A FLUORESCENT PHOTO-AFFINITY LABEL FOR CYCLIC AMP BINDING PROTEINS

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Summary

A fluorescent analog of cyclic AMP which contains the photoreactive azide group has been synthesized and characterized. Since upon photolysis the molecule becomes irreversibly bound to its acceptor site it should prove useful as a fluorescent probe for cyclic AMP binding sites.

In order to develop a fluorescent label for the active sites of proteins which bind cyclic AMP, we are engaged in an investigation of the azide derivative of $1,N_6$-ethenoadenosine 3':5' cyclic monophosphate. Haley et al. (1-3) have recently succeeded in using the azide derivatives of ATP and cyclic AMP to label the binding sites of proteins. The possibility of combining the photosensitive activity of the azide group with the fluorescent properties of the ethenoadenosine derivatives has led to the synthesis of the molecule described here. The fluorescence of the ethenoadenosine derivatives has been studied and described extensively by Leonard and coworkers (4-7). We propose to use these fluorescent properties to probe the active sites of proteins which bind cyclic AMP.

Materials and Methods

Adenosine 3':5'-cyclic monophosphoric acid was obtained from Sigma Chemical Co. and chloroacetaldehyde diethyl acetal from Aldrich Chemical Co.

Thin-layer chromatography was performed on Eastman cellulose sheets (13255) and developed in either solvent system A (n-butanol-acetic acid - H_2O, 5:2:3, v/v), B (isopropanol - NH_3(aq)-H_2O, 7:1:2, v/v) or C (isobutyric acid-

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NH_3(aq)-H_2O, 75:1:24, v/v). Ultraviolet spectra were determined on a Perkin Elmer - Coleman Model 124D double - beam spectrophotometer and infrared spectra on a Perkin Elmer Model 700 spectrophotometer. Fluorescence measurements were made on a Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer. Proton magnetic resonance spectra were obtained on a Varian XL100 spectrometer. Water was redistilled in glass vessels. Dimethylformamide was distilled under vacuum and stored over molecular sieves. Other materials were of reagent grade and used without futher purification.

8-Bromoadenosine 3':5'-cyclic monophosphate (1).

8-Br-cAMP* was prepared according to the procedure of Muneyama (8) as follows: 0.2510 g (0.76 mmoles) of cyclic AMP was dissolved in 0.4 ml of 2M NaOH and 0.50 ml of 1M acetate buffer was added. 0.1736 g (2 mmoles) of bromine was dissolved in 6.0 ml of 1M acetate buffer and added to the cyclic AMP solution. The reaction was allowed to stir at room temperature overnight. The resulting precipitate was filtered and washed with water. yield 0.2193 g (70.2%)

8-Azidoadenosine 3':5'-cyclic monophosphate (2).

8-N_3-cAMP was prepared according to the procedure of Muneyama (8) as follows: 0.1199 g (0.29 mmoles) of 8-Br-cAMP and 0.0422 g (0.65 mmoles) of sodium azide was dissolved in 12 ml of dry dimethylformamide and stirred overnight at 75°C. The solvent was removed on a rotary evaporator. The residue was dissolved in a minimal amount of 1M NH_3 and the pH adjusted to <2 with 2M HCl. The product crystallized upon standing at 4°C. The crystals were filtered, washed with water and dried under vacuum. yield: 0.0614 g (57.4%)

8-Azido-1,N_6-ethenoadenosine 3':5'-cyclic monophosphate (3)

This reaction was carried out according to the procedure of Roberts et al. (9) with the following modifications. 0.0400 g(0.102 mmoles) of 8-N_3-cAMP was

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* Abbreviations: 8-Br-cAMP, 8-Bromoadenosine 3':5'-cyclic monophosphate; 8-N_3-cAMP, 8-Azidoadenosine 3':5'-cyclic monophosphate; ε-cAMP, 1,N_6-Ethenoadenosine 3':5'-cyclic monophosphate; cyclic AMP, Adenosine 3':5'-cyclic monophosphate; DSS, Sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

combined with 6 ml of ∿1M aqueous chloroacetaldehyde, which had been prepared according to the procedure of Secrist et al. (6) and stored below 5°C prior to use. The pH of the reaction mixture was brought to 4.5 and maintained at that value as the reaction proceeded by the addition of 0.1M NaOH from a pH stat (Radiometer TTT11 equipped with Autoburet ABU12 and Titration Assembly TTA3). The reaction was complete, judged by the cessation of base uptake, after 96 hrs. at 25°C. The reaction mixture was lyophilized, redissolved in water and extracted with diethyl ether to remove excess chloroacetaldehyde. The aqueous layer was lyophilized, redissolved in a minimal amount of water and the product was precipitated with isopropanol. The precipitate was filtered and washed with diethyl ether. yield: 0.0463 g (>99%).

Results and Discussion

Thin-layer chromatography in three different solvent systems (table I) was used to monitor the reactions and detect impurities of the nucleotide derivatives. In all cases products appeared as a single spot and were considered to be free of trace impurities. Both ε-cAMP and 8-N_3-ε-cAMP are intensely fluorescent and can be easily distinguished from the other cyclic AMP derivatives. ε-cAMP appears as a bluish fluorescent spot while 8-N_3-ε-cAMP fluoresces with a green-blue color.

The infrared spectrum of 8-N_3-ε-cAMP shows the characteristic bands for -N_3 stretching vibrations at 2171 cm^{-1} (s) and 1280 cm^{-1} (w).

The proton magnetic resonance spectra of cyclic AMP, ε-cAMP and 8-N_3-ε-cAMP are summarized in table II. The absence of an absorbance for the hydrogen on C_8 indicates substitution by the azide group at this position. Introduction of the etheno bridge is shown by the two doublets at 7.94 δ and 8.37 δ.

A comparison (table III) of the UV spectra of 8-N_3-cAMP and 8-N_3-ε-cAMP shows a shift of the absorbance maximum from 281 nm to 290 nm and a decrease in the molar extinction coefficient. This decrease is characteristic of etheno-adenosine derivatives (6). A plot of extinction coefficient at 290 nm vs. pH

TABLE I

R_f Values for Cyclic AMP and Derivatives

Compound	Solvent A[a] R_f	Solvent B[a] R_f	Solvent C[a] R_f
cyclic AMP	0.47	0.48	0.69
8-Br-cAMP	0.62	0.60	0.68
8-N_3-cAMP	0.63	0.61	0.67
8-N_3-ε-cAMP[b]	0.62	0.65	0.65
ε-cAMP[b]	0.42	0.53	0.56

[a] see text for composition of solvents
[b] fluorescent spots

TABLE II

Proton Magnetic Resonance Spectra
Chemical shifts (δ)

Compound	C_2-H	C_8-H	C_{10}-H	C_{11}-H	$C_{1'}$-H
cyclic AMP[a]	8.24 s(2)		-	-	6.16 s(1)
ε-cAMP[b]	9.21 s(1)	8.43 s(1)	8.06 d, J=1Hz(1)	7.68 d,J=1Hz(1)	6.32 s(1)
8-N_3-ε-cAMP[b]	9.37 s(1)	-	8.37 d, J=1Hz(1)	7.94 d,J=1Hz(1)	6.26 s(1)

[a] δ values relative to internal DSS.
[b] δ values relative to external DSS.

TABLE III

Ultraviolet Absorption Data

Compound	pH	λ_{max}(nm)	ε x 10^{-3}($M^{-1}cm^{-1}$)
8-Br-cAMP	6.0	264	35
8-N_3-cAMP	6.0	281	13
8-N_3-ε-cAMP	6.0	290	3.5
	2.0	290	5.5

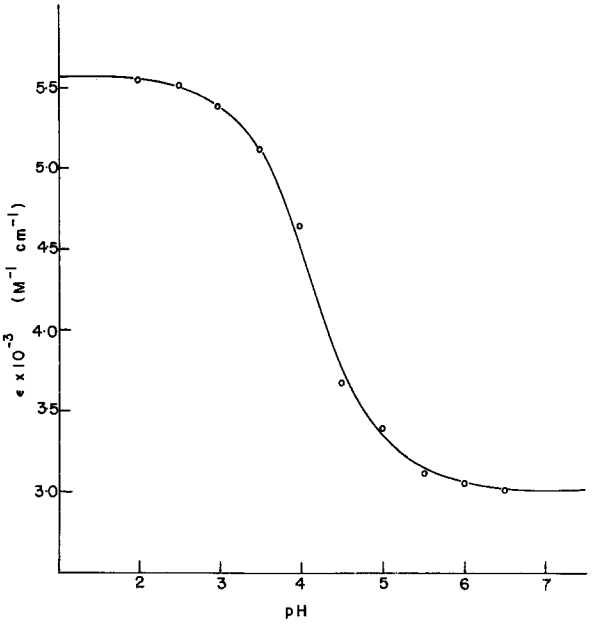


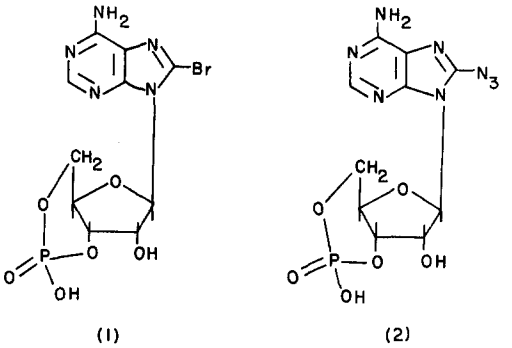
Figure 1. Cyclic AMP Drivatives

(figure 2) places the pK_a of 8-N_3-ε-cAMP at 4.17, slightly higher than that of ε-cAMP (6).

The fluorescence emission spectrum of (3) at pH 5.5 shows a single unresolved band with a maximum at 402 nm when the solution is excited at 300 nm. The excitation spectrum, monitored at 400 nm, shows maxima at 280 nm, 310 nm and 360 nm.

The photoreactivity of the molecule was demonstrated by its ability to bind irreversibly to cellulose thin-layer plates when irradiated at 253.7 nm. The 8-N_3-ε-cAMP fluorescent spot remained at the origin in all solvent systems after irradiation, whereas a nonirradiated control spot moved significantly in every system.

The good yields obtained and the ease of purification of products, as well as the fluorescent characteristics of the product, makes attractive the use of this molecule as an irreversibly bound fluorescent probe. Such studies of cyclic AMP binding proteins are now in progress in these laboratories.



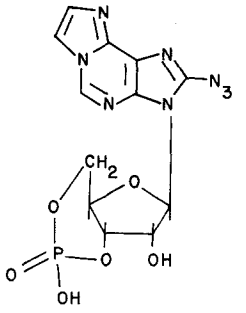


Figure 2. Spectrophotometric titration of 3 at 290 nm. The solid line is the theoretical curve for pK_a=4.17, ε(low pH) = 5580 $M^{-1}cm^{-1}$, ε(high pH) = 3000 M^{-1} cm^{-1}. Points are experimental values.

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