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Reversible Denaturation of *Aequorea* Green-Fluorescent Protein: Physical Separation and Characterization of the Renatured Protein†

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ABSTRACT: The green-fluorescent protein (GFP) that functions as a bioluminescence energy transfer acceptor in the jellyfish *Aequorea* has been renatured with up to 90% yield following acid, base, or guanidine denaturation. Renaturation, following pH neutralization or simple dilution of guanidine, proceeds with a half-recovery time of less than 5 min as measured by the return of visible fluorescence. Residual unrenatured protein has been quantitatively removed by chromatography on Sephadex G-75. The chromatographed, renatured GFP has corrected fluorescence excitation and emission spectra identical with those of the native protein at pH 7.0 (excitation λ_{max} = 398 nm; emission λ_{max} = 508 nm) and also at pH 12.2 (excitation λ_{max} = 476 nm; emission λ_{max} = 505 nm). With its peak position red-shifted 78 nm at pH 12.2, the *Aequorea* GFP excitation spectrum more closely resembles the excitation spectra of *Renilla* (sea pansy) and *Phialidium* (hydromedusan) GFPs at neutral pH. Visible absorption spectra of the native and renatured *Aequorea* green-fluorescent proteins at pH 7.0 are also identical, suggesting that the chromophore binding site has returned to its native state. Small differences in far-UV absorption and circular dichroism spectra, however, indicate that the renatured protein has not fully regained its native secondary structure.

The green-fluorescent proteins (GFP)[1] are a small class of unusual chromoproteins found only among certain bioluminescent coelenterates (Morise et al., 1974; Morin, 1974; Cormier et al., 1974; Prendergast & Mann, 1978; Ward & Cormier, 1979; Ward, 1979, 1981). These accessory proteins function as ultimate bioluminescence emitters, accepting energy from enzyme-bound, excited-state oxyluciferin (Ward & Cormier, 1976, 1978). Depending upon the species, the mechanism of energy transfer involves either radiationless transfer or trivial transfer (Ward, 1979). Two coelenterate species have been intensively studied as representatives of these two types of bioluminescence energy transfer systems. In the sea pansy, *Renilla reniformis* (and related anthozoans), energy transfer is clearly radiationless and a biochemical mechanism involving transient protein–protein interaction between luciferase and the *Renilla* GFP has been demonstrated in vitro (Ward & Cormier, 1976, 1978; Hart et al., 1979). In the jellyfish, *Aequorea aequorea*,[2] in vivo energy transfer appears to be a radiative process (Johnson et al., 1962; Ward, 1979, 1981) mediated by close physical packing of *Aequorea* GFP with the calcium-activated photoprotein aequorin (Shimomura et al., 1962, 1963) into specialized photocytes that line the perimeter of the jellyfish umbrella (Davenport & Nicol, 1955).

Green-fluorescent proteins from *Aequorea* and *Renilla* (abbreviated A-GFP and R-GFP, respectively) have been physically characterized as acidic, globular proteins with similar amino acid compositions and monomer molecular weights of 27 000–30 000 (Morise et al., 1974; Ward, 1979). *Aequorea* GFP exists as the monomer in dilute solution while the native form of R-GFP is a dimer of identical subunits stabilized by strong, noncovalent interactions. Their fluorescence emission spectra (λ_{max} = 508–509 nm), quantum efficiencies (78–80%), and fluorescence polarization values (0.4–0.5) are each very similar (Wampler et al., 1971; Cormier et al., 1973; Morise et al., 1974; Ward & Cormier, 1979; Prendergast, 1980), suggesting that both proteins contain the same chromophore. However, A-GFP has absorption and excitation maxima near 395 nm (Morise et al., 1974), and R-GFP has a major peak at 498 nm (Wampler et al., 1971; Ward & Cormier, 1979). Despite the large (103-nm) difference between absorption maxima, the fully denatured proteins (6 M guanidine hydrochloride, 90 °C) have identical absorption peaks in acid (383–384 nm) and base (447–448 nm) and a single isosbestic point in the visible region at 405 nm (Ward et al., 1980). It has been concluded that the spectral differences noted above for the native proteins are the result of unique noncovalent interactions between a common chromophore and differing apoprotein environments (Ward et al., 1980).

From papain digests of A-GFP, Shimomura has purified a chromopeptide with the same spectral characteristics as the

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[1] Abbreviations: GFP, green-fluorescent protein; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; CD, circular dichroism.

[2] The species of *Aequorea* common to the waters around Friday Harbor, WA, has been referred to both as *A. aequorea* and *A. forskalea*; see Johnson & Shimomura (1978) for a discussion of the nomenclature.

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guanidine-denatured protein (Shimomura, 1979). The A-GFP chromopeptide is proposed to be the blocked C-terminal tetrapeptide (Phe-Tyr-Glu-Val-$CONH_2$) cyclized between the carbonyl carbon of phenylalanine and the amino acid nitrogen of glutamic acid. The corresponding chromopeptide from R-GFP appears to contain a different complement of amino acids on the basis of its chromatographic behavior on HPLC (Cody & Ward, 1981).

Experimental Procedures

Reagents. Guanidine hydrochloride was ultrapure grade from Heico, Inc., Delaware Water Gap, PA. All other chemicals were reagent grade. Deionized, distilled water was used throughout.

Protein Purification. Specimens of the hydrozoan medusa, *Aequorea aequorea*, were hand collected at the University of Washington's Friday Harbor Laboratories, Friday Harbor, WA, during the month of Aug 1979. Dissection and preliminary biochemical processing were performed on site by the methods of Blinks et al. (1976, 1978). Crude extracts were returned to New Brunswick as frozen ammonium sulfate pellets and stored at −80 °C until further use. *Aequorea* GFP and the photoprotein aequorin were purified by the methods of Blinks et al. (1976, 1978) Johnson & Shimomura (1978), and Morise et al. (1974) as modified by A. F. Roth and W. W. Ward (unpublished experiments). All A-GFP samples used in these studies were purified to a final absorbance ratio (A_{395}:A_{280}) of 1.0 or greater as measured on the Cary 17D spectrophotometer at room temperature in dilute Tris–EDTA buffer at pH 7–8. An absorbance ratio of 1.0 has been used by others (Morise et al., 1974; Prendergast & Mann, 1978) as an indication of purity; however, we have recently achieved a ratio of 1.2 with a modified procedure (A. F. Roth and W. W. Ward, unpublished experiments).

Denaturation–Renaturation. Denaturation of A-GFP with 6 M Gdn·HCl at elevated temperature (2 min at 92 °C) or by acid treatment at room temperature (5 min at pH 2.0) was performed as previously described (Bokman & Ward, 1981a,b). Guanidine-denatured samples were routinely renatured by 48-h dialysis against 1 mM *N*-ethylmorpholine buffer (pH 8.0) at 4 °C. Following neutralization to pH 8.5, acid-denatured samples became renatured upon storage for 48 h at 4 °C (Bokman & Ward, 1981a,b). Alkaline denaturation was accomplished by treating the native protein with 0.1 N NaOH (pH 13) for 5 min at room temperature. The sample was neutralized with an equal volume of 0.1 N HCl, buffered at pH 8.2 with 50 mM Tris, and then stored for 48 h at 4 °C. The small amount of residual unrenatured protein was quantitatively removed by chromatography on a 0.7 × 15 cm column of superfine Sephadex G-75 (Pharmacia). Absorption spectra were taken of each fraction to verify purity.

Fluorescence Quantum Efficiency. The fluorescence quantum efficiency of renatured A-GFP (renatured from Gdn·HCl) was determined relative to native protein. A sample of the column-purified, renatured A-GFP was assayed spectrophotometrically and then diluted more than 100-fold with 10 mM Tris–EDTA buffer (pH 8.0) to an absorbance of 0.010 at 395 nm. A sample of native A-GFP was similarly diluted to the same final absorbance. Using narrow slits (0.5–2.0 mm), emission spectra were recorded of both samples at room temperature on the Aminco-Bowman spectrofluorometer with the excitation monochromator set at 395 nm.

Instrumentation. Routine fluorescence assays were performed at room temperature with a Turner 111 filter fluorometer equipped with a No. 110-853 blue excitation filter. On the emission side, two filters were used together—a Ditric 514-nm three-cavity interference filter and a Corning 3-70 color glass filter. This arrangement produced a full-scale meter deflection, on the 1X (pinhole) slit setting, with 6.5 μg/mL A-GFP or 1.8 μg/mL R-GFP while virtually eliminating light scattering interference with even the most turbid samples. The fluorometer was calibrated daily with a standard solution of fluorescein in 0.1 N NaOH that was otherwise stored in the dark at 4 °C.

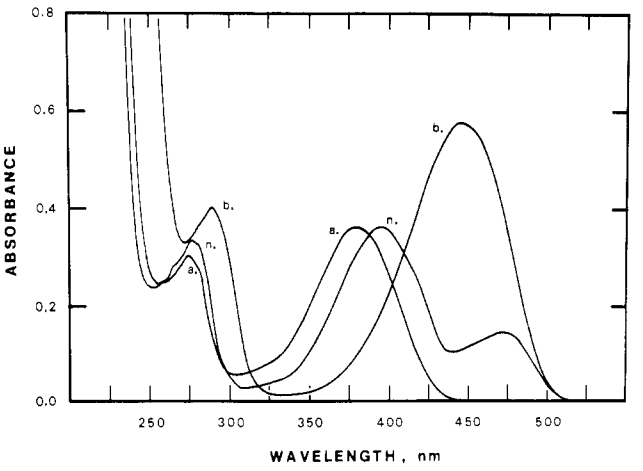

FIGURE 1: Absorption spectra of native and denatured *Aequorea* green-fluorescent protein. Each spectrum was run on the Cary 17D at room temperature in 1 mM sodium phosphate buffer at a protein concentration of 13.0 μM. The labels n, a, and b represent native (pH 7.2), acid-denatured (pH 2.4), and base-denatured (pH 12.2) A-GFP, respectively. The base-denatured sample was warmed to 50 °C for 1 min at pH 12.2 to promote denaturation.

Absorption spectra were measured at room temperature on a Cary 17D recording spectrophotometer (bandwidth ≤0.2 nm above 350 nm). Single wavelength determinations were made on the Cary 17D or a Gilford-updated Beckman DU. An Aminco-Bowman spectrofluorometer was used for routine excitation and emission spectra. Fully corrected spectra were obtained at room temperature on an SLM 4800 spectrofluorometer. Emission spectra were corrected by using correction factors supplied by the manufacturer. Excitation correction factors were generated with a Rhodamine B (0.3% in ethylene glycol) quantum counter by using front surface optics. Slits were adjusted to provide bandwidths of 4.0 nm. Circular dichroism measurements were made on a Cary 61 CD instrument with a 2-mm cell. Temperature was regulated with a programmable circulating water bath and monitored with a chemically inert thermistor immersed directly in the sample. Measurements of pH were made with a Radiometer pH meter equipped with a No. 9100 microelectrode (Broadley-James, Santa Ana, CA) with extended sensitivity in the alkaline range.

Results and Discussion

Native *Aequorea* GFP (Figure 1) has chromophore-derived absorption maxima at 395 and 475 nm in addition to an aromatic absorption band centered at 276 nm (Morise et al., 1974; Ward, 1979; Ward et al., 1980). Excitation with near-UV or blue light generates a brilliant green fluorescence with a wavelength maximum at 508 nm (Morise et al., 1974). Denaturation of A-GFP by treatment with acid (<pH 4), base (pH 12–13), elevated temperature (>65 °C), or strongly denaturing solvents such as 6 M Gdn·HCl abolishes the green fluorescence and markedly alters the absorption spectrum (Ward et al., 1980), as shown in Figure 1. Following treatment with 6 M Gdn·HCl at 92 °C or acidification to pH 2.0, the denatured protein can be partially renatured (up to 90%) as evidenced by the return of fluorescence and native absorption

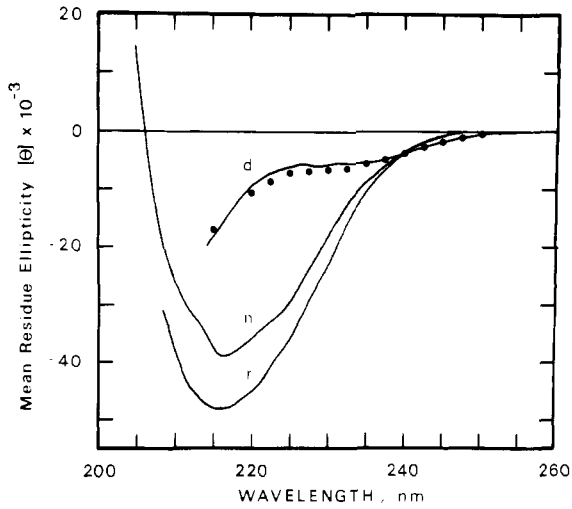

FIGURE 2: Circular dichroism spectra of native (n), denatured (d), and renatured (r) *Aequorea* green-fluorescent protein. Each spectrum was run on the Cary 61 at room temperature at a protein concentration of 6.9 μM. The native sample (n) was dissolved in 1 mM Tris–0.1 mM EDTA (pH 8.0). The denatured sample was dissolved in a 6 M Gdn·HCl solution (pH 8.0) containing 1 mM Tris and 0.1 mM EDTA. The sample was heated to 90 °C for 2 min, rapidly cooled to room temperature, and scanned within 10 min [curve d (—)]. (●) represents a CD spectrum of the same sample after 52 h of standing at room temperature. The renatured sample (r) was processed as described under Experimental Procedures (with Gdn·HCl as the denaturant) and finally chromatographed on Sephadex G-75 in 0.1 mM Tris buffer (pH 7.8).

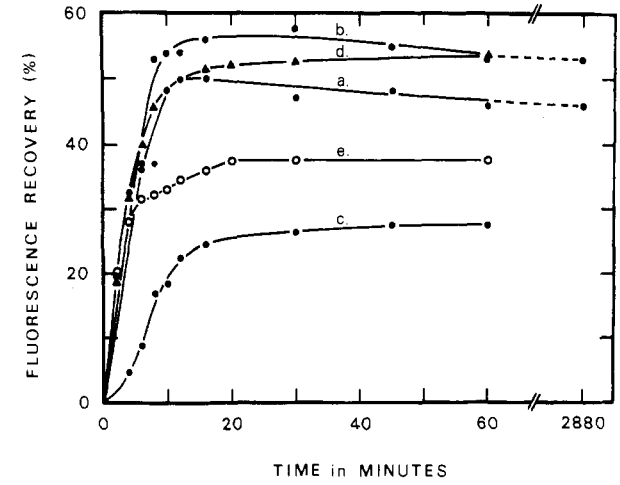

FIGURE 3: Kinetics of renaturation of *Aequorea* green-fluorescent protein. Samples of pure A-GFP were denatured with 6 M Gdn·HCl, acid (pH 2), or base (pH 13) as described under Experimental Procedures. Renaturation of Gdn·HCl-denatured samples was initiated by rapid dilution into 1 mM *N*-ethylmorpholine buffer (pH 8.0) at room temperature to final Gdn·HCl concentrations of 0.04 (curve a), 0.24 (curve b), or 0.54 M (curve c). At final Gdn·HCl concentrations of 1.0 M or higher, renaturation was barely detectable, even after 60 min. Acid-denatured (curve d) or base-denatured (curve e) samples were neutralized to pH 8.0 by the addition of small volumes of 1 M NaOH or 10 M HCl, dispensed precisely from Hamilton syringes.

properties (Bokman & Ward, 1981a,b). We have now shown that base-denatured A-GFP (pH 13.0) will also spontaneously renature at 4 °C following neutralization to pH 8.0. By this procedure the maximum recovery of native A-GFP, based on spectrophotometric analysis following Sephadex G-75 chromatography, was 49%.

Figure 2 shows the effect of guanidine treatment (2 min at 92 °C) on the secondary structure of A-GFP as measured by circular dichroism. The native CD spectrum has a strong negative deflection near 215 nm, indicating significant spectral contribution from the β structure. This secondary structure is disrupted by guanidine treatment; the resulting CD spectrum is indicative of a disordered coil. No further change in the spectrum can be seen following prolonged (52-h) incubation in 6 M Gdn·HCl (Figure 2). This is consistent with a previous observation that, in dilute aqueous buffers, complete denaturation occurs upon rapid heating to 80 °C and that prolonged heating at 80 °C or incubating at higher temperature (86 °C) causes no additional CD change (Bokman & Ward, 1981b).

Guanidine-denatured A-GFP may be renatured by dialysis at 4 °C vs. 1 mM *N*-ethylmorpholine at pH 8.0 (Bokman & Ward, 1981a,b) or by dilution into aqueous buffer at 22 °C to a final Gdn·HCl concentration of 0.24 M or lower. The rate of renaturation, as followed by the recovery of native fluorescence properties, is very rapid, with more than half of the final fluorescence returning in the first 5 min following dilution. The return of fluorescence is equally rapid when acid- or base-denatured GFP is neutralized to a final pH of 8.0 at 22 °C (Figure 3). From guanidine-denatured GFP, up to 55% of the original fluorescence (Figure 3) and 64% of native absorbance (Bokman & Ward, 1981b) may occur if dilution or dialysis is begun within a few minutes of heating in Gdn·HCl. However, if a sample is allowed to stand for 24 h in 6 M Gdn·HCl at room temperature before dialysis, renaturation, as measured by fluorescence or absorption spectroscopy, is considerably diminished; after 48 h it is almost undetectable. It is possible that this inability to renature the GFP results from a much slower conformational change too subtle to be detected by UV circular dichroism (Figure 2). The absence of cystinyl residues (Prendergast & Mann, 1978) eliminates disulfide exchange as a factor in the slow conformational change.

Table I: Estimates of Protein Concentration in Native and Renatured A-GFP Samples Having the Same Absorbance at 395 Nanometers[a]

sample	absorbance 280 nm	395 nm	750 nm[b]
native GFP	0.036	0.040	0.103
renatured GFP	0.044	0.040	0.100

[a] The renatured sample was renatured from Gdn·HCl by dialysis and was then chromatographed on Sephadex G-75, as described in the text. Protein assays were performed by the method of Lowry et al. [b] Lowry et al. (1951).

Complete spectral renaturation has not as yet been possible. In our first report (Bokman & Ward, 1981b), simultaneous equations were used to calculate the fractional renaturation, a value that never exceeded 90%. We have subsequently found that the residual denatured component can be physically separated from the renatured component, thus allowing direct spectrophotometric quantitation. A small column of Sephadex G-75 (superfine) was used to achieve separation, taking advantage of the apparent tendency of denatured A-GFP to adsorb to the gel filtration matrix. (A bright yellow band usually remains at the top of the column. On a few occasions, denatured A-GFP appears to have formed aggregates that elute near the void volume.) In Figure 4 the absorption spectrum of native A-GFP is compared with that of column-purified A-GFP renatured from base. The spectra are very similar in the near-UV and visible range with identical A_{395}:A_{475} absorbance ratios of 2.3. Differences from native GFP are observed in the aromatic absorbance bands of all renatured samples (Figure 4 and Table I), indicating that the renatured protein is spectrally (conformationally?) distinct from native A-GFP. The smallest spectral differences are seen

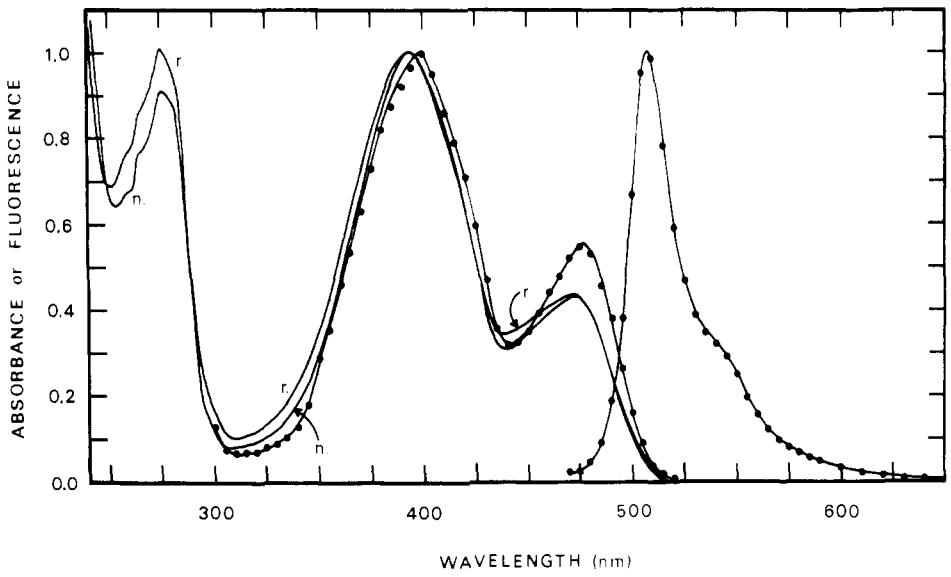

FIGURE 4: Absorption and fluorescence spectra of native and renatured A-GFP samples at neutral pH. Absorption spectra of native (n) and renatured (r) A-GFP were measured on the Cary 17D spectrophotometer in 10 mM Tris–EDTA buffer (pH 8.2) at room temperature. The renatured sample was renatured from base by neutralization and then chromatographed on Sephadex G-75, as described in the text. Fluorescence spectra of native (—) and renatured (●) A-GFP were measured on the SLM 4800 spectrofluorometer in 10 mM Tris–EDTA buffer (pH 7.0) at room temperature. Excitation spectra were run with excitation monochromator slit settings of 2 mm (4-nm band-pass) and emission wavelength fixed at 540 nm. Emission spectra were run with emission monochromator slit settings of 2 mm (4-nm band-pass) and excitation wavelength fixed at 397 nm. The renatured sample was renatured from 6 M Gdn·HCl by dialysis and was then chromatographed on Sephadex G-75, as described in the text. Excitation and emission spectra were fully corrected by using a Rhodamine quantum counter and SLM correction factors, respectively.

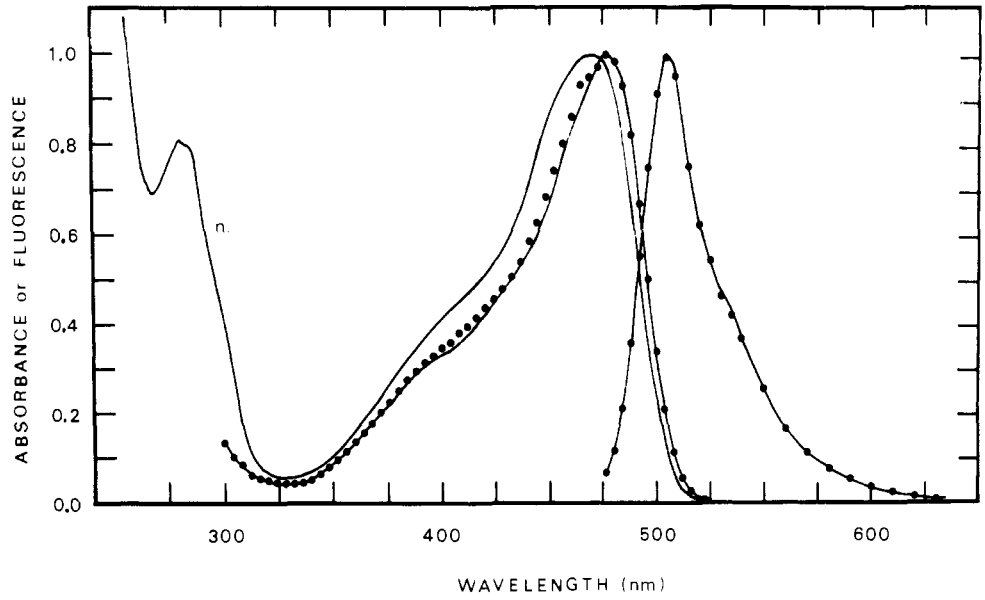

FIGURE 5: Absorption spectrum of native A-GFP and fluorescence spectra of both native A-GFP and renatured A-GFP at pH 12.2. The absorption spectrum of native A-GFP (n) was measured, at room temperature, on the Cary 17D spectrophotometer immediately after the sample was diluted into 10 mM sodium phosphate buffer at pH 12.2. Fluorescence spectra of native (—) and renatured (●) A-GFP were measured on the SLM spectrofluorometer in 10 mM sodium phosphate buffer (pH 12.2) at room temperature. Excitation spectra were run with excitation monochromator slit settings of 2 mm and a fixed emission wavelength setting of 540 nm. Emission spectra were run with emission monochromator slit settings of 2 mm and a fixed excitation wavelength setting of 468 nm. The renatured sample was renatured from 6 M Gdn·HCl by dialysis and was then chromatographed on Sephadex G-75, as described in the text. Excitation and emission spectra were fully corrected by using a Rhodamine quantum counter and SLM correction factors, respectively.

following renaturation from base as shown in Figure 4. The presumed conformational differences have not been observed in the aromatic region of the CD spectrum (250–300 nm). The only region where a large CD difference is apparent is within the peptide absorption band, below 240 nm (Figure 2).

Despite the fact that the renatured protein does not fully return to the native state, it cannot be distinguished from native A-GFP with regard to temperature and pH stability and resistance to trypsin digestion (Bokman & Ward, 1981a,b). Furthermore, the excitation and emission spectra are virtually identical for native and renatured A-GFP over a wide pH range from 7 to 12.2 (Figures 4 and 5). Because the shapes of the emission spectra are identical, it is possible to calculate the fluorescence quantum efficiency of renatured A-GFP (see Experimental Procedures) relative to that of the native protein simply by measuring, with fixed excitation wavelength, the ratio of emission peak heights (renatured:native). Using samples with identical absorbancies of 0.010 at the excitation

maximum (395 nm), we measured a peak height ratio of 0.80. Multiplying 0.80 by Morise's quantum yield of 0.78 for native A-GFP (Morise et al., 1974), we calculate a quantum efficiency for A-GFP renatured from Gdn·HCl to be 0.62. The lower fluorescence quantum yield and decreased A_{395}:A_{280} ratio (Figure 4) for renatured A-GFP cannot be explained on the basis of loss or chemical alteration of the chromophore. There is no indication in Figure 4 of a spectrally altered chromophore. In addition, the ratio of chromophore (as A_{395}) to total protein (based on the Lowry assay) is the same for native and renatured A-GFP (Table I). We believe, instead, that the chromophore environment is slightly altered in the renatured protein, resulting in a decreased fluorescence quantum yield.

A very interesting spectral change occurs in A-GFP under alkaline conditions. At pH 12.2 (Figure 5) the excitation maximum, as measured on the SLM 4800, becomes red-shifted by 78 nm, from 398 to 476 nm. This is not a spectral shift in the usual sense. Instead, the strength of the excitation shoulder (at 476 nm) increases nearly 2-fold while the major peak (at 398 nm) decreases in strength by 3-fold. In addition, the emission peak shifts slightly (3 nm) to the blue, the spectral bandwidth increases by about 10 nm, and the emission shoulder at 540 nm becomes less pronounced. These spectral changes are completely reversible. Returning the pH to 7.0 fully restores the original excitation and emission spectra. A corresponding reversible shift in the absorption spectrum of A-GFP at high pH has previously been observed (Ward, 1981; Ward et al., 1982), but conditions were slightly different (higher ionic strength) and the perturbation was not so pronounced as it is here. The excitation and emission spectra at pH 12.2 have mirror image symmetry, almost identical in shape with the GFP spectra from *Renilla reniformis* (Wampler et al., 1971; Ward & Cormier, 1979) and *Phialidium gregarium* (Levine & Ward, 1980, 1982). For three species of GFP (*Aequorea*, *Phialidium*, and *Renilla*) the corrected excitation maxima are respectively 476 (pH 12.2), 487, and 498 nm; corresponding corrected emission maxima are 505 (pH 12.2), 497, and 509 nm.

For some time we have been suggesting that the chromophores of *Aequorea* and *Renilla* GFP are chemically identical species (Ward, 1979) despite the large difference (103 nm) between absorbance (excitation) maxima of A-GFP and R-GFP in the native state. This spectral difference has been attributed to differences in the apoprotein environments (i.e., differences in noncovalent interactions between the apoprotein and a common chromophore), known to occur between opsin and retinal in the visual protein rhodopsin (Dartnall & Lythgoe, 1965) or between luciferase and luciferin in the firefly bioluminescence system (Seliger & McElroy, 1964; Biggley et al., 1967). Principal supporting data have included the near identity of fluorescence emission spectra (Ward, 1979) and the discovery that the fully denatured proteins have identical absorption properties in the 350–500-nm range, including a sharp isosbestic point at 405 nm (Ward et al., 1980). In the present work, we have demonstrated a new set of nondenaturing conditions (i.e., pH 12.2) under which the excitation spectrum of *Aequorea* GFP is strongly, yet reversibly, perturbed. The perturbed A-GFP excitation spectrum is strikingly similar to that of R-GFP, providing further evidence that the chromophores may, in fact, be chemically identical. Similar experiments have been attempted with *Renilla* GFP; however, R-GFP shows no spectral perturbation, short of denaturation, over its entire pH stability range (pH 5–12.5). Furthermore, reversible denaturation, of the type described here for A-GFP, has not yet been clearly demonstrated with *Renilla* GFP (renaturation yields of <5% have been observed). Should it be possible to efficiently renature R-GFP, an additional criterion of renaturation can be investigated—protein–protein interaction between *Renilla* luciferase and R-GFP during radiationless energy transfer.

Acknowledgments

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Structure of the Glycyl-L-histidyl-L-lysine–Copper(II) Complex in Solution†

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ABSTRACT: Optical, electron paramagnetic resonance, and electron spin-echo envelope spectroscopies were used to examine the structure of the Cu(II) complex of glycyl-L-histidyl-L-lysine (GHL) in solution. At neutral pH, GHL forms a mononuclear 1:1 Cu(II) compound having an EPR spectrum resembling that of Cu(II) equatorially coordinated by two or three nitrogen atoms. Electron spin-echo studies demonstrate that one of these is located in the histidyl imidazole ring. A pH titration of Cu(II)–GHL shows three optical transitions with apparent p*K*s of 3.6, 9.2, and 11.4 and molecularities, with respect to protons, of 2, 2, and 1, respectively. At the lowest p*K*, GHL binds Cu(II), forming the species present at physiological pH. At elevated pH, spectroscopic experiments suggest that an alteration of the Cu(II) structure occurs, yet the bound imidazole is retained. These solution studies are consistent with nitrogen coordination of Cu(II) in Cu(II)–GHL, but the solid-state polymeric structure, with oxygen-bridged Cu(II) pairs as previously determined by X-ray crystallographic analysis [Pickart, L., Freedman, J. H., Loker, W. J., Peisach, J., Perkins, C. M., Steinkamp, R. E., & Weinstein, B. (1980) *Nature (London) 288*, 715–717; C. M. Perkins, N. J. Rose, R. E. Steinkamp, L. H. Jensen, B. Weinstein, and L. Pickart, unpublished results], does not exist in solution.

We have previously reported the isolation and characterization of the human serum tripeptide glycyl-L-histidyl-L-lysine, which, in nanomolar concentrations, stimulates the growth or enhances the viability of a variety of cultured cells and organisms (e.g., fungi, hepatocytes, lymphocytes, fibroblasts, T-strain mycoplasma, and *Ascaris* larvae) (Pickart, 1981; Fouad et al., 1981; Castillo & Roberts, 1980; Schlesinger et al., 1977). GHL[1] coisolates from serum with copper in near equimolar quantities, and it is the Cu(II)–GHL complex that is believed to exert the observed bioactivity of the molecule (Pickart & Thaler, 1979, 1980).

X-ray diffraction analysis of crystalline Cu(II)–GHL prepared at near neutral pH (Pickart et al., 1980; C. M. Perkins, N. J. Rose, R. E. Steinkamp, L. H. Jensen, B. Weinstein, and L. Pickart, unpublished results) shows a polymeric structure, where GHL forms a planar, tridentate complex involving (1) the N-terminal amino group of glycine, (2) the nitrogen atom of the glycylhistidyl amide bond, and (3) the imino nitrogen of the histidyl imidazole ring. The fourth and fifth ligands are oxygenous and are bound to Cu(II) in an adjoining molecule forming a binuclear metal complex, reminiscent of crystalline copper(II) acetate (Bleaney & Bowers, 1952a,b; van Niekerk & Schoening, 1953). This structural assignment is in disagreement with that obtained from a potentiometric titration and a comparison of the relative binding constants of Cu(II) with glycyl-L-histidine, glycyl-L-histidylglycine, and GHL, where the metal ion coordination is believed to involve the ϵ-amino of lysine in addition to the histidyl imidazole nitrogen and the amide nitrogen (Lau & Sarkar, 1981).

In view of the broad physiological activity of this peptide and the ambiguous description of the complex as it exists in solution, we have determined the structure of Cu(II)–GHL by employing optical, electron paramagnetic resonance, and electron spin-echo envelope spectroscopies. Using these probes, we have shown that at neutral pH, the tripeptide forms a monomeric complex with a single bound Cu(II) atom. An analysis of the EPR spectrum (Peisach & Blumberg, 1974) suggests that three nitrogen atoms are coordinated to the copper as equatorial ligands. Electron spin-echo spectroscopy demonstrates that one of these is derived from a histidine imidazole. These experiments indicate that the structural assignment derived from spectroscopic measurements of the complex in solution is consistent with the three nitrogen

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[1] Abbreviations: GHL, glycyl-L-histidyl-L-lysine; GH, glycyl-L-histidine; EPR, electron paramagnetic resonance; DET, diethylenetriamine; shf, superhyperfine lines; NMR, nuclear magnetic resonance.

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