Thermostabilization of Firefly Luciferase by a Single Amino Acid Substitution at Position 217

Naoki Kajiyama* and Eiichi Nakano

Research and Development Division, Kikkoman Corporation, 399 Noda, Noda-city, Chiba 278, Japan

Received July 27, 1993; Revised Manuscript Received October 18, 1993®

ABSTRACT: Random mutagenesis of the luciferase cDNA from "Genji" firefly, *Luciola cruciata*, was induced by hydroxylamine in an attempt to isolate thermostable mutants. Three mutants were isolated, and the cDNAs encoding these proteins were sequenced. All mutant cDNAs carried the same C to T transition mutation that conferred an amino acid substitution of Thr by Ile at position 217. The wild-type luciferase and the thermostable variant (Thr217Ile) were purified to homogeneity, and their enzymatic properties were determined. Thr217Ile was superior to wild-type in thermal and pH stability. Furthermore, the specific activity of the Thr217Ile mutant was increased to 130% of that of the wild-type. In order to examine the effect of amino acid residue substitution at position 217 on the thermostability of luciferase, we replaced the Thr residue at position 217 with all of the rest of the possible amino acid residues by site-directed mutagenesis. The thermostability of these substitution mutants seemed to correlate with the hydrophobicity of the substituted amino acid residue.

Firefly luciferase catalyzes the oxidation of luciferin in the presence of ATP, Mg^{2+}, and molecular oxygen (DeLuca & McElroy, 1978). This enzyme efficiently converts chemical energy into light with a quantum yield of 0.88 (Seliger & McElroy, 1960). Due to its high sensitivity and extreme specificity for ATP, luciferase has been used to determine the amount of ATP in various biological samples (Ludin, 1981). Many nucleotides and enzymes can be measured with high sensitivity through coupling with reaction for ATP production. Furthermore, luciferase is now being used in various assays, including activated sludge (Neethling et al., 1985), food testing (Sharpe et al., 1970), and bioluminescence immunoassay (Wannlund & DeLuca, 1982).

One of the most important goals of protein engineering is to produce mutant enzymes which have greater thermostability than the parent proteins. Various approaches to enhance the stability of proteins have been made by many workers (Perutz & Raidt, 1975; Arbos et al., 1979; Imanaka et al., 1986), but no definite conclusion has been reached on the cause and effect of thermostability. A rational strategy for increasing the stability of a specific protein is by site-directed mutagenesis. However, this approach requires a thorough knowledge of the three-dimensional structure of the protein and the molecular mechanism responsible for its heat inactivation. There are only a limited number of proteins that fulfill these requirements, and examples of successful design of stabilized proteins are rare (Wetzel et al., 1988; Matsumura et al., 1989; Quax et al., 1991). In the absence of detailed information about the structure of the protein, random mutagenesis remains an alternate strategy but entails the problems associated with mutant screening.

The luciferase cDNA from *Luciola cruciata* ("Genji-botaru" in Japanese) has been cloned and analyzed in our laboratory (Masuda et al., 1989). The primary structure of this luciferase deduced from the nucleotide sequence consists of 548 amino acids, with a molecular weight of 60 024. Recently, we succeeded in isolating several color mutant luciferases by using random mutagenesis and an easy screening system (Kajiyama & Nakano, 1991). A similar random approach can be adopted for the generation of mutant proteins which show greater thermostability than the wild-type.

We report here the isolation of thermostable variants of "Genji" luciferase. We have confirmed that the alteration in the mutant gene is from ACA to ATA, resulting in the change of Thr to Ile at position 217. Furthermore, using site-directed mutagenesis, we have constructed mutants in which Thr at position 217 is substituted by each of 18 different amino acid residues.

EXPERIMENTAL PROCEDURES

Plasmid, Escherichia coli Strain, and Media. The luciferase cDNA from *Luciola cruciata* had been cloned and sequenced in our laboratory (Masuda et al., 1989). Plasmid pGLf37, which is a plasmid directing the synthesis of active luciferase in *E. coli* under the control of *trp* promoter, was also constructed from pGLf1 in our laboratory. *E. coli* strain JM101 [*SupE,thi,* Δ(*lac-pro*), [F'*traD*36, *lacI*ΔZ M15,*proAB*]] (Yanish-Perron et al., 1985) was used for the expression of luciferase cDNA. The *E. coli* cells were grown in LB broth (1% Difco Tryptone containing 0.5% yeast extract and 0.5% sodium chloride), and ampicillin was added to a final concentration of 25 μg/mL when necessary.

Mutagenesis and Screening of Thermostable Mutants. Plasmid pGLf37 was treated, according to the methods of Kironde et al. (1989), in a solution containing 0.8 M hydroxylamine, 0.1 M sodium phosphate, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 6.0, for 2 h at 65 °C. The mutagen-treated plasmid was precipitated with ethanol and redissolved in 10 mM Tris-HCl with 1 mM EDTA, pH 8.0, followed by transformation into *E. coli* JM101. After 12 h at 37 °C, colonies on LB/ampicillin plates were transferred on nitrocellulose filters. The filters with the colonies were put on new agar plates, and incubated at 60 °C for 30 min. The filters were then removed from the agar plates, soaked with 0.5 mM luciferin in 100 mM sodium citrate buffer, pH 5.0, for 5 min, and then exposed by contact to X-ray film for 1 h.

Assay of Luciferase Activity. Ten microliters of luciferase solution was added to 400 μL of substrate mix [25 mM

* Abstract published in *Advance ACS Abstracts,* December 1, 1993.

0006-2960/93/0432-13795$04.00/0

```
        Arg  Ala  His  Ser  Phe  Arg  Thr  Val  Xxx  Asn  Glu  His
5' — C TCT AGC ATG CGA AAA TCT AGT GAC NNN ATT TTC GTG A  — 3'
          •                          ***
        SphI
```

FIGURE 1: Sequence of the synthetic oligonucleotide primer used for site-directed mutagenesis. Nucleotide substitutions are indicated with asterisks. This oligonucleotide primer is designed to have a new restriction site, *Sph*I, which is a silent mutation. This site was used as a marker to identify the mutant. Abbreviation: N, A+C+G+T.

glycylglycine (pH 7.8)/5.4 mM $MgSO_4$/0.086 mM D-luciferin]. Then 80 μL of 10 mM ATP was injected into the mixed sample, and a number of photons was integrated in 20 s by a BLR-201 luminescence reader (Aloka, Tokyo). One light unit (LU) was defined as the emission of 1 count.

Purification of Luciferase. Purification of the wild-type and the thermostable luciferases produced in *E. coli* was carried out as described by Kajiyama et al. (1992). *E. coli* JM101 cells harboring the wild-type or the mutant plasmid were cultured in 3 mL of LB broth containing ampicillin at 37 °C for 12 h. Two microliters of the cultures was inoculated into 100 mL of LB broth containing ampicillin. After growth at 37 °C for 6 h, the cultures were harvested. *E. coli* pellets were resuspended in lysis buffer (100 mM potassium phosphate, pH 7.8, 2 mM EDTA, and 1 mg of lysozyme/mL), incubated on ice for 15 min, and then frozen on dry ice. The frozen pellets were allowed to thaw at 25 °C and cleared by centrifugation.

The lysate of *E. coli* was fractionated with ammonium sulfate; the fraction precipitated between 0.3 and 0.6 saturation was saved. The precipitate was dissolved with 25 mM Tris-HCl buffer, pH 7.8, containing 1 mM EDTA and 10%-saturated ammonium sulfate, and then loaded on an Ultrogel AcA34 gel filtration column (LKB). The fraction showing activity was applied to a hydroxyapatite column (Tosoh, Tokyo, Japan), followed by elution with a gradient of 10–100 mM sodium phosphate.

DNA Sequencing. Various restriction fragments derived from mutant luciferase cDNA were subcloned into pUC118 or pUC119, and sequenced using a DNA Model 373A sequencer (Applied Biosystems).

Site-Directed Mutagenesis. Plasmid pSD1 was constructed from pGLf37 and pUC119 for site-directed mutagenesis. Single-stranded DNA was isolated from *E. coli* JM101 containing pSD1 by using phage M13KO7. The DNA oligonucleotide primer synthesized on a DNA Model 382 synthesizer (Applied Biosystems) was designed to create a new restriction site, *Sph*I, adjacent to the amino acid replacement site (Figure 1). This site of restriction cleavage was used for screening of the desired mutants. Site-directed mutagenesis was performed with the Amersham oligonculeotide-directed in vitro mutagenesis system version 2–1 kit.

RESULTS

Isolation of Thermostable Mutants. From the bacterial colonies carrying hydroxylamine-treated pGLf37 plasmid, the mutants which appear to have thermotolerant luciferase were screened as described under Experimental Procedures. Three clones, T-M-1, T-M-2, and T-M-3, were chosen for further experiments. Figure 2 shows the thermostabilities of crude luciferases from these strains. At pH 7.8, the wild-type luciferase was completely inactivated by heat treatment at 50 °C for 40 min. In contrast, over 30% of luciferase activity still remained in extracts from T-M-1, T-M-2, and T-M-3 under the same conditions.

In order to determine the nucleotide sequences of the thermostable luciferases, we isolated three plasmids (pTM1, pTM2, and pTM3) from T-M-1, T-M-2, and T-M-3, respectively. From the sequences of these plasmid DNAs, we found that all three mutants carried the same C to T base change at base 650 of the luciferase coding sequence (Table 1). This base transition resulted in the substitution of a Thr-(ACA) by an Ile(ATA) at position 217 in the amino acid sequence. Besides this missense mutation, pTM1 and pTM2 carried silent mutations: C to T transitions at positions 460 and 468 for pTM1 and at position 1140 for pTM2. On the other hand, pTM3 contained another missense mutation from His to Tyr at position 246. These results strongly suggest that the substitution of Thr to Ile at position 217, which is common in all of these mutants, is important for enhancing the thermostability of luciferase.

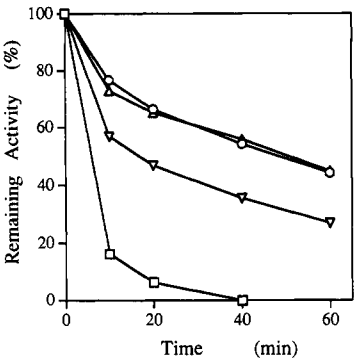

FIGURE 2: Thermostability of the crude wild-type and mutant luciferases. Cell-free extracts from the *E. coli* clone carrying wild-type luciferase (□) and those carrying the mutant enzymes T-M-1 (○), T-M-2 (△), and T-M-3 (▽) were incubated at 50 °C in 0.05 M phosphate buffer (pH 7.8) containing 10%-saturated ammonium sulfate and 0.2% (w/v) BSA. Remaining luciferase activities were determined at 10, 20, 40, and 60 min and are shown as the percentage of the activity at 0 min.

Table 1: DNA Sequence and Amino Acid Sequence Change in Mutants

mutant plasmid	base change	amino acid change
pTM1	C650 → T	Thr217 → Ile
	C460 → T	
	C468 → T	
pTM2	C650 → T	Thr217 → Ile
	C1140 → T	
pTM3	C650 → T	Thr217 → Ile
	C736 → T	His246 → Tyr

Comparison of the Thermostability of the Wild-Type and Thr217Ile Proteins. The wild-type luciferase and the mutant Thr217Ile luciferase from T-M-1 clone were purified to homogeneity as described under Experimental Procedures. The specific activities of the purified wild-type and Thr217Ile luciferases were 1.38×10^{11} and 1.81×10^{11} LU/mg, respectively. In the Thr217Ile mutant, the specific activity was 130% of the wild-type enzyme. To compare the thermostability of these enzymes, purified enzymes were incubated at 50 °C, pH 7.8, and residual luciferase activity was measured at various times. The Thr217Ile mutant showed greater thermostability than the wild-type luciferase (Figure 3). Its half-life at 50 °C was roughly 8–10 times loner than that of the wild-type luciferase. Moreover, we examined the pH stability of each luciferase. Thr217Ile luciferase was also much more stable than that of the wild-type over a wide pH range (data not shown).

Site-Directed Mutagenesis. In order to investigate the effect of amino acid residues at position 217 on the thermostability of enzyme, we replaced the Thr residue at position 217 of

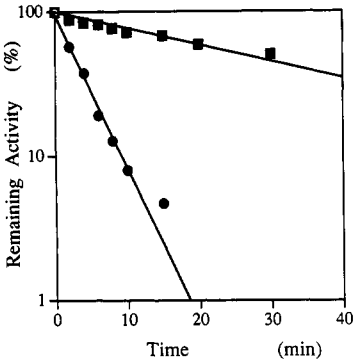

FIGURE 3: Time course of thermoinactivation at 50 °C of the purified wild-type and Thr217Ile mutant luciferases. The purified wild-type luciferase (●) and Thr217Ile variant (■) were incubated at 50 °C in 0.05 M phosphate buffer (pH 7.8) containing 10%-saturated ammonium sulfate and 0.2% (w/v) BSA. Remaining activities were determined and are shown as the percentage of the activity at 0 min.

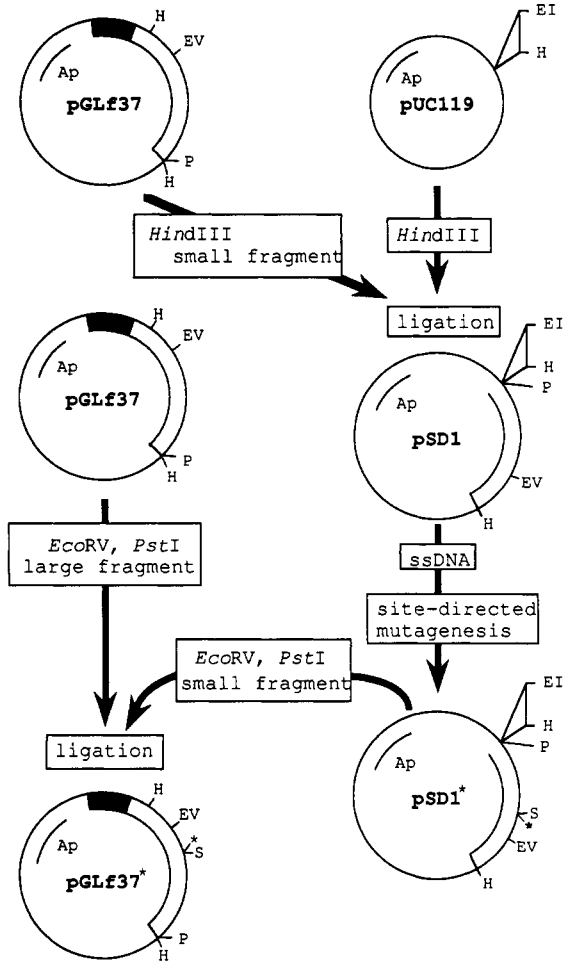

FIGURE 4: Construction of plasmids for site-directed mutagenesis and subsequent analysis. Details are discussed in the text. Clear and solid portions show the luciferase cDNA and the trp promoter, respectively. Mutants carrying mutations in luciferase cDNA are shown by asterisks. H, *Hin*dIII; EV, *Eco*RV; P, *Pst*I; EI, *Eco*RI; S, *Sph*I; ssDNA, single-stranded DNA; Ap, ampicillin resistance gene.

luciferase with all of the rest of the possible amino acid residues by site-directed mutagenesis. The general scheme of oligonucleotide site-directed mutagenesis is shown in Figure 4. We constructed pSD1, a plasmid which produces single-stranded DNA in *E. coli* JM101. Mutations were introduced at position 217 in the luciferase cDNA, using a synthetic oligonucleotide primer having the sequence shown in Figure 1. Mutants were identified by *Sph*I digestion, and their nucleotide sequences

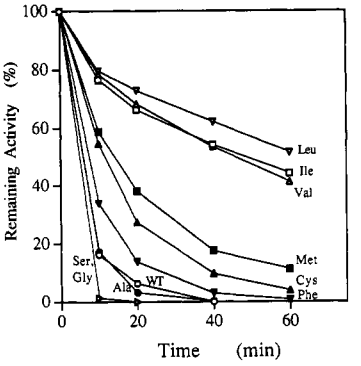

FIGURE 5: Thermostability of mutant luciferases. Cell-free extracts from *E. coli* harboring each of the mutant luciferases were incubated at 50 °C in 0.05 M phosphate buffer (pH 7.8) containing 10%-saturated ammonium sulfate and 0.2% (w/v) BSA. Remaining luciferase activities were determined at 10, 20, 40, and 60 min and are shown as the percentage of the activity at 0 min. Symbols associated with each experimental point are defined within the figure. The expression yields of 10 variants, which have Arg, Lys, His, Asp, Glu, Asn, Gln, Trp, Tyr, and Pro in place of Thr in the wild-type, were less than 0.1% of that obtained from the transformant harboring the wild-type plasmid pGLf37.

Table 2: Correlation between the Thermostability of the Wild-Type and Mutant Luciferases and the Hydrophobicity of the Substituting Residues at Position 217[a]

amino acid	remaining activity (%)	hydropathy index
isoleucine	76.6	4.5
valine	78.5	4.2
leucine	79.6	3.8
phenylalanine	33.9	2.8
cysteine	54.6	2.5
methionine	58.8	1.9
alanine	17.2	1.8
glycine	1.3	−0.4
threonine	16.2	−0.7
serine	2.4	−0.8

[a] Hydropathy index values are taken from Kyte and Doolittle's estimation (1982). The remaining activity after heating at 50 °C for 10 min was assayed as described under Experimental Procedures and is shown as the percent ratio to the activity at 0 min.

were determined. To examine the effects of these mutations on the thermostability of enzyme, we replaced the small fragment digested with restriction enzymes *Eco*RV and *Pst*I with that of the original plasmid pGLf37 (Figure 4). Ampicillin-resistant colonies were selected, and the mutations at position 217 were reconfirmed by sequencing. Thus, we constructed mutants in which the amino acid at position 217 was substituted by each of the remaining 18 different amino acid residues.

Thermostability of Mutant Proteins. The cells harboring each of the 18 kinds of mutant plasmids were cultured in LB broth containing ampicillin at 37 °C for 6 h, and luciferase activities in cell-free extracts were tested. For comparison of the thermostability of mutant luciferases, cell-free extracts of each mutant enzyme were incubated at 50 °C, and residual activity was measured at various times (Figure 5). After 10-min incubation, the wild-type enzyme was inactivated to only 16.2% of the initial activity. The Thr217Ala mutant was inactivated at the same rate as the wild-type. Under the same experimental conditions, the mutant enzymes with Leu, Val, Cys, Met, and Phe at position 217 were more thermostable than the wild-type luciferase. Especially Thr217Leu and Thr217Val luciferases still maintained over 75% of their initial activities after 10 min at 50 °C. Table 2 shows the correlation between the thermostability of the wild-type and mutant proteins and the hydrophobicity of the substituting residues

at position 217. When the amino acid at position 217 was replaced with hydrophobic residues, which have positive values of the hydropathy index, all of the mutant enzymes had increased thermostability compared with the wild-type enzyme. These data suggest that the stability of luciferase may have a high correlation with the hydrophobicity of the amino acid residues at position 217.

DISCUSSION

Mutagenesis of firefly luciferase cDNA in pGLf37 plasmid was induced with hydroxylamine, and from about 1000 transformants, 3 thermostable mutants were isolated. Sequencing of the cDNAs encoding these mutant proteins revealed that all of the mutants carried a C to T transition mutation at the same position. The distinct enhancement of the thermostability of luciferase was ascribed to the replacement of Thr217 by Ile among the 548 amino acid residues of the enzyme. In general, a globular protein is stabilized by hydrophobic residues in its interior (Janin, 1979; Yutani et al., 1987). The stability of luciferase may also correlate with the hydrophobicity of the amino acid residues at position 217. To confirm this, we constructed 18 mutants of luciferase by substituting Thr217 with 18 different amino acid residues and analyzed the effect of the mutations on thermostability. There are different scales for hydrophobity for amino acid residues (Janin, 1979; Rose & Roy, 1980; Hopp & Woods, 1981; Kyte & Doolittle, 1982). Using the scale as described by Kyte and Doolittle (1982), we compared the relationship of the hydrophobicity of residues at position 217 and protein stability (Table 2). We found that there is a clear correlation between residue hydrophobicity and protein thermostability. When Thr at position 217 was replaced with Ile, Leu, Val, Cys, Phe, and Met, which have greater hydrophobicity than Thr, each of the mutant enzymes had increased thermostability compared with the parent enzyme. These data suggest that the local hydrophobic interactions at position 217 directly contribute to the overall thermostability of firefly luciferase.

Yutani et al. (1987) constructed mutant proteins substituted by each of 19 amino acid residues at position 49 of the tryptophan synthase α subunit in order to elucidate the role of individual amino acid residues in the conformational stability of a protein. Very similar to our present results, they reported that the enzyme easily become more stable or less stable to denaturant as a result of single amino acid substitution and the stability of the enzyme tends to increase linearly with the hydrophobiity of the substituting residues.

The amino acid residues that interact to form the hydrophobic core of a protein clearly play a key role in determining the protein's structure. In the N-terminal domain of the λ-repressor, seven buried amino acids form a hydrophobic core. In order to elucidate the relationship between the core sequence and the protein's structure, Lim and Sauer (1989) constructed mutant proteins in which seven amino acid residues had been replaced with random sets of residues by using the technique of cassette mutagenesis. The cores of all mutants that retain some activities were composed almost entirely of the eight amino acids Ala, Cys, Thr, Val, Ile, Leu, Met, and Phe. Most of these residues have positive values of the hydropathy index. They suggested that hydrophobicity is the most essential feature of a core sequence. Since the firefly luciferase mutants with these eight amino acids at position 217 have some activities, residue 217 may contribute to the major hydrophobic core. Furthermore, the fact that hydrophilic and large hydrophobic (Trp and Tyr) substitutions resulted in an expression yield >1000-fold less than wild-type is also consistent with residue 217 being in a buried, hydrophobic environment.

In the absence of structural information on a specific protein, random mutagenesis is a very efficient method to obtain mutant proteins. However, the problem with most random mutations is that they are silent. Therefore, an approach that increases the probability of isolating the thermostable variants from a library of random mutants is necessary. The screening system used here, a method to detect luminescence photographically from colonies of *E. coli* in which thermostable firefly luciferase may be expressed, is very simple and effective. The ATP needed for the luminescent reaction is provided by the cells themselves, and O_2 is freely permeable into cells. Also, at acid pH, luciferin is also penetrable to the membranes of *E. coli*. Thus, the luminescent reaction can be initiated *in vitro* simply by adding an acid solution of luciferin (Wood & DeLuca, 1987). However, this luminescence was not bright enough to be observed directly. Therefore, we used X-ray film to record the dim luminescence. By the combination of random mutagenesis and this screening system, we succeeded in isolating three thermostable mutants efficiently and sensitively.

In this report, we have shown that replacement of Thr by a hydrophobic amino acid residue at position 217 is important for enhancing the thermostability of firefly luciferase. However, since hydroxylamine used in this study causes only GC to AT transition mutations, all possible amino acid substitutions could not be tested. To overcome this problem, Myers et al. (1985) used the method of treating single-stranded DNA with nitrous acid, formic acid, or hydrazine, followed by synthesis of the complementary strand with reverse transcriptase. Moreover, a simple *in vitro* mutagenesis method for the generation of random mutations using the polymerase chain reaction is described by Leung et al. (1989). Attempts at mutagenesis using these techniques may allow the isolation of other thermostable mutants.

The thermostability of the Thr217Leu mutant which was constructed by site-directed mutagenesis was superior to that of Thr217Ile. To our knowledge, the Thr217Leu variant is the most thermostable luciferase obtained so far. In Japan, there are two well-known fireflies, *Luciola cruciata* and *Luciola lateralis* ("Heike-botaru" in Japanese). We have already purified luciferases from these fireflies and found that the luciferase of *L. lateralis* is superior to that of *L. cruciata* (which was used in this study) in thermostability (Kajiyama et al., 1992). Recently, we have cloned and analyzed a cDNA encoding the luciferase of *L. lateralis* (Tatsumi et al., 1992). The primary structure of this luciferase deduced from the nucleotide sequence consists of 548 amino acids which is the same as that of *L. cruciata* luciferase. Also, the amino acid sequences of the two luciferases were 94% identical. Especially, the area from positions 190 to 238 including the amino acid residue at position 217, which is important for thermostability of *L. cruciata*, is well conserved between these two luciferases. If the amino acid residue at position 217 of *L. lateralis* luciferase is substituted by a hydrophobic amino acid, its thermostability may be superior to that of *L. cruciata* Thr217Leu mutant. The study to enhance the thermostability of *L. lateralis* luciferase is now in progress.

ACKNOWLEDGMENT

We thank Mrs. Kazuko Saitoh for her technical assistance.

REFERENCES

Argos, P., Rossmann, M. G., Grau, U. M., Zuber, H., Frank, G., & Tratschin, J. D. (1979) *Biochemistry 18*, 5698–5703.

DeLuca, M., & McElroy, W. D. (1978) *Methods Enzymol. 57*, 3–15.

Hopp, T. P., & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3824.

Imanaka, T., Shibazaki, M., & Takagi, M. (1986) *Nature (London) 324*, 695–697.

Janin, J. (1979) *Nature (London) 277*, 491.

Kajiyama, N., & Nakano, E. (1991) *Protein Eng. 4*, 691–693.

Kajiyama, N., Masuda, T., Tatsumi, H., & Nakano, E. (1992) *Biochim. Biophys. Acta 1120*, 228–232.

Kironde, F. A. S., Parsonage, D., & Senior, A. E. (1989) *Biochem. J. 259*, 421–426.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Leung, D. W., Chen, E., & Goeddel, D. V. (1989) *Technique 1*, 11–15.

Lim, W. A., & Sauer, R. T. (1989) *Nature (London) 339*, 31–36.

Ludin, A. (1981) in *Bioluminescence and Chemiluminescence* (DeLuca, M., & McElroy, W. D., Eds.) pp 187–196, Academic Press, New York.

Masuda, T., Tatsumi, H., & Nakano, E. (1989) *Gene 77*, 265–270.

Matsumura, M., Becktel, W. J., Levitt, M., & Matthews, B. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6562–6566.

Myers, R. M., Lerman, L. S., & Maniatis, T. (1985) *Science 229*, 242–247.

Neethling, J. B., Jhonson, K. M., & Jenkins, D. (1985) *J.—Water Pollut. Control Fed. 57*, 890–894.

Perutz, M. F., & Raidt, H. (1975) *Nature (London) 225*, 256–259.

Quax, W. J., Mrabet, N. T., Luiten, R. G. M., Schuurhuizen, P. W., Stanssens, P., & Laster, I. (1991) *Bio/Technology 9*, 738–742.

Rose, G. D., & Roy, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4643.

Seliger, H. H., & McElroy, W. D. (1960) *Arch. Biochem. Biophys., 88*, 136.

Sharpe, A. N., Woodrow, M. N., & Jackson, A. K. (1970) *J. Appl. Bacteriol. 33*, 758–767.

Tatsumi, H., Kajiyama, N., & Nakano, E. (1992) *Biochim. Biophys. Acta 1131*, 161–165.

Wannlund, J., & DeLuca, M. (1982) *Anal. Biochem. 122*, 358–393.

Wetzel, R., Perry, L. J., Baase, W. A., & Becktel, W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 401–405.

Wood, K. V., & DeLuca, M. (1987) *Anal. Biochem. 161*, 501–507.

Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103.

Yutani, K., Ogasahara, K., Tsujita, T., & Sugiono, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4441–4444.