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HETEROGENEITY IN THE BINDING OF CHYMOTRYPSIN AND RELATED PROTEINS TO AFFINITY CHROMATOGRAPHIC MEDIA

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The binding of a series of related proteins, namely, α-chymotrypsin, chymotrypsinogen A, DIP-chymotrypsin, and TPCK-chymotrypsin,[2] to affinity gels consisting of 4-phenylbutylamine or ε-aminocaproyl-D-tryptophanmethylester covalently attached to Sepharose 4B was investigated. Considerable heterogeneity in the binding was observed, both at the level of the affinity adsorbent and with respect to the proteins themselves. Both the aromatic moiety of the ligand and the positively charged isourea group introduced during the coupling of the ligand to the gel play a part in the stabilization of the binding of α-chymotrypsin. At least a part of the binding involves the substrate binding site of the enzyme. The existence of secondary binding sites on the proteins, capable of interaction with gel-bound ligand, is suggested by the observation that at fairly high levels of substitution of gel by ligand the zymogen and covalently modified enzyme species are also bound quite strongly. Since the level of gel substitution is difficult to control, the results emphasize the necessity of a thorough investigation of the binding properties of a given affinity medium prior to use in the separation of active chymotrypsin from inactive derivatives.

INTRODUCTION

Since the first detailed report in 1968 (1), the technique of affinity chromatography has become established as an extremely powerful tool in the isolation and purification of biomolecules (2–4). The method generally is based on the original observation that compounds containing free amino groups can be covalently attached to water-insoluble polysaccharides that

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[2]Abbreviations: ACTME, ε-aminocaproyl-D-tryptophanmethylester; PBA, 4-phenylbutylamine; DFP, diisopropylfluorophosphate; TPCK, L-(l-tosylamido-2-phenyl) ethyl chloromethyl ketone; ATEE, *N*-acetyltyrosine ethyl ester.

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have been activated by prior treatment with cyanogen bromide (5). By suitable choice of ligand, the biological specificities of a wide variety of cellular components may be exploited in order to facilitate their isolation from complex mixtures. Despite numerous successes, however, the physical and chemical characteristics of affinity gels are only just beginning to be understood. It has beome apparent that the complexity of the affinity process extends beyond that of a simple association–dissociation phenomenon. In addition to the biospecificity of the attached ligand, possible roles for the "spacer arm" through which the ligand is coupled to the gel, for the type of chemical linkage at the gel–ligand interface, and for the gel itself have been documented. Both hydrophobic (6, 7) and ion exchange (7, 8) effects may be operative and these may either diminish or obliterate the biospecificity of the ligand (9). Conversely, these effects may be exploited in a specific manner by appropriate manipulation of the chromatographic conditions (10–14).

The use of ε-aminocaproyl-D-tryptophanmethylester (ACTME) as an immobilized ligand for the affinity purification of chymotrypsin was an early application of the method (1). Subsequently, it was shown that Sepharose 4B-4-phenylbutylamine (PBA) was also an effective medium for this enzyme (15). Both ligands have proven useful in the separation of active chymotrypsin from enzymatically and chemically modified protein (16, 17). In an earlier report (18), brief mention of certain anomalous properties of these gels has been made. More recent studies have shown that this behavior occurred frequently enough to merit further investigation. In this communication we describe experiments designed to explore the relative contributions of both specific and nonspecific interactions in the binding of α-chymotrypsin and other proteins to these affinity chromatographic media. The results provide further insight into the binding properties of both the derivatized gels and the proteins themselves, and serve to emphasize the need for careful investigation of these properties prior to using a given gel preparation in the separation of α-chymotrypsin from inactive species.

MATERIALS AND METHODS

Materials

4-Phenylbutylamine, cyanogen bromide, and DFP were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin); Sepharose 4B was a product of Pharmacia (Canada) Ltd. (Montreal, Quebec). ACTME was synthesized as previously described (16). Two preparations of Sepharose 4B-ACTME gels were purchased from Miles-Yeda (Rehovot, Israel). The

proteins used in this study were purchased from either Worthington Biochemical Corp. (Freehold, New Jersey) or Sigma Chemical Company (St. Louis, Missouri). The latter supplier also provided ATEE and TPCK. DIP-chymotrypsin and TPCK-inhibited chymotrypsin were prepared in the laboratory according to published procedures (19, 20).

Preparation of Affinity Gels

The ligands were coupled to cyanogen bromide-activated Sepharose 4B as previously described (1, 4, 15, 18). Attempts to vary the extent of substitution of gel by ligand were made by varying the amount of cyanogen bromide used, either as the powdered solid or dissolved in *N*-methyl-2-pyrrolidone (21), or by varying the amount of ligand added to the activated Sepharose 4B during the coupling step. Over the past 4 years we have examined over 50 different preparations of the PBA gels and six of the ACTME gels. Two of the latter were commercial preparations.

Binding Studies

The ability of a given gel to bind the various proteins was determined by the following procedure: A 0.9×15 cm column of the gel was equilibrated with the starting buffer (see later). The protein sample (5–10 mg) dissolved in 1–2 ml of the same buffer was applied to the column and elution with starting buffer was continued at a flow rate of 10–30 ml/h until at least 10 column volumes (about 60 ml) of buffer had passed without emergence of protein, or, if this occurred earlier than 10 column volumes, until all the protein had been removed by the starting buffer. Nonemergence of protein within 10 column volumes of buffer was taken to indicate strong binding of the protein to the gel. In these cases, the eluent was changed to the second buffer (see later) to effect complete removal of protein from the gel. Detection and quantitation of protein was by measurement of the absorbance at 280 nm. In all cases a total recovery in excess of 95% of the applied protein was obtained.

The starting buffer used was 0.05 M Tris, pH 8.0. The second buffer was most frequently 0.1 M acetic acid, pH 3.0, which completely and rapidly removed all of the bound protein in all cases examined. In experiments to determine the nature of the binding process, 0.05 M Tris–0.1 or 1.0 M NaCl, pH 8.0 (high ionic strength buffers); 0.05 M Tris–ethylene glycol (1 : 1), pH 8.0 (low polarity buffer); 0.05 M Tris–0.01 M PBA, pH 8.0, or 0.05 M Tris, pH 8.0, containing both ethylene glycol and NaCl were used as the second buffer solution. The elution patterns of the following protein solutions were examined: α-chymotrypsin, chymotrypsinogen A,

DIP-chymotrypsin, TPCK-inhibited chymotrypsin, trypsin, ribonuclease, lysozyme, subtilisin, ovalbumin, and bovine serum albumin (BSA).

Miscellaneous Techniques

α-Chymotrypsin activity was measured using ATEE as substrate by spectrophotometric assay (22) or by pH-stat assay using a Radiometer TTTll titration unit, syringe buret SBUla, and Titrigraph SBR2 recorder. The titrant was 0.01 M NaOH contained in a 2.0-ml syringe.

The presence of ionizing groups on the substituted gels was quantitatively determined by potentiometric titration with 0.100 M NaOH. The titrant was added in 5- to 100-μl aliquots to about 10 ml of settled gel suspended with stirring in 10 ml of 0.10 M KCl. Two minutes was allowed between each addition of titrant. Blank titrations in the absence of gel were carried out. Qualitative identification of guanidino-containing compounds was obtained after prolonged exposure of the gels to aqueous ammonia solution followed by Sakaguchi determination of the supernatant (23). Comparisons were made between gels showing good affinity properties and those with anomalous binding properties.

RESULTS

Affinity Chromatography

The behavior of a "good" column of Sepharose 4B-PBA towards chymotrypsinogen A and α-chymotrypsin is shown in Figs. 1a and 1b, respectively. In the case of chymotrypsinogen A more than 90% of the applied protein was eluted by 0.05 M Tris buffer, pH 8.0. The splitting of the peak may be attributed to the rapid elution of a small amount of contaminant protein followed by elution of the slightly retarded zymogen, and has been observed earlier (15). In the case of α-chymotrypsin the situation was, as expected, reversed, with more than 90% of the protein being retarded until the buffer was changed to 0.1 M acetic acid. Similar results were obtained with all of the Sepharose 4B-ACTME gels with the exception of one of the commercial samples. None of the other cationic proteins used was significantly retarded by these "good" columns, although the anionic proteins BSA and ovalbumin were strongly bound. Gels exhibiting the behavior illustrated in Figs. 1a and 1b are clearly suitable for the separation of active α-chymotrypsin from inactive derivatives and other positively charged proteins, using the procedure described.

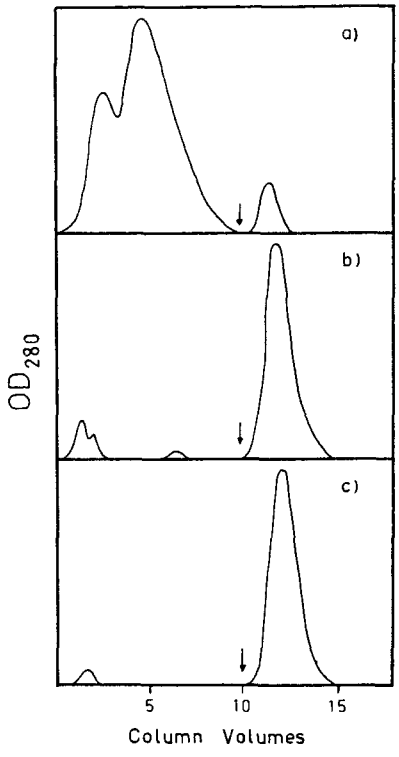

FIG. 1. Binding of chymotrypsinogen and α-chymotrypsin to Sepharose 4B-PBA. (a) "Good" column, chymotrypsinogen A; (b) "good" column, α-chymotrypsin; (c) anomalous column, identical elution profiles obtained with α-chymotrypsin, chymotrypsinogen A, DIP-chymotrypsin, and TPCK-chymotrypsin. Arrow indicates change of buffer from 0.05 M Tris, pH 8.0, to 0.1 M acetic acid.

Anomalous Gels

A number of preparations of Sepharose 4B-PBA and one of the commercial samples of Sepharose 4B-ACTME showed the anomalous binding properties illustrated in Fig. 1c. These columns were capable of strongly retarding both chymotrypsin and its zymogen, as well as the inactive DIP and TPCK derivatives of the enzyme. None of the other cationic proteins was retarded. All samples of bound protein were readily removed by elution with acetic acid in the usual manner.

Nature of Binding of Protein to Gels

In order to assess the contribution of biospecific interaction to the binding affinity, a "good" column of Sepharose 4B-PBA was run with an α-chymotrypsin sample under the same initial buffer conditions but with a second buffer of 0.05 M Tris, pH 8.0, containing 0.01 M PBA as a

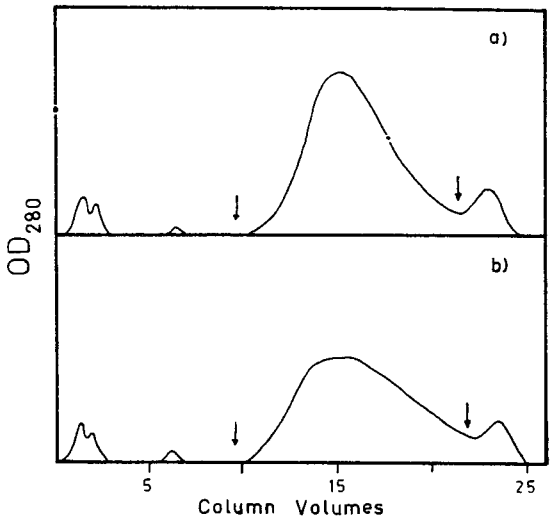

FIG. 2. Effect of 0.01 M PBA and 0.01 M NaCl on α-chymotrypsin binding to "good" PBA gel. (a) Second buffer 0.05 M Tris–0.01 M PBA, pH 8.0; (b) second buffer 0.05 M Tris–0.10 M NaCl, pH 8.0. First buffer was 0.05 M Tris, pH 8.0, in both cases.

competing ligand. Although a relatively poor inhibitor of the enzyme ($K_i = 2.8 \times 10^{-3}$ M) (15), Fig. 2a shows that the compound is quite effective in removing the bound enzyme from the gel. We conclude that added PBA competes with the gel-bound ligand for the same site on the enzyme and therefore that apparent biospecificity does contribute to the binding process. This is further supported by the apparently competitive inhibition produced by the gel when ATEE hydrolysis is measured by the pH-stat method (Fig. 3). However, bioaffinity is not the only factor involved since an elution pattern similar to that of Fig. 2a can be produced by using 0.05 M Tris–0.10 M NaCl, pH 8.0, as the second buffer, indicating that electrostatic interactions may also play a part (Fig. 2b). When these forces are suppressed by the inclusion of salt in the buffer, it appears that bioaffinity alone is insufficient to retard the enzyme strongly. Conversely, the ion exchange properties of the gel are also insufficient alone, as indicated by the ability of PBA to displace the enzyme from the gel. Both types of interaction seem to be necessary for the effective binding of α-chymotrypsin to Sepharose 4B-PBA.

The behavior of the anomalous gels with the solvents just mentioned was quite different. PBA-containing buffer was ineffective in removing bound proteins, nor could they be eluted with NaCl-containing buffers (Figs.

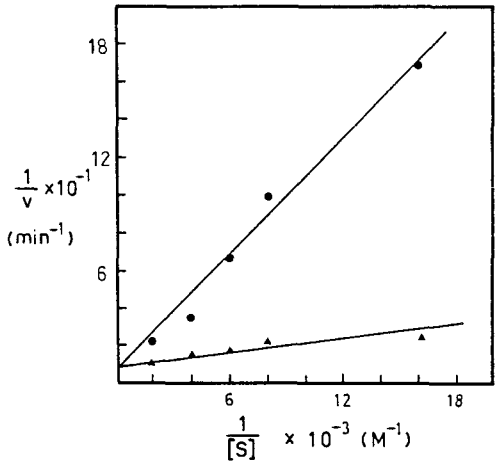

FIG. 3. Inhibition of α-chymotrypsin-catalyzed hydrolysis of ATEE by Sepharose 4B-PBA. All gels examined showed qualitatively similar behavior. ▲, No gel; ●, in presence of 3.0 ml gel. Total volume of reaction mixture was 40.0 ml.

4a and 4b). Although prolonged elution of "good" gels with the 0.05 M Tris, pH 8.0, starting buffer did result in the eventual removal of bound enzyme as a very broad peak beginning at about 25 column volumes and extending over a further 15–25 column volumes, the only protein to be similarly eluted from the anomalous gels was chymotrypsinogen A. Neither the active enzyme nor its inactive DIP or TPCK derivatives were eluted in detectable amounts, even after passage of 50 column volumes of the starting buffer. Thus, although the zymogen is less tightly bound than the other three proteins, the binding of all four is considerably stronger than in the case of a more typical affinity gel.

The binding of substrates and substrate analogs to α-chymotrypsin is largely hydrophobic in nature and therefore difficult to separate from nonspecific hydrophobic interactions with gel-bound ligands. Nevertheless, the relative contribution of the hydrophobic component toward the binding of the various chymotrypsin derivatives was examined by using buffers containing 50% ethylene glycol as a polarity-reducing agent in the second eluent. Typical results are shown in Figs. 5a and 5b. The low polarity medium proved capable of removing bound enzyme from the gel, but once again the stronger binding to the atypical gels is clearly manifest. Inclusion of 1.0 M NaCl in addition to ethylene glycol, however, produced virtually identical behavior with both types of gel (Fig. 5c). Similar effects on the

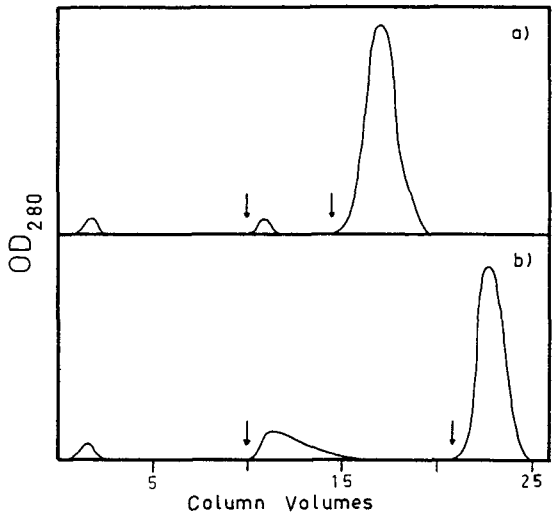

FIG. 4. Effect of PBA and NaCl on binding of α-chymotrypsin and its derivatives to anomalous PBA gel. (a) Second buffer 0.05 M Tris–0.01 M PBA, pH 8.0; (b) second buffer 0.05 M Tris–1.0 M NaCl, pH 8.0. First buffer was 0.05 M Tris, pH 8.0; third buffer was 0.1 M acetic acid, in all cases.

binding of inactive chymotrypsin species to the anomalous gels were also observed. As noted by Hofstee (7), the presence of salt in this medium would be expected to enhance any hydrophobic binding component should this be the predominant mode of binding. The fact that binding is further *reduced* by NaCl indicates that both hydrophobic and electrostatic forces contribute to the binding of these proteins to all of the gels studied.

Acid–Base Titrations

It seemed possible that the enhanced binding capabilities of the anomalous gels might be related to the level of substitution of gel by ligand, a parameter which is difficult to quantitate accurately. However, since it is likely that a large proportion of ligand molecules are attached to the gel via an isourea linkage (8, 24, 25), we felt that an estimate of the levels of isourea substitution should reflect at least relative levels of ligand substitution. Samples of the gels were therefore titrated with dilute NaOH over the pH range 3.0–11.0. A typical titration curve is shown in Fig. 6. All the gels examined showed qualitatively similar behavior, which is dominated by the ionization of a group with pK_a of about 9.2, attributable to the isourea

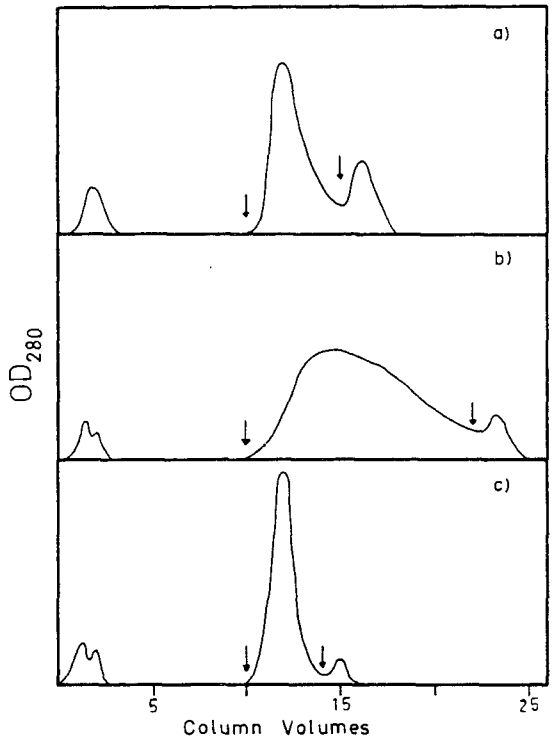

FIG. 5. Effect of mixed solvents on α-chymotrypsin binding to PBA gels (a) "Good" gel, second buffer 0.05 M Tris (pH 8.0)–ethylene glycol (1 : 1); (b) anomalous gel, second buffer as in part (a); (c) "good" or anomalous gel, second buffer 0.05 M Tris–1.0 M NaCl (pH 8.0)–ethylene glycol (1 : 1). First buffer was 0.05 M Tris, pH 8.0; third buffer was 0.1 M acetic acid, in all cases.

linkage. The extent of substitution is conveniently estimated from the first derivative of the titration curve, which further indicates the presence of a small amount of additional ionizable material with a relatively low pK_a. The latter is also present in unsubstituted Sepharose.

The range of isourea substitution was surprisingly narrow, given the marked differences in binding properties among the various preparations. Nevertheless, some correlation could be made between the ability to retard chymotrypsinogen A strongly and the level of isourea substitution (Table 1). In general, gels having a level of isourea substitution below about 15 μmol/ml showed high specificity for α-chymotrypsin binding; above this level the anomalous binding properties become apparent.

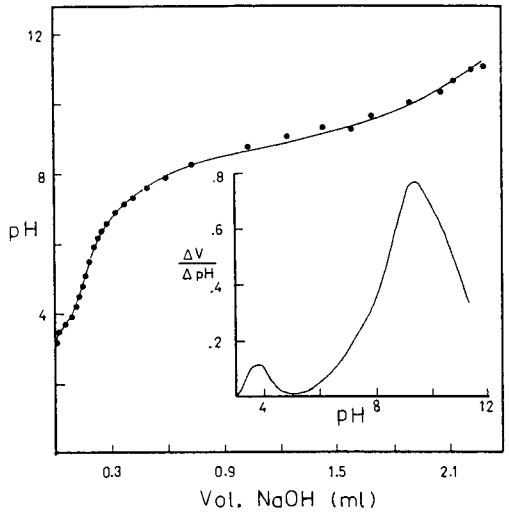

FIG. 6. Titration of Sepharose 4B-PBA with 0.100 M NaOH: 10.0 ml (settled volume) of gel suspended in 10.0 ml 0.1 M KCl. All gels showed qualitatively similar behaviour. Inset: First derivative of titration curve.

The presence of the isourea linkage in all the gels tested was confirmed qualitatively by treatment with aqueous ammonia solution at 35°C for up to 24 h, followed by application of the quantitative Sakaguchi test (23) to the supernatant solution. A positive result was obtained in all cases; however, the levels of substitution indicated by this procedure were very much lower than those obtained by direct titration, probably due to the instability of the released guanidino derivative of 4-phenylbutylamine. Similar behavior has been noted in the case of Sepharose-ornithine (6).

DISCUSSION

It has been suggested (3) that acceptable criteria for true affinity chromatography should include, among others, the following: (a) good correlation between the binding properties of the macromolecule–affinity matrix and the macromolecule–ligand system in free solution; (b) complete inhibition of enzyme activity should produce loss of ability of the enzyme to be bound to the affinity adsorbent; (c) inactivity of the bound enzyme; and (d) specific elution of enzyme by a substrate or other ligand known to bind to the substrate binding site of the enzyme.

TABLE 1. **Isourea Content of PBA Gels**

Gel	Chymotrypsinogen binding ability[a]	Isourea content (μmol/ml settled gel)[b]
PBA 25	−	8.25
PBA 26	−	14.50
PBA 27	+	19.25
PBA 28	+	17.75
PBA 29	+	24.25
PBA 30	−	15.05
PBA 31	+	20.25
PBA 32	+	22.75
PBA 33	−	10.60

[a] − means zymogen eluted within 10 column volumes of 0.05 M Tris, pH 8.0; +, zymogen remains bound to gel after passage of 10 column volumes of the same buffer.
[b] Determined by titration with 0.100 M NaOH (see text for details).

The "good" PBA and ACTME gels described here come quite close to satisfying these criteria. Both ligands are known competitive inhibitors of α-chymotrypsin. Irreversible inhibition of the enzyme produces a marked reduction in binding to the affinity adsorbent. The bound enzyme appears to be inactive, as indicated by the roughly competitive inhibition of ATEE hydrolysis in the presence of gel. Free ligand in solution can displace the bound enzyme. Furthermore, the inability of these gels to bind the zymogen strongly argues in favor of specific interaction with the enzyme.

Nevertheless, our results confirm previous suggestions (7, 30) that the binding of α-chymotrypsin to these adsorbents is via a combination of more or less specific hydrophobic interactions, which can be predicted *a priori* on the basis of the known specificity of the enzyme, and additional electrostatic interactions between negative charge(s) on the protein and positively-charged isourea groups introduced during the coupling of ligand to the gel (29, 30). The latter type of interaction is not so easily predicted from specificity considerations. The fact that both types of interaction are necessary for strong binding is evident from the independent ability to displace the enzyme shown by low concentrations of free ligand, low polarity solvent, or high ionic strength solvent. Whether these interactions take place at a single site on the protein (i.e., both hydrophobic and electrostatic forces provided a single ligand–protein interaction) or whether binding to more than one site on the protein is taking place is difficult to distinguish clearly. However, α-chymotrypsin is positively charged at pH 8.0, which should lead to weakening of binding because of electrostatic repulsions. That this does not

seem to be the case indicates some degree of specificity in the interaction of the enzyme with Sepharose 4B-PBA. Other positively charged proteins such as trypsin and lysozyme are not bound at all. On the other hand, the strong binding of anionic proteins such as ovalbumin and BSA illustrates that the ion exchange properties of the gels can function in a nonspecific manner. In any event, the apparent biospecific interaction of α-chymotrypsin with the "good" gels is certainly heterogeneous in nature.

The selectivity of a gel toward structurally related proteins (α-chymotrypsin, its zymogen, and irreversibly inhibited derivatives) appears to be critically dependent on the extent of substitution of gel by ligand. In general, lower levels of substitution produce the most selective affinity adsorbents, whereas higher levels tend to produce anomalous binding properties. One would of course expect that higher levels of substitution, particularly by groups containing potential ion exchangers, might lead to increased possibilities of nonspecific binding. In this case, however, the binding of cationic proteins other than α-chymotrypsin appears to be confined to proteins which are at least similar in structure to the native enzyme. Chymotrypsinogen A is slightly retarded by the "good" gels, which has been explained on the basis of the presence of a partially formed specificity pocket in this protein (15). The relatively strong binding of the zymogen to gels with greater than 15 μmol/ml of ligand seems hard to explain simply on the basis of higher substitution levels resulting in increased probability of binding to this low affinity site. Furthermore, the ability of anomalous gels to bind DIP-chymotrypsin and especially TPCK-chymotrypsin would appear to rule out this explanation. We therefore conclude that there is also heterogeneity among the protein binding sites, with each of these proteins having secondary PBA binding sites in addition to the (actual or potential) specificity site.

A modest increase in the extent of substitution of gel by ligand could conceivably enable the proteins to interact simultaneously with two or more ligands, which would have a synergistic rather than additive effect on the overall binding affinity. Not only would α-chymotrypsin bind even more strongly to the column but also the zymogen and the inhibited enzyme species could also bind moderately strongly through interactions with two or more of these peripheral sites. The idea of functional substites in α-chymotrypsin is by no means new. These appear to be involved in the binding and positioning of the protease substrates (26), and similar sites have been indicated in the case of the zymogen (27). A recent study of the reactions of a series of p-nitrophenylcarbamates (28) concluded that α-chymotrypsin indeed possesses a secondary binding site specific to positively charged side chains, and that the zymogen also has a secondary binding site capable of interacting with aromatic groups. Both of these structural fea-

tures are present when the affinity ligands studied here are covalently attached to Sepharose 4B.

In summary, the binding of chymotrypsin-like proteins to the affinity adsorbents Sepharose 4B-PBA and Sepharose 4B-ACTME is quite complex, exhibiting heterogeneity at the level of both the affinity medium and the proteins themselves. The appearance of anomalous binding properties appears to be critically dependent on the extent of substitution of the gel by ligand. Since the latter is quite difficult to control, despite careful attention to the experimental conditions during coupling, it is essential that the binding properties of individual preparations be carefully evaluated before use. We find that strong binding of α-chymotrypsin in the absence of strong binding of chymotrypsinogen A is a sufficient and convenient criterion of selectivity.

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