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RATIONAL ENHANCEMENT OF ENZYME PERFORMANCE IN ORGANIC SOLVENTS

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by

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DISCLAIMER

The overall goal of this project was to contribute to a scientific foundation needed to rationally improve the catalytic performance of enzymes in organic solvents. Below we summarize our main findings during each of the four years of the project. They are described in detail in our scientific publications in peer-reviewed journals listed at the end of this report.

During the *first* year, we investigated the dependence of enzymatic activity of several model hydrolases (lipases and proteases) in nonaqueous solvents on (i) physico-chemical properties of the solvent, and (ii) the presence of excipients in enzyme aqueous solutions prior to lyophilization and subsequent placement of the enzyme into the organic solvent.

With respect to (i), the question of whether the solvent's water-immiscibility is relevant to enzymatic activity was addressed by assaying four different hydrolases (three lipases and one protease) in nine anhydrous solvents of similar hydrophobicities of which four were infinitely miscible with water and five were not. For no enzyme was a jump in activity observed upon a transition from water-miscible to water-immiscible solvents. The relevance of solvent apolarity to enzymatic efficiency was also examined. To this end, three groups of isomeric anhydrous solvents were selected where within each group one solvent was apolar (i.e., lacked a permanent dipole moment). For none of the four enzymes studied was activity significantly higher in apolar solvents than in their polar counterparts. Thus we conclude that often-cited solvent's immiscibility with water and apolarity by themselves are irrelevant to enzymatic activity and instead solvent's hydrophobicity is the key pertinent characteristic.

With respect to (ii), we discovered that when seven different hydrolytic enzymes (four proteases and three lipases) were lyophilized from aqueous solution containing a ligand, N-Ac-L-Phe-NH_2, their catalytic activity in anhydrous solvents was far greater (one to two orders of magnitude) than that of the enzymes lyophilized without the ligand. This ligand-induced

activation was expressed regardless of whether the substrate employed in organic solvents structurally resembled the ligand. Furthermore, non-ligand lyoprotectants [sorbitol, other sugars, and poly(ethylene glycol)] also dramatically enhanced enzymatic activity in anhydrous solvents when present in enzyme aqueous solution prior to lyophilization. The effects of the ligand and of the lyoprotectants were non-additive, suggesting the same mechanism of action. Excipient-activated and non-activated enzymes exhibited identical activities in water. Also, addition of the excipients directly to suspensions of non-activated enzymes in organic solvents had no appreciable effect on catalytic activity. These observations indicate that the mechanism of the excipient-induced activation is based on the ability of the excipients to alleviate reversible denaturation of enzymes upon lyophilization. Activity enhancement induced by the excipients is displayed even after their removal by washing enzymes with anhydrous solvents. Subtilisin Carlsberg, lyophilized with sorbitol, was found to be a much more efficient practical catalyst than its "regular" counterpart.

In addition, we addressed the important question of the possibility of rationally manipulating enzyme chemoselectivity by the solvent. To this end, kinetics of monoacylation of different aminoalcohols with trifluoroethyl butyrate catalyzed by a dozen of proteases and lipases were investigated in a variety of anhydrous solvents. Chemoselectivity of these transformation (defined as the ratio of the rate of *O*-butyrylation to the rate of *N*-butyrylation, v_o/v_N) was found to be markedly dependent on the solvent under otherwise identical conditions. For example, with *N*-α-benzoyl-L-lysinol as the aminoalcohol, although *Pseudomonas* sp. lipoprotein lipase preferred the OH group and *Mucor meheii* lipase the NH_2 group in all solvents tested, the extent of this preference varied up to 20 fold depending on the solvent. Moreover, for porcine pancreatic lipase, a solvent-induced inversion of chemoselectivity was observed. A mechanistic

rationale was proposed which related the v_o/v_N values to physico-chemical properties of the solvent.

During the *second* year of this project, we focused on (iii) control of substrate selectivity of the protease subtilisin Carlsberg by the solvent, (iv) control of catalytic activity and enantioselectivity of this enzyme in organic solvents by immobilization support, and (v) lipase-catalyzed acylation of sugars in anhydrous hydrophobic media.

(iii) In our previous work, the substrate specificity of the protease subtilisin Carlsberg in the transesterification of *N*-Ac-L-Ser-OEt and *N*-Ac-L-Phe-OEt with propanol was found to be markedly dependent on the solvent and, in fact, directly proportional to the ratio of solvent-to-water partition coefficients of the substrates; a thermodynamic model was developed which explained this phenomenon. Now, the utility of this model has been broadened to include water-miscible solvents (for which the partition coefficients cannot be readily determined experimentally). Additionally, the need to experimentally measure the partition coefficients has been eliminated altogether through the use of a computer program which uses the UNIFAC algorithm to calculate the partition coefficient ratios. The calculated partition coefficient ratios satisfactorily predict subtilisin's substrate specificity in water-miscible, as well as in water-immiscible, solvents.

(iv) Subtilisin Carlsberg has been covalently attached to five macroporous polyacrylic supports of varying aquaphilicity (a measure of hydrophilicity). Kinetic parameters of the transesterification of *S* and *R* enantiomers of *sec*-phenethyl alcohol with vinyl butyrate, catalyzed by various preparations of immobilized subtilisin, have been determined in anhydrous dioxane and acetonitrile (these solvents have been previously found to afford extreme - the highest and

the lowest, respectively, - enantioselectivities of subtilisin). Enzyme enantioselectivity in acetonitrile (but not in dioxane) correlates with the aquaphilicity of the support: the greater the latter, the higher the enantioselectivity; a mechanistic rationale for the phenomenon has been proposed. While the catalytic activity of immobilized subtilisin in anhydrous solvents is a strong function of the mode of enzyme pretreatment (for example, depending on whether the immobilized enzyme was dried under air *vs.* lyophilized, or dried with *vs.* without subsequent washing, or lyophilized with *vs.* without excipients, its catalytic activity in the same organic solvent may vary up to 50 fold), the enantioselectivity is essentially conversed.

(v) By complexing glucose and other mono- and disaccharides with phenylboronic acid, they have been solubilized in many organic solvents, including hydrophobic ones. *Pseudomonas* sp. lipoprotein lipase readily acylates such solubilized sugars in these solvents (significantly, no reaction is observed without phenylboronic acid, i.e., when sugars are insoluble in the reaction medium). Activated esters of various carboxylic acids, a number of triglycerides (different plant oils), and even free fatty acids all can act as acylating agents in the enzymatic transesterification. Solubilized α-D-glucose has been enzymatically acylated with vinyl acrylate on a preparative scale, and the resultant 6-*O*-acryloylglucose has been chemically polymerized to yield a sugar-based polyacrylate material with a molecular weight of 14,000 Da.

During the *third* year of the project, we focused on (vi) the possibility of accelerating enzymatic processes in organic solvents by certain cosolvents; (vii) whether lipase catalysis in organic solvents can be enhanced by introducing interfaces in the reaction medium; and (viii) the structure of proteins suspended in organic solvents.

(vi) The rates of transesterification reactions catalyzed by the protease subtilisin Carlsberg suspended in various anhydrous solvents at 30°C can be increased more than 100 fold by the addition of denaturing organic cosolvents (dimethyl sulfoxide or formamide); in water, the same cosolvents exert no enzyme activation. At 4°C, the activation effect on the lyophilized protease is even higher, reaching 1,000 fold. Marked enhancement of enzymatic activity in anhydrous solvents by formamide is also observed for two other enzymes, α-chymotrypsin and *Rhizomucor miehei* lipase. In addition to lyophilized subtilisin, cross-linked crystals of the enzyme are also amenable to the dramatic activation by the denaturing cosolvents. In contrast, subtilisin solubilized in anhydrous media by covalent modification with poly(ethylene glycol) (PED) exhibits little activation. These observations are rationalized by postulating an enhanced protein flexibility in anhydrous milieu brought about by the denaturing organic cosolvents. The latter exert their lubricating effect largely at the interfaces between enzyme molecules in a solid preparation, thus easing flexibility constraints imposed by protein-protein contacts.

(vii) The question of whether lipases can be activated by adsorption onto an interface in organic solvents has been addressed by using *R. miehei* lipase as a model. In aqueous solution, this enzyme undergoes a marked interfacial activation. However, lipase (either lyophilized or precipitated from water with acetone) suspended in ethanol or 2-(2-ethoxyethoxy)ethanol containing triolein exhibits no jump in catalytic activity when the concentration of triolein exceeds its solubility in these solvents, thereby resulting in formation of an interface. To test whether the lack of interfacial activation is due to the insolubility of the enzyme in organic media, lipase has been covalently modified with PEG. The modified lipase, although soluble in nonaqueous media, is still unable to undergo interfacial activation, regardless of the nature of the interface. This inability is due to the absence of adsorption of lipase onto interfaces in organic

solvents, presumably because of the absence of the hydrophobic effect (the driving force of lipase adsorption onto hydrophobic interfaces in water) in such media. The uncovered lack of interfacial adsorption and activation suggests that the short α-helical "lid" covering the active center of the lipase remains predominantly closed in nonaqueous media, thus contributing to diminished enzymatic activity.

(viii) The structure of a model protein, bovine pancreatic trypsin inhibitor (BPTI), in organic solvents has been examined using hydrogen isotope exchange/high-resolution NMR methodology. When lyophilized deuterated BPTI is suspended in acetonitrile, tetrahydrofuran, ethyl acetate, or butanol, each containing 1% ^1H_2O, several protein amide protons that are buried and strongly hydrogen-bonded in aqueous solution are found to exchange with the solvent significantly within 24 h. In contrast, in water most of these protons do not exchange appreciably even after a week under otherwise similar conditions. The isotope exchange rates of the corresponding amide protons of BPTI are similar in these nonaqueous solvents. When solid BPTI is prepared by different methods, such as rotary evaporation, acetone precipitation, or lyophilization from a dimethyl sulfoxide solution, and subsequently suspended in acetonitrile containing 1% water, the exchange intensities of the amide protons vary greatly among the preparations. These data combined suggest that the structure of BPTI in the four aforementioned organic solvents is partially unfolded, but not more so than in lyophilized powder, i.e., that these solvents cause little additional protein denaturation beyond that brought about by lyophilization. Using the same methodology, the BPTI structure also has been studied in several protein-dissolving solvents containing 1% water. In dimethyl sulfoxide, dimethylformamide, or methanol, the same amide protons exchange almost completely within 24 h, while in glycerol (known to stabilize proteins and to function as a water mimic) they do not. These results

demonstrate that some protein-dissolving organic solvents strongly denature BPTI and that intermolecular contacts in the suspended protein are important in maintaining the protein conformation in organic solvents. Our findings, if general, explain the considerable but much reduced (compared to water) enzymatic activity in nonaqueous media.

During the *fourth* year of this project, we focused on (ix) affecting and improving enzymatic enantioselectivity in organic solvents by optimizing the mode of enzyme preparation; (x) mechanistically dissecting and analyzing the frequently observed plunge in enzymatic activity upon replacing water with organic solvents; (xi) the structure of an enzyme suspended in organic solvents; and (xii) the structural basis for the previously discovered phenomenon of molecular memory of imprinted proteins in organic solvents. Below, the technical highlights of these studies are outlined and summarized.

(ix) The enantioselectivities of the protease subtilisin Carlsberg and *Rhizomucor miehei* lipase in organic solvents have been found to strongly depend on the method by which the enzymes are prepared. For the transesterification between *sec*-phenethyl alcohol and vinyl butyrate in dioxane at 7°C, the enantioselectivity of subtilisin precipitated with isopropanol is more than twice that of the enzyme prepared by lyophilization from aqueous buffer. Furthermore, the temperature dependence of the enantioselectivity is influenced by the mode of enzyme preparation. For example, in the aforementioned process the enantioselectivities of subtilisin lyophilized from aqueous buffer and of cross-linked subtilisin crystals increase when the temperature is raised from 7 to 45°C. In contrast, the enantioselectivities decrease with temperature for the enzyme precipitated from aqueous solution with acetone or isopropanol and for the enzymatic hydrolysis in water. The temperature dependence of the enantioselectivity of

subtilisin lyophilized from buffer is markedly affected by the solvent: in acetonitrile and nitromethane the enzyme is more enantioselective at higher temperatures, while negligible temperature effects have been found in tetrahydrofuran and pyridine. Lyophilized lipases exhibits striking temperature dependencies of its enantioselectivity in dioxane, acetonitrile and nitromethane, while showing almost none in pyridine, triethylamine, and tetrahydrofuran. The results underscore the importance of the mode of enzyme recovery on enantioselectivity and its temperature dependence in enzymatic reactions in organic solvents (in contrast to those in water).

(x) Subtilisin Carlsberg dissolved in aqueous solution is several orders of magnitude more active than the enzyme suspended in anhydrous acetonitrile. In order to ascertain why, we have employed crystalline subtilisin lightly cross-linked with glutaraldehyde as a catalyst in both aqueous and organic media. The structure of this crystalline enzyme in acetonitrile had been previously found (by X-ray crystallography) to be virtually identical to that in water, thus ruling out solvent-induced conformation changes as the cause of the enzymatic activity drop. Titration of the competent active centers of subtilisin has revealed that the k_{cat}/K_m is solely responsible for this activity plunge. Quantitative mechanistic analysis of the 7-order-of-magnitude difference in k_{cat}/K_m values between subtilisin dissolved in water and cross-linked subtilisin crystals suspended in anhydrous acetonitrile has allowed accounting for at least 5.6 orders of magnitude. This drastic decline is due to (a) a marked shift in the activity *vs.* pH profile of the cross-linked crystalline enzyme compared to its soluble counterpart; (b) different (far less favorable in acetonitrile than in water) energetics of substrate desolvation; and (c) very low thermodynamic activity of water in anhydrous acetonitrile resulting in a much more rigid and thus less active enzyme.

(xi) We have developed an FTIR (Fourier-transform infrared) methodology for quantitatively assessing the secondary structure of proteins suspended in nonaqueous media. This methodology has been used to measure the percentages of α-helices and β-sheets of subtilisin Carlsberg, prepared under different conditions, placed in various organic solvents. The question of whether conformational changes can be responsible for solvent and excipient effects on the enzymatic activity has been addressed with respect to some instances of markedly influencing the subtilisin activation by KCl and N-Ac-L-Phe-NH_2 present in the aqueous solution of the enzyme prior to lyophilization may be due to their preservation of the secondary structure, otherwise altered by the dehydration. Likewise, subtilisin inactivation in the protein-dissolving solvent dimethyl sulfoxide is likely caused by enzyme denaturation (the loss of both α-helices and β-sheets). On the other hand, some other ligands, as well as protein non-dissolving organic solvents, while greatly affecting the subtilisin activity, have little effect on its secondary structure, thus ruling out the causal relationship between the two.

(xii) FTIR spectroscopy has been used to quantitatively examine the secondary structure of imprinted (i.e., lyophilized in the presence of multifunctional ligands, followed by removal of the latter) proteins in anhydrous media. Lysozyme, chymotrypsinogen, and bovine serum albumin, imprinted with L-malic acid, all exhibit significant differences in the secondary structure compared to that of their nonimprinted counterparts. A rise in the β-sheet content, which invariably occurs upon lyophilization, is substantially lower for imprinted proteins. Alterations in the α-helix contents of these proteins have also been observed upon imprinting, although these changes are specific to the protein. A structural explanation has been obtained for other previously observed aspects of the protein imprinting phenomenon, including the effects of the ligand and the solvent and the lack of enantioselectivity. Exposure to aqueous solution, but

not to anhydrous solvents, results in the disappearance of imprinting-induced changes in the secondary structure of proteins.

Publications Resulting From This Project

The following publications have resulted from this project and describe in great detail the findings outlined above.

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- K. Dabulis and A.M. Klibanov, "Dramatic enhancement of enzymatic activity in organic solvents by lyoprotectants". *Biotechnol. Bioeng.* **41**, 566-571 (1993).
- S. Tawaki and A.M. Klibanov, "Chemoselectivity of enzymes in anhydrous media is strongly solvent dependent". *Biocatalysis* **8**, 3-19 (1993).
- A.M. Klibanov, "Enzyme catalysis without water". *R&D Innovator* **2** (4), 1-17 (1993).
- C.R. Wescott and A.M. Klibanov, "Predicting the solvent dependence of enzymatic substrate specificity using semi-empirical thermodynamic calculations". *J. Am. Chem. Soc.* **115**, 10362-10363 (1993).
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- I. Ikeda and A.M. Klibanov, "Lipase-catalyzed acylation of sugars solubilized in hydrophobic solvents by complexation". *Biotechnol. Bioeng.* **42**, 788-791 (1993).
- C.R. Wescott and A.M. Klibanov, "The solvent dependence of enzyme specificity". *Biochim. Biophys. Acta* **1206**, 1-9 (1994).
- B. Orsat, G.J. Drtina, M.G. Williams, and A.M. Klibanov, "Effect of support material and enzyme pretreatment on enantioselectivity of immobilized subtilisin in organic solvents". *Biotechnol. Bioeng.* **44**, 1265-1269 (1994).

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- J.L. Schmitke, C.R. Wescott, and A.M. Klibanov, "The mechanistic dissection of the plunge in enzymatic activity upon transition from water to anhydrous solvents". *J. Am. Chem. Soc.* **118,** 3360-3365 (1996).
- P. Mishra, K. Griebenow, and A.M. Klibanov, "Structural basis for the molecular memory of imprinted proteins in anhydrous media". *Biotechnol. Bioeng.* **52**, 609-614 (1996).
- K. Griebenow and A.M. Klibanov, "Can conformational changes be responsible for solvent and excipient effects on the catalytic behavior of subtilisin Carlsberg in organic solvents?". *Biotechnol. Bioeng.* **53**, 351-362 (1997).