The interaction of chymotrypsin with an insoluble substrate*

This paper describes an attempt to develop a procedure for the isolation and purification of enzymes by exploiting the highly specific nature of enzyme–substrate interactions. The approach is based upon the hypothesis that an enzyme will be selectively adsorbed by substrates or competitive inhibitors which are insoluble in aqueous solutions, *i.e.*, by synthetic substances possessing hydrophobic groups in addition to the structural features found to be necessary for function as a substrate or competitive inhibitor**. The interaction of the proteolytic enzyme chymotrypsin with *N*-carbobenzyloxy-L-leucyl-D-phenylalanine benzyl ester (CLPB)*** is described here. CLPB possesses the general structure $R \cdot CO \cdot NH \cdot CH \cdot (CH_2 \cdot C_6H_5) \cdot CO \cdot OR'$, with the phenylalanine residue having the D configuration. It, therefore, meets the structural requirements for a competitive inhibitor of chymotrypsin[1] and, in addition, is insoluble in water.

The experimental procedure, described in detail in Table I, yielded results which can be summarized as follows:

(a) CLPB is capable of adsorbing various quantities of all of the proteins listed in Table I.

(b) CLPB shows the greatest affinity for chymotrypsinogen, chymotrypsin and pepsin, adsorbing as much as 85% of the dissolved protein.

(c) The adsorption of trypsin and trypsinogen was somewhat greater than that of the other proteins, excluding those listed in (b).

(d) CLPB, when exposed to a mixture of equal amounts of trypsin and chymotrypsin, adsorbed all of the chymotrypsin but less than 20% of the trypsin.

(e) Inactivation of trypsin and chymotrypsin by warming to 56° for 15 min markedly reduced their affinity for CLPB.

(f) DEP-chymotrypsin§ was adsorbed to the same extent as chymotrypsin, but warming to 56° for 15 min did not affect the interaction.

It was also found that adsorbed chymotrypsin could be eluted in 80% yield from CLPB by a cold 7 *M* urea solution in 0.05 *M* Tris buffer, pH 7.8, containing 2% NaCl. (Rapid denaturation occurs in the absence of NaCl.)

The above findings suggest that adsorption on CLPB or other insoluble substrates might be a feasible procedure for the isolation and purification of chymotrypsin and that this principle may be applicable to the isolation of many other enzymes.

Secondly, the data in paragraphs (e) and (f), taken together, lend support to the hypothesis that the adsorption of chymotrypsin by CLPB is the result of an interaction with the "active center" of the enzyme. According to VASLOW AND DOHERTY[2], this site in chymotrypsin consists of two parts: an attractive center (responsible for substrate specificity) and an activating center. Since CLPB is not hydrolyzed by chymotrypsin, its interaction would be only with the attractive center. The affinity of the insoluble substrate for chymotrypsinogen indicates that this center already exists in the zymogen, rather than being formed as part of the process of conversion of zymogen to active enzyme. This is in agreement with the findings of VASLOW AND DOHERTY[2]. The adsorption of pepsin could have been predicted on the basis of the similarity of its specificity to that of chymotrypsin.

If adsorption of chymotrypsin by CLPB is, indeed, a result of an interaction with the attractive center of the enzyme, a detailed study of this process could provide some understanding of the mechanism of substrate specificity. With this in mind, the adsorption of chymotrypsin by CLPB was determined at various hydrogen-ion concentrations. Within the pH range 1.5–8.5, the extent of adsorption remained constant (about 5.5 mg enzyme/g CLPB) but decreased rapidly between pH 8.5 and 10.2, becoming negligible at the latter pH and remaining so in the region of higher alkalinity. Since CLPB is uncharged, it follows that a change occurred in chymotrypsin between pH 8.5 and 10, destroying its affinity for CLPB. The descending portion of the curve resembles, in shape and pH range, the alkaline side of the pH-activity curve for the chymotryptic hydrolysis of proteins and certain synthetic substrates. It is suggested that the enzymic activity of chymotrypsin starts to decrease in the pH region 8–8.5 because of an alteration in the charge of an essential group (or groups) having a pK of approximately 9.5 located in the attractive center of the enzyme. The activating center of the enzyme may remain intact but is ineffectual

* This work is supported by the Office of Naval Research, under Contract N-onr-266(44).

** The term "insoluble substrate" will be used to describe this class of compounds. This term will apply even if the substance is structurally related to a competitive inhibitor.

*** The preparation of CLPB will be described in a later paper. It was used as a crystalline material, no attempt being made to grind it into a fine powder.

§ DEP-chymotrypsin was prepared by treating 60 mg crystalline chymotrypsin (Worthington) with 100 μg tetraethyl pyrophosphate in 3 ml 0.05 *M* Tris buffer, pH 8.0. No chymotryptic activity (with acetyl-L-tyrosine ethyl ester) was detectable in 10 min. The solution was then dialyzed and lyophilized.

TABLE I

ADSORPTION OF VARIOUS PROTEINS BY CLPB

2.1 mg of protein were dissolved in 2.1 ml 0.05 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.8, containing 2% NaCl. 0.1 ml (0.1 mg protein) of this cold solution was added to 15 mg of pre-cooled CLPB in a 15 ml tapered centrifuge tube, which was kept in an ice bath. The mixture was stirred by hand for 7 min, during which time adsorbed air was removed, and the mixture developed a paste-like consistency. 0.7 ml of the above Tris buffer (cold) was added, the mixture stirred and then centrifuged. The supernatant was assayed, as indicated below.

Protein	*mg adsorbed*	$\frac{\textit{mg adsorbed}}{\textit{mg unadsorbed}}$
Chymotrypsin	0.085 *, **, ***	5.66
Chymotrypsin (56°, 15 min)	0.018 **, ***	0.23
DEP-chymotrypsin	0.080 ***	4.00
DEP-chymotrypsin (56°, 15 min)	0.075 ***	3.00
Chymotrypsinogen	0.080 *, **, ***	4.00
Trypsin	0.047 *, **, ***	0.89
Trypsin (56°, 15 min)	0.017 ***	0.21
Trypsinogen	0.053 **, ***	1.12
Pepsin	0.080 **, ***	4.0
Chymotrypsin (0.1 mg) + Trypsin (0.1 mg)	0.100 (Chym) * 0.020 (Tryp) *	— 0.25
RNAase§, BSA§, β-lactoglobulin, HSA§	$\leq$ 0.035 ***	$\leq$ 0.54

* Assay by means of activity against substrate: Acetyl-L-tyrosine ethyl ester for chymotrypsin; benzoyl-L-arginine methyl ester for trypsin. Method of SCHWERT AND TAKENAKA[4]. Chymotrypsinogen was first activated by trypsin.

** Assay by absorption at 278 mμ.

*** Assay by FOLIN-CIOCALTEU (micro) method[5].

§ RNAase, Ribonuclease; BSA, Bovine serum albumin; HSA, Human serum albumin.

since no enzyme–substrate complex can form. This same mechanism may apply to other hydrolytic enzymes having similar pH-activity curves.

It should be noted in Table I that all of the proteins tested interacted with CLPB to some extent. One could postulate, therefore, that proteins, in general, have some ability to bind any substance capable of forming hydrogen bonds and that the enzyme–substrate interaction is merely a special case in two respects: (1) spatial considerations insure a closer "fit", allowing short range forces to come into play, and (2) an activating site is properly located in relation to the binding site. Enzymoid[3]-substrate and, perhaps, antigen–antibody systems could be considered to occupy an intermediate position, since they possess attribute (1) but lack (2).

The technical assistance of Messrs. R. EPAND, M. LACHE and N. KOKOWSKY is gratefully acknowledged.

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[1] H. NEURATH AND G. W. SCHWERT, *Chem. Revs.*, 46 (1950) 69.
[2] F. VASLOW AND D. G. DOHERTY, *J. Am. Chem. Soc.*, 75 (1953) 928.
[3] E. H. FRIEDEN, *J. Am. Chem. Soc.*, 78 (1956) 961.
[4] G. W. SCHWERT AND Y. TAKENAKA, *Biochim. Biophys. Acta*, 16 (1955) 570.
[5] E. A. KABAT AND M. M. MAYER, *Experimental Immunochemistry*, Charles C. Thomas, Springfield Ill., 1948, p. 322.

Received November 20th, 1957