Photoaffinity Labeling of a Protein Kinase from Bovine Brain with 8-Azidoadenosine 3′,5′-Monophosphate†

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ABSTRACT: 8-Azidoadenosine 3′,5′-monophosphate (8-N_3-cAMP) containing ^{32}P has been used as a photoaffinity label specific for the adenosine 3′,5′-monophosphate (cAMP) binding site(s) present in a partially purified preparation of soluble protein kinase from bovine brain. 8-N_3-cAMP and cAMP were found to compete for the same binding site(s) in this preparation, as determined by a standard filter assay. When this protein preparation was equilibrated with [^{32}P]-8-N_3-cAMP, and then irradiated at 253.7 nm, the incorporation of radioactivity was predominantly into a protein with an apparent molecular weight of 49,000, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. This labeled protein comigrated in the gel with the only protein which is endogenously phosphorylated by [γ-^{32}P]ATP, a protein which has been shown to be the regulatory subunit of the protein kinase (H. Maeno, P. L. Reyes, T. Ueda, S. A. Rudolph, and P. Greengard (1974), *Arch. Biochem. Biophys. 164,* 551). The incorporation of [^{32}P]-8-N_3-cAMP into this protein was half-maximal at a concentration of 7×10^{-8} *M*. In accordance with a proposed mechanism involving the formation of a highly reactive nitrene intermediate upon irradiation of the azide, the incorporation of radioactivity into protein was maximal within 10 min of irradiation, and was almost eliminated by preirradiation of the photolabile ligand. Moreover, this incorporation was virtually abolished by a 50-fold excess of cAMP, but not by AMP, ADP, ATP, or adenosine. We suggest that 8-N_3-cAMP may prove to be a useful molecular probe of the cAMP-binding site in receptor proteins and report its use in conjunction with sodium dodecyl sulfate–polyacrylamide gel electrophoresis as a highly sensitive and selective radiochemical marker for cAMP-binding proteins.

In their initial report of the synthesis of 8-N_3-cAMP,[1] Muneyama et al. (1971) observed that optimal concentrations of this compound mimicked the stimulatory effect of cAMP on the phosphorylation of histone by a protein kinase from bovine brain. Simon et al. (1973) determined the activation constant of 8-N_3-cAMP in this system and found the compound to be 0.7 times as effective as cAMP. In addition, both the stimulatory and inhibitory effects of cAMP on the endogenous phosphorylation of extracts from a variety of mammalian, amphibian, and avian tissues can be mimicked (A. H. Pomerantz, S. A. Rudolph, and P. Greengard, unpublished results) by a commercial preparation of 8-N_3-cAMP. These observations suggest that 8-N_3-cAMP interacts in vitro with the functional receptor for cAMP. Since the 8-azidopurine moiety possesses considerable aromaticity, 8-N_3-cAMP should readily photolyze under ultraviolet irradiation of appropriate energy to produce a highly reactive nitrene intermediate capable of covalent reaction with proteins (Fleet et al., 1969; Kiefer et al., 1970; Knowles, 1972). These characteristics of 8-N_3-cAMP suggested its potential as a photoaffinity reagent which is specific for cAMP-binding sites (Ruoho et al., 1973). (This possibility is supported by the recent observation of Haley and Hoffman (1974) that the analogous 8-azido derivative of ATP behaves as a photoaffinity label for an ATPase present in human erythrocyte membranes.) Ideally, such a compound should be stable under nonphotolytic conditions, bind selectively and reversibly to the cAMP effector site, and be photoactivated into a form which is covalently incorporated only into this site.

We have, therefore, attempted to demonstrate that 8-N_3-cAMP is such a photoaffinity analog of cAMP. To measure photoaffinity labeling, we have used [^{32}P]-8-N_3-cAMP. In order to characterize both the covalent and noncovalent binding of 8-N_3-cAMP, we have used a partially purified preparation of a soluble protein kinase from bovine brain. The cAMP-binding activity present in this preparation is associated with the protein kinase regulatory subunit, a polypeptide with an apparent molecular weight of 49,000 (Maeno et al., 1974). This polypeptide has also been shown to undergo endogenous phosphorylation by the catalytic subunit of the protein kinase (Maeno et al., 1974). This preparation thus allows us to identify independently the cAMP-binding subunit of the protein kinase and the site of photoaffinity labeling, and so to determine more clearly whether 8-N_3-cAMP can act as a photoaffinity reagent specific for cAMP-binding sites.

Experimental Procedure

Materials

Soluble protein kinase from bovine brain was purified through the step of hydroxylapatite chromatography as described by Miyamoto et al. (1973). Peak II was used in all experiments. [^{32}P]-8-N_3-cAMP (4–16 Ci/mmol) was prepared by the micro-method described by Haley (1975).

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[1] Abbreviations used are: 8-N_3-cAMP, 8-azidoadenosine 3′,5′-monophosphate; cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate.

[G-^{3}H]cAMP (16.3 Ci/mmol), diluted to a specific activity of 3.4 Ci/mmol with cold cAMP prior to use, and [^{32}P]cAMP were purchased from New England Nuclear. cAMP and cGMP were purchased from Boehringer-Mannheim. 8-N_3-cAMP, ATP, ADP, and 5′-AMP were purchased from Schwarz/Mann. Adenosine, morpholinoethanesulfonic acid, Pyronin Y, and bovine serum albumin were purchased from Sigma. [γ-^{32}P]ATP (5–20 Ci/mmol) was prepared by the method of Post and Sen (1967).

Methods

Procedures for Photoaffinity Labeling of the Protein Kinase. All photoaffinity incorporation experiments, except the irradiation time course, were performed in Pyrex spot plates containing nine wells. For the standard incorporation assay, each well contained 60 μg of protein in a total volume of 0.1 ml containing 50 m*M* sodium morpholinoethanesulfonate (pH 6.2) and indicated concentrations of [^{32}P]-8-N_3-cAMP. The well contents were mixed with an air stream and incubated at 4° in the dark for 60 min to allow reversible cyclic nucleotide binding to reach equilibrium. Following this prephotolytic incubation, the entire spot plate was subjected to irradiation at 253.7 nm with a Mineralite UVS-11 hand lamp supported at a distance of 8 cm. In the standard assay, irradiation lasted 15 min at 4°. The contents of each well was then mixed with 0.05 ml of a stop solution containing 9% (w/v) sodium dodecyl sulfate, 3% 2-mercaptoethanol, 15% glycerol, 0.03 *M* Tris-HCl (pH 8.0), 3 m*M* EDTA, and 0.2% Pyronin Y; 0.1-ml aliquots were then subjected to slab gel electrophoresis in the presence of sodium dodecyl sulfate as described by Fairbanks et al. (1971) and Johnson et al. (1972). The slab gels were then stained, destained, and dried, and autoradiographs were made as described by Ueda et al. (1973). The optical density profile of each channel of the exposed film was recorded using a Canalco Model-G-II microdensitometer. Under the conditions used, the peak areas of the densitometric tracings were proportional to the corresponding radioactivities of the bands resolved by the gel, as determined by liquid scintillation counting. Dried gel slices were digested prior to counting according to the method of Goodman and Matzura (1971).

We assume that the ^{32}P detected by this procedure is a direct measure of the amount of [^{32}P]-8-N_3-cAMP covalently incorporated into protein and have calculated molar amounts of incorporation from the specific activity of the photoaffinity label. Thus, we operationally define covalent incorporation of 8-N_3-cAMP as the amount of protein-associated radioactivity stable to sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The experiments determining the irradiation time course of photoaffinity labeling were performed in a quartz cuvet of 1-cm path length containing 0.8 ml of the standard assay mixture which was equilibrated at 4° as described above. The entire cuvet was then subjected to irradiation at 4° with the UVS-11 hand lamp supported at a distance of 4 cm; 0.1-ml aliquots of this solution were removed after periods of irradiation ranging from 0 to 10 min and were immediately mixed with 0.05 ml of the sodium dodecyl sulfate stop solution. The samples were then analyzed for covalent incorporation of [^{32}P]-8-N_3-cAMP as described above. Photolysis in a quartz cuvet was also employed in the preirradiation time course experiment in which 10^{-6} *M* [^{32}P]-8-N_3-cAMP in 50 m*M* sodium morpholinoethanesulfonate (pH 6.2) was photolyzed for various periods of time prior to incubation with protein kinase and subsequent standard irradiation for 15 min by the spot plate method.

Assays for the Noncovalent Binding of Cyclic Nucleotide. All determinations of the reversible binding of cAMP or 8-N_3-cAMP to protein kinase (binding which would be eliminated by sodium dodecyl sulfate polyacrylamide gel electrophoresis) were performed by a modification of the filter assay of Gilman (1970), without the heat stable inhibitor. In the study of 8-N_3-cAMP competition for cAMP-binding sites, the incubation mixture contained 50 m*M* sodium acetate (pH 4.0), 20 μg of protein, 10^{-8} *M* [G-^{3}H]cAMP, and up to 5×10^{-6} *M* 8-N_3-cAMP or cAMP in a total volume of 0.2 ml. The mixture was incubated for 2 hr at 0°. In the study of [^{32}P]-8-N_3-cAMP binding, incubations were performed in a 0.1-ml volume, using incubation conditions identical with those described above for the spot plate assays of photoaffinity labeling. With both types of incubation mixture, the protein kinase was the last component added.

In both cases, at the end of the incubation, samples were diluted with 4 volumes of a wash solution of 20 m*M* sodium acetate buffer (pH 6.0), applied to prewashed Millipore Type HA filters in a Millipore suction manifold, and rinsed four times with 4 ml of wash solution. Filters were then dissolved in 8 ml of a 1:1 Cellosolve–toluene scintillation cocktail and counted for ^{3}H or ^{32}P. Corrections for extraneous filter binding were made by subtracting values for control incubations performed exactly as described above but in the absence of protein kinase.

Endogenous Phosphorylation of Protein Kinase. Endogenous phosphorylation of the protein kinase regulatory subunit was carried out in a volume of 0.1 ml containing 50 m*M* sodium morpholinoethanesulfonate (pH 6.2), 60 μg of protein kinase, and 10 m*M* $MgCl_2$. After a 1-min preincubation of this mixture at 20°, 0.1 nmol of [^{32}P]ATP was added and the mixture allowed to incubate for an additional 15 sec at which point 0.05 ml of sodium dodecyl sulfate stop solution was added. Electrophoresis of the mixture and autoradiography were then performed as described above.

Other Methods. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Ultraviolet difference spectra of 8-N_3-cAMP before and after photolysis were obtained with a Bausch and Lomb Spectronic 200 uv scanning spectrophotometer operating in transmittance mode.

Results and Discussion

Binding Competition Experiments. Mimicry by 8-N_3-cAMP of the enzymological effects of cAMP suggests that 8-N_3-cAMP acts at the functional effector site for cAMP (Muneyama et al., 1971; Simon et al., 1973). The present binding competition experiments lead to the same conclusion. Figure 1 illustrates the binding of [G-^{3}H]cAMP to the kinase preparation in the presence of varying concentrations of nonradioactive 8-N_3-cAMP or cAMP. The 8-azido analog inhibited the binding of cAMP to the protein kinase preparation. Moreover, the concentration required for half-maximal inhibition by 8-N_3-cAMP, namely 3×10^{-8} *M*, was only threefold greater than that for cold cAMP. Conversely, nonradioactive cAMP blocked the binding of [^{32}P]-8-N_3-cAMP to the protein kinase almost quantitatively (Table I). These data indicate that cAMP and its 8-N_3 analog compete for the same binding sites, suggesting that the 8-azido derivative binds specifically and reversibly to the cAMP binding site in our protein kinase preparation,

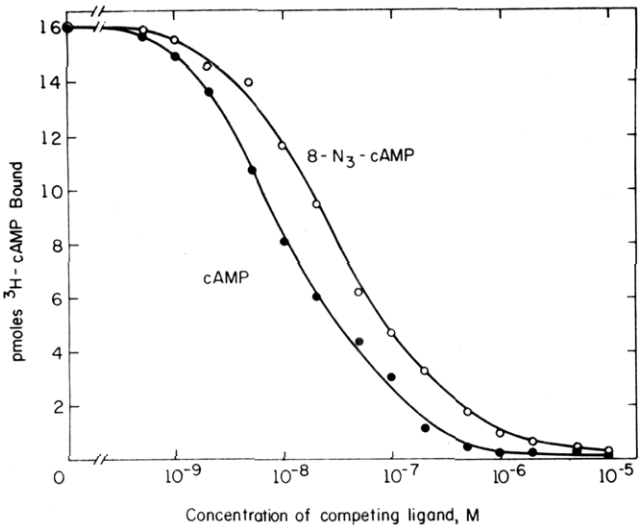

FIGURE 1: Inhibition by 8-N_3-cAMP of the binding of [^{3}H]cAMP to protein kinase. Reversible binding at pH 4.0 of 10 n*M* [G-^{3}H]cAMP (3.4 Ci/mmol) to protein kinase in the presence of the indicated amounts of nonradioactive 8-N_3-cAMP or cAMP was determined using the modified Gilman assay, as described in the text. Binding values are reported per milligram of total protein present in the incubation mixture.

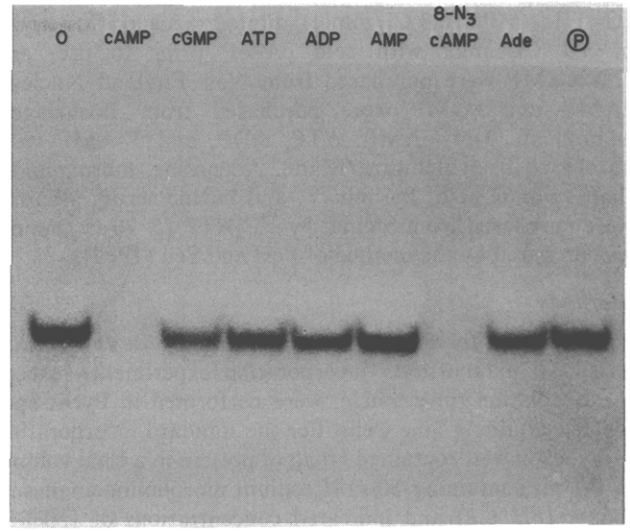

FIGURE 2: Autoradiograph of a sodium dodecyl sulfate–polyacrylamide gel showing the selective incorporation of 8-N_3-cAMP into the protein kinase regulatory subunit. The first eight channels of the autoradiograph show the results of photolysis by the spot plate method. Protein kinase, prior to irradiation, was incubated with 0.2 μM [^{32}P]-8-N_3-cAMP in 50 m*M* sodium morpholinoethanesulfonate (pH 6.2) and 10-μM amounts of the indicated nonradioactive nucleotides or adenosine (Ade). The last channel of the gel marks the regulatory subunit of the protein kinase which was endogenously phosphorylated with [γ-^{32}P]ATP as described in the text. Each channel of the gel contained 40 μg of total protein. The amounts of [^{32}P]-8-N_3-cAMP incorporated into the regulatory subunit were (in fmol per channel): none, 110; cAMP, <1; cGMP, 80; ATP, 93; ADP, 93; 5′-AMP, 106; 8-N_3-cAMP, <1; adenosine, 77.

Table I: Inhibition of the Binding of 8-N_3-cAMP to the Kinase.[a]

cAMP, *M*	fmoles of 8-N_3-cAMP Bound
0	180 ± 11
10^{-6}	71 ± 7
10^{-5}	17 ± 2
10^{-4}	7 ± 1

[a] Noncovalent binding of [^{32}P]-8-N_3-cAMP (3×10^{-7} *M*) to 40 μg of total protein in the presence of varying amounts of cAMP was determined by the modified Gilman assay described in the text. Values given are means of triplicate determinations.

and that its photolytic product would be selectively incorporated only into this protein site.

Selective Photoactivated Incorporation of [^{32}P]-8-N_3-cAMP into the Regulatory Subunit of Protein Kinase. An autoradiograph showing photoactivated incorporation of [^{32}P]-8-N_3-cAMP into protein under the standard conditions of photolysis is presented in Figure 2. Radioactivity is incorporated into a peptide which comigrates with the endogenously phosphorylated band. This phosphorylated peptide, with an apparent molecular weight of 49,000, has been previously identified by Maeno et al. (1974) as the regulatory subunit of the protein kinase in our preparation. Thus, the data indicate that, upon photoactivation, 8-N_3-cAMP was covalently incorporated into the regulatory subunit of the protein kinase, a known site of reversible cAMP binding.

The data of Figure 2 further indicate that the incorporation of radioactivity from ^{32}P-labeled 8-N_3-cAMP into the regulatory subunit is highly specific for this known protein receptor for cAMP. Under the conditions of the standard spot plate method of photolysis, greater than 98% of the total radioactivity incorporated into the preparation was associated with the regulatory subunit, a peptide which makes up less than 2% of the total protein present, as estimated from the protein staining pattern of the gel. The rest of the radioactivity detected is specifically associated with two proteins in the 110,000–130,000 molecular weight range. The fact that 8-N_3-cAMP, upon irradiation, labels its target receptor almost exclusively in a heterogeneous macromolecular mixture is an indication of its high selectivity. We have recently reported photolytic incorporation of [^{32}P]-8-N_3-cAMP into a 49,000 molecular weight peptide from both soluble and membrane fractions from a wide variety of tissues (Malkinson et al., 1975). This peptide was also found to undergo cAMP-dependent phosphorylation in a manner similar to the preparation used in the present study.

Incorporation of radioactivity (even that not associated with the 49,000 molecular weight peptide) is virtually eliminated by the presence in the incubation mixture of nonradioactive cAMP at a 50-fold molar excess over [^{32}P]-8-N_3-cAMP. As expected, nonradioactive 8-N_3-cAMP has a similar blocking effect. Identical concentrations of ATP, cGMP, ADP, 5′-AMP, and adenosine inhibited the photoactivated incorporation less drastically. For several reasons, the specific blocking effect by cAMP cannot be a result of uv light absorption and consequent interference with the photoactivation of 8-N_3-cAMP: the absorbances of all incubation mixtures at the 253.7-nm wavelength of photolysis were comparable; the absorbances were less than 0.1 OD unit relative to H_2O; and the 15-min period of irradiation used for these spot plate assays was shown to be sufficient to completely photolyze all 8-N_3-cAMP initially present.

Figure 3 shows the inhibitory effect of various concentrations of cAMP on the photoactivated labeling of the regulatory subunit. Increasing concentrations of cAMP in the prephotolytic incubation mixture appeared to titrate all the sites available for photolytic incorporation of 8-N_3-cAMP,

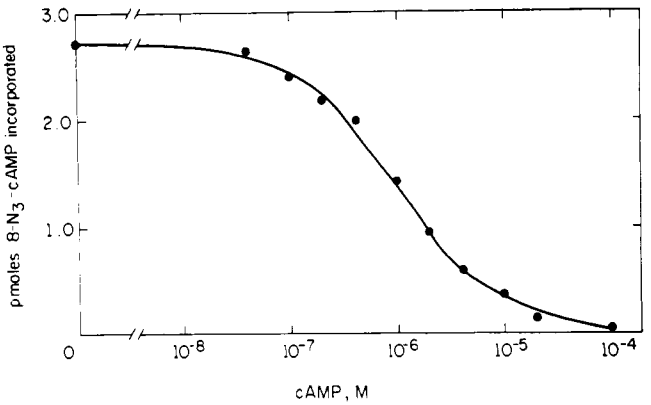

FIGURE 3: Concentration dependence of the inhibition by cAMP of the photoactivated incorporation of [^{32}P]-8-N_3-cAMP into protein kinase regulatory subunit. [^{32}P]-8-N_3-cAMP (0.3 μM) was preincubated with the protein kinase at pH 6.2 in the presence of the indicated amount of cAMP and photolyzed according to the spot plate method described in the text. Incorporation of [^{32}P]-8-N_3-cAMP into the protein kinase regulatory subunit is expressed per milligram of total protein applied to the gel.

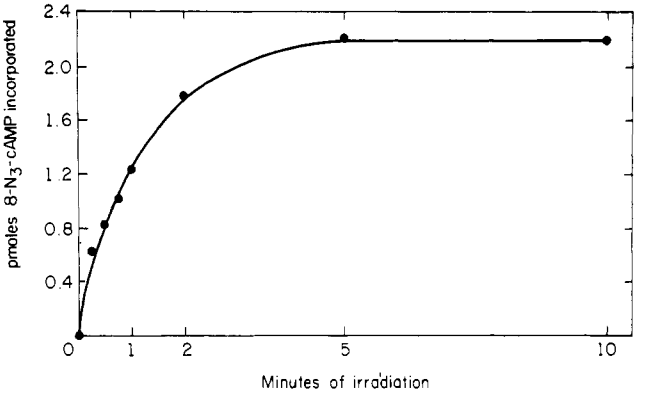

FIGURE 4: Irradiation time course of the photoaffinity labeling of the protein kinase regulatory subunit. [^{32}P]-8-N_3-cAMP (0.3 μM), in the standard assay mixture, at pH 6.2, was photolyzed at 253.7 nm for the indicated periods of time, according to the cuvet method described in the text. Incorporation of [^{32}P]-8-N_3-cAMP into the protein kinase regulatory subunit is expressed per milligram of total protein applied to the gel.

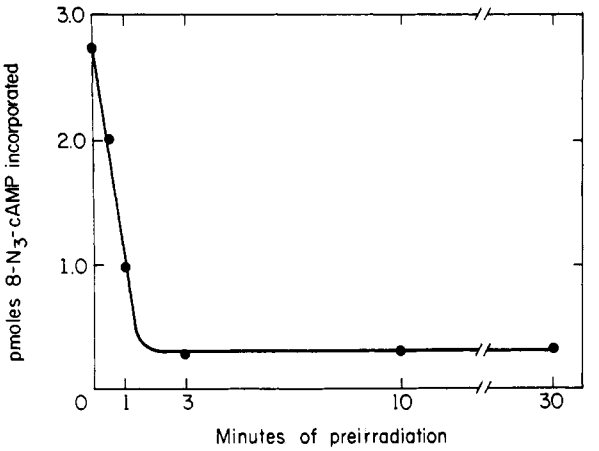

FIGURE 5: Effect of preirradiation on the photoaffinity labeling of the protein kinase regulatory subunit. A solution of [^{32}P]-8-N_3-cAMP (1 μM) in 50 mM sodium morpholinoethanesulfonate (pH 6.2) was photolyzed in a quartz cuvet at 253.7 nm. Aliquots were withdrawn after the indicated times of preirradiation and assayed for incorporation of radioactivity into protein kinase using the standard procedures of spot plate incubation and photolysis. Incorporation of [^{32}P]-8-N_3-cAMP into the protein kinase regulatory subunit is expressed per milligram of total protein applied to the gel.

half-maximally eliminating such covalent labeling at a concentration of 9×10^{-7} M, a threefold excess over the concentration of the photoaffinity reagent. The fact that a threefold excess of cAMP was required to half-maximally inhibit the photoincorporation of 8-N_3-cAMP, whereas the apparent affinity for the reversible binding of 8-N_3-cAMP was threefold lower than that of cAMP, may be explained as follows: For reversible binding, the ratio of cAMP bound to 8-N_3-cAMP bound will be determined only by their relative concentrations and affinities. Since the noncovalent binding of cAMP and 8-N_3-cAMP to the regulatory subunit is presumed to be in dynamic equilibrium, there should be continual exchange of cAMP and 8-N_3-cAMP molecules at the binding site. In contrast, in the case of covalent incorporation, there will be a "trapping" effect whereby 8-N_3-cAMP molecules which exchange with cAMP molecules during the irradiation period can be covalently incorporated. This will be reflected in a higher apparent affinity for covalent binding than for noncovalent binding. Thus the concentration of nonphotoactive ligand required to inhibit covalent incorporation would be determined not only thermodynamically, but kinetically as well.

Characterization of the Photoaffinity Labeling of the Regulatory Subunit. Figure 4 shows the results of the irradiation time course experiment performed in a quartz cuvet as described above. Photoactivated incorporation into the regulatory subunit is seen to be essentially complete after a 5-min period of photolysis, at which point it has reached a level of 2.2 pmol of [^{32}P]-8-N_3-cAMP incorporated/mg of total protein. In accord with our previous statements about reversible binding, no detectable incorporation of radioactivity occurred when the photolytic incubation mixture was not irradiated. The irradiation time dependence reported here markedly contrasts with the observations of Antonoff and Ferguson (1974) that photoactivated incorporation of [^{3}H]cAMP into a lamb testis preparation was nearly linear with irradiation time at 253.7 nm for at least 3 hr. The rapid time dependence found here is more in accord with the proposed mechanism of true photoaffinity labeling in which a photolabile functional group on the ligand is photolyzed to yield a short-lived, but highly reactive, intermediate which actually participates in the labeling.

Further evidence for the behavior of 8-N_3-cAMP as such a photoaffinity reagent was obtained by studying the effect of preirradiation, for various periods of time, on the incorporation of [^{32}P]-8-N_3-cAMP into the regulatory subunit (Figure 5). For this purpose, a 10^{-6} M solution of [^{32}P]-8-N_3-cAMP was preirradiated at 253.7 nm in a quartz cuvet; aliquots were removed after varying periods of photolysis, incubated with protein kinase, and subsequently assayed for photoactivated incorporation into the regulatory subunit during the standard 15-min irradiation period using the spot plate method. Incorporation fell within 3 min of preirradiation to 10% of maximum labeling. This decrease is attributable to photolysis of the azide group, and hence inactivation of the photoaffinity reagent. Indeed, by monitoring the azide chromophore of 8-N_3-cAMP at 282 nm using ultraviolet difference spectroscopy of photolyzed solutions, we found that 10 min of irradiation under the conditions used was sufficient to photolyze completely a 10^{-5} M solution of 8-N_3-cAMP, a tenfold higher concentration than that actu-

ally used in the preirradiation time course experiment. The basal level of incorporation which occurs during the standard irradiation, using preirradiated solutions of [^{32}P]-8-N_3-cAMP (Figure 5), must then occur in the absence of a highly labile, nitrene-generating group on the ligand, and is thus analogous to the phenomenon reported by Antonoff and Ferguson (1974), where the possibility remains that the photoactivated group resides on the receptor protein, i.e., as an aromatic amino acid residue. Our observation that 15 min of irradiation of the kinase preparation in a spot plate was sufficient to eliminate 29% of its cAMP binding activity, as determined by the Gilman assay, suggests the existence of such a photolabile chromophore on the protein.

The effect of pH on the incorporation of [^{32}P]-8-N_3-cAMP into protein kinase was also determined. The photoaffinity labeling of the regulatory subunit was almost independent of pH from pH 3.5 to 9.0 (2.5 ± 0.5 pmol/mg of protein of 8-N_3-cAMP incorporated). The buffers used were sodium acetate, pH 3.5–6.0; sodium morpholinoethanesulfonate, pH 6.2–7.0; and Tris-HCl, pH 8.0 and 9.0. This insensitivity to pH contrasts with the ethyldiazomalonyl derivatives of cAMP described by Brunswick and Cooperman (1973) and Guthrow et al. (1973).

In other experiments, the incorporation of [^{32}P]-8-N_3-cAMP was measured under the standard conditions except for varying the protein concentration or the [^{32}P]-8-N_3-cAMP concentration. Incorporation varied linearly with protein concentration over the range 0–600 μg/ml with a slope of approximately 2.7 pmol/mg of protein. Under the conditions used, half-maximal incorporation was attained at a concentration of 8-N_3-cAMP of 7×10^{-8} *M* and maximal incorporation was approximately 3.0 pmol/mg of protein.

In conclusion, 8-N_3-cAMP fulfills several criteria for a useful photoaffinity analog of cAMP: its affinity for the receptor binding site is similar to that of cAMP, its selectivity is high in a heterogeneous protein mixture, and covalent incorporation is rapid upon irradiation.

References

Antonoff, R. S., and Ferguson, J. J., Jr. (1974), *J. Biol. Chem. 249,* 3319.

Brunswick, D. J., and Cooperman, B. S. (1973), *Biochemistry 12,* 4074.

Cooperman, B. S., and Brunswick, D. J. (1973), *Biochemistry 12,* 4079.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.

Fleet, G. W. J., Knowles, J. R., and Porter, R. R. (1969), *Nature (London) 224,* 511.

Gilman, A. G. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 305.

Goodman, A., and Matzura, H. (1971), *Anal. Biochem. 42,* 481.

Guthrow, C. E., Rasmussen, H., Brunswick, D. J., and Cooperman, B. S. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3344.

Haley, B. E. (1975), *Biochemistry, 14,* preceding paper in this issue.

Haley, B. E., and Hoffman, J. F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3367.

Johnson, E. M., Ueda, T., Maeno, H., and Greengard, P. (1972), *J. Biol. Chem. 247,* 5650.

Kiefer, H., Lindstrom, J., Lennox, E. S., and Singer, S. J. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1688.

Knowles, J. R. (1972), *Acc. Chem. Res. 5,* 155.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Maeno, H., Reyes, P. L., Ueda, T., Rudolph, S. A., and Greengard, P. (1974), *Arch. Biochem. Biophys. 164,* 551.

Malkinson, A. M., Krueger, B. K., Rudolph, S. A., Casnellie, J. E., Haley, B. E., and Greengard, P. (1975), *Metab. Clin. Exp. 24,* 331.

Miyamoto, E., Petzold, G. L., Kuo, J. F., and Greengard, P. (1973), *J. Biol. Chem. 248,* 179.

Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971), *Biochemistry 10,* 2390.

Post, R. L., and Sen, A. K. (1967), *Methods Enzymol. 10,* 773.

Ruoho, A. E., Kiefer, H., Roeder, P. E., and Singer, S. J. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2567.

Simon, L. N., Shuman, D. A., and Robins, R. K. (1973), *Adv. Cyclic Nucleotide Res. 3,* 294.

Ueda, T., Maeno, H., and Greengard, P. (1973), *J. Biol. Chem. 248,* 8295.