Studies on the Bioluminescence of Renilla reniformis*

II. REQUIREMENT FOR 3',5'-DIPHOSPHOADENOSINE IN THE LUMINESCENT REACTION

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In earlier investigations on the bioluminescent system of the sea pansy, Renilla reniformis, we reported that adenine-containing nucleotides (either adenosine mono-, di-, or triphosphate), an oxidizable substrate (Renilla luciferin), molecular oxygen, and an enzyme (Renilla luciferase) were required for luminescence in crude extracts of this organism (1, 2). In addition, several lines of evidence indicate that adenosine triphosphate functions indirectly, i.e., by first being converted to the functional nucleotide (2). These are: (a) kinetics of the reaction with ATP is much slower than that with AMP or ADP, (b) relatively crude luciferase preparations respond to AMP, ADP, and ATP, whereas more purified fractions will emit light in the presence of AMP or ADP but not ATP. Crude preparations contain a phosphatase that converts ATP to ADP and AMP and, during purification of the enzyme, the phosphatase activity is removed; and (c) ATP activity in purified extracts can be restored by the addition of commercial alkaline phosphatase plus magnesium. These data were interpreted to mean that ATP was being converted to AMP and ADP and that one of the latter two nucleotides was responsible for driving the light reaction. This seemed to be a reasonable conclusion in view of the fact that rechromatography of ADP on formate-charged Dowex 1 yielded ADP fractions that were all active, and that the nucleoside mono-, di-, and triphosphates of guanosine, cytidine, uridine, and inosine were found to be inactive (2).

The data presented in this paper are intended to show, however, that the activity exhibited by AMP and ADP is due to contamination by trace amounts of an adenine-containing nucleotide that has been isolated from crystalline AMP preparations and found to be identical with 3',5'-diphosphoadenosine. On the other hand, it will be shown that the ATP preparations that we are using are not contaminated by 3',5'-diphosphoadenosine and thus the activity of ATP in crude extracts can be explained in terms of synthesis of DPA from ATP.

It will be shown that the luminescent reaction represents the most sensitive assay known for DPA, since concentrations of $5 \times 10^{-8} \text{M}$ are easily detected. A discussion of the possible relationship between the involvement of DPA in the Renilla bioluminescent system and its role in the transfer of active sulfate between phenolic compounds, as shown by Gregory and Lipmann (3), is presented.

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The abbreviation used is: DPA, 3',5'-diphosphoadenosine.
were combined and evaporated on a Rineo evaporator at 30°. The dried residue was taken up in a minimal volume of absolute ethanol to which had been added 1 ml Tris, pH 7.5, to make a final concentration of 0.001 M Tris. The suspension was kept at -20° overnight and centrifuged at 12,000 × g for 10 minutes at 0° to remove the suspended material. The clear, orange-colored alcohol solution contained at least 90% of the detectable luciferin from the ammonium sulfate precipitates, and since Renilla luciferin is quite stable at alkaline pH there is good reason to believe that this method gives good recovery. Alcoholic solutions of luciferin are stable for at least 3 months at -20°. Luciferin from 500 sea pansies is routinely concentrated in 4 ml of ethanol in the above manner and diluted in 0.005 M phosphate buffer, pH 7.5, for testing.

Assay Conditions—The complete system consists of potassium phosphate buffer, pH 7.5 (50 μmoles); mercaptoethanol (2 μmoles); CaCl₂ (5 μmoles); luciferin (0.1 ml); DPA (0.003 μmoles); enzyme (0.1 ml); and water to 1.6 ml. The assays were performed at 30°, and the reaction was initiated by the addition of enzyme unless otherwise stated.

Light Measurements—Light emission was measured by the system previously described (1) except that a Texas Instruments Company recorder, model RRIM, was used. This recorder was adjusted to give a response time of 0.25 second full scale which is sufficient to measure the kinetics illustrated in Fig. 5.

RESULTS

Detection and Isolation of Active Cofactor—When 2'-AMP, 3'-AMP, and 3',5'-cyclic-AMP were tested in the Renilla luminescent system for activity, it was found that 2'-AMP and 3'-AMP were both active and that 3',5'-cyclic-AMP was inactive. On an equimolar basis, the order of decreasing activity for the adenosine monophosphates was found to be as follows: AMP > 3'-AMP > 2'-AMP. To test the possibility that 3'-AMP and 2'-AMP were contaminated with AMP, all three compounds at equimolar concentrations were incubated for varying lengths of time with 5'-adenylate deaminase and aliquots were removed, heated at 100° for 3 minutes to destroy deaminase activity, centrifuged to remove denatured protein, and tested for nucleotide activity in the luminescent system. At the end of a 20-minute incubation with deaminase, spectral analysis showed a quantitative conversion of AMP to IMP, whereas the 2' and 3' compounds were unaffected. However, the activities of 2'-AMP, 3'AMP, and AMP in the Renilla luminescent system after 20 minutes incubation with 5'-adenylate deaminase remained unchanged. Since IMP is inactive in this system, the unchanged activity of the AMP incubation mixture was surprising.

One of the possible explanations was that we were actually dealing with a trace contaminant in our AMP and ADP preparations. Since earlier attempts to show this by rechromatography of AMP on formate-charged Dowex 1 had failed, we turned to paper chromatography.

By use of a solvent system consisting of ethanol-1.0 M ammonium acetate (7:3), pH 7.5, it was possible to separate the active material from AMP or ADP on paper. When 50 mg of AMP were placed along a 3-inch wide area of Whatman No. 3MM paper and the chromatogram allowed to develop by descending chromatography for 3 days, ultraviolet-quenching spots appeared on the dried chromatogram as illustrated in Fig. 1.

FIG. 1. Paper chromatogram patterns from commercial nucleotide preparations. The numbers in the outlined areas indicate ultraviolet-quenching regions. See text for details.

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Relative luminescence rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13</td>
</tr>
<tr>
<td>3'-AMP</td>
<td>13</td>
</tr>
<tr>
<td>2'-AMP</td>
<td>13</td>
</tr>
<tr>
<td>AMP</td>
<td>13</td>
</tr>
<tr>
<td>ADP</td>
<td>13</td>
</tr>
<tr>
<td>ATP</td>
<td>13</td>
</tr>
<tr>
<td>Unknown</td>
<td>477</td>
</tr>
</tbody>
</table>

A very intense quenching area appeared farthest from the origin (Spot 3) and, as expected, was AMP on the basis of the enzymatic assay for AMP which involved the use of creatine transphosphorylase coupled to the firefly luminescent system (4, 5). The eluted AMP was found to retain only approximately 1% of its original activity in the Renilla luminescent system, and, after rechromatography and elution twice more, had lost all of its activity as shown in Table I. Spot 3 from AMP was barely detectable, and the compound is not identical with ADP or ATP as seen from Fig. 1. It is not active in the firefly luminescent system, but is approximately 1000 times more active on an equimolar basis than commercial preparations of AMP in stimulating luminescence in partially purified extracts of Renilla reniformis. Spot 1 from AMP represents an unidentified ultraviolet-quenching material.

As seen from Fig. 1, ADP also shows three spots when chromatographed under these conditions. Spots 3 and 2 were AMP and
**Analytical data on isolated cofactor**

Table II

<table>
<thead>
<tr>
<th>Component</th>
<th>Relative amount per mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>1.00</td>
</tr>
<tr>
<td>Ribose</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>Total phosphate</td>
<td>2.01 ± 0.04</td>
</tr>
</tbody>
</table>

ADP, respectively, the AMP being determined as described above and the ADP by coupling adenylate kinase to the firefly luminescent system (4, 5). As shown in Table I, repeated chromatography of ADP results in a complete loss of activity. Spot 1 from ADP contained the active material. The results in Table I show that the activities exhibited by 3′- and 2′-AMP must also have been due to a contaminating nucleotide. In addition, ATP shows no activity above background levels, as seen in Table I. This is true regardless of whether the ATP has been rechromatographed.

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Identification of Isolated Cofactor—The isolated material contained adenosine on the basis of the ultraviolet absorption spectra. As shown in Fig. 2, the absorption peak at pH 2.0 is 257 μm, whereas at pH 12.0 it is shifted to 260 μm in accord with absorption maxima for adenosine in acid and alkali. Furthermore the 250:260 μm and 260:260 μm ratios approximate those reported for the adenine-containing mononucleotides (6).

Subsequent to the finding that the unknown contained adenine, the presence of ribose and phosphate were also detected by the orcinol procedure for pentose (7, 8) and by the method of Fiske and SubbaRow for organic phosphate (8, 9), respectively. The results of analytical determinations for adenosine, ribose, and phosphate in the unknown are presented in Table II. As shown in Table II, the relative content of adenosine, ribose, and phosphate in the unknown is 1:1:2, respectively. These data suggest that the isolated cofactor is an adenosine diphosphate. Since by chromatographic analysis (see Fig. 1) the unknown is not identical with ADP, it seemed likely that it could be either DPA or 2′,5′-diphosphoadenosine.

Identification of the unknown as DPA is based upon its cochromatographic behavior with authentic DPA² and a comparison of the activity of the unknown in the luminescence assay with known adenine-containing nucleotides. An illustration of a typical descending paper chromatogram of the unknown and a variety of known nucleotides is shown in Fig. 3. From Fig. 3, it can be seen that the unknown is easily distinguished from AMP, ADP, and ATP, and that it moves identically with DPA, and very close to 2′,5′-diphosphoadenosine.

Proof of identity of the unknown cofactor as DPA rests finally

²The author wishes to express his appreciation to Dr. Harry Peck of the Oak Ridge National Laboratory for generous gifts of 2′,5′-diphosphoadenosine, DPA, adenosine 5′-phosphosulfate, and 3′-phosphoadenosine 5′-phosphosulfate.
upon its equal activity with authentic DPA in the luminescent and sulfoxokinase assay systems. As seen from Fig. 4, the activity of DPA in the bioluminescence assay is identical with that of the isolated cofactor. The apparent Michaelis constant for DPA as calculated from Lineweaver-Burk plots of the data obtained in the bioluminescence assay (10) is $7.3 \times 10^{-9}$ M. This assay represents the most sensitive one known for this nucleotide in view of the relatively small apparent Michaelis constant, since concentrations of $5 \times 10^{-9}$ M of the compound are easily detected. This order of sensitivity approximates that for the well-known firefly assay for ATP (4, 5).

The isolated cofactor and DPA are also equally active in the transfer of active sulfate between phenolic compounds catalyzed by a sulfoxokinase system as reported by Gregory and Lipmann (3). $2',5'$-Diphosphoadenosine is approximately 1% as active as DPA in the luminescence assay, possibly because of contamination with the latter compound, which could come about by migration of the phosphate from the $2'$ to the $3'$ position (11). Adenosine $5'$-phosphosulfate is inactive whereas $3'$-phosphoadenosine $5'$-phosphosulfate shows 15 to 20% of the activity of DPA due primarily to contamination with DPA. As a matter of fact, a number of commercial preparations of nucleotides show varying degrees of contamination with DPA. For example, preparations of coenzyme A are approximately 7% as active as DPA, whereas TPN, AMP, and ADP are 0.1%, $3'$-AMP is 0.05%, and $2'$-AMP is 0.02% as active as DPA. No activity could be observed with DPN, $3',5'$-cyclic-AMP, or deoxy-AMP. We were able to obtain good activity with AMP and ADP in the first place because these nucleotides do not inhibit the luminescent reactions. For example, at saturating levels of DPA, the addition of 2000 times this level of AMP or ADP does not affect the rate of the reactions.

DPA does not appear to be converted to $3',5'$-diphosphoinosine before its action in the luminescent reaction, since prolonged incubation of the former compound, under conditions that support luminescence, with the crude enzyme does not change its spectral characteristics. In addition, synthetic $3',5'$-diphosphoinosine (3) is inactive in this system and does not inhibit DPA activity in equimolar concentrations. The luminescent reaction, therefore, is rather specific for DPA since $3',5'$-diphosphoinosine shows only 1% the activity of DPA, possibly because of contamination with the latter compound.

From the above data, it may be concluded that the functional nucleotide in luminescence from extracts of R. reniformis is DPA.

Function of DPA in Luminescent Reaction—It has been previously reported (2) that a heat-stable intermediate accumulates upon incubation of the complete system under anaerobic conditions. For example, if the complete system is incubated under the conditions described in Table III, followed by heating at 100° for 1 minute to destroy the enzyme, an intermediate can be detected by kinetic measurements as shown in Fig. 5. Fig. 5A illustrates the kinetics observed when the reaction is initiated with luciferin, whereas Fig. 5B illustrates that observed when luciferin is added to the system after first being preincubated under anaerobic conditions in the presence of DPA, Ca++, mercaptoethanol, and luciferase. With luciferin, the time required to reach one-half the maximal intensity is approximately 2 minutes, whereas with anaerobically preincubated luciferin this time is approximately 0.2 second. Although the amount of preincubated luciferin injected in Fig. 5B was prepared from the amount of luciferin added in Fig. 5A, it can be seen that the preincubated luciferin not only shows much faster kinetics but also gives approximately 4 times the maximal light intensity. Thus an intermediate is formed during anaerobic incubation of the complete system and possibly represents an activated form of luciferin.

The requirements for intermediate formation are given in Table III. The maximal light intensity was used as a measure of the relative amounts of intermediate formed since, at the concentrations used, there is a linear relationship between light intensity and intermediate added. It should be mentioned that no light was observed unless enzyme was present in the assay tube. From Table III it can be seen that formation of the intermediate requires the presence of DPA, luciferin, and enzyme. It has been difficult to remove the last traces of DPA from the enzyme preparations; this accounts for the relatively small light signal observed when the incubation was carried out in the absence of luciferase.
DPA. Thus a heat stable intermediate is formed by an enzyme-catalyzed reaction involving DPA and luciferin. Since crystalline ATP preparations do not support luminescence under the conditions used in these experiments, in the presence or absence of a variety of metal ions which include magnesium and manganese, it follows that ATP activity in crude extracts might be explained in terms of DPA synthesis from ATP. This viewpoint is supported by our earlier observation that ATP content of these compounds. It should be mentioned that whereas the ATP used in these experiments is inactive in the Renilla luminescent system, it gave the expected response when assayed in the firefly luminescent system (4, 5).

Effects of Metals—A calcium salt is included in the assay system since it has been found that Sephadex-treated enzymes show a partial dependency upon metal ions for maximal luminescence. For example, many divalent cations such as Ca++, Sr++, Ba++, Fe++, Pb++, Mg++, and Mn++ will stimulate the luminescent reaction to varying degrees. Calcium ion, the best activator of all the cations tested, gives a 5- to 7-fold stimulation of the luminescent rate. With the exception of Mg++, the addition of the above cations to the assay system results in the formation of a noticeable precipitate because of the insolubility of their phosphate salts. This slight precipitation does not, however, affect the light measurements, since removal of the precipitate by centrifugation does not change the luminescence rate. In addition, the same metal responses are obtained when arsenate buffer is used instead of phosphate, in which case no precipitation occurs upon the addition of any of the above cations. A detailed account of the effects of metal ions on the luminescent system will be reported elsewhere.

DISCUSSION

These results are interesting in view of the relatively recent progress which has been made in the field of sulfate activation. The activation of sulfate, as it occurs in yeast and mammalian tissue, has been reviewed by Lipmann (12). It has been shown by Gregory and Lipmann (3) that "active sulfate" (3'-phosphoadenosine 5'-phosphosulfate) will transfer its active sulfate group to phenols, in the presence of specific sulfokinases, to form sulfonated phenols and DPA as products. In addition, they showed that this reaction is reversible when sulfate is transferred from p-nitrophenyl sulfate to phenol. In this reaction, DPA acts as a coenzyme.

It is evident from our observations reported here that DPA also acts as a coenzyme in the bioluminescent reaction of R. reniformis. Whether the activation of Renilla luciferin by ATP involves a mechanism analogous to the reverse of a sulfokinase reaction, or an entirely different one, is an interesting question, but the answer to this will have to await future investigations. One apparent difference in the two reactions involves the requirement for metals. Evidence presented by Gregory and Lipmann (3), indicated no metal requirement for the sulfokinase reaction, whereas divalent metal ions activate the Renilla luminescent system. Whether this difference is apparent or real is not known, but if it is real one might expect the mechanisms of the two reactions to be different.

SUMMARY

We have isolated a nucleotide from commercial preparations of adenosine monophosphate that has been identified as 3',5'-diphosphoadenosine (DPA) and which has been shown to function in the bioluminescent reaction of the sea pansy, Renilla reniformis. Adenosine triphosphate (ATP) is not active under the conditions of these experiments. Thus the activity of ATP in crude extracts reported earlier (1, 2) can be explained in terms of synthesis of DPA from ATP. The system is quite specific for DPA since 3',5'-diphosphoisocitryne is inactive and since 2',5'-diphosphoadenosine is only 1% as active as DPA, possibly because of contamination by the latter compound. The luminescent reaction provides the most sensitive and rapid assay known for DPA since concentrations of $5 \times 10^{-9}$ M are easily detected. The apparent Michaelis constant for DPA, as calculated from Lineweaver-Burk plots of the data, is $7.3 \times 10^{-8}$ M. Studies on the function of DPA in this reaction show that DPA reacts with luciferin in the presence of luciferase to produce an intermediate which results in light emission upon the admission of molecular oxygen.

After passage through Sephadex, luciferase shows a partial dependency upon metal ions for maximal luminescence. For example, many divalent cations such as Ca++, Sr++, Ba++, Fe++, Pb++, Mg++, and Mn++ will stimulate the luminescent reaction to varying degrees. Calcium ion is the best activator tested: it gives a 5- to 7-fold stimulation of the luminescence rate.

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