ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 334, No. 2, October 15, pp. 406–414, 1996
Article No. 0472

Effect of Secondary Structure on the Activity of Enzymes Suspended in Organic Solvents

Aichun Dong,[1] Jeffrey D. Meyer, Brent S. Kendrick, Mark C. Manning, and John F. Carpenter[2]
Cooperative Program in Pharmaceutical Biotechnology and Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received May 13, 1996, and in revised form July 24, 1996

Despite the extensive use and study of enzymes suspended in organic solvents, whether activity differences between different preparations can be accounted for by differences in protein secondary structure is still unknown. To address this issue, in the current study two model enzymes, **α**-chymotrypsin and subtilisin Carlsberg, were lyophilized and suspended in both polar and nonpolar organic solvents. The secondary structures of the proteins in the initial aqueous solution, in the lyophilized powder, and in the subsequent suspensions in organic solvents were determined using infrared spectroscopy. Lyophilization perturbed the secondary structure of both enzymes. With **α**-chymotrypsin, lyophilization from buffer followed by suspension in ethanol, hexane, or pyridine did not alter the unfolded structure observed in the dried powder. In contrast, with subtilisin Carlsberg, suspension of the dried enzyme in ethanol led to further perturbation of structure, whereas in hexane, and more so in pyridine, there was some return toward native structure. Lyophilization of the aqueous protein solutions in the presence of either trehalose or sorbitol led to retention of more native-like structure of both enzymes in the dried solid. However, large structural perturbations arose when these samples were suspended in organic solvents. The only exception was the subtilisin–trehalose mixture, which regained some native structure in ethanol and hexane. The greatest changes were noted in samples suspended in pyridine, in which the infrared spectra indicated extensive intermolecular beta-sheet formation from protein aggregates. There was not any consistent correlation between activity in organic solvents and either the initial structure obtained in the dried powders or the final structure when suspended in organic solvents. Nor could differences in residual water contents in dried samples or the total water content in the organic solvent reaction system account for the activity differences. © 1996 Academic Press, Inc.

Key Words: nonaqueous enzymology; protein secondary structure; infrared spectroscopy; subtilisin Carlsberg; **α**-chymotrypsin; lyophilization; protein stabilization.

The ability of enzymes to function in low water environments, which are found in organic solvents, is well established (1). However, the structure–activity relationships of proteins in these systems are not well understood. This is especially true for enzymes that are first freeze-dried (lyophilized) and then suspended in organic solvents. It has been found that lyophilization conditions can profoundly affect catalytic activity of these systems (2, 3). Of greatest interest for the current study is the observation that lyophilizing enzymes in the presence of sorbitol or trehalose increased the activity noted in the subsequent enzyme suspension in organic solvents (4–6). It has been documented with infrared spectroscopy for numerous proteins that freeze-drying can induce unfolding and that stabilizing carbohydrate additives can inhibit this structural perturbation (7–9). Dabulis and Klibanov have proposed that the increased activity noted in organic solvents for enzymes lyophilized with carbohydrates is due to protection of protein structure during freeze-drying (4).

The purpose of the current study was to test this hypothesis directly. We used infrared spectroscopy to determine the effects of freeze-drying in the absence or presence of the stabilizing additives, sorbitol and trehalose, on the secondary structure of subtilisin

[1] Current address: Department of Chemistry and Biochemistry, University of Northern Colorado, Greeley, CO 80639.
[2] To whom correspondence should be addressed at Department of Pharmaceutical Sciences, School of Pharmacy, Campus Box C238, University of Colorado Health Sciences Center, Denver, CO 80262. Fax: (303) 270-6281. E-mail: John.Carpenter@UCHSC.edu.

0003-9861/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Carlsberg and α-chymotrypsin, in the initial dried solid and after suspension of lyophilized formulations in organic solvents. Differences in catalytic activities measured for different lyophilized subtilisin and α-chymotrypsin formulations in ethanol, hexane, or pyridine could not be accounted for by differences in protein secondary structure, either in the initial dried solid or after suspension in the organic solvents. Furthermore, neither residual water content in the dried samples nor total water content in the suspensions correlated with enzymatic activities.

MATERIALS AND METHODS

Materials. Both subtilisin Carlsberg and α-chymotrypsin were purchased from Sigma and used as received. *N*-Acetylphenylalanine methyl ester (*N*-Ac-Phe-OMe)[3] was purchased from Bachem. Acetonitrile (HPLC grade) was from Fisher. HPLC-grade hexane and pyridine were obtained from Aldrich. Sorbitol and trehalose were obtained from Pfanstiehl Laboratories, Inc. Trifluoroacetic acid was obtained from Sigma. Ethanol was purchased from AAPER Alcohols.

Lyophilization. Samples were lyophilized according to the methods of Dabulis and Klibanov (4). Briefly, the proteins were prepared at 10 mg/ml concentration in 10 mM potassium phosphate buffer (pH 7.5 at 23°C), in the presence or absence of sorbitol and trehalose. Aliquots (100 μl) were placed into 1.5-ml polypropylene Eppendorf test tubes, which were immersed for 2–3 min in liquid nitrogen. The sample tubes were then placed under vacuum (5–10 mTorr) on a benchtop Labconco lyophilizer (−50°C condenser temperature) for 18–24 h.

Infrared spectroscopy. Protein solutions were prepared for infrared measurement in a CaF_2 cell (Beckman FH-01) with a 6-μm spacer. To prepare homogeneous protein suspensions in organic solvents, 1 ml of organic solvent was added to the dried powder in the 1.5-ml Eppendorf test tube. Then, the resulting mixture was dispersed by sonication for 20 s. The suspensions were placed into a Perkin Elmer variable pathlength cell set to 6 μm. Dried samples (ca 0.2–0.4 mg protein) were ground with 300 mg KBr and pressed into pellets, as described previously (7, 8). This procedure for preparing KBr pellets does not alter structure of proteins in the dried solid (7).

Infrared spectra were recorded at 25°C using a Nicolet Magna 550 spectrometer equipped with a dTGS detector. For each spectrum a 512-scan interferogram was collected in single beam mode with a 4 cm^{-1} resolution. For aqueous samples and samples suspended in organic solvents, reference spectra were recorded under identical scan conditions with only the corresponding solutions (without protein) in the cell. The spectra of liquid water and organic solvents, and the spectra for water vapor, were subtracted from the observed protein spectra according to previously established criteria with a double subtraction procedure (9–11). Second-derivative spectra were obtained with the derivative function of Omnic software (Nicolet). The final protein spectrum was smoothed with a 7-point Savitsky–Golay function to remove white noise. The spectra for a given experimental sample and that for the native, aqueous protein were normalized for area and overlaid for comparison (9, 12).

Assay of enzyme catalytic activity. Enzymatic activity was measured by following the transesterification of the substrate *N*-Ac-Phe-OMe into its ethyl ester (13). The enzyme (1 mg) was suspended in 1 ml of the reaction mixture and sonicated for 20 s to disperse the sample. Each reaction mixture contained substrate concentrations giving V_{max} activity, based on previously published reports (2–4, 13–15). For reactions in ethanol, the substrate concentration was 500 mM and the reaction was terminated by the addition of glacial acetic acid. For reactions in pyridine, the substrate concentration was 100 mM and the ethanol concentration was 150 mM. The reaction was terminated by centrifuging (6700*g* for 5 min) the solid enzyme out of the reaction mixture and removing the supernatant. The hexane reaction mixture contained ethanol at 10% by volume and a *N*-Ac-Phe-OMe concentration of 60 mM. The reaction was terminated by the addition of glacial acetic acid. The extent of reaction at given time points was determined by analysis of *N*-Ac-Phe-OEt on a Beckman 110A HPLC equipped with a Spherisorb PhaseSep ODS2 (25 cm × 4.6 mm) column. After evaporating the sample to dryness, it was redissolved in acetonitrile and injected. All samples were eluted with an isocratic 32:68 acetonitrile:water mixture containing 0.1% trifluoroacetic acid, at a flow rate of 2 ml/min, while absorbance was monitored at 215 nm. Reported activities reflect the initial rate of the reaction for the substrate concentration listed and 1 mg of suspended enzyme in a total reaction volume of 1 ml. These rates are given in Tables I and II.

Water content measurements. Water content of the lyophilized samples were determined by Karl Fisher titration, using a Mettler DL37 coulimetric titrator. The values are the average of triplicate measurements on three independent lyophilized samples.

TABLE I

Rates for α-Chymotrypsin Suspended in Organic Media

Lyoprotectent	Solvent	Rate (μmol/h·mg)	Total water (μg)[a]
None	Ethanol	0.95	2020 ± 160
None	Hexane	2.8	330 ± 40
None	Pyridine	0.002	430 ± 40
10% trehalose	Ethanol	1.6	2420 ± 160
10% trehalose	Hexane	3.3	730 ± 20
10% trehalose	Pyridine	0.003	830 ± 20
2% sorbitol	Ethanol	0.97	2020 ± 160
2% sorbitol	Hexane	0.55	330 ± 30
2% sorbitol	Pyridine	0.002	430 ± 30

[a] Total water values reflect the mean and standard deviation of nine measurements of both solvents and lyophilyzed pellets.

RESULTS

It is now well established, using infrared spectroscopic analysis of protein structure in the dried solid, that freeze-drying in the absence of stabilizing additives induces protein unfolding (7–9). During rehydration some proteins aggregate and lose activity, whereas others refold and are fully native and active (7–9). To ascertain the behavior of the model proteins under conditions used in the current study, infrared spectroscopy was used to determine the secondary structure of α-chymotrypsin and subtilisin Carlsberg in the initial aqueous solution, in the dried solid, and after rehydration. As can be seen in Fig. 1, the second derivative infrared spectrum of lyophilized α-chymotrypsin, in the

[3] Abbreviation used: *N*-Ac-Phe-OMe, *N*-acetylphenylalanine methyl ester.

TABLE II

Rates for Subtilisin Carlsberg Suspended in Organic Media

Lyoprotectent	Solvent	Rate (μmol/ h · mg)	Total water (μg)[a]
None	Ethanol	37	2030 ± 170
None	Hexane	0.22	340 ± 50
None	Pyridine	0.38	440 ± 50
10% trehalose	Ethanol	65	2500 ± 200
10% trehalose	Hexane	0.088	840 ± 100
10% trehalose	Pyridine	7.6	940 ± 100
2% sorbitol	Ethanol	81	1980 ± 160
2% sorbitol	Hexane	0.10	300 ± 40
2% sorbitol	Pyridine	2.9	400 ± 40

[a] Total water values reflect the mean and standard deviation of nine measurements of both solvents and lyophilyzed pellets.

conformationally sensitive amide I region (7–12, 16, 17), is grossly altered relative to that for the initial native protein in aqueous solution. There are increases in bandwidths and shifts in band positions and relative absorbances, which are indicative of protein unfolding (7–9, 12). However, upon rehydration, the protein is fully soluble and its infrared spectrum is virtually identical to that for the initial native protein solution, indicating that α-chymotrypsin refolds during rehydration (data not shown).

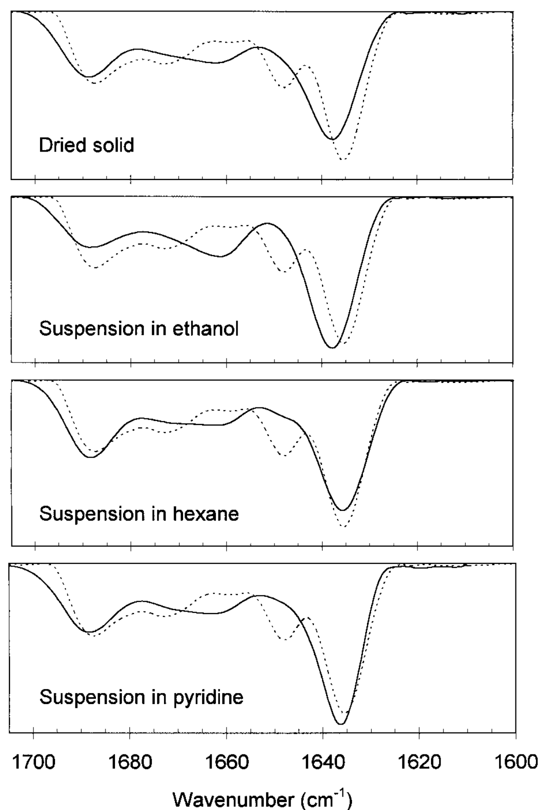

FIG. 1. Comparison of the second derivative spectra of α-chymotrypsin in aqueous solution (---) and in the dried state or suspended in an organic solvent. The protein was lyophilized from 10 mM potassium phosphate buffer (pH 7.5).

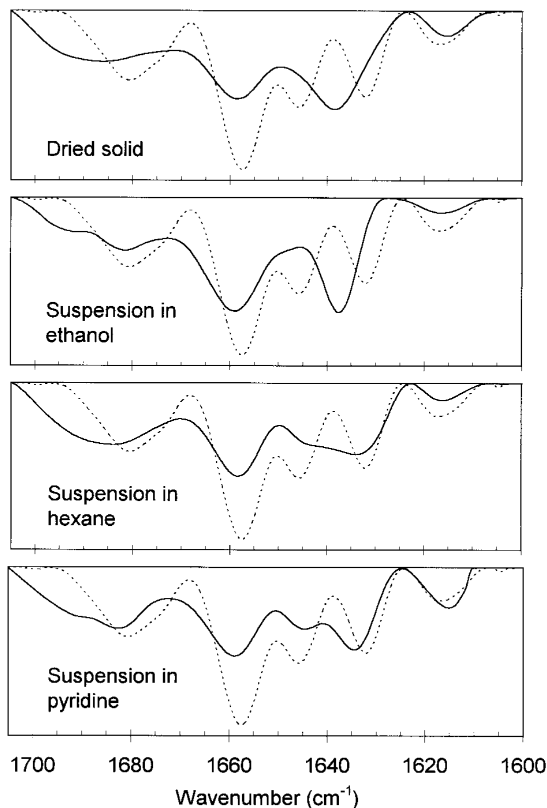

FIG. 2. Comparison of the second derivative spectra of subtilisin Carlsberg in aqueous solution (---) and in the dried state or suspended in an organic solvent. The protein was lyophilized from 10 mM potassium phosphate buffer (pH 7.5).

Similar results were obtained with subtilisin Carlsberg (Fig. 2). However, the degree of structural perturbation in the dried solid appears to be more severe than that noted with α-chymotrypsin, based on spectral differences between the dried and native aqueous protein samples. After rehydration, the protein is fully soluble and the infrared spectrum is essentially identical to that for the initial native protein solution (data not shown).

Suspension of the freeze-dried samples in organic solvents led to different structural behaviors for the two enzymes. Suspension of lyophilized α-chymotrypsin in ethanol, hexane, or pyridine led to only minor structural changes, as indicated by the small changes in the infrared spectra relative to that for the initial dried solid (Fig. 1). In contrast, subtilisin's structure

was altered upon contact with organic solvents to varying degrees, depending on the solvent (Fig. 2). The infrared spectrum of the sample suspended in ethanol was not changed much relative to that for the original dried protein. In hexane, the protein's structure appeared to be further perturbed, as evidenced by further broadening of the beta sheet (9–11, 16, 17) infrared band near 1632 cm^{-1}. Finally, suspension of subtilisin in pyridine led to partial refolding of the protein. The two components bands for beta sheet in the range of 1625–1645 cm^{-1} and that for turn structure near 1685 cm^{-1} (9–11, 16, 17) show improved resolution, which is more similar to that of the native protein than to that noted in the initial dried solid.

It has also been well established that lyophilization-induced structural alterations can be inhibited by the presence of stabilizing additives, e.g., trehalose and sorbitol (7–9). Also, lyophilization of enzymes in such stabilizers has been shown to increase greatly the catalytic activity measured in suspensions in organic solvents (4). To ascertain if structural stabilization during lyophilization could account for this effect, we lyophilized α-chymotrypsin and subtilisin in either 2% (w/v) sorbitol or 10% (w/v) trehalose. Sorbitol was assessed at a 2% concentration to mimic conditions used previously by Dabulis and Klibanov (4). The higher concentration of trehalose was tested because our previous research with protein stabilization by disaccharides had documented that this is often the minimal level needed to obtain optimum protection of proteins during freeze-drying (8, 18).

Figure 3 shows the second derivative infrared spectra for α-chymotrypsin lyophilized in the presence of 2% sorbitol. The structure of the protein in the dried solid is more native-like than that obtained in the absence of stabilizer (Fig. 1). The bands at 1645 and 1650 cm^{-1}, which are representative of beta sheet and disordered components, respectively, are resolved, although not as completely as in the spectrum of the native aqueous protein. Suspension of the enzyme prepared with sorbitol in all organic solvents tested led to extensive alterations in the protein's infrared spectrum, indicating that the structure was greatly perturbed (Fig. 3). The structural perturbation was most pronounced in the sample suspended in pyridine, for which the infrared spectrum has a large new band near 1608 cm^{-1}. This band is due to intermolecular beta sheet and its appearance indicates that protein molecules were aggregated extensively in pyridine (9).

Lyophilization of α-chymotrypsin in 10% trehalose led to an even greater retention of native structure in the dried solid than noted with 2% sorbitol (Fig. 4). The beta sheet band at 1635 cm^{-1} has almost the same frequency and intensity as seen in the spectrum of the native protein and the band for disordered elements at

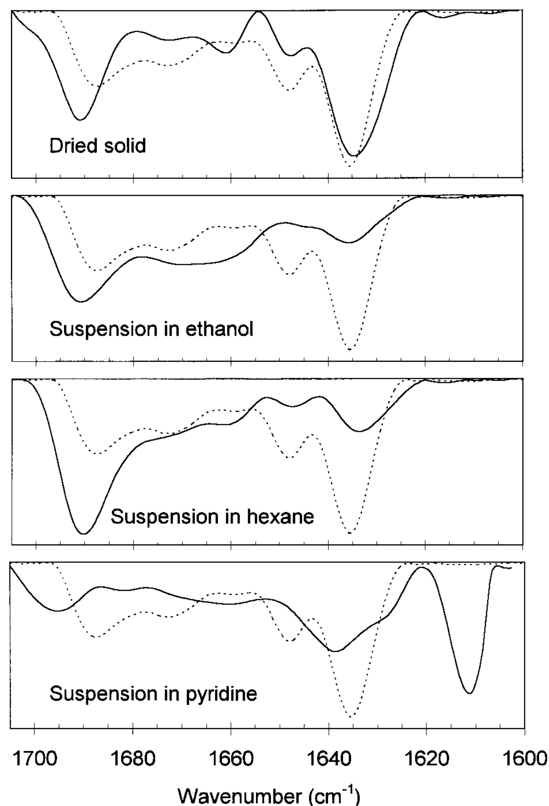

FIG. 3. Comparison of the second derivative spectra of α-chymotrypsin in aqueous solution (---) and in the dried state or suspended in an organic solvent. The protein was lyophilized from 10 mM potassium phosphate buffer (pH 7.5) containing 2% (by weight) sorbitol.

1648 cm^{-1} is well resolved. However, as noted with the samples lyophilized with sorbitol, the structures of samples dried with trehalose were greatly perturbed upon suspension in organic solvents, based on the loss of similarity of the respective samples' infrared spectra to those of the native protein (Fig. 4). Again, this effect was most dramatic in pyridine and the spectrum has a dominant intermolecular beta sheet band (1608 cm^{-1}) indicative of protein aggregation.

Lyophilization of subtilisin in either 2% sorbitol (Fig. 5) or 10% trehalose (Fig. 6) led to almost equivalent, partial retention of native structure in the dried solid. This is most evident by the retention of the three component bands in the region of 1625–1660 cm^{-1}. For samples prepared with sorbitol, large structural perturbations were noted in all three organic solvents (Fig. 5). The spectrum of the sample in pyridine is dominated by an intermolecular beta sheet band, indicating protein aggregation. For samples prepared with trehalose, suspension of the enzyme in ethanol or hexane had minimal effect on the protein's structure (Fig. 6). In contrast, suspension in pyridine grossly perturbed

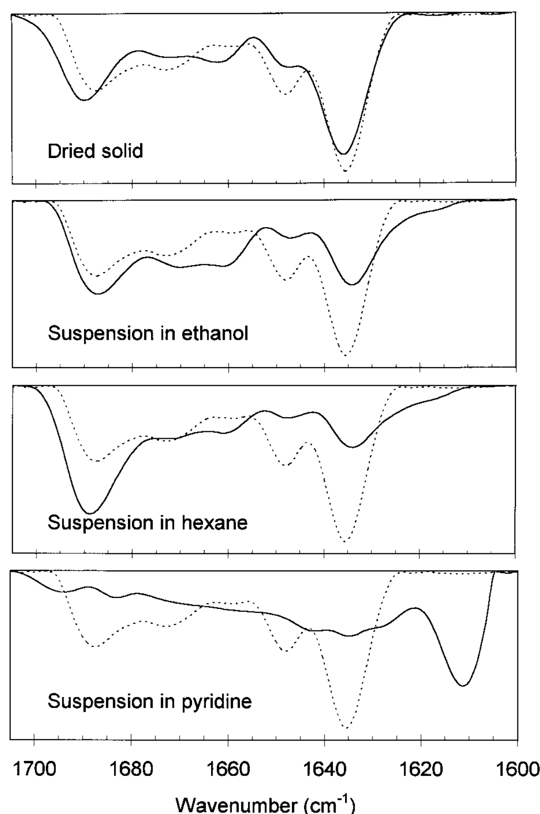

FIG. 4. Comparison of the second derivative spectra of α-chymotrypsin in aqueous solution (---) and in the dried state or suspended in an organic solvent. The protein was lyophilized from 10 mM potassium phosphate buffer (pH 7.5) containing 10% (by weight) trehalose.

structure and led to protein aggregation, as indicated by the predominant intermolecular beta sheet band.

In order to assess whether these structural alterations impacted enzymatic activity, the initial rate of reaction was measured by following the time course of the reaction at a fixed substrate concentration. Similar approaches for determining enzymatic activity have been reported previously (2–4, 13, 14). Choices of substrate concentration roughly correspond to that needed for V_{max} rates, based on work by Klibanov and co-workers (2–4, 13, 14) and our own work with subtilisin (15). While not providing a complete kinetic analysis, this information does provide insight into whether the substantial changes in secondary structure resulting from lyophilization and suspension in organic solvents affects activity to any significant degree. In general, there was not a clear correlation between retention of native-like structure (or lack of it) and the measured enzymatic activity of α-chymotrypsin (Figs. 1, 3, and 4 and Table I). In the case of reactions in ethanol, the activity was relatively constant for all samples; however, the structure was greatly disrupted when α-chymotrypsin was suspended in the presence of trehalose and sorbitol. With pyridine as the solvent, the activity was invariant, but very low, even though the protein was extensively aggregated in the presence of trehalose and sorbitol, but relatively native-like without those additives. Furthermore, the level of enzymatic activity in hexane (with a small amount of ethanol as a reactant) and ethanol was substantial, even when the secondary structure was significantly different than the native conformation.

As with α-chymotrypsin, the trends in the enzymatic activity of subtilisin Carlsberg do not reflect the gross differences in secondary structure (Figs. 2, 5, and 6 and Table II). For example, in the absence of additives, the sample in ethanol was arguably the least native-like, and yet it displayed activity more than 100-fold greater than samples in the other solvents. Samples suspended in pyridine had higher activity when greatly aggregated (in the presence of sorbitol and trehalose) than when subtilisin was not aggregated (in the absence of additives), contrary to what one might expect (19–21).

Another factor that could potentially account for the differences in activity noted for the different prepara-

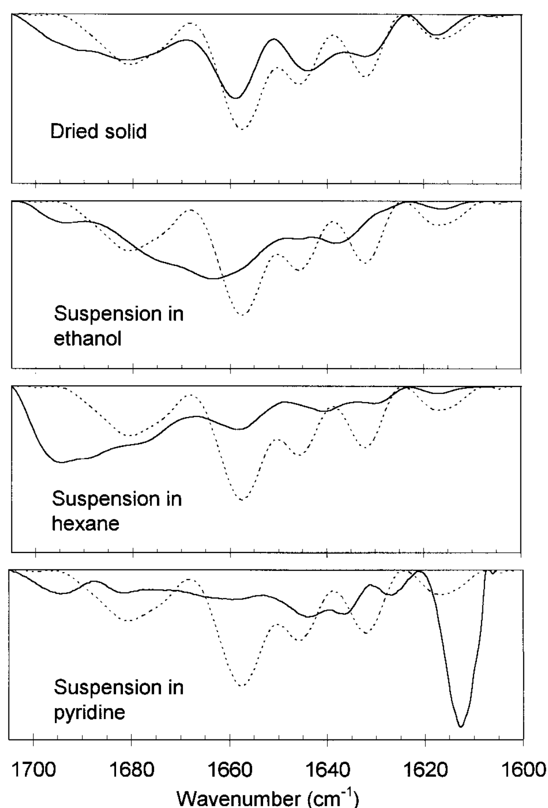

FIG. 5. Comparison of the second derivative spectra of subtilisin Carlsberg in aqueous solution (---) and in the dried state or suspended in an organic solvent. The protein was lyophilized from 10 mM potassium phosphate buffer (pH 7.5) containing 2% (by weight) sorbitol.

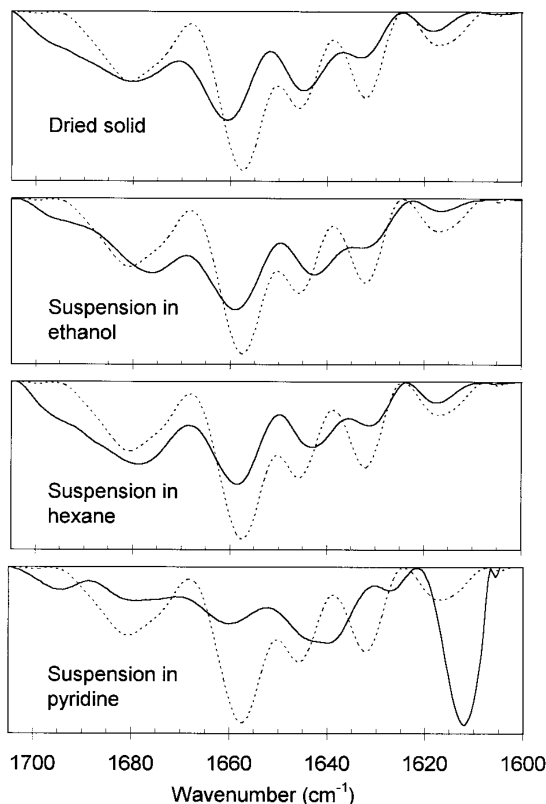

FIG. 6. Comparison of the second derivative spectra of subtilisin Carlsberg in aqueous solution (---) and in the dried state or suspended in an organic solvent. The protein was lyophilized from 10 mM potassium phosphate buffer (pH 7.5) containing 10% (by weight) trehalose.

tions in a given organic solvent is the amount of water present in the reaction mixture. Water is available from the solvent itself, added substrate, and the "dried" protein preparation. As shown in Fig. 7A, the residual water in the lyophilized samples as a percentage of the total sample mass was greatest in the samples prepared without stabilizers. However, in terms of the total amount of water present in the dried sample, the most water was measured in the samples with trehalose, which had the greatest dried weight (Fig. 7B). Actually, with regard to the catalytic activity in the reaction mixture, it seems that the most relevant parameter is the total amount of water present from all sources (Tables I and II). However, comparing the catalytic activity data for each sample type in a given solvent for any of the measurements of water content did not show a consistent correlation. Thus, it appears that neither secondary structure nor water content can account for the observed differences in catalytic activity.

DISCUSSION

The hypothesis tested in the current study was that the stabilizing additives, sorbitol and trehalose, should prevent lyophilization-induced unfolding of enzymes, such as α-chymotrypsin and subtilisin, and that such structural stabilization should account for the greater activity of these preparations upon suspension in organic solvents, relative to that for samples lyophilized without stabilizers (4, 22). The first part of this hypothesis is now well supported by studies on many lyophilized proteins (7–9). Similarly, in the current study, it was found that α-chymotrypsin and subtilisin unfold when lyophilized without stabilizers and that more native-like secondary structure was retained in the dried solid when sorbitol or trehalose was included in the formulation. However, no correlation was observed between the level of enzymatic activity noted in various organic solvents and either the initial secondary structure in the dried solid or the final structure arising after suspension in the solvents. Of course, simply monitoring secondary structure provides no detailed information about regions such as the active site. Furthermore, the measured enzymatic activity may be the result of a relatively small population of active molecules. Methods, such as infrared spectroscopy, can only observe an average conformation for the ensemble and cannot directly measure the number of individual competent enzyme molecules. In addition, when a sample appears relatively native-like at the level of secondary structure, it is possible that the active site structure could still be perturbed significantly. Yet, it seems unlikely that a native, functional active site would be retained in samples in which the secondary structure is grossly altered. Little information is available regarding the structural integrity of the active site of enzymes in organic solvents, other than from a few NMR spectroscopic (23, 24) and X-ray crystallographic studies (25). These suggest that the active site is not compromised if the overall structure is intact, but no clear example is reported of an enzyme with grossly altered secondary structure and a viable active site.

We also found, contrary to previous hypotheses (1, 4, 13, 22, 26), that large-scale alterations in a protein's structure can occur upon suspension of the lyophilized sample in organic solvents, whether the solvent is polar or nonpolar. These changes range from partial refolding of the protein to formation of intermolecular beta sheet associated with nonnative protein aggregates. The only exceptions were for α-chymotrypsin samples lyophilized without stabilizers, which retained about the same level of nonnative structure in ethanol, hexane, and pyridine as was seen in the initial dried solid.

A common theme in nonaqueous enzymology is that proteins are much less flexible in nonpolar organic solvents than they are in aqueous solution (13, 14, 24). Accordingly, the proteins are said to have "memory" of the structure present prior to suspension in the nonpolar solvent. Due to the low polarity, high conforma-

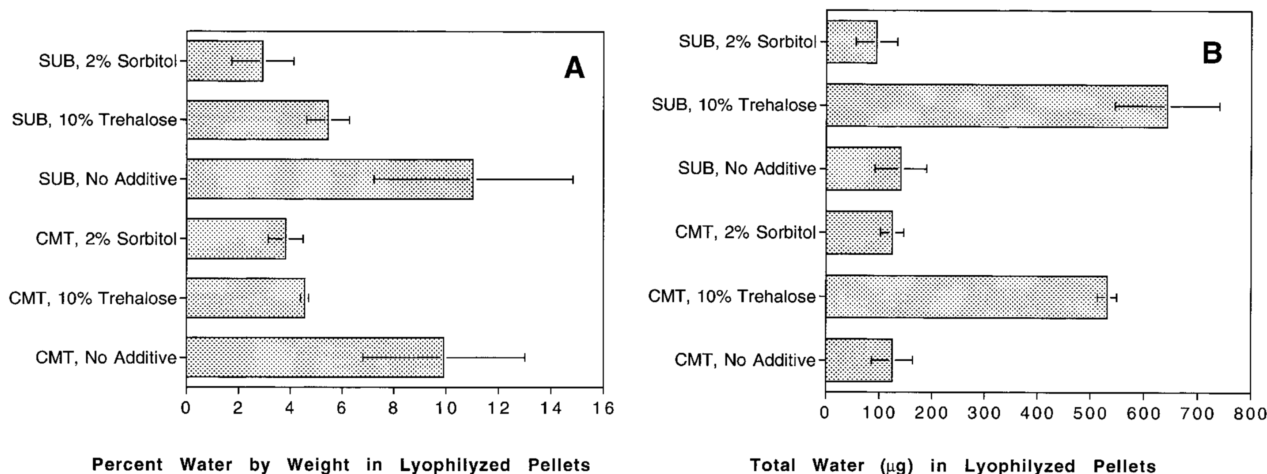

FIG. 7. (A) Total mass of residual water in the lyophilized samples as a percentage on the total sample mass. (B) Total mass of residual water present in the lyophilized samples. The mass of water in lyophilized samples was determined by Karl Fischer autotitration. The values shown are means ± SD for triplicate measurements on three independent lyophilized samples.

tional rigidity in the medium in which "enzyme memory" is displayed is expected. In contrast to these suggestions, the current results indicate that there can be substantial reorganization in enzyme structure when lyophilized powders are placed in organic solvents. This is consistent with the work of Farber and co-workers who found evidence of specific interactions between proteins and solvents as nonpolar as hexane (25). Hence, the initial structure in a dried solid is not predictive of the final structure in organic solvents.

One potential reason for the observed changes in structure was our finding that the "dried" samples contained a substantial amount of residual water (5–10 g H_2O/g dry wt). Thus, suspension in organic solvents would result in an extremely complicated milieu, in which water, carbohydrate, and organic solvent could all impinge on protein structure and mobility. With so many components, which alone or in concert could alter protein structure and stability, it is not possible to predict what would happen to protein conformation in a given mixture, nor even to explain rigorously the various behaviors observed in the current study. For example, sugars are known to stabilize native protein structure in aqueous solutions (26, 27), whereas, in the presence of "sufficient water," more polar organic solvents (e.g., alcohols) will foster denaturation, at least at relatively high temperatures (28). In contrast, it is well established that when the water activity coefficient is sufficiently low, the resistance of proteins to thermal denaturation is greatly increased in organic solvents (29), including alcohols (30).

The complexity of these systems could be reduced substantially if a more rigorous lyophilization protocol were used to prepare samples that have substantially lower residual moisture (e.g., < 0.01 g H_2O/g dry wt). The less effective drying method of the current study was chosen because it has been used most often by researchers preparing proteins for nonaqueous enzymology, including Dabulis and Klibanov (4). In terms of residual moisture, a more carefully controlled lyophilization cycle (31–33) will result in lower residual moisture because: (1) the sample can be maintained below the collapse temperature during sublimation of ice (i.e., primary drying); and (2) the final temperature of the sample can be adjusted to greater than ambient, which would foster an increased drying as water is desorbed from the amorphous phase (i.e., secondary drying) that contains protein and noncrystalline additives (e.g., carbohydrates). The final temperature of the dried product during secondary drying is most important in determining residual moisture; increasing the duration of this step will not have nearly as much effect (31–33). Therefore, with the current protocol, in which the final sample temperature is equal to room temperature (ca. 23°C), increasing the drying time even several more hours would not be expected to reduce the final water content substantially.

Furthermore, our results also did not indicate any correlation between enzyme catalytic activity and sample water content, although it has been shown previously that a higher water activity coefficient results in higher enzyme catalytic activity in organic solvents (34–36). It is important to note that for a given mass ratio of water in the resuspended lyophilized powder (e.g., compare subtilisin dried without stabilizes versus with sorbitol or trehalose, Table II), the water activity coefficient will be higher in the less polar solvents (e.g., hexane). However, for our samples, even in hexane, catalytic activity did not correlate with water content.

Considering the complexities of the systems in terms of composition and morphology, it is not surprising that numerous factors in addition to those discussed above have been proposed to be important for influencing catalytic activity of enzymes suspended in organic solvents. These include:

(1) that the polar organic solvents may bind at the active site, meaning the solvent in which the enzyme is most active might be the least inhibitory (18, 34). Also, as noted above infrared spectroscopy cannot determine site-specific changes in structure, such as disruption of the functional conformation of the active site. It is known that different solvents can alter regioselectivity (37) and enantioselectivity (38), possibly via changes in the nature of the active site.

(2) that the solvent alters the partitioning of the substrates between the bulk solvent and the enzyme/water complex (30, 34, 39). With the variety of substrates often used in transesterification assays, which are routinely used for studying enzyme activities in organic solvents, this effect could account for the differences in activity. The substrate used here is quite soluble in ethanol and much less so in hexane, which may account for the differences in activities we noted in these solvents (Tables I and II). Furthermore, the presence of a protein-stabilizing carbohydrate used during lyophilization may also limit accessibility to the active sites (4–6).

(3) that the thermodynamic activity of the alcohol reactant varies depending on the solvent. Partitioning is not the sole physicochemical parameter being affected by differences in solvent composition. For example, the thermodynamic activity of a reactant such as hexanol is approximately 10-fold larger in hexane than it is in 2-butanone, and it is about 2-fold greater than even in carbon tetrachloride (20).

(4) that catalytic activity in polar solvents versus nonpolar ones might be due to immediate loss in enzymatic hydration (3, 40). However, both α-chymotrypsin and subtilisin Carlsberg have been reported to remain active in methanol and tetrahydrofuran, even though little water remains around the protein (13). Subtilisin was found to maintain some activity in dimethylformamide, despite extensive water stripping (3). In other words, the solvent can affect the partitioning of water between the enzyme and the bulk solvent (32).

Most likely, each of these factors plays some role in controlling the activity of an enzyme in a low-water environment. Perhaps if all other parameters were controlled and kept equivalent, then differences in protein secondary structure could be shown to be important for differences in catalytic activity. If such were the case, it seems reasonable that increasing the fraction of native protein molecules in the total population should result in greater catalytic activity.

ACKNOWLEDGMENTS

We gratefully acknowledge support from the National Science Foundation (BES 9505301) to J.F.C. and from the American Foundation for Pharmaceutical Education for a fellowship award to B.S.K.

REFERENCES

1. Klibanov, A. M. (1990) *Acc. Chem. Res.* 23, 114–120.
2. Zaks, A., and Klibanov, A. M. (1988) *J. Biol. Chem.* 263, 8017–8021.
3. Schulze, B., and Klibanov, A. M. (1991) *Biotechnol. Bioeng.* 38, 1001–1006.
4. Dabulis, K., and Klibanov, A. M. (1993) *Biotechnol. Bioeng.* 41, 566–571.
5. Triantafyllou, A. O., Wehtje, E., Adlercreutz, P., and Mattiasson, B. (1995) *Biotechnol. Bioeng.* 45, 406–414.
6. Adlercreutz, P. (1993) *Biochim. Biophys. Acta* 1163, 144–148.
7. Prestrelski, S. J., Tedeschi, N., Arakawa, T., and Carpenter, J. F. (1993) *Biophys. J.* 65, 661–671.
8. Prestrelski, S. J., Arakawa, T., and Carpenter, J. F. (1993) *Arch. Biochem. Biophys.* 303, 465–473.
9. Dong, A., Prestrelski, S. J., Allison, S. D., and Carpenter, J. F. (1995) *J. Pharm. Sci.* 84, 415–424.
10. Dong, A., Huang, P., and Caughey, W. S. (1990) *Biochemistry* 29, 3303–3308.
11. Dong, A., and Caughey, W. S. (1994) *Methods Enzymol.* 232, 139–175.
12. Kendrick, B. S., Dong, A., Allison, S. D., Manning, M. C., and Carpenter, J. F. (1996) *J. Pharm. Sci.* 85, 155–158.
13. Zaks, A., and Klibanov, A. M. (1988) *J. Biol. Chem.* 263, 3194–3201.
14. Russell, A. J., and Klibanov, A. M. (1988) *J. Biol. Chem.* 263, 11624–11626.
15. Meyer, J. D., Kendrick, B. S., Matsuura, J., Ruth, J. A., Bryan, P. N., and Manning, M. C. (1996) *Int. J. Peptide Protein Res.* 47, 177–181.
16. Susi, H., and Byler, D. M. (1986) *Methods Enzymol.* 130, 290–311.
17. Surewicz, W. K., and Mantsch, H. H. (1988) *Biochim. Biophys. Acta* 952, 115–130.
18. Carpenter, J. F., Crowe, L. M., and Crowe, J. H. (1987) *Biochim. Biophys. Acta* 923, 109–115.
19. Monot, F., Borzeix, F., Bardin, M., and Vandersteele, J. P. (1991) *Appl. Microbiol. Biotechnol.* 35, 759–765.
20. van Tol, J. B. A., Stevens, R. M. M., Veldhuizen, W. J., Jongejan, J. A., and Duine, J. A. (1995) *Biotechnol. Bioeng.* 47, 71–81.
21. Yamamoto, Y., and Kise, H. (1993) *Biotechnol. Lett.* 15, 647–652.
22. Klibanov, A. M. (1995) *Nature* 374, 596.
23. Burke, P. A., Smith, S. O., Bachovchin, W. W., and Klibanov, A. M. (1989) *J. Am. Chem. Soc.* 111, 8290–8291.
24. Desai, U. R., and Klibanov, A. M. (1995) *J. Am. Chem. Soc.* 117, 3940–3945.
25. Yennawar, H. P., Yennawar, N. H., and Farber, G. K. (1995) *J. Am. Chem. Soc.* 117, 577–585.

26. Arakawa, T., and Timasheff, S. N. (1982) *Biochemistry* 21, 6536–6544.
27. Lee, J. C., and Timasheff, S. N. (1981) *J. Biol. Chem.* 256, 7193–7201.
28. Gerlsma, S. Y. (1968) *J. Biol. Chem.* 243, 957–961.
29. Volkin, D. B., Staubli, A., Langer, R., and Klibanov, A. M. (1991) *Biotechnol. Bioeng.* 37, 843–853.
30. Gandhi, N. N., Sawant, S. B., Joshi, J. B., and Mukesh, D. (1995) *Enzyme Microb. Technol.* 17, 373–380.
31. Pikal, M. J., Shah, S., Roy, M. L., and Putnam, R. (1990) *Int. J. Pharm.* 60, 203–217.
32. Pikal, M. J. (1990) *BioPharm* 3(8), 18–27.
33. Carpenter, J. F., and Chang, B. S. (1996) *in* Processing and Preservation of Biotechnology Products (Avis, K. E., and Wu, V. L., Eds.), pp. 199–264, Interpharm Press, Buffalo Grove, IL.
34. Clapes, P., Valencia, G., and Adlercreutz, P. (1992) *Enzyme Microb. Technol.* 14, 575–580.
35. Bovara, R., Carrea, G., Otoolina, G., and Riva, S. (1993) *Biotechnol. Lett.* 15, 937–942.
36. Hwang, K.-A., Lee, S. B., and Lee, K. H. (1995) *Biotechnol. Lett.* 17, 71–76.
37. Rich, J. O., Bedell, B. A., and Dordick, J. S. (1995) *Biotechnol. Bioeng.* 45, 426–434.
38. Kawashiro, K., Sugahara, H., Sugiyama, S., and Hayashi, H. (1995) *Biotechnol. Lett.* 17, 1161–1166.
39. Bemquerer, M. P., Adlercreutz, P., and Tominaga, M. (1994) *Int. J. Peptide Protein Res.* 44, 448–456.
40. Gorman, L. S., and Dordick, J. S. (1992) *Biotechnol. Bioeng.* 39, 392–397.