LIMITATIONS OF AFFINITY CHROMATOGRAPHY: SEPHAROSE-BOUND CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE*

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1. Introduction

The nucleotide cyclic 3′,5′-adenosine monophosphate (c-AMP) is a trigger for many biologically important reactions [1]. One of its main functions appears to be the activation of phosphokinase by associating with the regulatory proteins (R) of the inactive complex enzyme and release of the enzymatically active catalytic subunits (C) [2]:

$$\underset{\text{inactive phospho-kinase}}{\{R,C\}} \xrightarrow{\text{c-AMP}} \underset{\text{active phospho-kinase}}{\{R,\text{c-AMP}\}} + C$$

Many derivatives of c-AMP have been prepared and investigated, some being even more effective in this respect than the parent compound [3].

In this communication, we describe the synthesis of a new derivative of c-AMP ("Sepharose-bound c-AMP") designed for use in affinity chromatography. As the mean binding constant of c-AMP for "binding proteins" is rather high ($K_{ass} = 4.5 \times 10^8$ l/mole [4]), isolation of such proteins and of regulatory subunits from solutions containing inactive phosphokinases, and the activation of phosphokinases by mere filtration through this material were envisaged **.

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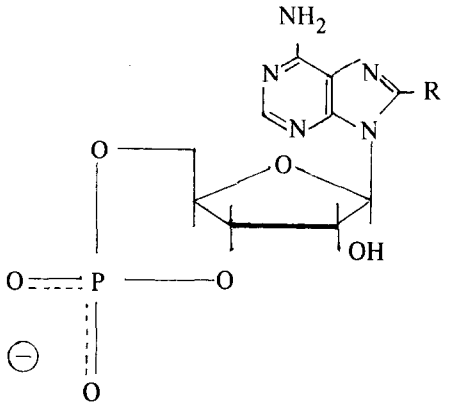
** After completion of the experimental part of this work, a paper by Wilchek et al. [5] on the same subject appeared, using different c-AMP derivatives. Some general observations on the limitations of affinity chromatography made by us might help to explain many of the difficulties encountered by those authors.

2. Materials and methods

In order to obtain sterically unhindered ligand substituted matrices, the basic concepts of affinity chromatography suggest the use of "spacers" between matrix and ligand [6]. To this end, acylation of the amino group in position 6 of the adenine base could be used [7]; however, because of their convenience with regard to purification and biological activity, derivatives of 8-bromo c-AMP [3,8] were preferred:



1 : R = $-S-CH_2CH_2-NH_3^+$

2 : R = $-S-CH_2CH_2-NH-CO(CH_2)_5-NHCO\cdot O\cdot C(Me)_3$

3 : R = $-S-CH_2CH_2-NH-CO-(CH_2)_5-NH_3^+$

2.1. *Chemical approach.*

c-AMP (Fluka AG, Buchs, Switzerland) was converted to 8-bromo c-AMP, originally described by Ikehara and Uesugi [8]. The thioether 1 was prepared by reac-

tion of the halogenated nucleotide with cysteamine in the presence of sodium methoxide. The side chain amino group of **1** proved to be very reactive towards *p*nitrophenyl N^{ϵ}-*t*-butoxycarbonylamino caproate, the reaction in dimethylformamide (DMF) being complete within $\sim$ 2 min. The heterocyclic 6-amino group proved to be unaffected by the active ester. The *t*-butoxycarbonyl protecting group (Boc–) of **2** was cleaved by trifluoroacetic acid (cf. [9]); the resulting trifluoroacetate was easily converted to the zwitterion **3** by drying in vacuo.

2.2. *Stability of* **3**

Thin-layer chromatographic investigations in different solvent systems revealed only one spot. The compound is very stable in neutral and basic solutions: after standing for 3 weeks in 1 N ammonia at 20°, not the slightest deterioration could be detected by chromatography and electrophoresis. Aqueous acid hydrolyzed the nucleotide very slowly (cf. [10]). The reason for the great stability, even of the phosphodiester ring, must be sought in the special *kinetics* of 6-membered ring hydrolysis ($\sim 10^8$ times slower than 5-membered rings [11]), because c-AMP was shown to be *thermodynamically* very unstable with respect to its products of hydrolysis [12].

2.3. *Reaction with c-AMP "binding proteins"*

Both **1** and **3** were able to compete with ^{3}H-c-AMP in the "Sepharose coupled binding protein" assay of Fisch et al. [4]; $K_{ass} \simeq 1.5 \times 10^8$ (**1**) and $2.3 \cdot 10^8$ (**3**) l/mole.

2.4. *Matrix-bound c-AMP and its stability limitations*

Sephadex G-10, Sepharose 2 B or 4 B, (Pharmacia AB, Sweden) and cellulose (Whatman, England) were activated with cyanogen bromide (cf. [13]). Coupling of the activated gels with **3** was perfomed at pH = 10.2, excess ligand **3** was easily removed by washing with water. The preparations, containing about 2–5 μmoles of c-AMP per ml suspension, appear to be reasonably stable below pH $\simeq$ 5, but a c-AMP-containing soluble product is continuously released at higher pH values. This compound, appearing in pmolar amounts per min (fig. 1) was assayed by the method of Fisch et al. [4]. Because of the intrinsic stability of **3** in alkaline solutions, the unknown product probably still contains the whole aminocaproylcysteamine side chain in position

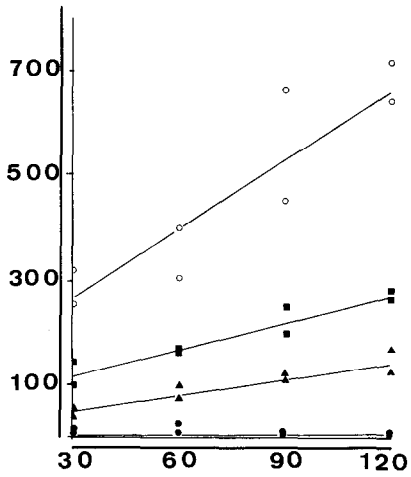


Fig. 1. Liberation of ligand from cAMP-spacer-agarose in dependence on time and pH. (●——●——●): pH 5 (sodium acetate); (▲——▲——▲): pH 6 (Imidazol-HCl); (■——■——■): pH 7 (Tris-HCl), (○——○——○): pH 8 (Tris-HCl) (Room temp.). The incubation medium contained 0.005 M of the mentioned buffers plus 0.015 M NaCl. Abscissa: time (min). Ordinate: conc. in pmoles per ml of ligand released in suspensions of gels in the pertinent buffer. (1:5 v/v).

8 of c-AMP. Its exact identity is being examined. Such a cleavage, if it were a general phenomenon characteristic of Sepharose derivatives prepared with BrCN, would place serious limitations on the use of affinity chromatography for isolating pmolar and nmolar amounts of proteins, because the ligand–protein complex might be formed in the non-stationary phase or be cleaved and therefore not stay fixed to the column. It would raise questions regarding the suitability of Sepharose (or Sephadex, or cellulose) hormone complexes for testing hormone actions and the location of hormone receptor molecules in or on the target cell, cf. [14].

Although the cleavage of the estradiol-Sepharose bond by base or neutral hydroxylamine has been reported [15], no attention has been drawn to the fact such cleavages can occur in neutral aqueous solution. It appears that (for statistical reasons?) "small" ligands that are monovalently bound to Sepharose are more prone to this limitation than polyvalently bound proteins (cf. [4]). We suggest that some of these difficulties might be alleviated by using "polyvalent handles" for hormones and similar small ligands. This aspect is under investigation in our laboratory.

A detailed description of the chemical synthesis of **1**–**3**, and the Sepharose derivatives of **3** will appear in Helv. Chim. Acta, and include a detailed investigation of the cleavage mechanism.

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