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# FIREFLY LUCIFERASE REACTS WITH $P^1,P^5$-DI(ADENOSINE-5'-)PENTAPHOSPHATE AND ADENOSINE-5'-TETRAPHOSPHATE

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SUMMARY: Purified luciferase from firefly tails produces light not only with ATP but also with adenosine-5'-tetraphosphate and $P^1,P^5$-di(adenosine-5'-)pentaphosphate. The latter compound is a potent and specific inhibitor of adenylate kinase. $P^1,P^5$-Di(adenosine-5'-)pentaphosphate produces light with an intensity of about 0.75 per cent relative to ATP and adenosine-5'-tetraphosphate produces light with an intensity of about 2.2 per cent relative to ATP, even if efforts were made to remove contaminating ATP.

The use of firefly luciferase to measure ATP (1, 2, 3) and compounds that can be converted to ATP (1, 3, 4, 5) has grown much in popularity because of the great sensitivity and specificity of luciferase to ATP (6). One of the main problems in this connection is to obtain a sufficiently pure preparation of luciferase because often small amounts of ATP have to be measured in the presence of large amounts of ATP (5), and even purified luciferase (5, 7, 8) may contain some adenylate kinase. If ADP and adenylate kinase present a problem then it is obvious to think of $Ap_5A$ as an inhibitor of adenylate kinase (9) that contaminates the luciferase preparation. However, the results presented here show that $Ap_5A$ and $Ap_4$ produces light with the same luciferase, luciferin, $Mg^{++}$ mixture used for ATP measurement

Abbreviations: $Ap_5A$, $P^1,P^5$-di(adenosine-5'-)pentaphosphate; $Ap_4$, adenosine-5'-tetraphosphate.

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(1), also when contaminating ATP was removed from the crude materials of $Ap_5A$ and $Ap_4$.

## MATERIALS

Chemicals of analytical purity were used throughout. Hexokinase, glucose-6-phosphate dehydrogenase, adenylate kinase, pyruvate kinase, phosphoenolpyruvate, NADP, ATP, ADP, and $P^1,P^5$-di(adenosine-5'-)pentaphosphate (90 per cent pure on basis of organic phosphate) were purchased from Boehringer, Mannheim. D-Glucose and EDTA were from Merck. Adenosine-5'-tetraphosphate (grade III, approx. 95 per cent), desiccated firefly tails, once recrystallized luciferase, D-luciferin, and DEAE-cellulose were from Sigma.

## METHODS

ATP, $Ap_4$ and $Ap_5A$ were measured by the absorption of light at 259 nm, and with luciferase by the method of Rasmussen and Nielsen (1).

4 mg of $Ap_5A$ was applied to a DEAE-cellulose column 1×30 cm and eluted with a linear gradient from 500 ml of 5 mM Tris, pH 7.5 (adjusted with HCl) to 500 ml of 300 mM KCl. 7 ml samples were collected at a speed of 35 ml per hour at 4°C.

In order to determine the ATP content and to remove ATP from $Ap_5A$ (or $Ap_4$), 500 µl of 1.3 mM $Ap_5A$ (or 2.5 mM $Ap_4$) were incubated in a semi micro cuvette with 10 µl 100 mM D-glucose, 10 µl 10 mM NADP, 10 µl of 1 IU/ml hexokinase and 1 IU/ml glucose-6-phosphate dehydrogenase and 10 µl 50 mM $MgCl_2$. Before addition of glucose, two 25 µl samples were taken out to measure the light response from luciferase before reaction. The reaction was followed by recording the absorbance of light at 365 nm. When the reaction stopped two 25 µl samples were taken out again to measure the light response from luciferase.

AMP and ADP were identified by incubating 100 µl of the solution to be tested with 100 µl of 10 IU/ml adenylate kinase (omitted if only ADP was tested for), 2 IU/ml of pyruvate kinase, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phosphoenolpyruvate for 30 min at room temperature. The production of ATP was measured with luciferase.

Identification of $Ap_5A$ was achieved by measuring the adenylate kinase activity in a crude extract of luciferase from firefly tails (1, 7). 25 µl of 0.1 mM ADP was mixed with 500 µl of crude extract together with varying amounts of test solution. The inhibition of ATP production was measured.

## RESULTS

The ultraviolet absorption spectrum of $Ap_5A$ in the 220 to 450 nm region showed no difference in shape from that of ATP (10).

The molar absorption coefficient at 259 nm was 21,000/M/cm for the crude material of $Ap_5A$ at pH 7.2.

Enzymatic removal of contaminating ATP from the crude material of $Ap_5A$ was achieved by treatment with hexokinase/glucose-6-phosphate dehydrogenase as described in methods. The light response from luciferase was measured relative to an ATP standard before and after reaction with hexokinase/glucose-6-phosphate dehydrogenase. To be shure that all ATP was removed, the production of NADPH was followed by recording the absorption of light at 365 nm and when no more NADPH was produced for 10 min the reaction had stopped and all ATP had been converted to ADP. When a solution of pure ATP was processed in this way the ATP concentration decreased to less than 1 nM measured with luciferase. The light response of $Ap_5A$ after enzymatic treatment to remove ATP was 0.75 per cent relative to ATP. The content of ATP in the crude material of $Ap_5A$ was about 1 per cent (w/w) determined both by the amount of NADPH produced and by the difference in light response from luciferase before and after treatment with hexokinase/glucose-6-phosphate dehydrogenase.

DEAE-Cellulose anion exchange chromatography of 4 mg $Ap_5A$ (Fig. 1) showed several impurities in the crude material. From the areas under the curve showing absorbance at 259 nm, it can be calculated that the crude product contained about 70 per cent (mol/mol) $Ap_5A$, and that the molar absorption coefficient of $Ap_5A$ at 259 nm, pH 7.5, is about 22,000/M/cm. For comparison, the molar absorption coefficient of ATP, ADP, and AMP at 259 nm under the same conditions is 15,400/M/cm (10). Fig. 1 also shows the light response from luciferase compared to an ATP standard. Qualitatively the two curves follow each other, indicating that all compounds in this region of fraction numbers produced light

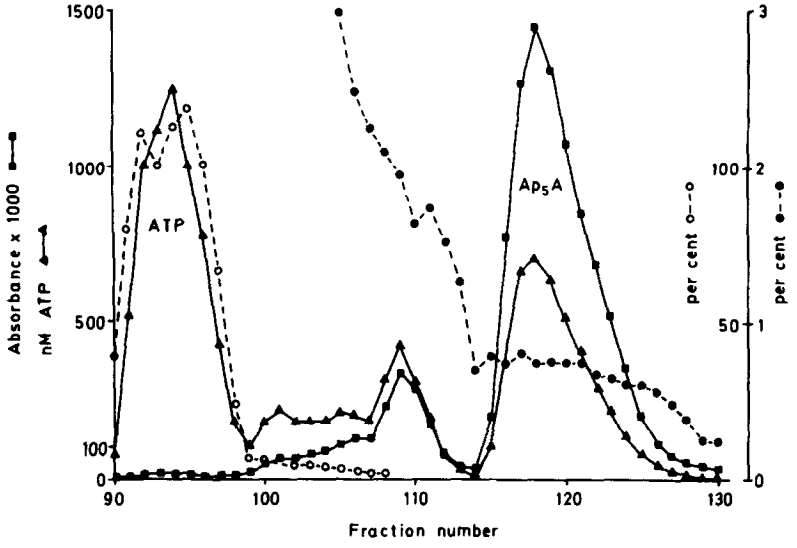


Figure 1. DEAE-Cellulose column chromatography of 4 mg $Ap_5A$. Column dimensions: 1 cm in diameter and 30 cm high. The column was eluted with a linear gradient from 500 ml of 5 mM Tris, pH 7.5 to 500 ml of 300 mM KCl. 7 ml samples were collected at a speed of 35 ml per hour at 4°C. The absorbance×1000 at 259 nm, 1 cm light path is shown (■—■). The light response from luciferase relative to an ATP standard is expressed as equivalent nM ATP (▲—▲) and does not necessarily represent true ATP. The dotted line (o--o) represents the relative light response defined as the light response (▲—▲) relative to the absorbance (■—■) divided by the molar absorption coefficient of ATP (see text). The dotted line (•--•) represents an extension of this curve in an expanded scale (see right hand ordinate).

with luciferase. The relative light response (dotted line in Fig. 1) as defined in the legend to Fig. 1 was about 100 per cent in the region of fraction numbers 91 to 97 (about 195 mM KCl) indicating that the compound was ATP. Before that peak two very small peaks appeared that could be identified as AMP and ADP (see methods). The other large peak in the region of fraction numbers 116 to 122 (about 250 mM KCl) could be identified as $Ap_5A$ by its action on adenylate kinase as described in methods. The smaller peaks from 99 to 114 were not identified but as stated later $Ap_4$ also produces light with luciferase therefore the compounds in this region may have been a mixture of different

adenosine polyphosphates. The relative light response in the region 116 to 122 was about 0.75 per cent and constant over the whole region. The compound in that region was identified as $Ap_5A$ and the light response is the same as measured when ATP was removed from the crude material of $Ap_5A$ by the enzymatic way (see above). The light response from the crude material should be only about 5 per cent because the content of $Ap_5A$ in the material was about 70 per cent. However, the impurities also seem to produce light with luciferase as shown by the column chromatography. In the region above fraction number 122 a few very small peaks appeared but they were not identified.

Adenosine-5'-tetraphosphate showed a light response both before and after treatment with hexokinase/glucose-6-phosphate dehydrogenase as described above for $Ap_5A$ and in the section on methods. The content of ATP in the crude material of $Ap_4$ was about 3 per cent. After removal of ATP with hexokinase/glucose-6-phosphate dehydrogenase the remaining light response was about 2.2 per cent relative to ATP (at the same concentration). The criteria for removal of ATP was the same as described under enzymatic removal of contaminating ATP from $Ap_5A$.

Phosphatase contaminating the luciferase preparation might have produced ATP under the conditions used for light emission measurements. However, by the method of Rasmussen and Nielsen (1) the light intensity is followed on a recorder after a rapid mixing of the sample and the luciferase-luciferin preparation (approx. 0.2 s). The light response from pure ATP reaches a maximum after a short delay (aprox. 0.3 s from the start of the mixing) whereupon the light intensity slowly decreases because of product inhibition of the luciferase. If the light response from $Ap_5A$ was caused by some phosphatase producing ATP, the response would have

been a slowly increasing light intensity with time (cf. ref. 1). This was actually observed with crude firefly extract. However, with purified luciferase the response from $Ap_5A$ was exactly like that of ATP in shape and 0.75 per cent of the intensity of ATP at the same concentration. This ratio, 0.75 per cent, was independent of the concentration of both $Ap_5A$ and luciferase, and the ratio was independent of the degree of purification of luciferase. In an experiment where a purified luciferase preparation was heat denaturated at 40°C, the light response from $Ap_5A$ was 0.75 per cent of ATP independent of the degree of denaturation. All these results can only be explained if the $Ap_5A$ reacts directly with luciferase under light emmision. The same holds for $Ap_4$.

## DISCUSSION

The results show that both $Ap_5A$ and $Ap_4$ give light response even with highly purified luciferase and if contaminating ATP is removed from the compounds. $Ap_5A$ produces light with an intensity of 0.75 per cent, and $Ap_4$ with an intensity of 2.2 per cent both relative to ATP at the same concentrations. $Ap_5A$ is therefore not a suitable agent for inhibition of the adenylate kinase in the luciferase preparations unless the ATP concentrations to be measured are high. The luciferase method may be used to measure the $Ap_4$ concentration in extracts from e. g. tissues after removal of ATP with the hexokinase/glucose-6-phosphate dehydrogenase method.

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