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NON-SPECIFIC BINDING IN THE AFFINITY CHROMATOGRAPHY OF CHYMOTRYPSIN

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SUMMARY

The strong and specific binding of chymotrypsin on chromatographic columns containing agarose substituted with N-ε-amino caproyl-D-tryptophan methyl ester is abolished when the ε-amino groups on the surface of the enzyme are reacted with acetic anhydride. Because the catalytic properties of the acetylated chymotrypsin are identical to those of the underivatized enzyme, it is concluded that the high affinity of chymotrypsin for this column is not due solely to biospecific inhibitor binding, which is by itself very weak, but requires reinforcement through weak non-specific interactions with the column support. It is postulated that these non-specific interactions include electrostatic interactions between agarose matrix and positively charged lysyl residues on the enzyme. The results demonstrate for the first time that residues on the surface of an enzyme not associated with its active site can play an important role in some chromatographic systems previously thought to be based on purely biospecific interactions.

INTRODUCTION

Affinity chromatography is a method of purification of an enzyme or receptor protein based upon its specific adsorption to an insoluble polymer or gel to which a specific protein ligand (*e.g.* a substrate analogue) has been covalently attached. When proteins lacking biological affinity are passed through a column containing the bound ligand, they pass through unretarded, while proteins forming a biologically specific enzyme–ligand complex will become strongly adsorbed by the affinity column. In contrast to conventional adsorption chromatographic procedures based on gross electrostatic, hydrophobic or other non-covalent interactions (non-specific adsorption), the high specificity of biological adsorption often leads to remarkably efficient separations.

To a large extent, the present interest in affinity chromatography for enzyme purification originates from the paper of Cuatrecasas *et al.*[1]. Among several examples cited, they reported the specific biological interaction of bovine α-chymotrypsin with the indolyl group of the inhibitor, ε-amino caproyl-D-tryptophan methyl ester coupled

on agarose (Sepharose). Non-active forms of this protein did not bind on the substituted agarose.

O'Carra[2] has recently stressed that some successful affinity chromatographic procedures are probably not purely biological affinity chromatography but are complicated by unavoidable non-biological adsorption effects. These conclusions were based upon investigations with affinity chromatographic systems where the matrix or the spacer-arms used to link the substrate analogue to the matrix were systematically altered.

We report here the preparation of an altered but active form of chymotrypsin having acetylated lysyl residues. Affinity chromatography of this enzyme derivative demonstrates that lysine groups not involved in the active site of the enzyme are essential for the proper performance of chromatography columns containing D-tryptophan methyl ester linked to agarose.

EXPERIMENTAL

Materials

Chymotrypsinogen A and δ-chymotrypsin were crystalline preparations purchased from Sigma (St. Louis, Mo., U.S.A.). The affinity matrix, N-ε-amino caproyl-D-tryptophan methyl ester coupled to Sepharose was a commercial preparation from Miles-Yeda (Rehovoth, Israel) and was identical to that described by Cuatrecasas *et al.*[1]. The stated capacity for the resin was 7 mg chymotrypsin per ml resin. Affinity chromatography was carried out in a column (10 × 1.2 cm) equilibrated with 0.05 *M* Tris–Cl buffer, pH 8 at 4°. Enzyme activity was measured spectrophotometrically using N-acetyl-L-tyrosine ethyl ester as the substrate[3].

Acetylated δ-chymotrypsin was prepared according to the method of Oppenheimer *et al.*[4] with little modification.

Acetylation of chymotrypsinogen

Chymotrypsinogen was acetylated at 4° in the water-jacketed glass cup of a Radiometer pH-stat. Chymotrypsinogen (300 mg) was added to 30 ml of 0.01 *M* $CaCl_2$ and 0.05 *M* sodium tetraborate buffer, pH 8. Approximately 2 mg of phenylmethylsulfonyl fluoride was also added to inhibit trace amounts of chymotrypsin present in the commercial preparation. Acetic anhydride (300 μl) was slowly added with constant stirring over 45 min. The pH was maintained at 6.7 ± 0.2 with 5 *M* NaOH. An excess of base was added after the final addition of acetic anhydride to ensure hydrolysis of unreacted acetic anhydride. The acetylated chymotrypsinogen was dialysed against three charges of 0.05 *M* sodium tetraborate buffer, pH 8 for 20 h at 4° and then fractionated with ammonium sulfate. The fraction which precipitated at 30–40% saturated ammonium sulfate was dissolved in sodium tetraborate buffer (0.05 *M*, pH 8) and any remaining ammonium sulfate was removed by passing the solution through a Sephadex G-25 column (40 × 2.5 cm) equilibrated with sodium tetraborate buffer. All free ε-amino groups on chymotrypsinogen were acetylated as determined quantitatively using trinitrobenzenesulfonic acid[5].

Acetylated chymotrypsinogen was activated to acetylated-δ-chymotrypsin by incubating with trypsin (ratio of 30:1) at 4° for 2 h. The buffer was then removed by passing the activated mixture through a Sephadex G-25 column (40 × 2.5 cm)

equilibrated with distilled water and trypsin was removed by batchwise adsorption at 4° on CM-Sephadex C-50 which was also equilibrated with distilled water. As expected, one free amino group was detected when this preparation was checked with the trinitrobenzenesulfonic acid assay.

RESULTS AND DISCUSSION

Cuatrecasas *et al.*[1] have shown that immobilized N-acetyl-D-tryptophan methyl ester promotes strong and apparently biologically specific retention of α-chymotrypsin on chromatographic columns. Fig. 1 shows that this also holds true for δ-chymotrypsin and that it is eluted only after addition of 0.1 *M* acetic acid, pH 3. These results are somewhat surprising because it is known[6] that the chymotrypsin inhibitor, N-acetyl-D-tryptophan methyl ester, has a inhibition constant, K_i, of only 0.10 m*M*. On theoretical grounds it can be argued that inhibitors with K_i values of this range should not display strong chromatographic retardation (R; see Appendix) and that efficient affinity chromatography where the enzyme binds tenaciously to the column should only be achieved using ligands having dissociation constants in the micromolar region or below[2].

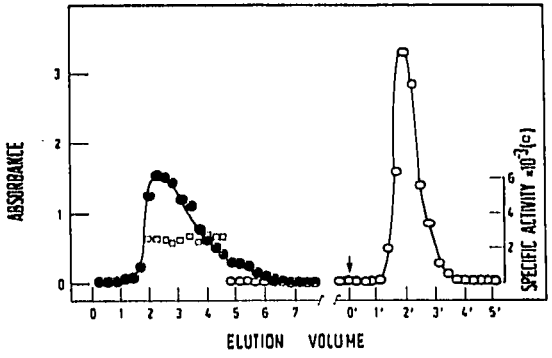

Fig. 1. Affinity chromatography of acetylated δ-chymotrypsin and δ-chymotrypsin on ε-caproyl-D-tryptophan methyl ester substituted agarose. Elution volume (in column volume units) was measured from the time of application of the enzyme or acetic acid irrigant. ●—●, Acetylated δ-chymotrypsin, eluted in 0.05 *M* Tris–Cl buffer, pH 8. δ-Chymotrypsin (○—○) was strongly bound to the column under these conditions and could only be eluted with 0.1 *M* acetic acid, pH 3.0 (arrow). The specific activity of the acetylated δ-chymotrypsin (□) was constant throughout the elution profile.

Acetylation of the lysine groups of δ-chymotrypsin had a marked effect on its binding to the affinity column. Despite the fact that acetylation of the ε-amino groups of δ-chymotrypsin does not alter its catalytic properties in any way[7,8] this derivative had very little affinity for ε-caproyl-D-tryptophan methyl ester substituted agarose. Acetylated chymotrypsin emerged from the column only slightly retarded ($R = 0.3$). The specific activity of the acetylated δ-chymotrypsin preparation was constant across its elution profile, showing that the enzyme preparation was free of acetylated chymotrypsinogen ($R = 0.0$) or inert proteins.

The data prove that a functional active site alone is not sufficient to give strong binding and that non-biological adsorption must play a significant role in the

affinity chromatographic system for chymotrypsin developed by Cuatrecasas *et al.*[1]. In agreement with theory[2] these results underscore the importance of taking into account non-biological mechanisms when assessing apparently successful affinity chromatography systems utilizing immobilized ligands known to have relatively weak binding affinities.

What is the nature of the non-specific binding between α- or δ-chymotrypsin and the ligand-coupled Sepharose column? Examining non-specific binding of proteins to agarose substituted with linear aliphatic hydrocarbons, Hofstee[9] found that both short-range hydrophobic and long-range electrostatic interactions were involved. Chymotrypsin, chymotrypsinogen, and other proteins which are positively charged at neutral pH bound only weakly. On the other hand, negatively charged proteins (including a negatively charged carbamylated derivative of chymotrypsinogen) bound very strongly at low ionic strength (0.05 *M* Tris–Cl buffer, pH 8) and could only be removed from the column by increased ionic strength (1 *M* NaCl). It appears that only at high salt concentrations the binding interaction with hydrophobic groups linked to agarose is purely hydrophobic[10]. The electrostatic interactions observed at low salt concentration may involve charges on the matrix introduced by side reactions of the CNBr activation and ligand coupling processes. The identity of these charged groups is not known.

Like carbamylated chymotrypsinogen, acetylated δ-chymotrypsin is negatively charged at neutral pH. Considering the findings of Hofstee[9], we might expect this active enzyme derivative to bind even more strongly to the hydrophobic tryptophan methyl ester column by non-specific electrostatic interactions. In fact, the opposite was observed. Retardation of this negatively charged protein on the affinity column was marginal.

Despite this apparent lack of agreement (and it must be stressed that the ligand coupled on Sepharose was not identical nor is it likely that the procedures used to prepare the gel derivatives were identical with those used by Hofstee) we are reluctant to discard long-range electrostatic interactions as a contributing factor in the non-specific binding implied from our experimental results. The adsorption of chymotrypsin on the inhibitor–Sepharose column is extremely sensitive to ionic strength[1]. The excellent retention, using 0.01 *M* Tris as the column irrigant is notably weakened when the concentration is increased to 0.05 *M*. These results indicate that electrostatic interactions are important.

Hydrophobic and steric factors may also play a role in the non-specific binding. While it is possible that acetylation of the lysine residues on chymotrypsin may perturb delicate steric or hydrophobic interactions between the enzyme and the matrix, we suggest that these effects are negligible by comparison with the interference caused by loss of the positive charge on the ε-amino group. Thus, when the same ε-amino groups are chemically masked by the method of reductive methylation[11], this fully active enzyme derivative still retains the strong affinity characteristics of the native enzyme[12]. Notably, this enzyme retains the positive charges on the modified lysine residues.

It is appropriate here to note the rather unusual arrangement of lysine residues on the surface of bovine chymotrypsin. As is shown schematically in Fig. 2, twelve out of the fourteen lysine residues are located on the surface of one side of this globular protein in a narrow band spanning one half of its circumference[13,14]. In relation to

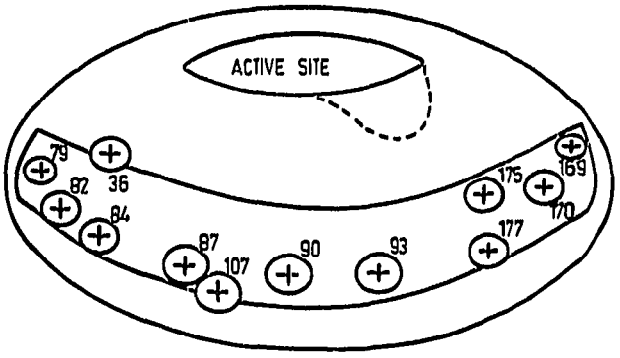

Fig. 2. Schematic distribution of positively charged lysyl residues on chymotrypsin. The sketch is based on X-ray crystallographic data of chymotrypsin[13,14]. The numbers correspond to the positions of the lysyl residues in the protein chain.

this half belt of positive charges around the middle of the protein, the active site is a shallow depression in the top of the molecule. Negatively charged carboxyl groups of glutamic acid and aspartic acid residues, in contrast, are fairly evenly distributed over the entire surface of the molecule. As a consequence of this arrangement of the charges on chymotrypsin, the molecule as a whole is electrostatically polar and should preferentially orientate itself as it approaches a charged Sepharose matrix. Such long-range electrostatically induced orientations may constitute the underlying factor in the somewhat surprising experimental observations that very weak specific interactions can cooperate synergistically with very weak non-specific interactions to produce strong and apparently specific binding of chymotrypsin to tryptophan methyl ester–Sepharose columns.

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APPENDIX

Quantitation of affinity column chromatographic behavior must consider both the enzyme-immobilized inhibitor dissociation constant (K_i) and the partition coefficient (K_p) of the chromatographic column. In the first instance:

$$E + I \rightleftharpoons EI \tag{1}$$

and

$$K_i = \frac{[E]\,[I]}{[EI]} \tag{2}$$

where E = units of enzyme unbound, EI = units of enzyme bound and I = effective immobilized inhibitor concentration.

The partition coefficient is defined as:

$$K_p = \frac{[EI]}{[E]} \tag{3}$$

and is related to other column parameters by the following expression:

$$V_e = V_0 + K_p \cdot V_0 \tag{4}$$

where V_e is the elution volume and V_0 is the void volume. Expressing the elution volume in column volume units, as suggested by O'Carra[2], eqn. 4 simplifies to:

$$V_e = 1 + K_p \tag{5}$$

which by substitution from eqns. 2 and 3 can be equated to:

$$V_e \text{ (column volume units)} = 1 + \frac{[I]}{K_i} \tag{6}$$

By defining retardation (R) as the elution volume, in column volume units, minus one, a very simple and useful equation relating such retardation to ligand concentration and dissociation constant is derived[2]:

$$R = \frac{[I]}{K_i} \tag{7}$$

For example, this equation, which is limited to enzymes with a single binding site, can be used to predict the retardation of chymotrypsin on our affinity column. The affinity column used in this work had an effective immobilized inhibitor concentration of 0.3 μmole per ml packed gel ($[I] = 3 \times 10^{-4}$ M). An inhibitor, whose dissociation constant is 10^{-4} M should cause a retardation by specific binding of only 3 (*i.e.* the enzyme should emerge after four column volumes of buffer have passed through the affinity column).

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