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COMPARISON OF THE INTERACTIONS OF HUMAN α_1-ANTICHYMOTRYPSIN WITH HUMAN LEUKOCYTE CATHEPSIN G AND BOVINE CHYMOTRYPSIN

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SUMMARY - The interactions of human α_1-antichymotrypsin with human leukocyte cathepsin G and bovine chymotrypsin were investigated by means of circular dichroism spectroscopy and concurrent polyacrylamide gel electrophoresis. The mixtures were made in inhibitor excess at 0°C and studied at different times. Circular dichroism analyses indicated that within 10 min significant modifications had occurred in the aromatic environment of the components upon interaction. By SDS-polyacrylamide gel electrophoresis a complex having a M_r near 80,000 was observed in both mixtures. By both methods it was demonstrated that these complexes were not stable.

INTRODUCTION

Human α_1Achy is a serum glycoprotein which is known to inhibit chymotrypsin and chymotrypsin-like enzymes such as leukocyte cathepsin G (1,2). Limited data were available concerning the formation of complexes between this inhibitor and the enzymes. Our previous work (3) dealt with the formation of complexes between human leukocyte cathepsin G and α_1Achy and showed that production of a modified inhibitor occurred concurrently with the formation of a 1:1 complex of M_r equal to about 80,000. When α_1Achy was allowed to react with bovine chymotrypsin in different molar ratios (4), a 1:1 complex and a modified inhibitor were also obtained.

CD spectroscopy is a direct method of observing the interaction between the components of an equilibrium mixture, without physical separation of the components such as electrophoresis which may perturb the equilibrium. CD spectra modifications have already been used to follow the minor conformational changes induced by the interaction of α_1-protease inhibitor with bovine trypsin and chymotrypsin (5-7). Recently complex formation between chymotrypsin and ratα_1 inhibitor$_3$ (8) was shown to lead to a significant modification of the CD spectra in the near UV.

Abbreviations. α_1Achy, $\alpha_1$antichymotrypsin ; M_r, relative molecular mass ; TLCK, N α-p-Tosyl-L-lysine chloromethyl ketone ; PAGE, polyacrylamide gel electrophoresis ; CD, circular dichroism ; PMSF, phenylmethylsulfonyl fluoride ; SDS, sodium dodecyl sulfate ; UV, ultraviolet.

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Such a study was thought to be helpful in following the α_1Achy interaction with human leukocyte cathepsin G or bovine α-chymotrypsin, since only protease inhibition measurement using a chromogenic substrate and analytical electrophoretic methods have been carried out in our other studies (3,4). In the present work the same enzyme-inhibitor mixtures (in inhibitor excess) were concurrently analyzed at different times by CD spectroscopy and electrophoretic methods.

MATERIALS AND METHODS

Human α_1Achy was isolated and kept according to a procedure previously described (2). Its concentration (1.35 mg/ml i.e. 2.33×10^{-5}M) was measured by electroimmunodiffusion and with the method of Lowry et al. (9)as described in (3). Leukocyte cathepsin G (EC 3-4.21.20) was obtained in our laboratory from purulent sputum using the procedure described by Martodam et al. (10). Bovine α-chymotrypsin (TLCK-treated, type VII) was from Sigma. Both enzymes were dissolved in 0.01 M sodium phosphate buffer, 0.3 M NaCl, 0.2% NaN_3 (pH 7.5). The solutions were 2.18×10^{-5}M for cathepsin G and 2.9×10^{-5}M for bovine chymotrypsin.

Analytical electrophoreses were carried out on polyacrylamide slab gels using the technique described by Kerckaert (11). Alkaline-PAGE was performed on 10% polyacrylamide gels at pH 8.3 using the gel buffer system of Davis (12). SDS-PAGE was performed on a 5-30% gel gradient using the buffer system of Laemmli (13). Samples treatment before electrophoresis, protein precipitation and staining, M_r determination were carried out as previously described (3). M_r were estimated to be 58,000, 27,500 and 26,500 for α_1Achy, leukocyte cathepsin G and bovine α-chymotrypsin respectively.

Chymotrypsin inhibitors were made visible in polyacrylamide gels using the slightly modified (2) method of Uriel and Berges (14).

The CD spectra were recorded using a Jobin-Yvon Mark III dichrograph in cells of 1 cm pathlength in the near UV (350 to 255 nm) and 0.01 cm in the far UV (260 to 200 nm) which were thermostatized at 4°C.

The buffer baseline was recorded before and after the measurement of the corresponding solution and automatically subtracted. The ellipticity was expressed as mean residual molar ellipticity **[θ]** in degree. $cm^2.dmol^{-1}$ taking 111, 112, 113 as mean residue weight for chymotrypsin, α_1Achy and cathepsin G respectively. The so-called theoretical spectrum of the mixture corresponds to the sum of individual spectra of its components at the concentrations present in each reaction mixture.

Enzyme-inhibitor incubation mixtures were made at 0°C. The inhibitor-enzyme molar ratio was equal to 1.6 for the mixture of α_1Achy and cathepsin G and to 1.36 for the mixture of α_1Achy and α-chymotrypsin, but due to more inactive material in commercial chymotrypsin (20%) than in cathepsin G (less than 10%) we considered that the active components molar ratio was about the same in both cases.

CD spectra were recorded after 10 min, 19 h, 48 h and 6 days for both incubation mixtures. At every time an aliquot of these mixtures was immediately added with a hundred-fold molar excess of PMSF over enzyme initially present for electrophoretic studies to be carried out a few hours later.

RESULTS AND DISCUSSION

The near UV CD spectra of cathepsin G, chymotrypsin and α_1Achy are presented in Fig. 1A and 1B. The spectrum of chymotrypsin is very similar to published spectra (5-7). Concerning the spectrum of cathepsin G, in accordance with Strickland (15) the positive band at 262 nm and the shoulder at 275 nm are assigned to Phe residues. The large negative band with its maximum at

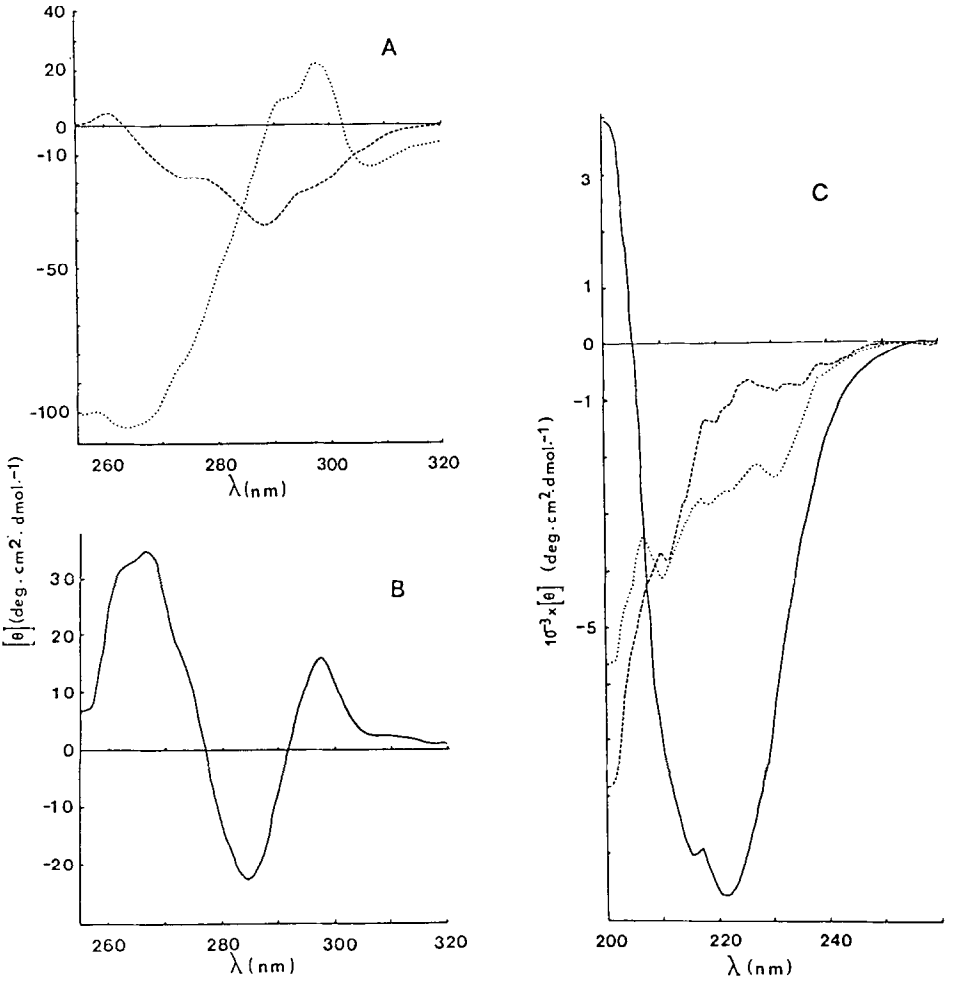

Figure 1 - CD spectra in 0.01 M sodium phosphate buffer, 0.3 M NaCl, 0.2 °/₀₀ NaN_3 (pH 7.5) of α_1Achy (———), human cathepsin G (- - - - -) and bovine chymotrypsin (·····) in the near UV (A and B) and in the far UV (C).

290 nm contains unresolved contributions from Trp and Tyr and the shoulder at 297 nm is due to Trp. The spectrum of α_1Achy exhibits three bands. The positive band at 298 nm is possibly due to the Trp residues, the negative one at 284 nm contains the contributions of Trp and Tyr. The positive band at 267 nm in addition to the shoulders at 262 and 272 nm correspond to the fine structure of Phe residues. The nullpoints are observed at 277 and 292 nm.

The far UV CD spectra of cathepsin G, chymotrypsin and α_1Achy are shown in Fig. 1C. Cathepsin G and chymotrypsin secondary structures consist of β sheet, unordered structure and only a weak percentage of α helical form. On the contrary α_1Achy has an appreciable α helical content in addition to unordered structure. The α_1Achy spectrum shows a minimum at 220 nm [θ] = 9,600 deg.cm^2.dmol^{-1}) and a shoulder at 214 nm which might be due to the

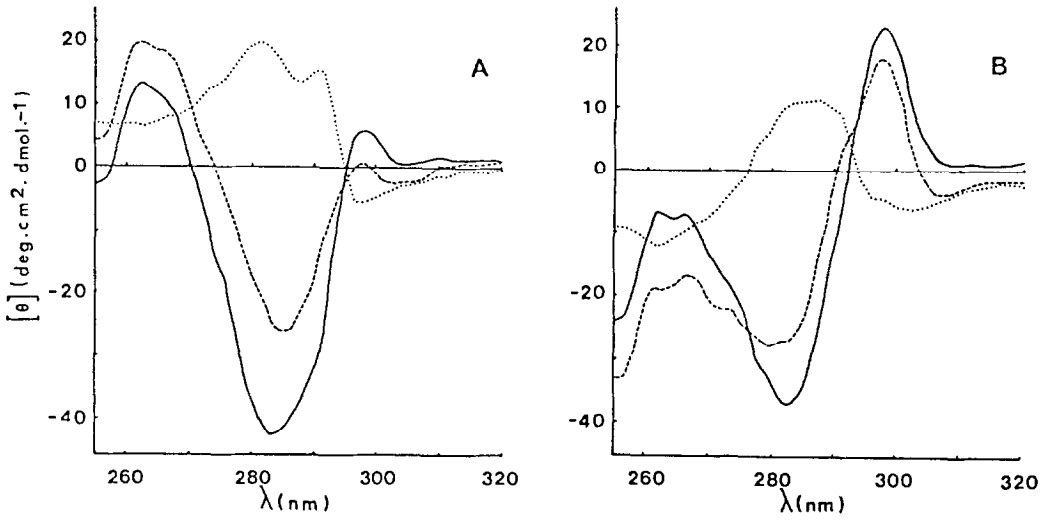

Figure 2 - Near UV spectra of the mixtures after 10 min incubation : (——) experimental spectrum ; (------) theoretical spectrum ; (·····) difference spectrum. A : α_1Achy-cathepsin G mixture ; B : α_1Achy-chymotrypsin mixture.

presence of slightly deformed α helix. The contribution to CD from acetamido groups in the carbohydrate part can be neglected as previously shown (16).

Studies of the mixtures after 10 minutes incubation

CD spectra of the 10 min-mixtures, prepared as described under "Materials and Methods", are shown in Fig. 2. The experimental CD spectra differ significantly from the theoretical spectra in the near UV region and the difference spectrum between the calculated and the observed spectra has been drawn for each mixture. The differences are thus reflections of perturbations that have occurred upon interaction. Both difference spectra show one extremum at about 295 nm which is due to the disturbance of at least one Trp residue. Other extrema are observed around 285 nm and at 280 and 290 nm for the α_1Achy-chymotrypsin and α_1Achy-cathepsin G mixtures respectively. In both cases these bands are reflecting changes in the Trp and Tyr residues. The changes in the 270-255 nm region are more difficult to interpret ; indeed a perturbation arising from S-S bond can also be involved in this domain (14).

No marked change was observed in the far UV spectra of both mixtures, indicating the lack of modification in the secondary structure consecutive to the interaction.

In alkaline-PAGE, excess of active inhibitor is seen in the 10 min mixtures (Fig. 3A, slots 4 and 7). A new component having a α-mobility is shown in the α_1Achy-chymotrypsin mixture (Fig. 3B, slot 7) : it is a complex since coexistence of α_1Achy and chymotrypsin has been demonstrated in this component (more details will be given elsewhere (4)). No visible complex can be seen in these conditions in the α_1Achy-cathepsin G mixture (3) (Fig. 3B,

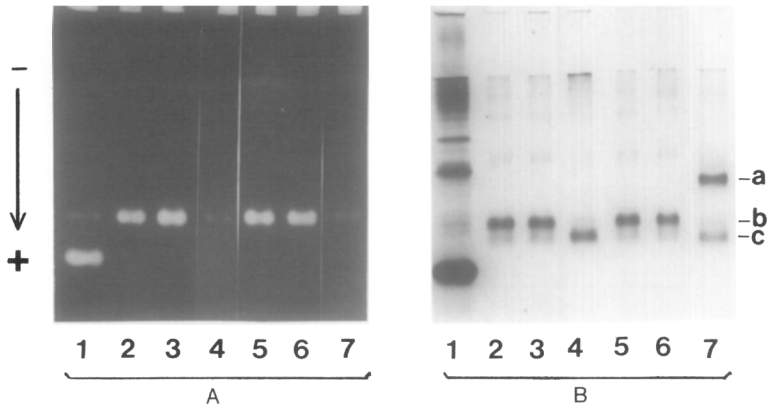

Figure 3 - Alkaline-PAGE. 1 : normal serum ; 2 and 5 : α_1Achy ; 3 : α_1Achy + inactivated cathepsin G ; 4: 10 min mixture of α_1Achy and cathepsin G ; 6 : α_1Achy + inactivated chymotrypsin ; 7 : 10 min mixture of α_1Achy and chymotrypsin. A ; visualization of chymotrypsin inhibitors. B : protein staining. a : α_1Achy-chymotrypsin complex ; b : active α_1Achy ; c : inactive α_1Achy. Some α_1Achy polymers are visible in slots 2, 3, 5 and 6 between the top of the gel and band b.

slot 4). Formation of a modified α_1Achy (band c more acid than active α_1Achy) is visualized in both cases and this modified form has no inhibitory activity (Fig. 3A and 3B, slots 4 and 7).

Free or complexed chymotrypsin is split when reduced, unlike cathepsin G (3), thus reduction before SDS-PAGE is avoided for chymotrypsin containing samples ; for valid comparison we do not reduce the α_1Achy-cathepsin G

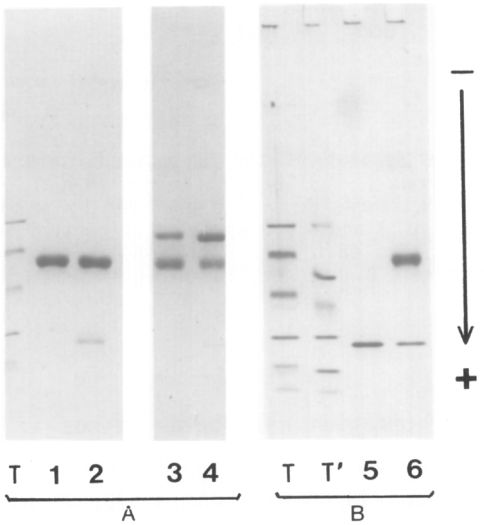

Figure 4 - SDS-PAGE. Only T, 1 and 2 were reduced before electrophoresis. T and T' : M_r markers : phosphorylase b (94,000), bovine albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), α-lactalbumin (14,400). Slab A : 1 : α_1Achy ; 2 : α_1Achy + inactivated cathepsin G ; 3 : 10 min mixture of α_1Achy and cathepsin G ; 4 : 10 min mixture of α_1Achy and chymotrypsin. Slab B : 5 : chymotrypsin ; 6 : α_1Achy + inactivated chymotrypsin.

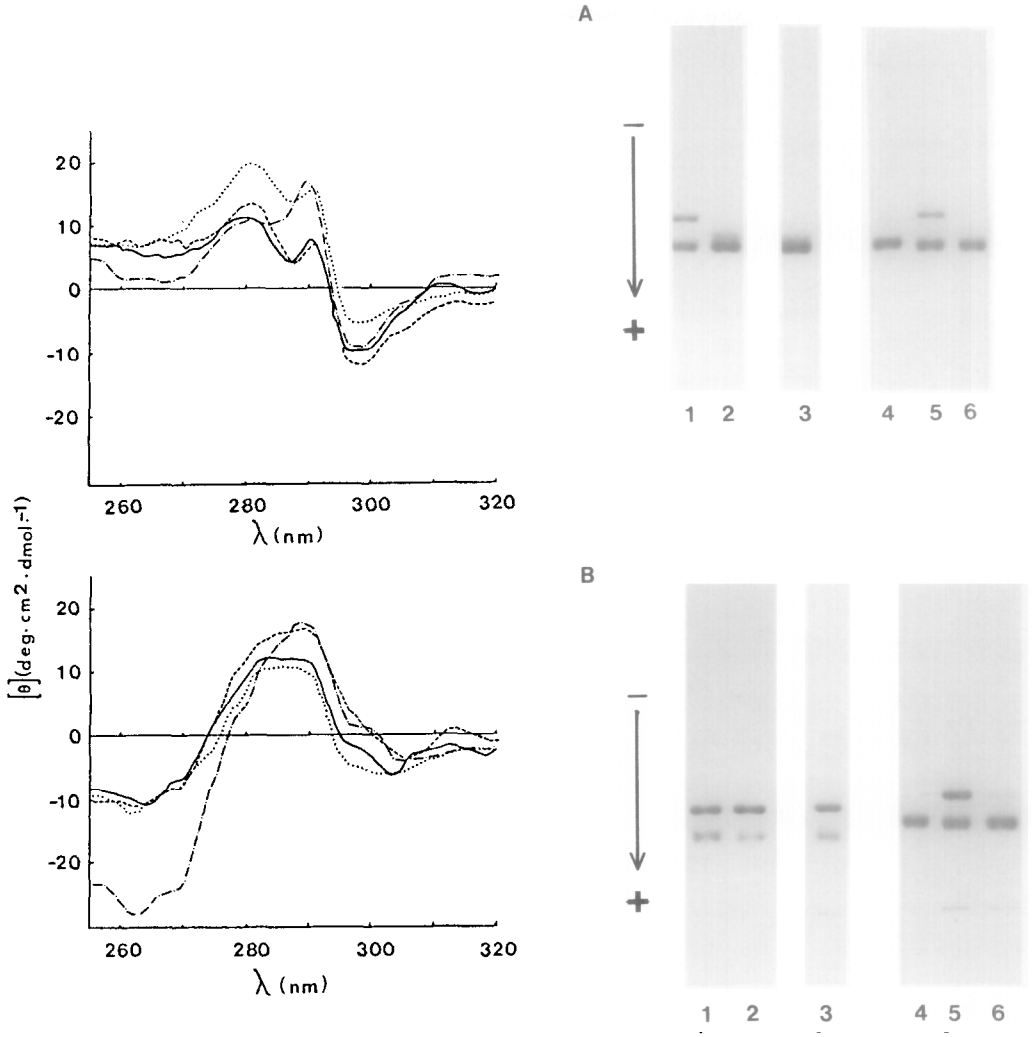

Figure 5 - Time-course studies of the incubation mixtures. The difference spectra were drawn after : (·····) 10 min ; (——) 19 h ; (-----) 48 h ; (—·—·—) 6 days incubation. The mixtures were also analyzed by SDS-PAGE without prior reduction after : 10 min (1 and 5 ; 5 was used as control in the gel made for the time 6 days), 19 h (2), 48 h (3), 6 days (6). 4 : native α_1Achy. A : α_1Achy-cathepsin G mixture ; B : α_1Achy-chymotrypsin mixture.

mixture either. Complex formation with both enzymes is seen in Fig. 4. The estimated M_r of α_1Achy-cathepsin G complex and of α_1Achy-chymotrypsin complex are respectively 80,000 and 79,000 instead of 85,500 and 84,500 as expected for 1:1 complexes. A diffuse band migrating as a component of M_r near 6,000 (not easily visible on the photograph) probably corresponds to the missing peptide (3,4). No free cathepsin G subsists (slot 3) and just a weak chymotrypsin band is seen (slot 4), it is probably due to inactive enzyme which is always significantly present in commercial preparations.

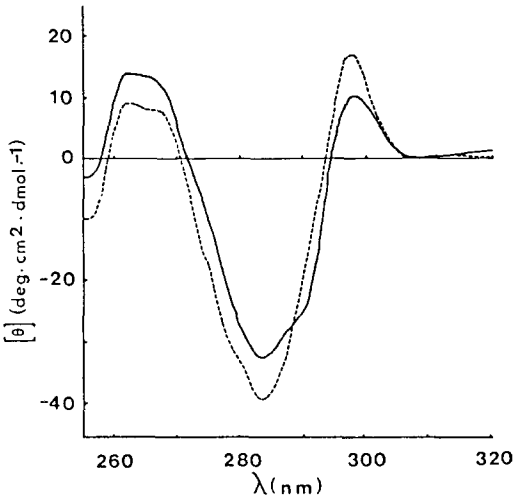

Figure 6 - Comparison of the experimental near UV spectra recorded after 19 h for the α_1Achy-cathepsin G mixture (———) and after 6 days for the α_1Achy-chymotrypsin mixture (-----).

Time-course studies of the mixtures

The mixtures kept at 0°C, were analyzed by CD spectroscopy and SDS-PAGE after 19 h, 48 h and 6 days. Only difference spectra, in the near UV, are presented in Fig. 5 for each time, with SDS-PAGE patterns. No evolving was observed in the far UV spectra of both mixtures.

Mixture with cathepsin G (Fig. 5A) - Difference CD spectra have slightly evolved between 10 min and 19 h but significant differences between theoretical and experimental spectra persist. Except in the range of 290 nm, a stationary state is observed as well after 19 h as 6 days indicating that no important modification has occurred in the aromatic environment of the components. However after 6 days a significant variation is observed at 290 nm in accordance with the modification of the environment of at least one Trp residue. In SDS-PAGE patterns, disappearance of the complex of M_r = 80,000 is observed after 19 h (slot 2) and a slower migrating component (of M_r near 67,000) is seen in addition to a band migrating just a bit faster than α_1Achy. After 6 days (slot 6) we observe the same results as in our previous work (3) : modified inhibitor (M_r = 55,000) and the component of M_r near 6,000 (not easily visible) are only present. No free enzyme is detected either by SDS-PAGE or by activity measurement.

Mixture with chymotrypsin (Fig. 5B) - Difference CD spectra show no important modification between 10 min and 19 h. Between 48 h and 6 days some differences appear in the range 255-275 nm and the experimental spectrum gets farther from the theoretical spectrum. As shown in SDS-PAGE a noticeable decrease in complex amount occurs only after more than 48 h. Just a few

complex remains after 6 days (slot 6). The most important component migrates just a bit faster than native inhibitor ; in alkaline-PAGE and in electrophoresis on agarose plate at pH 8.6 (data not shown) it has the same behaviour as the modified inhibitor previously described (3).

Comparison of the results obtained with the two enzymes

Using SDS-PAGE it has been possible to show complex formation between α_1Achy and both enzymes and to demonstrate that some spontaneous dissociation of these complexes occurs ; however α_1Achy-chymotrypsin complex dissociates more slowly than α_1Achy-cathepsin G complex.

Since in SDS-PAGE patterns the material observed in the 19 h α_1Achy-cathepsin G mixture (Fig. 5A, slot 2) and in the 6 days α_1Achy-chymotrypsin mixture (Fig. 5B, slot 6) consists mainly of modified inhibitor, it seems interesting to compare the experimental spectra of these mixtures. Similarities between the two spectra of Fig. 6 prove that the environments of the aromatic residues are alike in the two mixtures. It is, therefore, possible to think that the modified α_1Achy molecule would be responsible for these similar spectra since the CD spectra of the two proteases, as shown in Fig. 1A, are very different.

In conclusion, it has been pointed out that some interactions occur between α_1Achy and both enzymes leading to complexes ; furthermore these components evolve in time but do not go back to the initial state. CD spectroscopy results are in agreement with the results of electrophoretic studies. Therefore it appears that electrophoretic methods do not disturb the equilibrium and are properly usable in the study of interactions between proteases and antiproteases.

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