Comparison of x-ray crystal structures of an acyl-enzyme intermediate of subtilisin Carlsberg formed in anhydrous acetonitrile and in water

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ABSTRACT The x-ray crystal structures of trans-cinnamoyl–subtilisin, an acyl-enzyme covalent intermediate of the serine protease subtilisin Carlsberg, have been determined to 2.2-Å resolution in anhydrous acetonitrile and in water. The cinnamoyl–subtilisin structures are virtually identical in the two solvents. In addition, their enzyme portions are nearly indistinguishable from previously determined structures of the free enzyme in acetonitrile and in water; thus, acylation in either aqueous or nonaqueous solvent causes no appreciable conformational changes. However, the locations of bound solvent molecules in the active site of the acyl- and free enzyme forms in acetonitrile and in water are distinct. Such differences in the active site solvation may contribute to the observed variations in enzymatic activities. On prolonged exposure to organic solvent or removal of interstitial solvent from the crystal lattice, the channels within enzyme crystals are shown to collapse, leading to a drop in the number of active sites accessible to the substrate. The mechanistic and preparative implications of our findings for enzymatic catalysis in organic solvents are discussed.

Enzymes in organic solvents, instead of their natural aqueous milieu, remain synthetically useful catalysts (1–6). In addition, they display remarkable properties, e.g., solvent-dependent selectivity (7, 8). Such solvent dependences of prochiral selectivity (9) and enantioselectivity (10) of crystalline enzymes in nonaqueous media have been nearly quantitatively predicted using structure-based molecular modeling and thermodynamic calculations.

Despite recent advances (11–14), the question of why enzymatic activity is often much reduced in organic solvents compared with water is far from resolved. Such an understanding, like that concerning the selectivity, requires both structural and mechanistic information. Recently, structures of several enzymes in neat organic solvents, namely those of subtilisin Carlsberg in acetonitrile (15, 16) and dioxane (17), γ-chymotrypsin in hexane (18, 19), and elastase in acetonitrile (20), have been elucidated and found to be essentially the same as in water. Furthermore, the structures in hexane of a γ-chymotrypsin-product complex has been determined (19) and that of a tetrahedral complex claimed (18). However, to provide further insight into the enzyme mechanism in organic solvents, structures of covalent reaction intermediates should be determined. To the best of our knowledge, no such structures have been available, until now.

All the aforementioned enzyme structures are of serine proteases, for which the universal reaction intermediate in water is an acyl-enzyme. In organic solvents, however, the formation of such an intermediate in the catalysis by subtilisin is supported by kinetic but not direct structural data (21–24). Moreover, it is simply presumed that the structure of the enzyme-substrate intermediate formed in different solvents is the same (9, 10). The most direct way to experimentally test this assumption is to determine the corresponding structures.

At this point, three major pertinent questions remain unanswered: (i) Is there an acyl-enzyme intermediate formed in organic solvent? if so, (ii) Is the structure of the intermediate the same as that formed in water? and (iii) Are solvent molecules displaced from the active site by the substrate portion of the acyl-enzyme intermediate? In this study we address these questions, as well as some related issues.

MATERIALS AND METHODS

Crystal Preparation and Acylation. Subtilisin Carlsberg (protease from Bacillus licheniformis, EC 3.4.21.14) was purchased from Sigma. Crystals were grown from aqueous 330 mM cacodylate buffer (pH 5.6), with saturated (~13%) Na2SO4 (25). Single crystals (~0.8 × 0.1 × 0.08 mm) were cross-linked using 10% glutaraldehyde [aged for 3 days at room temperature before reaction (17)] containing 30 mM cacodylate buffer (pH 7.5) and 10% Na2SO4. We presume a relatively low extent of cross-linking: there are nine lysine residues in subtilisin of which only five are within 20 Å. The crystals were incubated in the cross-linking solution for 30 min and washed five times with 2 ml of distilled water with 5 min per wash. When preparing a crystal for acylation in acetonitrile the water was then removed and replaced with anhydrous acetonitrile by washing five times with 2 ml. We have shown that crystals similarly washed with organic solvent have a residual water content of 3% (wt/wt) (17). For acylation in water, the crystal was transferred to a 5 mM acetate solution (pH 5.0). Crystals were acylated with 100 mM N-trans-cinnamoylimidazole in acetonitrile (5 min) or 110 μM in cacodylate buffer (pH 5.0) (0.1 min with subsequent pH adjustment to 3.0 with 1 M HCl). The extent of acylation was assayed by measuring the catalytic activity of the subtilisin crystal toward the hydrolysis of the substrate N-acetyl-L-phenylalanine ethyl ester. In water, the disappearance of absorption at 335 nm for the N-trans-cinnamoylimidazole was also monitored (26). In both cases the acylation was complete.

Data Collection and Reduction. X-ray diffraction data were obtained at ambient temperature (23 ± 1°C) using a Rigaku RU200 rotating copper anode source, double-Franks focusing mirrors, and a RAXIS II detector. In acetonitrile, diffraction data of one crystal were measured to a nominal resolution of 2.15 Å within 20 hr of transfer to the solvent. In water, diffraction data of two crystals were collected to a nominal resolution of 2.20 Å. Reflections were indexed and integrated, and scaled using the HKL Package (27), and structure factors were calculated and

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; rmsd, root-mean-square displacement; DMF, dimethyl formamide.
truncated using the CCP4 Suite (28) (Table 1). Ten percent of the reflections were reserved for R-free calculations.

**Refinement and Model Building.** Rigid body refinement (10–4.0 Å) was performed using X-PLOR (29) for acetonitrile and water data and coordinates for the corresponding cross-linked nonacylated crystal structure PDB 1scb and Isca (15, 16).

A series of \([2F_{\text{obs}} - F_{\text{calc}}]^2\exp(-i\phi_{\text{calc}})\) maps were prepared using phases from the starting model for each solvent where one-eighth of the total residue was omitted in each map. These maps were examined only in the areas of omitted residues using the program O (30). Any region for which there was no clear density and all the residues within a 6-Å sphere of the active site Ser-221 were not included in the model in the first round of refinement.

For refinement in acetonitrile, no solvent correction was used. In water, a bulk solvent-mask corrected reflection file was used. The highest resolution limit was selected such that the R-free (31) improved after refinement in the low resolution shells when the high resolution data were included. The first round of refinement in X-PLOR consisted of a simulated annealing slow-cool followed by a restrained individual B-factor refinement. Later refinement rounds comprised only positional and B-factor refinements.

Potential solvent water molecules were located as peaks in the \(F_{\text{obs}} - F_{\text{calc}}\) electron density map at the 3.0σ contour level (32), which clearly exhibited the appropriate shape; any peaks that were possibly due to acetonitrile molecules in acetonitrile were excluded. Water molecules were retained if their electron density in subsequent \([2F_{\text{obs}} - F_{\text{calc}}]\) maps persisted after the positional refinement and if they had: (i) an oxygen atom within 3.4 Å of a hydrogen-bond donor with good hydrogen-bonding geometry, (ii) a B-factor of less than 65 Å², and (iii) a real-space-fit correlation coefficient above 70%. A total of 62 water molecules were included in the model in acetonitrile and 88 water molecules and 1 Ca²⁺ molecule in the model in water. Organic solvent molecules similarly were introduced into the model as appropriate using the acetonitrile model (15), and parameter and topology files generated with the aid of XPLOR (33). Acetonitrile electron density was recognized by comparison with \(F_{\text{calc}}\) maps calculated for a theoretical acetonitrile molecule between the resolution limits of 14–2.15 Å. Only the acetonitrile molecules with average B-factors of less than 65 Å² or real-space-fit correlation coefficients greater than 75% were kept. Twelve acetonitrile molecules were included in the final model of cinnamoyl–subtilisin in acetonitrile, as well as 74 water molecules and 1 Ca²⁺ molecule.

**Cinnamoyl Group.** The trans-cinnamoyl group model used was from the crystal structure of trans-cinnamic acid (34), with the parameter and topology files generated with the aid of XPLOR (35). The cinnamoyl group and Ser-221 were included in the models in later stages of refinement. Positional refinement followed by an

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### Table 1. Crystal properties, data collection, refinement, and model statistics for trans-cinnamoyl–subtilisin in anhydrous acetonitrile and in water

<table>
<thead>
<tr>
<th>Crystal properties</th>
<th>Acetonitrile</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>(P_2_1_2_1_2_1)</td>
<td>(P_2_1_2_1_2_1)</td>
</tr>
<tr>
<td>Cell dimensions ((a \times b \times c), Å)</td>
<td>76.4 × 55.3 × 52.8</td>
<td>77.0 × 55.0 × 53.6</td>
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<tr>
<td>Data collection statistics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of measurements</td>
<td>44,825</td>
<td>4,339</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>10,495</td>
<td>1,038</td>
</tr>
<tr>
<td>Completeness, %</td>
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<td>77.3</td>
</tr>
<tr>
<td>Mean (I/\sigma_I)</td>
<td>24.5</td>
<td>10.1</td>
</tr>
<tr>
<td>(R_{\text{sym}}), %</td>
<td>5.3</td>
<td>15.5</td>
</tr>
<tr>
<td>Resolution limits, Å</td>
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<td>2.3–2.2</td>
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<tr>
<td>Data collection statistics</td>
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<td></td>
</tr>
<tr>
<td>No. of solvent atoms</td>
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<td>88</td>
</tr>
<tr>
<td>rmsd bond length, Å</td>
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<td>0.010</td>
</tr>
<tr>
<td>rmsd bond angle, °</td>
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<td>1.5</td>
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<tr>
<td>Mean B-factor (standard deviation), Å²</td>
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</tr>
<tr>
<td>Protein</td>
<td>20 (9)</td>
<td>23 (8)</td>
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<tr>
<td>Solvent</td>
<td>43 (17)</td>
<td>35 (11)</td>
</tr>
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<td>rmsd of structure in acetonitrile vs. water, Å</td>
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<td></td>
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<tr>
<td>Protein backbone—0.25</td>
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<td></td>
</tr>
<tr>
<td>Active site atoms—0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamoyl group—0.24</td>
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</table>

*In the cinnamoyl acetonitrile data set, the resolution shell of 2.20–2.15 Å was also included in the refinement as described. This shell contained 2,557 measurements with 722 unique reflections, a completeness of 57.8%, a mean \(I/\sigma_I\) of 9.0, and an \(R_{\text{sym}}\) of 18.0%.

1\(R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum |I|\), where the free R-factor is calculated for a randomly chosen 10% of reflections and the crystallographic R-factor is calculated for the remaining 90% of reflections (\(F > 2.0\sigma_F\)) used for the refinement.

6Includes 10 atoms from the covalently bound cinnamoyl group and 1 Ca²⁺ atom.

7Root-mean-square-derivation (rmsd) from ideal geometric values (Enge and Huber parameter set).
occupancy refinement for the trans-cinnamoyl group and a restrained individual B-factor, 25 cycles, were performed in X-PLOR. The occupancy refined to 0.77 and 0.78 for the cinnamoyl residue in acetonitrile and water, respectively. The final values of R-factor and R-free were 0.20 and 0.23 (8.0–2.15 Å) in acetonitrile and 0.18 and 0.22 (14–2.2 Å) in water (Table 1).

Model Validation. The final models in acetonitrile and water contained no residues that were in disallowed regions of the Ramachandran plot, as determined with the program PROCHECK (35). The models in acetonitrile and water contained one (Thr-211) and two (Ser-161 and Asn-212) residues, respectively, in generously allowed regions. The r.m.s.d from ideal values for both structures can be found in Table 1. The real-space-fit ($2F_{\text{obs}} - F_{\text{calc}}$) correlation coefficients for the model in acetonitrile were 0.90 ± 0.05 for protein and 0.84 ± 0.10 for solvent; for water, the values were 0.90 ± 0.05 and 0.84 ± 0.07. The final models contained no peaks above 3.5σ in the $|F_{\text{obs}} - F_{\text{calc}}|$ electron density maps. The overall B-factors for the models in acetonitrile and water were 21 and 24 Å², respectively—i.e., comparable to Wilson Å², respectively. The average coordinate error predicted by model validation (25 cycles, were performed in X-PLOR).

Active Site Titration. The accessible active sites of subtilisin in the crystal were measured by titration with phenylmethylsulfonyl fluoride (PMSF) (12). Briefly, cross-linked crystals (10–50 mg/ml) were placed in 1 ml of acetonitrile containing 500 μM PMSF, and the suspension was shaken at 30°C and 300 rpm, with the disappearance of PMSF monitored in triplicate by HPLC. Crystal pretreatments were taken from Partridge et al. (41). Pretreated crystals were washed with anhydrous acetonitrile (3 × 1 ml), followed by incubation in either acetonitrile (10 ml) for 1.7 and 3 days or in air for 3 days in a sealed jar. Crystals with no pretreatment were washed with anhydrous acetonitrile (3 × 1 ml), recovered by centrifugation, and used immediately. The fraction of the catalytically competent and accessible cross-linked subtilisin crystals was as follows: 44 ± 2%, no pretreatment; 55 ± 1.3% and 1.1 ± 0.7% for the 1.7- and 3-day incubation in acetonitrile, respectively; and below 0.5% after the 3-day incubation in air.

Crystal Dye Soak. An aqueous solution of methylene blue was from Hampton Research (Laguna Hills, CA). The crystals with no pretreatment were washed with anhydrous acetonitrile (3 × 1 ml) and immediately placed in a solution of the dye in anhydrous acetonitrile (1 ml). After 3 hr, the crystals became blue and electron density, and used immediately. The fraction of the catalytically competent and accessible cross-linked subtilisin crystals was as follows: 44 ± 2%, no pretreatment; 55 ± 1.3% and 1.1 ± 0.7% for the 1.7- and 3-day incubation in acetonitrile, respectively; and below 0.5% after the 3-day incubation in air.

RESULTS AND DISCUSSION

Previously, we solved the x-ray crystal structures of lightly cross-linked subtilisin Carlsberg in dioxane (17), acetonitrile (15), and water (16) and found them virtually identical, with an r.m.s.d of the backbone atoms of 0.3 Å among the different structures. However, to determine whether the enzyme mechanism is the same in organic solvent as in water, one must examine the structure of a reaction intermediate formed in both media, not just the structure of the free enzyme. To this end, in this study, we set out to solve the structure of an acyl-enzyme reaction intermediate of subtilisin, specifically that of trans-cinnamoyl-subtilisin, formed in acetonitrile and in water. Its formation was achieved as follows. Crystals of subtilisin were grown from aqueous solution, lightly cross-linked, and placed in either anhydrous acetonitrile or water, to which N-trans-cinnamoylimidazole was subsequently added. The resulting cinnamoyl-subtilisin crystals were then mounted in capillaries, x-ray diffraction data were collected, and the structures were determined to 2.2-Å resolution in acetonitrile (Fig. 1) and in water (Fig. 2). The structures included the substrate cinnamoyl group, as well as 74 bound water and 12 bound acetonitrile molecules in acetonitrile, and 88 bound water molecules in water.

The enzyme portion of subtilisin does not change upon formation of the acyl-enzyme intermediate in either acetonitrile (Fig. 3A) or water (Fig. 3B). To estimate the average coordinate error inherent in the acyl-enzyme structures, Luzzati (36–38) or sigmaA (39) analyses were performed and a value of 0.3 Å was estimated by either method for both structures. The r.m.s.d of the backbone atoms between the free and acyl-enzyme structures are 0.30 Å in acetonitrile and 0.25 Å in water; these values are on the order of the average coordinate error inherent in each of the independent structures. In addition, only 3 of 274 amino acid residues have an average displacement above 0.5 Å between the acyl- and free enzyme structures in acetonitrile (Gly-157 and Ser-159 of a flexible surface loop remote from the active center, and Tyr-171, also on the surface). Similarly, only five residues have an average displacement greater than 0.5 Å between the two structures in water (Ala-129 and Ser-130, and Asn-158, Ser-221) of the flexible surface loop). Fig. 3C reveals

FIG. 1. Ribbon diagram of the protein structure of trans-cinnamoyl-subtilisin in acetonitrile. The catalytic triad (Asp-32, His-64, and Ser-221) is portrayed as sticks. The cinnamoyl group is shown in black. Water molecules and acetonitrile molecules are depicted by balls-and-sticks, with the nitrogen atoms of acetonitrile in black.

FIG. 2. Ribbon diagram of the protein structure of trans-cinnamoyl-subtilisin in water. The catalytic triad and cinnamoyl group (black) are portrayed as sticks. Water molecules are depicted by gray balls.
causing a conformational change in the active site or by affecting the mode of binding of the substrate.

As mentioned earlier, structure-based molecular modeling has been successfully used by us to predict the solvent dependence of enzymatic stereoselectivity (9, 10). In so doing, the minimum energy conformation of the tetrahedral intermediate of the enzymatic, e.g., acylation, reaction is determined in vacuum. The assumption is then made that this minimum energy structure will be the same in solvent. We tested this assumption and found that the conformation of the substrate’s cinnamoyl portion predicted by molecular modeling is indeed identical to that experimentally determined by x-ray crystallography (depicted in Fig. 4). Thus, the structure of the acyl-enzyme, which is the same in acetonitrile and water in our study, can be correctly predicted by molecular modeling.

An analysis of the enzyme-bound solvent molecules in the active site of the acyl- vs. free enzyme reveals that in acetonitrile (Fig. 4 A and C, respectively), the cinnamoyl moiety displaces one acetonitrile molecule, that (labeled b in Fig. 4C) from the P1 specificity pocket. In the free enzyme structure, an additional acetonitrile molecule (a in Fig. 4C) is bound in the location of the “catalytic water” molecule [this molecule is a hallmark of the active site of subtilisin and other serine proteases that act by means of a ping-pong mechanism (40)] found in the structure in water (a in Fig. 4D). This acetonitrile molecule is still present in the acyl-enzyme structure in acetonitrile (a in Fig. 4 A and C). Note that particular attention was paid to the issue of the inclusion of acetonitrile and water molecules in the acyl-enzyme structure in acetonitrile.

Examination of the active site situation in water shows that in the acyl-enzyme (Fig. 4B) the cinnamoyl group displaces one water from the specificity pocket and another from the oxygen hole (b and c, respectively, in Fig. 4D). This gives rise to the question; can the dissimilar solvation of the active site in different solvents be a contributor to the distinct subtilisin activities in them (21–24)? Serine proteases such as subtilisin act by means of a ping-pong mechanism (40), where the “pong” is the deacylation step. In enzymatic transesterification reactions in organic solvents (1–6), the nucleophile involved in the deacylation must first displace whatever molecule occupies the catalytic water site. In acetonitrile, an acetonitrile molecule resides in this site (a in Fig. 4 A and C), whereas in dioxane (17) the catalytic water molecule is present. Consequently, differences in the enzymatic deacylation rates observed in, e.g., these two solvents, may be partially due to the difference in displacement energies of an acetonitrile molecule in acetonitrile and a water molecule in dioxane.

The deacylation step is not the only one in which differential solvation of the active site could play a role. Because the cinnamoyl group in the acyl-enzyme displaces water molecules in water and an acetonitrile molecule in this solvent (Fig. 4), different energies are required to displace these molecules to allow substrate binding. In effect, the solvent acts as a competitive inhibitor of the substrate. In an earlier work (12), where we attempted to explain the decrease in activity of cross-linked crystals of subtilisin on transition from water to anhydrous acetonitrile, 1.4 out of some 7 orders of magnitude of activity loss remained unaccounted for. Perhaps the differential energies of displacing enzyme-bound water and acetonitrile molecules from the enzyme active site, not considered in that work, explain this shortfall.

The concept of specific interactions of solvent molecules with the enzyme can be used to rationalize some literature data. For instance, Partridge et al. (41) washed cross-linked crystals of subtilisin with either acetonitrile or propanol, then assayed the enzyme crystals in propanol, and found the resultant enzymatic activity lower in the former case. The acetonitrile introduced by washing could be acting as an inhibitor of the enzyme in propanol. Such specific interactions were also suggested by molecular dynamics studies performed.

that the structure of the cinnamoyl–subtilisin formed in acetonitrile is the same as that formed in water, with an rmsd of 0.25 Å for the backbone atoms. Likewise, only four residues have an average displacement larger than 0.5 Å, namely Gly-157 and Ser-161, located in the flexible surface loop, Ala-274 at the C terminus, and Ser-101, also on the enzyme surface remote from the active site.

Because subtilisin exhibits markedly different catalytic activity and other properties in different solvents (7–14, 21–24), the structure of the active site in these solvents is a critical issue. We find that not only is the overall structure of the crystalline acyl-enzyme the same whether formed in acetonitrile or in water, but it can be seen in Fig. 3C and by comparing Fig. 4A with Fig. 4B that the structure of the active site region, including that of the covalently bound cinnamoyl moiety, is also identical. The rmsd of all atoms within a 10-Å sphere of the active site catalytic triad nucleophile, the Ser-221 Oγ atom, is 0.29 Å in acetonitrile compared with water. Thus, it appears that the solvent does not change the activity of subtilisin by

![Fig. 3.](image-url)
on subtilisins immersed in dimethyl sulfoxide (43) and di-
methyl formamide (DMF) (44), which predicted that organic
solvent molecules would bind to the protein. Moreover, DMF
molecules were predicted to bind in the enzyme active site (44).
In particular, molecular dynamics simulations in DMF placed
at least one DMF molecule into the active site in both wild-type
subtilisin E and a mutant (45) thereof. Interestingly, similar
studies of these subtilisins in water showed that the water, but
not DMF, molecules did not remain bound in the active site
and diffused in and out during the simulations (44).
In the examination of reactions involving enzyme crystals in
organic solvents it may be important to consider the role of the
crystal itself, i.e., the overall physical presentation of the enzyme.
Different pretreatments of cross-linked crystals of subtilisin have
been shown to result in distinct enzymatic activities in organic
solvent. After a 3-day incubation of the crystals in anhydrous
acetonitrile or in air the result was a 3- and 200-fold drop in
activity, respectively, when subsequently assayed in acetonitrile
(41). These data can be explained by the following hypothesis:
When either immersed in organic solvents for long times (days)
or exposed to conditions that remove interstitial solvent, cross-
linked enzyme crystals experience a partial collapse of their
crystal lattice and concomitant closure of their solvent channels
to the substrates. Consequently, the enzymatic activity drops
because of that supramolecular phenomenon rather than to a
specific change on the molecular level.
We verified this hypothesis. First, the accessible active sites
of subtilisin in the crystal were determined by titration with
PMSF, an irreversible inhibitor of serine proteases (46), as a
function of the pretreatment described above. After a 1.7- and
3-day incubation in acetonitrile, the number of accessible
active sites dropped to 13 and 2.5%, respectively (see
Materials and Methods). Moreover, when the crystals were exposed to air
for 3 days, fewer than 1% of the initial active sites remained
accessible. In a second experiment, we investigated the crys-
tals’ ability to uptake methylene blue, an organic dye, as a
function of the pretreatment. If the solvent channels of the
crystal are open (intact), the dye should penetrate the crystal
and turn it blue. If they are not open, e.g., have collapsed, the
dye will fail to do so. As seen in Fig. 5A, crystals with no
pretreatment, placed in anhydrous acetonitrile containing
methylene blue, do turn blue. Conversely, crystals exposed to

![Fig. 4. Active site of trans-cinnamoyl-subtilisin in anhydrous acetonitrile (A) and in water (B), and that of the free subtilisin in anhydrous
acetonitrile (C) and in water (D). The catalytic triad, Asn-155 of the oxyanion hole, the cinnamoyl group (A and B), and solvent molecules are
shown as balls-and-sticks with carbon, oxygen, and nitrogen shown in white, light gray, and black, respectively. The semitransparent solvent-
accessible surface of the protein (calculated using the Connolly algorithm (42) with a probe radius of 1.4 Å in the INSIGHT II package from Biosym
Technologies, San Diego) in the active site region is shown in light gray.](image-url)
air overnight remain colorless (Fig. 5B). Furthermore, the latter crystals appeared damaged, curled up, and deformed. These data add another dimension to the question of catalytically competent active sites. In contrast to the situation of an enzyme dissolved in water where only the number of competent active sites is important, their accessibility is also an issue for a crystalline enzyme suspended in organic solvents. A similar predicament may also occur in lyophilized enzyme powders suspended in organic solvents, where prolonged incubation of subtilisin in acetoneitrile also led to a reversible loss of activity (47). Thus, not only is structural information on the molecular level of the enzyme important, but so is an understanding of what is occurring at the supramolecular, crystalline level, of the enzyme catalyst.

Concluding Remarks. That the structures of the crystalline acyl-enzyme intermediate formed in acetoneitrile and in water are the same verifies earlier kinetic evidence (21–24) that the enzymatic mechanisms in organic solvents and in water are similar. The observed distinct binding of solvent molecules in the enzyme active site between the acyl-enzyme intermediate and the free enzyme structures in different solvents may be responsible for at least some of the heretofore unexplained disparity in the activity of enzymes in organic solvents. This factor should be considered when endeavoring to manipulate enzymatic stereoselectivity by rational selection of the organic solvent (7–10). On a separate note, a decrease in the accessible active sites in crystalline (and amorphous) enzyme preparations because of the collapse of solvent channels causes a reduction in the observed enzymatic activity in organic solvents. Khalaf et al. (48) recently showed that when cross-linked crystals of subtilisin are first treated with certain detergents before drying to a constant water activity, a different catalytic activity in organic solvent ensues. Given our findings here, the addition of a detergent to the solvent in the crystal lattice may combat the phenomenon of channel collapse and preserve the uniformity of the crystal network. Thus, carefully designing the conditions to maintain the order of the solvent channels and protein crystal lattice should be explored as a strategy to achieve the full enzymatic potency.

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