Extensive counter-ion interactions seen at the surface of subtilisin in an aqueous medium†

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The extent of protein and counter-ion interactions in solution is still far from being fully described and understood. In low dielectric media there is documented evidence that counter-ions do bind and affect enzymatic activity. However, published crystal structures of macro-molecules of biological interest in aqueous solution often do not report the presence of any counter-ions on the surface. The extent of counter-ion interactions within subtilisin in an aqueous medium has been investigated crystallographically using CsCl soak and X-ray wavelength optimised anomalous diffraction at the Cs K-edge. Ten Cs$^+$, as well as six Cl$^-$ sites, have been clearly identified, revealing that in aqueous salt solutions ions can bind at defined points around the protein surface. The counter-ions do not generally interact with formal charges on the protein; formally neutral oxygens, mostly backbone carbonyls, mostly coordinate the Cs$^+$ ions. The Cl$^-$ ion sites are also found likely to be near positive charges on the protein surface. The presence of counter-ions substantially changes the protein surface electrical charge. The surface charge distribution on a protein is commonly discussed in relation to enzyme function. The correct identification of counter-ions associated with a protein surface is necessary for a proper understanding of an enzyme's function.

Introduction

Ions can interact at many places on a protein molecule. The mechanism of interaction between ions and a protein is far from being fully understood. It is also a matter of debate whether the nature of such interactions is site-specific, or nonspecific *i.e.* depending on interaction with nearby groups.[1,2]

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Some interactions take place at relatively strong and specific binding sites, and have been well studied.[3] At higher salt concentrations, more ions become associated, with important and significant effects on function associated with Hofmeister effects.[4–6] Properties following the Hofmeister series include enzyme activity, protein stability, protein–protein interactions, protein crystallization, optical rotation of sugars and amino acids and bacterial growth, as reviewed by Zhang and Cremer.[7] Further elucidation of the sites of ion interactions on protein surfaces is warranted so as to better rationalize ion-specific, Hofmeister, effects.

Molecular dynamics simulations have been used to elucidate the affinity of cations like K$^+$ and Na$^+$ on protein surfaces in aqueous solutions. These cations show a strong preference for aspartic and glutamic acid side chains, with minor contributions from other side chains or from carbonyl oxygens.[8]

The "law of matching water affinity"[9] suggests that K$^+$, a chaotrope, clusters water molecules only weakly, and, as such, it is mismatched in its water affinity to the strongly hydrated phosphates and carboxylates. A K$^+$ ion would form weak, labile interactions with phosphate and carboxylate.[10]

Molecular dynamics simulations have also shown that large soft anions such as SCN$^-$ and I$^-$ interact with the backbone of a folded protein *via* a hybrid binding site that consists of the amide nitrogen and an alpha-carbon, with a Cl$^-$ ion binding far more weakly to the site.[11]

Protein X-ray crystallography represents a source of information on these interactions with ions. If their identification is specifically targeted, confidence in ion assignments can be improved. The most common cations used in crystallisation, Na$^+$ and NH$_4^+$, are iso-electronic with water and hence will usually be falsely modelled in a protein X-ray crystal structure as waters, unless distinctive features of their coordination environment are clearly seen. Replacing these common counter-ions with ones based on heavier elements, but as similar as possible in their size and their chemistry, combined with anomalous diffraction measurements with softer X-rays[12,13] can be used to unambiguously identify their binding sites.[14,15] We

have been exploring such methods to give definitive information about such counter-ion interactions when investigating proteins that had been transferred to a predominantly non-aqueous environment (acetonitrile) and where interactions with counter-ions might be expected to become stronger. The enzyme subtilisin Carlsberg, in acetonitrile, was shown to have no fewer than eleven defined binding sites for Cs^+ cations and eight for Cl^- anions, with clear evidence from an enzyme assay that their binding affected function.[14]

We report here that the same enzyme, shown to retain activity in 3.5 M aqueous NaCl,[16] has a substantial number of clear binding sites for these counter-ions. Using the same approach of CsCl soak and optimised anomalous diffraction measurements with softer X-rays, ten Cs^+ and six Cl^- sites are unambiguously identified. This finding is rather unexpected, because the improved solvation of ions in water might be expected to reduce their binding to the protein in comparison with their behaviour in non-aqueous media. The electrical potential surface of the protein will be substantially affected by the presence of these counter-ions.

Experimental section

Crystallization, cryo-protection and Cs soaking of crystals

Subtilisin Carlsberg was purchased from Sigma (product code: P5380) and used for crystallization without further purification. The protein powder was dissolved in 330 mM Na cacodylate buffer at pH 5.6 to a concentration of 10 mg mL^{-1} and crystallized by the batch method from a buffer solution saturated with Na_2SO_4 ~13% (w/v) as precipitant.[14] Needle morphology crystals grew over a period of two weeks to typical dimensions $50 \times 50 \times 400$ µm^3. These 'native' crystals were cryo-protected using a solution of 25% glycerol and 75% buffer reservoir. The caesium derivative was prepared by soaking a single crystal with the above cryoprotectant containing 2 M CsCl for 1 minute.

X-ray diffraction data collection and processing and subsequent protein structure refinement

Data collection was performed on SRS BL100 [17] Daresbury Laboratory. Diffraction data were collected using softer X-rays[13]

Table 1 Data collection, data processing and refinement statistics for aqueous subtilisin, with and without Cs soak

	Aqueous	Cs-Aqueous
PDB deposition code	4c3v	4c3u
Crystallographic data		
Wavelength (Å)	2.070	2.070
Cell parameter (a, b, c)	51.89, 55.07, 75.31	52.17, 55.23, 75.85
Space group	$P2_12_12_1$	$P2_12_12_1$
$R_{merge}^{a,b}$	0.07 (0.236)	0.124 (0.210)
Completeness^a	100.0 (100.0)	99.9 (99.2)
Unique reflections	10 628 (1025)	10 346 (994)
Redundancy^a	10.1 (8.5)	5.8 (4.6)
Resolution^a	50.0–2.26 (2.34–2.26)	50.0–2.28 (2.36–2.28)
$I/\sigma(I)$	55.61 (14.3)	10.6 (6.9)
Average B_{factor} from Wilson plot^c (Å^2)	20.07	23.63
Matthews' coefficient (Vm)	1.98	2.01
Solvent content (Vs)	37.7	38.7
Refinement results		
R_{factor} (R_{free})	15.9 (22.6)	15.6 (23.4)
Bond lengths refined atoms (rms; weight)	0.016; 0.019	0.014; 0.019
Bond angles refined atoms (rms; weight)	1.840; 1.948	1.718; 1.941
Cruickshank's DPI for coordinate error (Å)	0.39	0.41
DPI based on free R factor (Å)	0.23	0.25
Number of bound waters and ions		
Waters	120	118
Cesium	—	12
Chlorine	—	6
Calcium	1	1
Sodium	3	1
Sulfate	3	—
Ramachandran plot^c		
Most favoured regions (%)	89.0	87.3
Additional allowed regions (%)	11.0	12.7
Generously allowed regions (%)	0.0	0.0
Disallowed regions (%)	0.0	0.0

^{a} The values in parentheses are for the highest-resolution shell. ^{b} $R_{merge} = \Sigma_{hkl}\Sigma_i |I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i |I_i(hkl)|$. ^{c} Data taken from PROCHECK.[25]
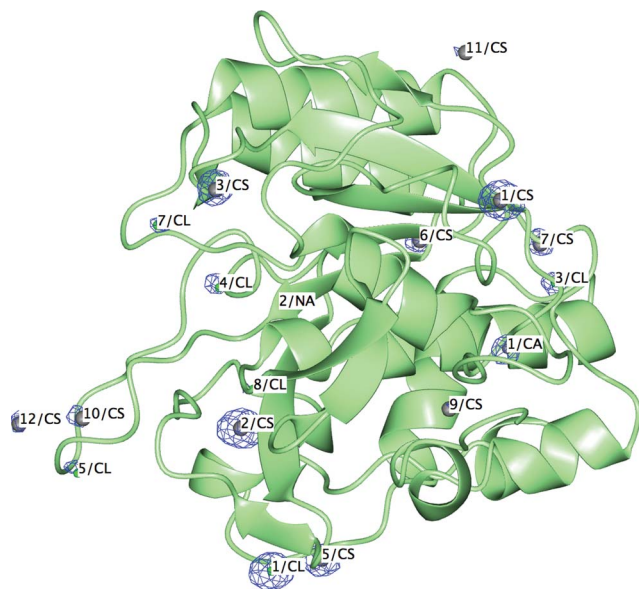
Fig. 1 Crystallographically detected counter-ion sites for subtilisin in water: Cs in grey, Cl in green. Anomalous difference electron density Fourier map contour level is at 4σ.

at an X-ray wavelength of 2.070 Å and these data processed using the HKL2000 software package.[18] Initial crystallographic phases were calculated from the PDB model code 2WUW[14] for the aqueous subtilisin model and 2WUV[14] for the Cs derivative subtilisin model. These protein molecular models were refined with the CCP4 Refmac 5.0 [19] and the protein structure regions displaying different conformations were rebuilt with COOT.[20] Anomalous difference Fourier electron density maps were calculated and those peaks contoured at a 4σ level or higher were considered significant. Ion assignments were made on the basis of coordination geometry at a peak, the map peak height, and finally the B-factor and occupancy of each site. The individual Cl$^-$ and Cs$^+$ ion occupancies were refined with a B-factor set equal to the average of their surrounding protein atoms within a radius of 7 Å of each ion. This adjustment was performed using the software program ION_GRINDER, a Python script which uses the Python interfaces of the Computational Crystallography Toolbox (CCTBX)[21] to work on PDB models. Final R_{factor} (R_{free}) were 15.9 (22.6) and 15.6 (23.4) for the aqueous subtilisin model and for the Cs derivative subtilisin model respectively. Overall X-ray diffraction data and protein model refinement statistics for both are reported in Table 1. The figures presented in this paper were made using CCP4MG.[22]

Table 2 Ion sites in the aqueous Cs structure. Ion site numbering follows the series reported in Cianci *et al.* (2010): Occ = ion site occupancy; B-fac = ion B-factor; σ contour level = maximum peak height of the anomalous difference electron density Fourier map[a]

Atom	Occ.	B-fac.	σ	Potentially charged groups within 0.8 nm	Coordinating atoms within 0.4 nm
Cs-1	0.73	30.31	29	His39 ND (0.68), Cl3** (0.76)	Leu42 O (0.30), His39 O (0.30), Ala37 O (0.30), W40 (0.34)
Cs-2	0.79	31.37	29	Cl8 (0.49), Glu195 OE (0.71)	Ala194 O (0.30), Leu196 O (0.31), Ser260 O (0.31), Gly193 O (0.33), Ser260 OG (0.39), W34 (0.35), W152 (0.37)
Cs-3	0.26	31.86	11	Cl7 (0.72), Asp60 OD (0.79)	Ser98 O (0.33), Gly61 O (0.33), W98 (0.25)
Cs-5	0.36	33.76	11	Glu54** OE (0.39)	Tyr256 ring C's (0.34–0.39), W127** (0.31), Glu54** OE (0.39), Ala52** O (0.29)
Cs-6	0.24	31.37	6	Arg247 NH (0.72)	Tyr143 O (0.33), Ser244 OG (0.37), Tyr143 ring C's (0.37–0.39), W113 (0.23)
Cs-7	0.24	35.68	7	Asp120 OD (0.57), Cl3 (0.71)	Leu241 O (0.28), Pro239 O (0.32), W46 (0.27), W62 (0.28), W119 (0.32)
Cs-9	0.22	29.49	4	Ala1 N (0.50), Asp41 OD (0.77)	Thr3 OG (0.28), Ala1 O (0.30), Gly80 O (0.33), Thr3 N (0.38), W53 (0.27), W107** (0.23)
Cs-10	0.12	34.61	5	Cl5 (0.42), Arg186 NH (0.71)	Ser156 O (0.33), Asn158 O (0.34)
Cs-11	0.12	29.94	4	None	Gly47 O (0.35), Tyr57 OH (0.40)
Cs-12	0.24	45.47	5	None	Ser159 O (0.31), Thr162 OG (0.31), Ser159 OG (0.37)
Cl-1	1.00	16.00	22	Asp181 OD (0.76)	Asn185 ND (0.30), Ser184 O (0.33), Tyr263 OH (0.38), Leu257 O (0.38), W123 (0.34), Gln2** O (0.28), W54** (0.36)
Cl-3	0.74	39.36	6	Cs7 (0.71), Cs1** (0.76)	Asn240 O (0.30), Gln245 NE (0.32)
Cl-4	0.64	31.88	5	Glu195 OE (0.41), Asp172 OD (0.51), Na2 (0.63), Lys170 NZ (0.67*), Lys136 NZ (0.73*), Tyr167 OH (0.61)	Lys170 O (0.30), W3 (0.35), W87 (0.40)
Cl-5	0.48	44.62	5	Lys15** NZ (0.38), Cs10 (0.42), Arg186 NH (0.66)	Asn158 O (0.29), Asn158 ND (0.29)
Cl-7	0.39	32.75	5	Lys265** NZ (0.69), Cs3 (0.72)	Ser99 O (0.30), W147 (0.33), Asn248** ND (0.31)
Cl-8	0.27	25.81	4	Cs2 (0.49), Glu195 OE (0.66), Glu197 OE (0.76)	Ala194 O (0.30), W22 (0.32)

[a] Atoms are identified by the usual PDB codes, with O, being the alpha-carbonyl oxygen; * – the amino groups of Lys136 and Lys170 appear to be in salt bridges with the carboxylates of Asp140 and Glu195 respectively; ** – these groups are in an adjacent protein molecule in the crystal.

Results and discussion

The clear anomalous signal shows very well defined sites (>4σ level Fig. 1 and Table 2). All the caesium sites, and most of the chloride ones, had partial occupancies. The total occupancies for Cs$^+$ and Cl$^-$ sum to +3.3 and −3.5 respectively, indicating almost zero (−0.2) net charge from the counter-ions. In contrast, the caesium and chloride counter-ions in the previously determined subtilisin crystal structure in acetonitrile had a net charge of −1.7.

In protein crystallography ion's partial occupancies and ion's B_{factor} are a measure of ion mobility likelihood. It is reasonable to think that the protein molecule has only some of the sites fully occupied, and perhaps some are mutually exclusive.

The distribution of cesium ions on the subtilisin surface in an aqueous medium was predicted by molecular dynamics simulations in a preliminary study and showed good agreement with the cation sites observed here crystallographically.[23] The electrical potential surface of subtilisin is altered by the protein–counter-ion interactions at high salt concentrations (Fig. 2). Patches of positive electric potential appear to increase
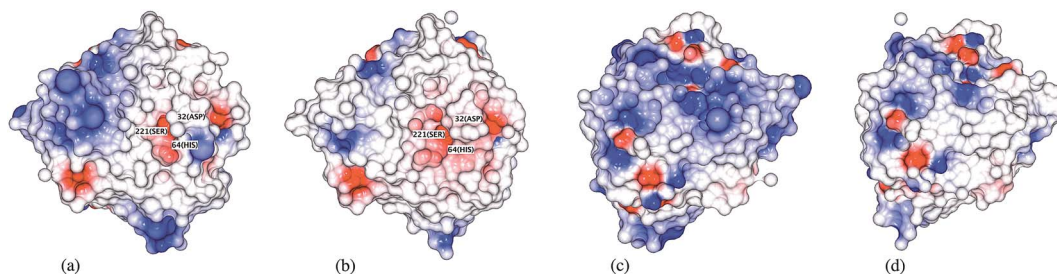

Fig. 2 Change of electrostatic potential of the protein surface. Red areas have a negative potential, blue areas a positive potential, white areas are neutral. (a) Subtilisin soaked in a CsCl salt aqueous solution; (b) in aqueous solution; (c and d) views at 180°. Electrostatic potential of the protein surface were computed using CCP4MG.[22]
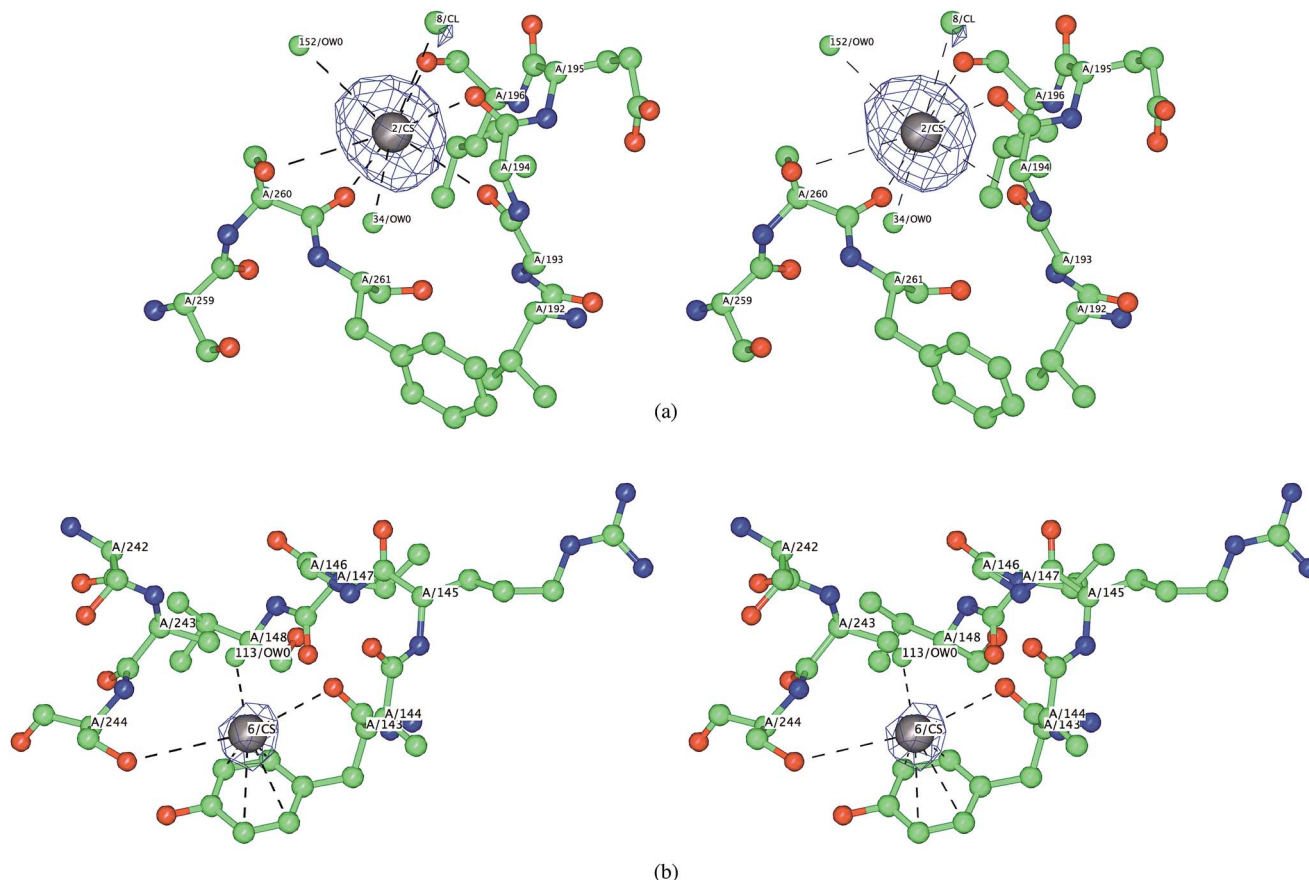

Fig. 3 Stereo view of two representative Cs$^+$ ion binding sites. Atoms colored according to atom type: blue = N; red = O; green = C, grey = Cs. Anomalous difference Fourier map contour level at 4σ. (a) Cs site 2 coordination by carbonyls; (b) Cs site 6 interacting with tyrosine ring.

in area on the protein surface overall and in close proximity of the catalytic triad (Fig. 2). Molecular dynamics simulations show a poor agreement of the distribution of chloride ions on the subtilisin surface in an aqueous medium compared with the anion sites observed crystallographically.[23] It was calculated that the surface potential of subtilisin is more negative in aqueous solution than in the crystal, limiting the binding of anions.[23] In fact, patches of negative electric potential do not show an extension of the areas in a high salt condition (Fig. 2).

Protein electrical potential surfaces are commonly calculated and discussed in structure–function relationships. However, such calculations normally do not consider any counter-ions, as well as the intrinsic difficulty of predicting ionisable amino acid side chain pKas,[24] and electric potentials are very different and even opposite in sign if counter-ions are included.[14,23] Clearly, an experimental determination of the cation and anion substructures should be sought for any of those systems where electrical potential surfaces are studied, especially at high salt concentrations.

Previous suggestions are that cations are expected to be coordinated mainly by hydrated protein side-chain carboxylates, with a minor contribution from carbonyl oxygens and other side chains.[8,10] In contrast, we observe that, most of the Cs^+ ions are predominantly coordinated by formally neutral oxygens, mostly backbone carbonyls: Fig. 3a shows Cs site 2 as an example. Two of the Cs^+ ions appear to show coordination by the aromatic rings of Tyr residues, presumably in a charge–π

interaction (Fig. 3b). Only Cs site 5 is closely associated with a charged protein group, and since it interacts with two different protein molecules, the site is probably an artefact of the crystal packing. The "law of matching water affinity"[9] would suggest relatively weak association of large cations like Cs^+ with protein carboxylates.

Most of the Cl^- ion sites have relatively few of the potential H-bond donors and they are also not near likely protein +ve charges. All the sites show coordinating interactions with an atom in an adjacent protein molecule in the crystal. Cl site 5, for instance, is placed between a Lys amino group and a Cs^+ ion associated with the symmetry related molecule and it might be a crystal packing site. In these cases we have to accept the possibility that Cl-sites are a result of crystal packing, as confirmed by the results of the molecular dynamics simulations,[23] and would not have particular affinity for counter-ions when the enzyme is in solution. This possibility is also in agreement with the observation that Cl^- binds far more weakly to the protein surface.[11]

We can also compare the counter-ion sites found in this study with those in previously reported studies, and also with one for our aqueous crystals not soaked with CsCl (Table 2). The tightly bound Ca-1 is conserved across all reported subtilisin structures.

In the Cs-1 site, three of the previously determined crystal structures reported a Ca^{2+} (Table 3). In our aqueous structure that has not had a CsCl soak, we confirm a sodium atom (Na-1)

Table 3 Equivalent ion sites in related PDB structures of subtilisin Carlsberg

Cs ion structure	Native structure	1CSE	1C3L	1SCA	1SCN	2SEC	2WUV	2WUW	1SCB	1SCD	1AF4	1BFU
Water	Water	Water	Water	Water	Water	Water	Acetonitrile	Acetonitrile	Acetonitrile	Acetonitrile	Dioxane 100%	Dioxane 20%
Cs-1	Na-1	—	—	Ca-403	Ca-403	Ca-278	Cs-1	Na-1277	W371	W598	—	W315
Cs-2	Na-3	W57	W398	W578	W582	W382	Cs-2	W2114	W357	W539	—	—
Cs-3	—	W795	—	W681	—	—	Cs-3	—	—	—	—	—
—	—	—	—	—	—	—	Cs-4	—	—	W597	—	—
Cs-5	—	—	—	W577	—	—	Cs-5	—	—	—	—	—
Cs-6	—	—	W389	—	—	W433	Cs-6	—	W113	—	—	—
Cs-7	—	W602	—	—	—	—	Cs-7	—	W398	—	—	—
—	—	W519	—	—	—	—	Cs-8	—	—	W511	—	—
Cs-9	W12	W427	W351	W627	—	W422	Cs-9	W2039	—	W629	W322	W336
Cs-10	—	W698	—	—	—	—	Cs-10	—	—	—	—	—
Cs-11	W125	—	—	—	—	—	Cs-11	—	—	—	—	—
Cs-12	—	—	—	—	—	—	—	—	—	—	—	—
Ca-1	Ca-1	Ca-1	Ca-1	Ca-1	Ca-1	Ca-1	Ca-1	Ca-1279	Ca-1	Ca-1	Ca-1	Ca-1
Na-2	Na-2	Ca-401	Ca-284	Na-402	—	Ca-277	Na-2	Na-1278	W359	Ca-403	W305	W294
Cl-1	W18	W885	—	—	—	W489	Cl-1	—	—	W582	W299	—
—	W97	W503	W404	W662	W665	W484	Cl-2	—	W285	W518	W326	—
Cl-3	—	W577	—	—	—	—	Cl-3	—	—	—	—	—
Cl-4	—	—	—	—	—	—	Cl-4	—	—	—	—	—
Cl-5	—	—	—	—	—	—	Cl-5	—	—	—	—	—
—	SO4	—	W334	—	W590	—	Cl-6	SO4	—	—	—	W304
Cl-7	—	W492	—	—	—	—	Cl-7	—	W384	—	—	—
Cl-8	—	—	—	—	W640	W463	Cl-8	—	W372	—	—	—

in this position distinguishing it from a water or from bivalent cation site. In site Cs-2, a water molecule is modelled in all five previous structures. Four carbonyl oxygens, a side chain hydroxyl and a water molecule coordinate the site. This appears more like octahedral coordination rather than a tetrahedron of waters. For all but one of the other Cs^+ sites in our structure, a water molecule has been modelled in at least one of the other aqueous structures. In many of these sites the coordination geometry does not obviously indicate a counter-ion rather than a water molecule, so without the optimized anomalous difference electron density data it would not be easily recognized. Our structure also contains a site clearly assigned to a Na^+ (Na-2). The same site has been reported as containing Na^+ in some other structures, but as a second Ca^{2+} in others (Table 3). This site showed no anomalous difference Fourier map peak at 2.070 Å wavelength, which excluded a Ca^{2+} (Ca^{2+} f" at this wavelength is 2.95 e$^-$). The assignment as a calcium is not supported by our findings.

Turning to the Cl^- sites we identify, four of the six sites have been modelled as containing waters in one or more of the previously reported crystal structures.

We can also compare the counter-ion sites with those found in our previous subtilisin crystal structure soaked with CsCl and acetonitrile (2WUV).[14] All but one Cs^+ site, and all the Cl^- sites, were also found in this acetonitrile structure (Table 2). In addition, the acetonitrile based crystal structure had further counter-ion sites, 2 Cs^+ and 2 Cl^-, which are not found in the aqueous crystal structure. The favoured sites for ion interactions are not very different in acetonitrile and aqueous media in subtilisin.

Conclusions

The present study extends the understanding association of counter-ions with the surface of subtilisin and of proteins in general. Subtilisin shows significant numbers of defined counter-ion sites even in aqueous conditions i.e. with its large dielectric constant. Most counter-ion sites did not involve close interaction with formal protein charges. The presence of these counter-ions substantially changes the protein electrical potential surface, and which is an effect commonly discussed in relation to function.

The interactions between proteins and counter-ions may have important influences on enzyme function, especially in systems where quite high salt concentrations are encountered. To understand these effects, it is important to have information about the sites of interaction, and which we provide in this case study.

Acknowledgements

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