THE PURIFICATION OF GUINEA-PIG LUNG PHOSPHODIESTERASE BY AFFINITY CHROMATOGRAPHY

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1. Introduction

A series of compounds exemplified by doxantrazole [1], synthesised as potential anti-asthmatic drugs showed anti-allergic activivity, demonstrated by their inhibition of a reagin mediated passive cutaneous anaphylaxis (PCA) reaction in rats. These compounds also inhibited cyclic 3′,5′-adenosine monophosphate (cAMP) phosphodiesterase from guinea-pig or human lung [2]. There may be some direct correlation between these two effects [3–6] although recently the pharmacological effect has been related to the ratio of inhibition of hydrolysis of cAMP relative to that of cGMP [7].

Examination of CPK molecular models indicates a striking resemblance between doxantrazole and one conformation of cAMP, which is unexpected from a consideration of the two dimensional structures [8].

One of the aims of the present work was to investigate the implications of this structural similarity using affinity chromatography. In the course of this study a novel purification of guinea-pig lung cAMP phosphodiesterase was effected which compares favourably with those described in the literature [9,10].

2. Materials and methods

The tricyclic compounds 495C and 575C (fig.1) were provided by Dr H. F. Hodson of these laboratories. Sepharose 4B, CH–Sepharose, AH–Sepharose and Sephadex G-25 were purchased from Pharmacia, Uppsala, Sweden. [8-^{3}H]cAMP ammonium salt, (spec. act. 27.5 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, England. All other reagents were of the highest commercial grade available.

The guinea-pig lung phosphodiesterase was provided as the supernatant from a centrifuged (10 000 × *g* for 30 min at 0°C) crude homogenate in 50 mM Tris, 250 mM sucrose, pH 7, by Dr J. E. Tateson of these laboratories. Protein concentrations were measured using the method of Lowry et al. [11].

cAMP Phosphodiesterase activity was measured using a modification of the radioactive assay of Brooker, Thomas and Appleman [12]. The units of specific activity of enzyme are reported in nmol substrate hydrolysed/mg protein/h.

2.1. *Synthesis of affinity gel with 495C*

Sepharose 4B was washed and activated with cyanogen bromide and subsequently coupled to 4-azaheptane-1,7-diamine following the method of Cuatrecasas [13]. This material (10 ml packed volume) suspended in water (10 ml) was coupled to 495C (0.50 g) dissolved in dimethylformamide (50 ml) in the presence of ethyl dimethylaminopropylcarbodiimide hydrochloride (EDC·HCl) (0.75 g) in water (15 ml). The mixture was agitated by upward nitrogen gas flow via a glass sinter and the pH was adjusted to 4.9. This remained constant while the reaction mixture was left overnight at room temperature. The yellow gel was filtered under slight suction, washed with 1 M K_2HPO_4 solution until the washings were colourless (600 ml) and then with water. The product, whose structure is shown in fig.1, was stored at 4°C as a suspension in water. The concentration of ligand attached to the gel was determined from the ultraviolet spectrum of the conjugate measured as a

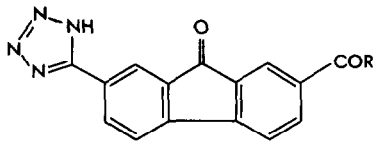

Fig.1. Compound 495C, R = OH; Compound 575C, R = NEt_2; Affinity gel, R = $NH(CH_2)_3NH(CH_2)_3NH$ – Sepharose.

suspension and after aqueous hydrolysis, on a Double Monochromator Beckmann Acta CV.

2.2. *Affinity chromatography*

The appropriate washed gel was packed in a column (5.0 X 1.0 cm) and prepared for use by washing with 50 mM Tris containing 5 mM $MgCl_2$, pH 7.5 (100 ml).

Enzyme samples (2.0 ml or multiples thereof) containing 5–7 mg protein/ml, were applied to the column and washed through with the Tris buffer. Flow rates used were 56 ml/h with collection of 0.2 ml, 0.5 ml or 0.75 ml fractions. Effluent protein concentration was monitored continuously at 280 nm. The elution buffers used are described in the text.

Inhibitor elution of the affinity column was by application of 2×10^{-3} M 575C (4.5 ml) to the column. This solution was left on the column overnight and then eluted. The fractions containing 575C (monitored by ultraviolet absorption) were combined and applied to a column of Sephadex G-25 (11 X 1.0 cm) previously calibrated for void volume using blue dextran. The flow rate was 56 ml/h with collection of 1.25 ml fractions. The column eluate was continuously monitored for ultraviolet absorption at 280 nm and fractions were assayed for phosphodiesterase activity. All chromatographic procedures were carried out at 4°C.

3. Results

The affinity gel exhibited a ultraviolet spectrum ($\lambda_{max}^{H_2O}$ 277.8 nm) identical with the spectrum of 575C (and 495C also). Using the molecular extinction coefficient of 575C ($\epsilon_{277.8}$ 70 200) the degree of coupling of ligand to Sepharose was calculated as 1.4 μmol/ml settled gel.

Several control experiments on binding to functionalised Sepharose columns were carried out. Application of crude enzyme (2.0 ml) to a column of commercial Sepharose 4B gave a 94% recovery of phosphodiesterase activity in the initial eluate. No purification was achieved.

A similar procedure applied to CH–Sepharose gave almost quantitative recovery of activity in the void volume fractions, again no purification was observed. Complete retention of activity was observed on application of enzyme (2.0 ml) to AH–Sepharose with the large protein peak emerging in the void volume fractions being inactive. Subsequent stepwise elution with 0.5 M KCl/Tris (10.0 ml) and 2.0 M KCl/Tris (10.0 ml) removed 79% of the enzyme activity with a purification of 1.75X.

Figure 2 shows high ionic strength elution of the 495C affinity column. Some leakage of activity was observed under this double loading technique. Stepwise elution with the high ionic strength buffers recovered 68% of the retained activity with a purification of 11 fold. (spec. act. 958 units). The profiles of inhibitor elution of the affinity column and of the subsequent Sephadex G-25 separation required to assay the enzyme activity are shown in fig.3(a) and 3(b). 55% of the retained enzyme activity was recovered in the void volume fractions from the Sephadex column with a purification factor of 422 (spec. act. 13 900 units).

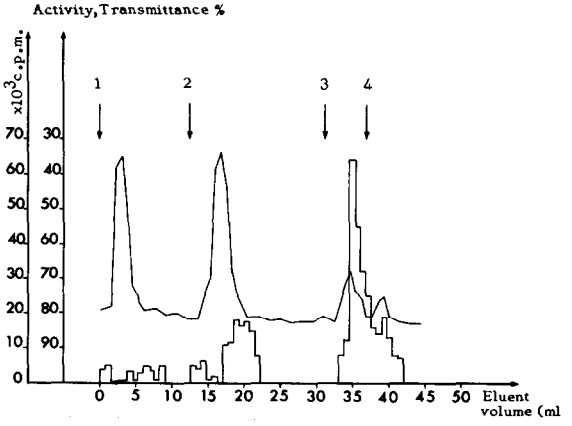

Fig.2. Elution of phosphodiesterase from the affinity column using high ionic strength buffer. 2.0 ml crude enzyme (spec. act. 87 units) applied at 1 and 2. Elution with 0.5 M KCl/Tris at 3. Elution with 2.0 M KCl/Tris at 4. Protein concentration (——). Enzyme activity (⊓).

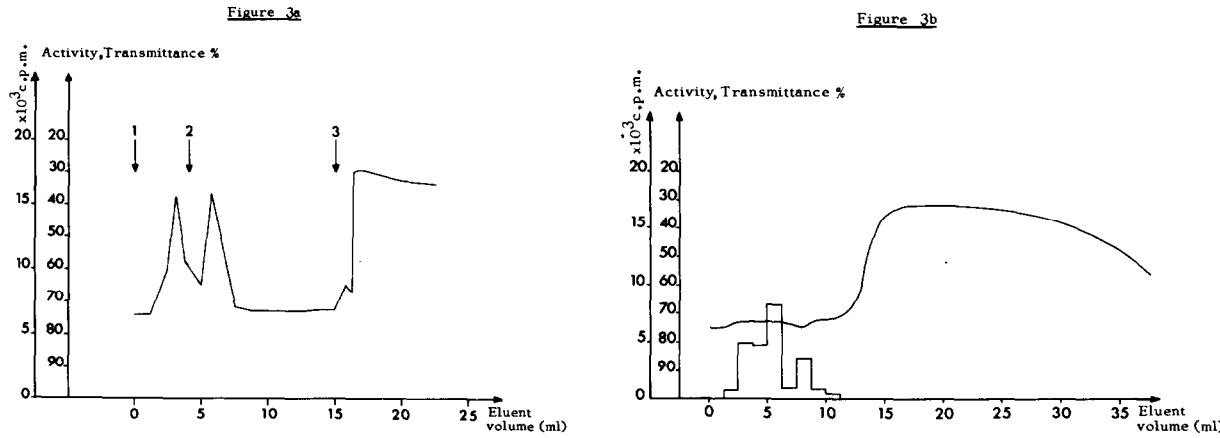

Fig.3a. Elution of phosphodiesterase from the affinity column using inhibitor elution. 2.0 ml crude enzyme (spec. act. 33 units) applied at 1 and 2. Elution with 2×10^{-3} M 575C at 3. A_{280} nm (——). Fig.3b. Elution from Sephadex G-25. A_{280} nm (——). Enzyme activity (⊓).

Attempted elution of the enzyme from the affinity column with a saturated solution (approx. 0.5 mM) of 495C was unsuccessful as were attempts to elute activity using the substrate cAMP (2 mM) in Tris/$MgCl_2$ buffer.

4. Discussion

The readily available fluorenone 495C was chosen as the affinity chromatography ligand for two reasons. It contains a carboxyl group which is used as the attachment point to the solid support and, although it is only a weak inhibitor of cAMP phosphodiesterase itself (20% inhibition at 1 mM [2], its diethylamide 575C, a more direct analogue of the immobilised ligand, is a good inhibitor (57% inhibition at 0.1 mM [2]).

In order to distinguish between 'ion-exchange' binding effects and true 'biospecific binding' of the enzyme to the affinity column [14] several control columns were run.

Sepharose and CH–Sepharose (carboxyl functionalised) did not retain, retard or purify the crude enzyme which was recovered quantitatively from these columns. In contrast AH–Sepharose (amino-functionalised) retained the phosphodiesterase. The enzyme was subsequently eluted with high ionic strength buffer giving a purification of 1.75-fold, suggesting an ionic binding of the enzyme to this 'cationic' column.

Because of the method of synthesis of the affinity support (from an AH–Sepharose-type precursor) it was expected that this non-specific ionic binding of enzyme could occur with the affinity column also. Phosphodiesterase was retained by the 495C affinity column (with some leakage) but 68% of this retained activity was removed by high ionic strength buffer elution thus implying that the support is indeed contaminated by the intermediate amino-functionalised Sepharose. Biospecific elution (using 575C), in contrast removed 55% of the retained activity with, however, a great increase in purification (422-fold against 11-fold). This simple purification procedure contrasts favourably with the 8-step purification (20 000-fold with 9.5% recovery) of the enzyme from Baker's yeast [10] and also with the 20-fold purification of the rabbit aorta enzyme on a Sepharose 6B column [9].

Attempted elution of the enzyme from the affinity colum using the weak inhibitor 495C was unsuccessful and, more significantly, substrate elution did not remove the phosphodiesterase from the column. As cAMP and 575C are approximately equipotent in binding to the enzyme [2,15] this latter result could be interpreted as a binding of substrate and inhibitor at different sites on the enzyme, (c.f. disodium cromoglycate which exhibits competitive inhibition

kinetics for cAMP phosphodiesterase [4]). A corollary to this implication is that the proposed structural similarity between cAMP and the tricyclic anti-allergic phosphodiesterase inhibitors (as exemplified by 575C) may be fortuitous in terms of binding to this receptor.

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