Reversible Acetonitrile-Induced Inactivation/Activation of Thermolysin

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Thermolysin is catalytically inactive in mixtures of 10–15% acetonitrile in aqueous buffer. Unexpectedly, dilution of the inactive enzyme with acetonitrile leads to complete recovery of the catalytic activity in a similar way to dilution with aqueous buffer. Circular dichroism and fluorescence studies of thermolysin in the same solvent mixtures reveal discontinuous changes in the overall secondary and tertiary protein structure that correlate well with the reversible differences in catalytic activity. The spectra on either side of the minimum activity point are different from each other, a fact indicating that the enzyme may be able to access two active conformations which are thermodynamically stable in different solvent environments.

KEYWORDS:

activity studies · biocatalysis · enzymes · protein structures · solvent effects

Introduction

Enzymes are increasingly used as efficient catalysts in synthetic chemistry. Due to the unfavourable equilibria commonly observed in aqueous solutions, many applications involve the use of enzymes in 'low-water' media. These can be organic solvents,[1] highly concentrated substrate suspensions,[2] supercritical fluids[3] or ionic liquids.[4] It is remarkable that enzymes are effective as catalysts in these nonnatural environments, since they have evolved to catalyse reactions in aqueous media, where correct protein folding to form a thermodynamically stable active protein is possible. To increase the understanding of enzyme activity in low-water media, it is of interest to look at structural changes in enzymes when they are removed from their natural aqueous environments. In this article we report investigations into the effects of increasing amounts of acetonitrile on the catalytic activity and structure of a commonly used protease, thermolysin. Thermolysin is widely employed for peptide synthesis[5] and is particularly well-known for the industrial synthesis of the low-calorie sweetener aspartame.[6] The catalytic activity of the enzyme is remarkably unaffected by various pretreatments and a variety of organic solvents.[7] Thermolysin is, however, inactived in mixtures of 10–15% acetonitrile in aqueous buffer. Here we investigate the nature of this inactivation in detail and show that thermolysin can be reactivated not only by addition of aqueous buffer but also by addition of acetonitrile.

When enzyme activity is plotted against water content in organic solvents, U-shaped curves are often obtained, where high catalytic activity is observed in both high- and low-water compositions.[8–10] In intermediate solvent/water regions the activity is much lower, and in many cases this behaviour could be correlated to structural changes in the protein (partial or complete denaturation). This was shown by several spectroscopic techniques including fluorescence[9–11] and circular dichroism (CD).[12] CD spectroscopy in the far UV region detects changes in the secondary structure of the protein. Near UV CD and fluorescence spectroscopy give insight into changes in tertiary structure and the environments of aromatic amino acid side chains in the protein.

Denaturation by intermediate levels of organic solvents is thought to be due to disruption of the driving forces for protein folding, particularly the hydrophobic effect. The water content at which enzyme denaturation can be expected depends on the physico-chemical properties of the solvent, and quantitative correlations between these parameters have been shown for organic solvents.[13]

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The high levels of catalytic activity commonly observed at low-water content (in nearly dry organic solvents) are usually attributed to the restricted conformational mobility of the enzyme,[1] which allows the enzyme to remain in the active conformation for considerable periods of time. This active conformation is usually not thought to be the thermodynamically most stable conformation in the organic solvent/water mixture; the enzyme is said to be 'kinetically trapped' in this active form.[14]

Upon dilution of the inactive enzyme in the intermediate solvent/water region with water or aqueous buffer, the catalytic activity is usually completely recovered. Any incomplete reactivation can be attributed to nonspecific enzyme aggregation and can be avoided by using low concentrations of enzyme. In contrast, upon dilution with organic solvent the catalytic activity is usually not recovered;[8] however in some cases a limited regain of activity was observed from partially denatured enzyme.[12, 15]

Results and Discussion

The effects of acetonitrile on the catalytic activity of thermolysin

Figure 1 shows the variation of thermolysin catalytic activity in the presence of increasing amounts of acetonitrile. Both the synthesis and hydrolysis rates of a model peptide, Z-Phe-Leu-NH_2, were measured (Z = benzyloxycarbonyl). As expected, the

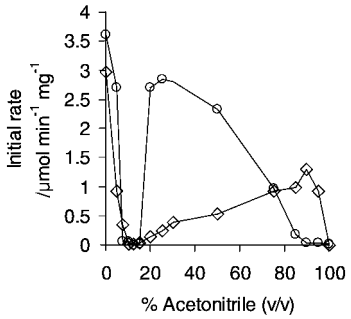

Figure 1. *Initial rates of synthesis (diamonds) and hydrolysis (circles) of Z-Phe-Leu-NH_2 catalysed by thermolysin at varying water contents in acetonitrile. Substrate amounts present were 0.025 mol per kg of reaction mixture in all cases. At acetonitrile contents below 40% the peptide substrate was suspended.*

catalytic activity in both synthesis and hydrolysis is highest in aqueous medium, where the enzyme is in its native conformation. At the other end of the scale the peptide synthesis rate increases to reach a local maximum at around 90% acetonitrile. The peptide hydrolysis curve shows a second maximum at 20–25% acetonitrile and then decreases again at higher levels of acetonitrile. These observations can be explained in terms of changes in the thermodynamic activities (or availability to the enzyme) of water and the other reactants. These phenomena have been described in detail elsewhere[16] and have also been observed in other systems, such as for lipase-catalysed esterifications.[17]

The pronounced decrease in both synthetic and hydrolytic activity observed at 10–15% acetonitrile cannot be correlated with changes in water activity or substrate solvation, and it must be due to specific inactivation of the enzyme in this region. The most obvious explanation would be one involving a change in enzyme conformation leading to loss of activity. A complete loss of native structure is however unlikely at a level of 10–15% acetonitrile. For a number of enzymes, such as α-chymotrypsin,[8, 9] lysozyme and subtilisin, inactivation is commonly observed at acetonitrile/water levels of around 50–60%.[14]

Is acetonitrile-induced thermolysin inactivation reversible?

To investigate the nature of the enzyme inactivation at 10–15% acetonitrile, we went on to check whether the observed inactivation was reversible. Figure 2 shows that thermolysin was completely inactive in the presence of 10% acetonitrile. When acetonitrile was added after 20 minutes to reach a final concentration of 40%, the catalytic activity was completely recovered immediately, giving rise to a linear peptide-synthesis profile with a rate of 0.4 μmol min^{-1} mg^{-1} (Figure 2). Note that the substrate concentration in this experiment was lower (0.01 M) than that used in Figure 1.

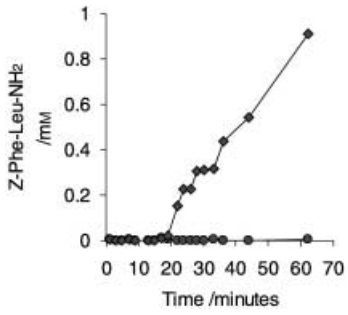

Figure 2. *Thermolysin activity recovers upon addition of pure acetonitrile to inactive thermolysin in a mixture of 10% acetonitrile in water (diamonds). After 20 min acetonitrile was added to a final content of 40%. Circles show a control in which no addition was made.*

As expected, addition of aqueous buffer to the reaction mixture containing inactive enzyme in 10% acetonitrile also resulted in complete recovery of catalytic activity. In previous studies[12, 15] it was found that some regain of native-like conformation and activity of partly denatured enzymes could be brought about by addition of organic solvent for α-chymotrypsin and subtilisin. In these cases, however, enzyme reactivation was not observed when the inactive enzyme solution was left for longer periods of time prior to addition of more organic solvent. These findings suggest that the enzyme that could be reactivated was probably in a metastable state.

For thermolysin in 10% acetonitrile, however, even after overnight incubation at either room temperature or 40 °C significant reactivation of the enzyme was observed upon addition of pure acetonitrile. In addition, no difference was observed when the enzyme was either first dissolved in water and acetonitrile was then added (leading to a clear solution) or

the enzyme was suspended in the 10:90 acetonitrile/water mixture (in which the enzyme molecules slowly dissolved overnight). These different routes leading to inactive enzyme that could be reactivated suggest that the inactive thermolysin represents a thermodynamically stable state.

Effects of acetonitrile on thermolysin structure

To investigate the possible conformational changes brought about by addition of acetonitrile, CD spectra in the far UV and near UV regions were recorded from 0.5 mg ml^{-1} solutions of thermolysin (Figure 3a). For thermolysin dissolved in different compositions of acetonitrile and aqueous buffer, distinct differences could be observed in the far UV spectra in the range of 10 – 15% acetonitrile (Figure 3b). At concentrations of acetonitrile above 30% thermolysin precipitates at concentrations of 0.5 mg ml^{-1} or above, precluding reliable spectroscopic measurements. The use of lower enzyme concentrations to minimise precipitation adversely affected the reliability of the spectra. For these reasons, mixtures containing more than 30% acetonitrile could not be analysed satisfactorily. The far UV CD spectra obtained over the range of 0 – 30% acetonitrile were analysed by the SELCON procedure,[18] with the results shown in Figure 3c. The most important structural changes in the range of 10 – 15% acetonitrile appear to be a decline in the proportion of α helices and an increase in the proportions of antiparallel β sheets and β turns. Although there could be potential complications in the application of SELCON to spectra of proteins in the presence of organic solvents, the data indicate that there may be some significant differences between the overall secondary structures of thermolysin in the two "active regions" (that is, <10% and >15% acetonitrile).

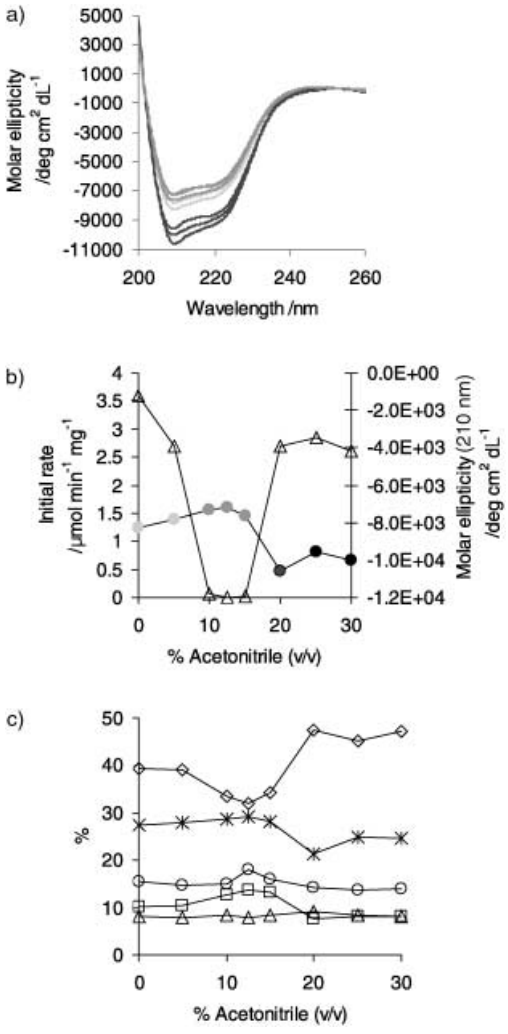

Figure 3. a) CD spectra of thermolysin (0.5 mg ml^{-1}) in the far UV region. From top to bottom the lines represent: 12.5 and 10 (essentially identical), 15 and 5 (ditto), 0, 25, 30 and 20% acetonitrile in aqueous buffer. b) The CD signals observed at 210 nm (circles) and the initial rate of Z-Phe-Leu-NH$_2$ hydrolysis by thermolysin (triangles) in the presence of different concentrations of acetonitrile. c) Percentages of secondary structural features in thermolysin. Diamonds = α helices, squares = antiparallel β sheets, triangles = parallel β sheets, circles = β turns, stars = remaining regions.

The near UV CD spectra of thermolysin (Figure 4a and b) showed a decrease in signal intensity with increasing acetonitrile content with a prominent minimum at 12.5%. This decrease could reflect a reduction in the interactions between aromatic residues within the enzyme core and/or an increase in their mobility. The absence of catalytic activity in 10 – 15% acetonitrile could therefore be caused by partial denaturation of the enzyme molecule. At higher concentrations of acetonitrile the signal increased again, a fact suggesting increased interactions and/or decreased mobility in this active conformation. Again, these observations might suggest that thermolysin exists in two

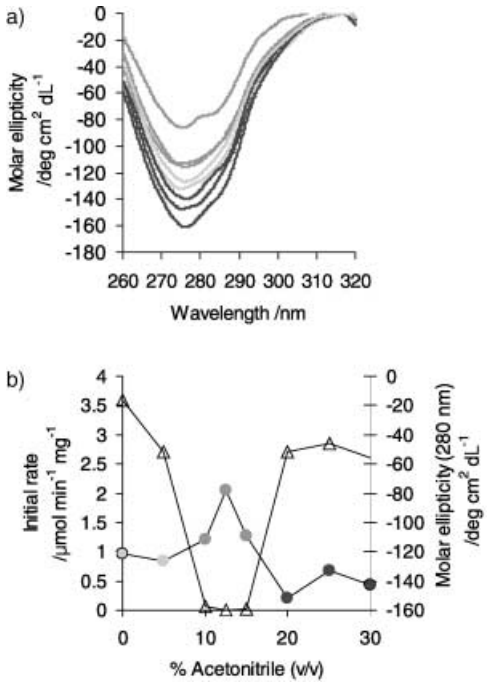

Figure 4. a) CD spectra of thermolysin (0.5 mg ml^{-1}) in the near UV region. From top to bottom the lines represent: 12.5, 10, 15, 0, 5, 25, 30 and 20% acetonitrile in aqueous buffer. b) The CD signals observed at 280 nm (circles) and the initial rate of Z-Phe-Leu-NH$_2$ hydrolysis by thermolysin (triangles) in the presence of different concentrations of acetonitrile.

different active conformations at higher or lower acetonitrile levels.

A further examination of the effects of acetonitrile on the enzyme was carried out by using fluorescence measurements. Fluorescence of proteins is mainly observed due to the presence of Trp and, to a lesser extent, of Tyr residues. Thermolysin has a total of 3 Trp and 28 Tyr residues per polypeptide chain. It is well known that both the intensity and wavelength of the maximum emission (λ_{max}) of Trp fluorescence depend on solvent polarity. When studying fluorescence at different solvent compositions these effects have to be taken into account. This can be done by comparing enzyme fluorescence with the fluorescence of model Trp derivatives such as *N*-acetyltryptophan ethyl ester (Ac-Trp-OEt).[12] For acetonitrile/water mixtures the λ_{max} of Ac-Trp-OEt decreases steadily with increasing acetonitrile content (Figure 5).

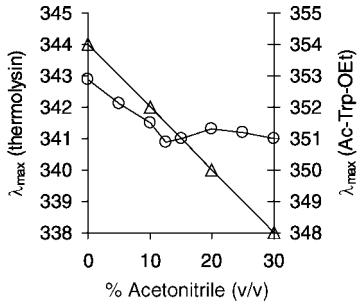

Figure 5. *Wavelength of the emission maximum of* N*-acetyltryptophan ethyl ester (right axis, triangles)[12] and thermolysin (left axis, circles) in aqueous buffer/acetonitrile mixtures.*

In purely aqueous buffer, the emission maximum shows that at least some Trp residues in thermolysin are in a less polar environment, that is, not fully exposed to the solvent medium. On addition of increasing concentrations of acetonitrile, the emission maximum does not change in a continuous fashion, but shows a clear break at about 12.5% acetonitrile. At lower acetonitrile levels, the Trp environment becomes progressively less polar. However, at higher levels of acetonitrile, changing the medium has only a very small further effect on the emission maximum; this suggests that the Trp residues are now almost completely inaccessible to the medium. Their constant environment in the protein corresponds in terms of polarity to about 20% acetonitrile in water. Trp fluorescence measurements on other enzymes such as α-chymotrypsin and subtilisin have shown an increase in Trp exposure that correlates with the loss of catalytic activity on increasing organic cosolvent concentration.[10–12] For thermolysin, the transition to unexposed Trp above 15% acetonitrile correlates with the regain of catalytic activity.

In summary, the loss of thermolysin activity in the presence of 10–15% acetonitrile is reversible and correlates well with changes in spectroscopic data. The conformation in the 10–15% region appears to be significantly different from that in the two regions where thermolysin is catalytically active (<10% and >15% acetonitrile).

Conclusions

In mixtures of 10–15% acetonitrile in water thermolysin is inactive in both the synthesis and hydrolysis reactions. Upon addition of either pure acetonitrile or aqueous buffer to a solution containing inactivated thermolysin, catalytic activity of the enzyme could be completely recovered. CD and fluorescence spectroscopic analysis of the enzyme in the presence of varying amounts of acetonitrile revealed significant changes in the enzyme conformation that could be correlated with loss of catalytic activity. The secondary and tertiary structures of thermolysin appear to be significantly different in the 10–15% acetonitrile range from those in the two regions where thermolysin is catalytically active (<10% and >15% acetonitrile).

Experimental Section

Enzymes, substrates, chemicals and solvents: Thermolysin (EC 3.4.24.2; protease type X) and benzyloxycarbonyl-L-phenylalanine (Z-Phe) were obtained from Sigma Chemical Company (UK). L-leucine amide (Leu-NH_2; free base) and Z-Phe-Leu-NH_2 were purchased from Bachem (UK) and NovaBiochem (UK), respectively. Acetonitrile was of HPLC grade from Aldrich (UK) and was dried over 3 Å molecular sieves. The aqueous buffer used was tris(hydoxymethyl)aminomethane (Tris)/HCl (25 mM, pH 7.8) containing $CaCl_2$ (10 mm).

Synthesis and hydrolysis experiments: In a typical synthesis experiment, equimolar (0.1 mmol) amounts of substrates Leu-NH_2 and Z-Phe were mixed together in 5-mL screw-capped vials with a total of 4 mL of the appropriate amounts of water and acetonitrile. In the case of hydrolysis experiments the starting material was Z-Phe-Leu-NH_2 (0.1 mmol). The reaction mixtures were incubated for about 1 h at 35 °C to ensure equilibration. Reactions were started by addition of thermolysin powder (3 mg). In mixtures of up to 20% acetonitrile in water the enzyme dissolved readily, while it remained partly suspended at higher acetonitrile concentrations. Reactions were performed, under constant stirring, in a temperature-controlled orbital shaker operating at 35 °C and 200 rpm.

Enzyme reactivation experiments: The time-course of the synthesis reaction (shown in Figure 2) was studied as follows: A solution (15 mL) of both amino acid substrates (0.01 M) was prepared in 10% acetonitrile and 90% water. Thermolysin powder (4 mg) was added to this solution. The mixture was sampled over a 20-min period, then 4 mL of the reaction mixture was removed, which was continuously sampled to obtain a 'blank'. To the remaining mixture (10 mL), acetonitrile (5 mL) with substrates (0.01 M) was added. This mixture was sampled over a further 60 minute period. The final acetonitrile concentration in this mixture was 40%.

Sampling: The reactions were sampled (100 μL) every 2 minutes over a 20-min period and dissolved in a mixture of 50:50 acetonitrile/water (900 μL) to which trifluoroacetic acid (Aldrich; final concentration 0.1%) was added to inactivate the enzyme.

HPLC analysis: Samples were analysed by means of a Waters Alliance HPLC system equipped with a reversed-phase column (0.46 × 25 cm; Hichrom HIRPB-250A). The mobile phase consisted of a 50% (v/v) mixture of deionised water and acetonitrile. This mixture was acidified with 0.1% trifluoroacetic acid. The flow rate was 1 mL min^{-1}. The Z-Phe substrate and the dipeptide product were detected at 254 nm while Leu-NH_2 was detected at 225 nm. The reactant

concentrations were calculated by using appropriate calibration curves.

CD and fluorescence spectroscopy: Samples for both CD and fluorescence spectroscopy were prepared at 0.5 mg ml^{-1} concentrations in appropriate mixtures of acetonitrile and 25 mM Tris/HCl buffer (pH 7.8) containing $CaCl_2$ (10 mm). CD spectra were recorded with a Jasco J-600 spectropolarimeter. The cell pathlengths used for near UV and far UV CD measurements were 0.5 cm and 0.02 cm, respectively. Fluorescence spectra were recorded with a Perkin Elmer LS50-B spectrofluorimeter. The pathlength was 1 cm and the excitation wavelength used was 295 nm.

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