Biochimica et Biophysica Acta, 614 (1980) 553—563

BBA 69033

PURIFICATION AND PHYSICOCHEMICAL CHARACTERIZATION OF A NEW RAT PLASMA PROTEINASE INHIBITOR, α_1-INHIBITOR III

FRÉDÉRIC ESNARD and FRANCIS GAUTHIER

Laboratory of Biochemistry, Faculty of Medicine, 2 bis, Boulevard Tonnellé, 37032 Tours Cedex (France)

(Received December 18th, 1979)

Key words: α_1-inhibitor III; Proteinase inhibitor; Rat plasma

Summary

A three-stage procedure was used to isolate an additional proteinase inhibitor in rat plasma tentatively called α_1-inhibitor III. A 20% yield was obtained after two successive gel filtrations on Ultrogel Ac-A. 33—4 followed by an ion-exchange chromatography on DEAE-cellulose. This method was chosen since it permits further study of the enzyme binding properties of the isolated molecule. The purified material was first controlled to retain an inhibiting capacity towards serine proteinases using bovine chymotrypsin. The isolated molecule has an apparent molecular weight of 215 000, a p*I* of 4.65, an $E^{1\%}_{1cm,280nm}$ of 7.50 and a sedimentation coefficient of 8.6 S. It contains approx. 15% carbohydrates and is made up of a single peptidic chain. Study of the periodic structure by circular dichroism has demonstrated a low α-helix content (4—5%) whereas the β-sheet conformation accounts for approx. 30% of the peptidic moiety. Tryptophan residues have been shown to be mainly responsible for the molecular fluorescence most of them being non-accessible to the solvent since only 25% of the tryptophanyl fluorescence was quenched in presence of I^-.

Studies on the mechanism of proteinase inhibitors have been carried out in this laboratory using rat serum, to determine the competitive or cooperative effects occurring between the main proteinase inhibitors [1,2]. During the course of purification of these inhibitors i.e. α_1-macroglobulin, $\alpha_1$proteinase inhibitor and α_2 acute phase macroglobulin an additional quantitatively important inhibitor was demonstrated and isolated [3]. This inhibitor tentatively called α_1-inhibitor III (since it represents the third inhibitor with an α_1 electrophoretic mobility described in rat plasma), seems to be identical with a trypsin and chymotrypsin rat plasma binding factor previously mentioned [4].

The study of the enzyme binding properties of this inhibitor have shown it to bind and inhibit a variety of heterologous as well as homologous pancreatic proteases including anodal and cathodal trypsins, chymotrypsins and elastase [1,2].

The present paper deals with the physicochemical characterization of this protein in order to determine its biological significance in the general mechanism of binding and elimination of proteases.

Experimental

Isolation procedure

Modifications of a previously described method [3] were used to enhance the preparation stability. These modifications, including a change in pH conditions and avoiding the use of $(NH_4)_2SO_4$, were necessary to study the enzyme binding properties of isolated α_1-inhibitor III.

Citrated blood was collected from four normal adult rats and was made 10^{-5} M with soybean trypsin inhibitor (Sigma), $3 \cdot 10^{-3}$ M NaN_3, $5 \cdot 10^{-3}$ M EDTA. After centrifugation, about 25 ml of plasma were submitted to the first step of gel filtration on Ultrogel Ac.A. 3—4 (IBF reactants Pharmindustrie). This step as well as the following chromatographic fractionations were carried out at 4 °C. The Ultrogel column (5 × 50 cm) was equilibrated with 0.05 M Tris-HCl, (pH 7.4)/0.15 M NaCl (flow rate, 20 ml/h). Chymotrypsin-inhibiting capacity of eluted fractions was determined using bovine α-chymotrypsin (Sigma 3-times crystallized) and *N*-acetyl-L-tyrosine ethyl ester as a substrate according to the method of Berthillier [5]. Experimental conditions were applied as described earlier [6] with the exception of the following change: 10 µl of eluted fractions were incubated with $8.5 \cdot 10^{-8}$ mol of chymotrypsin before recording spectrophotometrically the enzymatic activity. α_1-inhibitor III was identified in the third eluted peak (Fig. 1a) by electroimmunoassay [7] using specific antibodies prepared from the previously described inhibitor isolation [3]. α_1-inhibitor III containing fractions were then analysed by polyacrylamide gel electrophoresis [8] and these fractions in part, corresponding to about 70% of the total inhibitor content (shaded area of Fig. 1a) were pooled then concentrated to 10 ml by ultrafiltration using an Amicon UM 10 membrane. This volume was then submitted to the second step of gel filtration using the same experimental conditions. Eluted fractions (Fig. 1b) were analysed electrophoretically as noted before and a part of the eluted peak (shaded area of Fig. 1b) corresponding to about 40% of the initial inhibitor content was dialyzed against 0.05 M Tris-HCl buffer, (pH 7.4)/0.05 M NaCl and applied to a column (2 × 10 cm) of DEAE-cellulose (Whatman DE-52) equilibrated in the same buffer. A stepwise NaCl gradient (0.05 M, 0.1 M and 0.15 M) was used to carry out the elution.

Fractions of the second eluted peak (Fig. 1c) were pooled, concentrated and then controlled for purity and protease-binding properties. Polyacrylamide gel electrophoresis [8] and crossed immunoelectrophoresis [9] using anti-whole-rat serum (Behringwerke Marburg/Lahn) were chosen as criteria of purity; the chymotrypsin-inhibiting property was analysed according to the aforementioned method of Berthillier [5].

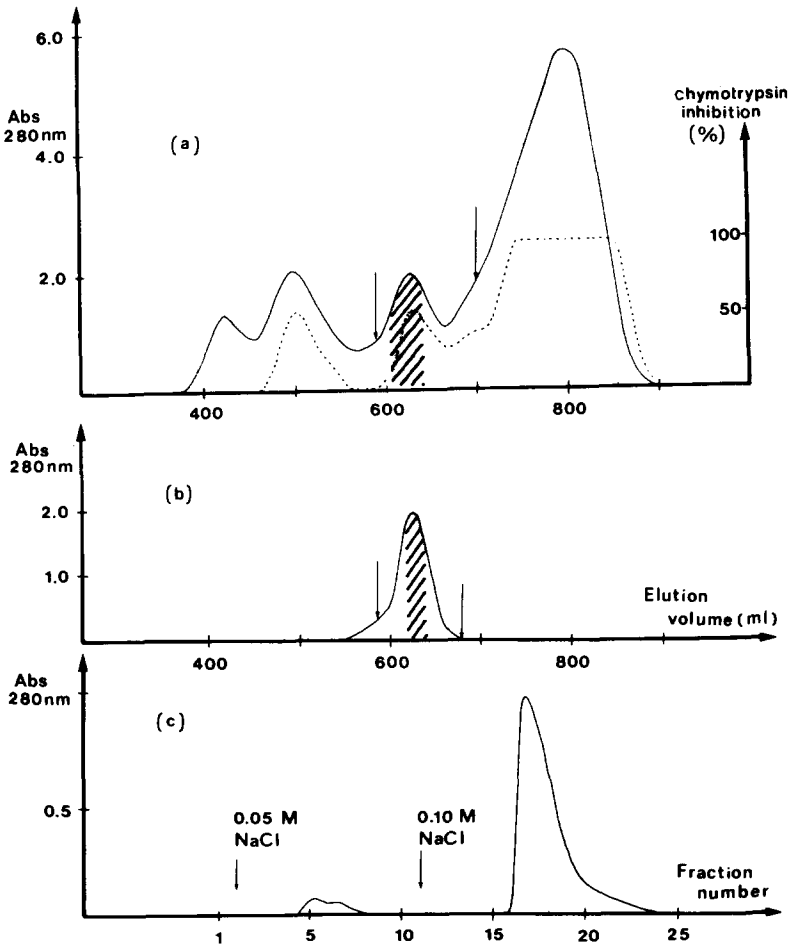

Fig. 1. Purification of α_1-inhibitor III. (a) (———) Elution pattern at 280 nm of whole plasma after Ultrogel Ac.A. 3—4 chromatography and (- - - - -) percentage of chymotrypsin inhibition. (b) Elution pattern of the shaded fraction of (a) after concentration using the same chromatographic medium. (c) Elution pattern of the shaded fraction of (b) after ion-exchange chromatography. The distance between arrows in (a) and (b) indicates the area in which α_1-inhibitor III was detected by electroimmunoassay.

Analytical methods

Isoelectric focusing. Experiments were carried out according to Wrigley [10] on polyacrylamide gels (4.7% acrylamide, 2.6% bisacrylamide) containing 2.4% ampholytes (LKB Ampholine pH range 4.0—6.5). Four identical samples were analysed simultaneously, two were stained after the protein had focused. The other two were cut into 4 mm thick slices and allowed to diffuse for several hours in 1 ml of deionized water before we measured their pH. An alternative procedure was to use the commercially available Ampholine plates (LKB pH 4.0—6.5) following the recommended procedure. pH measurements were made using a contact electrode.

Reduction and alkylation were made according to Raftery and Cole [11] using β-mercaptoethanol and ethyleneimine in 8 M urea.

Molecular weight. The molecular weight of α_1-inhibitor III was calculated

using polyacrylamide gel electrophoresis with sodium dodecyl sulfate as described by Weber and Osborn [12] using 4.4% gels. All marker proteins were commercially available (Pharmacia electrophoresis calibration kit, Boehringer combiteck) and were over the M_r range 18 500—330 000.

Extinction coefficient. This was measured using a Beckman 25 spectrophotometer at 280 nm. The protein solution (1 mg/ml) was dialysed extensively against deionized water before determining its absorbance. A precise volume of 6 ml was then dessicated at 60°C under an increasing vacuum and weighed until the minimal value was obtained.

Sedimentation values. Values were obtained as described earlier [13]. α_1-inhibitor III solutions (1.6—6 mg/ml) were dialyzed against 0.05 M Tris-HCl, pH 7.8/0.1 M NaCl prior to ultracentrifugation.

Circular dichroism spectra. Spectra were measured with a Mark III dicrograph (Jobin-Yvon) equipped with a temperature controlled sample cell holder. 2 mm cells were maintained at 20°C.

The protein solution (0.17 mg/ml in 0.1 M Tris-HCl buffer, pH 7.8) was filtered through a 0.45 μm membrane (Millipore). The ellipticity curves were drawn taking average values from two recordings and expressed as mean residue ellipticities ($deg \cdot cm^2 \cdot dmol^{-1}$) taking a mean residue weight of 110 as calculated from amino acid composition.

The helical and β-structure contents of α_1-inhibitor III were first determined as described by Chen et al. [14] and then by using a similar method with nine reference spectra [15].

Fluorescence. The emission and absorption spectra were recorded on a double beam spectrofluorimeter Fika 55 MK coupled to a Houston recorder 2000. α_1-inhibitor III (0.06 mg/ml) was centrifugated at 12 000 $\times g$ for 20 min prior to use. Fluorescence quenching by I^- and guanidinium chloride was performed according to Lehrer [16].

Amino acid and carbohydrate analyses. Amounts of 1 mg of α_1-inhibitor III were hydrolysed for periods of 24, 48 and 72 h in 1 ml of 5.6 M HCl at 110°C. Half-cystine was determined as cysteic acid as described by Hirs [17]. Tryptophan was calculated after hydrolysis by *p*-toluene sulfonic acid according to Liu and Chang [18].

N-terminal amino acid was determined by the dansyl technique described by Gros and Labouesse [19]. Dansyl amino acids (Serva) were used as standards. Sugar analysis was made by GLC according to Reinhold et al. [20]. Sialic acid was determined by the method of Aminoff [21].

Results

Purity of α_1-inhibitor III. The Fig. 2a shows the electrophoretic patterns of the preparation on polyacrylamide gels after each stage of purification. α_1-inhibitor III (3 mg/ml) appears as a single band after the last step of ion-exchange chromatography. The recovery of α_1-inhibitor III after this last step is about 20% as evaluated by electroimmunoassay [7]. The crossed immunoelectrophoretic pattern of purified α_1-inhibitor III shows only one peak of precipitation with an α_1-mobility against anti-whole-rat serum (Fig. 2b).

Proteases inhibiting capacity. The percentage of chymotrypsin inhibition

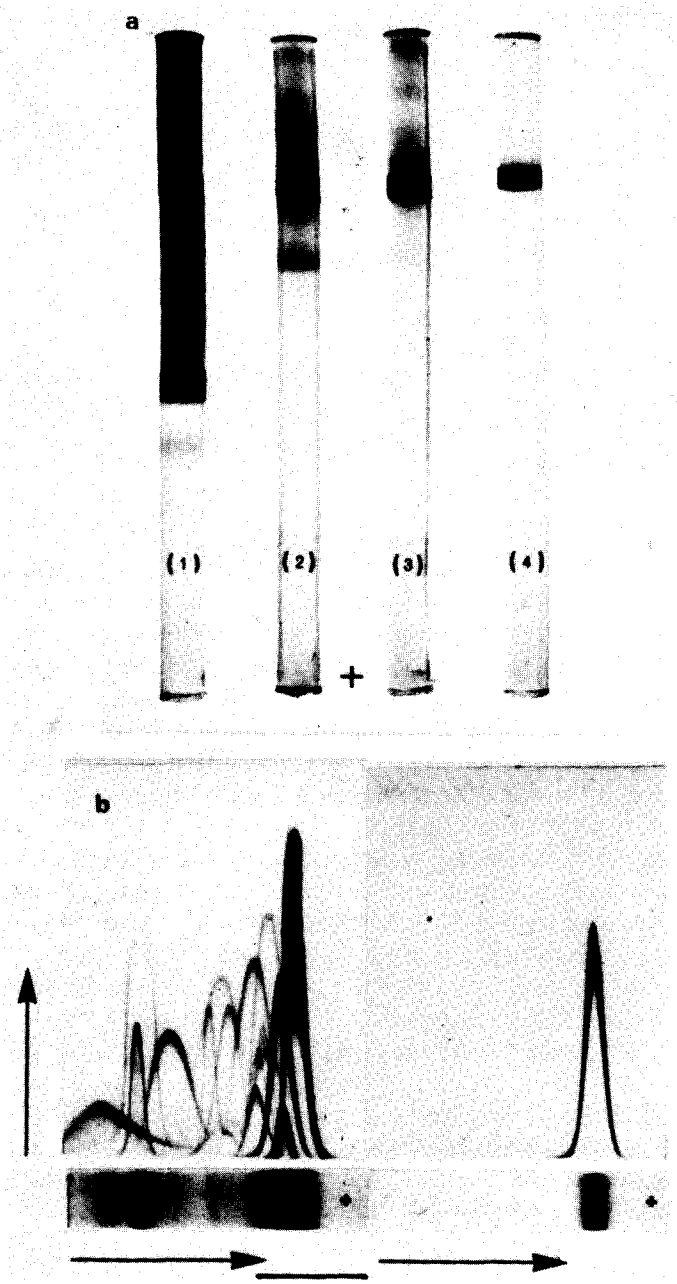

Fig. 2. Purity of α_1-inhibitor III. (a) Disc electrophoresis of the preparation after the different stages of purification: (1) whole plasma; (2) α_1-inhibitor III after the Ultrogel Ac.A. 3—4 chromatography and (3) after second Ultrogel Ac.A. 3—4 chromatography. (4) purified material after ion exchange chromatography. (b) Crossed immunoelectrophoresis of whole plasma and purified α_1-inhibitor III against anti-whole-rat serum. Electrophoretic patterns of normal plasma and α_1-inhibitor III are given for reference.

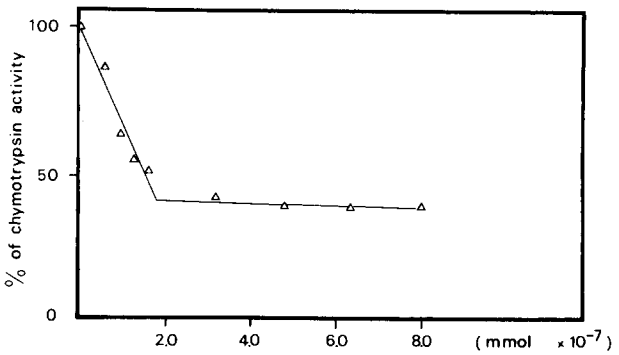

Fig. 3. Chymotrypsin inhibition by increasing amounts of α_1-inhibitor III using *N*-acetyl-L-tyrosine-ethyl-ester as a substrate. $1.7 \cdot 10^{-7}$ mmol of chymotrypsin were incubated for 2 min at 37° C with α_1-inhibitor III (0—$8.0 \cdot 10^{-7}$ mmol) in 0.05 M Tris-HCl buffer, pH 7.4/0.05 M $CaCl_2$/0.15 M NaCl to give a final volume of 3 ml. The reaction was started by the addition of 0.2 ml of substrate and the hydrolysis rate was followed spectrophotometrically for 3 min at 237 nm.

with respect to increasing amounts of inhibitor is plotted on Fig. 3. No total inhibition of chymotrypsin was recorded even with a 4-fold molar excess of inhibitor, thus suggesting the complex to be esterolytically active. The confirmation of this hypothesis was given by the demonstration of an esterolytic activity associated to the inhibitor after an electrophoretic separation of the complex on polyacrylamide gel (unpublished data). The observation on Fig. 3 that the maximal inhibition (about 60% of the free enzyme activity) is reached when $1.78 \cdot 10^{-7}$ mmol of inhibitor were reacted with $1.7 \cdot 10^{-7}$ mmol chymotrypsin suggest the existence of a 1 : 1 binding ratio.

Molecular weight. The apparent molecular weight of the protein was found to be 215 000 after SDS polyacrylamide gel electrophoresis. The same value was obtained after prior reduction by 0.1 M β-mercaptoethanol or after reduction-alkylation in 8 M urea (Fig. 4) indicating the protein to be a single chain.

Isoelectric point. After focusing on polyacrylamide gel, two close bands were seen on the stained samples. The most important was located at pH 4.65 and the minor band at pH 4.60. Evidence for heterogeneity of the α_1-inhibitor III was not, however, demonstrated since the minor band when compared to the other appeared as being very faint and consequently could be a contaminant.

Extinction coefficient. The dessicated protein was weighed several times and only the minimal value was taken into account. A sample containing the same volume of the last dialysis bath was treated under the same conditions to correct the weight value of the dessicated protein. $E^{1\%}_{1cm,\,280nm}$ was found to be 7.50.

Sedimentation coefficient. The values of $S^0_{20,w}$ were determined at three different concentrations of α_1-inhibitor III. Extrapolation to zero concentration gave a value $S^0_{20,w} = 8.60$ S.

Amino acid and carbohydrate compositions. The N-terminal amino acid analysis has permitted the identification of only one amino acid, e.g. glycine, in the dansylated protein hydrolysate after comparison with standard dansyl amino acids. This is in agreement with the presence of a single peptidic chain after SDS polyacrylamide analysis of the reduced and alkylated protein.

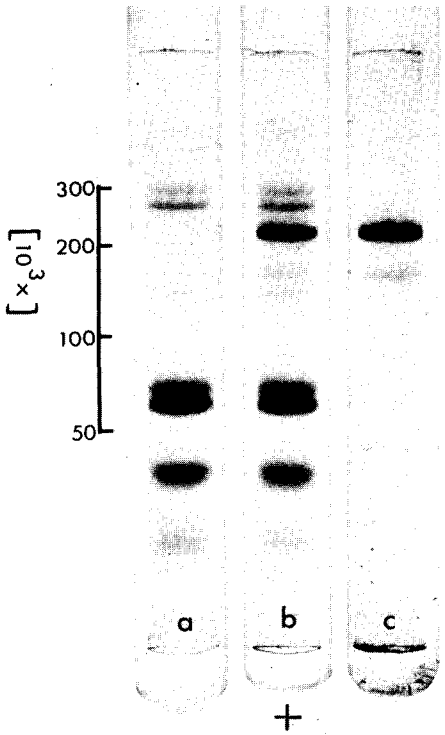

Fig. 4. Disc gel electrophoresis in presence of sodium dodecyl sulfate. (a) Marker proteins (Pharmacia high M_r calibration kit). (b) Marker proteins added to α_1-inhibitor III. (c) α_1-inhibitor III. All the samples were reduced by β-mercaptoethanol. Electrophoresis was carried out on 4.4% polyacrylamide gels containing 0.1% sodium dodecyl sulfate.

The overall amino acid composition is given in Table I; values were calculated from duplicate analyses at 24, 48 and 72 h.

Carbohydrates accounted for 15% of the dry weight of the α_1-inhibitor III molecule (Table I).

Circular dichroism. Fig. 5 shows the characteristic far ultraviolet CD spectrum of the glycoprotein with a maximum at 216 nm indicating a β-sheet conformation. Four successive analyses have been carried out without taking into account the carbohydrate moiety. Whatever the reference spectra used, the α-helical content appears to be very low (4—5%). The estimation of the β-sheet conformation was much more imprecise since in addition to the proper limitation of the technique in protein structure determination, the glycoprotein contains a rather high level of sialic acid and acetylglucosamine (Table I) the acetamido and carboxylate groups of which contribute to the CD spectrum [22,23]. A mean value of 30% was obtained for the β-sheet content of the protein.

Fluorescence. The analysis of the fluorescence spectra gives valuable information on the tryptophan environment of the inhibitor since this amino acid is mainly responsible for the protein fluorescence. The maximal emission was recorded at 338 nm after the inhibitor solution was excited at 280 nm and 290 nm. This indicates a relatively non-polar environment. By studying protein

TABLE I

AMINO ACID AND CARBOHYDRATE COMPOSITION OF α_1-INHIBITOR III. RESULTS IN RESIDUES PER MOL WERE OBTAINED FROM THE MEANS 24, 48 AND 72 h HYDROLYSES IN 5.6 M HCl EXCEPT WHERE NOTED

Figures in parentheses indicate the number of times that each analysis was performed.

Component	Time of hydrolysis			
	24 h (2)	48 h (2)	72 h (2)	residues/molecule of 215 000
Lysine	104.4	101.9	108.3	105
Histidine	37.2	39.7	40.0	39
Arginine	39.9	42.0	38.3	40
Aspartic acid	161.4	155.0	153.3	157
Threonine	102.5	104.5	101.0	103 [a]
Serine	139.6	127.3	114.0	152 [a]
Glutamic acid	190.7	193.5	196.0	193
Proline	116.3	112.1	119.9	116
Glycine	87.5	90.4	100.1	93
Alanine	109.0	102.2	116.3	109
1/2-Cystine (4)	—	—	—	47 [c]
Valine	108.6	118.6	134.9	121
Methionine	19.3	17.9	19.5	19
Isoleucine	64.8	69.9	77.7	78 [b]
Leucine	151.2	156.0	163.7	164 [b]
Tyrosine	56.7	58.2	46.1	54
Phenylalanine	61.8	67.9	71.0	67
Tryptophan (2)	—	—	—	9 [d]
N-Acetyl glucosamine (4)	—	—	—	58 [e]
Galactose (4)	—	—	—	39 [e]
Mannose (4)	—	—	—	28 [e]
Fucose (4)	—	—	—	traces
Sialic acid (2)	—	—	—	33 [f]

[a] By extrapolation to zero hydrolysis time.
[b] Mean value of 72 h hydrolysate.
[c] After performic oxidation.
[d] Determined by paratoluene sulfonic acid hydrolysis.
[e] Determined by GLC.
[f] Determined according to Aminoff [19].

fluorescence-quenching in the presence of I^- (Fig. 6), only 20—25% of the fluorescence intensity was quenched, indicating a large part of tryptophan residues to be nonaccessible to the solvent, whereas in 6 M guanidinium chloride all the fluorescence intensity was quenched.

Discussion

The purification of α_1-inhibitor III was carried out using a rapid procedure which permits a further investigation of its enzyme binding capacity. This property appears to be at the moment the only known biological function of this molecule. The purity of the protein after the last step of purification was similar to that obtained using the previously described method [3], but the stability was greatly enhanced. Polymeric forms of the inhibitor which appeared spontaneously with time by using the former technique, necessitated a repurification, whereas a stable preparation available for 6—8 days when kept

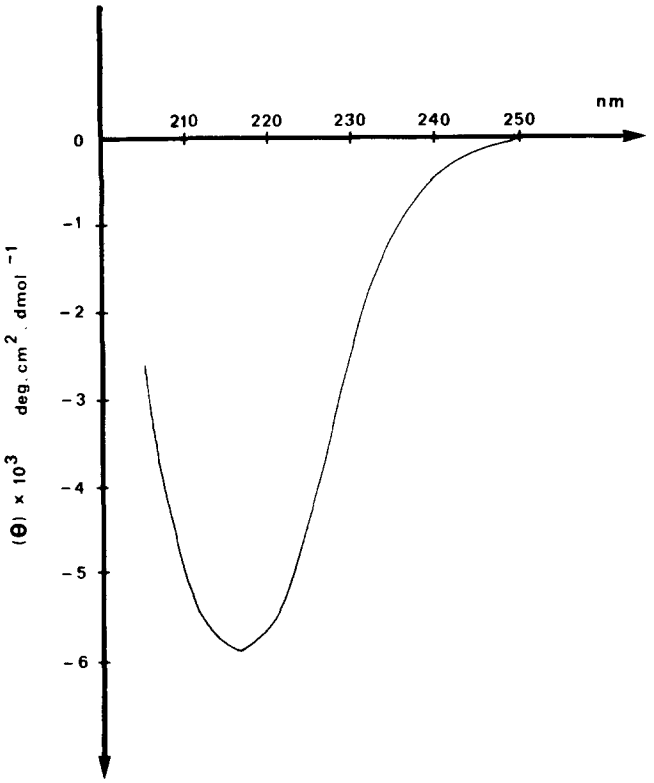

Fig. 5. Circular dichroism spectrum of a solution of α_1-inhibitor III in the far ultraviolet zone. The curve was calculated from four recordings. For further details see under Experimental.

at 4°C was obtained using this new procedure. This type of storage was used to study the enzyme binding properties of the α_1-inhibitor III molecule. The main physicochemical properties of the molecule are summarized in Table II. A characteristic of this glycoprotein is that it is made of a single peptidic chain in spite of its rather high molecular weight. This fact was deduced from SDS

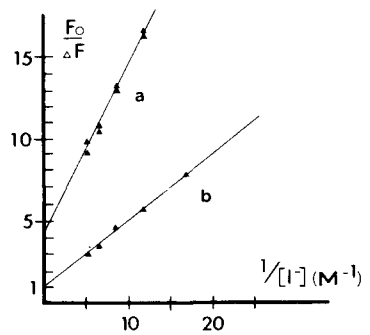

Fig. 6. Modified Stern-Volmer plot of the quenching of α_1-inhibitor III fluorescence by iodide in 0.05 M Tris-HCl buffer, (pH 7.4)/0.15 M NaCl/3.10^{-3} M NaN_3 (a) and in 6 M guanidinium hydrochloride (b) (excitation, 290 nm 20°C; emission, 350 nm, α_1-inhibitor III concentration 0.15 mg/ml).

TABLE II

Molecular weight	215 000
Isoelectric point	4.65
Specific absorption coefficient ($E^{1\%}_{1\ cm,\ 280\ nm}$)	7.5
Sedimentation coefficient ($S^{o}_{20,w}$)	8.6
Number of peptidic chains	1
N-terminal amino acid	Gly
Carbohydrate content (%)	15

electrophoresis after prior reduction or reduction and alkylation in 8 M urea of the protein. This polypeptide chain possesses a N-terminal residue identified as glycine after analysis of the dansylated protein hydrolysate. Such a single chain in the presence of reducing agents was also found for human inter-α-inhibitor [24], whose molecular weight is close to that of α_1-inhibitor III and that we have previously mentioned as being the possible human homologue of this rat inhibitor [3].

CD analysis has demonstrated the low α-helix content in the inhibitor molecule whereas the β-sheet accounts for about 30% of its peptidic moiety; 65% of the molecule corresponds to an aperiodic conformation. When compared to other proteinase inhibitors studied using CD, this type of structure ressembles more that of α-macroglobulin than that of α_1-proteinase inhibitor; the former was found to have a very low α-helix content [25] whereas the latter contains 16—20% of α-helix [26]. No clear relationship between the structural analysis and the proteinase binding ability can be however established, but advantage can be taken from the CD analysis to study the conformational changes of the inhibitor when it reacts with proteolytic enzymes.

Acknowledgements

This work was carried out at the laboratory of Professor H. Mouray, Faculty of Medicine, and was supported by a grant from INSERM No. CRL-76-1070-3. We are indebted to Dr. M. Leng and Dr. J.C. Maurizot (CBM, Orleans-La Source) for their contribution to the CD and fluorescence analyses. The technical assistance of Mrs. G. Serros who carried out the ultracentrifugations is also greatly acknowledged.

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