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Bioluminescence Is Produced from a Trapped Firefly Luciferase Conformation Predicted by the Domain Alternation Mechanism

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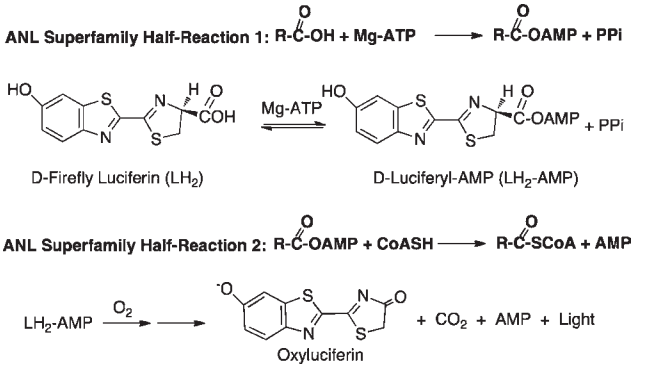
Supporting Information

ABSTRACT: According to the domain alternation mechanism and crystal structure evidence, the acyl-CoA synthetases, one of three subgroups of a superfamily of adenylating enzymes, catalyze adenylate- and thioester-forming half-reactions in two different conformations. The enzymes accomplish this by presenting two active sites through an ~140° rotation of the C-domain. The second half-reaction catalyzed by another subgroup, the beetle luciferases, is a mechanistically dissimilar oxidative process that produces bioluminescence. We have demonstrated that a firefly luciferase variant containing cysteine residues at positions 108 and 447 can be intramolecularly cross-linked by 1,2-bis(maleimido)ethane, trapping the enzyme in a C-domain-rotated conformation previously undocumented in the available luciferase crystal structures. The cross-linked luciferase cannot adenylate luciferin but is nearly fully capable of bioluminescence with synthetic luciferyl adenylate because it retains the ability to carry out the oxidative half-reaction. The cross-linked luciferase is apparently trapped in a conformation similar to those adopted by acyl-CoA synthetases as they convert acyl adenylates into the corresponding CoA thioesters.

The relationships between the conformational dynamics and functions of a large group of proteins known as the "ANL superfamily of adenylating enzymes" are the subject of an excellent recent review by Gulick.[1] The ANL superfamily enzymes that were first recognized by Conti and Brick[2] share ~20% sequence identity, are structurally similar, and include the *a*cyl-CoA synthetases, the adenylation domains of the *n*onribosomal peptide synthetases (NRPSs),[3] and the (beetle) *l*uciferases. All ANL enzymes catalyze two half-reactions (Scheme 1), the first of which converts carboxylate-containing substrates into the corresponding adenylates. With the exception of the luciferases, the second half-reaction involves the substitution of AMP by coenzyme A (CoA) or protein-bound 4′-phosphopantetheine to produce a thioester. The second half-reaction catalyzed by luciferase, a multistep oxidative process that produces bioluminescence, is quite different (Scheme 1).[4,5] Interestingly, luciferase can use CoA, a cofactor not required for light emission, to convert dehydroluciferyl-AMP (L-AMP), a potent inhibitor formed in a side reaction, into the less inhibitory thioester (L-CoA).[6]

On the basis of four crystal structures of ANL enzymes, only one of which contained CoA and a rotated C-domain, Gulick proposed[7] a domain alternation mechanism to account for the catalysis of the half-reactions (Figure 1). Substantial support for this mechanism in the case of the acyl-CoA ligase subgroup is

Scheme 1. Half-Reactions Catalyzed by the ANL Superfamily



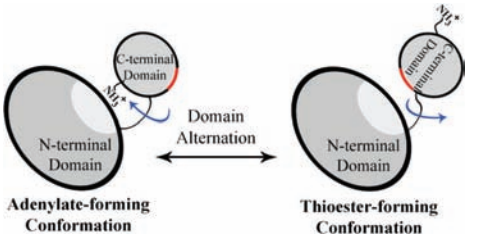


Figure 1. Schematic of the domain alternation mechanism. The acyl-CoA synthetases and NRPSs in the ANL superfamily use an ~140° rotation to present opposite faces of the C-terminal domain to catalyze two different half-reactions.

now available from sets of crystal structures of two enzymes[8,9] in both the adenylate- and thioester-forming conformations, which are related by ~140° rotations of the C-domain. However, the NRPSs and luciferases have been crystallized only in the adenylation conformation. This is especially problematic for luciferase biochemistry because (1) CoA is not a required substrate and (2) the oxidative half-reaction is mechanistically dissimilar to the thioester-forming reaction. However, we have reported[10,11] results of mutagenesis studies with *Photinus pyralis* luciferase (Ppy WT) that are consistent with a mechanism requiring two conformations. Specifically, we found that alanine replacement of Lys529 or Lys443, residues that are ~20 Å apart and on opposite faces of the C-domain, disrupts only the adenylation or oxidative half-reaction, respectively. With our results and crystal structures

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of luciferase in the adenylation conformation in hand,[2,12,13] we undertook this study to produce structural evidence for the existence of the rotated (thioester-forming) conformation of luciferase and to determine its relevance to the oxidative half-reaction that produces bioluminescence.

Our experimental approach was designed to trap the elusive rotated luciferase conformation using cysteine thiol chemistry. This required the introduction of Cys residues on each domain that could approach each other closely in the rotated conformation but not in the adenylate-forming one. N-domain residue Ile108 and C-domain residue Tyr447 were initially chosen for mutation to Cys on the basis of our observation that a crystal structure[14] of a highly thermostable long-chain fatty acid CoA synthetase in the thioester conformation contained, among 35 salt bridges not found in Ppy WT, a single interdomain interaction involving Arg108 and Glu447 (Ppy WT numbering). Indeed, a luciferase variant containing the changes Ile108Arg and Tyr447Glu displayed 6-fold-enhanced stability at 37 °C relative to Ppy WT [Table S1 in the Supporting Information (SI)]. Because these residues are ~40 Å apart in the luciferase structures, it appeared that transient salt bridge formation had occurred in a rotated conformation. Next, we considered making a Ppy WT variant containing the changes Ile108Cys and Tyr447Cys with the intention of trapping the rotated conformation through intramolecular disulfide formation or chemical cross-linking. However, this plan was abandoned because significant inactivation of Ppy WT by ferricyanide ion (accompanied by partial oxidation of the native Cys residues) occurred under the mild conditions required for disulfide formation (Table S2). Likewise, activity loss and partial intrinsic Cys modification were observed when Ppy WT was treated with a maleimide cross-linking reagent in another control experiment (Table S2). To circumvent these problems, we prepared the Ppy WT variant Ppy 9$^-$ (see page SI-4 in the SI), in which the four Cys residues were eliminated by mutation to Ser or Ala. To accomplish this, it was first necessary to make five additional amino acid changes[15] to overcome protein stability problems resulting in the formation of inclusion bodies that were encountered when variants containing multiple Cys changes were expressed. Ppy 9$^-$ produced strong bioluminescence with excellent specific activity based on total light emission, but it displayed a 2-fold longer rise time and an extended decay time relative to Ppy WT (Table S3). Similar emission characteristics with more prolonged rise and decay times were observed with the addition of the Ile108Cys and Tyr447Cys changes that produced the protein Ppy 9$^-$ C108/C447.

While attempts to trap Ppy 9$^-$ C108/C447 in a rotated conformation through ferricyanide ion-mediated disulfide bond formation produced ~70% cross-linked enzyme (Table S4), we were unable to find suitable reaction conditions to accomplish the conversion cleanly because the reagent partially inactivated the enzyme by reaction at residues other than Cys. Instead, we pursued a chemical trapping approach using the symmetrical bifunctional reagent 1,2-bis(maleimido)ethane (BMOE) that we anticipated would function as shown in Figure 2 (and/or by conjugate addition first at Cys108 followed by reaction at Cys447). Upon incubation of a 20 μM solution of enzyme with 24 μM BMOE at 20 °C and pH 7.0, a rapid loss of bioluminescence activity was observed that was nearly complete (98%) in 1 h, following kinetics typical of irreversible enzyme inactivation (Figure S1 in the SI). Exhaustive dialysis did not restore the activity, and LC/ESI-MS analysis (Figure 3A) indicated a major

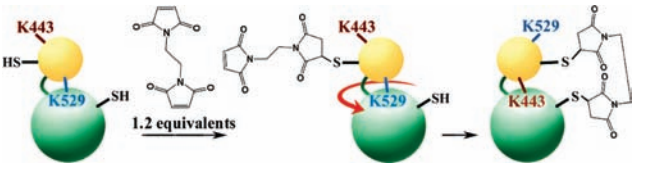

Figure 2. Chemical trapping of Ppy9$^-$ C108/C447 with BMOE. The schematic shows the attachment of BMOE to Cys447 in the adenylate-forming conformation and then to Cys108 after C-domain rotation.

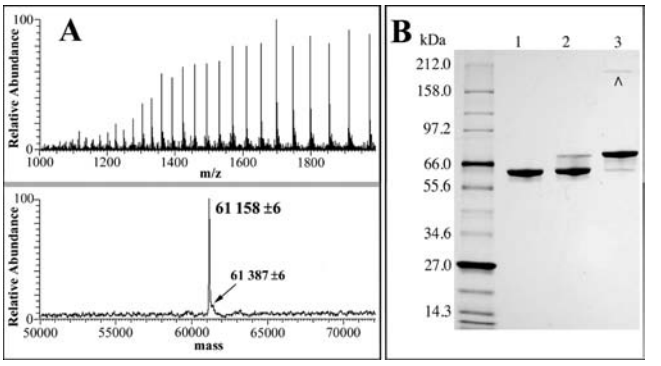

Figure 3. ESI-MS and SDS-PAGE analysis of Ppy 9$^-$ C108/C447 treated with BMOE. (A) Mass spectrum (top) and spectrum following BIOMASS deconvolution using the algorithm of the BioWorks 3.0 software (bottom). (B) Nonreducing SDS-PAGE gel (4−20%) of Ppy 9$^-$, Ppy 9$^-$ C108/C447, and BMOE-linked Ppy 9$^-$ C108/C447 in lanes 1−3, respectively.

component with a mass of 61 158 ± 6 Da, in agreement with the value of 61 161 Da expected for BMOE (220 Da) covalently attached to Ppy 9$^-$ C108/C447 in a 1:1 molar ratio. Additionally, a minute peak at 61 387 ± 6 Da corresponding to the covalent incorporation of 2 mol of BMOE/mol of protein was detected (Figure 3A). In a control experiment, treatment of BMOE-modified Ppy 9$^-$ C108/C447 with an excess of the sulfhydryl blocking reagent *N*-ethylmaleimide (NEM) produced no change in mass, indicating the absence of detectable free thiols. Taken together, the MS results are consistent with Ppy 9$^-$ C108/C447 being mostly intramolecularly cross-linked by BMOE, with a minor amount of the protein labeled twice, presumably at Cys108 and Cys447. We further analyzed the BMOE-labeled luciferase using SDS-PAGE (Figure 3B). Both Ppy 9$^-$ and Ppy 9$^-$ C108/C447 exhibited intense bands corresponding to the expected masses of the enzymes. The Cys mutant also gave a minor (~6%) band with an apparent mass of ~74 500 Da. The higher-mass band in the BMOE-labeled sample accounted for ~96% of the protein applied to the gel. On the basis of the MS data (Figure 3A), the higher-mass band must represent the BMOE-modified luciferase. Apparently, it migrates more slowly, possibly because it takes up an abnormally low amount of SDS, consistent with our expectation of having trapped a single and different luciferase conformation. The minor band, corresponding to enzyme with two BMOE molecules attached, would be expected to run normally because it could not be locked into a single conformation. In the unmodified Ppy 9$^-$ C108/C447 sample, the slower-moving minor band represents disulfide cross-linked enzyme that formed on standing in the absence of a reducing agent (Figure S2). Traces of protein linked intermolecularly by BMOE were also observed in the gel analysis (indicated by a caret in lane 3 of Figure 3B).

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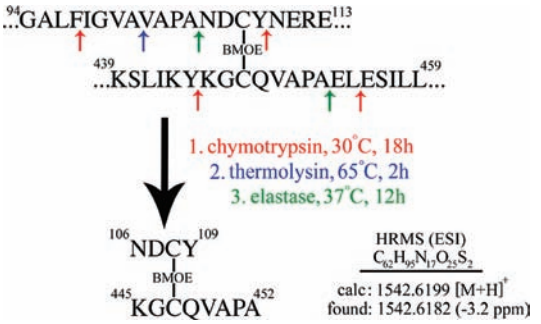

Figure 4. Proteolytic sequencing of Ppy 9$^-$ C108/C447 covalently labeled with BMOE. A 0.8 mg sample of the chemically modified protein was sequentially digested with immobilized chymotrypsin, thermolysin, and elastase (see page SI-4). After each digestion, masses corresponding to the calculated values for BMOE-cross-linked peptides cleaved at the sites indicated by the colored arrows were detected by LC/ESI-MS.

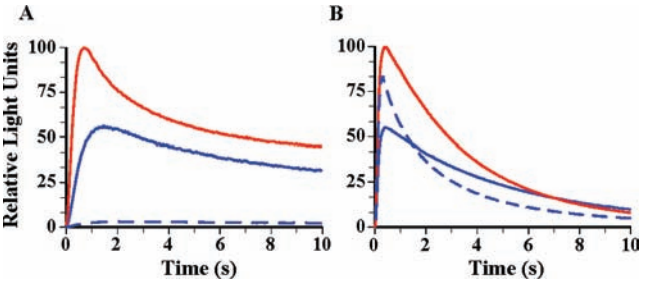

Figure 5. Bioluminescence time courses with natural substrates or synthetic LH_2-AMP. In addition to 0.6 μg of protein (Ppy 9$^-$, solid red line; Ppy 9$^-$ C108/C447, solid blue line; Ppy 9$^-$ C108/C447 cross-linked with BMOE, dashed blue line), the 0.5 mL reaction mixtures in 50 mM glygly buffer (pH 7.8) contained (A) 100 μM LH_2 and 2 mM Mg-ATP or (B) 75 μM LH_2-AMP. Light emission was initiated by injection of Mg-ATP or enzyme.

Table 1. Relative Rates of the Adenylation and Oxidation Half-Reactions

		relative rates (%)[a]		
Ppy 9$^-$ variant[b]	BMOE[c]	overall	1st half-rxn	2nd half-rxn
Ppy 9$^-$	–	100 ± 3	100 ± 15	100 ± 9
I108C/Y447C	–	56 ± 1	113 ± 12	52 ± 6
I108C/Y447C	+	1.7 ± 0.05	–[d]	84 ± 9
I108C/Y447C/K443A	–	0.5 ± 0.01	93 ± 14	0.6 ± 0.06
I108C/Y447C/K443A	+	0.02 ± 0.001	–[d]	0.4 ± 0.04

[a] Overall and oxidation (2nd half-reaction) rates (expressed in % relative to the Ppy 9$^-$ values) were estimated using flash-height-based activity values obtained with LH_2 and Mg-ATP or LH_2-AMP, respectively. Adenylation (1st half-reaction) rates were estimated from the rates of L-AMP formation. All of the relative rate values in the table were obtained from at least three trials and are reported as mean ± standard deviation. For additional experimental details, see the SI. [b] Amino acid changes to Ppy 9$^-$ are indicated. [c] A + sign indicates that the enzyme was treated with 1.2 equiv of BMOE for 1 h at 20 °C. [d] The rate was below the level of detection for the assay.

Having demonstrated that Ppy 9$^-$ C108/C447 was covalently modified with BMOE, we used proteolysis and LC/ESI-MS to confirm that the reagent had in fact cross-linked the protein through the Cys thiols at positions 108 and 447, as envisioned (Figure 2). Control experiments (see page SI-4) were first performed with unmodified and BMOE-treated Ppy 9$^-$ C108/C447 samples that were incubated with excess NEM. After digestion with chymotrypsin, the expected masses for the two NEM-containing peptides ^{98}IGVAVAPANDCY^{109} and ^{445}KGCQVAPAEL^{454} were readily detected in the unmodified sample but not in the BMOE-treated one. The BMOE-modified Ppy 9$^-$ C108/C447 sample was then sequentially digested with chymotrypsin, thermolysin, and elastase (Figure 4). The masses 2425.9, 2123.0, and 1543.0 Da that were detected after the protease treatments correspond to the values of 2425.7, 2122.8, and 1542.6 Da expected from sequential cleavage at the sites indicated by the red, blue, and green arrows, respectively, in Figure 4. We note that the calculated masses subsequent to the thermolysin treatment at 65 °C and pH 8.0 are based on hydrolysis of both succinimide rings (+36 Da), as verified in a control reaction. HRMS analysis of the final digest confirmed the molecular formula of a unique peptide (Figure 4) that substantiated the cross-linking of Cys108 and Cys447. Since there was only trace evidence for intermolecular protein cross-linking and because BMOE can span only ~8 Å, it is not possible that the cross-linking occurred in the adenylate-forming conformation captured in the luciferase crystal structures, in which residues 108 and 447 are ~40 Å apart. We conclude that luciferase was covalently cross-linked by BMOE as shown in Figure 2, thus providing strong supporting evidence for the existence of a rotated luciferase conformation predicted by the domain alternation mechanism.

We next carried out light-emission activity measurements (Figure 5) to investigate the role of the rotated conformation in luciferase bioluminescence. With the natural substrates LH_2 and Mg-ATP, light is emitted as a result of an overall process that requires both half-reactions (Scheme 1). The bioluminescence activities of Ppy 9$^-$ and Ppy 9$^-$ C108/C447 (Figure 5A) reflect their overall flash-height-based specific activities (Table 1). After Ppy 9$^-$ C108/C447 is cross-linked with BMOE, however, it is essentially incapable of light production. The remaining activity can likely be ascribed to the small amount of enzyme labeled twice with BMOE. Luciferase bioluminescence also can be initiated by replacing the natural substrates with synthetic LH_2-AMP, thereby bypassing the adenylation half-reaction and requiring only that the oxidative second half-reaction be functional. The relative light production from Ppy 9$^-$ and Ppy 9$^-$ C108/C447 generated with the synthetic adenylate (Figure 5B) is similar to that observed with the natural substrates. In marked contrast to the result with LH_2 and Mg-ATP, the light intensity observed with Ppy 9$^-$ C108/C447 after cross-linking with BMOE was restored with LH_2-AMP and approached the level obtained with Ppy 9$^-$.

Additional supporting evidence for the importance of the rotated conformation of luciferase to oxidation but not adenylation was accumulated by estimation of the relative rates of the half-reactions (Table 1). The introduction of Cys residues into Ppy 9$^-$ at positions 108 and 447 reduced the overall reaction rate 2-fold as a result of a similar drop in the oxidative half-reaction. BMOE cross-linking nearly (84%) restored the LH_2-AMP oxidation rate to the level of Ppy 9$^-$, evidently eliminating the deleterious effect of the Cys substitutions on the oxidation rate. The greatly reduced overall rate of light production (Figure 5A and Table 1) for the BMOE-trapped enzyme is predominantly due to the ~100-fold reduction in the rate of adenylate formation, as expected for a luciferase trapped in the

oxidative conformation. The bioluminescence results strongly suggest that the trapped luciferase conformation closely (but not exactly) resembles the conformation populated during the course of the natural bioluminescence process. Another indication that the trapped conformation is perturbed is the red-shifted (612 nm) bioluminescence observed with the LH_2-AMP-initiated reaction (Figure S3). Additionally, using methods developed by Fraga, da Silva, and co-workers,[6,16] we demonstrated that the BMOE-cross-linked enzyme used CoA to convert L-AMP into the corresponding L-CoA thioester, albeit at a somewhat lower rate than Ppy 9^- C108/C447 (Figure S4).

The previously documented[11] importance of Lys443 to only the oxidative chemistry was confirmed by introducing the Lys443Ala change into Ppy 9^- C108/C447 (Table 1). Reassuringly, this variant was nearly fully capable of catalyzing the adenylation half-reaction but incapable of oxidizing LH_2-AMP. After it was cross-linked with BMOE, however, the Lys443Ala-containing protein displayed a greatly reduced adenylation rate and remained incapable of producing light from LH_2-AMP. These results confirmed the importance of the highly conserved luciferase β-hairpin motif ^{442}IKYKGYQV^{449} to only the oxidative luciferase reactivity. Interestingly, this motif is part of the pantetheine channel in the acyl-CoA thioester conformation.[9] It is likely that the measured properties of the trapped conformation are not identical to those of Ppy 9^- C108/C447 because the β-hairpin motif structure is slightly altered by the linkage through Cys447.

We have demonstrated that a firefly luciferase variant containing Cys residues at positions 108 and 447 can be intramolecularly cross-linked by BMOE, trapping the enzyme in a C-domain-rotated conformation previously undocumented in the available luciferase crystal structures.[2,12,13] The BMOE-modified protein cannot adenylate luciferin but is nearly fully capable of both CoA thioester formation and bioluminescence as a result of the retained ability to carry out the half-reaction in which LH_2-AMP is converted into oxyluciferin in an electronically excited state. The cross-linked luciferase is apparently trapped in a conformation quite similar to those adopted by acyl-CoA synthetases during the process of converting acyl adenylates into the corresponding CoA thioesters.[8,9] These results provide support for the proposal of Oba[17] that firefly luciferase evolved from an ancestral fatty acyl-CoA synthetase that acquired oxygenase activity. It is the rotated conformation that maintained the CoA activity and acquired a new oxidative function.

While the strategy of cross-linking Cys residues has been applied to investigations of protein folding,[18] subunit interactions,[19] and trapping of proteins in non-native conformations,[20] our study represents an uncommon example in which a previously inaccessible active conformation of an enzyme has been captured covalently. The methodology described herein should be readily applicable to the ANL superfamily of enzymes and perhaps may be of more general utility. The availability of cross-linked proteins should enable mechanistic and crystal structure studies of previously unattainable conformations. It is possible that our methodology can be adapted to trapping of the active adenylate-forming conformations of the ANL enzymes as well. Work on determining the crystal structure of the BMOE-trapped luciferase described herein and elucidating the details of the luciferase oxidative chemistry and the vexing issue of bioluminescence color determination is currently in progress. Since it is very unlikely that domain rotation can occur during the lifetime of the electronically excited state of oxyluciferin, the bioluminescence must occur in the rotated conformation documented in our study and not in the conformation captured in the crystal structures. In the future, the rotated luciferase conformation should be taken into account in any mechanistic proposal of bioluminescence emission color.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, enzyme characterization, chemical modification controls and HPLC analyses of L-AMP reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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