CHAPTER 13

Effects of Water and Non-aqueous Solvents on Enzyme Activity

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13.1 Introduction

Water is largely considered to be an indispensable ingredient to life. Living organisms can adapt to a surprisingly broad range of harsh conditions, including for example the glacial temperatures found in the Himalayas, the very hot (up to 400 °C) hydrothermal vents at the bottom of oceans, the very acidic or very basic conditions found in geysers and volcanic environments, and the high salinity of the Dead Sea.[1] However, despite the harshness of these extreme conditions, the presence of liquid water seems to remain an essential requirement. Even for organisms like seeds, which may survive in a dormant state in dry conditions, water is necessary to grow and develop. One may then ask what is so special about water. What are the molecular properties that make it so important for the functioning of living organisms? Is it for example its high polarity, or rather its ability to form an extended hydrogen-bonded network, or the lability of this network?

The layer of water molecules encasing a biomolecule is considered to play a large number of roles in biochemical systems, all essential to the proper

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functioning of biomolecules.[2–4] The presence of water is for example important for the structural integrity of proteins and DNA, as illustrated by the conformational change undergone by DNA at low hydration levels from the canonical B-form of the double helix to the A conformation.[5] The hydration shell is also a protection against any excess of energy that could denature the fragile scaffolding of biomolecules, held together by weak hydrogen bonds. Such an excess of energy due to, for example, a photon absorption can be quickly dissipated in the numerous vibrational modes available in the hydration shell,[6] which acts as a thermostat.

Many experimental and theoretical studies have investigated the properties of biomolecular hydration layers (see, for example, ref. 7 and 8). One key point has been to determine how much the hydration shell differs from bulk water, and especially to what extent its dynamics is perturbed by the presence of the biomolecular interface. In the limit of a very slow and viscous hydration shell, this could hinder the protein conformational motions. While some aspects are still debated, there is now a growing consensus that the water rotational and hydrogen-bond dynamics in most of the hydration shell is only moderately retarded with respect to the bulk, by a factor of 2 to 5, with some water molecules being more slowed[9–11] (Figure 13.1). This picture is supported both by NMR experiments[9,12] and by molecular dynamics simulations.[10,11] (We refer the interested reader to recent reviews on this topic.[12,13]) However, even though the perturbation induced by the biomolecule on its hydration shell appears to be limited, the presence of this hydration shell has a major impact on the functioning of the biomolecule.

In this chapter, we will focus on the effects of the hydration shell on the properties of proteins, and more specifically on the catalytic activity of enzymes. We will first review the widespread image that considers water as a lubricant of the protein motions necessary for catalysis: we will describe its

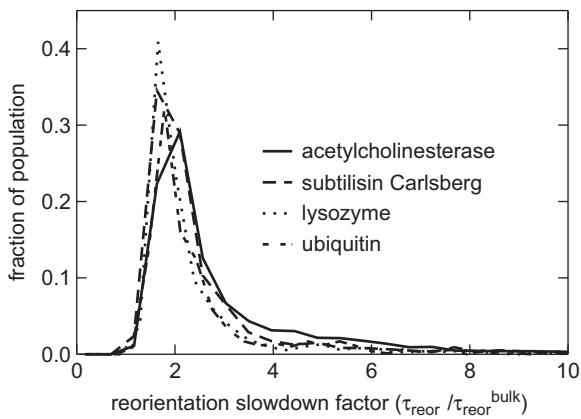

Figure 13.1 Distribution of retardation factors for the reorientation dynamics of water molecules in the first hydration shell of a series of proteins with respect to the bulk.[11]

origin and some recent experimental results that question this model. We will then discuss how some enzymes can retain a catalytic activity in non-aqueous conditions, thus opening a tremendous range of potential applications for biocatalysis. We will in particular review how changing the solvent can be used to tune the activity and the specificity of enzymes. We will then examine the different molecular pictures that have been proposed to rationalise these solvent effects on enzyme catalysis, and we will close with some concluding remarks.

13.2 Traditional Picture: Water Lubricates the Protein Motions

A widespread[2,3,14] picture sees water as a lubricant of the protein dynamics. The water molecules would act as a plasticiser that facilitates the protein conformational rearrangements necessary for the catalysis (we note that this image of water as a lubricant is also employed outside of the biochemical context, for synthetic objects including, for example, molecular machines[15]). The hydration layer would thus help proteins maintain the delicate balance between rigidity – without which the protein would lose its specificity – and flexibility – that is required to undergo conformational changes.

Protein motions take place on a rough high-dimensional free energy surface characterised by a succession of local minima corresponding to different conformations.[16] On this energy landscape, the different conformations are separated by barriers which range from low barriers for local rearrangements, as is the case for example for a simple rotation of a methyl group, up to very high barriers for large-amplitude, collective motions, including, for example, domain motions (Figure 13.2). This leads to a very broad spectrum of protein conformational motions, occurring on timescales ranging from picoseconds to milliseconds (see, for example, ref. 17).

During catalysis, the enzyme needs to rearrange from a conformation adapted to the reactant state to a conformation stabilising preferentially the reaction transition state, and in the traditional picture, water is considered to facilitate these conformational transitions.

13.2.1 Hydration, Protein Flexibility and Enzymatic Activity

Starting in the late 1970s, pioneering experimental studies investigated the effect of decreasing hydration levels on the activity of enzymes in hydrated powders (see ref. 18 for a review). For a series of enzymes including chymotrypsin, lysozyme, subtilisin Carlsberg, urease and amylase, a minimum amount of water was found to be necessary to detect a catalytic activity (see Figure 13.3). This critical hydration level was measured to range from 0.1 to 0.3 g of water per g of protein. For lysozyme, this amounts to close to 300 water molecules,[19] *i.e.* approximately the quantity of water necessary to form a monolayer around the protein. Above this minimum hydration, enzymatic

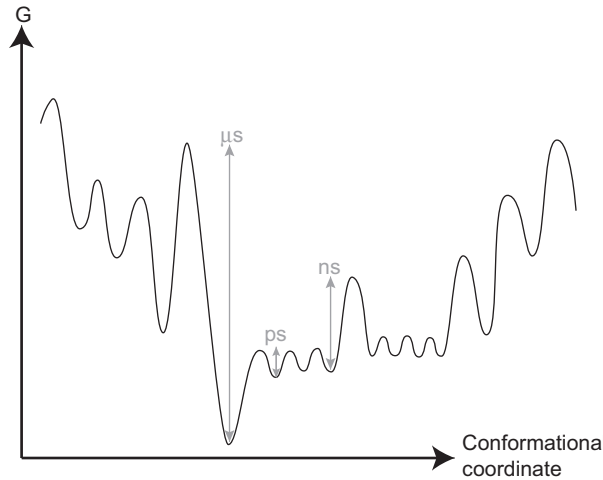

Figure 13.2 Schematic protein free energy landscape with different barrier heights, leading to a broad range of conformational rearrangement timescales.

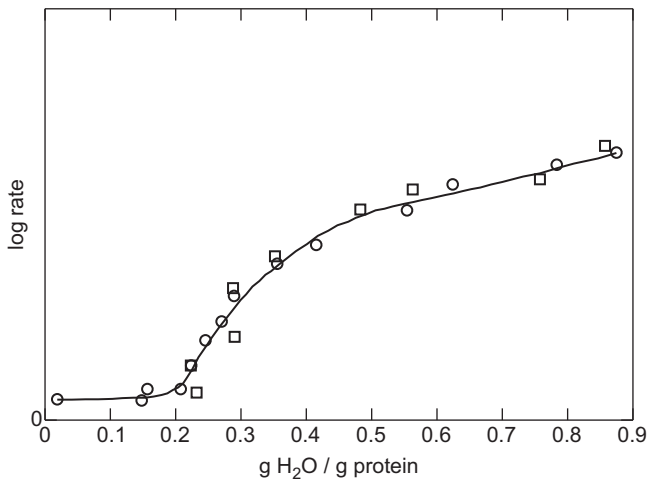

Figure 13.3 Hydration dependence of the lysozyme enzymatic activity (squares) and protein relaxation dynamics measured by ESR (circles) (data from ref. 19; the line is shown as a guide to the eye).

activity rises abruptly to reach a plateau value at high hydration levels (see Figure 13.3).

In addition, for systems like lysozyme,[18] the increase in the protein dynamics (measured here by the reorientation relaxation time of an ESR spin probe[18,19]) was measured to closely parallel the sigmoidal shape of the increase in the enzymatic activity (see Figure 13.3).

This correlation thus led to a picture[18] where the hydration shell gives the enzyme the mobility that is required to undergo the conformational rearrangements necessary to bind the substrate and convert it to a product (however, we stress that the experiments only showed a correlation between these properties, and did not establish an actual causality). In this image, the progressive addition of water is considered to enhance the protein flexibility for example by replacing the protein–protein hydrogen bonds with protein–water hydrogen bonds that can be broken and formed more easily, and by partly screening the electrostatic interactions between charged sites in the protein. According to this picture, a minimum amount of water is necessary to induce the protein flexibility, and once the protein is covered by approximately a full layer of water, the activity reaches a plateau value (Figure 13.3).

The relationship between protein hydration and protein dynamics was examined with a broad range of techniques. In addition to the above-mentioned ESR measurements, these include for example Mössbauer spectroscopy that showed that myoglobin does not exhibit significant motions at low hydration levels and ambient temperature, while a marked increase in the dynamical amplitudes is measured at a higher relative humidity.[20] The mean-square displacement of protein atoms was also measured by neutron scattering[21,22] (Figure 13.4). In dry protein powders, the amplitude of the protein motions remains very limited at every temperature. In contrast, for hydrated powder samples, a sudden increase in the amplitude of the protein motions is measured as the temperature is increased above approximately 200 K. This has been suggested to correspond to a dynamical transition[21] and to the onset of larger amplitude anharmonic protein motions, while at lower temperature the motions would be limited to harmonic fluctuations around a single conformation.

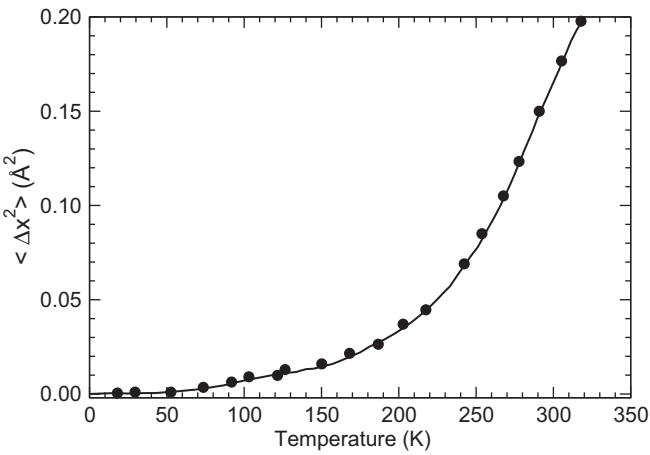

Figure 13.4 Mean-square displacements of hydrogen atoms in myoglobin measured by neutron scattering as a function of temperature (data from ref. 21).

13.2.2 Inconsistencies

Although the simplicity of this picture connecting the hydration level, the protein flexibility and the enzymatic activity is very appealing, subsequent experimental results revealed some inconsistencies, which suggest that a more elaborate description is required to understand the molecular-level effect of water on enzyme catalysis.

Some concerns were raised[23] about the experimental procedure that is employed to prepare the samples and measure the activity (see a description and references in ref. 18). In these experiments, the enzyme and the substrate are mixed quickly together in solution and immediately frozen and lyophilised to stop the reaction. The powder is then rehydrated at a controlled hydration level and the enzyme activity is measured at ambient temperature.[18] However, if the freeze-drying step is not fast enough with respect to the reaction time, there is a possibility that what is actually measured is the product release rather than the catalytic step.[23]

To overcome these difficulties, different experimental conditions can be employed, that do not require the freezing and unfreezing steps for the enzyme–substrate mixture, during which reactions can occur. They involve either enzymes in non-aqueous organic solvents with trace amounts of water, or enzymes with gaseous substrates where the relative humidity can be varied. Experiments performed on the pig liver esterase enzyme and the *Candida rugosa* lipase B in the vapor phase showed that an enzymatic activity can still be detected at very low hydration levels,[24] below 0.03 g of water per g of protein, *i.e.* with typically less than 30 water molecules per protein (see Figure 13.5). No sign of a minimum hydration level below which the enzymatic activity ceases completely was found in these experiments. This is further supported by very recent experiments, where myoglobin and a lipase enzyme were found to remain active in solvent-free environments when a polymer corona is attached to the biomolecule and replaces the solvent.[25,26] This series of experimental results at very low hydration levels therefore question the existence of a minimum hydration threshold for activity.

The connection between catalytic activity and protein flexibility may also need to be revised. At the very low hydration levels used in a study of the pig liver esterase enzyme and of the *Candida rugosa* lipase B,[24] the water molecules can cover only on the order of 10% of the protein exposed surface (we further note that this assumes that water molecules do not form droplets, which would lead to an even smaller coverage of the protein interface). Therefore, they cannot have a significant effect on its flexibility. In addition, measurements at temperatures below the protein dynamical transition have shown that while the protein anharmonic structural dynamics are arrested, a residual catalytic activity can still be detected.[27,28] This shows that although protein flexibility may facilitate the catalysis, it is not indispensable to the catalytic activity.[29]

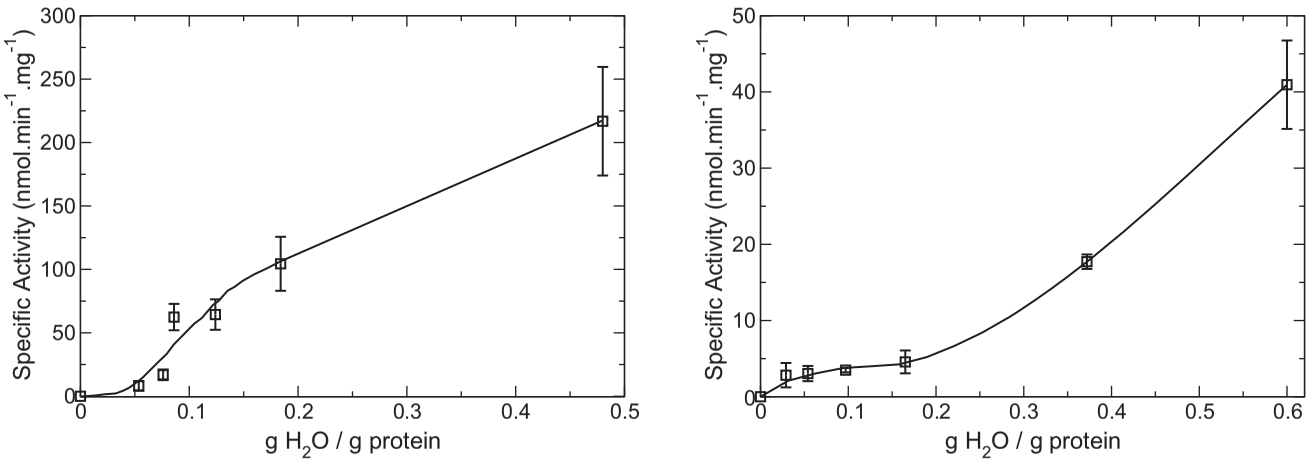

Figure 13.5 Changes in the vapour-phase catalytic activities of *Candida rugosa* lipase B (left) and of pig liver esterase (right) with hydration level (data from ref. 24).

13.3 Enzyme Catalysis in Non-aqueous Organic Solvents

13.3.1 Overview

Although the presence of water may not be a prerequisite for an enzyme to be catalytically active, the presence and the nature of the solvent surrounding the protein both have a strong effect on the functioning of the enzyme. And the range of solvent conditions in which enzymes can catalyse reactions is surprisingly broad![30]

This was realised a few decades ago when enzymes were found to remain active in non-aqueous organic solvents,[31–35] which paved the way for exciting new developments in biocatalysis. Indeed, enzymes are remarkably efficient catalysts, perfectly suited to supporting life, but usually not so well suited for technology. In their natural aqueous environment, exploiting their excellent catalytic power for synthetic chemistry and to catalyse 'non-natural' reactions is greatly limited by several obstacles, including the low water solubility of most compounds and the presence of unwanted degradation side reactions such as hydrolysis.[34,36] Upon transfer to non-aqueous media, it was long believed that enzymes' folded structures would not survive. However, recent studies have firmly established that a large range of enzymes, including lipases, esterases and proteases, not only retain their structural integrity[34,37] but also display a catalytic activity in the absence of bulk water, for example in organic solvents[1,34] and ionic liquids.[1] While the unfolded state may be more stable than the folded structure (because of favourable interactions between the hydrophobic protein core and the organic solvent), these proteins are kinetically trapped in their folded form, and their melting temperature can be even higher than in aqueous solution.[38] (However, we note that some organic solvents including, for example, acetonitrile tend to denature the proteins by 'stripping off' the residual water molecules at the protein interface.[39]) This therefore opened up a tremendous range of potentialities in chemistry, to catalyse non-natural reactions. New reactions that were suppressed in water can be catalysed, and the enzyme activity and selectivity can be finely tuned through the choice of organic solvent.[1,34] For example, hydrolase enzymes that catalyse a bond cleavage by hydrolysis in aqueous conditions can then catalyse transesterification reactions when used in organic solvents[31] (Figure 13.6). Enzyme catalysis in organic solvents is now widely used by the chemical and pharmaceutical industries, as illustrated for example by the synthesis of derivatives of the widely used antitumoral drug taxol that are more soluble in water than the parent molecule[40,41] (Figure 13.7).

13.3.2 Solvent Effects on Enzyme Activity and Specificity

Over recent decades, a broad range of experimental studies have established that for a given catalysed reaction, the enzymatic activity, *i.e.* the catalytic rate constant, is very sensitive to the nature of the solvent conditions.[34,42]

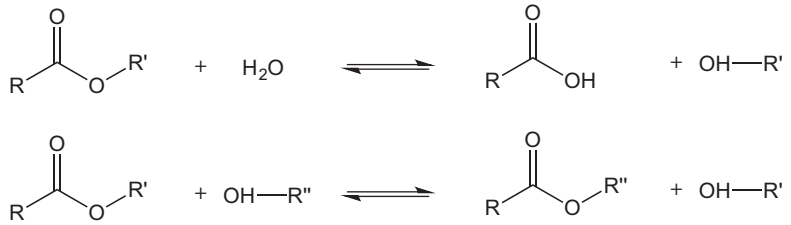

Figure 13.6 Mechanisms of hydrolysis (top) and transesterification (bottom) reactions of a carboxylic acid ester.

This has been a topic of major interest and a key challenge for the practical application of enzymes in non-aqueous conditions, because the enzymatic activity is usually greatly reduced in these solvents compared to that in aqueous conditions (up to a 10^4 decrease).[1,34] A series of empirical strategies have then been developed to activate the catalysis, including for example the addition of small amounts of water,[43] ions[44] or water mimics such as glycerol or ethylene glycol.[1] This trial and error approach has successfully recovered activities similar to those in water in some cases,[1] but a molecular-level understanding of the mechanisms underlying these rate enhancements has remained elusive, which precludes a systematic optimisation of the solvent conditions to maximise the catalytic activity.

Even if a residual catalytic activity remains at very low hydration levels, the addition of trace amounts of water in the non-aqueous organic solvent (*e.g.* iso-octane) can enhance the catalytic rate constant by more than one order of magnitude[43] (see Figure 13.8). It has also been found empirically that the addition of some ions such as sodium fluoride (NaF) or sodium acetate ($NaOOCCH_3$) can lead to a large catalytic enhancement, while some other ions (like sodium iodide NaI) only have a limited effect on the catalytic efficiency.[45]

In addition to the catalytic activity, the specificity of a given enzyme vis-à-vis two different substrates has been shown to be affected by the nature of the solvent conditions.[34] While in an aqueous solvent, such a change in the specificity would require a full site-directed mutagenesis study, in non-aqueous solvents it can be tuned by altering the solvent conditions. Examples include changes in the chemoselectivity between two chemically distinct substrates,[46] but also changes in the regioselectivity and in the enantioselectivity.[34] The latter is particularly interesting for applications in synthetic chemistry, in order to distinguish chiral molecules which otherwise have identical chemical and physical properties in an achiral environment. While an (over-)simplified approach could consider that the enzymatic catalytic rates for two enantiomers would be affected by a solvent change in the same way, thus keeping the enantiomeric ratio unchanged,[47] experimental measurements showed a pronounced solvent dependence of the enantioselectivity.[48] However, while the enantiomeric ratio exhibits some correlation with solvent parameters including for example the solvent dipole moment, its relative permittivity and its hydrophobicity,[48] recent

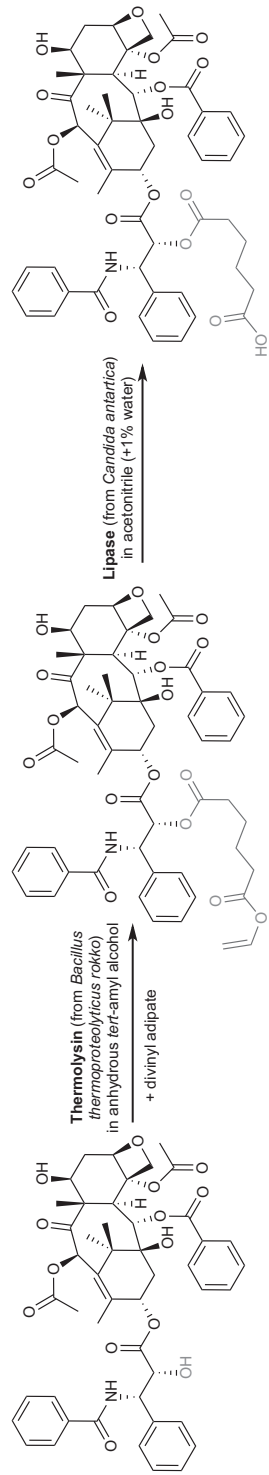

Figure 13.7 Biocatalytic synthesis of paclitaxel (taxol) derivatives.[41]

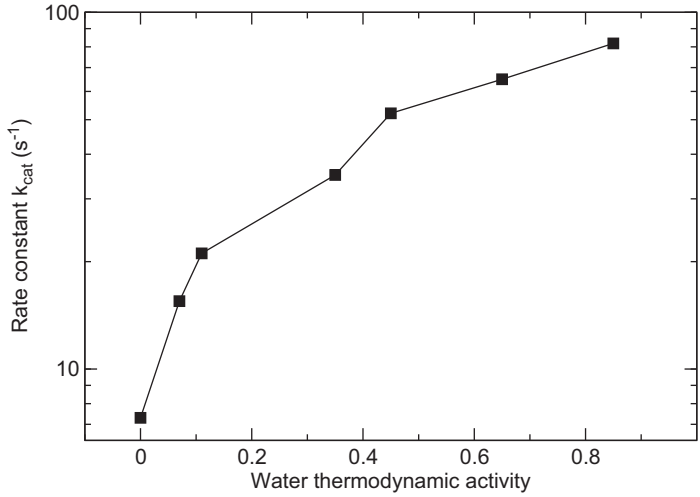

Figure 13.8 Catalytic rate constant for the transesterification reaction catalysed by Subtilisin Carlsberg in isooctane with increasing quantities of water (data from ref. 43).

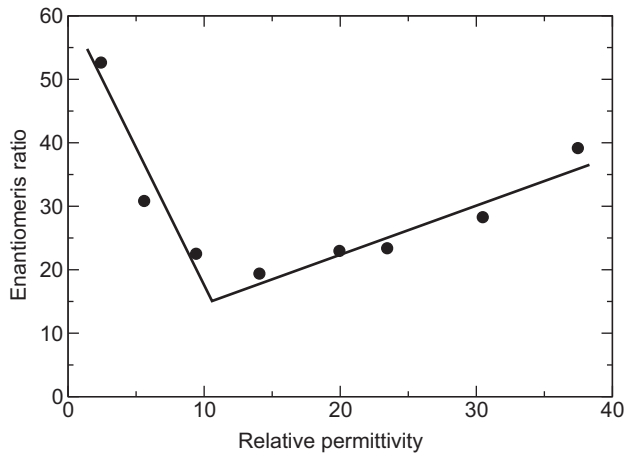

Figure 13.9 Ratio of the k_{cat}/K_M ratios for the *R* and *S* enantiomers for the acetylation of an alcohol catalysed by the *Candida antarctica* Lipase B enzyme for a series of solvent mixtures with different dielectric permittivities (data from ref. 49).

experiments revealed a puzzling non-monotonic dependence of the enantiomeric ratio with the composition of a mixed solvent for the resolution of primary and secondary alcohols by the Candida Antarctica Lipase B[49] (see Figure 13.9). Whether the solvent could affect the enzyme enantioselectivity by changing the protein flexibility is still unclear, since experimental results have lead to different, sometimes seemingly contradictory explanations.[50,51]

A decrease in the protein flexibility may be argued either to increase the enantioselectivity by providing a better discrimination of the two enantiomers, or on the contrary to decrease the enantioselectivity by hindering the accommodation of the fast reacting enantiomer more than that of the slow reacting one.

Therefore, here again, while some first studies have started to investigate the solvent effects on the enantiospecificity,[52] a full molecular-level understanding of the key effects at play is still missing.

13.4 Towards a Molecular Picture of Solvent Effects on Catalysis

We now review several possible molecular mechanisms that have been suggested to explain the effect of water on the enzymatic activity and specificity, together with some experimental results that are in agreement or in contradiction with these pictures.

As a preliminary remark, we stress that while increasing the fraction of water in the solvent usually leads to an increase in the enzymatic rate constant (as found, for example, for proteases in apolar organic solvents,[43] for an esterase in the gas phase[53] and for a reductase in water–cosolvent mixtures[54]), the opposite behavior has also been observed. It can be due to the 'stripping off' of the residual water molecules at the protein interface and the denaturation of the protein,[55,56] but the rate constant of the chemical step in the catalysis has also been measured to decrease for a structurally intact thermophilic reductase when the water fraction is increased.[57]

13.4.1 Solvent Polarity

Gradually replacing the aqueous solvent with another less polar solvent leads to a decrease in the solvent dielectric constant. Since the charge distributions of the reactant and transition states differ, this could affect differently the two states and modify the reaction free energy barrier. For example, a series of experiments performed on different DHFR homologues from mesophilic and thermophilic organisms in various water–cosolvent mixtures[54,58] were shown to yield a good correlation between the measured catalytic rate constant and the effective solvent dielectric constant (see Figure 13.10a). However, it is not fully clear whether a simple dielectric continuum picture that ignores the molecular structure of the active site is applicable to these systems.

13.4.2 Lubrication Picture

Another popular picture involves the lubricating effect due to the solvent.[34,36] This idea was already mentioned in Section 13.2.1 in connection with protein dynamics, and we focus here on its effect on the catalytic rate

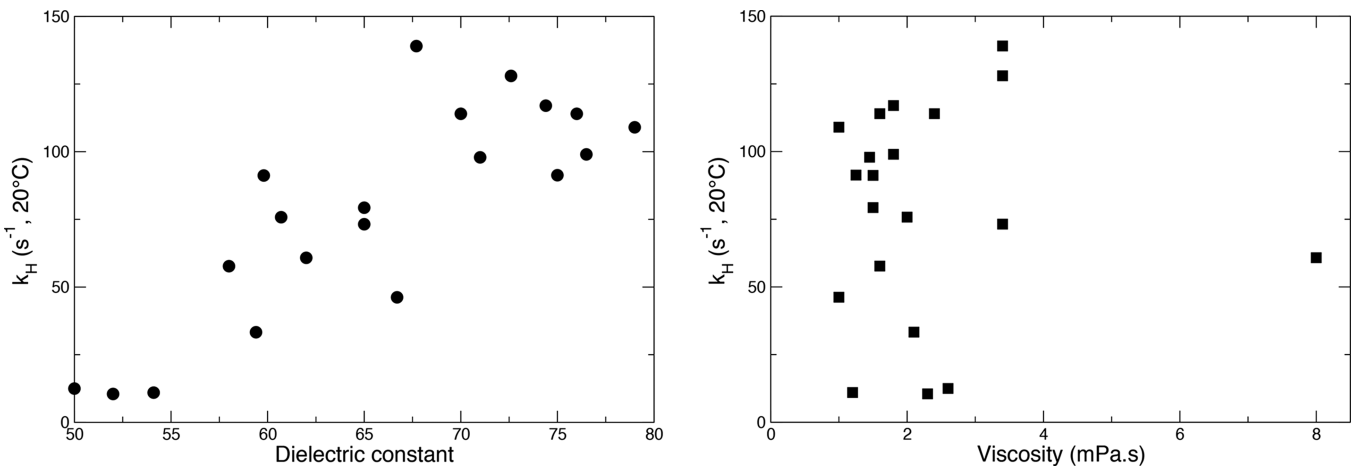

Figure 13.10 Rate constant of the hydride transfer reaction catalysed by BsDHFR in aqueous mixtures with different dielectric constants and different viscosities (data from ref. 58).

constant. The 'lubrication' terminology has been used with different meanings. It is sometimes employed in an equilibrium perspective, where the solvent would reduce the free energy barriers between different conformations (*e.g.* the open and closed M20 loop configurations in DHFR[59]) and especially the free energy cost to access enzyme conformations where the barrier for the chemical step is lower. But it also has a dynamical meaning, where the lubricating effect would imply a reduced friction on the reaction coordinate governing the chemical step (while the equilibrium free energy profile remains unchanged).

Experimental and simulation studies concluded that a decrease in the hydration level does lead to a decreased flexibility in the protein.[56] However, these studies did not specifically probe the motions involved in the reaction coordinate. While a change in the hydration level may strongly affect the flexibility of exposed protein sites, it may not have a significant effect on the active site residues directly involved in the chemical step of the catalysed reaction. For example, experimental measurements of the catalytic rate constant for a series of dihydrofolate reductase enzymes did not reveal any correlation with the viscosity of the solvent mixture[54,57,58] (see Figure 13.10b), thus suggesting that the protein flexibility may not be a key factor. In addition, other experiments have shown that adding large quantities of ions to an enzyme in an apolar organic solvent with less than a monolayer of residual water can greatly enhance the catalytic activity of a protease.[44] This was suggested to arise from the structure-breaking character of these ions, which would increase the lability of the hydrogen-bond network formed by the residual water molecules.[44] However, extensive experimental and simulation studies of the effect of ions on the dynamical properties of aqueous solutions have shown that the structure-breaker and -maker classification is often ambiguous[60] and that at the concentration levels used in these enzymatic systems, the ions actually increase the solution viscosity.[61] It thus seems difficult to explain the enhanced enzymatic activity by an enhanced lubrication, since the addition of the ions makes the solvation shell more viscous. Finally, reducing the friction on the reaction coordinate governing the chemical step would only be important if dynamical effects at the transition state played an important role in the catalytic rate constant, but recent works suggest that this effect is certainly limited.[62]

13.4.3 Competitive Inhibition

The two previous descriptions ignored the molecular structure of the solvent and focused exclusively on its relative permittivity and viscosity properties to interpret the solvent effects on catalysis. However, different solvent molecules have different abilities to penetrate into the enzymatic active site, where they can compete against the substrate for binding. It has been suggested that in some cases both the solvent and substrate molecules can be simultaneously present in the active site and the solvent molecule thus effectively changes the size of the enzymatic pocket.[63] This interplay could then lead to solvent

effects that depend on the shape[64] and on the size[65] of the solvent molecule, and that would thus be very specific to each solvent molecule.

13.5 Concluding Remarks

In this chapter, we have reviewed the available experimental data and molecular models describing the effect of different solvents, including water, on the catalytic activity of enzymes. Enzymes are found to remain catalytically active in surprisingly harsh environments, including almost dry vapors and organic solvents, but the addition of water usually enhances the catalytic activity. A popular picture suggests that water acts as a lubricant of the protein conformational motions required for catalysis but this dynamical picture is not supported by recent results. Further experimental and theoretical studies will thus be needed to provide a consistent molecular interpretation of the solvent effects on the catalytic activity and specificity of enzymes.

Acknowledgements

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