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Mechanistic enzymology in non-aqueous media

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Abstract - A variant of subtilisin Carlsberg thiolsubtilisin, in which the active site serine residue is replaced by a thiol-containing cysteine has been synthesized in our laboratory. From the results of analysis of the resulting enzyme it can be argued that the mechanism of subtilisin suspended in organic solvents involves Ser221, undoubtedly in the formation of an acyl-enzyme intermediate. Further, the active site of the enzyme is not interacting with bulk solvent, and the environment of Ser221 is not radically altered by changing the solvent in which the enzyme is suspended. The enzyme structure has also been analyzed with FT-IR to demonstrate that the global secondary structure is the same in organic dispersants as in water. Finally, the morphology of subtilisin particles has been studied during hydration in the gas phase using ESEM.

In the last decade the use of enzymes in anhydrous organic solvents has evolved from investigations in a few laboratories, to commercialized biocatalytic reactions with over 300 publications per year in the field. The need for a detailed understanding of the relationships between enzyme structure, function, and environment has never been greater. The first, and perhaps the most important task of this paper, however, is to stress that enough is now known about essentially non-aqueous enzymology to design careful kinetic and structural experiments which can help to answer key questions.

Lyophilized enzyme powders, suspended in anhydrous organic solvents, have already been used to catalyze ester synthesis, transesterification, peptide synthesis, racemate resolution, epoxidation, hydroxylation, dehydrogenation, polymerization, and depolymerization reactions. The reasons why it is advantageous to use biocatalysts in the absence of water have been listed many times before, and we will proceed with the assumption that biocatalysis in anhydrous environments is an established fact. We do however stress that although the solvents used are essentially

anhydrous, the catalyst particles can be hydrated to a level corresponding to about a monolayer of water coverage per molecule of enzyme.

Before embarking on any kinetic investigations of enzymes in organic solvents it is necessary to understand any effect of particle morphology (one must always remember that non-aqueous biocatalysis is generally heterogenous) and protein structure on the kinetic behavior of the protein. We have presented evidence of the effect of organic solvents on protein particles previously (Rozeiwski & Russell, in press) . Suffice it to say here that proteins suspended in organic solvents do not undergo drastic morphological changes as a result of the presence of non-aqueous media. The particle shape and porosity depends to some degree on the solvent, although most protein powders appear to be either flaky or spherical particles with dimensions of around 100 um in diameter. The particles appear to be both macro- and microporous. Importantly, the addition of water to dry protein powders results in agglomeration and swelling of the particles, combined with a decrease in the microporosity as viewed by environmental electron microscopy.

Interpretation of electron micrographs enables one to determine what types of diffusion must be considered when dealing with heterogenous biocatalysis. Diffusional limitations on heterogenous reactions have been considered for many years, and are the subject of many excellent texts. There are two major diffusional constraints in the non-aqueous enzyme systems which are used most frequently. We have previously published the relationship between enzyme activity, particle size, and diffusional limitations and have determined that the most significant parameter in determining whether a reaction is diffusionally limited is, of course, the activity of the enzyme particle (Kamat et al., in press). This is particularly important when attempting to derive correlations between solvent properties

and enzyme activity and specificity over a large range of enzyme activities. One must insure that as the enzyme becomes more active, there is no change in the diffusional limitations on the reaction. Another reason that diffusional limitations must be elucidated before kinetic experiments is that attempts to optimize activity by either solvent or protein engineering are futile if the reaction is under diffusional limitation. Thus, optimization of enzyme function in anhydrous environments must address mass transfer constraints fully. Unfortunately, for those who work in the field there can be no short cuts. A demonstration that one enzyme in a given environment is not diffusionally limited does not infer that the same enzyme will remain kinetically controlled in another environment. Each powdered enzyme preparation must be tested for external and internal diffusional limitations in each different environment. To make the process simpler we have published a series of charts which give an indication, based on the activity of the preparation, whether there will be a problem.

A typical comment of classical enzymologists when considering enzyme-catalyzed reactions in non-aqueous media is that the enzyme suspended in the solvent must have a different structure than the same enzyme in water. Indeed, it has been suggested on many occasions that a structural difference accounts for changes in the activity of the enzyme when transferred to non-aqueous environments. Experimentation has shown, however, that any structural changes are minimal. We have recently investigated the effect of organic solvents on the global structure of proteins. FT-IR has been used to determine the secondary structure content of subtilisin and myoglobin in a variety of environments. There is no discernable change between subtilisin solubilized in buffer, lyophilized subtilisin, lyophilized subtilisin suspended in mineral oil, and lyophilized subtilisin suspended in carbon tetrachloride. The same held true for myoglobin. Importantly, equivalent experiments on enzyme solubilized in dimethylsulfoxide demonstrate

that organic solvents that solvate proteins denature the protein considerably. The type of denaturation referred to here is markedly different than denaturation by boiling the protein. Finally, we have shown that the local structure of the myoglobin active site has only minor changes in structure when the protein is placed in anhydrous environments. These experiments were performed by using FT-IR on azidometmyoglobin.

Given that enzymes function in organic solvent without a drastic change in structure and that there are significant advantages for such uses, there are still two drawbacks for kinetic experimentation. The most significant is that in most cases the Km of the enzyme for most substrates is increased upon transfer to the solvent. Most hydrophobic substrates, which are those with which non-aqueous enzymology would be advantageous, bind to enzyme active sites via hydrophobic bonding. When the bulk solvent is not water, the entropy gain of forming a hydrophobic bond will be significantly decreased and thus Km is expected to increase. For commercial biocatalysis this represents an advantage since high substrate concentrations will not lead to enzyme inactivation. Detailed kinetics cannot be performed unless the enzyme can be saturated with substrate. The solubility of substrates is often reached before saturation, and in addition if it is possible to saturate the enzyme the use of high concentrations of substrate may alter the physical properties of the solvent relative to pure solvent. Such effects have largely been overlooked in the literature. Because of these limitations it is often impossible to separate the turnover number and Michaelis constant from the specificity constant. Thus, we must identify model enzymes which can approach saturation with a number of substrates, and then attempt to understand how the enzyme environment can be modified to predictably alter enzyme activity, specificity and stability.

Subtilisin, a serine protease, is an ideal model enzyme. In water subtilisin catalyzes hydrolysis via the acyl-enzyme

mechanism, whereas in organic solvent alcoholysis reactions can be catalyzed. The mechanism is well understood in aqueous solutions, the structure of the enzyme has been determined, and subtilisin is readily available. Furthermore, it is possible to perform site-directed mutagenesis on subtilisin since the gene encoding the protein has been cloned previously. The mechanism of the enzyme in organic solvents has been investigated before. We have also compared the activity of an active-site mutant, thiolsubtilisin, to that of native subtilisin in an attempt to further understand the function of the enzyme in anhydrous environments.

During the acyl-enzyme mechanism a covalent bond is formed between the serine-221 hydroxyl group of the enzyme, and the substrate. Protein engineering studies have shown that when this group is removed the enzyme functions a million times less efficiently. Coincidentally, this is also the reduction in activity when comparing the specificity constants of hydrolysis and alcoholysis (transesterification). By converting chemically Ser-221 to a cysteine we have been able to evaluate the role of this amino acid in organic solvents. Thiolsubtilisin (Ser-Cys 221) has been investigated for many years and it is known that the overall structure of the enzyme does not change upon synthesis. Thiolsubtilisin is, however, very susceptible to oxidation and must be treated with great care. Simply comparing the activity of subtilisin and thiolsubtilisin yields important information about how subtilisin functions in organic media.

We have reported the calculation of the difference in the free energy of transition state stabilization between native and thiolsubtilisin (Chatterjee & Russell, in press). This free energy does not change significantly when the enzyme is placed in hexane, acetonitrile or dioxane, indicating that the active site of the enzyme must be protected from bulk solvent by either water or salt molecules in these environments.

Given that subtilisin utilizes the acyl-enzyme mechanism for transesterification, it is now important to elucidate the effect of

different solvents on the relative rates for acylation and deacylation of the enzyme. Clearly, if we are to optimize catalysis in extreme environments we must first understand which steps in the mechanism are rate limiting, and what the rate of each step is. This is not a simple procedure for the use of enzymes in hetrogenous biocatalytic reactions. We will present a number of different approaches which may be used to determine the microscopic rate constants for alcoholysis. Importantly, each of these methods has been used before to either study enzyme-catalyzed reactions in water or classical hetrogenous catalytic reactions. As such the methods themselves are novel only in their application to this system. We have described previously the use of steady-state isotopic transient kinetic analysis (SSITKA) for determination of microscopic rate constants of the subtilisin-catalyzed alcoholysis of N-acetyl-L-phenylalanine ethyl ester by propanol. In summary, it is possible to perform steady state experiments with radiolabelled substrates in order to investigate each step of the reaction. The main drawback of this approach is that before it is possible to determine actual rate constants one must first quantitate the role of diffusion as described above.

We introduce here the use of added nucleophiles for microscopic rate constant determinations. For this approach to be successful it is necessary to be able to saturate the enzyme with both substrates, and the rate constants for acylation and deacylation should not differ by more than an order of magnitude. Under these circumstances determination of kcat and Km for each substrate, in the presence of varying concentrations of a second added nucleophile allows the determination of the microscopic rate constants for acylation and deacylation as has been described previously for chymotrypsin in mixtures of water and butane-1,2-diol. Our experiments to date suggest that this is a viable method for the separation of k2 and k3. Thus we are now able to generate details of how changing the solvent for the reaction will affect

the binding and chemical steps of the reaction. This data should enable further optimization and increased predictability of this biocatalytic reaction in organic solvents.

Solvent engineering is one approach for increasing activity of enzymes in non-aqueous media. Much can be achieved, however, utilizing the lessons learned from protein engineering studies in aqueous media. Just as it is possible to measure the strength of interactions within a protein in aqueous media via site-directed mutagenesis, it is possible to evaluate the important criteria for rational control of activity by measuring the activity of engineered proteins in extreme environments. In collaboration with Dr. F. Arnold (California Institute of Technology) we are evaluating the activity of engineered subtilisin E variants. The mutant proteins have been selected for increased activity in the presence of high concentrations of water miscible organic solvents. It is interesting that proteins not specifically designed for use in anhydrous environments show considerably increased activities. This suggests that selection of proteins in water based environments for increased activity in solvents is a reasonable approach.

In conclusion, the use of enzymes in organic solvents is a practicle route for synthesis of many compounds. However, there is still a need to understand fully the interaction between solvent and enzyme. During our attempt to dissect the catalysis of subtilisin in different environments we have shown that the enzyme undergoes no major conformational changes when suspended in chloroform or miner oil. The enzyme retains activity, and we are beginning to investigate why the enzyme suffers such a significant loss in overall activity.