FLAVIN BINDING BY BACTERIAL LUCIFERASE:

AFFINITY CHROMATOGRAPHY OF BACTERIAL LUCIFERASE

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Immobilized FMN covalently attached to Sepharose-6B-hexanoate binds bacterial luciferase. Immobilized flavin is also effective in its reduced form as a substrate in the light emitting reaction catalyzed by luciferase.

Bioluminescence catalyzed by bacterial luciferase utilizes $FMNH_2$ and aldehyde as substrates in a mixed function oxidation by molecular oxygen (1,2,3). Although the affinity of oxidized FMN for luciferase is much lower than that of the reduced form (4,5,6), its association constant ($K = 4 \times 10^3\ M^{-1}$) is high enough to suggest that affinity chromatography with bound FMN would be successful. Using a modification of the procedure of Mosbach and co-workers (7), we have immobilized FMN by covalent attachment to a Sepharose-6B-hexanoate matrix and found that it binds luciferase. In its reduced form it is effective as a substrate in the reaction.

MATERIALS: Sepharose 6B was purchased as the pre-swollen gel from Pharmacia Fine Chemicals. FMN, riboflavin and cytochrome c were obtained from Sigma, dicyclohexylcarbodiimide and 6-aminohexanoic acid from Aldrich, cyanogen bromide (CNBr) from Eastman, crystallized bovine serum albumin (BSA) from Miles Laboratories and ovalbumin from Worthington. 5'-Phosphopentyl flavin, Li salt, was a gift of Drs. D.B. McCormick and M.N. Kazarinoff. The two luciferases used in these studies, designated as Pf and MAV, were isolated from two species of luminous bacteria assigned by Reichelt and Baumann (8) to Photobacterium fischeri (Pf, ATCC 7744) and Beneckea harveyi (MAV, Strain 392).

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METHODS AND RESULTS: A settled volume of 30 ml of Sepharose 6B was washed with 0.1 M $NaHCO_3$ to remove the azide preservative, resuspended in the same buffer, cooled to 4° and adjusted to pH 11. Activation was initiated by the dropwise addition of 3 g CNBr in 6 ml N,N'-dimethylformamide, and the slurry was maintained at pH 11. After 30 minutes at 4°, the material was filtered and washed with cold 0.2 M sodium borate buffer, pH 8.5.

The washed, activated gel was resuspended in 40 ml of 0.2 M sodium borate buffer, pH 8.5, containing 2.6 g 6-aminohexanoic acid and stirred for 18 hours at 4°. The gel was then washed sequentially with 500-ml each of 0.2 M sodium borate buffer, pH 8.5, 0.1 M $NaHCO_3$, 0.01 M HCl, 0.5 M NaCl and H_2O, followed by extensive washing with 80% aqueous pyridine (v/v). After filtration the gel was resuspended in 6 ml distilled H_2O containing 0.4 g FMN and transferred to a 250-ml light-shielded Erlenmeyer flask. A solution of 10 g dicyclohexyl-carbodiimide in 24 ml pyridine was added and the slurry gently agitated at 37° for 4 days.

In order to remove byproducts and unbound FMN, the FMN-Sepharose was sequentially washed with 95% ethanol, H_2O, warm (45°) 1-butanol, 95% ethanol, H_2O, 10^{-3} M HCl, cold 0.1 M $NaHCO_3$, 0.5 M NaCl and H_2O and stored in 0.2% NaN_3 at 4° in the dark until used. The 450 nm absorbance of the acid hydrolysates of the flavin-substituted Sepharose gels indicated that 2.4 μmoles of FMN were bound per ml of gel. The gel was stable for at least one year.

Riboflavin-Sepharose conjugates were prepared using the same procedure, but only about 0.1 μmoles were bound per ml of gel. Control gel with the hexacarbon spacer alone was made by omitting the steps utilizing FMN. Attempts to covalently link 5'-phosphopentyl flavin, an FMN derivative in which the 5'-phosphoribityl side chain is replaced by a 5'-phosphopentyl group were unsuccessful. It is possible that the

coupling reaction between FMN and the Sepharose-hexanoate involves esterification of a ribityl side chain hydroxyl group with the terminal carboxyl group of the hexanoate spacer. The FMN phosphate group may function as an effective acylating agent through the production of a mixed phosphate-carboxylate anhydride intermediate (A.M. Michelson, personal communication). This may account for the lower yield in immobilizing riboflavin by the same treatment.

Chromatography was carried out at 4° in columns (0.9 x 2.6 cm) using a bed volume of 2 ml at a flow rate of 18 ml/hr. The FMN-Sepharose was regenerated by washing with 2 M NaCl in buffer (0.02 M phosphate, pH 7, containing 5×10^{-4} M dithiothreitol (DTT), referred to as buffer A), and then equilibrated in the same buffer without NaCl. The protein sample was applied and the column developed with buffer A until the A_{280} returned to baseline. Bound luciferase was eluted by increasing the concentration of either NaCl or FMN in the same buffer.

The A_{280} of each fraction (1.2 ml) was determined with a Cary Model 15 spectrophotometer. Conductivity was measured at 0° with a Radiometer conductivity meter. Protein concentrations in cell extracts were determined by the method of Lowry (9).

Luciferase was purified as described by Gunsalus-Miguel et al. (10). It's activity was measured at $21^{\circ} \pm 2^{\circ}$ with decanal as the aldehyde, either initiating the reaction with catalytically reduced $FMNH_2$ (10), or using the "dithionite" assay (11), in which the flavin and luciferase are reduced together in a vial by the addition of solid dithionite, followed by the injection of aldehyde with excess oxygen to initiate the reaction. Luciferase activity is expressed in quanta$\cdot$sec^{-1} (q$\cdot$sec^{-1}), using the standard of Hastings and Weber (12).

Both MAV and Pf luciferases bind to FMN-Sepharose. Fig. 1a illustrates the elution pattern of MAV luciferase using a linear gradient of FMN. In an experiment using the Pf enzyme the elution pattern was

similar, except that the Pf eluted slightly later (5×10^{-3} M FMN for Pf compared to 3×10^{-3} M FMN, for MAV). This is somewhat surprising in view of the fact that the binding of free FMN to Pf luciferase has been estimated to be considerably (2 to 20 times) weaker (5). This may indicate that the affinity of luciferases for this gel is not due solely to their interaction with the FMN ligand. The bound enzymes can also be eluted with NaCl, as shown in Fig. 1b for Pf enzyme. With MAV, the

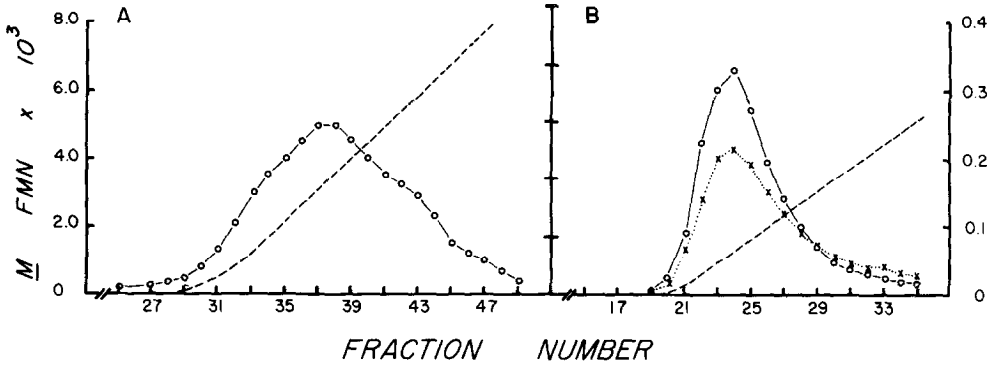

Figure 1a. FMN-Sepharose chromatography of MAV luciferase using a gradient of FMN for elution MAV luciferase (4 mg) in 0.25 ml of buffer A, having a specific activity of 1.4×10^{14} q·sec^{-1}·mg^{-1}, was applied to a column pre-equilibrated with the same buffer and eluted with FMN. 1b. Pf luciferase. The sample (3 mg) in 0.3 ml of buffer A, having a specific activity of 1.2×10^{14} q·sec^{-1}·mg^{-1}, was applied to a column pre-equilibrated in the same buffer and eluted with a linear NaCl gradient as shown (dashed line). Absorbances at 280 nm (dotted line, x) and luciferase activities (o) are plotted for each fraction. Specific activity of a pool of fractions 21-30 was 1.5×10^{14} q·sec^{-1}·mg^{-1}. Center ordinate scale, applicable to solid lines in both panels, luciferase activity in units of q·sec^{-1}·ml^{-1}·10^{13} (one division equals one unit).

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activity eluted slightly earlier in the NaCl gradient than did Pf. The luciferases do not bind appreciably to the Sepharose-hexanoate lacking FMN; of the 4×10^{14} activity units of each enzyme loaded onto such a control column, 90% of the Pf activity and 98% of the MAV activity eluted in the buffer wash.

Luciferase can also be separated from a mixture of non-flavoproteins. Purified MAV luciferase was mixed with BSA, cytochrome c and ovalbumin,

applied to the FMN-Sepharose column and chromatographed. The non-flavoproteins came through in the buffer wash while the luciferase eluted at its characteristic position in the gradient. The specific activity of eluted luciferase was 1 x 10^{14} q·sec^{-1}·mg^{-1}, similar to the starting material.

A partial purification of luciferase was achieved using this column. Crude cell extracts of strain MAV were batch-adsorbed on DEAE-Cellulose. The luciferase activity was eluted with 0.35 M phosphate buffer, pH 7, and dialyzed against a large volume of buffer A. This enzyme (specific activity of 8 x 10^{12} q·sec^{-1}·mg^{-1}) was chromatographed on a Sepharose-hexanoate gel to remove proteins which bind to the hexacarbon spacer. Luciferase activity came through in the buffer wash and was applied to the FMN-Sepharose column, and eluted with a linear NaCl gradient (Fig. 2).

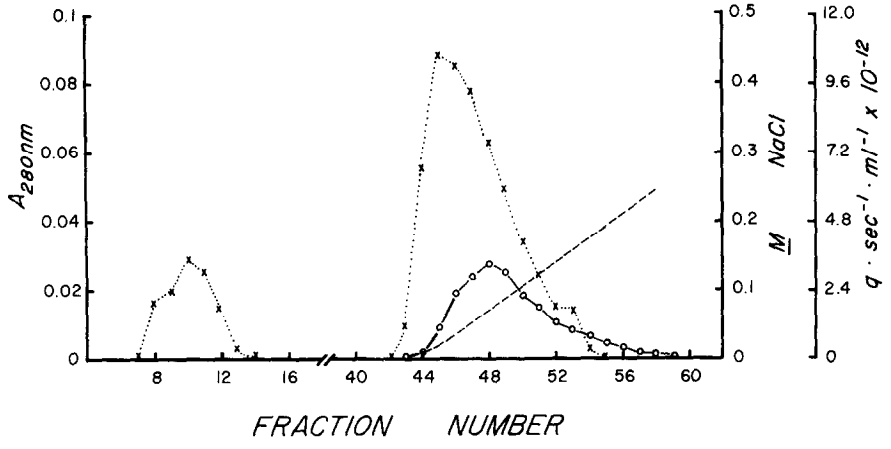

Figure 2. Partially purified luciferase was applied to the FMN-Sepharose column. Some contaminating proteins came through in the wash (tubes 8-12). Luciferase, as well as some other unidentified proteins, was eluted with NaCl.

The pooled luciferase had a specific activity of 6 x 10^{13} q·sec^{-1}·mg^{-1}. Since highly purified MAV luciferase has a specific activity of about 1.4 x 10^{14} q·sec^{-1}·mg^{-1}, the MAV luciferase activity after the FMN-Sepharose step is estimated to be about 40% pure.

Immobilized FMN, reduced either catalytically with H_2 or with dithionite, is effective as a substrate in the luminescence reaction,

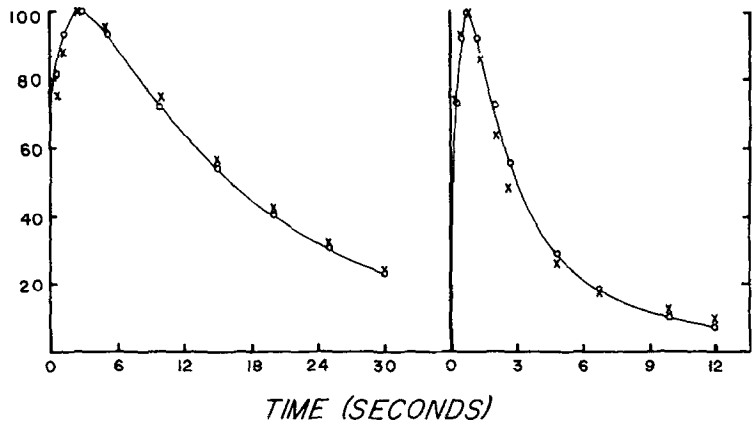

Figure 3. The kinetics of the reaction, measured by the time course of the bioluminescence, are the same with immobilized $FMNH_2$ (x) as with soluble $FMNH_2$ (o), both with dodecanal (left) and decanal (right). Curves for reactions with soluble and insoluble $FMNH_2$ are normalized to the maximum initial intensities in each case. Note that the turnover time is quite different with the different aldehydes. Apparent first order rate constants for decay of luminescence: decanal = 0.37 sec^{-1}; dodecanal, 0.056 sec^{-1}. MAV luciferase, 25°C.

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in spite of the fact that the competitive autoxidation of $FMNH_2$ occurs in less than one second (13). The apparent maximum rate is only about 2% of that with free $FMNH_2$, perhaps because of immobilization. But the turnover time for the reaction initiated by insoluble $FMNH_2$ is the same as that for soluble $FMNH_2$, both with decanal and dodecanal (Fig. 3), unexpected in view of the fact that substitutions on the flavin molecule (13) and also the chain length of the aldehyde (14), may alter turnover time substantially.

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REFERENCES

1. Hastings, J.W. (1968) Annu. Rev. Biochem. 37, 597-630.
2. Hastings, J.W., Eberhard, A., Baldwin, T.O., Nicoli, M.Z., Cline, T.W., Nealson, K.H. (1973) in Chemiluminescence and Bioluminescence

(M.J. Cormier, D.M. Hercules, J. Lee eds.) pp. 369-380, Plenum Publishing Co., New York.
3. Dunn, D.K., Michaliszyn, G.A., Bogacki, I.G., and Meighen, E.A. (1973) Biochem. 12, 4911-4918.
4. Gibson, Q.H., Hastings, J.W., Weber, G., Duane, W., and Massa J. (1966) in Flavins and Flavoproteins (E.C. Slater ed.) pp. 341-359, Elsevier Publishing Co., Amsterdam.
5. Baldwin, T.O. (1974) Biochem. Biophys. Res. Comm., in press.
6. Nicoli, M.Z. (1972) Ph.D. Thesis, Harvard University, Cambridge, Mass.
7. Mosbach, K., Guilford, H., Ohlsson, R., and Scott, M. (1972) Biochem. J. 127, 625-631.
8. Reichelt, J.L., and Baumann, P. (1973) Arch. Mikrobiol. 94, 283-330.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-276.
10. Gunsalus-Miguel, A., Meighen, E.A., Nicoli, M.Z., Nealson, K.H., and Hastings, J.W. (1972) J. Biol. Chem. 247, 398-404.
11. Meighen, E.A., and Hastings, J.W. (1971) J. Biol. Chem. 246, 7666-7674.
12. Hastings, J.W., and Weber, G. (1963) J. Opt. Soc. Am. 53, 1410-1415.
13. Gibson, Q.H., and Hastings, J.W. (1962) Biochem. J. 83, 368-377.
14. Mitchell, G.W., and Hastings, J.W. (1969) J. Biol. Chem. 244, 2572-2576.
15. Hastings, J.W., Spudich, J.A., and Malnic, G. (1963) J. Biol. Chem. 238, 3100-3105.