Using Sugar and Amino Acid Additives to Stabilize Enzymes within Sol−Gel Derived Silica

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The inclusion of additives during the immobilization of proteins into sol−gel processed materials has been widely explored as a route to stabilize proteins against the denaturing stresses encountered upon entrapment. In this report, we explore the effects of sorbitol and *N*-methylglycine (collectively referred to as osmolytes) on both the conformational stability and biological activity of the enzymes α-chymotrypsin and ribonuclease T1 in solution and when entrapped into sol−gel derived silica. In each case, the encapsulation of the enzymes into sol−gel derived silica in the absence of additives led to a moderate decrease in the thermodynamic stability of the proteins. However, entrapment in the presence of the osmolytes produced significant increases in the thermal stability and biological activity of the encapsulated proteins. We show that the observed enhancements in enzyme stability are likely based on a combination of increases in the pore size of the silica material (which improves substrate delivery and thus activity) and changes in the thermal stability of the entrapped enzymes in the presence of osmolytes. Our results suggest that these additives stabilize the two proteins by altering the hydration of the entrapped protein, hence this stabilization method may prove to be applicable to a wide variety of proteins.

Introduction

The characteristics of proteins entrapped in glasses formed by the sol−gel processing method have been extensively studied during the past few years.[1,2] Numerous reports have appeared describing the function,[3] structure,[4] dynamics,[5] accessibility,[4,6] reaction kinetics,[4a,7] initial stability,[8] and long-term stability[9] of entrapped proteins. These studies have established that, in the majority of cases, entrapped biological molecules retain their characteristic biochemical functionality and remain stable over periods of months. However, in cases where proteins are destabilized by either low pH or high alcohol levels, encapsulation can result in a substantial loss of function,[10] and can cause significant structural changes in proteins immediately upon entrapment and as the materials age.[3,11,12]

A variety of additives have been examined as stabilizers of entrapped proteins, including ligand-based stabilizers (Cod III parvalbumin,[8d] oncomodulin[8e]), the use of methyltrimethoxysilane-based materials to stabilize atrazine chlorohydrolase,[13] the incorporation of organosilanes and polymers into lipase-doped silica (which produced an 88-fold enhancement of activity compared to free lipase for esterification reactions[14,15,16]), the use

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of a graft copolymer of polyvinylimidazole and polyvinylpyridine to improve the stability of entrapped glucose oxidase and horseradish peroxidase,[17,18,19] incorporation of charged polymers such as poly(vinylimidazole) and poly(ethyleneimine) to stabilize lactate oxidase and glycolate oxidase,[20] and the addition of poly(ethylene glycol) to silica to stabilize acetylcholinesterase and butyrylcholinesterase.[21] Such studies clearly show that interactions between the additive and the protein can be used advantageously to maximize the stability and function of some proteins; however, at present there is very limited information available on the mechanism(s) by which the proteins are stabilized. Furthermore, none of these methods of stabilizing entrapped proteins appear to be universally applicable, as might be expected given the large variability in native protein properties.

Recent experimental and theoretical work has attempted to address the basis of changes in protein stability upon entrapment. Both our group[4a,8d,e] and Valentine's group[11] have noted that the conformational stability of some proteins is improved upon entrapment, although recent results from our group suggest that the enhanced stability may in some cases be transient.[12] Eggers and Valentine have explored both excluded volume and alterations in protein hydration as possible causes of the enhanced stability of entrapped proteins, both in undoped glasses[11] and in wet-aged glasses that had protein-stabilizing dopants such as sugars and amino acids added after gelation,[22] and this effect has been elaborated at the theoretical level by Minton[23] and by Dill and co-workers.[24]

In this work, we have examined how the presence of the osmolytes sorbitol and sarcosine (*N*-methylglycine) in the buffered protein solution that is used to form the glass affects both the thermodynamic stability and the activity of enzymes entrapped into tetraethyl orthosilicate (TEOS) derived silica. The osmolytes were chosen on the basis of their high solubility in aqueous solution and because both compounds have been used previously to stabilize proteins in aqueous solution.[25] The enzymes chosen for this study were α-chymotrypsin and ribonuclease (RNAse) T1. These proteins both show significant changes in the emission intensity and spectral characteristics of the Trp residue(s) upon protein denaturation, which can be used to examine changes in the tertiary structure of the proteins upon entrapment and to derive the unfolding temperatures of proteins.[8d,e] These proteins also have convenient colorimetric activity assays to allow for determination of protein activity as a function of entrapment conditions. Furthermore, the two proteins are significantly different in size, structure, and isoelectric point (α-chymotrypsin has a molecular weight of 25 000 and an isoelectric point of 9.1,[26] whereas RNAse T1 has a molecular weight of 11 000 and an isoelectric point of 2.9[27]). Hence, these proteins should provide good test cases to establish the effects of osmolytes on protein stability.

We show that the unfolding temperatures, and hence the thermodynamic stability, of both free and entrapped enzymes are improved upon addition of increasing levels of either sorbitol or sarcosine. The improvements in conformational stability correlate to improvements in the activity of the enzymes at elevated temperatures in the presence of osmolytes, suggesting that these additives may be useful for improving the shelf life of entrapped enzymes. Measurement of the physical properties of the osmolyte-doped materials show that the addition of osmolytes produces increases in the average pore size, which result in greater diffusion of analytes into the matrix and thus higher overall enzymatic activity. Taken together, our results suggest that osmolytes may provide a robust and generic method for improving the performance of entrapped enzymes, and may be useful in the development of second-generation silane precursors that can produce sol–gel glasses designed to stabilize entrapped biomolecules.

Experimental Section

Chemicals. α-Chymotrypsin (Type II: lyophilized powder from bovine pancreas), *Aspergillus oryzae* ribonuclease T1 (suspension in 3.2 M ammonium sulfate solution, pH 6), benzoyl-L-tyrosine ethyl ester (BTEE), guanylyl (3′ → 5′) uridine (GpU), and *N*-methylglycine (sarcosine, SigmaUltra grade) were purchased from Sigma (Oakville, ON). Tetraethyl orthosilicate (TEOS, 99.999 + %) and D-sorbitol (98%) were purchased from Aldrich (Oakville, ON). All reagents were used as received without further purification. All solutions were made with water that was distilled and deionized through a Milli-Q Synthesis A10 4-stage water purification system.

Procedures. *Preparation of Protein and Osmolyte Solutions.* α-Chymotrypsin solutions were prepared fresh for each use by dissolving a small amount of the lyophilized protein into a few milliliters of Tris buffer (0.08 M, pH 7.8) containing 0.1 M $CaCl_2$. The enzyme concentration was determined by UV–Vis spectroscopy using an extinction coefficient of $\epsilon_{280} = 51\,000$ M^{-1} cm^{-1}.[26] RNAse T1 solutions were prepared in a 5 mM phosphate buffer containing 50 mM KCl, pH 7.2. The concentration of RNAse T1 in the stock solution was determined by absorbance measurements using an extinction coefficient of $\epsilon_{278} = 20\,900$ M^{-1} cm^{-1}.[27] All protein solutions were diluted as needed for further experiments.

In cases where proteins were tested or entrapped in the presence of osmolytes, the osmolyte was dissolved in the appropriate buffer at a level of between 0.25 and 4.0 M for fluorescence studies or at a level between 0.5 and 4.0 M for the activity studies, and this solution was used to dilute the protein stocks to prepare working solutions. Typical dilution factors were 100:1 or greater to achieve concentrations of 2–10 μM of protein in the working solution (as determined by UV–

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Vis absorbance measurements) with less than 1% dilution of the osmolyte solution.

Preparation of Sol–Gel Derived Monoliths. A 4.5-mL portion of TEOS, 1.4 mL of ddH_2O, and 0.1 mL of 0.1 N HCl were mixed and sonicated for an hour until a homogeneous mixture was obtained. Hydrolyzed TEOS (300 μL) was mixed with 300 μL of a buffer containing the appropriate protein and between 0 and 4.0 M of a given osmolyte. The mixture was placed in a disposable polymethacrylate cuvette that was then sealed with Parafilm paraffin wax film and laid on its side to allow gelation to occur. Upon gelation, several small holes were punched in the Parafilm and the monoliths were aged at room temperature for 21 days, at which time they were removed from the cuvettes and slowly rehydrated to avoid cracking. Over this time the materials generally shrank to about 25% of their initial volume (initial dimensions were 37 × 10 × 1.6 mm, and final dimensions were ca. 27 × 7 × 0.8 mm), however it is important to note that the mass of entrapped enzyme did not change (note, leaching studies after 21 days indicated no loss of either of the entrapped enzymes). Protein concentration within the aged monoliths was assessed directly by UV–Vis absorbance spectroscopy and was used to correct all fluorescence intensity data for changes in protein concentration, as described below.

Thermal Denaturation of Proteins. Thermal denaturation of free and entrapped protein was assessed by measuring the emission of the tryptophan residue(s) within the proteins as a function of temperature, using instrumentation that is described in detail elsewhere.[4a] Samples were excited at 295 nm and the emission was collected from 305 to 450 nm in 1-nm steps using a 4-nm band-pass on the excitation and emission monochromators and a 0.3-s integration time. An appropriate protein-free blank was subtracted from each sample spectrum, and the resultant spectra were corrected for variations in emission monochromator throughput and PMT response, and for any changes in protein concentration that resulted from shrinkage of the sol–gel derived monoliths to obtain a relative molar emission value.

Experiments involving proteins in solution were done using osmolyte concentrations between 0.0 and 4.0 M. In the case of entrapped proteins, the samples were tested with no osmolyte in either the monolith or buffer, with 0.0–4.0 M of osmolyte in the monolith but not in the surrounding buffer (resulting in a dilution of osmolyte to ~10% of the original concentration in the glass), and with 0.0–4.0 M of osmolyte present in both the monolith and the buffer to maintain the osmolyte concentration at the level initially present in the glass. All samples were purged with nitrogen for 15 min and immediately capped to remove dissolved oxygen, which caused irreversible alterations in the fluorescence signals of thermally unfolded proteins.[16] Emission spectra were collected in approximately 3 °C increments, starting at 20 °C and going up to at least 70 °C. All samples were allowed to equilibrate at each temperature for 15 min before a spectrum was collected (note: longer equilibration times did not produce any changes in T_{un} values). The spectra were corrected for changes in protein concentration, as described above, integrated over the entire emission band, and the molar intensity at each point was used to determine the percentage of native enzyme remaining at each temperature according to the following equation:

$$f_N(T) = 1 - \left(\frac{F_{max} - F(T)}{F_{max} - F_{min}}\right) \quad (1)$$

where F_{max} is the maximum fluorescence intensity (at 20 °C), F_{min} is the minimum fluorescence intensity, generally obtained at the highest temperature, and $F(T)$ is the fluorescence intensity at a given temperature T. The fraction of native enzyme was plotted against temperature to generate intensity-based unfolding curves. Unfolding temperatures (T_{un}) were determined from the maxima in first-derivative plots of the unfolding curves, which typically corresponded to the temperature where the fluorescence signal had decreased to 50% of its initial value. All unfolding curves for both free and entrapped proteins were normalized to the value obtained for the given protein in osmolyte-free buffer.

Temperature Effects on Enzyme Activity. The activity of the two enzymes was examined spectrophotometrically at various temperatures. For α-chymotrypsin, 1.4 mL of 0.001 M benzoyl-L-tyrosine ethyl ester (BTEE) in 50% (w/w) methanol was mixed with 1.5 mL of 0.08 M tris·HCl, pH 7.8, containing 0.1 M calcium chloride and the desired level of osmolyte. To this solution, either 0.10 mL of a 1.6 mM solution of α-chymotrypsin in 0.001 N HCl (for solution studies) or the intact monolith containing the enzyme was added. The protein solution was incubated at the desired temperature in the presence of the osmolyte for 15 min before addition to the substrate solution. The absorbance at 256 nm was measured for 30–60 min, and the relative activity of the protein was determined from the slope of the absorbance vs time curve using ϵ_{256} (benzoyl tyrosine) = 964 $M^{-1}\cdot cm^{-1}$.[28]

Activity assays of RNAse T1 involved the addition of 0.1 mL of a 0.4 μM solution of guanylyl (3′ → 5′) uridine (GpU) to 2.8 mL of buffer solution (5 mM PBS, pH 7.2).[29] This solution contained varying levels of osmolyte and was incubated at the desired temperature before addition of the enzyme. A 0.1-mL portion of a 0.05 μg/mL solution of RNAse T1 (solution assays) or an intact monolith containing the enzyme (each incubated for 15 min at the desired temperature) was added, and the absorbance at 260 nm was measured for 30 min. The activity of the protein was determined from the slope of the absorbance vs time curve using ϵ_{260} (products) = 23 800 $M^{-1}\cdot cm^{-1}$.[30]

For both enzymes the relative activity per milligram of enzyme was determined by normalizing the activity to the value obtained in solution in the absence of osmolyte at 25 °C. The relative activity was plotted against temperature to establish temperature effects on activity.

Quenching Studies. Sol–gel derived monoliths containing 10 μM of α-chymotrypsin were placed into a quartz cuvette containing 1.5 mL of Tris buffer with between 0.0 and 2.0 M acrylamide and were allowed to equilibrate for 1 day before measurements were done. Longer equilibration times did not produce any further quenching for the samples. At each quencher concentration, time-resolved fluorescence intensity decay data were acquired in the time-domain using a PTI LaserStrobe fluorimeter (Photon Technologies Incorporated, London, ON), as described elsewhere.[31] Samples were excited at 295 nm and the emission intensity data were collected at 340 nm using 4-nm band-passes under magic angle conditions into 25-ps time windows, starting 2 ns before the laser pulse arrived and covering a 25 ns range. The instrument response function was collected by measuring the Rayleigh scattering of the laser pulse from water, and was used to deconvolute the instrument response from the experimentally determined decay trace. The decay was fit to a discrete decay model using methods that are described elsewhere.[31] Intensity-weighted mean lifetimes were plotted against quencher concentration, and the quenching data were fit using a modified version of the Stern–Volmer equation which accounted for the possibility of there being a fraction of protein that was not accessible to the quencher (f_i):[32,33]

$$\frac{\langle\tau\rangle_0(1 - f_i)}{\langle\tau\rangle - f_i\langle\tau\rangle_0} = 1 + K_{SV}[Q] = 1 + k_q\langle\tau\rangle_0[Q] \quad (2)$$

where $\langle\tau\rangle_0$ is the intensity-weighted mean lifetime in the

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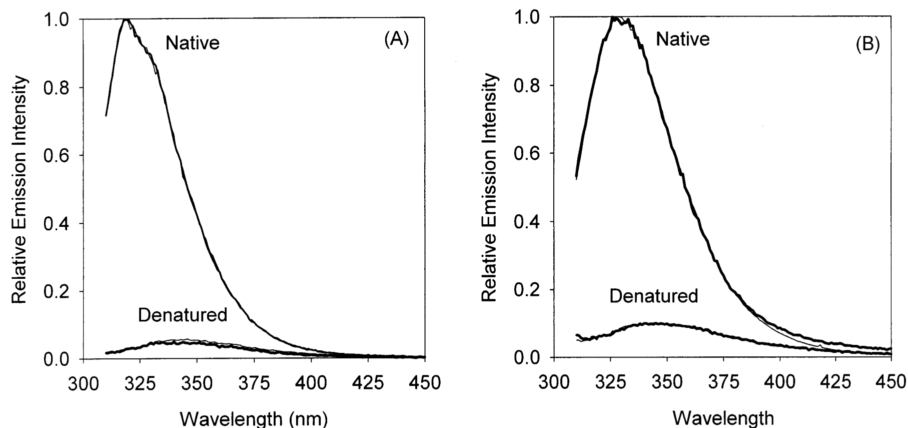

Figure 1. Fluorescence spectra as a function of temperature for RNAse T1 (panel A) and α-chymotrypsin (panel B) in the native state at 20 °C and in the denatured state at 70 °C. The thin lines show the spectra of proteins in solution, and the thicker lines show the spectra of entrapped proteins.

absence of quencher, $\langle\tau\rangle$ is the intensity-weighted mean lifetime in the presence of quencher, $[Q]$ is the molar concentration of the quencher, K_{SV} is the Stern–Volmer quenching constant for the collisional process (M^{-1}), and k_q is the bimolecular quenching constant ($M^{-1}\cdot s^{-1}$).

Pore-Size Analysis. Pore-size analysis of completely dried monoliths was performed on a Quantachrome Nova 2200 surface area/pore-size analyzer. Before analysis, the monoliths were washed copiously to remove any entrapped osmolytes, crushed to a fine powder, freeze-dried, and outgassed at 120 °C for 4 h to remove air and bound water from the surface of the powder. The pressure was measured as nitrogen was adsorbed and desorbed at a constant temperature of −196 °C. Using the desorption branch of the resulting isotherm the average pore-size and distribution of pore-sizes was determined using the Barrett, Joyner, and Halenda (BJH) calculation.[34]

Results and Discussion

Initial Conformation of Entrapped Enzymes. Upon entrapment of a protein into a sol–gel derived glass it is possible that the protein may undergo significant conformational changes that are consistent with partial denaturation. To examine how entrapment affected the initial conformation of the enzymes, three separate experiments were performed. First, the molar fluorescence intensity of the two enzymes was examined in solution and in TEOS-derived glasses in the absence and presence of the two osmolytes at a level of 2.0 M. The molar fluorescence intensity of the enzyme in a buffer solution containing no osmolytes is taken as corresponding to 100% native enzyme. As shown in Table 1, the molar fluorescence of the two enzymes was in all cases within 5% of the value in buffer solution, suggesting that the two enzymes were fully native in all cases. The only system that was slightly altered upon entrapment was α-chymotrypsin in sorbitol, which showed a slight decrease in molar intensity in solution and an slight increase in intensity upon entrapment in the presence of sorbitol. These data suggest that the conformation of this enzyme may have been slightly altered upon entrapment.

Emission wavelength data, shown in Table 1, provide further support that the proteins do not undergo significant conformational changes upon denaturation.

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Table 1. Emission Properties of RNAse T1 and α-Chymotrypsin in Solution and When Entrapped in the Absence and Presence of 2.0 M Sarcosine or Sorbitol

sample	molar fluorescence intensity (±2%)	initial emission wavelength (±1 nm)	change in intensity upon unfolding (±3%)
RNAse T1			
solution	100	320	95
sarcosine	100	319	94
sorbitol	98	318	94
TEOS	103	319	95
TEOS/sarcosine	100	320	95
TEOS/sorbitol	98	319	96
α-chymotrypsin			
solution	100	328	90
sarcosine	99	330	91
sorbitol	96	331	88
TEOS	104	330	90
TEOS/sarcosine	98	329	90
TEOS/sorbitol	105	331	89

Although the emission wavelength of α-chymotrypsin is slightly red-shifted in all cases relative to the value in undoped solution, this likely reflects subtle changes in the polarity of the external solvent that perturb the Trp residues.

As a last test to confirm entrapment of the proteins in a native form, the change in intensity upon thermal denaturation was examined under all conditions for the two enzymes. Figure 1 shows the emission spectra obtained at 20 and 70 °C during the denaturation of RNAse T1 (panel A) and α-chymotrypsin (panel B) in solution and in undoped sol–gel derived materials. Increases in temperature caused similar trends for the two proteins. These included a decrease in intensity (95% loss for RNAse T1, 90% loss for α-chymotrypsin), and a red-shift in the maximum emission wavelength (319 → 346 nm for RNAse T1, 328 → 345 nm for α-chymotrypsin,) as temperature was increased. These trends are consistent with changes in the tertiary structure of the two proteins which resulted in the exposure of the buried tryptophan residue(s) to the surrounding solvent as the proteins unfolded, and confirmed that fluorescence measurements were capable of monitoring the unfolding of the two proteins both in solution and when entrapped in sol–gel derived materials. As shown in Table 1, the change in intensity upon

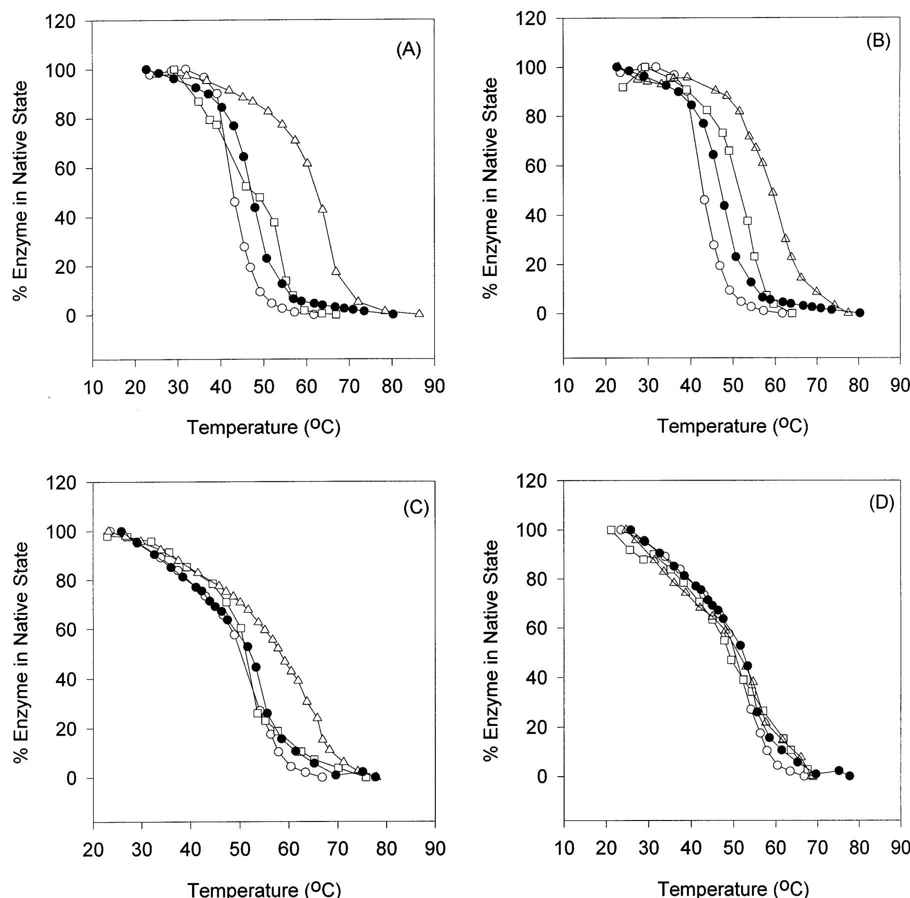

Figure 2. Fluorescence-based unfolding curves for RNAse T1 in the presence of sarcosine (panel A) and sorbitol (panel B), and for α-chymotrypsin in the presence of sarcosine (panel C) and sorbitol (panel D): (●) proteins in solution; (○) entrapped proteins in the absence of osmolyte; (□) entrapped proteins tested in the presence of 0.25 M osmolyte; (△) entrapped proteins tested in the presence of 2.0 M osmolyte.

denaturation is within error under all conditions for the two proteins (i.e., 95 ± 1% for RNAse T1, 90 ± 2% for α-chymotrypsin). The similarity in the change in intensity indicates that both the initial and final conformation of the two proteins is similar under all conditions.

It must be noted that some entrapped proteins may never have similar fluorescence properties relative to those in solution.[3] However, both of the entrapped proteins examined in this work showed spectral properties that were essentially identical to those obtained from the proteins in solution. This is not unexpected, given that in both proteins the Trp residue(s) are sequestered within the protein structure and thus do not respond to the external solvent environment unless the protein conformation changes. The similarity in the emission profiles for free and entrapped proteins strongly suggests that the entrapment method did not perturb the initial conformation of the protein. Furthermore, the spectral properties of the denatured proteins were similar in solution and when entrapped, indicating that full unfolding of the entrapped protein was possible, and allowing for direct comparisons of the unfolding behavior of free and entrapped proteins.

Thermal Stability of Enzymes. Figure 2 shows the percentage of native enzyme remaining as a function of temperature ($f_N(T)$) for RNAse T1 in the presence of varying levels of sarcosine (panel A) or sorbitol (panel B), and for α-chymotrypsin in the presence of sarcosine (panel C) or sorbitol (panel D). Closed symbols show unfolding curves for the free enzymes in solution, and open symbols show unfolding curves for entrapped enzymes in the presence of varying levels of osmolyte. The calculation of the fraction of native enzyme used emission intensity values that were normalized for the concentration of protein within each sample, as shown in Table 1. The unfolding curves show that both proteins have a pre-unfolding baseline which gently slopes downward owing to direct quenching of Trp fluorescence at increased temperatures, followed by a rapid decrease in intensity as the protein unfolds, and finally a post-unfolding baseline. The figures clearly show that the presence of increasing levels of sarcosine leads to improvements in the unfolding temperature for both entrapped proteins, while sorbitol improves the stability of RNAse T1 only.

Figure 3 shows the unfolding temperatures as a function of osmolyte concentration for both the free (open symbols) and entrapped (closed symbols) proteins, and confirms that the thermal stability of both the free and entrapped proteins increases in the presence of osmolytes in a concentration-dependent manner, and that sarcosine tends to be the more potent stabilizer. It is also clear that low levels of sorbitol and sarcosine lead to an apparent *destabilization* of α-chymotrypsin (even though fluorescence results show no changes in the initial conformation), which suggests that careful control over the concentration of this osmolyte is crucial for obtaining significant enhancements of thermal stability.

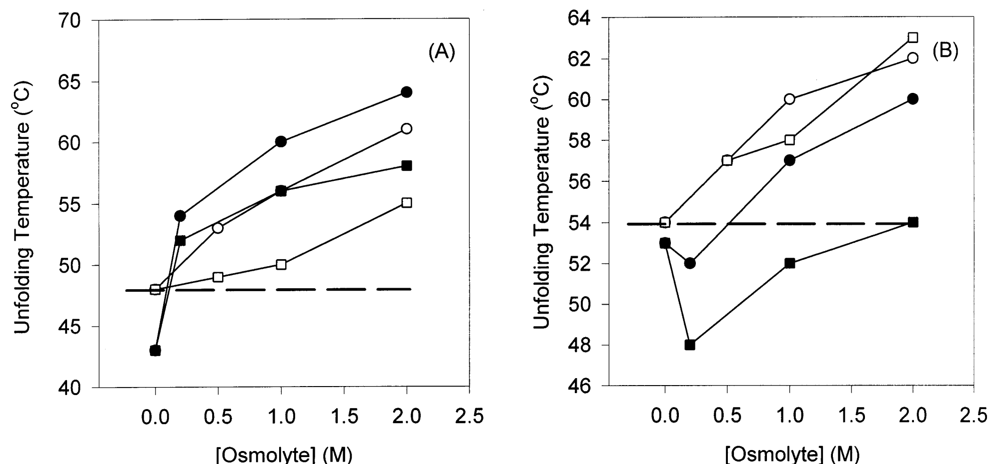

Figure 3. Changes in unfolding temperature as a function of osmolyte concentration for free and entrapped RNAse T1 (panel A) and α-chymotrypsin (panel B): (□) enzyme in solution containing sorbitol; (○) enzyme in solution containing sarcosine; (■) entrapped enzyme in the presence of sorbitol; (●) entrapped enzyme in the presence of sarcosine. Typical errors are ±2 °C.

On the other hand, the presence of sarcosine produced enhancements in the stability of both proteins, at all concentration levels, suggesting that this is the more potent stabilizing agent.

Enzyme Activity. To further explore the stabilizing effects of the two osmolytes, activity assays were done for each protein as a function of temperature to allow direct comparisons of temperature effects on protein conformation and activity. Figure 4 shows the changes in relative activity per mg of protein vs temperature for RNAse T1 in solution (panel A) and when entrapped (panel B), and for α-chymotrypsin in solution (panel C) and when entrapped (panel D) in the presence of sarcosine and sorbitol. All results are normalized to the activity obtained for a given protein in aqueous solution in the absence of osmolytes at 25 °C. For ribonuclease T1 in solution, the activity of the enzyme was significantly enhanced at all temperatures when either sorbitol or sarcosine were present. As shown in Figure 4, the activity of RNAse T1 peaks at a similar point in all cases (approximately 35−40 °C), but then decreases rapidly for the protein in aqueous buffer, becoming inactive beyond 60 °C. The T_{un} of RNAse T1 is approximately 50 °C, which is in good agreement with that obtained from fluorescence measurements. Addition of osmolytes results in a more gradual decrease in activity, and it is apparent that a significant fraction of the enzyme activity is retained even out to 70 °C (approximately 60% with respect to the activity of RNAse T1 in solution at 25 °C, and about 20% with respect to the maximum activity obtained at 45 °C in the presence of the osmolytes). The activity values at 70 °C remain constant for at least 24 h at this temperature.

As shown in panel B, a similar trend is obtained for RNAse T1 in sol−gel derived glasses, where the addition of the osmolyte produced increased activity for the entrapped protein relative to the protein in undoped glasses. As was the case in solution, the most dramatic increases again occur for sarcosine-doped glasses, which show up to a 2-fold enhancement of activity relative to undoped glasses at higher temperatures. These results are in accordance with those obtained from fluorescence studies, and confirm that both sarcosine and sorbitol can stabilize the entrapped enzyme against denaturation. It is noteworthy that the activity of the entrapped enzyme is usually lower than the value obtained for the corresponding sample in aqueous solution. This result suggests that the diffusion of the substrate into the glass is hindered so that the observed activity, which is diffusion dependent, is lowered.

The activity of α-chymotrypsin was also enhanced in the presence of osmolytes, and by sarcosine in particular, both in solution and when entrapped. The activity of the enzyme increased to a maximum value at ca. 35−40 °C, and then decreased significantly as temperature increased. In solution, the enzyme showed no activity at 50 °C or higher, in agreement with the unfolding temperature of the protein (53 °C), suggesting that most of the activity is lost early in the unfolding transition. In the presence of different osmolytes, the temperature corresponding to the loss of activity did not change appreciably, suggesting that the osmolyte did not prevent the subtle changes that were associated with denaturation, even though large-scale conformational changes, as reported by Trp fluorescence, were stabilized. Even so, some residual activity was retained, even at 65 °C, when sorbitol or sarcosine were present in the buffer solution.

In the case of the entrapped enzyme two general trends were observed. First, the overall activity (relative to undoped glass) was significantly higher in the presence of both osmolytes. Second, the activity of the entrapped enzyme was generally *higher* than the corresponding value in solution, particularly at higher temperatures (>40 °C) although it eventually decreased to a baseline value similar to that in solution beyond 65 °C. The increased activity upon entrapment is unusual, particularly given the results obtained for RNAse T1. One possible explanation for this discrepancy is that the substrate for the RNAse T1 reaction is anionic, and thus is likely to be hindered in its entry into the anionic silica material,[35] whereas BTEE is neutral and thus may enter the silica matrix more easily. Once again, sarcosine was observed to be the more potent stabilizer, and produced up to a 2-fold enhancement in the activity of the entrapped enzyme

(35) Badjic, J. D.; Kostic, N. M. *Chem. Mater.* **1999**, *11*, 3671.

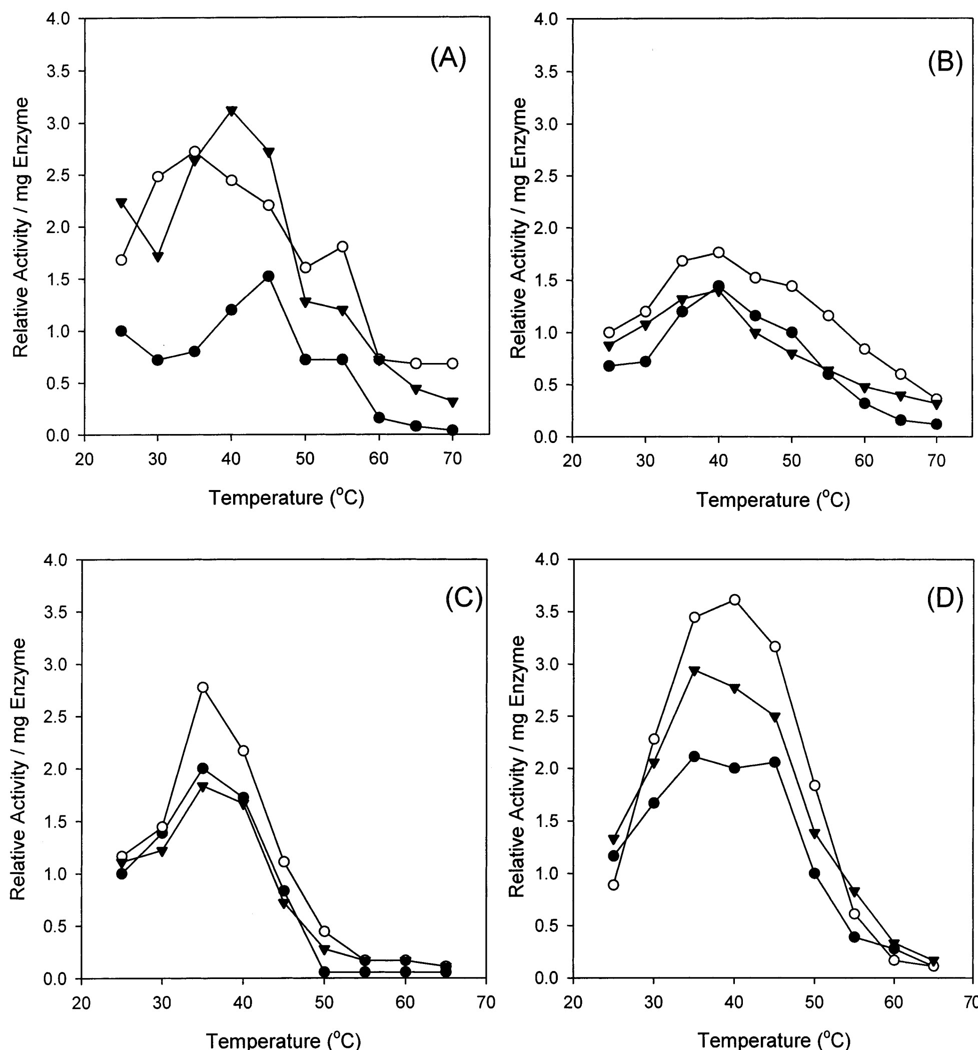

Figure 4. Changes in enzyme activity as a function of temperature for RNAse T1 in solution (panel A) and when entrapped (panel B), and for α-chymotrypsin in solution (panel C) and when entrapped (panel D) in the presence of sorbitol and sarcosine: (●) no osmolyte; (▼) 2.0 M sorbitol; (○) 2.0 M sarcosine. Typical errors are 10% RSD.

Table 2. Pore Size Data Obtained for Doped and Undoped Silica after Aging for 15 days Followed by Removal of Dopants by Washing

sample	average pore diameter (nm)[a]	pore size distribution (nm)[a]	total pore volume ($cm^3 \cdot g^{-1}$)	total surface area ($m^2 \cdot g^{-1}$)
TEOS[b]	2.2 ± 0.3	1.5	0.192 ± 0.010	352 ± 22
sarcosine	7.1 ± 0.4	4.9	0.389 ± 0.023	218 ± 18
sorbitol	6.8 ± 0.4	4.8	0.373 ± 0.028	220 ± 21

[a] Obtained from the desorption branch. [b] TEOS samples were washed in a manner identical to that used for sorbitol- and sarcosine-doped samples.

relative to undoped glasses. Overall, the results clearly show that the osmolytes provide significant improvements in activity for both free and entrapped enzymes.

Effect of Additives on Pore Size and Protein Accessibility. The average pore size of the doped and undoped materials and the accessibility of enzymes entrapped in these materials were examined to further assess the possible origin of the observed changes in protein stability and activity upon entrapment. Table 2 shows the pore-size data obtained from TEOS-derived silica that was prepared and aged in the presence and absence of 2.0 M sorbitol and sarcosine. It must be noted that we were not able to obtain meaningful pore-size data when the additives were present during the measurement, thus all measurements were done using samples that had the additives removed via exhaustive washing. As shown in Table 2, the addition of either sarcosine or sorbitol to the glasses led to significant alterations in the pore morphology, including a larger pore diameter (~2 nm for TEOS-derived samples, but up to 7 nm for sarcosine-doped samples), a doubling of the total pore volume, and approximately a 40% decrease in total surface area. These results suggest that the pore walls are both larger and smoother when the additives are present, and suggest that the improvements in enzyme activity upon addition of osmolytes may be due to either greater accessibility of the entrapped enzyme or more rapid diffusion of the substrates into the matrix (which is consistent with the higher initial rate of substrate conversion obtained for

Table 3. Acrylamide Quenching Data for α-Chymotrypsin Entrapped in TEOS Derived Materials Containing 2.0 M Sorbitol or Sarcosine

sample	K_{sv} ($M^{-1}\cdot s^{-1}$) (±0.1)	τ_o (ns) ± 0.2 ns	k_q (s^{-1}) (±0.3 × 10^8)	fraction of accessible protein (%)	r^2
solution	1.6	3.0	5.3 × 10^8	100	0.99
TEOS	0.6	3.0	2.0 × 10^8	92 ± 3	0.98
TEOS + sorbitol	2.5	3.2	7.8 × 10^8	92 ± 5	0.94
TEOS + sarcosine	2.6	3.1	8.4 × 10^8	94 ± 5	0.98

osmolyte-doped glasses relative to undoped glasses). It must be noted that the pore sizes reported in Table 2 are those that are present after removal of entrapped water by incubation of samples at 120 °C followed by freeze-drying. It is likely that the pore sizes of the hydrated materials are significantly larger, perhaps even as large as 20 nm in diameter,[2,11,22] thus it is not likely that the entrapped enzymes were significantly constrained in the hydrated materials.

To determine whether enzyme accessibility was altered by the additives, the accessibility of entrapped α-chymotrypsin to the quencher acrylamide was examined. As shown in Table 3, the additives had no significant effect on the accessibility of the enzyme, with over 90% of the enzyme remaining accessible under all conditions. These results are in general agreement with previous results that show full accessibility of HSA to acrylamide in both wet-aged and dry-aged TEOS-derived materials.[11] Interestingly, the k_q value obtained in undoped glasses dropped by approximately 2.5-fold with respect to the value in solution, reflecting hindered diffusion of the quencher through the glass matrix.[4a] However, the incorporation of osmolytes led to an increase in the k_q to a value that was similar to or even slightly greater than that in solution. The increase in the k_q value reflects an enhanced rate of diffusion of the quencher to the entrapped protein, and indicates that the larger pores that exist in the presence of osmolytes led to greater diffusion of analytes through the matrix. It is likely that the enhanced mobility of the analyte contributed to the enhanced rates of substrate conversion that were observed for entrapped enzymes in samples that contained sarcosine or sorbitol.

Origin of Stabilization Effect. The entrapment of proteins into sol−gel derived silicate materials produced from TMOS or TEOS can lead to significant changes in their conformation, thermal stability, and activity. Even in the absence of osmolytes, some proteins show improvements in thermal stability, although as shown in this and other studies, it is also possible for proteins to be destabilized upon entrapment. For example, Eggers and Valentine[11] show that the proteins lysozyme, α-lactalbumin, and metmyoglobin have improved thermal stability upon entrapment even in the absence of osmolytes, in agreement with our findings for HSA[32] and monellin.[4b] However, apomyoglobin has been shown to be destabilized upon entrapment in TMOS derived silica,[11] in keeping with our observations of lowered T_{un} values for RNAse T1 and α-chymotrypsin entrapped in TEOS-derived glasses. More importantly, the enzymatic activity of many proteins is significantly altered upon entrapment,[13−21] and in the absence of dopants there is almost always a decrease in activity owing to limitations in diffusion rates, restricted accessibility of the entrapped protein, and/or alterations in binding constants.[1,2]

Eggers and Valentine suggest that there are three possible phenomena for stabilization of sol−gel entrapped proteins: (A) molecular confinement; (B) adsorption to silica; and (C) alterations in protein hydration. It is interesting to note that neither of our enzymes showed improvements in thermal stability upon entrapment into sol−gel derived silica in the absence of osmolytes. These results argue against excluded volume as a predominant effect in the stabilization of either RNAse T1 or α-chymotrypsin, and in fact it appears that the smaller pore sizes obtained in the absence of dopants may restrict the diffusion of analytes into the matrix and hence reduce the activity of entrapped enzymes. It is also likely that the presence of ethanol during the initial stages of entrapment may have destabilized the enzymes, and RNAse T1 in particular, producing a lower unfolding temperature.

Adsorption to the silica may also alter the stability of the protein. The isoelectric point of α-chymotrypsin is 9.1,[26] thus this protein is positively charged at neutral pH, whereas RNAse T1 has an isoelectric of 2.9[27] and thus is negatively charged at neutral pH. Given that silica is isoelectric at pH 2.0, the matrix is expected to be negatively charged at neutral pH.[36] On the basis of the differences in protein and matrix charge, one might expect that α-chymotrypsin should undergo electrostatic interactions with the matrix, whereas RNAse T1 should not. Even so, RNAse T1 is more destabilized upon entrapment, arguing against adsorption as a major destabilizing factor. Furthermore, adsorption does not appear to occur for these proteins as the emission properties of both proteins are similar in solution and in glass (Figure 1). Furthermore, in the presence of additives adsorption is likely to be negated due to high ionic strength (for sarcosine) or possible hydrogen bonding of the osmolyte to the silica surface (for sorbitol), which should alleviate this situation and result in a more nativelike structure.

On the basis of these results it is likely that alterations in protein hydration play a significant role in protein stability, and, as shown herein, may also alter enzyme activity. It is well-known that there is a significant change in the structure of entrapped water owing to templating of water to the silica wall.[37] The more ordered structure of the water has a higher free energy and may lead to a decrease in the strength of the hydrophobic effect that destabilizes the entrapped protein (in the absence of osmolytes).[22] The incorporation of either a sugar-based or a methylamine-based osmolyte can lead to disruption of the ordered water structure within the silica and thus may lower the free energy of the bulk phase such that the hydrophobic effect is enhanced, and thus protein folding is promoted, in agreement with the results of Eggers and Valentine.[22]

It is important to note that rinsing of the osmolyte out of glass removed the stabilizing effects (results not

(36) Hench, L. L.; West, J. K. *Chem. Rev.* **1990**, *90*, 33.
(37) (a) Xu, S.; Ballard, L.; Kim, Y. J.; Jonas, J. *J. Phys. Chem.* **1995**, *99*, 5787. (b) Korb, J.-P.; Delville, A.; Xu, S.; Demeulenaere, G.; Costa, P.; Johas, J. *J. Chem. Phys.* **1994**, *101*, 7074.

shown), showing that the changes in pore morphology were not the major cause of the enhanced thermal stability of the proteins, although the enhanced pore size was perhaps more important for achieving the improved enzyme activity as this requires good mobility of analytes through the silica matrix. These results also suggest that this method of protein stabilization may be useful primarily for enhancing the shelf life of entrapped proteins, but may not be as useful for enhancing stability during use unless methods can be found to permanently retain osmolytes in glass.

Conclusions

The addition of osmolytes to enzymes during and after entrapment can have significant effects on both the thermal stability and enzymatic activity. The present study suggests that sarcosine provides significant improvements in the stability of entrapped enzymes, but sorbitol is useful only for some enzymes, and can in some cases lead to decreased thermal stability upon entrapment. The entrapment of either RNAse T1 or α-chymotrypsin into TEOS-derived materials without osmolyte present resulted in decreases in thermal stability as compared to that in solution, and resulted in decreased enzyme activity for RNAse T1 owing to restricted diffusion of the anionic analyte through the silica matrix. Addition of 2.0 M sorbitol or sarcosine during encapsulation increased the thermal stability of the encapsulated proteins by up to 17 °C, and also improved enzyme activity by up to 2-fold relative to that of undoped glasses. The results suggest that the use of osmolytes, and particularly methylamines, should be a good method to increase the stability of proteins during or after entrapment (i.e., during storage).

The current work builds upon the observations of Eggers and Valentine, and shows that not only thermal stability, but also enzymatic activity, can be improved through the addition of osmolytes. Our work also shows that osmolytes can be added directly to the protein solution before mixing with the sol to form a glass, rather than after formation of the glass, as reported by Eggers and Valentine.[11] On the basis of our thermal unfolding data, it is clear that proteins are stabilized in the presence of osmolytes even when in solution, thus the most prevalent role of osmolytes is the alteration of hydration effects, although secondary effects including the alteration of protein–silica interactions and changes in pore morphology also play a role in enhancing the performance of entrapped enzymes.

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