AFFINITY AND HYDROPHOBIC CHROMATOGRAPHY OF THREE VARIANTS OF CHLORAMPHENICOL ACETYLTRANSFERASES SPECIFIED BY R FACTORS IN *ESCHERICHIA COLI*

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1. Introduction

The high level resistance of *E. coli* to chloramphenicol (CM) is the result of antibiotic acetylation by the enzyme chloramphenicol acetyltransferase (EC 2.3.1.99) [1,2]. Three distinguishable types of chloramphenicol acetyltransferase (CAT) have been found in *E. coli* strains bearing an episomal (R factor) determinant for chloramphenicol resistance [3,4]. Two out of the three variants have been found to be associated with so-called fi^- R factors and one with fi^+ factors. Each variant is composed of four identical subunits of approx. 20 000 mol. wt. [5]. Differences in electrophoretic mobility, inhibition by sulfhydryl reagents, K_M for chloramphenicol, and immunological behaviour have been observed [4]. Although purification of the CAT variants by conventional methods has been successful [6,7], their use has resulted in variable overall yield, especially when purification on a large scale has been attempted.

In this report we describe the use of the technique of affinity chromatography [8] to achieve purification to homogeneity of CAT in high yield. The approach taken was to attach the free amino of chloramphenicol (CM base) or of *p*-amino chloramphenicol (fig.2) to the free carboxyl group of substituted Sepharose [Seph-NH-$(CH_2)_n$-COOH] by formation of an amide bond following activation by a water soluble carbodiimide.

The three variants of CAT mediated by R factors behave differently in affinity chromatography as regards the amount of each enzyme bound to any given affinity support and the ionic strength requirements for elution from such columns. Additional differences in behaviour include the length of spacer arms and the extent of their substitution by ligand for optimal adsorption of the enzymes. Although hydrocarbon spacer arms have been used for some time to situate affinity ligands some distance from the matrix [9–12], it has only recently been appreciated that the hydrocarbon spacer arms may themselves contribute to the binding of proteins through hydrophobic interactions [13–15]. The systematic exploitation of this phenomenon has led to the concept of 'hydrophobic chromatography' [13]. The studies on R factor CAT to be described suggest that the interactions involved in the adsorption of CAT variants to affinity resins are of two types; a non-specific and probably hydrophobic one which is a function of the spacer arm and another which is more or less dependent on substrate recognition. The varying contributions of such effects among the naturally occurring types of R factor CAT offer means both of characterising and of purifying the variants.

2. Materials and methods

The bacterial strains used in the present study are derivatives of *E. coli* K12 which contain plasmids (R factors) specifying the constitutive synthesis of CAT variants which were classified by the procedures

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described previously [4]. *E. coli* strain W677 carries the JR66 plasmid [16] which mediates the synthesis of Type I enzyme. The plasmids designated s-a and R387 dictate the synthesis of Type II and Type III variants of CAT respectively [4] and were carried independently in *E. coli* strain J5-3. Bacterial cells were grown to stationary phase, at 37°C (rotary shaker), in Oxoid nutrient broth supplemented with 1% glycerol, were broken by extrusion in a French pressure cell, and the crude cell-free extract was obtained by centrifugation at 20 000 *g* for 20 min.

CAT activity was assayed by the spectrophotometric method [6] with a Unicam SP 1800 spectrophotometer. Acetyl-S-CoA was prepared from Grade I CoA (Boehringer Co.) and acetic anhydride. Protein concentrations were determined by the Lowry method [18]. Analytical polyacrylamide disc gel electrophoresis was performed for native CAT in 7.5% polyacrylamide as described previously [6].

Sepharose-$NH(CH_2)_n$-COOH was prepared by coupling CNBr-activated Sepharose with H_2N-$(CH_2)_n$-COOH [13]. Titration of the substituted Sepharose products yielded approximately 10 μequiv of carboxyl groups per ml of gel in each case. Coupling of CM-base or *p*-amino CM as the substrate analogue to the above resin was performed by adding a volume of the ligand (dissolved in water) to a volume of settled gel, the pH was adjusted to 4.5 and 100 mg *N*-cyclohexyl *N'*-[2-(4-morpholinyl)-ethyl] carbodiimide - methyl *p*-toluene sulfonate (Fluka & Buchs) were added per 1 ml of gel. Two types of columns were prepared to study the effect of the degree of substitution of the affinity ligand. A high substitution was achieved by mixing 5 mol of ligand per mole of agarose-bound carboxyl group, and the low substitution support was prepared by mixing 2 mol of ligand per mole of carboxyl spacer arm groups. The coupling reaction mixtures were allowed to stand with gentle stirring for 24 h at room temperature and were washed by deionized water and 100 mM Tris-HCl (pH = 7.8) containing 1 M NaCl. Before use, the resins were equilibrated with 50 mM Tris-HCl (pH = 7.8).

CM and CM-base were obtained from Sigma Chemical Co. L-erythro CM and *p*-amino CM were obtained from Parke, Davis Co., Detroit. *p*-Amino CM was also prepared by reduction with titanous chloride [17]. A kit of alkyl and ω-aminoalkyl

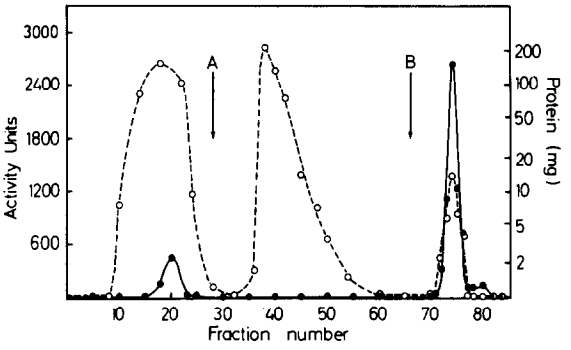

Fig. 1. Chromatography of *E. coli* type III CAT (R387) extract on Seph-$NH(CH_2)_5$-CO CM-base column. The column (2.5 × 16 cm) was pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.8) at 22°C. The extract (23 mg protein, containing 11 000 units of CAT) was applied to the column and the unadsorbed proteins were eluted by 50 mM Tris-HCl (pH 7.8) containing 0.3 M NaCl (A). Elution of CAT was achieved with a buffer containing 50 mM Tris-HCl (pH 7.8), 0.3 M NaCl and 5 mM CM (B). Fractions of 15 ml were collected and their enzymatic activity (•——•) and protein concentration (○---○) were determined.

Sepharose columns were kindly provided by Dr S. Shaltiel.

Experiments using purified CAT variants were performed with enzymes prepared by previously described methods [7] and found to be homogeneous by native and SDS electrophoresis. The experiment described in fig.1 was performed with a CAT containing cell-free extract that had been subjected to the heat and ammonium sulfate steps of the conventional purification procedure [7]. Large scale purification of CAT has been carried out by the pilot plant of the Department of Biochemistry, Imperial College, using appropriate modifications for 300 litre batch cultures.

3. Results and discussion

CM-base or *p*-amino CM were chosen as suitable ligands for the CM substituted agarose because they possess a free amino group and because the nature of the *N*-acyl or *p*-nitro substituents are less important determinants of substrate affinity for CAT variants than are the 1,3 propanediol side chain or the presence of the 1-phenyl ring [19]. Purification of CAT by affinity chromatography was achieved by applying

Table 1
Effect of ionic strength on the recovery of CAT from affinity resins prepared with high and low substitution ratios of CM base to matrix[a]

Resin type	CAT type	R factor	Enzyme bound per ml resin	Enzyme activity recovered (per cent)			
				Elution by 0.3 M NaCl	Elution by 0.3 M NaCl + CM	Elution by 0.6 M NaCl + CM	Elution by 1.0 M NaCl + CM
High substitution	I	JR66	1450	0	20	45	100
	II	s-a	220	0	36	100	–
	III	R387	120	0	100	–	–
Low substitution	I	JR66	1100	0	36	100	–
	II	s-a	180	0	100	–	–
	III	R387	85	100	–	–	–

[a] Columns of 1 ml bed volume were prepared from Pasteur pipettes using the high and low substitution resins described in Materials and methods. Each of 24 columns was equilibrated with 50 mM Tris-HCl (pH 7.8) and samples of purified CAT were applied in the same buffer in a volume of 0.5 ml. The specific activities of the electrophoretically homogeneous preparations were 240, 225, and 195 (units per mg) respectively for the Type I, II and III variants of CAT. The amount of enzyme applied in each case was in excess of the binding capacity of the column as judged by elution of activity in a preliminary wash of each column with 4 ml of the equilibration buffer. Four experiments were performed with each CAT variant and any one type of resin. Fractions of 1 ml were collected in each experiment. The results are tabulated as per cent of total activity applied which was eluted in the first four fractions collected with each eluant. No significant elution of activity was observed in later fractions. CM was present at a concentration of 5 mM where noted. All steps were performed at 22°C.

the extracts obtained (see Materials and methods) on a substituted CM-base affinity resin. Fig.1 shows a characteristic chromatographic pattern of purification on CM-base resin. Similar patterns are obtained for the other CAT types purified either on CM-base resin or *p*-amino CM resin. The three types of *E. coli* CAT differ mainly in their requirements for a high ionic strength in buffer (B) to achieve their elution. Specific elution of type III CAT (R387) requires 0.3 M NaCl in Buffer B as shown in fig.1 whereas type II (s-a) and type I (JR66) require 0.5 and 1.0 M NaCl respectively. Differences in capacity as shown by the amount of enzyme units bound per ml of resin were also found (table 1).

It is clear from the data in table 1 that purification of both the type I or type II variants of CAT can be achieved with low substitution affinity columns using buffer of lower ionic strength for the elution of enzyme. The same phenomenon has been observed with CAT variants adsorbed to *p*-amino CM substituted columns. The recoveries of pure enzyme from large scale purification (more than 1 kg *E. coli* cells) have been consistently of the order of 80–90% and the final product has in all cases given a sharp single band in polyacrylamide disc gel electrophoresis (native and SDS). In general, the concentration of NaCl used for the pre-elution of non-CAT proteins prior to the specific elution of the enzyme (Buffer A in fig.1) was always the same as that required in the specific step (Buffer B), the sole difference between buffers A and B being the presence of CM in the latter. Variation of the concentration of CM from 1 to 10 mM in buffer B gave no significant change in the final recovery when ionic strength of the eluant was limiting. Only in the presence of a critical ionic strength (for example, 0.3 M for the type III enzyme and the highly substituted resin) will CM effect elution of CAT.

We wish to stress that the length of the spacer arms is as important for binding and elution as are the ionic strength of the buffer and the extent of ligand substitution. Reduction of the length of the spacer arm leads to marked decreases in the adsorption of enzyme to resin. For example, whereas the type III CAT is not significantly adsorbed on Seph-$NH(CH_2)_3$-CO [CM-base], the type I variant, although adsorbed, is eluted from the resin by 0.3 M NaCl

without CM. The same situation obtains with the *p*-amino CM substituted affinity support.

Our failure to detect significant adsorption of all variants of CAT by supports with short spacer arms or those obtained by the direct coupling of CM analogues to CNBr activated sepharose should be contrasted with results reported by Guitard and Daigneult [17]. In particular, we have failed to confirm the adsorption of the type I (JR66) variant of CAT by the affinity matrix prepared by coupling of *p*-amino CM to activated Sepharose. Although we have not examined in a systematic manner the effects of extreme degrees of substitution of Sepharose by either affinity ligand, it seems likely that some adsorption of CAT might be expected on affinity supports lacking a spacer arm but possessing levels of ligand substitution substantially higher than those used in our experiments. It should be mentioned, however, that an extreme degree of direct substitution of Sepharose by CM analogues yields a support with marked aromatic and hydrophobic properties. As discussed below, a distinction should be made between such non-specific factors and ligand-active site interactions typical of substrate affinity chromatography.

The behaviour of CAT indicates that the interactions involved in the adsorption of the enzymes involve factors other than simple ligand affinity. We have, in fact, used hydrophobic chromatography as an alternative means to survey CAT variants, and their behaviour under such conditions is broadly similar to that seen on affinity resins. The type I CAT is more hydrophobic in nature than the type III enzyme, and the type II variant shows intermediate behaviour (table 2).

It is obvious that the hydrocarbon spacer arm interposed between the ligand and the Sepharose matrix has a profound effect on the properties of the 'affinity' column [13–15, 20]. Although the unsubstituted Seph-NH$(CH_2)_5$-COOH does not adsorb or retard the CAT enzymes, the substitution of the spacer group by a ligand that is itself hydrophobic in nature can increase the hydrophobic interactions.

The difference between the hydrophobic and affinity interactions is that the former are non-specific while the other should be dependent on recognition of the substrate analogue ligand. Elution profiles of type III CAT from a variety of columns by specific and non-specific eluants are shown in fig.2. Elution from the affinity resins is specific since only the D-threo isomer of CM, which is the substrate for CAT,

Table 2
Elution of CAT variants from Seph-NH$(CH_2)_n$-CH_3 columns[a]

Column	CAT type	Enzyme activity recovered (percent)		
		0.2 M NaCl	0.2 M NaCl + CM (5 mM)	0.5 M NaCl
Seph-NH$(CH_2)_3$-CH_3	I	9	24	65
	II	55	95	87
	III	100	100	100
Seph-NH$(CH_2)_5$-CH_3	I	0	3	0
	II	0	10	4
	III	2	100	10
Seph-NH$(CH_2)_7$-CH_3	I	0	0	0
	II	0	0	0
	III	0	17	0

[a] Samples of 35–40 units (0.15–0.20 mg) of pure CAT were applied to 1 ml bed volume columns of the resins indicated. Each column was then washed with 4 ml of 50 mM Tris-HCl (pH 7.8) without the appearance of activity in the eluate. Bound enzyme was then eluted with one of the solutions indicated and the recoveries were tabulated as described in table 1.

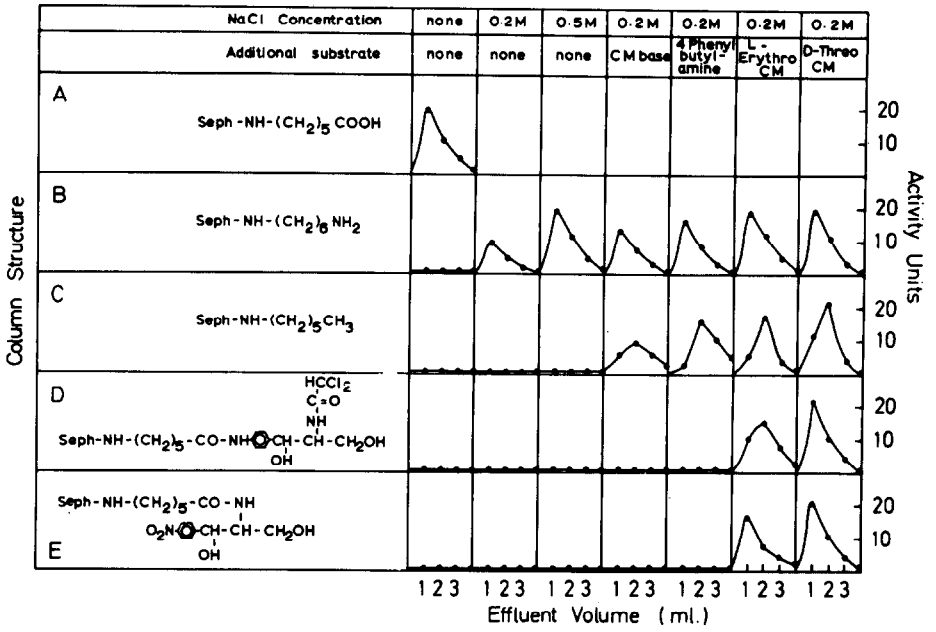

Fig. 2. Effect of various elution conditions on the behaviour of type III CAT following application to various hydrophobic and affinity supports. Samples of 35 units of purified CAT were applied in each case to small columns (1 ml bed volume) equilibrated with 50 mM Tris-HCl (pH 7.8) at 22°C. Immediately following application of the sample, each column was washed with the eluant specified in 50 mM Tris-HCl (pH 7.8). Fractions of 1 ml were collected and assayed for CAT. The concentration of the added substrate was 5 mM in each case.

and the L-erythro isomer, which is a competitive inhibitor (with respect to CM), are effective eluants. Various other CM analogues fail to elute the enzyme under conditions of ionic strength which are adequate for elution in the CM resins.

Taken together, the results of the experiments described form a model for CAT binding to 'affinity' supports which incorporate both substrate recognition and important non-specific interactions which are hydrophobic and sensitive to changes in ionic strength. Although it is impossible to describe the relative contributions of each type of interaction for all combinations of CAT variant and affinity support, it is clear that the use of an approach similar to that outlined in table 1 is a powerful one in characterising and purifying CAT variants. The method is a general one which should be useful in characterising naturally-occurring enzyme variants and mutants, especially those which exhibit minimal differences in net charge and are not easily differentiated by electrophoresis or other methods.

We anticipate that an approach to characterising plasmid-mediated enzymes which is based upon the results described for CAT should be useful for genetic and epidemiologic studies. The rapid purification of such enzymes and their characterisation should permit a more precise assessment of chloramphenicol resistance genotype, and the ease with which the method may be scaled up for preparative work should facilitate structural studies on the family of CAT proteins.

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