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Effects of 3′ End Deletions from the *Vibrio harveyi luxB* Gene on Luciferase Subunit Folding and Enzyme Assembly: Generation of Temperature-Sensitive Polypeptide Folding Mutants†

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ABSTRACT: Ten recombinant plasmids have been constructed by deletion of specific regions from the plasmid pTB7 that carries the *luxA* and *luxB* genes, encoding the α and β subunits of luciferase from *Vibrio harveyi*, such that luciferases with normal α subunits and variant β subunits were produced in *Escherichia coli* cells carrying the recombinant plasmids. The original plasmid, which conferred bioluminescence (upon addition of exogenous aldehyde substrate) on *E. coli* carrying it, was constructed by insertion of a 4.0-kb *Hin*dIII fragment of *V. harveyi* DNA into the *Hin*dIII site of plasmid pBR322 [Baldwin, T. O., Berends, T., Bunch, T. A., Holzman, T. F., Rausch, S. K., Shamansky, L., Treat, M. L., & Ziegler, M. M. (1984) *Biochemistry 23*, 3663–3667]. Deletion mutants in the 3′ region of *luxB* were divided into three groups: (A) those with deletions in the 3′ untranslated region that left the coding sequences intact, (B) those that left the 3′ untranslated sequences intact but deleted short stretches of the 3′ coding region of the β subunit, and (C) those for which the 3′ deletions extended from the untranslated region into the coding sequences. Analysis of the expression of luciferase from these variant plasmids has demonstrated two points concerning the synthesis of luciferase subunits and the assembly of those subunits into active luciferase in *E. coli*. First, deletion of DNA sequences 3′ to the translational open reading frame of the β subunit that contain a potential stem and loop structure resulted in dramatic reduction in the level of accumulation of active luciferase in cells carrying the variant plasmids, even though the *luxAB* coding regions remained intact. Second, the C-terminal ca. 10–15 residues of the β subunit appeared to have little to do with the structure or stability of the active heterodimeric form of the luciferase, but deletion of amino acid residues from this region resulted in greatly reduced levels of accumulation of active heterodimeric luciferase, especially at higher temperatures. The active luciferase that did form from the truncated β subunit (β^t) constructions had essentially normal activity and stability but impaired ability to refold from urea. As with the apparent temperature sensitivity of accumulation of active luciferase in vivo, these variants showed temperature sensitivity in refolding from urea in vitro. We conclude that the carboxyl-terminal region of the β subunit has little to do with the bioluminescence reaction or stability of the dimeric structure per se, but it does appear to play a critical function in proper folding and/or assembly into the active dimeric structure.

Many proteins exhibit structural domains that appear to fold independently within the constraints imposed by the covalent continuity of the peptide chain. That is, individual domains appear in many cases to contain the necessary information to fold properly, independent of the remainder of the protein. Extension of these ideas to a multisubunit protein would suggest that the individual subunits should fold independently, thereby generating interacting surfaces that lead to subunit interfaces in much the same way that structural domains of a single polypeptide interact (Wetlaufer, 1981). One of the more exciting developments over the past decade in the protein folding field comes from the work of King and his colleagues (Smith et al., 1980; Goldenberg & King, 1982; Yu & King, 1984) with the temperature-sensitive folding mutants of the phage P22 tail spike protein, a homotrimer that forms first as an unstable "protrimer" that can be trapped in the cold prior to rearrangements which lead to the highly stabilized trimeric tail spike. The temperature-sensitive folding mutants fail to form the normal yield of the stable trimeric structure at the nonpermissive temperature but do form the mature tail spike structures having normal stability if the cells are grown at the permissive temperature. These temperature-sensitive folding mutants are distinctly different from the classic temperature-sensitive mutant for which the protein formed at the permissive temperature is thermally labile. The temperature-sensitive folding mutants that have been inves-

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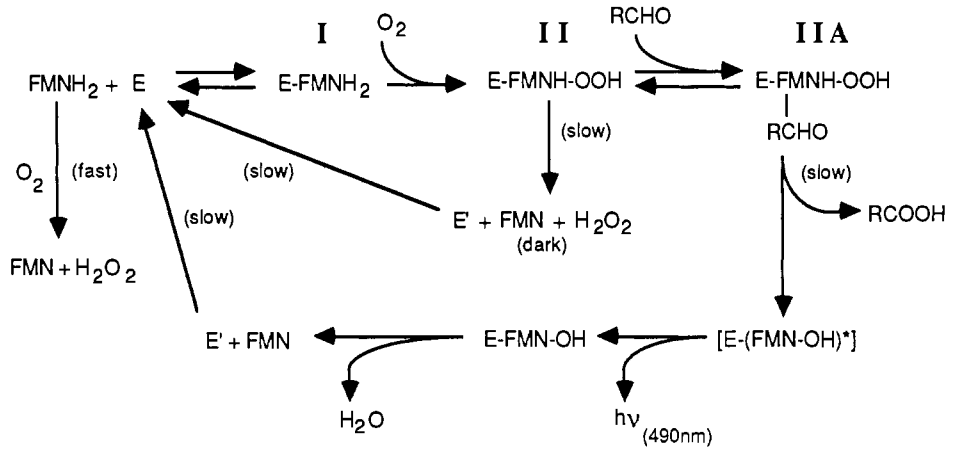
Scheme I



tigated by King and his colleagues apparently suffer an accelerated off-pathway aggregation process that effectively competes with the normal folding pathway at the nonpermissive temperature (Yu & King, 1984).

We have recently begun to study the folding and association of the subunits of bacterial luciferase and have obtained evidence that the two subunits, α and β, interact initially as partially folded structures, with the final steps of folding occurring in the heterodimeric structure (Waddle et al., 1987). Bacterial luciferase is a flavin monooxygenase that catalyzes the light-emitting reaction in luminous bacteria. Three substrates, molecular oxygen, a long-chain aldehyde, and reduced flavin mononucleotide, are required in the overall reaction (Ziegler & Baldwin, 1981). AbouKhair et al. (1985) have obtained evidence that following enzymatic turnover luciferase is in an altered conformational state (E′ in Scheme I), which slowly relaxes to the native conformation.

The enzyme consists of two apparently homologous subunits, α and β, of similar size [40 108 and 36 349 daltons, respectively (Cohn et al., 1985; Johnston et al., 1986)]. Analyses of mutant enzymes and chemical modification studies indicate that the single active center resides primarily, if not exclusively, on the α subunit [see Ziegler and Baldwin (1981) for a review]; however, the β subunit is absolutely required for the bioluminescence activity. The luciferase structural genes from *Vibrio harveyi* have been cloned (Cohn et al., 1983; Baldwin et al., 1984; Belas et al., 1982) and the complete nucleotide sequences of *luxA* (Cohn et al., 1985) and *luxB* (Johnston et al., 1986) reported. The two subunits appear to be translated from polycistronic mRNA (Miyamoto et al., 1985).

The important question of subunit folding and dimerization can be approached by studying bacterial luciferase since the enzyme is a heterodimer; folding of the individual subunits and assembly of the biologically active heterodimer can be studied as independent, experimentally distinguishable steps. Recent experiments from this laboratory show that the processes of subunit folding and dimerization in bacterial luciferase occur by a concerted mechanism (Waddle et al., 1987). That is, the encounter complex formed between α and β subunits appears to occur between partially folded subunits, with the final steps of folding that lead to active dimeric enzyme occurring in the heterodimeric state. Subunits that are allowed to complete the folding process in vivo as separate subunits are effectively incapable of dimerization without prior treatment with urea.

The purpose of this investigation was to begin to develop a detailed understanding of the processes of subunit folding and assembly of dimeric luciferase in vivo. The experiments reported here were initiated to answer the following specific questions: First, can sequences from the carboxyl terminus of the β subunit be deleted without loss of enzymatic activity? Second, can deletion of sequences from the carboxyl terminus of the β subunit reduce or eliminate enzymatic activity without eliminating the ability of the α subunit to associate with the truncated β subunit (β^t)? These questions stem from earlier work in which we reported that cells carrying the *luxA* gene and only a short region of the *luxB* gene did not accumulate free α subunit (Baldwin et al., 1984). We suggested two possible explanations: (a) free α subunit might not be stable in *Escherichia coli* or (b) the mRNA produced might not be stable. Other investigators agreed with the former suggestion (Gupta et al., 1985). However, our recent investigations (Waddle et al., 1987) as well as the studies reported here disprove the former hypothesis: free α subunit *is* sufficiently stable in *E. coli* to accumulate to levels that can be detected by gel electrophoresis. The second hypothesis, instability of the mRNA with 3′ untranslated sequences deleted, is supported by the observations reported here.

To modify the sequence of the β subunit, we used plasmid pTB7 (Baldwin et al., 1984) which contains the intact *luxA* and *luxB* genes from *V. harveyi* on a 4.0-kb *Hin*dIII fragment in pBR322 as the parent plasmid for construction of a family of deletion variants. In this paper, we report the importance of the carboxyl-terminal region of the luciferase β subunit and its downstream untranslated DNA sequences to the expression of luminescence in *E. coli*.

Materials and Methods

Enzymes and Chemicals. Enzymes for the construction of recombinant plasmids were purchased from New England Biolabs, Bethesda Research Laboratories, and Boehringer Mannheim Biochemicals. Reagents for DNA sequence analysis by the procedure of Maxam and Gilbert (1977) were purchased from Aldrich and radiolabeled nucleotides from New England Nuclear and ICN Biomedicals. FMN and *n*-decanal were obtained from Sigma, 1-chloro-4-naphthol was from Bio-Rad, sodium dithionite was from Fisher Scientific, TPCK-treated trypsin and α-chymotrypsin were from Worthington, dithiothreitol (DTT) was from Aldrich, and Ultra Pure urea was from Schwarz/Mann. All other reagents, salts, and materials were of the highest quality commercially available.

Construction of Recombinant Plasmids and Strains. The *Escherichia coli* strain TB1 was used in all cloning work. TB1 is an r^-m^+ derivative of JM83 (Vieira & Messing, 1980) constructed in this laboratory by P1 transduction with MM294

(Meselson, 1980) as donor. Plasmid pJS2 was constructed by digestion of pTB7 (Baldwin et al., 1984) with *Cla*I, treatment with Klenow fragment to generate blunt ends, and religation of the plasmid. The plasmid picked for characterization had suffered apparent exonuclease activity, since two bases were deleted from the pBR322 side of the fusion (see Figures 1 and 2). The plasmid pJS4 was constructed from pTB7 by deleting ca. 1.4 kbp from the *Cla*I site (141 bp downstream from the *luxB* termination codon) to the *Cla*I site in pBR322. The plasmid pJS9 was constructed from pJS4 by deleting the 84-bp fragment from the *Hin*pI site (57 bp downstream from *luxB*) to the ClaI site (141 bp downstream from *luxB*). The plasmid pJS22 was constructed from pJS4 by deleting the 137-bp fragment from the *Mae*II site (4 bp downstream from *luxB*) to the *Cla*I site (141 bp downstream from *luxB*). Plasmid pJS23 was constructed from pJS4 by deleting the 177-bp *Cla*I to *Cla*I fragment and inserting the 49-bp *Mae*II to *Cla*I fragment in reversed orientation. All other plasmids were constructed from pJS9. Plasmid pJS12 was constructed by filling in the ends generated by *Cla*I with Klenow fragment and religating. The plasmid pJS17 was constructed by deleting the single-stranded ends generated by *Sac*II with S1 nuclease and religating. Plasmid pJS18 was constructed by deleting the 34-bp *Taq*I to *Cla*I fragment. The modified regions of all constructions were verified by DNA sequence analysis according the method of Maxam and Gilbert (1977).

Assay of Luciferase. Luciferase activity was measured with a photomultiplier–photometer (Mitchell & Hastings, 1971) calibrated with the light standard of Hastings and Weber (1963). Light measurements of cells during growth were made following injection of 1 mL of a sonicated suspension of 0.001% (v/v) *n*-decanal in LB medium into 1-mL samples removed from the flasks. Luciferase assays were performed in vitro by the standard $FMNH_2$ injection assay (Hastings et al., 1978). The modified dithionite assay (Meighen & Hastings, 1971; Meighen & MacKenzie, 1973) was employed to determine the dissociation constant for reduced flavin mononucleotide. Bioluminescence decay rates were determined from light intensities monitored by an IBM AT computer system.

Western Blot Analysis. Anti-luciferase IgG was prepared as previously described (Reeve & Baldwin, 1982). Lysates from *E. coli* were prepared by collecting cells by centrifugation of 20-mL samples at 20 °C, resuspending cells in 2.0 mL of 0.2 M phosphate buffer, pH 7.0, and disrupting the cells in a French pressure cell (SLM/AMINCO). Lysates were clarified by centrifugation. Samples were removed and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Laemmli, 1970). Protein bands were transferred electrophoretically from the acrylamide gel to nitrocellulose (Bittner et al., 1980). The nitrocellulose was equilibrated with the anti-luciferase IgG and then with IgG-conjugated horseradish peroxidase. The peroxidase activity was detected by use of 4-chloro-1-naphthol.

Purification of Mutant Luciferases. Lysates were prepared as described above, and protein precipitating upon addition of ammonium sulfate between 40% and 75% of saturation at 4 °C was collected and dialyzed against 0.25 M phosphate, 0.5 mM DTT, pH 7.0. The protein was then subjected to ion-exchange chromatography on DEAE-Sephadex A-50 and on aminohexyl-Sepharose 4B, as described (Hastings et al., 1978). A high molecular weight contaminating protein was largely removed by a final Sephadex G-100 chromatography step.

Renaturation from Urea and Subunit Complementation. Luciferases (either pure or in lysates) were denatured by dilution (1:4) into 10 M urea, yielding final solution conditions of 8 M urea and 1 mM DTT. Samples were incubated 30 min at 20 °C and then diluted 1:50 into renaturation buffer [1 mM DTT, 0.1% bovine serum albumin (BSA) in various concentrations of phosphate buffer, pH 7.0]. Renaturation reactions were allowed to proceed for 24 h at 20 °C or 45 min at 37 °C.

To determine the relative levels of expression of α subunit from the various constructions, lysates were prepared as described and mixed with a 10-fold excess (relative to the luciferase content of cells carrying plasmid pTB7) of luciferase purified from AK-6, a mutant of *V. harveyi* having a lesion in the α subunit resulting in a dramatic loss in bioluminescence activity but possessing wild-type β subunit (Cline & Hastings, 1972, 1974). The AK-6 luciferase and the variant luciferase being analyzed were denatured by treatment with 8 M urea and allowed to renature for 72 h at 8 °C, as described above.

Analysis of Protease Lability and Thermal Stability. Luciferases from all species of luminous bacteria that have been studied are extremely sensitive to the action of proteases (Holzman & Baldwin, 1980a). To determine whether the altered luciferases studied here had altered protease sensitivity, wild-type and variant luciferases were treated with trypsin and with chymotrypsin by methods that have been described in detail (Holzman & Baldwin, 1980a,b; Baldwin & Riley, 1980; Holzman et al., 1980). Protease inactivation studies were carried out at 25 °C in 0.02 M phosphate and in 0.2 M phosphate at pH 7.0 (Baldwin & Riley, 1980; Holzman et al., 1980). Luciferase concentrations were 0.1 mg/mL, and protease concentrations were 16 μg/mL. Aliquots were removed as a function of time, and the luciferase activity remaining was determined by the flavin injection assay.

Thermal inactivation studies were conducted by incubation of the wild-type and variant luciferases in phosphate buffers (0.02–0.5 M, pH 7.0) at elevated temperatures. Reactions were initiated by dilution of luciferase 1:100 (final concentration of 16 μg/mL) into buffer preequilibrated at the experimental temperature. The inactivation process was monitored by dilution of luciferase into room temperature assay buffer; the remaining luciferase activity was determined by the flavin injection assay.

RESULTS

A series of 10 variant plasmids were constructed from pTB7 as described under Materials and Methods. They have been divided into three groups, A, B, and C. Group A variants (pJS4, pJS9, and pJS22) have intact *luxB* coding sequences; deletions were made in the regions 3′ to the β subunit coding region, in the region of *luxE*. Group B variants (pJS12, pJS13, pJS17, pJS18, and pJS19) have deletions in the 3′ end of the *luxB* coding region but leave the sequences immediately downstream from the coding region intact. These deletions result in luciferases having β subunit C-terminal sequences of varying lengths that differ from the normal β subunit C-terminal amino acid sequence, due to fusions of sequences containing translation termination signals downstream from the point of fusion. Group C variants (pJS2 and pJS23) lack both the downstream sequences and the normal translational stop signal. The construction of these plasmids is shown schematically in Figure 1, the nucleotide sequence of the modified portion of each is given in Figure 2, and the encoded amino acid sequence of each is given in Figure 3.

Effects of Temperature on Luminescence in Vivo, Accumulation of α Subunit, and Luciferase Stability in Vitro.

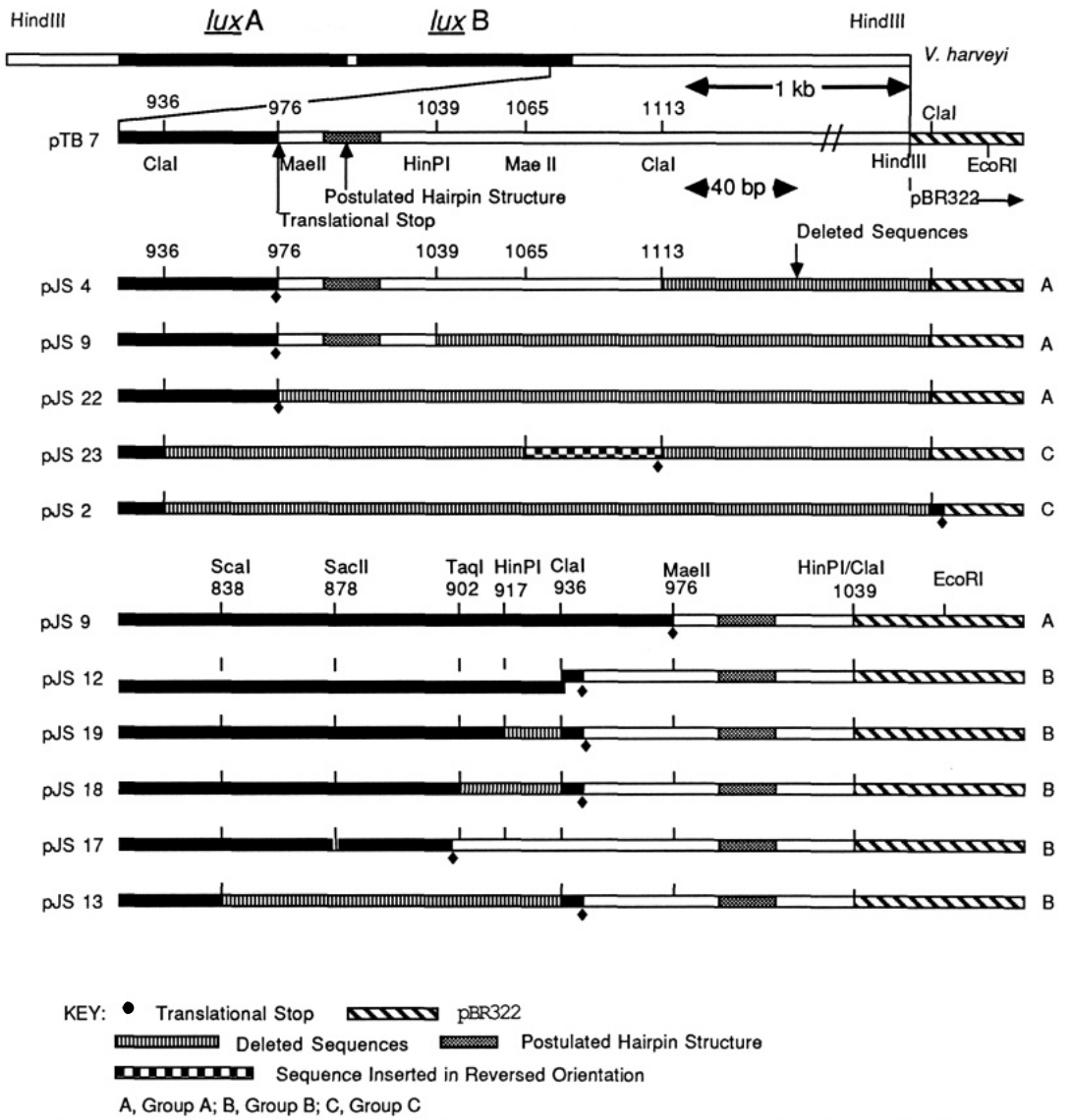


FIGURE 1: Schematic representation of the deletion strategy that was employed to construct the plasmids used in this study. Plasmids pJS4, pJS9, pJS22, pJS23, and pJS2 were constructed by deletion of the indicated regions from pTB7. Plasmids pJS12, pJS19, pJS18, pJS17, and pJS13 were constructed by deletion of the indicated sequences from pJS9. The β subunit coding region is indicated by filled bars. All fusions were determined by DNA sequencing; the sequence of the fused regions is presented in Figure 2.

Relative peak luminescence in vivo of TB1 cells carrying the variant recombinant plasmids grown at 20 and at 37 °C is presented in Table I, column 2. All of the variants produced less luminescence than pTB7 at both temperatures except pJS4 and pJS9, which were identical with pTB7 in luminescence. The luminescence of pJS22 was decreased by 75% at both temperatures relative to pTB7, even though the luciferase encoded by the variant plasmid was the same as that encoded by pTB7 (group A), suggesting that sequences 3′ to the β subunit coding region are critical for high-level expression. The expression of luminescence from cells carrying pJS22 was similar to that from cells carrying pJS23, even though the carboxyl-terminal 12 residues of the β subunit encoded by pJS23 were different from those of the wild type. The β subunit encoded by pJS23 was 1 residue longer than the 324-residue wild-type β subunit encoded by pJS22 and pTB7. A similarity between pJS22 and pJS23 is that they both lack a sequence 3′ to the *luxB* coding region, spanning bases 992-1011 (Johnston et al., 1986), that has a strong potential for secondary structure formation (Figure 4). It would appear that it is these sequences, and/or others contained within the *Mae*II (base 976) to *Hin*PI (base 1029) fragment that was deleted from pJS9 to construct pJS22, that result in reduction in pJS22 of ca. 75% of the luminescence expression in vivo relative to that of pJS9 at both 20 and 37 °C. The ratio of luminescence at 37 °C to the luminescence at 20 °C is recorded in column 3 of Table I. While pJS23, with an altered carboxyl-terminal amino acid sequence one residue longer than that of the wild type, showed lower luminescence at both temperatures than did the wild-type enzyme (as did pJS22, which encoded the wild-type sequence), the ratio of luminescence at 37 °C to luminescence at 20 °C was essentially the same for pJS23 (group C), pJS22 and the other group A variants, and the wild type. All of the variants in groups B and C except pJS23 showed a marked temperature effect, relative to the wild type, in expression of luminescence in vivo. All of the group B and C variants except pJS23 had truncated β subunits (β^t). It is interesting to note that cells carrying pJS12, which encodes a β^t subunit of 315 amino acid residues (cf. wild-type 324 residues), exhibit ca. 75% of the wild-type (pTB7) level of bioluminescence at 20 °C, while the luminescence at 37 °C is less than 10% that of cells carrying the wild-type plasmid.

The results presented in Table I could be due to changes in levels of luciferase accumulation and/or differences in luciferase activity. Decreased accumulation of active luciferase

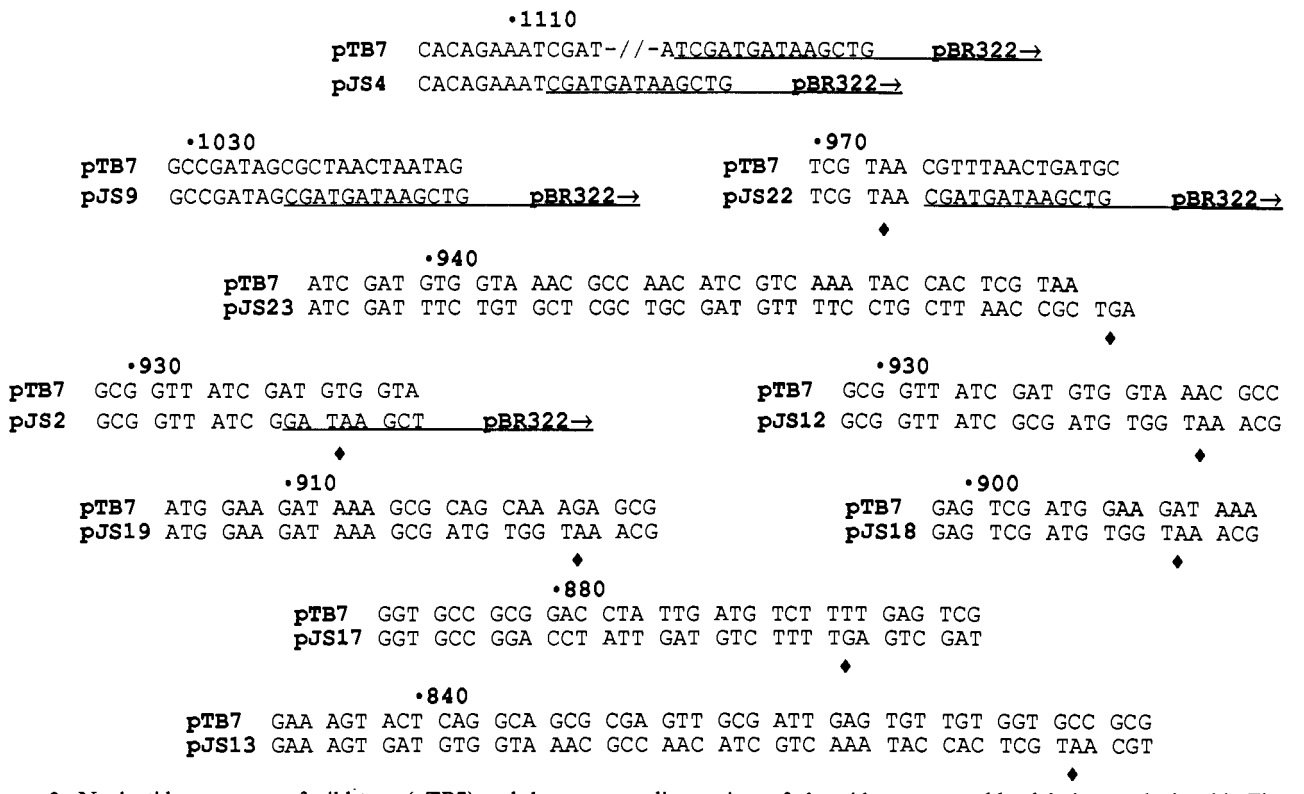


FIGURE 2: Nucleotide sequences of wild-type (pTB7) and the corresponding regions of plasmids constructed by deletion, as depicted in Figure 1. The numbers above the pTB7 sequences are relative to the ATG sequence signaling the beginning of the *luxB* gene (Johnston et al., 1986).

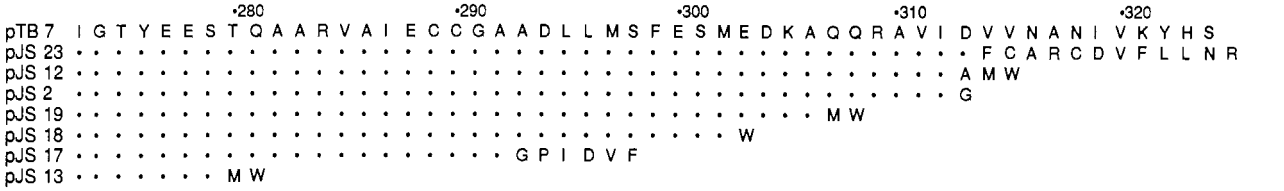


FIGURE 3: Sequence of amino acid residues at the carboxyl-terminal end of the wild-type and mutant β subunits. The sequence of residue 273 through the carboxyl terminus (residue 324) of the wild-type subunit is given in the single-letter code. For the mutants, the residues that are identical with those of the wild-type are designated by (•), with the altered positions resulting from the fusions presented in the single-letter abbreviations.

Table I: Comparison of Relative Peak Luminescence in Vivo with α Subunit Accumulation and Enzyme Stability in Vitro

(1) plasmid	group[a]	(2) peak luminescence[b] 20 °C	(2) peak luminescence[b] 37 °C	(3) ratio (37 °C/20 °C)	(4) relative accumulation of α subunit[c] 20 °C	(4) relative accumulation of α subunit[c] 37 °C	(5) half-life in vitro (h, 37 °C)[d]
pTB7	wild type	100	20	0.20	100	100	8.5
pJS4	A	96	19.6	0.20	96	96	
pJS9	A	100	19.4	0.19	98	86	
pJS22	A	27.8	7.4	0.27	23	30	
pJS23	C	27.2	4.4	0.16	29	27	8.0
pJS12	B	74.9	1.6	0.02	76	42	5.8
pJS2	C	0.25	0.01	0.04	30	18	5.3
pJS19	B	0.24	0.004	0.02	69	43	4.8
pJS18	B	0.08	0.002	0.03	63	40	6.2
pJS17	B	0.02	ND[e]		32	22	
pJS13	B	ND	ND		55	43	

[a] Group A plasmids have intact *luxB* coding sequences, with deletions in the 3′ untranslated region; group B variants have deletions in the 3′ end of the *luxB* coding region, with downstream untranslated sequences intact; group C variants lack both the downstream sequences and the normal 3′-terminal *luxB* coding sequences. See Figure 1 for plasmid constructions. [b] Luminescence was stimulated by injection of 1 mL of 0.001% *n*-decanal in LB medium into 1 mL of cell suspension. All measurements are relative to the wild-type enzyme at 20 °C. [c] The amount of α subunit present in a lysate was determined by addition of excess β subunit, denaturation by addition of urea, and renaturation of luciferase by dilution of the urea. At each growth temperature, the expression of α subunit for each mutant at each temperature was taken relative to that of wild type at that temperature. (See Materials and Methods for details.) [d] Centrifuged lysates in 0.2 M phosphate, 0.5 mM DTT, pH 7.0, of cells grown at 20 °C were incubated at 37 °C and luciferase activity measurements recorded over a 24-h period. [e] ND, not determined.

could be due to decreased synthesis, increased degradation of either the α or β subunit (or both), or thermal instability of the variant forms of the luciferase. To distinguish between the temperature dependence of luciferase synthesis and luciferase thermal stability, extracts were prepared of group B and group C cells, both grown at 20 °C. The extracts were

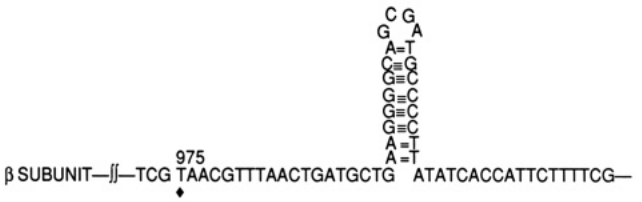


FIGURE 4: Region of potential secondary structure 3′ to the β subunit open reading frame in *V. harveyi*. The sequence shown is from base 970 to base 1029 [base 1 is at the start codon for *luxB* (Johnston et al., 1986)]. TAA at bases 973–975 signals the stop of the β subunit.

then incubated at 37 °C and luciferase activity measurements taken for a period spanning over 24 h. The approximate half-times for inactivation of the enzymes at 37 °C are presented in Table I, column 5. It is clear from the results of this experiment that decreased thermal stability of the luciferases is not a primary contributor to the lower level of expression of luminescence in vivo at 37 °C displayed by these variants; rather, the decreased level of luminescence must be due to some other factor related to luciferase synthesis or folding.

Since the activity of bacterial luciferase requires the collaboration of both subunits, a lower level of activity could be due to decreased levels of a single subunit. To investigate the possibility that the lower level of activity was due to decreased expression of the variant β subunits relative to α subunit, the lysates of cells carrying the various plasmids were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis. Photographs of the blots are presented in Figure 5. Panel A is the blot of lysates of cells grown at 20 °C, while panel B is the blot from cells grown at 37 °C. Lanes 1 and 11 contain lysates from cells carrying pTB7, lanes 2–10 contain lysates from cells carrying variant plasmids, and lane 12 contains lysate from *E. coli* strain TB1, demonstrating that the bands labeled N.L. (non-luciferase) were from *E. coli* proteins. For rough quantitation of the relative amounts of α and β subunits, the blots were analyzed with a scanning densitometer. The level of accumulation of β subunit in several of the constructions was too low to detect with confidence; only pTB7, pJS9, pJS22, pJS23, and pJS12-carrying cells accumulated sufficient antibody-precipitable β subunit to allow quantitation. The most notable point from the densitometric scanning of the blots is the apparent preferential reduction in β subunit from lysates of cells carrying pJS12 when grown at 37 °C rather than at 20 °C; the amount of β subunit at 37 °C was about $^1/_4$ of the 20 °C level, while for pTB7, pJS9, pJS22, and pJS23 the ratio of the two subunits was not strongly dependent on temperature. These results suggest that the β^t subunit was either expressed at a lower level or was less stable than the wild-type β subunit or the β^t subunit encoded by pJS23. It should also be noted that the level of accumulation of α subunit was not the same for all constructions just as the level of bioluminescence was not the same, even though the *luxA* gene was the same for all constructions.

To further investigate the relative levels of synthesis of α and β subunits in the various constructions, lysates were prepared, proteins were denatured by urea, excess β subunit (in the form of purified luciferase from an α-defective mutant, AK-6) was added, and the mixture was allowed to refold at 8 °C for 72 h following dilution of the urea (Cline & Hastings, 1972). Since the added β subunit was in about 10-fold excess (relative to the amount of β subunit produced in cells carrying pTB7), the activity obtained following refolding should be proportional to the amount of α subunit present in the initial lysates. The results show that even in the very worst cases the level of α subunit available for complementation with

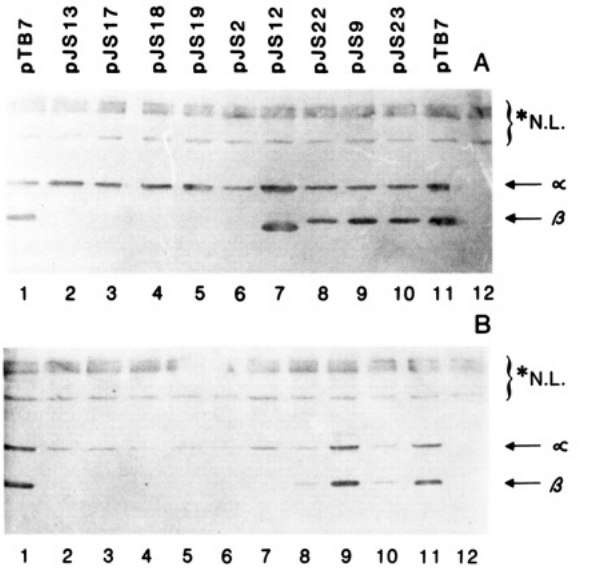


FIGURE 5: Western blots (Bittner et al., 1980) of lysates of cells carrying recombinant luminescence plasmids following electrophoresis in sodium dodecyl sulfate (Laemmli, 1970). Lane 1 contains lysate from cells carrying pTB7, lanes 2–6 contain lysates from cells carrying pJS13, pJS17, pJS18, pJS19, and pJS2, lanes 7 and 8 contain lysates from cells carrying pJS12 and pJS22, and lanes 9–11 contain lysates from cells carrying pJS9, pJS23, and pTB7. Lane 12 contains lysate from cells of *E. coli* strain TB1, demonstrating that the bands labeled N.L. (non-luciferase) were from *E. coli* proteins. Photograph A was of lysates from cells grown at 20 °C and photograph B was of lysates from cells grown at 37 °C.

exogenous β was about $^1/_4$ the level available from the pTB7 grown at that temperature (Table I, column 4). These results, together with those summarized above, suggest that the level of accumulation of α subunit is reduced by about 75% when sequences downstream from the β subunit coding region, within 54 bp of the translational stop signal, are deleted (lysates of cells containing pJS22, pJS23, and pJS2, compared with pTB7, pJS4, and pJS9). At 20 °C, the reduced amount of α subunit in cells carrying pJS22 and pJS23 correlates well with the reduced level of luminescence in vivo (Table I, columns 2 and 4). However, the levels of luminescence in cells carrying pJS2 and the group B deletions (except pJS12), all of which code for truncated β subunits, are far lower than can be accounted for by the reduced levels of α subunit found in these cells (Table I, column 4, and Figure 5).

Properties of Purified Variant Luciferases. To better understand the significance of the levels of luciferase activity in lysates of cells carrying the various plasmids, we purified the enzymes from four different plasmid constructions: pTB7, for wild-type enzyme; pJS23, an enzyme with altered β subunit carboxyl-terminal sequence but nearly normal length; pJS12, an enzyme with a truncated β subunit, lacking 9 amino acid residues from the carboxyl terminus; and pJS2, an enzyme lacking 11 amino acid residues from the β subunit carboxyl terminus. Cells were grown at 20 °C to maximize expression of luminescence, and the enzymes were purified as previously described (Hastings et al., 1978). The highest specific activities obtained during the purification of the variant luciferases were essentially the same as the specific activity obtained for the wild-type enzyme (Table II). We also determined the lifetime of the C4a peroxydihydroflavin intermediate with *n*-decanal (intermediate IIA) and the $FMNH_2$ binding affinity (Meighen & Hastings, 1971; Meighen & MacKenzie, 1973). The results, presented in Table II, indicated that the intermediates (IIA) of the variant enzymes have the same stability as the wild-type intermediate and that there is no significant dif-

Table II: Kinetic Parameters of Wild-Type and Mutant Luciferases

source of luciferase	specific activity of pure enzyme (quanta s^{-1} mg^{-1} × 10^{-14})[a]	apparent first-order rate constant for luminescence decay (s^{-1})[a]	dissociation constant for $FMNH_2$ (M × 10^7)[b]
pTB7	2.45	0.34	4.2
pJS 23	1.96	0.34	6.9
pJS 12	2.25	0.34	5.7
pJS 2	2.55	0.33	3.8

[a] Specific activity and the rate constant for decay of luminescence were determined by the $FMNH_2$ injection assay (Hastings et al., 1978) with *n*-decanal at 22 °C. [b] The $FMNH_2$ dissociation constant was determined kinetically according to the procedure of Meighen and Hastings (1971).

Table III: Thermal Inactivation of Wild-Type and Mutant Luciferases[a]

source of luciferase	phosphate concn (M)	apparent first-order rate constant for thermal inactivation (min^{-1}) 45 °C	50 °C	55 °C	60 °C
pTB 7	0.02	0.14	1.02		
	0.2	0.037	0.44		
	0.5			0.023	0.32
pJS 23	0.02	0.15	1.22		
	0.2		0.048	0.4	
	0.5			0.041	0.40
pJS 12	0.02	0.22	1.35		
	0.2		0.083	0.65	
	0.5			0.069	0.55
pJS 2	0.02	0.19	1.22		
	0.2		0.076	0.62	
	0.5			0.058	0.55

[a] Purified luciferases were used to compare the thermal stability of the wild-type and mutant forms. The enzymes were added to the indicated phosphate buffer preequilibrated at the specific temperature. Aliquots were removed, diluted (10 μL/mL) into room temperature assay buffer, and assayed immediately by the flavin injection assay. (See Materials and Methods.)

ference in $FMNH_2$ binding affinity between the variants and the wild-type enzyme.

To determine whether the purified luciferases were differentially heat labile, we determined the apparent first-order rate constants for denaturation at temperatures between 45 and 60 °C (Table III). The wild-type enzyme is stabilized by phosphate (Holzman & Baldwin, 1980a,b), so the effect of phosphate (0.02, 0.2, and 0.5 M) on the rate of thermal inactivation was determined for the variant luciferases. While the three variant luciferases did show slightly faster rates of thermal inactivation than the wild type, the differences were minor. All three variants showed stabilization by phosphate comparable to that afforded the wild-type enzyme.

It seemed possible that the reduced levels of accumulation of luciferase in cells carrying the variant plasmids pJS2, pJS12, and pJS23 were due to increased protease susceptibility of the variant enzymes. To test this hypothesis, the purified enzymes were exposed to trypsin and to chymotrypsin, and the apparent first-order rate constants for the inactivation process were determined in the presence of 20 mM phosphate and 200 mM phosphate for each enzyme (Table IV). The luciferases encoded by pJS2 and pJS12 were somewhat more susceptible than the wild type to trypsin in 20 mM phosphate; in 200 mM phosphate, the three variants did not differ significantly from the wild type in protease lability. None of the three variants differed significantly from the wild-type enzyme in sensitivity to the action of chymotrypsin at either phosphate concentration. It thus seemed unlikely that the lower levels of active luciferase in cells carrying the variant plasmids resulted from increased protease susceptibility.

Table IV: Protease Lability of Wild-Type and Mutant Luciferases[a]

source of luciferase	phosphate concn (M)	apparent first-order rate constant for inactivation (min^{-1}) trypsin	chymotrypsin
pTB7	0.02	0.22	1.29
	0.2	0.064	0.20
pJS 23	0.02	0.25	1.24
	0.2	0.060	0.19
pJS 12	0.02	0.41	1.38
	0.2	0.074	0.21
pJS 2	0.02	0.38	1.24
	0.2	0.071	0.18

[a] Purified luciferase (1 mg/mL) was equilibrated in phosphate buffer at 25 °C. Inactivation was initiated by addition of protease (either trypsin or chymotrypsin, 16 μg/mL final concentration); activity remaining was determined by dilution of aliquots 1:100 into assay buffer and analysis by the flavin injection assay. The apparent first-order rate of inactivation was determined graphically from semilog plots of activity remaining versus time. (See Materials and Methods for details.)

Table V: Recovery of Activity following Denaturation in 8 M Urea[a]

temp (°C)	phosphate concn (M)	recovery (%) pTB 7	pJS 23	pJS 12	pJS 2
37	0.02	0.01	0.04	0.01	0.02
	0.05	0.12	0.15	0.03	0.03
	0.10	0.31	0.43	0.06	0.03
	0.20	1.8	1.4	0.33	0.02
	0.50	14.6	12.6	3.0	0.08
20	0.02	1.4	1.9	0.50	0.05
	0.05	10.5	7.5	2.0	0.17
	0.10	22.6	15.2	5.0	0.31
	0.20	33.7	33.4	11.7	0.89
	0.50	43.2	43.2	33.7	4.2

[a] Purified luciferases were denatured by dilution into urea-containing buffers. The enzymes were renatured by dilution (final concentration 0.2 μg/mL) into buffers of various phosphate concentrations. Following incubation at either 20 or 37 °C, the recovered activity was determined by the flavin injection assay and is presented as a percent of an identical control that had been diluted into a buffer without urea. (See Materials and Methods for details.)

The decreased levels of bioluminescence in cells carrying the variant plasmids, especially pJS2 and pJS12 when grown at 37 °C, could be due to failure of the β^t subunit to fold properly and to interact productively with the α subunit. The ability of the β^t subunits to refold and interact properly with the α subunit was analyzed by allowing luciferases to renature following dilution out of 8 M urea into recovery buffers comprised of 0.1% BSA and 1 mM DTT, pH 7.0, with varying concentrations of phosphate. The results of these experiments are presented in Table V. For luciferase from *V. fischeri*, recovery of luciferase activity following dilution from urea has been shown to be more rapid at higher temperatures, but the extent of recovery is greater at lower temperatures (Friedland & Hastings, 1967), an observation which also holds for the enzyme from *V. harveyi*. Under optimal solution conditions (500 mM phosphate) at 37 °C, 14.6% of the initial wild-type activity and 12.6% of the initial activity of pJS23 was recovered, while only 3% and 0.08% of the activity were regained for pJS12 and pJS2, respectively. By comparison, at 20 °C, the extents of recovery of activity of pJS23 and wild-type enzyme were identical (43% of initial activity), while the pJS12 enzyme recovered about 33% of its initial activity and pJS2 enzyme recovered only about 4% of its initial activity.

Discussion

The recent experiments of Waddle et al. (1987) have shown that the structure of one or both of the subunits of bacterial luciferase that exist in the active heterodimer ($\alpha\beta$) is not the

structure that is formed if folding is allowed to occur in the absence of dimerization. This observation suggests that the heterodimeric structure, the kinetically favored structure that forms when the two subunits fold together, may not be the thermodynamically favored structure. We have proposed that intermediate structures form during the folding of the α and β subunits, designated [α] and [β], which are competent to interact, ultimately yielding active, heterodimeric luciferase (Waddle et al., 1987). However, if [α] and [β] are obliged to fold *without* formation of the heterodimeric structure, it appears that one or both fold into structure(s), α' and β', that are incapable of dimerization without prior unfolding, for example, with urea (Waddle et al., 1987). This model predicts that mutations or environmental parameters such as temperature that alter the relative concentrations of [α] and [β] within the cell, for example, by accelerating the [β] $\rightarrow \beta'$ process, should alter the second-order complexation reaction between [α] and [β].

Effect of Deletion of the Region of Dyad Symmetry. The 3′ end deletion experiments reported here suggest two mechanisms by which the level of bioluminescence is reduced in the variants. Deletions of the 3′ untranslated sequences closer than 54 bases to the *luxB* gene (plasmid pJS22) resulted in a ca. 4-fold decreased level of active luciferase (both α and β subunits), even though the coding regions for both subunits remained intact, suggesting that sequences had been deleted that were critical for luciferase mRNA stability. Deletion of *V. harveyi* sequences more than 54 bases downstream of the translational stop signal of *luxB* (plasmid pJS9) had no effect on the level of accumulation of active luciferase in *E. coli*. The translational stop codon of *luxB* is bases 973–975. Plasmids pJS9 and pJS22 both encode intact β subunits; the two plasmids differ in that the fragment from base 976 to base 1029 was deleted from pJS9 to obtain pJS22. Inspection of the DNA sequence within the deleted region shows a region of dyad symmetry, suggesting the possibility of secondary structure in the transcript. The potential structure is shown in Figure 4. There is at this time no evidence that this structure exists, but it is interesting that deletion of the region of DNA containing this possible structure element results in a decrease in the level of accumulation of both the α and β subunits.

Effect of Sequence Variation of the Carboxyl Terminus of the β Subunit. The plasmid pJS23, like pJS22, lacks the potential secondary structural element, and the level of luminescence from cells carrying the two plasmids is essentially indistinguishable. Plasmid pJS23 encodes a modified β subunit that is actually one amino acid residue longer than the native β subunit, with an altered sequence for the carboxyl-terminal 12 residues. This observation shows that even though deletion of sequences outside the *luxB* coding region can cause a dramatic decrease in bioluminescence, alteration of the carboxyl-terminal 12 residues has little or no effect on the ability of the β subunit to properly fold and interact with the α subunit. It is interesting in this context that a comparison of the amino acid sequences of luciferase subunits from *V. harveyi*, *V. fischeri*, and *Photobacterium leiognathi* shows that the C-terminal region of the β subunits is one of the least precisely conserved regions of either subunit (J.-W. Lin and T. O. Baldwin, unpublished data).

Effect of Carboxyl-Terminal Truncation of the β Subunit. Plasmid pJS12 encodes a truncated β subunit having a modified carboxyl-terminal sequence. In pJS12, residues 313–315 are Ala-Met-Trp, rather than Asp-Val-Val (wild-type sequence), and nine residues, 316–324, have been deleted. At 20 °C, pJS12-carrying cells accumulated ca. 75% as much luciferase activity as pTB7-carrying cells, while at 37 °C the level of luciferase accumulated in pJS12-carrying cells was only 10% that of pTB7-carrying cells. The luciferase produced at 20 °C by cells carrying pJS12 had normal thermal stability, normal specific activity, $FMNH_2$ binding affinity and intermediate IIA lifetime, and only slightly increased sensitivity to trypsin. Furthermore, the enzyme was capable of refolding at 20 °C following denaturation in 8 M urea to about 75% of the level of activity reached by wild-type enzyme, while at 37 °C the enzyme encoded by pJS12 reached only about 20% the level of activity of wild-type enzyme. These observations suggest that the amino acid residues deleted and altered do not function in maintaining quaternary structure but rather function in folding of the β subunit and/or productive interaction with the α subunit. The defect in the β subunit of pJS12 appears to be a temperature-sensitive folding defect.

The β subunit of the luciferase encoded by pJS2 differs from that of pJS12 by being two residues shorter and having the carboxyl-terminal sequence Ile-Gly rather than Ile-Ala-Met-Trp. Cells carrying pJS2 accumulated only 0.25% of the activity of pTB7 (0.33% of the activity of pJS12) when grown at 20 °C, while cells carrying pJS12 accumulated about 300 times more activity than pJS2, ca. 75% of the wild-type level. The plasmid pJS2 lacks the potential secondary structural element which is 3′ to the *luxB* coding region, which according to the observation made with plasmid pJS22 may be responsible for an approximately 4-fold decrease in luciferase activity; the remaining 75-fold reduction observed with pJS2 relative to pJS12 thus appears to have resulted from the difference in the C-terminal sequence of the β subunit. The accumulation of activity at a 37 °C growth temperature was 0.05% of the wild-type level and 0.13% of the level reached by cells carrying pJS12. The luciferase encoded by pJS2 had normal flavin binding and intermediate IIA lifetime and had normal specific activity. The enzyme had slightly decreased thermal stability and was slightly more sensitive to trypsin than the wild-type enzyme. The enzymes encoded by pJS2 and pJS12 were identical with respect to thermal stability and protease lability. The decrease in stability relative to the wild type does not appear to be sufficient to account for the dramatically lower accumulation of active enzyme in cells carrying the variant plasmid. The primary distinction between the wild-type enzyme and the luciferase encoded by the pJS2 plasmid appears to be the impaired ability of the β^t subunit to properly fold and interact with the α subunit. The small proportion of the β^t subunit that does properly fold with the α subunit forms an enzyme that does not differ substantially from the wild-type enzyme. We therefore feel that the plasmid pJS2 encodes a form of bacterial luciferase that is deficient in subunit folding. It is not clear whether the mutant should be regarded as temperature sensitive or nonconditional. The level of activity reached when refolded from urea was ca. 50-fold greater at 20 than at 37 °C, yet even at 20 °C the absolute level of activity reached was about 10% that of the wild-type enzyme, whereas pJS12 enzyme reached about 75% of the activity of the wild-type, in good agreement with the levels of luminescence in vivo (Table I).

Summary. For measurements in vitro, we focused primarily on the enzymes encoded by plasmid pTB7 (wild-type control), pJS23, pJS12, and pJS2. These three β subunit variant proteins were chosen for study in purified form because the principal effects of the deletions apparently were revealed by deletions within the carboxyl-terminal 12–15 amino acid residues. Plasmid pJS19 encodes an enzyme 16 amino acid

residues shorter than the wild-type enzyme, with the carboxyl-terminal 2 residues being different from the wild type (see Figure 3). This enzyme, like that encoded by pJS18, which was shortened by 21 residues relative to the wild-type enzyme, was readily detectable in lysates of cells grown at 20 °C as well as at 37 °C, and the activity was not dramatically temperature sensitive in lysates (Table I). It therefore appears that deletion of as many as 21 carboxyl-terminal residues from the β subunit does not alter the activity of the $\alpha\beta^t$ dimer per se, but lack of the carboxyl-terminal residues alters the ability of the β^t subunit to fold and/or productively interact with the α subunit.

The only published studies of folding and dimer assembly in bacterial luciferase rely on recovery of activity as the sole parameters for the assembly process (Friedland & Hastings, 1967; Gunsalus-Miguel et al., 1972). These limited studies suggest a possible nonproductive folding pathway for the β subunit if allowed to refold from urea in the absence of the α subunit. With luciferase from both *V. fischeri* (Friedland & Hastings, 1967; Gunsalus-Miguel et al., 1972) and *V. harveyi* (Gunsalus-Miguel et al., 1972), allowing the β subunit to refold from urea prior to interaction with the α subunit results in a significant decrease in the level of activity reached when the α subunit is added, relative to the level of activity reached when the two subunits are allowed to refold together. Prior refolding of the α subunit has no effect on the level of activity reached after addition of β subunit, but prior refolding of the α subunit may result in a faster recovery of activity when the β subunit is added (Gunsalus-Miguel et al., 1972). These results suggest that the two subunits must interact prior to the final stages of folding. That is, the final stages of folding must involve a dimeric structure.

The experiments reported here indicated that the carboxyl-terminal sequence of the β subunit, the last portion of the subunit to be constructed on ribosomes in the cell, plays a crucial role in directing the final stages of assembly of the active dimeric structure of the enzyme, suggesting that the processes of folding may be slow compared with the rate of protein synthesis. It is apparent that the precise amino acid sequence of the carboxyl-terminal region of the β subunit is comparatively unimportant, in that alteration of the carboxyl-terminal 12 residues had little, if any, effect on the stability or activity of the dimer. Deletion of the residues spanning the same region, on the other hand, had a profound effect on the ability of the dimeric structure to form, in vitro and apparently in vivo, but had little effect on the stability of the structure once it had formed. A detailed analysis of these mutants may be very useful in developing a more precise understanding of the mechanism of polypeptide folding and dimer assembly in bacterial luciferase.

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