Structure and Function of Subtilisin BPN′ Solubilized in Organic Solvents

Pramod P. Wangikar,[†] Peter C. Michels,[‡,§] Douglas S. Clark,*[,‡] and Jonathan S. Dordick*[,†]

Contribution from the Department of Chemical and Biochemical Engineering, and Center for Biocatalysis and Bioprocessing, University of Iowa, Iowa City, Iowa 52242, and Department of Chemical Engineering, University of California, Berkeley, California 94720

Received July 29, 1996[⊗]

Abstract: Enzyme structure and function have been studied for subtilisin BPN′ solubilized in organic solvents by ion pairing with low concentrations of an anionic surfactant (Aerosol OT) in the absence of reversed micelles. Soluble subtilisin shows strikingly different behavior in octane and tetrahydrofuran (THF). In octane, the k_{cat}/K_m for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) is 370 M^{-1} s^{-1}, within one order of magnitude of the enzyme's hydrolytic activity in water. Moreover, the observed half-life of the soluble enzyme in octane is nearly three orders of magnitude greater than in water, presumably because of the absence of autolysis in the organic solvent. In contrast, the catalytic efficiency of the enzyme dissolved in the polar solvent THF is 0.04 M^{-1} s^{-1}, and the enzyme loses 99% of its activity within 10 min. Comparable enzyme inactivation could also be observed in octane, but only at elevated temperatures such as 70 °C. Therefore, the mechanisms of deactivation of the soluble enzyme were investigated in both octane and THF. Kinetic and spectroscopic (CD and EPR) studies support the existence of multiple inactive forms of the soluble enzyme in THF at 25 °C and in octane at 70 °C. Notably, in both cases a denatured form can be renatured in anhydrous octane at 25 °C, the first demonstration of enzyme renaturation in a bulk organic solvent. A model explaining the THF- and thermally-induced inactivation processes of soluble subtilisin BPN′ is proposed, and the apparent reasons for the exceptionally high activity and stability of the soluble enzyme in octane are discussed.

Introduction

Enzymatic catalysis in organic solvents has resulted in important advances in enzyme technology.[1] Nearly all nonaqueous enzyme systems involve heterogeneous suspensions, and the catalytic efficiencies of enzymes in such systems are often up to four orders of magnitude lower than in aqueous solutions.[2] The low activity has been attributed to several different phenomena including ground-state stabilization of hydrophobic substrates in hydrophobic solvents.[2,3] solvent-induced structural perturbation of the enzyme's active site,[4] dehydration of critical regions of the enzyme,[5] and lyophilization-induced structural changes.[6]

Several techniques have been developed to enhance enzyme activity in nearly anhydrous organic solvents. Most of these procedures have utilized hydrolytic enzymes, mainly because so much is known about the structure and function of these enzymes. For example, lyophilization in the presence of a substrate analogue has been shown to activate enzymes by up to two orders of magnitude, presumably because the enzyme's active site retains its catalytically competent structure through lyophilization.[7] Even more significant activations have been achieved by lyophilizing enzymes in the presence of nonbuffer salts.[8] This activation has been ascribed to the formation of a polar matrix surrounding the enzyme, thereby helping to maintain the catalytically competent structure. Protein engineering has also proven to be successful in activating enzymes in organic solvents. Through specific site-directed mutations in the active site of subtilisin BPN′, activations of up to two orders of magnitude have been achieved.[3a,9]

Although native enzyme molecules are insoluble in organic solvents, Paradkar and Dordick recently demonstrated that enzymes could be solubilized in nearly anhydrous organic solvents via ion-pairing with surfactant molecules.[10] By maintaining a low concentration of surfactant, reversed micelle formation is prevented, and stable, individual enzyme molecules are obtained in organic solutions. These ion-paired enzyme−surfactant complexes enable rapid spectroscopic analysis and can be used to evaluate the effect of solvent on enzymic secondary and tertiary structure.[11] This is particularly important in evaluating the effect of the organic solvent on the stabilization of the enzyme. Thus, use of organic solutions is fortuitous in that it enables detailed investigation of enzyme structure and

[†] University of Iowa.
[‡] University of California.
[§] Present address: EnzyMed Inc., Iowa City, Iowa 52242.
[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1996.

(1) (a) Dordick, J. S. *Biotechnol. Prog.* **1992**, *8*, 259. (b) Klibanov, A. M. *CHEMTECH* **1986**, *16*, 354. (c) Zaks, A.; Russell, A. J. *J. Biotechnol.* **1988**, *8*, 259. (d) Dordick, J. S. *Applied Biocatalysis*; Blanch, H. W., Clark, D. S., Eds; Marcel Dekker: New York, 1991; pp 1−51, Vol. 1.

(2) Ryu, K.; Dordick, J. S. *Biochemistry* **1992**, *31*, 2588.

(3) (a) Wangikar, P. P.; Rich, J. O.; Clark, D. S.; Dordick, J. S. *Biochemistry* **1995**, *34*, 12302. (b) Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 10362.

(4) Burke, P. A.; Griffin, R. G.; Klibanov, A. M. *J. Biol. Chem.* **1992**, *267*, 20057.

(5) Gorman, L. S.; Dordick, J. S *Biotechnol. Bioeng.* **1992**, *39*, 392.

(6) (a) Desai, U. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1995**, *117*, 3940. (b) Desai, U. R.; Osterhout, J. J.; Klibanov, A. M. *J. Am. Chem. Soc.* **1994**, *116*, 9420.

(7) Dabulis, K.; Klibanov, A. M. *Biotechnol. Bioeng.* **1993**, *41*, 566.

(8) Khmelnitski, Y. L.; Welch, S.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 2647.

(9) Wangikar, P. P.; Graycar, T. P.; Estell, D. A.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1993**, *115*, 12231.

(10) (a) Paradkar, V. M.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 5009. (b) Paradkar, V. M.; Dordick, J. S. *Biotechnol. Bioeng.* **1994**, *43*, 529.

(11) (a) Matsuura, J.; Powers, M. E.; Manning, M. C.; Shefter, E. *J. Am. Chem. Soc.* **1993**, *115*, 1261. (b) Meyer, J. D.; Matsuura, J. E.; Kendrick, B. S.; Evans, E. S.; Evans, G. J.; Manning, M. C. *Biopolymers* **1995**, *35*, 451.

S0002-7863(96)02620-0 CCC: $14.00

function in organic solvents. Moreover, the inherent activity and stability of enzymes in organic solvents can be determined free from potential diffusional limitations. In the present work, we examine enzyme structure, function, and stability in homogeneous organic solutions using subtilisin BPN′ (from *Bacillus amyloliquefaciens*) as a model enzyme. Different states of subtilisin during solvent-induced inactivation are described, and a model is proposed to explain the nature of subtilisin in organic solvents as unfolding occurs.

Materials and Methods

Materials. Semipurified subtilisin BPN′ was generously provided by Thomas Graycar (Genencor International, S. San Francisco, CA). The enzyme was further purified by cation exchange chromatography,[12] desalted on a Sephadex G-25 column using a 10-mM ammonium bicarbonate running buffer, pH 6.5, and then lyophilized for 40 h. The buffer was evaporated during lyophilization to give an essentially salt-free enzyme preparation. All solvents were purchased from Aldrich (Milwaukee, WI) and were of the highest grade commercially available. The solvents were dried over molecular sieves for at least 24 h prior to use. All amino acid and peptide-based substrates and Aerosol OT (AOT) were purchased from Sigma (St. Louis, MO). Active-site titration of the enzyme preparations was performed in aqueous buffer with *N*-*trans*-cinnamoylimidazole as the titrant.[13] The subtilisin BPN′ preparation contained 43% active enzyme in water, ca. 95% of which was found to be active when solubilized in octane.

Solubilization of Subtilisin BPN′ in Organic Solvents. The enzyme was extracted from an aqueous solution (pH 7.8) into an equal volume of isooctane containing 2 mM AOT as described for α-chymotrypsin by Paradkar and Dordick.[10a] The aqueous phase consisted of 1 mg/mL subtilisin BPN′ and 10 mM bis-Tris propane buffer, pH 7.8, containing 2 mM $CaCl_2$. The solutions were contacted by stirring at 250 rpm at 25 °C for 2 min. The phases were allowed to settle and then centrifuged to obtain clean phase separation. The organic soluble enzyme was then dried by bubbling N_2 through the solution to give an enzyme−surfactant ion-paired complex which can be dissolved in different organic solvents. The concentration of subtilisin in the organic phase was determined spectrophotometrically at 280 nm.

Kinetic Measurements. Kinetics of hydrolysis of *N*-Ac-L-Phe-OEt (0.1−1.0 mM) in water was followed using a Mettler DL-21 pH-Stat using the method of Zerner et al. (1964).[14] A typical hydrolytic reaction mixture contained 0.5% (v/v) CH_3CN, 0.1 M NaCl, and 10−50 μg/mL of enzyme at 25 °C. Transesterification reactions of *N*-Ac-L-Phe-OEt (APEE) (0.1−250 mM) with 0.5 M 1-propanol were performed in nearly anhydrous organic solvents with 14−140 μg/mL of solubilized enzyme. Gas chromatography [25-m capillary column with 530−μm fused silica gum (Hewlett-Packard), N_2 as carrier gas (30 mL/min), and injector and detector port temperatures of 250 °C] was used to measure the initial rate of formation of *N*-Ac-L-Phe-OPr. Values of k_{cat}/K_m were determined by nonlinear fits of initial rate vs substrate concentration data.

Circular Dichroism. CD measurements were performed on an AVIV instrument (Model 62 DS, Lakewood, NJ) equipped with a thermoelectric temperature control unit at temperatures ranging from 0 to 25 °C. All temperatures were measured to within ±0.2 °C. Far-UV CD spectra (205−250 nm) were collected at protein concentrations of 5−8 μM in a 0.1-cm cell. Near-UV CD spectra (250−300 nm) were measured at protein concentrations of 10−12 μM in a 1-cm cell. The spectra were collected at 1-nm intervals, averaged over three scans, corrected for the appropriate (solvent, buffer, or solvent−buffer mixture) base line, and smoothed by a three point moving average.

Electron Paramagnetic Resonance Spectroscopy. Subtilisin was spin-labeled at the active site serine with 4-(methylfluorophosphinyloxy)-TEMPO as described by Morrisett and Broomfield (1972).[15] The labeled enzyme was separated from free label using a Sephadex G-50 size exclusion column and concentrated to 1 mg/mL using a YM-10 ultrafiltration membrane (Amicon) in the same buffer as used in the extraction experiments. The spin-labeled enzyme was extracted into isooctane, as described above, dried by bubbling a stream of nitrogen through the solution, and transferred to glass capillary tubes via a syringe. To obtain the initial spectrum at a given temperature, the sample cavity was first equilibrated at the desired temperature using a heated nitrogen stream. A control sample of labeled subtilisin in the appropriate solvent was used to tune the EPR signal. Then, a fresh protein sample was placed into the sample cavity, and the signal was quickly fine tuned after the sample reached temperature equilibration. Trials with a thermocouple inserted in an EPR capillary filled with water or octane and tuning the EPR spectrometer at the desired temperature both indicated that less than 90 s was required for thermal equilibration from room temperature to 70 °C. Conventional ESR spectra were immediately recorded at the indicated temperature on a Bruker ER200D-SRC spectrometer with a microwave power of 12.6 mW, a modulation amplitude of 2.0 G, and a scan width of 150 G. Samples could be rapidly cooled by removal from the cavity and immersion of the capillary in a water bath. Thus, the entire heating, sample collection, and cooling of the labeled soluble enzyme in solvent could be accomplished for strong EPR signals in ca. 4 min.

Table 1. Catalytic Constants for Soluble Subtilisin BPN′ in Various Solvents

solvent	k_{cat}/K_m (M^{-1} s^{-1})[a]	observed half-life (h)[b]
octane	370 ± 40	1400[d]
tert-amyl alcohol	1.2 ± 0.2	1.67
tert-amyl alcohol + 0.2% (v/v) H_2O	2.8 ± 0.4	n.d.
tetrahydrofuran	0.04 ± 0.01	0.17
tetrahydrofuran + 0.2% (v/v) H_2O	0.36 ± 0.06	0.67
aqueous buffer	3500 ± 200[c]	2.17
octane (suspended)	0.6 ± 0.1	n.d.

[a] Catalytic constants determined for transesterification between N-Ac-L-Phe-OEt (0.1−250 mM) and 0.5 M *n*-PrOH using 14−140 μg/mL soluble enzyme. Reaction mixtures shaken at 250 rpm at 25 °C. [b] Observed half-life (25 °C) measured by incubating soluble subtilisin in the given solvent and then assaying the activity in water by transferring small aliquots of subtilisin solution to water; n.d. indicates not determined. The enzyme concentration was 0.05 mg/mL. [c] Catalytic efficiency determined for hydrolysis of N-Ac-L-Phe-OEt (0.1−1.0 mM) in aqueous buffer (10 mM bis-Tris propane buffer, pH 7.8, containing 2 mM $CaCl_2$) using 10−50 μg/mL enzyme at 25 °C. [d] Observed half-life reported in octane is an estimated value, because soluble subtilisin lost only ca. 35% activity in 2 months when incubated in dry octane.

Results

Soluble Enzyme Activity and Stability. The k_{cat}/K_m for the transesterification of APEE with *n*-PrOH catalyzed by subtilisin BPN′ dissolved in octane is nearly three orders of magnitude greater than that of the suspended enzyme in the same solvent (Table 1). This exceptional activation of the enzyme results in a value of k_{cat}/K_m in octane that is within one order of magnitude of the value for ester hydrolysis in aqueous buffer, and demonstrates that the organic soluble enzyme is remarkably active in nonpolar, homogeneous organic solutions. Moreover, the enzyme is ca. three orders of magnitude more stable to irreversible inactivation in octane than in aqueous buffer, possibly because of the absence of autolysis in the organic solvent. Therefore, over its active lifetime the productivity of the soluble enzyme should be higher in octane than in water. These results for organic-solvent soluble subtilisin BPN′ are similar to those reported for soluble α-chymotrypsin,[10a] highlighting the general utility of the soluble enzyme form.

The benefits of solubilization, however, are not observed in all organic solvents. For instance, values of k_{cat}/K_m are much lower in the more polar *tert*-amyl alcohol and tetrahydrofuran

(12) Estell, D. A.; Graycar, T. P.; Wells, J. A. *J. Biol. Chem.* **1985**, *260*, 6518.

(13) Schonabaum, G. R.; Zerner, B.; Bender, M. L. *J. Biol. Chem.* **1961**, *236*, 2930.

(14) Zerner, B.; Bond, R. P. M.; Bender, M. L. *J. Am. Chem. Soc.* **1964**, *86*, 3674.

(15) Morrisett, J. D.; Broomfield, C. A. *J. Biol. Chem.* **1972**, *247*, 7224.

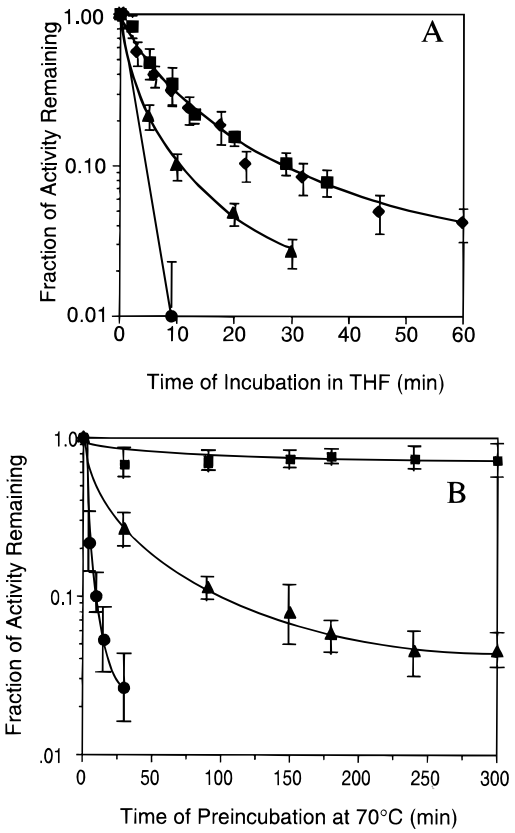

Figure 1. (A) Inactivation of subtilisin BPN′ solubilized in dry THF. Soluble subtilisin was incubated in THF for different lengths of time and the activity was assayed in dry THF (●), and after transferring small aliquots to dry octane (▲) and water (■). Also, subtilisin was incubated in THF for different lengths of time, then transferred to octane, extracted into water after 30 min more in octane, and the activity assayed in water (◆). For the enzyme transferred from THF to octane, no further change in activity was observed after 24 h in octane. The enzymatic activities are assayed for transesterification between N-Ac-Phe-OEt and 1-propanol in organic solvents, and for hydrolysis of suc-Ala-Ala-Pro-Phe-*p*-nitroanilide in water. Error bars are standard deviations of at least three measurements. (B) Inactivation of soluble subtilisin in octane at 70 °C. The fraction of residual activity is reported for the soluble enzyme preincubated in octane at 70 °C for the indicated times and then aliquots assayed in octane at 70 °C (●), 25 °C (▲), and 25 °C in aqueous buffer (■). Error bars are standard deviations of at least three measurements.

(THF) solutions (Table 1). Indeed, the difference of k_{cat}/K_m values between octane and THF corresponds to a 7.7 kcal/mol destabilization of the transition state of subtilisin in THF as compared to octane.[16] Moreover, application or further study of the soluble enzyme in polar solvents is greatly hindered by the extremely low enzyme stability in these systems (Table 1). Whereas the measurable activity of soluble subtilisin is retained after several months of storage in octane, activity in THF is lost in minutes. Furthermore, incubation in THF induces severe irreversible inactivation of soluble subtilisin. Specifically, less than 35% of the activity can be recovered if soluble subtilisin is incubated in THF for 10 min and then transferred to water (Figure 1A). The effect of organic solvents on enzyme structure and function, then, is an important parameter for catalysis in organic solutions and is a major focus of this study.

Effect of Solvent Hydration on Enzyme Activity. Stripping of water from the enzyme in polar solvents is known to cause lower catalytic efficiencies in heterogeneous organic suspensions.[5] To that end, we observed that adding small amounts (0.2%, v/v) of water to both THF and *tert*-amyl alcohol increased the activity of soluble subtilisin (Table 1). The addition of 0.2% (v/v), water to THF also improves the stabilitity of soluble subtilisin by ca. 4-fold. Thus, addition of water to polar solvents improves activity and stability of soluble subtilisin, at least in the polar THF.

Reversible and Irreversible Inactivation of Soluble Subtilisin in Organic Solvents. The rapid loss of activity in THF is depicted in Figure 1A. When assayed in THF, subtilisin loses 99% of its activity in less than 10 min. Much of this inactivated enzyme can be regenerated, however, upon transfer of the soluble enzyme to either water or octane. For example, transfer of subtilisin to water (by taking an aliquot of enzyme from the THF solution and diluting it into aqueous buffer) after 10 min resulted in the recovery of over one-third of the native enzyme activity (Figure 1A). Thus, soluble subtilisin inactivates quickly in dry THF, but can be partially renatured by transferring the enzyme to water. Similarly, transferring the enzyme from THF to *octane* also restores a portion of the active subtilisin, albeit a lesser fraction than in water (Figure 1A). After the rapid, partial reactivation, the fraction of active enzyme in octane does not measurably change further for up to 30 min. Subsequent transfer of the partially reactivated enzyme in octane to water, however, results in a fraction of active enzyme similar to that observed for the direct transfer of enzyme from THF to water (Figure 1A).

Thermal Inactivation of Soluble Subtilisin BPN′ in Octane. Similar inactivation and reactivation behavior was observed using elevated temperature as the denaturant in octane. Subtilisin was incubated in octane at 70 °C for various lengths of time, and the activity of the enzyme was assayed in octane at 70 °C, in octane at 25 °C, and following extraction into water at 25 °C. Three distinct trends were observed as shown in Figure 1B. First, in contrast to the high stability of soluble subtilisin at 25 °C, over 98% of enzymatic activity is lost after 30 min of incubation at 70 °C in octane. Second, the majority of the inactivated enzyme can be renatured by extraction back into aqueous buffer at 25 °C. Third, a significant fraction of the enzyme can also be renatured *in octane* by rapid cooling of the enzyme solution to 25 °C, albeit a smaller fraction than in water.

Thus, the thermal denaturation of soluble subtilisin BPN′ is qualitatively similar to the solvent-induced denaturation in THF. Soluble subtilisin BPN′ undergoes rapid inactivation both in octane at 70 °C and THF at 25 °C, and the enzyme can be partially renatured upon transferring the enzyme to buffer or octane at 25 °C. In both cases, the extent of renaturation depends on whether the enzyme is transferred to aqueous buffer or octane, strongly suggesting the existence of multiple inactivated soluble enzyme forms, each exhibiting different renaturation characteristics. To probe further the mechanisms of enzyme deactivation in organic solutions and the subsequent partial enzyme reactivation in aqueous and organic solutions, the structure of soluble subtilisin BPN′ was examined in organic solvents.

Structural Characterization of Subtilisin BPN′ in Organic Solutions. The solubility of subtilisin in organic solvents provided the opportunity to investigate the secondary and tertiary structure of the enzyme. The secondary structure (as determined

(16) It known that differences in substrate ground-state free energy can cause differences in the activities of enzyme suspensions in organic solvents.[2,3a] In the present case, differential ground-state calculations of N-Ac-L-Phe-OEt in THF and octane provide a value of $\Delta\Delta G_s$ ($\Delta\Delta G_s = (\Delta G_s)_{octane} - (\Delta G_s)_{THF} = 2.1$ kcal/mol, accounting for only a single order of magnitude difference in the activities of soluble subtilisin in these solvents. Thus, the effect of substrate ground-state free energy differences does not account for much of the deactivation of subtilisin BPN′ in going from octane to THF.

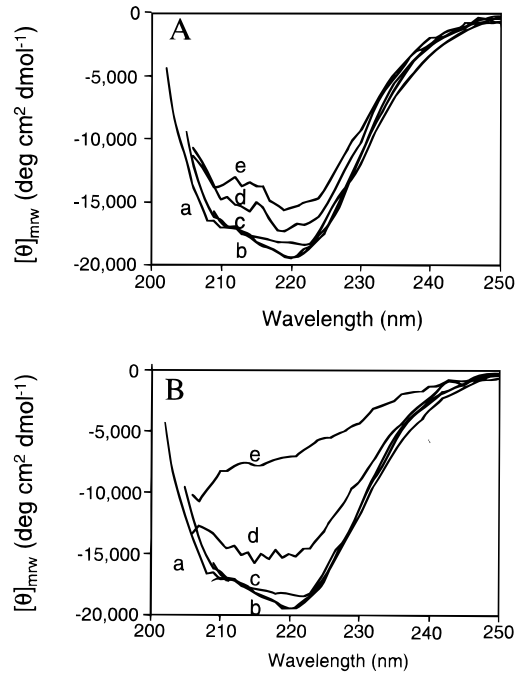

Figure 2. Far-UV circular dichrosim (CD) spectra of subtilisin BPN′ dissolved in water (a), octane (b), THF (c), and first dissolved in THF and then transferred after 1 min into octane (d) or water (e). Spectra were collected (A) immediately after solubilization (or transfer from THF for (d) and (e)), and (B) after 30 min in the respective solvent.

by the far-UV circular dichroism spectrum (205−250 nm)) of subtilisin dissolved in dry THF and octane was found to be nearly identical with that of the native enzyme in aqueous buffer (Figure 2A). Immediate transfer (within 1 min) of the dissolved enzyme in THF to either octane or water did not result in loss of secondary structure, as evidenced by the nearly identical shape and signal magnitude of the CD spectra at 220 nm. During a 30-min incubation in dry THF, subtilisin maintains its secondary structure, even though this is a long enough incubation period to impart significant and irreversible loss of activity. Hence, deactivation of subtilisin in THF is not necessarily accompanied by a change in the secondary structure of the enzyme. Subsequent transfer of this inactivated enzyme to octane and water provided strikingly different results. When this THF-inactivated enzyme is transferred to octane, the secondary structure remains substantially intact; however, transfer to water results in nearly complete loss of secondary structure (Figure 2B).

Near-UV circular dichrosim (250−300 nm) was used to probe the bulk tertiary structure of subtilisin dissolved in different solvents. As shown in Figure 3, the near-UV CD spectrum of soluble subtilisin in octane is comparable to that of the native enzyme's in water. In THF, an immediate loss in tertiary structure is evident as measured by a significantly smaller CD signal at 280 nm. Immediately transferring subtilisin from THF to either octane or water results in partial restoration of tertiary structure, an observation consistent with the partial recovery of catalytic activity.

These results imply that incubation of subtilisin in THF significantly alters the tertiary structure of the enzyme. The structural damage caused by THF is partially reversible in water and, interestingly, in octane as well. Furthermore, large secondary structural changes do not take place without substantial changes in the tertiary structure of the enzyme, and in any event, secondary structural changes are only observed in aqueous buffer.

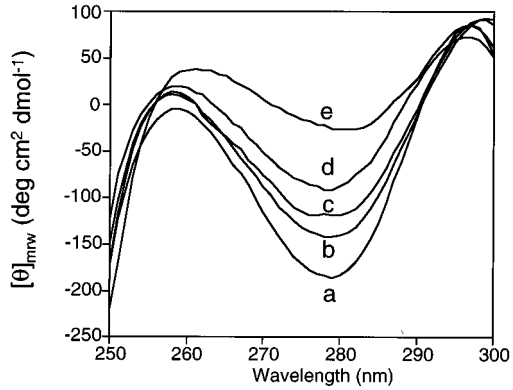

Figure 3. Near-UV CD spectra of soluble subtilisin BPN′ collected immediately after solubilization in octane, THF, and water. Also, soluble subtilisin was transferred from THF to octane or water immediately after dissolving in THF.

Stabilization of Subtilisin Dissolved in THF upon Addition of Water. Near-UV CD spectroscopy can be sufficiently quantified to examine the role of solvent and added water on the tertiary structure of subtilisin and to test the correlation between the kinetics of structural change and activity loss. Specifically, the near-UV CD spectra include major contributions from aromatic−aromatic interactions in the interior of the protein. Each interaction should contribute a distinct peak based on the environment of the aromatic groups.[17] The intensity of the peak is sensitive to the distance between the aromatic groups and in turn to the compactness of the protein. It is not possible to resolve the peaks for different aromatic interactions at room temperature; however, the area under the CD spectrum (between 260 and 290 nm) can approximate the fraction of native-like enzyme.[18]

Figure 4 depicts the fraction of native enzyme as a function of incubation time in both dry THF and THF supplemented with 0.2% (v/v) water. The rapid loss of catalytic activity in dry THF is concomitant with the loss of native tertiary structure of the enzyme. Hence, the loss of native tertiary structure *does* appear to correlate with the loss in catalytic activity in THF, supporting the hypothesis that THF inactivates soluble subtilisin by disrupting the native tertiary structure. Furthermore, the addition of a small concentration of water helps to maintain the tertiary structure of subtilisin, and increases the half-life of the enzyme (Table 1). Hence, water appears to stabilize subtilisin in THF by helping to preserve its native structure.

EPR Spectroscopy of Soluble Subtilisin BPN′. EPR spectroscopy of subtilisin spin-labeled with an active site (Ser_{221}) probe was used to provide more detailed information about the high activity and stability in octane, as well as the nature of the solvent- and thermally-induced structural changes causing inactivation. A simple measure of the mobility of the enzyme-bound spin label is the hyperfine splitting constant (A_{max}), which describes the separation of the upfield and downfield spectral peaks. Figure 5A reveals that soluble subtilisin in octane (Figure 5A, line b) has a narrower hyperfine splitting constant than lyophilized (suspended) subtilisin in octane (Figure 5A, line c), and broader hyperfine splitting than soluble subtilisin in water

(17) Kahn, P. C. *Methods Enzymol.* **1979**, *61*, 339.

(18) Base-line spectra were collected in water for subtilisin immediately transferred from dry THF (native spectrum) and for subtilisin transferred from dry THF following a 60-min incubation (presumed to be the spectrum of fully denatured enzyme). The fraction of native tertiary structure (and, hence, native enzyme) can then be estimated from the areas under the near-UV CD spectra according to eq 1, where A_t, A_f, and A_u are the areas under the near-UV CD spectra for soluble subtilisin at time t, for the folded (native) enzyme, and for the unfolded enzyme, respectively, and $[N]_0$ is the initial concentration of native enzyme. $[N]/[N]_0 = (A_t - A_u)/(A_f - A_u)$

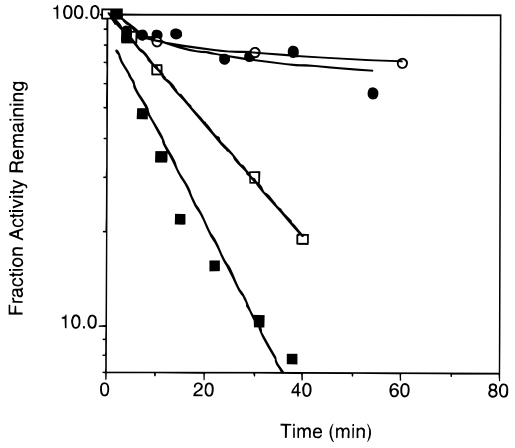

Figure 4. Relative stability of soluble subtilisin in dry THF (squares) and THF containing 0.2% added water (circles). Irreversible inactivation was followed by incubating soluble subtilisin in THF (dry or containing 0.2% water), transferring aliquots to water and measuring activity (filled symbols), and recording near-UV CD spectra in water (empty symbols). Fractions of native-like enzyme were estimated from the areas under the near-UV CD spectra.[35]

(Figure 5A, line d). This implies that the flexibility of the organic solvent-soluble enzyme is intermediate between that of the soluble enzyme in water and the insoluble enzyme in octane.

Further experiments to probe the structural changes of the organic-soluble subtilisin are summarized in Figure 5B. Even at short times (3 min) after solubilization, the spectrum of soluble subtilisin in THF (Figure 5B, line b) reflects greater mobility than that of the native enzyme in octane (Figure 5B, line a). These spectral differences are consistent with significant structural perturbation around the enzyme-active site in THF. The spectrum of soluble subtilisin in THF remains roughly constant for at least 900 min of incubation. In contrast, when subtilisin solubilized in THF for 30 s is transferred into octane at 25 °C, the native-like spectrum of subtilisin is regained (Figure 5B, line c).

Likewise, at 70 °C in octane the spectrum of soluble enzyme (Figure 5B, line d) is significantly different in line shape compared to the enzyme in octane at 25 °C. This implies an increase in spin-label mobility and probable perturbation of the active site structure. When subtilisin in octane at 70 °C is rapidly cooled to 25 °C in the EPR capillary, a hybrid spectrum containing features of both the "native" and "non-native" octane spectra is obtained. These data support the presence of a detectable structural change in subtilisin solubilized in THF at 25 °C and octane at 70 °C that is partially reversible by transfer to octane at 25 °C.

Discussion

Proposed Model of Subtilisin in Organic Solvents. Based on the kinetic and structural information obtained in this study, a model of subtilisin inactivation in homogeneous organic solutions is proposed (Figure 6). Specifically, it is hypothesized that four distinct enzyme species are present at different stages of the inactivation process. These enzyme species include the native (N) form, a denatured form renaturable in octane or water (D_1), a denatured form renaturable only in water (D_2), and the irreversibly inactivated (I) form of soluble subtilisin. A fifth, unfolded (U) form of soluble subtilisin, devoid of any secondary and tertiary structure, exists only in water. The D_1, D_2, and I forms in organic solvents have no equivalents (or counterparts) for the proposed model of inactivation of subtilisin in water.[19]

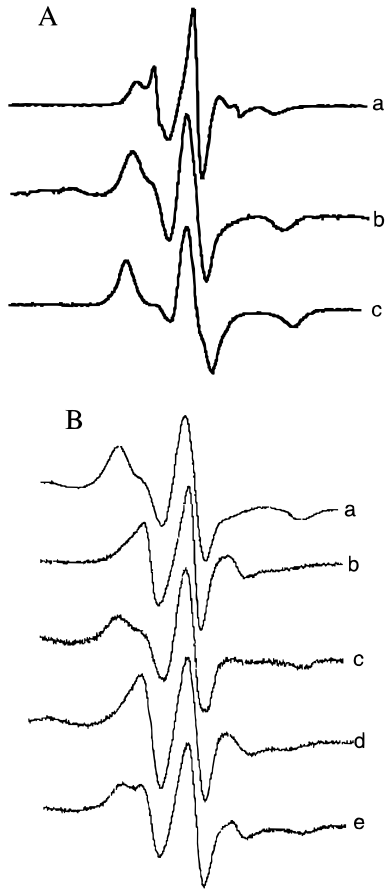

Figure 5. (A) EPR spectra of soluble subtilisin BPN′ spin labeled at Ser_{221}. Spectrum (a) is of native enzyme at 25 °C in aqueous buffer, spectrum (b) is of soluble enzyme at 25 °C in octane, and spectrum (c) is of lyophilized enzyme suspended in octane at 25 °C. Hyperfine splitting (A_{max}) values suggest that the active site of soluble subtilisin (A_{max} = 63 Gauss) is more flexible than that of insoluble subtilisin (A_{max} = 72 G) in octane. (B) EPR spectra of spin-labeled subtilisin BPN′. Spectrum (a) is of the native enzyme at 25 °C in octane. Spectrum (b) is the inactive enzyme form present after a short (~3 min) exposure to THF. This spectrum becomes similar to that of the native spectrum upon dilution into octane at 25 °C after very short (~30 s) exposure to THF (spectrum (c)). Dilution in octane after longer preincubations in THF also result in a largely native-like spectrum, although the signal strength rapidly decreases as enzyme visibly precipitates from solution. Spectrum (d) is observed for soluble subtilisin incubated in octane for 15 min at 70 °C, and the structural changes indicated by this spectrum are apparently reversed by cooling to 25 °C (spectrum (e)).

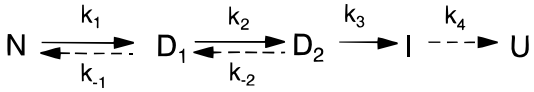

Figure 6. Proposed model for inactivation of soluble subtilisin BPN′ in THF and reactivation in octane and water. The dashed arrows represent transformations that do not occur in THF, but were apparent in octane and/or water. The details are described in the text.

The N form in organic solvents is proposed to be similar to the native structure present in aqueous solutions. Both CD and EPR spectroscopic studies support the native-like, compact structure of the soluble enzyme in octane. Although spin-labeled subtilisin is less flexible when dissolved in octane than in water, it is still more flexible than the suspended enzyme in the organic solvent. The N form is the only active form of enzyme, but is stable in a polar organic solvent such as dry THF for only a very short time.

(19) (a) Eder, J.; Rheinnecker, M.; Fersht, A. R. *Biochemistry* **1993**, *32*, 18. (b) Schulze, B.; Klibanov, A. M. *Biotechnol. Bioeng.* **1991**, *38*, 1001.

The D_1 form is devoid of any measurable catalytic activity, yet some tertiary structure is retained. D_1 is formed by short-time (several minutes) incubation in dry THF at 25 °C or in octane at 70 °C, and has measurably less tertiary structure (as measured by near-UV CD spectroscopy) than the native enzyme. The D_1 form produced by thermal denaturation in octane may not be structurally identical with the D_1 form produced by THF-induced denaturation at 25 °C. However, the D_1 form produced by both denaturing conditions has more conformational mobility than the N form, suggesting significant structural perturbation of active site structure (see Figure 5). The reversible transformation of the D_1 form into the N form in water is not surprising as it is well known that enzymes can refold in aqueous solutions as long as no irreversible inactivation has occured.[20] In the case of subtilisin BPN′, the maintenance of the complete secondary structure and partial tertiary structure in the D_1 form enables refolding to the native protein without the need for the pro sequence.

A significant finding is that the *partially unfolded subtilisin is capable of refolding in an organic solvent.* This demonstrates that bulk water is not required for refolding of a partially denatured enzyme. Moreover, the reversibility of the N to D_1 transformation in octane implies that the native subtilisin BPN′ in octane (N form) is thermodynamically stable. Possibly, the neutralization of some of the charged residues and/or the introduction of dioctyl tails of AOT on the surface of the enzyme stabilizes the folded enzyme structure in nonpolar solvents.

The relative fractions of enzyme renatured upon transferring from THF to water is greater than that renatured in octane. For example, after a 10-min incubation in THF, less than 35% of the enzyme can be renatured upon transfer to water, whereas, only ca. 10% of the enzyme can be renatured upon transfer to octane (Figure 1A). This difference is not due to continued deactivation of soluble enzyme upon transferring the enzyme to octane (Figure 1A). This suggests the existence of the D_2 form which does not renature in octane, but can undergo renaturation upon transfer to water. The concentration of the D_2 form can be estimated by the difference in the fractions of activity recoverable in water and octane. Based on CD spectroscopy, it is proposed that D_2, unlike D_1, has lost almost all native tertiary structure, but retains full secondary structure.

Still longer incubation in THF results in the formation of the I form which cannot be renatured to active enzyme even upon transfer to aqueous buffer. This enzyme form maintains its secondary structure in THF and octane, but is devoid of any significant tertiary structure (similar to the D_2 form). Thus, maintainence of secondary structure in organic solutions is not necessarily an indication of active enzyme. In contrast to the D_2 form, which can be renatured by transfer to water, the I form undergoes nearly immediate transformation to the U form upon transfer to water. The U form exists in the random coil configuration and has no activity. It is important to note that complete secondary unfolding of subtilisin could be observed only in water and not in organic media.

The time-dependent denaturation of subtilisin BPN′ in THF can be understood with the help of a simplified physical model of the enzyme in the polar organic solvent. Based on the crystal structures of proteins in organic solvents, it is known that polar organic solvents can penetrate into the protein structure.[21] It may be speculated that this would result in a solvent-swollen, less compact protein molecule. Evidence of a less compact structure comes from near-UV CD spectroscopy in which aromatic−aromatic interactions are weakened by incubation of the enzyme in THF, presumably a consequence of solvent-induced perturbations of the enzyme molecule. At very short times (<5 min), THF may only be capable of penetrating into the outer hydrophilic shell of the enzyme. Placing the enzyme into water at this time may serve to extract THF molecules into the bulk solvent. This would enable re-formation of the native enzyme structure. A similar event can also occur when the solubilized enzyme in THF is transferred to octane; enzyme-bound THF molecules will be washed from the enzyme and partition into the bulk solvent. Because octane molecules are not likely to be thermodynamically stable in a polar environment (the outer shell of the enzyme), no octane molecules are expected to replace THF, and the native structure of the enzyme is regained. Thus, the D_1 form can be converted back to the N form in either water or octane.

Further penetration of THF molecules deeper into the enzyme molecule results in the D_2 form. It may be speculated that these THF molecules displace structural water molecules, which are presumably in relatively hydrophilic environments within the enzyme's structure.[21a] When the D_2 form of the enzyme is placed in water, the THF molecules may eventually be displaced by water molecules. The stabilization of the native subtilisin upon the addition of small concentrations of water (0.2%, v/v) to bulk THF may indicate that water favorably competes with THF for these hydrophilic sites in the protein's structure. On the other hand, octane cannot displace THF molecules that have penetrated farther from the enzyme surface. Thus, the D_2 form cannot be transformed into the N form in octane.

After sufficient incubation time in THF (>30 min), solvent penetration results in the complete loss of tertiary structure of the enzyme, presumably by the solvent interacting with a sufficient number of peptide residues. However, THF cannot break the intramolecular hydrogen bonding patterns composing the secondary structures of subtilisin. Precedence exists for the presence of a molten globule state of protein (specifically recombinant human growth hormone) in 40% 1-propanol, where tertiary structure is lost but the secondary structure is maintained.[22] Moreover, model peptides tend to have greater propensities for helical structures in organic solutions than in aqueous solutions.[23] Water is capable of forming stable hydrogen bonds, and therefore, complete unfolding of the enzyme (loss of secondary structure) is only observed in a predominantly aqueous solution. Once the I form predominates in organic solvents, the enzyme is unable to re-fold under any circumstances as subtilisin BPN′ requires a pro sequence for correct folding from the random coil state in water.[19a,24]

Increasing the solution temperature in octane results in increased fluctuation of the enzyme conformation, which disrupts the native tertiary structure, producing the D_1 form. At lower temperature, decreased thermal perturbations allow re-formation of the active enzyme from D_1, even in octane. The D_2 and I forms generated by incubation at elevated temperature of the soluble enzyme in octane may be kinetically trapped from refolding in octane at 25 °C.

High Intrinsic Activity of Organic Solvent-Soluble Subtilisin. The marked activation of the soluble enzyme in nonpolar

(20) Rossman, M. G.; Argos, P. *Annu. Rev. Biochem.* **1981**, *50*, 497.

(21) (a) Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.*, **1993**, *90*, 8653. (b) Yennawar, N. H.; Yennawar, H. P.; Farber, G. K. *J. Am. Chem. Soc.* **1995**, *117*, 577. (c) Yennawar, H. P.; Yennawar, N. H.; Farber, G. K. *Biochemistry* **1994**, *33*, 7326.

(22) Wicar, S; Mulkerrin, M. G.; Bathory, G.; Khundkar, L. H.; Karger, B. L. *Anal. Chem.* **1994**, *66*, 3908.

(23) (a) Jasanoff, A.; Fersht, A. R. *Biochemistry* **1994**, *33*, 2129. (b) Waterhous, D. V.; Johnson, W. C. *Biochemistry* **1994**, *33*, 2121. (c) Zhang, Y.-P.; Lewis, R. N. A. H.; Henri, G. D., Sykes, B. D.; Hodges, R. S.; McElhaney, R. N. *Biochemistry* **1995**, *34*, 2348.

(24) Straussberg, S.; Alexander, P.; Wang, L.; Schwarz, F.; Bryan, P. *Biochemistry* **1993**, *32*, 8112.

solvents relative to the suspended enzyme preparation is intriguing. This activation cannot be explained by differences in mass transfer between the two enzyme forms because suspended subtilisin shows no significant intraparticle diffusional limitations.[3b] A major difference between the two preparations is that the suspended enzyme form undergoes lyophilization, whereas the soluble enzyme form undergoes a liquid−liquid extraction followed by gaseous drying. It is well known that lyophilization can induce conformational changes in a protein.[6] These conformational changes are often reversible, but reverse to the native structure only in aqueous solutions.[6] The suspended enzyme, therefore, may be much less active than its soluble counterpart because it exists in a partially denatured form in organic solvents. The soluble enzyme is also more conformationally flexible than its insoluble counterpart. Such increased flexibility, particularly at the active site of the enzyme, has been shown to promote enzyme activity in organic solvents.[25]

Conclusions

The high catalytic activity of subtilisin BPN′ in nonpolar organic solutions contrasts greatly with the activity of the suspended enzyme, and this appears to be due to maintenance of the secondary and tertiary structure of the soluble enzyme when extracted from an aqueous solution into a nonpolar organic solvent. Subsequent drying of the extracted protein without freezing appears to minimize perturbation of the native enzyme structure. One factor that has not been considered in this study is the influence, if any, of the AOT surfactant other than in the formation of ion pairs with subtilisin to aid in the solubilization in organic solvents. Such an influence is the subject of continuing investigations.

Soluble subtilisin is very stable in octane, and, presumably because of the absence of autolysis in octane and decreased flexibility, the enzyme is substantially more stable than in aqueous solutions. A surprising feature of organic solvent-soluble subtilisin is the reversible denaturation in octane. This is the first report of partial renaturation of an enzyme in a nonaqueous media and demonstrates that bulk water is not a prerequisite for this fundamental process.

Assuming substrate saturating conditions, a practical measure of the operational value of an enzyme is the number of turnovers accomplished in a single half-life of the enzyme. This dimensionless term (represented by $k_{cat} \times t_{1/2}$) provides a general measure for the utility of a biocatalyst. For the soluble enzyme in octane, this value is 1.0×10^7 (based on a half-life of 2 months) and is greater than the value in water (1.2×10^6). The activity and stability of soluble subtilisin are far lower in polar organic solvents. In THF, major structural changes occur during incubation of the enzyme in the solvent at room temperature. The rapid deactivation and unfolding of subtilisin in THF demonstrates that the active structure of the enzyme molecule is strongly distorted by such a polar organic solvent.

Acknowledgment. This work was supported by grants from the Army Research Office (DAAH04-94-G-0307) and the Biotechnology Research and Development Corporation. P.P.W. acknowledges partial support of a fellowship from the Center for Biocatalysis and Bioprocessing at the University of Iowa.

JA962620Z

(25) (a) Xu, Z-F.; Affleck, R.; Wangikar, P. P.; Suzawa, V.; Dordick, J. S.; Clark, D. S. *Biotechnol. Bioeng.* **1994**, *43*, 515. (b) Affleck, R.; Xu, Z.-F.; Suzawa, V.; Focht, K.; Clark, D. S.; Dordick, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 1100.