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# Protonation linked equilibria and apparent affinity constants: the thermodynamic profile of the $\alpha$ -chymotrypsin–proflavin interaction

Gilles Bruylants · René Wintjens · Yvan Looze ·  
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**Abstract** Protonation/deprotonation equilibria are frequently linked to binding processes involving proteins. The presence of these thermodynamically linked equilibria affects the observable thermodynamic parameters of the interaction ( $K_{\text{obs}}$ ,  $\Delta H_{\text{obs}}^0$ ). In order to try and elucidate the energetic factors that govern these binding processes, a complete thermodynamic characterisation of each intrinsic equilibrium linked to the complexation event is needed and should furthermore be correlated to structural information. We present here a detailed study, using NMR and ITC, of the interaction between  $\alpha$ -chymotrypsin and one of its competitive inhibitors, proflavin. By performing proflavin titrations of the enzyme, at different pH values, we were able to highlight by NMR the effect of the complexation of the inhibitor on the ionisable residues of the catalytic triad of the enzyme. Using ITC we determined the intrinsic thermodynamic parameters of the different equilibria linked to the binding process. The possible driving forces of the interaction between  $\alpha$ -chymotrypsin and proflavin

are discussed in the light of the experimental data and on the basis of a model of the complex. This study emphasises the complementarities between ITC and NMR for the study of binding processes involving protonation/deprotonation equilibria.

## Introduction

The concept of molecular recognition is generally used to describe the relationship between interacting partners in a receptor–ligand complex, whether the receptor is a biological macromolecule or an organic host molecule. The stability of the complex is usually characterised by its affinity constant,  $K_a$ , expressed as the ratio of the molar concentration of receptor–ligand complex to the molar concentrations of the free receptor and free ligand:

$$K_a = \frac{[RL]}{[L] \cdot [R]}. \quad (1)$$

This affinity constant can be obtained by measuring the concentration of one of the above-mentioned species and determining the concentration of the other two from the known total receptor and ligand concentrations. This supposes that the only equilibrium that needs to be taken into account is the  $R + L \rightleftharpoons RL$  one. Various other equilibria linked to the binding process can however be present and  $K_a$  should then be considered as an apparent (or observed) affinity constant (Eftink et al. 1983; Weber 1972; Wyman 1965). The receptor and/or ligand can for example exist in different aggregation or protonation states which are in equilibrium with each other. A priori, each state of the ligand can interact, with its own intrinsic affinity, with each

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state of the receptor. However, most experimental techniques are, by and large, not able to distinguish between the different states of the different interacting partners and the affinity constant is generally derived considering only the three quantities indicated in (1). Consequently, the affinity constants derived as mentioned above will be a function of the intrinsic affinities and of the equilibria between the various states of the interacting molecules. The free energy of interaction ( $\Delta G^0$ ) that can be derived from the apparent affinity constant will contain contributions from all these equilibria and does not correspond to the change in free energy of the single binding equilibrium.

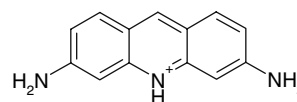
In this paper we focus on protonation/deprotonation equilibria coupled to binding events. Such events lead to a pH dependence of  $K_{\text{obs}}$ . This case is frequently met when one of the interacting partners is a protein. If a ligand binds preferentially to certain protonation states of a protein, a shift in the distribution of the states of the ionisable residues will occur and this will lead to a release or uptake of protons upon complex formation. If the ligand can only interact with one specific protonation state of the receptor, i.e. if all the titratable groups have to be in a specific ionisation state in order for the complex to form (to avoid electrostatic repulsion or to be able to form hydrogen-bonds for example), the coupling between the protonation equilibria of the titratable groups and the binding process can be considered to be mandatory. In this case an intrinsic affinity constant, characteristic of the interaction between the partners in the right protonation state, can be derived by measuring the apparent affinity constant at different pH values and taking the  $pK_a$  of the titratable groups into consideration (Eftink et al. 1983; Gomez and Freire 1995; Hendrickson 1999; Waelbroeck 1982). If, on the other hand, the ligand can interact, to a certain extent, with different protonated states of the receptor, the coupling between the protonation equilibria of the titratable groups and the binding process can be considered to be non-mandatory. In this case, several intrinsic affinity constants should be considered when analysing the experimental data (Baker and Murphy 1996; Doyle et al. 1995; Eftink et al. 1983; Weber 1972; Wyman 1965). It is clear that the ligand itself can also be an ionisable molecule and undergo a modification of the distribution of its protonation state upon complexation.

The number of protons linked to a binding event, that is the number of protons released or taken up upon ligand binding, can be obtained from the tangent to the curve representing the logarithm of the observed association constant versus pH (Doyle et al. 1995; Waelbroeck 1982). It is, however, extremely difficult to determine an association constant with good precision and the number of protons linked to the binding event can only be obtained, in this way, with poor precision. On the other hand, this

quantity can be obtained with a good precision by titration calorimetry. The advent of microcalorimeters sensitive enough to study biological systems has enabled the thermodynamic characterisation of protonation equilibria linked to protein–ligand interactions (Baker and Murphy 1997; Gomez and Freire 1995).

We report here on the structural and thermodynamic characterisation by nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC), of the interaction between  $\alpha$ -chymotrypsin and one of its competitive inhibitors, proflavin (Fig. 1). The affinity of proflavin for the enzyme has been determined by several authors using differential absorption or emission spectroscopy (Bernhard et al. 1966; Feinstein and Feeney 1967; Glazer 1965; Havsteen 1967; Shiao and Sturtevant 1969; Sturgill et al. 1978; Wallace et al. 1963). The observed affinity constant is reported to increase with pH: from approximately  $4,000 \text{ M}^{-1}$  at pH 4 to approximately  $30,000 \text{ M}^{-1}$  at pH 8 (Bernhard et al. 1966; Feinstein and Feeney 1967). The pH dependence of the affinity constant is a clear indication of the presence of at least one linked protonation equilibrium.

$\alpha$ -Chymotrypsin is a member of the serine protease family, a class of enzymes characterised by an Asp-His-Ser catalytic triad and found in essentially all living organisms, from eukaryotes to prokaryotes and viruses. The ubiquitous nature of these enzymes and their extreme efficiency have given rise to much interest in the elucidation of their catalytic mechanism. Many studies are devoted to the determination of the nature and energy of the different non-covalent interactions stabilising the catalytic triad in serine proteases and in the different complexes formed between these enzymes and their substrates. NMR has contributed to a great extent to the picture emerging for their detailed catalytic mechanism. The side-chain NH protons of the catalytic histidine of different serine proteases, such as chymotrypsin,  $\alpha$ -lytic protease, trypsin and subtilisin, appear as extremely deshielded signals in their  $^1\text{H}$  NMR spectra (Robillard and Shulman 1974). For  $\alpha$ -chymotrypsin, signals are observed at low pH for each of the  $\text{N}^{\delta 1}\text{-H}$  and  $\text{N}^{\epsilon 2}\text{-H}$  protons of the catalytic histidine (His57) at around 18 and 13 ppm, respectively. In the mature form of the enzyme, it has furthermore been observed that the signals characterising these protons are influenced by the protonation state of the catalytic aspartic acid (Asp102), with which the  $\text{N}^{\delta 1}\text{-H}$  proton of His57 forms a highly energetic hydrogen bond (Bruylants et al. 2007; Zhong



**Fig. 1** Structure of proflavin, presented in its protonated form

et al. 1995). Two signals are indeed observed in the 600 MHz  $^1\text{H}$  NMR spectrum of the fully active enzyme for the  $\text{N}^{\delta^1}\text{-H}$  and  $\text{N}^{\epsilon^2}\text{-H}$  protons of His57 at low pH: one set of signals corresponding to the NH protons when Asp102 is protonated (respectively 18.2 and 13.3 ppm for the  $\text{N}^{\delta^1}\text{-H}$  and  $\text{N}^{\epsilon^2}\text{-H}$  protons) [19]. At higher pH values, when His57 is fully deprotonated, only one signal, corresponding to the  $\text{N}^{\delta^1}\text{-H}$  proton, which is the tautomer favoured in the enzyme, is observed at around 15 ppm (Bachovchin 1985; Robillard and Shulman 1972). The  $pK_a$  of His57, determined, by different experimental techniques, in the active form of the enzyme is reported to be around 6.7 (Bachovchin 2001; Blow et al. 1969), while the  $pK_a$  of Asp102 has been reported to be close to 4 (Fersht and Sperling 1973). It has been observed that the binding of a competitive inhibitor can lead to a change in the His57  $pK_a$  (Bachovchin 1986; Bender and Kezdy 1964; Caplow 1969; Fersht 1972; Fersht and Renard 1974; Fersht and Requena 1971; Lucas et al. 1973; Zhong et al. 1995).

Proflavin is an effective competitive inhibitor of the chymotryptic hydrolysis (Wallace et al. 1963). Upon complexation, a quenching of the fluorescence of proflavin and a bathochromic shift of its absorption spectrum are observed (Bernhard et al. 1966). Proflavin is positively charged in this pH range—the  $pK_a$  of its central cyclic nitrogen is reported to be 9.65—and remains charged in the complex since its emission spectrum is not modified upon complexation (Glazer 1965). The protonation equilibrium/equilibria linked to the interaction between proflavin and  $\alpha$ -chymotrypsin must therefore be associated with titratable amino acids in the protein. The effect of the protonation/deprotonation equilibria of the catalytic residues of serine proteases on the binding of a competitive inhibitor has, to our knowledge, never been completely characterised from a thermodynamic point of view.

## Materials and methods

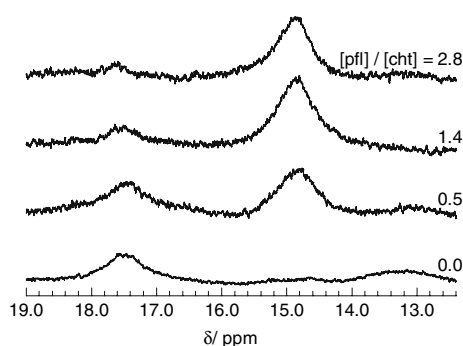
Bovine pancreatic  $\alpha$ -chymotrypsin was purchased from Fluka. Stock solutions of the enzyme were dialysed at 4°C against a large quantity of the desired buffer. The dialysis buffer was changed twice, each time after approximately 6 h. Enzyme concentrations were determined spectrophotometrically at 280 nm using a molar extinction coefficient of 50,100  $\text{cm M}^{-1}$ . Solutions were stored at 4°C and used within 2 days. Proflavin (3,6-diaminoacridine) hemisulphate dihydrate was purchased from Fluka. Solutions were made using the last dialysis buffer. Concentrations were determined by absorption spectroscopy at 444 nm using an extinction coefficient of 35,000  $\text{cm M}^{-1}$ . All buffers were prepared at a concentration of 100 mM. The pH was

adjusted by addition of concentrated HCl or NaOH. Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Mes (2-(N-morpholino)-ethanesulfonic acid), BisTris (bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane) and Imidazole were purchased from Fluka, Tris (Tris(hydroxymethyl)aminomethane) from Merck and BisTris Propane (1,3-Bis[tris(hydroxymethyl)methylamino]-propane) from Sigma. Sodium azide (BDH) and calcium chloride (Fluka) were added to all solutions in 1 and 10 mM concentrations, respectively.

Differential absorption titration experiments were undertaken using a 10  $\mu\text{M}$  reference solution of proflavin. The solution in the titration cell was 10  $\mu\text{M}$  in proflavin and the enzyme/proflavin ratio varied from 20 to 0. The spectra were recorded from 300 to 600 nm to ensure the presence of an isosbestic point. The maximum difference in absorption between proflavin and complexed proflavin was observed at 465 nm and data were analysed at this wavelength. For the analysis of the data, an  $\alpha$ -chymotrypsin dimerisation constant of 350  $\mu\text{M}$  was taken into account. All absorption measurements were undertaken on a Perkin–Elmer Lambda 40 spectrophotometer.

Calorimetric experiments were performed at 25°C on an ITC-4200 from Calorimetric Science Corporation. Aliquots of 10  $\mu\text{l}$  of a proflavin solution of a known precise concentration (in the 100  $\mu\text{M}$  range) were injected into the 1.3 ml titration cell containing the stock solution of  $\alpha$ -chymotrypsin (in the 300  $\mu\text{M}$  range). A minimum of 10 injections were performed for each set of experimental conditions at intervals of 300 s to ensure the return to equilibrium. A blank experiment was performed each time in order to confirm that proflavin dilution was negligible.

1D  $^1\text{H}$  NMR spectra were recorded on a Varian Unity 600 spectrometer at the University of Brussels. Water suppression was obtained using the jump-return sequence (Plateau and Gueron 1982)  $((\pi/2)x - \tau - (\pi/2)x - \text{acq})$ , with delays  $\tau$  of 34  $\mu\text{s}$ . Spectra were recorded at 5°C with a minimum of 2,048 transients, an acquisition time of 2 s, a recycle delay of 2 s, a spectral width of 18,000 Hz and a digital resolution of 0.5 Hz/pt. The FIDs were weighted by a 5 Hz line-broadening prior to Fourier transformation. All NMR samples were prepared in 90%/10%  $\text{H}_2\text{O}/\text{D}_2\text{O}$  and the pH was adjusted by addition of HCl or NaOH at 5°C. The pH meter was calibrated at this temperature using standard calibrations buffers (Consort). Small amounts of concentrated 3-(Trimethylsilyl)propionic acid were added to the NMR tubes for referencing. Protein concentrations were around 0.5 mM for all NMR experiments. Proflavin titrations were undertaken at different pH values by adding successively small volumes of concentrated solutions of proflavin at a known concentration in the NMR tubes containing a known volume of protein solution.

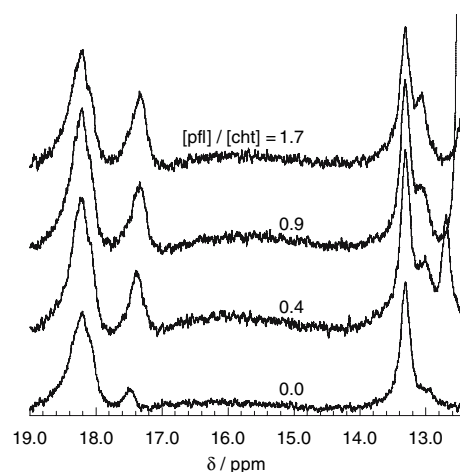


**Fig. 2** Low field region of the 600 MHz  $^1\text{H}$  jump-return NMR spectra of  $\alpha$ -chymotrypsin at pH 7 in presence of different concentration of proflavin.  $[\text{Pfl}]/[\text{Cht}]$  represents the ratio between the concentration in proflavin and  $\alpha$ -chymotrypsin. The signal around 17.5 ppm has been assigned to the  $\text{N}^{\delta^1}\text{-H}$  proton of His57 in the protonated residue and the signal around 15 ppm to the same proton when the residue is deprotonated

## Results and discussion

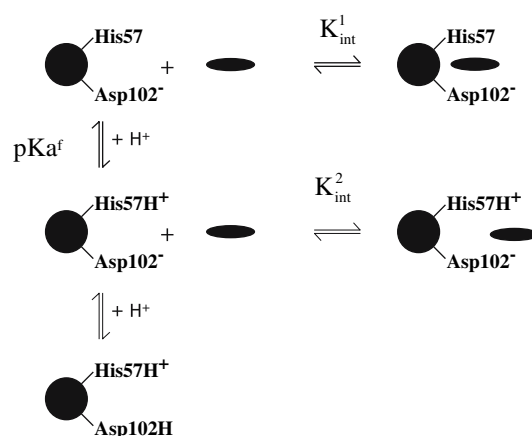
The low field region of the  $^1\text{H}$  NMR spectrum of  $\alpha$ -chymotrypsin in the presence of different concentrations of proflavin was monitored at pH values between 3 and 7. Figures 2 and 3 show, respectively, the results obtained at pH 7 and 3. At pH 7, a significant proportion of His57 is still protonated and a broad signal is observed around 17.5 ppm for the  $\text{N}^{\delta^1}\text{-H}$  proton in the protonated histidine. The signals of the  $\text{N}^{\delta^1}\text{-H}$  proton in the deprotonated histidine (around 15 ppm) and the  $\text{N}^{\epsilon^2}\text{-H}$  in the protonated histidine (around 13 ppm) are, due to exchange with the solvent protons, too broad to be clearly observed. When proflavin is added to the enzyme solution, the relative intensity of the signal corresponding to the  $\text{N}^{\delta^1}\text{-H}$  proton in the deprotonated His57 increases and the signal at 17.5 ppm disappears. The  $pK_a$  of this residue, which is reported to be around 6.7 in the free enzyme (Bachovchin 2001; Blow et al. 1969), seems to be shifted to a lower value in the complex. At pH 3, when proflavin is added to the solution an increase in the relative intensity of the signals corresponding to the  $\text{N}^{\delta^1}\text{-H}$  and  $\text{N}^{\epsilon^2}\text{-H}$  protons of His57 in presence of deprotonated Asp102 is observed. The  $pK_a$  of Asp102, which has been shown to be lower than 4 in the free enzyme (Fersht and Sperling 1973), also seems to be shifted to a lower value in the complex.

The different possible species to consider when studying the interaction between proflavin and  $\alpha$ -chymotrypsin are represented in Scheme 1. Each complexation equilibrium may be characterised by an intrinsic affinity constant: (1)  $K_{\text{int}}^1$  when both His57 and Asp102 are deprotonated (2)  $K_{\text{int}}^2$  when His57 is protonated and Asp102 deprotonated. When both His57 and Asp102 are protonated, it is known that the interaction of  $\alpha$ -chymotrypsin with proflavin does not occur (Feinstein and Feeny 1967).



**Fig. 3** Low field region of the 600 MHz  $^1\text{H}$  jump-return NMR spectra of  $\alpha$ -chymotrypsin at pH 3 in presence of different concentration of proflavin.  $[\text{Pfl}]/[\text{Cht}]$  represents the ratio between the concentration in proflavin and  $\alpha$ -chymotrypsin. The signal at 18.2 and 13.3 ppm have been assigned respectively to the  $\text{N}^{\delta^1}\text{-H}$  and the  $\text{N}^{\epsilon^2}\text{-H}$  protons of His57 in the presence of protonated Asp102 and the signals at 17.5 and 13 ppm to the same protons in the presence of deprotonated Asp102. The signal which appears at high field for the higher proflavin concentrations belongs to proflavin

The  $pK_a$  of His57 and Asp102 in the free enzyme are sufficiently different so that only one of the protonation equilibria has to be taken into consideration at a time when studying the proflavin/ $\alpha$ -chymotrypsin binding process at different pH values: the protonation/deprotonation of His57 around pH 7 and the protonation/deprotonation of Asp102 around pH 4. In the pH range close to the  $pK_a$  of His57 ( $pK_a^f$ ) the observed affinity constant can be written in the following way:

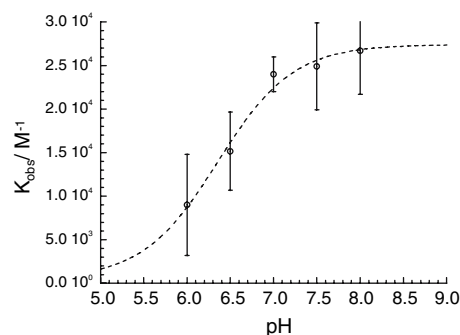


**Scheme 1** Equilibria that have to be considered for the interaction between  $\alpha$ -chymotrypsin (represented as a *sphere*) and proflavin (represented as a *disc*)



$$K_{\text{obs}} = K_{\text{int}}^1 + \frac{(K_{\text{int}}^2 - K_{\text{int}}^1)}{(1 + 10^{\text{pH} - \text{p}K_{\text{a}}^f})} \quad (2)$$

The different affinity constants cannot be determined by ITC because of proflavin aggregation. The dissociation of proflavin clusters is highly endothermic and concentration dependant, which makes the determination of the precise value of  $K_{\text{obs}}$  impossible. However, as mentioned above, the observed affinity constant of proflavin for the enzyme can be determined using differential absorption or emission spectroscopy (Bernhard et al. 1966; Feinstein and Feeney 1967; Glazer 1965; Havsteen 1967; Shiao and Sturtevant 1969; Sturgill et al. 1978; Wallace et al. 1963). We measured the observed affinity constant for the proflavin/ $\alpha$ -chymotrypsin interaction at different pH values using differential absorption spectroscopy. In order to avoid any proflavin aggregation, proflavin concentrations were kept below 10  $\mu\text{M}$  for all the experiments (Glazer 1965).  $\alpha$ -Chymotrypsin is known to form dimers in solution and the dimer to monomer dissociation constant is reported to be on the order of 350  $\mu\text{M}$  at low pH and to be negligible at pH values above 7 (Shiao and Sturtevant 1969). Since proflavin binds only to the monomeric form of the enzyme (Shiao and Sturtevant 1969), the protein dimer/monomer equilibrium should be taken into consideration when determining the observed affinity constant. The observed  $\alpha$ -chymotrypsin/proflavin affinity constants were measured in different buffers, at 5 different pH values (Table in supporting material). Within experimental error, the buffer has no influence on the observed affinity constant. The measured values are in good agreement with those reported in the literature (Bernhard et al. 1966; Feinstein and Feeney 1967; Glazer 1965; Havsteen 1967; Shiao and Sturtevant 1969; Sturgill et al. 1978; Wallace et al. 1963). Equation 2 was fitted to the experimental data with the two equilibrium constants and  $\text{p}K_{\text{a}}^f$  as parameters (Fig. 4). Values of  $28,000 \pm 2,500 \text{ M}^{-1}$ ,  $500 \pm 8,000 \text{ M}^{-1}$  and  $6.4 \pm 0.7$  (95% confidence interval) are obtained for  $K_{\text{int}}^1$ ,  $K_{\text{int}}^2$  and  $\text{p}K_{\text{a}}^f$ , respectively. The value obtained for the  $\text{p}K_{\text{a}}^f$  is within the range of values reported in the literature. The small value, accompanied by large error, for  $K_{\text{int}}^2$  suggests that the interaction with the enzyme where His57 is protonated is negligible compared to the interaction when His57 is deprotonated. It is interesting to point out that if the two intrinsic affinity constants differ by two orders of magnitude ( $K_{\text{int}}^1/K_{\text{int}}^2 > 100$ ), the effect of  $K_{\text{int}}^2$  on the observed affinity constant (2), is less than 10% which is below the experimental error. This certainly explains the large confidence interval on  $K_{\text{int}}^2$  derived from the absorption spectroscopy data. The modifications observed in the NMR spectra upon proflavin titration highlight the presence of a weak interaction when His57 is protonated. It is, however,



**Fig. 4** pH dependence of the observed affinity constant. Values plotted are the average of the values obtained in different buffers (values reported in supporting material). Error bars represent two times the standard deviation without taking into account the experimental errors on the observed constants. The dotted line corresponds to the fitting of (2) to the experimental data

extremely difficult to estimate binding constants by NMR as the complexation by the ligand in the active site more than certainly modifies the exchange rates of the different protons with the solvent protons. It is consequently not certain that the peak areas are directly proportional to the concentrations of the different forms of the enzyme in solution.

Despite the fact that ITC cannot be used to determine  $K_{\text{obs}}$ , the observed enthalpy of interaction ( $\Delta H_{\text{obs}}^0$ ) associated to the binding of proflavin to  $\alpha$ -chymotrypsin can be determined using this technique by injecting the inhibitor into the titration cell which contains a large excess of enzyme. The measured heats correspond to the product of  $\Delta H_{\text{obs}}^0$  and the number of moles of complex formed. The excess of enzyme in the titration cell cannot be increased enough to ensure complete complexation and the number of moles of complex formed can be calculated, for each titration, using the value of the observed affinity constants obtained by absorption spectroscopy. At pH 8, up to 90% of the injected ligand will be complexed while at pH 6, approximately 70% is complexed. The value of  $\Delta H_{\text{obs}}^0$  was determined in several buffers at the same pH values as those used for the absorption measurements (Table in supporting material). The measured enthalpy is dependent on the nature of the buffer for experiments run below pH 8. This is due to the fact that the protons released upon proflavin complexation are accepted by the buffer. The observed enthalpy will therefore contain a contribution due to the protonation of the buffer (Sturtevant and Beres 1971):

$$\Delta H_{\text{obs}}^0 = \Delta H_0^0 + n_{\text{H}^+} \cdot \Delta H_{\text{ioniz-buffer}}^0 \quad (3)$$

where  $\Delta H_0^0$  is the enthalpy that would be measured in a buffer with an ionisation enthalpy equal to zero,  $n_{\text{H}^+}$  is the number of moles of protons exchanged per mole of

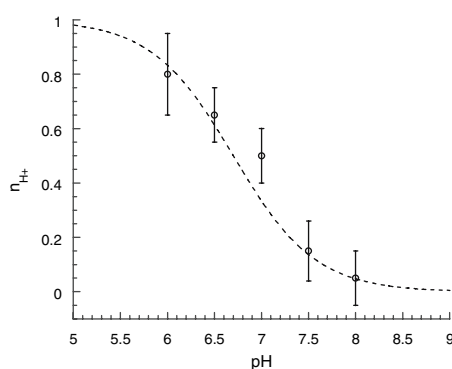
complex formed ( $n_{H^+} > 0$  if protons are accepted by the enzyme) and  $\Delta H_{\text{ioniz-buffer}}^0$  is the ionisation enthalpy of the buffer (Goldberg et al. 2002).  $n_{H^+}$  at each pH was determined from the slope of  $\Delta H_{\text{obs}}^0$  as a function of  $\Delta H_{\text{ioniz-buffer}}^0$ . At each pH, the number of moles of protons released per mole of complex formed is approximately equal to the percentage of free enzyme that presents a protonated histidine considering a  $pK_a$  of 6.7 for this residue (see Fig. 5). This means that the formation of the complex is linked to a quasi complete deprotonation of the catalytic histidine. These results confirm that proflavin interacts essentially with the protein when His57 is deprotonated and that the second interaction is negligible.

If only the protonation of His57 and the interaction with the protonated enzyme are considered,  $\Delta H_0^0$  may still be decomposed as:

$$\Delta H_0^0 = \Delta H_{\text{int}}^0 - n_{H^+} \cdot \Delta H_{\text{ioniz-a.a.}}^0 \quad (4)$$

where  $\Delta H_{\text{int}}^0$  is the intrinsic enthalpy of interaction between the enzyme and the inhibitor when His57 is deprotonated and  $\Delta H_{\text{ioniz-a.a.}}^0$  is the ionisation enthalpy of this residue in the free enzyme. The plot of  $\Delta H_0^0$  as a function of  $n_{H^+}$  yields a value of  $-34.5 \pm 5 \text{ kJ mol}^{-1}$  for  $\Delta H_{\text{ioniz-a.a.}}^0$  which is larger than the reported value of  $-29.5 \text{ kJ mol}^{-1}$  for the ionisation enthalpy of a free histidine (Goldberg et al. 2002). The highly energetic hydrogen bond that His57 forms with the negatively charged Asp102 must be, to a great extent, responsible for the higher deprotonation enthalpy of this histidine in the active site of the enzyme compared to an aqueous medium.

The intrinsic enthalpy of interaction between proflavin and  $\alpha$ -chymotrypsin, where the two partners are in the most favourable protonation state before the interaction, is determined to be  $25 \pm 5 \text{ kJ mol}^{-1}$ . Using this value and the value of the intrinsic affinity constant ( $K_{\text{int}}^1$ ) determined



**Fig. 5** Number of moles of protons released per mole of complex formed as a function of the pH. The curve corresponds to the percentage of free enzyme that presents a protonated histidine in its active site, based on a  $pK_a$  of 6.7 for this residue

from the absorption spectroscopy data (which corresponds to a  $\Delta G^0$  of  $25 \pm 2 \text{ kJ mol}^{-1}$ ), the entropic contribution ( $-T \cdot \Delta S^0$ ) is estimated to be approximately null at 298 K. The interaction is clearly enthalpy driven, which could seem surprising for the complexation of a hydrophobic molecule in a hydrophobic binding site. Some structural and thermodynamic studies on the interaction between proteases and their inhibitors have been reported in the literature. A study undertaken by Dullweber et al. (2001) pertaining to the binding of known inhibitors by two serine proteases (trypsin and thrombin) has shown that a wide variation exists in the thermodynamic profiles of these systems. Some of the profiles are similar to the one observed in this study (a large enthalpic contribution and a small entropic contribution to the free energy of binding). For each protease-inhibitor system, there are multiple factors affecting  $\Delta H^0$  and  $\Delta S^0$  and conclusions obtained for one system are difficult to transpose to other systems.

In order to correlate the experimental thermodynamic data to structural information, a model for the structure of the interaction complex was built. The structure of  $\alpha$ -chymotrypsin has been resolved by X-ray crystallography (PDB code 6CHA) (Tulinsky and Blevins 1987) but no structure of the  $\alpha$ -chymotrypsin/proflavin complex is reported in the protein data bank (PDB). A structure of the thrombin/proflavin complex has, however, been reported (PDB code: 1BCU) (Conti et al. 1998).  $\alpha$ -Chymotrypsin and thrombin are serine proteases that exhibit close to 50% sequence similarity (35% sequence identity) and their superimposed structures using the SoFiST software give a backbone RMSD of less than 0.5 Å for 56% of the residues aligned (Boutonnet et al. 1995). After superimposition of the structures, the coordinates of the proflavin atoms and of the water molecules within 15 Å of the inhibitor, extracted from the thrombin-proflavin complex, were introduced into the  $\alpha$ -chymotrypsin structure. Hydrogen atoms were added to the heavy atoms and their position optimised leaving the heavy atoms fixed using the DISCOVER module of INSIGHT II (ACCELRYS Technology, San Diego, CA, USA) with the consistent valence force-field (Dauber-Osguthorpe et al. 1988; Hagler et al. 1974). The same software was used to minimise the energy of the  $\alpha$ -chymotrypsin/proflavin complex, using different minimisation strategies to ensure convergence to similar model complexes. Minimisations were performed by 100 to 500 dynamics steps at 500 K followed by 200 steps of the steepest descent and 1,000 steps of the VA09A method.

The correlation of the model of the interaction complex with the experimental results highlights some of the determinant factors of the interaction. Water molecules remain buried at the interface of the interacting partners. This may explain the thermodynamic profile observed for the interaction between  $\alpha$ -chymotrypsin and proflavin when

both Asp102 and His57 are deprotonated. Indeed, the immobilisation of water molecules in complexes is frequently reported in the literature and is generally characterised by an unfavourable entropic term (Ladbury 1996; Tame et al. 1996). This may explain that, despite the fact that proflavin and the binding cavity in  $\alpha$ -chymotrypsin are both hydrophobic, the net entropy term for this interaction is approximately null. The good complementarity between the surfaces of the inhibitor and the binding pocket could probably lead to a particularly favourable enthalpic term due to London dispersion forces (more favourable in the complex than in solution) (Barratt et al. 2005). The fact that the intrinsic affinity constant characterising the interaction between the protonated enzyme (His57 protonated) and proflavin is at least 2 orders of magnitude smaller than the intrinsic affinity constant characterising the interaction between the deprotonated enzyme (His57 deprotonated) and proflavin can also be explained by analysis of the model complex. Indeed, the distance between the proflavin cyclic nitrogen and the N<sup>δ1</sup>-H proton of His57 in the model is less than 10 Å. In a proteinic environment, the Coulombic repulsion ( $\epsilon_r = 4$ ) between two localised charges separated by 10 Å, leads to an unfavourable energy contribution of approximately 18 kJ mol<sup>-1</sup>. This value is considerably larger than the  $\Delta\Delta G^0$  resulting from a ratio of two orders of magnitude between two affinity constants (11.4 kJ mol<sup>-1</sup>). Of course, the Coulombic repulsion estimated in this way only represents a rough estimate of the repulsion interaction because delocalised charges should be considered and the dielectric constant in the active site is difficult to determine. However, electrostatic repulsion certainly participates to the decrease of the affinity constant of  $\alpha$ -chymotrypsin for proflavin when His57 is protonated.

## Conclusions

Quantitative information pertaining to the energy of interaction between proteins and ligands is essential for understanding the selectivity of the interaction. It is important to keep in mind that the receptor and ligand can be involved in various equilibria linked to the binding process, like protonation/deprotonation or dimerisation equilibria. Protonation equilibria are often linked to the interaction of proteins with ligands or inhibitors (proteinic or not) due to the frequent presence in the binding site of titratable groups. It is theoretically possible that each protonation state of the ligand can interact with each protonation state of the receptor with its own specific affinity. However, the presence or absence of charges in the interaction site can have a significant effect on the possible non-covalent interactions between the partners and several orders of magnitude may be expected between the different intrinsic affinity constants.

By the combined use of NMR and ITC, we were able to characterise the interaction between  $\alpha$ -chymotrypsin and a competitive inhibitor, proflavin. NMR enabled us to determine the equilibria which had to be considered for this system: the protonation/deprotonation of His57 and of Asp102 and the complexation/decomplexation equilibria between proflavin and  $\alpha$ -chymotrypsin, where both Asp102 and His57 are deprotonated or where His57 is protonated and Asp102 is deprotonated. The extreme sensitivity of the NMR parameters to a change in local environment also permitted us to confirm the presence of an interaction between the inhibitor and the enzyme when His57 is protonated, which affinity is however several orders of magnitude less favourable than when this residue is deprotonated. The intrinsic parameters of the interaction between proflavin and the protein when His57 is deprotonated were determined by ITC and absorption spectroscopy. The determination of these intrinsic parameters, and not only of the observed parameters in one set of experimental conditions, is essential for the understanding and rationalisation of the driving forces responsible for an interaction.

The results obtained clearly highlight the complementarities between ITC and NMR for the study of complexation events when protonation/deprotonation equilibria are linked to the interaction. It is important to take the protonation state of ionisable residues into consideration, especially for the analysis of complex structures obtained by X-ray crystallography. Indeed, no information on the presence of protons can be extracted from X-ray data and the *pKa* changes of the ionisable residues upon complexation may be difficult to determine. Only experimental studies by NMR or ITC can provide this fundamental information.

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