Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1180-1186

SUBUNIT EXCHANGE BETWEEN AND SPECIFIC ACTIVITIES OF MUTANT BACTERIAL LUCIFERASES[1]

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Received June 20,1980

SUMMARY: Complementation has been demonstrated between two mutant bacterial luciferases which possess low activities due to different structural defects in different subunits. Activity characteristic of the wild type dimer was obtained by incubating under non-denaturing conditions. The specific activities of the two mutant enzymes were determined by using specific antiserum made against the wild type luciferase.

Some oligomeric enzymes, such as lactate dehydrogenase, exhibit classical subunit exchange in buffer (1). In other cases, where a high affinity exists between subunits, extreme - even denaturing - conditions are required to achieve separation. Bacterial luciferase, a 79,000 M.W. $\alpha\beta$ heterodimer (2), appears to be in the latter category. This conclusion is based on the absence of detectable quantities of free subunit in chromatographic, ultracentrifugal, and subunit titration analyses (3,4), and on the fact that activity is directly proportional to dilution over many orders or magnitude (5). Individually, subunits are completely devoid of activity (6,7).

In order to demonstrate subunit exchange in bacterial luciferase under nondenaturing conditions we used enzymes isolated from two dim mutants, AK-6 and FB-1, both of which have lower specific activities than that of the wild type enzyme (8,9,10). The AK (altered kinetics) mutant has a defect in the α

[1]Supported in part by a grant from the National Science Foundation to J.W.H. (PCM 77-19917) and by NIH postdoctoral research fellowships to S.-C.T. (1 F32 GM00348-02,03). We are grateful to Drs. T.O. Baldwin, T.W. Cline and C. Waters for their valuable contributions to this study, including the mutants (T.O.C.) and the antisera (C.W.) used.

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0006-291X/80/151180-07$01.00/0

subunit of luciferase and a slower turnover rate than the wild type, whereas the FB-1 (flavin binding) mutant possesses a lesion in the β subunit and has a lower affinity than wild type luciferase for $FMNH_2$.[3]

In the experiments reported here wild type activity was obtained by incubating these two mutant luciferases under non-denaturing conditions. In addition, we have been able to determine their specific activities by using antibody made against the wild type luciferase.

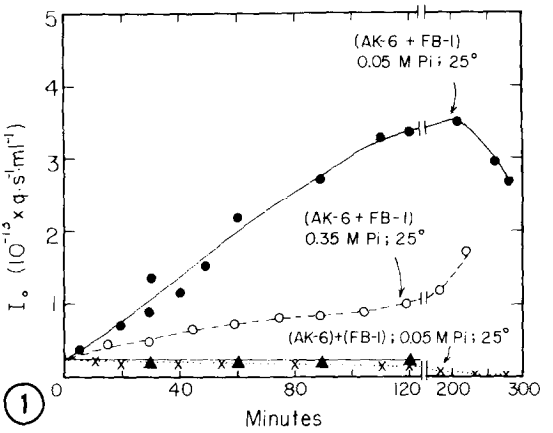
MATERIALS AND METHODS: Beneckea harveyi, strain 392 (MAV) (11) was used as the wild type. Strains AK-6 and FB-1 were derived by mutagenesis and shown to possess defects in α and β subunits, respectively (8,9). Cells were grown in a 250 l New Brunswick Fermacell fermentor, harvested and stored at -20°C prior to use. Luciferase purified as previously described (7,12) had a specific activity of 1.8×10^{14} q/sec/mg. AK-6 and FB-1 luciferases (each about 8 mg/ml) were stored in 0.35 M phosphate, pH 7, 1 mM DTT. Protein was determined (13) using bovine serum albumin as a standard.

Activity was assayed by injecting 1 ml $FMNH_2$ (5×10^{-5}M) into a vial with luciferase, 0.001% decanal (v/v), 0.2%[2] bovine serum albumin, 0.02 M phosphate buffer, pH 7 in 1 ml (14). This is a "nonturnover" assay: any $FMNH_2$ not reacted with luciferase at the outset is promptly autoxidized (15,16). The kinetics of the decay of luminescence therefore provide a measure of the turnover time and altered kinetics can be determined directly by the decay rate. The initial maximum velocity (light intensity expressed in quanta/sec) was used to determine the specific activity of the luciferase.

Antisera were raised in rabbits using purified wild-type luciferase as the antigen (17,18). Measurements of the specific activities of luciferases were carried out by mixing different amounts of luciferase (FB-1, AK-6, wild type, crude or pure) with a constant amount of antiserum and incubating for 4 hours at 23°C. The remaining luciferase activity was measured and plotted against the amount of activity added. Normal rabbit serum was used in controls. DTT at a final concentration of 1 mM had no inhibitory effect upon the antigen-antibody reaction.

RESULTS AND DISCUSSION: The activity of a mixture of AK-6 and FB-1 luciferases increased promptly and substantially upon incubation in 0.05 or 0.35 M phosphate buffer (pH 7.0) at 25°C (Figure 1). At the higher phosphate concentration the increase in activity was considerably slower but the total activity ultimately obtained was about the same. High phosphate concentration has been previously shown to stabilize the wild type luciferase against proteolysis (19) and denaturation by heat (unpublished); it may

[3]Abbreviations: $FMNH_2$, reduced flavin mononucleotide; DTT, dithiothreitol.



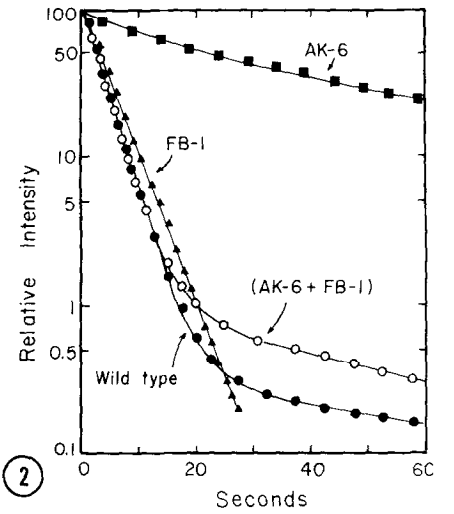


Figure 1. Increases in activity upon mixing low activity mutant luciferases. Equal volumes (0.5 ml) of AK-6 and FB-1 luciferases were combined, incubated in either 0.05 M (solid circles) or 0.35 M (open circles) phosphate buffer, pH 7.0 with 1 mM DTT, at 25°, and assayed at later times for luminescence activity. Samples of AK-6 (X) and FB-1 (triangles) in 0.05 M phosphate buffer were mixed individually with equal volumes of buffer and incubated separately at 25°. The sum of the activities of these two control samples is also plotted (triangles) as a function of time. Each data point is an average of three measurements.

Figure 2. Kinetics of in vitro reactions at 23°C for luciferases purified from AK-6 (squares), FB-1 (triangles), and wild type (solid circles) B. harveyi, as well as the activity formed as in Figure 1 in a mixture of FB-1 and AK-6 luciferases (open circles). The initial maximal intensities were normalized for plotting.

impede subunit exchange by stabilizing the dimeric form of mutant luciferases. At 0°C the increase was much slower, but the total activity obtained was comparable to that obtained at 25°C (data not shown). A similar increase also occurred in crude preparations, but the time required to obtain maximum activity at 25°C in 0.05 M was somewhat longer (about 8 hours).

The kinetics of the in vitro luminescence reaction for each of the purified luciferases are shown in Figure 2. At 23°C the half times for decay of luminescence were about 35, 3, and 2 seconds for AK-6, FB-1, and wild-type luciferases, respectively.[4] Purified AK-6 and FB-1 luciferases

[4]In some cases biphasic decay was observed; no suitable interpretation for the second slower phase is available at the present time, but it constituted only about 1% of the reaction.

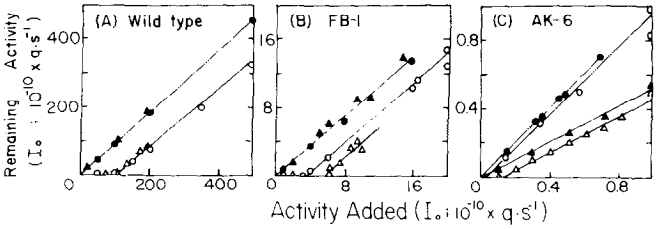


Figure 3. Titrations of antiluciferase serum (open symbols) with purified (circles) and crude (triangles) luciferases. Different amounts of purified and crude luciferases from B. harveyi (A, wild type; B, FB-1; and C, AK-6) were admixed with 50 ul of antiserum in a final volume of 0.2 ml with 0.2 M phosphate, pH 7, 0.2% bovine serum albumin, 1 mM DTT. After 4 hours of incubation at room temperature, the activity remaining in each sample was determined and plotted against the activity added. For controls (solid symbols), various amounts of purified (circles) and crude extract (triangles) of each of the luciferases were added to the same buffer containing no antiserum, incubated and assayed as described above. Controls using normal rabbit antiserum gave similar results. Ordinate, remaining luciferase activity; one unit equals 10^{-10} q/sec; abscissa activity added same units.

were incubated together in 0.35 M phosphate buffer for 4 hours at 25°C followed by 36 hours at 0°C. The activity increased as in Figure 1 and exhibited decay kinetics (Figure 2) and a molecular weight by gel filtration corresponding to the wild-type luciferase. The phenomenon is attributed to subunit exchange between mutant luciferases and the consequent formation of wild type luciferase. It does not seem surprising that this would occur. Both of the mutant enzymes have been shown to be less stable than the wild type (8), and a subunit equilibrium would be strongly pulled towards the formation of wild type enzyme if, as expected, the association constant for its formation is much greater than for the mutant enzymes.

In order to determine and compare the specific activities of the wild-type and mutant luciferases, different amounts of luciferase were added to a constant amount of antiluciferase antiserum (Figure 3). With small amounts, the added luciferase was completely inactivated by the antiserum. With greater amounts the excess luciferase was active; by extrapolation one can calculate the amount of luciferase (in activity units) which was complexed and inactivated by the antibody. Assuming that the antigen-antibody reaction is the same irrespective of the exact type of luciferase (wild-type, AK-6 or

FB-1) and its state of purity, then any differences in the total luciferase activity inactivated by a constant level of antibody can be attributed to differences in the specific activities of these luciferases.

Figure 3A shows that 10^{12} q sec^{-1} activity units of wild type luciferase, both with crude and purified enzyme, were neutralized by 50 µl of antiserum. With crude and purified FB-1 mutant luciferases 4×10^{10} and 5.4×10^{10} activity units respectively were inactivated by 50 µl of antiserum (Figure 3B). Thus in crude and purified preparations, the FB-1 specific activities were 9.7×10^{12} and 7.2×10^{12} q $sec^{-1}mg^{-1}$, respectively, about 20 fold less than for the wild type luciferase. The difference between crude and purified preparations might have been due to a loss of activity but retention of the integrity of the luciferase polypeptide during purification, assuming that the method estimated enzymatically inactive but immunologically reactive luciferase. In fact, some such material may have already been present in crude extracts, thereby contributing in part to the low specific activity of the mutant luciferases.

AK-6 mutant luciferases had even lower specific activities: in crude and purified preparations 1×10^{9} and 2×10^{8} q sec^{-1} activity units, respectively, were inactivated, representing specific activities of 1.8×10^{11} and 3.6×10^{10} q $sec^{-1}mg^{-1}$. It is interesting to note that although purified AK-6 luciferase exhibited a lower specific activity than the activity in crude extracts, the purified enzyme was more stable during a 4 hour incubation at room temperature than the latter sample (Figure 3C).[5] With the wild-type luciferase there was no difference in specific activity

[5] A slope of 1 means that the amount of activity measured is equal to the amount of activity added (as in the controls). The fact that the slope is less than 1 in the AK-6 crude luciferase experiments, both with and without antibody, is due to loss of activity during the 4 hour incubation at 25°C. The reason for this is not known; it may have been due to the presence of an endogenous protease which specifically attacks the α subunit. Since AK-6 is an α subunit mutant, it may be more susceptible to proteolysis than wild-type or β mutant luciferases.

between the crude and purified preparations. Crude extracts of wild-type cells did not contain immunologically cross-reacting but inactive luciferase, unless it failed to separate from luciferase during purification.

Cline (8) had demonstrated that subunit exchange could be induced by thermal inactivation of a pair of temperature sensitive luciferases (TSL) possessing defects in different subunits. The present experiments show clearly that with mutant luciferases an exchange of subunits may occur under non-denaturing conditions to produce wild-type luciferase. The use of mutant luciferases for the study of subunit exchange offers the possibility for determining quantitative parameters of subunit equilibria and how these are altered in various mutant luciferases. This would be an especially interesting investigation with temperature sensitive luciferases (10), where one could study the possible relationship between the temperature of thermal inactivation and the equilibria for subunit dissociation.

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