

Why are enzymes less active in organic solvents than in water?

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In order to exploit fully the biotechnological opportunities afforded by nonaqueous enzymology, the issue of often drastically diminished enzymatic activity in organic solvents compared with that in water must be addressed and resolved. Recent studies have made great strides towards elucidating causes of this phenomenon of activity loss. None of these causes is insurmountable; by designing strategies that systematically target them, enzymatic activity in organic solvents can be readily enhanced by multiple orders of magnitude and ultimately brought to the aqueous-like level.

Over the past decade, nonaqueous enzymology has emerged as a major area of biotechnology research and development¹. Although the notion of enzymatic processes in organic solvents was initially met with scepticism by the scientific community, nonaqueous enzymology is now studied and exploited by numerous academic and industrial laboratories worldwide, and the number of papers devoted to this topic has surged into the hundreds¹.

The reason why nonaqueous enzymology has generated so much excitement is that enzymes exhibit striking new properties in organic solvents. For example, in organic solvents enzymes often catalyze reactions impossible in water and are very stable¹. Furthermore, enzyme selectivity in organic solvents is not only distinct from that in water but can be markedly controlled, and even reversed, by the solvent^{2,3}; this 'solvent engineering' thus provides an alternative to protein engineering.

A number of applications for enzymes in organic solvents have been developed in chemical processing (particularly for the synthesis of optically active intermediates), food-related conversions and analyses¹. The only dark cloud hanging over nonaqueous enzymology is the notion (largely, albeit not always, correct) that enzymes in organic solvents are far less active than in water. For instance, the proteases α -chymotrypsin and subtilisin have activities 10^4 – 10^5 -times lower in anhydrous octane than in water; the two enzymes are less active still in most other organic solvents⁴. Such numbers seem to be typical, although two comments must be made. First, while handicapped by transfer from water to organic solvents, enzymes nevertheless remain impressive catalysts in organic solvents. For

example, in the case of α -chymotrypsin and subtilisin, the specificity constant, k_{cat}/K_M , of an enzymatic transesterification reaction in anhydrous octane exceeds the bimolecular rate constant of the same reaction without enzyme by as much as 10^{11} -fold⁴. Second, whereas the rates of enzymatic processes in anhydrous solvents are indeed dwarfed by those in water, this is not necessarily so in hydrous ones. For example, polyphenol oxidase in octanol containing 3% water retains more than a third of its activity in water⁵. Likewise, the activity of yeast alcohol dehydrogenase in isopropyl ether containing 0.5% water is about a quarter of that in aqueous solution⁵.

These comments notwithstanding, the fact that enzymatic activity in anhydrous or nearly anhydrous solvents falls short of that in water clearly threatens the practical potential of nonaqueous enzymology (although it should be kept in mind that from the industrial standpoint the real question is whether the enzymatic activity is high enough for the intended application, not how it compares with that in water). Therefore, the issue of activity loss must be confronted and, hopefully, alleviated. The only way to do so rationally is to elucidate why enzymatic activity plummets on transition from water to anhydrous solvents. This elucidation, much advanced by recent studies, is the subject of the present article. The discussion will involve, primarily, the model enzyme subtilisin Carlsberg, for which the bulk of the relevant work has been done and the greatest insights have been attained. Two enzyme forms introduced into organic solvents will be considered: amorphous (e.g. lyophilized, which is the most popular form among researchers¹⁻³) and crosslinked crystalline (CLC) enzymes⁶.

Diffusion and accessibility factors

Enzymes are soluble in water but insoluble in nearly all organic solvents. Hence, the addition of enzymes

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leads to solutions in water but, usually, suspensions in organic solvents. Consequently, it is intuitively plausible that enzymatic activity in nonaqueous solvents may be diminished due to diffusional limitations on the substrates. This phenomenon, common in heterogeneous, including immobilized enzyme, catalysis⁷, results in the under-utilization of enzymatic power and thus decreased observed activity. Theoretical analysis⁸ shows that such mass transfer limitations may indeed slow down enzymatic catalysis in organic solvents. We experimentally tested this possibility in the specific case of subtilisin. If subtilisin catalysis in organic solvents is suppressed by diffusional limitations, then the dependence of the enzymatic activity in such media, or that in water, should be nonlinear (and should eventually level off); by contrast, a linear dependence should ensue in the absence of diffusional restrictions^{7,9}. In fact, we observed linear dependences in all three organic solvents examined: dodecane, carbon tetrachloride and acetonitrile⁹. Therefore, the 5000–100 000 times slower catalysis in these solvents compared with that in water⁴ cannot be caused by mass transfer limitation.

Even in the absence of diffusional limitations *per se*, it is conceivable that in a lyophilized enzyme particle or CLC enzyme, some enzymatic active centers may be sterically shielded by the neighboring enzyme molecules and thus be inaccessible to the substrate. This would effectively prevent those enzyme molecules from participating in catalysis, thereby cutting the observed catalytic activity. This possibility was tested by titrating the competent active centers in subtilisin and α -chymotrypsin suspended in organic solvents. The data obtained^{4,10} reveal that in octane the number of centers is between one- and two-thirds of that in water. For CLC subtilisin in organic solvents it is even higher – essentially unity¹¹. Therefore, steric blockage can be responsible for only a tiny fraction of the loss of enzymatic activity on transition from water to organic solvents.

Structural changes

Another obvious suspect among the possible causes of the diminished enzymatic activity in anhydrous solvents compared with that in water is a change in enzyme conformation. Such protein denaturation in aqueous–organic mixtures is well known¹², and thus it would not be unreasonable to expect an even graver situation in neat organic solvents. For CLC enzymes suspended in anhydrous solvents, this issue can be addressed experimentally by means of X-ray crystallography. Several years ago we succeeded in solving the crystal structure of CLC subtilisin in anhydrous acetonitrile¹³. Comparison of this structure with its counterpart in water^{13,14} revealed that the two are virtually identical. The same holds true for the recently solved crystal structure of CLC subtilisin in dioxane (see the cover of this journal issue). Likewise, the X-ray crystal structure of uncrosslinked chymotrypsin in hexane was found to be very similar to that in water¹⁵. Therefore, factors other than conformational change

must underlie, for example, the 10^7 -fold difference in enzymatic activity (k_{cat}/K_M) between subtilisin dissolved in water and CLC subtilisin suspended in acetonitrile¹¹.

X-ray crystallography, unfortunately, cannot be applied to lyophilized enzymes suspended in organic solvents, thus making complete structure characterization of such systems impossible. Nevertheless, some structural information can be derived by other biophysical methods, such as Fourier-transform infrared (FTIR) spectroscopy. Using this methodology we have recently ascertained that placing lyophilized subtilisin in a variety of anhydrous solvents, such as octane, acetonitrile and dioxane, has no appreciable effect on its secondary structure (as reflected by the α -helix content)¹⁶. However, the preceding step, lyophilization, does result in a significant (and reversible if lyophilization is done carefully) denaturation of subtilisin¹⁶ and many other proteins^{17,18}. In other words, somewhat ironically, it is not the contact with an organic solvent but the prior dehydration that alters the enzyme structure and, undoubtedly, lowers catalytic activity. This deleterious effect can be prevented, or at least minimized, by lyoprotectants, as discussed in a later section.

Energetics of substrate desolvation and transition state stabilization

The energy of binding between the enzyme and the substrate is the major driving force for enzymatic catalysis and the source of huge rate enhancements afforded by enzymes¹⁹. In order for binding to occur, the substrate must first undergo desolvation upon its journey from the reaction medium to the enzyme active center. The more energetically favorable this desolvation is, the greater the net binding energy (resulting in catalysis) becomes. Subtilisin, α -chymotrypsin and many other enzymes have hydrophobic active centers; they work best with hydrophobic substrates because there is a large energetic incentive for the latter to partition from water into the active center. When water is replaced with an organic solvent, the substrate is no longer 'squeezed out' of the medium owing to the hydrophobic effect and the energetic advantages of the partitioning drop – or, in thermodynamic language, the ground state of a hydrophobic substrate is stabilized in organic solvents relative to water²⁰. This ought to raise the activation barrier and hence slow down the enzymatic reaction¹⁹.

Using CLC subtilisin (where, as discussed above, there are no conformational changes upon replacement of water with anhydrous reaction media), we have quantified the decline in enzymatic activity stemming from this less favorable substrate desolvation. In the case of the substrate N-Ac-L-Phe-OEt, this effect accounts for more than a 100-fold reduction in k_{cat}/K_M in anhydrous acetonitrile compared with that in water¹¹.

Another term affecting the activation energy (and thus enzymatic reactivity) is the energy of the transition state. For subtilisin and many other hydrolases, this

reaction transition state is highly polar (resembling a charged tetrahedral intermediate)¹⁹. If it is fully enveloped by the enzyme active center (i.e. shielded from the solvent), a solvent change should not affect its energy. However, full shielding does not always occur – for example, at least one-third of the transition state of subtilisin with N-Ac-L-Phe-OEt is exposed to the solvent². Since water stabilizes tetrahedral intermediates much better than less polar solvents, the enzymatic activity in the latter should be lower as a result of this phenomenon²¹. Enzyme–substrate interactions in partially-exposed transition states may also be affected by the solvent²⁰.

Conformational mobility

Interaction of proteins with water (hydration) is critical for a plethora of biological functions, particularly enzymatic activity²². Water, acting as a lubricant or plasticizer²³, allows enzymes to exhibit the conformational mobility required for optimal catalysis. Organic solvents, in general, are not nearly as accommodating, if accommodating at all, because they lack water's ability to engage in multiple hydrogen bonds (and, because of their lower dielectric constants, lead to stronger electrostatic interactions and hence more rigid proteins²⁴). These considerations dictate that, other things being equal, enzymes should be less active in anhydrous solvents than in water owing to restricted conformational mobility. In fact, enzymes exhibit significant catalytic activity in such media only because

they strongly bind some 'essential' water and retain it even when suspended in anhydrous solvents⁴.

There are numerous lines of evidence supporting this analysis. There is a general inverse correlation between enzymatic activity in an organic solvent and the latter's hydrophilicity – the highest activity is usually observed in hydrophobic solvents^{4,25}. This is because hydrophobic solvents possess a lesser ability than their hydrophilic counterparts to strip the essential water off enzyme molecules⁴. Addition of water to enzyme suspensions in anhydrous solvents⁵ or increasing the thermodynamic activity of water (a_w) in such systems by other means²⁶ can dramatically enhance enzymatic activity. To some extent, water can be replaced by water-mimicking organic solvents, such as glycerol or ethylene glycol, which can also form multiple hydrogen bonds^{5,27}.

Note that CLC subtilisin loses almost two orders of magnitude in catalytic activity solely due to the decline in its conformational mobility in anhydrous acetonitrile compared with that in water¹¹.

pH situation

In aqueous solution, enzyme catalysis profoundly depends on the pH and has a pH optimum¹⁹. Since the notion of pH has no meaning in organic solvents, the protonation state of enzyme ionogenic groups must be controlled by other factors. It was discovered^{4,25} that enzymes have a 'pH memory' – i.e. their catalytic behavior in organic solvents reflects the pH of the last

Table 1. Causes of lower enzymatic activity in organic solvents compared with that in water and ways to alleviate them^a

Cause ^b	Comments	Remedies ^b
Diffusional limitations	Not as likely as widely claimed	Vigorously agitate enzyme suspensions; use small enzyme particles
Active center blockage	Responsible for no more than a few-fold activity reduction	If matters, use crystalline rather than amorphous enzyme particles
Conformational change	Occurs upon lyophilization and other modes of dehydration. Is avoided with CLC enzymes. Is usually not caused by contact with the solvent	Use lyoprotectants; alternatively, prepare enzyme complexes with amphiphiles soluble in organic solvents; use enzyme CLCs
Unfavorable energetics of substrate desolvation	Severe with hydrophobic substrates; also, likely with natural substrates. May be responsible for at least a 100-fold activity reduction	Select the solvent expected to yield unfavorable solvent–substrate interactions
Transition state destabilization	Likely to be pronounced when the transition state is at least partially exposed to the solvent	Select the solvent expected to yield favorable interactions with the transition state
Reduced conformational mobility	Severe in anhydrous, hydrophilic solvents owing to the stripping of the essential enzyme-bound water. May be responsible for at least a 100-fold activity reduction	Optimize water activity (a_w); hydrate the solvent; use hydrophobic solvents; use water-mimicking and denaturing cosolvent additives
Suboptimal pH situation	May be responsible for at least a 100-fold activity reduction	Dehydrate from aqueous solution of the pH optimal for enzymatic activity; use organic-phase buffers

^aThe table refers to lyophilized (or other amorphous preparations) or crosslinked crystalline (CLC) enzymes suspended in neat organic solvents.

^bBoth proven and hypothetical (see text).

aqueous solution to which they were exposed (e.g. from which they were lyophilized). Therefore, unless enzyme powders are obtained from an aqueous solution of the pH affording maximal activity, enzymatic performance in organic solvents is doomed to be suboptimal.

An alternative to recovering enzymes from aqueous solutions at appropriate pH values is to employ organic-phase buffers – i.e. suitable mixtures of organic-soluble acids and their conjugate bases²⁸. This approach has been successfully used to increase markedly the catalytic activity of lyophilized²⁸ and CLC (Ref. 29) enzymes in organic solvents.

How to enhance enzymatic activity in organic solvents

The foregoing analysis shows that there are several physicochemical factors that may lower enzymatic activity in organic solvents compared with that in water (Table 1). Gratifyingly, virtually all examples of enzyme activation in nonaqueous media reported in the literature to date can be explained in terms of minimizing these individual factors.

The most popular strategy has been to prevent enzyme denaturation during dehydration. This has been achieved by lyophilizing enzymes in the presence of lyoprotectants such as sugars³⁰, poly(ethylene glycol)³⁰, inorganic salts (notably KCl)³¹, substrate-resembling ligands^{30,32}, and crown ethers³³. An alternative is to preform organic-soluble complexes of enzymes with detergent (or other amphiphile) molecules^{34,35}; enzymes are apparently in native-like conformations in such complexes^{16,35}. These approaches have resulted in increasing enzymatic activities in organic solvents by up to three to four orders of magnitude.

Another effective strategy has been to 'loosen up' enzymes in anhydrous solvents. Enzymatic activity has been increased up to two to three orders of magnitude by adding small quantities of water to organic solvents⁵, maximizing a_w (Ref. 26), and adding water mimics^{5,27} or denaturing cosolvents³⁶ to anhydrous media.

Concluding remarks

The reasons why enzymatic activity in organic solvents usually does not measure up to that in water have been elucidated (Table 1). In some instances, their contributions to the overall activity loss have been quantified, and the sum thereof comes close to the observed decline in the catalytic performance in nonaqueous media compared with water¹¹. None of these factors is insurmountable and, as the aforementioned examples illustrate, can be prioritized, systematically tackled (Table 1) and, perhaps, eliminated. Indeed, 'aqueous-like' activities of enzymes in nonaqueous solvents have thus been achieved^{5,35} and less spectacular but nonetheless quite dramatic activations are now commonplace^{4,10,11,26–36}. There is every reason to be optimistic that, by judiciously combining the remedies discussed above, enzymatic activities in organic media can be routinely made comparable with those

in water. In fact, it may even be possible to make enzymes more active in organic solvents than in water. For example, particularly for unnatural substrates, the energetics of substrate desolvation may be made more favorable. Also, there is the intriguing possibility of molding enzyme active centers and thus optimizing enzymatic activity in organic solvents for a desired substrate by molecular imprinting^{32,37}.

There is now convincing evidence dispelling the popular cliché that enzymes should be more active in water than in organic solvents because that is where Nature intended to use them. Regardless of Nature's intention, if optimally used, enzymes may be able to work as well in nonaqueous as in aqueous media. The real question thus becomes: what novel mechanistic knowledge and useful practical applications will be derived from this opportunity?

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Atomic force microscopy in analytical biotechnology

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The past decade has seen the atomic force microscope evolve not only as a high resolution imaging tool, but also as an instrument capable of measuring forces between surfaces and the material properties of samples. Here, the use of atomic force microscopy for surface force measurement is reviewed, highlighting the considerable progress that has recently been made in the area of biotechnology. Particular emphasis is placed on how the instrument can be used to probe directly biomolecular interactions, illustrating the potential of the technique to impact on our fundamental understanding of molecular structure and processes.

Since its invention in 1986, the atomic force microscope (AFM)¹ has evolved as a valuable imaging technique with resolution in the micrometre to sub-nanometre range. The ability of the AFM to image both insulating and conducting surfaces in a variety of environments has facilitated molecular resolution images of a wide range of biomolecules, including proteins², lipids³ and DNA (Ref. 4). In addition to its imaging abilities, the AFM can also be employed to probe spatial variations in surface properties, such as adhesiveness and elasticity⁵. The use of the AFM as an imaging technique in the study of biomolecules has frequently been the subject of review^{6,7}. The aim of this article is to outline the use of an AFM as an instrument for surface force measurements, with a particular emphasis on how the technique can be employed in the areas of biotechnology and biological science.

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The AFM as a force sensing instrument

Intermolecular forces govern the fundamental properties of solids, liquids and gases, the properties of colloids and the organization of biological structures⁸. Numerous biophysical methods have been employed to investigate specific and non-specific molecular interactions, including optical trapping⁹, magnetic force experiments¹⁰, pipette suction¹¹ and the surface force apparatus¹². Atomic force microscopy complements these techniques by uniquely combining an extended force range with high spatial resolution. The AFM has a theoretical force sensitivity of 10^{-15} N (Ref. 13), and employs probes with a tip apex of 10–50 nm radius¹⁴, to produce contact areas as small as 10 nm²; therefore providing a means to overcome the limitations of other techniques.

By recording force measurements between different probe-sample combinations in various ionic media, it was first illustrated that the AFM could be used to measure forces, such as van der Waals and electrostatic forces¹⁵. Ducker and colleagues¹⁶ also employed the technique to measure colloidal forces, by attaching spherical particles to the apex of AFM cantilevers. The