Stability and Activity Modulation of Chymotrypsins in AOT Reversed Micelles by Protein–Interface Interaction

Interaction of α-Chymotrypsin with a Negative Interface Leads to a Cooperative Breakage of a Salt Bridge That Keeps the Catalytic Active Conformation (Ile^{16}–Asp^{194})

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Abstract: The stability of α-chymotrypsin and δ-chymotrypsin was studied in reversed micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) in isooctane. α-Chymotrypsin is inactivated at the interface and at the water pool, while δ-chymotrypsin is inactivated only at the water pool. The mechanism of inactivation at the interface is related to the interaction of N-terminal group alanine 149 (absent in δ-chymotrypsin) with the negative interface. The dependence of enzyme activity on water content of these two enzymes in reversed micelles of AOT is also related with the interface interaction, since δ-chymotrypsin does not have a bell-shaped curve as observed for α-chymotrypsin. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **59:** 360–363, 1998.

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1. INTRODUCTION

α-Chymotrypsin is a 25 kDa enzyme formed by three chains linked by disulfide bonds. The enzyme is activated by proteolytic cleavage of an inactive precursor (chymotrypsinogen). α-Chymotrypsin has three N-terminal residues: Cys^{1}, Ile^{16}, and Ala^{149} (Blow, 1972; Hess, 1972; Kraut, 1972; Sharma and Hopkins, 1978, 1979). Ile^{16} forms a salt bridge with Asp^{194} critical for the enzymatic activity. δ-Chymotrypsin is an active intermediate in the chymotrypsinogen activation process, without the cleavage responsible for the formation of the N-terminal Ala^{149}. Although Ala^{149} is not essential for activity, it is related to a cooperative effect on protein inactivation at alkaline pH (Ghéllis et al., 1970; Oppenheimer et al., 1966; Sharma and Hopkins, 1982; Valenzuela and Bender, 1970, 1973).

α-Chymotrypsin has been widely used in organic solvents or immobilized in solid surfaces, being mostly used for peptide synthesis (Gupta, 1992; Pileni, 1989). The enzymatic activity retained is these systems is much lower than in aqueous media. Upon incorporation of α-chymotrypsin in reversed micelles, the enzymatic activity remains equal or higher than in aqueous media. As a water soluble globular protein, α-chymotrypsin is incorporated in the water pool of the micelle in a catalytic active form (Barbaric and Luisi, 1981; Fletcher et al., 1984; Garcia-Carmona et al., 1992; Luisi and Steinmann-Hoffman, 1987; Luisi et al., 1988; Ruckenstein and Karpe, 1990). Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) is the most suitable and widely used surfactant to form reversed micelles. α-Chymotrypsin solubilized in AOT reversed micelles presents a bell-shaped curve of activity as a function of the water content. In reversed micelles the water content is usually expressed as the water/AOT molar ratio (W_o). The maximal activity of α-chymotrypsin is at W_o close to 10 (Barbaric and Luisi, 1981; Fletcher et al., 1984).

Upon incorporation of α-chymotrypsin in AOT reversed micelles the enzyme inactivates faster than in aqueous medium. The stability of α-chymotrypsin as a function of W_o follows a bell-shaped profile, being the W_o of maximum stability the same of the maximum activity (Barbaric and Luisi, 1981; Fletcher et al., 1984). In this communication we studied the activity and stability of α-chymotrypsin and δ-chymotrypsin in reversed micelles of AOT in isooctane. δ-Chymotrypsin is as stable as in aqueous medium and does not present the bell-shaped behavior of activity with W_o. α-Chymotrypsin inactivation depends on pH and W_o. The analysis of the inactivation profiles of both enzymes gave

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insights in the mechanism of inactivation in AOT. The same protein group responsible for the higher sensitivity of α-chymotrypsin (if compared to δ-chymotrypsin) to alkaline pH in aqueous solutions (N-terminal Ala^{149}) is involved with the inactivation in AOT reversed micelles, by interaction with the negative interface.

2. MATERIALS AND METHODS

α-Chymotrypsin from bovine pancreas (Worthington, NJ, and Sigma Chem. Co., St. Louis, MO; EC 3.4.21.1) and δ-chymotrypsin from bovine pancreas (Sigma Chem. Co., St. Louis, MO; EC 3.4.21.1); *p*-nitrophenylacetate was double crystallized from dry ethyl ether; sodium bis(2-ethylhexyl)sulfosuccinate (AOT) 99% (Aldrich Chem. Co., Milwaukee, WI); isooctane (Riedel-de Haen, Hannover-Germany) was distilled over sodium. All other reagents were from Sigma (analytical grade).

Reversed micelles preparation. The reversed micellar solution of AOT in isooctane was 0.1 *M* in all experiments. Water solutions containing buffers, substrate, and enzyme were added to yield the desired W_o. Enzyme concentrations are referred to the overall volume. The concentration of other components are referred to the aqueous pool volume. The expressed pHs refer to the pH of the aqueous buffer solutions.

Enzymatic activity determination. The initial rate of *p*-nitrophenol formation (410 nm) was used to determine enzymatic activity. The absorbance at 410 nm of cells containing temperature-equilibrated (30°C) micellar solutions was followed as a function of time. From the enzymatic initial velocity was then subtracted the nonenzymatic hydrolysis of the substrate, measured at the same experimental condition but in the absence of enzyme. The molar quantities were obtained by the measured coefficient of molar absorption (ε) of the *p*-nitrophenol 0.1 *M* AOT/isooctane, 50 m*M* Tris, pH 8.0, at 410 nm: $W_o = 5.0 - \varepsilon = 800\ M^{-1}\ cm^{-1}/W_o = 10 - \varepsilon = 1{,}600\ M^{-1}\ cm^{-1}/W_o = 20 - \varepsilon = 3{,}700\ M^{-1}\ cm^{-1}/W_o = 30 - \varepsilon = 5{,}600\ M^{-1}\ cm^{-1}/W_o = 40 - \varepsilon = 7{,}200\ M^{-1}\ cm^{-1}$. The ε at the same condition in bulk water is $16{,}000\ M^{-1}\ cm^{-1}$.

Enzyme stability measurements. Enzyme in aqueous solution or in reversed micellar solutions was pre-incubated at 30°C in the absence of substrate. Aliquots were withdrawn (at the indicated times) and activities were measured as described above. The rate constant and pre-exponential factor were obtained by fitting the kinetics as a monoexponential according the equation $V/V_o = e^{-kt}$, where V_o is the enzymatic activity at time zero and V is the enzymatic activity at an indicated time. The fitting of the biexponential kinetics was determined according equation $V/V_o = A_f\, e^{-k_f t} + A_s\, e^{-k_s t}$. A_f and A_s are pre-exponential factors of the two process ($A_f + A_s = 1$). The inactivation kinetics at different pHs were measured using two different buffers. From pH 8 to 8.75 Tris buffer was used. Ethanolamine buffer was used for higher pHs. For pH 8.75 both buffers were used.

3. RESULTS

The activity of hydrolysis of *p*-nitrophenyl acetate catalyzed by α-chymotrypsin and δ-chymotrypsin was measured in AOT/isooctane at pH 8.0 at substrate saturation (Fig. 1). α-Chymotrypsin activity as a function of W_o is bell-shaped as previously reported (Barbaric and Luisi, 1981; Fletcher et al., 1984). At W_o 5 or below there is no enzymatic activity. α-Chymotrypsin activity increases up to W_o 10 and then decreases, reaching a plateau at $W_o > 30$. The activity of δ-chymotrypsin as a function of W_o does not show a bell-shaped profile (Fig. 1). The maximum activity is obtained at W_o 5.0 and the same activity is maintained upon increase of W_o. The specific activity of both forms of chymotrypsin is approximately 42 nmol min^{-1} mg^{-1} in aqueous buffer at pH 8.0.

The deactivation of α-chymotrypsin was studied in reversed micelles of AOT as a function of W_o and pH (Fig. 2). As α-chymotrypsin is inactive at W_o 5.0 or below, the measurement of the kinetic of inactivation is not feasible. The activity at this W_o cannot be restored by further increase in W_o (the deactivation at this W_o is instantaneous). The kinetic of inactivation at pH 9.0 behaves as a monoexponential. At other pHs the kinetics could not be fitted as a monoexponential decay (Fig. 2A).

The fit of the kinetics of inactivation of α-chymotrypsin as biexponential decay permitted the evaluation of the rate constants and the pre-exponential factors. k_f, the fast component, increases linearly with W_o, being minimum at W_o 10 (Table I). The values of k_f and A_f change remarkably at pHs above 8.5 (Fig. 2B and 2C). At pH 9.0 the fast component vanishes and the measured rate constant is the same order of k_s. k_s, on the other hand, is independent of W_o and pH (Figure 2D and Table I).

The presence two components suggests two distinct mechanisms for the enzyme inactivation. Since the fast component is not present in water it is probably related with

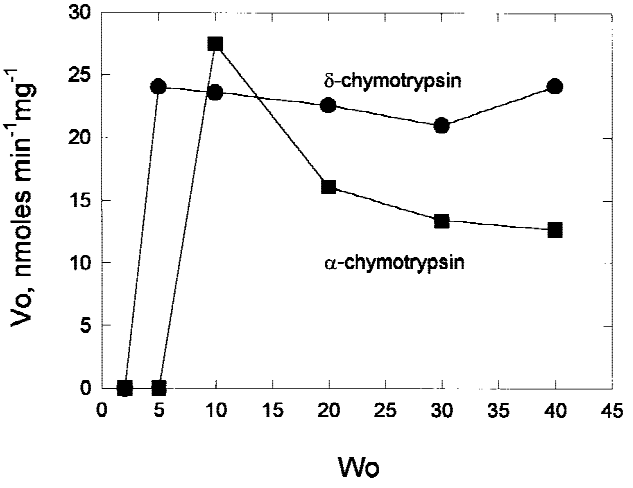

Figure 1. Specific activity of α-chymotrypsin and δ-chymotrypsin in 0.1 *M* AOT/isooctane at 30°C as a function of the W_o. [Tris] = 50 m*M*, pH 8.0; [substrate] = 10 m*M*; [δ- and α-chymotrypsin] = 40 μg/mL.

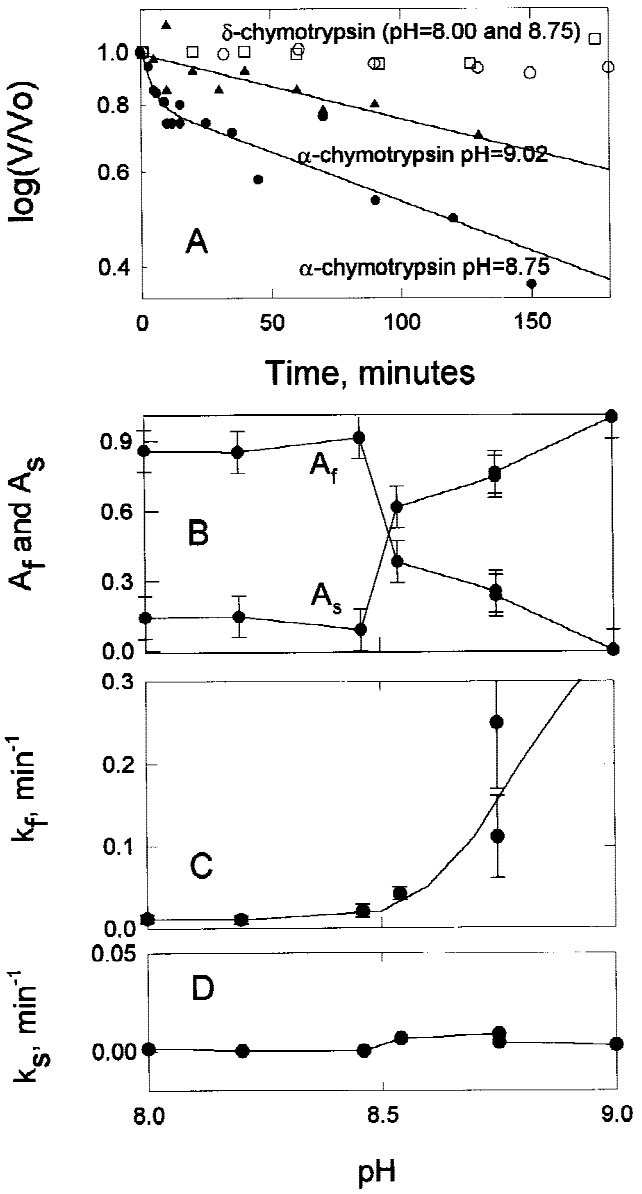

Figure 2. (A) Kinetic of inactivation of α-chymotrypsin in 0.1 *M* AOT/isooctane at 30°C at W_o = 10, pHs 9.02 and 8.75 ([ethanolamine] = 50 m*M*), and of δ-chymotrypsin at pHs 8.00 (empty circle, [Tris] = 50 m*M*) and 8.75 (empty square, [ethanolamine] = 50 m*M*); [α- and δ-chymotrypsin] = 60 μg/mL (2.4 μ*M*). *V* is the enzymatic activity at indicated time, and V_o is that at time zero. (B) Pre-exponential factors (A_f and A_s) and rate constants (k_f and k_s) obtained from inactivation kinetics of α-chymotrypsin in AOT reversed micelles at W_o = 10 as a function of pH. The vertical bars are an estimate of the experimental error. Note that as k_f increases and A_f decreases, the error in k_f increases substantially. All the kinetics were done using Tris · HCl buffer, with the exception of the pH 8.75 experiment with the larger k_f value that was carried out using ethanolamine buffer. The deactivation kinetics at pH 9.02 could be fitted as a monoexponential decay (A_f tends toward zero).

inactivation by the micellar system. Also, as this component of inactivation depends on pH, it involves a protonable group of the protein, with pK_a between 8.6 and 9. The other mechanism (slower) seems to be the same as the one present in bulk water. Inactivation mechanisms involving protein–protein interactions, like autolysis, are ruled out because of the independence of the inactivation kinetic constants on enzyme concentration (data not shown).

Table I. Pre-exponential factors (A_f and A_s) and rate constants (k_f and k_s) obtained from inactivation kinetics of α-chymotrypsin in AOT reversed micelles at pH 8.0 in several W_o's.

W_o	A_f	k_f (min^{-1})	A_s	k_s (min^{-1})
10	0.86	0.012	0.14	1.1×10^{-3}
20	0.61	0.025	0.39	2.5×10^{-3}
30	0.78	0.037	0.22	2.1×10^{-3}
40	0.76	0.056	0.24	1.7×10^{-3}

The kinetics of inactivation of δ-chymotrypsin was studied as a function of pH at W_o 10. At pHs 7.50, 8.00, 8.46, and 8.75 the enzyme does not deactivate for at least 300 min (Fig. 2A shows the kinetics of inactivation at pH 8.00 and 8.75). Different from α-chymotrypsin the kinetic of inactivation of δ-chymotrypsin in reversed micelles of AOT is independent on pH.

The only difference between δ-chymotrypsin and α-chymotrypsin is the Ala^{149} that is not present as a N-terminal in the δ intermediate. The changes in k_f and A_f for α-chymotrypsin inactivation (Fig. 3) is in the pH range of a typical pK_a of an N-terminal residue (Stryer, 1988). It suggests that the positive charge of the N-terminal is related to the fast inactivation in AOT reversed micelles and can be related to interaction with the negative interface.

4. DISCUSSION

In this communication we have shown that the inactivation of α-chymotrypsin in AOT reversed micelles is related to the protonation state of the N-terminal Ala^{149}. α-Chymotrypsin is rapidly inactivated when its N-terminal is positive. The deprotonation of Ala^{149} has a cooperative effect on the deprotonation of Ile^{16} and ultimately on the breakage of the salt bridge Ile^{16}–Asp^{194} (Sharma and Hopkins, 1982). The interaction of protonated Ala^{149} with the negative charge of the AOT head group could produce similar effect on the protein: a cooperative effect that leads to the breakage of the salt bridge Ile^{16}–Asp^{194}.

This is an evidence of a specific interaction of a protein group with the detergent that controls the stability and the enzymatic activity, as shown by the activity profile of δ-chymotrypsin as a function of W_o. The decrease in activity observed with the increase in W_o can be due to protein–interface interaction. The thermal stability of alkaline phosphatase of two sources (chicken intestine and *Escherichia coli*) in AOT micelles is also controlled by the contact with the AOT head group. Charged glycosilation residues (sialic acid) prevent the contact with the AOT head group (Valente, 1994).

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