Characteristics of nearly dry enzymes in organic solvents: implications for biocatalysis in the absence of water

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We have examined enzymes in nearly anhydrous organic solvents spanning a wide range of dielectric constants using a combination of electron paramagnetic resonance (EPR) spectroscopy, molecular dynamics simulations, high-pressure kinetic studies and the electrostatic model of Kirkwood. This approach enabled us to investigate the relationship between catalytic activity, protein flexibility and solvent polarity for an enzymatic reaction proceeding through a highly polar transition state in the near absence of water. Further insights into water–protein interactions and the involvement of water in enzyme structure and function have been obtained by EPR and multinuclear nuclear magnetic resonance studies of enzymes suspended and immobilized in organic solvents with and without added water. In these systems, correlations were observed between the water content and enzyme activity, flexibility, and active-site polarity, although the structural properties of suspended and immobilized enzymes differed markedly. These results have helped to elucidate the role of water in molecular events at the enzymic active site leading to improved biocatalysis in low-water environments.

Keywords: non-aqueous enzymology; salt activation; electron paramagnetic resonance; nuclear magnetic resonance; Kirkwood analysis

1. INTRODUCTION

Non-aqueous solvents, in which enzymes remain catalytically active and often exhibit enhanced thermal stability, are emerging as versatile media for fundamental studies of enzyme structure and function. In particular, nearly anhydrous organic solvents enable studies of protein properties in low-dielectric environments in the absence of bulk water. Such studies are an important step towards answering the question of whether enzymes can function in the complete absence of water, either in a gas atmosphere or in a non-aqueous liquid solvent. The present paper, based largely on work performed in our own laboratory or in collaboration with J. S. Dordick and co-workers, describes results that illuminate important determinants of enzyme function in low-water environments, as well as an effective strategy that compensates for the deleterious effects of an organic medium containing minimal water. In all, these findings suggest that enzymatic catalysis in a totally dry (i.e. water-free) environment would not be prohibited by the rigid nature of the protein or by the absence of water *per se*, provided that a catalytically competent conformation is maintained and adequate transition-state stabilization is achieved. Thus, it may be possible to expand the landscape of biocatalysis to include completely dry terrain.

2. ENZYME ACTIVITY AND PROTEIN DYNAMICS IN NEARLY ANHYDROUS SOLVENTS

In addition to their value as model systems for investigating enzyme function in non-aqueous media, enzymes in organic solvents are of practical interest for a variety of reasons. For example, despite its advantages as a non-toxic natural solvent, water is a very poor solvent for many synthetic organic reactions, and indeed many enzymatic reactions that do not occur to a significant extent in water proceed readily in organic solvents. However, enzymatic reaction rates in organic media are typically very low, often 4–5 orders of magnitude lower than their aqueous counterparts, as illustrated by the catalytic efficiencies presented in table 1, measured for a stereoselective transesterification reaction catalysed by the protease subtilisin BPN' in various nearly anhydrous solvents. Because these organic solvents contain only trace amounts of water, the enzyme does not dissolve; nonetheless, active-site titration assays indicate that the suspended enzyme presents a comparable number of active sites for catalysis as the same quantity of enzyme dissolved in water. However, compared with the corresponding hydrolysis reaction catalysed by enzyme dissolved in water, the reaction rates are almost negligible. Although the low activity of enzymes in non-aqueous media has been clearly recognized for some time, until recently it was not clear why the rates are so low, and what, if anything, can be done to improve them. As discussed below, our work in this area has addressed these particular issues, and in the process provided new insights into the importance and functions of water in enzymatic structure and function.

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Table 1. Catalytic efficiencies of subtilisin BPN' for the transesterification of acetyl-L-phenylalanine ethyl ester (APEE) with n-propanol in various organic solvents (Michels *et al.* 1997). (Each solvent was dehydrated by storage over 3 Å molecular sieves for at least 24 h immediately prior to use.)

solvent	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1} \text{s}^{-1}$)
water	2800
acetone	0.95
<i>t</i> -amyl alcohol	0.60
propyl ether	0.44
hexane	0.43
2-propanol	0.26
toluene	0.03
2-heptanone	0.018
cyclohexanone	0.016
3-heptanone	0.006

(a) **Kirkwood analysis: transition state maintained in predominantly non-aqueous media**

A mechanistic understanding of an enzyme's reaction rate requires knowledge of the transition state. To examine the nature of the enzymatic transition state in organic solvents, Michels *et al.* (1997) took the simple, yet novel, approach of considering Kirkwood theory as it applies to the kinetics of ionization. Kirkwood theory assumes that a solute molecule can be approximated as a collection of discrete point charges in a sphere of radius r , in which the dielectric constant is 1, surrounded by a solvent with a uniform dielectric constant. With this approach, which accounts only for electrostatic forces, the solvation energy of the solute is equal to the energy required to create the charge distribution of the solute in the dielectric cavity, which is given by equation (2.1), where μ is the dipole moment of the solute, N_{AV} is Avagadro's number, ϵ_0 is the vacuum permittivity and ϵ is the dielectric constant:

$$\Delta G_{\text{solv},i} = \frac{N_{\text{av}}}{4\pi\epsilon_0} \left(\frac{\mu^2}{r^3} \right) \frac{(1 - \epsilon)}{(2\epsilon + 1)}. \quad (2.1)$$

In the particular case of an enzymatic reaction, the enzyme molecule becomes the solvent, and the solute becomes the transition state. Straightforward application of transition-state theory then provides equation (2.2) for the natural log of $k_{\text{cat}}/K_{\text{m}}$, where μ_{TS} is the dipole moment of the transition state, and μ_{E} and μ_{S} are the dipole moments of the enzyme and substrate in their ground states, respectively. A more reliable relationship is obtained by taking the derivative of equation (2.2) with respect to pressure, which yields equation (2.3), an expression that relates the activation volume of the reaction to the transition-state dipole moment through the derivative of the so-called dielectric constant factor (abbreviated as q in figure 1). Despite dating back to 1934 (Kirkwood 1934), before the work of Michels *et al.* (1997) Kirkwood theory had never been applied to an enzymatic reaction, begging the question of whether it could aptly describe enzymatic catalysis in non-aqueous media.

$$\ln \left(\frac{k_{\text{cat}}}{K_{\text{M}}} \right) = \ln \left(\frac{k_{\text{cat}}}{K_{\text{M}}} \right)_{\text{int}} + \frac{N_{\text{av}}}{RT} \left(\frac{\mu_{\text{TS}}^2}{r_{\text{TS}}^3} - \frac{\mu_{\text{E}}^2}{r_{\text{E}}^3} - \frac{\mu_{\text{S}}^2}{r_{\text{S}}^3} \right) \left\{ \frac{\epsilon - 1}{2\epsilon + 1} \right\}, \quad (2.2)$$

Figure 1. Kirkwood plot for the apparent activation volumes of subtilisin-catalysed transesterification in organic solvents. (Reproduced from Michels *et al.* 1997, with permission.)

$$-RT \left(\frac{\partial \ln(k_{\text{cat}}/K_{\text{m}})}{\partial P} \right)_T = \Delta V^* = \Delta V_{\text{int}}^* - N_{\text{av}} \left(\frac{\mu_{\text{TS}}^2}{r_{\text{TS}}^3} - \frac{\mu_{\text{E}}^2}{r_{\text{E}}^3} - \frac{\mu_{\text{S}}^2}{r_{\text{S}}^3} \right) \frac{\partial \{(\epsilon - 1)/(2\epsilon + 1)\}}{\partial P}. \quad (2.3)$$

It transpires that the answer is yes, at least in the case of transesterification catalysed by subtilisin. Activation volumes for the transesterification reaction in various organic solvents are plotted in figure 1, revealing a linear relationship that is described remarkably well by equation (2.3). Furthermore, the slope of the line reveals a dipole moment for the transition state of 31.5 D, which compares very well with 35 D, the transition-state dipole moment for the hydrolysis reaction in water estimated using an entirely different approach (Lamotte-Brasseur *et al.* 1990). Thus, Kirkwood theory proved to be a useful tool for probing the transition state of subtilisin catalysis in low-water environments. Moreover, given the highly polar nature of the enzyme's transition state in organic solvents, it is easy to understand why the reaction rates in non-polar organic solvents (which are not well matched to the transition state) are so low. Other factors may also be important, however, such as the flexibility of subtilisin in organic media, a topic that is considered next.

(b) **EPR spectroscopy and spin-labelling studies of enzyme flexibility: does activity correlate with dynamics?**

An intriguing hypothesis put forth in the early days of non-aqueous enzymology was that proteins are much more rigid in organic media (Zaks & Klibanov 1988), although at the time there was little direct physical evidence to confirm this notion. A closely related question is whether the rigid nature of enzymes in organic solvents is largely responsible for their low activity. Therefore we set out to determine whether proteins are in fact more rigid in organic media, and whether protein flexibility does indeed have a major impact on activity.

Our initial efforts to study the dynamics of proteins in organic solvents relied on EPR spectroscopy and the spin-labelling technique. In this connection, figure 2 depicts

Figure 2. (a) Schematic of nitroxide spin label covalently attached to Ser-195 in the active site of α -CT. Experimental (solid lines) and simulated (dotted lines) room-temperature EPR spectra of immobilized (b) and suspended (c) α -CT spin-labelled at Met-192 with 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinyl-1-oxyl. Simulation of the EPR spectrum of spin-labelled α -CT suspended in THF containing 7% v/v water (not shown) indicated a rotational correlation time of 7 ns, further illustrating the role of water as a lubricating agent that increases enzyme flexibility.

the serine protease α -CT with a nitroxide spin label covalently attached to serine 195 within the enzyme's active site. By recording the EPR spectrum of the spin-labelled enzyme, one can probe the dynamics of the spin label, together with certain other features of the spin-label's microenvironment. Moreover, by modelling the motion of the spin label in a three-dimensional coordinate system defined with respect to the spin label itself, the motion of the spin label can be quantified in terms of two rotational correlation times, or an average rotational correlation time, τ_R , as shown in figure 2.

For example, the EPR spectrum of the spin-labelled enzyme suspended in dry THF reveals a very rigid structure characterized by an average rotational correlation time for the spin label equal to *ca.* 100 ns (figure 2c). However, when 7% v/v water is added to the organic solvent, the spectrum reveals much greater spin-label mobility, and the average rotational correlation time determined by simulations decreases to 7 ns (data not shown). For comparison, figure 2 also shows the EPR spectrum of spin-labelled immobilized (i.e. glass-adsorbed) chymotrypsin recorded in THF containing 7% water, for which τ_R equals 1.6 ns. Therefore, the enzyme does appear to be much more rigid in the dry organic solvent, and water seems to act as a plasticizer by increasing the spin label's mobility.

EPR results for α -CT spin-labelled at two separate locations within the enzyme molecule are shown in figure 3 for a series of dry organic solvents (Affleck *et al.* 1992a). These results reveal that the motion is highly sensitive to the solvent, and that the rotational correlation time decreases, i.e. the motion increases, as the dielectric constant of the solvent increases. Moreover, in each case, the dependence between τ_R and the dielectric constant ϵ follows a similar power-law relationship.

To test whether the motion of the spin label truly reflects the dynamics of the surrounding protein, we also

performed molecular dynamics simulations of the enzyme's carbon backbone atoms, and found that although the motions of the backbone carbon atoms were faster than the attached spin label, as expected, they followed a very similar dependence on the solvent dielectric constant (Affleck *et al.* 1992a). Therefore, these results confirm the expectation that the enzyme is more rigid in organic solvents than in water, and that the dynamics decrease as the dielectric constant of the solvent decreases. Water molecules remaining on a protein in an organic solvent may therefore increase the local dielectric constant near charged residues, thus reducing the net electrostatic force between charges and enabling greater flexibility.

The highly polar transition state and decreased overall flexibility revealed for subtilisin and α -CT in organic media suggest that enzymatic activity would be higher in more polar solvents (i.e. solvents of higher dielectric constant), which should afford greater transition-state stabilization as well as a more flexible active site. However, efforts to obtain the expected correlation between enzyme activity in organic solvents and either protein flexibility or solvent dielectric constant, using in this case α -CT, which uses the same general catalytic mechanism as subtilisin, produced a surprising result. As shown by the rate data in figure 4 and the corresponding plot of k_{cat}/K_m versus the backbone correlation time determined from molecular dynamics calculations, α -CT is most active in octane, the least polar solvent and the one in which it is most rigid, and is least active in acetonitrile, the most polar solvent, in which it is most flexible.

These results reveal an apparent paradox: contrary to expectations, the rate of an enzymatic reaction with a highly polar transition state *decreases* as solvent polarity increases and protein flexibility increases. More polar organic solvents have been shown to strip away more of the labile water remaining on freeze-dried enzymes

Figure 3. Rotational correlation times of enzyme-bound spin labels determined from spectral simulations of EPR spectra (a,b), and correlation times of α -carbon atoms determined from molecular dynamics simulations (c,d), for α -CT in solvents with dielectric constants ranging from 1.89 (hexane) to 46.7 (dimethyl sulphoxide). (Reproduced from Affleck *et al.* (1992a), with permission.)

Figure 4. (a) Values of $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ ($\text{M}^{-1} \text{s}^{-1}$) $\times 100$ for glass-adsorbed α -CT in various organic solvents (Suzawa *et al.* 1995); (b) Catalytic efficiencies versus correlation times of Met 192 α -carbon atom determined from molecular dynamics simulations performed for the same solvents. (Affleck *et al.* (1992a).)

(Gorman & Dordick 1992), and potential stripping effects have not been investigated for the samples included in figure 4; nor have ground-state stabilization effects, which can also be an important determinant of enzymatic activity across different organic solvents (Kim *et al.* 2000). None-

theless, these results indicate that relatively high enzyme activity can be obtained in non-aqueous solvents whose properties are non-conducive to transition-state stabilization and protein flexibility, two presumed activators of biocatalysis often thought to be facilitated by water.

3. HYDRATION OF ENZYMES IN ORGANIC SOLVENTS: LUBRICATING THE ACTIVE SITE TO IMPROVE FUNCTION

(a) EPR measurements of active-site polarity

We have also examined enzyme structure and function in a single organic solvent, namely THF, specifically as a function of added water. Investigating the effects of added water has provided insights into molecular events at the enzyme's active site leading to improved catalysis in low-water environments. In this regard, another important physico-chemical property that can be probed by EPR spectroscopy is the polarity of the local environment. Briefly, because the free energy of the nitroxide free radical is sensitive to the polarity of the spin-label's environment, a spectral parameter known as the hyperfine splitting constant, A_O , is a measure of the local polarity. A_O values for a free or enzyme-bound spin label can be determined from a simulation of the rigid-limit spectrum recorded at low temperature.

Having access to information on dynamics and polarity enabled us to make comparisons such as that shown in figure 5 for two subtilisins, subtilisin Carlsberg and BPN'. Figure 5 compares the catalytic activity, as indicated by the rate constant for irreversible enzyme inhibition by phenylmethylsulphonyl fluoride, the active-site polarity as measured by A_O , and protein flexibility as measured by the average rotational correlation time of an active-site spin label, all as a function of water added to THF. These results reveal that the addition of water affects all of these properties simultaneously, suggesting that the increase in enzyme activity is the result of higher active-site polarity, or greater flexibility, or both. In the case of subtilisin BPN', it appears that increased flexibility may play a dominant role, whereas for subtilisin Carlsberg, it is difficult to separate one effect from the other.

(b) Multinuclear NMR studies of subtilisin hydration

Clearly, water can have both direct and indirect effects that contribute to more efficient catalysis in organic media, but what about the water itself? To learn more about the interaction between added water and the enzyme, multinuclear NMR studies of subtilisin hydration were carried out in THF (Lee *et al.* 1998). For example, when ^{17}O NMR was used to measure the chemical shift of ^{17}O -enriched water added to THF, with and without enzyme present, the presence of enzyme had no effect on either the chemical shift or the line width of the added water, even though the isotherm for water adsorption to subtilisin Carlsberg indicated that a large fraction of the added water was adsorbed to the enzyme under these conditions (data not shown).

The isotherm for water adsorption also showed that freeze-dried subtilisin Carlsberg initially retained *ca.* 3% bound water (Lee *et al.* 1998). This residual water was not visible by liquid-state NMR measurements, and instead could be detected only by solid-state NMR. Such a spectrum was obtained by freeze-drying the enzyme from D_2O and recording the spectrum of the dry enzyme under nitrogen. In this case, the spectrum was consistent with 46 D_2O molecules and 135 exchangeable OD and ND deuterons, all of which collapsed into a single, much narrower peak when the dry enzyme was transferred into

THF (Lee *et al.* 1998). Therefore, there appeared to be two distinct populations of enzyme-bound water, one of which was visible only by solid-state NMR, corresponding to the residual water remaining on the enzyme after freeze-drying. The second population was evident from the isotherm of water adsorption but was indistinguishable in chemical shift and line width from unbound water.

These results can be explained by a two-state hydration model, in which one population of enzyme-bound water, denoted tightly bound water, consists of water molecules that do not exchange with other water molecules on the enzyme or in the bulk solvent. These water molecules have solid-like properties and are invisible to liquid-state deuterium NMR. The second population, denoted loosely bound water, exchanges freely with the bulk solvent, and has chemical and motional properties that are indistinguishable by NMR from those of free water. Thus, it is possible that the tightly bound water is necessary to maintain the enzyme's catalytically active conformation, whereas the loosely bound water induces an increase in enzyme activity by increasing enzyme flexibility and active-site polarity.

(c) Comparison of suspended and immobilized chymotrypsin: structure versus polarity

In an effort to distinguish the effects of hydration on polarity and flexibility, we have also compared the structure and function of α -CT suspended as an insoluble powder and immobilized by adsorption to controlled pore glass (Suzawa *et al.* 1995). As shown by table 2, which lists k_{cat}/K_m values for both preparations in solvents ranging from relatively non-polar *n*-octane to relatively polar acetonitrile, the catalytic efficiency of the glass-bound enzyme is consistently 1–2 orders of magnitude larger than that of the suspended enzyme.

Considering again the particular case of THF, adding water substantially increased k_{cat}/K_m for both preparations: *ca.* 1000-fold for the suspended enzyme, and over 100-fold for the immobilized enzyme (figure 6). However, two different mechanisms appear to be responsible for this effect; as shown by the A_O values plotted in figure 6, the active-site polarity increases significantly for the immobilized enzyme, but exhibits little change for the suspended enzyme. Therefore, these results suggest that the increased activity of the suspended enzyme is due to greater flexibility, whereas the increased activity of the immobilized enzyme is due more to an increase in active-site polarity.

Regarding the 13-fold difference in activity between the suspended and immobilized enzyme in dry THF, the corresponding EPR spectra are very revealing. A comparison of EPR spectra and rotational correlation times obtained in dry THF indicated that the suspended enzyme is much more rigid than the immobilized enzyme (table 3). Furthermore, on the basis of the ratio of rotational correlation times about the two primary axes of the spectral simulations, not only are the protein dynamics different, so too are the active-site conformations. Therefore, the higher activity of the immobilized enzyme in the dry solvent appears to arise from a combination of its active-site conformation and greater flexibility. Furthermore, as water is added to each sample, both enzymes become much more

Figure 5. Kinetic and structural data for subtilisins Carlsberg (filled circles) (Affleck *et al.* 1992*b*) and BPN' (open squares) in THF with added water: (a) rate constants for the inhibition with phenylmethylsulphonyl fluoride; (b) active-site polarity as measured by the hyperfine splitting constants for 4-ethoxyfluorophosphinyloxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) spin label attached to Ser-221; (c) rotational correlation times for the same nitroxide spin label. (From Suzawa (1995), with permission.)

Table 2. Values of $(k_{\text{cat}}/K_{\text{m}})_{\text{app}}$ (M⁻¹ s⁻¹) × 100 for freely suspended and immobilized (glass-adsorbed) α-chymotrypsin in various solvents (Suzawa *et al.* 1995).

solvent	freely suspended chymotrypsin	immobilized chymotrypsin	$(k_{\text{cat}}/K_{\text{m}})_{\text{immobilized}}/(k_{\text{cat}}/K_{\text{m}})_{\text{suspended}}$
n-octane	221	19120	87
carbon tetrachloride	15	2150	141
toluene	7.7	557	72
diethyl ether	33	1370	42
THF	7.2	91	13
acetonitrile	< 0.5	56	> 112

flexible, and both exhibit changes in their active-site conformations.

The effects of water on the two enzyme preparations can thus be summarized as follows. At 0% added water, the suspended enzyme is less active and less flexible. Between 0% and 0.5% added water, the activity of

the suspended enzyme increases sharply, which is accompanied by an increase in flexibility, as indicated by EPR, with little or no change in active-site polarity. By comparison, the activity of the suspended enzyme increases gradually, with no apparent change in protein dynamics but a sharp increase in active-site polarity.

Figure 6. (a) Catalytic efficiencies of suspended (open squares) and immobilized (glass-adsorbed; filled circles) α -CT measured in THF at various water contents. (b) Hyperfine splitting constants of spin-labelled CT as a function of water added to THF. (Reproduced from Suzawa *et al.* (1995), with permission.)

Table 3. Calculated rotational correlation times for a nitroxide spin label attached to Met-192 of immobilized and suspended chymotrypsin in THF (Suzawa *et al.* 1995).

enzyme sample	τ_R (ns)	$\tau_{\parallel}$ (ns)	$\tau_{\perp}$ (ns)	$\tau_{\parallel}/\tau_{\perp}$	symmetry axis
immobilized, dry	23	108	4.8	22	2p π orbital
immobilized, 7% v/v water	1.6	1.3	2.0	0.65	2p π orbital
suspended, dry	100	55	8.0	6.9	N–O bond
suspended, 7% v/v water	7	14	3.4	4.1	2p π orbital

Further addition of water produces a comparable improvement in activity for both preparations, with only a gradual increase in active-site polarity for the immobilized enzyme.

4. DRAMATIC ACTIVATION OF ENZYMES IN NEARLY DRY ENVIRONMENTS

The results described above point to the following general strategy for activating enzymes in nearly anhydrous organic solvents: shield the enzyme from the bulk solvent while increasing its active-site polarity (assuming that the transition state is fairly polar), possibly by partly hydrating the enzyme in a suitably protective matrix. To this end, Khmel'nitsky *et al.* (1994) devised a relatively simple strategy, which starts by dissolving the enzyme in a concentrated salt solution. Such conditions can lead to so-called preferential hydration (Timasheff 1993), whereby certain ions are excluded from the water near the protein's surface, and the protein is stabilized against unfolding. Thus, including salts with a strong propensity for preferential hydration (e.g. salts that comprise kosmotropic anions and chaotropic cations) may help protect the protein against destabilizing influences during freeze-drying, and promote hydration of the protein in the final freeze-dried formulation. Freeze-drying the enzyme removes most of the water, leaving behind a freeze-dried, partly hydrated enzyme in a salty matrix, which presumably affords some degree of protection when the salt-activated enzyme is transferred to an organic solvent. Furthermore, water pools that remain on the enzyme, which comprise up to 3 wt% or more of the protein, may have a very high ionic

strength. Therefore, it was anticipated that such a system—presumably through a combination of effects—would exhibit greater activity in organic media.

Indeed, the salt-activation strategy was highly successful (Khmel'nitsky *et al.* 1994; Ru *et al.* 1999, 2000, 2001; Lindsay *et al.* 2004). For example, when subtilisin Carlsberg was freeze-dried together with KCl, a dramatic activation effect was observed in several solvents, with a rate enhancement of nearly 4000-fold observed in hexane for a preparation containing 98% w/w KCl salt (Khmel'nitsky *et al.* 1994). Furthermore, scanning electron micrographs of the different preparations revealed that the salt-activated enzyme preparation has a very different microstructure from the unactivated enzyme (figure 7), indicating that the salt-activated enzyme is truly a unique material with distinct physico-chemical properties.

Recently the Clark and Dordick groups have begun to use a combinatorial approach to determine optimal combinations of activating salts for different enzymes. For example, figure 7 illustrates the initial rates of transesterification catalysed by penicillin amidase in hexane for different salts and 1 : 1 binary salt mixtures (Lindsay *et al.* 2004). Clearly, KCl is not the best salt formulation for penicillin amidase. Indeed, a 1 : 1 mixture of CsCl and KAc yielded a much higher rate and produced an activation factor of 35 000-fold.

5. CONCLUDING PERSPECTIVE: CAN ENZYMES RUN COMPLETELY DRY?

The results described herein point to several conclusions about enzyme structure and function in organic

Figure 7. (a) Scanning electron micrographs of subtilisin Carlsberg freeze-dried without (top) and with KCl, yielding a preparation that contains 98% w/w salt (bottom). (b) Initial rates of transesterification of phenoxyacetic acid methyl ester with propanol catalysed by penicillin amidase in hexane for different salts and of 1 : 1 binary salt mixtures. (Reproduced from Lindsay *et al.* 2004, with permission.)

solvents with and without added water. It is evident that proteins slow down when they run dry; that is, they indeed exhibit slower dynamics in nearly anhydrous organic solvents. Moreover, the reduced flexibility in organic solvents follows the expected dependence on dielectric constant based on electrostatic contributions to protein dynamics (Affleck *et al.* 1992a). However, enzymes can still function, and function reasonably well, in a relatively rigid state. It is thus tempting to speculate that whatever further rigidification may result from the *complete* removal of water would not, in itself, be sufficient cause to preclude catalytic activity completely.

As for water remaining on the enzyme after freeze-drying or added to the surrounding solvent, the NMR results indicate that it is either tightly bound—and is not visible by liquid-state NMR spectroscopy—or it is indistinguishable from bulk water. However, the freely exchanging water appears to loosen the protein, corresponding to faster protein dynamics and higher activity in a given organic solvent. An increase in active-site polarity with added water is also observed in some cases, depending on the enzyme formulation, which translates into more effective transition-state stabilization and enhanced activity.

Consistent with these observations, nearly dry enzymes can be activated for catalysis in organic media by formulating them in a salty matrix, which in some cases has produced spectacular degrees of activation. The activation appears to involve protection from the solvent in combination with transition-state stabilization, which may entail active-site hydration. In summary, these findings clearly illustrate that enzymes can exhibit high activity in environments containing *very little* water. Thus, should it not be feasible for an enzyme to show at least low activity in a suitable environment containing *no* water? This question still remains to be answered, but the evidence to date

suggests that truly anhydrous enzymatic catalysis is hardly an arid idea.

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Discussion

P. Ball (18 Hillcourt Road, East Dulwich, London SE22 0PE, UK). I can see how studies of enzymes in low-water environments can help us address the question ‘is terrestrial life possible without water?’—but this is not quite the question that is being asked. If we assume that highly selective and efficient catalysis is likely to be needed in any living system (whatever that might mean) to channel chemical information down a particular pathway, do you see any fundamental reason—based on what you have learnt about water’s role in the function of enzymes—why enzyme-like selectivities and activities should not be achievable in catalysts designed to work in wholly anhydrous media? (It seems that enzymes themselves cannot really be made wholly anhydrous.)

D. S. Clark. Certain synthetic small-molecule catalysts have already been shown to achieve high stereoselectivities under anhydrous conditions. Thus, assuming that a catalytically competent conformation can be maintained in the complete absence of water, I am unaware of any inherent limitations that would prohibit simple enzymatic reactions from occurring in a totally dry medium (setting aside any special requirements that may be imposed by cofactors, flexibility needs for large conformational changes or other limiting factors). The challenge, then, is to remove all the water from the enzyme while preserving its active conformation, which, as you point out, has not yet been done and may not be possible.

J. A. Littlechild (School of Biological Sciences and Chemistry, University of Exeter, Exeter EX4 4QD, UK). With reference to the different behaviour of suspended and

immobilized enzyme with regard to addition of water. What is the method of immobilization? Does this restrict or alter the flexibility of the protein? Could this explain the results? How does the very tightly bound water correlate with that found as ordered water in the crystal structure?

D. S. Clark. The method of immobilization was adsorption of the enzyme to controlled pore glass. The enzyme was adsorbed by drying a slurry of glass particles in buffered enzyme solution under vacuum in a rotary evaporator. The immobilized enzyme was more active and flexible in organic solvents than the freely suspended enzyme, possibly because physical adsorption of less than an enzyme monolayer onto the glass reduced unfavourable protein–protein interactions (that restrict the flexibility of the suspended enzyme) and/or because the glass mimics the role of water by forming favourable hydrogen bonds with the surface of the enzyme, thus allowing whatever water is present to lubricate the protein more effectively. As for where the water is or how it correlates to ordered water within the crystal structure, we are not sure.

D. W. Rice (Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, UK). Is there any correlation between the activation factor for catalysis that can be achieved with salt and enzymes whose catalytic functions either do or do not involve large-scale conformational changes such as domain movements?

D. S. Clark. The enzymes for which the highest degrees of salt activation have been achieved so far are monomeric enzymes that do not undergo large conformational changes, but there is not yet enough evidence to say whether the converse is true (i.e. conformational changes would be restricted by the salt matrix, thus leading to low activation factors for enzymes that normally experience them). This would be a very interesting line of inquiry, assuming the role of conformational changes in salt activation (or a lack of activation, as the case may be) could be separated from all other mechanistic considerations.

B. Halle (Department of Biophysical Chemistry, Lund University, SE-22100 Lund, Sweden). In your calculation of the electrostatic energy, you describe an extended charge distribution as a point dipole. Have you checked the convergence of the multipole expansion of which this is the first term?

D. S. Clark. In the expression for the energy of solvation, the dipolar term was assumed to dominate and higher-order terms were neglected for simplicity. Although not checked rigorously, the excellent agreement between the experimental results and the resultant expression supports the validity of this approach.

B. Halle. What limits can you establish on the residence times of the 46 ‘tightly bound’ water molecules and on what experiments are they based?

D. S. Clark. No such limits can be determined from the experimental data obtained to date; however, we hope to obtain such information from NMR measurements of spin-lattice relaxation times now underway.

GLOSSARY

α -CT: α -chymotrypsin
EPR: electron paramagnetic resonance
NMR: nuclear magnetic resonance
THF: tetrahydrofuran