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whether this is a phenomenon unique to hyperthermophilic enzymes must obviously await structural information on enzymes of other types, ideally those having a mesophilic counterpart that has been well characterized structurally. If changes in ratios of surface area to volume are a primary factor in determining thermal stability, it does not bode well for understanding the molecular basis for hyperthermostability, or for the chances of finding means to stabilize labile mesophilic enzymes, even if high resolution structures of them are available. It suggests that simple approaches such as just one or two site-specific amino acid changes in a complex enzyme are very unlikely to lead to a global change that would result, for example, in a significantly decreased surface area. Clearly, we have only begun to get the barest of glimpses of what stabilizes enzymes from hyperthermophilic organisms, and detailed structural data on a range of both hyperthermostable enzymes and on their mesophilic relatives are sorely needed.

See also BACTERIAL GROWTH AND DIVISION; ENZYMES, ENERGETICS AND REGULATION OF BIOLOGICAL CATALYSIS BY.

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ENZYMOLOGY, NONAQUEOUS

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Key Words

Enzyme A thermolabile proteinaceous biological catalyst.

Lyophilized Enzyme Powder A freeze-dried preparation of biological catalysts, which, if desired, can be suspended directly into a water-free organic solvent to promote activity.

Supercritical Fluid A material above its critical temperature and pressure, the physical properties of which lie in between those of liquids and gases

Modern day nonaqueous enzymology evolved in a number of distinct steps. Initially, water-miscible organic solvents, such as acetone and ethanol, were added to aqueous enzyme solutions to determine the maximal concentration of solvents that enzymes can tolerate ($\approx$ 50% for most conventional enzymes). Next biphasic mixtures in which aqueous solutions of enzyme were emulsified in water immiscible solvents, such as chloroform and ethyl acetate, were evaluated. This approach was further developed by the use of reversed micelles to stabilize enzymes in aqueous organic mixtures, and the application of freeze-dried enzyme powders suspended in anhydrous organic solvents and supercritical fluids. This article lists the advantages of nonaqueous enzymology over the aqueous form, as well as the disadvantages of the newer technology. It also reviews hydrolases, explores the effects of solvents on organic biocatalysts, and discusses commercial processes that use enzymes, as well as control aspects of solvent engineering.

1 INTRODUCTION

Enzymes are thermolabile, biological, proteinaceous catalysts capable of enhancing the rates of chemical reactions by up to 10^{12}-fold. They are distinguished from other catalysts by properties such as remarkable specificity and the ability to work at mild temperatures and pH. Conventional biocatalysis is carried out in aqueous solutions. Recently, however, interest has shifted from aqueous to nonaqueous enzymology, since most industrial chemistry takes place in organic solvents. Some of the advantages of nonaqueous enzymology over aqueous enzymology are as follows:

- Greater solubility of hydrophobic substrates in organic media
- Ease of recovery of enzymes from organic media due to their insolubility in organic solvents eliminating the need for enzyme immobilization
- Reduced microbial contamination of bioreactors
- Shifting of thermodynamic equilibria to favor synthesis over hydrolysis (e.g., in peptide and ester synthesis)

- Ease of product recovery from low boiling, high vapor pressure solvents
- Effect of solvent on substrate specificity

Hence, it is not difficult to envisage enzymes as ideal catalysts in industrial processes in the not too distant future. There are, of course, certain disadvantages such as:

- Inactivation of enzymes by many organic solvents
- Limited enzyme activity in most organic solvents (compared to their activity in water)
- Absence of any predictive model to describe the effect of solvents on enzyme activity, specificity, and structural stability

Hydrolases, such as lipases, proteases, and esterases, are the most widely used enzymes in organic solvents. In aqueous media, these enzymes catalyze reactions in which water acts as a nucleophile. In organic media, the hydrolases are able to accept other nucleophiles such as alcohols, amines, thio esters, and oximes. Thus, hydrolysis reactions in water can be substituted by synthetic reactions such as peptide synthesis, transesterification, esterification, and aminolysis. At present there are four commercial processes that utilize enzymes in organic media:

- Lipase-catalyzed interesterification of fats
- Thermolysin-catalyzed synthesis of the sweetener aspartame
- Lipase-catalyzed production of optically active 2-halopropionic acid
- Peroxidase-catalyzed production of phenolic resin

2 AREAS OF NONAQUEOUS ENZYMOLOGY

The various strategies that enable the utilization of proteins in nonaqueous media are shown in Figure 1. The areas covered range from homogeneous systems as represented by reversed micelles, soluble polyethylene glycol (PEG) modified enzymes and other soluble chemically modified enzymes, to heterogeneous systems as represented by enzyme powders dispersed in anhydrous organic solvents and supercritical fluids, enzymes in biphasic mixtures of immiscible organic solvents and water, and immobilized enzyme systems in organic solvents. The choice of any system is, of course, dictated by the relative advantages and disadvantages of each.

2.1 Enzymes in Reversed Micelles and Biphasic Mixtures

Reversed micelles (microemulsions) and biphasic mixtures are water–solvent mixtures that rely on protein interactions at the water–organic solvent interface. A reversed micelle is an aggregate of amphiphilic molecules in organic solvents which can encapsulate a water pool. The polar head groups of the surfactant molecules orient themselves toward the interior aqueous phase, whereas the hydrophobic chains arrange themselves in the continuous organic phase.

Reversed micellar systems provide a means for protein solubilization in organic media without direct interaction between enzyme and solvent. The presence of the surfactant layer between the aqueous and organic media effectively shields the enzyme from the dele-

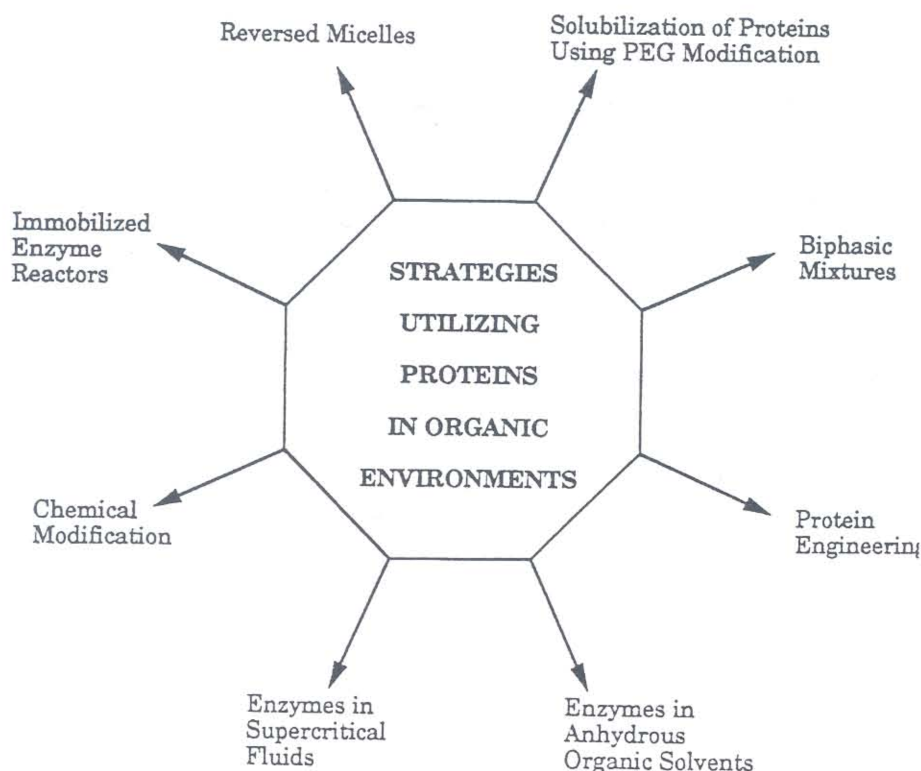

Figure 1. Methods enabling the use of proteins in organic environments.

terious effects of organic solvents, thereby enabling the protein to exist and function in its native aqueous conformation. Water-soluble proteins such as subtilisin and chymotrypsin, as well as integral membrane proteins such as cytochrome oxidase, rhodopsin, and ATPase, have been successfully solubilized in reversed micelles.

Biphasic mixtures are composed of two immiscible phases. Choice of the organic solvent phase is determined by a number of factors such as the differential solubility of substrates in the aqueous and organic phase, the partitioning of products between the two phases, and the extent to which the organic phase is able to influence enzyme stability and activity. Synthesis and hydrolytic reactions have been studied in biphasic systems using a wide variety of enzymes such as lipases, acid phosphatase, β-glucosidase, and β-fructofuranosidase. Such studies have demonstrated the potential of biphasic systems for increasing the thermostability, and in some cases the β-glucosidase and acid phosphatase activity, of hydrolytic reactions.

2.2 Enzymes Chemically Modified for Solubilization in Organic Media

A recent development in organic biocatalysis is the use of PEG-modified enzymes, which are soluble in a variety of organic solvents. Modification by means of polyethylene glycol involves the covalent attachment of a hydrophobic polymer to the surface of an enzyme, thus facilitating proteins solubilization. Enzymes such as PEG–lipase, PEG–chymotrypsin, PEG–catalase, and PEG–peroxidase have been used to catalyze a wide spectrum of reactions in organic media. Inada and co-workers have analyzed the structures of PEG–catalase and PEG–horseradish peroxidase in organic media. The absorption spectra for PEG–catalase and PEG–horseradish peroxidase in benzene were found to be similar to those of the unmodified enzymes in water, indicating that the association of prosthetic groups and protein was not affected by PEG modification. More recently Sakurai and colleagues have demonstrated the possibility of using PEG–chymotrypsin, PEG–papain, PEG–thermolysin, and PEG–pepsin for solid state peptide synthesis in organic media. In another study, Gaertner and Puigserver successfully used PEG–chymotrypsin for peptide synthesis in organic media.

Other examples of enzymes chemically modified for solubilization in organic media include lipases from *Pseudomonas fluorescens* modified with a copolymer of poly(oxyethylene allylmethyl diether) maleic anhydride and lipoxygenase modified using *N*-acyloxysuccinimide. The modification of lipases rendered the enzyme soluble and active in organic media and also improved its thermostability. The modification of lipoxygenase indicated an increase of hydrophobicity at positions distant from the active site and showed promise for future use of this enzyme in organic media.

An interesting variation on the concept of solubilization by chemical modification is the dissolution of hydrophilic chymotrypsin in nonpolar organic solvents (*n*-octane, cyclohexane, and toluene) up to micromolar concentration levels by prolonged shaking at room temperature.

2.3 Protein Engineering of Enzymes for Organic Biocatalysis

A recent trend in nonaqueous enzymology is the use of protein engineering techniques to redesign enzymes for improved activity and stability in organic media. Initial interest in this type of work was generated by the work done with crambin, a small plant protein, which is soluble in organic solvents. Arnold and co-workers recently modified subtilisin E and α-lytic protease by site-directed mutagenesis for enhanced compatibility with polar organic media. Both enzymes exhibited enhanced stability and activity in mixtures of water and dimethyl formamide. In a separate study, Wong et al. successfully engineered subtilisin BPN′ for enhanced stability in dimethylformamide. The results of these two studies augur well for adoption of protein engineering tactics for optimization of organic biocatalysis.

2.4 Freeze-Dried and Immobilized Enzymes in Organic Media

When an enzyme powder is suspended in an organic medium, the total concentration of water in the system can be as low as 0.01% ($V_{water}/V_{solvent}$). The total amount of water necessary to support activity is actually less than a monolayer per molecule of enzyme in many cases. Under these conditions, the enzyme is protected from solvent and therefore functions in an essentially anhydrous media. Suspensions of freeze-dried (lyophilized) enzyme powders and immobilized enzyme dispersed in anhydrous organic media are examples of heterogeneous biphasic systems of low water concentration (as opposed to immiscible organic solvent/water mixtures). As mentioned in Section 1, such systems offer the advantage of ease of enzyme recovery, improved enzyme thermostability, and the reduction of undesirable side reactions that require water as a substrate. However, a significant problem faced in heterogeneous systems is mass transfer limitation. Mixing and sonication are usually employed in such systems to overcome diffusional limitations.

Applications of enzymes in anhydrous organic media include the use of chymotrypsin, lipases, and subtilisin for peptide synthesis, regioselective acylation of carbohydrates, synthesis of biosurfactants, and lipolysis of fats and horseradish peroxidase for the manufacture of sensors used to detect unacceptable extremes in temperature.

Immobilized enzymes systems for use in organic solvents may be prepared either by physical adsorption on a matrix or by covalent attachment to a stationary phase. Stark and Holmberg have successfully immobilized lipase from species of *Rhizopus* on tresyl silica in hexane buffer and microemulsions based on hexane. The immobilized enzyme was found to catalyze both hydrolysis and transesterification reactions. In another study with immobilized lipases Rizzi and colleagues looked at the kinetics of synthesis of isoamyl alcohol via transesterification reactions in *n*-hexane. Proteases have been immobilized on porous chitosan beads. Compared to the free enzyme, the immobilized enzymes were found to exhibit enhanced stability and activity in ester and peptide synthesis in organic solvents such as methanol, dimethylformamide, and acetonitrile. Blanco et al. have evaluated the effect of immobilization of chymotrypsin on its activity and stability in anhydrous organic solvents. They, too, observed better activity with immobilized enzyme than with free enzyme.

Continuous reactors utilizing immobilized enzymes have been designed for hydrolysis of olive oil by lipases, peptide synthesis with thermolysin, and the resolution of racemic amines by subtilisin in organic media. The racemate resolution process was developed for the production of compounds such as (*R*)-1-aminoindan and (*R*)-1-(1-napthyl)ethylamine, which are considered to be important intermediates in the pharmaceutical industry.

2.5 Freeze-Dried Enzymes in Supercritical Fluids

The use of enzymes in supercritical fluids is a relatively new concept in nonaqueous enzymology. A supercritical fluid is described as a material above its critical temperature and pressure, exhibiting physical properties between those of a gas and a liquid. For biocatalytic processes these substances offer advantages such as high diffusivity, ease of downstream processing, recyclability, and low toxicity. Studies in supercritical fluids have been carried out in a number of solvents such as carbon dioxide, ethane, sulfur hexafluoride, and fluoroform.

3 EFFECTS OF SOLVENTS ON ORGANIC BIOCATALYSIS

Organic solvents may influence nonaqueous biocatalysis on a macroscopic level by interacting with the enzyme, with concomitant effects on its structure and morphology. In addition, interactions of the solvent medium with substrates and products of reaction may influence enzyme stability, kinetics, and reaction mechanism.

3.1 Effects of Solvents on Enzyme Morphology

Complete characterization of enzyme function in organic media requires a detailed study of the effects of environment on enzyme structure, activity, and specificity. Morphological studies on protein powders suspended in organic solvents have shown considerable changes in the powder as a function of the water associated with the protein. An environmental scanning electron micrograph (ESEM) showed the powder to be flaky (≈ 0.2 μm thick) with dimensions exceeding 100 μm at a vapor pressure of 9.2 torr. When the humidity of the analysis chamber was increased above 50%, the flakes were observed to swell, and at 85% humidity they coalesced to form branched structures. The effect of an organic solvent, toluene, on particle morphology was also investigated in these studies. Specifically, toluene was evaporated by slowly drying a suspension of lyophilized subtilisin. Comparison of the images of the dried enzyme and native enzyme showed that the exposure to toluene had no marked effect on enzyme morphology. Particle morphology becomes particularly important when one is attempting to understand the impact of organic solvents on enzyme-catalyzed reactions that proceed relatively fast but may be diffusionally limited.

3.2 Interaction of Solvent with the "Essential" Water of Suspended Enzymes

Noncovalent interactions such as hydrogen bonding, hydrophobic forces, and electrostatic and van der Waals interactions help to maintain an enzyme in its catalytically active form. Water plays an important role in all these interactions. Replacement of water by organic solvents should, therefore, directly affect the native structure and activity of an enzyme.

Organic solvents can interact with the water molecules bound to lyophilized enzyme powders as well as with the enzyme molecule itself. These interactions may take place in two steps. In the first step, water may be stripped from the enzyme. In the second step, the solvent may penetrate and interact directly with the protein, thereby affecting its native configuration and activity. In this regard, solvent hydrophilicity will determine the ability of the solvent to "strip" away the essential water. The more hydrophilic the solvent, the greater will be its tendency to strip water. Clearly, the direct interaction of a nonaqueous solvent with an enzyme would be expected to be deleterious to overall enzyme activity, and thus it is expected, a priori, that hydrophobic solvents are favorable when the goal is to minimize solvent–enzyme interactions.

Halling has analyzed the interaction between protein and water on a molecular level. His studies show that organic solvents have little effect on tightly bound water, since there is little penetration of the primary hydration layer in most solvents. Gorman and Dordick have looked at solvent-induced desorption of tritiated water from lyophilized chymotrypsin, subtilisin, and horseradish peroxidase suspended in anhydrous organic solvents. They report the highest degree of desorption by polar solvents such as *n*-propanol, *n*-butanol, methanol, and dimethylformamide. Wasacz et al., via Fourier transform infrared (FTIR) spectroscopy studies, have provided direct evidence of the stripping of water from albumin by methanol.

3.4 Interactions of Solvents with Substrates and Products

Organic solvents may reduce an enzyme's activity by adversely affecting either stability or partitioning of substrate or product. Chloroform, by acting as a phenoxy radical quencher, has been shown to reduce the activity of horseradish peroxidase catalyzed polymerization of phenols (a reaction initiated by the generation of phenoxy radicals). The partitioning of products between the enzyme and the reaction medium also depends on the relative hydrophobicities of the enzyme and all organic media. For instance, if the products formed are hydrophilic, they will tend to partition more onto the hydrated enzyme particle rather than into the bulk organic solvent. This may subsequently lead to product inhibition.

3.5 Structural Integrity of Enzymes Suspended in Organic Solvents

The first direct demonstration of structurally intact enzymes in anhydrous organic dispersants was made with electron paramagnetic resonance (EPR) spectroscopy. This study on an immobilized alcohol dehydrogenase indicated that the enzyme had not unfolded. While EPR can investigate only the environment and mobility of a spin label, the data nevertheless suggest that a protein can maintain its native conformation in an organic dispersant. Klibanov and colleagues have shown that the microenvironment of the active site histidine of α-lytic protease suspended in anhydrous acetone is identical in water and in organic dispersants. Solid state [^{15}N] nuclear magnetic resonance (NMR) spectroscopy was used to analyze the catalytic triad at the active site of this serine protease, and it was demonstrated that the hydrogen bond network at the active site of this serine protease does not change when the enzyme is suspended in organic solvents.

A complete understanding of structure–environment relationships requires the application of all possible techniques that provide information about protein structure. EPR and NMR are excellent techniques for investigating local regions of the protein. EPR, as stated earlier, is limited to the environment of a spin label, whereas NMR is limited by protein size and the type of investigation being performed. Many classical techniques for protein structure determination, such as circular dichroism and X-ray crystallography, are, of course, not possible for enzyme powders suspended in anhydrous organic solvents.

FTIR is a widely accepted method for the study of global protein structure in a variety of environments. The use of infrared spectroscopy in the study of proteins was pioneered by Elliott and Ambrose in 1950 and has been extensively applied since. Of particular relevance to this study is the use of FTIR techniques to detect small changes in global and local structure of proteins. The IR spectra of polymers such as proteins can be interpreted in terms of vibrations of structural repeat units. The vibration of a single repeat unit such as an α-helix or β-sheet can be separated from an otherwise complicated spectrum, enabling quantification of secondary structure. Nine groups of vibrational frequencies, manifested as characteristic bands in FTIR protein spectra, have been identified. Of these, amide I and amide II are the most useful infrared probes of protein structure. FTIR spectroscopy also is readily applicable to the study of enzymes in anhydrous environments, since the interference from the very strong absorption of water in the spectral region of interest is dramatically decreased. On the basis of a detailed study of conformationally sensitive infrared absorption frequencies, it is possible to detect changes in protein secondary structure that arise from alterations in the protein environments.

Earlier investigators have used FTIR methods to study the effect of organic solvents on global protein structure. In a series of papers, Wasacz and colleagues studied the effect of exchanging the solvent of a protein from water to alcohols. The expected findings were reported: namely, when water is gradually replaced by methanol (and other alcohols) as bulk solvent, structural changes are induced in the protein, and FTIR analysis indicates an increased helix content. This work does not contradict the long-recognized loss of activity of proteins that have been diluted with high concentrations of alcohols. In the 1970s solid protein samples were also dispersed in mineral oil, and their FTIR spectra were compared to protein dissolved in water. The authors expressed surprise that the spectra (for lysozyme) were similar, although their data agree with the earlier work of Dastoli and Price, who were the first investigators to measure kinetic constants for active lyophilized enzymes dispersed in a variety of solvents.

More recently Mantsch and colleagues have investigated the effect of halogenated alcohols on protein secondary structure. They report that partial unfolding, aggregation, and helix structure alterations result from increasing the mole fraction of solvent in the system. They also indicate that the helical structure of myoglobin in pure chloroethanol is indistinguishable from that in water, supporting the claim that protein secondary structure is not affected by neat organic dispersants.

Subtilisin is the most intensely studied enzyme that functions in organic media, although there is little information on its structural integrity in organic media. FTIR spectroscopy has also been used to analyze the global structure of subtilisin suspended in organic media and to determine the effect of organic dispersants on the global and local structure of myoglobin.

Figure 2 shows typical FTIR spectra for highly concentrated samples of subtilisin and myoglobin solubilized in aqueous solution and suspended in organic dispersants. The spectra were generated by deconvolution of spectra followed by subtraction of the water spectrum. The choice of organic solvent was guided by the need to match the density of dispersant and enzyme particle to prevent settling of the sample during data collection. As expected, subtilisin and myoglobin solubilized in aqueous media have different spectra, confirming that the FTIR technique is sufficiently sensitive to detect the structural differences between individual proteins. Table 1 presents a quantitative summary of the data. To test the validity of this method for the detection of protein unfolding, denaturation of subtilisin was induced by solvation with dimethyl sulfoxide (DMSO) followed by FTIR analysis. In a separate experiment, phenylmethylsulfonyl fluoride inhibited subtilisin dissolved in water was denatured by boiling. The use of the inhibitor was merely to prevent autolysis during the experiment. There is a clear difference in the spectra between the native and denatured proteins. In each case the amide I band splits into two discrete bands. It is interesting that the intensity of the bands is reversed for protein dissolved in aqueous and organic solvents. It has been recognized that solvents vary considerably in their helicogenicity. Since both DMSO and water can solubilize proteins, but have distinct helicogenicity, one would not expect the FTIR spectra to be similar for the same protein.

Figure 2 also presents the FTIR spectra for subtilisin (native and denatured) and myoglobin in carbon tetrachloride and mineral oil. As just described, differences in the shape, magnitude, and position of FTIR peaks indicate an altered structure of the protein. Clearly the global structures of subtilisin and myoglobin do not change radically when the solvent is changed from water to mineral oil or carbon tetrachloride. If subtilisin was unfolded in organic dispersants, or even if a fraction of the enzyme present was denatured, the global change in structure would be detectable by FTIR spectroscopy. In the organic dispersants tested one sees no such protein denaturation. Indeed, the spectra for both myoglobin and subtilisin in organic media are almost identical to those in aqueous solution.

There is a close correlation between the area of the amide I and II bands and protein secondary structure. Table 1 provides estimates for the total areas of each band for myoglobin and subtilisin in aqueous solution, and organic dispersant. In both environments there is a striking similarity not only between band positions but also with respect to the intensity of the amide peaks. Size and shape of the amide I and II bands are related to the α-helix, random coil, and β-pleated sheet content of proteins. An approximation (using the data shown) of the ratio of α-helix to β-sheet content supports the notion that the global structure of subtilisin does not change drastically upon suspension in an anhydrous solvent.

A separate experiment investigated the interaction between myoglobin and its prosthetic group in aqueous and organic environments. When the azide ion binds to the heme iron of hemoproteins the antisymmetric stretching frequency shifts from 2049 cm^{-1} to 2045 and 2023 cm^{-1}, which corresponds to azide bound to high and low spin irons, respectively. The half-width of the antisymmetric stretch also decreases from 22 cm^{-1} to about 10 cm^{-1}. The active site of lyophilized horse azidometmyoglobin suspended in mineral oil or carbon tetrachloride has been examined and compared to that of the acid denatured suspension of azidometmyoglobin in water.

In the organic dispersant studied, the positions of the azide antisymmetric stretches do not appear to shift relative to the aqueous protein, although the half-widths do increase to about 20 cm^{-1} (Figure 3A–C). It is also clear from our measurements that the iron spin state equilibrium, which favors the low spin species, is not significantly perturbed, since the shoulder corresponding to the high spin species appears to be only half as intense as the low spin peak. The acid-denatured azidometmyoglobin shows only a single peak at 2055 cm^{-1}, with a half-width of about 21 cm^{-1} (Figure 3D). This is higher in energy than the free azide (2049 cm^{-1}) but still lower than the aqueous hydrogen azide (2148 cm^{-1}).

From Figures 2 and 3 one can conclude that there is no change in the secondary structure of subtilisin and myoglobin upon exposure

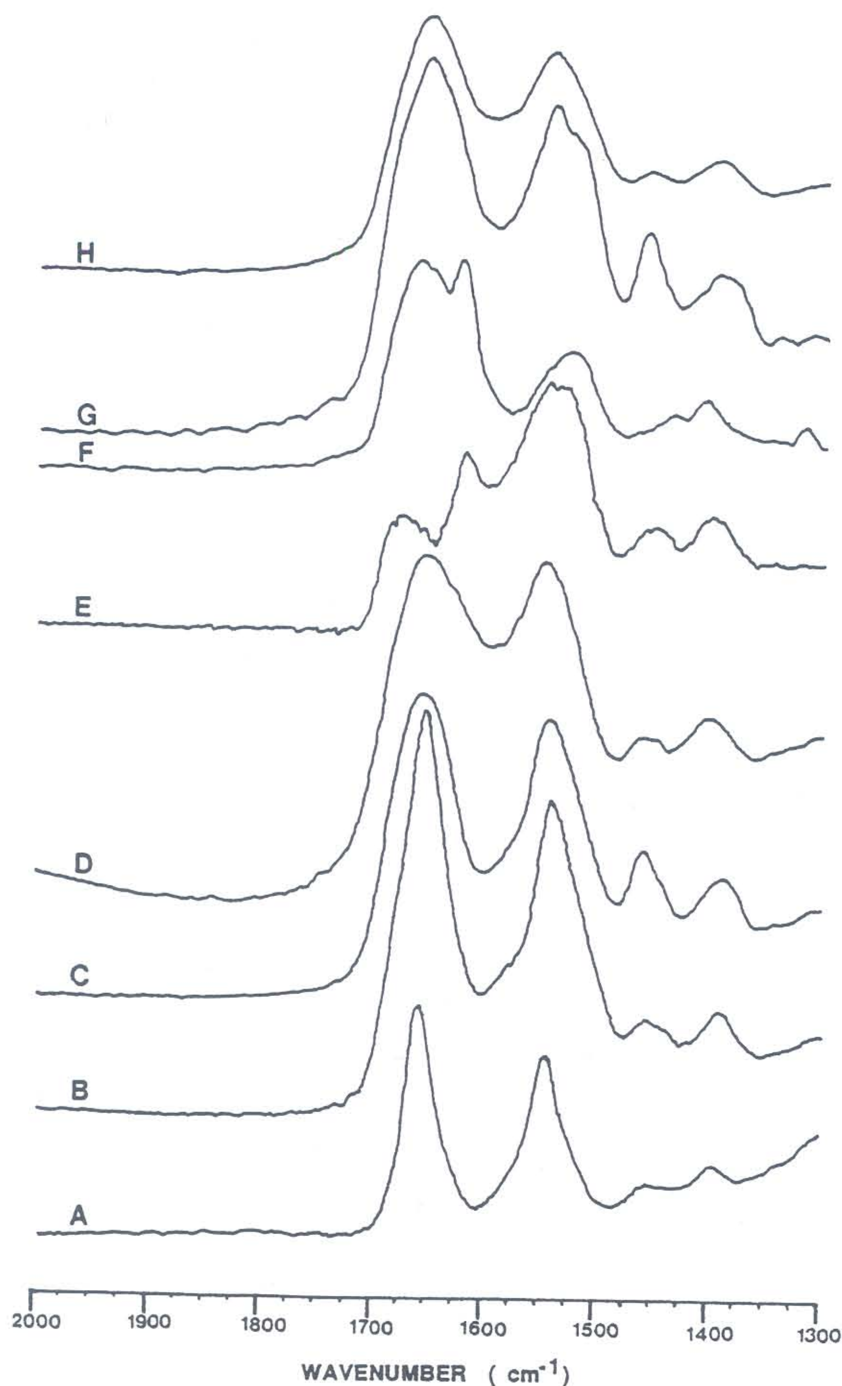

Figure 2. FTIR spectra of myoglobin and subtilisin in aqueous and organic media: (A) myoglobin in aqueous solution, (B) lyophilized myoglobin dispersed in carbon tetrachloride, (C) lyophilized myoglobin dispersed in mineral oil, (D) subtilisin in aqueous solution, (E) boiled phenazine methosulfate–subtilisin in aqueous solution, (F) lyophilized subtilisin in dimethyl sulfoxide, (G) lyophilized subtilisin dispersed in mineral oil, (H) lyophilized subtilisin dispersed in carbon tetrachloride.

of powdered preparations of the enzyme to carbon tetrachloride and mineral oil. In addition, the data for azidometmyoglobin suggest that there is no significant change in the protein structure around the heme-binding site, although protein structural changes that affect the bandwidths of the azide antisymmetric stretch may occur.

Subtilisin, a serine protease, is catalytically active in carbon tetrachloride, although the dispersant is more hydrophilic than those recommended for use with biocatalysts. It is interesting to note that when subtilisin is suspended in carbon tetrachloride, the water associated with the enzyme is partitioned into the dispersant, which hypothetically results in decreased activity of the enzyme. Clearly, the removal of water from the enzyme surface by the dispersant does not result in a significant structural alteration, and the lowered activity must be explained in other ways.

Table 1 Amide I and II frequencies for myoglobin and subtilisin in aqueous and nonaqueous environments.

Protein	Solvent/Dispersant	Amide I Frequency (cm^{-1}, (%))[a]	Amide II Frequency (cm^{-1}, (%))
PMS-Subtilisin	Water	1653.2, (62±1)	1547.1, (38±1)
Subtilisin (Denatured)[b]	DMSO	1662, 1626, (28±5)	1526, (72±10)
PMS-Subtilisin (Denatured)[c]	Water	1676, 1617, (80±10)	1539, (20±4)
Subtilisin	Mineral oil	1651.3, (62±1)	1537.4, (38±1)
Subtilisin	CCl_4	1649.3, (63±1)	1541.3, (37±1)
Myoglobin	Water	1655.1, (52±1)	1547.1, (48±1)
Myoglobin	Mineral oil	1655.1, (55±1)	1541.3, (45±1)
Myoglobin	CCl_4	1655.0, (57±1)	1541.0, (43±1)

[a]The individual areas for the spectra in Figure 1 were approximated using the trapezoidal rule.
[b]Subtilisin was resuspended in dimethylsulfoxide to facilitate denaturation.
[c]Subtilisin was first inactivated with phenylmethylsulfonyl fluoride, then denatured by boiling in buffer for 5 minutes prior to performing FT-IR.

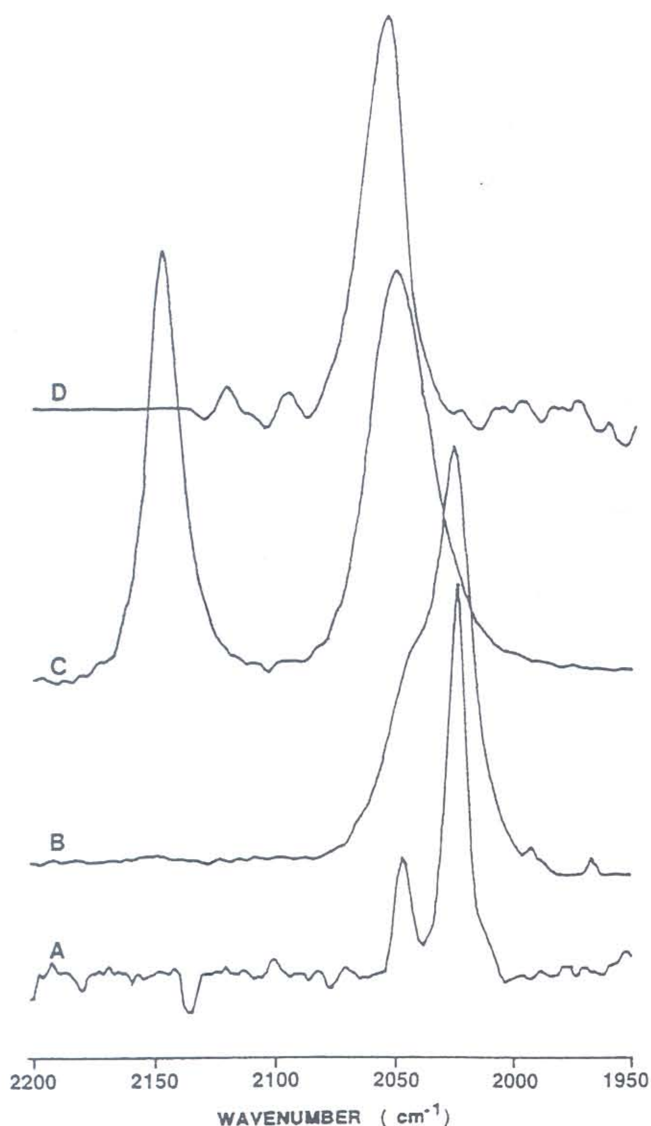

Figure 3. FTIR Spectra of azidometmyoglobin: (A) azidometmyoglobin in aqueous solution, (B) azidometmyoglobin in mineral oil, (C) free azide and hydrogen azide in aqueous solution, (D) acid-denatured azidometmyoglobin in aqueous solution.

3.6 Thermostability of Enzymes in Organic Media

At high temperatures, enzyme inactivation in aqueous media is caused by protein unfolding, covalent alterations in the primary structure of the protein, or both. Water plays a primary role in both types of inactivation by facilitating protein unfolding through increasing protein mobility; water also participates as a substrate in the processes of disulfide bond interchange, deamidation of glutamine and asparagine residues, and hydrolysis of peptide bonds. Hence, it is not surprising that the absence of water in nonaqueous environments positively affects enzyme thermostability.

Studies with lipases, mitochondrial F_1-ATPase, and cytochrome oxidase have shown these enzymes to be more active and stable in organic solvents at higher temperatures than when dissolved in water. Often, increased thermostability is accompanied by increased stability toward other denaturing effects such as proteolysis.

Volkin et al. have analyzed the thermoinactivation of three unrelated enzymes (ribonuclease, chymotrypsin, and lysozyme) between 110 and 145°C in anhydrous organic media. They found these enzymes to be more thermostable in hydrophobic solvents, such as butanol and nonane, than in hydrophilic solvents such as dimethylformamide. The thermostability of ribonuclease in nonane was attributed to increasing water content of the lyophilized enzyme powder. At high temperature the aggregation of ribonuclease molecules was found to be due to both physical association and chemical cross-linking. In another study, Ahern and Klibanov analyzed the mechanism of irreversible thermoinactivation of hen egg lysozyme at 100°C. Their results showed that deamidation of asparagine residue, hydrolysis of peptide bonds at aspartic acid residues, and the formation of incorrect (scrambled) structures all contribute to enzyme inactivation.

3.7 Effects of Organic Solvents on Enzyme Kinetics

As mentioned earlier, organic solvents may affect rates of enzymatic reactions either directly or indirectly. One can consider the binding of solvent molecules to be a direct effect; indirect effects include the partitioning of substrates and products between the solvent and enzyme, the shift of chemical equilibria, and certain mass transfer limitations that are due to the organic solvent. The overall effects are manifested in changes of kinetic parameters for biocatalyzed reactions upon manipulation of the solvent environment. In a study with methanol–water mixtures, methanol was shown to

compete with water for the binding sites in the active site of α-chymotrypsin, resulting in a decrease of enzyme activity during the hydrolysis of an ester of hydrocinnamic acid.

Serine proteases, such as α-chymotrypsin and subtilisin, are excellent model systems for studying the effect of solvent on biocatalyst kinetic properties. These enzymes catalyze the hydrolysis of ester and amide substrates via the acyl–enzyme mechanism. In the first step the enzyme and substrate associate noncovalently. Next the hydroxyl group of the serine residue attacks the carbonyl group on the substrate, leading to the formation of a tetrahedral intermediate, which collapses to release the first product, an alcohol or an amine, and the covalent acyl–enzyme complex. This is followed by the attack on the acyl-enzyme complex by the second nucleophile, water, to give rise to an enzyme–product complex, which subsequently breaks down to form the second product, an acid, and releases the free enzyme. It has been reported that in the α-chymotrypsin-catalyzed hydrolysis of an amide substrate (where acylation is rate determining), the addition of organic solvents to water has no effect on k_2, the acylation rate constant. This is because water is not involved in the rate-determining acylation step. However, for an ester substrate (where deacylation is rate controlling), the deacylation rate constant, k_3, was found to increase when the concentration of water in an organic solvent reaction system was increased, implicating the direct involvement of water in the rate-determining deacylation step.

4 SOLVENT ENGINEERING

Conventional biocatalysis is carried out in aqueous media; therefore, it is not surprising that most methods developed to optimize enzyme performance are water based. Nonaqueous enzymology offers a new route for control of enzyme properties by "solvent engineering," which affords a means for the control of enzyme function by changing reaction medium, rather than by changing the enzyme itself.

4.1 Control of Enzyme Activity by Solvents

Solvents can affect enzyme activity by affecting substrate partitioning between the enzyme's active site and the solvent. For example, in water, hydrophobic substrates like esters of phenylalanine and tyrosine easily partition into the active sites of the enzymes, which are lined with hydrophobic amino acid residues. In organic media, however, hydrophobic solvents reduce the substrate partitioning into the active site thereby causing a decrease in rate. Zaks and Klibanov have measured rates of transesterification of *N*-acetyl-L-phenylalanine ethyl ester with propanol in a series of solvents using both subtilisin and chymotrypsin. Their study showed enzyme activity to be a function of solvent hydrophilicity. It was hypothesized that the more hydrophilic the solvent, the greater will be its tendency to interact with the enzyme's essential water, and thus adversely influence enzyme structure and activity.

4.2 Control of Selectivity of Enzymes by Solvents

In general, enzymes retain their stereoselectivity in organic media. The release of water from a hydrophobic binding site upon correct binding of a substrate will be more energetically favored in water than in organic solvents. One would not expect incorrect binding of inappropriate enantiomers to be affected to such a significant extent. Hence, in some cases nonaqueous media relax an enzyme's enantioselectivity by weakening the interaction of an enzyme with its preferred substrate.

Changing of the reaction medium can also bring about an alteration in substrate specificity. In water, α-chymotrypsin catalyzes the hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester, a hydrophobic substrate, 10^5 times faster than that of *N*-acetyl-L-serine methyl ester, a hydrophilic substrate. However, in organic solvents the enzyme is five times more reactive toward the hydrophilic substrate. It has been suggested that the nature of the solvent can modulate enzyme specificity. In water, for instance, the binding of hydrophobic substrates to hydrophobic enzyme active sites is driven by the entropy increase resulting from the expulsion of the ordered water molecules surrounding both the enzyme pocket and substrate. In organic solvents this process will be unfavorable relative to organic media. The process will, however, be favored in hydrophilic rather than in hydrophobic solvents. Thus changing the solvent will have an effect on the binding of substrates to enzymes (in terms of binding, hydrophilic will be better than hydrophobic). In addition, by changing solvents it may be possible to fine-tune enantioselectivity to a predetermined degree.

Substrate specificity of α-chymotrypsin-catalyzed esterification in organic media was investigated by Clapes and Adlercreutz. The study shows that the specificity of chymotrypsin toward the side chains of the amino acid substrates is the same as that in water. However, the enzyme specificity toward the N-protecting group is the reverse of that in water. In water, the specificity constant of esterification was found to increase proportionally with the hydrophobicity of the N-protecting group, whereas in organic solvents, the reverse was found to be true. These studies demonstrate the potential of using solvent engineering to control enzyme selectivity and specificity.

4.3 Control of Enzyme Stability by Organic Solvents

Engineering of proteins to improve conformational stability and stability toward denaturants, oxidants, proteolysis, and high temperatures is an elusive goal. Protein engineering uses techniques such as site-specific mutagenesis to engineer disulfide bonds into globular proteins for the purpose of enhancing conformational and proteolytic stability. Phenotype screening of random mutants is often used to screen for mutants with enhanced thermostability. Enzymes in organic media have shown improvements in both catalytic and storage stability. α-Chymotrypsin incubated in anhydrous octane exhibited a half-life of 6 months, whereas in water the half-life is reduced to 1 week. At the same time, the half-life was observed to be a function of the nature of the medium. The enzyme had half-lives of 130, 80, 35, and 1.5 minutes in butyl ether, *tert*-amyl alcohol, dioxane, and pyridine, respectively. Obviously, in respect of stability, the best solvents will be those able to keep an enzyme in its native conformation via solvophobic interactions.

Khmelnitsky has evaluated the denaturation capacity of a series of organic solvents while working with α-chymotrypsin, trypsin, lactase, chymotrypsinogen, cytochrome *c*, and myoglobin. Denaturation capacities were reported in terms of a threshold concentration, which is defined as the concentration of added organic solvent at which the enzyme loses half its initial activity. For the enzymes studied, abrupt changes in spectra of the dissolved enzyme were noted at this threshold concentration. This implies that the drop in

enzyme activity was specifically due to protein denaturation. They also noted that there is a critical water content that is necessary for enzyme activity. Once the enzyme has dehydrated beyond this critical value, denaturation sets in. In a separate study, Zaks and Klibanov, while working with alcohol dehydrogenase, polyphenol oxidase, and alcohol oxidase, also noticed that the catalytic activity of the enzyme was a function of the water content of the enzyme.

Schulze and Klibanov have investigated the effects of solvents, substrates, water content of medium, and pH prior to lyophilization on the stability of subtilisin in acetonitrile and in *tert*-amyl alcohol. They observed that enzyme lyophilization at the pH optima for activity or incubation in the presence of substrates/ligands had a positive effect on enzyme stability. On the other hand, the addition to the reaction medium of exogenous water adversely affected enzyme stability. Other efforts at solvent engineering include the investigation of the effects of additives such as glycerols and polyols in aiding a protein to maintain its native structure in organic media via solvophobic interactions. Efforts have also been made to optimize esterification reactions by removal of water produced during reactions using loop reactors or reduced pressure. Finally, another example of solvent engineering is the alteration of enzyme activity by the addition of water. It has been observed that the addition of water to α-chymotrypsin and lipases elevates enzyme activity and influences enzyme stereoselectivity, respectively.

5 MECHANISTIC INTEGRITY OF ENZYMES IN ORGANIC MEDIA

Before comparing an enzyme's activities in different media, it is important to determine its structural and mechanistic integrity of enzyme in these media. For if the mechanism and structure were to change from solvent to solvent, it would be impossible to correlate activity to the properties of the solvent alone. Initial studies on mechanisms in organic solvents with porcine pancreatic lipase and PEG–chymotrypsin have indicated that the enzymes obey Michaelis–Menten kinetics in anhydrous organic solvents. Analyses of reaction mechanisms via the conventional parallel lines method have indicated that the acyl–enzyme mechanism holds true even in nonaqueous media.

Linear free energy relations have been used to prove the transition state structures in organic solvents by Hammett analyses. Hammett analysis, which investigates the charge distribution of the transition state, is very sensitive to reaction mechanism. For esters, it has been reported that the Hammett values are almost the same for hydrolysis in water, and for transesterification in tetrahydrofuran, acetone, butyl ether, acetonitrile and *tert*-amyl alcohol using subtilisin. This result not only provides evidence of the acyl–enzyme mechanism but also implies that the active site of subtilisin and the transition state structure are equivalent in water and organic media.

Investigation of kinetic isotope effects on labeled substrates of subtilisin in various organic solvents and in water has shown that the magnitude of the primary deuterium kinetic isotope effect is the same in different solvents. This study, too, indicates that the transition state structure formed during transesterification and hydrolysis are independent of the nature of the reaction medium. Although the above-mentioned mechanistic studies point toward an acyl–enzyme mechanism for subtilisin-catalyzed transesterification reactions in organic media, there is no direct proof of the mechanism, since the acyl–enzyme complex has never been isolated or detected in organic solvents. Hence, studies on the mechanism of subtilisin-catalyzed reactions in organic media are by no means complete.

The log *P* model, which relates enzyme activity to solvent hydrophobicity, is most frequently used to define the ability of a solvent to support catalytic activity. However, this simple model provides neither a kinetic nor a mechanistic basis for the explanation of biocatalysis, nor does it consider the diminished binding of substrate to enzyme in organic media. It has value only because it can be used as the first step to choose solvents that support catalytic activity and also to determine suitable reaction conditions.

To successfully control enzyme activity and specificity in organic media, the effects of other solvent properties (e.g., solvent viscosity, density, hydrophobicity, dielectric constant, dipole moment) also need to be considered. To date, all the kinetic studies in organic solvents have related enzyme function and structure to the apparent kinetic constant k_{cat}, K_m, and k_{cat}/K_m. The macroscopic kinetic constants are a function of the microscopic rate constants for the individual steps of a reaction mechanism. Determination of the individual rate constants is therefore necessary for an understanding of the effect of solvent on the individual steps of a multistep reaction mechanism.

6 THE FUTURE OF NONAQUEOUS ENZYMOLOGY

The opportunities presented by nonaqueous biocatalysis are limited only by our imaginations. In just one decade, this area of research has become one of the most active research areas in science and engineering. While the technology has already been commercialized, full-scale adoption of nonaqueous enzymology is dependent on the generation of a predictive model to correlate the effects of solvent properties to the binding and catalytic steps of an enzyme-catalyzed reaction. The design of bioreactors, and the development of upstream and downstream processing units for nonaqueous enzymology, are also central to further utilization of this core technology. The clear need for biocatalysts in industry should not obscure what the use of enzymes in extreme environments can teach us about the molecular basis of enzyme catalysis. Current research is already demonstrating how the delicate interaction between environment and biological molecules serves to modulate function.

See also CHIRALITY IN BIOLOGY; ENZYMES, ENERGETICS AND REGULATION OF BIOLOGICAL CATALYSIS BY.

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EPIDEMIOLOGY, MOLECULAR

Paul A. Schulte, Frederica P. Perera, and Nathaniel Rothman

1 **Principles**
- 1.1 Biologic Markers of Exposure
- 1.2 Biologic Markers of Effect
- 1.3 Biologic Markers of Susceptibility
- 1.4 Utility of Molecular Epidemiology

2 **Techniques**
- 2.1 Representational Validity of Molecular Biological Markers
- 2.2 Validation of the Behavior of Molecular Biological Markers
- 2.3 Etiologic Studies
- 2.4 Public Health Applications

Key Words

Biological Markers (Biomarkers) Biochemical, molecular, genetic, immunologic, or physiologic signals of events in biological systems.

Biologic Marker of Effect A measurable cellular, biochemical, or molecular alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease.

Biologic Marker of Exposure A xenobiotic chemical or its metabolite, or the product of an interaction between a chemical, physical, or biologic agent and some target cell or biomolecule.

Biologic Marker of Susceptibility An inherited or acquired indicator of the response of an individual or a population to a specific xenobiotic agent.

Molecular epidemiology is the use of molecular biological techniques to identify exposures, effects, or susceptibility factors in studies of human populations. Molecular epidemiology and traditional epidemiology utilize the same paradigm. However, the former presents the opportunity to use the enhanced resolving power of molecular biology in the assessment of exposure–disease relationships. The resolving power, to elucidate a continuum of events between xenobiotic exposure and disease, can provide stronger approaches to research, prevention, and intervention.

1 PRINCIPLES

The use of molecular biological techniques in epidemiology provides a potentially powerful tool for medical and public health researchers. These techniques allow for the identification of biological markers (Figure 1) that can indicate exposure to a xenobiotic agent, reveal a biological effect early in the natural history of disease, or represent unique disease subtypes or susceptibility to the development of disease. Although the use of biological markers in epidemiology is not new, the current generation of molecular biological markers enhances past approaches. Epidemiology is an observational science: one makes inferences about disease and health based on comparing groups of people in terms of disease incidence and mortality. Ideally, the groups being compared should be similar in all respects except for the risk factor in question. A benefit of molecular epidemiology is that instead of comparing two groups on the basis of environmental exposure, investigators can compare populations with respect to dose of an environmental agent as measured in critical macromolecules, such as DNA or surrogate proteins for DNA. That is presumably a more accurate means of classifying the subjects' true exposure. At the other end of the exposure–disease

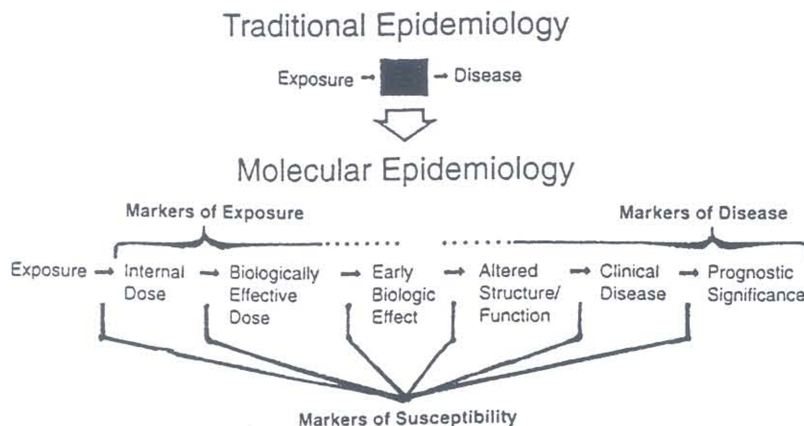

Figure 1. Enhancement of the traditional epidemiologic paradigm by the use of biological markers resulting in a molecular epidemiologic approach. In traditional epidemiology the mechanism of action is often a "black box," and associations between an exposure and disease are made by inference. In molecular epidemiology a continuum between an exposure and a disease is defined, and various markers are identified.