FEBS Letters 351 (1994) 211–214

FEBS 14472

Evidence for redox forms of the *Aequorea* green fluorescent protein

Satoshi Inouye**, Frederick I. Tsuji*

Marine Biology Research Division 0202, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, USA

Received 6 June 1994

Abstract Highly purified recombinant *Aequorea* green fluorescent protein is able to undergo a reversible oxidation-reduction reaction in the presence of molecular oxygen. In the oxidized form in near UV light, the protein is highly fluorescent, but when reduced with sodium dithionite, it becomes completely non-fluorescent. On exposure to molecular oxygen the reduced, non-fluorescent protein reverts to its original fluorescent state.

Key words: Oxidation–reduction; Electron transfer; Protein chromophore; Energy transfer; Bioluminescence; Aequorin

1. Introduction

The characteristic greenish bioluminescence of the jellyfish, *Aequorea victoria*, clearly visible as a ring of bright light along the margin of the umbrella [1], is due to the action of two closely associated proteins: aequorin, a small Ca^{2+}-binding protein (21.4 kDa) [2–4], and a green fluorescent protein (GFP, 27 kDa, fluorescence λ_{max} = 508 nm) [5–9]. The latter contains a chromophore that is the ultimate emitter in the bioluminescence reaction [8,10]. Aequorin is made up of a complex of apoaequorin (apoprotein), coelenterazine (an imidazole compound, mol.wt. = 423) and molecular oxygen. When aequorin is mixed in vitro with Ca^{2+}, a bluish light is observed due to an intramolecular reaction in which the coelenterazine substrate is oxidized to coelenteramide. The reaction is catalyzed by the protein, which is converted to a luciferase on the binding of Ca^{2+}. The products are light (λ_{max} = 470 nm), CO_2 and a blue fluorescent protein (BFP, coelenteramide + apoaequorin, fluorescence λ_{max} = 470 nm). The electronically excited state of BFP (coelenteramide) is the emitter in the reaction. If the reaction is carried out in the presence of excess GFP, a greenish luminescence is observed, identical to that seen in the living animal. The greenish luminescence results from an energy transfer from the excited state of BFP to GFP [6,11,12].

$$\text{Aequorin} \xrightarrow{Ca^{2+}} \text{Blue fluorescent protein} + h\nu_{\lambda_{max}\,470\text{ nm}} + CO_2$$
$$\downarrow + \text{GFP}$$
$$h\nu_{\lambda_{max}\,508\text{ nm}}$$

We previously reported on the PCR cloning, expression, fluorescence characteristics, and energy transfer capability of *Aequorea* GFP [13]. The results showed that the properties of recombinant GFP are similar, if not identical, to those of native GFP. A further investigation of this recombinant protein has now revealed that it may exist in either an oxidized or reduced form. This finding and the key role that the protein plays in *Aequorea* bioluminescence have prompted us to examine the previous PCR-generated cDNA clone, since errors are known to occur in PCR cloning and because variation in nucleotide sequence has been reported for the cDNA for GFP [14]. Thus, the *Aequorea* cDNA library was re-screened, additional cDNA clones isolated, the DNA analyzed and the cDNA expressed in *E. coli* to prepare high purity recombinant GFP for characterization studies. This paper reports on this work and the redox properties of the purified recombinant GFP.

2. Materials and Methods

2.1. Materials

The following were obtained from commercial sources: Chelating Sepharose-Fast Flow, Pharmacia-LKB, Piscataway, NJ; imidazole, sodium dithionite ($Na_2S_2O_4$, 84% purity), $FeSO_4$, $K_4Fe(CN)_6$, $NaHSO_3$, NaN_3, NaCN, EDTA, L-cysteine, glutathione (reduced), dithiothreitol, 2-mercaptoethanol, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), Sigma, St. Louis, MO; $NiCl_2$, Merck; hydrogen peroxide, platinum asbestos (5%), Fisher Scientific, Pittsburgh, PA; 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Calbiochem. San Diego, CA; sodium borohydride, Aldrich, Milwaukee, WI; bovine enterokinase (EK3), Biozyme, San Diego, CA; and SDS-PAGE gel and sample buffer, Novex, San Diego, CA.

2.2. Cloning of cDNA for GFP

The *Aequorea* cDNA library was screened using the *Bam*HI/*Sal*I insert of pAGP [13] as a probe. Subsequent isolation and sequence analysis of the cDNA clones for GFP were carried out as previously described [15].

2.3. Purification of rGFP

The *E. coli* expression system for the histidine-tagged GFP (His-GFP) and the procedure for purifying the expressed protein using a Ni-chelate affinity column were described previously [13]. The protein was eluted from the column using a linear gradient of imidazole (0–0.3 M). The fractions showing green fluorescence under near UV light (Model UVL-56, Ultraviolet Products, San Gabriel, CA) were collected and combined. The yield of this His-GFP was 1.3 mg from 300 ml of the cultured cells and the purity was greater than 95% as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The fused region of His-GFP was removed by incubating 200 μg of the protein with 185 units (1 μg protein) of enterokinase in 1 ml of 50 mM sodium phosphate buffer, pH 6.0, at room temperature (22–24 °C) for 24 h. An aliquot of this digest was analyzed by SDS-PAGE and the remainder was dialyzed against 100 mM ammonium bicarbonate, pH 8.0. The dialysate was then applied to a freshly prepared Ni-chelate column (500 μl of gel bed) equilibrated with the same buffer and the pass-through fractions showing a green fluorescence (rGFP or His-GFP lacking the histidine tail) were collected and combined.

2.4. Protein analysis

Protein concentration was determined by the dye-binding method of Bradford [16] using a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard. SDS-PAGE was carried out under non-reducing conditions using a 8–16% separation gel according to Laemmli [17]. The rGFP in the enterokinase digest mixture

*Corresponding author. Fax: (1) (619) 534 7313.

**On leave from the Yokohama Research Center, Chisso Corp., Kanazawa-ku, Yokohama, Kanagawa 236, Japan.

SSDI 0014-5793(94)00859-0

was further purified by reversed-phase HPLC and then subjected to N-terminal amino acid sequence analysis using an Applied Biosystems (Foster City, CA) Model 470A gas phase protein sequencer connected to an on-line Model 120A phenylthiohydantoin analytical system.

2.5. Reactions of rGFP with various chemical reagents

The reactions of rGFP with various chemical reagents were carried out at room temperature in a final volume of 0.5 ml. The reaction was started by adding the chemical dissolved in 1 M ammonium bicarbonate, pH 8.0, to 20 μg of rGFP dissolved in 100 mM ammonium bicarbonate, pH 8.0. A transilluminator (Fischer Scientific, Model FBTIV-88) was used as a source of short UV light (312 nm) to monitor changes in the green fluorescence of the solution. In the reduction experiments with $NaBH_4$ and H_2/Pt, 100 μg of rGFP was used.

2.6. Measurements of absorption and fluorescent spectra

Absorption spectra of the purified rGFP were measured at room temperature with a Beckman Model DU-50 recording spectrophotometer and the fluorescence emission spectrum was measured with a Perkin-Elmer (Norwalk, CT) MPF-4 Fluorescence Spectrophotometer.

3. Results and discussion

In the previous study [13], two full-length cDNA clones for GFP were isolated by PCR and were found to have identical DNA sequences. In the present study, five positive clones, designated pAGP11, pAGP21, pAGP31, pAGP51 and pAGP71, were obtained from 1.5×10^5 clones. Sequence analysis [18] of pAGP11 and pAGP31 showed that they contained the full-length cDNA of GFP [13] and identical DNA sequences, including the 5′- and 3′-non-coding regions. The other clones, pAGP21, pAGP51 and pAGP71, were truncated forms of the 5′-coding region of GFP cDNA, which have been reported previously in pAGP [13] and *gfp*10 [14]. Thus, our studies have shown only one complete nucleotide sequence for GFP (Fig. 1) and the same primary structure, deduced from the nucleotide sequence, as reported previously [13].

Fig. 2A shows the N-terminal region of the fused His-GFP and the cleavage site of enterokinase. The expressed His-GFP was purified by Ni-chelate affinity chromatography and the histidine segment removed by treatment with enterokinase. After purification by HPLC, N-terminal amino acids sequence analysis yielded two sequences: DRWIPKM^{1}SKG, accounting for 40% of rGFP, and WIPKM^{1}SKGEE, accounting for the remaining 60% (Fig. 2A). The first sequence indicates that the fused region was cleaved correctly [19], but the reason for the two cleavages is unknown, unless the enterokinase has two cleavage sites instead of one or a contaminating protease is present. SDS-PAGE of His-GFP, carried out under non-reducing conditions with heat treatment, gave an apparent molecular mass of 31 kDa (Fig. 2B, lane 1), whereas after cleavage with enterokinase the protein (rGFP) had an apparent molecular mass of 28 kDa (Fig. 2B, lane 2). An enterokinase band was not detected due to its low concentration. The calculated molecular weight of rGFP with 244 amino acid residues was 27,692.89. SDS-PAGE performed on rGFP under non-reducing conditions without heat treatment gave a greenish fluorescent band with an apparent molecular mass of about 40 kDa. While this band has been previously attributed to a partially denatured dimer [13], the true state of GFP in this band is unknown and it is also possible that the band is due to a monomer with an unusual electrophoretic mobility.

Fig. 3a shows the absorption spectrum of rGFP with peaks at 280, 395 and 478 nm. The spectrum was identical to that reported previously for native GFP [9]. His-GFP also gave the same absorption spectrum (data not shown). The fluorescence emission spectrum of rGFP (Fig. 3b) was virtually identical to

```
                                                                (EcoRI/NotI)tactacacacgaataaaagacaacaaag   -1

 M  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D  G  D  V  N    23
atg agt aaa gga gaa gaa ctt ttc act gga gtt gtc cca att ctt gtt gaa tta gat ggc gat gtt aat    69

 G  Q  K  F  S  V  S  G  E  G  E  G  D  A  T  Y  G  K  L  T  L  K  F    46
ggg caa aaa ttc tct gtc agt gga gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctt aaa ttt   138

 I  C  T  T  G  K  L  P  V  P  W  P  T  L  V  T  T  F  S  Y  G  V  Q    69
att tgc act act ggg aag cta cct gtt cca tgg cca aca ctt gtc act act ttc tct tat ggt gtt caa   207

 C  F  S  R  Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y    92
tgc ttt tca aga tac cca gat cat atg aaa cag cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat   276

 V  Q  E  R  T  I  F  Y  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E   115
gta cag gaa aga act ata ttt tac aaa gat gac ggg aac tac aag aca cgt gct gaa gtc aag ttt gaa   345

 G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G   138
ggt gat acc ctt gtt aat aga atc gag tta aaa ggt att gat ttt aaa gaa gat gga aac att ctt gga   414

 H  K  M  E  Y  N  Y  N  S  H  N  V  Y  I  M  A  D  K  P  K  N  G  I   161
cac aaa atg gaa tac aac tat aac tca cat aat gta tac atc atg gca gac aaa cca aag aat gga atc   483

 K  V  N  F  K  I  R  H  N  I  K  D  G  S  V  Q  L  A  D  H  Y  Q  Q   184
aaa gtt aac ttc aaa att aga cac aac att aaa gat gga agc gtt caa tta gca gac cat tat caa caa   552

 N  T  P  I  G  D  G  P  V  L  L  P  D  N  H  Y  L  S  T  Q  S  A  L   207
aat act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt   621

 S  K  D  P  N  E  K  R  D  H  M  I  L  L  E  F  V  T  A  A  G  I  T   230
tcc aaa gat ccc aac gaa aag aga gat cac atg atc ctt ctt gag ttt gta aca gct gct ggg att aca   690

 H  G  M  D  E  L  Y  K  *                                              238
cat ggc atg gat gaa cta tac aaa taa atgtccagacttccaattgacactaaagtgtccgaacaattactaaattctcagg   797

gttcctggttaaattcaggctgagacttttatttatatatttatagattcattaaaatttttatgaataatttattgatgttattaataggggg   888
ctatttttcttattaaataggctactggagtgtat(NotI/EcoRI)                                              922
```

Fig. 1. Nucleotide sequence of *Aequorea* GFP and primary structure of GFP deduced from the nucleotide sequence. The hexapeptide segement involved in the cyclic formation of the fluorescent chromophore is underlined [8,10,21]. GenBank Accession Number is L29345.

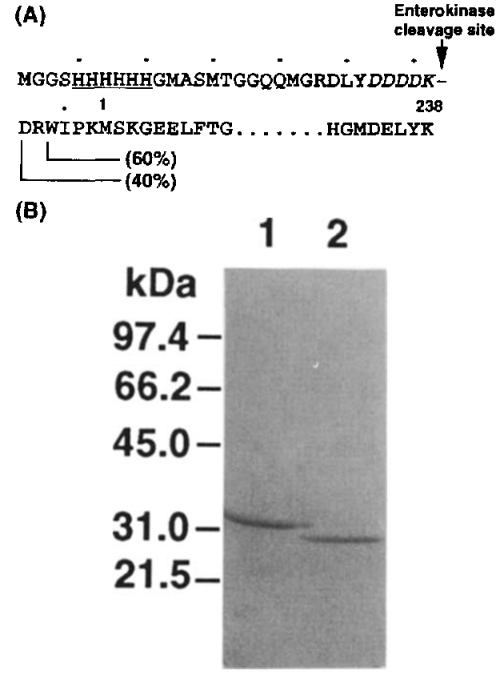

Fig. 2. N-Terminal amino acid sequence and SDS-PAGE of His-GFP. (A) N-Terminal amino acid sequence showing the 34 amino acid residues plus the 3 amino acid residues arising from use of the PCR primer, with the 6 histidine residues underlined and the enterokinase cleavage site indicated by an arrow. (B) SDS-PAGE of His-GFP before (lane 1: 5 μg of protein applied) and after (lane 2: 4 μg of protein applied) cleavage with enterokinase. Molecular weight markers (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

those reported previously for native GFP [9] and for His-GFP [13] and the fluorescence excitation spectrum (read at λ = 530 nm) gave peaks at 395 and 478 nm, closely matching the peaks of the absorption spectrum. However, the ratios of the absorbance peaks, 395 nm/280 nm, for rGFP and His-GFP were 0.43 and 0.40, respectively, which are approximately one-half of the value reported for native GFP [8,10]. The difference in the ratios is presumably due to the presence of a second tryptophan in the fused N-terminus of each of the proteins (Fig. 2A).

The expressed GFP in near UV light has been found to show strong intrinsic fluorescence, both intracellularly and extracellularly [13,20]. The fluorescence has been attributed to the presence of a chromophore in the protein, formed by the cyclization of three amino acid residues within an hexapeptide segment of the polypeptide chain by post-translational modification [8,10]. Considerable work has gone into the chemical structure of the chromophore [8,10,21], but the chemical basis for the fluorescence is still unknown. During the purification of His-GFP, a marked increase in fluorescence intensity was observed when the harvested cells and cell-precipitate were allowed to stand overnight at room temperature. Subsequently, the intensity remained unchanged for over a month. This suggested that an oxidative step may be involved in the initial increase in fluorescence.

On adding sodium dithionite ($Na_2S_2O_4$), a strong reducing reagent, to a solution of rGFP, the green fluorescence of the solution disappeared within a few minutes and, on standing for several hours, the fluorescence reappeared first as a thin band at the surface, which then spread slowly downward into the solution. On standing overnight the solution recovered its fluorescence completely. This result indicated that the rGFP was involved in a redox reaction with molecular oxygen, with the oxidized form being fluorescent and the reduced form being nonfluorescent.

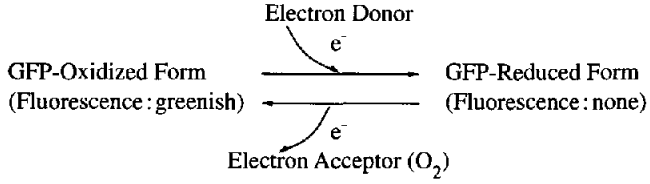

Table 1 summarizes the results of mixing various chemical reagents (reductants and oxidants) with rGFP. Some of the compounds were examined under both aerobic and anaerobic conditions, as noted in the table. The addition of either $FeSO_4$ or ABTS [22], to a solution of rGFP under argon atmosphere caused the greenish fluorescence to be quickly extinguished and, as with $Na_2S_2O_4$, the reaction was reversible, that is, on standing in air the greenish fluorescence returned once more. However, when rGFP was treated with $Na_2S_2O_4$, $FeSO_4$ or ABTS in an argon atmosphere and allowed to stand, the greenish fluorescence did not return until air was readmitted. The time required for the recovery of the greenish fluorescence was shortened if the solution was shaken in air or subjected to dialysis. In order to ascertain whether any spectral change occurs on reduction, 450 μl of a 0.18 mg/ml solution of rGFP in 50 mM ammonium bicarbonate, pH 8.0, was mixed with 25 μl of 100 mM sodium dithionite in 1 M ammonium bicarbonate, pH 8.0, and the mixture was scanned between 600 and 300 nm in a microcell using an Uvikon 810 (Kontron, Zurich, Switzerland) recording spectrophotometer. Controls consisted of (a) 450 μl of the same rGFP solution mixed with 25 μl of the

Table 1
Reactions of various chemical reagents with rGFP

Addition to rGFP	Green fluorescence	E_0', V
None	(+)	
$Na_2S_2O_4$ (5 mM)		
under Ar_2	(+) → (−)	−0.66
exposure to air/dialysis	(+) ← (−)	
$FeSO_4$ (2 mM)		
under Ar_2	(+) → (−)	0.77
exposure to air/dialysis	(+) ← (−)	
1/2 O_2 + 2H^+/H_2O		0.82
ABTS (0.2 mM)		
under Ar_2	(+) → (−)	
exposure to air/dialysis	(+) ← (−)	
H_2O_2 (1%) under Ar_2 or Air	(+) → (−)	0.30
H_2/Pt (~1 mg)	(+) → (−)	−0.42
$NaBH_4$ (~1 mg) under Ar_2 or Air	(+)	
2-Mercaptoethanol (2%)	(+)	
Dithiothreitol (10 mM)	(+)	−0.33
Glutathione, reduced (10 mM)	(+)	−0.23
L-Cysteine (10 mM)	(+)	
$K_4Fe(CN)_6$ (2 mM)	(+)	0.36
$NaHSO_3$ (10 mM)	(+)	
NaN_3 (10 mM)	(+)	
NaCN (10 mM)	(+)	
EDTA (10 mM)	(+)	

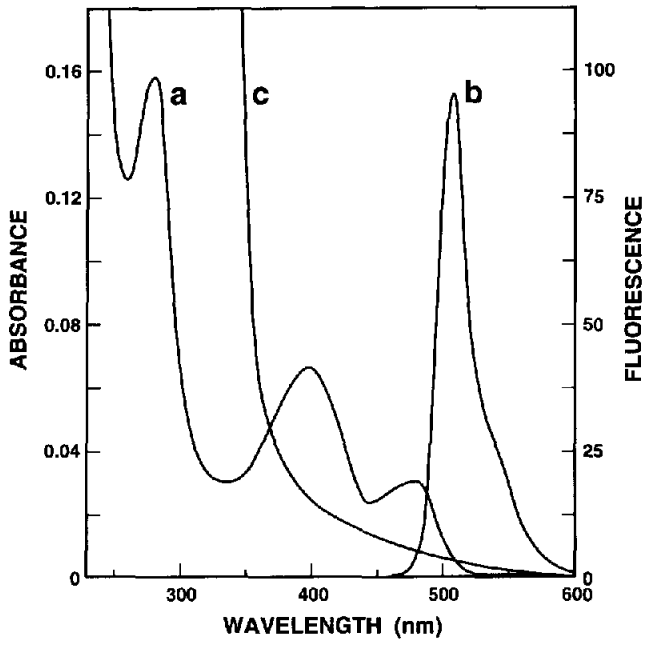

Fig. 3. Absorption and fluorescence emission spectra of rGFP. (a) Absorption spectrum of rGFP. (b) Fluorescence emission spectrum of rGFP. (c) Absorption spectrum of H_2O_2-treated rGFP. For (a), (b) and (c) the protein concentration was 0.18 mg/ml in 100 mM ammonium bicarbonate, pH 8.0.

same 1 M ammonium bicarbonate soution, and (b) 450 μl of the same 50 mM ammonium bicarbonate solution mixed with 25 μl of the same sodium dithionite solution. Except for the region between 400 and 300 nm, which was difficult to analyze due to the high absorbance of sodium dithionite, the absorption spectrum of the rGFP-sodium dithionite mixture did not show any major change in absorbance, shape or maximum (λ_{max} = 478 nm) between 600 and 400 nm when compared to the oxidized rGFP control. Thus, between 600 and 400 nm there appears to be no apparent change in absorption spectrum on reduction. If hydrogen peroxide was added to rGFP, the green greenish fluorescence slowly disappeared over a period of 1 h and never recovered even when exposed to air. This loss of greenish fluorescence was accompanied by a disappearance of the absorbance peaks at 395 and 478 nm (Fig. 3c), suggesting that a permanent structural change had taken place in the chromophore. Since rGFP is reduced by $FeSO_4$ and the reduced rGFP oxidized by O_2, with H_2O being a likely product, it appears that the standard reduction potential, E'_o, for rGFP lies somewhere between 0.77 V and 0.82 V (Table 1). Moreover, with the E'_os for rGFP and O_2 lying so close to each other, it is conceivable that GFP may be at the terminus of a series of electron carriers, as in the eukaryotic respiratory chain.

The rGFP has two cysteine residues at positions 48 and 70 in the primary structure [13,14]. Reducing reagents such as 2-mercaptoethanol, dithiothreitol, reduced glutathione and L-cysteine did not have any affect on the fluorescence of rGFP (Table 1), whereas treatment with the sulfhyryl reagent DTNB (1 mM) [23] caused the greenish fluorescence to disappear in a few minutes (data not shown). Thus, a free cysteine residue may be required for the greenish fluorescence of rGFP.

It is noteworthy that reversible photobleaching of GFP has been reported in vivo on exposure to UV light during the expression of the cDNA for GFP in *Caenorhabditis elegans* [20]. It is possible that the photobleaching may be due to a reduction taking place within the cell and the recovery of fluorescence to the transfer of electrons to molecular oxygen. The proposed imidazolone structure for the chromophore of GFP [8,10,21], however, does not appear to explain the redox behavior of rGFP in that the electron acceptor–donor group is not identifiable. Another interesting structure-related problem is that *Aequorea* GFP, when expressed in such diverse organisms as *E. coli*, *C. elegans* and COS cells, is fluorescent [13,20]. This must mean that a synthetic (enzymatic) mechanism must be present in all of these organism to form the complex chromophore by post-translational modification or a self-modification mechanism must exist within the protein.

Acknowledgements: The authors are grateful to Drs. M. Shimonaka and Y. Yamaguchi for their assistance in the HPLC purification of rGFP and to Dr. G. Fortes for help with the fluorescence measurements. The research was supported in part by Research Grant MCB91-04684 from the National Science Foundation.

References

[1] Harvey, E.N. (1952) Bioluminescence, Academic Press, New York.
[3] Inouye, S., Noguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T. and Tsuji, F.I. (1985) Proc. Natl. Acad. Sci. USA 82, 3154 3158.
[4] Charbonneau, H., Walsh, K.A., McCann, R.O., Prendergast, F.G., Cormier, M.J. and Vanaman, T.C. (1985) Biochemistry 24, 6762–6771.
[5] Shimomura, O., Johnson, F.H. and Saiga, Y. (1962) J. Cell. Comp. Physiol. 59, 223–239.
[6] Johnson, F.H., Shimomura, O., Saiga, Y., Gershman, L.C., Reynolds, G.T. and Waters, J.R. (1962) J. Cell. Comp. Physiol. 60, 85–103.
[7] Prendergast, F.G. and Mann, K.G. (1978) Biochemistry 17, 3448–3453.
[8] Shimomura, O. (1979) FEBS Lett. 104, 220–222.
[9] Ward, W.W., Cody, C.W., Hart, R.C. and Cormier, M.J. (1980) Photochem. Photobiol. 31, 611–615.
[10] Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G. and Ward, W.W. (1993) Biochemistry 32, 1212–1218.
[11] Morin, J.G. and Hastings, J.W. (1971) J. Cell. Physiol. 77, 313–318.
[12] Morise, H., Shimomura, O., Johnson, F.H. and Winant, J. (1974) Biochemistry 13, 2656–2662.
[13] Inouye, S. and Tsuji, F.I. (1994) FEBS Lett. 341, 277–280.
[14] Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G. and Cormier, M.J. (1992) Gene 111, 229–233.
[15] Inouye, S. and Tsuji, F.I. (1993) FEBS Lett. 315, 343–346.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[18] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[19] LaVallie, E.R., Rehemtulla, A., Racie, L.A., DiBlasio, E.A., Ferenz, C., Grant, K.L., Light, A. and McCoy, J.M. (1993) J. Biol. Chem. 268, 23311–23317.
[20] Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. (1994) Science 263, 802–805.
[21] McCapra, F., Razavi, Z. and Neary, A.P. (1988) J. Chem. Soc. Chem. Commun. 790–791.
[22] Childs, R.E. and Bardsley, W.G. (1975) Biochem. J. 145, 93–103.
[23] Ellman, G.E. (1959) Arch. Biochem. Biophys. 82, 70–77.