

Reprinted from

Proc. Natl. Acad. Sci. USA  
Vol. 75, No. 3, pp. 1199-1203, March 1978  
Biochemistry

## Fluorescent photoaffinity labeling: Adenosine 3',5'-cyclic monophosphate receptor sites

(specific binding/fluorescent probes/ethenoadenosine/photoattachment/protein kinases)

GIDEON DREYFUSS\*, KENNETH SCHWARTZ\*, ELKAN R. BLOUT\*\*†, JORGE R. BARRIO‡, FU-TONG LIU‡, AND NELSON J. LEONARD‡

\* Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115; and † Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

Contributed by Nelson J. Leonard and Elkan R. Blout, December 28, 1977

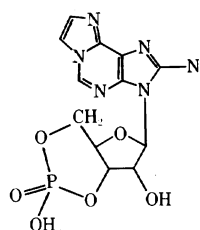
**ABSTRACT** An approach to the study of protein receptor sites in protein mixtures or supramolecular assemblies by using fluorescence spectroscopy is described. This approach, fluorescent photoaffinity labeling, combines the merits of photoaffinity labeling to attain site-directed reactivity with the probing power of fluorescent ligands. A fluorescent photoaffinity label for cyclic AMP receptor sites of cyclic AMP-dependent protein kinases was synthesized in both unlabeled and radioactive forms. The probe, 8-azido-1,*N*<sup>6</sup>-ethenoadenosine 3',5'-cyclic monophosphate, mimics cyclic AMP in its ability to stimulate the phosphotransferase activity of the protein kinases and strongly competes with cyclic AMP for its binding sites in all preparations so far tested. Photolysis, after equilibration of protein kinase and 8-azido-1,*N*<sup>6</sup>-ethenoadenosine 3',5'-cyclic monophosphate in the dark, effects binding of the intermediate nitrene irreversibly and specifically to the cyclic AMP sites with the development of fluorescence. Excess reagent and low molecular weight photolytic products are removable by dialysis. Studies of a crude beef heart preparation containing cyclic AMP-dependent protein kinase suggest that the cyclic AMP binding sites are hydrophobic in nature and strongly immobilize the adenine moiety of the cyclic nucleotide.

Fluorescence spectroscopy is among the most sensitive, versatile, and potentially informative methods available for studying the conformation and dynamics of macromolecules. Although much information relating to the structure and function of proteins has been obtained from the fluorescence of intrinsic fluorophores, notably tryptophan (1, 2), some inherent difficulties and limitations of this approach are apparent. Among these are the necessity of obtaining a pure, native protein and the difficulty of relating the observed spectral data to a particular site within that protein. In many cases, an "extrinsic" fluorophore can be introduced into a protein either by covalent coupling or by reversible interaction (1, 2). The use of such extrinsic fluorophores, although frequently advantageous in terms of sensitivity, often does not overcome the basic drawbacks of lack of specificity and lack of knowledge as to the precise location of the fluorescent probe.

The approach introduced in this communication, fluorescent photoaffinity labeling (FPAL), is designed to circumvent these obstacles. It combines the labeling achieved by site-specific photoaffinity analogs with the probing power of fluorescence techniques. Several recent publications have shown 8-azidoadenosine derivatives to be useful photoaffinity reagents for the nucleotide binding site of their biological receptors (3-7). The synthesis and utilization of a fluorescent photoaffinity label (FPAL), 8-azido-1,*N*<sup>6</sup>-ethenoadenosine 3',5'-cyclic monophosphate (8-*N*<sub>3</sub>-εcAMP), as a probe for cyclic AMP (cAMP)

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

receptor sites of several proteins is described herein. This compound combines the photoreactivity of the azide moiety



8-Azido-1,*N*<sup>6</sup>-ethenoadenosine 3',5'-cyclic monophosphate (8-*N*<sub>3</sub>-εcAMP)

(8-10) with the fluorescence of a substituted 1,*N*<sup>6</sup>-ethenoadenosine unit (11, 12) to yield a highly specific photoaffinity label that, upon photolysis, becomes covalently attached as an environment-sensitive fluorophore.

### MATERIALS AND METHODS

**Thin-Layer Chromatography (TLC).** Solvent systems used were: A, *n*-butanol/acetic acid/H<sub>2</sub>O, 5:2:3 (vol/vol); B, isobutyric acid/NH<sub>4</sub>OH/H<sub>2</sub>O, 75:1:24.

**8-*N*<sub>3</sub>-εcAMP.** All manipulations were carried out in the dark or under red light. To a solution of cAMP (16.5 mg, 50 μmol) in 5 ml of 0.2 M acetate buffer (pH 5.0) was added 0.6 ml of bromine water containing 0.26 mmol of bromine per ml. The reaction solution was kept at 25° for 20 hr and was followed by TLC (silica gel, solvent A). Excess bromine was eliminated with 50 μl of 1 M NaHSO<sub>3</sub> solution, and then 5 ml of 1.8 M aqueous chloroacetaldehyde was added and the reaction was followed by TLC (silica gel, solvent B). After 4 days at 25°, the mixture was evaporated to dryness *in vacuo* at 15°. The residue was applied immediately to a column of DEAE-cellulose (3 × 45 cm, HCO<sub>3</sub><sup>-</sup> form) and eluted with a linear gradient of 10 (1 liter) to 100 mM (1 liter) triethylammonium (TEA) bicarbonate (pH 8.0). The major peak, 8-Br-εAMP, was pooled and evaporated to dryness *in vacuo* at 15°. The material was then dissolved and coevaporated four times with 20-ml portions of absolute ethanol to remove excess TEA bicarbonate. To the dried 8-Br-εAMP product (yield: 75-80%), 2.5 ml of dimethylformamide containing 500 μmol of TEA azide was added,

Abbreviations: cAMP, cyclic AMP; εcAMP, 1, *N*<sup>6</sup>-ethenoadenosine 3',5'-cyclic monophosphate; 8-*N*<sub>3</sub>-εcAMP, 8-azido-1,*N*<sup>6</sup>-ethenoadenosine 3',5'-cyclic monophosphate; TLC, thin-layer chromatography; FPAL, fluorescent photoaffinity label(ing); TEA, triethylammonium.

† To whom reprint requests should be addressed.

and the mixture was heated at 70° for 12 hr. The reaction mixture was evaporated to dryness at 1 torr (133 Pa) and 20° and chromatographed as above. The major peak, 8-N<sub>3</sub>-εcAMP, was pooled and evaporated to dryness under the same conditions, and excess TEA bicarbonate was removed as before. Overall yield: 50–60%.

The 8-N<sub>3</sub>-εcAMP, fully characterized by elemental analysis, proton magnetic resonance, and infrared and ultraviolet spectroscopy, is nonfluorescent. Its photolytic activity was demonstrated by the irreversible binding to cellulose on a TLC plate and by the development of fluorescence with exposure to ultraviolet light (254 or 350 nm). 8-N<sub>3</sub>-εcAMP was also prepared from 8-N<sub>3</sub>-cAMP (P-L Biochemicals) and chloroacetaldehyde. 8-N<sub>3</sub>-εcAMP was found to be stable for at least a year when stored lyophilized and protected from light at –20° and for at least a few months in ethanol/H<sub>2</sub>O, 1:1 (vol/vol), at –20° or at 4°.

The same synthetic sequence can be used for the preparation of 8-N<sub>3</sub>-εAMP, 8-N<sub>3</sub>-εADP, and 8-N<sub>3</sub>-εATP, but these compounds are more sensitive to chemical breakdown. The radioactive compound, 8-N<sub>3</sub>-[<sup>3</sup>H]εcAMP, was prepared from [G-<sup>3</sup>H]cAMP by the same procedure described for the nonradioactive sample, except that added adenosine was used to monitor the bromination step and a larger excess of TEA azide was used. The identity of the 2-<sup>3</sup>H-labeled product, with approximately one-half the specific radioactivity of the original [<sup>3</sup>H]cAMP, was established by TLC (cellulose, solvents A and B) and by column chromatography, as described above, with detection of the compound by radioactivity. Additional precautions should be taken with 8-N<sub>3</sub>-[<sup>3</sup>H]εcAMP due to possible radiolytic processes (13).

**cAMP-Dependent Protein Kinase.** Preparations from beef heart, rabbit muscle DEAE-cellulose peak I, and rabbit muscle DEAE-cellulose peak II were from Sigma Chemical Company. The reversible binding of cAMP or 8-N<sub>3</sub>-εcAMP to the protein kinase was assayed in the dark according to a modification of the method of Gilman (14).

Stimulation of protein kinase activity was determined in the dark by measuring the amount of <sup>32</sup>P transferred from [γ-<sup>32</sup>P]ATP to protamine by a modification of the procedure of Rubin *et al.* (15). Additional details are described in the legends to the figures. Radioactive reagents were from New England Nuclear.

**Photolysis.** This was performed with a 15-W fluorescent “254-nm” lamp (Westinghouse G15T8 germicidal) or a “350-nm” lamp (General Electric F15T8-BLB) placed at a distance of 5 cm from the samples. Samples were photolyzed in multiple-well microtiter plates immersed in an ice-water bath. In the standard procedure, each well contained up to 1 mg of total protein in a final buffer volume of 200 μl and varying concentrations of 8-N<sub>3</sub>-εcAMP and of cAMP. The protein was the last component to be added. Subsequently, the contents of the well were gently mixed and equilibrated by incubation in the dark for 60 min. The entire plate was then irradiated for 10 min.

**Fluorescence Measurements.** Photolyzed samples were transferred to a multiple-chamber dialysis manifold and exhaustively dialyzed against the desired buffer at 4°. A known amount of [<sup>3</sup>H]cAMP was added routinely to samples after photolysis and prior to dialysis, and <sup>3</sup>H counts were monitored in the dialysate as an additional indicator to ensure complete removal of noncovalently bound cyclic nucleotide derivatives. Samples were then transferred to small-volume quartz cuvettes for fluorescence studies. Fluorescence measurements were performed at room temperature (22°) on an Hitachi-Perkin-

Elmer MPF-2A spectrofluorimeter operated in the ratio mode.

Steady-state fluorescence polarization measurements were performed under the same conditions using wavelengths of 320 and 400 nm for excitation and emission, respectively, and the “difference polarization” (P<sub>D</sub>) of the bound fluorophore was calculated as described in the legend to Table 2. All fluorescence measurements were performed on samples of identical (or within 5% and normalized to identical) protein concentrations. Protein concentrations were determined according to Lowry *et al.* (16) with bovine serum albumin as a standard and by protein fluorescence using wavelengths of 280 and 340 nm for excitation and emission, respectively.

## RESULTS

The synthetic procedure for 8-azido-1,N<sup>6</sup>-ethenoadenine derivatives, exemplified here with the synthesis of 8-N<sub>3</sub>-εcAMP, is simple, reproducible, and applicable to radiolabeled 8-N<sub>3</sub>-εcAMP as well as to mono-, di-, and triphosphate adenosine derivatives. Bromination of cAMP, using a bromine/cAMP molar proportion of ≤3:1 (4), and cyclization with chloroacetaldehyde were reduced to a “one-pot” reaction, because both steps proceed optimally at pH 4.0–4.5 (17, 18). We found it useful to introduce adenosine as a separable monitor for the bromination of tritiated cAMP, because the reactivities of adenosine and cAMP toward electrophilic substitution at C-8 are similar. Comparable reactivities toward chloroacetaldehyde were also observed in the cyclization step. TEA azide was used in the final nucleophilic displacement at C-8 of 8-bromo-εcAMP in dimethylformamide to produce the 8-N<sub>3</sub>-εcAMP product, either unlabeled or tritiated.

While the experiments with cAMP-binding protein were in progress, a communication by Keeler and Campbell (19) describing the synthesis of 8-N<sub>3</sub>-εcAMP was published in which 8-N<sub>3</sub>-εcAMP was described as an “intensely fluorescent” compound. Carefully prepared 8-N<sub>3</sub>-εcAMP (fully protected from light) is actually nonfluorescent. The lack of fluorescence is probably due to quenching of the 1,N<sup>6</sup>-ethenoadenine fluorescence (20, 21) by the long-wavelength n → π\* transition of the azido group (22, 23). Fluorescence develops only after photolysis, probably due to the formation of a substituted 8-amino-εcAMP derivative. This finding enables direct kinetic study and constant monitoring of the photolytic conversion of the azide to an amine by following the appearance of fluorescence. 8-Azido ethenoadenosine derivatives and, possibly, also other azido-quenched fluorophores may thus be interesting and useful tools for study of the kinetics and mechanisms of photolytic reactions. An additional advantage of 8-N<sub>3</sub>-ε-adenosine nucleotides over analogs lacking the 1,N<sup>6</sup>-etheno bridge (4–7, 24, 25) is that photolysis can be effected at long wavelengths (300–350 nm), where protein photodamage is minimized (9). Unlike the unlabeled analog, the radioactive compound, stored in 50% ethanol at 4°, was found to be unstable, yielding a single radioactive impurity according to two TLC systems and ion exchange chromatography. The impurity is nonactive photolytically, and its identity is currently unknown.

Fig. 1 *left*, which illustrates the binding of [<sup>3</sup>H]cAMP to rabbit muscle peak I protein kinase in the presence of varying concentrations of nonradioactive 8-N<sub>3</sub>-εcAMP or cAMP, shows that the 8-azido analog strongly competes with [<sup>3</sup>H]cAMP for binding to the protein kinase. Moreover, the apparent dissociation constant of 8-N<sub>3</sub>-εcAMP is only about 3 times higher than that of cAMP. Scatchard analysis (26) (Fig. 1 *right*) suggests that a single type of binding site for the cyclic nucleotides exists in

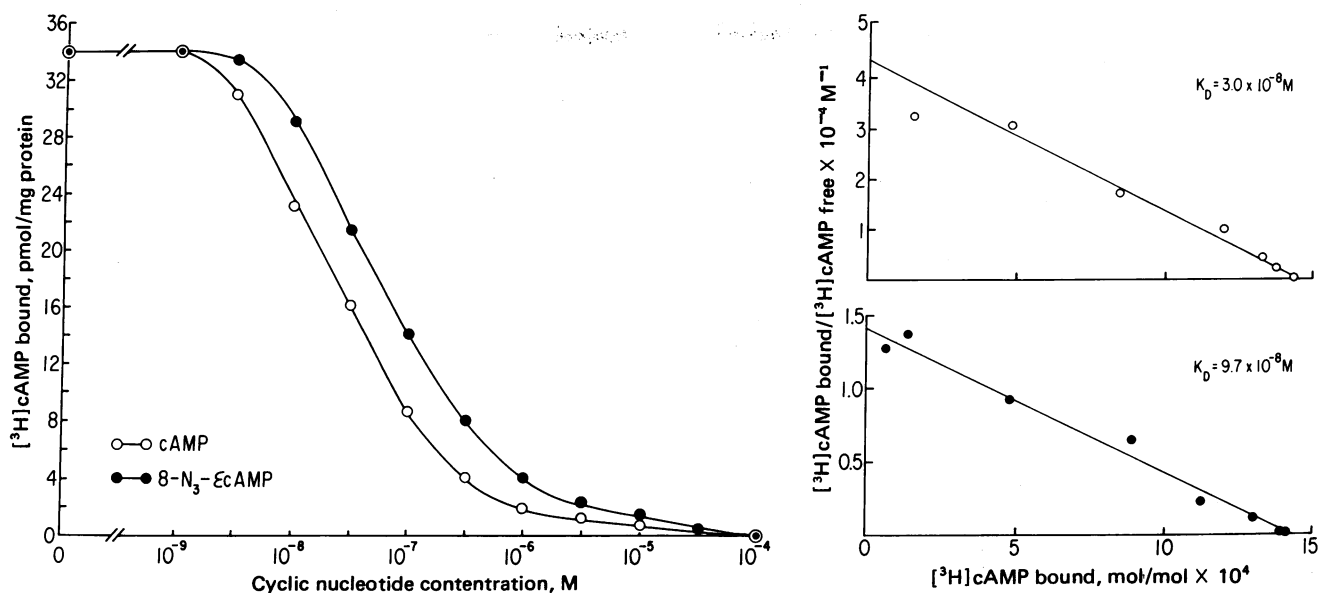


FIG. 1. (Left) Inhibition by 8-N<sub>3</sub>-εcAMP of the binding of  $[^3\text{H}]$ cAMP to rabbit muscle protein kinase (DEAE peak I). Each incubation mixture contained 5 μg of protein, 40 nM  $[^3\text{H}]$ cAMP (39.8 Ci/mmol), and the indicated concentrations of cAMP or 8-N<sub>3</sub>-εcAMP in 50 mM potassium phosphate buffer, pH 7.0, in a total volume of 200 μl. (Right) Scatchard plots (23) of the data for cAMP (Upper) and for 8-N<sub>3</sub>-εcAMP (Lower).

this preparation. Thus, in the dark, the FPAL binds reversibly and with high affinity to the cAMP binding sites in the protein kinase preparation. We have observed the same effect in all other protein kinase preparations so far tested—e.g., rabbit muscle protein kinase peak II, beef heart protein kinase, and the erythrocyte membrane cAMP receptor. The nature of the buffer or the pH (within the range 4.0–7.4) did not significantly affect the binding and competition profiles.

Typically, cAMP-dependent protein kinases are activated by cAMP to catalyze the transfer of the terminal phosphate group from ATP to a protein acceptor (27). Using  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , we have studied the incorporation of  $^{32}\text{P}$  into protamine in the presence of varying concentrations of the activator. Fig. 2,

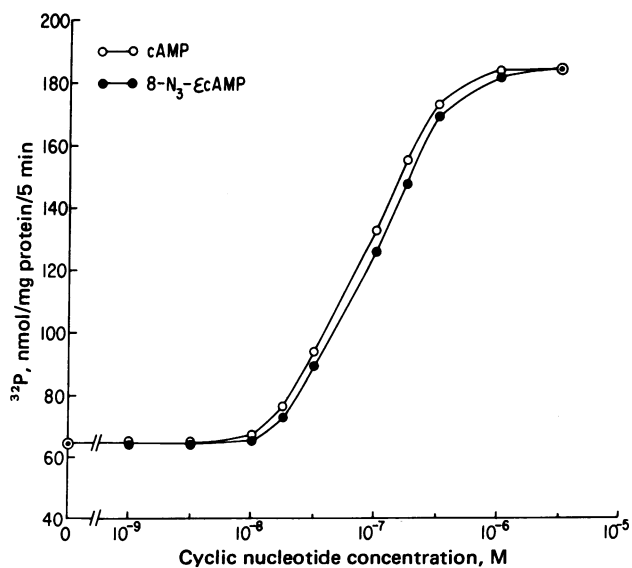


FIG. 2. Activation of beef heart protein kinase by cyclic nucleotides. The assay mixture (200 μl) contained 50 mM potassium phosphate buffer (pH 7.0), 50 μM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  ( $>10$  Ci/mmol), 10 mM MgSO<sub>4</sub>, 10 mM dithiothreitol, 2 mM theophylline, 0.25 mg of protamine sulfate, 0.5 mg of bovine serum albumin, 2 μg of the protein kinase preparation (added last), and the indicated concentrations of cAMP or 8-N<sub>3</sub>-εcAMP.

which illustrates the activation of beef heart cAMP-dependent protein kinase at varying concentrations of cAMP and of 8-N<sub>3</sub>-εcAMP, shows that the FPAL exhibits an activation profile close to that of cAMP itself. The mimicry by 8-N<sub>3</sub>-εcAMP of the enzymatic effects of cAMP suggests that 8-N<sub>3</sub>-εcAMP acts at the functional effector site for cAMP. Similar results were obtained with all other protein kinase preparations tested.

Photolysis experiments with 8-N<sub>3</sub>-εcAMP were carried out in Tris buffer as a potential radical scavenger to decrease nonspecific binding (9) and with the beef heart protein kinase preparation which, of the preparations available to us, contained the highest number of binding sites per milligram of protein.

Irradiation at 254 nm of the enzyme alone did not affect its cAMP binding or its catalytic activity. Fig. 3 shows the difference fluorescence spectrum of a beef heart protein kinase, samples A and B of Table 1. Raising the concentration of 8-N<sub>3</sub>-εcAMP to 0.5 μM did not alter the result. An identical difference fluorescence spectrum was obtained when samples of A and C were used. The fact that no fluorescence above that of the native preparation was observed when photolysis was performed in the presence of cAMP indicates that cAMP pro-

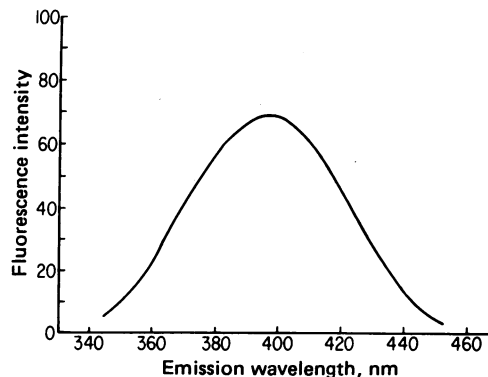


FIG. 3. Difference fluorescence spectrum of a beef heart protein kinase preparation photolabeled with 10 nM 8-N<sub>3</sub>-εcAMP in the absence and in the presence of 50 μM cAMP (samples A and B, respectively, of Table 1). Excitation wavelength was 320 nm. Excitation and emission slits were set at 5 nm.

Table 1. Photolysis experiments with a beef heart protein kinase preparation for fluorescence measurements

Sample	[8-N <sub>3</sub> -cAMP], nM	[cAMP], μM	Photolysis
A	10	0	+
B	10	50	+
C	0	0	+
D	10	0	-*

Each sample contained a total of 1 mg of protein in a final volume of 200 μl containing 50 mM Tris-HCl buffer, pH 7.4, and the indicated concentrations of cyclic nucleotides.

\* Sample was incubated and dialyzed in the dark.

protects the site from the photolytic covalent incorporation of the FPAL and that there is no detectable nonspecific labeling under the indicated conditions. This ceased to be the case when concentrations of 8-N<sub>3</sub>-cAMP exceeding 0.5 μM were used. Under such conditions, significant nonspecific labeling was observed. It is interesting to note that, when high concentrations of 8-N<sub>3</sub>-cAMP (>0.5 μM) were used, the labeled protein exhibited a (nonspecific) fluorescence emission maximum close to 420 nm, whereas at low concentrations (10 nM), at which the signal is specifically from the cAMP sites, the maximum was close to 395 nm, as shown in Fig. 3. Although possible contributions of 8-NH-substituent effects have not been evaluated in these experiments, the blue shift observed when the 8-NH fluorescent moiety is bound specifically to the cAMP receptor site of the protein is consistent with a hydrophobic environment at the cAMP binding site.

No fluorescence above that of the native protein was observed when the incubation mixture was not exposed to light and was dialyzed in the dark (sample D) or when the 8-N<sub>3</sub>-cAMP was exposed to light prior to the introduction of the protein. 8-N<sub>3</sub>-cAMP is, thus, a true photoaffinity label.

The results of the difference fluorescence polarization measurements (P<sub>D</sub>) are presented in Table 2. They indicate that: (i) samples B and C behave identically, in agreement with the fluorescence emission data, thereby confirming that cAMP protects the site from binding by the analog and that, with the specified low concentration of 8-N<sub>3</sub>-cAMP, no nonspecific fluorescent labeling could be detected; and (ii) the fluorescence of the nucleotide specifically bound at the cAMP binding site is strongly polarized. A likely interpretation of such P<sub>D</sub> values is that the cAMP receptor site in the beef heart protein kinase preparation strongly immobilizes the adenine moiety of the cyclic nucleotide.

Polyacrylamide gel electrophoresis of this protein kinase preparation on a 10% sodium dodecyl sulfate/polyacrylamide gel, according to Laemmli (28), resolved more than 30 protein bands. The FPAL apparently binds to a single site and probably to one protein within this mixture. The 8-N<sub>3</sub>-cAMP is, thus, a highly specific label that binds photolytically in sufficiently high yield to permit fluorescence studies of the particular designated site within a mixture without prior purification of the particular binding protein.

Studies on the cAMP receptor of the human erythrocyte membrane using 8-N<sub>3</sub>-[<sup>3</sup>H]cAMP also demonstrated the high binding specificity attainable with this FPAL. A single protein of apparent molecular weight (sodium dodecyl sulfate/polyacrylamide gel electrophoresis) of about 50,000 was labeled, and cAMP was absolutely competitive for the binding site.

## DISCUSSION

The basic information obtainable from fluorescence spectroscopy relates to the microenvironment around the fluorophore. Naturally occurring fluorescent constituents of macromolecular

Table 2. Difference fluorescence polarization (P<sub>D</sub>)

Samples*	P <sub>D</sub>
A vs B	0.35 ± 0.05
A vs C	0.35 ± 0.05
B vs C	0 ± 0.02

The "difference polarization" (P<sub>D</sub>) of the bound fluorophore was calculated (2) by using the expression:

$$P_D = \frac{(V_S - V_B) - t(H_S - H_B)}{(V_S - V_B) + t(H_S - H_B)}$$

in which V<sub>S</sub> and V<sub>B</sub> are the intensities of the vertically polarized component of emission from the sample and the blank, respectively, and H<sub>S</sub> and H<sub>B</sub> are the intensities of the horizontally polarized component of emission from the sample and the blank, respectively. The exciting light for V and H measurements was vertically polarized. The monochromator correction factor, t, was calculated from the ratio of vertically and horizontally polarized components of emission, exciting with horizontally polarized light. This correction factor was unity, within experimental error, for the wavelengths used.

\* Samples are as described in Table 1.

systems, intrinsic fluorophores, are usually of limited usefulness. Their frequent multiplicity, (e.g., of fluorescent amino acid residues) results in composite signals, and their precise locations within the system are difficult to determine and are beyond the control of the investigator. On the other hand, extrinsic fluorophores may be introduced into biological macromolecules either by a covalent reaction or by reversible binding due to their preferential affinity for certain domains. Most covalently bound extrinsic probes lack specificity for a single site. Of the noncovalently bound fluorophores, some show preference only for hydrophobic domains, whereas a few—being structural analogs of natural ligands or substrates—show greater specificity. An example of the latter are the ethenoadenosine derivatives (11, 12). This type of fluorophore often exhibits considerable specificity for a designated nucleotide binding site, yet the probe exists in an equilibrium of free and bound forms. In such a mixture the observed fluorescence signal is an average of the signals of the free and the bound ligand, weighted according to their contribution to the intensity, and the treatment of results is complex. One strategy to circumvent the inherent drawbacks of noncovalent extrinsic fluorophores is to develop fluorescent probes that can be attached to designated sites of proteins and satisfy desired spectroscopic requirements.

We have here illustrated FPAL in applications to cAMP receptor sites. In general, such labels should be structural analogs of natural substrates or ligands so designed that, while still retaining selective and high affinity of binding for the site to be studied, they also contain a reactive (or activable) functional group and a fluorophore (or a profluorophore). Probes of this type can achieve high specificity of probe attachment to the site without a need for purification of the target macromolecule prior to or after labeling. Provided that only one type of binding site exists, the FPAL, by virtue of its high affinity for that site, would bind specifically and irreversibly to that site even in complex mixtures.

Photoactivable reagents, first suggested by Singh *et al.* (29), that are chemically inert in the absence of light are of considerable potential usefulness (8). Light irradiation, after reversible binding in the dark, generates a reactive species (e.g., a nitrene from an azide) that does not require any particular reactive group in the macromolecular target site for covalent attachment. These properties of photoactivable labels eliminate the need for knowledge of the structure and composition of the residues in the target site.

This communication describes the synthesis and some ap-

plications of such a FPAL, 8-N<sub>3</sub>-cAMP, as a probe for cAMP binding sites. The data suggest that 8-N<sub>3</sub>-cAMP is a true and highly specific photoaffinity label that requires light irradiation for covalent binding to take place at the cAMP site. Even though a relatively large number of proteins were present in the several photolysis mixtures examined, no nonspecific labeling was detectable. Dialysis or gel filtration is effective in completely removing excess unreacted reagents. The fluorescence signal of the specifically bound FPAL is sufficiently strong to allow study of the nature of the binding site. The observed signal is blue-shifted and highly polarized, suggesting that the cAMP receptor sites of the beef heart protein kinase preparation are highly hydrophobic and immobilize the adenosine moiety of the nucleotide in a rigid conformation.

By using FPAL, one can, therefore, specifically attach an extrinsic fluorescent probe to a particular protein in a complex mixture of proteins or a supramolecular assembly—e.g., the plasma membrane. The observed fluorescence of such labeled proteins is more amenable to interpretation due to much better knowledge of the location of the reporter group within the studied assembly. Because, as noted above, 8-azido-*c*-derivatives of AMP, ADP, and ATP can be prepared, these compounds are potentially applicable as FPALs for a large number of adenine nucleotide-binding proteins.

We appreciate the help of Dr. Bruce A. Gruber, School of Chemical Sciences, University of Illinois, Urbana, IL. Research at the University of Illinois was supported by Research Grant GM-05829 from the National Institutes of Health, U.S. Public Health Service. The research at Harvard Medical School has been supported, in part, by U.S. Public Health Service Grant AM07300. G.D. is a recipient of a National Research Service Award, and K.S. is a National Institutes of Health Postdoctoral Fellow.

1. Stryer, L. (1968) *Science* **162**, 526–533.
2. Chen, R. F. & Edelhoch, H. (1976) *Biochemical Fluorescence: Concepts* (Dekker, New York), Vols. 1 and 2.
3. Haley, B. E. & Hoffman, J. F. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3367–3371.
4. Haley, B. E. (1975) *Biochemistry* **14**, 3852–3857.
5. Pomerantz, A. H., Rudolph, S. A., Haley, B. E. & Greengard, P. (1975) *Biochemistry* **14**, 3858–3862.
6. Koberstein, R., Cobianchi, L. & Sund, H. (1976) *FEBS Lett.* **64**, 176–180.
7. Koberstein, R. (1976) *Eur. J. Biochem.* **67**, 223–229.
8. Knowles, J. R. (1972) *Acc. Chem. Res.* **5**, 155–160.
9. Bayley, H. & Knowles, J. R. (1977) in *Methods in Enzymology*, eds. Jakoby, W. B. & Wilchek, M. (Academic, New York), Vol. 46, pp. 69–114.
10. Katzenellenbogen, J. A. (1977) in *Biochemical Actions of Hormones*, ed. Litwack, G. (Academic, New York), Vol. 4, pp. 1–84.
11. Leonard, N. J. & Tolman, G. L. (1975) *Ann. N.Y. Acad. Sci.* **255**, 43–58.
12. Thomas, R. W. & Leonard, N. J. (1976) *Heterocycles* **5**, 839–882.
13. Sheppard, G., Sheppard, H. C. & Stivala, J. F. (1974) *J. Labeled Comp.* **10**, 557–567.
14. Gilman, A. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 305–312.
15. Rubin, S. C., Ehrlichman, J. & Rosen, O. M. (1974) in *Methods in Enzymology*, eds. Hardman, J. G. & O'Malley, B. W. (Academic, New York), Vol. 38, pp. 308–315.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
17. Ikehara, M. & Uesugi, S. (1969) *Chem. Pharm. Bull.* **17**, 348–354.
18. Secrist, J. A., III, Barrio, J. R., Leonard, N. J., Villar-Palasi, C. & Gilman, A. G. (1972) *Science* **177**, 279–280.
19. Keeler, E. K. & Campbell, P. (1976) *Biochem. Biophys. Res. Commun.* **72**, 575–580.
20. Secrist, J. A., III, Barrio, J. R., Leonard, N. J. & Weber, G. (1972) *Biochemistry* **11**, 3499–3506.
21. Spencer, R. D., Weber, G., Tolman, G. L., Barrio, J. R. & Leonard, N. J. (1974) *Eur. J. Biochem.* **45**, 425–429.
22. Reiser, A. & Marley, R. (1968) *Trans. Faraday Soc.* **64**, 1806–1815.
23. Barrio, J. R., Sattsangi, P. D., Gruber, B. A., Dammann, L. G. & Leonard, N. J. (1976) *J. Am. Chem. Soc.* **98**, 7408–7414.
24. Brunswick, D. J. & Cooperman, B. S. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1801–1804.
25. Guthrow, C. E., Rasmussen, H., Brunswick, D. J. & Cooperman, B. S. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3344–3346.
26. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672.
27. Rubin, S. C. & Rosen, O. M. (1974) *Annu. Rev. Biochem.* **44**, 831–887.
28. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
29. Singh, A., Thornton, E. R. & Westheimer, F. H. (1962) *J. Biol. Chem.* **237**, PC3006.