Electronic Effects in the Interaction of Para-Substituted Benzamidines with Trypsin: the Involvement of the π-Electronic Density at the Central Atom of the Substituent in Binding[1]

Marcos Mares-Guia,* David L. Nelson, and Edyr Rogana

Contribution from the Departamento de Bioquímica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 30000 Belo Horizonte, CP 2486, Brazil. *Received July 30, 1975*

Abstract: The following para-derivatives of benzamidine were synthesized: methyl-, methoxy-, hydroxy-, amino-, fluoro-, chloro-, bromo-, carbethoxy-, and nitrobenzamidine. They are all competitive inhibitors of trypsin. *p*-Fluoro-, *p*-hydroxy-, and *p*-nitrobenzamidines are described for the first time as inhibitors of trypsin. The pK_a values of the inhibitors were determined spectrophotometrically; the π-electronic charge densities at the central atom of the para-substituent were calculated by the Hückel MO method using the ω technique. In the case of the para-substituted benzamidines, this charge would be located on the central atom of the substituent. Correlations were found between pK_i and normal Hammett substituent constants, σ_p, and between the charge density at the central atom of the substituent and pK_i. These results are interpreted in terms of a dipole–dipole interaction between enzyme and inhibitor that would reinforce or decrease binding as a consequence of intramolecular charge transfer in the inhibitor molecule. A CPK model of the active center region of the trypsin–benzamidine complex, built according to published coordinates, supports the above interpretation and allows the inference that the mesomeric dipole present at the para substituent in benzamidine interacts most probably with the negative end of the dipole of the active seryl-183 hydroxyl group in the catalytic site. The authors consider it important to emphasize that thermodynamical data when associated with quantum mechanical calculations, are important tools in reaching an understanding of interaction mechanisms at the submolecular level. In the present case, x-ray crystallography seems to support the results.

The competitive inhibition of trypsin by amidines and guanidines was first demonstrated by Mares-Guia and Shaw.[2] They proposed that a hydrophobic binding site was present in the active center of trypsin. The participation of hydrophobic bonding in tryptic complexes with alkyl amines had been suggested by the work of Inagami.[3] Further work demonstrated the participation of the hydrophobic interaction in the hydrolysis of synthetic substrates,[4a] as well as in the acylation of the enzyme.[4b] A thermodynamic investigation of the interactions of amidines and guanidines with trypsin characterized the entropic origin of the binding energy[5] and showed that in the tight complex formed with these inhibitors there was close approximation between a negative group in the enzyme anionic site and the positive group in the amidinium or guanidinium compounds.[6] Several other aspects of the interactions in the active center of trypsin were covered in a review by Inagami.[7]

In several papers dealing with trypsin inhibition, it was shown that substituted benzamidines, e.g., *p*-aminobenzamidine or *p*-methylbenzamidine could behave as better inhibitors than benzamidine itself[2,8] or could be less inhibitory than benzamidine, e.g., *p*-carboxybenzamidine.[9] Thus, ring substituents altered the affinity of the enzyme for the inhibitors.

The benzamidine–trypsin system appeared a convenient one to use as a model for the study of substituent effects on equilibria involving a biopolymer for the following reasons: (a) the dissociation constant for the enzyme–inhibitor complex is small enough to guarantee statistical significance for the changes caused by the introduction of substituents; (b) the inhibitors used are relatively simple, rigid molecules for which quantum mechanical calculations would not be extremely laborious to carry out; (c) the enzyme has a known primary structure, well-defined specificity, and it was known that its three-dimensional structure would soon become available through the x-ray diffraction studies of Stroud et al.[10] All these factors put together would probably allow a more encompassing interpretation of the substituent effects. We decided, then, to undertake a systematic study of the influences of substituents upon the properties of the inhibitors themselves, as well as upon the dissociation constants of the trypsin–*p*-X-benzamidine complexes. Free-energy relationships have been used quite extensively for the study of enzyme, receptor, and protein interactions with substrates, inhibitors, or drugs. A particular approach was developed by Hansch's school which is based upon a regression analysis that takes into consideration electronic, steric, and "hydrophobic bond" substituent constants.[11-13] This approach has been applied to a considerable number of enzyme-inhibitor systems: cholinesterase inhibitors;[14] malate dehydrogenase and phenols;[15] thymidine phosphorylase;[16] adenosine deaminase;[17] chymotrypsin.[18] Hansch's approach has been applied to the inhibition of neuraminidase by Tute.[19]

On the other hand, several studies of substituent effects on chymotrypsin catalysis based on the Hammett free-energy relationship have appeared in the literature.[20-22] Wang and Shaw[23] carried out work on deacylation of substituted benzoylchymotrypsins and -trypsins which represented the first investigation of substituent effects on trypsin catalysis.

Our approach to the investigation of substituent effects on the binding of para-substituted benzamidines to trypsin was different from that of Hansch's school in that our major interest was in understanding the role of the electronic component of the substituent effect. The hydrophobic interaction of benzamidines and guanidines with trypsin had been characterized with inhibitors[2,4a,5] and substrates. Steric effects in inhibitors with small substituents did not substantially affect overall binding.[8] The recent work on the x-ray diffraction of the benzamidine–trypsin complex[24] confirmed the interpretation proposed by Mares-Guia and Shaw[2] that the benzene ring of benzamidine was located in a hydrophobic pocket at the active center of trypsin, that the para hydrogen atom was oriented toward Ser-183 residue, and led to the inference that a para substituent on the ring of benzamidine would not be engulfed in the pocket.

In the present study several para-substituted benzamidines were synthesized, and their spectral and acid–base properties, as well as their binding to trypsin, were correlated with normal

Hammett substituent parameters and to quantum-chemical parameters with special emphasis on electron densities, which Pullman and Pullman[25] consider an important criterion in describing interactions between biological molecules. Indeed, of all electron densities investigated, those at the central atom of the substituents gave the best correlation with the Hammett substituent constants, leading to an interpretation of the mechanism of binding of para-substituted benzamidines to trypsin. The work on the spectral properties of the inhibitors used has been published.[26]

Experimental Section

Reagents. All chemicals used were reagent grade; the solutions were prepared in distilled, deionized water. Benzamidine was purchased from Aldrich and its molarity was determined as described by Mares-Guia and Figueiredo.[5] Twice crystallized bovine trypsin (E.C. 3.4.4.4.) was obtained from Sigma Chemical Col. β-Trypsin was prepared from the former according to Schroeder and Shaw.[27] Stock solutions in HCl at pH 3.0 were prepared weekly and stored cold. The active center molarity was routinely titrated with NPGB according to Chase and Shaw.[28] All the substituted benzamidines used were synthesized according to literature methods or to methods adapted from the literature. A detailed description of the properties of these substances appeared in a separate paper.[26]

Methods. The determinations of K_i values for the new inhibitors were carried out under conditions already described.[5] The determinations were carried out using trypsin in a concentration of 1 mg/mL with Bz-DL-Arg-NPA as substrate at two concentrations (1.8×10^{-4} and 2.7×10^{-4} M) at 15 °C in 0.10 M Tris, pH 8.0, containing 2.0 mM $CaCl_2$. At each of these concentrations, five duplicate concentrations of inhibitor were used. Dixon's kinetic formalism was used to calculate the K_i values and their respective standard deviations with the help of a program called INCOM. In order to determine the thermodynamic parameters (Table III), the K_i values were determined as described above at various temperatures (0, 15, 25, and 37 °C). $\Delta H°$ was determined from the slope of the plot of log K_i as a function of $1/T$ and $\Delta S°$ from $(\Delta H° - \Delta G°)/T$. Ultraviolet spectra were taken with a Perkin-Elmer Model 450 recording spectrophotometer at a constant temperature of 15 ± 0.1 °C. The pK_a values for the amidines were obtained by spectrophotometric titration in the ultraviolet, as will be described in the work of Rogana, Leite, Mares-Guia, and Nelson.[29] The calculation of the pK_a and the statistical analysis of the data were carried out with the help of a computer program called LINEA.[30] The binding of benzamidine to trypsin was also measured by titration using difference spectroscopy in the region of ultraviolet, with the Perkin-Elmer Model 450 instrument, using the technique described by East and Trowbridge.[33] The sample cell contained enzyme and benzamidine in one compartment and Tris buffer in the other. The reference cell contained benzamidine in one compartment and trypsin in the other. The results were analyzed in the following manner:

$$K_i = \{[E_0] - [EI]\}\{[I_0] - [EI]\}/[EI] \quad (1)$$

where

$$K_i = [E][I]/[EI] \quad (2)$$

and, from the law of conservation of mass,

$$[E] = [E_0] - [EI] \quad (3)$$

$$[I] = [I_0] - [EI] \quad (4)$$

$[E_0]$ is the molar concentration of total enzyme, $[I_0]$ is the molar concentration of total inhibitor, [EI] is that of the enzyme-inhibitor complex, and K_i is the dissociation constant of the complex. The value of [EI] in eq 1 may be substituted for its equivalent, given by eq 5 in which ΔA is the change in absorbance caused by addition of the inhibitor and $\Delta\epsilon$ is the difference in molar extinction coefficient between the enzyme-inhibitor complex and the sum of the coefficients of the enzyme and the inhibitor $\Delta\epsilon = \epsilon_{EI} - (\epsilon_E + \epsilon_I)$:

$$\Delta A = \Delta\epsilon[EI] \quad (5)$$

Equation 1, thus, becomes

$$[E_0][I_0](\Delta\epsilon)^2 - [E_0]\Delta A\Delta\epsilon - [I_0]\Delta A\Delta\epsilon + (\Delta A)^2 = K_i\Delta A\Delta\epsilon \quad (6)$$

Equation 6 was solved by two different methods. In the first, it was solved for pairs of points at different $[I_0]$ values, which yield the following equations:

$$[E_0][I_1](\Delta\epsilon)^2 - [E_0]\Delta A_1\Delta\epsilon - [I_1]\Delta A_1\Delta\epsilon + (\Delta A_1)^2 = K_i\Delta A_1\Delta\epsilon \quad (7)$$

$$[E_0][I_2](\Delta\epsilon)^2 - [E_0]\Delta A_2\Delta\epsilon - [I_2]\Delta A_2\Delta\epsilon + (\Delta A_2)^2 = K_i\Delta A_2\Delta\epsilon \quad (8)$$

where $[I_1]$ and $[I_2]$ are the initial molar concentrations of the inhibitor. Substituting the value of K_i given by eq 7 into eq 8, one obtains

$$\Delta\epsilon = \frac{b - (b^2 - 4ac)^{1/2}}{2a} \quad (9)$$

where $a = [E_0]([I_2]\Delta A_1 - [I_1]\Delta A_2)$, $b = \Delta A_1\Delta A_2([I_2] - [I_1])$, and $c = \Delta A_1(\Delta A_2)^2 - (\Delta A_1)^2\Delta A_2$. On the other hand, substitution of the $\Delta\epsilon$ value given by eq 9 into eq 7 yields

$$K_i = (f - g - (\Delta A_1)^2)/\Delta\epsilon\Delta A_1 \quad (10)$$

where $f = [E_0]\{[I_1](\Delta\epsilon)^2 - \Delta A_1\Delta\epsilon\}$ and $g = [I_1]\Delta A_1\Delta\epsilon$. Equations 9 and 10 were used to calculate K_i and $\Delta\epsilon$. A titration with nine points, taken two at a time, can yield 36 values for K_i and 36 for $\Delta\epsilon$. Evidently, because of experimental errors inherent in the titration, these groups of values of K_i and $\Delta\epsilon$ generally differ among themselves. The criterion used for choosing the most precise K_i value was that which gave the smallest variance in ΔA. This was estimated by the following expression:

$$V\Delta A = \sum_{i=1}^{n} (\Delta A_i(\text{theor}) - \Delta A_i(\text{exptl})^2/(n-2) \quad (11)$$

where $V\Delta A$ is the variance in ΔA, ΔA_i(theor) was calculated by the Lambert-Beer law and ΔA_i(expt) is the measured value. Equations 9, 10, and 11 were translated into a FORTRAN-IV program denominated EXCOK and the calculations executed on an IBM/360 computer.

In the second method, eq 1 was resolved for ΔA, giving eq 12:

$$\Delta A = aS - a(S^2 - 4P)^{1/2} \quad (12)$$

where $a = \Delta\epsilon/2$, $S = [E_0] + [I_0] + K_i$, and $P = [E_0][I_0]$. Equation 12 is linear in a (or $/\Delta\epsilon$) and nonlinear in S (or K_i). It was developed in a Taylor series and a nonlinear regression analysis by an iterative method was performed.[31] A computer program called EQUID was used for this purpose.

The first method is more accurate but does not supply standard deviations. It simply resolves two equations containing two unknowns by the substitution method. The second method is less accurate because it involves an approximation by the Taylor series, but supplies the standard deviation of K_i and $\Delta\epsilon$.

The charge densities, eigenvalues, and eigenvectors for each inhibitor molecule were calculated through use of Hückel's approximation by the ω technique, with use of a computer program called VANESSA.[32] All correlation coefficients were calculated with the help of the program called POLRG from the IBM Scientific Subroutine Package, after some convenient modifications. All computer programs were processed in an IBM/360 computer from the Computing Center of this University.

Results

The values of k_i for the different para-substituted benzamidines used in this work are shown in Table I, together with their respective standard deviations. Also included in Table I are the values of $\Delta G°$, at 15 °C, and the pK_a's of the respective amidinium ions. The following new inhibitors of trypsin were found to be competitive: *p*-fluoro-, *p*-hydroxy-, and *p*-nitrobenzamidines. Our data also support previous results of Markwardt et al.[9] who showed that *p*-methoxy-, *p*-chloro-, and *p*-bromobenzamidines act as competitive inhibitors of trypsin. It is obvious from Table I that *p*-aminobenzamidine is the best inhibitor in the group,[2] whereas *p*-nitrobenzamidine is the least effective one.

The spectrophotometric titrations were conducted with benzamidine as a type of probe with the objective of obtaining

Table I. Trypsin Inhibition by Para-Substituted Benzamidines and Acidity Parameters of the Inhibitors

Substituent (X)	K_i,[a] 10^{-5} M	SD, 10^{-5} M	$\Delta G°_{assoc}$ (kcal/mol)	pK_a'
NH_2	0.825[b]	—	−6.70	12.01[f]
OH	1.39	±1.48[e]	−6.40	12.69[f]
OCH_3	5.89	±0.71[e]	−5.55	11.40[f]
CH_3	2.65	±0.70[c]	−6.03	11.41[f]
H	1.66	±0.20[c]	−6.30	11.41[f]
F	4.26	±1.77[e]	−5.76	11.16[f]
Cl	5.40	±3.51[e]	−5.62	11.18[f]
Br	1.89	±0.44[e]	−6.22	10.74[f]
$COOC_2H_5$	15.18	±2.78[e]	−5.03	10.56[f]
COOH	20.0[d]	—	−4.87	—
NO_2	22.71	±4.70[e]	−4.80	10.14[f]

[a] Bz-DL-Arg-NPA as substrate 1.8×10^{-4} M and 2.7×10^{-4} M, at 15 °C in aqueous Tris buffer pH 8.0, 0.10 M. [b] M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240**, 1579 (1965). [c] M. Mares-Guia, *Arch. Biochem. Biophys.*, **127**, 317 (1968). [d] F. Markwardt, H. Landmann, and Walsmann, *Eur. J. Biochem.*, **6**, 502 (1968). [e] This work. [f] All the pK_a' data were taken from the work of E. Rogana, D. L. Nelson, L. F. F. Leite, and M. Mares-Guia (manuscript in preparation). Method: difference spectrophotometric titration in the UV, at 25 °C.

Table II. Binding Parameters for the Benzamidine-Trypsin Complexes Obtained by Difference Spectrophotometric Titration in the UV (0.10 M Tris, pH 8.0, 2 mM $CaCl_2$, at 15 °C)

Trypsin	No. of assays	λ (nm)	K_i ($M \times 10^5$)	Δε ($M\ cm^{-1}$)
Crystalline	*a* 9	287	1.66 ± 0.17[c]	−654 ± 55[c]
	b 14	287	3.90	−808
α	*a* 10	248	1.08 ± 0.10	736 ± 29
	b 9	248	1.37	755
β	*a* 10	252	2.81 ± 3.20	1581 ± 490
	b 10	248	0.45	2458

[a] EQUID. [b] EXCOK. [c] Standard deviation.

information about the microscopic molecular environment. The titrations by difference spectroscopy permit not only the determination of the K_i value by an independent technique, but can also supply valuable information on the state of the chromophores of the enzyme or of the probe in the presence of one another. The spectral properties of the probe have previously been studied.[26] The binding of benzamidine to trypsin causes a change in the inhibitor spectral properties which can be detected by difference spectroscopy, as shown in Figure 1. Trypsin has a λ_{max} at 280 nm and a minimum near 250 nm, while benzamidine alone absorbs at 225 nm with the greatest slope around 240 nm. Thus, the difference spectrum, which has a λ_{max} at 248 nm represents a bathochromic shift of the benzamidine spectrum.[32,40] The difference spectrum of benzamidine in butanol vs. benzamidine in aqueous medium is added for comparison with the enzyme-inhibitor difference spectrum in the 240-260-nm range. The form of the difference spectrum of benzamidine in butanol vs. benzamidine in aqueous medium is similar to that of the enzyme-inhibitor complex; i.e., neither presents electron-vibrational coupling, both are symmetrical, and the Δε's are of the same order of magnitude (Table II), although the λ_{max} are different. Table II contains the values of K_i and Δε obtained by difference spectrophotometric titrations at pH 8.0 of crystalline, β- and α-trypsins with benzamidine. The two methods (EQUID and EXCOK) give different values because different numbers of points and wavelength were used and because of the inherent differences in the methods. The orders of magnitude of K_i and

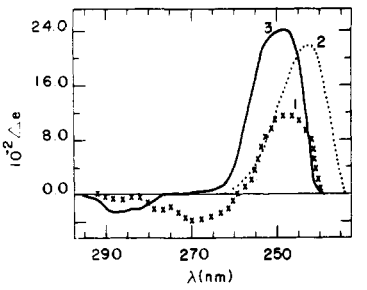


Figure 1. Difference spectra of benzamidine under different conditions: 1, benzamidine-HCl in the presence of α-trypsin; 3, benzamidine-HCl in the presence of β-trypsin (Both spectra were determined at pH 8.0 in 0.10 M Tris, 22 m $CaCl_2$, at 15.0 °C. For further details, see Methods.); 2, benzamidine-HCl in 1-butanol vs. benzamidine-HCl in 0.10 M Tris, pH 8.0, at 25.0 °C.

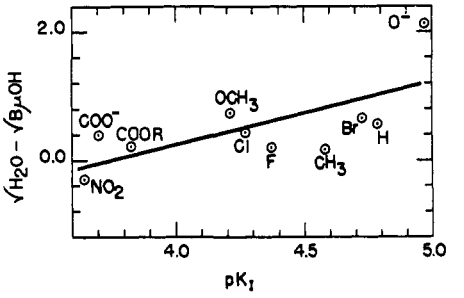


Figure 2. Correlation between bathochromic shift caused by solvent and pK_i of para-substituted benzamidinium ions. The solvents used were: aqueous Tris, 0.10 M, pH 8.0, and 1-butanol. The spectra were taken at 25.0 °C. The values of the bathochromic shifts were taken from the work of Rogana, et al.[26] The parameters are: $r = 0.6974$, slope = 0.9654 ± 0.3506; intercept = −3.6318.

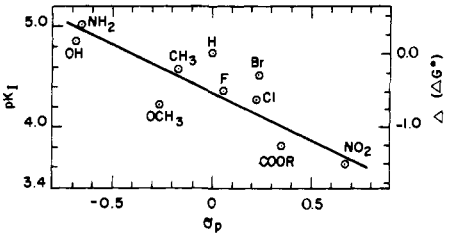


Figure 3. Hammett free-energy relationship for trypsin inhibition by para-substituted benzamidinium ions. The same plot also shows the corresponding changes in the standard free energy of dissociation at 15 °C. The parameters are: $r = -0.8182$; slope = −0.8813 ± 0.2063; intercept = 4.3965.

Δε are the same as those found by East and Trowbridge,[33] who also performed difference spectral titrations of crystalline trypsin chromatographed on Sephadex-G-50. A bathochromic shift was observed when spectra of the inhibitors were taken in aqueous medium and then in butanol. This shift correlates with pK_i values, as shown in Figure 2.

The substituent effect on the affinity of the inhibitors for trypsin is demonstrated by the correlation of pK_i with σ_p (Figure 3). It is clear from Figure 3 that the electron-donating substituents reinforce binding of benzamidine to trypsin, whereas the electron-withdrawing groups decrease it. This is more clearly shown by the correlation between $\Delta(\Delta G°)$ of binding and σ_p, also included in Figure 3. In order to obtain more information on the system in study, the thermodynamic parameters for the formation of trypsin-inhibitor complexes with *p*-F, *p*-Cl, and benzamidinium ions (Table III) were determined.

The σ_p is a thermodynamic parameter which does not depend on structural models and is a manner of representing the electron attracting or donating power of the substituent. This

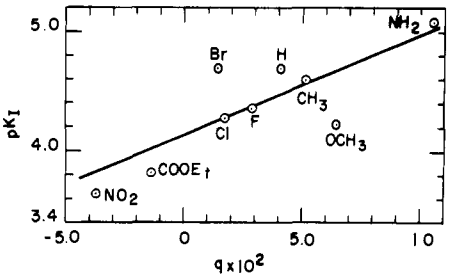


Figure 4. The values of pK_i for para-substituted benzamidinium ions as a function of q, the net π-electronic density at the central atom of each substituent. The π-electronic densities were calculated according to HMO theory. The p-hydroxyl derivative was omitted because, at pH 8.0, it is in equilibrium with the deprotonated form; the calculation of the electron density of the mixture does not correspond to a physical reality.

Table III. Thermodynamic Parameters for the Formation of Trypsin–Inhibitor Complexes with p-X-Benzamidinium Ions (Buffer: Tris, pH 8.0, 0.10 M, 2 mM $CaCl_2$, at 15 °C)

Substituent	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/(deg mol))
F	-5.90 ± 0.10	1.87 ± 2.53	23.8
Cl	-6.06 ± 0.18	1.39 ± 2.79	27.0
H	-6.43 ± 0.10	0.42 ± 1.00	25.9

power is also represented by the charge density at the central atom of the substituent (q), which does depend on the structural model and is used here in order to obtain information about the type of enzyme–inhibitor interaction. In order to be able to interpret the regularities in the pK_i values at the submolecular level, we calculated by quantum mechanical methods the reactivity indices for each inhibitor and tried to correlate them with the dissociation constants of the enzyme–inhibitor complex. There was no strong correlation with the bond order of each bond in the inhibitors or with the charge density of the atoms of the ring or of the amidinium group. The calculated ionization potential of the molecules, as well as the difference in π-energy of the protonated and unprotonated inhibitors also did not correlate well with pK_i. On the other hand, a strong correlation was found between the charge density at the central atom of the substituent and pK_i, as shown in Figure 4.

Discussion

In discussing our results, four possibilities should be considered.

First, in terms of the acid–base properties of the inhibitors, it would be reasonable to expect that electron-withdrawing groups, by turning the benzamidinium ion into a stronger acid, would decrease binding due to a decrease in the fraction of protonated species. The converse reasoning would be applied to benzamidinium ions carrying electron-donating groups. It should, therefore, be expected that a pK_i vs. σ_p correlation line would have a negative slope, which is indeed experimentally verified. However, the data in Table I show that while electron-withdrawing groups decrease the pK_a of para-substituted benzamidines, the values never fall below 10.1. Thus, at pH 8.0, all the amidines used are practically fully protonated; that is, they carry a +1 charge at the amidinium end that allows maximum interaction with Asp-177, the anionic site. Thus, the correlation shown in Figure 3 must not be directly related to substituent effects on the acid–base properties of the inhibitors.

Second, the electron-withdrawing groups could act by decreasing the electron density at the amidinium group, thereby increasing their positive charges. This would give a pK_i vs. σ_p correlation line with positive slope, which is contrary to the results shown in Figure 3.

Third, the fact that the p-nitrobenzamidine is the weakest inhibitor and the p-amino derivative is the strongest suggests that the inhibitor acts as a donor of electrons in a process of charge transfer from the inhibitor to the enzyme. In this case, there should be a correlation between the ionization potential of the inhibitors and the pK_i values. Since the ionization potentials for the inhibitors under study are not available, they were calculated by the Huckel MO method using the ω technique. No correlation was found between these two parameters. Also, it would be necessary to have an electron acceptor in the hydrophobic site, and there is no such group in this region, nor does the spectral band in Figure 1 present the characteristics of charge transfer (broad, with vibrational fine structure).

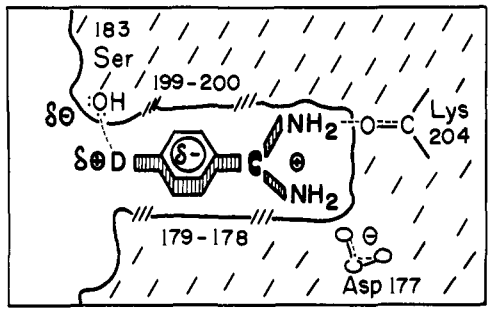
A fourth possibility, the existence of an additional type of interaction, is the one that seems to find experimental support. The data can be interpreted in terms of an enzyme–inhibitor interaction of the dipole–dipole type. A dipole will appear in the inhibitor as a consequence of an intramolecular charge transfer from the substituent to the ring or vice versa.

According to Hammett,[33] one should expect to find a linear free-energy relationship in a reaction series when $\Delta H°$ is constant, when $\Delta S°$ is constant, or when $\Delta H°$ is a linear function of $\Delta S°$. From the analysis of Table III one can infer that, for the substituent groups Cl and F, the $\Delta S°$ values do not vary significantly (isoentropic). The substituents Cl and F caused the formation of the complex to be more endothermic, raising the potential energy barrier between reactants and products. However, this assertion would be valid only if the reaction were to proceed in the gaseous state, without the influence of solvent, and it may not be valid for the other groups even in the gaseous state. Studies leading to the determination of the thermodynamic parameters for the remaining inhibitors and the effect of solvent on these parameters are in progress in this laboratory.

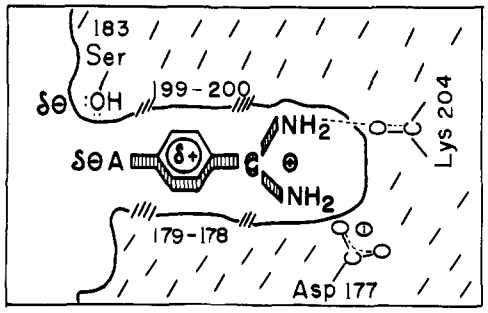
It is quite probable that the substituents affect very little the hydrophobic interaction, since they are located outside the hydrophobic site in the enzyme. In addition, the intramolecular charge transfer from the substituent to the ring places a rather small charge on the ring (a maximum of -0.10 in atomic units of charge for p-NH_2), a charge that is spread over six atoms. This should change the ring interaction with the amino acid residues in the hydrophobic site very little. The calculated electron densities on the amidinium group atoms are even less affected by the substituent. Since the substituent affects the pK_a of the amidinium group in a range of 2.5 pH units (Table I), it would appear that the substituent must have a large effect on the electron density of that group. However, the pK_a is not determined only by the electron density of the amidinium group but also by the electron densities on the atoms of the ring and on the para substituent because of field and solvation effects. Since the benzamidinium ion is bearing a full positive charge, the small effect of the substituent on the electron density of the amidinium group atoms would have little effect on the electrostatic interaction at the anionic site.

Thus, the small values of $\Delta(\Delta G°)$ of binding found (Figure 3) are interpreted as being due to an interaction between the substituent itself and a specific site on the enzyme surface. This suggestion is fully supported by the finding that, whereas there was no correlation between the electron densities on the ring or amidinium group atoms with pK_i, a strong correlation was observed between pK_i and the π-electronic charge at the central atom in the substituent (Figure 4). The data in Figure 4 agree with the model in which intramolecular charge transfer renders positive the electron-donating substituents, thereby giving origin to a dipole which is able to interact with a site in the enzyme, according to Scheme I. This interaction reinforces binding. On the other hand, electron-withdrawing groups

Scheme I. Enzyme–Inhibitor Complex with Electron-Donating Para Substituent. A New Dipole–Dipole Attraction Improves Binding



Scheme II. Enzyme–Inhibitor Complex with Electron-Withdrawing Para Substituent. The Dipole–Dipole Repulsion Decreases Binding



generate a dipole of opposite polarity which decreases binding by a dipole–dipole repulsion, as in Scheme II. A CPK model of the active center region was built according to the coordinates given by Krieger et al.,[24] and its study indicates the hydroxyl group of the reactive serine-183 as the most probable candidate for the dipole of the enzyme which interacts with the dipole at the para position in substituted benzamidines. The hydrolysis, as well as the inactivation experiments indicate that the *p*-carbethoxy group or the *p*-halomethylcarbonyl find themselves in the proximity of the reactive γ-hydroxy group of the Ser-183 residue. This contention is closely related to the results of Mares-Guia, Shaw, and Cohen,[4a] who studied hydrolysis of *p*-amidinobenzoates and phenylacetates by trypsin, as well as to the work of Schroeder and Shaw,[34] who inactivated trypsin with phenacyl halides derived from benzamidine and phenylguanidine.

The possibility of the seryl hydroxyl group donating a proton as a Bronsted acid or in the form of a hydrogen bond is not in accord with the sign of the ρ obtained in Figure 3, with the values of q calculated for the para substituents, or with the order of decreasing electronegativity (i.e., F, Cl, Br, CH_3 = H). The seryl hydroxyl group already has a hydrogen bond to the imidazole of His- and there is no reason for breaking this bond and forming a hydrogen bond with a less acceptable atom, with the possible exception of the *p*-oxybenzamidinum ion. In the case of the *p*-hydroxy- and *p*-aminobenzamidinium ions, there exists the possibility of the dipole–dipole interaction involving a hydrogen bond from the para-substituent to the hydroxyl group of Ser-183.

The transfer of electron density from the substituent to the ring (or vice versa) is directly related to the properties of the para-substituted benzamidines, whether free[26,29] or complexed to trypsin. The intramolecular charge transfer has been shown to cause a shift in ν_{max} and a change in pK_a.[26,29] Since it also provokes a change in pK_i (Figure 4), it is not at all unexpected that pK_a should correlate with pK_i, as indicated by the data in Table I, or that $\Delta\nu$ should also correlate with pK_i, as shown in Figure 2. A good correlation was also obtained between pK_i and ν_{max}, the frequency of maximal absorption of the inhibitors in the UV.

The appearance of the difference spectra of benzamidine in butanol vs. aqueous medium is due to a bathochromic shift of the ^1A-^1L_a band.[26] Since in the formation of the enzyme-inhibitor complex the inhibitor passes from an aqueous to a hydrophobic environment, one would also expect a bathochromic shift in the difference spectrum of this complex. This shift actually occurs although the λ_{max} is greater than that observed in butanol. Since the difference spectra are not superimposable, there must be additional factors influencing the spectra, such as: (a) the polarity in the hydrophobic site is lower than that in butanol; (b) there exist other interactions, one of which could be the interaction of the Ser-183 hydroxyl group with the para position of benzamidine; (c) a mixture of the two. The data available do not allow one to distinguish between these possibilities, but one can suppose that the last of the three is the most probable. The spectral shifts observed in the difference spectra (Figure 1) are thus due to a change in solvent polarity caused by the transfer of the inhibitor side chain from an aqueous to an organic medium (the hydrophobic site) as well as a change in the intramolecular charge transfer brought about by the dipole–dipole interaction between enzyme and inhibitor. In the special case of benzamidine, the intramolecular charge transfer is from the ring to the amidinium group, leaving C-4 of the ring with a positive π-electronic charge (Figure 4). Indeed, the data of Krieger et al.[24] and Stroud et al.[36] show a close approximation between C-4 of benzamidine and the Ser-183 residue.

Final Comment. The major features of trypsin specificity and the mechanism of binding to substrates and inhibitors that have been investigated by physicochemical techniques in aqueous medium have been fully confirmed by the x-ray diffraction studies of Stroud et al.,[24] Rühlmann et al.,[37] and Sweet et al.[38] These include the existence of a specificity site in trypsin, as described by Gutfreund,[39] and the existence of a hydrophobic binding site, indicated by Mares-Guia and Shaw[2] and later characterized in more detail by investigations carried out in Shaw's laboratory and in ours. The existence of two positively charged groups (lysyl side chains) in the neighborhood of the active center was suggested by the work of Bechet et al.,[40] who studied ionic strength effects upon ester hydrolysis by trypsin. The closeness of the interacting positive charge of the benzamidinium ion to the negative charge of Asp-177 at the anionic site was concluded from the work of Mares-Guia and Figueiredo.[6] The results presented and discussed in the present paper are also supported by x-ray crystallographic work. We wish, therefore, to emphasize that, at least with trypsin, the critical application of very basic thermodynamics to enzyme reactions does indeed provide a basis for reaching trustful conclusions about the mechanism of interaction.

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References and Notes

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