Solid-state NMR Assessment of Enzyme Active Center Structure under Nonaqueous Conditions*

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By using solid-state NMR spectroscopy, the integrity of the active center of α-chymotrypsin was investigated under a variety of nonaqueous conditions. Specifically, 13C cross-polarization/magic angle spinning NMR was used to analyze the ability of α-chymotrypsin to stabilize a transition state intermediate analog after freezing, drying, and addition of organic solvents (both anhydrous and hydrated) to the resultant powder. Lyophilization disrupted 42 ± 5% of the active centers; it was determined that this occurred during drying, as opposed to freezing. Seven anhydrous solvents caused 0–50% additional disruption, which occurred immediately on addition of the solvent to the enzyme powder. The extent of structural integrity loss correlated with the solvent hydrophobicity, indicating that further dehydration, i.e., stripping of water retained by the enzyme during lyophilization, was the cause. Enzyme samples prepared with lyoprotecting additives, sucrose and ammonium sulfate, exhibited varying degrees of stabilization against the drying step of lyophilization. Moreover, when hydrophilic anhydrous solvents, which had the highest propensity to strip bound water, were added to the resultant enzyme powders, no additional damage occurred.

One of the most insightful developments in nonaqueous enzymology (Klibanov, 1989; Dordick, 1989) is the recent application of biophysical methods, specifically magnetic resonance spectroscopies (Burke et al., 1989; Guinn et al., 1991; Affleck et al., 1992), to fundamental questions concerning enzyme structure and dynamics under these conditions. For example, why is enzyme activity lower in organic solvents than in water and why does it vary widely among solvents? Because conventional wisdom dictates that organic solvents denature proteins (Singer, 1962), one may expect that the extent of this denaturation depends on the properties of the solvent. Enzyme active center titrations in organic solvents (Zaks and Klibanov, 1988a) have dealt with this issue. However, because enzymes in organic solvents exist as suspensions (the particles of which contain many enzyme molecules each), the interpretation of such titrations is ambiguous. Specifically, when the fraction of titrated active centers compared to the same amount of enzyme in aqueous solution is less than 100% (Zaks and Klibanov, 1988a; Affleck et al., 1992), is it because some active centers are kinetically incompetent due to denaturation induced by the solvent, or because protein-protein contacts block some competent active centers (or both)?

Enzymes also can undergo denaturation during lyophilization (Fishbein and Winkert, 1979; Franks, 1985). If irreversible dehydration of the powder, this would lower the fraction of intact active centers in organic solvents compared to that for the same amount of protein dissolved in aqueous solution. Thus fractions of the biocatalyst may be nontitratable in organic solvents simply because of the manner of preparation of the enzyme powder rather than the effect of the solvent itself.

Subtilisin (a serine protease from Bacillus licheniformis) gradually inactivates in some anhydrous solvents due to a stripping of water retained by the enzyme during lyophilization (Schulze and Klibanov, 1991). With many anhydrous solvents, much of this dehydration occurs immediately upon addition of the solvent to the enzyme powder (Gorman and Dordick, 1992). Therefore the question arises of how the magnitude of the time-dependent inactivation compares to the loss of activity when the enzyme first sees the solvent (and most water-stripping occurs (Zaks and Klibanov, 1988b; Gorman and Dordick, 1992)). Also, due to low activities in some solvents, active center titrations can take days (Affleck et al., 1992); any activity loss during this period would confound the result.

Questions such as these can only be answered by examining enzyme active center structure at each step in the process: freezing, drying, initial addition of solvent to the resultant powder, and subsequent incubation. Because enzymes are amorphous and insoluble in nearly all organic solvents, physical characterization is possible by few techniques. In the present study, we address the aforementioned issues by means of solid-state NMR.

EXPERIMENTAL PROCEDURES

Materials

Bovine pancreatic α-chymotrypsin (EC 3.4.21.1), unlabeled TPCK,1 phenylmethylsulfonil fluoride, and L-phenylalanine were purchased from Sigma. 3H,SO4 and 13C-Phe were purchased from Cambridge Stable Isotopes (Woburn, MA). Synthetic reagents used in the preparation of the labeled TPCKs, as well as 3H-depleted water, were obtained from Aldrich. All organic solvents, which were of the highest purity commercially available, were also obtained from Aldrich and were dried over 3 Å molecular sieves. N-Ac-Ala-Ala-Pro-Phe-p-nitroanilide was from Bachem (Philadelphia, PA).

1 The abbreviations used are: TPCK, tosylphenylalanyl chloromethyl ketone; CP/MAS, cross-polarization/magic angle spinning; NMR, nuclear magnetic resonance; Tos, p-toluenesulfonil.

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Methods

Tos-L-[2',3',4',5',6'-H]Phe-CH₂Cl and Tos-L-[2',3',4',5',6'-H]Phe-CH₂Cl were synthesized according to the method of Schoellman and Shaw (1963), using the appropriately labeled starting material. L-[2',3',4',5',6'-H]Phe was prepared as described by Matthews et al. (1977).

The structures of the products were confirmed by 'H NMR. Melting points for these TPCKs were 104-106 °C (uncorrected; literature 102-103 °C (Schoellmann and Shaw, 1963)).

Preparation of Tos-L-[2',3',4',5',6'-H]Phe-CH₂-chymotrypsin and Tos-L-[2',3'-H]Phe-CH₂-chymotrypsin—All procedures were conducted at room temperature. Enzymatic activity was measured by following the hydrolysis of N-Ac-Ala-Ala-Pro-Phe-p-nitroanilide (2 mM) in 80 mM Tris-HCl buffer, pH 7.8. After the enzymatic solution of a-chymotrypsin was added (0.7 mg/ml) in 5 mM potassium phosphate, pH 7.0. Then 50 mg of the inhibitor dissolved in 15 ml of methanol/dimethyl sulfoxide (50:50 mixture by volume) was added dropwise with continuous stirring. After 4 h, when the residual activity was about 4% of the initial (an identical sample to which no inhibitor was added showed no loss of activity over this period), the pH was adjusted to 7.8, and a solution of the irreversible covalent inhibitor phenylmethylsulfonyl fluoride (Farney and Gold, 1963) in n-propyl alcohol (14 mg/ml) was added. After the enzymatic activity further declined to less than 0.02% of its initial value, the pH was adjusted to 6.0, and solid ammonium sulfate (561 g) was added to 80% saturation. (That no irreversible conformational changes occurred on (NH₄)₂SO₄ precipitation of a-chymotrypsin was shown by complete recovery of enzymatic activity on dissolution of the precipitate of uninhibited a-chymotrypsin.) The mixture was centrifuged, and the pellets (with entrained supernatant) were combined. The precipitate of uninhibited a-chymotrypsin was washed this way three times. (That no irreversible conformational changes occurred on (NH₄)₂SO₄ precipitation of a-chymotrypsin was shown by complete recovery of enzymatic activity on dissolution of the precipitate of uninhibited a-chymotrypsin.) The mixture was centrifuged, and the pellets (with entrained supernatant) were combined. The precipitate of uninhibited a-chymotrypsin was resuspended in 10 mM cacodylate, pH 5.6, containing 50% saturated, 'H-depleted (NH₄)₂SO₄ (obtained by lyophilizing the salt from 'H-depleted water four times), prepared from 'H-depleted water. The pellet was washed this way three times.

Deuterium Quadrupole Echo NMR Spectroscopy—Deuterium spectra were recorded on a home-built spectrometer at 9.34 tesla (9 MHz) of 'H frequency of 61.0 MHz using a deuterium-echo pulse sequence (Davis et al., 1976) with a pulse spacing of 30 μs and a π/2 pulse of 2.2-2.8 μs. Except for the crystals (about 60 mg of chymotrypsin), samples consisted of 200-250 mg of labeled protein, with at least 0.5 g of the appropriate solvent. Anhydrous solvents contained less than 0.01% water. Hydrated solvents were prepared gravimetrically using 'H-depleted water. Recycle delays were 3-5 s, such that echo heights were approximately 95% of their fully relaxed values. In solvent-containing samples, the isotropic spectral component (arising from natural abundance solvent deuterons) was removed by fitting the base line of both the real and imaginary channels of the time domain to the appropriate model. NMR Spectroscopic Data Analysis System (New Methods Research Inc., Syracuse, NY), and subtracting the resultant function prior to transformation. Spectra consist of 10-20 thousand acquisitions (80,000 in the case of the crystals) with 2048 real data points, and 4 kHz line broadening applied.

RESULTS AND DISCUSSION

The preeminent, defining characteristic of an enzyme is that rate acceleration is achieved by stabilization of its complex with the substrate in the transition state (Jencks, 1975). Thus, compounds which model the transition state structure are excellent competitive inhibitors (Kraut, 1977). Furthermore, they may analogously to transition state analogues can catalyze the corresponding chemical reactions (Schultz et al., 1990). In serine proteases, the tetrahedral transition state is stabilized by an "oxyanion hole," a pocket which forms hydrogen bonds to the tetrahedral intermediate in the enzymatic hydrolysis of esters and amides (Kraut, 1977; Steitz and Shulman, 1982). (Serine protease-catalyzed transesterification in anhydrous solvents likely occurs by the same mechanism (Kanerva and Klibanov, 1989; Adams et al., 1990).) Recent NMR studies (Finucane et al., 1989) have shown that reaction of TPCK with a-chymotrypsin in aqueous solution results in the complex depicted in Fig. 1. This complex is analogous to the transition state in serine protease catalyzed hydrolysis. Similar structures have been observed for complexes of a trifluoromethyl ketone inhibitor with a-chymotrypsin (Liang and Abeles, 1987) and a chloromethyl ketone inhibitor with trypsin (Malthouse et al., 1983 and 1985; Scott et al., 1986). As shown in Fig. 1, Ser-Ile attacks the carbonyl carbon of the trifluoromethyl residue of TPCK forming a tetrahedral hemiketal. Because of the high stability of the chloromethyl ketone carbon-carbon bond, the normal displacement observed with amides and esters does not ensue.

In addition to the covalent link between the inhibitor and the Ser-195 hydroxyl group, N² of the His-57 imidazole side chain displaces chloride from the chloromethyl group of the inhibitor, giving a complex which is covalently attached to...
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FIG. 1. Structure of the active center of Tos-L-[2-13C]Phe-CH2-chymotrypsin in the intact state (tetrahedral 13C) and in the disrupted state (trigonal 13C). In aqueous solutions of denatured Tos-L-[2-13C]Phe-CH2-chymotrypsin, the labeled carbonyl does not become hydrated (Finucane et al., 1989), and thus remains in the trigonal configuration. While the figure shows only the unprotonated (oxyanionic) hemiketal, the "oxyanion hole" stabilizes the protonated hemiketal as well.

the enzyme at two positions (Fig. 1). While the histidyl-inhibitor bond is highly stable, even to amino acid hydrolysis (Schoellman and Shaw, 1963; Ong et al., 1965), the hemiketal is relatively unstable. It forms only because of the proximity of Ser-195 and the inhibitor in the intact active center and the stabilizing effect of the oxyanion hole. Upon enzyme denaturation, the hemiketal collapses to re-form the ketone (Finucane et al., 1989), but the label is still covalently linked to the enzyme at His-57 (Schoellman and Shaw, 1963; Ong et al., 1965). Thus the hybridization state, trigonal (in the ketone) or tetrahedral (in the hemiketal), of the carbonyl carbon of the phenylalanyl residue of TPCK serves as a probe of the defining structural feature of the serine protease α-chymotrypsin. This hybridization state tests the enzyme’s ability to stabilize the tetrahedral intermediate resulting from attack, by the catalytic Ser-195, of the carbonyl of the residue in the P1 binding site. The hemiketal carbon chemical shifts are far from the natural abundance carbon chemical shifts in proteins (especially important in solid-state NMR, where line widths are broader than in solution (Mehring, 1983)). The hemiketal structure in the complex of a chloromethyl ketone inhibitor with a related serine protease, trypsin, was shown to be at least partially retained during lyophilization (Scott et al., 1986). Consequently, we chose to investigate the hemiketal complex of α-chymotrypsin as a probe of the integrity of the active center under nonaqueous conditions, using 13C CP/MAS NMR spectroscopy.

Preparation of Solid Enzyme Samples—At the outset, we investigated the effect of lyophilization on the enzyme-inhibitor complex depicted in Fig. 1. Subtraction of the natural abundance 13C CP/MAS spectrum of Tos-L-Phe-CH2-chymotrypsin from the spectrum of lyophilized Tos-L-[2-13C]Phe-CH2-chymotrypsin yields a difference spectrum (Fig. 2a) with three signals: at 205, 103, and 99 ppm. The 205 ppm peak corresponds to a trigonal hybridization state of the labeled carbon (Finucane et al., 1989), indicating that there is a substantial portion of the label which is not in the tetrahedral form. The peaks at 103 and 99 ppm arise from the intact active center complex shown in Fig. 1, with the downfield and upfield peaks attributable to the unprotonated and protonated hemiketal species, respectively. Thus, unlike in solution (Finucane et al., 1989), proton exchange between these two forms is slow on the NMR time scale in the lyophilized powder. The ratio between the forms is approximately that expected for an acid/conjugate base pair with the hemiketal’s pKₐ of 8.7 (Finucane et al., 1989), in a pH 8.0 solution. That the pH of the enzyme solution prior to lyophilization was 8.0 indicates

FIG. 2. 13C CP/MAS NMR difference spectra of Tos-L-[2-13C]Phe-CH2-chymotrypsin in the following states: lyophilized powder (a); frozen solution (b); co-lyophilized with sucrose (c) or ammonium sulfate (d); precipitated with ammonium sulfate (e) or acetone (f). Spectrum c was obtained by subtracting that of an unlabeled sample containing the same fraction of sucrose. The additional signal in f arises from residual acetone. Samples contained approximately 80 (a), 25 (b, c, d), or 60 (e, f) mg of chymotrypsin. The signals at 152 and 258 ppm are spinning side bands.
that no significant pH shifts occurred during freezing.

We subsequently examined the same sample in a frozen aqueous solution and found (Fig. 2b) that some of the labeled carbon is in the ketone form. The approximate fraction in this form is consistent with that observed in Tos-L-\([2-\text{\textsuperscript{13}}\text{C}]\) Phe-CH\(_2\)-chymotrypsin in solution (Finucane et al., 1989) and corresponds to TPCK which alkylated Met-192 rather than His-57. Thus, freezing did not result in disruption of any active centers. The ratio of the integrated intensities of the hemiketal resonances in Fig. 2, a and b, indicates that the lyophilized powder is 58\% intact (Table I). Fig. 2b also indicates that in the frozen solution sample the hemiketal exists predominantly in the protonated form (99 ppm). This is due to the lower pH of the concentrated solution prior to freezing, which would give approximately 95\% protonated hemiketal. A small peak at 103 ppm suggests that freezing did not further alter the ratio between protonated and unprotonated forms.

Because 42\% of the enzyme was disrupted during lyophilization, we explored other ways of preparation of a solid enzyme sample. First, we prepared samples from solutions which contained two commonly used lyoprotecting additives (Fishbein and Winkert, 1979; Franks, 1985). Lyophilization of the labeled protein (5 mg/ml) from solutions containing sucrose and ammonium sulfate (10 mg/ml in each case) gave the spectra shown in Fig. 2, d and f, respectively. As one can see in Table I, both additives afforded substantial stabilization during lyophilization, although in neither case was the resultant sample fully intact. In addition, both these samples showed pH shifts during the sample preparation. In the case of ammonium sulfate, while the pH of the protein solution was adjusted to 8.0 after addition of the salt, the freeze concentration effect (Franks, 1985) would further increase the ionic strength, thereby causing a decrease in the pH prior to freezing (Perrin and Dempsey, 1974). It is unclear why the pH shifts in the sucrose-containing sample.

Besides lyophilizing with additives, we also prepared solid enzyme samples by ammonium sulfate (Fig. 2e) and acetone (Fig. 2f) precipitation. The fraction of intact active centers in the former precipitate was similar to that observed in the lyophilized powder which was prepared with this salt (albeit in a much lower concentration) as a stabilizing additive (Table I). In contrast, acetone precipitation proved worse than lyophilization with no additive. Both the ammonium sulfate and acetone precipitates experienced pH shifts during preparation, as evidenced by the exclusive appearance of a protonated hemiketal peak (at 99 ppm) in Fig. 2, e and f. In the former sample, the observed shift is again attributable to ionic strength effects. As for the latter sample, alterations of the pH\(_K\), of the protein ionogenic groups, caused by the organic solvent, may be responsible. Also, other physical properties of the solvent will change during acetone addition, making the effective pH impossible to predict (Fink, 1986).

A more fundamental question is what causes active center disruption in the aforementioned samples. The retention of the aqueous protonation state in the lyophilized powder with no additives, and the similarity of the solution (Finucane et al., 1989) and frozen solution spectra, indicate that disruption in the absence of lyoprotectants occurs during the dehydration step of lyophilization. The spectrum in Fig. 2a was invariable with the storage time of the powder, ruling out deleterious molecular events subsequent to dehydration (Liu et al., 1991).

The \(^{13}\)C probe used here assays only the enzyme's ability to stabilize a transition state analog. While, from a pragmatic viewpoint, this is the salient enzyme feature, the present experiment says nothing about the precise nature of the conformational change leading to its loss. This structural change is likely minor in terms of the overall conformation; Przybycien and Bailey (1989) observed that the secondary structure content of \(\alpha\)-chymotrypsin does not change on lyophilization. Also, Rupley et al. (1980, 1983) showed that lysozyme does not unfold on dehydration (the inter-spin distance of doubly spin-labeled lysozyme remained constant).

In conclusion, while the methods employed here for preparation of solid enzyme samples for use in organic solvents result in varying percentages of intact enzyme, even in the worst case (acetone precipitation) the difference between the solid sample and the aqueous solution from which it came is only 2-fold (Table I). Considering that the only similarly studied enzyme, \(\alpha\)-lytic protease, remains fully intact during ordinary freeze drying (Huang et al., 1984), the method of preparation of the dehydrated enzyme should not be a major concern for routine applications of inexpensive enzymes.

**The Effect of Organic Solvents on the Lyophilized Enzyme**—Next we turned to the question of what happens to the fraction of intact enzyme active centers in the lyophilized powder when an anhydrous solvent is added. To this end, we examined the effect of seven commonly used organic solvents on the powder which was prepared in the absence of lyoprotectants (and was thus 58\% intact prior to the addition of solvent).

Solvents are listed in Table II along with the corresponding fraction of intact active centers, determined from difference spectra (not shown) qualitatively similar to those in Fig. 2. The spectra obtained were invariable with time, thus indicating that any decrease in the percentage of intact protein compared to the lyophilized powder occurred immediately upon addition of the solvent. In contrast, with two additional

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Fraction of intact (\alpha)-chymotrypsin active centers in solid enzyme preparations</td>
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</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction intact*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen solution</td>
<td>100%</td>
</tr>
<tr>
<td>Lyophilized powder</td>
<td>58%</td>
</tr>
<tr>
<td>Lyophilized powder/sucrose</td>
<td>83%</td>
</tr>
<tr>
<td>Lyophilized powder/ammonium sulfate</td>
<td>76%</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate</td>
<td>82%</td>
</tr>
<tr>
<td>Acetone precipitate</td>
<td>53%</td>
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</tbody>
</table>

* Based on integration of protonated and unprotonated hemiketal peaks of difference spectra of Tos-L-\([2-\text{\textsuperscript{13}}\text{C}]\)Phe-CH\(_2\)-chymotrypsin. Normalized to frozen solution. See text for details. Error, estimated from the variation in peak area with frequency domain phasing and the duplication of several samples, is \(\pm 5\%\).

**Table II**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Fraction of intact (\alpha)-chymotrypsin active centers in various organic solvents</th>
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<tbody>
<tr>
<td></td>
<td>Sample</td>
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<tr>
<td>Tetrahydrofuran</td>
<td>41%</td>
</tr>
<tr>
<td>Dioxane</td>
<td>29%</td>
</tr>
<tr>
<td>Acetonitrile, 5% (\text{H}_2\text{O})</td>
<td>32%</td>
</tr>
<tr>
<td>Dioxane, 5% (\text{H}_2\text{O})</td>
<td>35%</td>
</tr>
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</table>

The fraction was calculated as in Table I. Solvents, which were anhydrous (\(<0.01\% \text{ water}\) except where noted, were added to the lyophilized Tos-L-\([2-\text{\textsuperscript{13}}\text{C}]\)Phe-CH\(_2\)-chymotrypsin powder, which was initially 58\% intact.
solvents tested, pyridine and dimethylformamide, the intensity of the hemiketal peak decreased over time. In the case of pyridine, the half-life, estimated by integrating the hemiketal peak for specific time blocks over the course of a multi-hour acquisition, was approximately one hour. Dimethylformamide affected the enzyme even faster. While difficult to estimate accurately because the hemiketal peak disappeared before sufficient acquisitions had accumulated, the half-life was less than 10 min.

We suspected at the outset of this work that a cause of active center disruption in the enzyme powder by an anhydrous solvent would be water stripping (Schulze and Klibanov, 1991). Our conclusion that dehydration caused disruption during lyophilization supported this notion. A plot (Fig. 3) of the data from Table II versus log P (Rekker, 1977) shows an excellent correlation between the percentage of active centers remaining intact in a given solvent and the solvent's hydrophobicity; the more hydrophobic a solvent (characterized by a higher log P value), the lower its dehydration effect, thus leading to a higher percentage of active centers remaining intact in the sample.

Since the extent of active center disruption correlated with the solvent’s propensity to act as a dehydrating agent, we examined the effect of two hydrated solvents, acetonitrile, 5% water and dioxane, 5% water, on the lyophilized powder. One can see in Table II that hydrated dioxane was less harmful than its dry counterpart, but with acetonitrile the opposite occurred. The latter effect has been observed by Schulze and Klibanov (1991) who showed that small amounts of water (1-2%) had a stabilizing effect on subtilisin in tert-amyl alcohol; however, at higher water concentrations, stability decreased. Similarly, water concentrations above 1% lower the activity of subtilisin in tetrahydrofuran (Affleck et al., 1992). We hypothesized that subtilisin unfolded at sufficiently high water contents in the experiments of Schulze and Klibanov (1991) and Affleck et al. (1992) and that our samples in hydrated dioxane and acetonitrile may also have undergone partial unfolding.

Why does water exhibit opposite effects in the cases of dioxane and acetonitrile? To answer this question we consider the observation (Guinn et al., 1991) that enzyme flexibility in organic solvents is related to the solvent dielectric constant—enzymes are more flexible in solvents with high dielectric constants than in those with low. (This has also been inferred from subtilisin kinetic data (Fitzpatrick and Klibanov, 1991).) The dielectric constants of dioxane and acetonitrile hydrated to 5% (w/w) of water are 4 (Janz and Tomkins, 1972) and 37 (Akhadov, 1980), respectively. Thus, we expect that the degree of flexibility (and hence of unfolding) in the latter will be higher than in the former.

In order to test our hypothesis that unfolding of a fraction of the enzyme is occurring, we took advantage of the fact that our structural probe has a built-in indicator of dynamics, the aromatic ring of the phenylalanyl side chain. In the complex shown in Fig. 1, the phenylalanyl side chain of the inhibitor fills the hydrophobic specificity pocket (the P1 binding site) of chymotrypsin (Segal et al., 1971). The motions of aromatic rings in proteins have been the subject of seminal studies of protein dynamics by NMR (Wagner et al., 1976; Wagner, 1983). Tyr and Phe aromatic side chains undergo ‘flips’ (180° rotations, where the period of the rotation is short compared to the residence time in the new orientation). These flips average the quadrupolar powder pattern in the deuterium spectrum of static solids in which the protons of the aromatic ring of interest have been substituted with deuterium (Rice et al., 1981; Keniry et al., 1984). Line shapes in the quadrupole echo experiment are sensitive to flipping rates in the range of 10^3–10^7 Hz (Fig. 4) (Griffin et al., 1988). In the case of Tos-L-

![Fig. 3. The dependence of the fraction of intact Tos-L-[2-13C]Phe-CH2-chymotrypsin active centers on the solvent hydrophobicity, represented by log P (where P is the solvent partition coefficient between n-octyl alcohol and water; Rekker, 1977). Solvents: octane (a), toluene (b), tert-butyl methyl ether (c), octanol (d), tetrahydrofuran (e), acetonitrile (f), and dioxane (g).](image)

![Fig. 4. Theoretical 2H quadrupole echo NMR line shapes for an aromatic ring as a function of the flipping rate. Calculated as described by Wittebort et al. (1987).](image)
Phe-CH₂-chymotrypsin, the rate of ring flipping of the Phe side chain is expected to be at the slow end of this frequency range when the ring is in the intact enzyme binding pocket, but at the fast end when the pocket is disrupted (based on flipping rates for buried versus exposed aromatic rings in other proteins (Wagner et al., 1976; Wagner, 1983)). To determine whether disruption of the chymotrypsin binding pocket occurred in our system, we prepared samples identical to those used in the above ¹³C CP/MAS NMR experiments, only using Tos-L-[2',3',4',5',6'-²H]-Phe-CH₂Cl.

It was necessary to demonstrate that in Tos-L-[2',3',4',5',6'-²H]-Phe-CH₂-chymotrypsin, even under aqueous conditions (where dynamics are expected to be the fastest), the ring flipping in an intact binding pocket is sufficiently constrained to produce a rigid powder pattern in the deuterium spectrum. For this purpose, we prepared protein crystals of the Tos-L-[2',3',4',5',6'-²H]-Phe-CH₂-chymotrypsin, an inhibitor complex which, to our knowledge, has not been crystallized before. The deuterium spectrum of crystallized Tos-L-[2',3',4',5',6'-²H]-Phe-CH₂-chymotrypsin is presented in Fig. 5a. While the signal-to-noise ratio of the spectrum is suboptimal (due to the difficulty of obtaining large quantities of crystals), it is clear that there is no fast component in the intact enzyme, even in the (high-dielectric constant) aqueous crystallization solution.

Next, we examined the deuterium spectrum of the lyophilized powder (Fig. 5b). From the CP/MAS spectrum of the ¹³C-labeled powder prepared in an identical manner (Fig. 2a), we know that only 58% of the enzyme molecules are intact in this sample. As discussed above, the conformational change which resulted in disruption of the remaining active centers likely involved no unfolding of the protein. The deuterium spectrum (a rigid powder spectrum, Fig. 5b) of the lyophilized powder confirms that this is largely the case. (The presence of a small fast component indicates that a minor portion of the denatured protein has unfolded.) To confirm that this result was not an artifact of dehydration, which could in principle lead to a lower flipping rate for liberated aromatic rings, we examined the deuterium spectrum for the lyophilized powder in 50% saturated, ²H-depleted ammonium sulfate solution (in which it is insoluble). The spectrum was essentially identical to that in Fig. 5b.

Subsequently we examined the effect of organic solvents on the enzyme binding pocket. Based on the hypothesis delineated above, we anticipate that in anhydrous dioxane, due to its hydrophilicity, water stripping should be the sole mechanism of active center disruption. Because of the low dielectric constant of dioxane (ε = 2.2), the protein flexibility is likely to be insufficient to allow unfolding of the enzyme binding pocket. As shown in Fig. 5c, the deuterium spectrum is indeed characteristic of a highly constrained aromatic ring.

The deuterium spectra of the enzyme in anhydrous acetone, dioxane, 5% water, and acetonitrile, 5% water (ε of 36, 4, and 37, respectively) were studied next. In the first case, both mechanisms of active center disruption are expected. Because of its hydrophilicity, anhydrous acetone should inflict significant damage due to water stripping. In addition, acetone has a high dielectric constant, which will allow enhanced protein flexibility and, in turn, some protein molecules to unfold (Guinn et al., 1991). In the second two samples, only one of the two mechanisms of active center disruption will occur; the presence of 5% water precludes inactivation by dehydration. Water is expected, however, to increase enzyme flexibility (Finney and Poole, 1984; Zaks and Klíbanov, 1988b), and thus the likelihood that the enzyme will unfold, liberating the Phe side chain from the confines of the enzyme-binding pocket.

The deuterium spectrum in anhydrous acetone clearly shows a significant population of fast-flipping Phe rings (the center component in Fig. 5d with quadrupolar splitting of 30 kHz). Nevertheless, there is still a major rigid component in the spectrum (quadrupolar splitting of 125 kHz). Thus in anhydrous acetone both mechanisms of disruption are indeed at play: dehydration by the solvent (this was confirmed by the titration of 0.074% water in the supernatant after addition of the anhydrous solvent to the protein powder) and unfolding due to enhanced flexibility of the protein in this high-dielectric constant medium.

Fig. 5, e and f, indicates that hydrated dioxane and acetonitrile caused substantial unfolding. The spectra show that the majority of rings, no longer constrained by an intact binding pocket, are flipping at the fast frequency limit of the line shape experiment. Nevertheless, a significant fraction of rings flipping at the slow frequency limit remains (at least 35% in the case of dioxane, 5% water, and 13% in the case of acetonitrile, 5% water, based on the ¹³C data in Table 1).

The hydrophobic side chain of the deuterated Phe residue is expected to partition into the solvent upon unfolding. The unfolded protein molecules will have a distribution of conformations, and the liberated rings will exhibit a range of flipping rates. Thus it is difficult to quantify precisely the portion of intact binding pockets (as opposed to the fraction of intact catalytic centers, which was determined by the ¹³C experiments) in each of the samples discussed above. However, the following is clear: 1) The number of intact enzyme binding
pockets in anhydrous, low dielectric dioxane is essentially the same as in the lyophilized powder. 2) Anhydrous, high dielectric acetonitrile results in the unfolding of some binding pockets, but not as many as hydrated acetonitrile. 3) The molecular lubricant water increases the fraction of unfolded binding pockets in both solvents. The substantial increase in the fast component in the acetonitrile spectrum (Fig. 5, d compared with f) and the dioxane spectrum (Fig. 5, c compared with e) on addition of water indicates that water’s effect exceeds that predicted from the net dielectric constant. This is probably due to partitioning of water (Zaks and Klibanov, 1988b) to the enzyme surface, which will raise the local dielectric constant.

The Effect of Organic Solvents on Enzyme Prepared with Lyoprotectants—The cause of active center disruption in most of the solvents was dehydration. Because the lyoprotectants sucrose and ammonium sulfate were effective in stopping dehydration-induced damage during preparation of enzyme powders (Table I), we investigated the possibility that this beneficial effect might carry over into the dehydrating environment of the anhydrous solvents. To this end, we examined the 13C-labeled powders prepared with ammonium sulfate (Fig. 6a) and sucrose (Fig. 6b) in dioxane. Comparison with the spectra of the corresponding samples in the absence of solvent revealed that these lyoprotectants eliminated the deleterious effect of dioxane, confirming their ability to stop damage induced by dehydration.

In addition, we hypothesized that the lyoprotecting additives would lessen the extent of enzyme unfolding in solvents where flexibility was sufficiently high to permit it. The rationale behind this hypothesis is as follows. Both ammonium sulfate and sucrose are insoluble in the solvents examined and have low dielectric constants in the solid state (ε of 7.0 and 3.3, respectively). Hence they will lower the effective ε in the enzyme particles, according to the obstruction effect of particles suspended in a solvent of a different dielectric constant (Robinson and Stokes, 1959). This will occur regardless of the water content of the solvent, as long as its dielectric constant exceeds that of the solid particles. (Similarly, aqueous solutions of ammonium sulfate (Robinson and Stokes, 1959) and sucrose (Harned and Owen, 1964) have lower ε than pure water.) While in dioxane, 5% water, the net dielectric constant is lower than that of ammonium sulfate, the effect is still anticipated due to the partitioning of water to the enzyme surface (Zaks and Klibanov, 1988b).

We examined the lyoprotectant-containing enzyme powders in anhydrous acetonitrile, which had been shown to disrupt enzyme active centers by both dehydration and unfolding. The spectra of powders prepared with ammonium sulfate and sucrose (Fig. 6, c and d, respectively) show that the lyoprotecting additives markedly stabilized the enzyme active center; acetonitrile, which previously reduced the fraction of intact active centers to 32%, now has virtually no effect.

The sucrose-containing powders were also examined in the two hydrated solvents where unfolding was responsible for disruption of the active center. In these solvents, sucrose can only stop active center disruption by lowering the flexibility of the enzyme and thus keeping it in its kinetically trapped, native conformation. As shown in Fig. 6, e and f, there is no discernible disruption of the α-chymotrypsin active center in either dioxane, 5% water or acetonitrile, 5% water when the enzyme is lyophilized from a sucrose solution.

CONCLUSIONS

Using solid-state NMR spectroscopy, we have shown that the transition-state stabilizing capability of the α-chymotrypsin active center can be disrupted by two mechanisms: 1) Dehydration, which leaves the hydrophobic binding pocket intact, but can cause enough distortion of the catalytic center to destroy its transition-state stabilizing power. This mechanism occurs during lyophilization and on addition of water-stripping anhydrous solvents to lyophilized powders containing no protecting additives. 2) Unfolding, which occurs in organic solvents when the enzyme acquires sufficient flexibility to escape from its kinetically trapped conformation in the lyophilized powder.

Comparison of our results with active center titration data (Zaks and Klibanov, 1988a) suggests that in octane nonitrifiable active centers (about 35%) were incompetent due to a reversible structural change caused by the dehydration step of lyophilization. The present study also offers new mechanistic insights into the phenomenon of enzyme memory (Russell and Klibanov, 1988) which involves co-lyophilization of an enzyme and a ligand, followed by the removal of the latter with anhydrous solvents dissolving the ligand but not the protein. In addition to their effect of inducing beneficial conformational changes in the enzyme in water and preserving them during lyophilization, the imprinting ligands prob-
ably play a lyoprotecting role as well. In some cases, they may even protect intact active centers during freeze drying, without actually binding in them.

Finally, we turn to the questions of why enzymes are usually far less active in anhydrous solvents than in water (Zaks and Klibanov, 1986) and why activity markedly varies from one solvent to another (Klibanov, 1986). This underscores the notion (Zaks and Klibanov, 1988b) that, other factors, such as a decrease in molecular organic solvents (Affleck, 1986) and why activity markedly varies from one solvent to another (Klibanov, 1986), are primal. Even protect intact active centers during freeze drying, with-

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