A FLUORESCENT METHOD FOR THE DEMONSTRATION OF CYCLIC 3′, 5′-AMP PHOSPHODIESTERASE ACTIVITY IN POLYACRYLAMIDE GEL

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1. Introduction

The importance of adenosine 3′,5′-monophosphate (cyclic AMP) as a mediator for hormonal action needs no emphasis [1]. The level of cAMP is regulated by both the synthetic enzyme adenylate cyclase and the hydrolytic enzyme cAMP phosphodiesterase. While many useful methods have been developed for the biochemical assay of cAMP phosphodiesterase activity [2–6], only a few of them have been adapted for the localization of its activity in gel electrophoresis. Thus, an elegant but involved fluorescent method on starch gel was developed by Monn and Christiansen [7]. This method utilizes NADH in a cyclic coupling procedure and requires the addition of three auxiliary enzymes. Hrapchak and Rasmussen [8], on the other hand, used a gel method developed by Bikle for staining this enzyme. Only one enzyme, 5′-nucleotidase, is added to hydrolyze the primary product 5′-AMP to adenosine and P_i, which is trapped as a white precipitate of calcium phosphate. Both of these methods are indirect and pose problems in their application to tissue extracts where other nucleotide hydrolyzing enzymes are unavoidably present.

Recently, a new fluorescent analog of cyclicAMP, 1,N^6-etheno-2-aza-adenosine 3′,5′-phosphate (cyclic 2-aza-ϵ-AMP) was synthesized in our laboratory [9] and found to be an efficient substrate for cAMP phosphodiesterase [10]. The adaptation of this new substrate for the direct fluorescent visualization of cAMP phosphodiesterase should be possible without the addition of any other enzyme. The present communication reports the new fluorescent method and its application, as well as a comparison to the two known methods on beef heart cAMP phosphodiesterase.

While cyclic 2-aza-ϵ-AMP has met all of the substrate requirements we sought for this purpose, both this substrate and the primary product of enzymatic hydrolysis, 2-aza-ϵ-AMP, are soluble. Therefore, it was necessary for us to insolubilize 2-aza-ϵ-AMP with a mixture of $ZnSO_4$ and $Ba(OH)_2$, first developed by Caputto [11] for adenosine kinase and later used by Krishna et al. [12,13] for adenylate cyclase. A recent modification by Chen et al. [14] showed that $ZnCO_3$ could also be used. Like 5′-AMP, 2-aza-ϵ-AMP was found to be quantitatively insolubilized by either system, whereas both cyclic 2-aza-ϵ-AMP and cAMP are soluble.

2. Materials and methods

Beef heart cAMP phosphodiesterase, *Crotalus atrox* venom and cAMP were obtained from Sigma Chemical Co. Cyanogum 41 was purchased from the E-C Apparatus Corp. Bromophenol blue was a product of La Motte Chemical Product Co. Cyclic 2-aza-ϵ-AMP was synthesized as described previously [9,10].

2.1. *Polyacrylamide gel electrophoresis*

Electrophoreses were run at 0–4°C with 7% Cyanogum 41 in running buffer (0.09 M Tris–boric acid, pH 9.4), and a current density of 2 mA per tube as described previously [10]. Unless otherwise stated, each tube was loaded with 10 μl (0.2 unit) of cAMP

phosphodiesterase. One enzyme unit is defined by the supplier as the amount of enzyme that will convert 1 μmole of cAMP to 5′-AMP per min at pH 7.5 and 30°C. Electric field was applied for about 60 min. Bromophenol blue and human serum albumin were used as markers.

2.2. *Visualization of cAMP phosphodiesterase activity*

2.2.1. Fluorescent method with $ZnCO_3$

The gels were incubated in succession at room temperature (ca. 22°C) with: (1) 0.1 M $NaHCO_3$ containing 14 mM Na_2CO_3 (pH 9.2), 15 min.; (2) 0.1 M $ZnSO_4$ (pH 5.3), 15 min; (3) 0.1 M $NaHCO_3$ containing 14 mM Na_2CO_3, 15 min.; (4) 40 mM Tris–HCl, pH 7.8, 3 X 30 min each. They were then incubated at 37°C with 0.5 mg/ml cyclic 2-aza-ε-AMP in 40 mM Tris–HCl buffer, pH 7.8, containing 1 mM $MgCl_2$ for 18 hr. The gels were destained with frequent changes of H_2O. Destaining required 8–12 hr for maximal visualization under UV light (366 nm). The gels were also scanned on a Turner Fluorometer model 111 equipped with a TLC Scanner, model 2. The excition filter was 7–60 and the emission filters were 2A and neutral density 20% T. The gels could be stored in H_2O with a few crystals of thymol. For the determination of the sensitivity of this method serial dilutions of the enzyme were used.

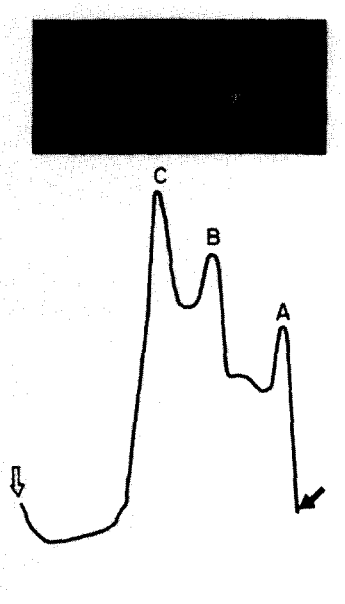

Fig. 1. *Top:* Polyacrylamide gel electrophoresis of 0.2 unit of partially purified beef heart cyclic nucleotide phosphodiesterase (Sigma). After electrophoresis, the gel was coated with $ZnCO_3$ as described in Materials and methods, incubated with cyclic 2-aza-ε-AMP, and destained with water. *Bottom:* Recorder trace of the gel scan. Solid arrow = top of gel; Open arrow = bottom of gel; Excitation filter = 7–60; Emission filter = 2A + 20% T; and Slit = 30X.

2.2.2. Fluorescent method with $BaSO_4$

The gels were incubated in succession at room temperature with 3.2 mM $Ba(OH)_2$ (pH 11.5), 15 min; 0.1 M $ZnSO_4$, 15 min; and 40 mM Tris–HCl (pH 7.8), 3 X 30 min. The gels were then incubated with cyclic 2-aza-ε-AMP and destained as above.

2.2.3. Bikle's Method

The gels were incubated at 37°C in a solution containing 40 mM Tris–HCl (pH 7.8), containing 15 mM $CaCl_2$, 1.8 mM $MgSO_4$, 1.5 mM cAMP and 200 μg of *Crotalus atrox* venom in a total volume of 2.8 ml.

3. Results

The fluorescent bands obtained by the present method with $ZnCO_3$ are shown in fig. 1. Fluorescence is observed on top of the gel and two other bands can be easily seen. The fast moving band (band C) is slightly diffuse. The relative intensity of these bands as obtained from scanning the gel is: band C > slow moving band (band B) > top of the gel (band A). The fluorescent bands correspond in position with the calcium phosphate bands obtained by the method of Bikle, except that the calcium phosphate bands are very weak and much more diffuse. Therefore, the fluorescent bands do represent the known activities of cyclic nucleotide phosphodiesterase, even though a synthetic substrate is used. The visual detection limit by this method is about 0.005 enzyme units. When the $BaSO_4$ method is used, only bands A and B are seen at much lower intensities. The disappearance of band C suggests that this band may be more labile to the alkaline condition of $Ba(OH)_2$ or that it is more susceptible to inhibition by Ba^{2+} ions.

4. Discussion

Two forms of cAMP phosphodiesterase from heart

have been reported by various workers. These forms differ in affinity for cAMP. Thus, Beavo et al. [15] reported two apparent K_m values of 25 μM and 0.8 μM for cAMP in a 1000 g particulate fraction of beef heart, although the partially purified enzyme had only the high K_m value. Thompson and Appleman [16] also found in a sonically disrupted 20 000 g supernatant fraction of rat heart two activities for cyclic AMP with K_m values of 8.67×10^{-5} M (fraction II) and 3.85×10^{-6} M (fraction III). In our own study [10] of the beef heart preparation similar to that of Butcher and Sutherland [17], two apparent K_m values of 503 μM and 15 μM were found for the new substrate cyclic 2-aza-ϵ-AMP. Since the concentration of this fluorescent analog used in the gel staining mixture (1.35 mM) was higher than the K_m of either form, both forms should be visualized. The present, simple and sensitive staining technique gives sharper and more easily discernible bands that can be photographed, than the calcium phosphate method of Bikle. The advantage of the present method over the Monn and Christiansen method lies also in that it does not show non-specific staining with adenosine triphosphatase, in addition to the sharper bands. Furthermore, since no auxiliary enzymes are required, this method can be easily adapted to the localization of cAMP phosphodiesterase in tissue extracts. In addition, the study of this enzyme in pathological tissues, (which are often available only in limited quantities), is made easier.

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