A NEW cAMP AFFINITY MATRIX FOR THE RAPID PURIFICATION OF PROTEIN KINASE REGULATORY SUBUNITS

Wolfgang WEBER, Carl-Wilhelm VOGEL and Helmuth HILZ
Institut fur Physiologische Chemie, Universitat Hamburg, Martinistraße 52, 2000 Hamburg 20, FRG

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1. Introduction

Affinity chromatography has been described as a useful step in the purification of protein kinase subunits (cf. [1–5]). Coupling of the cAMP derivatives to Sepharose was usually performed with CNBr-activated matrices. The stability of the linkages formed appears reasonably good when ligation involved more than one bond, as in the coupling of proteins. If, however, the ligand is bound via a single linkage, stability of the conjugate is drastically reduced as in the case of Sepharose-coupled 8-substituted cAMP derivatives [6]. Such matrices, then, may lead to significant losses of cAMP binding proteins when present in low concentrations. An additional disadvantage of most cAMP affinity matrices resides in the hydrophobic spacer region, which tends to bind unrelated proteins leading to contamination of the final product.

This paper describes the coupling of an 8-substituted thio-cAMP derivative to epoxy-activated Sepharose via a hydrophilic spacer. The resulting cAMP affinity matrix was rather stable and could be used successfully to isolate protein kinase I and II regulatory subunits in pure form and high yield. R II prepared in this way exhibited two binding sites for cAMP corresponding to an R_2·cAMP$_4$ formula.

2. Materials and methods

2.1. *Synthesis of the cAMP affinity matrix*

Epoxy-activated Sepharose 6B (Sepharose–O–CH_2–CHOH–CH_2–O–$(CH_2)_4$–O–CH_2–CH(O)–CH_2, Pharmacia), 10 g, was suspended in a solution of 700 mg 8-(2′-hydroxyethyl)-thio-cAMP (prepared as in [7]) in 30 ml 0.2 mM sodium carbonate buffer (pH 11) and stirred for 20 h at 45°C. To block excess of reactive groups, the matrix was allowed to react with 1 M ethanolamine·HCl (pH 9) for 3 h at 45°C. After extensive washing with 0.1 M borate/0.5 M NaCl buffer (pH 8.5) and 0.1 M acetate/0.5 M NaCl buffer (pH 4.0) the epitope density of the matrix could be determined by hydrolysis of an aliquot and subsequent quantification by ultraviolet absorption. In this experiment, a value of 3.7 μmol/ml matrix was found. The product was stored in 50 mM borate buffer (pH 8.5)/0.5 M NaCl at 4°C, using 0.05% toluene as a preservative.

2.2. *Preparation of protein kinase II regulatory subunit (R II)*

The first step of the procedure described [8] for the preparation of protein kinase I from rabbit muscle was adapted with the following modifications: The extract was prepared from 3.4 kg bovine heart and diluted to 30 l with cold water. Pre-equilibrated (5 mM morpholinoethane sulfonate, 9 mM NaCl, 15 mM β-mercaptoethanol, pH 6.5) DEAE cellulose (DE 52, Whatman) 1.8 l, was added and stirred for 1 h. The suspension was allowed to settle for another hour, separated and washed on a large Büchner funnel. A column (5 × 90 cm) was packed under pressure, and a linear gradient (2 × 2 l; 9–500 mM NaCl) was applied. Fractions were analyzed for cAMP binding capacity (see later). The major peak corresponding to protein kinase II eluting at about 150 mM salt was pooled (750 ml) and used for affinity chromatography.

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2.2.1. Affinity chromatography

Pooled protein kinase II fraction 371 ml were stirred with 10 ml affinity matrix for 4 h at 0°C. The matrix was then collected and washed with 150 ml buffer (5 mM morpholinoethane sulfonate, 0.1 mM EDTA, 100 mM NaCl, 15 mM β-mercaptoethanol, pH 6.5) and with 150 ml 2 M NaCl in the same buffer. The matrix was then transferred to a column (1.5 × 6 cm) and eluted with 2 vol. 1 mM 5′-AMP and 2 vol. 1 mM cAMP. In some tissues, this procedure separated out contaminating low affinity binding proteins [4]. The regulatory subunit of protein kinase II was eluted by slow passage of 30 mM cAMP at room temperature. Under these conditions, the major part of the bound R protein was eluted with about 2 column vol. of the buffered cAMP solution. Residual R protein may be desorbed by prolonged elution with 30 mM cAMP, or with 6 M urea/12 mM methylamine in TG buffer (10 mM Tris·HCl, 1 mM EDTA, 6 mM β-mercaptoethanol, 10% glycerol (pH 7.4, cf. [4]). Excess cAMP in the fractions was removed by dialysis or, especially when rapid determination of cAMP binding capacity was indicated, by double charcoal treatment (see below).

2.4. *Preparation of protein kinase I regulatory subunit (R I)*

Rabbit skeletal muscle (4.5 kg) was processed as above except that elution of R I by slow passage of a 30 mM cAMP solution at room temperature was preceded by a quick passage of 1 column vol. 30 mM cAMP at 0°C (removal of a contaminating protein).

2.5. *cAMP binding capacity*

c[^{3}H]AMP (75 pmol, 0.06 μCi) was mixed with the samples. An equal volume of 10 M urea/20 mM methylamine was added (final vol. 60 μl). After 1 min at 0°C, samples were diluted to 150 μl with assay mixture (50 mM Tris·acetate (pH 7.4), 5 mM EDTA, 5 mM theophylline, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, final concentration) and incubated at 0°C for 90 min. Charcoal suspension (10% charcoal, 2% bovine serum albumin in assay mixture) 80 μl, was added, mixed, and the suspension was centrifuged for 4 min at 10 000 × *g*. Aliquots of the supernatant were counted for radioactivity.

2.6. *Removal of excess cAMP*

Samples (150 μl) were mixed with 80 μl charcoal suspension (cf. above). After centrifugation (4 min; 10 000 × *g*), 170 μl aliquots of the supernatants were again mixed with 80 μl charcoal suspension, and centrifuged. Aliquots of the supernatant were subjected to the cAMP binding assay described above.

2.7. *Determination of 8-hydroxyethyl-thio-cAMP by radioimmunoassay*

The assay was performed with an antibody raised against an 8-substituted cAMP–serum albumin conjugate (M. Schumacher, unpublished). It allowed the determination of 0.1 pmol cAMP derivative.

2.8. *Protein determination*

This was by the Lowry method [11], using bovine serum albumin as a reference standard.

3. Results and discussion

3.1. *Coupling of 8-hydroxyethyl-thio-cAMP to epoxy-activated Sepharose*

Coupling of low molecular weight ligands to CNBr-activated Sepharose results in linkages which proved to be rather labile, even at neutral pH values (cf. [6]). In order to avoid bleeding of the ligand, 8-hydroxyethyl-thio-cAMP was coupled to an epoxy-activated Sepharose 6B which provides a hydrophilic spacer and a reactive oxirane ring for direct binding to nucleophilic groups of the ligand. Formation of a stable ether linkage proceeded with high yield under the conditions used leading to an epitope density of 3.7 μmol cAMP derivative/ml Sepharose.

The matrix exhibited a high affinity and capacity for cAMP binding proteins. Purified regulatory subunits R when passed even in the form of dilute solutions (< 25 μg/ml) showed complete (> 99%) retention (not shown). Also, considerable quantities (e.g., 500 ml) of concentrated tissue extracts (e.g., thyroid extract containing 120 mg protein/ml) could be chromatographed on a 3 ml cAMP column without breakthrough of the R proteins.

Bleeding of the coupled cAMP derivatives was studied with the aid of antibodies raised against an 8-substituted cAMP derivative. When the matrix was washed with various buffers at different pH values, only trace amounts of the cAMP derivative could be detected in the eluates (0.5 pmol/ml matrix at pH 6;

1.5 pmol at pH 7, and 1.0 pmol at pH 8). This represents a loss of < 0.00005% of total cAMP content per hour. When stored at 4°C in borate buffer for 6 months, the matrix lost < 0.04% of the ligand, and not more than 1.4% when kept for 6 months at 25°C.

The cAMP derivative coupled to epoxy-activated Sepharose offers additional advantages. It provides a spacer which is long enough to allow free binding even of bulky proteins. Its oxygen content renders the spacer region still hydrophilic enough to avoid unspecific binding of proteins as encountered with affinity matrices containing hydrophobic spacers (cf. [12]). This was evident from a comparison of serum albumin binding: While an 8-aminohexyl-amino-cAMP derivative coupled to Sepharose-bound serum albumin to a significant degree (not shown), no binding of serum albumin even at high concentrations was observed with the new cAMP affinity matrix (cf. fig.1, main band in D versus C).

The new affinity matrix should also be rather stable against phosphodiesterase, since 8-thio-cAMP derivatives are generally resistant to the enzyme (cf. [7]). This property, together with the stability of the spacer linkage, allows multiple reuse of the same affinity column for the preparation of cAMP binding proteins even from crude extracts.

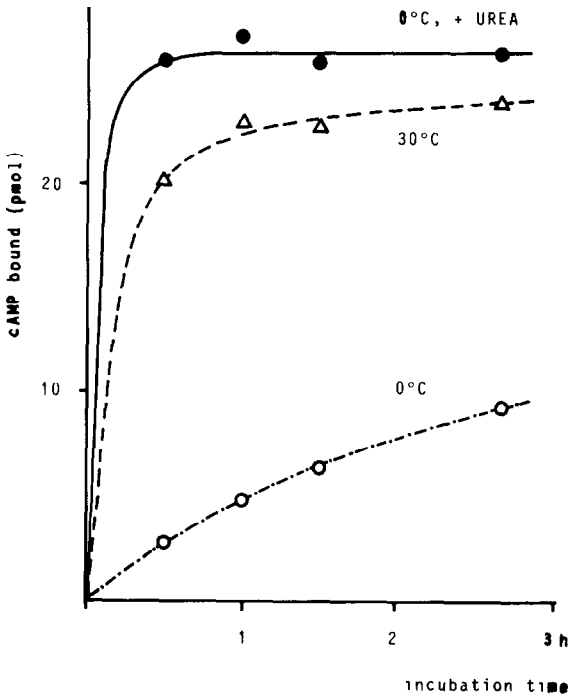

Fig.1. Time-dependent exchange of c[^{3}H]AMP with R·cAMP in the presence and absence of urea. R II·cAMP (0.68 μg) was incubated with 75 pmol c[^{3}H]AMP ± 5 M urea at 0°C or 30°C. At the times indicated, samples were analyzed for bound radioactivity by the charcoal procedure (see section 2).

3.2. *Application of the cAMP matrix to the isolation of protein kinase regulatory subunits*

The predominant form of cAMP-dependent protein kinase in bovine heart is type II isoenzyme [13]. Isolation of its regulatory subunit R II by affinity chromatography requires prior separation of other high affinity cAMP-binding proteins like R I or the products of limited proteolysis (cf. [4,14]. A clear separation of protein kinase II from type I holoenzyme was achieved by fractionation on DEAE cellulose. When protein kinase peak II (371 ml) (table 1) was stirred with 10 ml cAMP affinity matrix, 76%

Table 1
Purification of protein kinase II regulatory subunit from bovine heart

Fraction	Volume (ml)	Protein (mg)	cAMP-binding activity (nmol)	Specific activity (nmol/mg)	Yield (%)
Supernatant	14 800	30 945	800	0.026	
DEAE-cell. peak II	371	2325	495	0.21	100
cAMP matrix					
not retained	511	–	119	–	24
30 mM cAMP eluate	21.3	8.5	298	35	62
6 M urea eluate	10.6	–	10	–	

For details see section 2

of the binding activity were tightly bound to the derivatized Sepharose, which could be collected by filtration, transferred to a column and washed with 2 M NaCl, 1 mM 5′-AMP and 1 mM cAMP solution to remove contaminating proteins. Specific release of the regulatory subunit R II was finally effected by the slow passage of 2 bed vol. 30 mM cAMP solution (pH 6.5) at room temperature. This extract contained 8.5 mg electrophoretically homogeneous* cAMP binding protein R II (fig.1). Additional small amounts were released by elution with 6 M urea. It should be pointed out, that the only protein released by 6 M urea was the R II protein, thus excluding unspecific binding of unrelated proteins to the spacer. The overall yield of cAMP binding units from the crude peak II was 62% (cf. table 1). The yield may be increased when R II adsorption from the DEAE cellulose fractions is done by column chromatography instead of the time-saving batch-wise adsorption to the cAMP matrix. The entire preparation of pure R II from bovine heart could be performed within 2 days.

The procedure was applied with similar success to the isolation of the regulatory subunit of protein kinase I from rabbit skeletal muscle. When starting with 4.5 kg tissue 12.5 mg homogeneous regulatory subunit R I could be obtained (cf. fig.1).

Quantification of cAMP binding capacity in the various fractions deserves a special comment. Binding of cAMP to the isolated regulatory subunits of both protein kinases is tight enough to resist exhaustive dialysis or treatment with charcoal [5,9,10]. Quantitative removal of excess cAMP from fractions eluted with high concentrations of cAMP was not the only problem of a reliable evaluation of cAMP-binding sites. The tightly bound cAMP had to be released and exchanged against labeled cAMP. When purified regulatory subunit R II was freed from excess cAMP by charcoal treatment, then exposed to excess c[^{3}H]-AMP in the presence of 5 M urea, reversible denaturation led to a mixing of released (cold) and added c[^{3}H]AMP. Subsequent dilution to 2 M urea effected renaturation of R II with an immediate tight rebinding of c[^{3}H]AMP. In the absence of urea, c[^{3}H]AMP exchange with R·cAMP was very slow (fig.2). It could, however, be accelerated by raising the temperature to 30°C. Under these conditions equilibrium was reached not before 8 h of incubation. Both procedures, reversible denaturation with urea and exchange at elevated temperature led to the same maximal value of binding sites.

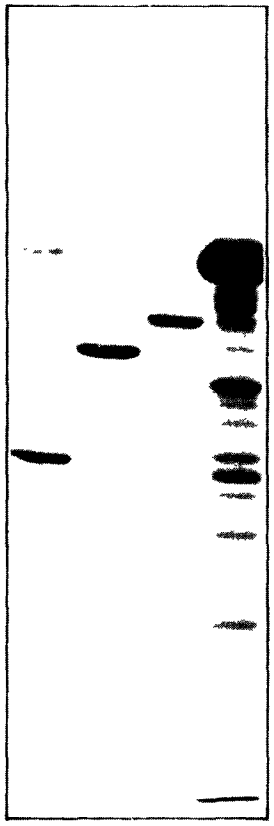

Fig.2. SDS–gel electrophoresis of DEAE-cellulose peak I (A) of rabbit skeletal muscle, of peak II (D) of bovine heart, and of the isolated regulatory subunits I (B) and II (C). Electrophoresis was performed as in [19], using the Tris–glycine system.

The high efficiency of the procedure presented here is also shown when applied to the problem of cAMP-binding stoichiometry. The urea-facilitated c[^{3}H]AMP exchange reaction and the charcoal procedure for the separation of free and protein-bound cAMP were applied to measure the cAMP-binding capacity of the regulatory subunit R II prepared with the aid of the new affinity matrix. In contrast to [15–18], but in accordance with [5] binding of 4

* Contains phospho- and dephospho-forms

(± 0.36) cAMP molecules instead of 2/R II dimer (= R_2·$cAMP_4$) was consistently found, and confirmed by independent procedures.

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