
Correction. In the article “Photo-Affinity Labels for Adenosine 3’:5’-Cyclic Monophosphate”, by D. J. Brunswick and Barry S. Cooperman, which appeared in the June 1971 issue of Proc. Nat. Acad. Sci. USA, 68, 1801–1804, in the structure shown on p. 1801, the fourth (IV) derivative of cAMP had a subscript 2 omitted and should read: IV $R_1 = R_2 = -CO(CH_2)_2CH_3$. 

Correction. In the article entitled “Organelle Mutations and Their Expression in Chlamydomonas reinhardii”, by Stefan J. Suraycki and Nicholas W. Gillham, which appeared in the June 1971 issue of Proc. Nat. Acad. Sci. USA, 68, 1301–1306, the following changes should be made. On p. 1303, Table 2, footnote e, should read: “Streptomycin-dependent”. On p. 1304, left-hand column, beginning 14 lines from bottom, should read: “should exhibit a wild-type phenotype …”, not “should inhibit a wild-type phenotype …”. On p. 1305, Table 4, column head should read: “Location of mutation (Chlp./Mit./Nuc.)”, not “Location of mutation (Chlp./Mit./Nuc.).”
Photo-Affinity Labels for Adenosine 3':5'-Cyclic Monophosphate
(diazomalonyl derivatives/cAMP-binding site of phosphofructokinase)

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ABSTRACT Three derivatives of cyclic AMP that are potentially useful as photo-affinity labels were synthesized. One of these derivatives is, upon photolysis, specifically incorporated into the cyclic AMP-binding site of rabbit muscle phosphofructokinase. The use of these derivatives in isolating and identifying cyclic AMP receptor sites is discussed.

Affinity labels have found widespread application as a method for covalently marking the binding sites of proteins (1–4), and their potential usefulness in the isolation and identification of biochemically important receptor-site macromolecules has been discussed (3, 4). Although Westheimer and his coworkers first introduced the use of photolytically generated carbones to probe the structure of the active site of chymotrypsin in 1962 (5, 6), it is only recently that carbones, and the chemically related nitrenes, have begun to find application in affinity-labeling studies (7–9). Recently, Singer (9) has used the term photo-affinity label to describe an affinity reagent which, although unreactive under ordinary conditions, is converted upon photolysis to a highly reactive intermediate capable of covalent bond formation with residues at the binding site.

Adenosine 3':5'-cyclic monophosphate (cAMP) has been shown to be a widespread intracellular 'second messenger' in hormone action (10), although, in all but a few cases (11–15), little is known about its detailed role. In order to provide a general tool for the isolation and identification of receptor macromolecules for cAMP, we have synthesized three diazomalonyl derivatives of cAMP that are of potential use as photo-affinity labels. We have specifically incorporated one of these derivatives into the cAMP-binding site of rabbit muscle phosphofructokinase (PFK), (ATP:1-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), which we have used as a test protein.

MATERIALS AND METHODS

[3H]cAMP was obtained from New England Nuclear. Other reagents, with the exception of bovine-serum albumin (Pentex), were purchased from Sigma or Calbiochem. Three different preparations of PFK have been used: Calbiochem (70 units/mg at 30°C) and Sigma (55 and 170 units/mg at 37°C). The three preparations exhibited similar properties. In the photolysis experiments, the extent of incorporation of radioactive material into PFK was virtually identical for both lower-activity preparations and was 50% higher for the higher-activity preparation.

Abbreviation: PFK, phosphofructokinase.

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Ethyl 2-diazomalonyl chloride was prepared by the method of Vaughan and Westheimer (16), and was purified by vacuum distillation (17) (bp 40°35°C).

3H[Diaza]malonyl derivatives of cAMP (I–III) were prepared by modifications of the method of Falbriard et al. (18) for the acylation of cAMP. O'2-(Ethyl 2-diazomalonyl) cAMP (I) was prepared by reaction of a 4-fold molar excess of ethyl 2-diazomalonyl chloride with a pyridine solution of the triethylammonium salt of cAMP, for 30 min, at room temperature. A yield of 70% was obtained. N',O'-Di(ethyl 2-diazomalonyl) cAMP (II) was similarly prepared, in 30% yield, by reaction of a 15-fold molar excess of the acid chloride for 18 hr at 4°C. N'- (Ethyl 2-diazomalonyl) cAMP (III) was prepared in 65% yield by alkaline hydrolysis of II in 1 M NaOH for 3 min at 0°C. The diazomalonyl cAMP derivatives were purified by either paper or ion-exchange chromatography, and were characterized by their ultraviolet spectra both before and after photolysis (18, 19). In addition, II was characterized by its 100-MHz proton NMR spectrum, in which the peaks due to adenyl protons were virtually identical to those in N',O'-dibutyl cAMP (IV).

Photolysis experiments were performed as follows. PFK was dissolved in a solution containing 25 mM sodium glycerophosphate, 25 mM glycyglycine, 1 mM dithiothreitol and 0.2 M ammonium sulfate (pH 7.0), and bound ATP was removed by treatment with activated charcoal. Enzyme thus treated had an A260/A280 ratio of 1.7. Final protein concentrations were routinely 0.8-1.6 mg/ml. Preparations were used within 2 hr of charcoal treatment.

After additions, enzyme solutions were photolyzed in quartz tubes, in a Rayonet photochemical reactor, at either 253.7 or 350 nm. Routinely the times of irradiation were...
After photolysis, bovine-serum albumin was contained in a solution and precipitated with 10% trichloroacetic acid. 0.2 M NaOH was added to the precipitate, and the mixture was heated on a boiling water bath for 15 min. After cooling, 1 ml of this solution was added to 9 ml of a toluene–Triton X-100 (v/v) emulsion that contained 10 g of 2,5-diphenyloxazole of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene per liter. After the addition of the alkaline protein solution, 0.3 ml of 1 M acetic acid was added to quench chemiluminescence, and the sample was counted for radioactivity in a liquid scintillation spectrometer (Interteknique).

Effects of cAMP and its derivatives on the activity of PFK were studied by use of a modification of the standard assay procedure of Racker (20). Measurements were made at 30°C and pH 6.7–6.9. The reaction mixture contained 0.1 M imidazole; 0.05 M potassium acetate; 0.67 mM magnesium sulfate; 2 mM sodium phosphate; 1 mM dithiothreitol; 2.1 mM ATP; 0.16 mM fructose 6-phosphate; 0.16 mM NADH; 0.4 mg/ml of bovine-serum albumin; 0.2 unit/ml fructose-1,6-diphosphate aldolase (EC 4.1.2.6); 1.0 unit/ml triosephosphate isomerase (EC 5.3.1.1) and 2.0 unit/ml glycerol-3-phosphate dehydrogenase (EC 1.1.1.8).

RESULTS

Covalent binding to phosphofructokinase

Photolysis experiments utilizing the trichloroacetic acid-precipitation procedure are presented in Table 1. Expts. 1–5 show that incorporation reaches a saturating value as the concentration of compound I is increased. Under the conditions used, these observed values extrapolate, at infinite concentration of compound I, to about 35% of the cAMP-binding sites present (see footnote to Table 1). The apparent $K_p$ is 1.2 ± 0.2 × 10⁻⁷ M. The extent of incorporation of radioactive material into PFK at 253.7 nm was not limited by destruction of the cAMP-binding site on irradiation, since irradiation of PFK for 1 min before the addition of I resulted in no significant loss in its ability to incorporate it on photolysis. Expts. 6 and 7 show that incorporation is specific for the cAMP site, since incorporation is abolished in the presence of cAMP. Expt. 8 shows that the wash procedure (see Methods) is valid, since “previously photolyzed” I (see footnote to Table 1) resulted in virtually no incorporation of radioactive material. Expt. 9 shows that carbene formation is necessary for incorporation, since incubation without photolysis gives no incorporation. Finally, experiments 10 and 11 show that incorporation is irreversible, since addition of excess cAMP after photolysis of a solution of PFK and I has no effect on incorporation. Expts. 12–18 measure the effect of added ligands on incorporation. Mg²⁺ and fructose 6-phosphate are apparently without effect, whereas ATP decreases incorporation, which

**Table 1. Covalent binding of compound I to PFK* on ultraviolet irradiation**

<table>
<thead>
<tr>
<th>No.</th>
<th>Concn of cmpd. I (µM)</th>
<th>Other ligands (µM)</th>
<th>PFK† of irrad. (µM)</th>
<th>Percent incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2</td>
<td>—</td>
<td>253.7</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>7.9</td>
<td>—</td>
<td>253.7</td>
<td>11.6 ± 1.0</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>—</td>
<td>253.7</td>
<td>16.0 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>—</td>
<td>253.7</td>
<td>23.9 ± 2.0</td>
</tr>
<tr>
<td>5</td>
<td>79</td>
<td>—</td>
<td>253.7</td>
<td>30.6 ± 2.6</td>
</tr>
<tr>
<td>6</td>
<td>16 cAMP, 40</td>
<td>9.0</td>
<td>253.7</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>16 cAMP, 400</td>
<td>9.0</td>
<td>253.7</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>16†</td>
<td>9.0</td>
<td>253.7</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>9.0</td>
<td>—</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>2.8</td>
<td>7.5</td>
<td>253.7</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>11</td>
<td>2.8 cAMP, 100‡</td>
<td>7.5</td>
<td>253.7</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>16</td>
<td>253.7</td>
<td>8.5 ± 0.9</td>
</tr>
<tr>
<td>13</td>
<td>15 F6P, 39</td>
<td>15</td>
<td>253.7</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>14</td>
<td>15 F6P, 370</td>
<td>14</td>
<td>253.7</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>15</td>
<td>15 ATP, 39</td>
<td>15</td>
<td>253.7</td>
<td>7.3 ± 0.7</td>
</tr>
<tr>
<td>16</td>
<td>15 ATP, 370</td>
<td>14</td>
<td>253.7</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>17</td>
<td>15 MgSO₄, 740</td>
<td>17</td>
<td>253.7</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>18</td>
<td>15 MgSO₄, 740</td>
<td>17</td>
<td>253.7</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>19</td>
<td>7.7</td>
<td>16</td>
<td>350</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>20</td>
<td>66</td>
<td>14</td>
<td>350</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>21</td>
<td>7.7†</td>
<td>16</td>
<td>350</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

* Expts. 1–9, Sigma PFK (170 units/mg). Expts. 12–18, Sigma PFK (55 units/mg). Expts. 10 and 11, 19–21, Calbiochem PFK (70 units/mg).
† Based on an equivalent weight of 90,000 for cAMP binding (21) and $E_{\text{eq}}$ cAMP = 10.2 at 270 nm (22).
‡ Previously photolyzed I, i.e., I was photolyzed, then added to the enzyme solution and the entire solution was photolyzed.
§ cAMP added after photolysis of a solution containing PFK and I.
Table 2. Activation of PFK by cAMP and I

<table>
<thead>
<tr>
<th>cAMP, μM</th>
<th>I, μM</th>
<th>Relative activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>—</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>—</td>
<td>1000</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>60</td>
<td>1000</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>100</td>
<td>—</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>—</td>
<td>2000</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>100</td>
<td>2000</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
</table>

* Activity in the absence of both compound I and cAMP taken as 1.0.

is consistent with the results of Kemp and Krebs (21) that ATP competes for the cAMP site. Expts. 19–21 show that irradiation at 350 nm leads to incorporation of I into PFK, although the extent of incorporation is lower than that observed at 253.7 nm under similar conditions. In experiments not shown in Table 1, the extent of incorporation upon irradiation at 253.7 nm was found to be relatively insensitive to changes in pH (in the range 6.3–7.5), buffer (Tris, imidazole, phosphate), and temperature (0–30°C).

Additional confirmation that incorporation is not an artifact of the precipitation procedure comes from the following experiment. After photolysis of a solution containing I and PFK at 253.7 nm, the sample was divided into two portions. One portion was treated with trichloroacetic acid and the radioactivity was counted in the usual manner, while the other was passed through a Sephadex G-25 column. As seen in Fig. 1A, radioactivity is associated with the protein peak. The number of counts in the pooled fractions corresponds quantitatively (within 10%) to the amount of incorporation measured by the trichloroacetic acid procedure. As a control, previously photolyzed I was added to PFK, and the solution was photolyzed and passed through a G-25 column. As seen in Fig. 1B, no appreciable radioactivity is associated with the protein peak, so that the protein labeling seen in Fig. 1A must be due to covalent binding after carbene formation. The small ultraviolet-absorbing peak at 9 μl is unidentified, but is not an adenine nucleotide, since A280/A260 is approximately 2. Photolyzed I is eluted slowly from the column starting at 9 ml.

Photolysis of solutions of PFK containing either II or III led to no significant amounts of incorporation of radioactive material.

Effects on PFK activity

It has been previously demonstrated that under appropriate conditions, rabbit muscle PFK is subject to substrate inhibition with respect to ATP, and that this inhibition can be reversed by cAMP (23, 24). A comparison of the stimulatory effects of I and cAMP is shown in Fig. 2. Activation by I is only about one-fifth as effective as by cAMP. I is also bound somewhat less tightly, having an S1/2 value of 1 × 10⁻⁴ M as compared with 4 × 10⁻⁴ M for cAMP. Evidence that I and cAMP compete for the same site comes from the data in Table 2, which shows that addition of excess I to a solution containing cAMP and PFK reduces the observed activation to a level characteristic of I when added alone to the enzyme.

DISCUSSION

The PFK activity and incorporation studies clearly show that I binds both noncovalently, and on photolysis, covalently, to the cAMP site of PFK. The Kp for I measured from the photolysis experiments (1.2 × 10⁻⁴ M) and the S1/2 value measured from the PFK activity experiments (1 × 10⁻⁴ M) differ, but this is not surprising in view of the differences in the reaction mixture for the two experiments—for example, the ATP concentration. Preliminary studies with II and III have shown no significant covalent binding on photolysis with PFK, which indicates that substitution at N² is unfavorable for binding at the cAMP site of PFK.

Photo-affinity labels acylated at the N² and O² positions were synthesized because of the findings that acyl derivatives of cAMP are more potent than cAMP itself in provoking hormonal responses in intact cells (25). Although it has been noted that the increased effectiveness might be due to increased permeability, and the derivatives once inside the cell may be deacylated before exerting their action, recent experiments in rat epididymal adipocytes (26) have shown that, in this tissue at least, IV is active without becoming deacylated. Moreover, N²- and O²-alkylated cAMP derivatives, where there is little possibility for conversion to cAMP, are active in some intact cells (27). It would thus appear that in many tissues, compounds I–III will bind to the cAMP receptor site and function as photo-affinity labels. In preliminary experiments we have been able to demonstrate photolytic incorporation of I into a fraction of rat testes extract that is precipitated by trichloroacetic acid. The incorporation is abolished in the presence of excess cAMP. Further experiments to identify the site(s) labeled are planned.

The advantages that photo-affinity labels have over other affinity labels have been discussed previously (9), with respect to (a) the very high reactivity of the photo-generated intermediates, which are capable of insertion even into aliphatic side chains (28), and (b) the possibilities they afford for very specific reactions with protein-binding sites. In addition, we would like to point out their special suitability for work on intact cells, since, in the absence of light, they are stable for the long periods of time that may be necessary to achieve equilibration across cell walls and membranes.
Incorporation of the carbene into protein is limited by two factors: (a) direct reaction with water, and (b) Wolff-type rearrangement to yield a ketene, which, in the absence of a good nucleophile at the binding site, would react entirely with water. We have used diazomalonyl derivatives because of the recent findings that substitution with electron-withdrawing groups tends to minimize rearrangement (29).

For studies directed toward isolating cAMP receptors, incomplete labeling could have distinct advantages. If the labeled protein has similar chromatographic or electrophoretic properties to the unlabeled protein, labeling to the extent of a few percent could provide the means for identifying and purifying the protein, without appreciably changing its properties.

We envisage extending our syntheses to other nucleotides, particularly 5'-AMP, which is widely involved in regulating the activity of allosteric enzymes (30). For studies directed toward establishing the stoichiometry of nucleotide sites, peptide mapping of the sites, and the freezing of allosteric equilibria, quantitative labeling will be either desirable or necessary. Experiments to achieve quantitative labeling are underway.

This work was supported by research grants from the National Institute of Health (AM-13212) and the National Science Foundation (FR-07083).