability of the native tailspike trimer.

An even stronger argument can be made that inclusion bodies containing GFP result from folding intermediates, and not monomeric protein which had previously folded. Like P22 tailspike trimer, native GFP is very stable and would resist denaturation *in vivo*. Through chromophore formation, GFP leaves a permanent indicator of once having obtained the native form. However, no chromophore was detected in the ibS65T-GFP. Furthermore, the kinetics of fluorescence acquisition from ibS65T-GFP also argue that the chromophore had not yet formed, most likely because the protein never properly folded. Two hundred seconds into a fluorescence acquisition time course, ibS65T-GFP has only generated 0.4% of its eventual 80% fluorescence yield, whereas mature S65T-GFP refolding has nearly completed fluorescence acquisition.

Once folding is complete, the tripeptide chromophore motif is buried in the central helix of GFP (Ormo et al., 1996; Yang et al., 1996). The three-dimensional conformation of the protein presumably promotes the next step in GFP maturation, a covalent rearrangement that results in cyclization of the chromophore tripeptide (step 2 in Figure 8). The rate constant derived herein for the intermediate step in chromophore maturation (k_c) should be regarded as an estimate and probably represents the lower limit to the rate of cyclization. The temporal resolution of our methods does not allow for a more precise derivation of the rate of tripeptide cyclization.

It is not known what possible constraints the full native structure of GFP places on the tripeptide in order to promote its cyclization. Cyclization, however, is presumably a prerequisite for the third step in chromophore maturation. The third and slowest phase in chromophore maturation is oxidation of the cyclized chromophore (step 3 in Figure 8), which is shown conclusively by comparison of the rates of fluorescence acquisition from renaturations of S65T-GFP and of drS65T-GFP. The only difference between these reactions is the oxidative potential of the denaturant solution. S65T-GFP renatured from these two different initial states exhibits two very different rates of fluorescence acquisition, which can only be due to slow reoxidation of the reduced form of S65T-GFP. At the level of an individual molecule of GFP, the process of going from denatured protein to mature fluorescent protein is an ordered reaction comprised of three distinct steps. The native structure must be present in order for efficient cyclization to occur, and the cyclized chromophore must, in turn, be present before oxidation can occur.

Heim et al. (1994) have proposed a mechanism of chromophore formation in wtGFP involving nucleophilic attack of the amino group of Gly 67 on the carbonyl carbon of Ser 65. This is followed by dehydration of the cyclized intermediate which yields an imidazolin-5-one species. The presence of a threonine at position 65 in place of the wild type serine most likely has little effect on the cyclization reaction, even though S65T-GFP is reported to have a faster rate of chromophore formation *in vivo*. Since oxidation is the slow step in chromophore formation, the S65T mutation probably facilitates more rapid fluorescence acquisition than in wtGFP through an increase in the rate of chromophore oxidation. The mechanism by which the rate of oxidation increases is unknown.

Specific mechanistic details, such as the chemical structure and lifetimes of any intermediates in the cyclization reaction, are not addressed in this work. Structural studies on anaerobically expressed GFP should help to test the proposed structural model of the cyclized chromophore prior to oxidation. Further biophysical studies may also confirm the presence of any early intermediates in the cyclization reaction.

The methods used in this paper for the derivation of rates for each step in chromophore formation in S65T-GFP can also be used on any other mutant form of GFP, and, of course, on wtGFP. It will be interesting to see how other chromophore mutations affect the formation of active chromophore. Mutations of Tyr 66 are associated with dramatic spectral shifts in the mature protein (Heim et al., 1994), and now these and other mutations can be analyzed for changes in the pathway of chromophore formation.

ACKNOWLEDGMENT

We thank R. Tsien for the S65T-GFP plasmid, M. Ormo and F. Dahlquist for advice, and J. Hutchison, B. Branchaud, S. J. Remington, P. von Hippel, and R. Wachter for critical evaluation of the manuscript.

REFERENCES

Bokman, S. H., & Ward, W. W. (1981) *Biochem. Biophys. Res. Commun. 101*, 1372−1380.
Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994) *Science 263*, 802−805.
Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., & Ward, W. W. (1993) *Biochemistry 32*, 1212−1218.
Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., & Tsien, R. Y. (1995) *Trends Biochem. Sci. 20*, 441−488.
Fersht, A. (1984) in *Enzyme Structure and Mechanism*, pp 118−119, W. H. Freeman and Company, New York.
Heim, R., Prasher, D. C., & Tsien, R. Y. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12501−12504.
Heim, R., Cubitt, A. B., & Tsien, R. Y. (1995) *Nature 373*, 663−664.
Inouye, S., & Tsuji, F. I. (1994) *FEBS Lett. 351*, 211−214.
King, J., Haase-Pettingell, C., Robinson, A. S., Speed, M., & Mitraki, A. (1996) *FASEB J. 10*, 57−66.
Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., & Remington, S. J. (1996) *Science 273*, 1392−1395.
Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., & Cormier, M. J. (1992) *Gene 111*, 229−233.
Siemering, K. R., Golbik, R., Sever, R., & Haseloff, J. (1996) *Curr. Biol. 6*, 1653−1663.
Skerra, A. (1994) *Gene 151*, 131−135.
Surpin, M. A., & Ward, W. W. (1989) *Photochem. Photobiol. 49*, 62S.
Ward, W. W., & Bokman, S. H. (1982) *Biochemistry 21*, 4535−4540.
Ward, W. W., Cody, C. W., Hart, R. C., & Cormier, M. J. (1980) *Photochem. Photobiol. 31*, 611−615.
Weissman, J. S., Rye, H. S., Fenton, W. A., Beechem, J. M., & Horwich, A. L. (1996) *Cell 84*, 481−490.
Yang, F., Moss, L. G., & Phillips, G. N. (1996) *Nat. Biotechn. 14*, 1246−1251.

BI970281W