Food Chemistry **48** (1993) 231–235

A test for the two-stage thermoinactivation model for chymotrypsin

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(Received 16 September 1992; revised version received and accepted 26 January 1993)

Chymotrypsin was irreversibly inactivated at 30–130°C, pH 1·0–7·0 and in the presence of 0–4·0 M guanidine hydrochloride (GnHCl). The activation enthalpy for enzyme thermoinactivation ($\Delta H^{\#}$) at moderate temperatures, pH 4·0–7·0 and in ≤ 2 M GnHCl was 175–322 kJ mol^{-1}. The activation entropy ($\Delta S^{\#}$) was 244–734 J mol^{-1} K^{-1}. Such results are compatible with enzyme unfolding being the rate-determining step for the thermoinactivation of native chymotrypsin. For chymotrypsin pre-unfolded at low pH, high temperature and/or in Gn/HCl, $\Delta H^{\#}$ was 30–40 kJ mol^{-1} and $\Delta S^{\#}$ was between −182 and −191 J mol^{-1} K^{-1}. Therefore, thermoinactivation of pre-unfolded chymotrypsin is likely to involve covalent bond lysis as the rate-determining step. A biphasic Arrhenius plot was obtained for chymotrypsin thermoinactivation in 1·0–1·5 M GnHCl. Taken together, these results provide strong support for the two-stage model for enzyme thermoinactivation.

INTRODUCTION

The irreversible thermoinactivation of the majority of enzymes proceeds via a reversible unfolding of the native tertiary structure (N). The heat-unfolded/ enzyme (D) subsequently undergoes one or more irreversible reactions to form an irreversibly inactivated enzyme (I). The two reactions leading to irreversible thermoinactivation are in series; N $\rightleftharpoons$ D and D $\rightarrow$ I (Klibanov & Mozhaev, 1978; Zale & Klibanov, 1983; Mozhaev & Martinek, 1984; Tombs, 1985; Ahern & Klibanov, 1988; Kistjansson & Kinsella, 1991).

The kinetics of two-stage enzyme thermoinactivation will lead to an Arrhenius plot with a break at the enzyme melting or unfolding temperature (T_m). Below T_m, the rate of enzyme conformation change will be rate-limiting for thermoinactivation, i.e. the time-course for enzyme unfolding should be the same as for irreversible activity loss. At temperatures exceeding T_m, the rate of thermoinactivation is likely to be determined by the rate of the D $\rightarrow$ I step (Owusu *et al.*, 1992). Thus, the time-course for the heat-unfolding of fungal aspartic proteinases is coincident with that for irreversible activity loss (Brown & Yada, 1991). For *Bacillus amyloliquefaciens* chimeric alpha-amylase the rate of irreversible thermoinactivation appeared to be the same as the rate for the D $\rightarrow$ I reaction (Tomazic & Klibanov, 1988; Suzuki *et al.*, 1989). A biphasic Arrhenius plot has been obtained for the heat-denaturation of whey proteins (Dannenberg & Kessler, 1988) and for the thermoinactivation of psychrotroph-derived lipases (Owusu *et al.*, 1992). Direct evidence for two-stage thermoinactivation, as provided by a biphasic Arrhenius plot, is unavailable for most enzymes.

The objective of this study was to examine the relationship between the reversible and irreversible thermoinactivation of chymotrypsin. In an attempt to obtain direct evidence for the two-stage thermoinactivation scheme, chymotrypsin was thermoinactivated in ≤ 2 M GnHCl. The kinetics of thermoinactivation for chymotrypsin in the native state or in the pre-unfolded state were also examined based on a determination of the inactivation reaction rate constant (k), activation energy ($\Delta E^{\#}$), enthalpy ($\Delta H^{\#}$), entropy ($\Delta S^{\#}$) and free energy ($\Delta G^{\#}$) at temperatures between 30 and 130°C.

MATERIALS AND METHODS

Chymotrypsin (Type I S; free of autolysis products and low-molecular weight contaminants) and melting-point

Food Chemistry 0308-8146/93/$06.00

tubes (0·9 mm × 100 mm) were from Sigma Chemical Co., UK. Guanidine hydrochloride (> 99·5% purity) was from Fluka, Switzerland. tri-hydroxymethylaminomethane (Tris) and all other chemicals were of AnalaR grade from the British Drug Houses, Poole, UK. Solutions were made using double-distilled water.

Chymotrypsin was dissolved directly in Tris–HCl buffer (0·1 M, pH 7·0) or in aqueous sodium chloride (0·1 M) adjusted to pH 1·0–5·0 and containing 0–4 M GnHCl. Samples of enzyme were sealed in boiling-point tubes and heated using a thermostat oil-bath (Grant model TD heater; Grant Instruments, Cambridge, UK) at 30–130°C; the heating medium was silicone fluid (Dow Corning). After heating, the tubes were cooled in an ice-bath and 50 μl of enzyme was transferred to an Eppendorf tube.

Residual enzyme activity was assayed using azocasein (1 mg/ml in Tris–HCl buffer, pH 7·0) as substrate. Azocasein (1 ml) was added to 50 μl of enzyme sample and the mixture was incubated at 37°C for 30 min. Trichloroacetic acid (20% w/v; 0·4 ml) was added to precipitate unreacted azocasein, and the sample was centrifuged using a microcentrifuge (Eppendorf model 5414C; Eppendorf–Netherler–Hinz GmbH, Germany) at 12 000 rpm. The supernatant was analysed by spectrophotometry at 410 nm. The level of enzyme activity remaining after heat-treatment was expressed as a percentage of the initial activity. In control experiments, it was shown that dissolving chymotrypsin at low pH, in 4 M GnHCl, or the process of sealing enzyme samples within boiling-point tubes, did not cause significant errors in the assay of enzyme residual activity or subsequent data analysis.

RESULTS AND DISCUSSION

The thermoinactivation of chymotrypsin followed first-order kinetics (Fig. 1(A)). A plot of log (residual activity) vs time was linear, usually with a correlation coefficient of >0·98 (Fig. 1(B)). The apparent first-order inactivation rate constant (k) was determined from the slope of such semi-log-plots. The temperature dependence of k is given by the Arrhenius equation $\ln k = \Delta E^{\#}/(RT + C)$. Hence, a plot of $\ln k$ vs $1/T$ gave one or more straight lines with a slope of $\Delta E^{\#}/R$ (Figs 2 and 3). Other activation parameters, e.g. $\Delta G^{\#}$, $\Delta H^{\#}$ and $\Delta S^{\#}$, were determined using standard thermodynamic relationships, e.g. $\Delta G^{\#} = RT \ln (k'h/KT)$, $\Delta H^{\#} = \Delta E^{\#} - RT$ and $\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T$, where K and h are the Boltzmann and Planck constants, respectively (More, 1985). A summary of results is given in Tables 1 and 2.

For native chymotrypsin, the apparent first-order rate constant for thermoinactivation was $1{\cdot}5 \times 10^{-3}$ s^{-1} ($t_{1/2}$ = 448 s) at 50°C and 256×10^{-3} s^{-1} ($t_{1/2}$ = 27 s) at 70°C. In this study, the minimum heating time which could be determined manually with accuracy is about 10 s. Therefore, at temperatures exceeding about 80°C, the rate constant for thermoinactivation could not be determined accurately. Arrhenius plots were therefore determined over a limited temperature range.

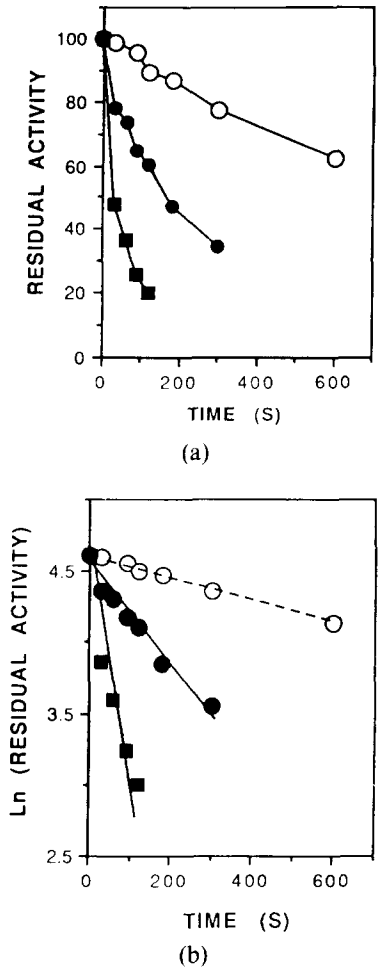

Fig. 1. Thermoinactivation of chymotrypsin at 40°C (○), 50°C (●) and 65°C (■). (a) A plot of residual activity vs heating time. (b) Plot of ln (residual activity) vs time. Solvent is Tris–HCl buffer (0·1 M, pH 7·0).

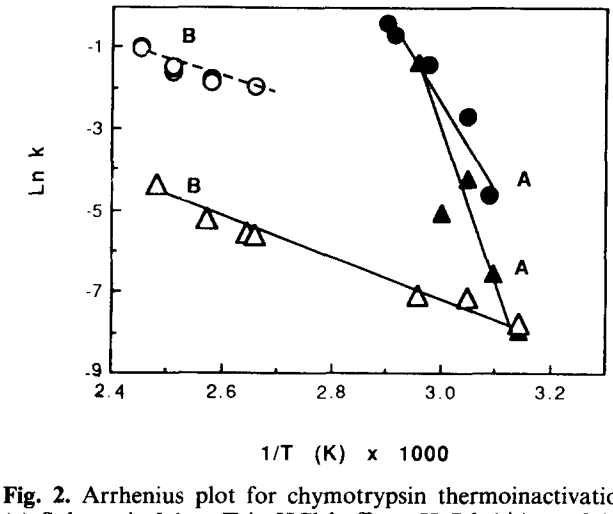

Fig. 2. Arrhenius plot for chymotrypsin thermoinactivation. (a) Solvent is 0·1 M Tris–HCl buffer, pH 7·0 (▲), or 0·1 M aqueous sodium chloride, pH 5·0 (●). (b) Solvent is 4 M guanidine hydrochloride in 0·1 M Tris–HCl buffer, pH 7·0 (Δ), or 0·1 M aqueous sodium chloride, pH 5·0 (○).

At temperatures below 75°C the Arrhenius plot for chymotrypsin heat-inactivation is monophasic or has a single slope (Fig. 2). However, experiments performed in 1·0 or 1·5 M GnHCl resulted in a bi-phasic Arrhenius plot (Fig. 3), corresponding to two distinct

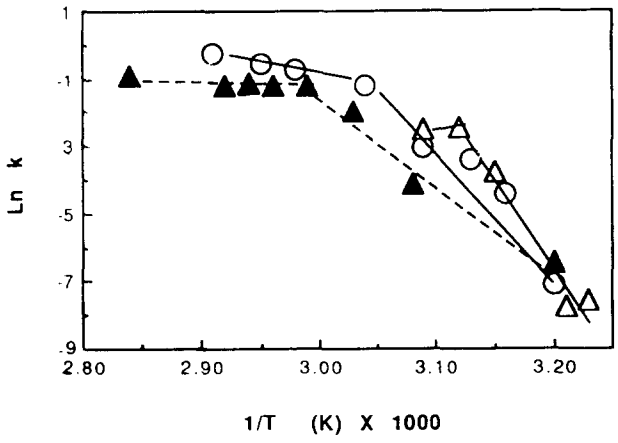

Fig. 3. Arrhenius plot for chymotrypsin thermoinactivation in 1·0 M (▲), 1·5 M (○) or 2·0 M (Δ) guanidine hydrochloride. Solvent is 0·1 M aqueous sodium chloride, pH 5·0.

rate-limiting reactions for thermoinactivation at 40–80°C.

Dilute GnHCl reduces T_m for chymotrypsin (Owusu & Berthalon, 1993). The rate of enzyme thermoinactivation at temperatures below T_m will be determined by the rate of enzyme unfolding (N $\rightleftharpoons$ D). At temperatures exceeding T_m, D $\rightarrow$ I reactions are expected to become rate-limiting (Owusu *et al.*, 1992). Therefore, a biphasic Arrhenius plot (Fig. 3) is support for the existence of two distinct rate-limiting reactions for chymotrypsin thermoinactivation at low or high temperatures.

For chymotrypsin dissolved in 4 M GnHCl (see below) or at pH 1·0, i.e. under conditions where the enzyme is initially in the unfolded (D) state before heating, an Arrhenius plot with a single slope was obtained (Fig. 2).

Activation parameters

At low temperature, moderate pH and/or in the presence of a low concentration of denaturant (e.g. T = 45–75°C, pH 4·0–7·0 and/or [GnHCl] ≤ 2 M), $\Delta H^{\#}$ was 175–301 kJ mol^{-1} (Tables 1 and 2). Such large activation enthalpy values are characteristic of protein denaturation reactions (Stearn, 1949; Walstra & Jenness, 1984; Brown & Yada, 1991). Therefore, enzyme unfolding may be the rate-determining step for the irreversible thermoinactivation of chymotrypsin under the conditions specified.

$\Delta H^{\#}$ for protein unfolding is attributable to the heat change associated with the disruption of a large number of weak non-covalent bonds in the heat-unfolding reaction, N $\rightleftharpoons$ N$^{\#}$ $\rightarrow$ D. N$^{\#}$ is an unfolding transition state with a structure intermediate between that of native chymotrypsin (N) and a partially unfolded (D) state. The number of non-covalent bonds broken to form the N$^{\#}$ state is difficult to assess. There is some uncertainty connected with the bond energies and relative importance of various non-covalent bonds in the N state. Assuming a hydrogen or hydrophobic bond (removal of a —CH_2 group from solvent contact) strength of about 5·4 kJ mol^{-1} (Pace, 1992; Shirley *et al.*, 1992), the formation of the N$^{\#}$ state at pH 5·0–7·0 is accompanied by the disruption of 40–50 noncovalent bonds.

However, the structural change leading to the N$^{\#}$ state, based on the value for the activation heat capacity ($\Delta C_p^{\#}$) for heat unfolding, appears very slight (Lumry & Biltonen, 1969; Segawa & Sugihara, 1984). $\Delta C_p^{\#}$, an index of the degree of exposure of hydrophobic groups, is zero for chymotrypsin and lysozyme. That is, for these enzymes the degree of apolar group exposure to solvent is the same in the N and N$^{\#}$ states (Lumry & Biltonen, 1969; Segawa & Sugihara, 1984). The structure of the N$^{\#}$ state lies closer to the N state than to the D state.

Positive $\Delta S^{\#}$ values also suggest that enzyme unfolding is the rate-determining step for the irreversible thermoinactivation of native chymotrypsin at pH 4·0–7·0 and [GnHCl] ≤ 2 M (Tables 1 and 2). There is an increase in entropy during the conversion of the N state to the N$^{\#}$ state.

Table 1. Calculated activation parameters and pH/temperature conditions

pH	Temperature °C	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
7·0	45–65	219 (0·1)	320 (2·3)	95·2–87·0
5·0	50–72	175 (0·08)	244 (3·7)	92·0–86·1
4·0	45–75	229 (0·1)	414 (4)	97·3–86·0
1·0	45–75	30 (0·1)	−191 (0·2)	91·0–96·4

Data are mean values with SD in parentheses (n = 4–5).

Table 2. Calculated activation parameters and pH/temperature/GnHCl conditions

Conditions	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
pH 5·0, 1 M GnHCl[a]			
40–60°C	267 (0·1)	547 (6)	93·4–85·6
65–80°C	18 (0·05)	−202 (0·3)	86·4–89·4
pH 5·0, 1·5 M GnHCl[a]			
40–50°C	301 (0·1)	660 (8)	95·1–88·4
56–70°C	55 (0·12)	−86 (0·1)	84·1–85·3
pH 5·0, 2 M GnHCl			
35–50°C	322 (0·04)	734 (4)	95·4–86·4
pH 5·0, 4 M GnHCl (n = 10)			
30–130°C	30 (0·01)	−182 (2)	99·0–104·5
pH 7·0 4 M GnHCl (n = 7)			
45–130°C	40 (0·31)	−186 (4)	98·5–114·5

n = 4–5, except where stated otherwise; SD in parentheses.
[a] Two-phase Arrhenius plot observed (see Fig. 3).

Effect of guanidine hydrochloride and low pH on activation parameters

Moderate concentrations of GnHCl destabilized chymotrypsin towards irreversible thermoinactivation at low temperatures. The half-life for thermoinactivation at 50°C was 448 s (0 M GnHCl), 103 s (1·0 M GnHCl), 20 s (1·5 M GnHCl) and 29 s (2·0 M GnHCl). In contrast, unfolding of chymotrypsin in 4 M GnHCl stabilized this enzyme towards irreversible inactivation (Fig. 2).

The destabilizing effect of ≤2·0 M GnHCl can be discussed in terms of reductions in the stability of the N# state and the value of $\Delta G^{\#}$ (Segawa & Sugihara, 1984). Increases in $\Delta H^{\#}$ and $\Delta S^{\#}$ values observed in 0–2 M GnHCl (Table 1) are consistent with a more expanded N# state in dilute GnHCl. The number of non-covalent bonds broken and the entropy change associated with generating the N# state increases in dilute GnHCl. It is noted that the proposed changes in the structure of the N# state may invalidate the two-state assumption ($N \rightleftharpoons N^{\#}$) underlying the transition-state formalism (Bloomfield, 1988). In any case, the reported changes in $\Delta H^{\#}$ and $\Delta S^{\#}$ values (Table 2) lead to real reductions in the value of $\Delta G^{\#}$ and a decrease in enzyme thermostability in 0–2 M GnHCl.

Effect of enzyme pre-unfolding on activation parameters

The unfolding of chymotrypsin in GnHCl was measured by UV difference spectroscopy essentially as described previously (Owusu, 1992). Figure 4 shows UV difference changes at 293 nm (E_{293}) and the fraction of enzyme unfolded (F) plotted against [GnHCl]. At room temperature, chymotrypsin begins to unfold at 2 M GnHCl and is completely unfolded in 4 M GnHCl. These results are similar to those previously reported (Greene & Pace, 1974).

$\Delta H^{\#}$ was 30–40 kJ mol^{-1} for the thermoinactivation of chymotrypsin unfolded in 4 M GnHCl (Tables 1 and 2). Similar values are obtained for thermoinactivation studies performed at pH 1·0. Under such conditions, irreversible inactivation probably involves a D → I reaction (Ahern & Klibanov, 1988). The particular reaction is apparently acid-catalysed because the rate of irreversible thermoinactivation was greater at pH 5·0 than at pH 7·0 (Fig. 2).

Irreversible thermoinactivation of pre-unfolded chymotrypsin leads to negative $\Delta S^{\#}$ values (Tables 1 and 2). These are characteristics of a second-order rate-determining reaction step that involves the bringing together of two reactants to form a transition (D#) state with the loss of translational and rotational degrees of freedom (Jencks, 1969). As discussed below, the rate-limiting step for the thermoinactivation of pre-unfolded chymotrypsin could be peptide bond hydrolysis (Ahern & Klibanov, 1988).

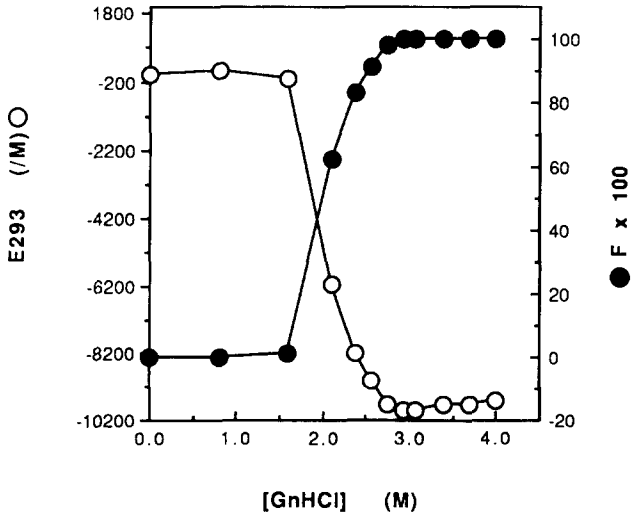

Fig. 4. Guanidine hydrochloride (GnHCl) unfolding of chymotrypsin monitored by UV difference change at 293 nm (E_{293}). E_{293} and the fraction of enzyme unfolded (F) plotted vs [GnHCl].

The end-products (I states) generated by the thermoinactivation of chymotrypsin at ≤60°C (pH 5·0–7·0) or 125°C (in 4 M GnHCl) have been assessed by high-pressure gel filtration chromatography. After heating at pH 5·0–7·0 for 0·5–5 h, chymotrypsin is thermoinactivated mainly by a process of autolysis and incorrect refolding. Thermoinactivation of pre-unfolded enzyme at 125°C appeared to involve non-specific peptide bond breakdown (Owusu & Berthalon, 1993). There was no evidence for base-catalysed sulphur-disulphide mediated aggregation. No large molecular weight aggregates were detected by gel-filtration analysis.

For acid, alkali- or enzyme-catalysed hydrolysis of model peptide compounds, $\Delta H^{\#}$ is 40–60 kJ mol^{-1} and $\Delta S^{\#}$ is between −104 and −133 J mol^{-1} K^{-1} (Escombe & Lewis, 1927; Stearn, 1949). These values are comparable with the activation parameters observed for the thermoinactivation of unfolded chymotrypsin (Tables 1 and 2).

The hydrolysis of a peptide bond can be presented as $D \rightleftharpoons D^{\#} \rightarrow I$; where D# is the classical tetrahedral transition state produced by nucleophilic attack of water at a carboxyl carbon. In general, there is an increase in charge separation in the product acid and amine compared with reactants (Jencks, 1969). Increased solvation of the D# state (compared with the D state) would also account for the negative $\Delta S^{\#}$ values observed for the thermoinactivation of unfolded chymotrypsin (Tables 1 and 2). At high water activity, covalent bond hydrolysis would be pseudo first order, consistent with the observed first-order thermoinactivation kinetics for unfolded chymotrypsin.

In conclusion, the present results are consistent with a two-state thermoinactivation process for chymotrypsin. In 0·1 M Tris–HCl buffer (pH 7·0) a biphasic Arrhenius plot was not observable. However, thermoinactivation of chymotrypsin dissolved in ≤2·0 M GnHCl produced a biphasic Arrhenius plot. A comparison of activation parameters for the inactivation of native and unfolded chymotrypsin also supports the view that there are two distinct rate-limiting reactions for irreversible heat-inactivation at the temperatures examined.

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