INDUCTION OF FATTY ALDEHYDE DEHYDROGENASE ACTIVITY DURING THE DEVELOPMENT OF BIOLUMINESCENCE IN BENECKEA HARVEYI

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SUMMARY: Bioluminescence rises very rapidly in the later stages of growth of Beneckea harveyi due to the induction of luciferase activity. This enzyme catalyzes the in vitro oxidation of $FMNH_2$ and a long chain aliphatic aldehyde resulting in the emission of light. The present experiments report the discovery of an aldehyde dehydrogenase in Beneckea harveyi which is remarkably similar to luciferase in its specificity for long chain aliphatic aldehydes. Furthermore, the activity of this enzyme is shown to be induced at the same time as luciferase thus providing strong evidence for a functional implication of aldehyde dehydrogenase in the bioluminescent system of Beneckea harveyi.

Bioluminescent bacteria are well known for their specialized enzyme function involved in the production of light. Although the chemical nature of the luciferase reaction has been relatively well characterized in vitro (1-6), the identity of the in vivo substrate and enzyme(s) involved in its generation have remained unresolved. Some recent evidence has suggested that a long chain aliphatic aldehyde may indeed be the true in vivo substrate (7,8). In our attempts to characterize the enzyme(s) which might be responsible for the in vivo biosynthesis of aliphatic aldehyde for the luminescence reaction, it was observed that crude extracts of Beneckea harveyi could reduce NAD^+ in the presence of exogeneously supplied dodecanal, indicating the presence of an aliphatic aldehyde dehydrogenase. Although aldehyde dehydrogenases have been observed in many diverse tissues and microorganisms with a wide range of substrate specificities (9), those which have been reported to act on long chain aldehydes have generally been induced by the addition of long chain alkanes, alcohol, aldehydes or acids to the growth medium (10,11). In the present work, the induction and specificity of the aldehyde dehydrogenase in Beneckea

harveyi has been investigated and evidence is presented relating this enzyme to the bioluminescent system.

MATERIALS AND METHODS: Bioluminescent bacteria, designated as Beneckea harveyi (12), were grown at 27° in 10 litres of complex media (13) under vigorous aeration. In vivo bioluminescence was monitored using a photomultiplier photometer (14) calibrated in quanta sec^{-1} with the standard of Hastings and Weber (15). Bacterial growth was followed by the optical density (A_{660}) at 660 nm; one unit of cell growth is defined to be the amount of bacteria in 1 ml of culture medium which results in an optical density of 1.0. Bacterial cells (225 units) at various stages of growth were collected by centrifugation, stored frozen overnight, and then lysed by vortexing for 1 min in 3 ml of 0.01 M EDTA, 10^{-3} M dithiothreitol, 10^{-3} M phosphate, pH 7.0. Lysis was judged to be complete since sonication of the lysate did not result in any increase of enzyme activity. Cellular debris was removed by centrifugation (9,000 g, 10 min) and the soluble extract assayed for enzyme activity. All phosphate buffers were made by mixing appropriate amounts of NaH_2PO_4 and K_2HPO_4.

Enzyme activities. Bacterial luciferase activity was determined from the maximal light intensity obtained upon injection of 1 ml of 5 x 10^{-5} M $FMNH_2$ (catalytically reduced) into 1.0 ml of 0.02 M phosphate, pH 7.0, containing 0.2% bovine serum albumin, 0.001% dodecanal and enzyme (16). Enzyme activity is given in units of quanta/sec. The activities of all other enzymes (listed below) are given in units of μmole/min (U).

FMN reductase activity was determined from the initial rate of decrease in absorbance at 340 nm upon addition of enzyme to 1.0 ml of 0.05 M phosphate, pH 7.0, containing 0.1% bovine serum albumin, 10^{-4} M NADH and 5 x 10^{-6} M FMN.

Aldehyde dehydrogenase activity was measured from the initial rate of increase in absorbance at 340 nm upon addition of enzyme to 1.0 ml of 0.05 M phosphate, pH 7.0, containing 0.0005% dodecanal and 7 x 10^{-5} M NAD^+.

Alkaline phosphatase activity was based on the increase in absorbance at 400 nm upon addition of enzyme to 1 ml of 1.0 M Tris-chloride, pH 8.0, containing 2 x 10^{-4} M p-nitrophenylphosphate (17).

Glucose-6-phosphate dehydrogenase activity was based on the initial rate of increase in absorbance at 340 nm upon addition of enzyme to 0.05 M Tris-chloride, pH 7.8, containing 2 x 10^{-4} M $NADP^+$, 3 x 10^{-3} M $MgCl_2$ and 3 x 10^{-3} M glucose-6-phosphate (18).

RESULTS AND DISCUSSION: The specificity of the aldehyde dehydrogenase in Beneckea harveyi towards aldehydes of varying carbon chain lengths is given in Table I. The highest activity was found with the longest aldehyde (dodecanal) that was used in the present experiment, and the activity decreased successively as shorter chain aldehydes were employed. The basic requirement seems to be for a long chain aliphatic backbone since there is complete absence of activity with aldehydes shorter than pentanal. Apparently, this is not due to non-saturating levels of substrate since a 100-fold increase in acetaldehyde concentration had no effect on activity. The specificity also

TABLE I

Specificity of Aldehyde Dehydrogenase and Luciferase

Aldehyde	Relative activity	
	Aldehyde dehydrogenase[a]	Luciferase[b]
Acetaldehyde	0	-
Propanal	0	1
Pentanal	0	3
Heptanal	33	4
Octanal	44	14
Nonanal	83	92
Decanal	100	100
Dodecanal	156	14

[a]Aldehyde dehydrogenase activities were measured as described in Materials and Methods and are expressed as the percentage of activity observed with decanal. Maximal activities were obtained with 0.0005% aldehyde for all the long chain aliphatic aldehydes. The initial rate of NAD^+ reduction was linear with respect to the amount of extract added. The measurement of the specificity of aldehyde dehydrogenase in crude extracts may not give a completely accurate representation since there is always the possibility of interfering side reactions particularly with assays involving NAD^+. However, no alcohol dehydrogenase (i.e. aldehyde reductase) activity was detected in the crude extracts and a change in absorbance at 340 nm was only observed on mixing all components in the assay.

[b]Relative activities for luciferase were measured at optimal aldehyde concentration and are expressed as percentages of the maximum light intensity obtained with decanal.

appears to be unusually strict in that aliphatic aldehyde dehydrogenases of other systems (9,10) have been shown to have a relatively high activity with short chain aldehydes. This is even more interesting when the specificity is compared with that of bacterial luciferase (Table I). The remarkable similarity in the specificities of both luciferase and aldehyde dehydrogenase for aliphatic aldehydes (except perhaps for dodecanal) strongly suggests that the latter enzyme may play an important role in the bioluminescent system of

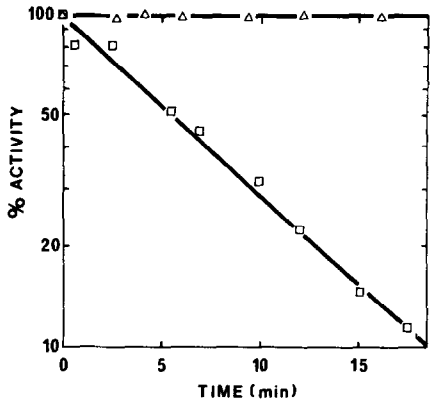

Figure 1. Temperature sensitivity of aldehyde dehydrogenase and luciferase activities. An aliquot (1.0 ml) of the soluble bacterial extract was mixed with 4.0 ml of 0.10 M phosphate, 0.1% bovine serum albumin, 10^{-3} M EDTA, pH 7.0, and incubated at 37°. Aliquots were assayed with dodecanal for aldehyde dehydrogenase and luciferase activity as a function of time.

Beneckea harveyi, perhaps by controlling the levels of substrate available for the luciferase reaction.

Since the aldehyde specificities are so similar it is necessary to establish that different enzymes are being investigated rather than a single enzyme with both functions (i.e. luciferase with aldehyde dehydrogenase activity). The temperature sensitivity at 37° of these two activities (Fig. 1) clearly shows that aldehyde dehydrogenase is rapidly inactivated (t 1/2 = 5 min) compared to luciferase. Moreover, incubation of the extract at 37° for 60 min completely destroyed aldehyde dehydrogenase activity whereas luciferase remained fully active showing not only that a component in the crude extract is necessary for the aldehyde dependent reduction of NAD^+ but also providing evidence that aldehyde dehydrogenase and luciferase are different enzymes. This difference was further demonstrated by separation of the two activities by chromatography on DEAE-cellulose (Fig. 2). The major difficulty encountered was the instability of the aldehyde dehydrogenase which consequently resulted in a low recovery of activity (18%) in this experiment and makes further purification difficult. However, the results clearly show that the two

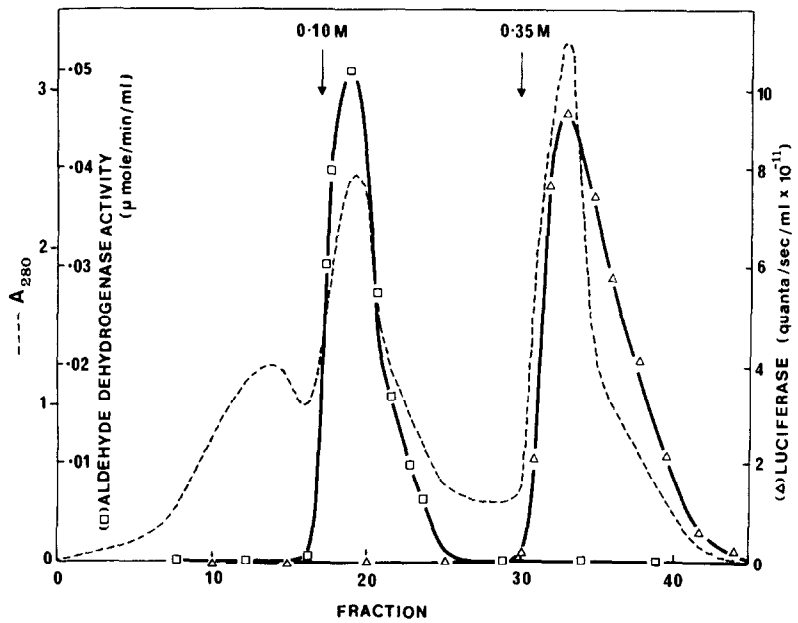

Figure 2. DEAE-cellulose chromatography of 50 ml of the soluble extract obtained from lysis of Beneckea harveyi cells grown to an optical density at 660 nm of 2.5. The crude extract was applied to a 2.5 x 7 cm column of DEAE-cellulose at 4°, preequilibrated with 0.01 M mercaptoethanol, 0.02 M phosphate, pH 7.0, and then washed with 50 ml of the same buffer. Aldehyde dehydrogenase activity was eluted with 100 ml of 0.01 M mercaptoethanol, 0.10 M phosphate, pH 7.0, and luciferase activity then eluted with 0.01 M mercaptoethanol, 0.35 M phosphate, pH 7.0, as indicated above. Fractions of eight ml were collected and assayed for aldehyde dehydrogenase and luciferase activity as described in Materials and Methods using dodecanal as the aldehyde substrate.

activities are eluted in different positions providing strong evidence that the activities can be attributed to different enzymes.

It is well established that bacterial luciferase activity is induced at a later stage of exponential cell growth (13). Since we have suggested involvement of aldehyde dehydrogenase in the luminescent system, it would be interesting to determine if its activity is coordinately controlled with that of luciferase. In order to investigate this possibility, the bioluminescent bacteria were analyzed for several different enzymic activities as a function of their growth (Fig. 3). In this experiment, a constant amount of bacteria (Volume (ml). A_{660} = 225) was harvested at different culture densities and then lysed in a fixed volume of buffer. An aliquot was then assayed for each

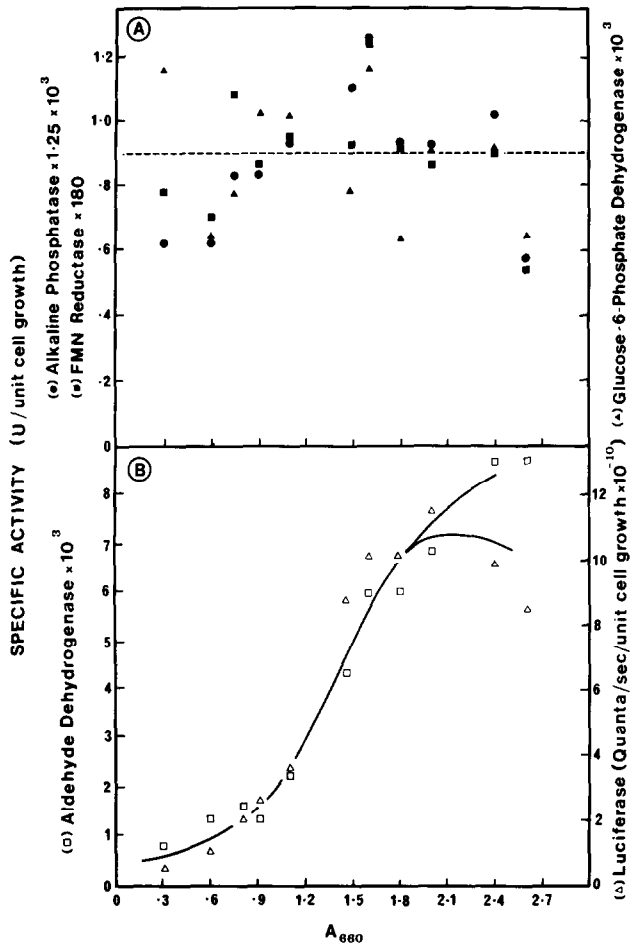

Figure 3. Specific activity of noninduced (A) and induced (B) enzyme activities in Beneckea harveyi as a function of cell growth (A_{660}). Specific activity is given in units of activity (U) per unit of cell growth. Complex medium was innoculated with bacteria to an optical density at 660 nm of 0.01 and a constant amount of bacteria (225 units of cell growth) collected at different stages of cell growth. The cells were lysed and the centrifuged extract assayed for the different enzyme activities as described in Materials and Methods.

enzyme function and the specific activity (activity per unit of cell growth) determined.

Two enzymes, FMN reductase and glucose-6-phosphate dehydrogenase, whose activities have been reported to parallel the exponential growth of the cells (13,19), are shown to have relatively constant specific activities at all stages of growth (Fig. 3A). Furthermore, the specific activity of alkaline

phosphatase is now shown to follow the same pattern and remains constant with cell growth. In contrast, the specific activities of both luciferase and aldehyde dehydrogenase remain very low during the early period of exponential cell growth but then rise dramatically and very rapidly during a later period (Fig. 3B). It is, however, quite clear, using the specific activities of alkaline phosphatase, FMN reductase, and glucose-6-phosphate dehydrogenase as internal controls, that the enzymic function of aldehyde dehydrogenase is induced as is that of luciferase. Furthermore, the simultaneous and parallel development of functional activity in both enzymes suggests a coordinate control.

Although aldehyde dehydrogenase is implicated as a functional part of the bioluminescent system in Beneckea harveyi, it is not immediately clear whether the true in vivo function is the generation of aldehyde as substrate for the luciferase reaction or the removal of aldehyde. Analysis of five dark mutants, which are stimulated in light emission upon addition of aldehyde, has shown that each contained normal levels of aldehyde dehydrogenase activity. Studies are therefore in progress to stabilize and purify this enzyme in order to investigate its properties, specificity, and possible functional activities as well as to establish its mechanism of induction.

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