Thermodynamics of the Hydrophobic Interaction in the Active Center of Trypsin. Investigation with Amidines and Guanidines*

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ABSTRACT: The binding to trypsin of amidines and guanidines of rigid structure was measured at four different temperatures with the intention of characterizing the hydrophobic interaction through the measured thermodynamic parameters of complex formation. It was found that the $\Delta H°$ values are close to zero and independent of inhibitor structure for acetamidine, benzamidine, and β-naphthamidine, thus demonstrating that the driving force for binding is an increase in the unitary entropy. A similar conclusion was reached through the investigation of guanidine and methyl-, cyclohexyl-, and phenylguanidines. In addition, an excellent agreement was observed between the free energies of transfer of benzene from an aqueous medium to the pure liquid with the free energies of binding of the phenyl ring of benzamidine to trypsin, at 0, 15, 25, and 37°. Together, the results demonstrate the existence of a hydrophobic binding site in the trypsin active center. The possible involvement of a tyrosyl residue in its structure is also discussed.

The investigation of the inhibition of trypsin by amidines and guanidines has led to the proposition that the active center of the enzyme contains a hydrophobic binding site, probably in the form of a slit or crevice (Mares-Guia and Shaw, 1965). The participation of a hydrophobic force in the binding process of trypsin has also been indicated by Inagami (1964) in his work on the inhibition of the enzyme by alkylammonium ions. Mares-Guia *et al.* (1967) were able to demonstrate the involvement of the hydrophobic binding site in the binding of substrate side chains. Thus, it was found that methyl *p*-amidinophenylacetate and ethyl *p*-guanidinophenylacetate behaved as trypsin substrates, displaying $K_{m\,(app)}$ values only three times higher than those for Bz-L-ArgOEt or α-*N*-tosyl-L-arginine methyl ester. The observation that ethyl *p*-guanidinobenzoate was able to acylate trypsin but that the acyl-enzyme had a very small rate of deacylation indicated that binding to the hydrophobic site might play an important role in the proper orientation of the substrate in relation to the catalytic groups of the enzyme (Mares-Guia and Shaw, 1967).

Further work on the thermodynamics of binding of alkylammonium inhibitors (Heidberg *et al.*, 1967) confirmed the existence of a hydrophobic binding site of limited size at the trypsin active center. In addition, the authors found evidence for a secondary hydrophobic site, which would become cccupied when compounds of longer side chains (>C7) are used.

In the work now to be described we used compounds with structures as rigid as possible, in order to render the interpretation of entropy data more straightforward. The changes to be observed would be mostly dependent on solvent changes or enzyme conformational adaptations, and the least dependent on the "freezing" of inhibitor conformation during complex formation. We were able to show that the process of binding to trypsin of the phenyl side chain of benzamidinium or the naphthyl side chain of β-naphthamidinium is qualitatively and quantitatively similar to the process of transfer of benzene from an aqueous medium to the pure liquid, thus demonstrating the hydrophobic nature of the interaction.

Experimental Procedure

Reagents. All chemicals used were reagent grade. The solutions were prepared in distilled deionized water. The hydrochlorides of guanidine and acetamidine as well as methylguanidine sulfate (all Eastman) were recrystallized from ethanol–ether, and gave the literature melting points (Mares-Guia, 1968a, and Heilbron and Bunbury, 1953). Benzamidine-HCl (Aldrich or K & K Laboratories) was found by its melting point to be a mixture of anhydrous and a small fraction of the hydrated species. A 0.1 M stock solution was prepared in water, and the exact molarity was evaluated after suitable dilution, from the absorbancy (Mason, 1954) at 2680 (ϵ 813) and at 2290 Å (ϵ 9120). The molarity thus determined was usually within 10% of that calculated by weight. β-Naphthamidine-HCl, cyclohexylguanidine sulfate, and phenylguanidine sulfate were synthesized by methods already quoted in previous publications (Mares-Guia and Diniz, 1967; Mares-Guia, 1968a). A stock trypsin (Sigma, lot 117B-8030) solution was prepared weekly in HCl, pH 3.0, and was stored at 4°. Its active center molarity was routinely titrated with the *p*-nitrophenyl ester of *p*-guanidinobenzoic acid, according to Chase and Shaw (1967).

Methods. The K_i values for the inhibitors were determined at 0, 15, 25, and 37° in 0.10 M Tris-HCl buffers. The pH of the buffer was so adjusted that it would reach pH 8.15 when equilibrated at the desired temperature. At the

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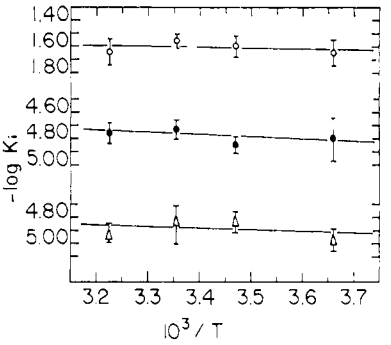

FIGURE 1: The effect of temperature on the enzyme–amidine dissociation constants: (-O-O-) acetamidine-HCl; (-●-●-) benzamidine-HCl; (-Δ-Δ-) β-naphthamidine-HCl. Experiments in 0.10 M Tris, pH 8.15.

end of each set of experiments we routinely checked the pH of sample tubes. At 0 and 37° the tubes were preincubated for 30 min, in order to assure thermal equilibrium. The conditions of the assays were essentially those described by Mares-Guia (1968a). α-*N*-Benzoyl-L-arginine-*p*-nitroanilide was used as a substrate. At lower temperatures we allowed longer incubation times for the enzyme with substrate, whereas at 37° the stock trypsin solution was conveniently diluted in pH 3 HCl before use. The evaluation of K_i was carried out according to Mares-Guia and Shaw (1965), and the calculations were carried out with an IBM-1130 digital computer. The computer program has been published (Mares-Guia, 1968b).

In a few instances the K_i values were measured with a Radiometer titrator TTT-lc (electrodes G-202B and K-401), equipped with the syringe unit SBU-1, a SBR-2 recorder, and a manual temperature compensator. The calculation of K_m, K_i, and k_{cat} as well as the statistics are described in a paper to follow. α-*N*-Benzoyl-L-arginine ethyl ester was the substrate, contained in a total volume of 200 ml of 0.10 M NaCl–20 mM $CaCl_2$, at 37°. In all assays at 25 and 37° the temperature was maintained within 0.05°, within 0.10° at 15°, and within 0.20° at 0°.

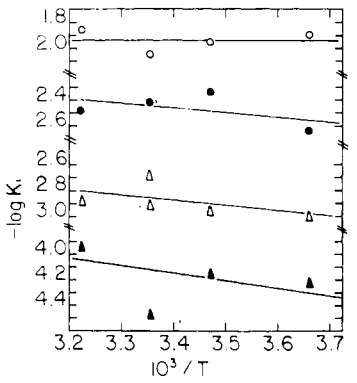

FIGURE 2: The effect of temperature on the enzyme–guanidine dissociation constants: (-O-O-) guanidine-HCl; (-●-●-) methylguanidine sulfate; (-Δ-Δ-) cyclohexylguanidine sulfate; (-▲-▲-) phenylguanidine sulfate. Experiments in 0.10 M Tris, pH 8.15.

Results

The values of the standard free energies of binding at each temperature were calculated from the equation

$$\Delta G^\circ = -RT \ln K_i \quad (1)$$

The variance in ΔG° was evaluated as indicated by Wilkinson (1961) through the equation

$$V(\Delta G^\circ) = V(K_i)\,(d\Delta G^\circ/dK_i)^2 \quad (2)$$

that yields for the standard error:

$$SE(\Delta G) = RT \times \frac{SE(K_i)}{K_i} \quad (3)$$

The standard enthalpy of binding is given by

$$\log K_i = -\frac{\Delta H^\circ}{2.303R} \times \frac{1}{T} + \text{constant} \quad (4)$$

Plots of log K_i as a function of $1/T$ for the competitive inhibitors were fitted to straight lines, as shown in Figures 1 and 2. The values of ΔH° were calculated from least-squares treatment.

The standard error in ΔH° was found from the equation

$$SE(\Delta H^\circ) = 2.303RSE\,(\text{slope}) \quad (5)$$

The values for the standard entropy changes were calculated from the equation

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (6)$$

The values of K_i for each inhibitor at different temperatures are included in Table I for the amidines, and in Table II for the guanidines. The results for the changes in standard free energy, enthalpy, and entropy at 25°, calculated with eq 1–6, are given in Table III for amidines, and Table IV for guanidines. The subscript u in ΔG_u and ΔS_u refers to unitary changes as defined by Kauzmann (1959); they depend only on those factors which involve the interaction of the inhibitor and the active center of trypsin with each other and with the solvent, and they do not depend on the contribution due to randomness of the mixing with the solvent, to almost quote Kauzmann.

Although the pH of the Tris buffer used was carefully checked before and at the end of each experiment, inhibition studies were carried out at different pH values as further controls. Bz-L-ArgOEt was used as substrate and the release of acid was titrated as described in the previous section. The results are shown in Figure 3.

The middle section contains $-\log K_i$ for phenylguanidine (full circles) and benzamidine (open circles). Within the range pH 7.5–8.4 there is no significant difference in binding of each inhibitor to the enzyme. Béchet *et al.* (1966) have arrived at a similar conclusion for the inhibition by *n*-butyl-

TABLE I: Dissociation Constants, K_i, for the Trypsin–Competitive Inhibitor Complexes with Inhibitors of the Amidinium Type (measured in 0.10 M Tris-HCl, pH 8.15, with DL-Bz-DL-Arg-NA[a] as substrate).

Temperature (°K)	Acetamidinium (7.7–69.0 mM)[b]	Benzamidinium (3.7–127.0 μM)[b]	β-Naphthamidinium (3.0–55.0 μM)[b]
273.2	23.1 ± 6.2 (20)[c]	17.1 ± 6.3 (20)	10.8 ± 2.0 (20)
288.2	25.5 ± 4.8 (16)	14.1 ± 2.0 (16)	14.6 ± 2.9 (20)
298.2	27.4 ± 4.0 (18)	18.8 ± 3.1 (18)	14.6 ± 4.8 (20)
310.2	23.1 ± 5.2 (18)	17.4 ± 3.1 (16)	11.9 ± 1.9 (20)

[a] Bz-DL-Arg-NA = α-*N*-benzoyl-DL-arginine-*p*-nitroanilide [b] Inhibitor concentration range. [c] Figures in parentheses beside K_i values are the numbers of assays carried out for each K_i evaluation.

TABLE II: Dissociation Constants, K_i, for the Trypsin–Competitive Inhibitor Complexes with Inhibitors of the Guanidinium Type (measured in 0.10 M Tris-HCl, pH 8.15, with DL-Bz-DL-Arg-NA as substrate).

Temperature (°K)	Guanidinium (8.10–74.0 mM)[a]	Methylguanidinium (2.20–20.0 mM)[a]	Cyclohexylguanidinium (0.80–7.60 mM)[a]	Phenylguanidinium (24.7–223 μM)[a]
273.2	12.6 ± 2.3 (16)[b]	2.28 ± 0.85 (16)	1.02 ± 0.38 (20)	60.0 ± 12.4 (16)
288.2	11.2 ± 1.4 (20)	4.40 ± 1.00 (16)	1.11 ± 0.10 (20)	70.2 ± 10.3 (20)
298.1	8.82 ± 0.2 (16)	3.78 ± 1.40 (20)	2.13 ± 0.42 (20)	33.2 ± 12.6 (20)
310.1	14.0 ± 2.4 (16)	3.28 ± 0.65 (20)	1.34 ± 0.23 (20)	114.3 ± 23.3 (20)

[a] Inhibitor concentration range. [b] Figures in parentheses beside K_i value are the numbers of assays carried out for each K_i evaluation.

TABLE III: Thermodynamic Parameters for the Formation of Trypsin–Inhibitor Complexes with Inhibitors of the Amidinium Type (all data at 25.0°).

Inhibitor	$\Delta G°$ (kcal/mole)	$\Delta G°_u$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (cal/deg mole)	$\Delta S°_u$ (cal/deg mole)
Acetamidinium	−2.12 ± 0.09	−4.50 ± 0.09	−0.16 ± 0.6	6.59 ± 2.4	14.6 ± 2.4
Benzamidinium	−6.43 ± 0.10	−8.81 ± 0.10	0.42 ± 1.0	23.0 ± 3.8	31.0 ± 3.8
β-Naphthamidinium	−6.57 ± 0.19	−8.95 ± 0.19	−0.56 ± 1.1	20.2 ± 4.3	28.2 ± 4.3

amine of the tryptic hydrolysis of *N*-acetylphenylalanine methyl ester.

Discussion

Change in Enthalpy. The data in Table III fully support the hypothesis raised by Mares-Guia and Shaw (1965) that with inhibitors of increasing chain size, as in this series, the increase in the free energy of binding was a function of the size of the inhibitor side chain. This effect was attributed to an interaction of the side chain with a hydrophobic binding site in the enzyme active center. The fact that the values of $\Delta H°$ are very small and independent of inhibitor side-chain structure demonstrates that the drive for binding does indeed originate from the increase in entropy which accompanies complex formation. A similar conclusion is reached from the analysis of similar data for the complex formation between several guanidinium compounds and trypsin (Table IV). The differences observed in the $\Delta H°$ values in Table IV, although proportional to side-chain size, are not statistically significant. Large values for the standard deviation in $\Delta H°$ have been observed by other authors for protein reactions in water. Table V in the paper by Kauzmann (1959) shows a series of values for protein reactions where the deviations in $\Delta H°$ are of the same order as those shown in Tables III and IV.

Inagami and York (1968) have measured the thermodynamic constants for the binding of alkylguanidines to trypsin from methyl- to butyl-, and of benzyl- and cyclohexylguanidines. The $\Delta H°$ values found were about the same for all

TABLE IV: Thermodynamic Parameters for the Formation of Trypsin–Inhibitor Complexes with Inhibitors of the Guanidinium Type (all data at 25.0°).

Inhibitor	$\Delta G°$ (kcal/mole)	$\Delta G°_u$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (cal/deg mole)	$\Delta S°_u$ (cal/deg mole)
Guanidinium	−2.80 ± 0.14	−5.18 ± 0.14	−0.01 ± 1.4	9.36 ± 5.5	17.3 ± 5.5
Methylguanidinium	−3.30 ± 0.22	−5.68 ± 0.22	−1.6 ± 1.8	5.73 ± 6.8	13.7 ± 6.8
Cyclohexylguanidinium	−3.65 ± 0.12	−6.03 ± 0.12	−1.8 ± 1.7	6.55 ± 6.1	14.5 ± 6.1
Phenylguanidinium	−6.11 ± 0.22	−8.49 ± 0.22	−2.7 ± 2.9	11.6 ± 10	19.6 ± 10

TABLE V: Contribution of the Side Chain of Aromatic Amidinium Compounds to $\Delta G°$ of Formation of the Complexes Trypsin–Inhibitor as Compared to $\Delta G°$ of Transfer of Benzene from Water to Pure Benzene.

	Benzamidinium		β-Naphthamidinium		$-\Delta G°$ of Transfer $C_6H_6(aq) \rightarrow C_6H_6(l.)$[c]
Temp (°C)	$\Delta(\Delta G°)$ Obsd[a]	$\Delta(\Delta G°)$ Cor[b]	$\Delta(\Delta G°)$ Obsd[a]	$\Delta(\Delta G°)$ Cor[b]	
0	3.64 ± 0.35	3.99 ± 0.35	3.91 ± 0.35	4.26 ± 0.35	4.24
15.0	4.28 ± 0.18	4.68 ± 0.18	4.26 ± 0.22	4.66 ± 0.22	4.51
25.0	4.31 ± 0.18	4.73 ± 0.18	4.47 ± 0.27	4.89 ± 0.27	4.65
37.0	4.56 ± 0.27	5.00 ± 0.27	4.66 ± 0.24	5.10 ± 0.24	4.80

[a] Obtained by subtracting $\Delta G°$ for binding of acetamidinium from $\Delta G°$ for binding of benzamidinium or β-naphthamidinium. [b] To $\Delta(\Delta G°)$ Obsd was added the methyl group contribution (see text). Thus, the $\Delta(\Delta G°)$ Cor values are now referred to the H_- of formamidinium. [c] Taken from Némethy (1962), p 330.

their compounds, and of the order of 4.5 kcal/mole. This figure is about twice the figure given in this paper.

It is not probable that the difference is due to the different pH of the experiments, 8.15 in our case, and 6.6 in the work of Inagami and York (1968), for there are no major differences in trypsin tertiary structure at pH 6.6 and at pH 8.5, as demonstrated by a constancy in the degree of optical rotation in this pH range (Neurath *et al.*, 1956), both in the absence and in the presence of the competitive inhibitor butylamine (D'Albis and Béchet, 1967). As expected, there is good agreement between both sets of data for $\Delta G°$ at 25° for methylguanidine and cyclohexylguanidine. There is also a discrepancy between the $\Delta H°$ data for the formation of trypsin–alkylammonium complexes given by Inagami (1964), $\Delta H°$ in the range of −11 kcal/mole, and those of Heidberg *et al.* (1967), $\Delta H°$ in the range of −4 kcal/mole, although

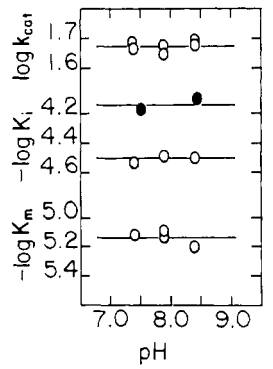

FIGURE 3: The effect of pH on the tryptic hydrolysis of Bz-L-ArgOEt and on the dissociation constants of competitive inhibitors. Legend for the middle section: (-○-○-) benzamidine-HCl; (-●-●-) phenylguanidine sulfate. The experiments were carried out in 0.10 M NaCl–20 mM $CaCl_2$, at 37°.

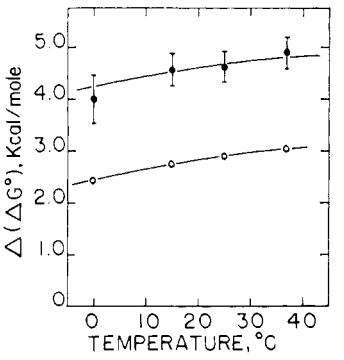

FIGURE 4: A comparison between $\Delta G°_u$ of transfer of benzene from water to pure liquid and the phenyl side-chain contribution ($\Delta(\Delta G°_u)$) to the binding of benzamidinium to trypsin. Upper line: data from Némethy (1962), p 330; points: phenyl side-chain contribution plus standard error; lower line and points: approximate values for the hydrophobic bond between three phenylalanyl side chains, one of which "sandwiched" between the other two (data from Némethy (1962), p 352, multiplied by 2).

the experimental conditions are similar in both cases, that is, pH 6.6 in 0.1 M KCl. Since the value of $\Delta H°$ is very sensitive to slope variations in the $-\log K_i$ *vs.* $1/T$ plot, it is possible that these differences arise from the number of points used in the plot. Thus, Inagami and York (1968) worked at three different temperatures, whereas the data in this paper and those of Heidberg *et al.* (1968) were obtained at four different temperatures.

Hydrophobic Interaction. The results shown in Tables III and IV demonstrate the entropic origin of the association between the active center of trypsin and the hydrocarbon side chain of inhibitors. This is in agreement with the initial interpretation that this interaction is hydrophobic (Mares-Guia and Shaw, 1965). It is interesting to compare the parameters of binding of the side chain of benzamidine and β-naphthamidine to the hydrophobic site in trypsin with the parameters of transfer of benzene from water to the pure liquid. The side-chain contribution to the free energy of binding was calculated by subtracting from the $\Delta G°_u$ value for benzamidine or β-naphthamidine the $\Delta G°_u$ value for acetamidine, and adding to the result the calculated contribution of the methyl group, at each temperature. The methyl group contribution was estimated from the difference in the binding of acetamidinium and formamidinium to trypsin at 15° (Mares-Guia, 1968a). The values were then adjusted to the other temperatures by comparison with the free energy of solution of methane in water as given by Némethy (1962). The results are shown in Table V. It is apparent that the phenyl and β-naphthyl side chains contribute the same value to the binding of the respective amidines to trypsin. What needs to be emphasized is the perfect agreement between the contribution to binding of the side chain of benzamidine and the free energy of transfer of benzene from water to pure liquid, as given by Némethy (1962), at the four temperatures investigated. These aspects are clearly demonstrated in Figure 4. The upper line represents the negative of the free energy of transfer of benzene from water to pure liquid; the points are the negative values of the side-chain contributions, with standard error indicated. The lower line is an approximation to the free energy of formation of a hydrophobic sandwich between three isolated side chains of phenylalanine. The values in Némethy's work (1962) were multiplied by 2, just to yield the order of magnitude to be expected.

The observed close agreement is suggestive of the presence of one or more aromatic amino acid residues as constituents of the site. More direct evidence points already toward such possibility: Mares-Guia (1964) and Mares-Guia and Shaw (1967) observed difference spectra during the formation of *p*-guanidinobenzoyl-trypsin, and the spectra are compatible with the involvement of a tyrosyl residue; Inada *et al.* (1964) measured the ionization of tyrosyl residues in trypsin and concluded for the participation of one of them in the active center. Quite recently Herskovits and Villanueva (1969), using the solvent pertubation technique of difference spectroscopy, concluded for the burial of tyrosyl residues upon phosphorylation of the active center of trypsin with DFP.

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