Conformational change in elastase following complexation with α_1 -proteinase inhibitor: a CD investigation

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The CD spectrum of porcine pancreatic elastase in complex with α_1 -proteinase inhibitor (α_1 -PI) was calculated by subtracting the CD spectrum of the proteolytically cleaved inhibitor from that of the elastase- α_1 -PI complex. Elastase undergoes a moderate secondary structure change: its β -structure is partially disordered while its α -helix content is poorly affected. In contrast, its tertiary structure undergoes a significant structural loosening upon complexation. These alterations have been compared with those following chemical and thermal unfolding of free elastase. Inhibitor-bound elastase and the denaturation intermediate of

free elastase share secondary but not tertiary structural features. On the other hand, both free and complexed elastases undergo a single-step transition in tertiary structure upon thermal unfolding. These data are discussed in terms of the inhibition and structural modification of elastase induced by α_1 -PI observed by previous investigators.

Key words: α_1 -antitrypsin, porcine pancreatic elastase, proteinase unfolding intermediate.

INTRODUCTION

Proteolysis, a biologically important event, is regulated by protein proteinase inhibitors that belong to two major classes: the so-called canonical inhibitors and the serine proteinase inhibitors, called 'serpins'. The former belong to numerous structural families and their reactive-site loop forms a reversible 'lock-and-key' complex with the substrate-binding crevice of the proteinase. In contrast, all serpins belong to a single superfamily with highly conserved secondary structural elements (nine α -helices and three β -sheets) and inhibit their target proteinase using a multi-step process that leads to an irreversible complex [1]. The first step is a reversible Michaelis-like complex [2] that resembles the lock-and-key complex formed with canonical inhibitors [3]. The proteinase then undergoes a translocation [4], cleaves the $P_1-P'_1$ bond of the inhibitor and forms an ester bond between its active-centre serine residue and P_1 [5]. This ester bond resists hydrolysis for several days, which accounts for the irreversible character of the inhibition. The crystal structure of the complex formed by trypsin and α_1 -proteinase inhibitor (α_1 -PI; or α_1 -antitrypsin) [6] shows that the C-terminal part of serpin's proteolytically cleaved reactive-site loop has deeply inserted into its A β -sheet and dragged the proteinase to the opposite pole of the serpin. The tertiary structure of the complex also shows that the catalytic triad of the proteinase is distorted, explaining why the ester-bond-linking enzyme and inhibitor is so resistant to hydrolysis.

Evidence has been provided that not only the serpin but also the proteinase undergoes a conformational change during complex formation. As early as 1980 we have shown that the maximum luminescence intensity and the circularly polarized luminescence spectrum of the porcine pancreatic elastase (PPE)-terbium complex significantly changes following reaction with α_1 -PI, indicating a serpin-induced conformational change in the enzyme [7]. More recently, Plotnick et al. [8] provided NMR

evidence that the active site of chymotrypsin is distorted within the serpin-proteinase complex. Other workers found that the serpin-bound proteinase is more susceptible to proteolysis [9,10], that some of its disulphide bonds are more sensitive to reduction [11] and that its thermal stability increases [11] while it undergoes a partial unfolding [12].

The crystallographic data on the trypsin- α_1 -PI complex elegantly confirmed the conformational disorder induced in the proteinase [6]. However, about 40 % of trypsin's structure was crystallographically disordered so that the changes in secondary and tertiary structure of the enzyme could not be detailed. In the present paper we attempt to solve this question by using CD spectroscopy of PPE in complex with α_1 -PI.

MATERIALS AND METHODS

Materials

The source and active-site titration of α_1 -PI and PPE were the same as described previously [2]. *Pseudomonas aeruginosa* elastase was used to inactivate α_1 -PI by proteolytic cleavage of its P_2-P_1 peptide bond [13].

CD

CD spectra were recorded using a Jobin-Yvon CD VI nitrogen-flushed dichrograph fitted with a thermostat-controlled cell holder interfaced with a temperature-controlled water bath. The protein concentration of the samples, in 10 mM sodium phosphate buffer/100 mM NaCl, pH 7.0, ranged between 0.7 and 5 μ M for measurements in the far-UV domain and was 20 ± 5 μ M for measurements in the near-UV domain. Cell pathlengths of

Abbreviations used: GdnHCl, guanidine hydrochloride; α_1 -PI, α_1 -proteinase inhibitor; PPE, porcine pancreatic elastase.

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0.02, 0.1 or 0.2 cm were used in the far-UV domain and a 1 cm cell pathlength was used in the near-UV domain. Calibration was done with (+)-camphorsulphonic acid ($\Delta\epsilon_{290.5}$, $2.37 \text{ M}^{-1} \cdot \text{cm}^{-1}$; $\Delta\epsilon_{192.5}$, $-4.95 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [14]. Temperature was controlled at $25 \pm 0.2^\circ \text{C}$ for all experiments, unless otherwise mentioned. Spectra were acquired by averaging 4, 9 or 16 scans and corrected for buffer signal. The secondary structure content was estimated using the program CD PROT [15] as well as the Johnson variable-selection method [16], with a wavelength interval of 0.5 nm. Units of molar mean residue ellipticity ($\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) are per backbone amide. Throughout denaturation measurements, samples were allowed to equilibrate for 30 min with the denaturant before recording spectra. As the mean residue ellipticity of the native and unfolded states of PPE did not significantly depend on denaturant concentration in the pre- and post-transition regions, no correction for curve sloping was applied.

To evaluate denaturation parameters, the CD data were interpreted using a three-state reactional scheme, as in [17]:

where N is the native conformation, and I and U are the intermediate and the completely unfolded forms, respectively. K_1 and K_2 are the equilibrium constants for the transitions. The CD signal at a given wavelength, Y , can be expressed as:

$$Y = \frac{Y_N + K_1 Y_I + K_1 K_2 Y_U}{1 + K_1 + K_1 K_2} \quad (2)$$

where Y_N , Y_I and Y_U are the CD signals assigned to N, I and U, respectively. Assuming the linear extrapolation model, the constants K_1 and K_2 can be written as follows:

$$K_1 = \exp\{(m_1[D] - \Delta G_{0,1}^{\text{H}_2\text{O}})/RT\} \quad (3)$$

$$K_2 = \exp\{(m_2[D] - \Delta G_{0,2}^{\text{H}_2\text{O}})/RT\} \quad (4)$$

where $[D]$ is the guanidine hydrochloride (GdnHCl) concentration, $\Delta G_{0,1}^{\text{H}_2\text{O}}$ and $\Delta G_{0,2}^{\text{H}_2\text{O}}$ are the free energy changes associated with the transitions, in the absence of GdnHCl, and m_1 and m_2 are constants, also called m -factors in denaturation studies. Substitution of eqns (3) and (4) in eqn (2) provides the equation with which the experimental data were fitted.

RESULTS

CD data and spectral resolution of the α_1 -PI-PPE complex

As indicated in Table 1 the CD structure data obtained for intact α_1 -PI and PPE compare well with those calculated from X-ray crystallographic data [18,19]. The spectra of the α_1 -PI-PPE complex in the far- and near-UV regions were deduced ex-

Table 1 CD secondary structure content of intact α_1 -PI and PPE

For comparison, X-ray data are given in parentheses. CD data related to the virtual intact α_1 -PI-intact PPE complex are compared with the experimental complex. All experiments were performed in 10 mM phosphate buffer/100 mM NaCl, pH 7, at 25°C .

	CD secondary structure content (%)			
	α -Helix	β -Sheet	β -Turn	Unordered
Intact α_1 -PI	32 (30)	25 (27)	15	28
Intact PPE	5 (5)	37 (35)	22	36
Virtual complex	22	30	17	31
Experimental complex	21	25	22	32

Figure 1 Far-UV CD spectra of PPE

Intact PPE (curve a), PPE complexed with α_1 -PI (curve b; calculated as reported in the Results section) and the intermediate in the chemical unfolding pathway (curve c; dashed line). All spectra were recorded in 10 mM phosphate buffer, pH 7, in the presence of 100 mM NaCl at 25°C .

Table 2 CD secondary structure content of α_1 -PI and PPE in free and complexed states

All experiments were performed in 10 mM phosphate buffer/100 mM NaCl, pH 7, at 25°C .

	CD secondary structure content (%)			
	α -Helix	β -Sheet	β -Turn	Unordered
Intact PPE	5	37	22	36
Complexed PPE	4	20	12	64

perimentally by titrating the inhibitor with the proteinase. The proteinase concentration required to saturate the inhibitor was determined by following the changes in the near-UV spectra at 275 and 280 nm. The intensity change in the near-UV region is larger than that in the far-UV region, allowing greater precision. It is worth noting that the spectrum of the experimental complex differs from that of the virtual intact α_1 -PI-intact PPE (1:1) complex, obtained by adding the spectra of intact inhibitor and proteinase (results not shown). That is reflected by the related CD secondary structure contents (Table 1). It appears distinctly that the discrepancy mainly concerns the global β -structure. Furthermore, as the structure of α_1 -PI in the complex with a serine proteinase [6] was shown to be identical to that of the proteolytically cleaved free inhibitor [20], the far- and near-UV CD profiles of PPE complexed with α_1 -PI were calculated by subtracting the experimental CD spectra of the cleaved serpin from that of the α_1 -PI-PPE complex. To perform the subtraction and resolve the complex spectrum, CD signals of both proteinase and cleaved inhibitor were weighted by their fractional content in amino acids.

The calculated spectrum of the complexed PPE in the far-UV domain (Figure 1, curve b) was found to differ significantly from that of the intact enzyme (Figure 1, curve a). It can be seen that the intensity of the negative band at 195 nm is largely increased while that of the shoulder at 205 nm is reduced. Estimation of the

Figure 2 Near-UV spectra of PPE

Intact PPE (curve a), PPE complexed with α_1 -PI (curve b; calculated as reported in the Results section), completely unfolded PPE (curve c; 6 M GdnHCl), the intermediate in the chemical unfolding pathway (curve d), and the molten globule obtained by heating at 65 °C (curve e). All spectra were recorded in 10 mM phosphate buffer, pH 7, in the presence of 100 mM NaCl at 25 °C.

secondary structure of PPE (Table 2) shows that the complexation with the serpin induces a partial conversion of the β -structure of PPE into a disordered structure, while the α -helix content is poorly affected.

In the near-UV domain, the intensity of the broad negative band centred at 275 nm is drastically reduced in the calculated spectrum of complexed PPE (Figure 2, curve b). Thus both secondary and tertiary structures of the enzyme appear to be affected upon complexation with the inhibitor. In contrast, only a slight alteration was observed in the near-UV CD profiles of complexed PPE upon salt addition up to 1 M NaCl (results not shown).

Chemical denaturation

To determine whether the structure of complexed PPE has common features with a possible protein intermediate, the chemical unfolding of PPE was studied using GdnHCl, as a typical chaotropic agent. In the far-UV domain, where the CD signal at 210 nm has been monitored (Figure 3, far-UV curve), two transitions can be distinguished unambiguously. The first, at low denaturant concentrations, has a midpoint around 0.12 M. It is related to a low Gibbs free energy and a large m value that is correlated with the change in hydrophobic surface exposed to solvent [21] (Table 3). The second, at higher denaturant concentrations, has a midpoint around 4.2 M. It is associated with a greater Gibbs energy and a markedly smaller m value. Interestingly, the transition domains are well separated by a broad flat plateau. This suggests a sequential unfolding process, involving a stable protein intermediate as the only populated state in the range of denaturant concentration defining the plateau. Similar denaturation curves and thermodynamic parameters were obtained in the near-UV domain by monitoring the CD signal at 275 nm (Figure 3, near-UV curve, and Table 3). Nevertheless, when compared with the far-UV domain, transition midpoints are shifted towards lower values, suggesting that the

Figure 3 GdnHCl-induced chemical unfolding of PPE

Plot of the denaturation fractional extent F versus denaturant concentration [$F = (\Theta - \Theta_N) / (\Theta_U - \Theta_N)$ where Θ_N and Θ_U are the mean residue ellipticities of the native and completely unfolded states, respectively]. CD signals were monitored at 210 nm in the far-UV domain (curve marked far-UV) and at 275 nm in the near-UV domain (curve marked near-UV). Inset: details at low denaturant concentrations, in the first transition. Midpoints are labelled for all curves (ball and stick structures) in accordance with column headings in Table 3. All spectra were recorded in 10 mM phosphate buffer, pH 7, in the presence of 100 mM NaCl at 25 °C. C_{MX} are the concentration midpoints, the subscript X (1 or 2) denoting the involved transition.

tertiary structure of free PPE is more sensitive to chemical denaturation than its secondary structure.

In the far-UV domain, the CD profile of the complexed PPE (Figure 1, curve b) resembles that of the chemically unfolded intermediate (Figure 1, curve c). The spectral similarity can only be observed above 204 nm because of absorption of the denaturant. However the similarity is significant since this wavelengths domain includes the region where CD bands due to α -helix or β -structure are observable. Therefore, it can be inferred that the α_1 -PI-bound PPE and the denaturation intermediate of PPE share secondary structure features. In contrast, such a spectral similarity is not observed in the near-UV domain, where the CD spectra indicate that the tertiary structure change is more substantial in the complexed elastase (Figure 2, curve b) than in the denaturation intermediate (Figure 2, curve d).

Thermal unfolding

The secondary structure of intact PPE at 25 °C (Figure 4, inset) is not significantly different from that obtained at 65 °C (Figure 4, inset), while a distinct single-step transition is observed ($T_m \approx 57.5$ °C) for the dramatic change undergone by the tertiary structure (Figure 4, curve marked intact PPE). This change was not reversed by cooling, suggesting the formation of a molten globule-like state, as frequently observed in globular proteins [22]. The CD spectra of complexed PPE in the near-UV domain were also calculated at increasing temperatures. A single-step transition was also observed, with a midpoint temperature of 63 °C (Figure 4, curve marked complexed PPE), revealing a

Table 3 Thermodynamic parameters describing the unfolding of PPE in the far- and near-UV domains

GdnHCl was used as a denaturing agent. All experiments were performed in 10 mM phosphate buffer/100 mM NaCl, pH 7, at 25 °C. Data were fitted to a three-state model. Subscripts 1 and 2 refer to the first and second transitions, respectively. $C_{M,X}$ are the concentration midpoints, the subscript X (1 or 2) denoting the involved transition.

	$\Delta G_{0,1}^{H_2O}$ (kcal · mol ⁻¹)	$\Delta G_{0,2}^{H_2O}$ (kcal · mol ⁻¹)	m_1 (kcal · mol ⁻¹ · M ⁻¹)	m_2 (kcal · mol ⁻¹ · M ⁻¹)	$C_{M,1}$ (M)	$C_{M,2}$ (M)
Far-UV	1.5 ± 0.05	2.5 ± 0.1	12.4 ± 0.1	0.6 ± 0.04	0.12 ± 0.01	4.2 ± 0.15
Near-UV	1.2 ± 0.05	2.3 ± 0.1	20.1 ± 0.1	0.8 ± 0.04	0.06 ± 0.01	2.9 ± 0.15

Figure 4 Thermal unfolding of PPE studied in the near-UV domain

Experimental CD signal of intact PPE and calculated CD signal of α_1 -PI-bound PPE (complexed PPE), as a function of temperature ($\lambda = 275$ nm). Midpoints are labelled (with ball and stick structures) T_{MI} for intact PPE and T_{MC} for complexed PPE. Spectra were recorded in 10 mM phosphate buffer, pH 7, in the presence of 100 mM NaCl. Inset: far-UV CD spectra of intact PPE at 25 and 65 °C.

slightly increased thermal stability of PPE after complexation with α_1 -PI.

DISCUSSION

Our CD data show that complexation with α_1 -PI changes both the secondary and tertiary structures of PPE. The secondary structure is moderately altered, with only part of the β -structure being disordered. This could however be sufficient to modify the β -barrel motif in which the catalytic triad is embedded, thus preventing deacylation of the acylenzyme. The tertiary structure of PPE is more substantially modified upon complexation, as revealed by changes in the environment and interaction of aromatic residues, particularly the two tryptophans located near the catalytic site. This change also indicates a loosening of the tertiary structure of PPE upon complexation.

To get further information on the conformation of the complexed enzyme, a comparison was drawn with that adopted by the free enzyme, upon both chemical and thermal denaturation. Thus, common secondary structure properties

were found with a chemical unfolding intermediate. The chemical unfolding is sequential, which may indicate the existence of two independent domains [23]. In relation to the low value of the associated Gibbs free energy, the observed intermediate probably results from the reversible unfolding of one of these domains.

Similarly, the secondary structure change of PPE in the complex might concern only a part of the proteinase and might be reversible. Moreover, as also observed in the unfolding intermediate, an increase in the solvent exposure of some residues might be suggested. This might account for the enhanced susceptibility to limited proteolysis displayed by some loops in the complexed proteinase [10,11]. In contrast, the tertiary structure of the PPE markedly differs from that of the compact chemical unfolding intermediate, suggesting specific protein–protein contacts. This is consistent with the observed increase in thermal stability of the complexed enzyme and supports the stabilizing effect of this kind of interaction in the inhibitory complex [11,24]. Taking into account the observed low structural sensitivity of the inhibitory complex to increasing ionic strength, electrostatic interactions probably play a minor role.

Furthermore, denaturation studies indicate that the secondary and the tertiary structure of PPE behave independently. This property might argue for a sequential structure change in the proteinase, during the steps of complexation. Given that no significant conformational change was observed in the Michaelis-type non-covalent complex [3], it might be suggested that the secondary structure change of the proteinase occurs during its translocation to the opposite pole of the serpin. This view agrees with that suggested in recent work focused on the fluorescence study of kinetics of proteinase deformation [12]. The tertiary structure change in PPE might be due to strong protein–protein interactions when the proteinase bypasses the F-helix which lies across the β -sheet A [6]. The tertiary structure change of PPE might thus accompany the irreversible reactive loop conformational change that traps the proteinase in the covalent complex.

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