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Activity and dynamics of an enzyme, pig liver esterase, in near-anhydrous conditions

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Abstract

Water is widely assumed to be essential for life, although the exact molecular basis of this requirement is unclear. Water facilitates protein motions, and although enzyme activity has been demonstrated at low hydrations in organic solvents, such nonaqueous solvents may allow the necessary motions for catalysis. To examine enzyme function in the absence of solvation and bypass diffusional constraints we have tested the ability of an enzyme, pig liver esterase, to catalyze alcoholysis as an anhydrous powder, in a reaction system of defined water content and where the substrates and products are gaseous. At hydrations of 3 (52) molecules of water per molecule of enzyme, activity is several orders-of-magnitude greater than nonenzymatic catalysis. Neutron spectroscopy indicates that the fast (%nanosecond) global anharmonic dynamics of the anhydrous functional enzyme are suppressed. This indicates that neither hydration water nor fast anharmonic dynamics are required for catalysis by this enzyme, implying that one of the biological requirements of water may lie with its role as a diffusion medium rather than any of its more specific properties.

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Activity and Dynamics of an Enzyme, Pig Liver Esterase, in Near-Anhydrous Conditions

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ABSTRACT Water is widely assumed to be essential for life, although the exact molecular basis of this requirement is unclear. Water facilitates protein motions, and although enzyme activity has been demonstrated at low hydrations in organic solvents, such nonaqueous solvents may allow the necessary motions for catalysis. To examine enzyme function in the absence of solvation and bypass diffusional constraints we have tested the ability of an enzyme, pig liver esterase, to catalyze alcoholysis as an anhydrous powder, in a reaction system of defined water content and where the substrates and products are gaseous. At hydrations of 3 ($\pm$2) molecules of water per molecule of enzyme, activity is several orders-of-magnitude greater than nonenzymatic catalysis. Neutron spectroscopy indicates that the fast ($\leq$nanosecond) global anharmonic dynamics of the anhydrous functional enzyme are suppressed. This indicates that neither hydration water nor fast anharmonic dynamics are required for catalysis by this enzyme, implying that one of the biological requirements of water may lie with its role as a diffusion medium rather than any of its more specific properties.

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An understanding of the role of hydration in enzyme activity is a central question in molecular biophysics (1–10). Previous work has indicated that the hydration required for activity is below the monolayer coverage (11–17). For instance, pig liver esterase (PLE; molecular mass of monomeric form ~60,070 Da) has been found to have hydrolytic activity at a hydration level of 0.03 g water/g enzyme (*h*) at room temperature; i.e., ~100 water molecules per molecule of protein (15). PLE is useful for low hydration studies because water is neither a substrate nor a product in the alcoholysis reactions catalyzed. For our study, the acyl transfer between methyl butyrate and propanol was followed by headspace analysis. The isotopic labeling of water molecules and its quantification by mass spectrometry is one of the most sensitive (18) methods of water determination. This method is used here to quantify low levels of PLE hydration and, accompanied by activity measurements and neutron spectroscopic experiments, has allowed the correlation of protein hydration with flexibility and activity. The role of water as a reactant or as a diffusion medium for the products and substrates of the reaction is precluded here by the use of a gas phase transesterification catalytic system.

Fig. 1 shows that enzyme activity is observed at all hydration levels investigated.

The lowest hydration achieved (see the *inset* to Fig. 1) is 3 ($\pm$2) water molecules per molecule of protein. This hydration level may relate to the presence of internal water molecules that cannot be removed by the method we have used, but with current analytical methods this is difficult to verify experimentally, and there is a significant possibility that the enzyme is actually anhydrous at this reported hydration. The hydration level at which activity is observed is thus very much lower than the 0.2 g of water per g of protein (*h*), i.e., a mole ratio >600, conventionally taken to be necessary for enzyme activity, and represents a qualitatively lower hydration regime. The first stage of any protein sorption isotherm consists of the hydration of the ionized groups at the protein surface, up to ~0.05 *h* (19). The data here show that enzyme activity occurs and increases up to this level of PLE hydration. Although the enzyme rates are low, they are at least one-order-of-magnitude higher than the uncatalyzed rate. At very low hydrations there is no clear correlation between activity and hydration, so although completely anhydrous enzyme may not have been achieved, enzyme activity at zero hydration seems likely. Water that interacts directly with the protein surface has been generally thought to play a major role in protein function (4). Because a water content as low as 3 $\pm$ 2 water molecules per molecule of PLE represents an insignificant coverage of the charged groups of the protein surface, the evidence here indicates

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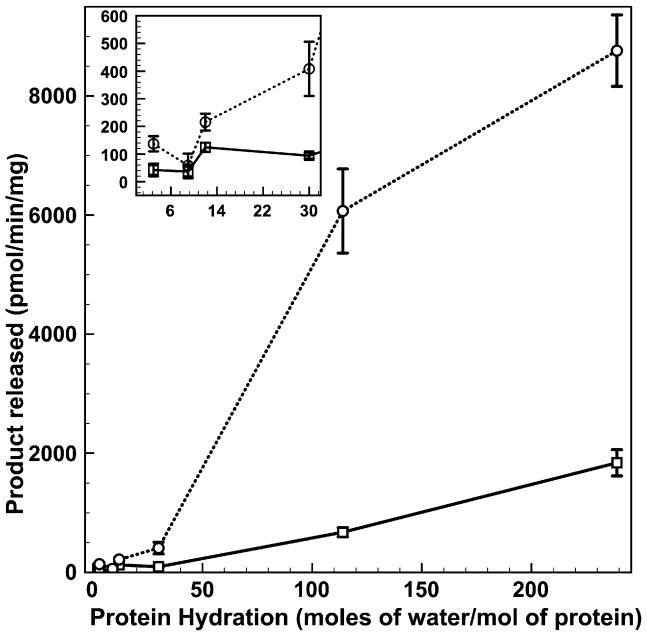

FIGURE 1 Enzyme activity with respect to propyl butyrate (*lines*) and methanol (*dashed lines*) production in the gas phase, as a function of the protein hydration. (*Inset*) Blow-up of the very low hydration region of the plot.

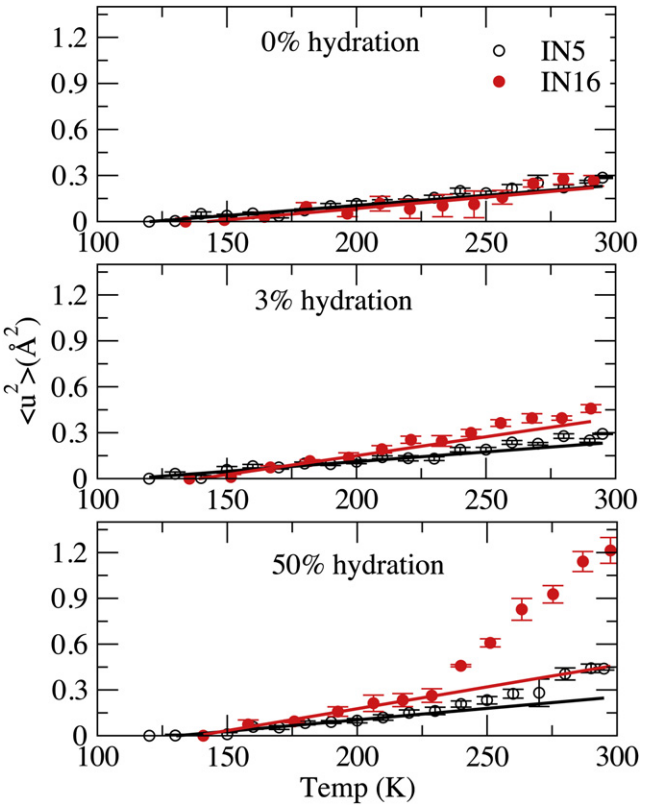

FIGURE 2 $\langle u^2 \rangle$ of PLE as a function of the temperature for the three hydrations measured and obtained from data collected with IN16 and IN5 (23).

that surface hydration water is not essential for PLE activity, although it may facilitate it.

With PLE being active at hydration levels close to zero, any motions required for the onset of enzyme activity are not likely to be dependent on hydration. Although water seems to play a major role in protein dynamics, previous work on xylanase in cryosolvent has revealed that this enzyme may be active while apparently rigid (20).

Thus, any correlation among enzyme hydration, dynamics, and activity is still not clear (21,22). To examine the fast motions of the enzyme, the average internal atomic mean-square displacement of PLE, $\langle u^2 \rangle$, was determined by neutron scattering with the IN5 time-of-flight spectrometer (23) and the IN16 backscattering spectrometer (see the Supporting Material) at the Institut Laüe-Langevin, Grenoble, France. These measurements were performed on dried or hydrated powders for activity measurements.

In Fig. 2, $\langle u^2 \rangle$ is shown as a function of temperature for three different hydrations.

The curve for the fully hydrated control, (0.5 h), exhibits a change in slope at ~220 K—this is the so-called dynamical transition or glass transition of the protein, where the protein motions apparently pass out of the timescale window of the instrument (24,25). The activation of motions at the dynamical transition has been associated with protein function. For the two other lower-hydration samples, the anharmonic motions that are reflected in the increased slope above the dynamical transition are strongly suppressed, consistent with their being largely solvent-driven (7,26,27). These results are consistent with an interpretation that water decreases the energy barriers between local minima, as is required for the onset of diffusive motions of the protein atoms (7,28). However, we note that NMR has shown that the inherent inhomogeneous temperature dependence of motion predicts the dynamical transition, consistent with it not being a product of solvent slaving per se (29). Because of the differing energy resolutions of the respective instruments, IN16 (Fig. 2) probes motions on a nanosecond timescale whereas IN5 (Fig. 2) probes motion on a picosecond timescale. With IN16, a steeper change in slope with hydration is observed than for IN5 (23), indicative of the effect of the energy resolution on the mean-square displacement: IN16 has a finer resolution and thus incorporates additional, slower motions into the mean-square displacement.

CONCLUSIONS

Our work shows clear evidence that the activity of PLE does not necessarily require that the enzyme be significantly hydrated: within the limits of the water detection method used, activity at very near zero hydration has been observed. It is important to realize that the hydration level of 3 ± 2 is an average, and that those enzyme molecules in the sample exhibiting the residual activity might be significantly more highly hydrated. Whether our results can be generalized to all enzymes is an open question. Perhaps pig liver esterase

is comparatively rigid, requiring only stabilization of the transition state of the catalyzed reaction, consistent with the idea that electrostatic preorganization accounts for the observed catalytic effects of enzymes, rather than dynamical effects (30). Other enzymes such as those involving mechanical displacements may require higher hydration levels.

Our results raise general questions concerning the role of surface hydration in enzyme activity. Clearly hydrolysis reactions require the participation of water molecules, and some proteins contain strongly-bound structurally important water molecules that may be difficult to remove by drying. However, the results show that, in principle, although hydration facilitates activity (probably due to the dynamical effects manifested above the glass transition in the neutron spectra in Fig. 2), significant solvation is not an absolute requirement.

Given that water is the only readily-available terrestrial liquid solvent, it is unsurprising to find its incorporation in proteins, and dependence upon it as diffusion medium. However, our results are consistent with the main role of water in enzymology being a (nonspecific) solvent and diffusion medium rather than a chemically unique essential component.

SUPPORTING MATERIAL

One figure and additional details are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00975-6.

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Supporting Material

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Supplementary material

Protein preparation

Pig liver esterase (PLE) (150 units/mg, EC 3.1.1.1) was obtained from Sigma, and further partially purified using Fast Flow Q Sepharose. The enzyme powder was dried over high grade phosphorus pentoxide $P_2O_5 \geq$ 99.99 % (Aldrich). To reach different hydration levels, the equilibration time of the protein over P_2O_5 has been extended (one, two or four weeks) and the temperature changed eventually from room temperature to 65°C over two weeks drying for the second lowest hydration point.

Water quantification

The protein powder was equilibrated against 1 g of pure ^{18}O-labeled (^{18}O atom ≥95%), Cambridge Isotope Laboratories (CIL, USA) water for 2 to 3 days in a confined environment until a hydration level of 30-40 % (w/w) was reached. After drying, the enrichment in ^{18}O of the protein sample was determined by mass spectrometry.

Gas phase activity measurements

The alcoholysis reaction studied here is the transfer of a butyl group between methyl butyrate and propanol catalysed by the pig liver esterase:

Methyl butyrate + propanol = methanol + propyl butyrate

The mechanism of this reaction catalysed by PLE implies the formation of an acyl-enzyme intermediate and the release of the product alcohol, followed by the release of the product ester as described in the figure below.

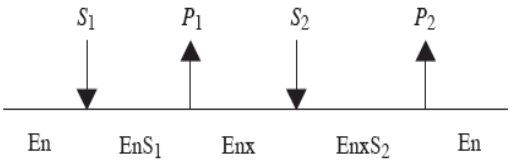

Figure 1: Alcoholysis supposed reaction mechanism.

In a elementary reaction, the ratio of the number of molecules of products produced should be one BUT the alcoholysis reaction is a two step réaction and each step has its own rate and its own regulation. Usually the second step is the limiting step. It is not surprising to find that water act differently on each step of the reaction. In addition, methanol which is the first product released is known to activate the first step. Thus methanol probably promotes its own synthesis here. Another reason to this observation is that we are measuring initial rates (Greenzaid P. & Jencks W.P. 1971. *Biochemistry*.10(7):1210-22)

The enzyme catalysed rate of butyl transfer between the methyl butyrate and propanol was measured using gas phase chromatography in a dual-mininert® system, allowing the drying of 5 mg of enzyme powder and isolation of the drying agent before measurement of the enzyme activity. The gas phase chromatograph (Varian 3000) was equipped with a flame ionization detector and a slightly polar packed column (Chromosorb 101, Supelco). The column was maintained at 170 °C. The flow rates were 30 mL/min for the dry N_2 and H_2, and 300 mL/min for the dry air. The control (non-enzymic) rate was zero in the conditions of our experiments (less than 0.5 pmol/min/mg).

Neutron Scattering

The samples were prepared and analysed in the manner described in (24). The incident neutron wavelengths were 5.1 Å and 6.27 Å on IN5 and IN16, respectively. All data were collected with the sample holder oriented at 135º relative to the incident beam. The samples were contained in aluminium flat-plate cells, of 0.3 mm thickness. Spectra were measured with a temperature ramp starting at 80 K and increasing to 300 K in steps of 10 K every half an hour. The measured transmission for all the samples was 0.96 indicating that multiple scattering was negligible.