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Circular dichroism studies of different aspartyl proteinases and their interactions with pepstatin

TAMARA LAH[1], IGOR KREGAR[1], ANDREJ ŠALI[1], BRIGITA LENARČIĆ[1], MATJAŽ KOTNIK[1], VLADIMIR KOSTKA[2] and VITO TURK[1]

[1]*Department of Biochemistry, J. Stefan Institute, E. Kardelj University, 61000 Ljubljana, Yugoslavia*

[2]*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, ČSSR*

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SUMMARY. – Circular dichroism (CD) of proteases from the molds Asperqillus Niger and Claviceps Purpurea, chicken pepsin A, chicken cathepsin D, calf chymosin, bovine cathepsin D and porcine pepsin was measured in free and pepstatin (pentapeptide inhibitor) – bound enzymes in the near and far uv regions. Far uv spectra revealed 55–84% of beta-structure thus justifying the grouping of all the above proteases into the beta-type of proteins. Near uv CD spectra revealed a high evolutionary conservation of assymetry around Trp/Tyr and Phe, while their absorbtion peaks, although of varying intensities in different enzymes, were at nearly identical wavelengths. Differential CD spectra upon pepstatin binding, although showing the perturbations of the same aromatic absorbance peaks in all enzymes, were different for each protease, which indicates rough topological conservation in the active site cleft. An interesting observation was the intensified far uv CD absorbance in the enzyme: inhibitor complex compared to the free enzyme, reflecting changes in peptide bond absorbance and induced structural order in the enzyme and/or the ligand after binding. In conclusion, neither CD spectra nor differential CD spectra upon pepstatin binding could reveal the structural relationship among evolutionary closer proteases nor could be the basis for classification of the enzymes into different subclasses, as was suggested on the basis of their distinct specificities.

INTRODUCTION

Aspartyl proteinases are widely distributed in living organisms. They originate from viral and microbial to mammalian species and exhibit their action intra – and extracellularly *(27)*. High homologies in primary structure *(2, 22)* have been observed in all aspartyl proteinases, but only five of them have been studied by high resolution X-ray crystallographic analysis *(1, 4, 6, 10, 11, 25)*. The supersecondary structure of hog pepsin revealed symmetrical packing of two topologically equivalent structural subunits within each of the two lobes. Comparing different aspartyl proteinases by X-ray studies, the lack of high homology of the primary structure in the topologically equivalent positions in the two domains was observed, indicating that the secondary and tertiary structure was conserved during evolution of aspartyl proteinases to a greater extent that the primary structure *(19)*.

Abbreviations: CD. circular dichroism: Mes. 2–(N-morpholino) ethanesulfonic acid: uv. ultraviolet.

The active site, which is located in the deep and extended cleft between the lobes (~ 30Å), can accomodate the side chains of eight residues in a polypeptide substrate or inhibitor. In spite of the common catalytic mechanism based on the high homology next to the two catalytically important Asps *(2, 20–22, 27)*, discrete differences exist in the subsite regions in the various enzymes. This and the great flexibility in segments neighbouring the active site region *(9)*, could explain the striking differences in their substrate and inhibitor specificities *(6, 12)*. Based on these, the discrimination between pepsin-like and chymosin-like aspartyl proteinases was proposed *(18)*.

The aim of our work was to elucidate the conformations of several aspartyl proteinases from evolutionarily distant sources, namely, microbial, avian and mammalian, as well as the structural changes upon binding of the pentapeptide inhibitor pepstatin.

MATERIALS AND METHODS

Aspartyl proteinase from *Aspergillus niger* was isolated from culture broth as described previously *(14)* and *Claviceps purpurea* aspartyl proteinase was obtained according to the method of Kregar et al *(15)*. Cathepsin D (EC 3.4.23.5) from bovine spleen and chicken liver was isolated using affinity chromatography on haemoglobin Sepharose as described by Smith and Turk *(23)*. Porcine pepsin A (EC 3.4.23.11) was a generous gift from Dr. A. Ryle, UK. Chicken pepsin A (EC 3.4.23.1) was isolated as described previously *(13)*. Chymosin (EC 3.4.23.4) from calf stomach *(7)* was a gift from Dr. B. Foltmann, Denmark. Pepstatin was obtained from the Peptide Institute, Japan. All other chemicals were of analytical grade.

Circular dichroic spectra were recorded at room temperature. Pathlengths of the cells were 1 cm or 2 cm in the near uv region, and 0.5 mm or 0.1 mm in the far uv region. Results are expressed as mean residue ellipticities, [Q] mrw, (deg cm^2 $dmol^{-1}$) or molar absorption values, ε (M^{-1} cm^{-1}), when spectra of free enzymes or differential spectra between free and complexed enzymes were presented, respectively. A value of 110 for the mean residue mass (Mr) was used for all enzymes in the formula for calculation of ellipticity:

$$[Q]\ \text{mrw} = \frac{S \cdot M \cdot l \cdot d}{c \cdot 1} \times 3300,$$

where s is the sensitivity which was 1×10^{-6} △ A/cm, d is the difference in the heights of protein spectra and buffer or pepstatin/buffer base line in (mm): c is the protein concentration in (g/1) and 1 is the path length in (cm). Differential spectra in the near and far uv region were obtained by substracting spectra of proteinase-pepstatin complexes from the spectra of free enzymes at the same pH value, i.e. 3.5. Estimates of the secondary structural content were calculated by the method of Chen et al *(5)*.

Enzyme solutions were prepared by dissolving or diluting the enzyme in buffer and/or buffer containing at least equimolar amounts of pepstatin. The following buffers were used: 0.05 M acetate buffer, pH 3.5, 0.05 M piperazine buffer, pH 5.5, 0.05 M Mes buffer, pH 6.6, and 0.05 M Tris buffer, pH 8.4.

Protein concentration was determined either by the method of Lowry et al *(17)* or by measuring the absorbance, using the following values for molar absorption coefficients: 4.39×10^4 M^{-1} cm^{-1} for cathepsin D : 5.10×10^4 M^{-1} cm^{-1} for pepsin and 5.72×10^4 $M^{-1}cm^{-1}$ for chymosin at wavelength of 278 nm.

Proteolytic activity and pepstatin inhibition of aspartyl proteinases were measured on the haemoglobin substrate at pH 3.5 by a modification of Anson's method as described *(23)*.

RESULTS

Comparison of circular dichroic absorbance of aspartyl proteinases

The spectra of six aspartyl proteinases in the near uv region are shown in Figure 1 (A–G). Positive circular dichroic absorbance with peaks at characteristic wavelengths was observed with all tested enzymes with maximum ellipticity values ranging from 30–100 deg cm^2 $dmol^{-1}$ for various proteinases. The spectra of some enzymes change when the pH is distant from that of their optimum stability (16, 28). Therefore, the spectra were followed at pH values where enzymes are in their most stable, native conformation which is at neutral to slightly acid pH. The possibility of autolytic change was avoided below pH 5.0 and alkaline denaturation was prevented above pH 6.6 for all enzymes except for cathepsins, which were stable up to pH 8.0.

The electronic ^1L_b transition of Trp at wavelength 290 nm is recognized in all spectra. In *Claviceps purpurea*, chicken and bovine cathepsin D and chicken pepsin, the band has a lower intensity and is separated by a deeper trough from the rest of the spectrum. This indicates that ^1L_b characteristic vibronic transition in these proteinases is less overlapped by the Trp ^1L_a transition than in other enzymes. The

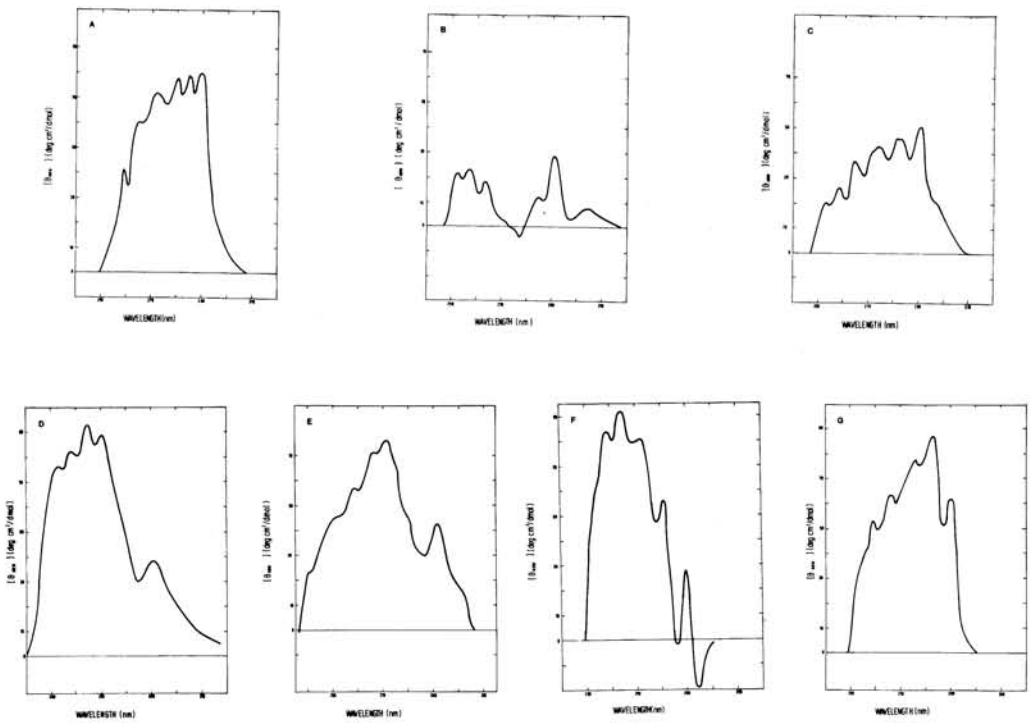

FIGURE 1. Near uv CD spectra of aspartyl proteinases of different origin. A) hog pepsin, acetate buffer, pH 5.0, c = 0.7 g/l; B) chicken pepsin, acetate buffer, pH 5.0, c = 1.59 g/l; C) calf chymosin, Mes buffer, pH 6.6, c = 1.4 g/l; D) chicken cathepsin D, Tris buffer, pH 8.0, c = 0.45 g/l; E) bovine cathepsin D, Mes Buffer, pH 6.6, c = 0.9 g/l; F) ***Aspergillus niger*** *aspartyl proteinase, acetate buffer, pH 5.0, c = 0.89 g/1; G) Claviceps purpurea aspartyl proteinase, piperazine buffer, pH 5.5, cp = 0.23 g/l. Spectra were measured at maximum sensitivity in 1 cm or 2 cm quarz cells as described in Material and Methods.*

Trp ^1L_a band probably contributes strongly to the CD absorption band at 285 nm and to the absorbance at around 270 nm, together with the tyrosyl ^1L_b transition band. In chicken pepsin exceptionally low absorbances of these bands are observed and they probably reflect the –S–S– absorbance under wavelengths of 285 nm. Slight red shifts of 290 nm and blue shifts of 285 nm band in some enzymes (Figure 1) could probably be due to electrostatic interactions with polar groups more or less distant to the indolyl rings in these enzymes.

Phe transitions exhibited well distinquished bands at wavelengths around 265 nm and 259 nm in all aspartyl proteinases. The intensity of Phe absorption is due, on one hand, to the high occurrence of Phe in aspartyl proteinases, but on the other hand, it could be due to strong dipole coupling with neighbouring groups, having appreciable electronic transitions and/ or to their hydrophobic environment *(24)*.

Far uv CD spectra are not illustrated, but their minima are presented in Table 1. They ranged from 210–218 nm. Structural analysis of spectra *(5)* revealed a very low content of helices and a high fraction of beta structure which is in accordance with the few available crystallographic data *(1, 4, 8, 10, 11, 22)* and is in agreement with the classification of aspartyl proteinases to the class of beta proteins *(27)*.

Conformational changes on pepstatin binding

Differential spectra in the near and far uv region were measured at pH 3.5. The spectra of free enzymes at this pH (not shown) were the same as at neutral pH for pepsins, chicken cathepsin D and *Asperqillus niger* proteinase. However, in the case of chymosin, bovine cathepsin D and *Claviceps purpurea* proteinase the intensities of some absorption bands changed immediately after acidification. These spectral changes could probably be ascribed to protonation of certain side chains and subsequent changes in the environments (polarity) around absorbing aromatic residues. In the far uv CD no significant spectral changes were observed after acidification. Dif-

TABLE 1

Far uv CD spectral data of aspartyl proteinases: position of minima and secondary structure

Enzyme	Wavelength[1] of minimum ellipticity	Fractions of secondary structure (%)[b] helix α	β-structure	unordered form
Pepsin. hoo	213.5 nm	5	67	28
Chymosin. calf	217 nm	3	55	42
Cathepsin D. bovine	215.5 nm	4	57	40
Aspergillus niger	212.5 nm	3	84	13
Claviceps purpurea	210 nm	4	63	33

[1]CD spectra were measured at pH values of 5.0 (pepsin, Aspergillus niger), pH 6.6 (chymosin) and pH 8.0 (cathepsin, Claviceps purpurea), protein concentrations ranging from 0.2 mg/ml to 0.9 mg/ml in 0.1 mm or 0.5 mm cells, as described in MATERIALS AND METHODS. [b]The structural fractions were determined by the linear least square method of Chen et al (5) with the constraints of $\Sigma_i f_i = 1$ and $n = 10.4$ (the average number of residues per helical segment).

ferential near uv CD spectra due to pepstatin binding are shown in Figure 2 (a–d) and (a–c). It is difficult to see much similarity in the shapes of these spectra among the different enzymes. Increased Trp ^1L_b absorbance with positive band around 290 nm is commonly observed, while the band at 285 nm is shifted to red or blue and is of varying intensity. The Trp ^1L_a transition is probably also increased, overlapping with its ^1L_b transition. Increased Tyr CD absorption in complexes would also be reflected in the 285 and 280 nm bands, as seen in the spectra in Figure 2 (a, b, d) and Figure 3 (a). The band is blue shifted in other spectra and overlaps with increased Phe absorption; this is especially recognizable in the spectra in Figure 2 (b, d) and Figure 3 (b, c).

In the far uv the binding of pepstatin generally lowered the ellipticity of all aspartyl proteinases. Figure 4 shows differential spectra of six enzymes. They exhibited minima in region from 210–220 nm of different shapes and intensities. Some enzymes (Figure 4 c–f) exhibit positive peaks around 230 nm indicating increased Trp/Tyr absorption in the complexes which is superimposed on the increased peptide bond aborption; the contribution of each is difficult to differentiate.

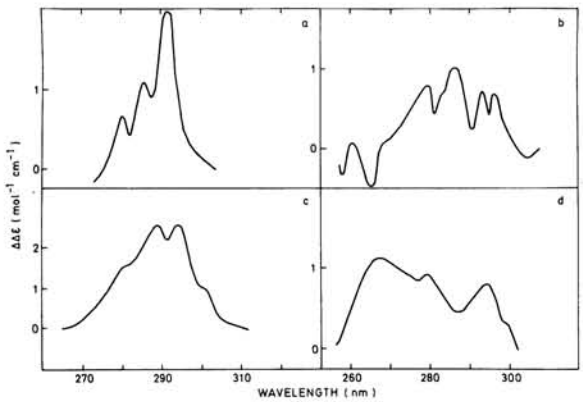

FIGURE 2. Differential spectra in the near uv region. Spectra were calculated by substraction of the absorbances of free proteinases from the absorbances of proteinase-pepstatin complexes. All spectra were measured in solutions of proteinases and proteinase complexes in acetate buffer, pH 3.5, at about the same concentrations as in Figure 1, a) hog pepsin, b) chicken pepsin, c) bovine cathepsin D, and d) chicken cathepsin D.

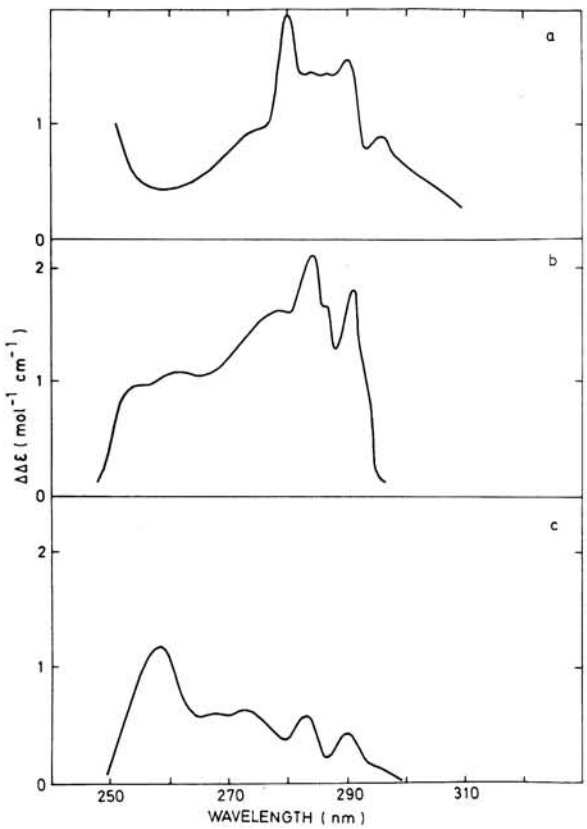

FIGURE 3. Differential spectra in the near uv region. Details are described under Figure 1 and Figure 2 a) calf chymosin, b) Aspergillus niger aspartyl proteinase, c) Claviceps purpurea aspartyl proteinase.

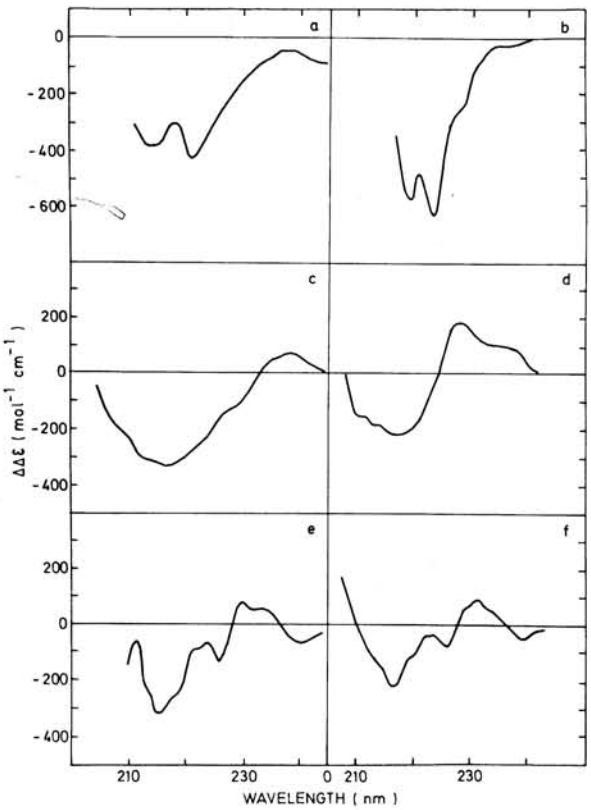

FIGURE 4. Differential uv CD spectra in the far uv region. Spectra were obtained by substraction of mean residue ellipticities of free proteinase from mean residue ellipticities of complexes of the proteinases and pepstatin. Protein concentrations were the same as described in Figure 1. The solutions were prepared in acetate buffer, pH 3.5, and measured as described in Materials and Methods. a) hog pepsin, b) chicken cathepsin D, c) bovine cathepsin D, d) calf chymosin, e) Aspergillus niger aspartyl proteinase, and f) Claviceps purpurea aspartyl proteinase.

DISCUSSION

The results presented here show that aspartyl proteinases of different origin exhibit different shapes of their near uv CD spectra, although the absorption maxima are at strikingly similar wavelengths. Since these enzymes differ in their net charge (pI) and pH stability, the measurements were done under conditions of optimum pH stability of the enzymes, which varied for different enzymes. This indicates that not only the sequence positions *(22)* but also the spatial microenvironment around Trp, Tyr and Phe, is to a great extent conserved during evolution. No particular similarities in the two microbial proteinases as opposed to gastric proteinases or the two cathepsins could be observed in their aromatic, that is near uv, CD spectra, nor far uv CD spectra. Calculations of secondary structures only revealed that the beta structure (55–84%) dominates over alpha helices in all measured enzymes justifying their classification into the beta type of proteins.

Pepstatin, a pentapeptide of microbial origin and the specific inhibitor of all aspartyl proteinases, increased the CD absorption of aromatic amino acids to different extents in different proteinases: Trp and Tyr absorption is intensified in the complexes of all proteinases, while a significant contribution of Phe occurred in chicken cathepsin D, *Aspergillus niger* and *Claviceps purpurea* proteinases. This suggests that the conformational change is not limited to one or two side chain orientations but is spread over a larger region of the molecule, comprising mainly the active site groove.

Crystallographic data obtained recently on the complexes of endothiapepsin (8) and previously on penicillopepsin *(10, 11)* and *Rhizopus chinensis (4)* with inhibitory peptides could serve as a model for the interpretation of differential CD spectra. Generally, the conformational changes were observed in the major substrate pocket (S1) involving Tyr (75), Trp (39) and Phe (111) (pepsin numbering) *(8)*. The flap, comprising a large beta-loop with Tyr (75) at the turn position moves deeper into the active site upon ligand binding *(20–22)* and assumes the general mechanisms of interaction in all aspartyl proteinases. We can speculate that the observed absorption bands of the Tyr ^1L_b transition (270–285 nm) reflect the transfer of the Tyr (75) residue into a more assymetric environment in the active site cleft. It was also observed *(8, 26)* that there was less flexibility in the enzyme-pepstatin complexes as in the free enzyme and that the ligand was aligned along the active site pocket in a way which would allow formation of beta sheets. The increased ellipticities of the complex in the far uv region, therefore might be ascribed to the induced interactions of the aromatic amino acids with n-Π^* and Π-Π^* transitions of peptide bonds *(24)*. On the other hand, formation of a short beta strand would also contribute to the observed increased order in the complexes.

The differences in the differential spectra in various enzymes, however, probably reflect subtle differences in their topology. Several specificity studies using natural and synthetic substrates and inhibitory peptides, failed in attempts to classify aspartyl proteinases into

distinct subclasses *(12, 18)*. Our data on conformations and conformational changes upon inhibitor binding could also not give a clear insight into the evolutionary relationship of aspartyl proteinases nor could these be the basis for classification of the enzymes into distinct subclasses. We can only conclude that they have evolved slightly different conformations in order to operate under specific physiological conditions.

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STUDIJ CIRKULARNEGA DIHROIZMA RAZLIJNIH ASPARTILNIH PROTEINAZ IN NJIHOVE INTERAKCIJE S PEPSTATINOM

IZVLEČEK. – Merili smo bližnje in daljnje spektre cirkularnega dihroizma (CD) aspartatnih proteaz evolucijsko različnega izvora: protease iz gljiv Aspargillus Niger in *Claviceps Purpurea, piščančjega pepsina A, piščančjega katepsina D, telečjega himozina, govejega katepsina D in svinjskega katepsina D, tako v prosti obliki kakor tudi po vezavi s pepstatinom (pentapeptidnim inhibitorjem). Dalnjnji uv spektri kažejo od 55–84% beta strukture, kar omogoča uvrstitev gornjih proteinaz v razred beta proteinov. Bližnji uv spektri pa odkrivajo visoko stopnjo ohranitve asimetrije v okolici Tyr/Trp in Phe, saj se njihovi absorpcijski vrhovi, čeprav različno intenzivni, pojavljajo ob istih valovnih dolžinah. Diferencialni CD spektri so različni za posamezne proteaze, vendar pa lahko opazimo, da so moteni isti absorpcijski vrhovi, kar vse skupaj kaže na podobno topologijo aktivnega mesta. Posebno zanimivo je, da je absorbcija v daljnjem uv intenzivnejša v kompleksu encima z inhibitorjem kakor v prostem encimu, kar odraža spremembe v absorbciji peptidnih vezi in večjo urejenost strukture v encimu ali/in inhibitorju po vezavi. Ugotavljamo, da niti primerjava konformacij aspartatnih proteinaz z merjenjem CD spektrov niti diferencialni spektri ob vezavi pepstatina ne odražajo značilnejših podobnosti med encimi, ki so si evolucijsko manj oddaljeni. S spektri tudi ni možno dokazati obstoj strukturnih podrazredov aspartatnih proteinaz, kot je bilo predlagano glede na razlike v specifičnosti.*