Methanol dramatically enhances serine protease activity under anhydrous conditions

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The catalytic activity of rigorously dried lyophilised subtilisin Carlsberg and α-chymotrypsin in anhydrous solvents is enhanced dramatically on addition of dry methanol, while ethanol and propanol produce no effect.

Enzymes are being increasingly used for catalysis of organic reactions in non-aqueous media. Hence, there is considerable interest in understanding the factors affecting their activity, selectivity, and stability under these conditions.[1] The catalytic activity of lyophilised enzyme powders suspended in organic solvents can often be increased substantially if the protein is hydrated above a critical level.[2] The precise reasons for this are not known but it has been proposed that water acts as a 'molecular lubricant' and enhances the conformational flexibility of the protein.[2] Certain solvents or 'additives' including dimethyl sulfoxide, formamide and ethylene glycol have been reported to increase enzyme activity when added into the organic media along with small amounts of water.[3,4] However, the effects of additives on rigorously dried enzyme preparations, where possible activation by water has been eliminated, have not been well explored. Here we show, for the first time, that methanol is a remarkably efficient promoter of both subtilisin Carlsberg and α-chymotrypsin activity under anhydrous conditions. This effect could be of considerable synthetic utility in enzyme catalysed reactions where the build up of hydrolytic side-products needs to be avoided.

The starting point for this work was a study of subtilisin Carlsberg activity at low hydration levels. Rate comparisons were made between the enzyme catalysed transesterification of acetylphenylalanine ethyl ester (APEE) by propanol or methanol, in hexane, at various known hydration levels.† Hydration was controlled by equilibrating the system with saturated salt solutions of known thermodynamic water activity, a_w.[5] Anhydrous conditions were obtained by lyophilisation of the protein followed by repeated dehydration with freshly activated molecular sieves and the use of completely dry solvents.‡ This treatment appears to leave only internally bound water molecules associated with the protein.[6]

As anticipated, under anhydrous conditions the transesterification rate with propanol was low but measurable (see Table 1). Initial hydration of the system to a_w = 0.11 led to an unexpected small drop in catalytic activity but on further hydration to a_w = 0.43 there was a slight increase in enzyme activity as reported previously.[2,7] In sharp contrast, using methanol as the nucleophile, even rigorously dried enzyme exhibited good catalytic activity and higher transesterification rates than with propanol were observed at all hydrations levels, with a 65-fold difference at a_w = 0.43. These remarkable enhancements for methanol compared to propanol were also observed when the ester concentration was varied from 5–15 mm.

Since it was possible the differences arose simply because deacylation was the rate limiting step under these conditions§ a competitive nucleophile experiment was carried out under anhydrous conditions. Fig. 1(*a*) shows the time course of a reaction mixture which initially contained anhydrous subtilisin, APEE and propanol in dry hexane. For the first 50 min very little reaction was observed whereupon 5 μl of dry methanol was added. Immediately rapid formation of both the methyl and propyl esters commenced. A similar effect was seen with another serine protease, α-chymotrypsin, Fig. 1(*b*). The observation of a dramatic increase in the rate of formation of the

Table 1 Effect of water activity on initial rates of transesterification

	Initial rate[b]/nmol min^{-1} mg^{-1}	
Water activity	0.5 m Methanol	0.5 m Propanol
Anhydrous[a]	24.5	1.5
0.11	47.8	1.4
0.22	64.4	1.1
0.43	102.2	1.6

[a] Enzyme dried over regenerated molecular seives.[6] [b] Reaction conditions: 10 mm APEE, 0.5 m alcohol and 1 mg ml^{-1} enzyme in hexane.

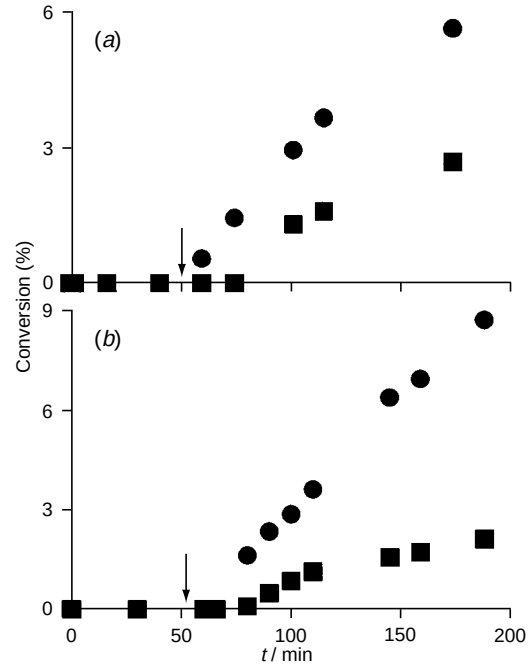

Fig. 1 Reaction course for the enzyme catalysed transesterification of APEE to the propyl ester (■) and methyl ester (●). The reaction mixture contained 5 mm APEE, 268 mm propanol, 1 mg lyophilised anhydrous enzyme and 1 ml of dry hexane (< 0.005% water). After 50 min (↓) 5 μl of anhydrous methanol, (125 mm) was added. The reaction mixture was shaken at 200 rpm at 20 °C and analysis was by gas chromatography. HPLC analysis of the reaction mixture showed that none of the hydrolysis product, *N*-acetyl-l-phenylalanine, was formed under these conditions. (*a*) Catalysed by subtilisin Carlsberg, (*b*) catalysed by α-chymotrypsin.

propyl ester shows that methanol cannot just be a better nucleophile since then only the rate of methyl ester production would be affected.¶ Rather the results suggest that solvation of the protein by methanol stimulates an increase in the intrinsic catalytic activity of both subtilisin Carlsberg and α-chymotrypsin under anhydrous conditions.‖ Since these serine proteases have very different secondary and tertiary structures similar effects might be expected with other related enzymes.

To eliminate the possibility that trace impurities (*e.g.* drying agent) or changes in particle size were responsible for the activation, a lyophilised powder sample of subtilisin Carlsberg was equilibrated with methanol through the vapour phase in the presence of molecular sieves. Gravimetric measurements showed that this procedure resulted in adsorption of low levels of the solvent onto the protein. Following resuspension in hexane, enzyme activity was assayed using APEE and 0.5 m propanol. Again a transesterification rate enhancement was observed compared to an untreated sample and with no lag period.** However, here the main reaction product was found to be the propyl ester. This suggests that the low levels of methanol adsorbed onto the enzyme were sufficient to activate it but the resultant concentration of methanol in the reaction mixture was not high enough for it to compete as a nucleophile. Importantly equilibration of subtilisin with ethanol or propanol vapour under identical experimental conditions gave absolutely no rate enhancement nor did the addition of ethanol to the reaction mixture. Thus, the effect of methanol is very specific and the activation mechanism cannot be simply ascribed to a hydrogen bonding phenomena.

Further study of the rates in Table 1 reveal an interesting effect when methanol and water were added to the enzyme together. With dry subtilisin and 0.5 m methanol the transesterification rate is 24.5 nmol min^{-1} mg^{-1} while with enzyme equilibrated to a_w = 0.43 and using 0.5 m propanol the rate is 1.6 nmol min^{-1} mg^{-1}. If we now compare the rate obtained at a_w = 0.43 and 0.5 m methanol, 102.2 nmol min^{-1} mg^{-1}, we see that it is closer to the product than the sum of the individual rates. This observation is not consistent with a single activation mechanism since then the contributions would be expected to be approximately additive. Instead it points to a cooperative effect whereby methanol and water activate the enzyme *via* two or more different processes. As a consequence the ubiquitous 'molecular lubrication model' will need to be revised and we are currently working on this problem.

Finally we sought to show that methanol could activate a serine protease without simultaneously acting as a substrate. Hence, a series of active site titrations were carried out on subtilisin in hexane using the irreversible inhibitor PMSF. Anhydrous enzyme was equilibrated for 3 h in hexane in the presence or absence of either methanol or propanol and with and without PMSF.†† Since in the inhibition process, no deacylation occurs, methanol will not be directly involved in the reaction scheme. Following equilibration the enzyme samples were repeatedly resuspended in pure dry hexane (which removed the PMSF if present) and then dried. The resultant enzymic activities were measured by monitoring transesterification of APEE with methanol in hexane‡‡ and are shown in Table 2. It can be seen that enzyme equilibrated with PMSF in either hexane or hexane–propanol gave very similar activities to the control samples showing only a small percentage of active sites had been titrated. This is consistent with the low activity of the enzyme under these condition (see Table 1). In comparison the presence of methanol during the PMSF equilibration resulted in significant inhibition of the enzyme preparation.§§ This is exactly what would be expected from the transesterification measurements and provides conclusive evidence that methanol activates anhydrous serine proteases *via* a solvation process.

Table 2 Effect of PMSF titration on subtilisin Carlsberg activity in hexane

	Initial rate[a]/ nmol min^{-1} mg^{-1}		
Alcohol	No PMSF[b]	PMSF[b]	Inhibition (%)
None	22.5	21.3	5.4
Propanol	11.5	10.5	13
Methanol	42	4.5	89

[a] Reaction conditions: 10 mm APEE, 0.5 m methanol and 1 mg ml^{-1} enzyme in hexane. [b] The enzyme was shaken with or without 10 mm PMSF and 0.5 m alcohol in hexane for 3 h, washed with hexane to remove unbound PMSF and dried over molecular sieves prior to rate analysis.

Footnotes

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† The variation in amount of enzyme bound water as a function of water activity in nonpolar solvents has been measured previously (ref. 8).

‡ Residual water levels in the dried solvents were measured *via* Karl–Fischer titration and found to be hexane < 0.005%, methanol 0.009%, propanol 0.01%.

§ The nucleophilic specificity of methanol relative to propanol has been shown to be 4.61 for this subtilisin catalysed reaction in hexane at a_w = 0.59.[9]

¶ The argument that the methyl ester product may be more reactive is not valid as the acyl intermediate will be identical whichever ester reacts with the enzyme and hence back reaction of the methyl ester cannot lead to an overall increase in rate.

‖ ^2H NMR difference measurements in hexane showed 50 deuterated methanol molecules bound per subtilisin molecule at a methanol concentration of 3 mm.

** Transesterification rates were; untreated enzyme, 1 nmol min^{-1} mg^{-1}, enzyme equilibrated with propanol, 1 nmol min^{-1} mg^{-1}, enzyme equilibrated with methanol, 6.8 nmol min^{-1} mg^{-1}.

†† PMSF is inert towards reaction with dry alcohols over the period employed (ref. 10).

‡‡ The exact conditions used for the assay will not affect the findings since it is the remaining activity following titration which is being measured. The assay used was convenient because of the known high activity of dry subtilisin in the presence of methanol.

§§ The increased activity of the control sample in the presence of methanol is reproducible and under study.

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