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The Binding of Phenylguanidinium Ion and Phenol to Trypsin*

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ABSTRACT: The technique of equilibrium dialysis was employed to investigate the nature of the interaction of phenylguanidinium ion and phenol with trypsin. [^{3}H]Phenylguanidinium ion bound at one site per catalytically active trypsin molecule with a dissociation constant of 1.4×10^{-4} M. Phenol affected the binding of phenylguanidine in a manner which was not competitive with $K_i = 4.3 \times 10^{-2}$ M. These results are consistent with the proposed model for ternary complex formation involving an auxiliary binding site on trypsin.

In a previous publication (Sanborn and Hein, 1968), kinetic evidence was presented for an auxiliary site on trypsin to which at least some neutral molecules preferentially bind. Confirmation of the existence of ternary complex formation was sought using the technique of equilibrium dialysis.

Equilibrium dialysis has been used to study the binding of virtual substrates to chymotrypsin and has yielded information on the groups involved in binding as well as thermodynamic data (Doherty and Vaslow, 1952; Vaslow, 1958; Loewus and Briggs, 1952; Johnson and Knowles, 1966). Competitive dialysis, where the displacement of a strongly bound molecule by a more weakly bound molecule is followed, has been employed to investigate the interaction of inhibitors with chymotrypsin (Weiner *et al.*, 1966). Most recently, the binding of thionine to trypsin, chymotrypsin, and modified forms of these enzymes has been reported (Glazer, 1967).

Experimental Details

Procedure. Lucite dialysis cells (E-1) of 2-ml total capacity were purchased from Technilab Instruments, Los Angeles, Calif. Squares of dialysis tubing (Visking Co., 1.87 stainless steel dialysis) were cut from tubing boiled for 7 hr in distilled water, stored in the cold, and soaked in the appropriate buffer for at least 1 hr prior to use. Eight cells were mounted on a Lucite holder and rotated in a Tamson-refrigerated bath at $10 \pm 0.1°$ for 7 hr unless otherwise indicated.

At the conclusion of the experiment, three 0.1-ml samples were withdrawn from each chamber of a given cell, added to 25 ml of Bray's (1960) scintillation solution, and counted in a Packard 3202 liquid scintillation spectrometer. Aliquots were reproducible within 1%; total counts were reproducible within 3%. Both an external standard provided with the counter and an internal [^{3}H]water standard (New England Nuclear Corp.) were used to normalize the counts.

The data were analyzed by the method of least squares with a t distribution percentage point of 0.05 and a constraint to retain 90% of the data. The errors are probable errors.

Materials. For this study, two structural analogs showing the kinetic behavior for compounds of different charge types predicted by the previous study were sought. Phenylurea and phenylguanidinium ion seemed likely choices of an isosteric pair since they each showed competitive behavior with substrates of the same charge type but deviated from it with substrates of a different charge type. Solubility considerations, however, necessitated the use of phenol in place of phenylurea. Phenol and phenylurea show similar kinetic behavior as modifiers of the trypsin-catalyzed hydrolyses of neutral and positively charged substrates (Sanborn and Hein, 1968).

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[^{3}H]Phenylguanidinium nitrate was prepared from [^{3}H]aniline hydrochloride (New England Nuclear Corp., generally labeled, 2 mCi/mmole) and 1-guanyl-3,5-dimethylpyrazole nitrate according to the method of Bannard *et al.* (1958), mp 120.5–121°. The concentration of a stock solution in 0.1 M sodium phosphate (pH 7.0) was determined by absorbance at 240 mμ using the optical factor 5010 ± 63 mole l.$^{-1}$ (OD)$^{-1}$; dilutions were estimated from a knowledge of the specific activity of the stock solution.

Neither phenylguanidinium nitrate nor phenol acted as a scintillation quencher under the conditions of the experiments. The enzyme also failed to show any inhibitory effect on the number of counts.

In a control experiment, it was shown that phenol required between 3.5 and 5 hr to distribute itself equally across the membrane in the dialysis cell; phenylguanidinium nitrate equilibrated in 4–6 hr. In the actual experiments phenylguanidine and phenol were added in equal concentrations to both sides of the membrane to shorten the time necessary to reach equilibrium.

Enzyme. The trypsin used (Worthington Biochemical Corp., lot 6402) was twice crystallized and salt free. Using enzyme which had been dialyzed against 0.001 N hydrochloric acid for 36 hr at 4° in size 20 Visking tubing, centrifuged for 15 min at 3000 rpm and 0°, and lyophilized, less than 2% of the protein (OD 280 mμ) diffused through the membrane under the conditions of a typical experiment. The enzyme did not adsorb to the membrane in detectable amounts.

Prior to use, an enzyme solution of 50 mg/ml in 0.1 M sodium phosphate was adjusted to pH 7.0 and centrifuged for 15 min at 10,000 rpm and 0°. Determination of total enzyme concentration was accomplished by measuring the optical density at 280 mμ using the optical factor 0.651 mg ml^{-1} (OD unit)$^{-1}$ (Worthington, 1961) and a molecular weight of 24,000 (Kay *et al.*, 1961). The active enzyme concentration was determined by the method of Bender *et al.* (1965) using *p*-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate hydrochloride (Cyclo Chemical Corp.) and extrapolating to infinite [S] concentrations.

The ionic strength in a typical experiment in pH 7.0 0.1 M sodium phosphate buffer with a total nitrate concentration of 2.62 × 10^{-2} M (phenylguanidinium nitrate plus sodium nitrate) was calculated to be 0.183. Under these conditions, the Donnan effect of a 2 × 10^{-4} M trypsin solution should be negligible assuming a net charge of +23 for trypsin (Duke *et al.*, 1952). The pH on both sides of the membrane remained essentially constant as did the volumes determined by weighing the cell contents withdrawn into plastic syringes.

Under the conditions of the experiments, trypsin solutions lost 10% of their activity toward benzoyl-arginine methyl ester (253 mμ) in 6.5 hr but only 3% in the presence of a representative concentration of phenylguanidine. Thus phenylguanidine protected the enzyme from autolysis, and since the corrections for binding of material to autolysis products would be rather arbitrary, none were made. The estimates of the active enzyme concentrations were made 4–5 hr after the experiments began and represent intermediate values.

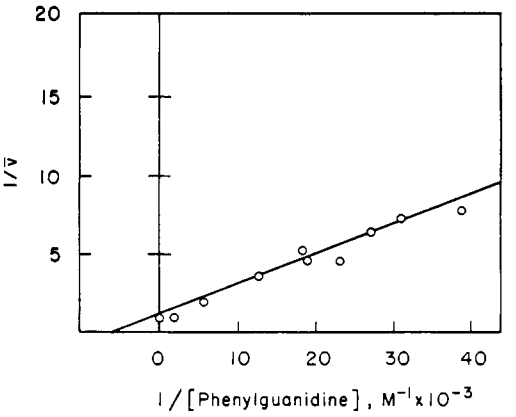

FIGURE 1: The binding of phenylguanidinium ion to trypsin based on the catalytically active enzyme concentration.

Tosyllysylmethyltrypsin was prepared according to Shaw *et al.* (1965).

Results

The Binding of Phenylguanidinium Ion to Trypsin. For *n* equivalent and independent sites the binding of a small molecule to a macromolecule can be represented by

$$\bar{v} = \frac{n[C]/K_0}{1 + [C]/K_0} \quad (1)$$

where $\bar{v}$ is the number of moles of small molecule bound per mole of macromolecule, [C] is the concentration of unbound small molecule, and K_0 is the intrinsic dissociation constant for a binding site (Tanford, 1956).

The expression $\bar{v}$ can be calculated from the binding data on the basis of either total or active enzyme concentration. Figure 1 shows the results of the latter method. The data were plotted as $\bar{v}$/[C] *vs.* $\bar{v}$ or $1/\bar{v}$ *vs.* 1/[C] to determine *n* and *K*. In Table I are presented the results of both types of plots and calculations. It is not reasonable that one molecule of phenylguanidine would bind to two molecules of trypsin. Therefore the conclusion of this experiment is that the substrate binds only to catalytically active trypsin. The binding constant from the double-reciprocal plot, 1.4 × 10^{-4} M, compares well with those measured kinetically.

Included in the data are several points which were measured after 9-hr dialysis. They do not differ significantly from those measurements taken at 7 hr and thus constitute evidence that true equilibrium conditions exist and that autolysis was negligible.

Attempts were made to extend the binding curve of phenylguanidine to higher concentrations in an effort to see the second binding site suggested by Trowbridge *et al.* (1963). The second dissociation constant is 100 times larger than the first constant, however, and for this reason any additional binding was obscured by experimental error.

Tosyllysylmethyltrypsin showed no measurable affinity for phenylguanidine (K = >10^{-3} M) under conditions comparable with those employed in the trypsin experiments. Thus the competition between tosyllysine chloromethyl ketone and phenylguanidine for the specificity

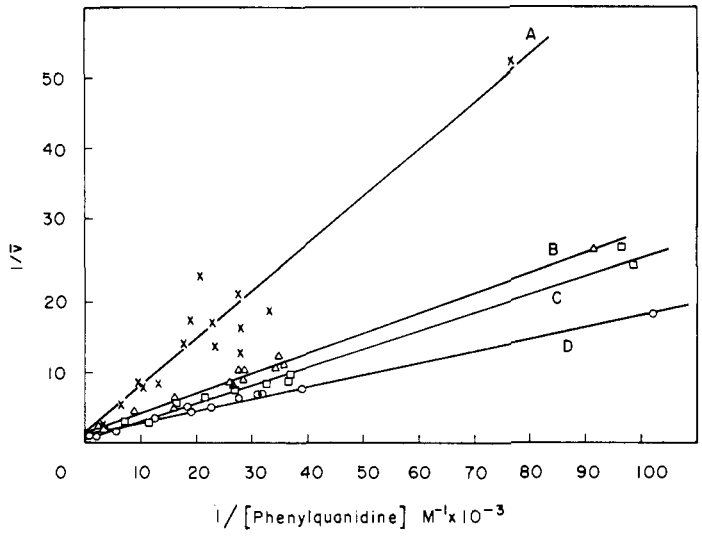

FIGURE 2: The effect of phenol on the binding of phenylguanidinium ion to catalytically active trypsin. Phenol concentrations are: (A) (X) 10.2×10^{-2} M, (B) (△) 5.69×10^{-2} M, (C) (□) 3.62×10^{-2} M, and (D) (○) 0.00. Solid lines are determined from a least-squares analysis of the data.

TABLE I: The Binding of Phenylguanidinium Ion to Trypsin.[a]

Type of Plot	Enzyme Concn Used in Calculation	n	K_0 (M)
$\bar{v}$/[C] *vs.* $\bar{v}$	Total enzyme[b]	0.60 ± 0.048	$2.2 \pm 0.16 \times 10^{-4}$
$1/\bar{v}$ *vs.* 1/[C]	Total enzyme[b]	0.34 ± 0.13	$1.2 \pm 0.49 \times 10^{-4}$
$\bar{v}$/[C] *vs.* $\bar{v}$	Active enzyme[c]	1.3 ± 0.088	$2.6 \pm 0.15 \times 10^{-4}$
$1/\bar{v}$ *vs.* 1/[C]	Active enzyme[c]	0.83 ± 0.077	$1.4 \pm 0.11 \times 10^{-4}$

[a] For the $\bar{v}$/[C] *vs.* $\bar{v}$ plot the x intercept is n; -1/slope is K_0. For the $1/\bar{v}$ *vs.* 1/[C] plot the y intercept is $1/n$; K_0 is the product of the slope $[K_0/n]$ and the reciprocal of the y intercept. [b] Determined by optical density at 280 mμ. [c] Determined by assay of Bender *et al.* (1965).

site observed in the kinetics of enzyme inactivation (Sanborn and Hein, 1968) has been substantiated by equilibrium dialysis.

Binding of [^{14}C]phenol to trypsin was detected, but the unfavorable dissociation constant ($\sim 10^{-2}$ M) and side effects made quantitative measurements impossible.

The Effect of Phenol on the Binding of Phenylguanidine to Trypsin. In the presence of a second small molecule, M, which can bind such that a binding site can accommodate C alone, M alone, or C and M together, eq 1 for the binding of C becomes

$$\bar{v} = \frac{n([C]/K_0 + [C][M]/\alpha K_0 K_i)}{1 + [C]/K_0 + [M]/K_i + [C][M]/\alpha K_0 K_i} \quad (2)$$

where $K_0 = [C][E]/[EC]$, $K_i = [M][E]/[EM]$, $\alpha K_0 = [C][EM]/[ECM]$, n represents the number of such sites, and α is a measure of the effect that the binding of one type of molecule has on the binding of the second molecule. If α were 1, phenol would not affect the binding of phenylguanidine; that such is not the case is shown in Figure 2 where data are presented using a linear form of eq 2 ($1/\bar{v}$ *vs.* 1/[C] at constant [M]). An α value approaching infinity would imply a competition between C and M for a particular site. Intermediate α values ($1 < \alpha < \infty$) imply that the presence of C on the enzyme affects the binding of M but does not exclude M and *vice versa.*

The scatter in the data at the highest phenol concentration is probably due to the effects of this substance on the enzyme itself. An activity study under the conditions of a typical experiment showed little loss of enzymatic activity in the 2×10^{-4} M trypsin control and no loss when the solution contained 10^{-4} M phenylguanidine. A 30% loss in activity occurred in a sample to which 10^{-1} M phenol was added; a sample containing enzyme, phenol, and phenylguanidine appeared to lose the same amount of activity.

Using eq 2 and the data from Figure 2, K_i, the dissociation constant for phenol binding, and α can be calculated. From the two lines representing the lower phenol concentrations, one obtains $K_i = 4.3 \times 10^{-2}$ M and α values of 2.6 (line B) and 2.2 (line C). This K_i compares favorably with the value of 5.4×10^{-2} M measured kinetically against benzoylcitrulline methyl ester (Sanborn and Hein, 1968).

An α value of 2 is indicative of an interaction involving a ternary complex. That is, phenol binding does not exclude phenylguanidine or *vice versa.* This result is an independent confirmation of the presence of an auxiliary binding site on trypsin.

Use of line A gave K_i values which were inconsistent with the above constant as well as kinetically determined contants (K_i = 7.0 M, −2.8 M). These data were considered in error because of the deleterious effect of 0.1 M phenol on trypsin over a period of 7 hr.

Discussion

Trypsin preparations used in the present study were found to contain only 56% catalytically active enzyme. The fact that phenylguanidinium ion binds only to catalytically active enzyme supports other evidence for the restrictive geometry of the active site (Mares-Guia and Shaw, 1965). Proper interactions favoring binding are apparently only present in the active conformation of the enzyme.

Glazer (1965) estimated a one-to one-stoichiometry for the binding of proflavin to trypsin based on total enzyme concentration. His criterion for trypsin concentration in a study of the binding of thionine to that enzyme is not immediately obvious (Glazer, 1967), but again a one-to-one correspondence is reported. It remains to be seen whether the stoichiometry relationship is a function of the type of molecule involved in binding.

The agreement between the dissociation constants obtained for both phenylguanidinium ion and phenol by the techniques of inhibition kinetics and equilibrium dialysis is encouraging. Complications introduced by the necessarily longer duration of the dialysis experiments prevent absolute confirmation of ternary complex formation; the data are consistent with this model, however.

Several models have been considered to reconcile the apparent deviation of line A in Figure 2 from the proposed behavior (Sanborn, 1968). Neither competitive behavior ($\alpha = \infty$, which would predict a linear relationship between $1/\bar{v}$ and [M]) nor models involving more than one binding site for phenol fit the data. It is concluded that line A is spurious due to the deleterious effects of high concentrations of phenol upon trypsin.

The simplest model which will explain both the kinetic information and the equilibrium dialysis results is that trypsin possesses two binding sites, one to which charged molecules preferentially bind and one to which neutral molecules preferentially bind. These sites are in reasonable proximity to one another and interaction between substances binding to each can be observed. Both sites orient substrates so that the same enzyme residues are operative in the catalytic mechanism for the two types of compounds.

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