

# Structure of bacterial luciferase

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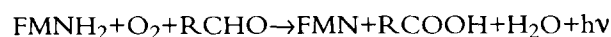
The generation of light by living organisms such as fireflies, glow-worms, mushrooms, fish, or bacteria growing on decaying materials has been a subject of fascination throughout the ages, partly because it occurs without the need for high temperatures. The chemistry behind the numerous bioluminescent systems is quite varied, and the enzymes that catalyze the reactions, the luciferases, are a large and evolutionarily diverse group. The structure of the best understood of these intriguing enzymes, bacterial luciferase, has recently been determined, allowing discussion of features of the protein in structural terms for the first time.

Current Opinion in Structural Biology 1995, 5:798–809

## Introduction

Luciferase is a generic name for any enzyme that catalyzes a reaction that results in the emission of light of sufficient intensity to be of biological consequence; that is, bright enough to be observed by another organism. Other than catalyzing light emission, different luciferases have little in common. All luciferases catalyze oxidative processes in which an intermediate (or product) is formed in an electronically excited state. Light is emitted when the excited state is converted to the ground state. Unlike proteases, for example, which all catalyze hydrolysis of peptide bonds, different luciferases utilize different substrates and catalyze very different reactions, the only similarities being the oxidative nature of the reaction and the production of an electronically excited state of a molecule capable of light emission.

The experiments of Robert Boyle [1] demonstrated that bioluminescence reactions require air. Oxygen was unknown at that time, and by the use of his air pump, Boyle demonstrated that removing the air around bioluminescent fungi resulted in the cessation of bioluminescence. Readmission of air to the chamber resulted in a resumption of bioluminescence. Oxygen is used by bacterial luciferase in a flavin monooxygenase reaction in which molecular oxygen, which has been activated by a reaction with reduced flavin mononucleotide (FMNH<sub>2</sub>), reacts with an aldehyde to yield the carboxylic acid, oxidized flavin (FMN), and blue-green light in the following reaction:



The reaction proceeds through a series of intermediates, some demonstrated and some proposed, leading to the

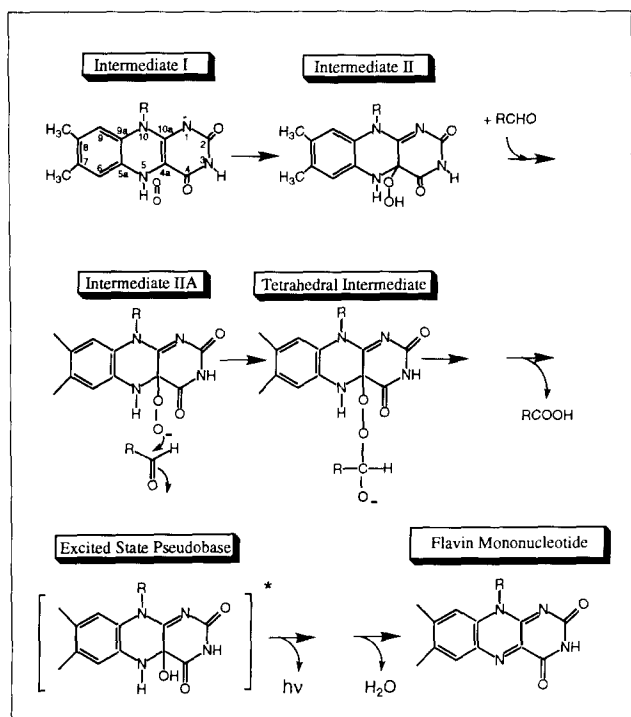
formation of C4a hydroxyflavin (the flavin pseudobase) in the excited state (Fig. 1). Light emission apparently occurs from the pseudobase, which then dehydrates to yield FMN, the flavin product, which dissociates from the enzyme. The reaction has been discussed in detail in a recent review [2]. The purpose of the present review is to discuss various features of bacterial luciferase and the luciferase-catalyzed reaction in the context of the recently-determined high-resolution structure [3••].

## Bacterial luciferases

All bacterial luciferases studied so far appear to be homologous, and all catalyze the same reaction. The only known variation on the common theme is that some bacteria emit light of different colors because they have secondary emitter proteins. For certain *Photobacterium* species and an isolate of *Vibrio fischeri*, light emission *in vivo* appears to occur not from a luciferase-bound electronically excited state, but from another protein. Some *Photobacterium* species utilize a 'lumazine protein' for light emission [4–7]. This protein appears to accept the energy from the primary excited state on the luciferase, resulting in an excited lumazine chromophore which emits light that is of a shorter wavelength (more blue) than that emitted directly from the luciferase. The yellow fluorescent protein (YFP) from one isolate of *V. fischeri* uses FMN as the chromophore and emits light that is red-shifted relative to that from luciferase [8–12]. These two 'antenna' proteins, the lumazine protein and YFP, constitute an interesting case of molecular evolution [2]. The

## Abbreviations

FMN—flavin mononucleotide; TIM—triose-phosphate isomerase; YFP—yellow fluorescent protein.



**Fig. 1.** The bacterial bioluminescence reaction. Bacterial luciferase is a flavin monooxygenase which reversibly binds FMNH<sub>2</sub> with 1:1 stoichiometry. The enzyme-bound flavin, Intermediate I, reacts with O<sub>2</sub>, forming the C4a peroxydihydroflavin Intermediate II. In the absence of the aldehyde substrate, the C4a peroxydihydroflavin decays without light emission to yield FMN and H<sub>2</sub>O<sub>2</sub> (not shown in the figure). In the light-emitting reaction, it is thought that the C4a peroxydihydroflavin reacts with the carbonyl carbon of the aldehyde substrate to yield the tetrahedral intermediate, which decays by an unknown mechanism to yield an electronically excited state (marked with an asterisk), probably of the flavin C4a hydroxide, and the carboxylic acid. Decay of the singlet excited state of the flavin to ground state is accompanied by light emission. The kinetic mechanism has been studied in detail [29,71,72].

two proteins are clearly homologous, as indicated by alignment of the amino acid sequences [7], and they have the same function—energy transfer and light emission following interaction with bacterial luciferase. However, they utilize different cofactors in accomplishing their function. In this respect they are unique: we know of no other example of two homologous proteins that have the same biological function in different organisms, but utilize different cofactors to carry out this function [2].

It is interesting that neither YFP nor the lumazine protein binds to the resting state of luciferase [5,11]. Rather, it appears that these proteins bind to an intermediate on the reaction pathway, probably the tetrahedral intermediate (Fig. 1), accelerating its conversion to the excited state [2]. It is known that bacterial luciferase undergoes a conformational rearrangement during catalysis [13,14], and it is likely that the two emitter proteins recognize and bind to an intermediate conformation, rather than the initial conformation.

Luciferases from all bacterial species studied so far consist of two subunits,  $\alpha$  and  $\beta$ , with molecular weights of ~40 000 and 35 000 respectively (355 and 324 residues

in the case of the luciferase from *V. harveyi*). The two subunits are clearly homologous (see below) [2,15], but the single active center is on the  $\alpha$  subunit (for a review, see [2]). The role of the  $\beta$  subunit is not yet clear, but it is essential for a high quantum yield reaction [2].

Alignment of the amino acid sequence of the  $\alpha$  subunit with that of the  $\beta$  subunit demonstrates that they share 32% sequence identity, and that the  $\alpha$  subunit has 31 amino acid residues that are not present in the  $\beta$  subunit [2]. The apparent homology of the subunits has suggested that they should have a similar three-dimensional structure, and that the two subunits may be related by a pseudo twofold rotation axis [2]. Furthermore, the apparent homology suggests that there should be two active sites, or at least a vestigial flavin-binding site on the  $\beta$  subunit [2]. However, numerous studies have shown that there is only one active site, and that a single flavin is involved in the bioluminescence reaction [16]. At very high protein concentrations, a second binding site for FMN has been observed in NMR experiments [17], but no functional significance of the second site has been demonstrated.

In the sequence alignment of the two subunits, there is a gap in the  $\beta$  subunit that corresponds to the 29 residues between residues 258 and 286 in the  $\alpha$  subunit [2,18,19]. This region of the  $\alpha$  subunit has a structural feature known as the protease labile region [18,20,21]. Luciferase is exquisitely sensitive to proteases [22], and inactivation of the enzyme can result from hydrolysis of a single peptide bond in the region of residues 272–291 on the  $\alpha$  subunit [18,20,23]. The  $\beta$  subunit is insensitive to proteases, and the quaternary structure of the  $\alpha\beta$  complex as a whole is not altered by treatment with proteases [23].

The protease labile region appears to move during the catalytic cycle. Binding of FMN, or of phosphate from the buffer, reduces the susceptibility to proteases [24–27]. Binding of FMNH<sub>2</sub> and reaction with O<sub>2</sub> results in the conversion of the enzyme to an altered conformational state that is not protease labile and in which the reactive thiol at position 106 of the  $\alpha$  subunit is no longer reactive [14,28]. This altered conformational state persists after the flavin has dissociated, slowly relaxing to the original structure. Such aspects of the structure are consistent both with the finding that the enzyme–FMNH<sub>2</sub> complex must undergo isomerization before reaction with O<sub>2</sub> [29], and with the apparent requirement for a conformational change in the luciferase to form a binding surface for YFP or the lumazine protein [5,11].

### Architecture of the enzyme

The structure of bacterial luciferase has recently been reported [3••] (Fig. 2). The structure was determined without the flavin substrate, so precise knowledge of

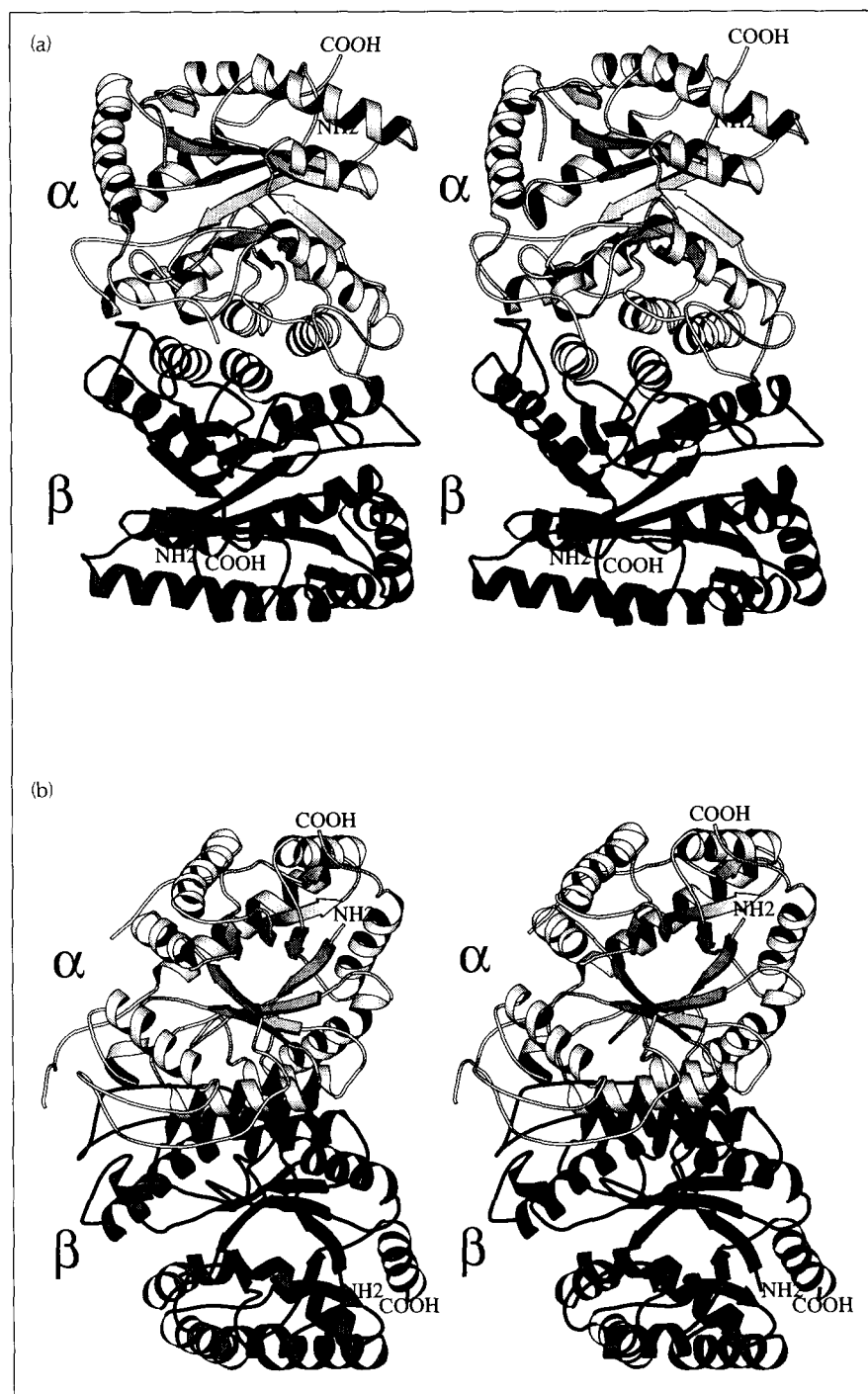


Fig. 2. Stereo views of bacterial luciferase. (a) View down the pseudo twofold axis. (b) View resulting from rotating the view in (a) 90° to the left. The locations of the amino and carboxyl termini of each subunit are indicated in both views.

the location of the active center is not yet available. As expected, the folds of the  $\alpha$  and  $\beta$  subunits are very similar: both assume the single-domain eight-stranded  $\beta/\alpha$  barrel motif ( $[\beta/\alpha]_8$ ) first identified in the crystal structure of triose-phosphate isomerase (TIM) [30]. This structural form (also called the TIM barrel) has a characteristic repeating pattern of  $\beta$ -strand-loop- $\alpha$  helix-loop, back to the next  $\beta$  strand, which is parallel to the preceding  $\beta$  strand. The pattern repeats eight times, with the eight  $\beta$  strands parallel to each other and forming a closed barrel and with the eight  $\alpha$  helices

forming the outside of the barrel. The N and C termini are usually adjacent in  $(\beta/\alpha)_8$  enzymes, residing at the end of the barrel where the amino ends of the  $\beta$  strands are located. It is common in  $(\beta/\alpha)_8$  enzymes for there to be a deviation from the repeating folding pattern following  $\beta$ -strand 7, resulting in a segment of the polypeptide folding over the carboxyl end of the barrel, prior to formation of  $\alpha$  helix 7 and completion of the structure. Such deviations are observed in both subunits of luciferase. In the  $\alpha$  subunit, the deviation is quite extensive, comprising ~55 residues, including

the protease labile region that is missing from the  $\beta$  subunit. In the  $\beta$  subunit, the corresponding excursion consists of ~35 residues. In the  $\alpha$  subunit, amino acid residues from Phe272 to Thr288 were not seen in the electron density map [3\*\*], consistent with the proposal that the protease labile region, of which this section is a part, has exceptional conformational flexibility, a factor contributing to protease lability [23–27].

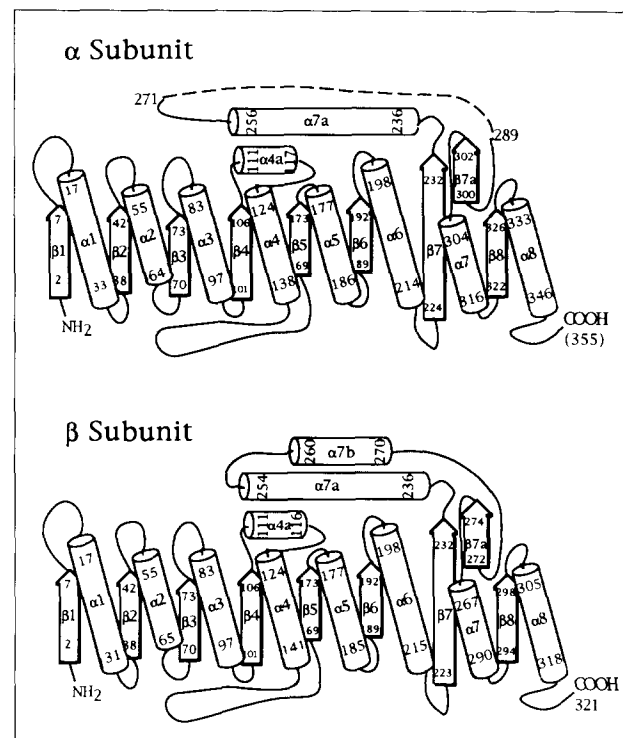
All known enzymes having the  $(\beta/\alpha)_8$  fold have active centers located at the carboxyl end of the barrel [31], and in most cases, the active center is composed of residues in the loops connecting the  $\beta$  strands to the  $\alpha$  helices. The  $(\beta/\alpha)_8$  fold is a common folding motif for flavoenzymes: old yellow enzyme [32], glycolate oxidase [33], trimethylamine dehydrogenase [34], and flavocytochrome *b<sub>2</sub>* [35] all have TIM barrels which bind FMN as a cofactor. Luciferase is composed of two TIM barrels, but has a single active center [2]. It has been proposed that the active center of luciferase resides at the subunit interface [36,37], but this suggestion has been challenged [2]. If the active center were to reside at the subunit interface, it would be a novel design for a TIM barrel, as the active center would consist of residues which are effectively on the outer surface of the barrel.

The two subunits associate through extensive surface contact, the center of which is occupied by an interesting form of parallel 4-helix bundle. At the center of the bundle is a pseudo twofold rotation axis that relates the  $\alpha$  subunit to the  $\beta$  subunit (Fig. 2a). Helices  $\alpha 2$  and  $\alpha 3$  of each subunit form the helix bundle, with the two  $\alpha 2$  helices packing very close together: the helix axes are 6.05 Å apart at the closest point and have a crossing angle of about 30°. The axes of the barrels of the  $\alpha$  and  $\beta$  subunits are related by a rotation of 80° and a translation of 34 Å, and the overall dimensions of the heterodimer are approximately 75 Å × 45 Å × 40 Å (see Fig. 2).

### The 'disordered loop' and the protease labile region

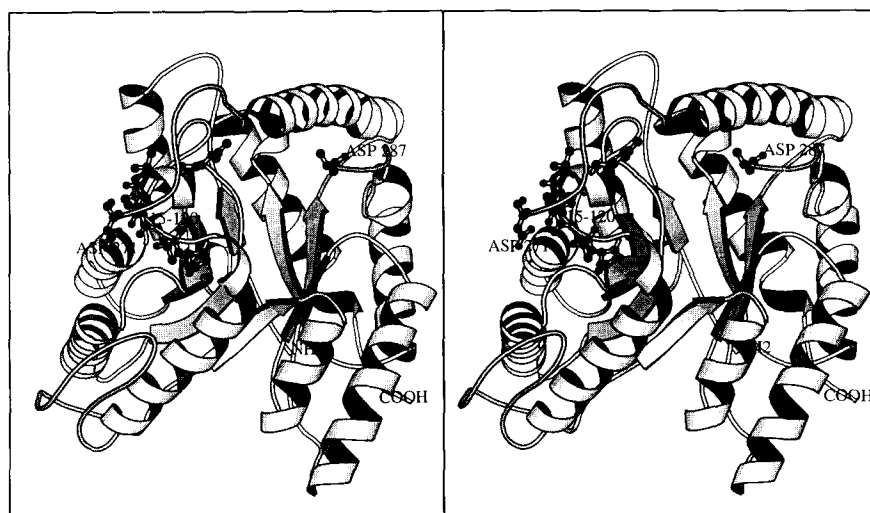
Luciferases from all bacterial species studied are exquisitely sensitive to inactivation by a variety of proteases [25,27,38]. This fact has led to the development of sensitive protease assay methods using bacterial luciferase as a substrate [22]. Upon exposure of bacterial luciferase to protease, the bioluminescence activity is lost at the same rate as the intact  $\alpha$  subunit is lost [23]. By densitometry of stained protein bands in polyacrylamide gels, it was shown [23,27] that the  $\alpha$  subunit is rapidly converted to two sets of fragments, designated  $\gamma$  and  $\delta$ . The precise molecular mass of each fragment is different for different proteases, but the similarity in size indicates that all proteases hydrolyze bonds in the same region on the  $\alpha$  subunit. Excision of the  $\gamma$  family of fragments from SDS gels and N-terminal sequencing have demonstrated that these fragments have an N-terminal sequence

identical to that of the  $\alpha$  subunit, indicating that the initial cleavage is in the region of residues 272–291 [21]. Following the initial, inactivating cut of the  $\alpha$  subunit, the  $\gamma$  fragments are slowly cleaved in a second region (between residues 116 and 117 in the case of chymotrypsin cleavage) to yield additional  $\delta$  fragments [21]. The consequence of limited proteolysis, therefore, is the conversion of the  $\alpha$  subunit to three groups of fragments, referred to collectively as  $\delta$  fragments. The locations of the protease labile regions of the  $\alpha$  subunit are depicted on the topological diagram in Figure 3 and in the stereo view in Figure 4.



**Fig. 3.** Topological diagrams depicting the secondary folding patterns of the  $\alpha$  and  $\beta$  subunits of bacterial luciferase. The  $\alpha$  helices are indicated by cylinders and the  $\beta$ -strands are indicated by flat arrows. The overall folding patterns are the same: the primary difference is that helix  $\alpha 7b$  of the  $\beta$  subunit has been replaced by a long loop consisting of residues 257–271 in the  $\alpha$  subunit. The region from 272–286 of the  $\alpha$  subunit, largely missing from the  $\beta$  subunit sequence, is disordered in the crystal and is indicated here by a dashed line. This region comprises the primary protease labile region of the  $\alpha$  subunit.

Cleavage and inactivation of luciferase does not result in dissociation of the fragments of the  $\alpha$  subunit from each other or from the  $\beta$  subunit, as demonstrated by equilibrium ultracentrifugation studies [23]. Extensive efforts to resolve the proteolytic fragments of the  $\alpha$  subunit from the intact  $\beta$  subunit under non-denaturing conditions have been unsuccessful. It appears that any treatment that results in dissociation of the fragments of the  $\alpha$  subunit from the intact  $\beta$  subunit will also cause unfolding of the  $\beta$  subunit (MM Ziegler, unpublished data). This observation is of significant interest because it has been demonstrated that when the



**Fig. 4.** Stereo view of the  $\alpha$  subunit showing the locations of the two protease labile regions of the  $\alpha$  subunit. The primary protease labile region is contained within a disordered region extending from residue 272 to residue 286. The side chains of the amino acid residues at the boundaries of the disordered region, Asp271 and Asp287, are shown in the drawing (Asp287 is modeled here as alanine due to low electron density). The secondary protease labile region, consisting of residues 115–120, appears to be located 'beneath' the primary protease labile region; the side chains of the amino acid residues in this region are also shown in the drawing.

$\beta$  subunit is folded alone, it forms a homodimer which is insensitive to proteases and does not unfold even after prolonged incubation in 5 M urea [39•], conditions that would cause rapid unfolding of the native heterodimer [40–42]. These biochemical studies, both with the proteolyzed enzyme and with the  $\beta$ -subunit homodimer, indicate that the subunit interface of bacterial luciferase contributes significantly to the overall conformational stability of the enzyme and of the  $\beta$ -subunit homodimer. These ideas are discussed in greater detail below.

Based on the protease lability of luciferase, it was proposed that the  $\alpha$  subunit of the enzyme has a disordered region which is missing from the  $\beta$  subunit [25–27]. The alignment of the amino acid sequences of the  $\alpha$  and  $\beta$  subunits [2,18,19] suggested that the region of the *luxA* gene that encodes residues 258–286 of the  $\alpha$  subunit has been deleted from the *luxB* gene (which encodes the  $\beta$  subunit), and N-terminal sequencing of the  $\delta$  fragments demonstrated that the sites of initial protease cleavage are between residues 272 and 291 [21]. The residues between Phe272 and Thr288 are not observed in the electron density map of the  $\alpha$  subunit [3•], consistent with a disordered structure in this region. After the initial cleavage of residues in this region, proteases cleave at residues in the region around residues 115–120 of the  $\alpha$  subunit [21]. The first cleavage results in loss of activity; the second cleavage is at a site that appears to be 'below' the location that is likely to be occupied by the disordered loop, and probably occurs after cleavage at the initial site. The resulting fragments, each comprising roughly one third of the  $\alpha$  subunit, contain sufficient structural information to ensure that they do not readily dissociate from each other [23], and the portion of the  $\alpha$  subunit that contains the subunit interface is not disrupted by the proteolysis, so that interaction with the  $\beta$  subunit contributes to the stability of the fragments of the  $\alpha$  subunit.

The protease labile region appears to move, as a consequence of binding of FMNH<sub>2</sub> and reaction with O<sub>2</sub>, to form an enzyme species that is insensitive to proteolysis [13,14]. Following decomposition of the C4a peroxydihydroflavin to yield free enzyme and FMN, the luciferase retains its protease insensitivity, slowly returning to the sensitive form with a  $t_{1/2}$  of about 20 min at 0–4°C [13,14]. Based on these observations, it has been suggested that the protease-labile loop functions as a flap that becomes less mobile, perhaps blocking water from coming into contact with the active center, as the reaction proceeds. The protease insensitive form of the protein that is released following a single catalytic cycle appears to be fully active; the conformational relaxation that renders the enzyme protease sensitive does not appear to affect the activity of the enzyme.

### Location of the reactive thiol

It has been known for two decades that luciferase from *V. harveyi* is rapidly inactivated by thiol-directed reagents as a result of modification of the cysteinyl residue at position 106 of the  $\alpha$  subunit [18,28,43]. The reactive thiol was shown to reside in or near a hydrophobic cleft [44,45], and is protected from reacting by the binding of FMN [28,43]. However, it has recently been clearly demonstrated that the reactive thiol is not involved in the bioluminescence reaction, and it is unlikely that aldehyde inhibition is due to reaction of the thiol with the aldehyde substrate. The luciferase from *V. fischeri* has a valyl residue at position 106 of the  $\alpha$  subunit, the position occupied by the reactive thiol of luciferase from *V. harveyi* [46–48], demonstrating that this thiol is not required for bioluminescence activity. This conclusion was confirmed by mutation of the reactive cysteinyl residue of *V. harveyi* luciferase to serine, which resulted in a fully active luciferase [46]; to alanine, resulting in a

mutant which is also fully active; and to valine, resulting in a mutant which is much less active than the wild-type luciferase [48], even though the enzyme from *V. fischeri* has valine at the same site [46].

Attempts by Rausch [21] to cross-link the reactive thiol at residue 106 on the  $\alpha$  subunit to residues on the  $\beta$  subunit using *p*-azidophenacylbromide were unsuccessful, suggesting that the reactive thiol resided more than 10 Å from the subunit interface. However, other chemical modification and cross-linking experiments [36,37] were interpreted as suggesting that the reactive thiol resided at the subunit interface. On the basis of the structure of the luciferase (Fig. 2 and Fig. 5), we can now state unambiguously that the reactive thiol is not at the subunit interface; the closest approach of the  $\beta$  subunit (at  $\beta$ Arg85) to the reactive thiol on the  $\alpha$  subunit is 11.0 Å, which is consistent with the results of Rausch [21] and inconsistent with the interpretations of chemical cross-linking data [36,37].

Investigation of the location of the reactive thiol on the surface of the enzyme using the program GRASP [49] revealed a crevice on the surface which communicates with a large internal pocket within the enzyme (Fig. 6). Ziegler-Nicoli and colleagues demonstrated that hydrophobic thiol-directed reagents react much faster with the reactive thiol than do less hydrophobic reagents, and suggested that the residue is located near or within a large hydrophobic pocket [43–45]. Their interpretation appears to be correct; the thiol is in contact with solvent, and is within the mouth of a narrow opening to a large hydrophobic cavity (Fig. 6). It should be noted that the reactive thiol on the  $\alpha$  subunit resides in a location that is probably 'below' the disordered protease-labile loop region. As discussed above, following binding of FMNH<sub>2</sub> and O<sub>2</sub>, the protease-labile loop becomes inaccessible to proteases [14]. The thiol also becomes unreactive [14,28], and remains so for much longer than the lifetime of the C4a peroxydihydroflavin [28]. As with the protease sensitivity, the reactivity of the thiol returns slowly, with a  $t_{1/2}$  of ~20 min at 0–4°C

[13,14]. These observations suggest that the disordered loop becomes less disordered as the luciferase reaction proceeds, becoming less protease sensitive and blocking access of thiol-directed reagents to the reactive cysteine.

### Locations of the mutations that alter kinetics

Cline and Hastings [50,51], using random mutagenesis, demonstrated that the active center of bacterial luciferase resides primarily, if not exclusively, on the  $\alpha$  subunit. All mutants detected in a screen for altered enzyme kinetics had lesions in the  $\alpha$  subunit, whereas mutants detected in a screen for thermal instability of the enzyme were roughly equally distributed between  $\alpha$ -subunit mutants and  $\beta$ -subunit mutants [51,52]. Several of the original mutant genes encoding proteins with altered kinetics have been cloned and the locations of the lesions determined [46,53,54]. The locations of these mutations, and several others created by site-directed mutagenesis, are marked on the structure of the wild-type enzyme shown in Figure 5. The mutation at position 113 on the  $\alpha$  subunit, Asp→Asn [46,53], was originally designated AK6 [51,55]. An enzyme with this substitution binds aldehyde with the same affinity as the wild type, but binds reduced flavin very weakly. The bioluminescence emission spectrum is red-shifted by about 12 nm, and the pH activity profile is acid-shifted about two pH units [50,55]. This mutation has been cloned and expressed in *E. coli* [53]. The mutation at position 227, Ser→Phe, was originally designated AK20 [51]. This enzyme binds the aldehyde substrate with lower affinity, but binds reduced flavin with slightly greater affinity than does the wild type [51,56].

In addition to the 'altered kinetics' mutants, the locations of the reactive thiol (at position 106 on the  $\alpha$  subunit), two histidyl residues implicated by Tu and colleagues [57] as being located in or near the active center, and two tryptophanyl residues thought to interact with the flavin

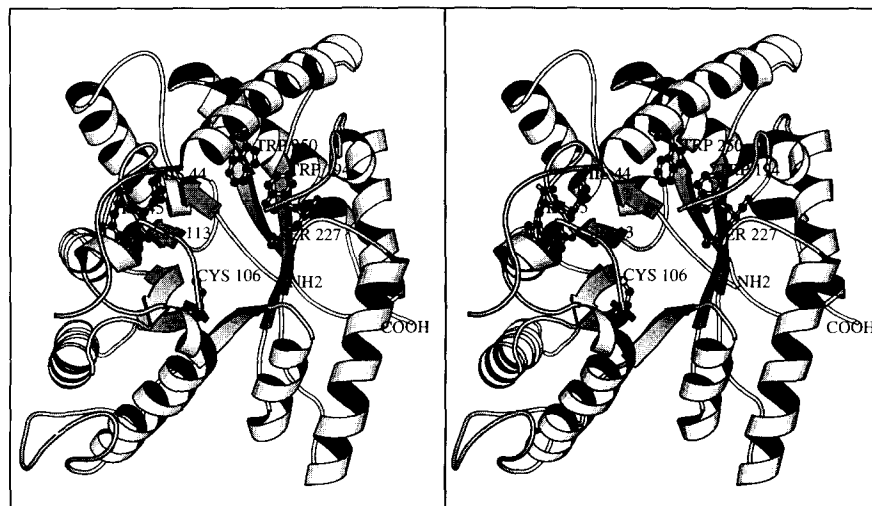
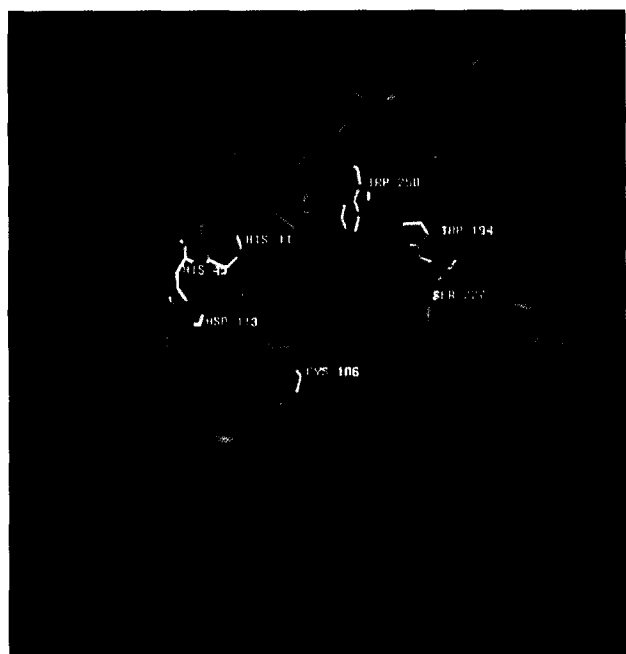
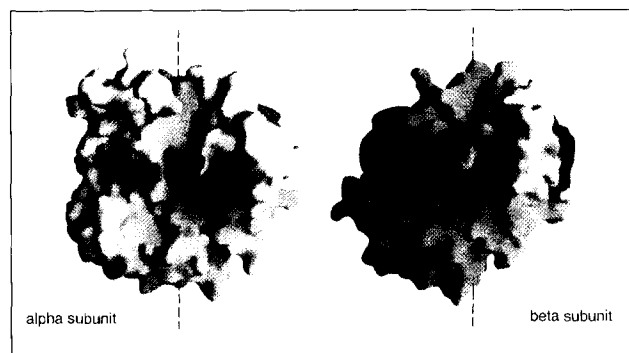


Fig. 5 Stereo view of the  $\alpha$  subunit showing the locations of amino acid side chains that are thought to reside in or near the active center. The view is approximately down the pseudo twofold symmetry axis shown in Figure 2a, from the carboxyl end of the barrel. In this orientation, the  $\beta$  subunit would be located to the left.



**Fig. 6.** Drawing of the  $\alpha$  subunit showing the location of a large internal cavity that communicates with solvent via a narrow opening. The surface of the opening and of the internal cavity is rendered using the program GRASP [49]. The reactive thiol at position 106 of the  $\alpha$  subunit resides in a surface depression near the opening of this cavity, as predicted from previous chemical modification studies. All of the residues indicated in Figure 5, with the exception of His45, contact the surface of this cavity.



**Fig. 7.** Interfacial region of the  $\alpha$  (left) and  $\beta$  (right) subunits rendered using GRASP [49]. The location of the pseudo twofold symmetry axis is indicated by the dashed lines. The subunits were separated and rotated such that the view shown is of the regions of each subunit that contact the other. Regions of positive potential are in blue and regions of negative potential are in red. The majority of the contact surface is hydrophobic. The central region of each interface has multiple potentially charged side chains that appear to interact across the subunit interface.

[58] are shown in Figure 5. The histidine at position 44 of the  $\alpha$  subunit was substituted with alanine by Xin *et al.* [57]. The resulting enzyme catalyzes the bioluminescence with  $\sim 10^{-5}$  the quantum efficiency of the wild-type enzyme. The histidine at position 45 extends away from the internal cavity and appears to interact with Glu88 in helix  $\alpha 3$  of the  $\beta$  subunit and with Glu43 of the  $\alpha$  subunit. Mutation of His45 to alanine [57] resulted in an enzyme that catalyzes the

bioluminescence reaction with a quantum efficiency  $\sim 10^{-6}$  that of the wild-type enzyme. Mutation of tryptophanyl residues 194 or 250 to phenylalanine resulted in greatly reduced bioluminescence activity, decreased affinity for flavin, and altered visible circular dichroism spectra of bound oxidized flavin [58]. These observations suggest a direct interaction of the bound flavin with these two tryptophanyl residues. Bound flavin, either oxidized or reduced, protects the cysteinyl residue at position 106 from modification by thiol-directed reagents [28]. It appears that the protection is attributable to a conformational change in the enzyme rather than direct steric protection: the disordered region of the  $\alpha$  subunit may cover the opening to the large internal cavity as a result of flavin binding. These residues are located in positions that are separated by much greater distances than would be expected for the dimensions of the flavin-binding pocket. Nonetheless, the locations of these residues in the enzyme implicate the internal pocket discussed above (Fig. 6) as being the most likely location of the flavin-binding pocket.

### Proposed location of the flavin-binding pocket

The flavin-binding pocket of luciferase is expected to be large enough to admit FMNH<sub>2</sub>, O<sub>2</sub> and a long-chain aldehyde. The aldehyde substrate used by the luminous bacteria is tetradecanal [59]. Furthermore, the pocket is expected to prevent the access of water to the C4a peroxydihydroflavin intermediate, and to the excited flavin that is formed following decay of the tetrahedral intermediate [2]. The data available at this time do not allow us to locate precisely the flavin-binding pocket, but we feel confident that the active center resides within the large internal cavity in the  $\alpha$  subunit (Fig. 6). It should be noted that every residue implicated as an active-center residue by mutagenesis or chemical modification contacts this internal cavity.

### Nature of the subunit interface

The nature of the subunit interface was of substantial interest in studies of the assembly of the heterodimer and of equilibrium dissociation of the enzyme [40–42]. We have shown that when the individual  $\alpha$  and  $\beta$  subunits are produced in different cultures of recombinant *Escherichia coli*, the subunits do not associate to form active luciferase upon mixing [60,61]. Further experiments demonstrated that proper assembly required that the subunits fold in the same reaction mixture [60]. The only published report of equilibrium dissociation of the  $\alpha$  and  $\beta$  subunits under non-denaturing conditions [62] showed that wild-type enzyme forms slowly when two mutant luciferases, one with a lesion in the  $\alpha$  subunit and one with a lesion in the  $\beta$  subunit, are mixed under

non-denaturing conditions. The halftime at 25°C for the exchange was about 12 hours [62]. One possible explanation of this observation was that the two subunits were intimately intertwined at the interface such that correct assembly could occur only if the two subunits were able to interact during the folding reaction. The structure of the enzyme does not support this hypothesis; indeed, the interface is rather flat and quite extensive, consisting of 3100 Å<sup>2</sup> of the  $\alpha$  subunit and 2950 Å<sup>2</sup> of the  $\beta$  subunit (see Fig. 7) with no instances of one polypeptide protruding into the other. As is common for subunit interfaces, the luciferase subunit interface is largely hydrophobic, with the exception of a patch of charged residues near the middle of the interface region of each subunit (Fig. 7).

This region of potentially charged side chains lies on the pseudo twofold rotational symmetry axis by which the two subunits are related, such that, for example, an arginyl side chain from one subunit that extends toward the other is related by a twofold rotation to an arginyl residue that extends from the second subunit toward the first. The side chains in this highly polar region of the interface are arranged relative to each other such that an intricate hydrogen bonding network appears to exist between the two subunits (Fig. 8). The cluster of potentially charged side chains at the interface appears to communicate with bulk solvent via a narrow channel that is largely attributable to a shallow cleft in the surface of the  $\alpha$  subunit aspect of the interface.

The inability of folded  $\alpha$  and  $\beta$  subunits to interact is the result of a slow homodimerization reaction of the  $\beta$  subunit to yield a kinetically stable species that does not unfold in 5 M urea [39•]. Preliminary structural data have recently been obtained from crystals of the

$\beta_2$  homodimer, the species formed when  $\beta$  subunits are allowed to fold in the absence of  $\alpha$  subunits (JB Thoden, HM Holden, JF Sinclair, TO Baldwin, I Rayment, unpublished data). It appears that the proposed solvent channel of the heterodimer has been occluded in the  $\beta_2$  homodimer as a result of several differences in the amino acid sequence of the  $\alpha$  and  $\beta$  subunits [18,19]. However, whether the kinetic stability of the  $\beta_2$  homodimer in 5 M urea is due to the inability of solvent water to access the charged residues buried at the subunit interface will require further experimentation.

### Role of the $\beta$ subunit

It is unclear from its structure why the  $\beta$  subunit is required for the high quantum yield reaction observed with the heterodimeric enzyme. As with the  $\alpha$  subunit (Fig. 6), there is an internal cavity located at the carboxyl end of the barrel of the  $\beta$  subunit. However, the cavity is much smaller than that of the  $\alpha$  subunit. It is possible that the cavity in the  $\beta$  subunit could constitute the second, low-affinity, flavin-binding site reported by Vervoort *et al.* [17]. The active center of the heterodimer seems to reside exclusively on the  $\alpha$  subunit, yet the  $\beta$  subunit is required for the high quantum yield bioluminescence reaction [2]. Individually both the  $\alpha$  and the  $\beta$  subunits are capable of only a very low quantum yield bioluminescence reaction [60,61,63•] and it is not clear that the  $\beta$  subunit contributes anything directly to the active center of the heterodimer. Numerous authors have proposed that the  $\beta$  subunit may be required to stabilize the high quantum yield conformation of the  $\alpha$  subunit

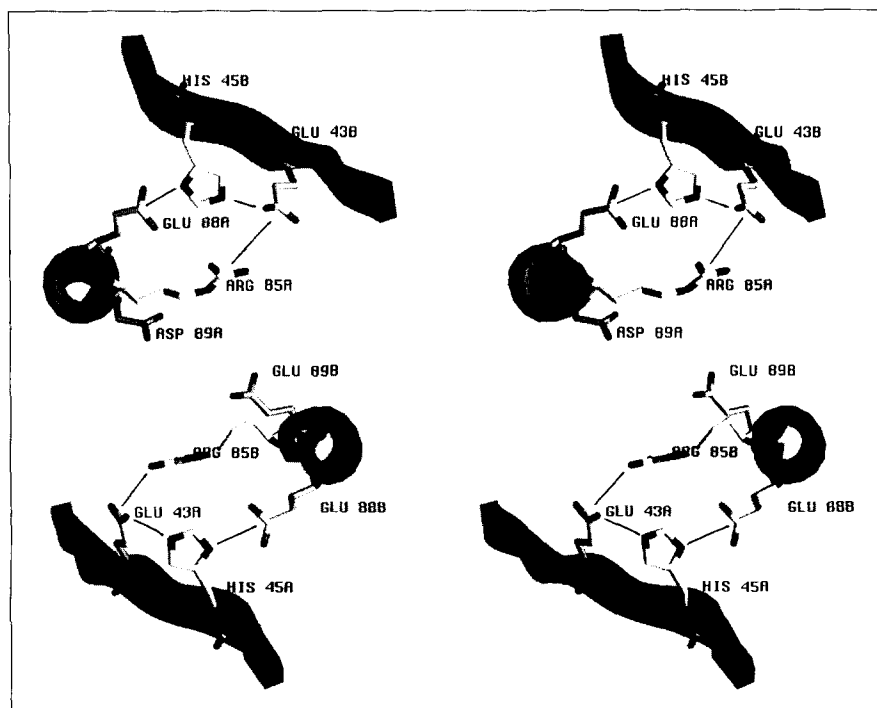


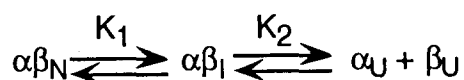
Fig. 8. Stereo drawing showing a portion of the proposed hydrogen-bonding network at the  $\alpha/\beta$  subunit interface. The view is down the pseudo twofold axis which is located between Asp89 of the  $\alpha$  subunit and Glu89 of the  $\beta$  subunit.

through interactions across the subunit interface (see [2] for further references). At this time, the only additional suggestion that we can make is that the disordered area of the  $\alpha$  subunit extending from residue 272 to residue 288 might interact with the  $\beta$  subunit, as residue 271 of the  $\alpha$  subunit is located  $\sim 4.5$  Å from Asn118 of the  $\beta$  subunit.

### Folding and assembly of bacterial luciferase

Bacterial luciferase has proved to be an interesting and informative subject for the study of the processes of subunit folding and assembly. Because the enzyme is a heterodimer, it has been possible to investigate the folding of the individual subunits as well as the assembly that occurs upon mixing of the refolding subunits. The exquisite sensitivity of the bioluminescence assay allows direct measurement of the formation of the heterodimer during refolding following denaturation [40,41,64–67] or folding during synthesis on a ribosome [68••].

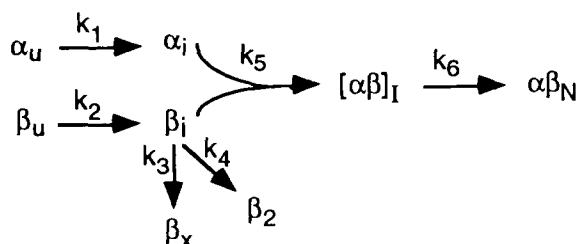
Equilibrium unfolding studies of the heterodimer have shown that the enzyme unfolds through a well populated non-native heterodimeric intermediate [42]. The data were fitted to a three-state mechanism as indicated below:



where N indicates the native, folded state, I indicates the intermediate, and U indicates the fully unfolded state.

The equilibrium constants  $K_1$  and  $K_2$ , extrapolated to water, were shown to be  $4.03 \times 10^{-4}$  and  $1.60 \times 10^{-15}$  M, respectively. The conversion from  $\alpha\beta_N$  to  $\alpha\beta_I$  was independent of protein concentration. The intermediate ( $\alpha\beta_I$ ) is enzymatically inactive, and it has a higher fluorescence quantum yield of the protein tryptophanyl residues and a lower circular dichroism at 222 nm than the native heterodimer ( $\alpha\beta_N$ ) [42]. The intermediate  $\alpha\beta_I$  was maximally populated at 18°C in the presence of  $\sim 2.2$  M urea [42], conditions that appear to cause partial unfolding of the protein.

Extensive refolding studies have shown that the folding and assembly of luciferase subunits to yield the heterodimeric enzyme can be well described by the following kinetic mechanism:



The conversions of  $\alpha_u$  and  $\beta_u$  to  $\alpha_i$  and  $\beta_i$ , represented here by single reactions with rate constants of  $k_1$

and  $k_2$  respectively, occur through multiple intermediates, but are shown as single kinetic processes for simplicity. The dimerization-competent forms of the two subunits,  $\alpha_i$  and  $\beta_i$ , associate with a bimolecular rate constant of  $\sim 2400 \text{ M}^{-1} \text{ s}^{-1}$  at 18°C in 50 mM phosphate buffer, pH 7.0. The resulting heterodimer appears to be inactive, and undergoes an isomerization process to become active. The  $\beta$  subunit has alternative folding pathways available to it: it can self-associate to form the homodimer, discussed above [39••], or it can isomerize to form a dimerization-incompetent form,  $\beta_x$ , which does not appear to be in equilibrium with the dimerization-competent form,  $\beta_i$  [58,69]. The formation of  $\beta_x$  appears to be highly temperature dependent, and predominates above 35°C. Mutants which are temperature sensitive with respect to folding [70] that have slow rates of formation of the heterodimer form large amounts of  $\beta_x$ , as expected from the kinetic mechanism discussed above.

In related studies, we have used the luciferase system to investigate whether protein folding occurs coincident with synthesis on ribosomes [68••]. By adding folded  $\alpha$  subunit ( $\alpha_i$ ) to a cell-free translation reaction in which the  $\beta$  subunit was being actively synthesized, we found that the newly synthesized  $\beta$  subunit must be released from the ribosome prior to association with the free  $\alpha$  subunit to form active enzyme. Furthermore, the newly synthesized  $\beta$  subunit requires only a brief interval in which to associate with the  $\alpha$  subunit and become active; much more time is required for fully synthesized but unfolded  $\beta$  subunit to fold in the same reaction mixture, which includes chaperones and other cellular constituents. These results demonstrate that the  $\beta$  subunit of bacterial luciferase folds during synthesis and is released from the ribosome in a nearly folded form that requires only a brief time to bind  $\alpha$  subunit and assume the active conformation [68••].

### Conclusions

Determination of the structure of bacterial luciferase has allowed interpretation of many observations documented during the past few decades, but knowledge of the structure has by no means answered all of the questions raised by these observations. Among the many unanswered questions are those concerning the locations of the binding sites for flavin and aldehyde. Possible locations are currently being investigated, and knowledge of the active center structure will surely assist in studies of the chemical mechanism of the enzyme. Investigation of the postulated roles of the charged residues at the subunit interface, and the proposed channel from this region to the bulk solvent in the  $\alpha\beta$  heterodimer *versus* the  $\beta_2$  homodimer, may provide insights into the structural basis of the exceptionally slow processes of association and dissociation of the  $\beta_2$  homodimer. The long-awaited structure of this

intriguing enzyme appears to have provided a starting point for detailed mechanistic studies rather than the answers to all of our questions.

## Acknowledgements

Research in the laboratories of the authors is supported by grants from the Office of Naval Research (N00014-93-1-0991 and N00014-93-1-1345 to TOB and MMZ), the National Institutes of Health (GM33894 to FMR, AR35186 to IR, Fellowship AR08304 to AJF and Traineeship T32GM08523 to JAC) and the Robert A Welch Foundation (A-865 to TOB and A-840 to FMR).

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Boyle R: **Experiments concerning the relation between light and air in shining wood and fish.** *Philos Trans R Soc Lond [Biol]* 1668, 2:581–600.
2. Baldwin TO, Ziegler MM: **The biochemistry and molecular biology of bacterial bioluminescence.** In *Chemistry and Biochemistry of Flavoenzymes, vol III*. Edited by Müller F. Boca Raton: CRC Press; 1992:467–530.
3. Fisher AJ, Raushel FM, Baldwin TO, Rayment I: **The three-dimensional structure of bacterial luciferase from *Vibrio harveyi* at 2.4 Å resolution.** *Biochemistry* 1995, 34:6581–6586.
- The structure of bacterial luciferase has been a subject of interest and active investigation for over two decades. The structure of the heterodimer exhibits the symmetry expected of homologous subunits, and the (β/α)<sub>8</sub> structure of each subunit and the mode of packing strongly suggests that the active center is not at the subunit interface.
4. Matheson IBC, Lee J, Müller F: **Bacterial bioluminescence: spectral study of the emitters in the *in vitro* reaction.** *Proc Natl Acad Sci USA* 1981, 78:948–952.
5. Lee J, O'Kane DJ, Gibson BG: **Bioluminescence spectral and fluorescence dynamic study of the interaction of lumazine protein with the intermediates of bacterial luciferase bioluminescence.** *Biochemistry* 1989, 28:4263–4271.
6. Prasher DC, O'Kane D, Lee J, Woodward B: **The lumazine protein gene in *Photobacterium phosphoreum* is linked to the lux operon.** *Nucleic Acids Res* 1990, 18:6450.
7. O'Kane D, Woodward B, Lee J, Prasher DC: **Borrowed proteins in bacterial bioluminescence.** *Proc Natl Acad Sci USA* 1991, 88:1100–1104.
8. Ruby EG, Nealson KH: **A luminous bacterium that emits yellow light.** *Science* 1977, 196:432–434.
9. Daubner SC, Astorga A, Leisman G, Baldwin TO: **Yellow light emission of *Vibrio fischeri* strain Y-1: purification and characterization of the energy-accepting yellow fluorescent protein.** *Proc Natl Acad Sci USA* 1987, 84:8912–8916.
10. Macheroux P, Schmidt KU, Steinerstauch P, Ghisla S, Colepicolo P, Buntic R, Hastings JW: **Purification of the yellow fluorescent protein from *Vibrio fischeri* and identity of the flavin chromophore.** *Biochem Biophys Res Commun* 1987, 146:101–106.
11. Daubner SC, Baldwin TO: **Interaction between luciferases from various species of bioluminescent bacteria and the yellow fluorescent protein of *Vibrio fischeri* strain Y-1.** *Biochem Biophys Res Commun* 1989, 161:1191–1198.
12. Baldwin TO, Treat ML, Daubner SC: **Cloning and expression of the luxY gene from *Vibrio fischeri* Y1 in *Escherichia coli* and the complete amino acid sequence of the yellow fluorescent protein.** *Biochemistry* 1990, 29:5509–5515.
13. Aboukhaire NK, Ziegler MM, Baldwin TO: **The catalytic turnover of bacterial luciferase produces a quasi-stable species of altered conformation.** In *Flavins and Flavoproteins*. Edited by Bray RC, Engel PC, Mayhew SG. Berlin: Walter de Gruyter; 1984:371–374.
14. Aboukhaire NK, Ziegler MM, Baldwin TO: **Bacterial luciferase: demonstration of a catalytically competent altered conformational state following a single turnover.** *Biochemistry* 1985, 24:3942–3947.
15. Baldwin TO, Ziegler MM, Powers DA: **The covalent structure of the subunits of bacterial luciferase: N-terminal sequence demonstrates subunit homology.** *Proc Natl Acad Sci USA* 1979, 76:4887–4889.
16. Becvar JE, Hastings JW: **Bacterial luciferase requires one reduced flavin for light emission.** *Proc Natl Acad Sci USA* 1975, 72:3374–3376.
17. Vervoort J, Müller F, O'Kane DJ, Lee J, Bacher A: **Bacterial luciferase. A carbon-13, nitrogen-15 and phosphorus-31 NMR investigation.** *Biochemistry* 1986, 25:8067–8075.
18. Cohn DH, Mileham AJ, Simon MI, Nealson KH, Rausch SK, Bonam D, Baldwin TO: **Nucleotide sequence of the luxA gene of *Vibrio harveyi* and the complete amino acid sequence of the α subunit of bacterial luciferase.** *J Biol Chem* 1985, 260:6139–6146.
19. Johnston TC, Thompson RB, Baldwin TO: **Nucleotide sequence of the luxB gene of *Vibrio harveyi* and the complete amino acid sequence of the β subunit of bacterial luciferase.** *J Biol Chem* 1986, 261:4805–4811.
20. Ziegler MM, Rausch SK, Merritt MV, Baldwin TO: **Active center studies on bacterial luciferase: locations of the protease labile regions and the reactive cysteinyl residue in the primary structure of the α subunit.** In *Analytical Applications of Bioluminescence and Chemiluminescence*. Edited by Schölmerich J, Andreesen R, Kapp A, Ernst M, Woods WG. New York: John Wiley; 1987:377–380.
21. Rausch SK: **Active center structure and sequence studies on bacterial luciferase utilizing the essential cysteine, protease-labile region, and delta fragments [PhD thesis].** Urbana: University of Illinois; 1983.
22. Njus D, Baldwin TO, Hastings JW: **A sensitive assay for proteolytic enzymes using bacterial luciferase as a substrate.** *Anal Biochem* 1974, 61:280–287.
23. Baldwin TO, Hastings JW, Riley PL: **Proteolytic inactivation of the luciferase from the luminous marine bacterium *Beneckea harveyi*.** *J Biol Chem* 1978, 253:5551–5554.
24. Baldwin TO, Riley PL: **Anion binding to bacterial luciferase: evidence for binding associated changes in enzyme structure.** In *Flavins and Flavoproteins*. Edited by Yagi K, Yamano T. Tokyo: Japan Scientific Societies Press; Baltimore: University Park Press; 1980:139–147.
25. Holzman TF, Baldwin TO: **The effects of phosphate on the structure and stability of the luciferases from *Beneckea harveyi*, *Photobacterium fischeri*, and *Photobacterium phosphoreum*.** *Biochem Biophys Res Commun* 1980, 94:1199–1206.
26. Holzman TF, Riley PL, Baldwin TO: **Inactivation of luciferase from the luminous marine bacterium *Beneckea harveyi* by proteases: evidence for a protease labile region and properties of the protein following inactivation.** *Arch Biochem Biophys* 1980, 205:554–563.
27. Holzman TF, Baldwin TO: **Proteolytic inactivation of luciferases from three species of luminous marine bacteria, *Beneckea harveyi*, *Photobacterium fischeri*, and *Photobacterium phosphoreum*: evidence of a conserved structural feature.** *Proc Natl Acad Sci USA* 1980, 77:6363–6367.
28. Nicoli MZ, Meighen EA, Hastings JW: **Bacterial luciferase. Chemistry of the reactive sulfhydryl.** *J Biol Chem* 1974, 249:2385–2392.

29. Abu-Soud H, Mullins LS, Baldwin TO, Raushel FM: **A stopped-flow kinetic analysis of the bacterial luciferase reaction.** *Biochemistry* 1992, **31**:3807–3813.
30. Banner DW, Bloomer AC, Petsko GA, Phillips DC, Pogson CI, Wilson IA, Corran PH, Furth AJ, Milman JD, Offord RE *et al.*: **Structure of chicken muscle triose phosphate isomerase determined crystallographically at 2.5 Å resolution using amino acid sequence data.** *Nature* 1975, **255**:609–614.
31. Farber GK, Petsko GA: **The evolution of  $\alpha/\beta$  barrel enzymes.** *Trends Biochem Sci* 1990, **15**:228–234.
32. Fox KM, Karplus PA: **Old yellow enzyme at 2 Å resolution: overall structure, ligand binding, and comparison with related flavoproteins.** *Structure* 1994, **2**:1089–1105.
33. Lindqvist Y: **Refined structure of spinach glycolate oxidase at 2 Å resolution.** *J Mol Biol* 1989, **209**:151–166.
34. Lim LW, Shamala N, Mathews FS, Steenkamp DJ, Hamlin R, Xuong NH: **Three-dimensional structure of the iron-sulfur flavoprotein trimethylamine dehydrogenase at 2.4 Å resolution.** *J Biol Chem* 1986, **261**:15140–15146.
35. Xia ZX, Mathews FS: **Molecular structure of flavocytochrome  $b_2$  at 2.4 Å resolution.** *J Mol Biol* 1990, **212**:837–863.
36. Tu SC, Henkin J: **Characterization of the aldehyde binding site of bacterial luciferase by photoaffinity labeling.** *Biochemistry* 1983, **22**:519–523.
37. Paquette O, Fried A, Tu SC: **Delineation of bacterial luciferase aldehyde site by bifunctional labeling reagents.** *Arch Biochem Biophys* 1988, **264**:392–399.
38. Ruby EG, Hastings JW: **Proteolytic sensitivity of the  $\alpha$  subunit in luciferases of *Photobacterium* species.** *Curr Microbiol* 1979, **3**:157–159.
39. Sinclair JF, Ziegler MM, Baldwin TO: **Kinetic partitioning during protein folding yields multiple native states.** *Nature Struct Biol* 1994, **1**:320–326.
- The  $\beta$  subunit of bacterial luciferase homodimerizes when allowed to fold independently. The homodimer forms slowly, with a rate constant of  $\sim 150 \text{ M}^{-1} \text{ s}^{-1}$  at 18°C in 50 mM phosphate, pH 7.0. The dissociation rate constant, determined following mixing with various concentrations of guanidinium chloride, is  $\sim 10^{-14} \text{ s}^{-1}$  in denaturant-free buffer. The dissociation half-life of about a million years explains the failure to form active enzyme upon mixing of folded  $\alpha$  subunit with folded  $\beta$  subunit. The kinetically preferred folding pathway yields the  $\alpha\beta$  heterodimer, but as the concentration of  $\alpha$  subunit is reduced, the rate for the pathway to the heterodimeric enzyme slows, so that the pathway to the  $\beta\beta$  homodimer becomes the kinetically preferred pathway.
40. Ziegler MM, Goldberg ME, Chaffotte AF, Baldwin TO: **Refolding of luciferase subunits from urea and assembly of the active heterodimer. Evidence for folding intermediates that precede and follow the dimerization step on the pathway to the active form of the enzyme.** *J Biol Chem* 1993, **268**:10760–10765.
41. Baldwin TO, Ziegler MM, Chaffotte AF, Goldberg ME: **Contribution of folding steps involving the individual subunits of bacterial luciferase to the assembly of the active heterodimeric enzyme.** *J Biol Chem* 1993, **268**:10766–10772.
42. Clark AC, Sinclair JF, Baldwin TO: **Folding of bacterial luciferase involves a non-native heterodimeric intermediate in equilibrium with the native enzyme and the unfolded subunits.** *J Biol Chem* 1993, **268**:10773–10779.
43. Nicoli MZ: **Active center studies on bacterial luciferase [PhD thesis].** Cambridge, MA: Harvard University; 1972.
44. Nicoli MZ, Hastings JW: **Bacterial luciferase. The hydrophobic environment of the reactive sulfhydryl.** *J Biol Chem* 1974, **249**:2393–2396.
45. Merritt MV, Baldwin TO: **Modification of the reactive sulfhydryl of bacterial luciferase with spin-labeled maleimides.** *Arch Biochem Biophys* 1980, **202**:499–506.
46. Baldwin TO, Chen LH, Chlumsky LJ, Devine JH, Johnston TC, Lin J-W, Sugihara J, Waddle JJ, Ziegler MM: **Structural analysis of bacterial luciferase.** In *Flavins and Flavoproteins*. Edited by McCormick DB, Edmondson DE. Berlin: Walter de Gruyter; 1987:621–631.
47. Baldwin TO, Devine JH, Heckel RC, Lin JW, Shadel GS: **The complete nucleotide sequence of the *lux* regulon of *Vibrio fischeri* and the *luxABN* region of *Photobacterium leiognathi* and the mechanism of control of bacterial bioluminescence.** *J Biolumin Chemilumin* 1989, **4**:326–341.
48. Baldwin TO, Chen LH, Chlumsky LJ, Devine JH, Ziegler MM: **Site-directed mutagenesis of bacterial luciferase: analysis of the 'essential' thiol.** *J Biolumin Chemilumin* 1989, **4**:40–48.
49. Nicholls A, Sharp K, Honig B: **Protein folding and association: Insights from the interfacial and thermodynamic properties of hydrocarbons.** *Proteins* 1991, **11**:281–296.
50. Cline TW: **Mutational alteration of the bacterial bioluminescence system [PhD thesis].** Cambridge, MA: Harvard University; 1973.
51. Cline TW, Hastings JW: **Mutationally altered bacterial luciferase. Implications for subunit functions.** *Biochemistry* 1972, **11**:3359–3370.
52. Cline TW, Hastings JW: **Temperature-sensitive mutants of bioluminescent bacteria.** *Proc Natl Acad Sci USA* 1971, **68**:500–504.
53. Chlumsky LJ, Chen LH, Clark C, Abu-Soud H, Ziegler MM, Raushel FM, Baldwin TO: **Random and site directed mutagenesis of bacterial luciferase.** In *Flavins and Flavoproteins*. Edited by Curti B, Ronchi S, Zanetti G. Berlin: Walter de Gruyter; 1991:261–264.
54. Chlumsky LJ: **Investigation of the structure of bacterial luciferase from *Vibrio harveyi* using enzymes generated by random and site-directed mutagenesis [PhD thesis].** College Station: Texas A&M University; 1991.
55. Cline TW, Hastings JW: **Mutated luciferases with altered bioluminescence emission spectra.** *J Biol Chem* 1974, **249**:4668–4669.
56. Chen LH, Baldwin TO: **Random and site-directed mutagenesis of bacterial luciferase: Investigation of the aldehyde binding site.** *Biochemistry* 1989, **28**:2684–2689.
57. Xin X, Xi L, Tu SC: **Functional consequences of site-directed mutation of conserved histidyl residues of the bacterial luciferase  $\alpha$  subunit.** *Biochemistry* 1991, **30**:11255–11262.
58. Clark AC: **Thermodynamic and kinetic studies of the polypeptide folding of bacterial luciferase from *Vibrio harveyi*: A mutational analysis [PhD thesis].** College Station: Texas A&M University; 1994.
59. Ulitzur S, Hastings JW: **Evidence for tetradecanal as the natural aldehyde in bacterial bioluminescence.** *Proc Natl Acad Sci USA* 1979, **76**:265–267.
60. Waddle JJ, Johnston TC, Baldwin TO: **Polypeptide folding and dimerization in bacterial luciferase occur by a concerted mechanism *in vivo*.** *Biochemistry* 1987, **26**:4917–4921.
61. Sinclair JF, Waddle JJ, Waddill EF, Baldwin TO: **Purified native subunits of bacterial luciferase are active in the bioluminescence reaction but fail to assemble into the  $\alpha\beta$  structure.** *Biochemistry* 1993, **32**:5036–5044.
62. Anderson C, Tu SC, Hastings JW: **Subunit exchange and specific activities of mutant bacterial luciferases.** *Biochem Biophys Res Commun* 1980, **95**:1180–1186.
63. Choi H, Tang CK, Tu SC: **Catalytically active forms of the individual subunits of *Vibrio harveyi* luciferase and their kinetic and binding properties.** *J Biol Chem* 1995, **270**:16813–16819.
- The homodimeric structure of the  $\beta$  subunit has been confirmed, as have the low bioluminescence activities of the individual subunits. The substrate binding affinities have also been determined.
64. Friedland J, Hastings JW: **The reversibility of the denaturation of bacterial luciferase.** *Biochemistry* 1967, **9**:2893–2900.
65. Friedland J, Hastings JW: **Nonidentical subunits of bacterial luciferase: their isolation and recombination to form active enzyme.** *Proc Natl Acad Sci USA* 1967, **58**:2336–2342.

66. Gunsalus-Miguel A, Meighen EA, Nicoli MZ, Nealson KH, Hastings JW: **Purification and properties of bacterial luciferases.** *J Biol Chem* 1972, **247**:398–404.
  67. Ruby EG, Hastings JW: **Formation of hybrid luciferases from subunits of different species of *Photobacterium*.** *Biochemistry* 1980, **19**:4989–4993.
  68. Fedorov AN, Baldwin TO: **Contribution of cotranslational folding to the rate of formation of native protein structure.** *Proc Natl Acad Sci USA* 1995, **92**:1227–1231.
- The rate of folding of the  $\beta$  subunit as it is synthesized on ribosomes is faster than the rate of refolding of the urea-unfolded  $\beta$  subunit under otherwise identical conditions. Folding of the polypeptide must occur during the process of synthesis.
69. Sinclair JF: **Equilibrium and kinetic studies of the folding of the subunits of bacterial luciferase [PhD thesis].** College Station: Texas A&M University; 1995.
  70. Sugihara J, Baldwin TO: **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.** *Biochemistry* 1988, **27**:2872–2880.

71. Abu-Soud H, Clark AC, Francisco WA, Baldwin TO, Raushel FM: **Kinetic destabilization of the hydroxyperoxyflavin intermediate by site-directed modification of the reactive thiol in bacterial luciferase.** *J Biol Chem* 1993, **268**:7699–7706.
72. Francisco WA, Abu-Soud H, Baldwin TO, Raushel FM: **Interaction of bacterial luciferase with aldehyde substrates and inhibitors.** *J Biol Chem* 1993, **268**:24734–24741.

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