Biochimica et Biophysica Acta, 569 (1979) 145—152

BBA 68781

DETERMINATION OF BINDING PARAMETERS OF CYCLIC AMP AND ITS ANALOGS TO CYCLIC AMP-DEPENDENT PROTEIN KINASE BY THE FLUORESCENT PROBE METHOD

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(Received November 29th, 1978)

Key words: Protein kinase; Cyclic AMP; Fluorescent probe; Binding parameter

Summary

The method for determination of dissociation constants for cyclic AMP and its analogs bound to cyclic AMP-dependent protein kinase from pig brain is described. The technique for measuring the binding parameters of the ligands is based on the changes in the fluorescent spectrum of etheno cyclic AMP once it is bound to protein kinase. The dissociation constants for a number of non-fluorescent cyclic AMP analogs were determined in the competitive displacement of etheno cyclic AMP by these analogs. The number of cyclic AMP-binding sites in the pig brain protein kinase was found to be 2.2; no cooperativity was observed upon binding. The holoenzyme complex (M_r = 180 000) of the protein kinase under study was established to have the stoichiometry of R_2C_2 type under native conditions.

Introduction

The biological role of cyclic AMP as an allosteric regulator of cyclic AMP-dependent protein kinases is generally accepted. The cyclic AMP-dependent protein kinases have been reported [1—4] to consist of catalytic (C) and regulatory (R) subunits. In the mechanism of activation of cyclic AMP-depen-

Abbreviations: cyclic AMP, adenosine 3′,5′-(cyclic)phosphate; 8-bromo cyclic AMP, 8-bromoadenosine 3′,5′-(cyclic)phosphate; 8-aminomethyl cyclic AMP, 8-aminomethyladenosine 3′,5′-(cyclic)phosphate; 8-aminobenzyl cyclic AMP, 8-aminobenzyladenosine 3′,5′-(cyclic)phosphate; etheno cyclic AMP, 1,N^6-ethenoadenosine 3′,5′-(cyclic)phosphate; cyclic GMP, guanosine 3′,5′-(cyclic)phosphate; cyclic IMP inosine 3′,5′-(cyclic)phosphate; cyclic dAMP, 2′-deoxyadenosine 3′,5′-(cyclic)phosphate; TEMED, *N′,N′,N′,N′*-tetramethylethylenediamine.

dent protein kinases, cyclic AMP is believed to cause a shift in the equilibrium towards the preferential dissociation of the holoenzyme into free regulatory and active catalytic subunits [5—8]. This shift can be accounted for by the formation of the stable noncovalent R-cyclic AMP complex.

Hitherto, the stoichiometry of R_nC_n complexes, the number of cyclic AMP-binding sites in the holoenzyme and cooperativity of binding have still remained obscure. One of the approaches to elucidating some of these problems can be the utilization of the fluorescent probe method; the fluorescent cyclic AMP analog (etheno cyclic AMP) being used as such a probe.

Since the first publications on the synthesis of $1,N^6$-ethenonucleotides [9—11], the successful applications of the latter as fluorescent analogs of nucleotides in numerous enzyme systems have been reported [12—16].

The possibility of selective excitation of the etheno chromophor, negligible distortions of the results due to inner filter effects at high protein concentrations, the long fluorescent lifetime (20 ns) [17], high sensitivity of the method and, at last, sufficiently high affinity permit the use of etheno cyclic AMP as an allosteric effector of the cyclic AMP-dependent protein kinase.

Materials and Methods

Cyclic AMP derivatives

The following cyclic AMP analogs were used in the experiments: 8-bromo cyclic AMP, 8-aminoethyl cyclic AMP, 8-aminobenzyl cyclic AMP, cyclic GMP, cyclic IMP, cyclic dAMP purchased from Sigma; cyclic AMP and etheno cyclic AMP from P.L. Biochemicals.

Enzyme purification and phosphotransferase assay

Cyclic AMP-dependent protein kinase from pig brain was isolated and purified by the previously described method [18]. Purification of the regulatory subunit was carried out according to Ref. 19.

Phosphotransferase activity of the protein kinase was measured as described in Ref. 18.

Molecular weight determination

Molecular weights of the holoenzyme and its subunits were determined by polyacrylamide gradient gel electrophoresis technique according to Margolis and Kenrick [20]. The linear gradient of the gel concentration within the range from 4 to 26% was prepared on a Gradient Mixer GM-1 and a gel slab casting apparatus (Pharmacia). Electrophoresis was conducted in 0.1 M Tris/borate buffer (pH 8.3) at 150 V, 50 mA for 14 h using a gel electrophoresis apparatus GE-4 (Pharmacia). The protein markers (Protein Calibration Kit, Serva) and the protein kinase preparations were applied to the slab in 30% sucrose solution.

After electrophoresis, the gels were stained with 0.2% Coomassie brilliant blue (B.D.H.) for 1 h. Thereupon, the gel was electrophoretically destained in a methanol/acetic acid/water mixture on a gel destainer GD-4 (Pharmacia). To determine molecular weights of the holoenzyme and its subunits, the electrophoresis data were plotted in the coordinates: log M_r versus the migration distance of the protein on the slab, in cm.

Fluorescence measurements

All fluorescence measurements were performed on a SPF-1000CS spectrofluorimeter (Aminco) at 20° C.

To increase the accuracy of the measurements, the spectra were scanned in the differential mode. Titration of the protein kinase holoenzyme was carried out by adding constant-volume aliquots (2 μl) of the probe solution, and the variations in the intensity of fluorescence were recorded.

The process of binding of a small ligand to a macromolecule is known to be described by the equation of adsorption isotherm:

$$r = \frac{n \cdot 1/K \cdot A}{1 + 1/K \cdot A}, \tag{1}$$

where r is the relative concentration of the bound ligand, n is the number of binding sites on the protein molecule, K is the protein-ligand dissociation constant, and A is the molar concentration of the unbound ligand.

For plotting the fluorescent titration data obtained in our experiments in Scatchard coordinates [21], Eqn. 1 is transformed to:

$$r/A = \frac{n}{K} - \frac{r}{K}. \tag{2}$$

When in the system there exists a second ligand, nonfluorescent in the region of the first and competing for the binding sites on the protein, Eqn. 2 can be written as follows:

$$r/A = \frac{n}{K}\left(\frac{K_L}{K_L + L}\right) - \frac{r}{K}\left(\frac{K_L}{K_L + L}\right), \tag{3}$$

where K_L and L are the dissociation constant and the concentration of the nonfluorescent ligand, respectively.

While in the absence of the competitive ligand (Eqn. 2) the intercepts of the straight line with the abscissa and ordinate axes of Scatchard plots give K and n values, respectively, the presence of the competitive ligand in the system and the change in the concentration of the latter in accordance with Eqn. 3 will give (in Scatchard coordinates) a number of straight lines with different intercepts with abscissa and different slopes.

If we designate the intercept with the ordinate axis by

$$Y = n/K\left(\frac{K_L}{K_L + L}\right), \tag{4}$$

the data of the competitive binding studies permit to obtain a secondary plot $1/Y$ vs. L.

From the slope of the secondary plot, the value of K_L can be calculated.

Results

Fig. 1 shows the slab of polyacrylamide gradient gel electrophoresis of the holoenzyme and its subunits of the pig brain protein kinase together with a number of protein markers of the known molecular weights. The molecular weight values for the holoenzyme, its regulatory and catalytic subunits were

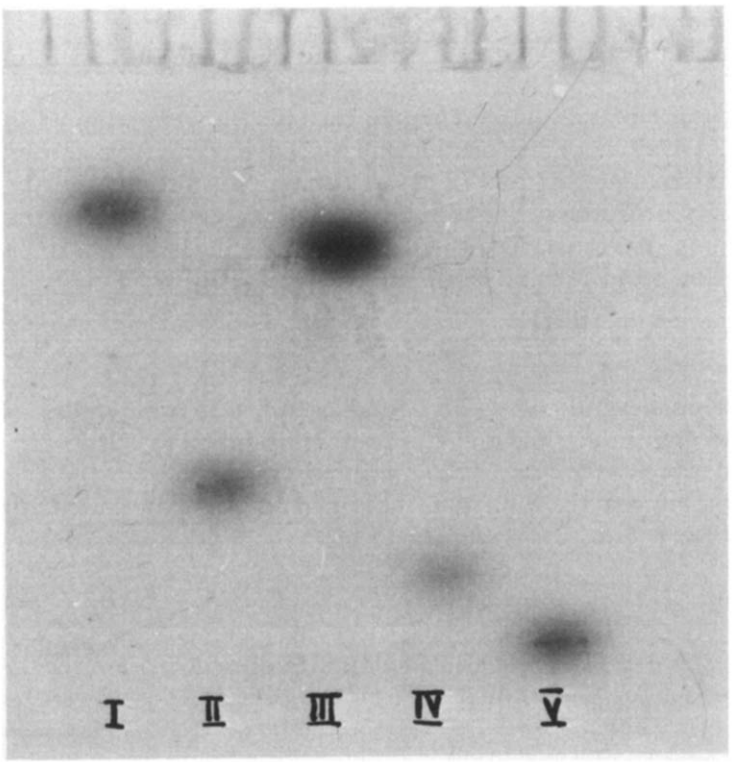


Fig. 1. Polyacrylamide gradient gel electrophoresis of protein kinase together with protein markers. Bands on the slab correspond to: (1) catalase (M_r = 240 000); (2) bovine serum albumin (M_r = 68 000); (3) holoenzyme; (4) regulatory subunit; (5) catalytic subunit.

found from the electrophoresis data plot (see Materials and Methods) to be 180 000; 50 000 and 40 000, respectively.

From our kinetic experiments, the activation constants (K_α) for etheno

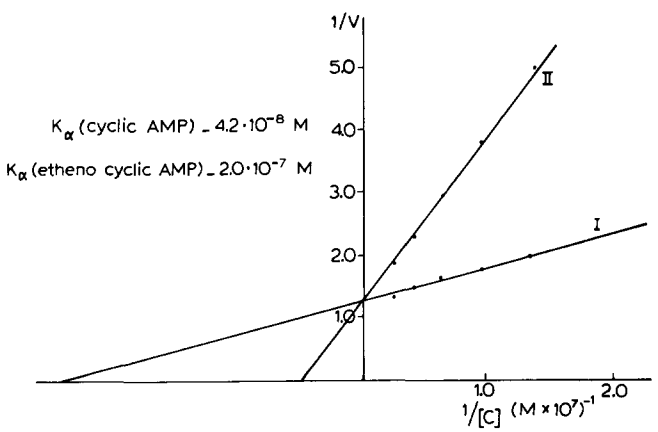


Fig. 2. Determination of K_α of cyclic AMP (I) and etheno cyclic AMP (II).

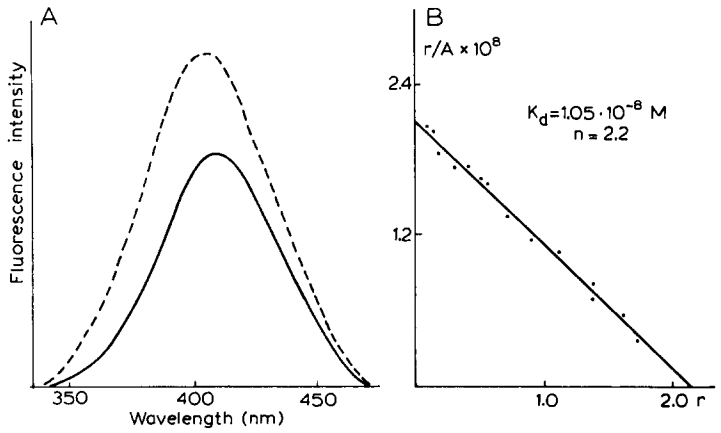


Fig. 3. Etheno cyclic AMP binding to protein kinase. A. Emission spectra of etheno cyclic AMP (10^{-7} M) (———) and etheno cyclic AMP (10^{-7} M) in the presence of 10^{-7} M protein kinase (- - - - -) . Excitation at 310 nm, slits 5 nm. B. Scatchard plot of etheno cyclic AMP binding to protein kinase.

cyclic AMP and cyclic AMP with respect to the protein kinase were calculated as $2.0 \cdot 10^{-7}$ M and $4.2 \cdot 10^{-8}$ M, respectively (Fig. 2).

Fig. 3 shows the results of fluorescent titration of the protein kinase with etheno cyclic AMP.

As seen in Fig. 3a, the addition of the fluorescent cyclic AMP analog to the

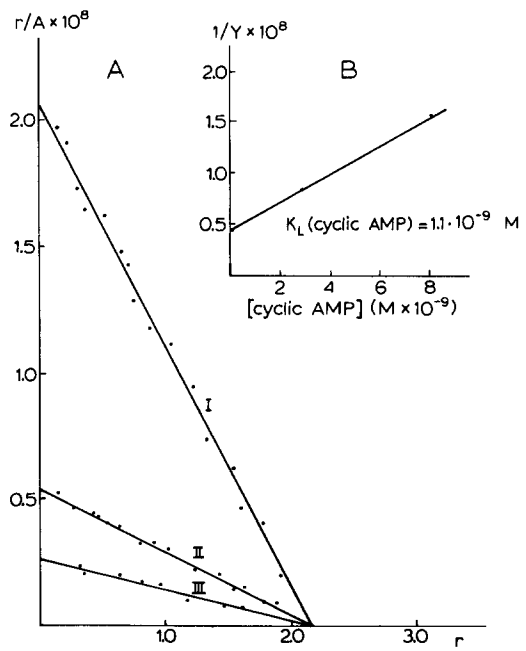
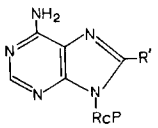
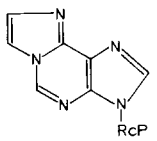
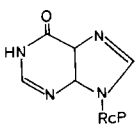
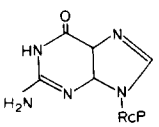
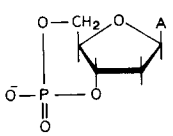


Fig. 4. Competitive displacement of etheno cyclic AMP by cyclic AMP. A. Scatchard plot. Cyclic AMP concentrations: I, 0; II, $3 \cdot 10^{-9}$ M; III, $8 \cdot 10^{-9}$ M. B. Secondary plot $1/Y$ versus cyclic AMP concentration.

TABLE I

THE DISSOCIATION CONSTANTS FOR CYCLIC AMP ANALOGS

RcP, ribose 3′,5′-phosphate.

Formula	R′	No.	$K_d \times 10^8$
	-H	I	0.11 ± 0.01
	-Br	II	4.70 ± 0.20
	$-NHCH_3$	III	2.92 ± 0.15
	$-NHCH_2C_6H_5$	IV	1.43 ± 0.10
		V	1.05 ± 0.15
			
		VI	4.02 ± 0.20
		VII	11.90 ± 0.10
		VIII	31.00 ± 0.20

protein kinase caused the small blue shift (4—5 nm) and enhancement of the fluorescence intensity of etheno cyclic AMP.

The fluorescent titration data of the protein kinase with etheno cyclic AMP provided the basis for plotting the adsorption isotherm in Scatchard coordinates (Fig. 3b). From this plot, the dissociation constant value for etheno cyclic AMP ($1.05 \cdot 10^{-8}$ M) and the number of binding sites in the enzyme (2.2) were determined.

In Fig. 4a, the competitive displacement of the fluorescent analog by cyclic AMP from the binding site of the enzyme is demonstrated. Further, the secondary plot (Fig. 4b) obtained according to Eqn. 4 permitted to determine the dissociation constant for the cyclic AMP-protein kinase interaction. The dissociation constants for a number of cyclic AMP analogs were calculated in a similar way.

These data are summarized in Table I.

Discussion

Determination of the binding parameters for the cyclic AMP analogs required the exact specification of the molecular weight of the holoenzyme

which in its turn allowed us to elucidate the stoichiometry of the holoenzyme complex. At present, the dimer R_2C_2 which dissociates into two free catalytic subunits (2C) and the dimer of the regulatory subunit (R_2) is believed to be the main form of protein kinases in solution [22,23]. The property of the holoenzyme to change its structure in solution under different conditions creates difficulties for determination of its molecular weight. Thus, in our previous gel filtration experiments [18], the molecular weight of the holoenzyme equal to 120 000 was determined; the molecular weights of the regulatory dimer and the catalytic subunit amounting to 90 000 and 40 000, respectively. Hence, the protein obtained seemed to be the complex of R_2C stoichiometry that was, probably, due to the dissociation of one catalytic subunit. In the present study, polyacrylamide gradient gel electrophoresis which is a widely used method in current investigations [20,24—26] has been employed for the molecular weight determination. This method is advantageous owing to the possibility to characterize the enzyme under native conditions. The data obtained in our experiments indicated that the molecular weights of the holoenzyme, its regulatory and catalytic subunits were 180 000; 50 000 and 40 000, respectively. Therefore, it becomes apparent that the enzyme in solution exists in the form of R_2C_2 complex which is in good conformity with the data obtained on the subunit composition of protein kinases from other tissues [23,27].

The main requirement for etheno cyclic AMP as a specific probe which should be checked before the fluorescent measurements is its ability to activate the holoenzyme.

The effect produced by etheno cyclic AMP on the histone H1 phosphorylation (Fig. 2) evidences high activating capacity of the former, thus suggesting the identity of the mechanisms of activation of the phosphotransferase reaction by cyclic AMP and its fluorescent analog.

According to the data of fluorimetric titration of the protein kinase with etheno cyclic AMP (Fig. 3b), the constant for the enzyme-ligand dissociation was equal to $1.05 \cdot 10^{-8}$ M; 2.2 ligand molecules binding per one mole of the enzyme (M_r = 180 000). The number of binding sites found provides an additional support for the dimeric structure R_2C_2. The Scatchard plot represented an unbroken straight line that was indicative of the lack of cooperativity of cyclic nucleotide binding.

The experimental results enabled us to utilize etheno cyclic AMP as a fluorescent probe competitive with cyclic AMP analogs. As follows from Table I, the dissociation constant for cyclic AMP is equal to $1.12 \cdot 10^{-9}$ M which is in agreement with the value obtained by the method of cyclic [^{3}H]-AMP binding by filter technique [28]. The data of the table led us to a number of conclusions about the effect produced by modification of some fragments of the cyclic AMP molecule on the affinity of the analog for the binding site. As should be expected, cyclic AMP had the highest affinity for the protein kinase. The modification of any fragment of the cyclic AMP molecule caused a change in the value of dissociation constant at least by an order. The affinity of the 8-substituted analogs (compounds II—IV) for the enzyme increased with the increase in hydrophobicity of the corresponding substituent. Substitution of the oxy-group for the exo-amino group of the purine ring or of the guanine ring for the adenine ring significantly decreased the affinity. In this connection, the

observed difference between the values of the constants for cyclic AMP and cyclic GMP can be assumed to be essential for the regulation of biological processes. On several occassions, cyclic AMP and cyclic GMP were demonstrated to be antagonists in various metabolic processes [29]. The greatest change in the affinity is observed upon substitution of deoxyribose for the ribose ring (VIII). It may be of interest to note the interrelation between the variations in binding properties of the analogs I—VIII and changes in their activation constant values [30].

Our investigations allowed us to propose the method of competitive displacement of etheno cyclic AMP by cyclic AMP analogs from the allosteric site of protein kinase. This method was found to be very convenient for determination of the dissociation constants for different cyclic nucleotide analogs and investigation of the cyclic AMP-binding site of the enzyme. Moreover, the suggested technique permits to assay the cyclic AMP-binding activity of protein kinase. The method in question proved to be less labour-consuming and more accurate as compared with the known method for determination of binding parameters using cyclic [^{3}H]AMP and allowed to estimate the true equilibrium values of the dissociation constants directly in solution as well as to develop some new approaches to investigating the process of protein kinase dissociation under the action of cyclic AMP.

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