RANDOM AND SITE DIRECTED MUTAGENESIS OF BACTERIAL LUCIFERASE

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Introduction

We have undertaken an investigation of the structure of the active center of luciferase through the use of a combination of random and site directed mutagenesis and chemical modification of the wild-type and variant enzymes. The sites for mutagenesis were within the α subunit at positions 106, the site of the reactive thiol (1), 113, the site of the lesion of AK-6 (Asp→Asn; 2) and 227, the site of the lesion of AK-20 (Ser→Phe; 3). Our approach has been to focus on residues or regions that appear to contribute to the structure, function or folding and assembly of the enzyme.

Results and Discussion

Position α106. The potential role of the reactive thiol at position 106 of the α subunit of luciferase has been the subject of much speculation. We have demonstrated that the thiol at this position plays no role in the chemistry of the luciferase-catalyzed reaction (4, 5). Luciferases from *V. fischeri* (5), *Photobacterium leiognathi* (5) and *P. phosphoreum* have valine at the same position. Furthermore, by site directed mutagenesis, we have previously constructed *V. harveyi* luciferase variants with serine, alanine and valine at this position, and all function in the bioluminescence reaction (4). It is interesting that the α106 valine variant has a greatly reduced quantum yield in the bioluminescence reaction relative to the wild-type luciferase, even though the homologous position in the other three wild-type luciferases is occupied by valine (4, 5). To investigate the cause of this reduced quantum yield, in the initial study of the α106Val variant, we reported the affinity of the enzyme for the reduced flavin and aldehyde substrates. The $FMNH_2$ binding affinity is not greatly altered for the α106Val enzyme, but is decreased by about 50-fold for the α106Ser variant; the

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effect of α106Ala is intermediate (4). The α106Ser and Ala mutant enzymes have somewhat lower apparent affinities for the substrate aldehyde than the wild type, but the α106Val variant is a very poor aldehyde binding mutant, having apparent dissociation constants for *n*-octanal, *n*-decanal, and *n*-dodecanal greater than millimolar, thus leading to difficulties in obtaining saturation (4).

To further investigate the cause of the reduced quantum yield of the α106 valine luciferase, we have measured the rate constants for reaction of oxygen with the enzyme-bound reduced flavin to form intermediate II and the rate of breakdown of intermediate II to form FMN for both wild-type luciferase and the α106Val variant. Under the conditions of the measurements (pH 7, 25°, 75 μM enzyme, 15 μM $FMNH_2$, 120 μM O_2), the rate of formation of the intermediate II was indistinguishable for wild-type luciferase and for the α106Val variant, but the rate of breakdown of the C4a peroxydihydroflavin intermediate was about 150 times faster for the α106Val variant than for the wild type. The reduced quantum yield of the α106Val variant thus may be the result of the combined effects of the more rapid breakdown of the intermediate II and the reduced affinity for the aldehyde substrate.

Position α113. The mutant AK-6, isolated and described by Cline and Hastings (6), has very interesting functional properties. The $FMNH_2$ binding affinity of AK-6 luciferase is weaker than for any of the other mutants isolated by Cline and Hastings, but the aldehyde binding affinity is unaltered. The bioluminescence emission spectrum is red shifted both *in vivo* and *in vitro*, demonstrating that light emission comes from the luciferase, not from any secondary emitter (7). We have found that the lesion of AK-6 resulted in substitution of Asp→Asn at position α113 (2). By site directed mutagenesis, we have constructed variants with Glu, Cys, Ala, Ser, Thr and Lys, as well as others, at this position. The α113Glu and α113Cys variants were similar to the wild-type luciferase, while the α113Ala, Ser and Thr variants were similar to the original AK-6 mutant. The α113Lys variant emitted detectable bioluminescence, but the quantum yield relative to enzyme was reduced by more than 10^5.

To further investigate the environment of α113, we constructed a double mutant with α106Ala and α113Cys, hoping to attach spectroscopic probes to position α113. The rate of reaction of N-ethylmaleimide was determined by monitoring the inactivation of the enzymes as a function of time. The enzyme with Cys at both α106 and α113 was inactivated by NEM at a slightly *slower* rate than the wild type enzyme, while the αC106A variant was much less reactive with the thiol directed reagent, comparable to the double mutant αC106A, αD113C. We were surprised to find that a cysteinyl residue at position α113 *did not react* with NEM. These findings, while disappointing from the perspective of using the α113Cys to attach

spectroscopic probes, indicate that the functional group of the cysteinyl residue, and by inference the wild-type aspartyl residue, is oriented away from solvent, such that reaction with alkylating groups is not possible. A buried aspartyl residue suggests the existence of another residue within the luciferase structure which interacts with the anionic functional group of the aspartyl residue (and presumably with the glutamic acid and cysteine, since the luciferase will function with these residues at position α113). The cationic functional group of a lysyl residue could serve such a role, and could explain the dramatic deleterious effect of the α113Lys mutation.

Position α227. One of the more interesting mutants isolated by Cline and Hastings was AK-20, which produces a luciferase with a reduced quantum yield when *n*-decanal is used as the aldehyde substrate, a reversal of the aldehyde chainlength dependence of the decay of bioluminescence, and a slightly higher affinity for the flavin substrate (6). We have determined that the lesion is Ser→Phe at position α227, and have constructed several other variants, including α227Ala, α227Tyr and α222Trp (3). The kinetic features are similar for AK-20 (α227Phe) and the α227Tyr and α227Trp variants, while the α227Ala variant shows essentially the same activity as the wild-type.

We measured the production of the chemical product of the reaction, the aliphatic acid, and compared the chemical yield with the yield of light (Table I). The α227Phe enzyme (AK20) and the α227Tyr variant both produce the acid product with essentially the same yield as the wild-type enzyme. This observation demonstrates clearly that the production of light and the chemistry of the luciferase-catalyzed reaction are not obligatorily coupled.

Table I: Quantitation of *n*-decanoic acid production by *V. harveyi* wild-type and α227 mutant luciferases[a].

Enzyme Source	k[b] (sec^{-1})	I_{max} [c] (Q/sec, 10^{10})	Total Quanta [d] (Q, 10^{12})	RCOOH[e] (molecules, 10^{14})	Q/molecule[f]
wild type	0.018	162	82.4	4.4 ± 1.9	0.187
AK-20	0.053	0.88	0.12	3.4 ± 2.5	0.0005
αS227Y	0.116	0.93	0.27	5.7 ± 1.6	0.0005

a ^{14}C-labelled *n*-decanal was produced from ^{14}C *n*-decanol by the action of alcohol dehydrogenase, essentially as described (8).

b Decay rate of bioluminescence under conditions of limiting aldehyde and in the presence of *n*-decanol, the source of the *n*-decanal.

c Initial light intensity in quanta/sec based on a liquid light standard (9).

d Total quanta emitted during one turnover of the luciferase enzyme.

e Number of acid molecules produced per turnover calculated based on the specific radioactivity of the label, the definition of a Curie, and Avogadro's number.

f The quantum yield of the reaction based on carboxylic acid production.

Conclusions

1. The reactive thiol at position α106 is not required for bioluminescence, but it does reside in a critical location in or near the active site.
2. The reduced quantum yield of the α106Val variant is a consequence of weak aldehyde binding and an increased rate of breakdown of the intermediate II (the 4a peroxyflavin), which forms at the same rate as for the wild type, and thus does not involve any fundamental change in mechanism, e.g., to an oxidase (10).
3. It appears that a functional group capable of ionization to an anion is required at position α113 for a high quantum yield reaction. Residues at this position are not exposed to solvent.
4. The residue at position α227 is not involved mechanistically in the bioluminescence reaction, but the structure of residues at this site does have a strong impact on the quantum yield of the bioluminescence reaction without altering the ability of the enzyme to carry out the chemistry of the reaction.

Acknowledgments

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References

1. Cohn, D. H., Mileham, A. J., Simon, M. I., Nealson, K. H., Rausch, S. K., Bonam, D., and Baldwin, T. O. (1985) J. Biol. Chem. 260, 6139-6146.
2. Baldwin, T. O., Chen, L. H., Chlumsky, L. J., Devine, J. H., Johnston, T. C., Lin, J.-W., Sugihara, J., Waddle, J. J., & Ziegler, M. M. (1987) In Flavins and Flavoproteins, eds. D. B. McCormick and D. E. Edmondson (Walter de Gruyter, Berlin) pp. 621-631.
3. Chen, L. H. and Baldwin, T. O. (1989) Biochemistry 28, 2684-2689.
4. Baldwin, T. O., Chen, L. H., Chlumsky, L. J., Devine, J. H. and Ziegler, M. M. (1989) J. Biolumin. Chemilumin. 4, 40-48.
5. Baldwin, T. O., Devine, J. H., Heckel, R. C., Lin, J.-W. and Shadel, G. S. (1989) J. Biolumin. Chemilumin. 4, 326-341.
6. Cline, T. W. and Hastings, J. W., (1972) Biochemistry 11, 3359-3370.
7. Cline, T. W. and Hastings, J. W., (1974) J. Biol. Chem. 249, 4668-4669.
8. Dunn, D. K., Michaliszyn, G. A., Bogacki, I. G. and Meighen, E. A. (1973) Biochemistry 12, 4911-4918.
9. Hastings, J. W. and Weber, G. (1963) J. Opt. Soc. Am. 53, 1410-1415.
10. Xi, L., Cho, K.-W., Herndon, M. and Tu, S.-C. (1990) J. Biol. Chem. 265, 4200-4203.