ESSENTIAL SULFHYDRYL MODIFICATION OF BACTERIAL LUCIFERASE

FROM *Vibrio harveyi*

APPROVED

Dean of the Graduate School

ESSENTIAL SULFHYDRYL MODIFICATION OF BACTERIAL LUCIFERASE FROM *Vibrio harveyi*

by

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ABSTRACT

The reagents, methyl methanethiosulfonate (MMTS), N-ethylmaleimide (NEM), iodoacetamide (IAM) and 2-bromo-2', 4'-dimethoxyacetophenone (Br-DMOAP), are thiol specific and potent reagents for luciferase alkylation. Pseudo first-order analysis showed that reaction of luciferase with MMTS, NEM and IAM followed strictly second-order kinetics; that is, the observed rate constant for inactivation was directly proportional to the concentration of the alkylation reagent. However, Br-DMOAP modification did not follow simple second-order kinetics. Studies of Br-DMOAP in aqueous solution showed that Br-DMOAP hydrolyzed in water. Thus the alkylation reaction of luciferase with Br-DMOAP was a complex reaction consisting of Br-DMOAP hydrolysis and alkylation. Flavin mononycleotide (FMN) protected luciferase against inactivation by these reagents. The degree of protection observed was greater, the greater the FMN concentration. Furthermore, the protective effect of flavin was enhanced in a low versus a high concentration of phosphate buffer, ph 7.0. Compare with the rate of IAM inactivation in the absence of flavin (7.6 $M^{-1}min^{-1}$), in the presence of 50 mM FMN the observed rate of IAM inactivation was 16 times slower in 0.25 M phosphate and 62 times slower in 0.02 M phosphate. By comparison, the rate of

NEM inactivation of luciferase in 0.25 M phosphate was reduced by about 3.4 fold in the presence of 50 mM FMN versus its absence. Unexpectedly, an unidentified product which resulted from FMN (50 mM) reaction with DTT (50 mM) led to substantial luciferase inactivation (about 20%). Chromatography of this sample led to further inactivation (a total of 75% inactivation). The reason for this inactivation is not known. In an attempt to block all of the non-essential thiols while maintaining full catalytic function, luciferase was reacted with 10 mM IAM in the presence of 50 mM FMN in 0.02 M phosphate, ph 7.0. The enzyme, which retained greater than 90% activity by this treatment, was chromatographed to remove flavin and thiol reagent and secondarily modified by treatment with Br-DMOAP. Ellman's reagent (5,5-dithiobis-(2-nitrobenzoic acid)) was used to calculate the number of unreacted thiols remaining on luciferase after each of these stages. Native enzyme contained about 13 sulfhydryl residues, blocked-active enzyme contained 3.1 to 3.4 residues, and modified enzyme contained 1.2 to 1.4 residues.

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INTRODUCTION

Luciferase: Physical Properties and Function

Bacterial luciferase is the enzyme responsible for bioluminescence in some bacteria. This enzyme of molecular weight 79,000-80,000 is a metal-free dimer of unequal subunits, α and β. The molecular weights of α and β are approximately 40,000-42,000 and 37,000-39,000 respectively (Hastings et al., 1969; Yoshida and Nakamura, 1973).

This enzyme binds reduced flavin mononucleotide ($FMNH_2$) at the α subunit and, on addition of O_2 and a long chain aldehyde, catalyzes light formation with λ_{max} from 480 nm to 505 nm (Hastings, Eberhard et al., 1973).

$$RCHO + FMNH_2 + O_2 \xrightarrow{\text{luciferase}} RCOOH + FMN + H_2O + h\nu$$

Luciferase is an unusual flavoenzyme, because it binds oxidized FMN poorly (10^3-fold less well than $FMNH_2$) and can not be isolated as a holoenzyme.

In the intact luminous bacterium, a feeder enzyme supplies the $FMNH_2$ at the expense of NADH oxidation. This NADH-FMN oxidoreductase is also unusual in using flavin as freely dissociating substrates rather than as nondissociable bound coenzyme.

In view of the rapid autooxidation rate of free $FMNH_2$ to FMN ($t_{1/2}$=100 sec), it is likely that oxidoreductase and luciferase from a functional complex *in vivo*.

The complete reaction stoichiometry of luciferase catalysis appears to be established as a flavin-linked monooxygenase, and thus luciferase is a one turnover enzyme. The aldehyde undergoes 2-e^- oxidation to the carboxylic acid. No H_2O_2 is detected, rather O_2 appears to yield H_2O. This would correspond to a 4-e^- reduction with the second electron pair coming from $FMNH_2$.

The luciferase dimer from *Vibrio harveyi* (formerly *Beneckea harveyi*) has a single binding site for $FMNH_2$ with k_d values of 0.1-0.2 μM (Meighen and Hastings, 1971; Watanabe *et al.*, 1974; Becvar and Hastings, 1975; Meighen and Bartlet,1980). Enzyme-bound $FMNH_2$ is weakly fluorescent (Becvar *et al.*, 1976).

From the effect of pH on binding of $FMNH_2$ to *V. harveyi* luciferase, two ionizable groups appear to be involved and have pK_a values about 6.2 and 6.8 (Nicoli *et al.*, 1974). There are two such groups on $FMNH_2$ itself: the N-10 ribityl phosphate and the N-1 of the reduced isoalloxazine, both of which have pK_a values of about 6.2 (Ehrenberg and Hemmerich, 1968; Theorell and Nygaard, 1954).

Alternatively, either observed pK_a (6.2 or 6.8) could be contributed by an amino acid on the protein, the "essential" histidyl with an apparent pK_a of about 6.8, being a reasonable candiate (Cousineau and Meighen, 1976). There is also a single binding site on luciferase for the product FMN (Baldwin, 1974; Baldwin et al., 1975b), which is bound much more weakly than $FMNH_2$, the k_d value being 0.4 mM for V. harveyi luciferase at 25°C (Baldwin et al., 1975b).

The enzyme-bound oxidized flavin is non-fluorescent and shows remarkable structure in its optical absorbance and circular dichroism spectra (Baldwin, 1974; Baldwin et al., 1975b).

Chemical Modification

Although there are no disulfide groups, there are 13 sulfhydryl residues within the covalent structure of luciferase from Vibrio harveyi (Hastings et al., 1969; Tu et al., 1977a). One sulfhydryl is most likely in or near the active center of luciferase and appears to be significantly more reactive than the others with respect to a variety of alkylation reagents. Alkylation of this single "essential" sulfhydryl residue results in complete inactivation (Ziegler and Baldwin, 1981).

The reactive thiol, which has a pK_a of about 9.4, has been labeled with N-(1-C^{14}) ethylmaleimide and shown

to be located on the α subunit (Nicoli et al., 1974) in a tryptic peptide, whose sequence is Phe-Gly-Ile-Cys-Arg (Nicoli, 1972). The alkylated enzyme has a greatly reduced binding affinity for $FMNH_2$ ($>10x$) and is inactive in the luminescent reaction. Long-chain aldehydes protect the native enzyme from inactivation, as does a cycle of enzymic oxidation of $FMNH_2$ (Nicoli et al., 1974).

Bound FMN protects the reactive thiol completely against alkylation (Ziegler and Baldwin, 1981), but the alkylated enzyme appears to bind FMN with nearly the same affinity as the native enzyme (Nicoli et al., 1976).

No direct participation of the sulfhydryl group in substrate binding or catalysis has yet been demonstrated. However, modification by methyl methanethiosulfonate (MMTS) which introduces only a small uncharged nonpolar group ($-SCH_3$) in a mixed disulfide linkage to the essential thiol, results in complete inactivation of the enzyme (Ziegler and Baldwin, 1981).

Modification of the reactive, essential sulfhydryl group of V. harveyi luciferase with long-chain N-alkyl-maleimides indicated that the vicinity of the reactive cysteine, and thus presumably of the active center, must include a region of great hydrophobicity (Nicoli and Hastings, 1974), as might be expected for an enzyme with a long-chain aliphatic substrate.