THE ROYAL SOCIETY

Published online 9 July 2004

What can we learn by studying enzymes in non-aqueous media?

Peter J. Halling

Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, UK (*p.j.halling@strath.ac.uk*)

What is the role of water in enzyme structure and function? One approach to answers should come from studies in which the amount of water present is a variable. In the absence of bulk liquid water, effective monitoring of enzyme action requires an alternative fluid medium through which substrates and products may be transported. The past 20 years have seen quite extensive study of enzyme behaviour when reactants are transferred via a bulk phase that is an organic liquid, a supercritical fluid or a gas. Some lipases, at least, remain highly active with only a few, if any, residual water molecules. Many enzymes seem to require larger amounts of water, but still not a liquid water phase. There are hysteresis effects on both the amount of bound water and the observed catalytic activity. Increasing hydration promotes mobility of the enzyme molecule, as revealed by various techniques, and there are correlations with catalytic activity. There are other plausible roles for hydration, such as opening up proton conduction pathways.

Keywords: enzymology; low-water media; organic media; hydration

1. INTRODUCTION

What is the role of water in enzyme structure and function? The literature offers many attempts at answers to this question, which is obviously of considerable interest. In tackling such a question about almost any other factor, we think first about the effects of varying it experimentally. Such evidence is much less sought in the case of water, in the past probably because of the belief that enzyme action required dilute aqueous solution. However, water levels can be made a variable in studies on enzymes, and this should surely be a source of useful information about the role of water.

One approach is to examine the effects of reducing the availability of water in aqueous solution by addition of osmotically active but otherwise inert solutes. This 'osmotic stress' method has been particularly exploited by Rand and co-workers, and is reviewed in the accompanying paper by Rand (2004). Even high osmotic pressures correspond to fairly small reductions in the mass-action effects of water (e.g. 1 osmolar gives *ca.* 25 atm, but the thermodynamic water activity is only reduced from 1 to 0.98). Hence the osmotic stress method gives information about the large number of relatively weakly associated water molecules. Tightly bound waters remain in place whether or not the osmotic stress is applied.

A complementary approach is to move away from aqueous solution completely, and study systems where even the strongly bound waters can be removed. This requires experiments on protein molecules with, at most, a few hundred surrounding water molecules. Such a protein–water system will normally be in the solid state. A wide variety of physical measurements have been made on such solid-state proteins with adsorbed water. These have led to many conclusions about their behaviour (Rupley & Careri 1991; Gregory 1994). However, one critical measurement, that of enzymatic activity itself, is extremely hard in such systems (containing only protein and water). Simple and reliable measurements of catalytic activity require a suitable fluid phase for the transport of substrates and products. Over the past 20 years or so, there has been quite extensive study of enzyme action in systems where such an alternative to liquid water is used for reactant transfer. The most work has been on systems with a fluid phase based on organic liquids, but it is also possible to use supercritical fluids, ionic liquids or volatile reactants in the gas phase. The main focus of this article is on the systems with organic liquids, but much of the behaviour is similar whichever of these non-aqueous phases is employed. The accompanying paper by Dunn & Daniel (2004) concentrates on systems with gas phase transfer, while that by Clark (2004) offers another perspective on organic media. For some recent reviews of enzyme action in organic solvents see Halling (2000), Carrea & Riva (2000), Klibanov (2001) and Lee & Dordick (2002).

The last paragraph may have given the impression of a protein molecule with some adsorbed water, surrounded by the non-aqueous fluid. However, this simple picture is not strictly correct. There will always be other things in the immediate molecular environment of the protein. These may be other molecules of the enzyme in a solid particle. Often a more uniform environment for all the protein molecules may be achieved by dispersing them over the surface of another solid particle, at the expense of introducing this additional component. Alternatively, the protein may be molecularly dispersed in solution in an organic liquid, but only by introducing solubilizing agents such as surfactants or polymers. Figure 1 shows some schematic diagrams of the types of system used. Note that

One contribution of 16 to a Discussion Meeting Issue 'The molecular basis of life: is life possible without water?'.

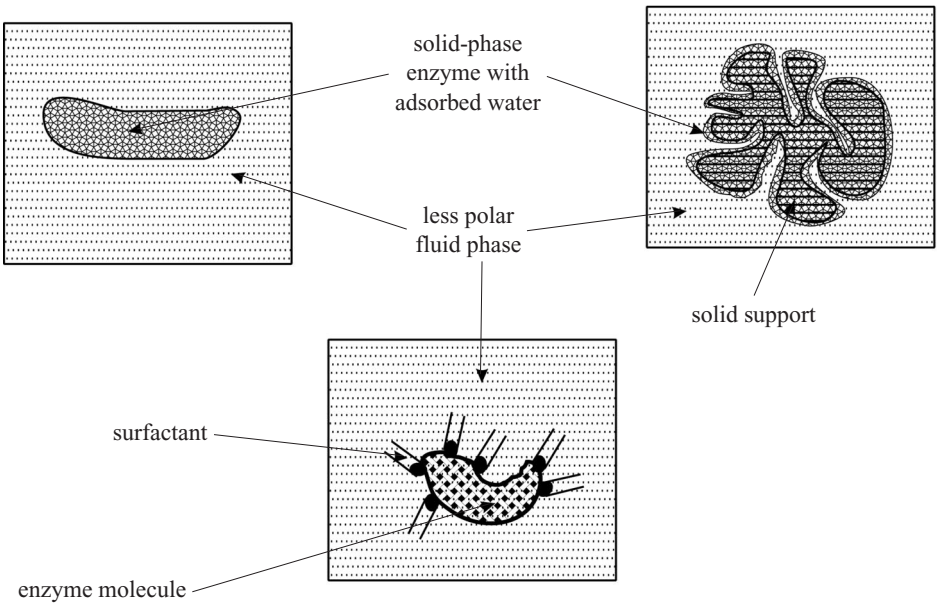

Figure 1. Some typical systems for low-water biocatalysis. In all of these there is no residual liquid aqueous phase. Note that the top two diagrams show structures on a microscopic scale, whereas the lower one is at a molecular scale.

the two upper panels show a microscopic scale view, whereas the lower one is magnified to a molecular scale. More information about all these systems can be found in the reviews cited above and references therein.

Most biologists and chemists are now aware that we can abandon the former dogma that enzymes are not active in organic solvents. However, there is a tendency to replace it with a principle that their catalytic activity in organic media is always much lower than in water. This idea is equally wrong. Anyone who works with enzymes will be aware that by using the wrong conditions it is possible to get very low catalytic activity. This remains true for organic media, with the added complication that the rules for identifying the right conditions are less well understood. However, if the best methods for preparing the biocatalyst and medium are used, it is quite possible to get catalytic activities that are within an order of magnitude or less of those in optimal aqueous conditions. It is not easy to make an exact comparison because the reactions catalysed tend to be different in the two media, because of thermodynamic effects on the equilibrium position. In a review in 1987, I pointed out that comparable rates were possible in organic media (Halling 1987). With increased understanding since then, it is easier to select the right conditions. Here is one example from our own work of a respectable (but not record) specific activity: an immobilized lipase catalysed esterification in organic media at 0.9 μmol min^{-1} (mg protein)$^{-1}$ (Janssen *et al.* 1999). This is *ca.* 100 times slower than a typical specific activity for this lipase in an aqueous emulsion. However, note that we are comparing opposite directions of reaction, and at least part of this difference reflects the general kinetic problems of activating the free carboxyl group. Even in organic media, transesterification is typically about 10-fold faster than an esterification such as this.

Those who state that enzymes in organic solvents have low activity will commonly cite a classic paper from Klibanov's laboratory (Schmitke *et al.* 1996). However, this is to misinterpret completely the key message of this paper. It certainly gives an example of enzyme activity seven orders of magnitude lower in an organic solvent than in water. But the paper proceeds to dissect in detail the reasons for this difference in activity. It turns out that most of the differences can be substantially reduced or eliminated by correctly adjusting the conditions in the organic medium.

Perhaps the best evidence that quite acceptable rates can be found for enzyme reactions in organic media is that there are now over 10 commercially operating processes (Straathof *et al.* 2002). As usual, precise details of commercial operations are hard to come by, but we can assume that a catalytic activity many orders of magnitude less than normal would be uneconomic.

2. HOW MUCH WATER IS REQUIRED FOR CATALYTIC ACTIVITY?

This is an obvious question to ask. More generally, we would like to know the relationship between the quantity of residual water and the enzymatic activity. The first issue that must be considered is how to quantify the amount of water present. For mechanistic interpretation we would ideally like to know the amount of water bound to the enzyme molecules, and indeed the location of the individual water molecules. Even the former is not so easy to measure or control experimentally, while the latter is known for only a few cases where diffracting crystals show activity while suspended in organic solvents. The most obvious measure, total water content of the reaction system, is of rather limited value. Some fraction of this water, often most of it, will not be associated with the enzyme. Instead it will be dissolved in the bulk fluid phase, or bound to other species present such as a solid support material.

In general, a useful parameter is the thermodynamic activity of water in the system. If water equilibrates between the various phases present, they will all come to the same water activity (provided all are referred to the

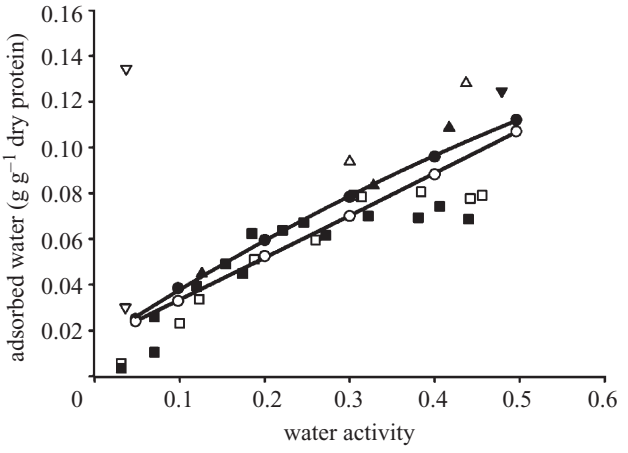

Figure 2. Water adsorption by proteins suspended in organic solvents. Water adsorption by β-lactoglobulin (open symbols) and bovine serum albumin (closed symbols) in air (circles), ethanol (squares), benzene (upright triangles) and ethylacetate (inverted triangles). (Data for organic media from Yamane *et al.* (1988), replotted on water activity basis (Halling 1990).)

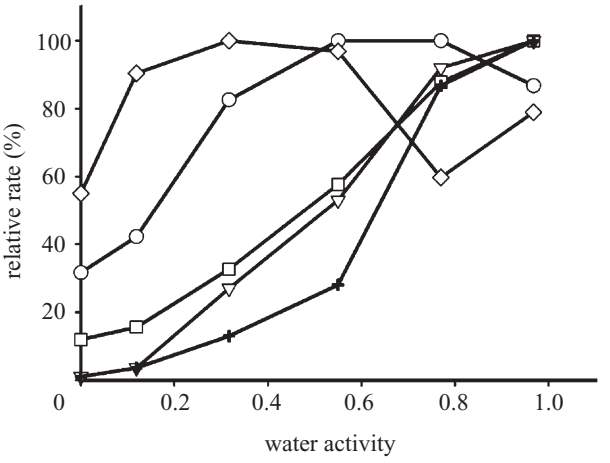

Figure 3. The effect of water activity on lipase catalytic activity. Lipases from *Rhizomucor miehei* (circles), *Rhizopus niveus* (diamonds), *Humicola lanuginosa* (squares), *Candida rugosa* (inverted triangles), and *Pseudomonas cepacia* (plus symbols). (Valivety *et al.* (1992*a*).)

same standard state, which conventionally is taken as pure liquid water at the same temperature). The enzyme molecules will tend to equilibrate in this way, so the quantity of water adsorbed to them will reflect the system water activity, however much water is also present in other phases. There are some fairly good methods available to fix or control the water activity of a mainly organic reaction mixture, for example by equilibrating with saturated salt solutions or salt hydrate pairs. Water activity may also be measured as the relative humidity of an equilibrated vapour phase, for example. It can also be calculated from a measured water concentration dissolved in the organic liquid, using the appropriate activity coefficient. (Such measurements are, however, rather inaccurate for the very low water concentrations dissolved in non-polar solvents.) The water activity of any water-saturated non-polar solvent will be very close to one. For a full description of the use of water activity, see Halling (1994).

An early example of the value of using water activity as a parameter in these systems came from measured water adsorption isotherms. Measurements had been made of the quantity of protein-bound water as a function of the concentrations dissolved in the organic phase (Yamane *et al.* 1988; Zaks & Klibanov 1988). To obtain the same amount of water associated with the protein required very different water concentrations in different solvents. However, when these water concentrations were converted to water activity (using known relationships), very similar isotherms were obtained for all the solvents. Figure 2 shows an example. Furthermore, the isotherms produced were essentially the same as that determined for adsorption of water by protein powders from the gas phase (the line in figure 2), at least in the lower water-activity range. Subsequently, this general picture has been confirmed by new experimental measurements of adsorbed water levels (although they are limited in number as the measurements are technically challenging (McMinn *et al.* 1993; Borisover *et al.* 1995; Lee & Kim 1995; Parker *et al.* 1995; Condoret *et al.* 1997)). There remains some disagreement about whether at higher water activities there are deviations above or below the gas-phase isotherm.

When we come to enzyme behaviour, it is found experimentally that analysis in terms of water activity often does simplify the picture. Thus, very similar profiles of catalytic activity as a function of water activity will be found for different solvents, despite large variations in total water contents and concentrations dissolved in the organic phase. In the same way, the use of water activity can discount many of the apparent effects on water dependence of changing the support and other factors (e.g. Halling 1994).

The dependence of catalytic activity on water activity has been determined for a variety of enzymes. The most obvious approach has been to dry the enzyme preparation and then add back water to the desired level. To obtain the required water activity in the test reaction system, it is necessary to make sure that all phases are pre-equilibrated at the desired value. Most of the earlier studies were made with lipases and revealed a wide range of profiles of catalytic activity as a function of water activity. An example is shown in figure 3. Some lipases retain good catalytic activity even at very low water activity, with optima well below 0.5. Others, however, show little or no activity at the lowest water activities, with a steep rise at higher values, to an optimum not too far below 1.

Subsequent studies with a variety of enzymes other than lipases have usually shown the pattern of higher water requirements. Thus, they show the steep rise in activity around water activities somewhere between 0.5 and 0.9. The most comprehensive studies have come from Adlercreutz's laboratory, and some examples (Adlercreutz 1991; Hansson *et al.* 2001) are shown in figure 4.

Figure 3 shows an example of one lipase that retained good activity at a water activity plotted as zero. This raises the issue of exactly how low is the level of hydration, a question that is very relevant to the theme of this meeting. To give a clearer answer, samples of the lipase biocatalyst were dried exhaustively over a variety of agents for two months, and then assayed in a dry-box with thoroughly dried reaction mixture components. The activity was

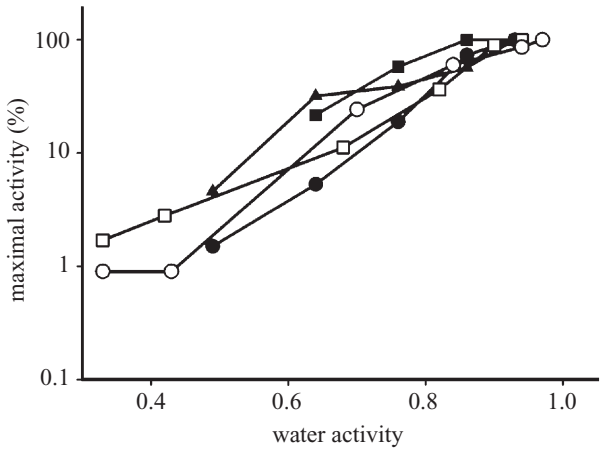

Figure 4. The effect of water activity on the catalytic activity of some enzymes other than lipases. Activity in hexanol of β-galactosidase from *S. solfataricus* (closed circles), β-glucosidase from *C. saccharolyticum* (closed squares) or almond (closed triangles), Hansson *et al.* (2001). Activity in di-isopropyl ether of horse liver alcohol dehydrogenase (open circles) or chymotrypsin (open squares). (Adlercreutz (1991).)

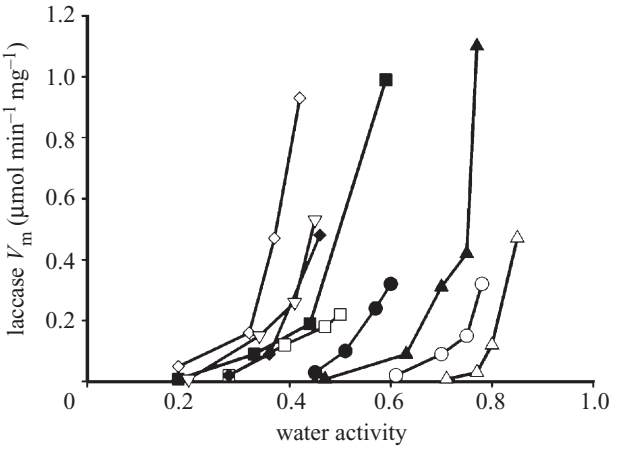

Figure 5. The relationship of laccase activity with water activity in miscible organic solvents. (Data from van Erp *et al.* (1991) for methanol (open diamonds), 2-propanol (inverted triangles), 2-butanol (closed diamonds), ethanol (open squares), 1-butanol (closed squares), dioxane (closed circles), acetonitrile (closed triangles), tetrahydrofuran (open circles) and acetone (upright triangles). Converted to water activity basis by Bell *et al.* (1997).)

found to be still *ca*. 30% of that at the optimal water activity (Valivety *et al.* 1992*b*). But how much water is left after this drying treatment? Measurement of trace amounts of water is remarkably difficult, because of the problems of avoiding interference from environmental water. The best standard method, coulometric Karl–Fischer analysis, can detect *ca*. 1 μmol of water: for most other analytes this would be considered poor sensitivity. To estimate the amount of residual water on the dried lipase, we used an ^{18}O labelling method previously applied to several dried protein powders (Dolman *et al.* 1997). This method gave an answer of 2 ± 3 waters per lipase molecule (M. Dolman, S. Waldron, B. D. Moore and P. J. Halling, unpublished data). Thus we can say confidently that there were very few residual water molecules in this reasonably active enzyme preparation. The previous studies had suggested an agreement between the number of residual waters after extensive drying and the number found buried in the three-dimensional structure of the protein (Dolman *et al.* 1997).

Although behaviour as a function of water activity may remain similar when some features of the system are changed, this is not always true. Figure 5 shows how catalytic activity for a single enzyme (laccase) is affected by water activity in various miscible solvents. It is clear that the critical water activity for high activity differs markedly between solvents. In water-miscible solvents, variation of water concentration or activity necessarily involves significant changes in the concentration or thermodynamic activity of the organic solvent itself, as well as substantial changes in the nature and polarity of the medium as a whole. It is not surprising if these have their own impact on enzyme behaviour.

Hysteresis effects are also important in preventing a unique relationship between water activity and enzyme behaviour. It is well known that water adsorption isotherms of proteins show significant hysteresis, with more bound water retained at a given water activity during desorption (Rupley & Careri 1991). Hence it is not too surprising that catalytic activity and other properties may show dependence on the history of water activity as well as on the final value reached. Furthermore, besides direct effects of hydration, it is clear that conformational changes of proteins become slow under low-water conditions. This has been shown to lead to a variety of effects of history (e.g. Ke & Klibanov 1998; Lee & Dordick 2002). There may be links between hysteresis in conformational changes and in hydration. The conformation may not remain in its lowest energy state throughout hydration and dehydration. Instead it may require forcing conditions to close the loop and return the protein to its original conformation, which may itself be the direct cause of hysteresis in enzyme behaviour. Such conformational changes have also been suggested as one of the possible reasons for hysteresis in the amount of bound water.

Table 1. Hysteresis effects on the catalytic activity of subtilisin cross-linked crystals.
(Water-wet cross-linked subtilisin crystals were washed three times in dry 1-propanol, then further treatment as shown, before assay in acetonitrile (1% water, a_w 0.22). (Partridge *et al.* (1996).))

additional treatment	activity (nmol min^{-1} mg^{-1})
1. none	575
2. acetonitrile, a_w 0.22, 3 days	187
3. air, a_w 0.22, 3 days	14
4. as 3, then aqueous and 1-propanol washes	505

Whatever the precise mechanism, substantial effects of hysteresis on catalytic activity can be demonstrated. Table 1 shows a pronounced example, where subtilisin catalysts have been taken through different hydration pretreatments before final assay, all at the same water activity (0.22). Good activity is obtained after drying by quick rinsing with the water-miscible solvent 1-propanol, but is some 40-fold

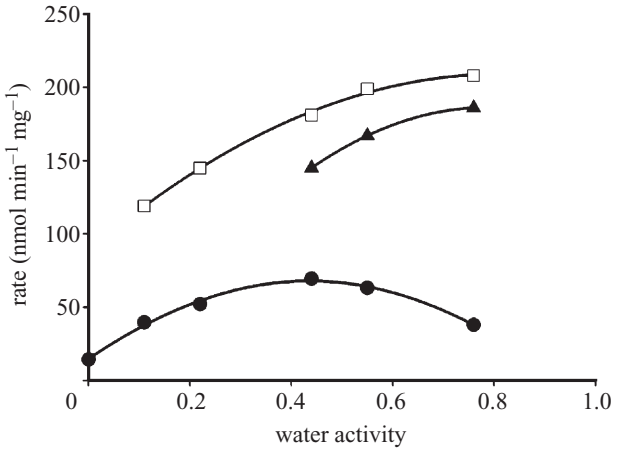

Figure 6. The effect of water activity and enzyme pre-treatment on catalytic activity of immobilized subtilisin Carlsberg in tetrahydrofuran. Rates in tetrahydrofuran were measured for immobilized biocatalyst which had been treated by: dry 1-propanol washing (closed squares): 1-propanol washing at the same water activity as the reaction (open squares) and no 1-propanol washing (closed triangles). Each reaction was carried out at least in triplicate with errors of less than 10%. The rate was determined as the sum of the initial rates of transesterification and hydrolysis. (J. Partridge, B. D. Moore and P. J. Halling, unpublished data.)

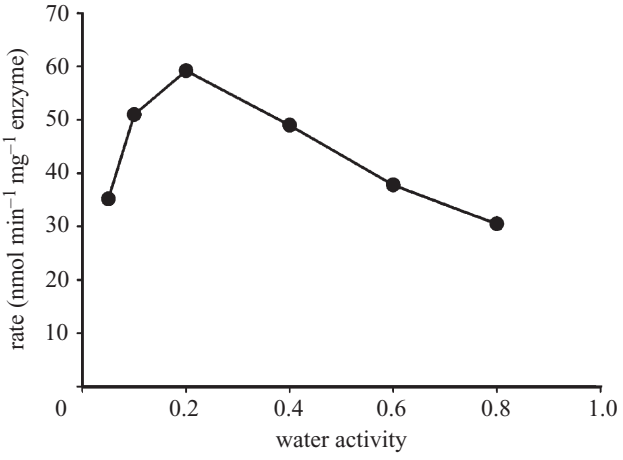

Figure 7. Water activity dependence of silica-papain activity after dehydration by rapid propanol rinse. (Theppakorn *et al.* (2004).)

smaller after holding at the same water activity in air. If, however, the air-dried catalyst is rehydrated in an aqueous medium, then re-dried, it can be seen that most of the activity is recovered again.

Here are two more examples of how hysteresis effects can dramatically influence the water activity dependence of catalysis. Figure 6 shows the behaviour of subtilisin (this time in a silica-adsorbed form) when dried by rinsing with a water-miscible solvent, as in the last example. When prolonged exposure to the lowest water activity is avoided before assay, the enzyme clearly retains good catalytic activity down to low water activities in the reaction mixture. Figure 7 shows similar data for the same type of preparation and treatment on papain. Both these proteases would show good catalytic activity only at higher water activity values when prepared in the traditional manner. (An example of the profile with lyophilized subtilisin powders re-hydrated from the dry state can be seen in figure 11, shown below for a different main purpose.) With these and other enzymes, hydration hysteresis can be exploited to get good catalytic activity at low water activity. This offers the benefits of low water activity in suppressing hydrolytic side reactions or maximizing equilibrium yields in synthesis by reversal of hydrolysis.

Relying on hysteresis effects for a desired behaviour always carries a risk, however. By definition, on at least one arm of the hysteresis loop, proteins are not in hydration equilibrium. Hydration may slowly change towards the other arm, with the resulting effects on enzyme behaviour. The consequence might be rather poor operational stability of any catalyst that has exploited hysteresis to obtain high initial activity. Just such poor operational stability has been observed for two types of protease catalyst that most strongly exploit this hysteresis (figure 8). However, this poor stability does not represent hydration changes alone, because the lost enzyme activity was not recovered, even on transfer of the enzyme back to an aqueous medium (Fernandes & Halling 2002). If dehydration is the first stage, it must be followed by a further irreversible effect.

3. HOW DOES HYDRATION PROMOTE CATALYTIC ACTIVITY?

One role of hydration is almost certainly to promote increased mobility or flexibility in the enzyme molecules. Several physical measurements have demonstrated that mobility of parts of the protein increases with increasing hydration, in the range that accelerates catalysis. Table 2 lists a number of methods that have led to this conclusion.

As an example of what can be done, I will present a method we have used, based on solid-state ^{1}H-NMR relaxation (Partridge *et al.* 1998). This method does not require pre-labelled protein, but uses treatment with D_2O to replace all protons in water and in the exchangeable hydrogens of the protein molecule. NMR measurements of both spin–lattice and spin–spin relaxation of the remaining protons (in the protein) probe the mobility of their environment. Easiest to interpret is the apparent T_2 (or peak width) of the solid-state signal. As shown in figure 9, at low water activity the signal consists of a broad hump characteristic of protons in an essentially solid immobile environment. As the hydration is increased (with D_2O, we are still looking at the protein protons), a much sharper line grows in the centre of the broad signal. This indicates that the mobility of the environment of a fraction of the protons is substantially increased. By integration of the signal it is possible to estimate the fractions of protons that experience the high and low mobility environments, with the result shown in figure 10. There is a steep rise in the fraction in the mobile environment between water activity 0.45 and 0.75. The catalytic activity of this enzyme preparation (hydrated in the same way) shows a steep rise in the same range of water activity (figure 11). However, we should note that the number of points is not enough to be sure that this quantitative link is much more than coincidence.

While hydration and increasing flexibility are usually associated with increased catalytic activity, their effects on

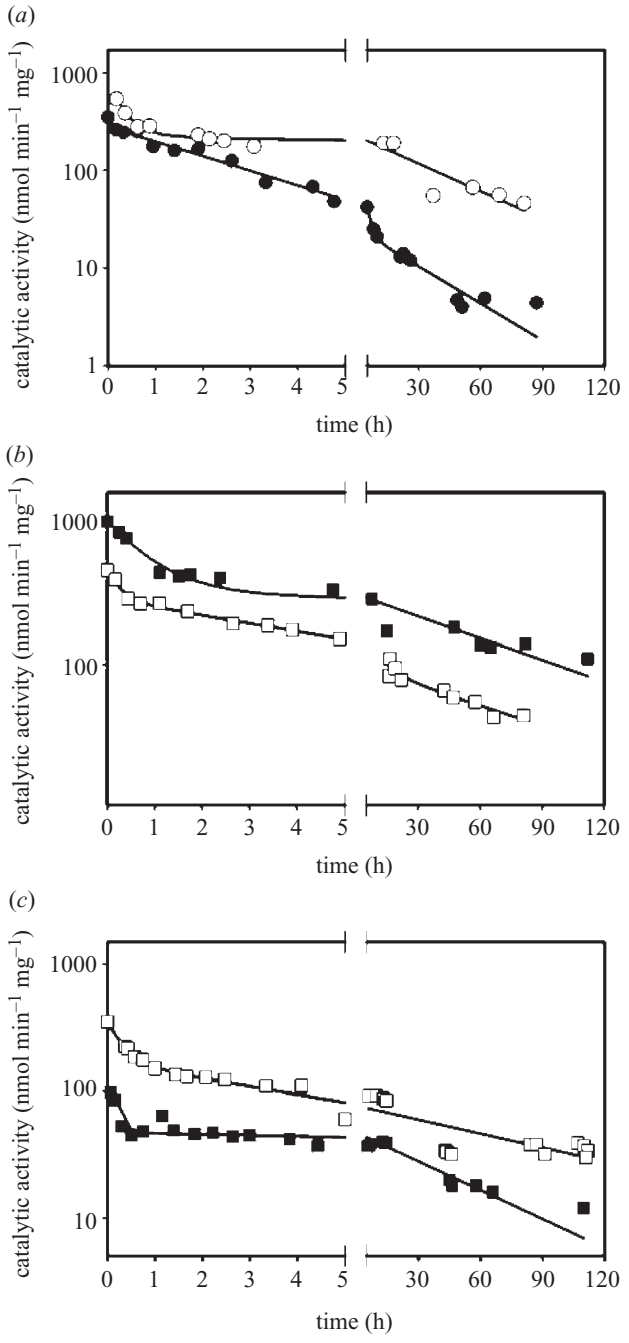

Figure 8. Poor operational stability of subtilisin dehydrated by rapid propanol rinse. Continuous reactors with acetonitrile and tetrahydrofuran. (*a*) Propanol-rinsed enzyme preparations (PREPs) at water activity of 0.22 (closed circles) and 0.76 (open circles); (*b*) and (*c*) cross-linked enzyme crystals (CLECs) at water activity of 0.22 (closed squares) and 0.76 (open squares); (*b*) with acetonitrile; (*c*) with tetrahydrofuran. (Fernandes & Halling (2002).)

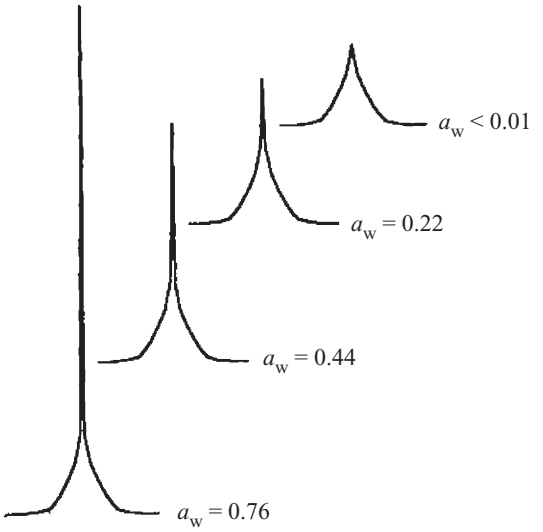

Figure 9. ^{1}H-NMR spectrum of subtilisin powder suspended in dichloromethane. (Partridge *et al.* (1998).)

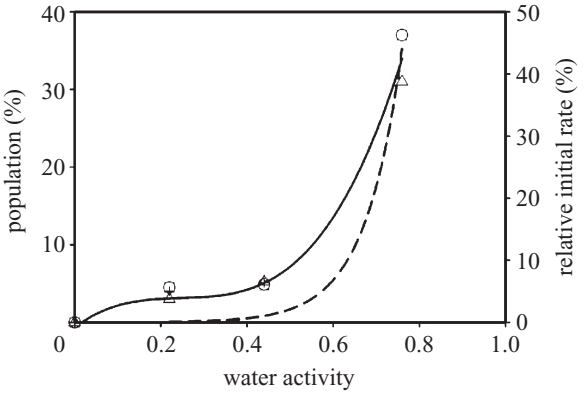

Figure 10. The effect of hydration on population of high-mobility protons in subtilisin. Fraction of narrower solid-state NMR component (longer T_2). The medium was air (open squares), cyclohexane (closed circles), dichloromethane (triangles) or acetonitrile (plus symbols). The dashed line shows the effect on catalytic activity. (Partridge *et al.* (1998).)

enzyme specificity are not so predictable. Broos (2002) has set out an interesting analysis that may account for the opposite dependences observed in different cases.

Increased mobility is not the only mechanism by which hydration could stimulate activity. It might promote structural changes in the protein. The relatively limited study here suggests that any changes are quite small over the lower water activity range, consistent with the idea that the structure is quite rigid (Griebenow & Klibanov 1997). Larger changes may sometimes occur at higher water activities, particularly in lyophilized powders, where they can reverse some of the (partial) denaturation that occurs during freezing and/or drying (Griebenow *et al.* 2001). Of course, even a very small conformational change, undetectable by the usual physical methods applied to solid-state proteins, could have a major effect on activity.

A second possibility has been advocated on the basis of dielectric measurements on hydrated protein powders. These indicate the appearance of proton conduction pathways through the hydration water as its amount increases (Rupley & Careri 1991). Proton movements close to the active site are part of the catalytic cycle for those enzymes (most of them) that use acid–base catalysis as part of their mechanism. It is very plausible that proton conduction around the active site may become limiting to turnover in dehydrated enzymes.

One group of findings is very relevant to the general topic of this meeting. It is clear that some other molecules can substitute for the role of water in activating low-water enzymes, at least in part. Their addition has been shown

Table 2. Methods indicating that hydration increases the mobility of protein structures in organic solvents.
(For more recent references on these techniques see Ueji *et al.* (2003) (electron paramagnetic resonance) and Soares *et al.* (2003) (molecular dynamics).)

electron paramagnetic resonance of active site label	Guinn *et al.* (1991); Affleck *et al.* (1992)
NMR lineshape of D-labelled Trp	Burke *et al.* (1993)
amide NH exchange by NMR	Wu & Gorenstein (1993)
molecular dynamics simulation	Hartsough & Merz (1992, 1993)
fluorescence depolarization	Broos *et al.* (1995)

Table 3. Water as a competitive inhibitor of lipase-catalysed esterification.
(Esterification of decanoic acid and dodecanol catalysed by immobilized lipases, analysed by full Ping–Pong model, to give Michaelis constants for the acid (K_{ac}) and alcohol (K_{al}). Note that the effect of water activity on the apparent K_m for the alcohol will reflect competition for the acyl-enzyme intermediate, essentially product inhibition under the Ping–Pong mechanism. In the case of K_m for the acid, however, there should be no effect from water as a reactant, so a probable explanation is competition with other (non-reactant) water molecules at the acid binding site. (Valivety *et al.* (1993).))

enzyme source	water activity	V_m (mmol s^{-1} kg^{-1})	K_{ac} (M)	K_{al} (M)
Rhizomucor miehei	0.12	3.0	0.37	0.089
	0.76	5.5	0.55	0.31
Candida rugosa	0.32	0.59	0.13	0.027
	0.76	5.9	0.57	0.28

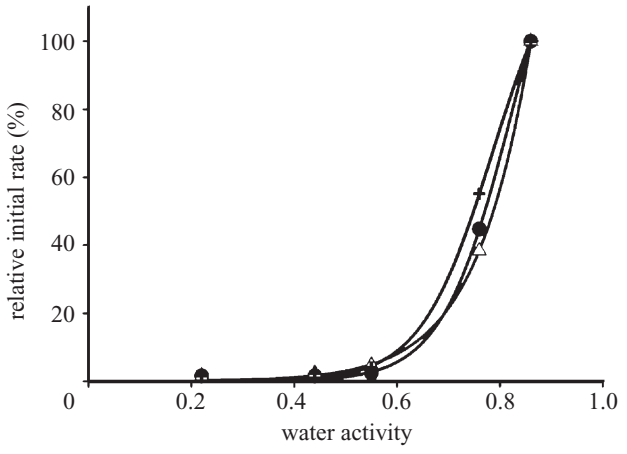

Figure 11. The effect of hydration on activity of lyophilized subtilisin powder suspended in organic solvents. *N*-acetyl-tyrosine ethyl ester transesterification with propanol. Maximal rates (100%) were 413, 3.52 and 18.6 nmol min^{-1} mg^{-1} in cyclohexane (circles), dichloromethane (triangles) and acetonitrile (plus symbols). (Partridge *et al.* (1998).)

to stimulate activity, often quite substantially. Usually the experiment involves an addition of the 'water mimic' without a change in water content. Thus water activity will be reduced if anything, as the additive interacts with what residual water there is. Such activating effects have been demonstrated for various glycols (Tanaka *et al.* 1981), formamide (Kitaguchi & Klibanov 1989; Kitaguchi *et al.* 1990), dimethyl sulphoxide (Almarsson & Klibanov 1996) and methanol (Hutcheon *et al.* 1997), but appear absent for compounds such as ethanol.

For some low-water enzymes, full kinetic studies have been made, allowing separate identification of water effects on V_{max} (or k_{cat}) and K_m values. In most cases it has been noted that K_m values *increase* with increasing water activity, so the enzyme shows less affinity for the substrates. An example taken from our own work is shown in table 3. If increasing quantities of any other component caused an increase in apparent K_m, we would describe it as a competitive inhibitor, and there seems no reason why we should not see water in this way. The usual mechanistic deduction would be that the inhibitor must be displaced from the active site on binding the substrate, and again it seems quite reasonable to apply this picture to water. This is exactly analogous to the treatment of water activity effects in aqueous solution studies, as presented in the accompanying article by Rand (2004). As in these studies, the quantitative dependence of apparent binding constants on water activity can be used to count the numbers of water molecules displaced. The apparent dissociation constant will increase with water activity raised to the power of the number of waters displaced on binding. In low-water systems the only data so far are for K_m values, rather than true dissociation constants, and there are few different water activities used (just two in table 3). Hence it is dangerous to suggest precise numbers of waters, although it is clear that these are much smaller than in aqueous solution. This is naturally expected when all but fairly tightly bound waters have already been removed from the protein.

It could be of interest to extend these studies to generate more precise counts of waters displaced. Measurement of true dissociation constants would be wise in such studies. This would not be easy in a simple binding experiment, as the fraction of bound sites would probably have to be estimated by an error-prone difference method. An attractive option may be to use kinetic measurements to estimate K_i values for a second known competitive inhibitor, as these are true dissociation constants. There may also be value in estimating elementary rate constants by competing substrate methods, as exploited by Wangikar *et al.* (1993).

4. HOW USEFUL IS UNDERSTANDING OF LOW-WATER ENZYMOLOGY, AND HOW COULD IT BE IMPROVED?

It is now clear that the use of organic and other low-water media allows convenient studies of the action of enzymes while varying the amount of tightly bound water. Thus we can explore directly the role of these water molecules, by examining the effects of removing them.

How relevant are these studies to biology? We know that the minimum water activity at which cell growth has been observed is *ca*. 0.6 (see Grant 2004). Hence it is arguable that studies at lower water activity do not tell us about crucial life processes. Essentially the proteins in these growing cells will still retain most of their tightly bound water, so the effects of its removal will be of rather academic interest. However, many living organisms have dormant states with much lower water activity, in which continued enzymatic activity may play important roles. Furthermore, low-water enzymology can potentially show which (if any) of the tightly bound waters are essential for catalytic activity. This may help us to understand and predict the effects of other changes, such as mutations in the amino acid sequence of the enzyme, and in this way be relevant to growing cells. Of course, further studies should help us get closer to an answer for the overall question of this meeting, by defining the essential roles of water in enzymatic catalysis.

So what do we need for better fundamental understanding of low-water enzymology? A first requirement is apparently rather trivial, but remains an unsolved problem. We need a simple method for accurate monitoring of the amount of water bound to proteins in an organic environment. Karl–Fischer analysis is available, but its restricted sensitivity means that errors rise at low levels of protein-bound water. With the usual solid-state protein samples, sensitivity is limited by entry of environmental water into the analysis chamber along with the sample. At the lowest levels there is also the need for a completely dry protein to check for interference in the Karl–Fischer reaction, which of course, requires a method to show that it is completely dry. Various studies have used water labelled with ^{3}H or ^{2}H, but these fail at lower levels because of the large correction required for the exchangeable hydrogens in the protein structure itself. Exchange problems are eliminated by the use of ^{18}O, but very accurate mass spectrometry is needed to quantitate the small enrichments after label is recovered in an aqueous medium.

True understanding needs more than just the numbers of bound waters: it needs molecular-scale structural information about the protein and bound waters. It would be useful to relate the location of waters and associated structural changes to enzyme behaviour (activity, specificity and stability).

A number of crystal structures have been published for protein crystals transferred to an organic environment (Yennawar *et al*. 1995; Schmitke *et al*. 1998; Gag *et al*. 1999; Zhu *et al*. 2001; and references cited therein). Crystals grown in aqueous media have been rinsed thoroughly with organic solvents, sometimes after mild cross-linking. In many cases non-polar solvents have been used, but this probably does not produce a low water activity: the very low solubility of water in these solvents means that even extensive washing will not remove most of the water from the crystal. However, in some instances polar solvents such as acetonitrile have been used, giving an eventual organic phase of undoubtedly low water activity. The reported structures show a substantial number of water molecules still associated with the protein, many in similar locations to those found in the original aqueous crystals. All of these crystallographic studies have shown only limited changes in protein structure from that previously determined in an aqueous environment. However, it seems to me that the approach used could hardly give any other answer. If there were to be a major change in conformation, it seems unlikely that the existing crystal packing arrangement could be maintained. Changes in this would normally result in a loss of order and of high-resolution diffraction. There have been reports that some attempts to transfer crystals to organic media did indeed lead to loss of diffraction or even fracture of the crystals.

NMR methods can give structural information without needing crystalline order. However, as noted, most enzyme forms usable in organic media are solid. High-resolution NMR in the solid state is much more difficult than in solution. There is active research on examining the structures of membrane proteins by solid-state NMR: when embedded in membrane fragments, they behave as essentially solid state in an NMR experiment (Luca *et al.* 2003). As these methods become established, they may also be applicable to low-water biocatalysts. Solid-state NMR can of course provide useful structural information even when full detail is not possible (e.g. Lee *et al.* 1998). It should be rather easier to carry out high-resolution NMR experiments on the systems where protein molecules are individually dispersed in organic solvents. However, even here the solubilizing agents necessary may further restrict the rotation of the already large protein molecules, making acquisition of high-resolution data harder.

Several methods can give some structural information about enzymes in these systems, although not full atomic-scale detail. Infrared spectroscopy has been applied quite extensively and can quantify the contributions of different secondary structural elements (Griebenow & Klibanov 1997). Raman measurements have also been shown to be possible, and offer complementary vibrational signals (Guo & Mabrouk 2002). In solution, circular dichroism is the most trusted method for determining secondary structure contributions. We have recently been finding that with the latest generation of circular dichroism instrumentation, it is possible to get useful spectra even from solid-state biocatalysts suspended in organic media. Fluorescence measurements have also been shown to be possible on enzyme suspensions in organic solvents (Broos *et al.* 1995), and the energy transfer method (fluorescence resonance energy transfer) can provide distance measurements within the structure.

In closing, I should note what has been a major obstacle to gaining better fundamental understanding of the role of water during enzyme action in low-water media. Nearly all the funding for research in this area has been driven by the applications of low-water biocatalysis in industrial and laboratory synthesis. Hence fundamental questions have tended to be addressed only where they are clearly relevant to improving these practical applications. If it is

believed that studies in low-water systems do have important messages for enzymology as a whole, basic science funding for such work will generate improved understanding more quickly.

I thank Dr Geeta Fuglevand for redrawing or replotting all the figures in this article.

REFERENCES

Adlercreutz, P. 1991 On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. *Eur. J. Biochem.* **199**, 609–614.

Affleck, R., Haynes, C. A. & Clark, D. S. 1992 Solvent dielectric effects on protein dynamics. *Proc. Natl Acad. Sci. USA* **89**, 5167–5170.

Almarsson, O. & Klibanov, A. M. 1996 Remarkable activation of enzymes in nonaqueous media by denaturing organic cosolvents. *Biotechnol. Bioengng* **49**, 87–92.

Bell, G., Janssen, A. E. M. & Halling, P. J. 1997 Water activity fails to predict critical hydration level for enzyme activity in polar organic solvents: interconversion of water concentrations and activities. *Enzyme Microbiol. Technol.* **20**, 471–477.

Borisover, M. D., Sirotkin, V. A. & Solomonov, B. N. 1995 Isotherm of water sorption by human serum-albumin in dioxane: comparison with calorimetric data. *J. Phys. Organ. Chem.* **8**, 84–88.

Broos, J. 2002 Impact of the enzyme flexibility on the enzyme enantio-selectivity in organic media towards specific and non-specific substrates. *Biocatalysis Biotransform.* **20**, 291–295.

Broos, J., Visser, A. J. W. G., Engbersen, J. F. J., Verboom, W., Vanhoek, A. & Reinhoudt, D. N. 1995 Flexibility of enzymes suspended in organic-solvents probed by time-resolved fluorescence anisotropy: evidence that enzyme-activity and enantioselectivity are directly related. *J. Am. Chem. Soc.* **117**, 12 657–12 663.

Burke, P. A., Griffin, R. G. & Klibanov, A. M. 1993 Solid-state nuclear-magnetic-resonance investigation of solvent dependence of tyrosyl ring motion in an enzyme. *Biotechnol. Bioengng* **42**, 87–94.

Carrea, G. & Riva, S. 2000 Properties and synthetic applications of enzymes in organic solvents. *Angew. Chem. Int. Edn* **39**, 2226–2254.

Clark, D. S. 2004 Characteristics of nearly dry enzymes in organic solvents: implications for biocatalysis in the absence of water. *Phil. Trans. R. Soc. Lond.* B **359**, 1299–1307. (DOI 10.1098/rstb.2004.1506.)

Condoret, J. S., Vankan, S., Joulia, X. & Marty, A. 1997 Prediction of water adsorption curves for heterogeneous biocatalysis in organic and supercritical solvents. *Chem. Engng Sci.* **52**, 213–220.

Dolman, M., Halling, P. J., Moore, B. D. & Waldron, S. 1997 How dry are anhydrous enzymes? Measurement of residual and buried 18-O labelled water molecules using mass spectrometry. *Biopolymers* **41**, 313–321.

Dunn, R. V. & Daniel, R. M. 2004 The use of gas-phase substrates to study enzyme catalysis at low hydration. *Phil. Trans. R. Soc. Lond.* B **359**, 1309–1320. (DOI 10.1098/rstb.2004.1494.)

Fernandes, J. F. A. & Halling, P. J. 2002 Operational stability of high initial activity protease catalysts in organic solvents. *Biotechnol. Prog.* **18**, 1455–1457.

Gag, X. G., Maldonado, E., Perez-Montfort, R., Garza-Ramos, G., De Gomez-Puyou, M. T., Gomez-Puyou, A. & Rodriguez-Romero, A. 1999 Crystal structure of triosephosphate isomerase from *Trypanosoma cruzi* in hexane. *Proc. Natl Acad. Sci. USA* **96**, 10 062–10 067.

Grant, W. D. 2004 Life at low water activity. *Phil. Trans. R. Soc. Lond.* B **359**, 1249–1267. (DOI 10.1098/rstb.2004.1502.)

Gregory, R. B. (ed.) 1994 *Protein–solvent interactions*. New York: Marcel Dekker.

Griebenow, K. & Klibanov, A. M. 1997 Can conformational changes be responsible for solvent and excipient effects on the catalytic behavior of subtilisin carlsberg in organic solvents? *Biotechnol. Bioengng* **53**, 351–362.

Griebenow, K., Vidal, M., Baez, C., Santos, A. M. & Barletta, G. 2001 Nativelike enzyme properties are important for optimum activity in neat organic solvents. *J. Am. Chem. Soc.* **123**, 5380–5381.

Guinn, R. M., Skerker, P. S., Kavanaugh, P. & Clark, D. S. 1991 Activity and flexibility of alcohol-dehydrogenase in organic-solvents. *Biotechnol. Bioengng* **37**, 303–308.

Guo, W. & Mabrouk, P. A. 2002 Raman evidence that the lyoprotectant poly(ethylene glycol) does not restore nativity to the heme active site of horseradish peroxidase suspended in organic solvents. *Biomacromolecules* **3**, 846–849.

Halling, P. J. 1987 Rates of enzymic reactions in predominantly organic, low water systems. *Biocatalysis* **1**, 109–115.

Halling, P. J. 1990 High affinity binding of water by proteins is similar in air and in organic solvents. *Biochim. Biophys. Acta* **1040**, 225–228.

Halling, P. J. 1994 Thermodynamic predictions for biocatalysis in non-conventional media: theory, tests and recommendations for experimental design and analysis. *Enzyme Microbial Technol.* **16**, 178–206.

Halling, P. J. 2000 Biocatalysis in low-water media: understanding effects of reaction conditions. *Curr. Opin. Chem. Biol.* **4**, 74–80.

Hansson, T., Andersson, M., Wehtje, E. & Adlercreutz, P. 2001 Influence of water activity on the competition between beta-glycosidase catalysed transglycosylation and hydrolysis in aqueous hexanol. *Enzyme Microbial Technol.* **29**, 527–534.

Hartsough, D. S. & Merz, K. M. 1992 Protein flexibility in aqueous and nonaqueous solutions. *J. Am. Chem. Soc.* **114**, 10 113–10 116.

Hartsough, D. S. & Merz, K. M. 1993 Protein dynamics and solvation in aqueous and nonaqueous environments. *J. Am. Chem. Soc.* **115**, 6529–6537.

Hutcheon, G. A., Parker, M. C., James, A. & Moore, B. D. 1997 Methanol dramatically enhances serine protease activity under anhydrous conditions. *Chem. Commun.* **1997**, 931–932.

Janssen, A. E. M., Sjursnes, B. J., Vakurov, A. V. & Halling, P. J. 1999 Kinetics of lipase-catalysed esterification in organic media: correct model and solvent effects on parameters. *Enzyme Microbial Technol.* **24**, 463–470.

Ke, T. & Klibanov, A. M. 1998 On enzymatic activity in organic solvents as a function of enzyme history. *Biotechnol. Bioengng* **57**, 746–750.

Kitaguchi, H. & Klibanov, A. M. 1989 Enzymatic peptide synthesis via segment condensation in the presence of water mimics. *J. Am. Chem. Soc.* **111**, 9272–9273.

Kitaguchi, H., Itoh, I. & Ono, M. 1990 Effects of water and water-mimicking solvents on the lipase-catalyzed esterification in apolar solvents. *Chem. Lett.* **7**, 1203–1206.

Klibanov, A. M. 2001 Improving enzymes by using them in organic solvents. *Nature* **409**, 241–246.

Lee, C. S., Ru, M. T., Haake, M., Dordick, J. S., Reimer, J. A. & Clark, D. S. 1998 Multinuclear NMR study of enzyme hydration in an organic solvent. *Biotechnol. Bioengng* **57**, 686–693.

Lee, M. Y. & Dordick, J. S. 2002 Enzyme activation for non-aqueous media. *Curr. Opin. Biotechnol.* **13**, 376–384.

Lee, S. B. & Kim, K. J. 1995 Effect of water activity on enzyme hydration and enzyme reaction-rate in organic-solvents. *J. Ferment. Bioeng.* **79**, 473–478.

Luca, S., Heise, H. & Baldus, M. 2003 High-resolution solid-state NMR applied to polypeptides and membrane proteins. *Acc. Chem. Res.* **36**, 858–865.

McMinn, J. H., Sowa, M. J., Charnick, S. B. & Paulaitis, M. E. 1993 The hydration of proteins in nearly anhydrous organic-solvent suspensions. *Biopolymers* **33**, 1213–1224.

Parker, M. C., Moore, B. D. & Blacker, A. J. 1995 Measuring enzyme hydration in nonpolar organic-solvents using NMR. *Biotechnol. Bioengng* **46**, 452–458.

Partridge, J., Halling, P. J. & Moore, B. D. 1996 Very high activity of cross-linked subtilisin crystals in acetonitrile by exploiting hydration hysteresis. *J. Am. Chem. Soc.* **118**, 12 873–12 877.

Partridge, J., Dennison, P. R., Halling, P. J. & Moore, B. D. 1998 Subtilisin mobility in hydrophobic organic media measured by proton NMR relaxation: hydration is more important than solvent dielectric. *Biochim. Biophys. Acta* **1386**, 79–89.

Rand, R. P. 2004 Probing the role of water in protein conformation and function. *Phil. Trans. R. Soc. Lond.* B **359**, 1277–1285. (DOI 10.1098/rstb.2004.1504.)

Rupley, J. A. & Careri, G. 1991 Protein hydration and function. *Adv. Protein Chem.* **41**, 37–172.

Schmitke, J. L., Wescott, C. R. & Klibanov, A. M. 1996 The mechanistic dissection of the plunge in enzymatic-activity upon transition from water to anhydrous solvents. *J. Am. Chem. Soc.* **118**, 3360–3365.

Schmitke, J. L., Stern, L. J. & Klibanov, A. M. 1998 Comparison of x-ray crystal structures of an acyl-enzyme intermediate of subtilisin Carlsberg formed in anhydrous acetonitrile and in water. *Proc. Natl Acad. Sci. USA* **95**, 12 918–12 923.

Soares, C. M., Teixeira, V. H. & Baptista, A. M. 2003 Protein structure and dynamics in nonaqueous solvents: insights from molecular dynamics simulation studies. *Biophys. J.* **84**, 1628–1641.

Straathof, A. J. J., Panke, S. & Schmid, A. 2002 The production of fine chemicals by biotransformations. *Curr. Opin. Biotechnol.* **13**, 548–556.

Tanaka, T., Ono, E. & Takinami, K. 1981 Method of producing improved by lipase. US Patent 4 275 011.

Theppakorn, T., Kanasawud, P. & Halling, P. J. 2004 Activity of immobilized papain dehydrated by *n*-propanol in low-water media. *Biotechnol. Lett.* **26**, 133–136.

Ueji, S., Taniguchi, T., Okamoto, T., Watanabe, K., Ebara, Y. & Ohta, H. 2003 Flexibility of lipase brought about by solvent effects controls its enantioselectivity in organic media. *Bull. Chem. Soc. Jpn* **76**, 399–403.

Valivety, R. H., Halling, P. J., Peilow, A. D. & Macrae, A. R. 1992*a* Lipases from different sources vary widely in dependence of catalytic activity on water activity. *Biochim. Biophys. Acta* **1122**, 143–146.

Valivety, R. H., Halling, P. J. & Macrae, A. R. 1992*b* *Rhizomucor miehei* lipase remains highly active at water activity below 0.0001. *FEBS Lett.* **301**, 258–260.

Valivety, R. H., Halling, P. J. & Macrae, A. R. 1993 Water as a competitive inhibitor of lipase-catalysed esterification in organic media. *Biotechnol. Lett.* **15**, 1133–1138.

van Erp, S. H. M., Kamenskaya, E. O. & Khmelnitsky, Yu. L. 1991 The effect of water content and nature of organic solvent on enzyme activity in low-water media—a quantitative description. *Eur. J. Biochem.* **202**, 379–384.

Wangikar, P. P., Graycar, T. P., Estell, D. A., Clark, D. S. & Dordick, J. S. 1993 Protein and solvent engineering of subtilisin BPN' in nearly anhydrous organic media. *J. Am. Chem. Soc.* **115**, 12 231–12 237.

Wu, J. & Gorenstein, D. G. 1993 Structure and dynamics of cytochrome-*c* in nonaqueous solvents by 2D NH-exchange NMR-spectroscopy. *J. Am. Chem. Soc.* **115**, 6843–6850.

Yamane, T., Kojima, Y., Ichiryu, T. & Shimizu, S. 1988 Biocatalysis in a microaqueous organic solvent. *Ann. N. Y. Acad. Sci.* **542**, 282–293.

Yennawar, H. P., Yennawar, N. H. & Farber, G. K. 1995 A structural explanation for enzyme memory in nonaqueous solvents. *J. Am. Chem. Soc.* **117**, 577–585.

Zaks, A. & Klibanov, A. M. 1988 The effect of water on enzyme action in organic media. *J. Biol. Chem.* **263**, 8017–8021.

Zhu, G. Y., Huang, Q. C., Zhu, Y. S., Li, Y. L., Chi, C. W. & Tang, Y. Q. 2001 X-ray study on an artificial mung bean inhibitor complex with bovine beta-trypsin in neat cyclohexane. *Biochim. Biophys. Acta* **1546**, 98–106.

Discussion

J. L. Finney (*Department of Physics and Astronomy, University College London, London, UK*). Two points. First, can you expand on the 'partly' when you say that some other molecules can (partly) substitute for water? Second, would you comment on what you think might be relevant common aspects or properties of the molecules that *can* (partly) substitute?

P. J. Halling. First, I include 'partly' to indicate that we cannot be sure that water was totally absent in any of the systems reported. It is very hard to remove the last traces of water from enzyme preparations, and perhaps even harder to measure how much remains (at the level of a few molecules per protein molecule). So we cannot exclude the possibility that a few water molecules are absolutely irreplaceable. Second, when the first of these 'water mimics' were identified, glycols and formamide, it seemed that the formation of multiple hydrogen-bonded networks could be relevant: these are the compounds that are said to show in bulk a 'solvophobic effect' that parallels the hydrophobic effect in water. However, subsequent studies showed that methanol and dimethyl sulphoxide could be effective in systems where, for example, ethanol had no effect. That makes it much harder to me to see a clear dividing line in molecular properties.

M. W. Ho (*Institute of Science in Society, London, UK*). I have two questions. First, you mention that water provides flexibility and mobility for enzymes. Are you aware of the old mobile-defect theory that not all the bonds in the protein can be satisfied all at once and so the protein has to move around and satisfy the bonds on average over a certain time? Second (this has been asked already), does the ability of molecules to substitute for water depend on their hydrogen-bonding capability?

P. J. Halling. First, I have not seen the term 'mobile defect' applied to proteins, but I do know of its analogous application to bulk water structure. It does give a reasonable picture of the model that is generally believed to underlie water effects on protein flexibility. Significant changes in conformation require changes in hydrogen bonding networks, with a transition state in which some bonds are broken before the new ones can form. Water molecules can catalyse such exchanges by acting as a temporary hydrogen-bond partner, perhaps using the bifurcated bonds we heard about in the context of bulk water structure. Second, most of them are good hydrogen bond donors and acceptors, of course, but dimethyl sulphoxide

has at most, weak donor ability. Of course, we cannot be sure that all these 'water mimics' act by the same mechanism to stimulate activity.

F. Franks (*BioUpdate Foundation, London, UK*). How do you arrive at the suggested 'hydration' levels of less than 300 mol mol^{-1} protein? What counts as 'hydration water'? Nearest neighbours, second shell? Do you know their spatial coordinates, interaction energies or exchange rates? If not, then how are 'hydration' water molecules tagged? Are these water molecules essential for a structural or kinetic role, e.g. proton transfer? It is my understanding that there are no water molecules or amino acid residues in a protein that will not exchange H for D more or less rapidly. Thermodynamics provides no information about *molecules*; so how can we distinguish 'hydration' water from any other water molecules? In drying technology, the removal of water, even from labile systems, is usually quite crude, with little attention being paid to possible inactivation processes. Would you agree that more information is needed about the location and function of specific water molecules integral to a protein structure? Given that such almost dry systems cannot be crystallized, what methods can be used to provide spatial coordinates, always on the assumption that unique locations do in fact exist?

P. J. Halling. I think you are referring to the problems of defining hydration water in aqueous solution, and I fully agree that this is very ambiguous. However, in most of the systems I am discussing, 'hydration water' can be defined simply as all the water associated with the protein in the solid phase. These water molecules must be interacting quite strongly with the protein, otherwise they would not be there. Apart from these relatively few waters, the protein will be surrounded by different molecules: other proteins, a bulk organic solvent or similar, other solid-state species. The associated water can be defined rigorously on an excess basis, analogous to the Gibbs surface excess. With a relatively non-polar bulk phase, a reasonably reliable measurement is possible as well: the correction for the low concentration of water dissolved in the bulk phase will not be too big. If we are not dealing with a pure solid protein, of course, we cannot exclude the possibility that water may be bound to the other components that are in turn bound to the protein. However, reasonable assumptions may often exclude a major contribution here: for example, we have used a hydrophobic polypropylene support that itself binds almost no water in the absence of protein. As for the role of water molecules in stimulating catalysis, I think we can rule out major structural changes as a general explanation. A number of studies, mainly using Fourier-transform infrared, have shown no large structural changes. Increased flexibility of the structure is a commonly suggested mechanism, as I described. Other kinetic roles might include proton transfer. Certainly, plenty of protein groups are also able to transfer protons, but a chain of water molecules might be required for essential long-distance transfers. It is also not possible to rule out an essential structural role for a few water molecules that are never removed even in the most exhaustively dried enzymes. Better understanding of the role of these remaining water molecules would, of course, be greatly aided if we knew exactly where they were. As I noted, although no one has crystallized proteins from non-aqueous systems, there are a number of structures known from crystals prepared in aqueous and then transferred to largely organic environments (and also at least one from crystals dried in a gas phase). These structures show substantial numbers of residual waters, necessarily at defined locations. However, as I argued, such crystal treatment approaches are only ever likely to show structures that do not differ much from the initial aqueous ones. So we should be cautious about drawing general conclusions from them. Perhaps the typical behaviour is that shown by the crystals that lose order or fracture when attempts are made to transfer them to another medium. As I mentioned, NMR is the only other method that might give coordinates. However, we need to wait for the further development of high-resolution solid-state NMR of proteins.

J. A. Littlechild (*School of Biological Sciences and Chemistry, University of Exeter, Exeter, UK*). I am interested in K_i values that increase in the absence of water. Systems where this has been measured are restricted to proteins that require a hydrophobic environment for their activity. For example, with lipases and with a hexokinase where there is a need to carry out the reaction in a hydrophobic environment in order to eliminate water so that a phosphate group from ATP is not transferred to water. When measuring protease/esterase reactions in the reverse direction in organic solvents, the mechanism is pushed to the synthetic side as a result of the enzymatic mechanism that requires water to regenerate the enzyme for hydrolysis. Have the K_i measurements you describe only been restricted to these enzymes? Is the result of K_i values increasing a general effect seen with other types of enzyme?

P. J. Halling. Sorry, I was not very clear here. I do not think there have been any measurements reported of K_i values as a function of water activity: I was suggesting that this would be a good approach to measure true binding constants by (easier) kinetic measurements. What have been reported are various measurements of K_m values as a function of water activity or concentration. Even these are too few, with too few water levels, to make reliable estimates of numbers of water molecules displaced, and hence to comment on how these depend on the type of enzyme. Even in enzymes with relatively hydrophobic binding sites, I think crystal structures will show some waters in the binding site of the free enzyme. If these are bound strongly enough to remain in place in the low-water medium, they will need to be displaced on substrate binding. But when we do have more data, it could well prove you right, with systematic differences in numbers of water displaced on binding in different categories of enzymes.

GLOSSARY

NMR: nuclear magnetic resonance