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On the Salt-Induced Activation of Lyophilized Enzymes in Organic Solvents: Effect of Salt Kosmotropicity on Enzyme Activity

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Abstract: The dramatic activation of enzymes in nonaqueous media upon co-lyophilization with simple inorganic salts has been investigated as a function of the Jones−Dole *B* coefficient, a thermodynamic parameter for characterizing the salt's affinity for water and its chaotropic (water-structure breaking) or kosmotropic (water-structure making) character. In general, the water content, active-site content, and transesterification activity of freeze-dried subtilisin Carlsberg preparations containing >96% w/w salt increased with increasing kosmotropicity of the activating salt. Degrees of activation relative to the salt-free enzyme ranged from 33-fold for chaotropic sodium iodide to 2480-fold for kosmotropic sodium acetate. Exceptions to the general trend can be explained by the mechanical properties and freezing characteristics of the salts undergoing lyophilization. The profound activating effect can thus be attributed in part to the stabilizing (salting-out) effect of kosmotropic salts and the phenomenon of preferential hydration.

Introduction

Nonaqueous enzymatic catalysis offers a means of organic synthesis in the presence of minute quantities of water, exhibiting improved substrate specificity and selectivity compared to traditional means of chemical synthesis. Yet despite potential applications in chiral resolution,[1] enantioselective synthesis,[2] combinatorial chemistry,[3] and drug discovery,[4] the application of enzymes in organic solvents has been handicapped by markedly low catalytic efficiencies.[5,6] Compared to reactions carried out in aqueous solution, reaction rates in nearly anhydrous organic solvents are typically several orders of magnitude lower. For example, the transesterification activity of lyophilized subtilisin Carlsberg in hexane is over 5 orders of magnitude less than the hydrolytic activity of the soluble enzyme in water.[7] To overcome the insolubility of enzymes in organic solvents, early investigations involved either water-miscible solvents such as ethanol or acetone,[8] biphasic mixtures where water is emulsified by solvents such as chloroform or ethyl acetate,[9] or reversed micelles where the enzyme is dissolved in small pools of water entrapped within surfactant

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molecules.[10] The stability of enzymes dissolved in these systems is often poor, however, especially in comparison to lyophilized enzyme powders suspended in more hydrophobic solvents with high log *P* values.[6,11]

Advances in this field have led to an improved understanding of the role of water in enzyme catalysis in nearly anhydrous organic solvents. Previous studies demonstrated that the amount of water adsorbed to an enzyme in organic solvents is a more important determinant of enzyme activity than the water content of the solvent itself.[12,13] For subtilisin Carlsberg, a certain population of this essential water is intricately associated with the enzyme and does not exchange with the water in the bulk organic solvent.[14] Electron spin resonance (ESR) spectroscopy showed that further water bound to the enzyme effects an increase in active-site polarity, which correlates closely with a sharp increase in enzyme activity.[13] The correlation between increased activity and active-site polarity prompted the addition of simple inorganic salts during lyophilization of the enzyme. It was found that the inclusion of excess KCl in an aqueous enzyme solution prior to freeze-drying afforded a dramatic 3750-fold enhancement in the catalytic efficiency of subtilisin Carlsberg in hexane over that of salt-free enzyme powders.[15] Subsequent work clearly demonstrated that this activation was intrinsic, and not due to reduced diffusional limitations.[16] Further optimization of lyophilization time and water content produced catalysts with k_{cat}/K_m values of the same order of magnitude as that for the enzyme in aqueous buffer.[17] Salt-induced activation has also been observed for other enzymes such as chymotrypsin,[15,18] thermolysin,[19] and lipases,[17,18] suggesting the generality of this phenomenon for a variety of enzymes.

The exact mechanism of salt-induced activation of enzymes in organic solvents is still unclear, although it has been suggested that increased active-site polarity afforded by the charged state of salt ions may stabilize the charged transition state for subtilisin Carlsberg.[17,20] Despite the wealth of information that has been gathered on the influence of salts on enzyme activity and protein stability in aqueous environments, it is unknown whether the effects of solvated salt ions on enzyme activity and structure translate to systems where salts exist in a predominantly insoluble form. Although it has been shown that water content is a critical parameter in the activity of enzymes in organic solvents,[13] and in certain cases salts have been used to control the thermodynamic water activity in nearly anhydrous solvent systems,[21] the role of water−salt interactions in the nonaqueous environment remains to be elucidated.

A thermodynamic parameter for characterizing such interactions is the Jones−Dole viscosity *B* coefficient, which measures the degree to which an additive increases or decreases the viscosity of water.[22] Additives that decrease the viscosity have negative values of the *B* coefficient, exhibit weaker interactions with water than does water with itself, and are known as chaotropes. On the other hand, additives that increase the viscosity of water have positive values of the *B* coefficient, exhibit relatively strong interactions with water, and are designated kosmotropes. The kosmotropic and chaotropic properties of salts have previously been shown to impact both the stability and solubility of proteins in solution. [23] In general, kosmotropes tend to increase the stability of proteins as well as decrease their solubility; the opposite is true of chaotropes.[23,24] The effects of chaotropic and kosmotropic salts have not been extended, however, to studies with lyophilized, salt-activated enzymes in organic solvents.

Following the results of earlier investigations on the dramatic activation in organic solvents exhibited by lyophilized samples of subtilisin Carlsberg containing a final dry weight of 98% KCl salt,[17,19] the catalytic efficiency of this protease activated by various sodium and potassium salts has been systematically investigated. Of particular interest is whether enzyme activation in hexane correlates with the *B* coefficient of the salt, and whether salt activation in organic solvents can be explained by established mechanisms of salt stabilization in aqueous solution. Moreover, kosmotropic salts bind water more strongly in solution and retain more water after lyophilization; thus, the extent of salt activation has also been examined in relation to the water content of the lyophilized enzyme powder.

Experimental Section

Materials. Subtilisin Carlsberg (EC 3.4.21.14; alkaline protease from *Bacillus licheniformis*; specific activity of 12 units/mg of solid), phenylmethanesulfonyl fluoride (PMSF), succinyl-ala-ala-pro-phe-*p*-nitroanilide (suc-AAPF-*p*Na), and *N*-acetyl-*L*-phenylalanine ethyl ester (APEE) were obtained from Sigma Chemical Co. (St. Louis, MO). *N-trans*-Cinnamoylimidazole (NTC) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Karl Fisher titrant and solvent were purchased from GFS Chemicals, Inc. (Powell, OH). Various potassium and sodium salts and all organic solvents were purchased from Fisher Scientific (Pittsburgh, PA) and were of the highest grade commercially available. The solvents were stored over oven-dried (140 °C) molecular sieves (3 Å Linde) at least 24 h prior to use.

Enzyme Preparation. The enzyme was obtained as a three-times crystallized and lyophilized powder. It was activated prior to use in organic solvents by lyophilization from an aqueous phosphate buffer. For enzyme samples containing the various sodium and potassium salts, the phosphate buffer concentration (2.87 mM or 0.5 mg/mL) and enzyme concentration (0.5 mg/mL) remained the same for all samples, whereas the salt concentration was adjusted to give the same solution ionic strength, $I = 0.658$ mol/kg H_2O. For each salt, a 1 mL aliquot of a concentrated enzyme solution (10 mg/mL) in phosphate buffer solution (pH 7.8) was added to 19 mL of the salt solution (pH 7.8) in a 50 mL Falcon tube. The pH of all enzyme solutions was adjusted to

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7.80 using a few drops of 100 mM KOH or 100 mM H_3PO_4; in all cases the amount added did not significantly alter the buffer concentration, ionic strength, or solution volume.

In freezing the samples, the Falcon tube was immersed in a liquid nitrogen (LN_2) bath laterally inclined at an angle of 10° and spun at 600 rpm about its longitudinal axis for 1 min, and then without spinning for 1 min or until LN_2 bubbling has subsided. Lyophilization on a Labconco Freezone 6 freeze-drier (Fisher Scientific) was carried out at a condenser temperature of −49 °C and a pressure of 50 μm Hg. All samples were then placed on the freeze-drier for 44 h and the resultant powder immediately assayed for activity as well as active-site and water content. When not in use, the enzyme powders were stored at −20 °C under N_2 in the Falcon tubes and then placed over $CaSO_4$ in a desiccator. The closed Falcon tubes were warmed to room temperature prior to exposing the contents to air.

In experiments investigating the effect of KCl content on catalytic efficiency, 20 mL solutions of salt−enzyme solutions (pH 7.8) of varying concentrations were prepared and lyophilized in the same manner as above to achieve final salt contents of 40, 70, and 98% (w/w). For samples without added salt, dissolved enzyme solution (pH 7.8, 5 mg/mL) was frozen and lyophilized in the same manner as above. The phosphate buffer content in the resultant powder was 1% (w/w).

Kinetic Measurements. The catalytic efficiencies of subtilisin Carlsberg (SC) activated by various salts were determined in dry hexane containing less than (0.010 ± 0.003)% w/w H_2O, as determined by the limit of Karl Fischer titration. The reaction studied was the transesterification of APEE with 1-propanol. In a typical experiment with KCl-activated SC in hexane, 10 mg of lyophilized enzyme powder was added to 5 mL of hexane containing 1−40 mM APEE, 0.85 M 1-propanol, and 1.5 mM nonadecane (nonreacting internal standard for gas chromatography assays). In experiments with SC activated by other salts, the amount of catalyst added contained 0.1 mg of total enzyme and 0.1 mg of phosphate buffer salt, the same enzyme content as in experiments with KCl-activated samples. The reaction was carried out at 30 °C in 20 mL glass scintillation vials with Teflon-lined screw caps and shaken at 250 rpm in a constant temperature incubator. Initial rate measurements were performed over a 40−100 min period for lyophilized salt−enzyme samples, during which time the rates remained linear. For enzyme samples activated by NaI, NaBr, and NaI, the slower initial rates were monitored over an 80−300 min period, during which time the rates remained linear. Similar preparations were made for all the salts without any enzyme and assayed for background reaction rates. All salt preparations gave <0.1% conversion over a reaction period of 6 h, negligible when compared to the reaction rates exhibited by the salt-activated enzyme preparations.

Gas Chromatography Analysis. The reaction mixtures were analyzed by sampling 500 μL aliquots of the homogeneous suspension and spinning the suspension in Eppendorf tubes at 14 000 rpm in a microcentrifuge for 25 s. The formation of the transesterification product *N*-acetyl-*L*-phenylalanine propyl ester (APPE) in the supernatant was measured using a gas chromatograph (Model 3800, Varian Instruments, Sugar Land, TX) equipped with a VA-5MS, 250 μm capillary column (15 m length and 0.25 μm inside diameter), a constant He carrier gas pressure of 15 psi (1.3 mL/min), 250 °C injection and detection temperatures, and an isothermal column temperature of 215 °C. All GC measurements were performed in triplicate, and initial rates were determined from straight-line fits of the average values. The kinetic parameters $(V_{max})_{app}$ and $(K_m)_{app}$ were obtained by fitting the initial rate data to the Michaelis−Menten equation (Kaleidograph). The intrinsic catalytic efficiencies $(k_{cat}/K_m)_{app}$ were obtained by normalizing $(V_{max}/K_m)_{app}$ by the concentration of active enzyme determined in active-site titration measurements.[25] Reproducibility of kinetic parameters was established from kinetic data obtained from four independent preparations of enzyme powders.

Measurement of Water Content. The water content of the lyophilized enzyme powders was determined by the Karl Fischer method. Prior to introducing the powder, the sealed Karl Fischer titration apparatus was equilibrated with Karl Fischer titrant to react with any water that was present. Upon equilibration, a known amount of catalyst powder was immediately transferred into the Karl Fischer titration vessel equipped with a magnetic stir bar. The vessel was then quickly resealed with a glass stopper and the titration commenced. The rapid transfer provided minimal exposure to environmental water. Values of water content for each lyophilized sample represent averages of four independent measurements.

Active Site Titration. To ensure the purity of subtilisin Carlsberg purchased from the vendor, a standard BCA assay was performed on a 1 mg/mL, aqueous enzyme solution, yielding >95% total protein. The percentage of active sites was determined for the various salt-activated SC preparations in organic solvents according to the previously published procedure of Wangikar et al. with minor modifications.[27] The experimental approach monitors the initial rate of enzyme inhibition using the serine protease inhibitor PMSF in the organic solvent and the initial rate of hydrolysis of the peptide substrate suc-AAPF-*p*Na. As described by Wangikar and co-workers, a set of six initial rate equations can be reduced to give the ratio of the active-site concentration in organic solvent to that in aqueous buffer. Lyophilized enzyme powder (10 mg/mL) was incubated in dry hexane at 30 °C containing 25 mM PMSF. Enzyme activity in aqueous buffer was measured for the hydrolysis of suc-AAPF-*p*Na by following the release of *p*-nitroaniline spectrophotometrically at 410 nm. Hydrolysis was performed in 100 mM Tris−HCl buffer, pH 7.8, using a substrate concentration of 400 μM and catalyst concentrations of 20 μg/mL (80 μg/mL for PMSF-inhibited samples). Product formation in the transesterification of APEE (20 mM) with 1-propanol (0.85 M) was measured via gas chromatography as described above. All experiments were performed in triplicate.

The percentage of active sites in organic solvents can then be calculated by measuring the active-site concentration in aqueous buffer by the procedure of Schonbaum, which observes spectrophotometrically the depletion of *N-trans*-cinnamoylimidazole (NTC) from its unimolecular reaction with the enzyme to form a relatively stable acyl-enzyme compound.[28] The amount of NTC consumed is equivalent to the amount of competent active sites available in an enzyme sample. The active-site concentration in aqueous buffer for samples prepared by the method of Wangikar et al. was determined as follows: 50 μL of a 1.5 mM stock solution of NTC in CH_3CN was added to 950 μL of a 100 mM acetate buffer (pH 5.0) containing 3.15% v/v CH_3CN (final CH_3CN content is 8.0% v/v), and the absorbance at 335 nm was monitored over 1 min to determine the baseline rate of NTC depletion. The extinction coefficient of NTC in acetate buffer containing 8.0% v/v CH_3CN was measured to be 2.28 cm^{-1} mM^{-1}. Extrapolation to the time of NTC addition ($t = 0$) yielded the initial absorbance of NTC in acetate buffer. NTC stock solution (50 μL) was then added to 950 μL of the enzyme solution (final catalyst concentration is 50 mg/mL) and the NTC consumption monitored over 2 min or until the baseline rate was achieved. Extrapolation of the baseline to $t = 0$ yielded the final NTC absorbance, and the difference with the initial absorbance yielded the amount of competent active sites available in aqueous buffer.

Results and Discussion

Effect of Salt on Water Content. Table 1 lists the Jones−Dole *B* coefficients for the anions of the various sodium and potassium salts selected in this study.[26] Those ions with positive values of the *B* coefficient bind relatively strongly to water, whereas those ions with negative values of the *B* coefficient bind relatively weakly to water. The ions with *B* coefficients near zero have a similar binding energy as that between adjacent

(25) The apparent rate constants are defined as per the convention for the protease-catalyzed reactions involving competing nucleophiles. All subsequent references to the kinetic parameters represent their apparent values $(k_{cat})_{app}$, $(K_m)_{app}$, and $(k_{cat}/K_m)_{app}$ as defined in the following reference: Wangikar, P. P.; Graycar, T. P.; Estell, D. A.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1993**, *115*, 12231−12237.

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(28) Schonbaum, G. R.; Zerner, B.; Bender, M. L. *J. Biol. Chem.* **1961**, *236*, 2930−2935. The percentage of active sites in aqueous solution for each of the salt-activated enzyme preparations ranged from a low of (11 ± 3)% for NaI to a high of (59 ± 7)% for $NaCH_3COO$.

Table 1. Jones−Dole *B* Coefficients of Cations and Anions

cation	*B* coefficient	anion	*B* coefficient
		$CH_3CO_2^-$	0.250
Na^+	0.086	SO_4^{2-} [a]	0.208
		F^-	0.100
↑ Kosmotropes			
↓ Chaotropes			
		Cl^-	−0.007
K^+	−0.007	Br^-	−0.032
		I^-	−0.068

[a] SO_4^{2-} is the predominant species at pH 7.8 prior to freeze-drying despite the diprotic nature of its conjugate acid. This can be verified by the Henderson−Hasselbalch equation and the pK_a values of sulfuric acid.

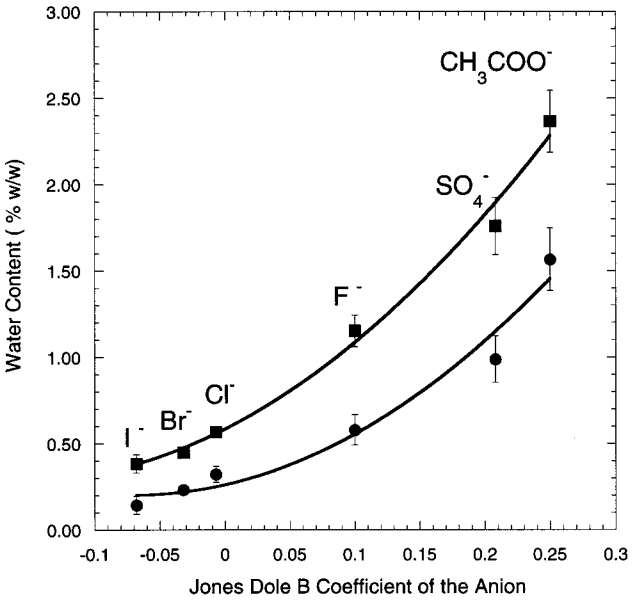

Figure 1. Water content (wt % H_2O/wt catalyst) of subtilisin Carlsberg activated by various sodium (■) and potassium (●) salts as measured by the Karl Fischer method. Values represent the average of four trials from lyophilized enzyme samples prepared independently, and the error bars represent one standard deviation from the mean. The samples were prepared from salt solutions of equal ionic strength (I = 0.658 mol/kg H_2O) and the final weight percent of salt ranged from 96.5 to 99.1% of the total catalyst. The solid lines represent least-squares fits to the data using a second-order polynomial (Kaleidagraph).

water molecules. To determine whether more kosmotropic salts retain more water after freeze-drying, catalyst powders of SC containing the selected salts were prepared from salt−enzyme solutions of equal ionic strength. The solutions were subsequently flash frozen using LN_2 and lyophilized together for 44 h. Figure 1 shows the clear correlation between the *B* coefficient of the anion and the catalyst water content as measured by Karl Fisher titration. The kosmotropic salts such as acetate and sulfate harbor more water than the chaotropic salts such as iodide and bromide. In addition, with sodium being a more kosmotropic cation than potassium, all the sodium salts retained more water after freeze-drying than their potassium counterparts.

Effect of Salt on Active Site Content. To determine the catalytic efficiencies of the catalysts activated by the various salts, the percentage of active sites in the enzyme powders was determined in the organic solvent (see Experimental Section). The procedure was adapted from that described by Wangikar and co-workers using the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) to provide the ratio of active centers in organic solvent to that in aqueous buffer.[27] The percentage of active sites in aqueous buffer was then independently

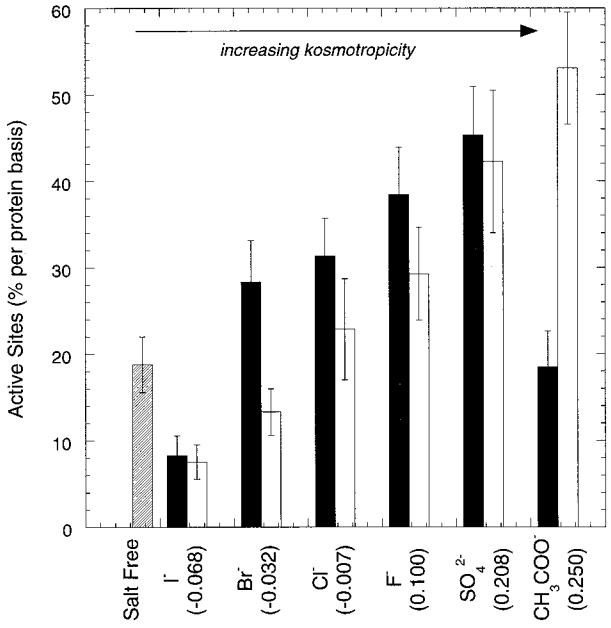

Figure 2. Percentage of catalytically competent active sites for subtilisin Carlsberg lyophilized from a buffered solution (pH 7.8, 0.5 mg/mL of K_2HPO_4 initial buffer concentration) containing various potassium (black bars) and sodium (white bars) salts of increasing kosmotropicity as represented by the Jones−Dole B coefficient of the anion (in parentheses). The percentage of active sites for the enzyme lyophilized without added salt is indicated by the diagonally striped bar for comparison. The values represent the average of four trials from lyophilized enzyme samples prepared independently, and the error bars represent one standard deviation from the mean.

determined by the method of Schonbaum using the inhibitor *N-trans*-cinnamoylimidazole (NTC).[28] Figure 2 illustrates that the percentage of competent catalytic centers in hexane tended to increase with increasing kosmotropicity of the anion. For salt-free preparations, the percentage of enzyme active sites in hexane was (19 ± 3)%, while enzyme preparations activated by kosmotropes such as $NaCH_3COO$ retained as much as (53 ± 6)% of competent active sites. Enzymes prepared with chaotropes such as NaI, on the other hand, showed active-site contents as low as (8 ± 2)%. In general, kosmotropic salts were more effective at preserving enzyme active sites, although the higher active-site content measured for salts of the more chaotropic potassium cation would suggest that the effect of the anion is more important. Thus, more kosmotropic salts may act as a lyoprotectant and may be more effective at diminishing possible denaturing effects of the organic solvent on the enzyme. If the percentage of active sites is viewed as a measure of enzyme activity and stability, then the trend is also consistent with the Hofmeister series. Kosmotropic salts are generally the salting-out salts in the series and therefore may stabilize the enzyme's native conformation against adverse solution perturbations. The chaotropic salts (salting-in salts) are not as effective at stabilizing the enzyme in solution and therefore produce samples with significantly reduced active-site contents.[29]

Effect of Salt on Catalytic Efficiency. To compare accurately the catalytic efficiencies of subtilisin Carlsberg activated by various salts, the solution volume and the enzyme and buffer concentrations were kept constant prior to lyophilization. However, to take into account the different molecular weights of the salts, the ionic strength of the various salt solutions was adjusted to I = 0.658 mol/kg H_2O. The enzyme and buffer concentrations were chosen to give a final dried preparation containing 98% w/w salt for samples activated by KCl. The

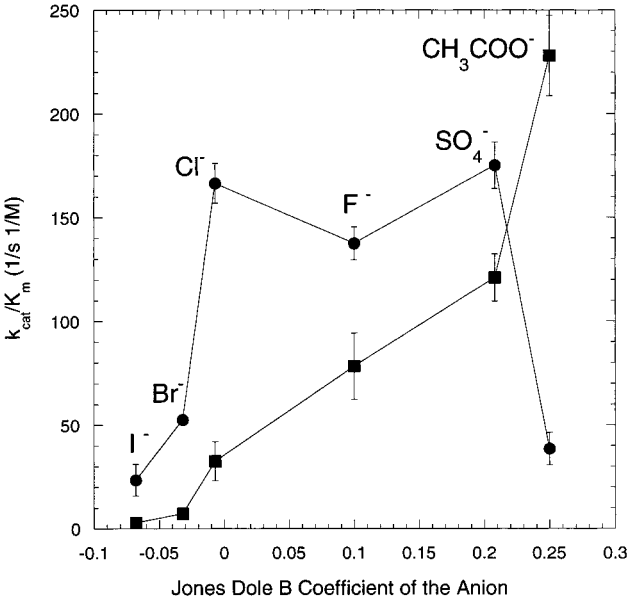

Figure 3. The catalytic efficiency, k_{cat}/K_m (s^{-1} M^{-1}), of subtilisin Carlsberg activated by various sodium (■) and potassium (●) salts, prepared as described for Figure 1. Values represent the average of four trials from lyophilized enzyme samples prepared independently, and the error bars represent one standard deviation from the mean.

final weight percentage for the various sodium and potassium salts varied from 96.5 to 99.1% w/w. All salt−enzyme solutions were adjusted to a pH of 7.8 and subsequently flash-frozen by LN_2 and lyophilized.

Figure 3 summarizes the catalytic efficiency, k_{cat}/K_m, of subtilisin Carlsberg activated by various salts. For sodium salts, increasing kosmotropicity clearly yielded salt-activated catalysts with increasing catalytic efficiency. For potassium salts, a similar pattern was observed, except that KCl and KCH_3COO fell above and below the expected trend, respectively. More kosmotropic salts may therefore act as cryo- and lyoprotectants during freeze-drying, resulting in a more robust catalyst resistant to any denaturing effects of the organic solvent. The low activity of the KCH_3COO-activated sample can be attributed to eutectic melting during freeze-drying, whereas the unusually high activity of the KCl-activated enzyme may be ascribed in part to favorable physical properties of the sample. In this connection, Chang and Randall conducted a detailed investigation using subambient thermal analysis to examine how additives such as salts affect proteins during lyophilization.[30] It was found that one of the major stress factors that contributes to the denaturation of proteins during lyophilization is the loss of cake structure. It was concluded that the addition of salts with eutectic melting temperatures (T_e) above −20 °C would promote rapid crystallization of the salt upon freezing and prevent the collapse of the frozen fraction during the freeze-drying process. In addition, the collapse temperature of protein solutions generally corresponds to the glass transition temperature (T_g) of the amorphous phase containing the protein. A high collapse temperature is desired to facilitate maintenance of the sample temperature below T_g during the lyophilization process.[31] For these reasons, KCl, which has no glass transition temperatures and a relatively high T_e of −11 °C, should be an excellent additive for the successful lyophilization of proteins.

We have shown previously that optimizing the lyophilization time and water content leads to an optimal catalytic efficiency for KCl-activated subtilisin Carlsberg in hexane that is nearly 20% of that for the enzyme in water.[17] Optimizing the lyophilization conditions for samples activated by K_2SO_4 and $NaCH_3COO$, which are shown in Figure 3 to have equal or higher k_{cat}/K_m values, respectively, than that of KCl, may therefore yield higher catalytic efficiencies in organic solvents closer to that observed in water.

The individual catalytic parameters k_{cat} and K_m are shown in Figures 4a and 4b, respectively. Increasing the kosmotropicity of the activating salt tends to increase k_{cat}, with KCH_3COO and KCl being clear exceptions due to reasons proposed above. By comparison, K_m decreases with increasing kosmotropicity, except for the acetate salts. Whereas the high K_m for KCH_3COO-activated enzyme is most likely due to the melting during freeze-drying, the cake integrity of the $NaCH_3COO$-activated sample appeared to be intact upon visual inspection at the end of the freeze-drying process. According to the results of Chang and Randall, however, the T_g for $NaCH_3COO$ is −64 °C, clearly too low for the commercially available lyophilizer used in our studies to maintain during freeze-drying. As a result, the cake integrity of the lyophilizing sample may have been partially lost on the microscopic scale, leading to the abnormally high K_m. Despite this, however, the high kosmotropicity of $NaCH_3COO$ in solution may have helped stabilize the enzyme, yielding an enzyme powder with high activity in organic solvents (mostly as a result of the high k_{cat}).

Effect of Salt Content and Water Content on Catalytic Efficiency. The final salt content of each activated catalyst was relatively high, ranging from 96.5 to 99.1% weight of salt per total weight of catalyst. As was first reported by Khmelnitsky and co-workers, increasing the final KCl content to above 40% w/w produced dramatic increases in the catalytic efficiency of subtilisin Carlsberg.[15] Specifically, the catalytic efficiency of a 98% w/w preparation of KCl-activated subtilisin Carlsberg was 3750-fold higher than that of salt-free enzyme. However, in that particular study the percentage of active sites was not directly measured and was assumed to be 10% for all enzyme preparations. Figure 5 illustrates the effect of final salt content on the catalytic efficiency of KCl-activated subtilisin Carlsberg. As was observed previously, the preparation containing 98% w/w KCl exhibited a significant increase in the catalytic efficiency over salt-free enzyme (1810-fold in this case). The apparent decrease in activation relative to the results of Khmelnitsky et al.[15] was primarily the result of a 2.6-fold increase in the percentage of active sites from 18.8% in the salt-free case to 48.9% for the 98% KCl-activated samples. Given that increased weight ratios of stabilizers such as sugars and carbohydrates are known to enhance protein stability during lyophilization,[30] the higher percentage of active sites in samples containing more KCl sug-

(29) It is also apparent from Figure 2 that the KCH_3COO-activated sample exhibited an unexpectedly low active-site content, inconsistent with the trend observed with samples activated by the other potassium salts. This exception is most likely explained by the melting of KCH_3COO-activated samples during the course of lyophilization (KCH_3COO has a T_g of −76 °C). Although the freeze-drying yielded a dry, brittle solid, the sublimation-induced cooling of the sample during freeze-drying was not able to maintain the sample temperature low enough to prevent eutectic melting. This phenomenon was also observed for enzyme samples activated by NaI and NaBr, but the more kosmotropic nature of KCH_3COO may have helped to stabilize the enzyme in the melted solution during drying, producing a catalyst with a higher active-site content. Nevertheless, the inconsistency suggests that although the Jones−Dole *B* coefficient of the salt and the water content of the freeze-dried powders are important parameters in determining the catalytic efficiency of salt-activated catalysts, there may be other significant factors such as the glass transition temperature and the mechanical properties of the additives during lyophilization that affect enzyme performance in organic solvents.

(30) Chang, B. S.; Randall, C. S. *Cryobiology* **1992**, *29*, 632−656.

(31) Carpenter, J. F.; Pikal, M. J.; Chang, B. S.; Randolph, T. W. *Pharm. Res.* **1997**, *14*, 969−975.

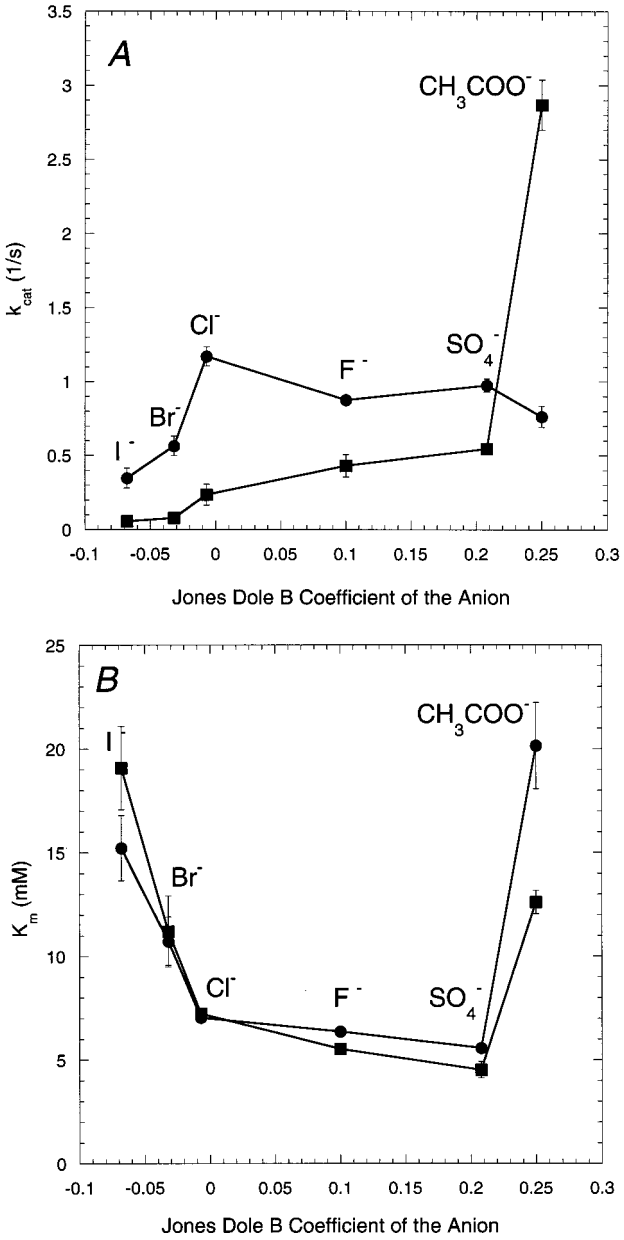

Figure 4. Kinetic parameters k_{cat} (s^{-1}, A) and K_m (mM, B) of subtilisin Carlsberg activated by various sodium (■) and potassium (●) salts as a function of the Jones−Dole *B* coefficient of the anion.

gests that the salt may act in part as a lyoprotectant. Nevertheless, the 2.6-fold increase in active sites cannot account for the 1810-fold salt-induced activation, indicating an inherent salt-induced activation of the enzyme. Figure 5 also illustrates the effect of salt content on the individual catalytic parameters, k_{cat} and K_m. Increased salt content decreased K_m and dramatically increased k_{cat}, both effects contributing positively to a high catalytic efficiency.

The water content, whether it is adsorbed to the enzyme or dissolved in the organic solvent, has been shown repeatedly to be an important factor affecting enzyme catalytic efficiency in organic solvents. In general, low levels of water are necessary to induce enzyme activity in anhydrous organic solvents.[12,13,32] It is possible that the higher catalytic efficiency observed for more kosmotropic salts is due, at least in part, to the greater affinity of the salt for water. The stronger binding may,

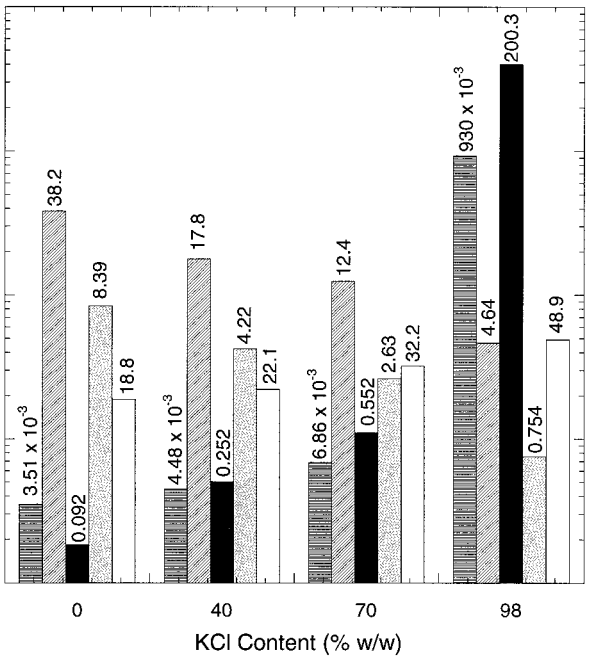

Figure 5. The effect of final salt content on the catalytic parameters k_{cat} (s^{-1}, horizontally striped bars), K_m (mM, diagonally striped bars), and k_{cat}/K_m (s^{-1} M^{-1}, black bars), and on the percentage of active sites (white bars) and water content (% w/w, gray bars) for subtilisin Carlsberg activated by KCl. For all preparations the final buffer content was 1% w/w.

therefore, result in more water adsorbed within the dried salt matrix, and in effect, increase the water content in the local environment of the enzyme. On the other hand, if water content were the overriding factor responsible for the increased catalytic efficiency with increased kosmotropicity, the more kosmotropic sodium salts should exhibit higher catalytic efficiencies than their potassium counterparts. However, as shown by Figure 3, potassium salts in general activated the enzyme to a greater degree than sodium salts.

We have shown previously that increased lyophilization time led to further dehydration of KCl-activated enzyme powders.[17] This dehydration accompanied an *increase* in the catalytic efficiency up to an optimum, after which the catalytic efficiency plummeted as the water content plateaued. Likewise, in Figure 5, higher weight fractions of KCl salt coincide with lower water contents, and the dehydration may in fact help to stabilize the enzyme and/or *enhance* catalytic efficiency. It is possible that to achieve optimal catalyst performance, a compromise must be made between providing the enzyme with sufficient water to achieve high activity and at the same time not so much water as to undermine the stability of the final enzyme preparation in the organic solvent. Moreover, low water content (<1% w/w) is generally desirable in the final lyophilized product to ensure the mechanical strength of the cake structure and avoid product collapse.[30]

Mechanism of Protein Stabilization by Kosmotropic Salts. Salt−protein interactions in aqueous solution have been thoroughly investigated by Melander and Horvath, who used the chaotropicity and kosmotropicity of salts to explain the salting-in and salting-out effects observed by Hofmeister.[33] At low concentrations of salt (0.1−0.3 M), the added salt ions may form ion pairs with charged residues on the protein surface, replacing protein−protein ion-pair interactions to disrupt pro-

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(33) Melander, W.; Horvath, C. *Arch. Biochem. Biophys.* **1977**, *183*, 200−215.

tein aggregates and increase solubility (salting-in). The salt can also decrease the solubility of the protein in the process (salting-out), by neutralizing the net surface charge on the protein. At higher concentrations, however, the interaction of salts is largely with neutral parts of the protein. As discussed in detail by Collins, kosmotropic salts also increase the surface tension of water, while chaotropic salts decrease the surface tension of water.[34] In general, kosmotropes at high concentrations stabilize proteins while chaotropes at high concentrations destabilize proteins. These observations were, nevertheless, macroscopic and empirical, and it was through the investigations of Timasheff, Arakawa, and co-workers that a more detailed, microscopic picture of protein stabilization by salts was developed.[35] They observed experimentally a lower concentration of protein stabilizing solutes in the immediate vicinity of the protein relative to the bulk solution, and coined the phrase "preferential hydration" to describe this phenomenon.[36] In effect, stabilizing solutes are preferentially excluded from interacting with the surface of the protein due to the increased surface tension of water associated with the solute. It is energetically unfavorable to disrupt solute−water interactions to form protein−water interactions, and therefore the high surface-tension solute-associated water and its solute are excluded from contact with the protein. Protein unfolding, which increases the protein surface area and increases the unfavorable free energy of interaction, is thus disfavored and the protein is stabilized. Furthermore, for denaturing solutes such as guanidine HCl and urea, the lack of solute near the protein also reduces the likelihood of adverse solute−protein binding. Because kosmotropic salts have strong interactions with water and increase its surface tension, the addition of kosmotropes stabilizes the native conformation of the enzyme against denaturation.

Timasheff and others have provided ample evidence that several additives, including salts, preferentially hydrate and stabilize proteins in solution against a variety of solution perturbations such as thermally induced unfolding and pH-induced dissociation.[37] Thus, it is plausible that including a high concentration of kosmotropic salt in the protein solution prior to freeze-drying may protect the protein against the subzero temperatures, the osmotic shock during ice formation, alterations in solution pH, and other destabilizing conditions that may occur during lyophilization. Indeed, Carpenter and Crowe used a similar argument to explain the effects of various cryoprotectants on the activity of lactate dehydrogenase after freeze-thawing and found that high concentrations (>1 M) of sodium acetate, potassium phosphate, and various sulfate salts (all kosmotropes) provided significant cryoprotection of the enzyme.[38] On the other hand, the more chaotropic NaCl yielded a much lower level of activity after freeze-thawing.

These observations suggest that kosmotropic salts exert a stabilizing influence during freeze-drying through the mechanism of preferential hydration, increasing the active-site content and/or catalytic efficiency of the enzyme in organic solvents. However, previous investigations have found that the degree to which known lyoprotectants such as poly(ethylene glycol) (PEG) and sugars enhance the activity of enzymes in organic solvents falls far short of that observed for salt-induced activation.[39] Thus it appears clear that protein stabilization is not the only mechanism by which salts effect dramatic increases in the activity of enzymes in organic solvents. Nevertheless, this theory of kosmotropic stabilization may partially explain the activity enhancement of lyophilized salt-activated enzyme powders suspended in organic solvents. This in turn may lead to further improvements in enzyme activity in organic solvents.

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