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Crystal Structure of Firefly Luciferase in a Second Catalytic Conformation Supports a Domain Alternation Mechanism

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Supporting Information

ABSTRACT: Beetle luciferases catalyze a two-step reaction that includes the initial adenylation of the luciferin substrate, followed by an oxidative decarboxylation that ultimately produces light. Evidence for homologous acyl-CoA synthetases supports a domain alternation catalytic mechanism in which these enzymes' C-terminal domain rotates by ∼140° to adopt two conformations that are used to catalyze the two partial reactions. While many structures exist of acyl-CoA synthetases in both conformations, to date only biochemical evidence supports domain alternation with luciferase. We have determined the structure of a cross-linked luciferase enzyme that is trapped in the second conformation. This new structure supports the role of the second catalytic conformation and provides insights into the biochemical mechanism of the luciferase oxidative step.

Firefly luciferase is a member of a large superfamily of adenylating enzymes. This ANL superfamily, named after the acyl-CoA synthetases, the adenylation domains of the modular non-ribosomal peptide synthetases (NRPSs), and luciferase, is present throughout all kingdoms of life and plays critical roles in both primary and secondary metabolism.[1] The three subfamilies catalyze two-step reactions, sharing an initial adenylating step to produce an acyl-AMP intermediate. The adenylate then serves as a substrate for a second step that is specific to the particular subfamily. The acyl-CoA synthetases and NRPS adenylation domains both use the adenylate for a thioester-forming reaction, using the pantetheine thiol of either CoA or a *holo*-acyl carrier protein domain as an acceptor of the activated acyl group. In contrast to these reactions, luciferase catalyzes a multistep oxidative decarboxylation of the luciferyl-AMP intermediate (LH_2-AMP) to produce bioluminescence.

ANL enzymes contain a 400−500 residue N-terminal domain and a smaller C-terminal domain of ∼110−130 amino acids. The active site of these enzymes is located at the interface between these two domains. Ten conserved regions of these proteins have been termed the A1-A10 motifs;[2] several of these motifs play critical roles in either or both partial reactions.[1] ANL enzymes use a domain alternation catalytic strategy in which the initial adenylation reaction is catalyzed by one conformation. Upon formation of the adenylate and release of pyrophosphate, a ∼140° rotation of the C-terminal domain allows the enzymes to adopt a conformation that is used for the second partial reaction. This strategy is supported by crystal structures of multiple acyl-CoA synthetases bound to CoA or CoA analogues[3−6] and, recently, of NRPS adenylation domains bound to the acyl-carrier proteins.[7,8] Examination of mutations to residues on opposing faces of the C-terminal domain[9,10] and the hinge residue[7,9,11] also supports this mechanism.

For luciferase, however, only biochemical evidence supports this catalytic strategy. Two lysine residues, one on each face of the C-terminal domain, are required for each partial reaction in a manner that suggests that a similar domain rotation is required for catalysis of the full reaction.[12,13] Mutation of Lys529, the A10 lysine, impairs only the adenylation reaction, while mutation of Lys443 in the A8 region disrupts the oxidative reaction. Recently, an engineered luciferase enzyme containing cysteine substitions was designed to test the domain movement hypothesis.[14] The two targeted residues are 37 Å apart in *Luciola cruciata* luciferase in the adenylate-forming conformation[15] but should be susceptible to cross-linking if the enzyme can adopt the second conformation. Indeed, the luciferase variant could react with a chemical cross-linker. Importantly, this trapped enzyme was competent for catalysis of the oxidative reaction when provided with the luciferyl-adenylate intermediate (LH_2-AMP), and the activity is dependent on the side chain of the A8 motif residue, Lys443.

We have successfully crystallized this trapped luciferase enzyme[14] and present here the first crystal structure of luciferase in this second catalytic conformation.

Structural Determination of the Chemically Cross-Linked Luciferase Enzyme. Ppy 9[−] C108/C447 is a *Photinus pyralis* luciferase variant in which the four native cysteine residues were eliminated by mutation to serine or alanine and two surface cysteines were introduced with the I108C and Y447C changes.[14] The structure of this enzyme, bound to the adenylate analogue 5′-*O*-[*N*-dehydroluciferyl)-sulfamoyl]-adenosine (DLSA)[16] and treated with the chemical cross-linker 1,2-*bis*(maleimido) ethane, was solved by molecular replacement using the N-terminal domain of luciferase.[17] The C-terminal domain was identified through manual model building

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and refinement. The two chains of the asymmetric unit are very similar, with a root-mean-square displacement of all Cα positions of 0.4 Å. For the purpose of comparison, we also solved a new crystal structure of the wild-type *P. pyralis* luciferase enzyme in the adenylate-forming conformation bound to DLSA (Figure 1A).

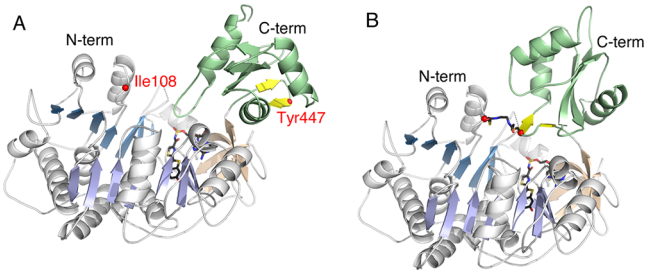

Figure 1. Ribbon diagrams of luciferase in both conformations. (A) The wild-type luciferase in the adenylate-forming conformation bound to DLSA. The location of Ile108 and Tyr447 are highlighted in red. (B) The cross-linked luciferase bound to DLSA crystallized in the second conformation.

Comparison of Luciferase Enzymes in Adenylate-Forming and Second Conformations. The Ppy 9$^-$ C108/C447 luciferase enzyme crystallized in the conformation identified previously to be used by thioester-forming ANL enzymes to catalyze the second partial reaction (Figure 1B). Following the conserved A8 motif harboring the hinge residue at Lys439, the antiparallel two stranded β-sheet is directed into the active site of the enzyme. The ϕ/ψ angles of Lys439 change from $-73°/-12°$ in the structure of wild-type luciferase in the adenylate-forming conformation to $-69°/158°$ in the cross-linked structure. As with other members of the ANL family of enzymes, this illustrates that a large component of the conformational change occurs with a rotation of the ψ angle of the hinge residue. Additional torsion angle changes are seen in ϕ angles for Arg437 and Leu441, although the magnitude of the change is not as large as at the hinge residue Lys439.

The active sites of the two luciferase structures illustrate conserved interactions that have been seen in other adenylate-forming enzymes of the ANL superfamily. In the adenylate-forming conformation, Lys529, the catalytic lysine for the initial adenylation reaction interacts with the carbonyl oxygen of the adenylate, the O5 atom that bridges the ribose and the sulfamate moiety, and the main chain carbonyl of Gly316. In the second conformation, observed with the cross-linked luciferase enzyme, the side chain amine of Lys443 adopts a nearly identical position as Lys529 (Figure 2). Additionally, from the C-terminal domain, Gln448 also rotates into the binding pocket where it interacts with a sulfamate oxygen. These interactions, along with an ionic interaction between Glu479 and Arg437, are the major interactions that stabilize the new C-terminal conformation.

His245 is part of the A4 motif that exhibits distinct side chain torsional rotations in the two conformational states.[1] In the adenylate-forming conformation, this residue points toward the active site, preventing access of water and perhaps stabilizing the approach of the negatively charged luciferyl carboxylate and ATP. In the thioester-forming members of the enzyme family, the rotation of the C-terminal domain withdraws the side chain out of the active site, clearing a tunnel for CoA or pantetheine to approach the adenylate intermediate. This side chain motion

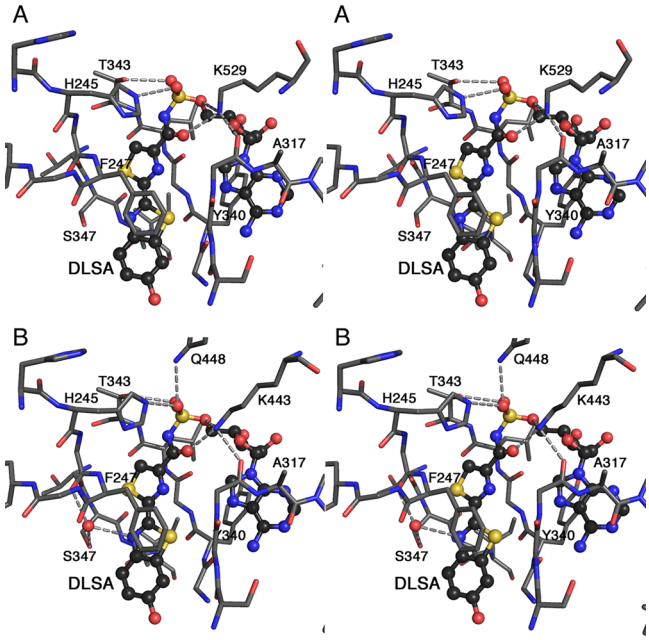

Figure 2. Stereoview of the active site of luciferase complexes with DLSA in the (A) adenylate-forming and (B) second catalytic conformation observed with the cross-linked enzyme.

appears to be induced by a residue from the C-terminal domain.[10] In the Ppy 9$^-$ C108/C447 luciferase structure, electron density for the side chain of His245 is weak, suggesting that His245 is adopting both side chain torsional conformations. On the basis of comparisons to other ANL enzymes, the residue from the C-terminal domain that would normally stabilize the rotated His245 position is Tyr447, which was mutated to cysteine for the cross-link formation. The missing side chain of Tyr447 most likely prevents the full rotation of His245 and contributes to the torsional disorder seen in the crystal structure.

Insights into the Biochemical Mechanism of LH_2-AMP Oxidation. The structure provides insights into several interesting features of the luciferase-catalyzed reaction. Like the homologous acyl-CoA synthetases, luciferase can catalyze thioester formation between CoA and luciferin, using LH_2-AMP as an intermediate.[18] In the cross-linked luciferase, the pantetheine tunnel seen in other ANL enzymes is intact and leads to the C4 carbon of DLSA. Luciferase contains in the A8 motif a conserved glycine residue, Gly446, that lines this tunnel. In the thioester-forming enzymes of this family, this glycine forms a distorted β-sheet interaction with the amide nitrogens of the pantethene moiety and likely facilitates CoA binding in this alternate reaction. A G446I mutation in luciferase specifically impairs the oxidative reaction[13] suggesting that O_2 approaches the intermediate through this same tunnel. Side chain rotation of His245, as discussed above, further expands the tunnel for access to C4 (see Supporting Information).

The structure also provides an explanation for the stereochemical requirements of luciferase. It is reported that while the enzyme is able to catalyze thioester-formation between CoA and both D- and L-luciferin, only D-luciferin is able to support the generation of light.[19] This observation is explained by the accessibility of the carbonyl of the adenylate of both diastereomers to the pantetheine tunnel to be attacked by the CoA thiol. In contrast, the C4 proton must be available for removal, possibly as a hydrogen atom (see below), in order to

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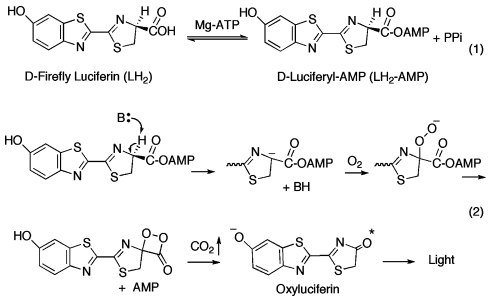
initiate the oxidative reaction required for the generation of light. The structure demonstrates that the adenylate with L-luciferin would project the C4 hydrogen toward the core of the protein and away from the pantetheine tunnel, preventing proton or hydrogen atom abstraction.

The generally accepted mechanism of firefly luciferase-catalyzed light production, as advanced primarily by the work of White and co-workers,[20] is initiated by abstraction of the C4 proton of LH_2-AMP by a presumed active site nucleophile to produce a carbanion (Scheme 1). The involvement of a peroxy

Scheme 1. Generally Accepted Mechanism of Firefly Bioluminescence



anion/hydroperoxide intermediate leading to a highly reactive dioxetanone intermediate, while not directly observed, is very reasonable and consistent with the general current view of excited state formation in bioluminescence.[21] It is, however, problematic that peroxide formation from unactivated molecular O_2 is a spin forbidden process.[22] Perhaps an even more serious shortcoming of an enzyme mechanism requiring C4 carbanion formation is the absence of a required nucleophile proximal to C4 (Figure 2B). While it might be tempting to suggest that the side chain imidazole of H245 is the critical residue, our prior finding[23] that the H245A and H245F luciferase mutants retained ~20% of the activity of the wild-type enzyme make it quite unlikely. The absence of an active site base near C4 certainly does not disprove this mechanism; however, it may be worthwhile to consider alternatives that do not require carbanion formation. We therefore propose for consideration another mechanism for formation of the key LH_2-AMP hydroperoxide intermediate that involves O_2 abstraction of a H atom producing C4 and hydroperoxide radicals as the initiating and rate determining step. This proposal is discussed further in the Supporting Information. Additional mutagenesis and chemical model studies are in progress to evaluate the radical-based mechanism.

Structural Data. The structure factors and coordinates for the wild-type (4G36) and cross-linked (4G37) luciferase models bound to DLSA have been deposited with the Protein Data Bank.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of protein structure determation, analysis of the active site tunnel, and the proposed alternate mechanism. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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