INSOLUBLE BACTERIAL LUCIFERASES : A NEW APPROACH TO SOME PROBLEMS IN BIOLUMINESCENCE

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SUMMARY. Insoluble bacterial luciferases were prepared and used to investigate the mechanism of bacterial luminescence. It could be shown that (a) the long-lived intermediate which leads to generation of the emitting product is an enzyme complex, (b) particulate enzyme requires the presence of aldehyde for maximal light production, (c) the enzyme is capable of turn-over without any regeneration step except reduction by $FMNH_2$.

Bacterial luminescence requires the presence of a luciferase, reduced FMN, oxygen, and, for maximal light production, a long chain aldehyde. The role of the aldehyde is not understood although the reaction sequences leading to the production of light have been studied in considerable detail with Photobacterium fischeri (1). It has been proposed that the enzyme is first reduced by $FMNH_2$, then oxidized (or oxygenated) by oxygen to give a long-lived intermediate which, in the presence of aldehyde, ultimately gives rise to the light-emitting product.

Despite persistent efforts there are still a number of unknown factors in the bioluminescence reaction. First of all, the nature of the emitter molecule is unknown. Some evidence (2,3) implicates FMN but its fluorescence spectrum does not coincide with that of bioluminescence (4). This may be due to changes induced by its association with enzyme, the implication being that the enzyme itself is part of the emitting product. Participation of the enzyme in the emission step is supported by the high efficiency of the luminescence and the fact that differences in emission spectra exist among the various microorganisms. However, no direct evidence exists.

With respect to aldehyde function, one possibility is that it is consumed (oxidized) during the reaction (5,6). Other studies have been interpreted as indicating that the aldehyde is not consumed but that it induces conformational changes

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in the enzyme favorable for the efficient production of light. In support of this concept, Hastings et al. have presented evidence (7) that the enzyme of Photobacterium fischeri in the frozen state (i.e. in ice) is almost as efficient a light producer in the absence of aldehyde as it is at ordinary temperatures in the presence of aldehyde. Furthermore, additional aldehyde produces relatively little stimulation. He proposed that the enzyme in ice, being in a particulate state, has a conformation favorable for maximal production of light and suggests that this condition exists in vivo where the enzyme is bound to a particulate structure. Cormier et al. (8) attribute his findings to a "puddling" effect which concentrates a small amount of aldehyde already present in the enzyme preparation.

Finally, there is the question of turnover of bacterial luciferase. Hastings (1) has established that the life time of $FMNH_2$ is much shorter than the emission cycle. As a result, the enzyme undergoes no turnover during an assay procedure. Since the repetitive participation of a single enzyme molecule has never been shown, the suggestion was made that systems for the regeneration of the enzyme exist in vivo, but do not accompany the luciferase during purification (4).

This paper describes a new approach that can be used to answer some of the above questions. Insoluble derivatives (9) of two bacterial luciferases were prepared by reaction with the azide of polyacrylic acid. The two enzymes successfully coupled to the polymer were obtained from Photobacterium fischeri and P. leiognathi by standard purification methods. The preparation and some properties of these derivatives, as well as their use in studies on the mechanism of bioluminescence, are given.

EXPERIMENTAL

Preparation of the insoluble luciferases (Similar procedures were used for both preparations). Polyacrylic hydrazide was obtained from Sandoz Ltd, Basle, Switzerland. Before use, the fibrous polymer was suspended in water and homogenized in a Waring blender for about a minute to produce a uniform powder that could be centrifuged easily. The powder was collected on a Buchner funnel, and washed with large quantities of water, ethanol and methanol (in that order) and then allowed to dry in air.

The soluble enzyme preparations had been stored as ammonium sulfate precipitates. A portion containing about 15 mg of protein was dissolved in 3 ml of 0.1 M K_2HPO_4 adjusted to pH 8.7 with dilute NaOH and dialyzed against 100 ml of the same solution for two hours at 4°C. Dialysis was continued for 18 hours in the cold room against a fresh portion of 500 ml of the K_2HPO_4 solution, pH 8.7.

Polyacrylic hydrazide (200 mg) was suspended in 75 ml of 0.67 N hydrochloric acid and cooled to 4° in an ice bath. A cold 3 % solution of sodium nitrite (4.5 ml) was added dropwise, with stirring and cooling, over a period of about three minutes. The suspension was allowed to stir, with cooling, for one hour.

It was then collected on a fritted glass funnel using suction and washed with 25 ml of cold 0.1 M K_2HPO_4 solution, pH 8.7. In the meantime the dialyzed enzyme was placed in a wide-mouth test tube containing a magnetic stirrer and cooled by an ice bath. The washed polyacrylic azide was added to the enzyme solution in one portion and stirring was begun. Throughout the reaction, the pH was maintained at 8.5 - 9 (pH paper) by the addition of small quantities of 2 N NaOH. Periodically, 0.1 ml portions were withdrawn, diluted to 3 ml with water and examined for 280 mμ absorbance (i.e. protein) (Centrifugation was required for clarification). After about 5 hours about 60 % of the protein was bound to the polymer. Stirring was stopped and the reaction mixture was collected on a small buchner funnel. The insoluble enzyme was then washed five times with 5 ml portions of 0.2 M phosphate buffer pH 7.0 and stored in the refrigerator.

For assay, the stock suspension was well mixed and 0.1 ml withdrawn for transfer to the cuvette.

Assay Procedure. ReducedFMN was prepared by hydrogenation in the presence of platinized asbestos of a solution containing 5×10^{-5} M FMN, 5×10^{-3} M EDTA in 0.01 M phosphate buffer pH 7.0.

To a cuvette which was silvered on one side was added 1.35 ml of "assay buffer" (containing 0.2 ml M phosphate buffer, pH 7.0, 0.95 ml of 5×10^{-3} M mercaptoethanol and 0.2 ml of 1 % bovine serum albumin). This was followed by the aliquot of enzyme and, when applicable, by 0.1 ml of an aldehyde suspension. The cuvette was inverted to mix the contents and then placed in the photomultiplier compartment. The reaction was initiated by adding 1.0 ml of reduced FMN solution by hypodermic syringe.

Aldehyde suspensions were prepared by adding 0.05 ml of redistilled aldehyde to 10 ml of water which had been de-aerated with argon and shaking the suspension vigourously on a vibrator.

Measurement of light emission and spectra were carried out as described previously (10).

Photobacterium fischeri luciferase was a commercial preparation from the Sigma Chemical Company, labelled type 1, purified, lot 125 B-0880. Its properties with respect to thermal stability, pH optimum, aldehyde stimulation and luminescence spectrum were consistent with the description of the authentic enzyme (11).

Photobacterium leiognathi luciferase was purified (J.P. Henry and A.M. Michelson, unpublished) by techniques similar to those used for the purification of the fischeri enzyme (1).

RESULTS

Properties of the insoluble enzymes. Coupling of the enzymes to the solid support had only a small effect on specific activity and did not change the emission spectra or the pH optima. On the other hand, the heat stabilities were affected. In the

case of the P. fischeri preparation, the particulate enzyme was considerably more stable, having a half-life at 33°C of 26 minutes compared to 2.8 minutes for the soluble preparation. The reverse was true for the P. leiognathi enzyme, which had a half-life of 4.6 minutes at 40° in the insoluble form compared to 28.8 minutes for the soluble form. (The denaturation curves for both the soluble and insoluble forms were biphasic, as was reported (12) for P. fischeri). Both insoluble enzymes could be kept at room temperature (about 23°) for many hours without loss of activity and indefinitely at 4°C.

Stimulation by Aldehyde. Stimulation by aldehyde occurred to an equal extent as with the parent soluble preparations. Neither the maximum intensities nor the quantum yields changed and there was no change in the apparent kinetics of the reaction, the decay time being the same for the soluble and insoluble enzyme. Moreover the order of effectiveness of various aldehydes did not change for the particulate enzymes. In all cases, $C_{14} > C_{12} > C_{10} > C_8$.

Using dodecanal, the light intensity of the insoluble P. fischeri preparation was increased 50-75 fold ; the quantum yield about 20-40 fold. For the leiognathi preparation, the values were about 300 fold for the intensity maximum and 120 fold for the quantum yield. As with the respective soluble preparations, addition of aldehyde changed the kinetics of the reactions, causing a decrease in the decay time[1].

Enzyme turnover. Both insoluble preparations can be repeatedly recovered by centrifugation and, with or without washing, used over again in the assay procedure. We have done this more than twenty times in some experiments without a decrease in quantum yield or intensity. There is apparently no upper limit.

Luminescence cycles can also be repeated without intervening centrifugation. Fresh $FMNH_2$ was added at the end of each cycle. Luminescence occurred each time, although some inhibition probably by FMN becomes apparent.

The question of the necessity of aldehyde. If repeated runs in the absence of aldehyde were made with either insoluble preparation (each followed by washing of the enzyme with buffer), the quantum yield decreased to a very low limitting level. When aldehyde was incorporated in the assay procedure at this time, an enormous stimulation of light output occurred with the leiognathus preparation : with dodecanal the intensity increased by a factor of 4,250 , the quantum yield by 1820. Increased light output also occurred with P. fischeri preparations treated similarly, but the results were not so dramatic : 270 fold for the intensity

[1] It is by no means a rule that aldehyde decreases the decay time. In the case of Photobacterium phosphoreum, aldehyde causes an increase.

and 135 fold for the quantum yield[2]. It was noted that low light levels in the absence of aldehyde were much more difficult to obtain if the insoluble enzymes were once exposed to dodecanal.

Participation of the enzyme in the emission step. Although previous work indicates that the long lived intermediate "complex II" immediately responsible for the formation of the emittor product, is an enzyme-substrate complex, there is no direct evidence for this. The enzyme might function, for example to oxidize a low molecular weight substance to give a product in an excited state in solution. If this were true we would not have to be concerned about the life-time of the intermediate, which is unusually long for an enzyme-substrate complex. This possibility was tested directly with the insoluble enzymes.

A suspension of enzyme (0.1 ml) was added to 1.7 ml of the assay buffer solution (see experimental). The resulting suspension was cooled in an ice bath : to this was added 0.2 ml of a dodecanal suspension. The mixture was immediately taken up into a glass hypodermic syringe (5 ml capacity) which had been previously chilled. The filled syringe was carefully placed on cracked ice until all preparations for the assay were made. Then 1.3 ml of cold $FMNH_2$ solution was drawn up into the syringe and, working as quickly as possible, the syringe was fitted with a Millipore filter through which the solution was forced into the photomultiplier compartment. Control experiments were run in which all operations were repeated identically but for which the filter disc was omitted from the Millipore chamber. No light was detectable if the Millipore cellulose acetate filter disc (MF, 0.45 μ pore size) or a filter paper (Durieux-111) disc were present. The controls, on the other hand, gave the expected luminescence.

DISCUSSION

Highly active insoluble derivatives of two bacterial luciferases have been obtained by coupling them to the azide of polyacrylic acid. Presumably the coupling is via the ε-amino groups of the enzymes' lysine residues. A third bacterial enzyme has also been coupled successfully indicating that the procedure should be generally applicable to bacterial luciferases.

Except for thermal stability, the properties of the insoluble enzymes (emission spectra, aldehyde stimulation and pH-optima) do not differ from those of the soluble enzyme from which they were derived. Thermal stability could be either greater or less for the insoluble enzyme, providing evidence for differences among various bacterial luciferases and serving to emphasize that experimental findings obtained with one bacterial luciferase need not apply to others.

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[2] Preliminary experiments with 5×10^{-3} M Girard P reagent in 0.2 M phosphate buffer pH 7.1 decreased the light output in "absence" of aldehyde by only about 50 %.

Using the insoluble enzymes, we have shown that bacterial luciferases are capable of turnover without an intervening regeneration step other than a possible reduction by $FMNH_2$. Moreover, the particulate enzymes can be re-used many times without loss of activity and should be particularly useful for enzymes which have been isolated in small quantity.

We have also been able to investigate whether luciferase in the particulate state does not require aldehyde for maximal light output. Our experiments show that conversion of the enzyme to a particulate state has no effect upon its stimulation by aldehyde. We cannot, of course rule out the possibility that a certain kind of particle bound to a certain kind of an membrane in vivo may not be able to dispense with aldehyde for maximal light output.

Repeated recycling of the insoluble enzymes through the luminescence cycle (with intervening washings) resulted in enzymes extremely sensitive to the presence of aldehyde. However, the P. fischeri preparation could not be brought down to the low output (high aldehyde sensitivity) obtainable with P. leiognathi. This may only reflect binding differences. On the other hand, we must be careful not to exclude the possibility that other, more important differences exist in their respective mechanisms.

We have not been able to determine unequivocally whether aldehyde is necessary for the production of light because of possible contamination of reagent solutions. On the other hand, the extremely low light yield obtainable with repeatedly recycled P. leiognathi enzyme indicates that aldehyde may indeed be a requirement for this enzyme. Another observation made during the course of this work is consistent with this suggestion. We have found that repeated recycling of the insoluble enzymes in the absence of added aldehyde is a more efficient way of obtaining a low level of luminescence than is repeated washing with buffer. This could indicate that aldehyde is consumed during luminescence reaction.

Using the insoluble enzymes, we have also investigated directly the participation of luciferase as part of the long-lived intermediate which gives rise to the excited product. We have found that it is. This result is consistent with the high efficiency of the luminescence reaction and with the shift in spectra which occurs with some bacterial luciferases.

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