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Enzyme crystal structure in a neat organic solvent

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ABSTRACT **The crystal structure of the serine protease subtilisin Carlsberg in anhydrous acetonitrile was determined at 2.3 Å resolution. It was found to be essentially identical to the three-dimensional structure of the enzyme in water; the differences observed were smaller than those between two independently determined structures in aqueous solution. The hydrogen bond system of the catalytic triad is intact in acetonitrile. The majority (99 of 119) of enzyme-bound, structural water molecules have such a great affinity to subtilisin that they are not displaced even in anhydrous acetonitrile. Of the 12 enzyme-bound acetonitrile molecules, 4 displace water molecules and 8 bind where no water had been observed before. One-third of all subtilisin-bound acetonitrile molecules reside in the active center, occupying the same region (P_1, P_2, and P_3 binding sites) as the specific protein inhibitor eglin c.**

The past several years have witnessed the emergence of nonaqueous enzymology (1–5); it has become clear that not only can ordinary enzymes [and antibodies (6–8)] function in organic solvents containing little or no water, but when placed in such an utterly unnatural milieu they exhibit unusual properties, including the ability to catalyze additional reactions, markedly altered specificity, and "molecular memory." To understand the mechanistic basis of these phenomena, it is critical to elucidate protein conformation in organic media and compare it with that in aqueous solution. This task has been achieved in the present work by means of high-resolution x-ray crystallography; remarkably, the three-dimensional structure of the serine protease subtilisin Carlsberg in anhydrous acetonitrile has been found to be essentially indistinguishable from that in water.§ Moreover, much of the water structure around the protein is identical to that in aqueous medium. In addition, several acetonitrile molecules are uniquely bound to the protein, mapping an interaction surface distinct from that for water.

METHODS

Crystallization. Subtilisin Carlsberg, an alkaline serine protease from *Bacillus licheniformis* (EC 3.4.21.14) purchased from Sigma (type VIII), was crystallized by batch precipitation from aqueous Na_2SO_4 as described (9). The long prismatic crystals obtained were divided into two portions. The first was used to determine the enzyme structure in water at a higher resolution than is currently available (10), so that the number and positions of bound water molecules could be established. The second batch of crystals was used for nonaqueous structure analysis.

Solvent Infusion. Attempts to gradually replace the interstitial water with acetonitrile failed; when the organic solvent content reached ≈50% (vol/vol), the enzyme crystals invariably fractured. To fortify the crystals, we cross-linked them lightly with glutaraldehyde following the general methodology of Quiocho and Richards (11–13). [Cross-linked enzyme crystals have been recently used as practical biocatalysts (14).] Gratifyingly, treatment of large crystals with 1.5% glutaraldehyde solution in 30 mM aqueous sodium cacodylate buffer (pH 7.5), containing 15% Na_2SO_4, for 20 min resulted in subtilisin crystals that remained intact and diffracted to 2.3 Å resolution even in anhydrous acetonitrile. Conditions of cross-linking are critical. For example, at 1% glutaraldehyde the subtilisin crystals diffracted x-rays in water but not in acetonitrile, indicating that cross-linking was too light to protect the crystals in acetonitrile, while at 2% the crystals lost their ability to diffract even in water, indicating that cross-linking was so heavy that the crystals became disordered.

The crystals were subsequently removed and washed 10 times with an excess of the same buffer but without glutaraldehyde and 5 times with an excess of distilled water. The specific activity of uncross-linked and cross-linked subtilisin in aqueous solution (pH 7.8; 25°C; 0.7 mM *N*-acetyl-L-phenylalanine ethyl ester as the substrate) was, respectively, 2.3 and 1.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ in 15% Na_2SO_4 (where both crystal samples are insoluble) and 10 and 6.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ in 0.1 M NaCl (where the uncross-linked crystals are soluble and the cross-linked ones are not).

After removal of the water, the crystals were placed in anhydrous acetonitrile (>99% pure) for 5 min and then thoroughly washed with this solvent. The acetonitrile that now constituted the mother liquor contained <0.01% water, and the interstitial water content of the crystal was <0.5%, as measured by the optimized Fischer method (15).

Data Collection. One large crystal (≈0.5 × 0.1 × 0.05 mm) in acetonitrile was mounted in a quartz capillary tube, and x-ray diffraction data were collected at 4°C to 2.3 Å resolution with a Xentronics area detector using CuKα radiation from a rotating anode. One uncross-linked crystal in aqueous solution was similarly mounted on the same instrument, and data were collected to 2.0 Å. Reduction of data was carried out with the XDS program (16). The structure of subtilisin in acetonitrile was refined against the previously determined structure in water (10) by the method of restrained least-squares (17). The first stage of the refinement, which included only an average overall *B* factor and no solvent structure, yielded an *R* factor of 23.1% after 15 refinement cycles. The structure was refined further after applying individual *B* factors yielding an *R* factor of 21.3% after 25 more refinement cycles. Water and acetonitrile molecules were added manually by using the FRODO program (18, 19), and manual correction of refinement errors was performed at this stage. Another 15 refinement cycles yielded a structure with a final *R* factor of 18.4%. Refinement of the subtilisin structure in water was carried out similarly, except that the TNT program (20) was used.

The glutaraldehyde cross-linking did not interfere with refinement because the aldehyde did not contribute to the

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§The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (references 1SCA and 1SCB).

observed electron density, presumably due to a random distribution of the cross-links in the crystal lattice and/or their low population.

Acetonitrile and Water Placement. Using the electron density map, calculated with coefficients $(2|F_o| - |F_c|)$ and α_c phases, both water and acetonitrile molecules were located where they would have at least one hydrogen-bonding partner. Water molecules were distinguished from acetonitriles in the electron density map because at the 1.0 σ contour level water molecule electron densities are spherical, whereas for acetonitrile they are quite ellipsoidal. The existence of subtilisin-bound acetonitrile molecules was further confirmed when we overlaid the $(2|F_o| - |F_c|)$ electron density map for the enzyme in water with the refined coordinates of subtilisin in acetonitrile and found no electron density corresponding to acetonitrile. Overlapping the structure of the subtilisin/eglin c complex with that of subtilisin in acetonitrile was accomplished by using the HYDRA program (Molecular Simulations, Waltham, MA).

RESULTS AND DISCUSSION

Since proteins are insoluble in almost all organic solvents (21), one cannot directly crystallize them from such media [in those few solvents that dissolve enzymes (22), the latter are catalytically inactive (1–5)]. Therefore, our strategy was to crystallize a protein from aqueous solution and then displace the interstitial water with a water-miscible organic solvent. We selected the serine protease subtilisin Carlsberg as a model because its crystal structure in water is known (10, 23, 24) and its catalytic behavior in anhydrous solvents has been extensively studied (25–31). Acetonitrile was selected because this water-miscible, typical organic solvent has been used as the reaction medium for many enzyme-catalyzed (1, 3–5), including subtilisin-catalyzed (25–31), processes.

Subtilisin Carlsberg was crystallized from water, followed by light cross-linking with glutaraldehyde and subsequent displacement of the interstitial water with acetonitrile, as described in *Methods*. Fig. 1 shows the completely refined crystal structure of subtilisin in neat acetonitrile with bound water and acetonitrile molecules located. Refinement parameters for both the acetonitrile and the independently solved aqueous enzyme structures are presented in Table 1. Using these data, we overlaid the C^α polypeptide chain of subtilisin in acetonitrile with that in water; even a visual inspection of Fig. 2*A* reveals that the two structures are strikingly similar. The rms backbone and side-chain shifts in atomic positions between the two coordinate sets are 0.21 and 0.47 Å, respectively, and the overall average shift is 0.32 Å. For comparison, the average shift in atomic positions between the three-dimensional structure of subtilisin in water determined here and that previously reported (10) is 0.91 Å [and that of a subtilisin complex with the inhibitor eglin c is 0.47 Å (23, 24)]. Thus, the difference between subtilisin structures in water and in acetonitrile is less than between two independently determined structures of the enzyme in water.

A plot of the average shift in atomic positions for each amino acid residue of subtilisin is depicted in Fig. 3. There are only 5 (of 274 total) residues with shifts above 0.91 Å; of these, Gln-275 has by far the greatest deviation (3.4 Å). This amino acid residue is the C terminus of the protein, which may explain its elevated rotational freedom. Note, however, that the electron densities for all 5 amino acid residues, in both water and acetonitrile, are consistent with dual or multiple side-chain conformations [i.e., their electron densities, although similar, can reflect two or more distinct conformations; this effect has been observed before with different subtilisin structures in water (P.A.F., unpublished results)]. Therefore, it is unclear whether the differences observed are in fact caused by the change in solvent. All but one of the residues possibly affected are on the surface of the enzyme and none is in the vicinity of the active center. Note that subtilisin in water has three putative calcium-binding sites, whereas in acetonitrile it has only one such site while the other two are occupied by water. One of the calciums in subtilisin in aqueous solution is coordinated with water molecules, one of which has been stripped from subtilisin in acetonitrile. Consequently, it is tempting to speculate that this calcium has been lost as a consequence of the loss of a nearby water molecule.

Given the special significance of the enzyme active center in catalysis, we subjected this region to particular scrutiny. Fig. 2*B* shows that there is little change in the structure of the

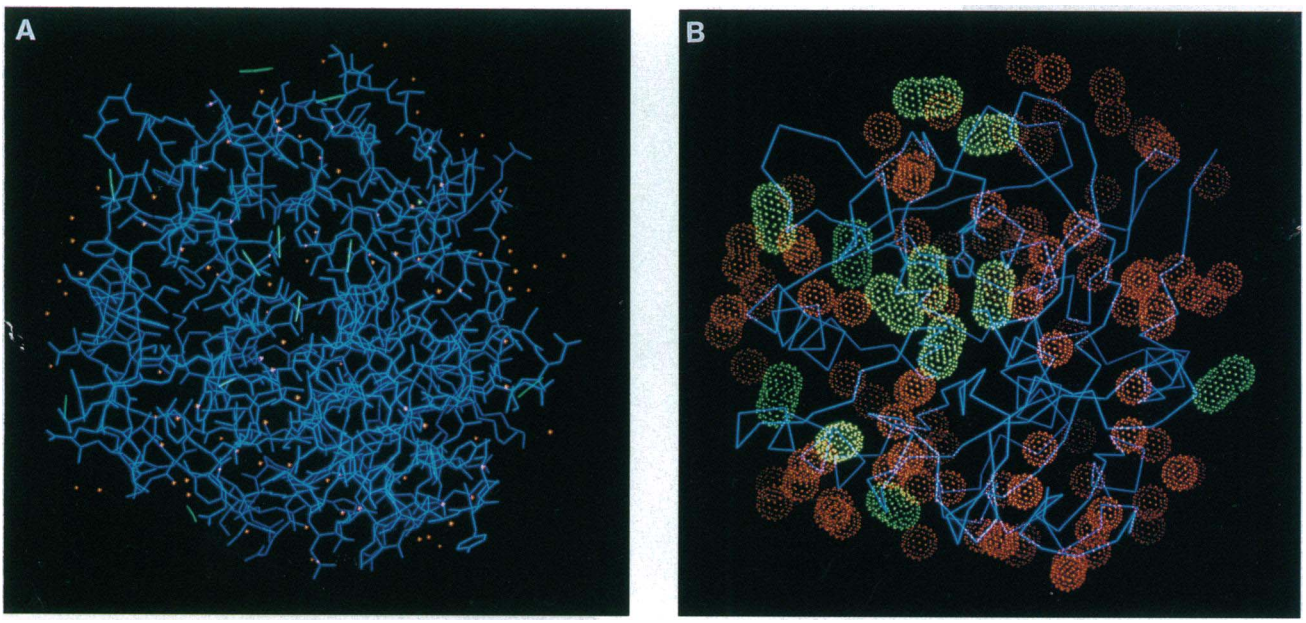

FIG. 1. The complete structure (*A*) and the C^α backbone trace (*B*) of subtilisin Carlsberg (blue) in anhydrous acetonitrile. Bound water (orange) and acetonitrile (green) molecules are also displayed (dot surfaces in *B*). The enzyme active center is approximately in the middle of the figure and faces the viewer. Displayed with the FRODO program (18, 19).

Table 1. Refinement parameters of subtilisin Carlsberg in water and in anhydrous acetonitrile

Parameter	Medium	
	Water	Acetonitrile
Crystallographic data		
Number of observed reflections	37,495	28,973
Number of unique reflections retained	14,163	9512
Completeness, %	89	87
R_{merge}, %	6.8	6.3
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions $a \times b \times c$, Å	76.5 × 55.3 × 53.4	77.1 × 55.4 × 53.6
Molecules/asymmetric unit	1	1
Number of water molecules bound	119	99
Number of acetonitrile molecules bound	0	12
Refinement results		
Resolution, Å	20–2.0	10–2.3
Final *R* factor, %	15.6	18.4
Restraints (σ applied) rms deviations observed		
Bond length, Å	(0.020) 0.020	(0.020) 0.016
Bond angle, °	(3) 3	(4) 1.8
Deviation from planarity, Å	(0.020) 0.020	(0.025) 0.018
Average overall *B* factor, Å^2 (protein only)	13.8*	10.4*

*Fitzpatrick and Klibanov (27) hypothesized that the conformational mobility of subtilisin Carlsberg is a function of the solvent dielectric (ε). These *B* factors seem to confirm this; subtilisin in water (ε = 78) has a higher average *B* factor than in acetonitrile (ε = 36), and a higher *B* factor is associated with greater fluctuations in atomic position (32). The structure of subtilisin in water derived by Neidhart and Petsko (10) yielded an average *B* factor of only 9.2 Å^2 but was refined at lower resolution, which is known to reduce the average *B* factor. The structure of the enzyme inhibited by eglin c (23, 24) gave a *B* factor of 10.4 Å^2; however, binding of the inhibitor to the enzyme is likely to restrict some of the latter's atomic mobility. Complicating the matter is that changes of the *B* factor may depend on lattice disorder, possibly affected by cross-linking of the crystal. Therefore, although these data do not allow definite conclusions regarding the conformational flexibility of subtilisin in acetonitrile vs. that in water, they are consistent with a reduction in mobility in the organic solvent.

subtilisin active center upon transition from water to neat acetonitrile. The rms shift in position for all amino acid residues within 10 Å of the hydroxyl O of the head nucleophile Ser-221 is 0.29 Å. The average shifts in position of the catalytic triad [the cornerstone of serine protease catalysis (34, 35)] residues (Asp-32, His-64, and Ser-221) are only 0.17, 0.14, and 0.13 Å, respectively. The hydrogen bond lengths between O^{δ} and $N^{\delta 1}$ of the first two residues and $N^{\varepsilon 2}$ and O^{γ} of the second two vary from 2.59 and 2.80 Å in water to 2.45 and 2.92 Å in acetonitrile, respectively. All these lengths are well within the range typical for hydrogen bonds in proteins (36), suggesting that the catalytic triad of subtilisin is intact in acetonitrile. This conclusion is in agreement with solid-state NMR evidence that the catalytic triad of a sister serine protease, *Lysobacter enzymogenes* α-lytic protease, retains its structural integrity in anhydrous octane and acetone (37). Furthermore, the results of Hammett analysis and kinetic isotope effect studies of subtilisin in anhydrous solvents, including acetonitrile, also support the native structure of the catalytic site (29–31).

Examination of the structure of subtilisin in acetonitrile depicted in Fig. 1 reveals 99 water and 12 acetonitrile molecules bound to the enzyme, as compared to 119 water molecules bound to it in aqueous solution (Table 1). Of the 99

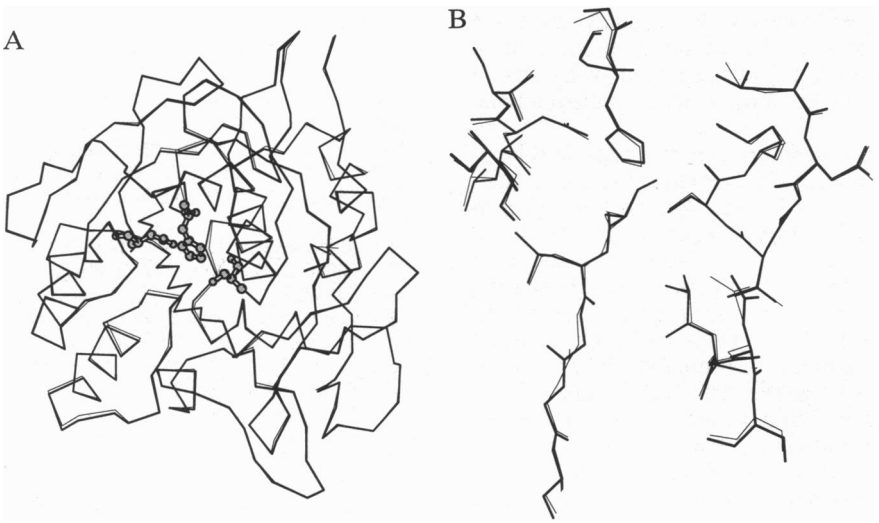

FIG. 2. Display of the C^{α} backbone trace (*A*) and the active center region (*B*) of subtilisin Carlsberg in acetonitrile (heavy lines) overlaid with those in water (light lines). Amino acid residues of the catalytic triad in *A* are presented in ball-and-stick fashion to provide orientation. Drawing was made with the MOLSCRIPT program, v1.1 (33).

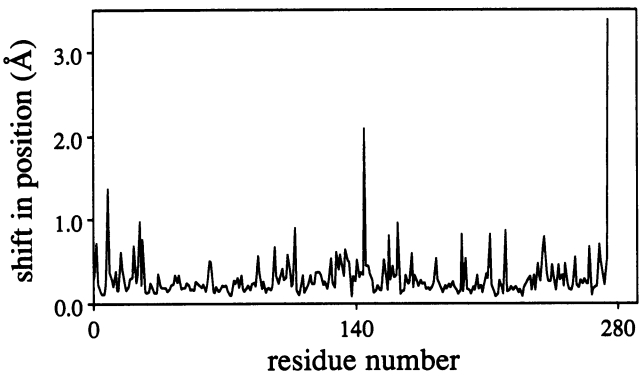

FIG. 3. Shift in atomic positions between subtilisin Carlsberg in acetonitrile and in water averaged over each amino acid residue calculated by the rms formula: shift = $[(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2]^{1/2}/N$, where x, y, and z are the orthogonal coordinates of the atoms in the residue in Å, subscripts refer to the two coordinate files (the enzyme in acetonitrile and in water, respectively), and N is the number of atoms in the residue.

water molecules found in the structure in acetonitrile, 54 are analogous (i.e., located within 1.5 Å) to those bound to the enzyme in aqueous solution. Of the 12 bound acetonitrile molecules, 1 replaces an enzyme-bound water and forms a hydrogen bond with the same group (NH_2 of Asn-155) in subtilisin. Three more displace waters (1 from the hydrophobic cleft) but form new interactions with the enzyme. The remaining 8 acetonitrile molecules reside in subtilisin sites where no water had been observed before. One of these resides in the position formerly occupied by the side chain of Arg-145, which is now hydrogen bonded to the carbonyl of Thr-115. Note that in subtilisin in water there is weak electron density corresponding with placement (albeit with low occupancy) of the arginine side chain in the same position as in subtilisin in acetonitrile. Apparently, binding of acetonitrile excludes the arginine side chain from the more highly occupied position and forces complete occupation of the previously low-populated site. We used computer modeling to superimpose the structures of the subtilisin/eglin c complex in water (23, 24) and subtilisin in acetonitrile (Fig. 1). Four (i.e., one-third of the total) acetonitrile molecules were located in the P_1, P_2, and P_3 sites of the active center, thus occupying the same region as eglin c. Interestingly, half of them, one of the two bound in the P_1 site (to NH_2 of Asn-155 and NH of Ser-221) and one in the P_3 site (to NH of Gly-127), form the same hydrogen bonds with the enzyme as the protein inhibitor at these sites. The second acetonitrile molecule in the P_1 site forms a hydrogen bond with OH of Ser-221, and that in the P_2 site forms a weak hydrogen bond with NH of Leu-126.

That a protein does not denature in a neat organic solvent implies that either, paradoxically, the latter is far less damaging than aqueous/organic mixtures [known to cause protein denaturation (38)] or protein crystals are far more resistant to denaturation by organic solvents than dissolved proteins. Cross-linking with glutaraldehyde (11–13) and the individual features of subtilisin do not appear to be significant, for we found (P.A.F., D.R., and A.M.K., unpublished data) that the crystal structure of uncross-linked bovine pancreatic RNase A in 94% acetonitrile is also indistinguishable from that in water. Furthermore, we ruled out the possibility that the cross-linking prevents subtilisin from denaturation in acetonitrile by establishing that the specific activities of the uncross-linked (and hence fractured) and cross-linked enzyme crystals in acetonitrile (measured as described in ref. 27) were comparable—13 and 9.8 $nmol \cdot min^{-1} \cdot mg^{-1}$, respectively (this modest reduction in activity, which is the same as indicated above for water, is due to the chemical cross-linking effect). Finally, since the subtilisin structure in anhydrous acetonitrile (and presumably in other organic solvents) is essentially the same as in water, the dramatic differences in behavior observed, such as catalytic activity (25, 28), substrate specificity (39, 40), and enantioselectivity (27, 41, 42), must be due to other factors, such as conformational dynamics and solvation variations.

That a protein still retains structural water, even when the medium is a water-miscible organic solvent, is remarkable and indicates that the bound water is an integral part of the structure of the protein. In addition, many of the waters identified in the electron density map of subtilisin in acetonitrile are in approximately the same locations as in the aqueous medium. Regions of the protein surface where no waters are observed in the aqueous structure are not devoid of bound water molecules, but the interaction of water with those parts of the surface is not tightly ordered; discrete positions are therefore not observed in an electron density map. Hence, acetonitrile molecules that occupy positions on the surface where no water had been previously observed are not necessarily associating with vacant sites but may be interacting with sites at which water normally interacts more weakly.

Acetonitrile is a polar, amphiphilic molecule. That it binds to a protein at all implies that the surface has complementary binding sites. The observation that acetonitrile binds in the active center suggests that this cleft has amphiphilic character, consistent with the preference of subtilisin for substrates having a polar backbone and hydrophobic side chains. The other sites at which acetonitrile binds on the surface of the enzyme may have similar character. Thus, acetonitrile may act as a probe to map the amphiphilic regions of the enzyme surface, which would suggest an experimental approach to mapping the complete binding surface of any crystalline protein. By the methods described here, crystals would be transferred to a series of organic solvents, each designed to mimic a particular functional group (e.g., benzene can be used to map binding sites for aromatic groups). Such experiments are directly analogous to computational methods that map the interaction energies of small probe molecules to protein surfaces (43, 44), thus providing a direct experimental test of such theoretical methods. Once the interaction surface has been mapped by a series of solvent experiments, the various functional groups can be connected to provide specific lead compounds for drug design. Unlike conventional substrate analogues, which interact only with the active center, compounds designed by solvent mapping can exploit additional regions of the protein surface to provide greater specificity and affinity.

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