CIRCULAR DICHROISM AND OPTICAL ROTATORY DISPERSION OF NATIVE AND CHEMICALLY MODIFIED PANCREATIC TRYPSIN INHIBITOR*

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The native pancreatic trypsin inhibitor shows a negative circular dichroic band of the aromatic chromophore of tyrosine side chains at 276 nm; two negative (at 216 and 202 nm), and one positive dichroic band (at 190 nm) correspond to electronic transitions of the peptide bond. The evaluation of the circular dichroic, rotatory dispersion, and infrared spectra points to the conclusion that the trypsin inhibitor contains in addition to the unordered structure approximately 19% of α-helical conformation. Reduction and carboxymethylation of the disulfide bonds affect all conformationally conditioned dichroic bands. Nitration of the tyrosine residues is without effect on the amide dichroic bands; a new circular dichroic band at 355 nm (pH 4·8) appears whose intensity is proportional to the number of substituted tyrosine residues.

In our preceding papers[1-3] the results of studies on the chemical topography of the pancreatic trypsin inhibitor have been reported. These papers also included the results of optical rotatory dispersion (ORD) measurements carried out both with the native and the modified trypsin inhibitor. In an effort to gain a deeper insight into the structure of the protein, we undertook a more detailed study on both the native and the chemically modified trypsin inhibitor by means of circular dichroism (CD) measurement. The results of these studies, together with infrared spectra and additional ORD data are discussed in this report.

EXPERIMENTAL

Material

Native pancreatic trypsin inhibitor was prepared by the method described by Dlouhá and coworkers[4]. The preparation, isolation, and characterization of chemically modified trypsin inhibitors has been described in preceding papers[1-3]. The model compound, cyclic dipeptide cyclo(3-

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nitro-L-tyrosyl-glycyl) was prepared by Dr K. Bláha[5], Department of Peptide Synthesis, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences.

Methods

CD spectra were measured in Roussel-Jouan Dichrograph CD 185 at a temperature of approximately 22°C in cells of 1–0·01 cm of length. The concentration of the protein was approximately 0·05 g/100 ml. The CD data are not corrected for the refractive index of the solvent and are given as molar residual ellipticities in deg cm^2 $dmol^{-1}$. The ORD values in the range 600–310 nm for the calculation of constants of the Moffitt-Yang equation[6] were measured in Jasco ORD/UV-5 spectropolarimeter using a 1-dm cell at 25°C and a protein concentration of 0·4 g per 100 ml. For the correction of dispersion values for the refractive index of the solvent the data tabulated by Fasman[7] were used. A mean molecular weight 113·5 of one amino acid residue was used in the calculation of reduced molar ellipticities and rotations. The solutions were prepared by weighing of dry products. The IR spectra were measured in Perkin-Elmer 621 Spectrophotometer. The protein film and solution in deuterium oxide (concentration 40 mg/ml) were prepared according to Timasheff and Susi[8]. The film was prepared on a KRS-5 plate and the solution was measured in CaF_2-cells 0·024 mm long.

RESULTS AND DISCUSSION

The CD spectrum of the native trypsin inhibitor in 0·1M Tris-HCl buffer, pH 8·0, is shown in Fig. 1. There is a negative dichroic band in the long-wave part of the spectrum at 276 nm, which shows vibrational structure and undergoes a bathochromic shift toward 294 nm in an alkaline medium (0·01M-NaOH, Fig. 1). This band belongs[1] to the long-wave $\pi-\pi^*$ transition $A_{1g} \leftarrow B_{2u}$ of the *p*-hydroxyphenyl group in the tyrosine side chains. The shape of the spectrum in the wavelength range below 250 nm is determined by the prevailing dichroic bands of electronic transitions of the peptide bond. The position and size of these bands permit a rough estimate of the type and distribution of various conformations in the trypsin inhibitor. The positive band at 190 nm is typical of the α-helical conformation, which, however, in view of the low ellipticity of this band can be present in a low amount only. The position of the negative band at 202 nm is close to the band characteristic of the unordered state of the peptide chain which is obviously prevalent in the protein.

On the origin of the broad shoulder in the 215 to 217 nm range may participate, besides the negative maximum of the α-helical conformation (222 nm), also the negative maximum of the β-pleated sheet conformation lying at 217 nm. The presence of both these conformations is suggested also by the position of the trough at 229 nm on the ORD curves[1].

The most suitable method of identification of the β-conformation in polypeptides and proteins is[9] infrared spectroscopy in the absorption range of amide I band. The spectra of the inhibitor both in film and also in deuterium oxide solution are shown in Fig. 2. When these spectra are interpreted according to Timasheff and coworkers[9], then the main band in the spectrum of the solution at 1644 cm^{-1} reflects

the unordered conformation, the shoulder at 1652 cm^{-1} the presence of the α-helix. The spectrum of the film displays a broad maximum involving two bands lying obviously close to each other: the band of the unordered conformation, which in the film is shifted toward 1658 cm^{-1}, and the band of the α-helical conformation, which in the film lies at the same frequency as in deuterium oxide. The absorption band at 1632 cm^{-1} typical of the β-conformation was not found in the spectrum of the inhibitor, either in solution or in film. It cannot therefore be assumed that the protein contains the undistorted β-pleated sheet conformation characterized by this frequency of amide I band in an amount greater than 5%. The weak band at about 1615 cm^{-1} indicates that as well a small amount of a different form of β-conformation could be present.

We estimated[1] a 25% content of α-helical conformation in the molecule of the inhibitor from the value of molar residue rotation $[R]_{229}$. The additional rotatory dispersion parameters were obtained by treatment of dispersion data in the range 310–600 nm using the Moffitt-Yang[6] equation. Since the aromatic chromophores of the tyrosine residues show a relatively high optical activity we had to correct the experimental $[R']_\lambda$-values for the rotatory contribution of these groups[9,10]. The corrections were calculated from simple relations[11] for molar residue rotation $[R']_\lambda$ and for the A_1-value, which is related to the rotational strength of the given electronic transition:

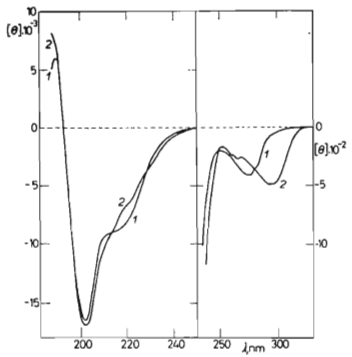

FIG. 1

Circular Dichroic Spectra of Native Trypsin Inhibitor *1* in 0·1M Tris-HCl Buffer, pH 8·0; *2* in 0·1M-NaOH.

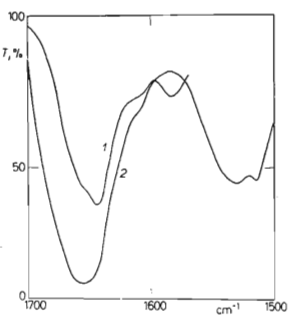

FIG. 2

Infrared Spectra of Native Trypsin Inhibitor *1* In deuterium oxide; *2* in film.

$$[R']_\lambda = \frac{A_i \lambda_i^2}{\lambda^2 - \lambda_i^2} \qquad (1)$$

and

$$A_i = \frac{2[\Theta']_i \Delta_i}{\pi^{1/2} \lambda_i} . \qquad (2)$$

For wavelength λ_i of the band, its half-width Δ_i, and molar residue ellipticity $[\Theta']_i$, the values of the aromatic B_{2u}-band obtained from the CD spectrum were substituted, *i.e.* 276 nm, 19 nm, and -327 deg cm^2 dmol^{-1} respectively. The calculated rotational contributions of the tyrosine chromophores $[R']_\lambda^{tyr}$, were subtracted from the experimental $[R']_\lambda$ values. This correction, based on simple Drude's approximation, was found to be sufficiently exact for the given wavelength range and considerably improved the linearity of the Moffitt-Yang plot. The value of 212 nm was used for constant λ_0 in the Moffitt-Yang equation.

The found rotatory dispersion parameters a_0 and b_0 of the inhibitor in 0·05M acetate buffer, pH 4·8 and in 0·1M Tris-HCl buffer, pH 7·8, show very close values, the average being $a_0 = -611$ and $b_0 = -119$ deg cm^2 dmol^{-1}. The value of b_0 corresponds to a content of 19% of α-helical conformation (for $b_0^{100\%} = -630$ and $b_0^{0\%} = 0$ deg cm^2 dmol^{-1}) (ref.[6,12]). The high negative value of parameter a_0 is adequate to the content of α-helical conformation and indicative of the prevailing unordered structure and at the same time also of the absence of any significant amount of the antiparallel β-pleated sheet structure, whose a_0 has usually high positive values (400 – 700 deg cm^2 dmol^{-1}) (ref.[9]). In trifluoroacetic acid the inhibitor shows $a_0 = -487$, $b_0 = -13$ deg cm^2 dmol^{-1} and therefore the protein does not contain the α-helical conformation in this medium.

The content of the α-helical conformation is also suggested by the value of the molar residue ellipticity in the maximum of CD bands characteristic of this conformation, *i.e.* at 222 and 191 nm. The negative maximum of the 222 nm band cannot be observed in the CD spectrum of the inhibitor (*cf.*, *e.g.* Fig. 1 and 3) due to superposition of the neighboring bands. The value $[\Theta]_{222} = -7800$ deg cm^2 dmol^{-1} read from the experimental curve corresponds to a 20% content of α-helical conformation and is thus in very good agreement with the content calculated from parameter b_0 (for $[\Theta]_{222}^{100\%} = -38000$ deg cm^2 dmol^{-1}, poly-L-glutamic acid. ref.[10]). As reference constant for random coil state we chose $[\Theta]_{222}^{0\%} = 0$. As follows from the discussion (see below), the ellipticity of the unordered state of the peptide chain of the protein in this part of the spectrum has rather zero or a weakly negative value, never, however, a positive value as is the case of polylysine or polyglutamic acid in the random coil state[9,10]. The ellipticity value in the positive maximum at 191 nm leads to a lower helical content, round 10%, which, however, in view of the high experimental error in this region of the spectrum is still in an acceptable agreement with the values determined by the remaining procedures.

The theoretical CD curve constructed for a polypeptide containing 19% of α-helical conformation and 81% of random coil state, does not reproduce well the experimental spectrum of the inhibitor. The nature of the differences becomes obvious on the curve obtained by subtraction of 19% of α-helical conformation (Fig. 3, curve *2*; poly-L-glutamic acid, ref.[10]) from the experimental spectrum of the protein (Fig. 3, curve *1*). Provided that the dichroic characteristics of the α-helical region in the protein are identical with the characteristics of the same conformation of the polypeptide, then the resulting difference spectrum (Fig. 3, curve *3*) belongs to the remaining structure of the peptide chain of the protein and also to the dichroic bands of the chromophores in the side chains.

The small positive maximum in the 225 to 230 nm region can be ascribed to the aromatic $A_{1g} \leftarrow B_{1u}$ band of tyrosine chromophores. Another important chromophore, the disulfide group, can participate[13] by its short-wave CD band on the negative maximum at 200 nm. The other long-wavelength band of the same chromophore at 260 nm (ref.[13]) has a low intensity and is obviously overlapped by the neighboring band of *p*-hydroxyphenyl groups. The negative maximum at 202 nm of $\pi - \pi^*$ transition of the peptide bond, however shows an ellipticity $[\Theta]_{202} = -16500$ deg cm^2 dmol^{-1}, *i.e.* considerably lower than would correspond to the random coil content of the protein (for poly-L-glutamic acid $[\Theta]_{200} \sim -28000$ deg cm^2 dmol^{-1}) (ref.[10]). This difference cannot be accounted for by the short-wavelength dichroic

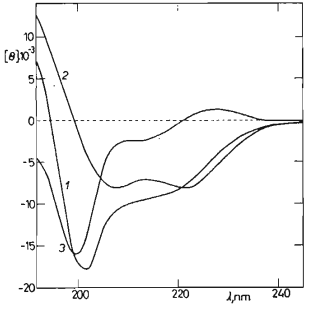

FIG. 3

Experimental Circular Dichroic Spectrum of Trypsin Inhibitor in 0·1M Tris-HCl Buffer, pH 8·0 *1*; Spectrum of 19% of α-Helical Conformation *2*; Difference Curve *3*

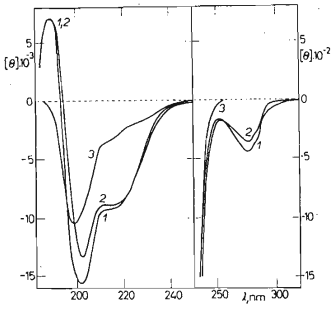

FIG. 4

Circular Dichroic Spectra in 0·01M-HCl *1* of Native Trypsin Inhibitor; *2* of Its Di-S-carboxymethylcysteinyl Derivative; *3* of Its Hexa-S-carboxymethylcysteinyl Derivative

band of disulfide groups which is – as follows from the characteristics of the trypsin inhibitor after reduction (see p. 2018) – obviously also negative. Another maximum on the difference curve, at 212 to 215 nm, cannot be ascribed to the chromophores in side chains and obviously belongs to $n-\pi^*$ transition of the peptide bond. The antiparallel β-pleated sheet conformation shows[9,10] a negative band in this region. However, it is not present in the trypsin inhibitor, as follows from the IR-spectra, in an amount greater than 5%. Therefore the negative difference band belongs most probably to the prevailing unordered structure. The CD spectrum of this part of the peptide chain of the trypsin inhibitor, which is in unordered, amorphous state, is thus characterized by a negative maximum at 200 nm and by a weak negative maximum in the 215 nm region. This spectrum differs from the spectrum of the random coil state of synthetic polypeptides in lower ellipticity of the short-wavelength band and by the opposite sign of the long-wave band.

It would thus appear that a similar type of the CD spectrum is characteristic generally of the unordered state of the peptide chain of the protein. It has been shown before[14–16] that experimental CD or ORD spectra of proteins often cannot be replaced merely by the sum of curves of known structures of synthetic polypeptides. One of the causes of this fact is the obvious difference between the conformation of the peptide chain in random coil state of polypeptides and in unordered, amorphous region of proteins. We may expect[15,17] that the compared structures will possess a different distribution of probability of conformation angles φ and Ψ. Tiffany and Krimm[18,19] were able to show that polypeptides of the polylysine and polyglutamic acid type contain in random coil state locally arranged regions (left-handed extended helix). Under the conditions when the extended helix regions undergo transition to the random coil state (by eliminating of electrostatic interactions of side chain charges), the polypeptide shows a CD spectrum of a type analogous to the difference spectrum shown in Fig. 3 : the ellipticity of the negative band at 200 nm decreases and a negative ellipticity in the region round 220 nm appears. A similar spectrum has been observed also with denaturated fibrous and globular proteins[19] and with phosvitin[20] in unordered state.

The results of our measurements show that the trypsin inhibitor contains a small amount of α-helical conformation. It would also appear that the α-helical regions bear no direct relation to the inhibitory activity of the protein. Thus, *e.g.* the time profile of activity[1] decrease in alkaline media is not paralleled by significant changes of ORD curves. A similar relation becomes apparent also after the reduction of one disulfide bond[3] (see p. 2018). The secondary structure of the pancreatic trypsin inhibitor is considerably stable to various external effects. The CD spectrum is independent of pH in the range between 2 and 8 (Fig. 1, 4, and 5). A more marked change is observable only in a strongly alkaline medium (0·01M-NaOH, Fig. 1). Neither does the CD spectrum of the trypsin inhibitor change in 8M urea at room temperature nor in 0·1% sodium dodecyl sulfate which cause merely a small decrease in the ellip-

ticity of the aromatic band. The ordered conformation of protein is, however, entirely destroyed by trifluoroacetic acid (*cf.* p. 2015) in which disappears also the aromatic band at 276 nm (ref.[3]).

Obviously, the disulfide bonds participate significantly on the stabilization of the secondary structure of the trypsin inhibitor. From this viewpoint interest deserve the characteristics of the reduced and carboxymethylated inhibitors. Fig. 4 shows the CD spectra of these derivatives which were measured in 0·01M-HCl because of the low solubility of the completely reduced protein at higher pH. The di-S-carboxymethylcysteinyl derivative (*i.e.* with reduced and carboxymethylated bond Cys^{14}-Cys^{38}), whose CD spectrum has been reported elsewhere[3], no longer has the ability to inhibit the enzymatic activity of trypsin[3], the position of dichroic bands and the general course of the curve, however, change only little after the chemical modification. The differences, which are rather insignificant with respect to the relative error of the measurement, seem to be inadequate to an essential change in biologic activity. A larger drop in ellipticity of the band at 202 nm can be accounted for by a decrease in the intensity of the negative dichroic band of disulfide group[13] after the reduction of one bond. It may thus be assumed that the site essential for the inhibitory activity of the pancreatic trypsin inhibitor is localized in that relatively small part of the molecule which is directly affected by the reduction and carboxymethylation of the corresponding disulfide bond. The ellipticity of the aromatic band at 276 nm is for

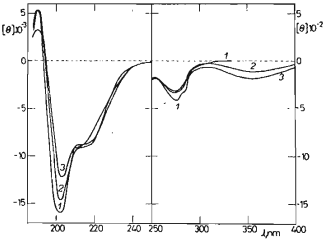

FIG. 5

Circular Dichroic Spectra in 0·05M Acetate Buffer, pH 4·8 *1* of Native Trypsin Inhibitor, *2* of Inhibitor with Tyr^{10} Nitrated; *3* of Inhibitor with Tyr^{10} and Tyr^{21} Nitrated

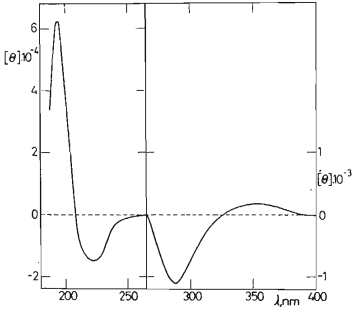

FIG. 6

Circular Dichroic Spectrum of Cyclo(3-nitrotyrosyl-glycyl) in Methanol

the di-S-carboxymethylcysteinyl derivative by 20% lower than for the native protein. On the other hand, substantial changes in dichroic absorption can be observed with the hexa-S-carboxymethylcysteinyl derivative of the inhibitor (Fig. 4). The positive maximum at 190 nm disappears, the negative short-wave band is bathochromically shifted toward 199 nm, and the ellipticity in the region of the $n-\pi^*$-band significantly decreases. These changes indicate a complete destruction of the α-helical conformation. The curve roughly corresponds to the expected type of spectrum characteristic of a unordered protein chain (*cf.* Fig. 3, curve *3*). The reduction of the remaining disulfide groups can again be responsible for an additional drop in the intensity of the negative band close to 200 nm. The hexa-S-carboxymethylcysteinyl derivative of the trypsin inhibitor does not show circular dichroism in the region of the aromatic band at 276 nm.

The results of our measurements show that the rotational strength of the aromatic dichroic band at 276 nm is conformationally conditioned. After the complete breakdown of the native secondary structure of the inhibitor, such as in trifluoroacetic acid or after complete reduction of the protein, the aromatic band entirely disappears. This band reacts, however, sensitively by changes in its intensity to a treatment which does not affect substantially the secondary structure of the protein (treatment with urea or detergent, partial reduction). Obviously in these cases only the immediate neighborhood is affected (*e.g.* the conformational rigidity is decreased) of a limited number, *i.e.* of one or two tyrosine residues situated at an accessible site on the surface of the protein molecule or in the region which becomes involved in the reduction of the disulfide bond (in the neighborhood of half-cystine residue 14 or, alternatively, 38). This would mean tyrosine residues 10 and 21 (ref.[3]). The ellipticity of the aromatic dichroic band in the native inhibitor as calculated for the molar concentration of the tyrosine residues is -6100 deg cm^2 dmol^{-1}, it is thus – regardless of the sign – 4·5-times higher than would correspond to the value released by amino acids (1320 deg cm^2 dmol^{-1}, ref.[21]) and approximately twice higher than the ellipticity of cyclic dipeptides[22] which contain a tyrosine or *p*-methoxyphenylalanine residue (3000 to 4000 deg cm^2 dmol^{-1}). The increased rotational strength of the aromatic chromophore in cyclic dipeptides can be explained by the fact that the backbone of their molecule has very rigid conformation and that the aromatic group is most likely in fixed parallel position with respect to the piperazinedione ring of the peptide[22]. Similarly, in the native inhibitor the mechanism increasing the rotational strength of the tyrosine chromophore will involve probably the high rigidity of its molecular neighborhood. A role may play moreover its specific interactions with other functional groups.

The CD spectra of nitrated derivatives of the pancreatic trypsin inhibitor (Fig. 5) in 0·05M acetate buffer, pH 4·8, confirm the results of ORD measurements[3]. The nitration of tyrosine 10 does not affect either the biological activity of the inhibitor or its secondary structure. The nitration of both tyrosines, 10 and 21, does not

affect the activity either but alters slightly the shape of the CD curve eventhough the positions of the amide bands are retained. This phenomenon might reflect a certain conformational rearrangement conditioned on the release of the conformation – stabilizing specific bonds of tyrosine (*e.g.* of hydrogen bonds) by nitration. The same relation between the intensity of the tyrosine dichroic band (around 276 nm) and the nitrotyrosine band (335 nm) which we have found[3] for the ORD spectra at pH 7·8, is observable also with the CD spectra at pH 4·8 (Fig. 5). While the ellipticity of the nitrotyrosine band increases essentially with the number of nitrated tyrosine residues and the two nitrated side chains are therefore equivalent as regards the rotational strength, a marked decrease in the ellipticity occurs in the region of the dichroic band at 276 nm after the first nitration step. The ellipticity at 276 nm does not practically change, however, after the nitration of the next tyrosine residue. In experiments with a model cyclic dipeptide, cyclo(3-nitro-L-tyrosyl-glycyl)[5] (Fig. 6) we have been able to show that the two long-wavelength absorption bands of the nitrophenol group at 356 and 279 nm show circular dichroism with opposite signs. It is possible that the dichroic absorptions of the tyrosine and the short-wavelength nitrotyrosine band are compensated in the region around 276–279 nm where these both bands overlap. When the contribution of the nitrotyrosine band at 279 nm in the protein is positive (*i.e.* when the relation between the signs of the two bands is the same as in the cyclic dipeptide) then the compensated change is negative and therefore the sign of the dichroic absorption of tyrosine 21 in the native inhibitor is opposite to that of dichroism of tyrosine 10, *i.e.* it is positive. Such a situation when the same chromophores in the side chains display dichroic bands of opposite signs is to be expected to exist in a number of proteins which in spite of their high content of aromatic amino acids or disulfide bonds do not show detectable dichroism in the region of their absorption.

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