Vol. 87, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 27, 1979 Pages 1080-1086

BIOSYNTHESIS OF ALIPHATIC ALDEHYDES FOR THE BACTERIAL BIOLUMINESCENT REACTION STIMULATION BY ATP AND NADPH

E.A. Meighen

Department of Biochemistry
McGill University
Montreal, Quebec

Received February 23, 1979

SUMMARY: Although the activity of bacterial luciferase is dependent on the presence of a long chain aliphatic aldehyde as well as $FMNH_2$ and O_2, the mechanism of biosynthesis of aliphatic aldehydes in bioluminescent bacteria is not yet known. The present paper reports the stimulation of luminescence by ATP and NADPH on injection of $FMNH_2$ into extracts of Photobacterium phosphoreum containing no added aldehyde. Over a 300-fold stimulation of luminescence was observed after incubation of the extract for 10 min with NADPH and ATP, increasing to 500-fold if myristic acid was also present. These results provide evidence in vitro for the existence of an enzyme dependent on ATP and NADPH and capable of synthesizing the aliphatic aldehydes necessary for bacterial luminescence.

Bacterial luciferase catalyzes the oxidation of $FMNH_2$ and a long chain aliphatic aldehyde resulting in the emission of a blue green light at 490 nm (1, 2). Although the involvement of aliphatic aldehydes in bacterial bioluminescence has been known for over twenty-five years (3, 4), both the mechanism and the enzymes involved in the synthesis of the aliphatic aldehydes remains unknown. The recent discovery that long chain fatty acids, and myristic acid, in particular, can stimulate the in vivo luminescence of some dark mutants lacking the in vivo aldehyde factor (5) has provided evidence that fatty acids may be the direct precursors of aliphatic aldehydes in bioluminescent bacteria.

The conversion of a fatty acid to an aldehyde has only been studied in a limited number of systems. These reactions have involved either α-oxidation of the fatty acid followed by decarboxylation (6-8) or activation of the fatty acid with ATP to form a high energy intermediate which can then be reduced to the aldehyde (9, 10). Since ATP levels drop in the bioluminescent bacterium

0006-291X/79/081080-07$01.00/0

Beneckea harveyi, during the induction of the bioluminescent system and this decrease is not detected in dark "aldehyde stimulable" mutants it was suggested that ATP may be involved in the synthesis of the aldehyde factor (11).

The present paper reports evidence for the existence of an enzyme in extracts of the luminescent bacterium, Photobacterium phosphoreum, that is dependent on ATP and NADPH and which can catalyze the synthesis of the aldehyde factor for the bioluminescent reaction. These studies are relevant not only with respect to the mechanism of aldehyde synthesis but also with regard to defining the function of the specific enzymes involved in the induction of the bioluminescent system (12, 13) and their mechanism of regulation.

EXPERIMENTAL PROCEDURE

Growth of cells. Photobacterium phosphoreum (NCMB 844) and Beneckea harveyi were grown in complex media containing 5 g of Difco Bacto tryptone, 5 g Difco yeast extract, 2 ml glycerol, 30 g NaCl, 3.7 g Na_2HPO_4, 1 g KH_2PO_4, 0.5 g $(NH_4)_2HPO_4$ and 0.1 g $MgSO_4$ in one liter. The B. harveyi cells were grown at 27° whereas the P. phosphoreum cells were grown at 19° with limited aeration.

Cells lysis. The cells at maximum luminescence ($A_{660} \sim 3.0$) were harvested by centrifugation and then lysed by sonication for 60 sec at 0° in H_2O containing 10^{-3} M mercaptoethanol (0.25 ml per ml of culture). The cellular debris was removed by centrifugation and the supernatant used for future analysis.

Luciferase assay. Luciferase activity was determined by injection of 1.0 ml of 5×10^{-5} M $FMNH_2$ (prepared by catalytic reduction of FMN over 5% platinized asbestos) into 1.0 ml of 0.05 M phosphate, pH 7.0, 0.2% bovine serum albumin, containing enzyme and dodecanal; the initial light intensity being a measure of the activity. Light intensity was measured with a photomultiplier photometer and is expressed in light units (LU) where 1 LU equals 6×10^9 quanta per sec based on the standard of Hastings and Weber (14). All phosphate buffers were made by mixing appropriate amounts of NaH_2PO_4 and K_2HPO_4.

RESULTS AND DISCUSSION

Extracts of bioluminescent bacteria containing the enzyme, luciferase, on injection of $FMNH_2$, will emit a low level of luminescence, apparently due to the presence of endogeneous levels of the aldehyde substrate for the bioluminescent reaction. A much higher light intensity can be obtained by addition of an aliphatic aldehyde to the extract as shown in Table I for two different bioluminescent strains, B. harveyi and P. phosphoreum.

TABLE I

Luminescence in Extracts of Bioluminescent Bacteria[a]

Components Added	B. harveyi	P. phosphoreum
None	0.8	0.35
Dodecylaldehyde	20[b]	130
Myristaldehyde	180[b]	1,200
Myristic acid	0.6	1.5
Myristic acid, ATP, NADPH	0.6	34

[a] Luminescence was measured after injection of 5 x 10^{-5} M $FMNH_2$ into 0.20 ml of extract and 1.0 ml of 10^{-3} M mercaptoethanol, 0.05 M phosphate, pH 7.0, preincubated for 10 minutes at 22°, with the added components. The indicated components were present in the assay mixture at the following levels; aldehyde, 0.1 μmole; myristic acid, 0.01 μmole; ATP, NADPH, 1.0 μmole.

[b] The levels of light emission were 2 to 3-fold higher if assayed immediately after the addition of aldehyde.

Over the last few years, our attempts to stimulate the level of light emission in extracts of B. harveyi by preincubation with potential precursors of the aliphatic aldehydes (e.g. aliphatic acids, alcohols, amines, plasmalogens, etc.) prior to injection of $FMNH_2$ have been unsuccessful. However, recently we have initiated investigations on a highly luminescent bacterium, P. phosphoreum, and have found evidence in vitro that fatty acid activation may be the pathway involved in aldehyde synthesis at least in this bacterium.

Table I shows that a 100-fold increase in light emission is obtained if extracts of P. phosphoreum are incubated with ATP, NADPH and myristic acid prior to addition of $FMNH_2$. A small increase in luminescence can also be obtained with myristic acid in the absence of ATP and NADPH. Under the same conditions of incubation, luminescence could not be stimulated in extracts of B. harveyi (Table I) or a dark, aldehyde stimulable, mutant of B. harveyi whose light emission in vivo could be increased about 100-fold by myristic acid. Different assay conditions or extraction methods or addition of other potential substrates and cofactors (coenzyme A, NADH, Mg^{++}, GTP, etc.) had no significant effect on luminescence with extracts of either B. harveyi or P. phosphoreum.

TABLE II

Stimulation of Light Emission in Extracts of P. phosphoreum

Components Added[a]	Light Intensity[b]
None	0.07
Myristic Acid	0.13
ATP	0.09
NADPH	0.38
Myristic Acid, ATP	0.16
Myristic Acid, NADPH	1.8
NADPH, ATP	24.1
Myristic Acid, ATP, NADPH	38.4
Myristic Acid, ATP, NADH	1.5
Myristic Acid, GTP, NADPH	1.7

a Compounds were added to the assay mixture at the following levels: myristic acid, 0.01 μmole; ATP, GTP, NADH, NADPH, 1.0 μmole.

b Maximum luminescence (LU) was measured after injection of 1.0 ml of 5×10^{-5} M catalytically reduced $FMNH_2$ into 0.20 ml of extract preincubated for 10 minutes at 22° with 1.0 ml of 10^{-3} M mercaptoethanol, 0.05 M phosphate, pH 7.0 containing the indicated components. The values are the average of two independent determinations.

Table II gives the effects of different combinations of myristic acid, ATP and NADPH on the levels of light emission in extracts of P. phosphoreum. Since a relatively low background level of light emission was obtained with this extract (Table II), incubation with NADPH, ATP and myristic acid resulted in over a 500-fold stimulation. Addition of any one of the three components alone leads to stimulation of light emission over background levels, however, the final level of light emission is much lower (< 1%) than with all three components. Addition of myristic acid and NADPH together stimulates luminescence, however the level obtained is still only 5% of that obtained if ATP is also present. Only the combination of NADPH and ATP can lead to light levels comparable (∿60%) to the complete mixture with ATP, NADPH and myristic acid. Repeated experiments with different extracts have shown that light emission

is increased 1.2 to 1.8-fold by myristic acid and that it appeared to be the most effective fatty acid for stimulation of luminescence. However, any definite conclusions about the fatty acid specificity must await experiments to lower the level of luminescence stimulation obtained with ATP and NADPH alone due apparently to the presence of endogeneous levels of fatty acids (or similar components) in the extract.

The results, however, clearly indicate that the stimulation of light emission in extracts is specific for both ATP and NADPH. Substitution of NADH for NADPH gave light levels only 4% of those obtained with NADPH whereas substitution of GTP for ATP gave a level of light emission identical to that obtained in the absence of ATP.

The dependence of the level of light emission on the time of incubation of the extract with ATP, NADPH, and myristic acid is given in Figure 1. A linear dependence is obtained over a period of 20 minutes with the level of luminescence reacting about 1,000 times (77 LU) that of the control (0.07 LU) containing no added components. The control during this period (20 minutes) decreased from about 0.19 light units (LU) to 0.07 LU with the greatest change occurring in the first two minutes of incubation.

Figure 2 compares the thermal stability of the stimulability of light emission in the P. phosphoreum extracts by ATP, NADPH, and myristic acid to the luciferase activity. After only one hour of incubation at 21°, about 80% of the stimulability of activity by the mixture of ATP, NADPH and myristic acid has been lost, and by three hours, the stimulation is only about 10% of that observed at the start of the experiment. In contrast, luciferase activity only decreased to 75% of the original activity after three hours. Extract stored at 4° for 20 hours retained 100% of its luciferase activity but only 15% of its ability to be stimulated by ATP, NADPH and myristic acid. These results suggest that an enzyme(s) other than luciferase is responsible for the stimulation of luminescence by ATP, NADPH, and myristic acid.

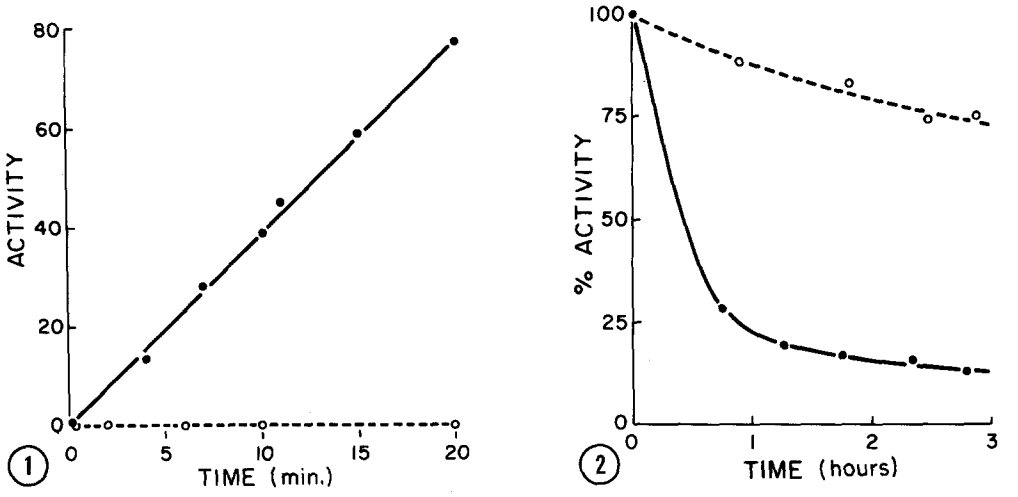

Figure 1. Dependence of the stimulation of luminescence on time of incubation with ATP, NADPH, and myristic acid. Aliquots of the P. phosphoreum extract (0.20 ml) were incubated at 22° with 1.0 ml of 10^{-3} M mercaptoethanol, 0.05 M phosphate, pH 7.0 containing 1 μmole of each of ATP and NADPH, and 0.01 μmole of myristic acid. After different extents of time, 1.0 ml of 5 x 10^{-5} M $FMNH_2$ was injected and the maximum luminescence determined (•). A control is also given (o) for the extract (0.20 ml) incubated for different periods of time with 1.0 ml of buffer containing only 10^{-3} M mercaptoethanol and 0.05 M phosphate at pH 7.0.

Figure 2. Comparison of the thermal stability at 21° of the luciferase activity (o) and the stimulability of light emission by ATP, NADPH, and myristic acid (•) in extracts of P. phosphoreum. The extract was incubated at 21° for the indicated periods of time prior to assay of aliquots for luciferase activity (with dodecanal) and stimulability of light emission with ATP, NADPH, and myristic acid as described in Table I. The activities are plotted as percentages of the activities obtained at the start of the incubation.

The present experimental data thus provide evidence for the existence of an enzyme in extracts that can catalyze the synthesis of aliphatic aldehydes for the bacterial bioluminescent reaction. Furthermore, the dependence on ATP and NADPH might suggest that the enzyme may function by activation of a fatty acid with ATP followed by reduction with NADPH to the aldehyde. Work is in progress to determine whether conditions can be established to detect this activity in extracts of B. harveyi and other bioluminescent bacteria as well as to separate it from luciferase and determine its mechanism of action particularly with regard to its specificity for fatty acids.

ACKNOWLEDGEMENTS: This work was supported by a grant (MT-4314) from the Medical Research Council of Canada.

REFERENCES

1. Hastings, J.W. (1968) Annu. Rev. Biochem. 37, 597-630.
2. Hastings, J.W. and Nealson, K.H. (1977) Annu. Rev. Microbiol. 31, 549-595.
3. Strehler, B.L. and Cormier, M.J. (1953) Arch. Biochem. Biophys. 47, 16-33.
4. Cormier, M.J. and Strehler, B.L. (1953) J. Am. Chem. Soc. 75, 4864-4865.
5. Ulitzur, S. and Hastings, J.W. (1978) Proc. Natl. Acad. Sci. USA 75, 266-269.
6. Galliard, T. and Mathew, J.A. (1976) Biochim. Biophys. Acta 424, 26-35.
7. Martin, R.O. and Stumpf, P.K. (1959) J. Biol. Chem. 234, 2548-2554.
8. Shine, W.E. and Stumpf, P.K. (1974) Arch. Biochem. Biophys. 162, 147-157.
9. Kolattakudy, P.E. (1970) Biochemistry 9, 1095-1102.
10. Johnson, R.C. and Gilbertson, J.R. (1972) J. Biol. Chem. 247, 6991-6998.
11. Ulitzur, S. and Hastings, J.W. (1978) J. Bacteriol. 133, 1307-1313.
12. Michaliszyn, G.A. and Meighen, E.A. (1976) J. Biol. Chem. 251, 2541-2549.
13. Ne'man, Z., Ulitzur, S., Branton, D. and Hastings, J.W. (1977) J. Biol. Chem. 252, 5150-5154.
14. Hastings, J.W. and Weber, G. (1963) J. Opt. Soc. Amer. 53, 1410-1415.