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The electrostatic fields in the active-site clefts of actinidin and papain

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The active sites of actinidin (EC 3.4.22.14) and papain (EC 3.4.22.2) display different reactivity characteristics to probes targeted at the active-site cysteine residue despite the close structural similarity of their active sites. The calculated electrostatic fields in the active-site clefts of actinidin and papain differ significantly and may explain the reactivity characteristics of these enzymes. Calculation of electrostatic potential also focuses attention on the electrostatic properties that govern formation of the active-site thiolate–imidazolium ion-pair. These calculations will guide the modification of the pH–activity profile of the cysteine proteinases by site-directed mutagenesis.

INTRODUCTION

The structures of actinidin (EC 3.4.22.14) and papain (EC 3.4.22.2) as determined by X-ray analysis are very similar. The root-mean-square deviation of the active-site residues is 0.03 nm (Kamphuis *et al.*, 1985). Despite this conformational similarity, reactivity probes to the active-site cysteine residue show differences between the active sites of the two enzymes (Brocklehurst *et al.*, 1984). The isoelectric points of these enzymes differ significantly; actinidin has pI 3.1, in contrast with pI 8.8 for papain. The pH–activity profile of these proteinases is bell-shaped and characterized by pK_a values of 3.2 and 10.1 for actinidin (McDowall, 1970) and 4.2 and 8.6 for papain (Lowe & Yuthavong, 1971). Ionization of the active-site cysteine thiol group and histidine side chain accounts for the pH-dependence of the catalytic activity. The close interaction of the cysteine and histidine residues results in the ionization state of one group affecting the pK_a of the other by about 4.2 pH units (Fig. 1; Lewis *et al.*, 1981). The active enzyme contains the thiolate–imidazolium ion-pair interacting electrostatically.

The electrostatic potential field in the active-site cleft of the enzyme will affect both a probe molecule with ionizable groups and the protein's own ionizable groups. The potential at the site of an ionizable group will modify the pK_a of that group. Positive potential will stabilize a negatively charged group and lower the pK_a of the cysteine residue by favouring the unprotonated thiolate ion. Conversely, negative potential will stabilize a positively charged group and increase the pK_a of the histidine residue by favouring the protonated imidazolium ion. The potential at the cysteine Sγ and histidine Nδ1 atoms indicates the contribution of the electrostatic properties of the protein to the stabilization of the active-site thiolate–imidazolium ion-pair.

In the present study the electrostatic potential fields of the two enzymes were calculated to investigate the effect of the different overall charges within the active-site clefts and to develop a rationale for modifying the pH–activity profile of the cysteine proteinases.

MATERIALS AND METHODS

The co-ordinates of actinidin (Baker, 1980) were taken from the protein data bank (Bernstein *et al.*, 1977) and papain co-ordinates (Kamphuis *et al.*, 1984) were provided by Professor J. Drenth of the Laboratory of Chemical Physics, Groningen, The Netherlands. The electrostatic fields of actinidin and papain were calculated by the finite-difference procedure of Warwicker & Watson (1982). Although there are theoretical objections to the application of dielectric constants at the molecular level, this method enables the electrostatic properties to be evaluated with some accuracy (Rogers *et al.*, 1985). This method calculates the potential from Poisson's equation, which relates electrostatic potential to the distribution of charges and dielectric media. It is an iterative finite-difference procedure in which infinitesimal

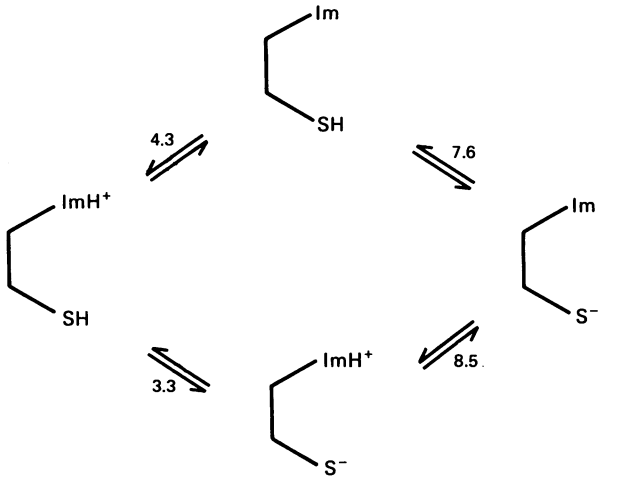

Fig. 1. Close interaction of the cysteine thiol group and the histidine imidazole group results in the ionization state of one group affecting the pK_a of the other by about 4.2 pH units

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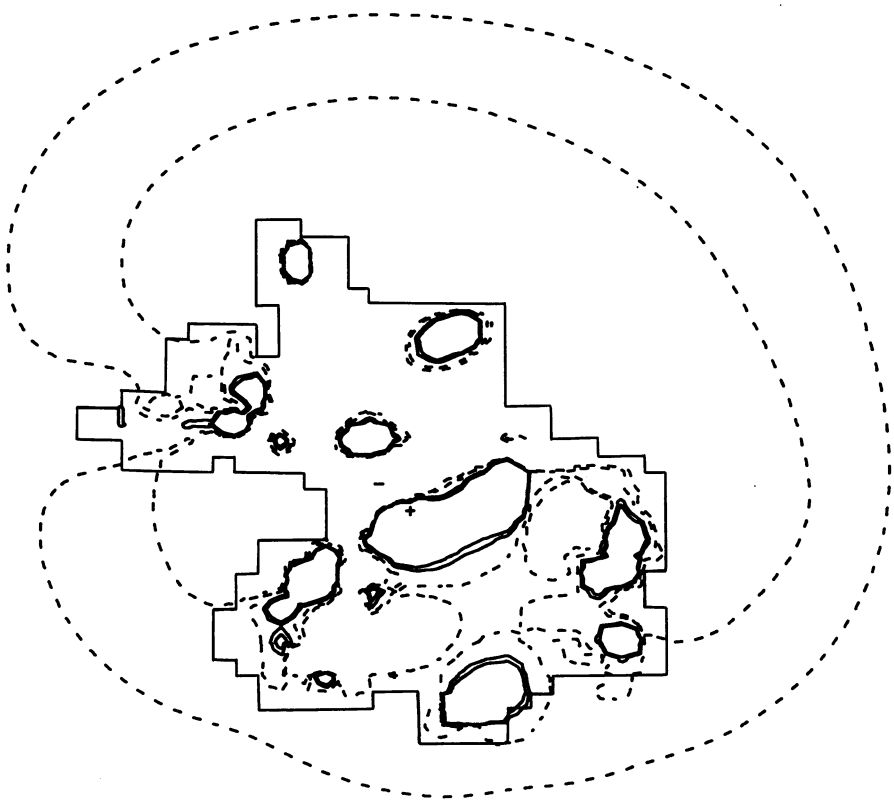

Fig. 2. Section through the active site of actinidin and its electrostatic potential at pH 7

The charges of the thiolate–imidazolium pair are included in the calculation. The protein–solvent boundary is the straight line and the active-site cleft is on the left of the Figure. The positions of the Sγ and Nδ1 atoms are indicated by the − and + respectively. The potential was calculated on a 0.14 nm grid and the contours are ±1.25 and ±2.50 kT/e; positive contours are continuous and negative contours are broken lines.

changes in potential are replaced by finite differences over a regular three-dimensional grid. Charges were assigned to grid points in accordance with Edmonds *et al.* (1984). The charges assigned to the main chain were those given by Hol *et al.* (1978); no charge was assigned to the active-site cysteine or histidine residues except for the calculations shown in Figs. 2 and 3. The method allowed the shape of the protein and the charge distribution to be modelled at close to the atomic level.

The electrostatic potential was calculated at two pH values, 7 and 3, and for the main-chain partial charges only. The side-chain charges assigned at pH 7 were as follows: lysine and arginine residues were assigned a positive electronic charge, and aspartic acid and glutamic acid residues a negative electronic charge (Table 1). Charges were also assigned to the *N*- and *C*-termini. The isoelectric point of actinidin is close to pH 3, and hence at this pH 16 of the 27 acidic residues will be protonated, so as to give no net charge. Solvent accessibilities were calculated by using the algorithm of Lee & Richards (1971), and only the most inaccessible acidic groups in actinidin were assigned negative charge at this pH on the assumption that the exposed groups would be protonated first. The sum of the solvent accessibilities of the carboxy oxygen atoms assigned negative charge was less than 0.17 nm^2. This accessibility value was used to assign negative charge to the side-chain carboxy groups in papain for the calculation at pH 3 (Tables 1 and 2).

RESULTS AND DISCUSSION

The equi-potential electrostatic contours reveal a striking difference in the electrostatic fields in the active-site clefts of actinidin and papain at pH 7 (Figs. 2 and 3). There are no equi-potential contours in the active-site cleft of papain, at this contour level, in contrast with those in the active-site cleft of actinidin. A reactivity probe would experience a considerably different electrostatic potential field in the active-site cleft of actinidin compared with that in the active-site cleft of papain. The values of the potential at the active-site residues are also different (Table 3) and thus the pK_a values will differ. Together these may explain the measured differences in the reactivity characteristics of actinidin and papain (Brocklehurst *et al.*, 1983) and circumvent the need to invoke conformational or mechanistic differences between actinidin and papain to explain their different reactivities. The different electrostatic fields may also subtly affect the conformation or dynamics of the active-site ion-pair; this cannot be ruled out as the cysteine residue is oxidized in the crystals.

The value of the potential at the position of the histidine Nδ1 and cysteine Sγ atoms (Table 3) reveals the stabilization of the active-site thiolate–imidazolium ion-pair due to the electrostatic properties of the enzyme. The pH range over which the ion-pair is formed determines the pH–activity profile. The factors involved in ion-pair formation are: (i) the interaction of the

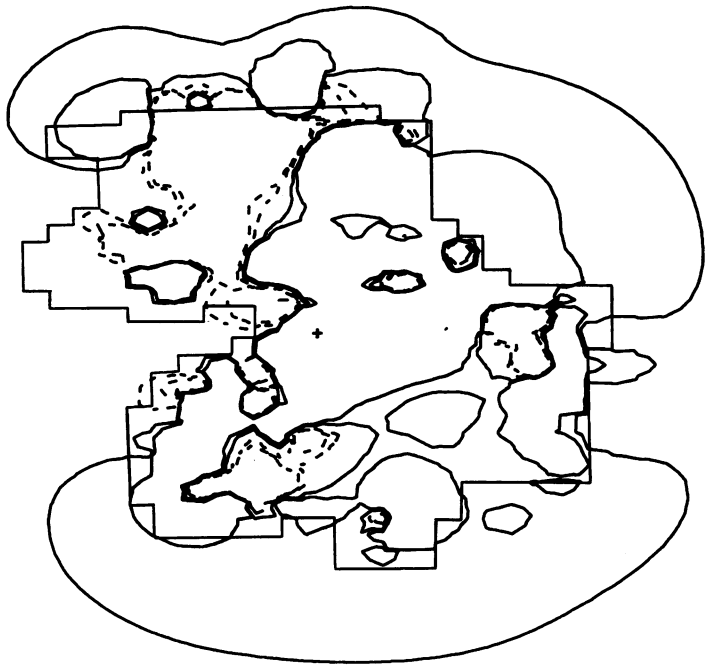

Fig. 3. Section through the active site of papain, in the same orientation as Fig. 2, and its electrostatic potential at pH 7

The charges of the thiolate–imidazolium pair are included in the calculation. The protein–solvent boundary is the straight line and the active-site cleft is on the left of the Figure. The positions of the Sγ and Nδ1 atoms are indicated by the − and + respectively. The potential was calculated on a 0.14 nm grid and the contours are ±1.25 and ±2.50 kT/e; positive contours are continuous and negative contours are broken lines.

Table 1. Numbers of assigned side-chain charges for actinidin and papain

The numbers of side-chain charges assigned for calculation of the electrostatic potential experienced by the active-site pair at pH 3 and 7 are shown.

	No. of side-chain charges				
	Arg	Lys	Asp	Glu	His
Actinidin					
Main chain	–	–	–	–	–
pH 7	5	6	16	11	–
pH 3	5	6	5	6	–
Papain					
Main chain	–	–	–	–	–
pH 7	12	10	6	7	–
pH 3	12	10	4	5	1

Table 2. Side chains of actinidin and papain assigned negative charge at pH 3

Negative charge was assigned to carboxy oxygen atoms with combined solvent accessibilities less than 0.17 nm². The values given in parentheses are the distances to the Nδ1 atom of the active-site histidine residue.

Actinidin	Papain
Asp-6	Asp-6
Glu-35	Glu-35
Glu-50	Glu-50
Glu-52	Glu-52
Asp-55	Asp-55
	Asp-57 (1.73 nm)
Asp-80 (2.54 nm)	
Glu-86 (1.68 nm)	
	Glu-89 (2.13 nm)
Glu-121 (1.98 nm)	
Asp-138 (1.13 nm)	
Asp-161	Asp-158
Glu-190	Glu-183

cysteine and histidine residues; (ii) the potential difference between Sγ and Nδ1 atoms; (iii) the potential at the Sγ and Nδ1 atoms. The effect of the ionization of one of the active-site groups on the pK_a of the other is shown in Fig. 1. The potential difference between the Sγ and Nδ1 atoms (Table 3) causes the otherwise more stable thiol–imidazole pair to be converted into the thiolate–imidazolium ion-pair. The Sγ atom is about 100 mV more positive than the Nδ1 atom. Consequently, the proton is encouraged to leave the Sγ atom for the Nδ1 atom and hence form the ion-pair. At low pH the thiolate ion will tend to become protonated to yield the inactive thiol. The ion-pair can be formed in two ways: (*a*) the thiol may lose its proton to yield the ion-pair, or (*b*) the imidazolium ion may lose its proton resulting in the thiol–imidazole pair, which is rapidly converted into the ion-pair by the mechanism described above. Both enzymes encourage the thiolate ion at low pH by positive potential at the position of the Sγ atom. The importance

Table 3. Electrostatic potentials at active-site residues in actinidin and papain

The electrostatic potentials at the Sγ and Nδ1 atoms of the active-site cysteine and histidine residues are given. The difference in potential between these atoms is also given. (The electrostatic potential varies rapidly in the region of the active-site pair and hence these values are qualitative indicators of the potential experienced by the active-site pair. The values at the Sγ and Nδ1 atoms were obtained by eight-point interpolation of the potential grid.)

	Potential (mV)		
	Sγ atom	Nδ1 atom	Difference
Actinidin			
Main chain	157	44	112
pH 7	23	−91	114
pH 3	111	−4	115
Papain			
Main chain	107	51	56
pH 7	86	25	61
pH 3	96	37	59

of the main-chain partial charges, in particular due to the helix (residues 25–43), in stabilizing the thiolate ion is clear (Table 3). The positive potential will decrease the pK_a of the cysteine residue by favouring the unprotonated form. The negative potential at the Nδ1 atom of actinidin will increase the pK_a of the histidine residue by favouring the protonated form. According to these calculations papain does not encourage the protonated form of the histidine residue in this way, and because the ionizations are mutally dependent this could be the reason for papain's lower activity at low pH.

To extend the pH–activity profile of papain to lower pH values we propose to maintain the favourable positive potential at the Sγ atom while introducing negative charge to make the potential at the position of the Nδ1 atom negative. The position at which the charges are introduced relative to the orientation of the ion-pair is likely to be critical to the modification of the pH–activity profile of these enzymes. Examination of the position of the side-chain carboxy groups assigned charge at pH 3 (Table 2) shows that actinidin has two additional negatively charged groups on the histidine side of the cysteine–histidine pair. These are aspartic acid-138 and glutamic acid-121. Changing the corresponding residues in papain, glutamine-135 and glutamine-118, into glutamic acid residues is therefore expected, on the basis of this model, to increase the activity of papain at low pH. We expect to learn much more about the factors favouring ion-pair formation by site-directed mutagenesis of papain. These calculations provide the basis for our experiments to modify the pH–activity profile of papain and a model against which to test our results.

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