Enzyme activity and dynamics in near-anhydrous conditions

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Water is widely assumed to be essential for life [1], although the exact molecular basis of this requirement is unclear [2-4]. Water facilitates protein motions [5-9] and although enzyme activity has been demonstrated at low hydrations in organic solvents [10-13], such non-aqueous 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 esterase to catalyse alcoholysis as an anhydrous powder, using a closed reaction system in which the substrates and products of the enzyme reaction are gaseous [14-15], and where the water content can be well defined [16]. At hydrations equivalent to 3 (±2) molecules

of water per molecule of enzyme, activity is observed that is several orders of magnitude greater than non-enzymatic catalysis. Neutron spectroscopy indicates that the fast (≤nanosecond) global anharmonic dynamics of the anhydrous functional enzyme are heavily suppressed. The results indicate that neither hydration water nor the solvent-activated fast anharmonic dynamics are required for enzyme function. An implication of these results is that one of the essential requirements of water for life may lie with its role as a diffusion medium rather than any of its more specific properties.

Pig liver esterase (PLE) was used here to catalyse alcoholysis reactions where water is neither a substrate nor a product. The acyl transfer between methyl butyrate and propanol, giving rise to methanol and propyl butyrate, was followed by headspace analysis and gas chromatography. Previous work has indicated that any hydration required for activity is below the monolayer coverage [10,15,17-18]. For instance, PLE has been found to have hydrolytic activity at a hydration level of 0.03 grams of water per gram of dried enzyme (*h); i.e.,* about100 water molecules per molecule of protein. Thus, if we are to probe possible activity close to zero water content, accurate determination of very low levels of protein hydration is essential. Among the techniques now available [19], the isotopic labelling of water molecules and its quantification by mass spectrometry is one of the most sensitive [16] and is used here to quantify the average number of water molecules bound to PLE after extensive drying.

Figure 1 shows that enzyme activity is observed at all hydration levels investigated. The lowest hydration achieved (see the inset to Figure 1) is about 3 (±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 very much lower than the 0.2 *h* (a mole ratio of over 600) conventionally taken to be necessary for enzyme activity, and represents a qualitatively lower hydration regime in which experimental enzyme activity and dynamics studies can be made. The first stage of any protein sorption isotherm consists of the hydration of the ionised groups at the protein surface, up to about 0.05 *h* [20]. 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 several orders of magnitude higher than the un-catalysed 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. Surface water that interacts directly with the protein has been generally thought to play a major role in protein function [4]. Since a water content as low as 3 ± 2 water molecules per molecule of PLE represents an insignificant coverage of the charged groups of the protein surface the evidence here indicates that surface hydration water is not essential for activity, although it may facilitate it.

Figure 1: Enzyme activity with respect to propyl butyrate and methanol production in the gas phase, as a function of the protein hydration *h* (g of water/ g protein). The inset is a blow-up of the very low hydration region of the plot.

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 [21]. Thus, any correlation between enzyme hydration, dynamics and activity is still not clear [22-23].

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 and the IN16 backscattering spectrometer at the Institut Laüe-Langevin, Grenoble, France.

In Figure 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 ~220K – 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]. 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]. Because of the differing energy resolutions of the respective instruments, IN16 (Figure 2) probes motions on a nano-second timescale while IN5 (Figure 2) probes motion on a pico-second timescale. With IN16, a steeper change in slope with hydration is observed than for IN5 [29], indicative of the effect of the energy resolution on the mean-square displacement (MSD): IN16 has a finer resolution and thus incorporates additional, slower motions into the MSD.

The present work shows clear evidence that enzyme activity 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. These results raise questions concerning the role of hydration in enzyme activity. Clearly hydrolysis reactions require the participation in the chemical steps of at least one water molecule, and some proteins contain strongly-bound structurally-important water molecules that may be difficult to remove by drying. However, the present results raise

questions concerning the role of surface hydration water in protein function. The results show that, although hydration facilitates function, due possibly in part to the dynamical effects manifested above the "glass transition" in the neutron spectra in Figure 2, solvation is not an absolute requirement. 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. 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, the present results are consistent with the main role of water in enzymology being as a (non-specific) solvent and diffusion medium rather than as a chemically-unique essential component.

Figure 2: $\langle u^2 \rangle$ of PLE as a function of the temperature for the three hydrations measured and obtained [29] from data collected with IN16 and IN5.

Methods summary

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 $P_2O_5 \geq 99.99$ % (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 enzyme catalysed rate of butyl transfer between the methyl butyrate and propanol was measured using gas phase chromatography in a dual-mininert® system [30], 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.

Neutron Scattering

The samples were prepared and analysed in the manner described in [29]. 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.

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Author contributions:

All the authors equally participated in the discussions of the data interpretation. M. L. performed GC analysis, ^{18}O-labelled water quantification experiments, neutron scattering sample preparation and wrote the paper. V K-S. Performed the neutron scattering data analysis. M. T. Supervised the neutron scattering experimental work. RV D. Implemented and developed the ^{18}O-labelled experiments. JL F. participated in the experimental work, brought his expertise in protein hydration to the project and contributed to the writing of the paper. JC S. brought his expertise in protein dynamics to the supervision of parts of the research,participated in the analysis of the results and wrote the paper. RM D brought his expertise in enzymology under extreme conditions for research supervision, analysis of the results and wrote the paper.

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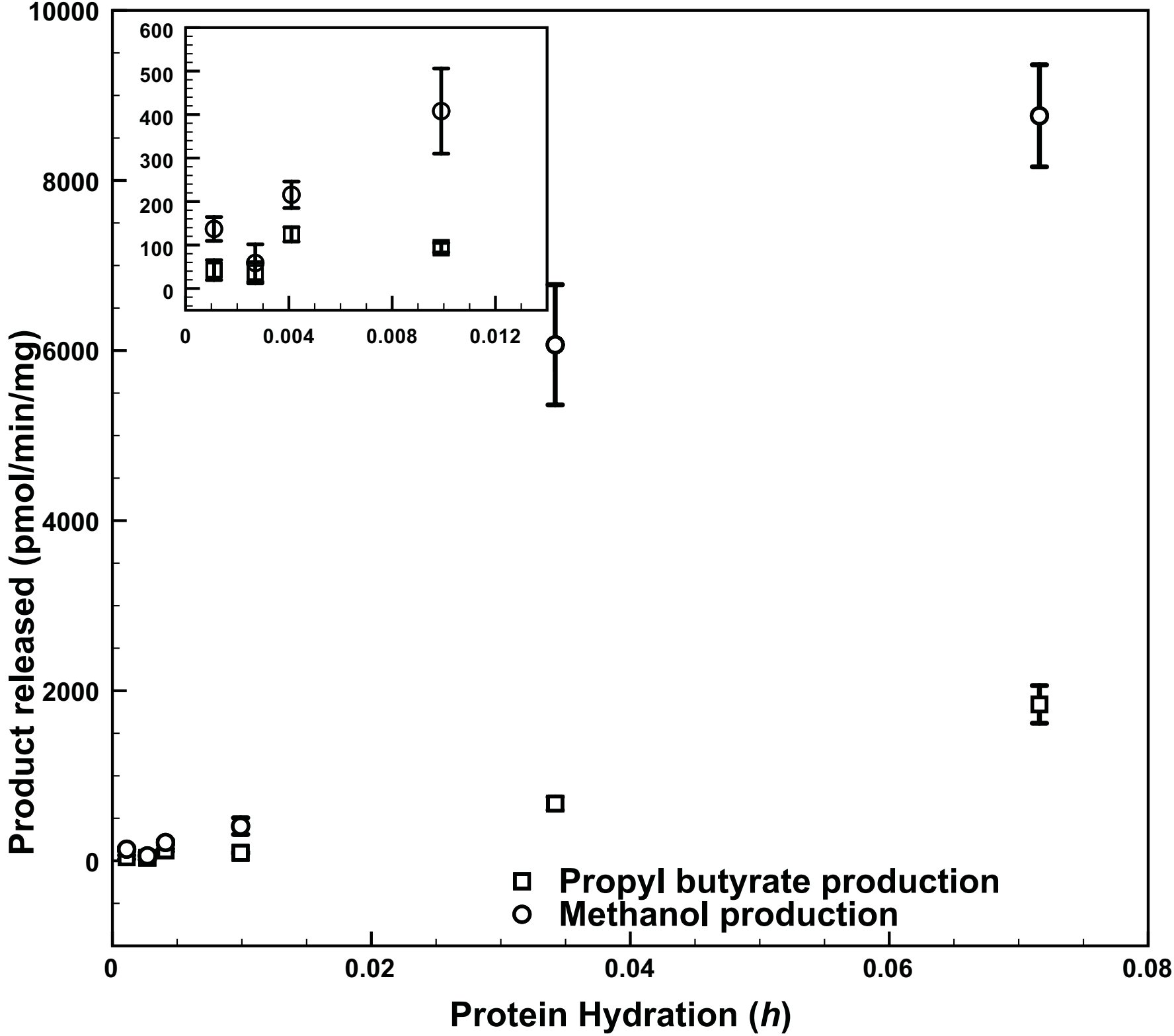
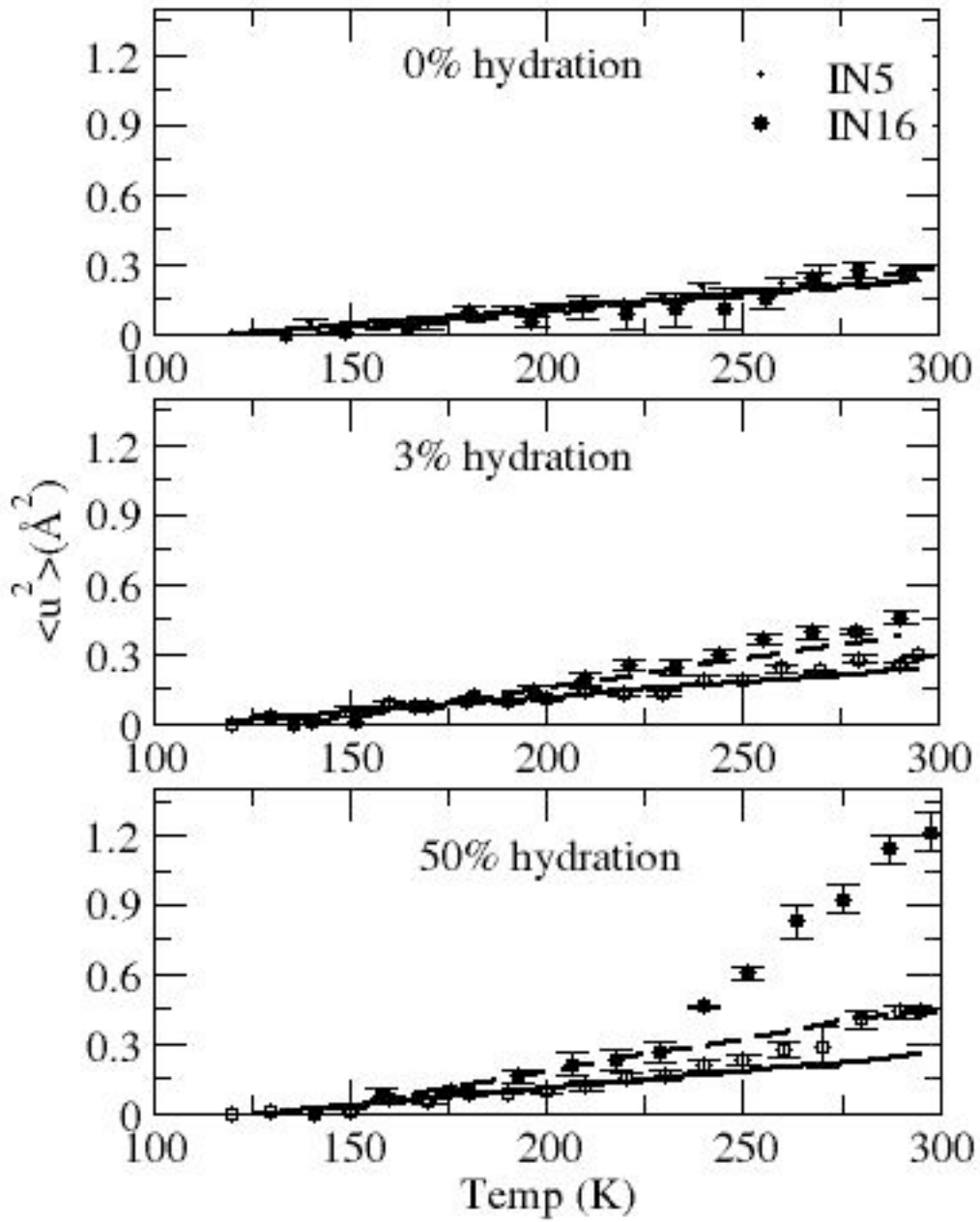

Product released (pmol/min/mg)
Protein Hydration (h)
Propyl butyrate production
Methanol production
0
2000
4000
6000
8000
10000
0.02
0.04
0.06
0.08
100
200
300
400
500
600
0.004
0.008
0.012


0% hydration
IN5
IN16
3% hydration
50% hydration
<u^2>(Å^2)
Temp (K)
0
0.3
0.6
0.9
1.2
100
150
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