Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 13549–13554, November 1998
Biochemistry

Chemical synthesis of the precursor molecule of the *Aequorea* green fluorescent protein, subsequent folding, and development of fluorescence

(*N*-[(9-hydroxymethyl)-2-fluorenyl]succinamic acid resin/segment condensation reaction/maximum protection strategy/protein folding/autocyclodehydration)

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Communicated by Andrew A. Benson, University of California at San Diego, La Jolla, CA, September 18, 1998 (received for review May 15, 1998)

ABSTRACT **The present paper describes the total chemical synthesis of the precursor molecule of the *Aequorea* green fluorescent protein (GFP). The molecule is made up of 238 amino acid residues in a single polypeptide chain and is nonfluorescent. To carry out the synthesis, a procedure, first described in 1981 for the synthesis of complex peptides, was used. The procedure is based on performing segment condensation reactions in solution while providing maximum protection to the segment. The effectiveness of the procedure has been demonstrated by the synthesis of various biologically active peptides and small proteins, such as human angiogenin, a 123-residue protein analogue of ribonuclease A, human midkine, a 121-residue protein, and pleiotrophin, a 136-residue protein analogue of midkine. The GFP precursor molecule was synthesized from 26 fully protected segments in solution, and the final 238-residue peptide was treated with anhydrous hydrogen fluoride to obtain the precursor molecule of GFP containing two Cys(acetamidomethyl) residues. After removal of the acetamidomethyl groups, the product was dissolved in 0.1 M Tris·HCl buffer (pH 8.0) in the presence of DTT. After several hours at room temperature, the solution began to emit a green fluorescence (λ_{max} = 509 nm) under near-UV light. Both fluorescence excitation and fluorescence emission spectra were measured and were found to have the same shape and maxima as those reported for native GFP. The present results demonstrate the utility of the segment condensation procedure in synthesizing large protein molecules such as GFP. The result also provides evidence that the formation of the chromophore in GFP is not dependent on any external cofactor.**

The bioluminescent jellyfish *Aequorea victoria* produces a greenish luminescence from the margin of its umbrella by using two proteins: a Ca^{2+}-binding protein, aequorin (21.4 kDa) and a chromophore-bearing green fluorescent protein (GFP; 27 kDa) (1). On binding Ca^{2+}, aequorin undergoes an intramolecular reaction, yielding a blue fluorescent protein in the singlet excited state, which transfers its energy by resonance to GFP. The acceptor of energy in GFP is a chromophore consisting of an imidazolone ring structure (2–4). A phenolate anion of the chromophore in the singlet excited state is the emitter of the green light (4). Chemical studies of GFP and expression of cDNA for GFP in prokaryotes and eukaryotes have shown that the chromophore is a product of a post-translational modification of the primary structure, involving (*i*) an autocyclodehydration ($-H_2O$) of the tripeptide segment, -Ser^{65}-Tyr^{66}-Gly^{67}-, and (*ii*) autoxidation ($-2H$) of the resulting dihydro-ring system (see scheme on next page) (2–7). With the formation of the chromophore, a greenish fluorescence develops in the protein. The cyclodehydration reaction is postulated to take place autocatalytically, but the mechanism is still unknown. Thus, it was of interest to chemically synthesize the precursor molecule of GFP and investigate the development of the green fluorescence *in vitro*.

Aequorea GFP consists of 238 amino acid residues in a single polypeptide chain (7–9). The native molecule has been shown to regenerate its intrinsic fluorescence from the totally denatured state (10). GFP is a relatively large molecule to serve as a target for chemical synthesis because a 99-residue HIV-protease analogue is the largest protein synthesized thus far in a highly homogeneous form by the solid-phase method (11) and the 136-residue human pleiotrophin (12) is the largest protein that has been synthesized by the solution method. A chemical procedure, first described in 1981 for the synthesis of biologically active peptides (13–15), was used in the present study to synthesize *Aequorea* GFP. It is based on carrying out segment condensation reactions in solution, using as building blocks Boc-amino acids developed for original solid-phase synthesis. In this procedure, all of the functional groups are protected (maximum protection strategy), and the free target molecule finally is generated by hydrogen fluoride (HF)-deprotection. The usefulness of the procedure has been confirmed by the syntheses of human parathyroid hormone, an 84-residue linear peptide (16), human angiogenin (14), human midkine (17), and human pleiotrophin (12). The syntheses of these proteins required the use of powerful solvent systems in which efficient coupling reactions could be carried out without danger of epimerization (18). The fully protected GFP precursor molecule was assembled from 26 segments (Fig. 1*B*) and was deprotected by the HF method. When the synthetic precursor molecule was allowed to fold in solution, the molecule developed fluorescence characteristics indistinguishable from those of native GFP.

MATERIALS AND METHODS

Materials. Reagents used for the synthesis of peptides and Boc-amino acids were the products of Peptide Institute (Osaka, Japan). Boc-Trp(cyclohexyloxycarbonyl) (19) and Boc-Tyr(3-pentyl) (20) were synthesized as described. *N*-[(9-

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Abbreviations: GFP, green fluorescent protein; Acm, acetamidomethyl; CHL, chloroform; TFA, trifluoroacetic acid; TFE, trifluoroethanol; DMF, *N*,*N*-dimethylformamide; HMFS, *N*-[(9-hydroxymethyl)-2-fluorenyl]succinamic acid.

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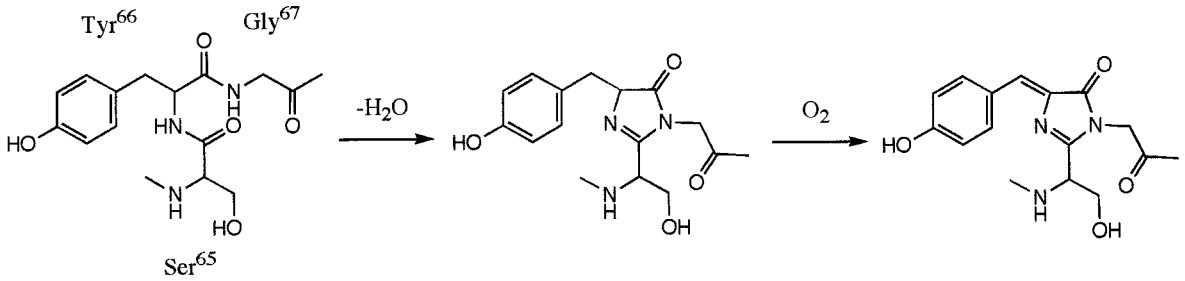

hydroxymethyl)-2-fluorenyl]succinamic acid (HMFS) was synthesized and attached to aminomethyl-polystyrene to form HMFS-resin (21). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate was purchased from Calbiochem–Nova Biochem, and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine was purchased from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Synthesis of Starting Segments. Fig. 1*A* shows the primary structure of *Aequorea* GFP synthesized in this study (9). The whole sequence was divided into 26 segments and was assembled through the routes shown in Fig. 1*B*. Each fully protected segment was synthesized by using a semiautomatic peptide synthesizer (Model ACT-90, Advanced ChemTech), with ordinary Boc-amino acids as building blocks on the HMFS-resin. The functional groups of the side chains were protected by using the following groups: benzyl for Ser and Thr, cyclohexyl for Asp and Glu, 9-xanthenyl for Asn and Gln, p-toluenesulfonyl for Arg, benzyloxymethyl for His, 2-cholorobenzyloxycarbonyl for Lys, acetamidomethyl (Acm) for Cys, cyclohexyloxycarbonyl for Trp, and 3-pentyl for Tyr. The C-terminal Boc-amino acid was coupled to the HMFS-resin after converting to the symmetric anhydride in the presence of 10% 4-dimethylaminopyridine in dichloromethane. Starting with Boc-aminoacyl-HMFS-resin, all of the 26 segments were synthesized by the stepwise elongation of the respective Boc-amino acids except for segments 18 and 26, which were synthesized in solution starting with H-Asp(O-benzyl)-O-phenacyl and H-Lys(2-cholorobenzyloxycarbonyl)-O-benzyl, respectively, following the procedures described (13–15). The

A

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MSKGEELFTG VVPILVELDG DVNGQKFSVS GEGEGDATYG KLTLKFICTT GKLPVPWPTL  60
VTTFSYGVQC FSRYPDHMKQ HDFFKSAMPE GYVQERTIFY KDDGNYKTRA EVKFEGDTLV 120
NRIELKGIDF KEDGNILGHK MEYNYNSHNV YIMADKPKNG IKVNFKIRHN IKDGSVQLAD 180
HYQQNTPIGD GPVLLPDNHY LSTQSALSKD PNEKRDHMIL LEFVTAAGIT HGMDELYK   238
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B

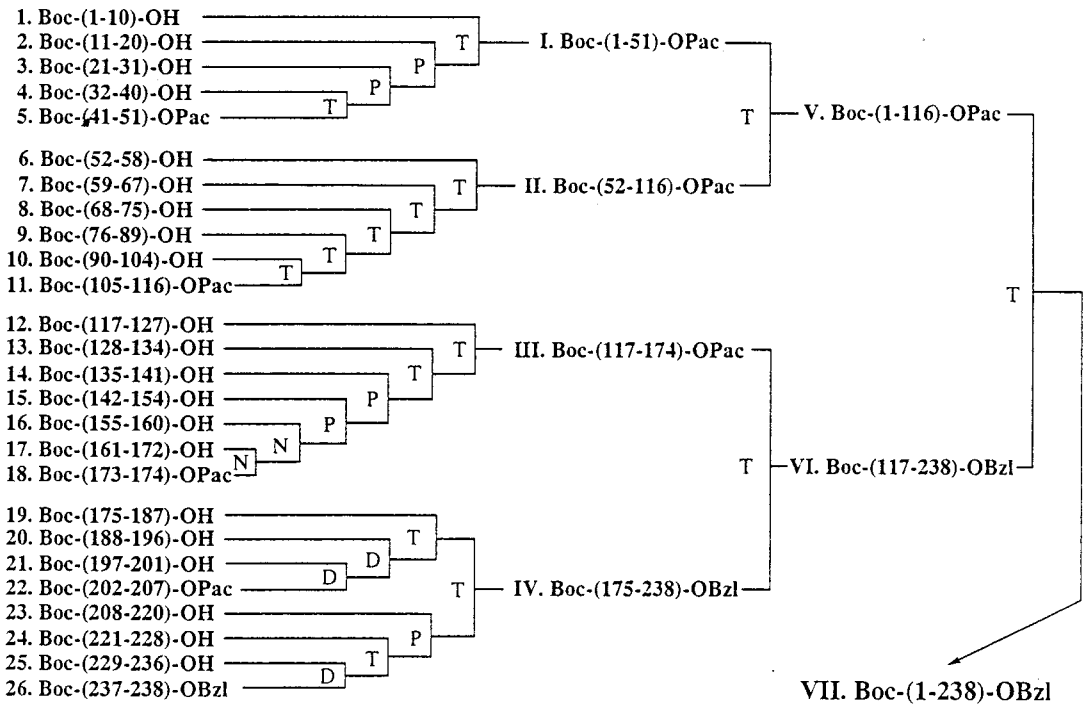

FIG. 1. (*A*) Primary structure of GFP. (*B*) Segments 1–26 and their coupling routes. Coupling solvents: D, DMF; N, *N*-methylpyrrolidone; P, phenol/CHL (1:3 vol/vol); T, TFE/CHL (1:3 vol/vol).

elongation reaction on the resin was performed in *N*-methyl-pyrrolidone by using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (22) as the coupling reagent in the presence of 1-hydroxybenzotriazole. After completing the elongation reaction, each segment was removed from the resin by treatment with 20% morpholine in *N,N*-dimethylformamide (DMF) for 30 min at room temperature. The C-terminal carboxyl groups of segments 5, 11, and 22 were converted to the phenacyl esters, and all segments thus obtained were purified by repeated precipitation from DMF. When checked by HPLC, each segment was found to be >95% pure. However, if the purity was <95%, the segment was purified further by silica-gel chromatography by using as a solvent system a mixture consisting of chloroform (CHL)/methanol/acetic acid or CHL/trifluoroethanol (TFE)/acetic acid.

Segment Condensation Reactions in Solution. Condensation reactions used to join the segments (Fig. 1*B*) were carried out in DMF, *N*-methylpyrrolidone, CHL/TFE, or CHL/phenol system using water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as the coupling reagent in the presence of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, as described for the synthesis of human midkine (17). The yield of the large intermediate segments synthesized were 2.4 g for compound I, 1.2 g for compound II, 2.4 g for compound III, and 0.9 g for compound IV. The purity of each product was checked by HPLC after removing the phenacyl group and carrying out HF-deprotection. The phenacyl group at the C terminus of each large protected segment was removed by heating with zinc powder in a mixture of 20% acetic acid in hexafluoroisopropanol at 40°C for 2 hr. The protected segments were assembled to form the complete GFP sequence by using CHL/TFE (3:1 vol/vol) as the coupling solvent. The final yield of fully protected GFP precursor molecule was ≈700 mg.

Removal of Protecting Groups. The fully protected GFP precursor peptide VII (465 mg) was treated with HF in the presence of cysteine hydrochloride (567 mg) and *p*-cresol (5 ml) as scavengers at −5°C for 1 hr. The precursor peptide was extracted with 50% acetic acid and was purified by gel-filtration chromatography by using Sephadex LH-20 (Pharmacia). The product (346 mg) was purified further by using a Model LC-6A or LC-8A HPLC system (Shimadzu, Kyoto, Japan) and a linear gradient (30–50%) consisting of CH_3CN in 0.1% trifluoroacetic acid (TFA) as eluting solvent. A single peak was obtained giving a yield of 33 mg. The ratios of the recovered amino acids, after acid hydrolysis, were as follows: Asp, 31.3 (31); Thr, 14.1 (15); Ser, 10.1 (11); Glu, 23.4 (23); Gly, 21.8 (22); Ala, 8.11 (8); $1/2(Cys)_2$, 1.8 (2); Val, 15.3 (16); Met, 6.4 (7); Ile, 12.7 (13); Leu, 18.1 (18); Tyr, 11.5 (12); Phe, 12.2 (12); His, 9.0 (9); Lys, 21.2 (21); Trp, 0.7 (1); Arg, 6.0 (6); and Pro, 10.7 (11). The molecular weight, $[M+H]^+$, measured with a Voyager Elite MALDI-TOF MS (PerSeptive Biosystems, Framingham, MA), was 27,037.2, which also agreed well with the theoretical value of 27,035.7 for the GFP molecule containing two Acm groups. Capillary zone electrophoresis also was carried out on the purified product by using a Model 270A Applied Biosystems apparatus under the following conditions: column, uncoated fused silica (50 μm × 72 cm); buffer, 20 mM sodium citrate (pH 2.5); voltage, 25 kV; detection, 200 nm; and temperature, 30°C. A single, sharp peak (observed migration time, 11.8 min) was obtained, indicating very high homogeneity. The final product was dissolved in 50% acetic acid and was treated with $Hg(AcO)_2$ at room temperature for 2 hr to remove the remaining two Acm groups. The mercury ions were removed by adding excess 2-mercaptoethanol followed by gel filtration of the mixture through a Sephadex G-25 column. The peptide-containing fractions were combined and lyophilized. The final product weighed 25 mg.

Folding of the Synthetic GFP Precursor Molecule. The completely deprotected peptide (28 μg) was dissolved in 28 μl of 6 M Gdn·HCl in 0.1 M Tris·HCl buffer at pH 8.0 containing 50 mM NaCl and 100 mM DTT. The solution was diluted with 2.8 ml of 0.1 M Tris·HCl buffer at pH 8.0, containing 1 mM EDTA and 50 mM NaCl. After standing at room temperature for 5 hr, the solution began to emit a greenish fluorescence under near-UV light (380–400 nm). The time course of the development of fluorescence and fluorescence excitation and emission spectra of the final solution were recorded with a Hitachi (Tokyo) Model F4500 fluorescence spectrometer at room temperature. In another experiment, 0.5 mg of the deprotected peptide was dissolved in 500 μl of the same buffer as described above, and the solution was diluted with 50 ml of 0.1 M Tris·HCl buffer at pH 6.5 containing 20% ethylene glycol, 1 mM EDTA and 50 mM NaCl. While standing at 3°C for several days, fluorescence intensity of the solution increased continuously and reached a maximum after 10 days. Based on the ratio of the UV-absorption maxima of the final mixture at 380 and 276 nm (measured under acidic condition), the conversion of the precursor molecule to native GFP was estimated to be ≈10% (data not shown). The homogeneity of the solution also was checked by taking an aliquot of the solution and analyzing by RP-HPLC using a Resource PRC column (Pharmacia).

RESULTS AND DISCUSSION

The strategy that was followed for synthesizing the GFP precursor molecule was to assemble the whole molecule in solution (13–15) from the 26 segments, as shown in Fig. 1*B*. To synthesize such a large number of segments within a reasonable period of time, the solid-phase method was chosen because the products could be checked for purity by TLC and/or HPLC after cleaving from the resin. Thus, any segment showing contamination was purified further before being used in the segment condensation work. From among the methodologies known for the solid-phase synthesis of fully protected segments, the method of Rabanal *et al.* (21) was used. Before starting the synthesis, the reliability of the method was tested with some model peptides. It was found that BrZ group for protecting Tyr, as well as For for protecting Trp, was too unstable under the basic conditions required for removing segments from the resin. For protecting Trp, the cyclohexyloxycarbonyl group was found suitable (19), but for the Tyr residue, a new protecting group, stable under basic conditions and removable by the standard HF method without side reaction, was required. From among several candidates, 3-pentyl was selected as the most appropriate protecting group for Tyr because it is completely stable under the conditions required for removing Boc-group (1 hr in TFA at room temperature) and for removing protected peptides from the resin (treatment with 20% morpholine in DMF for 30 min) (20). To increase the rate of the elongation reaction of Boc-amino acids on the resin, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (22) was used as the coupling reagent. However, under the reaction conditions with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, nitrile formation was considerable with Asn and Gln residues. The residues, therefore, were protected temporarily by 9-xanthenyl (23) during the coupling reactions because it is easily removed by TFA at room temperature. Some intermediate segments were only soluble in CHL/phenol (3:1 vol/vol) or CHL/TFE (3:1 vol/vol), and they were, therefore, coupled in these solvent systems. Use of the two solvent systems had the risk of forming TFE- or phenyl-esters, but the yield of the principal products was reasonable when excess 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine was added to the reaction mixture (18). The four big segments I, II, III, and IV, resulting from the syntheses, were found to be

>80% pure when checked for homogeneity by HPLC after HF-deprotection. The four segments were coupled one by one to obtain the larger segments V and VI, which then were coupled to obtain the final fully protected segment VII. Segment VII was treated with HF to obtain the GFP precursor molecule, with the two Cys residues still protected by Acm groups. At this stage, the GFP precursor product was purified further by HPLC and then was analyzed for homogeneity by amino acid and mass spectroscopic analyses. The observed values were in close agreement with the calculated values. Finally, the Acm groups were removed by treatment with $Hg(AcO)_2$ in 50% acetic acid (24, 25).

When 28 μg of the deprotected peptide was subjected to the refolding conditions described in *Materials and Methods*, the solution began to emit a green fluorescence under near-UV light after ≈5 hr at room temperature. The time course of the development of fluorescence is shown in Fig. 2*A*. The fluorescence excitation and fluorescence emission spectra of the synthetic product are shown in Fig. 2*B* and are seen to be the same as those reported for native GFP (1). Thus, the spectral data demonstrate that the fluorescent product derived from the synthetic precursor molecule is similar, if not the same, as native GFP and that the chromophore is formed by the cyclization of -Ser^{65}-Tyr^{66}-Gly^{67}- residues and subsequent autoxidation without the intervention of any external cofactor. The results also show that large proteins like GFP can be synthesized by chemical procedures described herein.

The UV-absorption spectrum of native GFP molecule shows chromophore-derived absorption maxima at 395 and 475 nm in addition to an aromatic absorption band at 276 nm. The ratio of the intensities of the two peaks at 395 and 276 nm in sodium phosphate buffer at pH 7.2 has been reported to be 1 to 1.2 with highly purified native GFP (10) and ≈0.4 in the case of recombinant GFP isolated from a soluble fraction of bacterial cells (26). Without the addition of ethylene glycol to the folding solution, the synthetic product was fluorescent but did not show any recognizable peak between 350 to 420 nm, as was found with protein isolated from insoluble aggregates (inclu-

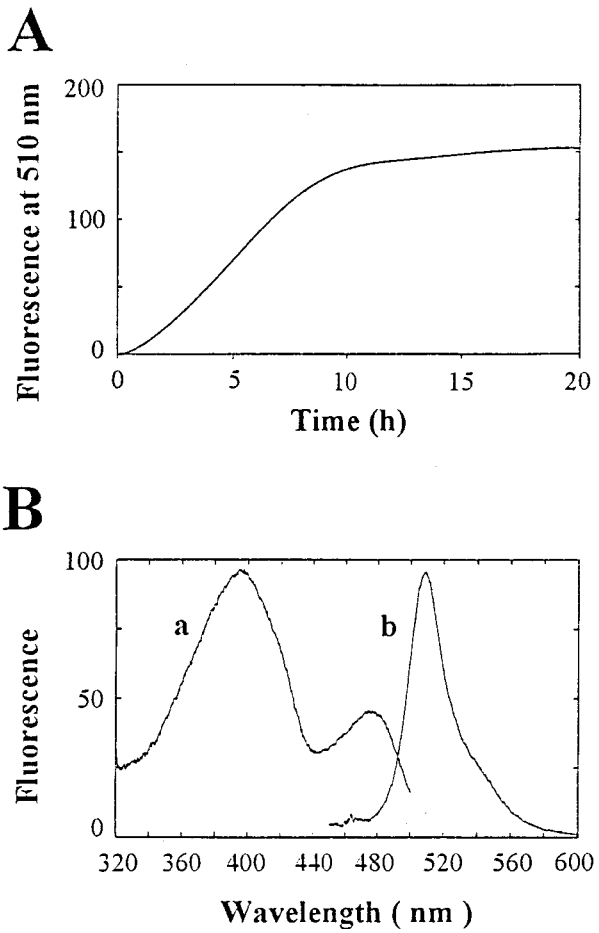

FIG. 2. (*A*) Time course of development of fluorescence in synthetic precursor molecule. Fluorescence intensity is expressed in relative units. (*B*) Fluorescence excitation and emission spectra of synthetic GFP. Curve a is excitation spectrum measured with excitation monochromator set at a band pass of 5 nm and an emission wavelength fixed at 510 nm; curve b is emission spectrum measured with emission monochromator set at band pass of 5 nm and an excitation wavelength fixed at 400 nm. Synthetic GFP was in 0.1 M Tris·HCl buffer (pH 8.0) containing 0.06 M Gdn·HCl, 1 mM DTT, 50 mM NaCl and 1 mM EDTA. Concentration of GFP: 3.7×10^{-7} M. Scan speed: 60 nm/min.

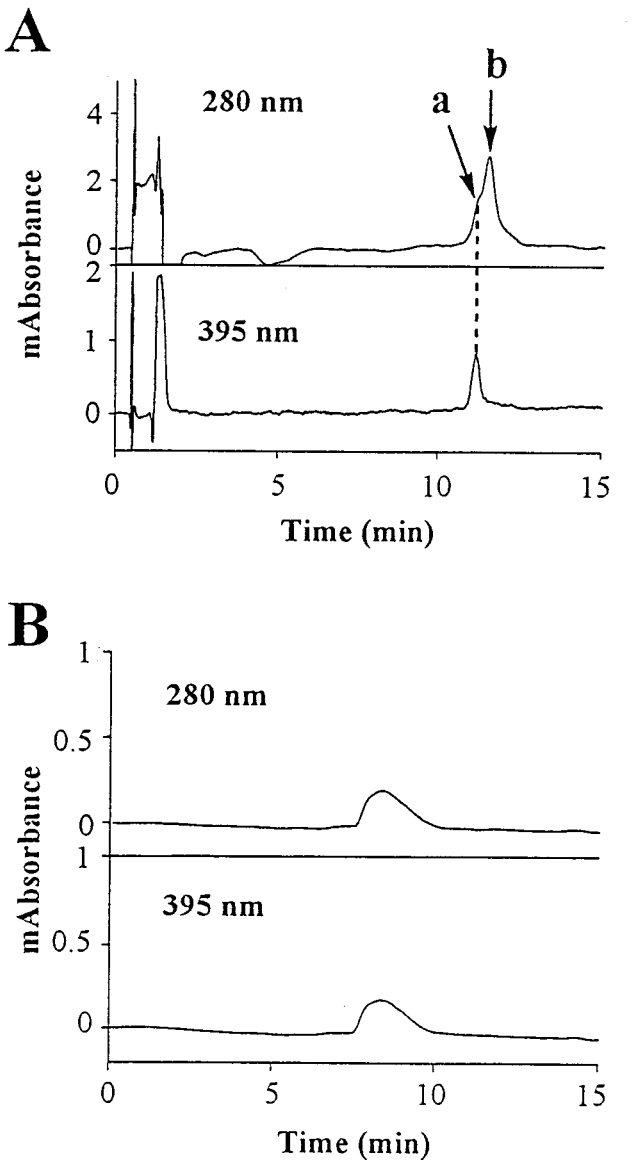

FIG. 3. HPLC elution profiles of synthetic precursor molecule of GFP after renaturation for 10 days in Tris buffer containing 20% ethylene glycol. (*A*) RP-HPLC. Column: Pharmacia Resource RPC (1 ml). Eluent: 20–50% CH_3CN in 0.1% TFA. Running conditions: 10 min gradient, 5-min holding; flow rate: 2 ml/min; temp: 25°C. Monitor: Shimadzu Model SPD-M10AVP Photodiode Array UV-VIS Detector attached to the HPLC. mAbsorbance, milli-absorbance unit. (*B*) Gel-filtration HPLC. Column: Bio-Rad Bio-Select SEC-125–5, 300 × 7.8 mm. Eluent: 0.1 M K_2HPO_4 buffer (pH 7.0). Running conditions: flow rate: 1.0 ml/min; temp: 25°C. Monitor: Same as in *A*.

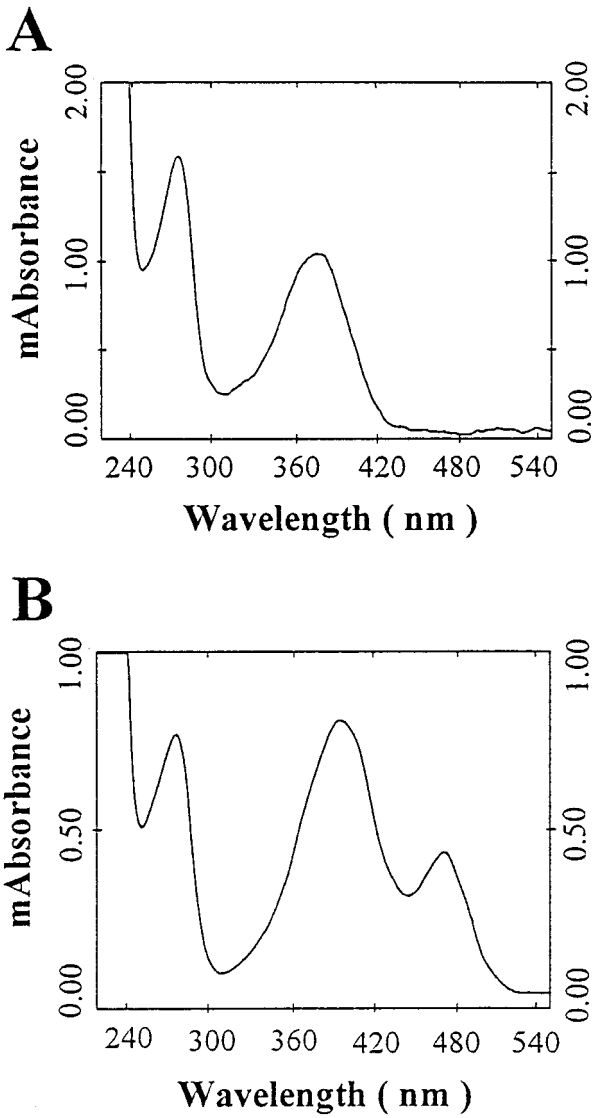

FIG. 4. (*A*) Absorption spectrum of fraction a from Fig. 3*A*. The spectrum was measured with the same instrument as in Fig. 3*A*. mAbsorbance, milli-absorbance unit. (*B*) Absorption spectrum of rechromatographed first eluting peak from Fig. 3*B*. The spectrum was measured with the same instrument as in Fig. 3*A*.

sion bodies) of *Escherichia coli* (26). Based on this observation, the estimated conversion of the synthetic precursor GFP to native GFP may have been at most ≈1%. Heim *et al.* (6) tried to isolate GFP expression products not only from the soluble part but also from the insoluble part (inclusion bodies) in *E. coli* and found that the proteins isolated from the soluble part underwent correct folding and chromophore formation with high efficiency, as in the jellyfish, whereas the proteins from the inclusion bodies, even though they were indistinguishable from the soluble protein on denaturing gel, were completely nonfluorescent and did not become fluorescent when solubilized and renatured from Gdn·HCl according to Ward and Bokman (10). In contrast, the nonfluorescent GFP protein, expressed in *E. coli* under anaerobic conditions and isolated from the soluble fraction, was found to develop fluorescence once atmospheric oxygen was admitted (6). Thus, it is conceivable that wild-type GFP precursor molecules isolated from a soluble fraction are already in a properly folded conformation, ready to undergo the dehydration and oxidative reactions, whereas those isolated from inclusion bodies may be folded improperly and extremely difficult to refold even when solubilized and renatured under conditions applicable to soluble recombinant GFP molecules. The synthetic GFP precursor protein may have a structure similar to that of proteins isolated from the inclusion bodies.

In view of the low efficiency of conversion discussed above, we tested various additives to see whether one would have a favorable effect on the folding of synthetic GFP precursor molecule. Of interest, the addition of ethylene glycol, a compound with a relatively low dielectric constant, to the folding medium enhanced chromophore formation up to 10%. Fig. 3*A* shows the result of renaturing synthetic precursor molecule in Tris buffer containing 20% ethylene glycol for 10 days, as described in *Materials and Methods*. An aliquot of the mixture was subjected to HPLC, and the solution eluting from the column was conducted into a flow cell of a Shimadzu Photodiode Array UV-VIS Detector and was monitored at 280 and 395 nm. The elution profile measured at 280 nm (Fig. 3*A Upper*) shows that the mixture consisted of two components: a fraction "a" and fraction "b," with retention times of 11.1 min and 11.5 min, respectively. In contrast, the elution profile measured at 395 nm (Fig. 3*A Lower*) shows only a single elution peak, in exact alignment with fraction a in the upper panel. Because the native GFP chromophore is characterized by an absorbance maximum at 395 nm, the results indicate that fraction a is the major folded GFP molecule whereas the following fraction b is the starting material. This conclusion is supported further by the absorption spectrum of a in 0.1% TFA shown in Fig. 4*A*, which is identical to the spectrum reported for acid-denatured GFP (10). Fraction b (Fig. 3*A*) did not show any UV absorbance at 395 nm, indicating that it consisted of the unfolded molecule. Based on the ratio of the peak intensities at 276 and 380 nm, the purity of a was estimated to be ≈50%.

Fig. 3*B* shows the results of subjecting the renaturation mixture to gel-filtration HPLC at neutral pH. The gel-filtration column was charged with 1.0 ml of the mixture and was eluted with 0.1 M phosphate buffer (pH 7.0). Under the elution condition, the unfolded precursor molecules remain at the top of the column because they are almost insoluble in the buffer. The peak eluting first from the column was collected and rechromatographed on the same column after washing the column thoroughly with buffer. The elution peak observed by measuring at 280 nm (Fig. 3*B Upper*) was found to be aligned exactly with the peak observed by measuring at 395 nm (Fig. 3*B Lower*). On measuring the UV absorption spectrum of the peak (Fig. 4*B*), the spectrum was found to be identical to the absorption spectrum of native GFP at pH 7.2 (10). Thus, the synthetic folded GFP molecule could be purified by this gel-filtration procedure. Studies are now in progress to find improved renaturation conditions for the synthetic GFP precursor molecule.

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