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α-Chymotrypsin stability in aqueous-acetonitrile mixtures: is the native enzyme thermodynamically or kinetically stable under low water conditions?

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Abstract

Like many proteins, α-chymotrypsin is denatured in 50% volume aqueous-acetonitrile mixtures. However, it also shows high catalytic activity in 70% or more acetonitrile. Good activity in two different aqueous organic composition ranges has been described for several other enzymes. The stability of the native protein under low water conditions is generally believed to be a kinetic phenomenon, though there are also arguments for thermodynamic stability. We have distinguished between these possibilities by studying the effects of changing medium composition at different times. In preliminary experiments, we found catalytic activity could be recovered by adding neat acetonitrile to chymotrypsin in a 50% mixture, suggesting that the enzyme could renature under these conditions. However, in the 50% mixture, the true initial activity at 30°C is not zero, as the literature suggests. Instead, there is an initial burst of product formation over a few minutes, after which the enzyme becomes inactivated. By pre-incubating a 50% aqueous-acetonitrile mixture at 30°C prior to enzyme addition, the product burst could be eliminated. Activity could not then be recovered by slow addition of acetonitrile to the denatured enzyme. In contrast, it was possible to renature by dilution with aqueous buffer so that regeneration of catalytic activity was achieved. Thus, the good practical performance at high acetonitrile concentrations almost certainly results from a high kinetic barrier towards denaturation. The kinetics of enzyme denaturation in 50% and 70% acetonitrile were also investigated both at 30 and 20°C. Loss of catalytic activity was faster at higher temperature and at lower acetonitrile concentrations.

Keywords: α-Chymotrypsin; Organic solvent; Aqueous-acetonitrile mixtures; Enzyme denaturation/renaturation; Kinetic stability

1. Introduction

A number of enzymes can show catalytic activity in two different composition ranges in aqueous organic mixtures [1–3]. At low organic solvent concentrations, the activity can be as high as in aqueous solution, or even slightly enhanced. At moderate concentrations of miscible solvents, usually in the 30–50% volume range, the catalytic rate is often more or less zero [4,5]. At these intermediate solvent levels, certain spectral characteristics of the protein are known to change, providing strong evidence of conformational changes in the protein structure, i.e., denaturation [4–8]. Previous research has shown that this reflects a reversible denaturation process, e.g., Ref. [5]. Immediate addition of

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water to the denatured enzyme allows refolding to occur, and thus, catalytic activity is recovered.

At higher solvent concentrations, typically in the range 70–99%, catalytic activity can often be observed again [9–11]. It is generally believed that in these low water systems, the observed stability reflects kinetic factors [12]. The native form is thought to be thermodynamically unstable to denaturation, partly because of the absence of the hydrophobic effect. The low water level is held to make the structure rigid, reducing the rate of unfolding to a negligible value under assay conditions. The position is sometimes paraphrased as 'the enzyme would like to unfold, but it cannot'. This hypothesis is supported by some recent FTIR spectroscopic studies on lysozyme in acetonitrile–water mixtures by Griebenow and Klibanov [8]. They found the secondary structure of the enzyme in 90% acetonitrile was significantly different depending on the method of preparation. When solvent was added to an aqueous solution of the enzyme, the α-helix content was lower than that for solid enzyme added directly to the aqueous-solvent mixture. The authors commented that this result was typical of a kinetically controlled system since the history of enzyme dictated its behaviour.

However, it could be argued that in some solvents, the native structure is thermodynamically stable in low water systems. This argument rests on bringing together some recent experimental and theoretical developments.

Experimental evidence comes from differential scanning calorimetry (DSC) measurements of protein denaturation temperature and the enthalpy (ΔH) for this transition [13–17]. Firstly, the entire DSC trace is very similar whether or not organic solvent is present, for a given water content of the enzyme powder. So, the differences from aqueous solution are due to the removal of water, rather than the introduction of organic solvent. When studies are made over the whole range of water contents from aqueous solution to extensively dried powder, there is a smooth increase in melting temperature and decrease in enthalpy. This applies equally in the presence of solvent. The transitions measured by DSC in aqueous solution are generally held to reflect thermodynamically reversible denaturation. The fact that the measured parameters change smoothly as water content is reduced, might suggest that the transition remains primarily under thermodynamic control. A change over to kinetic control might be expected to produce discontinuities in the behaviour. If this is so, it follows directly that the native form may still be thermodynamically stable below the melting temperature in low water systems.

A second line of evidence for this view comes from theoretical estimations of total hydration energies of proteins in various conformations [18,19]. Calculations on proteins of known structure all indicated that the hydration energy of the native structure is less than that of the unfolded, extended forms. By simple thermodynamic cycle, this difference equals that of the denaturation energies between solution and the unhydrated state (theoretically in vacuo). As pointed out by Ooi et al. [18,19], these hydration energies do not dispute the contribution of hydrophobic interaction tending to destabilise the unfolded form, where more of the non-polar protein groups are exposed to solvent. But the model suggests this is outweighed by an increase in favourable hydration of polar groups that are only exposed to solvent on unfolding. Another way of looking at this is that the effective energies of hydrogen bond and electrostatic interactions become greater on dehydration, as water is no longer a competitor for the groups involved. Properly, we should also consider the interactions of protein molecules in various conformations with organic solvent molecules. Solvents will offer some favourable solvation, compared with vacuo, but the effects are likely to be weak and non-discriminating. (There are no special interactions between non-polar groups.) The unfolded form may give a slightly higher total solvation energy, because of its greater surface area; but the difference is likely

to be much less than for hydration. The overall destabilising effect of water on the native conformation is likely to remain.

Recent studies by Winters et al. [20] suggest that water is not essential for the refolding of denatured lysozyme into a near native conformation, as assessed by Raman and FTIR spectroscopy. The solid untreated commercial powder and its aqueous solution were confirmed to be native. Dissolution in DMSO resulted in severe deterioration in the secondary structure. However, after precipitation from DMSO, by contact with supercritical CO_2, near-native secondary structure was restored.

Further direct evidence of refolding at low water content comes from work by Wangikar et al. [21], with subtilisin BPN' solubilised in organic solvents. The enzyme-surfactant complex lost 99% of its activity in less than 10 min in tetrahydrofuran at 25°C. By diluting some of this inactive enzyme into dry octane, a significant portion of catalytic activity could be restored. The authors concluded that water was not essential for refolding of a partially denatured enzyme, and that the native form is thermodynamically stable in octane. However, they pointed out that the surfactant present may stabilise the folded structure in octane.

The practical way in which water-miscible reaction systems are prepared also provides support for a thermodynamically stable form of the enzyme under low water conditions. In the literature, the common procedure is to add aqueous enzyme directly to the organic solvent (as opposed to adding solid enzyme to the water–solvent mix). Obviously, by mixing in this way, the water–solvent composition at the enzyme micro-environment must pass through an intermediate concentration where denaturation and loss of catalytic activity is possible. However, since loss of enzyme activity does not appear to occur, it might be argued that refolding of the biocatalyst occurs on further increase of solvent concentration.

With these views in mind, we set out to test this conventional position experimentally, in order to determine whether the native enzyme was thermodynamically or kinetically stable in a particular low water organic solvent.

2. Experimental

2.1. Chemicals

α-Chymotrypsin (EC 3.4.21.1), Type II from bovine pancreas, 52 units/mg of solid was purchased from Sigma Chemical and used without further purification. *N*-acetyl-L-tyrosine (AT) and its ethyl ester (ATEE) were also obtained from Sigma. Acetonitrile (ACN) of guaranteed HPLC grade was obtained from Rathburn Chemicals, stored over 3A molecular sieves, and used without further purification. All inorganic salts were of analytical grade from BDH.

2.2. Enzyme preparation

Prior to use in organic solvent, the enzyme was dissolved in 10 mM sodium phosphate buffer, pH 7.8 to a concentration of 5 mg/ml. The solution was then freeze-dried for 24 h. A large quantity of enzyme (approximately 600 mg) was lyophilised to eliminate any differences in morphology or enzyme activity arising from batch variation. The resulting enzyme powder was further dried by storing over 4A molecular sieves until required.

2.3. Typical reaction conditions

The reaction studied was the α-chymotrypsin (CT) catalysed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester. A solution of the enzyme in buffer (25 mM sodium phosphate, pH 7.8) was added to ATEE dissolved in anhydrous ACN to give the desired solvent: aqueous buffer ratio (total solution volume 20 ml, ATEE concentration 10 mM, CT at 0.025 mg/ml unless otherwise stated). After brief mixing at room temperature (20°C), the zero time sample was re-

moved. The mixture was then incubated at either 20°C or 30°C with constant shaking (150 rpm). Samples from the reaction mixture were taken at regular intervals. Analysis was by HPLC on a Gilson 715 equipped with an ODS2 reverse phase column (Anachem, UK). The mobile phase consisted of water from milli Q adjusted to pH 2 with orthophosphoric acid mixed with 50% volume acetonitrile.

2.4. Attempted renaturation at 30°C by addition of ACN

Neat ACN was added (10 ml/min) with stirring to a solution of the enzyme in 50% v/v ACN to bring the final concentration up to 70% by volume. On addition of the ester substrate, the hydrolysis reaction at 30°C was followed to see whether or not catalytic activity had been recovered.

2.5. Attempted renaturation at 30°C by addition of aqueous buffer

A 25 mM phosphate buffer (pH 7.8) was added (90 ml/min) to a solution of the enzyme in 50% ACN, giving a final water content of 95%. The substrate was then added to the enzyme solution and the rate of product formation at 30°C measured.

2.6. Pre-incubation of aqueous buffer–solvent mixture prior to enzyme addition and reaction at 30°C

Aqueous buffer–solvent mixture was incubated at 30°C for approximately 10 min. An aliquot of aqueous enzyme was added to the mixture, giving a final CT concentration of 0.025 mg/ml. The hydrolysis reaction at 30°C was monitored on addition of ATEE dissolved in 200 μl of dry ACN.

2.7. Pre-incubation of enzyme in aqueous buffer–solvent mixture prior to reaction at 30°C

Enzyme was dissolved in aqueous buffer and mixed with dry ACN to give the desired solvent concentration. The mixture was then pre-incubated at 30°C for 10 min. Subsequently, the reaction was initiated by addition of the substrate dissolved in 200 μl of dry ACN. The rate of hydrolysis at 30°C was followed as described above.

2.8. Pre-incubation of enzyme in aqueous buffer–solvent mixture prior to reaction at 20°C

Enzyme was dissolved in aqueous buffer and mixed with dry ACN (at room temperature, 20°C) to give the required solvent concentration. After pre-incubation at 20°C for various times, the rate of hydrolysis was followed on addition of ATEE dissolved in 200 μl of dry ACN.

2.9. SDS-PAGE sample preparation and analysis

Enzyme in ACN–aqueous buffer mixture was prepared as described above (at 0.025 mg/ml). A 1 ml portion was immediately pipetted into an eppendorf tube and frozen directly by suspension in liquid nitrogen. The remainder of the enzyme solution was then incubated at either 20°C or 30°C. After specific time intervals, 1 ml volumes were transferred into eppendorf tubes and frozen immediately. Samples were then lyophilised overnight. Subsequent to drying, a 50 μl aliquot of PMSF solution (1 mM) was added to each protein sample. Samples were left on ice for 15–20 min, after which time they were diluted with 50 μl of SDS-PAGE buffer and boiled for 5 min. SDS-PAGE on 10–20% gradient gels (Bio-Rad product no. 161-0966) was performed using standard methods [22].

3. Results and discussion

3.1. Activity and progress curves at different ACN concentrations, 30°C

As a model reaction, we chose to follow the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester by α-CT in aqueous-ACN mixtures [2]. Earlier

work on these systems has been carried out in either mixtures of organic solvent and pure water, or solvent and aqueous buffer solutions. We have carried out experiments both in the presence and absence of sodium phosphate buffer (pH 7.8). The results described here are those obtained in the presence of buffer species, since this system offers two major advantages. Firstly, the buffering capacity in ACN–H_2O mixtures will be low. Even low concentrations of the acidic hydrolysis product may cause the pH (effective) of the reaction mixture to fall below the optimum for catalysis. This is less likely to occur if buffer is included in the system. Secondly, we have found the rate of product formation is at least 10-fold higher in the presence of buffer so that much lower enzyme concentrations (0.025 mg/ml) can be utilised. In all experiments carried out here, there was no visible sign of enzyme precipitation.

Previous work has shown that at high concentrations of organic solvent, the buffer components precipitate, and chymotrypsin may form a suspension by adsorbing to these [2]. In view of these complications, we avoided precipitation at high ACN levels by using dilute phosphate buffer (25 mM).

Fig. 1 shows progress curves for chymotrypsin catalysed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester in acetonitrile–water mixtures at 30°C. Results obtained confirmed those of Kise and Tomiuchi [2]. However, since their study was carried out in ACN–H_2O mixtures, their rates of product formation were at least 10-fold lower than those obtained in this work.

The enzyme gives high catalytic rates at high water levels, but in 50% acetonitrile, it loses nearly all its catalytic activity. Interestingly, however, and previously unreported, reactions at this intermediate solvent level show a 3% (0.3 mM) product burst in the first 10 min, with no further product formation (Fig. 1). In higher acetonitrile mixtures, the enzyme shows significant catalytic activity at 30°C (as reported previously).

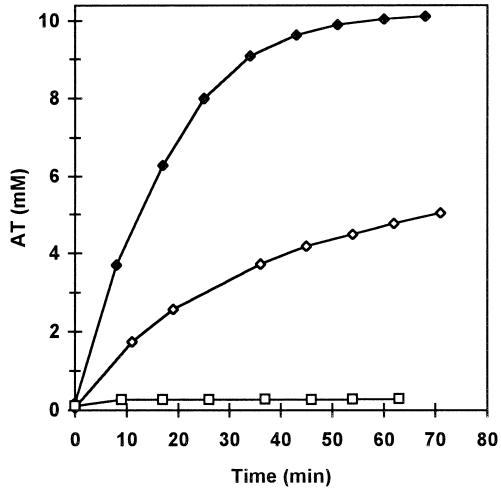

Fig. 1. Progress curves for α-chymotrypsin catalysed hydrolysis of ATEE in ACN-phosphate buffer mixtures, 30°C. Reactions in 5% ACN, 0.0025 mg/ml CT (♦); 50% ACN, 0.025 mg/ml CT (□) and 70% ACN, 0.025 mg/ml CT (◇). In all reactions, the enzyme in 25 mM phosphate buffer (pH 7.8) was added to substrate dissolved in ACN (final concentration of 10 mM ATEE in 20 ml reaction volume).

3.2. Can denatured enzyme in 50% ACN be renatured by adding more ACN?

It has previously been shown that the loss of biocatalyst activity at intermediate solvent concentrations is thermodynamically reversible by addition of water, e.g., Ref. [5]. We checked this observation for our system. A solution of α-chymotrypsin in 50% ACN (where the catalytic activity is almost absent), was diluted with aqueous buffer to 5% by volume. We observed full regeneration of catalytic properties at 30°C, as expected.

Although the enzyme appears to be denatured in 50% ACN, it shows significant catalytic activity at lower water contents in ACN. As discussed earlier, there is a general belief that this reflects kinetic stability in the enzyme there present. As a result, experiments have never been carried out to check whether the denatured enzyme in 50% ACN can be refolded by addition of ACN. Were this possible, it would show that the native form of the biocatalyst can be thermodynamically stable in systems of low

water content. This could be explained by an increase in the net favourable energetic interactions, including hydrogen bonds.

The 70% ACN reaction mixture in those experiments described so far was prepared by mixing of aqueous enzyme solutions and acetonitrile. It could be argued that the enzyme becomes dehydrated and fixed in conformation before it can be denatured. Hence, we studied another method to bring the enzyme to 70% acetonitrile. The enzyme was first prepared in 50% ACN–50% aqueous buffer at room temperature, conditions reported to give the mainly denatured form. The solution was then slowly diluted with neat acetonitrile, bringing the concentration up to 70% (CT, 0.015 mg/ml). There was no visible precipitation of either buffer or enzyme. On addition of ATEE, the reaction at 30°C was followed as before (Fig. 2). Results show a significant recovery in catalytic activity by comparison with the previous type of 70% ACN reaction at the same enzyme concentration (0.015 mg/ml).

This preliminary result appears to show that inactive enzyme in 50% ACN can be renatured by addition of ACN, i.e., the denaturation process would appear to be thermodynamically reversible. However, care needs to be taken since the initial burst seen in 50% ACN indicates the enzyme was not totally denatured before further ACN addition. Hence, the results were not conclusive and further evidence was sought.

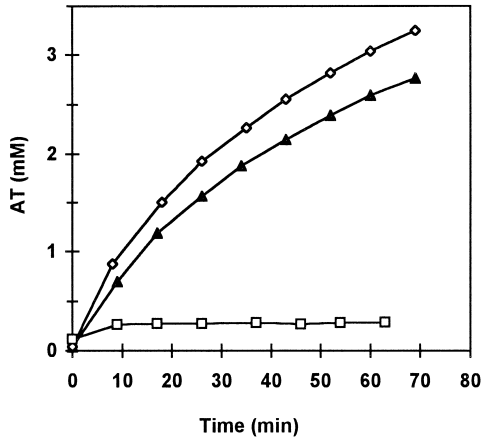

Fig. 2. Apparent recovery of catalytic activity 30°C. Neat ACN was slowly added to α-chymotrypsin in 50% ACN–50% phosphate buffer (25 mM, pH 7.8) to 70% by volume (0.015 mg/ml enzyme). On addition of ATEE, the hydrolysis reaction was followed (▲). Other points represent hydrolysis of ATEE in 50% ACN, 0.025 mg/ml CT (□) and in 70% ACN, 0.015 mg/ml CT (◇).

In the experiments discussed above, we followed the common procedure found in the literature: the substrate, enzyme, and solvent were mixed at room temperature (20°C), while reactions were carried out at 30°C. We found if mixing of the various reaction components was carried out at 30°C rather than room temperature, the product burst could be eliminated. This was achieved by either of two methods: (A) pre-incubating the 50% solvent–50% aqueous buffer mixture at 30°C for 10–15 min (allowing temperature adjustment to 30°C) prior to enzyme and substrate addition, or (B) pre-incubating the biocatalyst for 10–15 min in 50% ACN–50% aqueous buffer at 30°C prior to substrate addition.

Since these treatments evidently produce fully inactive enzyme, we proceeded to check whether this denaturation was thermodynamically reversible on addition of anhydrous ACN (to 70% v/v). In both cases, the biocatalyst was found to be catalytically inactive at 30°C after substrate addition. Renaturation was however possible by adding aqueous phosphate buffer to these pre-incubated 50% ACN–50% aqueous buffer mixes (final concentration of ACN, 5% v/v). More than 70% of the original enzyme activity could be recovered from enzyme treated according to method A. With the method where the enzyme was pre-incubated in the solvent (method B), the rate of product formation was only 40% that of the normal 5% ACN–95% aqueous buffer reaction. Several reasons may account for this observation. For example, some of the enzyme may have undergone irreversible denaturation during the 10 min incubation period. However, SDS-PAGE electrophoretic analysis of enzyme which had been incubated in 50% ACN at 30°C for 10 min showed a marked decrease in the native protein band and a greater

abundance of smaller autolysis products (Fig. 3). This strongly suggests that a significant fraction of the denatured enzyme in 50% ACN has been autolytically inactivated. In contrast, chymotrypsin which had not been pre-incubated in the 50% ACN mixture prior to analysis retained a greater fraction of native protein (regardless of mixing temperature). It is therefore apparent from these results that autolysis is the major reason why higher activity cannot be regained after addition of aqueous buffer to enzyme which has been pre-incubated in 50% ACN for 10 min at 30°C.

Nevertheless, these experiments demonstrate that catalytic activity can be regained when buffer is added to fully denatured enzyme in 50% ACN (whether treated by method A or B). This reconfirms that denaturation is thermodynamically reversible by addition of water. However, regeneration of the enzymes' catalytic properties cannot be attained on addition of ACN to the fully denatured catalyst in 50% ACN. Thus, we can confirm that the enzyme is thermodynamically unstable in 50% ACN, as previously known. In addition, we can now conclude with reasonable certainty that the same is true for an ACN concentration of 70%.

3.3. Effect of temperature / kinetics of denaturation

Having answered the question originally posed; we then turned our attentions to the product burst observed for enzyme which had been mixed with 50% aqueous-ACN at room temperature (20°C) and used to catalyse a reaction at 30°C. Our results seem to imply that temperature plays a crucial role in biocatalyst denaturation in 50% ACN.

Among other things, we were interested to see whether decreasing the reaction temperature would lower the rate of denaturation so that higher catalytic rates could be achieved. Recently, Bjorup et al. [23] have used immobilised

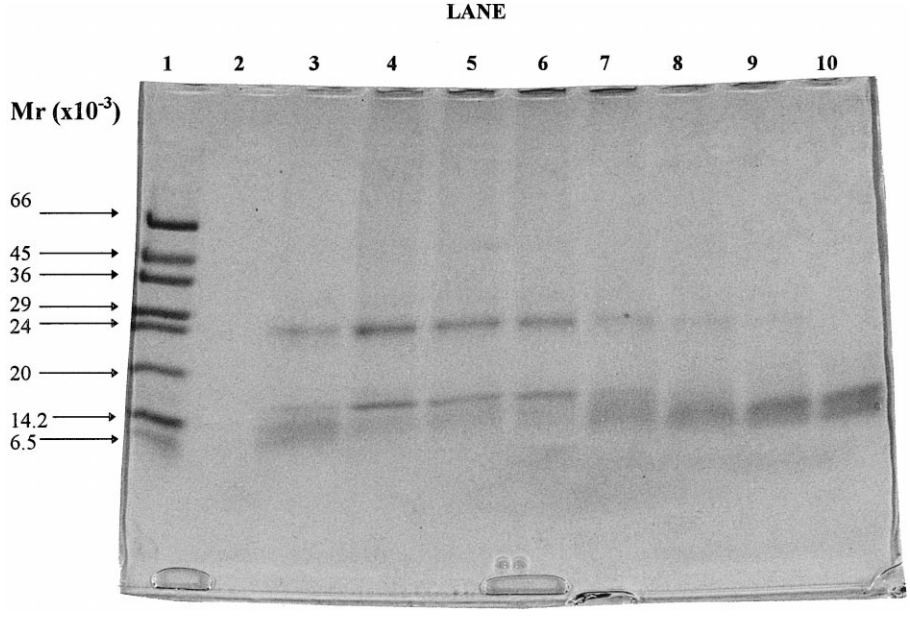

Fig. 3. A 10–20% gradient SDS-PAGE of α-chymotrypsin from 50% ACN–50% aqueous buffer mixtures. Molecular weight standards (lane 1). A 10-μg of protein was loaded into Lanes 3–10. Enzyme in 50% aqueous-ACN was prepared at 20°C, incubated for 10 min at 30°C, frozen and lyophilised for SDS-PAGE analysis (lane 3). Enzyme was mixed with 50% aqueous-ACN at 20°C (lane 4) or 30°C (lane 5) and immediately frozen and lyophilised for analysis. Enzyme in 50% ACN–50% aqueous buffer was prepared and incubated at 20°C; samples were taken for PAGE at times 0 min (lane 6), 8 min (lane 7), 15 min (lane 8), 30 min (lane 9) and 3 h (lane 10).

forms of CT to try and overcome this solvent inactivation at intermediate concentrations of ACN. The authors were successful to some extent in that inactivation was found to be lower for the immobilised enzyme than for the native form. However, it was not sufficiently low enough to make peptide synthesis at intermediate ACN concentrations attractive.

Here, we describe the effects of varying temperature on product formation both at 50 and 70% ACN. In the first instance, we studied the effect of pre-incubating enzyme in 70% ACN for 10 min at 30°C prior to substrate addition and reaction at the same temperature. Since we were aware that such treatment resulted in complete denaturation of the enzyme in 50% ACN, we were curious to determine the degree of denaturation at higher solvent concentrations. Fig. 4 shows the progress curves at 30°C for enzyme which has been pre-incubated at this temperature in comparison to that which has not. In 70% acetonitrile, the rate of product formation was reduced by approximately 30% by preheating the enzyme for 10 min at 30°C. Evidently, there is also some degree of denaturation in 70% ACN.

We then proceeded to study the progress of CT catalysed hydrolysis at 20°C in both 50 and

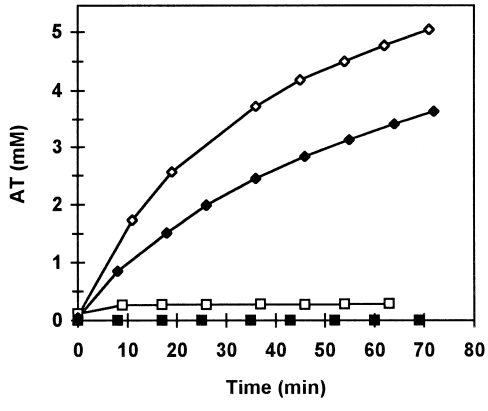

Fig. 4. Effect of pre-incubation at 30°C on α-chymotrypsin catalysed ATEE hydrolysis in ACN-phosphate buffer mixtures, 30°C. Hydrolysis of 10 mM ATEE in 50% ACN (■) and 70% ACN (◆) by CT (0.025 mg/ml) which has been pre-equilibrated for 15 min in the solvent–buffer mixture at 30°C prior to substrate addition; and hydrolysis in 50% ACN (□) and 70% ACN (◇) by enzyme (0.025 mg/ml) which has not been pre-equilibrated.

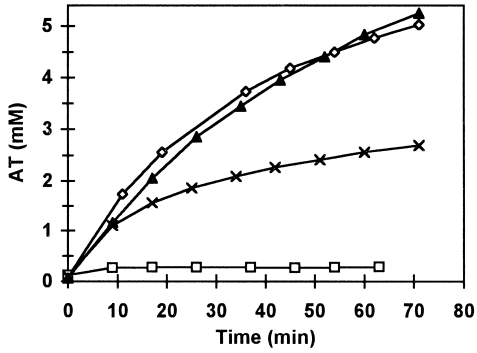

Fig. 5. Progress curves for α-CT catalysed hydrolysis of ATEE in ACN-phosphate buffer mixtures, 20°C and 30°C. Hydrolysis of 10-mM ATEE in 50% ACN (×) and 70% ACN (▲) by CT (0.025 mg/ml) at 20°C; hydrolysis in 50% ACN (□) and 70% ACN (◇) by CT (0.025 mg/ml) at 30°C.

70% ACN. Fig. 5 shows progress curves for enzyme which has been mixed at room temperature (20°C) and used directly to catalyse a reaction at 20°C. Corresponding reactions at 30°C are shown for comparison. The rate of catalysis at both solvent concentrations was found to be temperature dependent. At 20°C, the initial rate of catalysis in 70% ACN is slightly lower than that at 30°C (as expected elevated temperatures give increased rates). However, as the reactions proceed, the reaction at 30°C begins to tail off. This illustrates the classic temperature effect on enzymes and can be explained by the lower rate of biocatalyst denaturation in 70% ACN at 20°C. In 50% ACN at 20°C, the rate is higher than that at 30°C. There is no longer a product burst followed by inactivation. The product continues to be formed after 10 min, and the initial rate is remarkably close to that in 70% ACN at the same temperature. Evidently, the rate of denaturation is much lower at 20°C, allowing catalysis to proceed.

We studied the effect of incubation time on the rate of enzymatic hydrolysis at 20°C in 50% ACN mixtures. When the biocatalyst was pre-incubated for 15 min, before adding substrate, we found that the enzyme was not completely inactivated, but the catalytic rate was reduced significantly. With increasing incubation time, the rate of hydrolysis decreased. The enzyme

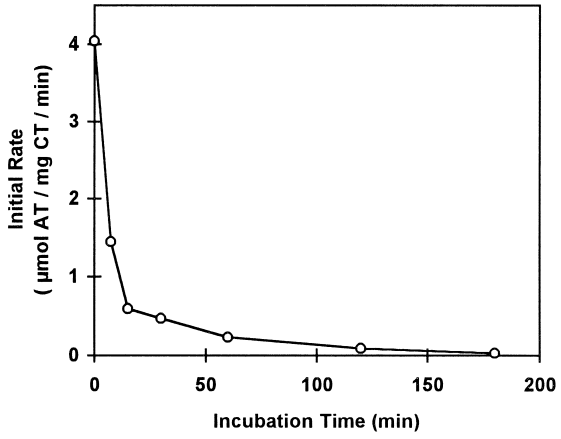

Fig. 6. Initial rate of ATEE hydrolysis in 50% ACN–50% phosphate buffer as a function of pre-incubation time, 20°C. α-CT (0.025 mg/ml) was incubated in the solvent–buffer mixture at 20°C for various time intervals, before addition of ATEE (10 mM). The hydrolysis reaction was then followed by HPLC.

was almost totally inactive after 3 h in the solvent mix (Fig. 6). From SDS-PAGE analysis (Fig. 3), we could conclude that autolysis was increasingly more apparent with incubation time at 20°C.

The effect of pre-incubation at 20°C on the biocatalyst in 70% ACN was also considered, though not so extensively. The catalytic activity was reduced slightly (about 15%) by incubating for 15 min (cf. much lower than at 30°C). A further reduction of 20% was observed on pre-incubating for 3 h. This appears to reflect a slow denaturation process. SDS-PAGE revealed that approximately 70% of the enzyme remained intact even after incubating for 3 h in 70% ACN.

Obviously, biocatalyst denaturation occurs in 70% or 50% ACN, but the rate constant for this process is much greater at 50%. Also, by comparison with those results obtained for CT at 30°C, unfolding appears to be slower at lower temperatures, regardless of solvent composition.

4. Conclusions

This research has provided experimental evidence that the stability of native chymotrypsin is simply a kinetic phenomenon in acetonitrile with low water content. Enzyme which is fully denatured (and thus, inactive) at intermediate solvent concentration, cannot be refolded by slow addition of organic solvent. This reflects a thermodynamically irreversible denaturation process by organic solvent. Although we have shown α-chymotrypsin is kinetically stable at low water content in acetonitrile, it is impossible to say whether the effects observed are due to low water conditions and would hold for other solvents. It is possible that the results obtained here are unique to aqueous-acetonitrile mixtures.

The studies also highlight the importance of temperature on the kinetics of denaturation and resultant catalytic rate. Enzyme denaturation occurs both at intermediate and high concentrations of ACN, and the rate of denaturation is greater with increased temperature. In 70% ACN, however, the rate is sufficiently low, both at 20 and 30°C, that enough catalyst remains for the reaction to proceed fairly rapidly.

These findings present important implications for the practical preparation of such CT catalysed reactions. It would appear that mixing conditions at 20°C are not critical, and addition of an aqueous enzyme solution to the solvent is a satisfactory procedure, i.e., a few seconds at intermediate ACN concentrations will not result in significant denaturation or loss of catalytic activity. However, at higher temperatures of 30°C or more, where rates of denaturation are greater, it is possible that significant loss of catalytic activity may result.

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