SEPARATION OF THE APOPROTEIN AND RECONSTITUTION OF THE HOLOPROTEIN FROM THE LONG-LIVED INTERMEDIATE IN BACTERIAL BIOLUMINESCENCE

Charles L. Murphy, George J. Faini and John Lee

Department of Biochemistry
University of Georgia
Athens, Georgia 30602

Received March 8,1974

Summary: A long-lived intermediate in bacterial bioluminescence, which has been suggested to be an FMN flavoprotein, has been separated as an apoprotein plus free FMN and the holoprotein reconstituted by addition of FMN (K_a = 7 x 10^5 M^{-1}). The apoprotein preparation reacts with long-chain aldehyde to give the full quantum yield observed for the complete system. Only after removal of all remaining FMN in the apoprotein preparation by prior dialysis of luciferase against KBr and inclusion of apoflavodoxin in the reaction mixture, can a dependence of the light output on FMN be observed. Bacterial bioluminescence therefore appears to be in the class of sensitized chemiluminescence with FMN acting as the specific sensitizing agent.

$FMNH_2$ reacts with bacterial luciferase in the absence of aliphatic aldehyde to form long-lived intermediates which can at later times be reacted with aldehyde to produce light (1). Lee and Murphy (2) identified two intermediates with "MAV" type luciferase (E)(3). The intermediate which appeared first after reaction of luciferase, $FMNH_2$ and oxygen was a reduced luciferase molecule to which one molecule of FMN (oxidized) was bound:

$$E + 2FMNH_2 + O_2 \rightarrow X_1 + FMN + H_2O_2.$$

The intermediate X_1 took up oxygen in a psuedo-first order reaction ($t_{\frac{1}{2}}$, 1.3 min 5° C) to form intermediate X_2:

$$X_1 + O_2 \rightarrow X_2.$$

The fact that X_2 was a flavoprotein was deduced from the fluorescence and absorption properties of the mixture (intermediate X_2 and FMN). In order to better characterize this intermediate it was decided to separate the free FMN from the mixture by Sephadex (G-25) chromatography at 0° C where the longer

lifetime of the intermediate ($t_{\frac{1}{2}}$, 14 min) allows this manipulation. It was found however, that not only free FMN but also most of the FMN bound to the intermediate is removed by this process, thus producing an apoprotein (apo-X_2). The holoprotein (holo-X_2) can be reconstituted by addition of FMN to the apoprotein.

Though the apo-X_2 preparation contained less than 5% the amount of flavin in the holoprotein, it was observed that the light yield on addition of aliphatic aldehyde to the apo-X_2 preparation was the same as that of holo-X_2. The light yield is not reduced until last traces of FMN are vigorously removed from the apo-X_2 mixture. Since under these conditions the quantum yield of flavin is greater than 100%, then in this step flavin must be acting as a sensitizing agent.

EXPERIMENTAL

Luciferase and FMN were purified and bioluminescence quantum yields (Q_B) were measured as previously described (2,6). Absorption spectra were taken with a Cary 14 recording spectrophotometer. Fluorescence measurements were made with an instrument previously described (4). Apo-X_2 was made by layering luciferase (>80 μM, 1cc) on top of a jacketed G-25 Sephadex column and injecting $FMNH_2$ (0.1 cc, 15 to 30 μM) into the luciferase with all components at 0° C. The mixture was then chromatographed at a rate of 3 ml/min. When a short column of G-25 (1 cm x 15 cm) or a longer column (2 cm x 30 cm) was used the protein eluted in 2-3 and 9-11 minutes respectively and the final results were essentially the same. Both columns completely separated FMN from luciferase or BSA in control experiments, where FMN rather than $FMNH_2$, was mixed with the proteins.

The equilibrium constant for the system (apo-X_2 + FMN $\rightleftarrows$ holo-X_2) was measured by the generation of apo-X_2 and addition back of FMN. The holo-X_2 concentration was determined by the amount of FMN fluorescence quenched. Apoflavodoxin was prepared from Desulfovibrio gigas flavodoxin (a gift from J. LeGall) by extensive dialysis at pH 3.9, 2 M KBr (5). The apoflavodoxin quenched the fluorescence of added FMN in the manner reported (5).

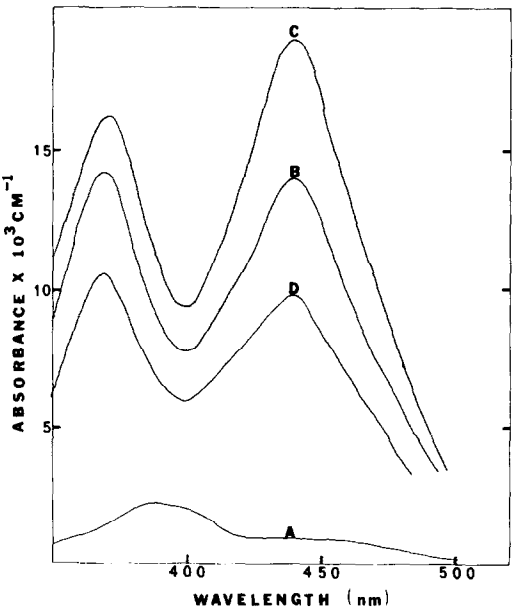

Figure 1. Curve A, absorption spectrum of the chromatographed intermediate (apo-X_2); B, apo-X_2 (3.6 µM) and added FMN (2.8 µM); C, after warming this mixture; D, the holo-X_2 estimated by subtracting the free FMN calculated from the binding constant.

Flavin from contaminating flavoproteins in the luciferase used was partially removed by dialysis against 1 M KBr but at pH 7.0 since luciferase is irreversibly denatured at low pH.

RESULTS

Absorption

Curve A, Figure 1, shows the absorption spectrum of luciferase reacted with $FMNH_2$ immediately after chromatography on G-25 Sephadex. No distinct absorptions can be seen even though the amount of intermediate X_2 present would require that if it contained bound FMN, an easily measurable absorption spectrum of a flavoprotein with an optical density greater than 20 x 10^{-3} cm^{-1} at 445 nm would result. On warming which carries all intermediates to final products, there is very little change in this spectrum. However, when FMN (2.8 µM, final concentration) is added to this chromatographed intermediate (3.6 µM) the absorption spectrum (curve B) of a mixture of the X_2 flavoprotein and free FMN results (2). On warming, an absorption spectrum identical to

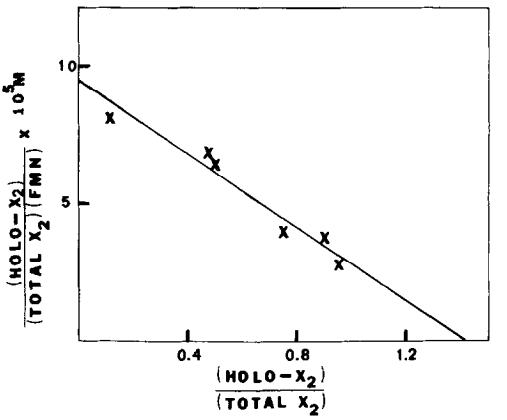

Figure 2. Scatchard plot of the binding to FMN to intermediate X_2 as determined by addition of FMN to apo-X_2. The amount of holo-X_2 was estimated by quenching of FMN fluorescence.

that of FMN returns (curve C). Using the equilibrium constant calculated from fluorescence quenching (below), the absorption spectrum of bound FMN (curve D) can be computed by substraction of the free FMN contribution from curve B. This absorption spectrum corresponds to that of intermediate X_2 reported before (2).

The chromatography, therefore, results in the removal of both free and bound FMN forming apo-X_2 from which, by addition of FMN, the holo-X_2 can be reconstituted.

Fluorescence

After warming, a detectable amount of FMN (0.1-0.3 μM) can be found in the apo-X_2 preparation by fluorescence measurements. This amount, not detectable by the less sensitive absorption measurements, varies from run to run and corresponds to less than 10% of that expected based on the amount of X_2 (2-3 μM) present. In control experiments (BSA + FMN or luciferase + FMN) no detectable FMN is carried down the column with the protein.

Apo-X_2 quenches the fluorescence of added FMN. This confirms our previous conclusion (2) that the bound FMN in X_2 was nonfluorescent. These results are shown in Figure 2 in the form of a Scatchard plot. The association constant is observed to be 7×10^5 M^{-1}. The somewhat greater than one

TABLE. Quantum yields on reaction of the apo-X_2 preparation with decanal and the additions indicated.

Addition	$Q_B(X_2)$[a]	$Q_B(FMN)$[b]
none	0.085	0.45
FMN (2.8 μM)	0.079	0.0052
FMN (28 μM)	0.093	0.00045
apoflavodoxin (12.8 μM)	0.056	>1
apoflavodoxin (64 μM)	0.056	>1
none[c]	0.079	1.2
apoflavodoxin[c] (13 μM)	0.036	>>1

a. Estimated X_2 as equal to one half the added $FMNH_2$ ($Q_B(FMNH_2)$ = 0.03, reference 6). b. Estimated FMN by fluorescence of the final reaction mixture after warming. c. Prior dialysis of luciferase against KBr (2 M, 0° C, 2 days).

to one binding may be due to additional formation of intermediate by photoreduction of FMN by room light necessary for these manipulations. This would lead to a greater concentration of intermediate than that estimated from the amount of reduced flavin added.

Quantum Yields

Quantum yield measurements of apo-X_2 were made by injecting 0.1 cc of the apoprotein solution into 1 cc of buffer solution (phosphate, pH 7, BSA 1 mg/ml) containing decanal (40 μM) at room temperature. This temperature is used since at 0° C, though the final quantum yield is the same, the rate of light production is greatly reduced. As seen in the Table, the quantum yield with respect to FMN can be much greater than any of the reactants while the quantum yield of X_2 is very nearly the same as aldehyde (0.05)(6). Added FMN does not affect the light yield or the rate of light emission. If how-

ever, apoflavodoxin is added before aldehyde the light yield is quenched but not completely. The amount of quenching can be increased if luciferase is first dialyzed against 1 M KBr for 48 hours, which results in the removal of some fluorescent material, perhaps FMN in a contaminating flavoprotein. This protein can apparently replace FMN in its role in the final steps leading to light emission (7).

The quantum yield of X_2 is probably the same as that of aldehyde but again its concentration may be slightly underestimated due to the photoreduction of FMN on the column.

DISCUSSION

These results support our previous conclusion (2) that the long-lived intermediate in the bacterial bioluminescence reaction is a flavoprotein of composition $E(H_2O_2)FMN$. In the absence of aliphatic aldehyde it breaks down to H_2O_2 and free FMN (2). The present results show that the FMN is not strongly bound to X_2 and equilibrium is rapidly established, which is the reason most of the FMN is separated on the Sephadex column to produce the apo-X_2. The holoprotein is reconstituted simply by adding back FMN, as indicated by the reappearance of the characteristic X_2 absorption spectrum and by the quenching of FMN fluorescence.

The light intensity and quantum yield of the reaction of apo-X_2 with decanal are independent of the concentration of FMN until it is reduced to very low levels, much less than 1% of the X_2 concentration or 10^{-8} M. This result does not support a recent claim that luciferase bound flavin hydroperoxide constitutes the intermediate (8), since removal of the flavin hydroperoxide from the protein would certainly lead to its breakdown.

Since there is little doubt that the light emission comes from a flavin molecule in some form or other, probably bound to the protein as a cation (9), then FMN must be a specific sensitizing chromophore in an enzymatic oxidation of aldehyde.

There are two possible roles for the apo-X_2 in the complete reaction

scheme. The first is that it reacts directly with RCHO and the FMN is a specific sensitizer.

$$\text{apo} - X_2 + \text{RCHO} \rightarrow \text{apo} - X_2 - \text{RCHO}$$

$$\downarrow \text{FMN}$$

$$E + \text{RCOOH} + h\nu$$

Alternatively the X_2 may react with RCHO directly in which case the FMN can be described as a catalyst as well as a sensitizer.

$$\text{apo} - X_2 + \text{FMN} \rightleftarrows \text{holo} - X_2$$

$$\text{holo} - X_2 + \text{RCHO} \rightarrow X_2 - \text{RCHO}$$

$$\downarrow$$

$$E + \text{RCOOH} + \text{FMN} + h\nu$$

REFERENCES

1. Hastings, J. W. and Gibson, Q. H., J. Biol. Chem. 238, 2537 (1963).
2. Lee, J. and Murphy, C. L., Biochem. Biophys. Res. Comm. 53, 157 (1973).
3. Hastings, J. W., Weber, K., Friedland, J., Eberhard, A., Mitchell, G. W., and Gunsalus, A., Biochemistry 8, 4681 (1969).
4. Wampler, J., and DeSa, R., Applied Spectroscopy 6, 623 (1971).
5. Mayhew, S. G., in "Flavins and Flavoproteins" Kamin, H., Ed., University Park Press, Baltimore, p. 185 (1971).
6. Lee, J. and Murphy, C., in "Chemiluminescence and Bioluminescence" Cormier, M. J., Hercules, D. M., and Lee, J., Plenum Press, New York, p. 381 (1973).
7. Lee, J., Murphy, C. L., Faini, G. J., and Baucom, T. L., in "Liquid Scintillation Counting: Recent Progress" Stanley, P. E., and Scoggins, B. A., Eds., Plenum Press, New York, in press (1974).
8. Hastings, J. W., Balny, C., Le Peuch, C., and Douzou, P., Proc. Nat. Acad. Sci. (USA) 70, 3468 (1973).
9. Eley, M., Lee, J., Lhoste, J.-M., Lee, C. Y., Cormier, M. J., and Hemmerich, P., Biochemistry 9, 2902 (1970).