Summary

Understanding enzymic binding affinity is of fundamental scientific importance as well as a prerequisite for structure-based drug design. In this study, the interactions of the serine proteinase trypsin with several artificial, benzamidinium-based inhibitors have been studied in aqueous solutions. In order to evaluate the contributions to binding of a specific part of the inhibitor, the influence of small structural variations in that part was studied. The relatively small variations employed in this study are quite unique compared to other studied in this field, where the often large structural variations lead to difficulties in inferring the precise reason for changes in the thermodynamics of binding. In this study, the complete thermodynamics of binding were obtained using isothermal titration calorimetry over a range of temperatures, which is also an advantage in comparison to studies were only the binding constant at one specific temperature has been assessed. Since the Gibbs energy of binding, unlike the enthalpy and entropy of binding, is often relatively unaffected when a modest structural variation is introduced in the inhibitor, only reporting the Gibbs energy may lead to the erroneous conclusion that the structural variation does not affect the binding. Crystal structures and, in particular, molecular dynamics simulations performed by Dr. Alessandra Villa, have been most helpful in interpreting part of the thermodynamic data.

All inhibitors studied bind to trypsin with a negative heat capacity change and show enthalpy-entropy compensation upon varying the temperature, phenomena that are hallmarks of the hydrophobic effect. Enthalpy-entropy compensation also occurs upon varying the structure of the inhibitor. As a result, the Gibbs energy of binding fluctuates much less with perturbations of the system, with respect to both the temperature of the surroundings and the structure of the inhibitor, than the enthalpic and entropic contributions. This protects an enzymic system against environmental and mutational challenges. The specific influence of small structural changes on the binding thermodynamics will be summarised below.

Chapter 2 describes the thermodynamics of binding of the benzamidinium parent compound to trypsin. No protons are transferred upon binding at the standard pH of 8.0. At 25 °C both the enthalpy and the entropy of binding are favourable, but this is only true in a narrow temperature range. The contribution to binding of the amidinium group, which forms a hydrogen-bond reinforced salt bridge to Asp189 and hydrogen bonds to Ser190, Gly 219 and an internal water molecule, is primarily enthalpy-driven. The lowered binding affinity of benzylammonium chloride, which lacks one amino group relative to benzamidinium, is due to a less favourable enthalpy of binding. This is most probably due to the loss of hydrogen bonds, which is consistent with the crystal structure of the benzylammonium-trypsin complex. The amidinium-Asp189 salt bridge in the benzamidinium-trypsin complex has also been modelled by studying the interaction between benzamidinium and the benzoate anion in DMSO, ethanol and water. The contribution to binding of the phenyl ring, which binds in the hydrophobic S₁ pocket, is primarily entropy-driven. The lowered binding affinity of cyclohexylcarboxamidinium chloride, which is more hydrophobic, larger and more flexible than benzamidinium, is due to a less favourable entropy of binding. This most probably originates from the restricted conformational freedom of the cyclohexyl ring in the binding
Chapter 3 reports the influence on the binding thermodynamics of the inductive and resonance effects of the \( p \)-substituent of a series of \( p \)-substituted benzamidinium chloride inhibitors. Upon changing the \( p \)-substituent, an example of strong enthalpy-entropy compensation is encountered: the enthalpic and entropic contributions of the different substituents to binding are linearly dependent. Electron-donating substituents increase 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. The observed trend is most likely caused by substituent-induced changes in the hydrophobicity of the phenyl ring and/or the fact that more polar inhibitors are more stabilised in water (and thus have a lower tendency to enter the binding pocket). As will be discussed in more detail in Chapter 4, 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.

Chapter 4 discusses the influence of steric and hydrophobic effects of the \( p \)-substituent on the binding thermodynamics. The hydrophobic and steric effects on the binding were separated from the inductive and resonance effects by studying systematically varied \( p \)-alkylbenzamidinium chlorides, of which all alkyl groups have similar inductive and resonance effects. The binding affinity increases upon elongating a linear tail. This, together with the magnitudes of the enthalpic and entropic contributions to binding, the changes in heat capacity upon binding, and the calculated changes in solvent-accessible surface area upon binding, indicates the importance of hydrophobic interactions between the inhibitor and the enzyme. Molecular dynamics simulations show that the alkyl tail is oriented towards the hydrophobic S3/S4 pocket and, in case of longer tails, is partly buried in that pocket. However, the observed changes in binding Gibbs energy on extending the chain length of the substituent are small compared to what might be expected for a purely hydrophobic interaction based on the burial of nonpolar surface area. An unfavourable effect, related to the steric properties of the substituent, is opposing the hydrophobic interaction. \( p \)-\( t \)-Butylbenzamidinium, which was experimentally found not to bind but was calculated to have a significant binding affinity, is thought to be unable to access the binding pocket. For inhibitors that do bind, the binding affinity decreases with increased branching at the first carbon. Structural analysis showed that the decrease in binding affinity upon increasing the steric bulk on the first carbon of the substituent is not correlated with a substantial disturbance of the binding pocket and its surroundings. Rather, it is correlated with the dehydration of the protein, as will be discussed in Chapter 5.

Chapter 5 deals with the influence of the \( p \)-alkyl substituent on the dehydration of the catalytic triad. Buffer-dependent thermodynamic experiments show that a larger substituent induces a larger decrease in the fraction of protonated residues, most likely due to a \( pK_a \) shift of His57 N2. Binding of \( p \)-\( n \)-hexylbenzamidinium at pH 7.4 is accompanied by a transfer of protons from the enzyme to the buffer equal to the number of protons calculated to be transferred upon binding of a natural protein inhibitor, suggesting that this relatively small inhibitor is a satisfactory model for a
natural protein inhibitor of trypsin. The transfer of protons upon binding of \( p-n \)-hexylbenzamidinium was larger than that found upon binding of unsubstituted benzamidinium chloride at pH 7.4. Since this proton transfer most likely reflects the penalty of dehydrating His 57, these data seem to indicate that His57 is more shielded from water upon binding of \( p-n \)-hexylbenzamidinium chloride than upon binding of benzamidinium chloride. Molecular dynamics simulations corroborated that for more sterically demanding \( p \)-alkyl substituents, less water molecules are found in the surroundings of His57 and Ser195, and that a hydrogen bond between these two residues is present for a larger fraction of the time. The formation of this hydrogen bond by binding of the substrate activates the enzyme and, therefore, dehydration of the catalytic triad is necessary for catalysis to occur. Dehydration is, however, thermodynamically unfavourable, primarily due to an unfavourable contribution to the enthalpy of binding, and thus lowers the binding affinity.

Chapter 6 describes the synthesis and aggregation behaviour of \( p-n \)-alkylbenzamidinium chloride surfactants. In order to solubilise \( p-n \)-decylbenzamidinium chloride, a surfactant with a high Krafft temperature, it was mixed with both cationic and anionic cosurfactants. For mixtures with \( n \)-alkyltrimethylammonium chlorides, the aggregation process is enthalpically more favourable than for the pure \( n \)-alkyltrimethylammonium chlorides, which is probably caused by the charge delocalisation in the amidinium headgroup leading to diminished headgroup repulsion. For \( p-n \)-decylbenzamidinium chloride at 40 °C, a critical aggregation concentration (cac) between 3 and 4 mM has been estimated. This cac is around two times lower than that of similar surfactants without charge delocalisation in the headgroup and well comparable to that of similar surfactants with charge delocalisation in the headgroup. In mixtures of \( p-n \)-decylbenzamidinium chloride with either sodium \( n \)-alkylsulfates or sodium dodecylbenzenesulfonate, bilayer aggregates seem to be formed by the pseudo-double-tailed catanionic surfactants composed of \( p-n \)-decylbenzamidinium and the anionic surfactant. These aggregates are solubilised to mixed micelles by excess free anionic surfactant at the measured cac.

Chapter 7 reviews the most important conclusions of this thesis and puts them into perspective. Inhibitor-enzyme interactions are best studied when both the structure of the complex and the complete binding thermodynamics over an appreciable temperature range are known. Specific inhibitor-enzyme interactions such as hydrogen bonds, salt bridges and hydrophobic effects are important, but elementary factors like the accessibility of the binding pocket, the affinity of the inhibitor for bulk water and decreased degrees of freedom of the inhibitor in the pocket, should not be overlooked. Furthermore, bound water molecules must be explicitly taken into account. Suggestions for new research projects in the field of binding of benzamidinium-based inhibitors to trypsin are made.