VOLUME 118, NUMBER 51
DECEMBER 25, 1996

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Exploiting Hydration Hysteresis for High Activity of Cross-Linked Subtilisin Crystals in Acetonitrile

Johann Partridge, Gillian A. Hutcheon, Barry D. Moore,* and Peter J. Halling

Contribution from the Department of Bioscience and Biotechnology and the Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XW, U.K.

Received April 26, 1996[⊗]

Abstract: The catalytic behavior of cross-linked subtilisin crystals in acetonitrile exhibits pronounced hysteresis. The transesterification activity of the crystals in anhydrous solvent varies as much as 80-fold, depending on the hydration history of the enzyme. In a comparison of drying methods, the highest rates of reaction were obtained with crystals dried by washing with organic solvent. Crystals dried over molecular sieves in air or solvent showed significantly lower activity. In all cases, full activity could be recovered in aqueous buffer. Evidence for hysteresis in hydration was obtained using a ^{2}H-NMR method. Crystals rinsed with anhydrous solvent were found to retain approximately 70 waters per enzyme molecule more than those dried over molecular sieves. When different solvents were used to dry the enzyme crystals, the catalytic rate in anhydrous acetonitrile was found to vary significantly—methanol-washed CLEC giving the lowest rates. The transesterification activity of the solvent-washed enzyme was found to be profoundly effected by the concentration of water in the system. Optimum conditions for ester production were obtained in **anhydrous** acetonitrile. Under these conditions, despite a lower initial rate, absence of the hydrolysis byproduct which inhibits the enzyme led to better synthetic yields.

Introduction

There is considerable current interest in the use of cross-linked enzyme crystals (CLECs) as biocatalysts in aqueous and organic media. As with enzymes immobilized on supports, CLECs offer advantages over the conventional freeze-dried preparations when used in organic solvents: access to individual enzyme molecules will be improved, while particle aggregation and diffusional limitation are considerably reduced. In addition, it has been reported recently that CLECs exhibit exceptional stability properties.[1] They remain active after prolonged exposure to high temperatures, near anhydrous organic solvents, and aqueous−organic solvent mixtures. They are also highly stable against autolysis and exogenous protease degradation. These characteristics make them potentially very attractive as catalysts for use in organic reaction mixtures. However, as yet there is limited information available on the optimal conditions for their use in such systems.

The method by which conventional lyophilized or supported enzyme is prepared for use in organic media often varies. In some cases the biocatalyst, which is essentially dry, is added directly to the organic solvent which contains a known amount of water.[2] However, in other studies the enzyme and solvent are preequilibrated to a known thermodynamic water activity (a_w),[3,4] either together or separately. Initial studies on the effect of hydration on catalytic activity of subtilisin CLECs in

* Correspondence on manuscript: Barry D. Moore, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XW, U.K. FAX: (+44) 141 552 5664. E-mail: b.d.moore@strath.uk.

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1996.

(1) (a) St. Clair, N. L.; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314−7316. (b) Lalonde, J. J. *Chim. Oggi* **1995**, *13*, 31−35. (c) Persichetti, R. A.; St. Clair, N. L.; Griffith, J. P.; Navia, M. A.; Margolin, A. L. *J. Am. Chem. Soc.* **1995**, *117*, 2732−2737. (d) Khalaf, N.; Govardhan, C. P.; Lalonde, J. J.; Persichetti, R. A.; Wang, Y. F.; Margolin, A. L. *J. Am. Chem. Soc.* **1996**, *118*, 5494−5495.

(2) (a) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194−3201. (b) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 8017−8021. (c) van Erp, S. H. M.; Kamenskaya, E. O., Khmelnitsky, Y. L. *Eur. J. Biochem.* **1991**, *202*, 379−384.

S0002-7863(96)01383-2 CCC: $12.00

acetonitrile have been reported recently.[5] The CLEC utilized was acetonitrile washed and subjected to no further equilibration. The authors demonstrated that the initial rate of transesterification increased when the system a_w was increased from <0.002 up to 0.006. However, this work did not include studies of possible hysteresis effects.

There have been some literature reports that different catalytic activity can be found in systems with the same water content or a_w, but different hydration histories.[6] A particular problem with studies in this area is that conventionally used freeze-dried preparations can be susceptible to significant batch to batch variations. This makes it difficult to make direct rate comparisons between laboratories and also to carry out meaningful studies of possible hysteresis effects arising from different pretreatments. It was anticipated that the more homogeneous nature of CLECs should reduce these problems, and we were hence interested in studying how different methods of treatment changed the catalytic efficiency.

Results and Discussion

ChiroCLEC-BL is supplied stored either in solid crystal form or as a suspension in aqueous buffer. The manufacturer's literature recommends using the solid form for reactions in organic media since the CLEC can then be used directly without further treatment. However, they also report, "by comparison to an aqueous suspension which can be stored indefinitely, the biocatalyst is fairly unstable in the dry state and significant loss in catalytic activity results after 3−4 days". This is intriguing since the more commonly used lyophilized powders generally exhibit very little change in activity even when stored for several weeks. We were therefore interested to investigate the cause of this effect and to see if it were possible to arrest it. Since we wished to begin with fully active preparations, in the present study we decided to routinely use CLEC stored in an aqueous suspension and prepare the samples for use in organic solvent as required. A number of routes can be envisaged for moving from an aqueous suspension to a "dry state", including washing with polar solvent and drying over molecular sieves in air or solvent.

As a starting point we explored the effect on enzyme activity of washing CLEC with either propanol or acetonitrile. The solvent-washed enzyme was used directly in a reaction or else preequilibrated in air or solvent (see Materials and Methods for details). We followed the rate of a standard transesterification in anhydrous acetonitrile using CLECs pretreated in different ways: the initial reaction rates are shown in Table 1. The highest initial catalytic rates were obtained with CLEC placed directly in the reaction mixture after solvent washing, while equilibration at a fixed water activity invariably reduced the rate. It is interesting to note that although CLEC preequilibrated in air gave by far the lowest rate, this is still greater than that obtained with a conventional freeze-dried preparation of subtilisin (0.17 nmol mg^{-1} min^{-1}).

Table 1. Effect of Enzyme Treatment on Catalytic Activity of ChiroCLEC-BL in Anhydrous Acetonitrile

washing solvent[a]	further enzyme treatment[d]	rate (nmol mg^{-1} min^{-1})
PrOH	none	224 ± 7
PrOH	1	34 ± 4
PrOH	2	2.9 ± 0.1
AcN	none	112 ± 4
AcN	1	33 ± 3
AcN	2	2.3 ± 0.2
PrOH[b]	none	187 ± 2
PrOH, AcN	none	84 ± 4
AcN, PrOH	none	102 ± 3
AcN, Buffer[c], PrOH	none	190 ± 14

[a] CLEC was rinsed 3 times with 1 mL of each solvent shown respectively (with the exception of *b* which was rinsed 6 times). After each wash, the solvent was removed by centrifugation. [b] See footnote *a*. [c] The aqueous buffer consisted of 100 mM sodium acetate, 20 mM calcium chloride, pH 5.7. [d] (1) Solvent washed CLEC was suspended in anhydrous AcN. The enzyme−solvent mixture was then equilibrated over molecular sieves for 3 days, 20 °C. (2) Solvent washed CLEC was equilibrated over molecular sieves for 3 days, 20 °C. After this time it was suspended in anhydrous AcN.

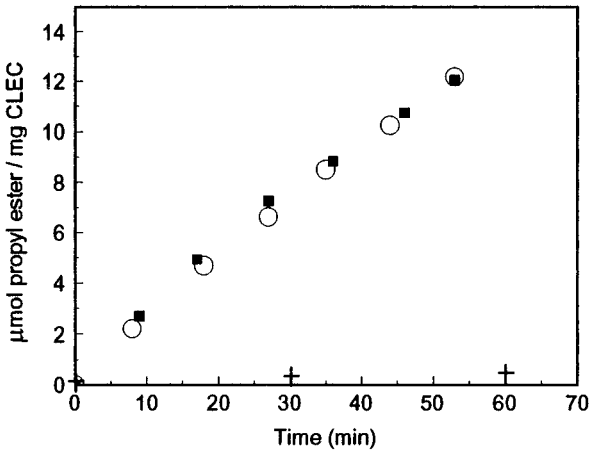

Figure 1. Rate of transesterification in anhydrous acetonitrile. Reactions were carried out with ChiroCLEC-BL which had been propanol washed (○); propanol washed and equilibrated over molecular sieves for 3 days (+); and propanol washed and equilibrated over molecular sieves for 3 days, before suspension in aqueous buffer and further propanol washing (■).

It has been shown previously using freeze-dried subtilisin powder in hexane that higher reaction rates were obtained on equilibration in solvent as opposed to equilibration in air.[7] However, the difference between the two treatments is much greater for the CLECs in acetonitrile.

From these results, it could be inferred that preequilibration through the vapor phase structurally damages the enzyme crystals, so that most of their catalytic activity is irreversibly lost. We were able to eliminate this possibility experimentally. Biocatalyst, which had been solvent washed and left to equilibrate through the vapor phase for 3 days, was returned to an aqueous environment. After this, it was solvent washed to remove excess water and used immediately to catalyze a reaction in anhydrous acetonitrile. The data in Figure 1 show that the CLEC can regain high catalytic activity after it has been subjected to the procedure described above. Furthermore, the rate observed is comparable to that of CLEC which has undergone solvent washing only.

The observations discussed above are not exclusive to reactions in acetonitrile. Experiments in propanol have con-

(3) Effects of residual water level on enzyme activity can often be explained by the following simple model. Catalytic activity is determined by the amount of water bound to the enzyme molecules, which is only a fraction of the total present.[2b] At equilibrium there will be a characteristic relationship between the amount of water bound and that dissolved in the organic phase. If the water level is expressed in terms of its thermodynamic activity (a_w), the relationship between catalytic activity and residual water level is similar for many different solvents.[4]

(4) For reference, see: (a) Valivety, R. H.; Halling, P. J.; Macrae, A. R. *Biochim. Biophys. Acta* **1992**, *1118*, 218−222. (b) Svensson, I.; Wehtje, E.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. Bioeng.* **1994**, *44*, 549−556.

(5) Schmitke, J. L.; Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 3360−3365.

(6) Halling, P. J. *Enzyme Microb. Technol.* **1994**, *16*, 178−206.

(7) Parker, M. C.; Moore, B. D.; Blacker, A. J. *Biocatalysis* **1994**, *10*, 269−277.

firmed that enzyme, which has undergone solvent washing only has a catalytic rate more than 12-fold higher than that which has been dried further using molecular sieves. Similarly, for reactions in propanol, enzyme preequilibrated through the vapor phase can regain high catalytic activity by exposure to an aqueous environment as described above.

Conventional enzyme preparations for use in organic media are usually dried by prolonged treatments (e.g. under vacuum or over water absorbents). Several days may be required to reach steady final water contents.[8] Washing with a polar organic solvent is relatively very quick, and hence, it would not be surprising if a higher bound water content was left. However, the differences we show are clearly not just a matter of time. Much more activity is retained with CLECs which have undergone prolonged equilibration in acetonitrile than for those left in air at the same a_w. Furthermore, it is interesting to note that the catalytic efficiency of CLECs extensively dried over molecular sieves cannot be improved significantly by further equilibration over a high a_w saturated salt. CLEC must be returned to the aqueous buffer if high catalytic activity is to be recovered.

In these experiments, the quantity of water that transfer to or from the organic phase will only be a small fraction of the total present. Thus, the organic phase composition and a_w will remain almost the same, however the enzyme is pretreated. Nevertheless, enzyme activity varies by 80-fold depending on its previous hydration history. It is therefore clear that the catalytic behavior of CLECs in acetonitrile exhibits pronounced hysteresis. One might speculate that the enzyme or enzyme crystal has a "memory" of how it has been treated, and this "memory" can be erased by returning the CLEC to an aqueous environment. Two possible hysteresis effects may contribute to this: (1) hysteresis in the relationship between bound water and a_w, which is well known for isotherms measured in air,[9] or (2) hysteresis in the dependence of catalytic activity on bound water. Interconversion of conformations is probably slow in low water enzymes[10] and a molecule initially at high hydration may be trapped in a more active state even when water is removed. Prolonged dehydration may however lead to a slow conformational change that is not readily reversed on rehydration (unless to very high a_w).

From Table 1 it is apparent that the difference in catalytic rate for CLEC dried over molecular sieves through the vapor phase is small, regardless of the solvent used to wash the crystals prior to equilibration. This is also the case for CLEC which is equilibrated in the reaction solvent prior to use. However, it is interesting to note that CLEC washed in propanol and used directly in the reaction gives a rate approximately double that of CLEC which was washed in acetonitrile. When the crystals were washed consecutively with propanol (3×) and acetonitrile (3×), regardless of the order in which the solvents were used, the rates of catalysis were less than half that for CLEC washed solely in propanol, but were close to that of AcN washed CLEC. When the number of washes is increased from three to six for propanol washed CLEC, the decrease in rate is barely significant. It is apparent that the effect of acetonitrile is dominant on the crystals and the catalytic efficiency of an AcN-washed CLEC cannot be increased by subsequent washing with propanol. To ascertain whether or not acetonitrile washing had a long-term detrimental effect on the crystals, we measured the catalytic activity of CLEC which was rinsed with acetonitrile, aqueous buffer, and propanol, respectively. The majority of catalytic activity was recovered, indicating that acetonitrile does not appear to damage the CLEC irreversibly. Furthermore, if acetonitrile washed CLEC was left overnight in buffer, 100% recovery in activity was attained.

It was considered that in order to interpret our results more fully, determination of the amount of water remaining on the crystal after each treatment was required. This is, however, extremely difficult to achieve.

The X-ray crystal structure of subtilisin−CLEC extensively washed with acetonitrile has recently been solved,[11] and the crystals were shown to retain at least 99 waters per enzyme molecule. However, the authors were unable to obtain crystal structure data when they immersed the crystals in other solvents (including propan-1-ol, acetone, and methanol).[12]

The coulometric Karl Fischer titration is commonly used for determination of water content and for the case of measuring water bound to solid samples two alternatives exist. Firstly, it may be possible to extract water from the enzyme using a suitable polar solvent. The concentration of water present in the solvent before and after contact with the enzyme can be measured, and the difference in the two values should represent the water associated with the enzyme. In the present case, however, it is not certain that solvents such as propanol will extract all water from the enzyme crystals. Secondly, the enzyme sample can be dissolved in solvent and the water content of the resultant solution determined using the solvent water content as a blank. Again, this method can pose problems because the solid enzyme may react with the Karl Fischer reagent, but more importantly here, no solvent tested has fully solubilized the CLEC.

As an alternative we have utilized a solution state ^{2}H-NMR method to determine differences in the amount of water remaining on the biocatalyst after drying. CLECs were first equilibrated in D_2O for several days, subjected to particular drying treatment, and then suspended in deuterium-depleted water for 24 h. This results in proton−deuterium exchange between the ^{2}H-depleted water and the deuterons on the protein. The intensity of the resultant ^{2}H NMR signal will reflect any D_2O associated with the protein, as well as deuterons exchanged onto the side chains and amide groups.

Unfortunately, it is difficult to apply this method to CLECs which have been washed in propanol: washing a D_2O equilibrated CLEC with propanol will result in loss of the deuterium label, since the solvent will readily exchange with any D_2O associated with the enzyme. We therefore decided to use CLECs washed with acetonitrile.

The ^{2}H-NMR spectra of crystals, that were washed with acetonitrile and those that had been washed and equilibrated further over molecular sieves were recorded. The number of deuterons titrated from the enzyme (mol/mol) were determined as 342 ± 9% and 206 ± 2%, respectively.

It is likely that crystals which have been dried over molecular sieves will still have a small number of water molecules tightly bound to the enzyme,[13,14] which will make a contribution to the number of deuterons titrated from the protein. However, the majority will be from the amides and exchangeable groups on the side chains. The number of exchangeable protons on the protein should be approximately the same regardless of the

(8) This may in part reflect bulk mass transfer effects rather than protein molecule behavior.

(9) Rupley, J. A.; Careri, G. *Adv. Protein Chem.* **1991**, *41*, 37−172.

(10) Russell, A. J.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 11624−11626.

(11) Fitzpatrick, P. A.; Steinmetz, A. C. U.; Ringe, D.; Klibanov, A. M. *Proc. Nat. Acad. Sci. U.S.A.* **1993**, *90*, 8653−8657.

(12) Fitzpatrick, P. A.; Ringe, D.; Klibanov, A. M. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 675−681.

(13) Fifteen or so waters per enzyme molecule remain bound to freeze-dried powders extensively dried in air.[14]

(14) Dolman, M.; Moore, B. D.; Halling, P. J.; Waldron, S. *Biopolymers*, in press.

Table 2. Effect of Different Solvent Washes on the Transesterification Activity of ChiroCLEC-BL in (A) Acetonitrile (Anhydrous) and in (B) Propan-1-ol (Anhydrous)

washing solvent	rate (nmol mg^{-1} min^{-1})
A. In Acetonitrile[a]	
EtOH	282 ± 32
BuOH	243 ± 23
PrOH	224 ± 7
Me_2CO	121 ± 11
AcN	112 ± 4
ethane diol	50 ± 12
CH_3 OH	39 ± 3
MeOH, buffer[b], PrOH	74 ± 1
B. In Propan-1-ol[c]	
PrOH	174 ± 9
Me_2CO	46 ± 5
AcN	26 ± 0.6
CH_3OH	17 ± 0.9

[a] CLEC was rinsed 3 times with 1 mL of each solvent shown, respectively. After each wash, the solvent was removed by centrifugation. The enzyme was then suspended in anhydrous acetonitrile. [b] The aqueous buffer consisted of 100 mM sodium acetate, 20 mM calcium chloride, pH 5.7. [c] CLEC was rinsed 3 times with 1 mL of each solvent shown. After each wash, the solvent was removed by centrifugation. The enzyme was then suspended in anhydrous propan-1-ol.

treatment used to dry the enzyme. Thus, CLEC dried by solvent washing only will have approximately 68 molecules of water/molecule of enzyme more than the CLEC dried over molecular sieves, i.e. (342−206)/2. As we anticipated, washing with solvent leaves a higher level of water bound on the enzyme which is evidently responsible for a 48-fold increase in catalytic rate.

Since solvent washing the CLEC gives the most efficient rates of catalysis, we went on to investigate the effect of washing with other solvents. Table 2A shows how transesterification rate in anhydrous acetonitrile varies as much as 7-fold depending on the organic solvent used to dry the catalyst. The alcohols generally gave the fastest rates of catalysis, followed by acetonitrile and acetone which gave intermediate rates. Interestingly, the smaller "water-like" solvents, ethane diol and methanol, gave the lowest rates of all solvents tested. With CLEC washed in methanol, only 33% of the maximum activity could be regained after washing with buffer and propanol (soaking in buffer overnight increased catalytic activity to 63% of the original propanol washed CLEC).

Nevertheless, the catalytic performance of all solvent washed CLECs was still higher than that which had been equilibrated in air or in solvent at $a_w < 0.01$. A plausible explanation for this variation in catalytic rate when different solvents are used to dry the CLEC is that each solvent has a specific capacity to displace the water within the crystal. Conformational changes may result on removal of more water from the crystal leading to a less active enzyme state and decreased rates of catalysis.

On the basis of these data one can conclude that care must be taken when choosing a solvent to wash the crystals if maximum enzymatic activity is to be achieved. Furthermore, initial studies of reactions in propanol have shown that a similar pattern of results arises when CLEC is washed with different solvents (Table 2B).

Studies with freeze-dried powders and immobilized preparations in organic media have shown that the amount of water present in the system plays an important role in controlling factors such as rate, stability, and hydrolytic equilibria.[15]

(15) (a) Bell, G.; Halling, P. J.; Moore, B. D.; Partridge, J.; Rees, D. G. *TIBTECH* **1995**, *13*, 468−473. (b) Koskinen, A.; Klibanov, A. M. *Enzymatic Reactions in Organic Media*; Chapman and Hall: Andover, 1995.

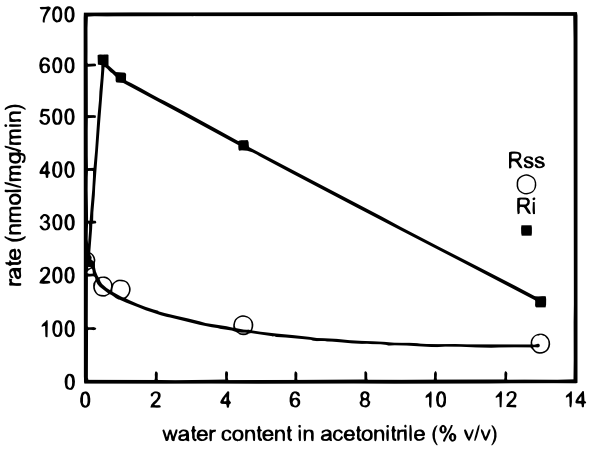

Figure 2. Effect of hydration on the transesterification activity of ChiroCLEC-BL in acetonitrile. The aqueous suspension of ChiroCLEC-BL was washed with anhydrous propanol and suspended in acetonitrile (with known water content). On addition of 10 mM *N*-acetyl-L-tyrosine ethyl ester and 1 M propanol-1-ol, the concentration of product produced was monitored by HPLC.

Table 3. Product Yields as Function of Water Content in Acetonitrile[a]

concentration of water in AcN (v/v)	% *N*-acetyl-L-tyrosine propyl ester[b]	% *N*-acetyl-L tyrosine[b]
0.0	65.5	0.0
0.5	59.2	2.9
1.0	59.1	5.9
4.5	33.4	15.5
13.0	23.5	24.1

[a] For the ChiroCLEC-BL catalyzed transesterification reaction between 10 mM *N*-acetyl-L-tyrosine ethyl ester with 1 M propan-1-ol. The aqueous suspension of ChiroCLEC-BL was washed with anhydrous propanol prior to use in reaction. [b] Yields of propyl ester and acid after 24 h were determined by HPLC.

Obtaining the correct water level is, therefore, necessary if the biocatalyst is to work efficiently and product yields are to be maximized.

Having established the most effective pretreatment for the CLECs, we proceeded to carry out a more detailed study on the variation in catalytic activity as a function of water content/water activity of the actual reaction mixture.

As shown in Figure 2, the maximum initial rate of reaction (R_i) was obtained when the water content in the solvent was 0.5% water v/v (a_w of 0.11). Increasing the water content of the acetonitrile further, resulted in a progressive decrease in R_i. In dry solvent, the rate of propyl ester formation is linear with time at least up to 12% conversion (1 h). However, at higher water, the rate decreases noticeably. Generally, there is a high conversion to the ester product in the first 10 min, R_i. The rate then decreases, and a slower steady-state rate, R_{ss}, is observed. This effect appears to be caused by formation of the hydrolysis acid byproduct. This was tested by adding 0.25 mM *N*-acetyl-L-tyrosine to a reaction in *anhydrous* acetonitrile. Directly after the acid addition, the rate of ester production was reduced from 226 to 30 nmol mg^{-1} min^{-1}. Thus, the hydrolysis product inhibits the enzyme.

Although the initial rate of reaction is lower when dry solvent is used, the absence of hydrolysis results in a steady-state rate (R_{ss}) higher than that at all other water contents studied. This means that the product ester yield after 24 h is highest with anhydrous acetonitrile (Table 3). Using ChiroCLEC-BL, optimal product yields can, therefore, be obtained by a very simple and convenient method: the CLEC is propanol washed and added directly, without further equilibration, to dry solvent.

Conclusions

With free enzymes, most of the water must be removed by freeze-drying or similar. Using enzymes as CLECs allows rapid drying by solvent rinse, leading to better specific activity. Exploitation of hysteresis in this way allows maintenance of high enzymatic activity in a low a_w medium. This is particularly valuable because it suppresses hydrolytic side reactions.

Materials and Methods

Chemicals. The cross-linked enzyme crystal ChiroCLEC-BL (Lot BL-95-005) was obtained from Altus Biologics Inc (Cambridge, MA). *N*-acetyl-L-tyrosine and *N*-acetyl-L-tyrosine ethyl ester were purchased from Sigma Chemical Co. (Poole, U.K.). All anhydrous organic solvents were obtained from Merck Ltd (Poole, U.K.), with the exception of ethane diol and butanol (Aldrich Chemical Co. (Gillingham, U.K.)). The solvents were stored over 3-Å molecular sieves and used without further purification. Their water contents, determined by Karl Fischer coulometric titration, were <0.007% v/v. All inorganic salts were of analytical grade from BDH. Deuterated water (99.9% D) was obtained from Goss Scientific Instruments. Deuterium-depleted distilled water (10^{-2} atom %) and acetone-d_6 (99.9% D) were purchased from Aldrich Chemical Co. (Gillingham, U.K.).

Enzyme Preparation. The enzyme crystals are supplied and stored in an aqueous buffer consisting of 100 mM sodium acetate, 20 mM calcium chloride, 0.02% sodium azide (pH 5.7), 69 mg of CLEC/mL of buffer. Prior to use, the aqueous CLEC suspension (15 μL) was washed with anhydrous propan-1-ol or acetonitrile (3 × 1 mL). After each wash, the solvent was removed by centrifugation. The water content of the solvent after the final wash by Karl Fischer was <0.01% v/v. The enzyme was then either used directly in a reaction, or preequilibrated to a fixed water activity in air or solvent.

Equilibration in Air. Solvent washed CLEC (ca. 1 mg) was placed in a sealed jar containing molecular sieves, and equilibrated for 3 days at 20 °C. After this time it was suspended in anhydrous acetonitrile.

Equilibration in Acetonitrile. Solvent washed CLEC (ca. 1 mg) was suspended in 10 mL of anhydrous acetonitrile. The enzyme-solvent sample was then equilibrated in a sealed jar containing molecular sieves for 3 days.

Typical Reaction Conditions. The reaction studied was the transesterification of 10 mM *N*-acetyl-L-tyrosine ethyl ester with 1 M propan-1-ol. A solution of the substrate in anhydrous propanol was added to a suspension of CLEC in 10 mL of AcN containing the desired amount of water. After a brief mixing, the zero time sample was removed. The mixture was then incubated at 20 °C with constant reciprocal shaking (150 min^{-1}). Samples from the reaction mixture were taken at regular intervals. After filtration to remove solid enzyme, they were analyzed by HPLC on a Gilson 715 equipped with an ODS2 reverse-phase column (Anachem, U.K.). The mobile phase consisted of 40% volume acetonitrile mixed with an aqueous phase (pH 2 with orthophosphoric acid).

Recovery of Catalytic Activity. A 15-μL sample of aqueous CLEC suspension was washed with propanol (3 × 1 mL) and left to equilibrate through the vapor phase over molecular sieves for 3 days. Subsequently, the enzyme was mixed with 1 mL of the aqueous storage buffer. After 3−4 h, the excess buffer was removed by centrifugation, and the CLEC was propanol washed as before. The enzyme was then suspended in anhydrous acetonitrile and used directly in catalysis.

Organic Solvent Washes. Solvents used were methanol, ethanol, propanol, butanol, acetone, acetonitrile, ethane diol, and aqueous storage buffer. CLEC (1 mg) was rinsed with one of the above solvents (3 × 1 mL), or a combination of them (3 × 1 mL of each). After each wash the solvent was removed by centrifugation. The crystals were suspended in anhydrous acetonitrile. On addition of the substrates, the catalytic activity was monitored.

Effect of Hydration on Catalytic Activity. The aqueous suspension of CLEC was washed with anhydrous propanol and suspended in acetonitrile of known water content. On addition of the substrates the transesterification reaction was followed.

Acid Inhibition. Propanol washed CLEC (1 mg) was suspended in 10 mL of anhydrous acetonitrile. On addition of the substrates (10 mM *N*-acetyl-L-tyrosine ethyl ester, 1 M propan-1-ol), the reaction was monitored by HPLC for 30 min. After this time, a 50-μL aliquot of *N*-acetyl-L-tyrosine in acetonitrile was added to the reaction mixture (to give a final concentration of 0.25 mM), and the subsequent rate of transesterification followed.

NMR Sample Preparation. Aliquots of CLEC (75 μL) were transferred into eppendorf tubes and washed with deuterated water (3 × 1 mL). The samples were equilibrated in D_2O for 48 h to allow deuterium exchange of most labile protein hydrogens. After this, excess D_2O was removed from each of the samples, which were then treated in one of two different ways: (A) washed three times with 1 mL of anhydrous AcN and (B) washed with AcN as above, and then *equilibrated through the vapor phase* over molecular sieves for 3 days.

A standard solution of acetone-d_6 in deuterium-depleted water was prepared (typically 5−10 mg of acetone in 30 mL of water), and 1-mL aliquot of the mixture was added to each of the protein samples. The mixture was shaken and left overnight. The aqueous suspension was centrifuged and 0.5 mL of supernatant transferred to an NMR tube. The ^{2}H-NMR spectrum shows a peak due to the isotopically enriched deuterium-depleted water after contact with the D_2O-equilibrated protein. The intensities of this peak (at 4.65 ppm) and that of the acetone-d_6 standard (at 2.05 ppm) were determined, and their ratio was used to calculate the amount of deuterons present on each protein molecule.

^{2}H-NMR measurements were carried out on a Bruker 400-MHz FT-NMR spectrometer. Typically 200 to 600 scans were collected using a sweep width of 922 Hz and an acquisition time of 9.999 s. The data was processed using 1-Hz line broadening and automatic baseline correction.

Acknowledgment. We thank J. J. Lalonde for helpful discussion. We are grateful to the Biotechnology and Biological Science Research Council for financial support.

JA961383X