CHAPTER 2

Thermodynamics of Binding of the Benzamidinium Chloride Parent Compound to Trypsin

The thermodynamics of binding of the inhibitor benzamidinium chloride to the serine proteinase bovine pancreatic trypsin has been studied using isothermal titration calorimetry. At 25 °C both the enthalpy and the entropy of binding are favourable, but this is only true in a narrow temperature range. Binding is characterised by a negative change in heat capacity and enthalpy-entropy compensation, both characteristic of hydrophobic interactions. No protons are transferred upon binding. Binding studies with the structurally related compounds benzylammonium chloride, α-methylbenzylammonium chloride and benzamide showed that hydrogen bonding between the amidinium group and the enzyme is primarily enthalpy-driven. Binding of cyclohexyl-carboxamidinium chloride and acetamidinium chloride showed that the hydrophobic bonding of the phenyl ring in the S1 pocket is primarily entropy-driven and that a rigid, flat hydrophobic binding site of the inhibitor is favourable. Also, the amidinium-Asp189 salt bridge in the benzamidinium-trypsin complex was modelled by studying the interaction between benzamidinium and benzoate in DMSO, ethanol and water. Benzamidinium-benzoate binding in an apolar, aprotic solvent yields thermodynamics similar to those for benzamidinium-trypsin binding. The polar, protic water is a too competitive solvent for binding to occur. This is still, but to a lesser extent, the case in the less polar, but also protic ethanol, where binding is entropy-driven.

"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 substrate or inhibitors."

2.1 Introduction

The guanidinium-carboxylate binding mode, with its strong binding interactions, is ubiquitous in enzyme-substrate binding as well as in the stabilisation of protein tertiary structures via internal salt bridges. Trypsin specifically cleaves the peptide bonds on the carboxyterminal side of the positively charged residues arginine and lysine, which bind in the specificity pocket to the negatively charged Asp189 carboxylate. Non-transition state inhibitors block binding of substrate by binding in the specificity pocket. Many natural proteinase inhibitors are small peptides with an arginine or lysine residue binding in the specificity pocket and synthetic proteinase inhibitors are also often based on these groups. In 1965, Mares-Guia and Shaw published an article entitled “Studies on the Active Center of Trypsin; the Binding of Amidines and Guanidines as Models of the Substrate Side Chain”. They concluded that, regarding the specificity requirements of the enzyme, amidines are good model systems for the arginine side chain ($pK_a = 12.48$) of trypsin substrate. Of the compounds studied, benzamidinium chloride ($pK_a = 11.41$) and $p$-aminobenzamidinium chloride ($pK_a = 12.69$) were the most effective. In fact, they were the most potent small molecular competitive inhibitors of trypsin reported until then. Apparently, the phenyl group is a good model for the hydrophobic part of the arginine side chain. The lengths of these groups are approximately the same, but the rigid phenyl group has only one single conformation. Arginine and benzamidinium are depicted in Scheme 2.1.

Figure 2.1A shows a cartoon representation of the crystal structure of the benzamidinium-trypsin complex. The benzamidinium ion is bound in the specificity pocket S1, making five hydrogen bonds with Asp189, Gly219, Ser190 and an internal water molecule. Both partially positively charged amidinium nitrogens bind to the carboxylate oxygens of Asp189 in an almost symmetrical manner, N1 to Asp189 OD1 (2.90 Å) and N2 to Asp189 OD2 (3.12 Å). In addition, N1 is hydrogen bonded to Gly219 O (2.83 Å), while N2 is hydrogen bonded to both Ser190 OG (3.06 Å) and the internal water molecule 416 (3.16 Å). The amidinium group is rotated out of the plane of the phenyl ring with an angle of $7^\circ$. The phenyl ring is mainly surrounded by nonpolar and neutral polar $\alpha$-amino acid residues and in van der Waals contact with the residues 214-216 and 190-191. It is also quite close to the His57 and Ser195 residues of the catalytic triad; the distance between Ser195 OG and the carbon on the $p$-position of the benzamidinium is 3.55 Å. The benzamidinium molecule does not occupy the hydrophobic S3/S4 groove, defined by the residues Trp215 and Leu99, as is the case for some larger, tailor-made benzamidinium derivatives, where hydrophobic groups bend back to this groove.

\[
\begin{align*}
\text{H} & \quad \text{NH}_2 \\
\text{OOC} & \quad \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{NH} & \quad \text{\textbullet N} & \quad \text{\textbullet NH}_2 \\
\text{NH}_3 & \quad \oplus & \quad \text{\textbullet NH}_2 & \quad \text{\textbullet NH}_2
\end{align*}
\]

*Scheme 2.1. Arginine (left) and benzamidinium (right).*
Figure 2.1. Cartoon representations of the crystal structures of the benzamidinium-trypsin complex (top, A), and the benzylammonium-trypsin complex (bottom, B) (3ptb and 2bza from the Brookhaven Protein Data Bank, respectively) generated using the program RasMol. Both inhibitors are bound in the S1 binding pocket, close to the catalytic triad. Also shown is the hydrophobic pocket S3/S4, defined by Trp215 and Leu99.
Just as amidinium ions are good structural analogues of the arginine side chain, alkyl ammonium ions are good model systems for lysine \( (pK_a = 10.53) \). The benzylammonium ion \( (pK_a = 9.50) \) was found to be the most efficient inhibitor of the compounds investigated, followed by the \( n \)-butylammonium ion with a three-fold lower affinity. Since the hydrophobic parts of these ions have similar lengths, the higher affinity of the phenyl ring is likely due to other specific properties of the phenyl ring, such as the aromatic and conformationally restricted character. Lysine and benzylammonium are depicted in Scheme 2.2.

It is obvious that the geometry of an amidinium group is more favourable than that of an ammonium group, which is not able to fully utilise all the hydrogen bonding sites provided by the enzyme. In comparison to benzamidinium, benzylammonium is expected to have less effective hydrogen bonding interactions because of the absence of one of the amino groups. This is indeed seen in the crystal structure of the benzylammonium-trypsin complex: Figure 2.1B shows a cartoon representation of the crystal structure of the benzylammonium-trypsin complex, which shows a binding mode different from that of benzamidinium-trypsin. A slight expansion of the binding pocket, involving residues 190-192 and 215-216, has been observed. The ammonium group exclusively occupies only one of the two possible locations, namely that corresponding to the N1 position of benzamidinium; around the position corresponding to the N2 position of benzamidinium, no electron density was observed for benzylammonium. The ammonium group makes an angle of 60° with the plane of the benzyl ring and forms hydrogen bonds with Asp189 OD1 (2.72 Å) and Ser190 O (2.67 Å), but not with Ser190 OG or Gly219 O, as is the case in benzamidinium-trypsin. Furthermore, it forms a hydrogen bond (2.99 Å) to a water molecule inserted between and forming hydrogen bonds with Asp189 OD1 (3.15 Å) and Gly219 O (3.12 Å). This hydrogen-bonding pattern may force the nitrogen of the ammonium group of benzylammonium in one specific position.

In this study, we investigated the thermodynamics of binding of benzamidinium chloride to trypsin by means of isothermal titration calorimetry (ITC). The influence of the amidinium group and the phenyl ring on the binding process was probed by studying binding of structurally related compounds: benzylammonium chloride, \( \alpha \)-methylbenzylammonium chloride, benzamide, cyclohexylcarboxamidinium chloride, and acetamidinium chloride. An attempt was made to model the amidinium-Asp189 salt bridge in the benzamidinium-trypsin complex by measuring the interaction between benzamidinium and model systems of the aspartate side chain in a solvent that approximates the dielectric constant of the active site cleft.
2.2 Thermodynamics of Binding of Benzamidinium Chloride to Trypsin

The thermodynamics of binding of benzamidinium chloride to trypsin was examined using ITC. Figure 2.2A shows the raw data of a typical binding experiment, the titration of benzamidinium chloride to trypsin in Tris buffer at 25 °C. Binding is clearly associated with an exothermic process. Dilution peaks are all endothermic and equally small, indicating that at this concentration, no aggregation of the inhibitor in water occurs. Figure 2.2B shows the accompanying enthalpogram, the enthalpy change upon binding for each injection as a function of the concentration of benzamidinium. This enthalpogram has been obtained by integrating the raw data of the binding experiment and correcting for the heat of dilution. A binding isotherm, that yields values for the binding constant \( K \) and the enthalpy of binding \( \Delta H \) (described in the Experimental Section), has been fitted to the data points.

The observed binding constant \( (K = 4.5 \times 10^4 \text{ M}^{-1}, 25 \degree \text{C}) \) compares well to the value of \( 5.4 \times 10^4 \text{ M}^{-1} \) obtained by Mares-Guia and Shaw at 15 °C and pH 8.15, by measuring the inhibition of the hydrolysis of \( N^\alpha\)-benzoyl-DL-arginine \( p \)-nitroanilide. From the observed \( K \) and \( \Delta H \), the Gibbs energy of binding \( \Delta G \) and the entropic component of binding \( T\Delta S \) have been calculated using

\[
\Delta G = -RT \ln K = \Delta H - T\Delta S
\]

(2.1)

where \( \Delta S \) is the entropy of binding and \( T \) is the temperature in K. This analysis yields values for \( \Delta G \), \( \Delta H \) and \( T\Delta S \) of \(-26.6, -18.9\) and \(7.7 \text{ kJ mol}^{-1}\), indicating that under these experimental conditions, both the entropic part and the enthalpic part of the change in Gibbs energy upon binding are favourable. As will be shown later in this chapter (Section 2.2.2), this is only true in a relatively small temperature interval.
Thermodynamics of Binding of Benzamidinium Chloride Inhibitors to Trypsin

**Figure 2.2.** (A) Raw data for titration of benzamidinium chloride into trypsin (lower trace) and buffer (upper trace) at 25 °C (all solutions in Tris pH 8.0). (B) Enthalpogram retrieved from A, corrected for the heat of dilution; the line represents the least-squares-fit to the single-site binding model.\(^\text{17}\)
2.2.1 Buffer Dependence

The observed enthalpy of binding is not necessarily only due to noncovalent interactions between the inhibitor and the enzyme, since all processes superimposed on the binding are included in the measured heat of reaction. Because the transfer of an inhibitor from the aqueous phase to the enzyme binding site can strongly affect the dielectrical properties of the local environment of groups involved in the binding, biological binding processes are often accompanied by a change in the protonation state of these groups. The change in the protonation state of residues involved in the binding process is equal to the number of protons exchanged between the buffer and the enzyme upon binding. When protonation/deprotonation effects are significant in the binding process, the observed enthalpy of binding $\Delta H$, as derived from the binding isotherm, not only consists of the intrinsic enthalpy of binding $\Delta H_{\text{int}}$ but also depends on the enthalpy change associated with ionisation of the buffer. This can be expressed by:

$$\Delta H = \Delta H_{\text{int}} + N_{\text{H}^+} \Delta H_{\text{ion}}$$

(2.2)

where $N_{\text{H}^+}$ is the number of protons from the buffer taken up by the enzyme and $\Delta H_{\text{ion}}$ the ionisation enthalpy of the buffer.

Figure 2.3. Enthalpy of binding of benzamidinium chloride to trypsin at pH 8.0 in five different buffers as a function of the ionisation enthalpy of the buffer.
In order to retrieve $N_{H^+}$ and $\Delta H_{int}$, titrations of benzamidinium in trypsin were performed at pH 8.0 in five buffers with different ionisation enthalpies, ranging from 20.2 to 47.3 kJ mol$^{-1}$: Hepes, Bicine, Tricine, Glycylglycine and Tris. Figure 2.3 shows the dependence of the observed enthalpy of binding on the ionisation enthalpy of the buffer at 25 °C. The linear regression of the data yields an abscissa, equal to $\Delta H_{int}$ of $-16.3 (1.9)$ kJ mol$^{-1}$ and a slope, equal to $N_{H^+}$, of $-0.03 (0.05)$ indicating that, within error limits, no protons are transferred upon binding of benzamidinium to trypsin at pH 8.0. Considering the spread of the data points in Figure 2.3 and the errors in the linear fit, it is clear that the contribution from the ionisation of the buffer to the observed enthalpy of binding is small and not systematic at pH 8.0. Therefore, at this pH, further titration experiments were performed only in the standard buffer Tris.

2.2.2 Temperature Dependence

Since the temperature dependence of the binding parameters gives a more detailed insight into the binding process, the binding of benzamidinium to trypsin was studied as a function of temperature. The titration experiments were performed at 20, 25, 30 and 37 °C in Tris pH 8.0; the resulting thermodynamic parameters are listed in Table 2.1. In Figure 2.4, the temperature dependence of $\Delta G$, $\Delta H$ and $T\Delta S$ for binding of benzamidinium chloride to trypsin is depicted. Over a narrow temperature range, the temperature dependence of $\Delta H$ is given by:

$$\frac{\partial \Delta H}{\partial T} = \Delta C_p$$  \hspace{1cm} (2.3)

where $\Delta C_p$ is the heat capacity change upon binding. From Figure 2.4, it is clear that in the temperature range studied $\Delta H$ is indeed linearly dependent on $T$. The value of $\Delta C_p$, determined from the slope of the linear fit to the data points, is $-400 \ (20)$ J mol$^{-1}$ K$^{-1}$.

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>$K \ (10^4 \ M^{-1})$</th>
<th>$\Delta G \ (kJ \ mol^{-1})$</th>
<th>$\Delta H \ (kJ \ mol^{-1})$</th>
<th>$T\Delta S \ (kJ \ mol^{-1})$</th>
</tr>
</thead>
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<td>3.1</td>
<td>$-26.7$</td>
<td>$-23.7$</td>
<td>3.0</td>
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</table>

Table 2.1. Thermodynamic parameters for binding of benzamidinium chloride to trypsin in Tris pH 8.0 at different temperatures.
CHAPTER 2: Thermodynamics of Binding of the Benzamidinium Chloride Parent Compound to Trypsin

Figure 2.4. ΔG, ΔH and -TΔS for binding of benzamidinium chloride to trypsin at different temperatures in Tris pH 8.0.

Arguments based on thermodynamic data of transfer of various compounds from a nonpolar phase to water,24-26 as well as protein folding data25-28 and binding of several ligands to their cognate proteins,27,29,30 suggest that the main contribution to a negative value of ΔCp stems from the burial of nonpolar surface area from water. By contrast, the removal of polar surfaces from the aqueous phase tends to increase ΔCp. Although the origin of the hydrophobic effect is still extensively debated, a reasonable explanation of this phenomenon is that hydration of a solute induces, relative to bulk water, a solvent reorganisation in its hydration shell (Section 1.1.6).31-35 It has been suggested35-37 that the solute causes a change in the amounts of two populations of hydrogen bonds, one with shorter and more linear bonds, the other with longer and more bent bonds. The first is favoured in case of nonpolar solutes and the second in case of polar solutes, which could explain the opposite effect of polar and nonpolar surface area on ΔCp.

A negative ΔCp results in the net thermodynamic driving force for association to shift from being entropic to enthalpic with increasing temperature. This implies that it is possible to define temperatures TH and TS at which the enthalpic and the entropic contributions to binding are zero (Section 1.1.6.1). TH and TS have been calculated from a linear fit to the temperature-dependencies of ΔH and TΔS. TH, the temperature at which the enthalpic contribution to the Gibbs energy of binding changes from unfavourable to favourable, is equal to −23 °C. TS, at which the entropic contribution to
the Gibbs energy of binding changes from favourable to unfavourable, is equal to 44 °C. Therefore, at temperatures below $T_H$, the binding process is completely entropy-driven, whereas at temperatures above $T_S$, the binding process is completely enthalpy-driven. Only in the temperature interval between $T_H$ and $T_S$ that is, from -23 to 44 °C, are both the enthalpic and the entropic part of the Gibbs energy of binding favourable.

Figure 2.4 shows that $\Delta G$ is practically temperature-independent as a result of the compensating temperature-dependence of $\Delta H$ on $T \Delta S$. This thermodynamic behaviour is characteristic for processes accompanied by a large, negative $\Delta C_p$ (Section 1.1.6.1) and thus, again, a consequence of the vanishing of the hydrophobic hydration of the inhibitor. This temperature-dependence is one of the main fingerprints indicating that the hydrophobic effect is involved in biological or chemical processes.\textsuperscript{30,34} Provided that $\Delta C_p$ is independent of temperature, together with $T_H$ and $T_S$ it gives a complete thermodynamic description of the binding process (Section 1.1.6).

### 2.3 Contribution of the Amidinium Group

In order to probe the contribution of the amidinium group to the binding of benzamidinium-based inhibitors to trypsin, the binding of benzylammonium\textsuperscript{38} chloride to trypsin was studied. The thermodynamic parameters obtained in Tris pH 8.0 are listed in Table 2.2. The value for the binding constant ($K = 5.5 \times 10^3 \text{ M}^{-1}$, 25 °C) is slightly higher, but well comparable to that reported by Markwardt et al.,\textsuperscript{39} who determined a value of $3.3 \times 10^3$ at 25 °C in a different buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl) by measuring the inhibition of the hydrolysis of $N$-$\alpha$-benzoyl-DL-arginine-4-nitroanilide by trypsin.\textsuperscript{40} At all temperatures studied, the binding constant of benzylammonium chloride to trypsin is more than six times lower than that of benzamidinium chloride (Table 2.1) and $\Delta G$ is between 4.7 and 5.0 kJ mol\textsuperscript{-1} less favourable. This less favourable $\Delta G$ is caused by a more than two times less favourable $\Delta H$ that is partly compensated by a more favourable $T \Delta S$.

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>$K$ (10$^4$ M$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$T \Delta S$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
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<td>8.6</td>
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</table>

*Table 2.2. Thermodynamic parameters for binding of benzylammonium chloride to trypsin in Tris pH 8.0 at different temperatures.*
It has been suggested that the enthalpy of binding primarily reflects the strength of the interactions of the inhibitor with the enzyme, whereas the entropy change mainly reflects two contributions: changes in hydration entropy and changes in conformational entropy.\textsuperscript{20} Therefore, the less favourable enthalpy of binding is most likely due to less strong interactions with the enzyme due to the less extensive hydrogen bonding in comparison to benzamidinium (Section 2.1). The more favourable entropy of binding is most probably due to enthalpy-entropy compensation, a phenomenon that has been described in detail in Section 1.1.7. We therefore propose that benzylammonium is less extensively conformationally restrained in the complex due to the less tight binding.

\[ \Delta C_p \text{, equal to } -254 \text{ J mol}^{-1} \text{ K}^{-1}, \text{ is less negative than that of benzamidinium chloride; } T_H \text{ is } 14 ^\circ \text{C and } T_S \text{ is } 71 ^\circ \text{C (values calculated as described in Section 2.2.2). The amidinium group has a lower charge density in comparison to ammonium.}^2 \text{ Since the removal of polar surfaces from the aqueous phase tends to increase } \Delta C_p \text{ (Section 2.2.2), the less negative } \Delta C_p \text{ could be caused by the more polar ammonium group in the benzylammonium ion. Although } \Delta C_p \text{ is less negative than that of benzamidinium chloride, it still has an appreciably negative value and results in enthalpy-entropy compensation with temperature, two factors that are considered hallmarks of the hydrophobic effect (Section 2.2.2).}

To even further disturb the hydrogen bonding capacities of the amidinium group, the binding affinities of \( \alpha \)-methylbenzylammonium and benzamide to trypsin have been tested. For both compounds, only peaks of dilution were observed at 25 °C when titrated in trypsin solution, which indicates that binding of these compounds to trypsin is negligible.

### 2.4 Contribution of the Phenyl Group

In order to probe the contribution of the phenyl ring to the binding of benzamidinium-based inhibitors to trypsin, titrations of acetamidinium chloride and cyclohexylcarboxamidinium chloride into trypsin were performed. In acetamidinium chloride, the large and hydrophobic phenyl group is replaced by the smaller and less hydrophobic methyl group, which is expected to have less favourable interactions with the hydrophobic \( S_1 \) pocket. The upper limit of the binding constant of acetamidinium chloride to trypsin at 25 °C is \( 10^2 \); more reliable binding constants are not retrievable for this poorly binding ligand. This value is significantly lower than the binding constant of benzamidinium to trypsin (\( K = 4.5 \times 10^4 \text{ M}^{-1} \) at 25 °C, Table 2.1), indicating that the more hydrophobic phenyl group increases the binding constant at least 450 times (\( \Delta G \) is more than 15 kJ mol\(^{-1}\) more favourable) relative to a methyl group.

In cyclohexylcarboxamidinium chloride, the phenyl group is replaced by the more hydrophobic, but also larger and more flexible cyclohexyl group.\textsuperscript{41} The polarisabilities of cyclohexane and benzene are, respectively, 10.99 and 10.39, which indicates that the dispersion interactions of both compounds will be comparable.
As described in Section 2.1, the phenyl ring sits really tightly in the binding pocket, in closer than Van der Waals contact with some hydrophobic residues of the enzyme. Therefore, for binding of the cyclohexylcarboxamidinium ion to trypsin to occur, it may be necessary to disturb the conformational equilibrium of the cyclohexyl ring and/or the conformation of the binding site in order to fit the cyclohexyl ring into the binding pocket. The thermodynamic binding parameters of cyclohexylcarboxamidinium chloride to trypsin are listed in Table 2.3. At all temperatures studied, the binding constant of cyclohexylcarboxamidinium chloride to trypsin is more than ten times lower than that of benzamidinium chloride and $\Delta G$ is more than 6 kJ mol$^{-1}$ less favourable (Table 2.1). This is an entropic effect; in fact, the enthalpy of binding is slightly more favourable at all temperatures studied.

In general, upon binding both the inhibitor and the enzyme lose conformational freedom, which is an entropic effect. To reduce the loss in conformational freedom, a strategy in inhibitor design is to conformationally constrain the inhibitor.$^{20,42}$ If the free inhibitor itself has little conformational freedom, the loss upon binding to the enzyme will be small. We therefore propose that the less favourable entropy of binding of the cyclohexylcarboxamidinium ion is due to the reduced flexibility of the cyclohexyl ring in the binding site of the enzyme as compared to that in the water phase. This will lead to a loss in conformational freedom for the inhibitor and therefore a reduced entropy change upon binding, which is not encountered for the already flat and constrained phenyl ring. Also, the enzyme may lose some conformational freedom. Furthermore, some adjustment of the binding pocket may be necessary to accommodate the cyclohexyl ring.

$\Delta C_p$ is equal to -458 J mol$^{-1}$ K$^{-1}$, $T_H$ is -17 °C and $T_S$ is 27.4 °C (values calculated as described in Section 2.2.2). The heat capacity change upon binding is slightly higher than that of benzamidinium chloride, probably due to the fact that the cyclohexyl ring is more hydrophobic than the phenyl ring. This is indicated by its higher $n$-octanol-water partition coefficient; $\Delta \log P$, the relative $n$-octanol-water partition coefficient of a substituent with respect to hydrogen, amounts to 2.82 for cyclohexyl and 1.96 for phenyl.$^{41}$ This large and negative $\Delta C_p$ results in enthalpy-entropy compensation with temperature (Section 2.2.2).

### Table 2.3. Thermodynamic parameters for binding of cyclohexylcarboxamidinium chloride to trypsin in Tris pH 8.0 at different temperatures.

<table>
<thead>
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<th>$T$ (°C)</th>
<th>$K$ ($10^4$ M$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
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2.5 Modelling of the Asp189-Amidinium Hydrogen Bonds

The nonpolar interiors of enzymes provide the living cell with the equivalent of an organic solvent, in which strong electrostatic interactions between substrate and specific polar groups of the enzyme can occur. In order to model a specific part of the interaction between benzamidinium and trypsin, namely the amidinium-Asp189 binding, the interaction between benzamidinium chloride and a model for the aspartate side chain has been studied in a solvent with a dielectric constant that resembles that of the active site cleft of the enzyme. Crystal structures of amidinium-carboxylate salts conform to a binding mode in which a salt bridge is reinforced by two almost linear hydrogen bonds between the oxygens of the carboxylate and the interfacial protons of the amidinium anti to the aryl group (NH\(_2\)), a binding mode that is the same as that of benzamidinium to Asp189 in the complex with trypsin (Section 2.1). In this model system, the other pair of amidinium protons (NH\(_2\)) is still available for additional one-point interactions. First, the choice of the model system for the Asp189 side chain will be discussed and next the appropriate solvent.

Aspartic acid itself is not a good model system, since there are two carboxylic acid groups present in this molecule. Instead, carboxylic acids with a pK\(_a\) similar to that of aspartic acid, which is 3.86, were considered: formic acid (pK\(_a\) = 3.7) and benzoic acid (pK\(_a\) = 4.2). The pK\(_a\) of formic acid most closely resembles that of aspartic acid, but formic acid is only poorly soluble in most organic solvents. Therefore, it was decided to use sodium benzoate, which is better soluble in organic solvents.

In an article by Evans et al., the behaviour of the fluorescent probe p-aminobenzamidinium chloride in several solvents and in trypsin solution has been described. This probe is weakly fluorescent and has excitation and emission maxima at, respectively, 293 and 376 nm in neutral aqueous buffer. The reported emission intensities at 22 °C in these solvents relative to water (I/I(H\(_2\)O)) are plotted as a function of the dielectric constant \(\varepsilon\) of the solvent in Figure 2.5; to guide the eye, a second-order exponential decay has been fitted to the data points. Also, the reported wavelengths of the emission maximum, \(\lambda_{\text{max}}\), in the different solvents are plotted as a function of \(\varepsilon\) in Figure 2.6. It is clear that a significant enhancement of emission intensity is observed, accompanied by a blue shift of the maximum emission intensity, as the solvent polarity decreases.

p-Aminobenzamidinium chloride is, like benzamidinium chloride, a competitive inhibitor for serine proteinases; fluorescence titration yielded a binding constant to trypsin of 1.6 \(\times\) 10\(^5\) M\(^{-1}\) at 25 °C. p-Aminobenzamidinium chloride could be completely displaced by benzamidinium chloride, which was found to bind with \(K = 5.1 \times 10^4\) M\(^{-1}\); this value is well comparable to the value reported in Section 2.2. For trypsin a 50-fold fluorescence enhancement has been reported at 25 °C, which corresponds to a solvent with a dielectric constant of around 14 (Figure 2.5). Furthermore, the wavelength of the emission maximum was reported to be 362 nm, which corresponds to a solvent with a dielectric constant of around 17 (Figure 2.6).
Figure 2.5. The relative emission intensities I/I(H₂O) of the fluorescent probe p-aminobenzamidinium chloride in different solvents (with emission intensity I) relative to water (with emission intensity I(H₂O)) at 22 °C versus the dielectric constant ε of the solvent; to guide the eye, a second order exponential decay has been fitted to the data points.

Figure 2.6. λ_max of the fluorescent probe p-aminobenzamidinium chloride in different solvents at 22 °C versus the dielectric constant ε of the solvent.
In conclusion, in trypsin solution the behaviour of the fluorescent probe p-aminobenzamidinium chloride is the same as that in a solvent with a polarity comparable to that of cyclohexanol ($\epsilon = 15$), indicating that the active site of trypsin is rather apolar in comparison to the bulk water surrounding the enzyme. This behaviour is consistent with values for the dielectric constant for enzymes reported in literature, which are, depending on the specific site studied, in the range of 2-30. For trypsin, a value of 12 has been calculated for the surroundings of both the oxyanion site and the site of His57. A solvent that approximates this polarity would therefore be most appropriate. However, sodium benzoate is poorly soluble in apolar solvents as cyclohexanol ($\epsilon = 15$) and even acetone ($\epsilon = 20.5$). Instead, DMSO ($\epsilon = 46.7$) and ethanol ($\epsilon = 24.3$) were used.

2.5.1 Aprotic Solvents

A $^1$H-NMR study on the benzamidinium-benzoate salt bridge in DMSO-$d_6$ by Papoutsakis et al. showed a substantial (>3 ppm) downfield shift of the amidinium protons involved in hydrogen bonding ($NH_{\parallel}$) to carboxylate upon addition of tetrabutylammonium benzoate to 5.2 mM benzamidinium chloride, whereas the amidinium protons external to the salt bridge ($NH_{\perp}$) are relatively insensitive (<0.5 ppm). This behaviour is a signature of salt-bridge formation.

For the binding of benzamidinium chloride to sodium benzoate at 25 °C in DMSO, the following thermodynamic parameters were found: $K = 5.6 \times 10^3$ M$^{-1}$, $\Delta G = -21.4$ kJ mol$^{-1}$, $\Delta H = -11.0$ kJ mol$^{-1}$, $T \Delta S = 10.4$ kJ mol$^{-1}$. The enthalpic and entropic contribution to the Gibbs energy of binding are of almost equal magnitude. The exceptionally high association of the salt bridge in DMSO is composed of the hydrogen-bonded interface, that directs the bond, and the reinforcing simple charge-charge electrostatic interaction between the negatively charged carboxylate and the positively charged amidinium. It is expected that the binding constant is even higher in more apolar solvents. Furthermore, DMSO is an extremely efficient hydrogen-bond acceptor, and therefore the desolvation of the amidinium group is expected to be unfavourable.

2.5.2 Protic Solvents

Next, an attempt was made to measure the binding in ethanol ($\epsilon = 24.3$). To this enthalpogram, no reliable curve could be fitted, but it was clear that the binding constant was small and that the enthalpy of binding was large and endothermic. The binding is entropy-driven due to the protic ethanol being a competitive solvent for the hydrogen bonds that are formed between benzamidinium and benzoate. This is consistent with an $^1$H-NMR study on the binding of dicarboxylates to cleft-type diamidinium receptors. The reported binding constants in the more competitive solvent CD$_3$OD are around 20 times lower than those in CD$_3$CN ($\epsilon = 36.0$)/CD$_3$OD ($\epsilon = 32.6$) 4:1. Also, higher-order associations are suppressed in CD$_3$OD. Binding was found to be entropy-driven in CD$_3$OD, which was explained by considering the solvation of the free and complexed states. In the unbound state, both the receptor and the guest are strongly solvated by
CD$_3$OD molecules that, upon complexation, are released from the solvation shell of the ionic groups into the bulk CD$_3$OD. This is an entropically favourable, but enthalpically unfavourable process.

When attempts were made to measure the strength of the salt bridge in water ($\varepsilon = 78.4$), upon titration of benzamidinium in aqueous benzoate solution, only peaks of dilution were found. This is consistent with titrations of sulphate ions into guanidinium receptors at 303 K. If performed in methanol, a binding constant of $2.7 \times 10^6$ M$^{-1}$ and an enthalpy of binding of $+22.84$ kJ mol$^{-1}$ was obtained. When these titrations were performed in aqueous solution, the heat effects were vanishingly small, and no titration curve could be derived; $^1$H-NMR spectroscopy yielded a binding constant of 84 M$^{-1}$ at 298 K in D$_2$O.

### 2.6 Conclusions

The thermodynamics of binding of benzamidinium chloride and structurally related compounds to trypsin has been studied at different temperatures using isothermal titration calorimetry. For those compounds measured over a range of temperatures, the observed thermodynamics of binding were characteristic of the hydrophobic effect: a large, negative $\Delta C_p$ and, as a consequence, enthalpy-entropy compensation with temperature. The influence of the amidinium group on the binding process has been probed by considering benzylammonium chloride. The lowered binding affinity of benzylammonium chloride is due to a less favourable enthalpy of binding that is partly compensated by a more favourable entropy of binding. This is in accord with the less extensive hydrogen-bond interactions observed in the benzylammonium-trypsin complex as compared to the benzamidinium-trypsin complex (Section 2.1). If the amidinium group is even further distorted, as in $\alpha$-methylbenzylamine and benzamide, the binding affinity is negligible.

The influence of the phenyl ring on the binding process has been probed by considering cyclohexylcarboxamidinium chloride and acetamidinium chloride. The lowered binding affinity of cyclohexylcarboxamidinium chloride is due to a less favourable entropy of binding and a similar enthalpy of binding. This is probably due to the restricted conformational freedom of the cyclohexyl ring in the binding pocket. Acetamidinium chloride, with its reduced hydrophobic area, is an inefficient inhibitor.

Furthermore, the benzamidinium-Asp189 hydrogen bonds have been modelled by considering benzamidinium-benzoate binding in solvents that approximate the dielectric constant of the binding pocket. Benzamidinium-benzoate binding in an apolar, aprotic solvent yields thermodynamics similar to that for benzamidinium-trypsin binding. The polar, protic water is too competitive a solvent for benzamidinium and benzoate for binding to occur. This is still, but to a lesser extent, the case in the less polar, but protic ethanol, where binding is entropy-driven.

These experiments point out that both the hydrogen-bond donating amidinium group and the hydrophobic phenyl ring of the benzamidinium molecule contribute considerably to the binding potency of the benzamidinium molecule to trypsin. Interactions of the amidinium group are
primarily enthalpically favourable, whereas the interactions of the phenyl ring are primarily entropically favourable.

2.7 Experimental Section

General remarks.
Bovine pancreatic trypsin was obtained from Fluka. The inhibitors were of the highest purity available and purchased from Aldrich (benzylamine hydrochloride, α-methylbenzylamine, benzamide), Fluka (acetamidine hydrochloride) and Sigma (benzamidine hydrochloride). Sodium benzoate was obtained from Merck. Starting materials for the synthesis were from Aldrich (cyclohexylcarbonitrile, 2M solution of trimethylaluminium in toluene). 1H-NMR spectra were recorded on Varian Gemini 200 (200 MHz) and VRX 300 (300 MHz) spectrometers. Elemental analyses were performed in the analytical department of our laboratory by Mr. E. Brussee.

Synthesis of cyclohexylcarboxamidinium chloride.
Cyclohexylcarboxamidinium chloride was synthesised as outlined in Scheme 2.3. An adapted literature procedure was used. A 2M solution of Me3Al in toluene (15.3 ml, 30.5 mmole) was slowly added to a magnetically stirred suspension of 1.8 g (33 mmole) of NH4Cl in 14 ml toluene (suspension dried by azeotropic distillation to 14 ml of 27 ml of toluene under a N2 atmosphere) at 0 °C under a N2 atmosphere. After the addition, the mixture was warmed to 25 °C and stirred until the gas evolution had ceased. 2.0 g (18 mmole) of cyclohexylcarbonitrile was added and the solution was heated to 80 °C under a nitrogen atmosphere until TLC indicated the absence of nitrile. The reaction mixture was slowly poured into a slurry of 10 g of silica gel in 30 ml of chloroform and stirred for 5 min. Next, 25 ml methanol was added and the suspension was stirred for two hours. The silica was filtered and washed with methanol. The filtrate and wash were combined and stripped to a residue of 17 ml, which was filtered to remove the precipitated NH4Cl and the solvent of the filtrate was evaporated.

Scheme 2.3. Synthesis of cyclohexylcarboxamidinium chloride.
The crude product was purified by stirring overnight in 10 ml i-propanol, which was filtered to remove the precipitated NH$_4$Cl and the solvent of the filtrate was evaporated. To remove organic contaminations, the product was dissolved in as little as possible i-propanol and titrated in 300 ml of ether, from which the precipitate was filtered off; this procedure was performed twice. The product was washed with ether and hexane and subsequently freeze-dried from 1M hydrochloric acid to yield 1.8 g (60%) of cyclohexylcarboxamidinium chloride,\textsuperscript{54} m.p. 181-182 °C (dec., lit.\textsuperscript{54} 184-185 °C). \textsuperscript{1}H-NMR (300 MHz, DMSO, ppm): 8.92 (s, broad, 2H), 8.81 (s, broad, 2H), 2.44 (t, 1H, J = 12.3 Hz), 1.78-1.10 (m, 10H). Elemental analysis: calc: C 51.69% H 9.29% N 17.22% found: C 51.63% H 9.43% N 17.23%.

\textbf{Preparation of trypsin solutions.}
Trypsin solutions were freshly prepared in the desired buffer and dialysed overnight at 4 °C before titration. Experiments were performed in Hepes, Bicine, Tricine, Glycyglycine or Tris (all 50mM, 10 mM CaCl$_2$, pH 8.0) buffer. Alternatively, enzyme solutions have been prepared at pH 3 in order to prevent possible autoproteolysis during the time before calcium stabilises the trypsin.\textsuperscript{55,56} After half an hour, the solutions were brought to pH 8.0 by adding sodium hydroxide solution and dialysed. Both thermodynamic and concentration measurement results were identical within error limits for the two different preparation methods, indicating that at 25 °C autoproteolysis is not significant in the preparation process. Trypsin concentrations were determined by measuring the optical absorbance at 280 nm using an extinction coefficient of 1.54\textsuperscript{55} for a 1 mg ml$^{-1}$ solution and a molecular weight of 24.0 kD.\textsuperscript{57}

\textbf{Isothermal titration calorimetry.}
Titration experiments were performed using either an Omega Isothermal Titration Calorimeter (Microcal, Inc., Northampton, MA) coupled to a nanovolt preamplifier in order to improve the signal to noise ratio or a MCS Isothermal Titration Calorimeter (Microcal, Inc., Northampton, MA). Both machines were connected to a waterbath for temperature control. The instruments were calibrated using standard electrical pulses. After a stable baseline (rms noise < 0.0050) was achieved, the inhibitor solution was titrated into the stirred (350 rpm) cell (1.3249 or 1.3496 ml, respectively) containing trypsin solution. Both solutions were degassed (ca. 10 min.) prior to use. The injection sequence consisted of a initial injection of 1 µl to prevent artefacts arising from the filling of the syringe (not used in data fitting), followed by injections of 5 µl each at 300 second intervals until complete saturation of the enzyme binding sites was reached. To correct for the heat of dilution and mixing, blank titrations of inhibitor into buffer were subtracted from the inhibitor-enzyme titration. Data were analysed using Origin Software (Microcal, Inc.) assuming a single site binding.\textsuperscript{17} This yielded $\Delta H$ (enthalpy of binding) and $K$ (binding constant). Measurements were repeated at least three times; $K$ was reproducible to within 10%, $\Delta H$ was reproducible to within 5% and the errors in $\Delta C_p$ are within 5%.
2.8 References and Notes


(16) The low temperature factor (~ 20Å²) is another indication that only one single conformation of the benzylammonium ion is present in the binding pocket. Interestingly, molecular dynamics simulations on the benzylammonium-trypsin complex showed two different conformations of benzylammonium in the pocket. One exactly matches the electron density map of the benzylammonium-trypsin crystals and the other forms two hydrogen bonds with the carbonyl oxygen of Ser190 and Asp189 OG2.
In fact, the intrinsic enthalpy of binding consists of contributions from both the noncovalent interactions involved in the binding process and heats from conformational changes upon binding. The latter, however, is not considered to be important for trypsin, since no significant conformational changes occur upon inhibitor binding.
Since the $pK_a$ of benzylammonium is 9.5, at pH 8.0, the pH at which the measurements are performed, 3.1% of the ammonium group is deprotonated, which is not considered significant. The percentage was calculated using the Henderson-Hasselbalch-equation (Fersht, A. Structure and Mechanism in Protein Science; Freeman: New York, 1999.):

$$\text{pH} = pK_a + \log \frac{[B]}{[BH^+]}$$

where $[B]$ is the concentration of deprotonated benzylammonium and $[BH^+]$ is the concentration of benzylammonium.


The same authors reported a binding constant of $2.86 \times 10^4$ for benzamidinium at 25 °C, which is also slightly lower than the value reported in Table 2.1.


The spectrum of pure benzamidinium chloride shows one joint peak for both types of amidinium protons ($NH_1$ and $NH_\perp$).


