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Bacterial luciferase $\alpha\beta$ fusion protein is fully active as a monomer and highly sensitive *in vivo* to elevated temperature

(bioluminescence/gene fusion/low light imaging/dynamic fluorescence measurement)

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ABSTRACT **A 2.2-kilobase-pair (kbp) DNA fragment from *Vibrio harveyi* contains the *luxA* and *luxB* genes separated by a 26-base-pair (bp) intergenic region. The two genes were converted to a single open reading frame by site-specific mutagenesis. A full-length fusion protein is obtained when the new gene is placed under transcriptional control of a T7 promoter in *Escherichia coli*. Bioluminescence of colonies containing the gene fusion is 0.002% of the wild-type luciferase [alkanal monooxygenase (FMN-linked); alkanal, reduced-FMN:oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] at 37°C. Growth at 23°C results in a >50,000-fold increase in light emission in cells containing fusion protein, whereas only a 3-fold increase is observed with cells containing the *luxAB* dicistron. Purified fusion protein isolated from *E. coli* grown at 19°C exists in both monomeric and dimeric forms with specific bioluminescence activities comparable to the heterodimeric wild-type enzyme at 23°C and 37°C. These findings show that the $\alpha\beta$ fusion polypeptide is functional as a monomer and suggest that its folding is drastically affected at elevated temperature. We hypothesize that the two-subunit bacterial luciferase may have evolved from a monomer as a result of a temperature increase in the environment.**

The luciferase [alkanal monooxygenase (FMN-linked); alkanal,reduced-FMN:oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] from the marine bioluminescent bacterium *Vibrio harveyi* is a heterodimeric enzyme that catalyzes the following reaction: $FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + 0.1\ h\nu_{490nm}$. The enzyme is composed of one α (40 kDa) and one β (36 kDa) subunit. Both the catalytic and substrate binding sites reside on the α subunit (1, 2). The β subunit is essential for activity but its function is unknown. The amino and carboxyl termini of the α-subunit polypeptide contribute differently to activity *in vivo*. Removal of the first four amino acids from the amino end of α subunit drastically decreases bioluminescence activity (3). In contrast, the addition of 11 amino acids from the major capsid protein of phage T7 to the amino terminus of α subunit results in a slight increase in activity (3). Furthermore, the addition of 24 amino acids to the carboxyl terminus of α subunit does not alter wild-type enzyme characteristics (unpublished data). The removal of 9 amino acids from the carboxyl terminus of the β subunit has little effect on luciferase activity (4). These data indicate the degree of flexibility present in the structure of both subunits.

The α- and β-subunit proteins are encoded by the *luxA* [1056 base pairs (bp)] and *luxB* (972 bp) genes, which are transcribed as part of a five-cistron transcript (5). Both genes have been introduced as two separate transcriptional units in prokaryotic (6) and in eukaryotic (7) organisms. The bioluminescence activities of cell homogenates obtained from *Escherichia coli* containing the *luxA* and *luxB* transcriptional units in the same cell is ≈40% of that observed from cells transformed with the *luxA* and *luxB* genes on a single transcriptional unit (8). When *luxA* and *luxB* are expressed separately in two different *E. coli* cultures and their homogenates are combined, ≈1% of the activity in homogenates of *E. coli* containing the *luxAB* single transcriptional unit is observed. Renaturation of the denatured α- and β-subunit polypeptides restores 20% of the wild-type activity, indicating that information stored in the amino acid sequence of one subunit is necessary for the correct folding and subunit assembly of the enzyme. These findings indicate that the cotranslational synthesis of α and β subunits results in the most efficient enzyme folding and assembly, probably due to the close proximity of the newly synthesized subunits. Therefore, immediate interaction of the α- and β-subunit polypeptides might be further improved by removing the 26-bp intergenic region or by direct linkage of the α and β luciferase subunits through a short amino acid sequence.

In this paper, we describe experiments in which the *luxA* and *luxB* genes have been fused into a single open reading frame encoding a new bacterial luciferase active as a monomer, with a specific activity and emission spectrum comparable to the wild-type heterodimeric enzyme. Furthermore, we report that the fusion protein is *in vivo* much more sensitive to elevation in temperature than the wild type.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* strain HB101 (9) was used for cloning and amplification of plasmids; strain DLT101 was used for the expression of the fusion protein—this strain is a recA⁻ derivative of BL21(DE)3 (10); strain JM101 (11) was used for site-specific mutagenesis and sequencing; strain CJ236 was used for site-specific mutagenesis (12).

Plasmid pT7/T3-19 contains the ϕ10 T7 promoter (10) and was used as a transcriptional expression vector. Plasmids pLX109-a, pLX1-b, and pLX203-ab (3) were used for site-specific mutagenesis and fusion construction.

Site-specific Mutagenesis and Construction of Gene Fusions. Site-specific mutagenesis was performed according to a protocol included with the Muta-gene kit from Bio-Rad. The presence of the mutation(s) was confirmed by DNA sequencing using the dideoxynucleotide chain-termination method (13). Three primers were designed. (*i*) Primer 2, a 31-mer, introduces one base change and one base addition. The base change causes the termination codon of *luxA* to be converted into a tyrosine codon. The base addition puts *luxB* gene in frame with *luxA*. (*ii*) Primer 8, a 17-mer, introduces one base change in the ATG start codon of *luxB* (ATG → CTG). (*iii*) Primer 9, a 23-mer, introduces two base changes in the ATG

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Abbreviations: LU, light unit(s); ANS, 8-anilino-1-naphthalene sulfonate; HPSEC, high-performance size-exclusion chromatography.

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start codon of *luxB* (ATG → AAA). The fusions were constructed as follows: primer 2 was used to modify pLX109-a, which is M13mp18 containing a 1.1-kilobase-pair (kbp) *Sal* I/*Eco*RI fragment encoding the *luxA* gene, the 26-bp *luxAB* intergenic region, and the first 38 bp of the *luxB* gene, yielding pLX109-fa2 (Fig. 1*A*). The mutagenized 1.1-kbp insert was cloned into the *Sal* I/*Eco*RI sites of pT7/T3-19, yielding pLX209-fa2. The rest of the *luxB* gene sequence was then excised as a 1.1-kbp *Eco*RI fragment from pLX1-b and cloned in the *Eco*RI site of pLX209-fa2, yielding pLX709-fab2. To increase translational expression of the fusion, plasmid pLX703-fab2 was constructed by replacing the 230-bp *Sal* I/*Pst* I DNA fragment corresponding to the 5′ end of the fusion gene, by the 256-bp *Sal* I/*Pst* I DNA fragment from pLX203-ab, which contains in addition the *V. harveyi* Shine–Dalgarno sequence of the *luxA* gene. Primer 8 was used to modify pLX109-fa2 as described above, yielding pLX703-fab8. Primer 9 was used to modify pLX109-fa2 (Fig. 1*A*), yielding pLX703-fab9 (Fig. 1*B*).

Bioluminescence Assay and Spectral Measurements. *E. coli* DLT101 cells transformed with various *luxAB* constructs were grown at 37°C to an OD_{600} of 0.8 in Luria–Bertani (LB) medium (14) with ampicillin (100 μg/ml). One microliter of each culture was resuspended in 20 μl of LB medium and applied onto a nitrocellulose filter with a multiwell manifold. The nitrocellulose membrane was transferred to a prewarmed LB plate containing ampicillin (100 μg/ml) and was incubated at 37°C for 2 hr. The number of photons emitted by each group of cells was then quickly measured with a video image analyzer. Cells were left to cool to room temperature (23°C) while light emission was continuously monitored for 6 hr. Bioluminescence measurements performed at other temperatures were obtained from the averaged number of photons emitted by 10 *E. coli* colonies of similar size. Luminescence activity from the purified φ9 fusion protein was assayed *in vitro* (15). The specific activity of the purified fusion protein [light units (LU) per A_{280} of enzyme] was estimated in a luminometer after quick injection of $FMNH_2$ and decanal [1 LU is equal to the emission of 10^{12} photons per s on a photometer calibrated with the luminol/dimethyl sulfoxide chemiluminescence reaction (15)].

Spectral measurements of individual colonies of transformed *E. coli* were made with a Spex spectrofluorimeter (16). The wavelength of maximum emission was determined from the first derivative of the emission spectrum.

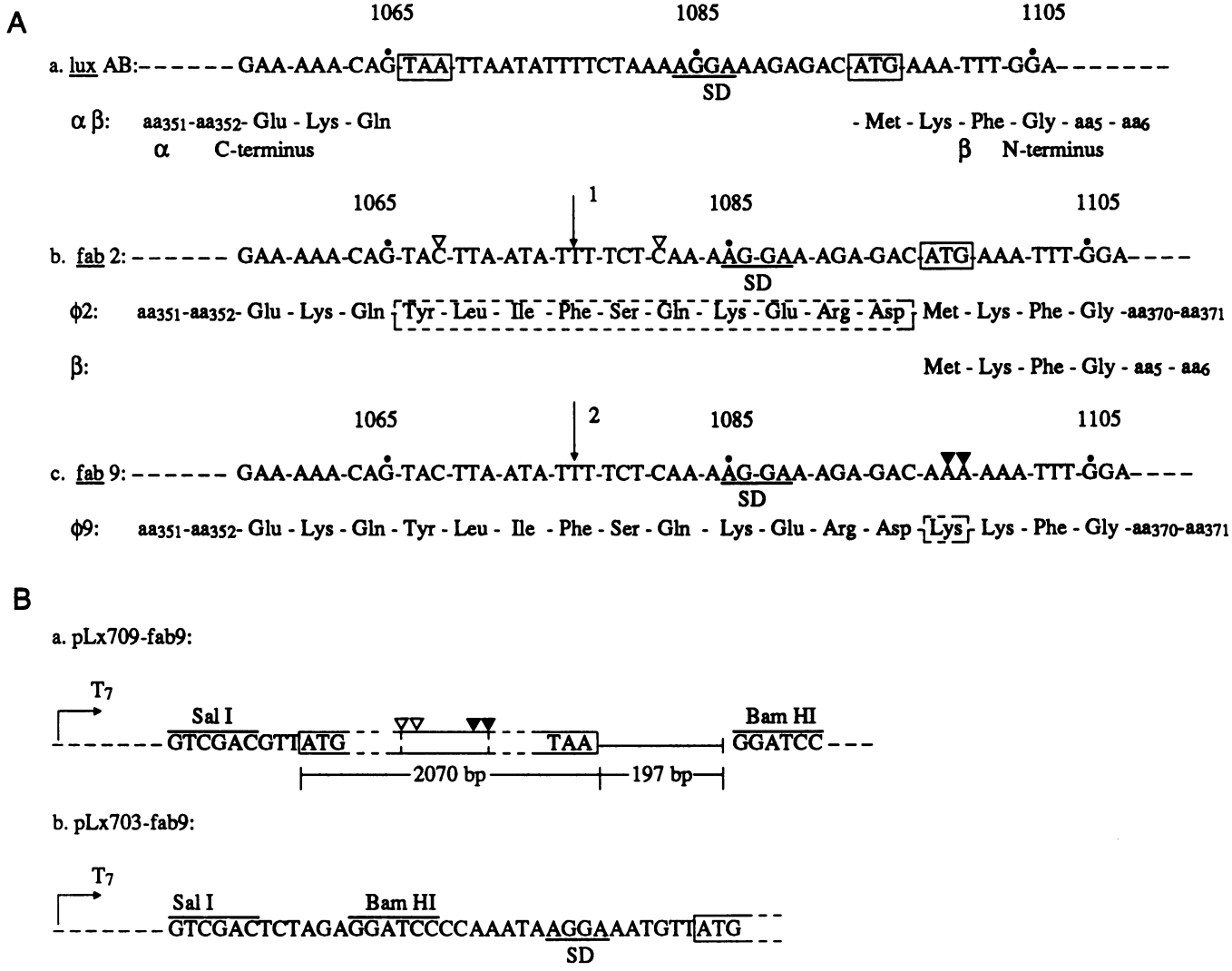

FIG. 1. (*A*) Intergenic region of the *luxA* and *luxB* structural genes. (*a*) wild-type DNA sequence and encoded amino acid sequences. Translational stop codon of *luxA* and initiation codon of *luxB* are boxed. Arrow 1, modification of *luxB* gene using primer 2. (*b*) Resulting DNA sequence of *fab2* and deduced amino acid sequences of encoded gene products. The modifications in the *luxAB* sequence are marked ▽ and the 10-amino acid linker peptide generated is boxed with dashed lines. Arrow 2, mutagenesis of *fab2* using primer 9. (*c*) Resulting DNA sequence of *fab9* and deduced amino acid sequence of encoded gene product. The modifications in *fab2* are marked ▼ and the amino acid change is boxed. aa, Amino acid; SD, Shine–Dalgarno sequence. (*B*) DNA sequence upstream of *fab9* gene in pLX709-fab9 (*a*) and pLX703-fab9 (*b*).

Protein Gel and Immunoblotting. *E. coli* transformants were grown in LB medium with ampicillin (100 μg/ml) at 23°C to an OD_{600} of 0.8 and then grown for 3 hr in the presence of 0.4 mM isopropyl β-D-thiogalactopyranoside. One hundred microliters of culture was then centrifuged and resuspended in 100 μl of an $NaDodSO_4$ loading buffer; 10-μl aliquots were boiled for 4 min and run on a 10% $NaDodSO_4$/polyacrylamide gel for 4.5 hr at 30 mA. Proteins were then transferred from the gel onto a nitrocellulose filter for 18 hr at 50 mA in a methanol/Tris/glycine transfer buffer. The nitrocellulose filter was exposed overnight to rabbit IgG raised against the denatured α and β subunits of *V. harveyi* luciferase. The filter was washed, exposed to goat anti-rabbit antibodies bound to alkaline phosphatase, and color reacted. All steps were according to a protocol of the Promega Protoblot kit.

Purification of Fusion Protein φ9. *E. coli* DLT101 cells transformed with pLX703-fab9 were grown at 19°C for 36 hr, and for an additional 3 hr in the presence of 0.4 mM isopropyl β-D-thiogalactopyranoside. The luciferase fusion protein was purified by preparative high-performance liquid chromatography using a three-column procedure (15).

Estimation of the Molecular Mass of the Native Fusion Protein by Rotational Measurements. The molecular mass of a protein can be determined from a measurement of the rotational correlation time (φ), which shows the extent of the rotational motion of a whole protein after laser excitation of protein bound 8-anilino-1-naphthalene sulfonate (ANS) (17). It can be shown that for a spherical body measured at 2°C, φ (in units of ns) is roughly equal to the molecular mass of the protein (in kDa). Dynamic fluorescence measurements of ANS bound to the wild-type luciferase and the fusion proteins in peak I and II were performed by laser excitation and single photon counting electronics (18).

RESULTS AND DISCUSSION

Expression of the *luxA* and *luxB* Gene Fusions in *E. coli*. Several gene fusions were designed to obtain a single bacterial luciferase fusion protein without the internal translation initiation of the β subunit (Fig. 1*A*). Gene fusion *fab2* was constructed by changing the termination codon of *luxA* to a TAC codon and by adding a cytosine residue in the intergenic region to create an open reading frame with *luxB*. We predicted a 78-kDa fusion protein band as a result of the fusion, and a 36-kDa β-subunit polypeptide band (Fig. 2, lane 3). Gene fusion *fab8* was constructed to eliminate the initiation of β-subunit protein translation by converting the ATG start codon of *luxB* to a CTG codon, which was thought to suppress protein synthesis initiation (19). To our surprise, the correct initiation of β-subunit polypeptide synthesis still occurred, although in a lower amount (lane 4). Gene fusion *fab9* was therefore constructed, in which the ATG start codon of *luxB* is changed to an AAA codon, which eliminates

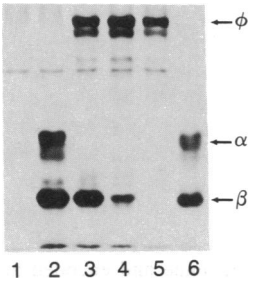

FIG. 2. Immunoblot analysis of luciferase fusion proteins from *E. coli* DLT101 grown at 23°C. Transformed with pT7/T3-19 (lane 1), pLX203-ab (lane 2), pLX703-fab2 (lane 3), pLX703-fab8 (lane 4), pLX703-fab9 (lane 5), *V. harveyi* luciferase (control) (lane 6).

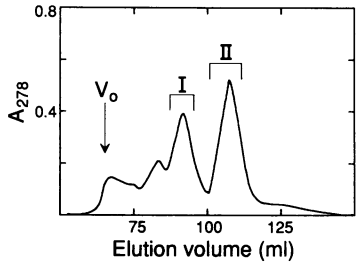

FIG. 3. HPSEC purification of the fusion protein. Indicated are the peak elution positions of blue dextran (V_o), the position of peak I fusion protein φ9, and the position of peak II fusion protein φ9.

β-chain initiation while allowing a full-length fusion protein (φ9) to be made (lane 5).

Purification of Fusion Protein φ9 and Estimation of Its Molecular Mass. Fusion protein φ9 was purified from an *E. coli* homogenate by successive high-performance hydrophobic interaction chromatography, high-performance anion-exchange chromatography, and high-performance size-exclusion chromatography (HPSEC). Two peaks containing bioluminescence activity were obtained (Fig. 3). Samples from each peak were run on a 12.5% $NaDodSO_4$/polyacrylamide gel and the results are shown in Fig. 4. The 78-kDa fusion protein is present in both peaks, as well as minor impurities, which do not account for >5% of the total protein in each sample. No α- and β-subunit bands were observed in either fraction.

Calibration of the HPSEC column with proteins of known Stokes radii and rotational measurements of bound ANS to the fusion protein were used to estimate the molecular mass of the fusion protein in each peak. The apparent hydrodynamic radii of the fusion proteins in peak I and peak II were different and were estimated to be 51 Å and 44 Å, respectively. The Stokes radii of peak I fusion protein and IgG (150 kDa) are similar, indicating that peak I consists of dimers of the αβ fusion. Peak II was eluted slightly earlier than the heterodimeric luciferase enzyme.

Molecular mass estimates were derived from rotational measurements of ANS bound to the wild-type enzyme and the fusion proteins from peak I and peak II (Table 1). The size of the *V. harveyi* luciferase was calculated to be 80.1 kDa, 4.5% higher than the molecular mass of the αβ wild-type enzyme deduced from the amino acid sequence. The peak I and peak II fusion proteins were, respectively, 146–152 and 89–97 kDa, determined under different conditions. These results confirm the dimer structure of the fusion protein present in peak I and indicate that peak II consists of monomer of the fusion protein. The larger than expected size of the fusion protein found in peak II could be due to a change in conformation of the protein caused by the linker peptide created between the α- and β-subunit polypeptides.

Emission Spectra and Specific Activities of Fusion Proteins. The emission spectra of light emitted by *E. coli* cells containing the three different fusion constructs were measured at

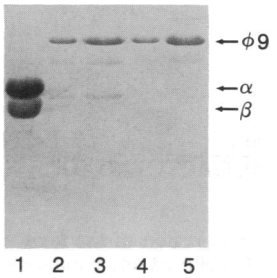

FIG. 4. Analysis of the fusion protein fractions by $NaDodSO_4$/PAGE. Lanes: 1, *V. harveyi* luciferase; 2 and 3, peak II fusion fraction (5 and 10 μg); 4 and 5, peak I fusion fraction (5 and 10 μg).

Table 1. Determination of molecular mass of fusion protein $\phi 9$ in each peak by rotational correlation time (ϕ_1) of bound ANS

	t, °C	Concentration, μM	ϕ_1, ns	kDa
$\alpha\beta$ wild	2	80	68 ± 3	76.6
type*	22†	130	38 ± 4	80.1
Peak I§	2	8	86 ± 1	96.8
	24	8	42 ± 2	89.1
Peak I§	2	8	135 ± 1	151.9
	24	8	69 ± 4	146.5

A limiting concentration of ANS was added to the luciferase and fusion protein (1:2 ratio) to eliminate free ANS contribution to fluorescence. The concentration is calculated as for all monomer. Molecular mass of the corresponding anhydrous sphere is shown.
*Isolated from an aldehyde-deficient mutant of *V. harveyi*.
†See ref. 16.
‡Fusion protein from peak II of the HPSEC column.
§Fusion protein from peak I of the HPSEC column.

23°C and were found to be identical to that of wild type (λ_{max}; *luxAB*, 487.4 nm; *fab2*, 488.1 nm; *fab8*, 488.4 nm; *fab9*, 487.7 nm). The specific activities of the monomer and dimer forms of the $\phi 9$ fusion protein purified from *E. coli* cells grown at 19°C were compared with each other and with that of the wild-type enzyme purified from *V. harveyi* cells grown at 23°C. The heterodimeric $\alpha\beta$ wild type has a specific activity of 430 LU/A_{280}, the $\phi 9$ fusion monomer has a specific activity of 365 LU/A_{280}, and the $\phi 9$ fusion dimer has a specific activity of 320 LU/A_{280}. Therefore, fusion of the α and β subunits of the luciferase enzyme does not significantly affect the light-emitting properties of the heterodimeric luciferase enzyme.

Effect of Temperature Change on Bioluminescence Activity of Luciferase Fusion Proteins. *E. coli* cells transformed with different luciferase fusion gene constructs were grown at 37°C and 23°C. Light emission was measured *in vivo* and the activities were compared with cells containing the *luxAB* dicistronic construct (wild type) fused to the same T7 promoter (Table 2). There are no significant variations in the relative amounts of fusion and free β-subunit proteins made at 37°C and 23°C (unpublished data). Cells containing *fab2*, *fab8*, or *fab9* genes at 37°C show 8%, 0.2%, and 0.002%, respectively, of the light emitted by *E. coli* cells containing the *luxAB* wild-type transcriptional unit and grown at 37°C. In contrast, at 23°C the same cells show 94%, 79%, and 46%, respectively, of the light emitted by *E. coli* cells grown at 23°C containing *luxAB*. The increase of bioluminescence activities starts within 1 min after exposure of the plates to 23°C, is gradual (e.g., *E. coli* cells grown at 30°C containing the *fab9* gene show 11% of the light emitted by cells grown at 30°C containing the *luxAB* genes), and stops after 3 hr.

The increase in light emission observed upon cooling the plates from 37°C to 23°C could have been due either to the *de novo* synthesis of fusion protein at a permissive temperature or to the correct folding of the fusion protein after its synthesis at 37°C. To determine the heat stability of the fusion protein, we measured the bioluminescence activities of the purified wild-type and fusion luciferase $\phi 9$ at 23°C and 37°C. At 37°C, the wild-type luciferase retains 62%, the fusion monomer retains 52%, and the fusion dimer retains 46% of its activity. The fusion protein is therefore not significantly more heat labile than the wild type. To determine further whether the increase in activity was due to *de novo* synthesis of fusion protein, protein synthesis in *E. coli* cells transformed with pLX703-fab9 grown in liquid LB medium at 37°C was stopped by adding chloramphenicol (30 μg/ml). The culture was grown for 1 hr and left for 3 hr to cool at 23°C. Little increase in luciferase activity was observed upon addition of $FMNH_2$ and decanal to sonicated cells of that culture, whereas cells not exposed to the antibiotic showed a >50-fold increase in light emission. These results indicate that the low activity of the fusion proteins observed at 37°C *in vivo* is not due to heat lability but to temperature-sensitive folding of the polypeptide.

Table 2. Comparisons of bioluminescence emitted from *E. coli* DLT101 cells containing different luciferase gene constructs and grown at different temperatures

t, °C	*fab2*	*fab8*	*fab9*	*luxAB*
37	600	20	0.13	7,500
23	19,000	16,000	9000	20,000

Measurement: number of photons emitted per s at 50% sensitivity of the photon counting tube.

The difference in the level of bioluminescence between the purified luciferase fusion synthesized at 19°C and the luciferase fusion synthesized at 23°C in *E. coli*, relative to the wild-type luciferase synthesized at 23°C, indicates that further decreases in growth temperature can increase the light emission from cells containing luciferase fusion protein relative to cells containing the wild-type luciferase. *E. coli* cells transformed with pLX703-fab9 and grown at 19°C and 15°C give, respectively, 60% and 80% of the light emitted by *E. coli* cells containing the *luxAB* genes grown at the same temperatures. The difference between the levels of bioluminescence measured *in vitro* and *in vivo* at 19°C may be due to formation of fusion aggregates or degradation of the fusion monomer in *E. coli*. These results demonstrate further the extreme sensitivity of the luciferase fusion monomer to elevation in temperature, which is observed when the linker peptide sequence is changed or lengthened up to four amino acids (unpublished data).

Furthermore, there is a correlation between bioluminescence activity of the fusions synthesized at 37°C and the amount of free β-subunit protein present in the cells. *E. coli* cells transformed with pLX709-fab2 contain the highest amount of free β-subunit protein and at the same time emits 30 times more light than *fab8*-containing cells, which make much less free β subunit. Cells containing *fab9* gene fusion make no detectable β subunit and emit 5000 times less light than cells containing *fab2*. At 23°C, however, there is only a 2-fold difference in light intensity between *fab2*- and *fab9*-containing cells, indicating that the effect of the β subunits on bioluminescence is less marked as the temperature is lowered. Cotransformation of *E. coli* cells with *fab9* in pACYC184 and *luxA* or *luxB* in pT7T3-19, all *lux* genes under transcriptional control of the T7 promoter, results in a 15- and 30-fold, respectively, increase in luciferase activity at 37°C as compared to cells containing the same plasmids but lacking the *luxA* or *luxB* gene. These data suggest that free α- and β-subunit polypeptides complement the fusion luciferase at elevated temperature.

CONCLUSION

Partially active subunit fusions of bacterial enzymes have been reported by different laboratories (19, 20). However, activities were not correlated to single polypeptide chains but rather to oligomers of fusion proteins. In the work described here, we have created a full $\alpha\beta$ fusion protein of *V. harveyi* luciferase to determine whether ensuring an immediate contact between the two subunits would have an effect on activity. The results presented indicate that the protein fusion is functional as a monomer with an activity comparable to wild-type enzyme when protein synthesis is allowed to occur at low temperatures. In contrast with the heterodimeric wild-type enzyme, the enzymatic activity of the fusion protein is extremely sensitive to elevated growth temperature. This allows us to put forward the hypothesis that the α- and β-subunit polypeptides of *V. harveyi* luciferase may have

existed originally in the form of a single protein chain. *V. harveyi* lives over a wide range of temperature in nature, generally between 5°C and 30°C. It is conceivable that the putative ancestral monomeric luciferase evolved into the present heterodimeric $\alpha\beta$-subunit enzyme due to temperature increases. It has been suggested that the luciferase *A* and *B* genes of *V. harveyi* arose by gene duplication. This hypothesis was based on extensive DNA sequence homologies located at the 5′ region of both *luxA* and *luxB* genes (21), with a 30% amino acid homology overall (22). We argue for an alternative possibility. Analysis of DNA sequences upstream of the *luxA* and *luxB* genes reveals the presence of highly conserved Shine–Dalgarno sequences, TATA boxes, and −35 regions (23), even though *luxB* was not shown to be transcribed separately [there may be transcription initiation from the *luxA* promoter (24)]. These homologies of the upstream regions may be the result of an insertion of the 5′ end of the monocistronic ancestral gene, or another promoter-like sequence, in a site generating a dicistronic luciferase gene encoding an enzyme with greater activity at higher temperatures. This hypothesis is indirectly supported by the complementation of the fusion protein by either the free α or β subunit at 37°C. α- and β-subunit complementation implies that the α- and β-subunit moieties of the fusion fold correctly at 37°C. This would suggest that the "initiation cassette" would have inserted in or in proximity of the DNA sequence encoding the temperature-sensitive amino acid sequence of the fusion. Such cassettes containing translational stop codons, promoter signals, and ribosomal binding sites would be an attractive way to fragment, at the DNA level, a single polypeptide into a multiple subunit enzyme if need arises.

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