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Crystallographic Analysis of Counterion Effects on Subtilisin Enzymatic Action in Acetonitrile

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Abstract: When enzymes are in low dielectric nonaqueous media, it would be expected that their charged groups would be more closely associated with counterions. There is evidence that these counterions may then affect enzymatic activity. Published crystal structures of proteins in organic solvents do not show increased numbers of associated counterions, and this might reflect the difficulty of distinguishing cations like Na^+ from water molecules. In this paper, the placement of several Cs^+ and Cl^- ions in crystals of the serine protease subtilisin Carlsberg is presented. Ions are more readily identified crystallographically through their anomalous diffraction using softer X-rays. The protein conformation is very similar to that of the enzyme without CsCl in acetonitrile, both for the previously reported (1SCB) and our own newly determined model. No fewer than 11 defined sites for Cs^+ cations and 8 Cl^- anions are identified around the protein molecule, although most of these have partial occupancy and may represent nonspecific binding sites. Two Cs^+ and two Cl^- ions are close to the mouth of the active site cleft, where they may affect catalysis. In fact, cross-linked CsCl-treated subtilisin crystals transferred to acetonitrile show catalytic activity several fold higher than the reference crystals containing Na^+. Presoaking with another large cation, choline, also increases the enzyme activity. The active site appears only minimally sterically perturbed by the ion presence around it, so alternative activation mechanisms can be suggested: an electrostatic redistribution and/or a larger hydration sphere that enhances the protein domain.

Introduction

There is interest, both preparative and fundamental, in the behavior of enzymes in low-water media, such as those based on organic solvents.[1−7] Some aspects can be qualitatively different from aqueous solution, such as the role of counterions to charged groups in proteins. Proteins carry a number of different charged groups, some of which are usually essential for proper function. Under most conditions, the molecule will have a net charge, and electroneutrality requires that this must be balanced by counterions. In aqueous solution, the high dielectric allows these counterions to be some distance from the protein, so their presence and identity can normally be disregarded in discussions of function. In low dielectric media, however, close approach and ion-pairing of counterions will be strongly favored. Hence, they may well have important effects on function. There is evidence[8,9] that counterions affect the activity of enzymes in mainly organic media. Essentially, protein ionization (and hence enzymatic activity) becomes dependent on the availability of counterions as well as of H^+.

However, direct evidence for the location of counterions has been lacking. A number of 3D structures have been determined for protein crystals that have been rinsed or soaked with organic solvents.[10−15] With nonpolar organic solvents having little capacity to dissolve water, like hexane, it is likely that much of the water in the crystal is not removed by the rinsing procedure. Hence, in these cases the protein environment remains substantially aqueous and counterions may be relatively distant and

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mobile. In contrast, with more polar solvents like acetonitrile or dioxane, the removal of weakly associated water is likely to be effective[16] (the soaking medium will reach a low water activity). Although we might expect ion-pairing to be promoted under these conditions, the structures reported in polar organic solvents have not shown this. However, the cations most commonly used in protein crystallization, Na^+ and NH_4^+, are isoelectronic with water and may often be modeled as water oxygens, although their coordination with other atoms is monitored. A further issue is that counterions may be rather more mobile, or perhaps have several alternative sites with partial occupancies, which would make them hard to resolve. Counterion sites can be located more clearly if heavier elements are used.[17−23]

The protease subtilisin Carlsberg has the status of a model in low-water enzymology, and structures have been reported for crystals soaked in dioxane[24] and acetonitrile.[25,14] It has a catalytic Asp-His-Ser triad, which must bear a net negative charge for good catalytic activity, as found in alkaline aqueous solutions. The possible counterions in low dielectric media and their effects have been discussed.[8] Counterion effects may also contribute to a much studied phenomenon when lyophilized enzyme powders are used as catalysts in organic media. Activity of subtilisin and other enzymes is increased enormously by drying with an excess of neutral salts like KCl,[26−31] and specificity can also be favorably altered.[29]

In this paper, the placement of ions in crystals of subtilisin Carlsberg soaked with CsCl and acetonitrile is investigated. In parallel, the catalytic activity of the crystals suspended in acetonitrile has been determined. Interestingly, treatment with CsCl was found to increase the activity several fold. Using X-ray crystallography with anomalous dispersion, clear sites for Cs^+ and Cl^- ions are identified around the protein molecule, where they may be affecting catalysis. Correct identification of the ion sites helps the modeling of electrostatic potential surfaces and, thereby, of the enhanced catalytic activity in the presence of CsCl in acetonitrile.

Material and Methods

Crystallization. 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. Subtilisin was crystallized by the batch method from buffer solution saturated with Na_2SO_4 ~13% (w/v) as precipitant.[32] Long needle-like crystals grew over a period of two weeks of typical dimensions $50 \times 50 \times 400\ \mu m^3$. They were stored in mother liquor for up to 6 weeks, with no evidence for difference in catalytic activity over this storage period.

Cs Soaking, Cross-Linking, and Acetonitrile Wash. Soaking with CsCl or other salts and glutaraldehyde cross-linking was performed in a single step, to reduce the number of washes and transfers. This reduces the chances of osmotic shocks to the crystals (very often fatal for the quality of diffraction). So the soak solution was prepared freshly by mixing 2 volumes of 25% aqueous glutaraldehyde with 3 volumes of 250 mM Na-cacodylate buffer pH 6.5 containing 25% v/v glycerol and, where required, 1.5 M salt. Crystals were soaked in this for 15 min, with vortexing in the case of suspensions of multiple crystals for activity assay. Washing in acetonitrile followed immediately. For crystallography, individual crystals were washed 5 times with acetonitrile for 10 s, again following the protocols described elsewhere.[25] For catalytic activity measurements, washing required vortexing and settling of the suspension, with each wash taking about 90 s. There were two washes in pure acetonitrile, then two in the reaction medium, acetonitrile containing 1% (v/v) H_2O and 1 M 1-propanol. Finally, crystals were resuspended in reaction medium and preincubated at 25 °C with shaking for 20 min, with vortexing and if necessary gentle poking to break up aggregated crystals.

Catalytic Activity Determination. A solution of *n*-acetyl tyrosine ethyl ester in the reaction medium was added to the suspension of crystals to give a final concentration of 10 mM. The reaction mixture was incubated with shaking at 25 °C, with periodic removal of small supernatant samples for analysis. Conversion to the propyl ester was followed by HPLC as described,[33] and the initial rate was estimated from at least 5 points on the linear progress curve. After the reaction, the mixture was centrifuged and the supernatant was decanted. The pellet of crystals was solubilized by heating in 1 M NaOH at 100 °C for 30 min, then the protein content was estimated from the A_{280} of the cooled solution. This value was used to estimate the initial rate in specific activity units.

Crystallography Data Collection and Structure Refinement. Data collection was performed on SRS BL10,[34] Daresbury Laboratory. A fluorescence scan showed a peak at 5721.51 eV. Diffraction data were collected using softer X-rays[35,36] at the Cs L1 edge (2.167 Å) and processed using the HKL2000 package.[37]

The structure was solved by molecular replacement using MolRep,[38] and refined with Refmac 5.0.[39] Finally, anomalous difference Fourier maps were calculated and electron density peaks contoured at 4sigma level or higher were taken into consideration. Ions were assigned on the basis of coordination geometry, map peak height, B-factor, and occupancy of each site. Final R_{factor} and R_{free} were 0.16 and 0.23 respectively for the Cs^+ ion model (Cs-ACN).

The plot of $\langle|\Delta F_{anom}|/\sigma\Delta F_{anom}\rangle$ versus resolution for the Cs-ACN model indicates a strong anomalous scattering signal across the

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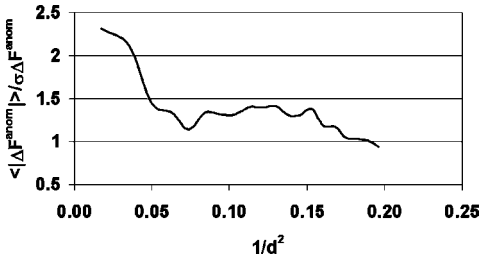

Figure 1. Plot of $\langle|\Delta F_{anom}|/\sigma\Delta F_{anom}\rangle$ versus $1/d^2$, where d is the interplanar spacing, for Cs-ACN. The diffraction anomalous signal is above one across the whole resolution range.

Table 1. Effect of Salt Soaks on Catalytic Activity of Subtilisin Crystals in Acetonitrile

added salt in soak solution	initial rate[a] (nmol $min^{-1}mg^{-1}$)
None	2.4 ± 0.8
KCl	5.6 ± 2.6
Choline-Cl	10.8 ± 3.7
CsCl	13.7 ± 5.2

[a] Rates are shown as mean ± standard deviation.

whole diffraction resolution range (Figure 1). A native model (ACN) was also collected and data refined as before. Native crystals were cross-linked and soaked in acetonitrile, preparing solutions as before without the addition of salt. Final R_{factor} and R_{free} were 0.18 and 0.23, respectively. Coordinates and structure factors have been submitted to the Protein Data Bank (2wuv and 2wuw PDB codes). Data collection, processing, and refinement statistics are reported in Supplementary Materials 1, Supporting Information.

Results and Discussion

Catalytic Activity Is Enhanced after Soaking in CsCl. Crystals for activity measurements were treated essentially identically to those used for crystallography, to allow comparison of the observations. Hence, crystals had a 15 min soak in a glutaraldehyde cross-linking solution, which also contained the added salts when tested, followed by washing in pure acetonitrile. As Table 1 shows, the inclusion of CsCl in the cross-linking soak solution led to significantly increased activity in acetonitrile. It appears that a KCl soak may also increase the rate somewhat, while choline-Cl has a larger effect. All of these specific activities are rather lower than for the cross-linked crystals normally used,[33] perhaps reflecting mass transfer limitations in the larger crystals grown for crystallography. The catalytic activity measured was also less reproducible than for other catalyst forms of subtilisin in acetonitrile. This may reflect variation in crystal sizes in different reactions, due to random sampling and/or the effect of soaking treatments. The crystals aggregated on transfer to acetonitrile, and the aggregates could be quite hard to disperse. Microscopic examination of crystals in the final acetonitrile suspensions showed that they were essentially dispersed back to individual crystals, but that the long needles observed for the original aqueous crystals had been broken to shorter lengths.

Soaking Crystals in CsCl Reveals Many Nonspecific Sites for Counterion Association. Using softer X-rays, Cs^+ ions give a strong anomalous signal from their L1 edge across the whole diffraction resolution range with an f″ of 10.42 e^- at 5767 eV. At the same energy a Ca^{2+} ion has an f″ of 2.68 e^- and Cl^- has an f″ of 1.57 e^-. An anomalous difference Fourier map was calculated using the phases of just the refined model without any ions. Display at the 4 sigma level allowed identification of a number of sites showing favorable association of ions with the protein, as expected. However, even the low occupancy sites could be modeled with confidence, because of the clear electron density derived from the anomalous dispersion measurements from these atoms. The sites were further confirmed by inspecting the isomorphous difference Fourier map. Given that Cs^+ and Cl^- atoms cannot be assigned only on the basis of the presence of an anomalous difference Fourier map peak, additional attention was paid to the coordination geometries, B-factors and occupancies in order to distinguish between Cs^+ and Cl^-. The overall occupancy was modeled to match the average B-factor of the residues surrounding the site, computed using the program BAVERAGE.[40] Rather surprisingly, there are no less than 11 Cs sites identified (full details in Supplementary Materials 2, Supporting Information). All of these sites are modeled with partial occupancy, varying from 0.6 to 0.1. Moreover, an additional 8 Cl^- sites are identified, with occupancies varying from 1.0 to 0.2. Cs^+ ions bound to subtilisin presenting two types of coordination. The most commonly present is octahedral often slightly distorted as shown by the Cs-1 for instance (Figure 2a). Another two Cs^+ ion sites present a cation-π coordination with the Cs^+ ion held above the aromatic ring of a tyrosine (Figure 2b). The coordination of chloride ions is mostly triangular or square pyramidal or octahedral where the chlorine occupies one vertex (Figure 2c).

The partial occupancies show that each individual site does not have a very high affinity. However, a plausible interpretation is that electroneutrality demands that several of these sites are occupied around every protein molecule but that there is some freedom for the ions to select between alternative sites. Ions with low occupancies also have a less defined geometry, probably due to a higher turnover of their ligands or a more variable position of the ion itself.

Counterion Sites Close to the Active Site. The active form of subtilisin has a negative charge on the catalytic triad, formally located on the Asp-32 carboxylate. There are no obvious balancing positive sites close by in the protein, so the need for associated counterions in nonaqueous media has been proposed.[8] Figure 3a,b shows that there are two Cs^+ sites (3 and 10) near the entrance to the active site cleft. The Cs-3 site is 6.6 Å away from the Cl-7 site, and Cs-10 is closely associated with Cl-5 with the overall net charge modeled as weakly negative. Under the relatively acidic conditions that are needed for subtilisin crystallization, the catalytic triad is likely to have only a partial negative charge. The presence of counterions near the entrance to the active site is however a plausible reason for effects on catalytic activity.

Most of the Closest Cation Interactions are with Carbonyl Oxygens, Not Formal Negative Charges. Turning to the remaining Cs^+ sites, the remarkable observation is how few of them are actually very close to potential formal negative charges. Only Cs-5 appears to be coordinated by a protein charge (the carboxylate of Glu-54). Cs-1 and Cs-10 are coordinated by chloride ions (Cl-8 and Cl-5), and Cs-8 is rather further (4.7 Å) from Cl-2. For all other sites negative charges are more than

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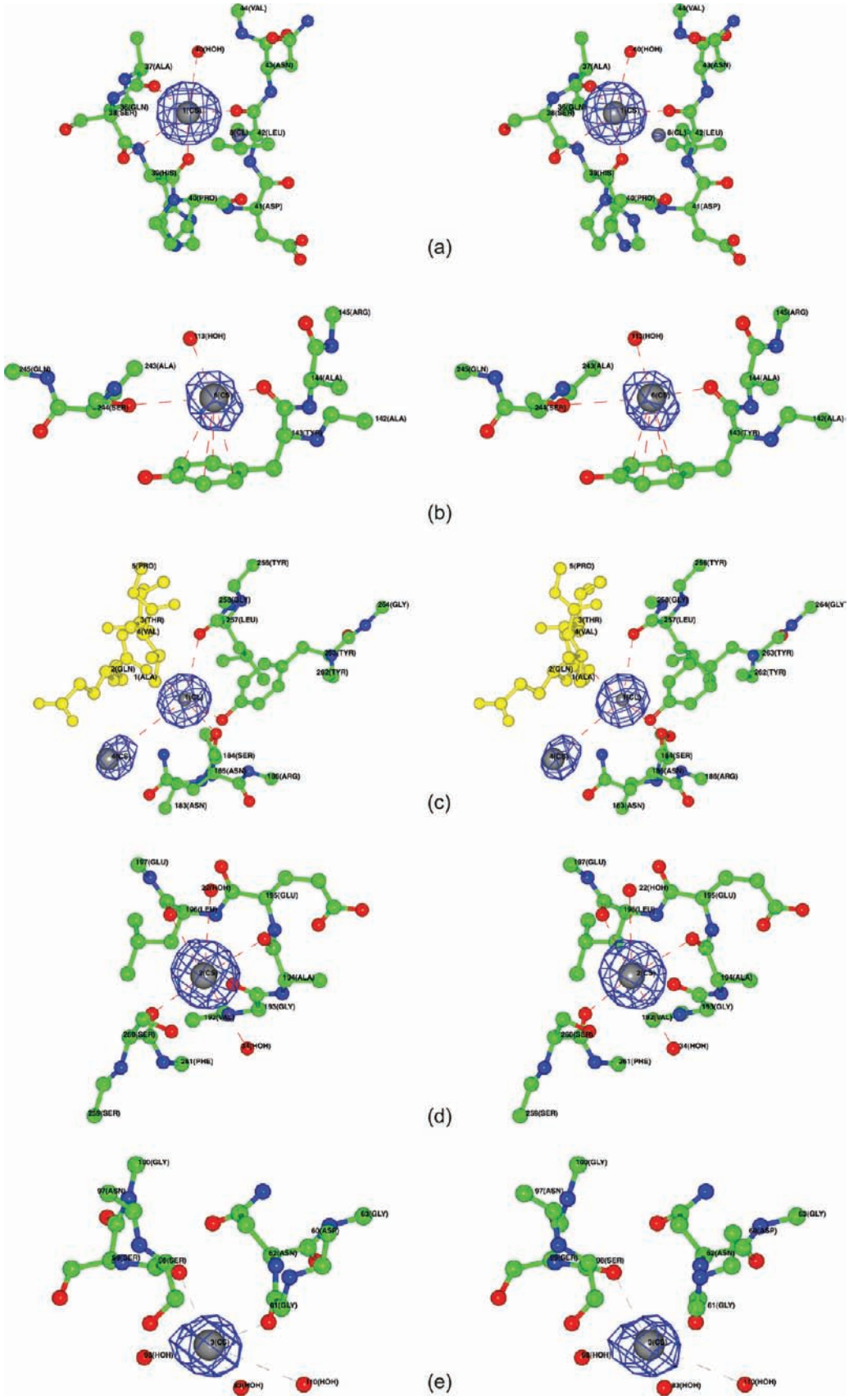

Figure 2. Stereoview of selected counterion sites identified. The anomalous difference Fourier map is shown cut at a 4 sigma contour level. Sites are Cs-1 (a); Cs-6 (2b); Cl-1 (2c); Cs-2 (2d); and Cs-3 (2e). The yellow atoms in (c) come from an adjacent protein molecule in the crystal. Graphics were made with Molecular Graphics.[40]

5 Å away, often more than 7 Å, with formally neutral atoms much closer. From Cs-7, water-46 may be bridging an interaction with the Asp-120 carboxylate at 5.3 Å. All the cations must be neutralizing partial negative charges spread around in the

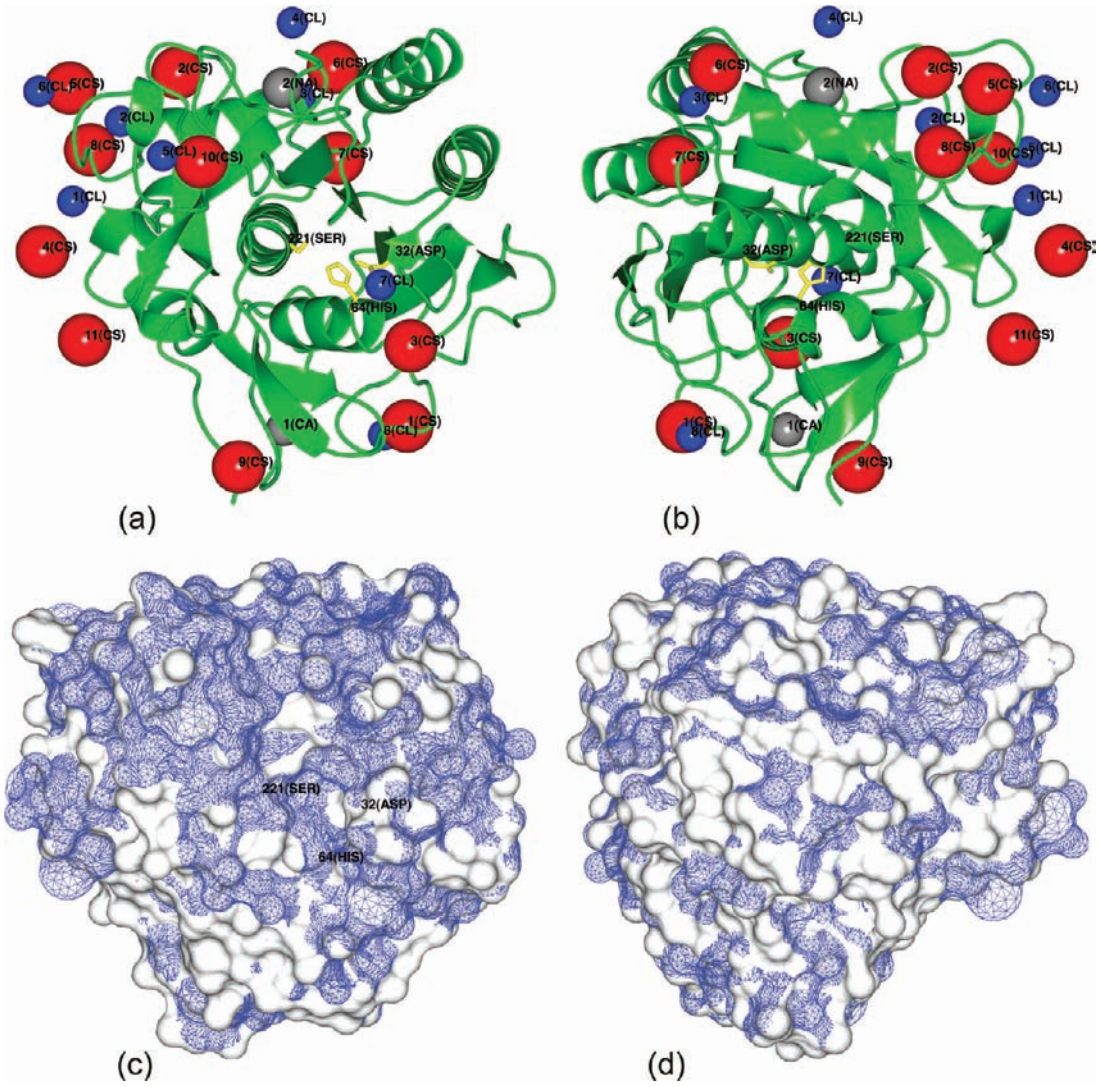

Figure 3. (a) Cesium and Chloride sites around the active site of subtilisin Carlsberg. View down the cleft to the catalytic triad (in yellow). Cs are in red, Cl in blue and the Ca and Na in gray, all as space-filling spheres; (b) view at 180°. (c) Increase in protein surface for the Cs soaked subtilisin (area 9988.7 Å^2, in blue) when compared to the native one (area 9959.5 Å^2, in white). In particular, the region around the active site is altered; (d) view at 180°.

crystal, but it is clear that reasonable separation of charges is possible even in the acetonitrile environment.

The most common close interaction with the protein is in fact with formally neutral backbone carbonyl oxygens. Figure 2a shows the strongest peak (Cs-1) appearing on the anomalous difference Fourier map at a 39 sigma level as a distorted octahedral coordination with four carbonyl oxygens, with a water and a chloride ion. The last of these was assigned in part because it showed a positive anomalous signal. Cs-2 (Figure 2d) again shows distorted octahedral coordination by four carbonyl oxygens, together with a Ser side-chain oxygen and one or two waters. For Cs-3 (Figure 2e) the geometry is again distorted octahedral, with coordination by two carbonyl oxygens and four waters. Similar coordination is seen with Cs-4 and Cs-8 and the remaining Cs sites with different degrees of distortion. Cation coordination by carbonyl oxygens appears to be a common feature of proteins.[41] Two cations, Cs-5 and Cs-6 (Figure 2b), appear to be interacting with aromatic rings of Tyr residues, with placement more or less over the face of the ring and distances to carbons below being 4 Å. This type of cation-π interaction in protein structure and function has been receiving more and more interest.[42,43]

In two cases, Cs^+ sites are found near the C-termini of alpha-helices, where the helix dipole would make this a favored position for positive charge. Cs-6 is close to the helix terminus at residues 142−145, and Cs-8 is close to the terminus at residues 251−254.

Cs-4, Cs-5, and Cs-8 show close interactions with two adjacent protein molecules in the crystal. These sites have generally a very distorted octahedral geometry. Thus they reflect the particular packing in the crystals studied, and may well not be favored sites for cation interactions in other crystal forms or noncrystalline protein, as often used catalytically in nonaqueous media.

WASP Analysis. The WASP program (WAter Screening Program)[44] was used to assess the specificity of the observed Cs ion positions. The observed Cs^+ ion positions were assigned to putative waters and Cs-specific valence values calculated. A

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valence value ≥1 is the condition necessary and sufficient to define a monovalent cation binding site.[45] The ligation distance was set to 4.0 Å, with only oxygen, waters and nonprotein atoms treated as ligating atoms. None of the Cs^+ sites has a valence value ≥1, meaning that they are not specific (values in Supplementary Material 2, Supporting Information). For instance Cs-3 site (Figure 2e), close to the active site, while it scores highly (valence value 0.685), is still likely to be nonspecific. This may indicate that these sites are not of very high affinity, in line with our findings that crystallographic occupancies are less than 1. Nevertheless, these sites could be occupied by similar monovalent cations like Na^+ or K^+, especially for sites with valence >0.5, and this needs to be considered in discussing function. This is particularly relevant when the enzyme is used in the solid state as a catalyst in nonaqueous media, especially as crystals.[46−49]

The ionic radius of Cs^+ (1.66 Å) is considerably larger than that for K^+ (1.33 Å) and particularly Na^+ (0.95 Å), the alkaline ions more likely to be relevant to normal enzyme behavior. However, there are indications that these relatively weaker counterion sites are quite flexible in terms of size that can be accommodated, as might be expected. In fact Rb^+ (1.48 Å) has been successfully used to replace and identify the position of Na^+ in thrombin,[44] while sites in RNA can be identified with Cs^+, albeit at lower occupancy than with the smaller Tl^+.[17]

Comparison with Other Structures. Data were collected and refined from crystals treated with ACN, with (model Cs-ACN) and without (ACN) CsCl soak. By superposing the 1SCB, the Cs-ACN and the ACN models, the calculated C-alpha rmsd's are: ACN vs 1SCB (25), 0.331 Å; Cs-ACN vs ACN 0.188 Å; Cs-ACN vs 1SCB, 0.332 Å. Considering that the average Cruickshank DPI or coordinate error[50] of these structures at 2.3 Å resolution is ∼0.39 Å we conclude that the peptide conformation of the backbone conformation is essentially identical in all 3 models.

There are a few changes in side-chain conformations between Cs-ACN and ACN, where, overall, their functional groups had moved between 2 and 4.4 Å. One move, of the carboxyl group of Asp-172 away from Cl-4, has a clear link to counterion interaction.

Ca^{2+} and Na^+ Ion Sites. Previous subtilisin crystal structures reported between two to three Ca^{2+} binding sites (pdb codes: 1CSE,[48] 1C3L,[49] 1SCA,[25] 1SCB,[25] 1SCN[25] and 2SEC[51]). By comparing the native and the Cs-soaked anomalous difference Fourier maps, it is possible to confirm only one genuine Ca^{2+} binding site, where strong peaks can be seen in the anomalous difference Fourier maps for both Cs-ACN and ACN. This site is modeled as a Ca^{2+} in all the previous structures. The second putative Ca^{2+} site (pdb codes: 1CSE,[48] 1C3L,[49] 1SCD[25] and 2SEC[51]) is, from our data, a possible Na^+ site (Na-2 in both our models), given that there is no peak in the anomalous difference Fourier maps. The second Ca^{2+} site in the previous structures has either partial occupancy or a B-factor a bit higher than the surrounding atoms, suggesting it may be a mistakenly assigned Na^+. Finally the assignments of sites equivalent to the Cs ion positions can be compared to other subtilisin Carlsberg structures. Site Cs-1 in Cs-ACN is confirmed as a genuine monovalent cation site, assignable to a Na^+ ion (Na-1) in the ACN structure. In the previously described acetonitrile structures 1SCB and 1SCD, a water is found in this position, while in the structure 1SCA, as well as 1SCN and 2SEC, this is assigned to a Ca ion. As noted, it is entirely possible that, in those structures, the site was occupied by Na^+ ions that were not clearly identifiable from the electron density maps. In our case the assignment is made easier by the presence or absence of anomalous difference Fourier map peaks in the data. Full details of the site comparisons are in Supplementary Material 3, Supporting Information.

Chloride Ion Sites. Cl-1 (Figure 2c) is placed between two protein molecules, at the vertex of a distorted triangular pyramid, given by the coordination with two side chain atoms (OH Tyr 263, NH Asn 185) and a water. Cl-5 and Cl-8 are clearly coordinating Cs^+ as counterions, while Cl-2 is also 4.7 Å away from Cs-8. Similarly to Cl-1, Cl-3 is on the vertex of a pyramid whose base is given by NH Gln 245 at 2.91 Å, and two waters at over 4 Å distance. The remaining Cl^- ions are organized in similar coordinations. Interestingly, all of the Cl^- ions have carbonyl groups in proximity making the ion identification process more difficult. Moreover, there appears not to be many comprehensive studies of negative ion coordination in proteins to assist in such distinction.

Counterion Total Charge. It is interesting to consider the total counterion charge found in the Cs-ACN model. The occupancies of the 11 Cs sites sum to a total of 2.90 positive charges, to which must be added the 3+ of one Ca and one Na. The 8 chloride sites have a summed occupancy of 4.65. In the acetonitrile soaked crystals it is most likely that there are no freely mobile counterions that are missed by the anomalous dispersion measurements. Hence, we estimate a net charge on the polypeptide of −1.25. From the amino acid composition of subtilisin Carlsberg, the ExPASy server calculates an expected isoelectric point of 6.57, close to the pH 5.6 used for crystallization. However, the net charge on the protein molecules in the crystal may be somewhat different after soaking with Cs-solution and washing with acetonitrile, reflecting preferential counterion association in the crystal. Note that the experimental p*I* of subtilisin Carlsberg is 9.4 (Sigma reference standard, http://www.sigmaaldrich.com/sigma/product%20information%20sheet/p5380pis.pdf), which presumably reflects one or two bound Ca^{2+} that comigrate with the protein in electrophoresis.

Acetonitrile Molecule Positions. The structure 1SCB[25] includes 12 acetonitrile molecules bound to subtilisin, while 5 acetonitriles have been identified in each of our own structures (Table 2). Relatively small molecules like acetonitrile often cannot be identified with certainty in medium resolution (2.3−2.4 Å) structures like these. We used the following approach, similar to guidelines already reported:[25] (a) 2Fo-Fc maps at one sigma level were searched for oblate shapes of 2.6−2.7 Å length (overall length of an acetonitrile molecule); (b) geometric and environmental constraints like proximity and type of surrounding residues were considered; (c) occupancy

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Table 2. Occupation of Acetonitrile Sites in the 3 Models[a]

1SCB	ACN	Cs-ACN
CCN-404	Empty	W-99
CCN-405	CCN-1[b]	CCN-1
CCN-406	CCN-2[c]	CCN-2
CCN-407	Empty	Empty
CCN-408	CCN-3[d]	CCN-3[d]
CCN-409	CCN-5	Cs-8 and W-113
CCN-410	CCN-6[e]	NH_2's of Arg-145
CCN-411	Empty	W-69
CCN-413	W-133	CCN-4[f]
CCN-414	Empty	Empty
CCN-415	Empty	Empty
CCN-416	Empty	Empty
W-383	W-107	CCN-5

[a] ACN is the usual abbreviation for acetonitrile in enzymatic studies and CCN is the conventional PDB id. Where there are significant differences in the positions of the acetonitrile molecules (by more than about 1 Å), this is indicated by footnotes. The electron density offers essentially no information about the direction of the linear acetonitrile molecule, and suggestions can only be made on the basis of putative interactions with other atoms. Hence reversal of assigned direction is probably not so significant. Refinement of some of the acetonitrile molecules in our structures led to significant deviations from linearity (with energetic cost), e.g. CCN-5 in model ACN. This may be an indication that the electron density is actually due to waters or counterions. [b] Reversed direction. [c] Moved about 2 Å in direction of CH_3. [d] Reversed direction and tilted (45° in ACN). [e] Tilted by 60° and moved 1.4 Å. [f] N in same place, but tilted at 90°.

was estimated on the basis of B-factors of the acetonitrile molecule (two carbons and one nitrogen) and their respective surrounding atoms, and no fractional occupancies. With these restraints reasonably reliable identification is possible, but the number of small molecules identified may well increase with the quality of the data as well as the resolution.

As Table 2 shows, three acetonitrile molecules are conserved across all 3 models, while three more are present in 1SCB and one of our models. The fully conserved sites include CCN-405 and CCN-406 (numbering scheme of 1SCB) which are present near the active site, with the former probably accepting a hydrogen bond from the catalytic Ser-221 hydroxyl.

CCN-413 (1SCB) and CCN-4 of model Cs-ACN have nitrogens in similar positions, but with the rest of the molecule in very different orientations. In 1SCB, there is a plausible hydrogen bond accepted from the backbone amide of Ala-52 (2.49 Å between nitrogens). Given that W-108 of Cs-ACN is in a similar position to the CH_3 of CCN-413 in 1SCB, it might be thought that the axis of the acetonitrile in Cs-ACN could be turned to match 1SCB. However, this interpretation did not fit the electron density as well. An interesting case is CCN-409 (1SCB), which agrees with CCN-5 (ACN), but where Cs-8 and W-113 are found in Cs-ACN (although not exactly in the same line as the CCN axis). This may be a real difference in site occupancy, but one can also speculate that a Na^+ ion and a water in the first two structures could have been modeled instead as an acetonitrile.

Sites at which organic solvent molecules are located may give clues about potential binding regions for natural effectors or designed ligands such as drug leads, as already shown in other studies.[13] The comparison above shows that careful attention to site identification is important if this approach is to be viable. Correct identification of counterion sites will have important implications in understanding organic ligand binding in soaked protein crystals.

Implications of the Presence of Counterions on the Electrostatic Potential Maps. Taking into account the counterions leads to substantial effects on calculated electrostatic potentials at the protein surface. In general there is an increase in the surface with weak positive potential. Figure 4 shows the electrostatic potential for the ACN and Cs-ACN models. A patch of weakly negative surface near the active site His-64 in the ACN model, becomes positive after taking into account counterions in the region (in particular Cs-3). It is also evident that the presence of negative or weakly negative patches on the unliganded protein model is not a sufficient condition for attracting positive counterions charges directly. It is evident that charge neutralization does not happen locally and directly, but rather with an averaged charge network spread over the whole surface.

Surface electrostatic potential maps and molecular dynamics are commonly used in discussing protein function. They are normally calculated on the basis of charges assigned to protein atoms, and perhaps the influence of associated waters. However, if there are actually associated counterions, these will have a major effect on electrostatic potentials. This should be taken into account if correct understanding of protein behavior in nonaqueous media is to be achieved.

Counterions will also affect protein interactions with hydration water. As shown above, Cs^+ and Cl^- ions find and exploit coordination sites not suitable for water molecules, like the cation-π ones, or short H bonds to anions. These ions, in turn, create new coordination opportunities for water molecules, thereby increasing the radius of the water binding shell around the protein. In Figure 3c and d, it is possible to see that the Cs^+ soaked protein shows a small increase (0.03%) in surface (in blue) when compared to the native one (in white). In particular, the region around the active site is affected.

Effect of Counterions on Catalytic Activity. As shown above, the type of counter-cations present has a significant effect on the catalytic activity of subtilisin crystals in acetonitrile. The crystallographic evidence points to the mechanism behind this—the counterions do indeed become closely associated with the protein in the nonaqueous medium. The clearly identified counterion sites not far from the active site are the most obvious candidates for the main effect. They might interfere with catalytic turnover either by electrostatic or steric effects, and these may influence approach of the substrate or the catalytic reaction.

It is less obvious why the rate should be increased when Cs^+ ions are bound. At first sight, their larger size would be more likely to interfere sterically with substrate binding, while their electrostatic effect would be similar to Na^+. It is possible that the large size of the Cs^+ ions forces them to lie rather further away from the active site, and hence actually interfere less. As noted, although Na^+ ions were not identified close to the active site in either our ACN model or the previously published 1SCB, the crystallographic data were not sufficient to distinguish the small cation from water. A link with countercation size is supported by the catalytic activity trend of crystals soaked with KCl and choline-Cl (Table 1).

It should be noted that acetonitrile has a relatively high dielectric (37.5) for an organic solvent. Hence, the driving force for close association of charged groups and counterions will not be as strong as in less polar solvents (although more than twice that in aqueous solution). The thermodynamics of separating balancing charges will also be influenced by the effective dielectric action of the protein structure itself.

Alternatively, it is possible to suggest a mechanism of electrostatic redistribution, in which polarization of water molecules or amino acid side chains cause effective spreading

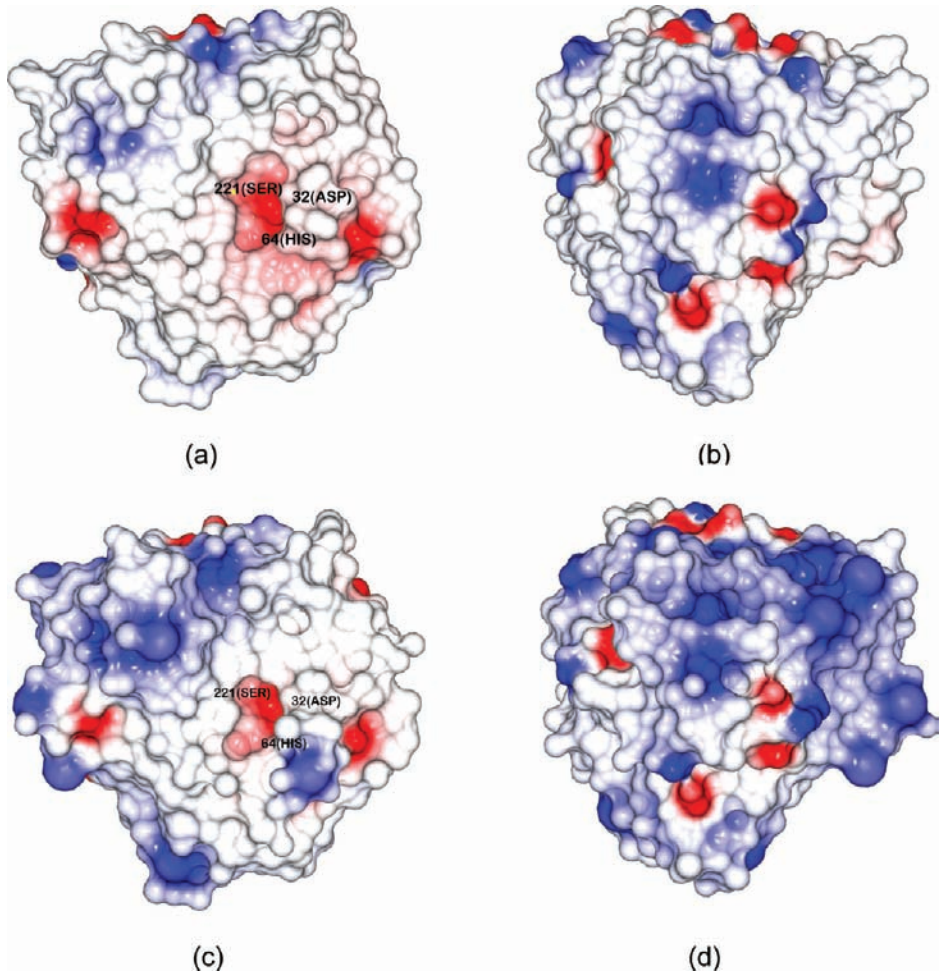

Figure 4. Change of electrostatic potential of the protein surface. Red areas have a negative potential, blue areas a positive potential, white areas are neutral. (a) subtilisin soaked in acetonitrile only, without Cs salt addition; (b) view at 180°; (c) subtilisin soaked in acetonitrile after Cs salt addition; (d) view at 180°.

of the counterion charge. The redistribution of the charge would be greater with ions like Na^+ and K^+ for which the water coordination sphere is more strongly polarized, while the polarization, and hence the redistribution of the charge, would be weaker for Cs^+ and choline ions. In a low dielectric medium a stronger redistribution of a positive charge for the smaller ions might partially compensate the overall negative charge of the catalytic triad thus reducing the enzymatic catalytic activity, as observed.

Conclusions

The enzymatic activity of subtilisin crystals in acetonitrile is sensitive to the type of counterions present before transfer to the organic solvent. Larger cations increase the enzyme activity. Anomalous dispersion from Cs^+ and Cl^- counterions at longer X-ray wavelength allows several of their sites to be much more confidently identified in protein crystals. This confirms that subtilisin Carlsberg in acetonitrile has a large number of associated ions. Correct identification of counterion sites appears to be important for a proper understanding of enzyme activity in nonaqueous media. Moreover, the knowledge of counterion sites allows for a better description of the electrostatic interactions of proteins. Although this study has been concerned with the nonphysiological situation of a protein surrounded by acetonitrile, similar effects could also apply in low-dielectric environments like lipid bilayers within the biological cell.

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Supporting Information Available: Supplementary materials 1−3 (tables). This material is available free of charge via the Internet at http://pubs.acs.org.

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