Understanding enzymic binding affinity
Talhout, Reinskje

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CHAPTER 3

Binding of Benzamidinium Chloride Inhibitors to Trypsin: Influence of the Electronic Properties of p-Substituents

The thermodynamics of binding of a series of p-substituted benzamidinium chloride inhibitors to the serine proteinase trypsin has been studied using isothermal titration calorimetry. The inhibitors differ in the electronic properties of the substituent. For each inhibitor, the heat capacity change upon binding is negative and the Gibbs energy of binding is relatively constant in the temperature range studied. This is thermodynamic behaviour characteristic of hydrophobic interactions. Furthermore, upon changing the substituent, the Gibbs energy of binding remains relatively constant in comparison to the changes in its enthalpic and entropic contributions. In fact, these contributions are linearly dependent. The relative magnitudes of the binding constants depend on the electronic properties of the substituent: the binding affinity is enhanced by electron-donating substituents and decreased by electron-withdrawing substituents, whereas an opposite trend would be expected for a binding governed by the acidity of the amidinium group. This trend is most likely due to changes in the hydrophobicity of the phenyl ring and a bulk solvation effect: more polar inhibitors are more stabilised in water. Additional interactions of the substituent with the enzyme, caused by for example the steric bulk of the substituent, also contribute significantly to the relative binding affinity.

“TO MY MIND A PARTICULARLY HAPPY ASPECT OF THE EXISTENCE OF LINEAR FREE ENERGY RELATIONSHIPS HAS BEEN THE PROOF IT SUPPLIES THAT ONE NEED NOT SUPPOSE THAT THE BEHAVIOR OF NATURE IS HOPELESSLY COMPLICATED MERELY BECAUSE ONE CANNOT FIND A THEORETICAL REASON FOR SUPPOSING IT TO BE OTHERWISE.”

3.1 Introduction

The benzamidinium ion is a potent competitive inhibitor of trypsin.² It has been shown that ring substituents alter the affinity of the inhibitor;²⁻⁴ for example, p-aminobenzamidinium is a more efficient inhibitor than benzamidinium, whereas p-nitrobenzamidinium is less efficient. In the crystal structure of the benzamidinium-trypsin complex, the amidinium group is rotated out of the plane of the phenyl ring by an angle of 7°.⁵,⁶ This angle is much smaller than the angle of 36.6° reported⁷ for the crystal structure of solid benzamidinium chloride, which is supposed to be large enough to prevent conjugation between the two π systems. Therefore, in inhibitor-enzyme binding, resonance interaction between the amidinium groups and the phenyl ring is likely. Strong electron withdrawal by electron-attracting substituents, such as the amidinium group, can decrease the hydrophobicity of neighbouring parts of a molecule. As the phenyl ring is mainly surrounded by nonpolar and neutral polar α-amino acid residues and in van der Waals contact with the residues 214-216 and 190-191 (Section 2.1), this is expected to be unfavourable.

A substituent at the p-position is able to influence both the amidinium and the phenyl ring via conjugation. It is known that the ionisation constant of p-substituted benzamidiniums is sensitive to the electronic effect of substituents; the reported pKₐ-values range from 10.14 (NO₂) to 12.69 (NH₂).⁸ The ¹H-NMR study on the benzamidinium-benzoate salt bridge in [D6]DMSO by Papoutsakis et al.,⁹ described in Section 2.5.1, also reports on the binding constants of p-substituted benzamidiniums to benzoate. In Figure 3.1, adapted from this study, these log $\frac{K}{K_H}$ values are plotted against the Hammett $\sigma_p$ parameter. From the almost perfect linearity ($r = 0.9997$) of the fit it can be concluded that the strength of the salt bridge directly correlates with the electronic properties of the p-substituent on the benzamidinium. This linear free energy relation with $\rho$ equal to 1.42 indicates that association is governed by the acidity of the amidinium group, which is dictated by the electron-accepting capacities of the substituent at the p-position.

In this study,¹ the thermodynamics of binding of five p-substituted benzamidinium chlorides, p-aminobenzamidinium chloride, p-methylbenzamidinium chloride, p-methoxybenzamidinium chloride, p-amidinobenzamidinium chloride, and p-bromobenzamidinium chloride, to trypsin has been examined by means of isothermal titration calorimetry (ITC). The differences in binding affinities are explained by considering the electronic (inductive and resonance) and additional properties of the substituent.
3.2 Thermodynamics of Binding of \( p \)-Substituted Benzamidinium Chlorides to Trypsin

The thermodynamics of binding of five \( p \)-substituted benzamidinium chlorides to trypsin has been studied using isothermal titration calorimetry. Table 3.1 lists the thermodynamic parameters for binding of these inhibitors, together with the parent compound benzamidinium chloride. In Figures 3.2A and 3.2B, the thermodynamic parameters \( \Delta G \), \( \Delta H \) and \( T \Delta S \) for all inhibitors are depicted at, respectively, 25 and 37 °C.
Table 3.1. Thermodynamic parameters of binding of p-substituted benzamidinium chlorides to trypsin in Tris pH 8.0 at different temperatures.

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<tr>
<th>R</th>
<th>T (°C)</th>
<th>$K \times 10^4$ 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>
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<tr>
<td>H$^b$</td>
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<td>3.0</td>
<td>-26.6</td>
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</tbody>
</table>

$^a$ Substituent at p-position. $^b$ From Table 2.1.
Figure 3.2. $\Delta G$, $\Delta H$ and $T\Delta S$ of binding of p-substituted benzamidinium chlorides at (A) 25 °C and (B) 37 °C in Tris pH 8.0.
Table 3.2. Heat capacity change, $T_S$ and $T_H$ for binding of $p$-substituted benzanidinium chlorides to trypsin in Tris pH 8.0.

<table>
<thead>
<tr>
<th>$R^a$</th>
<th>$\Delta C_p$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$T_S$ (°C)</th>
<th>$T_H$ (°C)</th>
</tr>
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<td>-23</td>
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<td>-19</td>
</tr>
<tr>
<td>OCH$_3$</td>
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<td>-13</td>
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<tr>
<td>CONH$_2$</td>
<td>-230</td>
<td>75</td>
<td>-26</td>
</tr>
<tr>
<td>Br</td>
<td>-600</td>
<td>37</td>
<td>-7.7</td>
</tr>
</tbody>
</table>

$^a$ Substituent at $p$-position. $^b$ Taken from Chapter 2.

For all inhibitors $\Delta G$ remains relatively constant at both temperatures, while $\Delta H$ and $T\Delta S$ vary with temperature in a compensating manner. This enthalpy-entropy compensation upon varying the temperature is characteristic for processes with a negative $\Delta C_p$. $^{10-12}$ $T_S$ and $T_H$, the temperatures at which respectively the entropy and enthalpy of binding equal zero, together with $\Delta C_p$, the change in heat capacity upon binding, are listed in Table 3.2. This thermodynamic behaviour is one of the main fingerprints indicating that hydrophobic interactions are involved in biological or chemical processes (Sections 1.6.1, 2.2.2). The main contribution to a negative value of $\Delta C_p$ stems from the burial of nonpolar surface area from water, whereas the removal of polar surfaces from the aqueous phase tends to increase $\Delta C_p$. In order to explain the difference in magnitude of $\Delta C_p$ for the different inhibitors, their hydrophobicity will be considered in Section 3.2.3.

Also, for the different inhibitors with respect to each other, $\Delta H$ and $T\Delta S$ vary to a larger extent than $\Delta G$. In other words, there is enthalpy-entropy compensation upon varying the substituent, which leads to $\Delta G$ being relatively constant in comparison to the changes in $\Delta H$ and $T\Delta S$ (Section 1.1.7) Here, we encounter an example of strong compensation: the correlation between $\Delta H$ and $\Delta S$ is a linear relationship (Figure 3.3) with a correlation coefficient of 0.9841 when CH$_3$ is included in the fit and 0.9991 when it is excluded. The compensation temperature, equal to the slope of the line, amounts to 172 (40) °C with CH$_3$ included in the fit, and 187 (10) °C with CH$_3$ excluded. These temperatures are well outside the range of experimental temperatures, ranging from 20 to 37 °C, which excludes$^{13}$ the possibility that this compensation temperature is an artefact due to highly correlated errors in $\Delta H$ and $\Delta S$. Furthermore, data plotted in the $\Delta H$-$\Delta G$ plane (not shown) with errors propagating in a random manner, also show a linear trend. At the compensation temperature, $\Delta G$ is equal to the abscissa of the line, $\Delta H_0$, which is equal to $-30$ (1) kJ mol$^{-1}$ with CH$_3$ included, and - $30.5$ (0.3) with CH$_3$ excluded. It is indeed shown, as anticipated in Section 1.1.7, that in the temperature range measured, which is well below the compensation temperature, a higher binding affinity can only be achieved by a more favourable enthalpy of binding and not by a more favourable entropy of binding.
The substituent influences the binding constants, which decrease in the order $K_{\text{NH}_2} > K_{\text{CH}_3} > K_{\text{H}} > K_{\text{Br}} > K_{\text{OCH}_3} > K_{\text{CONH}_2}$. This trend is illustrated by the Hammett plot at 25 °C depicted in Figure 3.4, where the value of $\log K/K_H$ is plotted against $\sigma_{p^+}$, the substituent constant that is used in case of direct resonance interaction of the substituent with an electron-poor reaction centre. The same trend is observed when a Hammett plot is made for the binding constants obtained by Markwardt et al. in a slightly different buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl) by measuring the inhibition of the hydrolysis of $N$-α-benzoyl-DL-arginine-4-nitroanilide by trypsin. Furthermore, a similar trend can also be observed using their data for plasmin and thrombin, although the ratios $K_{\text{trypsin}}/K_{\text{plasmin}}$ and $K_{\text{trypsin}}/K_{\text{thrombin}}$ do differ for the different substituents. This is due to the fact that the reaction centres of these enzymes are similar but not identical.

Since the data points for OCH$_3$ and CONH$_2$ are not in line with the trend of the other data points, two different linear fits have been performed. The solid line is obtained with the data points for OCH$_3$ and CONH$_2$ excluded, the dotted line with these points included. The slope is equal to $\rho$, the reaction constant, for which the negative value indicates that the process benefits from electron donating substituents. In both cases, the same slope, -0.31, is obtained.
It is therefore clear that electron-donating substituents enhance the binding constant, whereas electron-withdrawing substituents decrease it. There are several factors that could explain this trend. In Chapter 2, it was concluded that both the amidinium group and the phenyl group are important in the binding of benzamidinium to trypsin. The \( p \)-substituents might influence both groups via (direct) resonance interaction. However, if the electronic properties of the substituent would be the only factor determining the binding of benzamidinium derivatives to trypsin, a perfect correlation would be expected for the Hammett plot. However, the correlation is decent (\( r = -0.98 \)) when the data points for OCH\(_3\) and CONH\(_2\) are excluded, but only poor (\( r = -0.64 \)) when these data points are included. Therefore, additional interactions must play a role in the binding process. Both types of interactions, electronic (inductive and resonance) and additional, will be discussed.

### 3.2.1 Electronic Effects of the \( p \)-Substituent

First, the influence of the electronic (inductive and resonance) properties of the \( p \)-substituent on the amidinium group will be considered. In Section 3.1, it has been discussed that for the binding of \( p \)-substituted benzamidiniums to benzoate a perfect linear free energy relation was obtained with \( \rho \)}
equal to 1.42. This indicates that association is governed by the acidity of the amidinium group, which is dictated by the electron-withdrawing capacities of the substituent on the \( p \)-position. Interestingly, the effect of the substituents on the acidity of the amidinium group seems to play no role or is outweighed by other effects in the binding of \( p \)-substituted benzamidinium chlorides to trypsin, since \( \rho \) is of opposite sign.

Next, the effect of the electronic properties of the \( p \)-substituent on the hydrophobicity of the phenyl ring will be considered. Strong electron withdrawal by electron-attracting substituents can decrease the hydrophobicity of neighbouring parts of a molecule. Hence, it is conceivable that reduction in hydrophobicity by the strong electron-attracting amidinium group could be restored, at least in part, by strong electron-releasing groups at the \( p \)-position of the benzene ring.

In Figure 3.5, the chemical shift of the \( m \)-protons of the \( p \)-substituted benzamidinium chlorides (ortho to the \( p \)-substituent, DMSO-solutions) is plotted against \( \sigma_p^+ \). This is an indicator for the electronic effects of the substituent on the hydrogens in the phenyl ring. The linear fit to the data points yields a good correlation (\( r = 0.997 \)), which indicates that the electron density in the phenyl ring directly correlates with the electron-donating capacity of the substituent and that steric effects of the substituent on its \( o \)-hydrogen are not prominent. The electron density in the phenyl ring of benzamidinium is slightly decreased in comparison to benzene (the chemical shift of the \( m \)-proton in benzamidinium is 7.62, whereas that of a proton in benzene is 7.26)\(^{15}\). This effect can be compensated for by electron-donating substituents, and be enhanced by electron-withdrawing substituents. The phenyl ring in the benzamidinium-trypsin complex is surrounded by mainly nonpolar and neutral polar \( \alpha \)-amino acid residues, of which the residues 214-216 and 190-191 are in van der Waals contact (Section 2.1). The atoms in van der Waals contact are all carbon, and we therefore propose that the interactions between the phenyl ring and these carbons are more favourable in case of electron-donating substituents.

Another explanation for the substituent effect can be found in the polarity (expressed in the overall dipole moment of the molecule) of the inhibitor. The inhibitors with an electron-withdrawing substituent are more polar than those with an electron-donating substituent. On purely continuum electrostatic grounds, the Gibbs energy of hydration of a molecule should become more negative as its total dipole increases.\(^{16}\) This effect is far more pronounced in bulk water, with a dielectric constant of approximately 80, than at the binding site of the enzyme, with a dielectric constant that will be in the range of 2-30.\(^{16}\) Therefore, a more polar molecule will be preferentially stabilised in water, and will be a less efficient inhibitor than a less polar molecule. This was corroborated by quantum chemical calculations\(^{17}\) on the interaction energy of \( p \)-substituted benzamidinium ions to trypsin. The hydration energy of the free inhibitor was considered in terms of a simple model of the first hydration shell. A linear relation was found between the experimental Gibbs energy of binding and the hydration energy: the more favourable the hydration energy, the less favourable the Gibbs energy of binding.
3.2.2 Additional Effects of the $p$-Substituent

Here, the influence of properties of the $p$-substituent other than inductive and resonance effects will be considered. Plotting $\log K/K_H$ against $\Delta \log P$, the relative $n$-octanol-water partition coefficient of a substituent with respect to hydrogen,\textsuperscript{18,19} did not show any correlation (Figure 3.6). We therefore contend that the hydrophobicity of the substituent of this class of inhibitors does not play a dominant role in the binding to trypsin.

Another possible additional interaction, which was encountered in Molecular Dynamics calculations on the $p$-aminobenzamidinium-trypsin complex, involves an interaction between one of the hydrogens of the amino group and the oxygen of the Ser195 residue of trypsin.\textsuperscript{16} In this case, the hydrogen bonds of N2 of the amidinium group to the enzyme, present in benzamidinium (Section 2.1), are absent.

Furthermore, $p$-substituents are close to the hydroxy group of Ser195 of the catalytic triad, which, for larger substituents, may lead to steric interactions. This could well be the reason for OCH$_3$ and CONH$_2$ not to be in line with the trend for the other, smaller inhibitors that do not (or much less) encounter these steric interactions. In an attempt to account for this steric effect, the difference in the $\log K/K_H$ values of $p$-ethyl- and $p$-methylbenzamidinium chloride will be considered.
p-Ethylbenzamidinium chloride has the same $\sigma_{p^+}$-value as $p$-methylbenzamidinium chloride, but a much smaller value of $\log K/\K_{Hi}$ -0.19\(^{20}\) ($p$-methylbenzamidinium chloride: 0.19). As the electronic influence of the substituent on the binding constant is the same, we assume that the difference between their $\log K/\K_{Hi}$ values (0.38) is due to steric interactions. A set of steric parameters that is useful for intermolecular interactions was developed by Verloop and co-workers.\(^{18}\) This set consists of the parameters L, $B_1$ and $B_5$ that are a measure for, respectively, the length, the smallest width and the maximum width of a substituent. Since these parameters are very similar for the ethyl, the methoxy and the amidino group,\(^{19}\) it is possible to correct the data points for OCH$_3$ and CONH$_2$ in Figure 3.4 by adding the difference (0.38) between the $\log K/\K_{Hi}$ values of $p$-ethylbenzamidinium chloride and $p$-methylbenzamidinium chloride. This approach yields the data points indicated with the symbol □ (Figure 3.4), which are now much better in line with the trend for the other inhibitors.

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**Figure 3.6.** $\log K/\K_{Hi}$ values of $p$-substituted benzamidinium chlorides to trypsin at 25 °C in Tris pH 8.0 versus $\Delta\log P$,\(^{18,19}\) the change in the n-octanol-water partition coefficient upon introducing a substituent.

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3.2.3 Magnitude of the Heat Capacity change upon Binding

Based on the discussion of the effects of the substituents on the binding process, we will now try to explain the differences in magnitude of the $\Delta C_p$ values for the different inhibitors. Two factors will be considered: the hydrophobicity of the substituent (as expressed in their $\Delta \log P$, Section 3.2.2) and the influence of the substituent on the hydrophobicity of the phenyl ring (as expressed in their $\sigma_p^+$, Section 3.2.1). Linear fits were obtained for $\Delta C_p$ as a function of $\Delta \log P$ and as a function of $\sigma_p^+$. Also, a function was fitted in which $\Delta C_p$ is dependent on both $\Delta \log P$ and $\sigma_p^+$. In Figure 3.7, the experimental $\Delta C_p$ is depicted for all inhibitors together with $\Delta C_p$ calculated from each of these three functions. The trend for the dependence of $\Delta C_p$ on $\Delta \log P$ is, as expected, that polar substituents tend to decrease $|\Delta C_p|$, whereas nonpolar substituents tend to increase it. However, the experimental values of NH$_2$, and, to a lesser extent, CH$_3$, are not in line with this trend. The electronic properties of the substituents influencing the hydrophobicity of the phenyl ring could be the reason for this deviation. For that relation, the trend is, as expected, that electron donating substituents increase $|\Delta C_p|$, whereas electron-withdrawing substituents decrease it. However, in this case, the experimental values of CONH$_2$ and Br are not in line with the trend. For a function in which $\Delta C_p$ is dependent on both $\Delta \log P$ and $\sigma_p^+$, the calculated value agrees reasonably well with the experimental value of $\Delta C_p$. All calculated values are within 10 percent of the experimental value, except for Br, for which the calculated value is 20 percent less negative than the experimental value.

![Figure 3.7. Experimental and calculated heat capacity changes upon binding for p-substituted benzamidinium chlorides to trypsin.](image-url)
3.3 Conclusions

The thermodynamics of binding to trypsin of five \( p \)-substituted benzamidinium chlorides that differ in the electronic properties of their substituent has been studied using isothermal titration calorimetry. The thermodynamics for inhibitor binding are characteristic of hydrophobic interactions. Enthalpy-entropy compensation upon varying the substituent leads to \( \Delta G \) being relatively constant in comparison to the changes in \( \Delta H \) and \( T\Delta S \). The relative magnitudes of the binding constants of the inhibitors are mainly determined by the electronic properties of the substituent: electron-donating substituents enhance the binding affinity, whereas electron-withdrawing substituents decrease it. An opposite trend would be expected for a binding governed by the acidity of the amidinium group. Several possible explanations of this negative \( \rho \)-value have been considered. We propose that electron-donating substituents at the \( p \)-position enhance the binding affinity due to: a) an enhanced hydrophobicity of the phenyl ring and b) a bulk solvation effect. Furthermore, it is clear that additional interactions of the substituent with the enzyme contribute to the binding affinity. In Chapter 4, the importance of these additional effects on the binding of \( p \)-substituted benzamidinium chlorides to trypsin will be highlighted.

3.4 Experimental Section

General remarks.
Bovine pancreatic trypsin was obtained from Fluka. The inhibitors were of the highest purity available and purchased from Aldrich (\( p \)-amidinobenzamidinium hydrochloride), Avocado Research Chemicals (\( p \)-methylbenzamidinium hydrochloride) and Fluka (\( p \)-methoxybenzamidinium hydrochloride and \( p \)-aminobenzamidinium dihydrochloride). Trypsin solutions were prepared as described in Section 2.7. Starting materials for the synthesis were from Aldrich (\( p \)-bromobenzonitrile and 2M solution of trimethylaluminium in toluene). \(^1\)H-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. Harm Draaijer, Mr. Jan Ebels and Mr. Jannes Hommes.

Isothermal titration calorimetry.
Titration experiments were performed as described in Section 2.7.

\( p \)-Bromobenzamidinium chloride.
\( p \)-Bromobenzamidinium chloride\(^{21}\) was synthesised analogously to cyclohexylcarboxamidinium chloride (Section 2.7) using \( p \)-bromobenzonitrile. To the crude product, 10 ml of methanol saturated with HCl was added, followed by 100 ml of ether. The precipitate was filtered off and 40 ml of 4:1 \( i \)-propanol:acetone was added. This suspension was stirred overnight and filtered to remove the precipitated \( \text{NH}_4\text{Cl} \). The filtrate was concentrated to 10 ml, 100 ml of ether was added and the white
precipitate was filtered off. The crude product was purified by sublimation (2mm Hg, 200 °C), washed with aqueous HCl (pH 2) and dried under reduced pressure over P₂O₅ to yield 0.41 g (1.74 mmol, 18%) of p-bromobenzamidinium chloride, m.p. 268-269 °C (dec., sublimation around 220 °C). ¹H-NMR (200 MHz, DMSO, ppm): 9.16 (s, broad, 4H), 7.84 (d, 2H, J = 8.2 Hz), 7.75 (d, 2H, J = 7.8 Hz). Elemental analysis: calc: C 35.70% H 3.42% N 11.89% Cl 15.05%, found: C 35.46% H 3.62% N 11.66% Cl 15.26%.

3.5 References and Notes


(20) The binding constant to trypsin at 25 °C amounts to $2.9 \times 10^4$ M$^{-1}$ for $p$-ethylbenzamidinium chloride; the other thermodynamic parameters for this compound are reported in Chapter 4.
