Studies on the Active Center of Trypsin

THE BINDING OF AMIDINES AND GUANIDINES AS MODELS OF THE SUBSTRATE SIDE CHAIN*

MARCOS MARES-GUIA† AND ELLIOTT SHAW

From the Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112

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Progress in defining the active center of trypsin in structural terms has been made mainly in the direction of elucidating the catalytic site and its participation in the reactions promoted by the enzyme. The discovery by Jansen, Nutting, Jang, and Balls (1) that diisopropyl phosphofluoridate was able to inhibit the esterase and proteinase activity of trypsin led to the localization of the diisopropylphosphoryl group on a sequence containing a seryl residue which is especially reactive compared to other serine residues in the enzyme molecule (2). The studies of Gutfreund (3) on the pH dependence of the trypsin-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester indicated that a histidyl residue participates in the mechanism of trypsin catalysis. The development (4) in this laboratory of a new active center reagent for trypsin, the chloromethyl ketone derived from N™-tosyl-L-lysine, permits a clarification of the role of histidine in the catalytic process as will be described subsequently.

Here we present initial results on the specificity site at the active center of trypsin from studies on structural features that cause certain small molecules to possess high affinity for the active center of trypsin. Since trypsin catalyzes the hydrolysis of peptide and ester bonds formed with the carboxyl group of the basic amino acids, lysine and arginine (5), and since the side of peptide and ester bonds formed with the carboxyl group of the active center of trypsin from studies on structural features that cause certain small molecules to possess high affinity for the active center of trypsin, the chloromethyl ketone derived from N™-tosyl-L-lysine, permits a clarification of the role of histidine in the catalytic process as will be described subsequently.

Here we present initial results on the specificity site at the active center of trypsin from studies on structural features that cause certain small molecules to possess high affinity for the active center of trypsin. Since trypsin catalyzes the hydrolysis of peptide and ester bonds formed with the carboxyl group of the basic amino acids, lysine and arginine (5), and since the side chains of these amino acids are flexible and consequently are capable of existing in several conformations, model compounds of the side chains of the typical substrate amino acids were chosen which, besides fulfilling some of the specificity requirements of trypsin, also have a single or a limited number of sterically different structures.

The knowledge of the conformation of the substrate when located at the active center of an enzyme is essential for the understanding of enzyme specificity, since the conformation adopted by the substrate will disclose the stereochemistry of the active center in view of its complementarity to the substrate (6, 7).

The model compounds used all contained a positively charged group, either amidinium or guanidinium, and were shown to be competitive inhibitors of trypsin by the use of a kinetic treatment developed for the inhibition of an enzyme by two competitive inhibitors. The treatment was especially extended for the case in which the substrate is a racemic mixture of which only one of the optical isomers is attacked by the enzyme, the other being a competitive inhibitor. This was carried out, in part, to permit the use of the chromogenic substrate, N™-benzoyl-DL-arginine p-nitroanilide (8), for spectrophotometric assay, since other assays making use of the ultraviolet region of the spectrum were interfered with by the benzzenoid inhibitors.

Correlation between the contribution of the side chains of the inhibitors to the empirical standard free energy of binding and data on aqueous solutions of hydrocarbons, as well as calculated dispersion interaction energies, led to the proposition that the side chains of the inhibitors and substrates lie in a hydrophobic binding site, in the form of a slit or crevice, in the specificity site of the trypsin active center.

Preliminary reports of this work have appeared (4, 9).

EXPERIMENTAL PROCEDURE

Reagents—Trypsin was a Worthington twice crystallized, lyophilized, salt-free preparation, Lot TRL-6233. Benzamidine-HCl dihydrate was a product of the Aldrich Chemical Company. Cyclohexylguanidine and phenylguanidine hydrochlorides were gifts from the American Cyanamid Company. The melting points of these three compounds were in satisfactory agreement with their respective literature values (10–12). Acetamidine hydrochloride, p-aminobenzenamidine hydrochloride, and phenylguanidine sulfide were prepared by published procedures (10, 13, 14).

Cyclohexylcarboxamidine hydrochloride was synthesized by the standard method from the nitrile by way of the ethylinimid ether. Cyclohexitannitrile was prepared from cyclohexene-carboxylic acid, via the amide, as reported by Siegel and Komarny (15). The amidine hydrochloride, after recrystallization from ethanol and ether, melted at 192–192.8°.

C₁₅H₁₅N₄Cl (262.7)
Calculated: C 51.70, H 9.30, N 17.22, Cl 21.80
Found: C 51.05, H 9.62, N 17.28, Cl 21.70

Dimethyl sulfoxide was Baker Analyzed Reagent Grade. N™-Benzoyl-DL-arginine p-nitroanilide was a product of the Mann Research Laboratories, Inc.

Melting points were taken on a Fisher-Johns block. Micro-
trypsin were prepared after the weighed enzyme and the solvent, in separate flasks, had been brought to thermal equilibration at 0° was used to maintain the temperature of the system constant to within 0.1°.

Dreiding stereomodels were obtained from Swissgo Instruments, Greenville, Illinois.

Trypsin Solutions and Trypsin Concentration—All solutions of trypsin were prepared after the weighed enzyme and the solvent, in separate flasks, had been brought to thermal equilibration with an ice-water bath. Throughout the experiments the trypsin solutions were always kept in the ice-water bath. After centrifugation at 6,000 r.p.m., the concentration of the supernatant was calculated from the absorbance at 2,800 A, according to the formula

\[
\text{Trypsin concentration (m)} = \frac{0.0894 \times A(2,800 \text{ A})}{24 \times 10^8}
\]

where 0.0894 is the factor for conversion from absorbance (A) at 2,800 A to milligrams per ml (16), and 24,000 is the molecular weight of trypsin (17, 18).

Trypsin Assay—Spectrophotometric assays were carried out with Æ-BANA as substrate, as described by Erlanger, Kokowsky, and Cohen (8). Tryptic activity was measured by adding 0.10 ml of trypsin solution (9.00 mg in 10.0 ml of 0.0010 M HCl) at zero time to 2.90 ml of substrate and buffer, or substrate, inhibitor, and buffer solutions in the cuvette, after thermal equilibration with the cell compartment of the spectrophotometer had been achieved at 15°. The substrate, inhibitor, and buffer solutions were also maintained at 15° in a small water bath in series with the thermospacers and circulator. To the reference cuvette, 0.10 ml of 0.0010 M HCl was added instead of trypsin.

After addition of the enzyme to the sample cuvette, the change in absorbance at 4100 A was measured at 15-second intervals for 3 to 4 minutes. The values were plotted against time, and the slopes of the straight lines obtained gave the initial rates of reaction. In all experiments the rates were obtained in triplicate and the average values were used. In all cases the mean percentage deviation from the average was within 2 to 3%. The rates, in moles per liter per second, were obtained by multiplying the measured absorbance per minute by 1/(60 × 8800), where 8800 is the molar extinction coefficient of p-nitroaniline at 4100 A (8).

To improve the accuracy and decrease the time of the first reading, the enzyme solution was pipetted into an applicator made of a polyethylene stirring rod by opening a concavity in its base which held 0.1 ml. From this applicator the enzyme solution was transferred to the substrate solution at zero time, and mixing was simultaneously accomplished with 15 to 20 strokes of the applicator. In this manner the first 15-second reading was always readily obtained.

RESULTS

Kinetic Treatment—As shown by Erlanger et al. (8), Æ-BANA is cleaved by trypsin, whereas the D form acts as a competitive inhibitor of the enzyme. For the evaluation of the Kₐ values of inhibitors with Æ-BANA as a substrate, a kinetic treatment was developed which takes into account the presence of the p form when another inhibitor is added to the system.

For the system described by

\[
E + S \rightarrow ES \rightarrow E + P
\]

where I and I' are competitive inhibitors,

\[
K_I = \frac{(E)(I)}{(EI)}
\]

and the rate of reaction will be given by

\[
\frac{1}{v} = \frac{V(S)}{K_m} \left[ 1 + \frac{(I)}{K_I} + \frac{(I')}{K_{I'}} \right] + \frac{(S)}{V(S)K_{I'}}
\]

Equation 6 can be put in the inverse form

\[
\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V(S)} \left[ 1 + \frac{(I)}{K_I} + \frac{(I')}{K_{I'}} \right]
\]

and

\[
\frac{1}{v} = \frac{1}{V} \left[ 1 + K_m \left( \frac{(S)}{K_I} \left( 1 + \frac{(I)}{K_I} \right) \right) \right] + \frac{K_m(I')}{V(S)K_{I'}}
\]

From Equation 8 it is clear that a plot of 1/v against (I') at constant (I) and (S) will be a straight line of slope Kₐ/V(S)K_I.

In order to differentiate between competitive and noncompetitive inhibition, 1/v is plotted against (I') at several values of (S), and the differentiation will now be considered.

Let Sᵢ and Sⱼ be two concentrations of substrate (Æ-BANA) such that Sᵢ ≠ Sⱼ, and

\[
S_i = I_i
\]

where Iᵢ,j stands for Æ-BANA, a competitive inhibitor.

If the concentration of the second inhibitor, (I'), is varied at constant Sᵢ = Iᵢ, Sⱼ = Iⱼ, respectively, a plot of 1/v against (I') will give two straight lines which will intersect at the point

\[
(I') = -K_{I'}
\]

as can be calculated from Equation 8.

For the case of noncompetitive inhibition, the rate of reaction is given by

\[
\frac{1}{v} = \left( 1 + \frac{(I')}{K_{I'}} \right) \frac{1}{V} \left[ 1 + \frac{K_m}{V(S)} \left( 1 + \frac{(I)}{K_I} \right) \right]
\]

If the same discussion and experimental conditions are applied to Equation 11, two straight lines will also be obtained which intersect at

\[
(I') = -K_{I'}
\]

\[
\frac{1}{v} = 0
\]
TABLE I
Calculation of enzyme-inhibitor constants from plots of reciprocal initial rates against inhibitor concentration

<table>
<thead>
<tr>
<th>Equation</th>
<th>Competitive</th>
<th>Noncompetitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{1}{v} = \frac{1}{V} \left[ 1 + \frac{K_m}{(S)} \left( 1 + \frac{(I)}{K_I} \right) \right] + \frac{K_m}{V(S)} \left( \frac{t}{K_I} \right) )</td>
<td>( \frac{1}{v} = \left[ 1 + \frac{(I)}{K_I} \right] \frac{1}{V} \left[ 1 + \frac{K_m}{(S)} \left( 1 + \frac{(I)}{K_I} \right) \right] )</td>
<td>( \frac{1}{v} = \left[ 1 + \frac{K_m}{(S)} \left( 1 + \frac{(I)}{K_I} \right) \right] \frac{1}{K_I} )</td>
</tr>
<tr>
<td>Ordinate intercept</td>
<td>( \frac{K_m}{V(S)K_I} \left( 1 + \frac{(I)}{K_I} \right) )</td>
<td>( \frac{1}{v} \left[ 1 + \frac{K_m}{(S)} \left( 1 + \frac{(I)}{K_I} \right) \right] )</td>
</tr>
<tr>
<td>Slope</td>
<td>( \frac{1}{v} = \frac{1}{V} \left[ 1 + \frac{K_m}{(S)} \right] )</td>
<td>( \frac{1}{v} = 0 )</td>
</tr>
<tr>
<td>Intersection</td>
<td>( (I') = -K_I )</td>
<td></td>
</tr>
</tbody>
</table>

These results are summarized in Table I. This treatment can obviously be extended to any number of inhibitors in the system.

**DISCUSSION**

From the data in Table II it is possible to calculate the contributions of some of the groups to binding. Furthermore, by reference to calculated and measured values in the literature, it is possible to gain insight into the mechanism of binding of the inhibitors.

The data on benzamidine·HCl and cyclohexylcarboxamide·HCl will be considered first. Both molecules have approximately the same length and width, but differ in thickness and conformation. In addition, both have an axis of symmetry which passes through carbon atom 4 in the ring and the carbon atom of the amidine group. It may be added that each molecule has approximately the same length as the side chain of lysine or arginine, as measured from the nucleus of the \( \beta \) carbon atom.
TABLE II

Competitive inhibitors of trypsin

Experiments were performed at pH 8.15 and 15.0°.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_I$</th>
<th>$\Delta F^c$ kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$N       + C[-]</td>
<td>$8.25 \times 10^{-5}$</td>
<td>6.70</td>
</tr>
<tr>
<td>H$_2$N       + C[-]</td>
<td>$1.84 \times 10^{-5}$</td>
<td>6.24</td>
</tr>
<tr>
<td>H$_2$N       + C[-]</td>
<td>$7.25 \times 10^{-5}$</td>
<td>5.46</td>
</tr>
<tr>
<td>H$_2$N       + C[-]</td>
<td>$1.51 \times 10^{-5}$</td>
<td>2.40</td>
</tr>
<tr>
<td>H$_2$N       + C[-]</td>
<td>$3.65 \times 10^{-2}$</td>
<td>1.90</td>
</tr>
<tr>
<td>H$_2$N       + C[-]</td>
<td>$4.27 \times 10^{-4}$</td>
<td>4.44</td>
</tr>
<tr>
<td>H$_2$N       + C[-]</td>
<td>$1.54 \times 10^{-3}$</td>
<td>3.70</td>
</tr>
</tbody>
</table>

TABLE III

Contribution of phenyl and cyclohexyl moieties of respective Amidine hydrochlorides to binding to trypsin

<table>
<thead>
<tr>
<th>Group</th>
<th>$\Delta(\Delta F^c)$ kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>Phenyl...........</td>
<td>4.35</td>
</tr>
<tr>
<td>Cyclohexyl......</td>
<td>2.55</td>
</tr>
</tbody>
</table>

* Calculated.
† Observed.

to the positive group, when held fully extended, and with coincident centers of positive charge. Dreiding stereomodels were used to calculate these distances. The two amides therefore constitute good models of the side chains of the amino acids which form specific substrates of trypsin, with the added advantage of having a single, well defined conformation.

When measurements were taken with the Dreiding stereomodels, the center of positive charge of the amidinium group was considered at the intersection of the line joining the nuclei of the 2 nitrogen atoms with the longitudinal axis of the molecule, and the center of positive charge of the guanidinium group at the central carbon of the group.

If acetamidine-HCl is taken as reference, it is possible to obtain the contribution to binding made by the phenyl and cyclohexyl side chains, after the proper correction for the methyl group in acetamidine-HCl is added.

$$\Delta F_{(BZ)} - \Delta F_{(AcA)} = \Delta(\Delta F^c) \left[ (\text{C}_6\text{H}_5) - (\text{CH}_3) \right] = 4.35 \text{ kcal mol}^{-1}$$

$$\Delta F_{(CHA)} - \Delta F_{(AcA)} = \Delta(\Delta F^c) \left[ (\text{C}_6\text{H}_{11}) - (\text{CH}_3) \right] = 2.55 \text{ kcal mol}^{-1}$$

Here BZ represents benzamidine-HCl; AcA, acetamidine-HCl; and CHA, cyclohexylcarboxamidine-HCl.

If it is assumed that the contributions of the phenyl and cyclohexyl rings to binding of the inhibitors to trypsin are due essentially to dispersion forces, a very good agreement is obtained between the data and the calculated values for interactions of such rings with a protein.

Electronic van der Waals attraction (London dispersion forces) represents the principal force of attraction between neutral molecules. London (21) described this force as the result of the polarization of each molecule in the rapidly changing electrical field arising from the constantly changing configuration of the electrons and nuclei of the other molecule (22, 23).

Pauling and Pressman (22) studied the hapten inhibition of the precipitation of antisera homologous to o-, m-, and p-azo-phenylarsonic groups, and developed the equations for calculation of the contribution of substituents in the haptenic groups to the dispersion interaction energy, on the assumption that binding was determined by dispersion forces. This formulation was used by Webb (23), in an extensive review on interactions of inhibitors with enzymes, for calculations based on data available in the literature.

The calculated dispersion interaction energy of a benzene ring interacting on only one side with a protein surface is 2.35 kcal mol$^{-1}$, whereas for a cyclohexane ring the value is 1.47 kcal mol$^{-1}$ (23). As already emphasized, the $\Delta(\Delta F^c)$ values calculated from the data in the present work are to be interpreted as the difference between the contributions of the phenyl or cyclohexyl and the methyl groups, since acetamidine-HCl was used as reference. To obtain the contributions of the phenyl and cyclohexyl rings as related to hydrogen, the contribution of the methyl group to dispersion interaction has to be added to the $\Delta(\Delta F^c)$ values, and the figure calculated by Webb (23), 0.88 kcal mol$^{-1}$, was therefore added. The contribution of the phenyl or cyclohexyl groups to binding presumably can be obtained directly experimentally, if formamidine-HCl is taken as reference.

The agreement between the observed and calculated data for cyclohexylcarboxamidine-HCl is also an indication that inductive effects on the phenyl ring are not important for binding, nor is aromaticity the sole requirement for "good" inhibitors, since the binding of cyclohexylcarboxamidine to trypsin was maximum.
with the framework of the hypothesis. If any forces such as those determining dipole, dipole-induced ion-dipole interactions make important contributions to the observed binding of the inhibitor side chain to the enzyme, then cyclohexylcarbomamidine-HCl would be expected to be a poor inhibitor, and its empirical standard free energy of binding should not be much different from that of acetamidine-HCl (Table II).

From studies of inhibitors of carbamoylpeptidase, Smith, Luny, and Polglase (24) concluded that van der Waals forces were determinant in the binding of the inhibitors to the enzyme. It was also pointed out that with leucine aminopeptidase the dispersion forces are large enough to explain the action of the enzyme on its various substrates.

It would be interesting to compare the values in the present work with some values of standard free energies of binding calculated from $K_I$ values for other systems. Wilson (25) obtained a $K_I$ of $1.34 \times 10^{-3}$ for the inhibition of acetylcholinesterase by trimethylammonium ion. When phenyltrimethylammonium ion was used, the value of $K_I$ was $3.8 \times 10^{-2}$ (26). These values yield 2.10 kcal mole$^{-1}$ as the contribution of the benzene ring of the $\Delta F^0$ of binding of phenyltrimethylammonium ion to the active center of the enzyme. These results have been discussed extensively by Webb (23).

In a study on the interaction of aromatic compounds with $\alpha$-chymotrypsin, Wallace, Kurtz, and Niemann (27) measured $K_I$ values for many compounds, from which the following values for $\Delta F^0$ of binding were calculated: 2.19 kcal mole$^{-1}$ for benzene, 2.75 kcal mole$^{-1}$ for toluene, and 2.99 kcal mole$^{-1}$ for phenol. It should be kept in mind that $K_I$ defined as a dissociation constant (Equation 5), which gives the $\Delta F^0$ values a positive sign. Therefore, for the association process, which corresponds to the process of binding, the $\Delta F^0$ values will bear a negative sign.

Miles, Robinson, and Canady (28) also reported on the competitive inhibition of $\alpha$-chymotrypsin by aromatic compounds. They found $\Delta F^0$ values of 2.84 kcal mole$^{-1}$ for benzene, and 3.47 kcal mole$^{-1}$ for toluene. Although the empirical standard free energies of binding found for the interactions of aromatic compounds with $\alpha$-chymotrypsin suggest that interaction with the protein occurs on only one side of the aromatic ring, they cannot exclude the possibility of less than optimal interaction with a slit, crevice, or pocket in the enzyme binding site.

The interpretation of the binding data in terms of London dispersion forces alone faces the serious objection of neglect of solvent involvement in the process. As Luny pointed out (29), this interpretation neglects both entropy changes in water displacement and the hydrogen bonds that could be formed by the water molecules displaced from between the interacting groups.

A more encompassing interpretation of our results can be obtained if it is considered that binding of the inhibitor side chains occurs through the formation of a hydrophobic bond with a binding site in the enzyme active center.

Based on a model for the thermodynamic properties of liquid water and aqueous solutions of hydrocarbons (30, 31), Nemethy and Scheraga (32) and Nemethy (33) described the thermodynamic parameters for the formation of hydrophobic bonds in proteins, including estimates of hydrophobic bond formation involving side chains carrying polar groups.

If the binding of the side chains is visualized as a transfer from an aqueous environment to a nonpolar environment in the protein active center, it is possible to compare the contribution of the phenyl side chain of benzamidine to the $\Delta F^0$ of binding with data on the solution of benzene in water (31, 32), noting that the $\Delta F^0$ values in this paper were calculated from the dissociation constant of the enzyme-inhibitor complex.

It is seen that the uncorrected contribution of the phenyl group, 3.35 kcal mole$^{-1}$ (Table III), is entirely in agreement with the $\Delta F^0$ of solution of benzene in water, 4.61 kcal mole$^{-1}$ (31, 32). Although the effects of the charged group of the inhibitors on the parameters for hydrophobic bond formation remain unknown, the observed agreement is considered satisfactory.

In addition, recent data of Inagami (34) also support this conclusion. The contribution of the alkyl side chains of alkylammonium ions to the $\Delta F^0$ of binding to trypsin found by Inagami (34), although lower than expected by comparison with solution data, are within the range expected for hydrophobic bond formation with the protein (see, for instance, Tables III-XII and III-XIII in Reference 33). Inagami (34) also attributed the binding to a hydrophobic force. These data seem to indicate that the calculated free energies of binding based on dispersion forces, considered at the beginning of this section, represent a rather high estimate, since they are numerically very close to the parameters for hydrophobic bond formation.

The considerations presented above, and the good agreement between calculated and observed values, are taken as an indication that the carbon side chains of the inhibitors and substrates of trypsin form a very efficient hydrophobic bond with the enzyme, and might occupy a slit or crevice in the active center. The nature of the data discussed does not permit the interpretation that the substrate lies in a crevice or slit in its entirety, but only that the carbon side chain might do so.

This hypothesis leads to a more detailed picture of the "specificity site," as Gutjord (3) called this part of the active center. Besides an "anionic site," to which the substrate binds electrostatically through its positive charge, a "hydrophobic binding site" is also present, which, it is now proposed, exists as a slit or crevice, and which is responsible for binding the carbon side chain of the substrate molecule. The two sites must be adjacent, and conceivably they are continuous.

If the data for phenyl- and cyclohexyl guanidines in Table II are now examined, it is seen that these compounds have a $\Delta F^0$ of binding that is between 700 and 800 cal mole$^{-1}$ smaller than the $\Delta F^0$ of the corresponding amidines. Although phenylguanidine has the spectral characteristics of a planar molecule, it is not symmetrical about the axis which bisects the phenyl ring. The smaller binding energy observed for these two guanidines is considered as an indication that the slit or crevice in which the side chain lies is able to bind most efficiently a phenyl or cyclohexyl ring linked to a positively charged group when the latter is in the direction of the line passing through carbon atoms 1 and 4 of the ring, the molecules being viewed flat. With the guanidines it is possible that the guanidinium ion binds optimally to the anionic site, the side chain being partially displaced from the hydrophobic binding site, resulting in less than optimal binding.

The data for the inhibition of trypsin by 2-phenylacetamidine-HCl (Table II) show a decrease in the value of $\Delta F^0$ of binding of 3.84 kcal mole$^{-1}$ as compared to benzamidine-HCl, clearly indicating that, if a $-\text{CH}_2\text{C}=$ group is interposed between the ring and the amidinium group, binding is better than that of acetamidine by only 500 cal mole$^{-1}$. A model of this molecule not only shows the separation between the ring and the amidinium group, but also indicates that coplanarity may be difficulty
achieved. These facts and the data on the guanidines, just discussed, are taken as indicating that optimal binding of the positive group and partial displacement of the side chain from the slit or crevice represents the best explanation for the experimental observations. In the case of 2-phenylaetamidine-HCl, although the phenyl ring is free to assume several orientations by rotation of the bonds in the —CH₉— group, it is possible that the geometry of the molecule excludes those conformations which could favor optimum binding.

Further support for the hypothesis that the side chain of the substrates or inhibitors of trypsin lies in a slit or crevice in the binding site comes from the work of Inagami and Murachi (35). These authors reported Kᵣ values of 0.062 M and 0.0017 M, respectively, for the inhibition of trypsin by ethylamine and butylamine, measured with benzyl-L-arginine ethyl ester as substrate. From these values, ∆F° values of 1.66 and 3.78 kcal mole⁻¹, respectively, are obtained. This means that by increasing the side chain of the inhibitor by 2 carbon atoms, 2.13 kcal mole⁻¹ were contributed to the empirical standard free energy of binding, indicating very efficient interaction with the enzyme if attributed either to dispersion forces or to hydrophobic bond formation. Ethylammonium and butylammonium ions can assume the same conformations, at least in the parts of the molecule which are common to both. However, the entire butylammonium ion, in the fully extended conformation, might interact favorably with the enzyme. This is further supported by the observation that the distance between the center of charge of the amidinium group and carbon atom 4 of the cyclohexyl or phenyl ring in the respective amidines is almost equal to the internuclear distance between the ammonium group and carbon atom 4 of the fully extended n-butylammonium ion, approximately 5 Å, which is also the distance between the center of positive charge and the β carbon of the fully extended arginine or lysine side chains, as already mentioned. It can be added that Inagami and Murachi (35) also found that n-butylamine was the most potent of the trypsin inhibitors tested by them, being better than n-propyl- and n-hexylamine.

As mentioned at the beginning of this section, the molecules of cyclohexylcarbamidine-HCl and benzamidine-HCl have an axis of symmetry, passing through or close to carbon atoms 1 and 4 of the ring and through the center of positive charge. This symmetry has as a consequence a contribution of RT ln 2 to the standard free energy of binding, which, in the present case, amounts to about 400 cal mole⁻¹. This contribution is not enough to account for the smaller ∆F° of binding found for the cyclohexyl- and phenylguanidines, which have no axis of symmetry, and for which difference of the order of 800 cal mole⁻¹ were found, as compared to cyclohexylcarbamidine and benzamidine, respectively.

The high affinity of trypsin for aromatic rings indicated by these results is not in conflict with the familiar role of chymotrypsin as the proteolytic enzyme with high specificity for aromatic amino acid residues. The hypothesis that a slit or crevice in the active center acts as a polymethylene-binding site for the side chain of substrates of trypsin, as pictured here, has a rather exacting geometrical relationship to the anionic site and hydrolytic mechanism of the enzyme and is in agreement with the experimental data on the strict specificity of trypsin. That the slit or crevice is aligned with, and between, the anionic site and the catalytic site is shown by the fact that ethyl p-amidino-benzoate HCl is hydrolysed by trypsin (36). Such a binding region is perhaps not involved in the low level of chymotryptic activity said to be an inherent property of trypsin (37), and may or may not be related to the inactivation of trypsin by diphenylcarbamyl chloride (38) or to the superiority of benzohydroxamic acid over formohydroxamic acid in the reactivation of diethylphosphoryl trypsin (39).

It is not possible to indicate at present whether the slit or crevice pre-exists in the active center or forms during binding, as a result of a conformational change induced by the approaching positive charge during enzyme-substrate complex formation. However, suitable experiments are being designed to explore these possibilities.

SUMMARY

1. A kinetic treatment was developed for the inhibition of an enzyme by two competitive inhibitors. The treatment was especially extended for the case in which the substrate is a racemic mixture, from which only one of the optical isomers is attacked by the enzyme, the other being a competitive inhibitor.

2. p-Aminobenzamidine-2HCl, benzamidine-HCl, phenylguanidine sulfate, cyclohexylcarbamidine-HCl, cyclohexylguanidine-HCl, 2-phenylaetamidine-HCl, and acetamidine-HCl were found to be competitive inhibitors of trypsin. p-Amino benzamidine-2HCl and benzamidine-HCl are the most potent small molecular competitive inhibitors of trypsin described in the literature until now. These compounds are good models of the side chains of the substrates of trypsin.

3. Correlation between the contribution of the side chains of the inhibitors to the empirical standard free energy of binding, the calculated dispersion interaction energies, and the parameters for hydrophobic bond formation led to the hypothesis that the side chains of the inhibitors and substrates lie in a hydrophobic slit or crevice in the binding site of the active center of the enzyme.

4. The "specificity site" of trypsin is now proposed to be composed of an "anionic site," to which substrates or inhibitors bind electrostatically through their positive charge, and a hydrophobic binding site, located in line with, and between, the anionic and catalytic sites, in the form of a slit or crevice, which binds the carbon side chain of the substrates or inhibitors.

REFERENCES

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