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Probing the effect of the amidinium group and the phenyl ring on the thermodynamics of binding of benzamidinium chloride to trypsin

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The effect of the amidinium group and the phenyl ring on the thermodynamics of binding of benzamidinium chloride to the serine proteinase trypsin has been studied using isothermal titration calorimetry. Binding studies with benzylammonium chloride, α -methylbenzylammonium chloride and benzamide, compounds structurally related to benzamidinium chloride, showed that hydrogen bonding between the amidinium group and the enzyme is primarily enthalpy-driven. Binding of cyclohexylcarboxamidinium chloride and acetamidinium chloride showed that the hydrophobic binding of the phenyl ring in the S1 pocket is primarily entropy-driven and that a rigid, flat hydrophobic binding site for the inhibitor is favourable. The compounds that have been studied over a range of temperatures exhibit a negative change in heat capacity upon binding and enthalpy–entropy compensation, both characteristic of hydrophobic interactions.

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.¹ The serine proteinase 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 the 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 interactions.² Regarding the specificity requirements of the enzyme, it has been reported in 1965 that amidines are good model systems for the arginine side chain of trypsin substrates.³ Benzamidinium chloride and *p*-aminobenzamidinium chloride 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.

In this study, we investigated the influence of the amidinium group and the phenyl ring on the thermodynamics of binding of benzamidinium chloride to trypsin. To this purpose, the binding of the structurally related compounds benzylammonium chloride, α -methylbenzylammonium chloride, benzamide, cyclohexylcarboxamidinium chloride, and acetamidinium chloride, was examined by means of isothermal titration calorimetry (ITC).

Fig. 1a shows a cartoon representation of the crystal structure of the benzamidinium–trypsin complex.⁴ 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 Asp 189 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 Ser 190 OG (3.06 Å) and the internal water molecule 416 (3.16 Å). The phenyl ring is mainly surrounded by non-polar and neutral polar α -amino acid residues and is in van der Waals contact with the residues 214–216 and 190–191.⁵

(a)

(b)

Fig. 1 Cartoon representations of the crystal structures of the benzamidinium–trypsin complex⁴ (a), and the benzylammonium–trypsin complex⁷ (b) (3ptb and 2bza from the Brookhaven Protein Data Bank,²⁹ respectively) generated using the program RasMol.³⁰ Both inhibitors are bound in the S1 binding pocket, close to the catalytic triad (His57, Asp102, Ser195).

Just as amidinium ions are good structural analogues of the arginine side chain, alkyl ammonium ions are good model

Table 1 Thermodynamic parameters for binding of benzylammonium chloride, cyclohexylcarboxamidinium chloride, and benzamidinium chloride to trypsin in Tris buffer at pH 8.0 at different temperatures

Compound (chloride salt)	<i>T</i> /°C	<i>K</i> /10 ⁴ M ⁻¹	ΔG /kJ mol ⁻¹	ΔH /kJ mol ⁻¹	$T\Delta S$ /kJ mol ⁻¹
Benzylammonium	20.1	0.71	-21.6	-8.5	13.1
	25.1	0.55	-21.4	-9.9	11.5
	30.0	0.50	-21.5	-11.0	10.5
	37.1	0.42	-21.5	-12.9	8.6
Cyclohexylamidinium	20.1	0.42	-20.3	-17.0	3.3
	25.2	0.34	-20.2	-19.4	0.8
	30.1	0.32	-20.4	-21.5	-1.1
	37.0	0.28	-20.4	-24.8	-4.4
Benzamidinium ^a	20.1	6.2	-26.9	-17.0	9.9
	25.1	4.5	-26.6	-18.9	7.7
	30.0	4.1	-26.7	-21.4	5.4
	37.1	3.1	-26.7	-23.7	3.0

^aData taken from ref. 10.

systems for lysine.⁶ The benzylammonium ion 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 its aromatic and conformationally restricted character.

Concerning the binding to trypsin, 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 acceptor 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:⁷ the cartoon representation in Fig. 1(b) 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.⁸ The ammonium group 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 for 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 into one specific position.

Results and discussion

Effect 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 benzylammonium⁹ chloride to trypsin was studied. The thermodynamic parameters obtained in Tris buffer at pH 8.0 are listed in Table 1. The thermodynamics of binding of benzamidinium chloride to trypsin have been published before,¹⁰ and are, for the sake of comparison, listed in Table 1 as well.

At all temperatures studied, the binding constant of benzylammonium chloride to trypsin is more than six times lower than that of benzamidinium chloride (Table 1) and ΔG is between 4.7 and 5.0 kJ mol⁻¹ less favourable. This less favourable ΔG results from a more than two times less favourable ΔH that is partly compensated by a more favourable $T\Delta S$. 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 of inhibitor and enzyme and changes in con-

formational entropy.¹¹ 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. The more favourable entropy of binding is most probably due to enthalpy–entropy compensation, which is the phenomenon that small structural variations in an inhibitor often result in changes in the enthalpy and entropy of binding that are considerably larger than those in the Gibbs energy. Such enthalpy–entropy compensation is common when studying weak interactions and is important for understanding the nature of competing interactions.^{12–14} Compensation is the natural outcome of the fact that bound species will necessarily have less freedom of motion in a tighter complex.^{15,16} We therefore propose that benzylammonium is less extensively conformationally restrained in the complex due to the less tight binding.

Over a relatively narrow temperature range, the temperature dependence of ΔH is given by:

$$\delta\Delta H/\delta T = \Delta C_p$$

where ΔC_p is the heat capacity change upon binding. For benzylammonium chloride, the value of ΔC_p , determined from the slope of the linear fit to the data points, is equal to -254 J mol⁻¹ K⁻¹ (Table 2). A negative value of ΔC_p often correlates with the burial of non-polar surface area from water.^{13,17,18} By contrast, the removal of polar surfaces from the aqueous phase tends to increase ΔC_p . We contend that the ΔC_p of benzylammonium chloride is less negative than that of benzamidinium chloride (-400 J mol⁻¹ K⁻¹, Table 2) because of the fact that the ammonium group has a higher charge density than the amidinium group.¹ Since the removal of polar surfaces from the aqueous phase tends to increase ΔC_p , the less negative ΔC_p could be caused by the more polar ammonium group in the benzylammonium ion.

A negative ΔC_p results in a shift in the net thermodynamic driving force for association from being entropic to enthalpic with increasing temperature. Temperatures T_H and T_S can be defined, at which the enthalpic and the entropic contributions to binding are zero. Provided that ΔC_p is independent of temperature, together with T_H and T_S it gives a complete thermodynamic description of the binding process. T_H and T_S have been calculated from a linear fit to the temperature-dependencies of ΔH and $T\Delta S$ and are listed in Table 2.

ΔG is practically temperature-independent as a result of the compensating temperature-dependencies of ΔH and $T\Delta S$ (Table 1). This thermodynamic behaviour is characteristic for processes accompanied by a large, negative ΔC_p and thus, again, a consequence of the vanishing of the hydrophobic hydration of the inhibitor. A negative ΔC_p and enthalpy–entropy compensation with temperature are the main fingerprints indicating that the hydrophobic effect is involved in biological or chemical processes.^{19–21}

Table 2 ΔC_p , T_S and T_H for binding of benzylammonium chloride, cyclohexylcarboxamidinium chloride, and benzamidinium chloride to trypsin in Tris buffer at pH 8.0

Compound (chloride salt)	$\Delta C_p/\text{J mol}^{-1} \text{ K}^{-1}$	$T_S/^\circ\text{C}$	$T_H/^\circ\text{C}$
Benzylammonium	-254	71	14
Cyclohexylamidinium	-458	-17	27.4
Benzamidinium ^a	-400	44	-23

^a Data taken from ref. 10.

To even further disturb the hydrogen bonding capacities of the amidinium group, the binding affinities of α -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.

Influence of the phenyl ring

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 relatively large and hydrophobic phenyl group has been replaced by the smaller and less hydrophobic methyl group, which is expected to have less favourable interactions with the hydrophobic S1 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 inhibitor.¹⁰ 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 1), indicating that the more hydrophobic phenyl group increases the binding constant at least 450 times (ΔG 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 (six additional C–H bonds) and more flexible cyclohexyl group.^{22–24} The polarisabilities of cyclohexane and benzene are, respectively, 10.99 and $10.39 \times 10^{-24} \text{ cm}^3$ (20 °C), which suggests that the dispersion interactions of both compounds will be comparable.

As described in the Introduction, the phenyl ring of the benzamidinium ion sits really tightly in the binding pocket of trypsin, 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 1. At all temperatures studied, the binding constant of cyclohexylcarboxamidinium chloride to trypsin is more than ten times lower than that of benzamidinium chloride and ΔG is more than 6 kJ mol^{-1} less favourable. This is an entropic effect and 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.^{11,25} If the free inhibitor itself has little conformational freedom, the entropy loss upon binding to the enzyme will be small. We therefore propose that the less favourable entropy of binding of the cyclohexylcarboxamidinium ion is primarily due to the reduced flexibility of the cyclohexyl ring in the binding site of the enzyme as compared to that in the aqueous phase. This loss in conformational freedom is not encountered for the already flat and constrained phenyl ring. Also, the enzyme may lose some conformational freedom to adjust the binding site for the most favourable binding of the cyclohexyl group. Furthermore, we

cannot exclude that some adjustment of the binding pocket may be necessary to accommodate the cyclohexyl ring.

For cyclohexylcarboxamidinium chloride, ΔC_p is equal to $-458 \text{ J mol}^{-1} \text{ K}^{-1}$, T_H is -17°C and T_S is 27.4°C (Table 2). This large and negative ΔC_p results in enthalpy–entropy compensation with temperature. 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.²²

Conclusions

The effect of the amidinium group and the phenyl ring on the thermodynamics of binding of benzamidinium chloride to trypsin has been investigated by means of isothermal titration calorimetry measurements. The influence of the amidinium group on the binding process has been probed by comparison with 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. If the amidinium group is even further distorted, as in α -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, whereas the enthalpy of binding is rather similar. 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.

These experiments point out that both the hydrogen-bond donating amidinium group and the hydrophobic phenyl ring of the benzamidinium molecule contribute 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 appear to be primarily entropically favourable. For those compounds measured over a range of temperatures, the observed thermodynamics of binding were characteristic of the hydrophobic effect: a large, negative ΔC_p and, as a consequence, strong enthalpy–entropy compensation with temperature.

Experimental

General remarks

Trypsin solutions were prepared and titration experiments performed and analysed as described previously.^{10,26} Measurements were repeated at least three times; K was reproducible to within 10% and ΔH was reproducible to within 5%. Errors in ΔC_p were within 5%. 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). Starting materials for the synthesis were from Aldrich (cyclohexylcarbonitrile, 2 M 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 1. An adapted literature procedure²⁷ was

used. A 2 M solution of Me_3Al in toluene (15.3 ml, 30.5 mmol) was slowly added to a magnetically stirred suspension of 1.8 g (33 mmol) of NH_4Cl in 14 ml of toluene (suspension dried by azeotropic distillation to 14 ml of 27 ml of toluene under a N_2 atmosphere) at 0 °C under a N_2 atmosphere. After the addition, the mixture was warmed to 25 °C and stirred until the gas evolution had ceased. Cyclohexylcarbonitrile (2.0 g, 18 mmol) 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 silica gel in 30 ml of chloroform and stirred for 5 min. Next, 25 ml methanol was added and the suspension was stirred for 2 h. The silica was filtered off 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 NH_4Cl . Finally, the solvent of the filtrate was evaporated.

Scheme 1 Synthesis of cyclohexylcarboxamidinium chloride.

The crude product was purified by stirring overnight in 10 ml of *i*-propanol, which was filtered to remove the precipitated NH_4Cl and the solvent of the filtrate was evaporated. To remove organic contaminations, the product was dissolved in as little *i*-propanol as possible 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 *n*-hexane and subsequently freeze-dried from 1 M hydrochloric acid to yield 1.8 g (60%) of cyclohexylcarboxamidinium chloride,²⁸ mp 181–182 °C (dec., lit.²⁸ 184–185 °C). $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$, 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%.

References

- 1 F. P. Schmidtchen and M. Berger, *Chem. Rev.*, 1997, **97**, 1609–1646.
- 2 *Proteinase Inhibitors*, ed. A. J. Barrett and G. Salvesen, Elsevier, Amsterdam, 1986.
- 3 M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, 1965, **240**, 1579–1585.
- 4 W. Bode and P. Schwager, *J. Mol. Biol.*, 1975, **98**, 693–717.
- 5 M. Renatus, W. Bode, R. Huber, J. Stürzebecher and M. T. Stubbs, *J. Med. Chem.*, 1998, **41**, 5445–5456.
- 6 T. Inagami, *J. Biol. Chem.*, 1964, **239**, 787–791.
- 7 N. Ota, C. Stroupe, J. M. S. Ferreira-da-Silva, S. A. Shah, M. Mares-Guia and A. T. Brunger, *Proteins*, 1999, **37**, 641–653.

- 8 The low temperature factor (*ca.* 20 Å) is another indication that only a 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.
- 9 Since the pK_a of benzylammonium is 9.5 at pH 8.0 (C. H. Arrowsmith, H.-X. Guo and A. J. Kresge, *J. Am. Chem. Soc.*, 1994, **116**, 8890–8894), 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 (A. Fersht, *Structure and Mechanism in Protein Science*, Freeman, New York, 1999):

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

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

- 10 R. Talhout and J. B. F. N. Engberts, *Eur. J. Biochem.*, 2001, **268**, 1554–1560.
- 11 S. Leavitt and E. Freire, *Curr. Opin. Struct. Biol.*, 2001, **11**, 560–566.
- 12 C. T. Calderone and D. H. Williams, *J. Am. Chem. Soc.*, 2001, **123**, 6262–6267.
- 13 A. Cooper, C. M. Johnson, J. H. Lakey and M. Nöllmann, *Biophys. Chem.*, 2001, **93**, 215–230.
- 14 J. D. Dunitz, *Chem. Biol.*, 1995, **2**, 709–712.
- 15 W. C. Conner, *J. Catal.*, 2003, **78**, 238–246.
- 16 S. W. Benson, *Thermochemical Kinetics*, Wiley: New York, 1968.
- 17 *Structure-Based Drug Design: Thermodynamics, Modelling and Strategy*, ed. J. E. Ladbury, and P. R. Connelly, Springer: Berlin, 1997.
- 18 *Protein–Ligand Interactions: Hydrodynamics and Calorimetry*, eds. S. E. Harding, and B. Z. Chowdry, Oxford University Press, Oxford, 2001.
- 19 W. Blokzijl and J. B. F. N. Engberts, *Angew. Chem., Int. Ed. Engl.*, 1993, **32**, 1545–1579.
- 20 N. T. Southall, K. A. Dill and A. D. J. Haymet, *J. Phys. Chem. B*, 2002, **106**, 521–533.
- 21 J. H. Ha, R. S. Spolar and M. T. Record, *J. Mol. Biol.*, 1989, **209**, 801–816.
- 22 C. Hansch; A. Leo in *Exploring QSAR: Fundamentals and Applications in Chemistry and Biology*, ed. S. R. Heller, ACS, Washington, DC, 1995.
- 23 G. Graziano, *Can. J. Chem.*, 2000, **78**, 1233–1241.
- 24 W. P. Jencks, *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York, 1969, pp. 393–436.
- 25 M. J. Todd, I. Luque, A. Velásquez-Campoy and E. Freire, *Biochemistry*, 2000, **39**, 11876–11883.
- 26 R. Talhout, A. Villa, A. E. Mark and J. B. F. N. Engberts, *J. Am. Chem. Soc.*, 2003, **125**, 10570–10579.
- 27 R. A. Moss, W. Ma, D. C. Merrer and S. Xue, *Tetrahedron Lett.*, 1995, **36**, 8761–8764.
- 28 H. Schubert, *J. Prakt. Chem.*, 1956, **4**, 146–152.
- 29 F. C. Bernstein, T. F. Koetzle, G. J. B. Williams, E. F. Jr. Meyer, M. D. Brice, J. R. Rodgers, O. Kennard, T. Shimanouchi and M. Tasumi, *J. Mol. Biol.*, 1977, **112**, 542.
- 30 R. A. Sayle and E. J. Milnerwhite, *Trends Biochem. Sci.*, 1995, **20**, 374–376.