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Structural and circular-dichroism studies on the interaction between human C1-esterase inhibitor and C1s

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The reaction between complement factor C1s and C1-esterase inhibitor has been investigated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, *N*-terminal amino acid analysis and c.d. studies. It is confirmed that a very stable stoichiometric 1:1 complex with a molecular weight of about 180000 is formed, involving the light chain of C1s. On the sodium dodecyl sulphate/polyacrylamide gels a small peptide with a molecular weight of about 5000 can be seen, which may be released from the *C*-terminal portion of the inhibitor moiety in a manner analogous to that occurring in other similar proteinase–inhibitor reactions. By *N*-terminal amino acid analysis, a newly formed threonine residue is found in the complex, suggesting that the inhibitor peptide chain is cleaved in the complex between C1s and C1-esterase inhibitor. The stabilizing bond may therefore be an ester bond. C.d. studies of the native C1-esterase inhibitor indicated the presence of about 38% α-helix, about 24% β-structure and about 38% unordered structure. By gradual cleavage of the disulphide bridges under non-denaturating conditions, gradual changes in the c.d. spectra occurred, suggesting loss of ordered secondary structures. The c.d. spectra of the complex between C1s and C1-esterase inhibitor indicate that tryptophan residues are affected by the complex-formation.

Among the major proteinase inhibitors of human plasma, C1-esterase inhibitor has been proposed to play a role in the regulation of the classical pathway of the complement system (Harpel, 1976), and possibly in the kallikrein system (Gigli *et al.*, 1970; Shapira *et al.*, 1982) and the fibrinolytic system (Kluft, 1977). It has been shown that the complement factor C1s, when added to plasma, is mainly inhibited by the C1-esterase inhibitor (Sim *et al.*, 1979; Nilsson & Wiman, 1982*a*). The structural data provided so far have indicated that a very stable stoichiometric 1:1 inactive complex is formed during this reaction (Harpel & Cooper, 1975), and it has been speculated that an ester bond might be involved in the stabilization of the complex (Chesne *et al.*, 1982). In the present study we have investigated the structural events occurring on complex-formation by chemical and c.d. methods.

Abbreviations used: the nomenclature of complement components is that recommended by the World Health Organisation [(1968) *Bull. W.H.O.* **39**, 935–936]; SDS, sodium dodecyl sulphate; DABITC, 4-*NN*-dimethylaminoazobenzene 4′-isothiocyanate; DABTH, the thiohydantoin derivative of DABITC; S-2134, D-valylseryl-arginine *p*-nitroanilide.

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Materials and methods

Proteins

C1s was prepared by a modification of the method of Chapuis *et al.* (1977), as described previously (Nilsson & Wiman, 1982*a*). C1-esterase inhibitor was purified from human plasma by precipitation with poly(ethylene glycol), batch adsorption of the supernatant to DEAE-cellulose, elution with a NaCl gradient, and finally hydrophobic-interaction chromatography on hexyl-Sepharose, in which the C1-esterase inhibitor passed through the column unadsorbed (Nilsson & Wiman, 1982*c*). The concentrations of the proteins were determined either by their specific absorbances at 280nm, using an $A_{280}^{1g/l}$ of 0.94 for C1s (Gigli *et al.*, 1976) and 0.50 for

$C\bar{1}$-esterase inhibitor (Harpel, 1976), or by amino acid analysis after acid hydrolysis (see below). The molecular weights used were 83000 (7% carbohydrate) for $C\bar{1}s$ (Gigli *et al.*, 1976) and 104000 (35% carbohydrate) for $C\bar{1}$-esterase inhibitor (Harpel, 1976). $C\bar{1}s$–$C\bar{1}$-esterase-inhibitor complex for structural studies was obtained by incubating $C\bar{1}s$ (about 1 mg/ml) and $C\bar{1}$-esterase inhibitor (about 1.5 mg/ml in 0.1 M-sodium phosphate buffer, pH 7.3) in about 20% molar excess (25°C, 2h). The complex was thereafter diluted 2-fold with distilled water and purified by chromatography on a column (5 $cm^2 \times 4$ cm) of DEAE-cellulose [a linear gradient of NaCl from 0 to 0.3 M, in 0.04 M-sodium phosphate buffer, pH 7.0 (total volume 500 ml) being used]. The complex eluted as a symmetrical peak at about 0.16 M-NaCl. It was subsequently dialysed extensively in the cold against distilled water and freeze-dried.

Reagents

The chromogenic tripeptide *p*-nitroanilide substrate S-2314 was a gift from Dr. Petter Friberger (Kabi Peptide Research, Mölndal, Sweden). Dithioerythritol was from Sigma (St. Louis, MO, U.S.A.). DEAE-Sephacel was from Pharmacia, Uppsala, Sweden. All other reagents and chemicals used were of analytical grade.

Determination of $C\bar{1}s$ and $C\bar{1}$-esterase-inhibitor activities

$C\bar{1}s$ solution (5–25 μl) was added to a cuvette containing 0.1 M-sodium phosphate buffer, pH 7.3 (total volume 0.9 ml), at 25°C. Then 100 μl of S-2314 (5 mM in 0.1 M-sodium phosphate buffer, pH 7.3) was added, and the A_{410} was recorded with a Beckman model-35 double-beam spectrophotometer equipped with a thermostatically controlled sample holder and a Beckman recorder. The initial change in A_{410} was calculated graphically as a measure of the $C\bar{1}s$ activity. With this system, 1 μM-$C\bar{1}s$ produced an increase in A_{410} of about 2.0/min. Determination of C1-esterase-inhibitor activity was performed by mixing a specific amount of $C\bar{1}s$ with the purified inhibitor (5–50 μl) in the cuvette and incubating for 3 min at 25°C. Subsequently the residual $C\bar{1}s$ activity was measured after dilution with the phosphate buffer to 0.9 ml and addition of substrate, as described above. An inverse linear relation was obtained between residual $C\bar{1}s$ activity and the amount of $C\bar{1}$-esterase inhibitor added.

N-Terminal amino acid determination

This was performed by the manual DABITC double-coupling technique (Chang *et al.*, 1978), and the resulting DABTH-amino acids were identified by t.l.c. on polyamide sheets as described by Chang *et al.* (1978).

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed on reduced and non-reduced samples as described by Weber & Osborn (1969), with 7% (w/v) polyacrylamide gels. Molecular weights were estimated by using a kit (Protein-Standardmischung I; Merck, Darmstadt, Germany) consisting of CNBr fragments of horse myoglobin with molecular weights of 2512, 6214, 8159, 14404 and 16949.

Reduction and S-carboxymethylation of $C\bar{1}$-esterase inhibitor

This was performed under non-denaturating conditions in 0.1 M-sodium phosphate buffer, pH 7.3, containing about 2 mg of $C\bar{1}$-esterase inhibitor/ml. Dithioerythritol was added to a final concentration of 10 mM or 200 mM in order to obtain reduction of two or three disulphide bridges respectively, according to results obtained previously (Nilsson & Wiman, 1982*c*). After 30 min incubation under an N_2 atmosphere, iodoacetate was added to a final concentration of 80 mM or 450 mM respectively, and incubation was continued in the dark for another 10 min. The mixture was therefore dialysed extensively against 0.1 M-sodium phosphate buffer, pH 7.3, in the cold.

Circular dichroism

C.d. spectra were recorded by using a Jasco J 41 automatic spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) thermostatically controlled at 25°C. The instrument was calibrated with D-10-camphorsulphonic acid (7,7-dimethyl-2-oxobicyclo-[2.2.1]heptane-1-methanesulphonic acid). The protein concentrations (0.5–1.5 mg/ml) and the pathlengths of the cells (0.13–2 cm) were chosen to optimize the measuring conditions. Each spectrum was recorded at least twice. The proteins were dissolved in 0.1 M-sodium phosphate buffer, pH 7.3, and centrifuged to remove dust particles. Protein concentrations were determined as described above. The ellipticity is expressed as molar ellipticity above 250 nm, and as mean residue ellipticity, $[\theta]_{m.r.w.}$, below 250 nm. The mean residue weight used was 115 in all cases. The content of secondary structures of the protein moieties of the different glycoproteins was calculated from the ellipticity values between 204 and 240 nm as described by Chen *et al.* (1974).

Results

Formation of the stable complex between $C\bar{1}s$ and $C\bar{1}$-esterase inhibitor

The complex between $C\bar{1}s$ and $C\bar{1}$-esterase inhibitor was obtained by incubation of mixtures of

these proteins with a 20% molar excess of either the enzyme (on activity basis) or the inhibitor (see the Materials and methods section). The results from SDS/polyacrylamide-gel electrophoresis of non-reduced and reduced samples of these mixtures are shown in Figs. 1(*a*) and 1(*b*) respectively. A complex with a molecular weight of about 180000 is formed, which involves the inhibitor and the light chain of $C\bar{1}s$. In addition, a small peptide with a molecular weight of about 5000 is observed that is not present in the parent molecules. This peptide is found in both the non-reduced and reduced samples of the complexes, independently of whether an excess of inhibitor or enzyme was used. Fig. 1 also shows that the $C\bar{1}s$ preparation contains about 10% of inactive material. No proteolysis products other than the peptide with mol.wt. 5000 can be detected. The minor contaminants (Fig. 1*a*, gels 3 and 4) are already detectable in the $C\bar{1}s$ preparation under appropriate loading. Also, since plasminogen-depleted serum is always used, there is no detectable plasmin in the $C\bar{1}s$ preparation.

About 30nmol of the purified $C\bar{1}s$–$C\bar{1}$-esterase-inhibitor complex was subjected to *N*-terminal amino acid analysis. The expected amino acids, glutamic acid, isoleucine and asparagine, were found in approximately equimolar amounts as judged from the colour intensities of the corresponding t.l.c. spots. In addition, the presence of δ-threonine, also in about the same amount, was detected. No other amino acid could be detected.

Dissociation of the $C\bar{1}s$–$C\bar{1}$-esterase inhibitor complex with NH_3

The purified enzyme–inhibitor complex was dissolved in 100μl of distilled water or 2% (w/v) SDS. Then the solutions were diluted with 0.5ml of 0.2M-NH_3 to a final protein concentration of about 1mg/ml, incubated in sealed tubes at 37°C for $\frac{1}{2}$h and subsequently freeze-dried. SDS/polyacrylamide-gel electrophoresis of these samples (Fig. 2) shows that the treatment with NH_3 alone did not result in any appreciable dissociation. However, if the complex had been pretreated with 2% SDS, a dissociation of more than 50% of the enzyme–inhibitor complex occurs. The molecular weight of the fragments thus obtained are similar to those of the parent molecules. No significant decrease in molecular weight of the released inhibitor moiety as compared with the native molecule could be ascertained. However, the low-molecular-weight (5000) peptide was also seen, and it was unaffected by the NH_3 treatment. Moreover, two additional faint bands can be seen after the NH_3 treatment. On SDS/polyacrylamide-gel electrophoresis of reduced samples, these two bands exhibited a mobility indistinguishable from the light and heavy chains of reduced $C\bar{1}s$. If higher NH_3 concentrations were

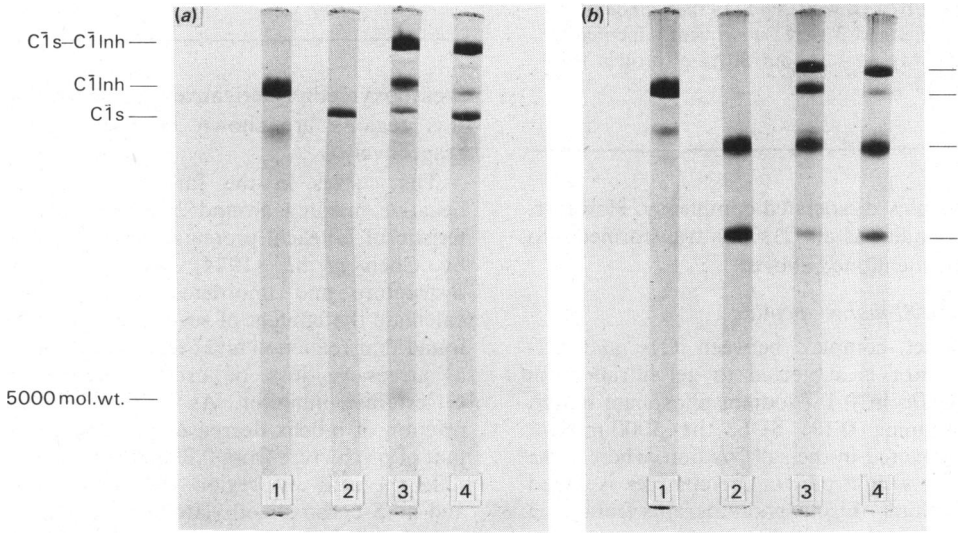

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of $C\bar{1}$-esterase inhibitor ($C\bar{1}Inh$) (1), $C\bar{1}s$ (2) and $C\bar{1}s$–$C\bar{1}$-esterase-inhibitor complex ($C\bar{1}s$–$C\bar{1}Inh$) obtained with inhibitor excess (3) or with enzyme excess (4)*

About 20μg of protein was applied on to each gel. (*a*) Non-reduced samples; (*b*) reduced samples (1%, dithioerythritol, 10min, 80°C).

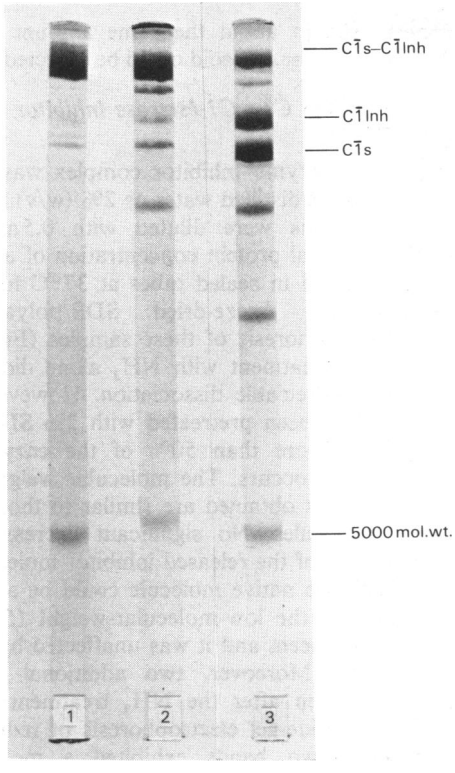

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of C1s–C1-esterase-inhibitor complex treated with NH_3*

1, Purified C1s–C1-esterase-inhibitor complex (C1s–C1Inh) dissolved in 2% SDS and incubated at 37°C for 30min; 2, the same complex dissolved in 0.2M-NH_3; 3, treated with 2% SDS then made 0.2M in NH_3. Both 2 and 3 were subsequently incubated in the same way as 1. About 30μg of protein was applied to each gel.

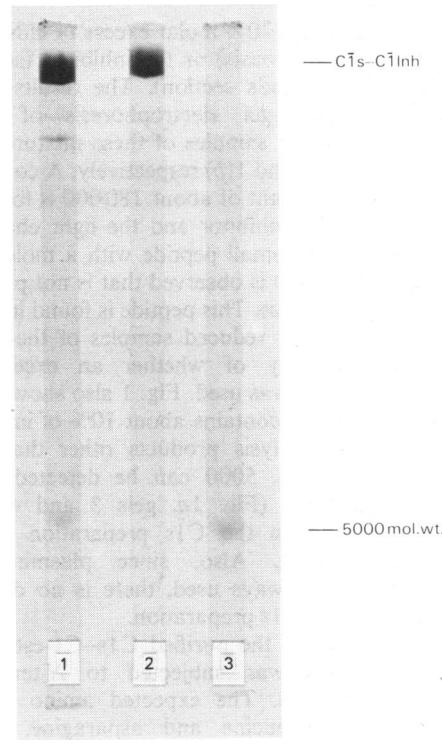

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of C1s–C1-esterase-inhibitor complex (C1s–C1Inh) (1), and the peptide material eluted in the void volume (2) or salt fraction (3) after gel filtration of the same complex on Sephacryl S-200 in the presence of 0.1% SDS*

About 20μg of protein applied to each gel.

used, the complex dissociated completely. However, under such conditions all C1s was transformed into the fragments mentioned above.

Isolation of 5000-mol.wt. peptide

If the intact complex between C1s and C1-esterase inhibitor is subjected to gel filtration on Sephacryl S-200 in 0.1M-sodium phosphate buffer, pH7.3, containing 0.1% SDS, the 5000-mol.wt. peptide is recovered in the salt fraction, whereas the high-molecular-weight part of the complex is eluted in the void volume. Electrophoretograms from such an experiment are shown in Fig. 3.

C.d. studies of native and reduced C1-esterase inhibitor

C.d. spectra of native C1-esterase inhibitor, as well as of its tetra-*S*-carboxymethyl and hexa-*S*-carboxymethyl derivatives, in the far- and near-u.v. regions are shown in Figs. 4(*a*) and 4(*b*) respectively.

The curves in the far-u.v. region show two negative maxima around 207 and 222nm, characteristic of α-helical proteins. When the values given by Chen *et al.* (1974) for normalized α-helix, β-structure and unordered forms were used to calculate the content of secondary structures, it was found that reduction and carboxymethylation causes a successive loss of ordered structures in the C1-esterase inhibitor. As shown in Table 1, the fraction of α-helix decreases from 0.38 to 0.25, and that of β-structure from 0.28 to 0.23.

In the near-u.v. region, the unmodified inhibitor and its *S*-carboxymethylated derivatives gave qualitatively similar c.d. spectra, in which contributions from all the aromatic amino acid side chains as well as from disulphide bridges overlap. The vibrational transitions originating from tryptophan residues (Strickland *et al.*, 1969, 1971) dominate, with maxima around 295–297, 287–289 and 285nm. The

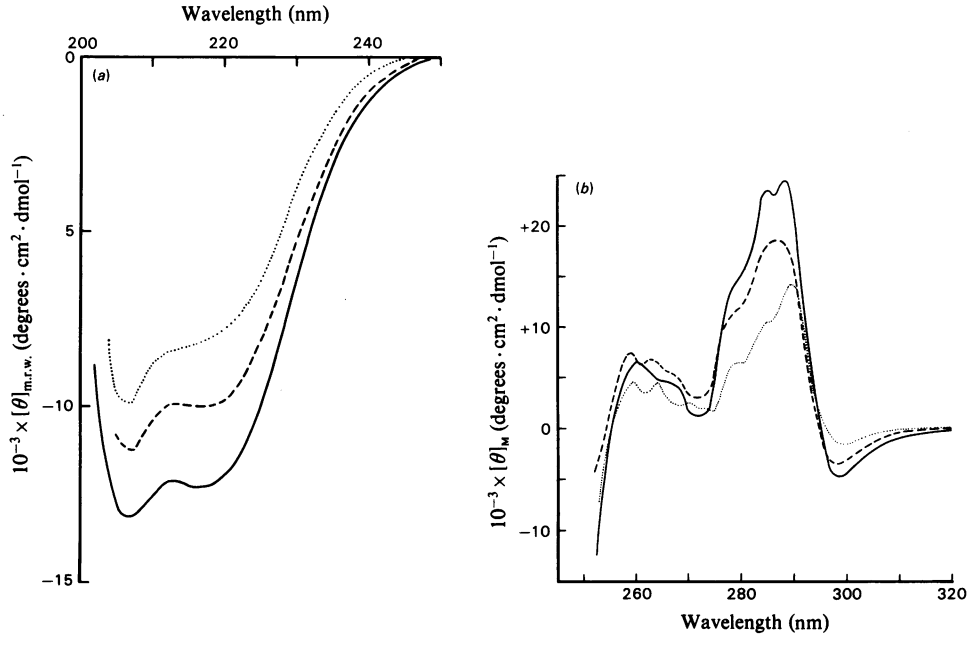

Fig. 4. *C.d. spectra of C$\bar{1}$-esterase inhibitor* (——), *tetra-S-carboxymethylated C$\bar{1}$-esterase inhibitor* (– – – –) *and hexa-S-carboxymethylated C1-esterase inhibitor* (· · · ·) *in the far- (a) or near- (b) u.v. regions*

Table 1. *Fractions of secondary structures as calculated from c.d. data by the method of Chen et al. (1974)*

Protein	Fraction α-Helix	β-Structure	Unordered forms
C$\bar{1}$-esterase inhibitor	0.38	0.28	0.34
Tetra-*S*-carboxymethylated inhibitor	0.31	0.24	0.45
Hexa-*S*-carboxymethylated inhibitor	0.25	0.23	0.52
C$\bar{1}$s	0.05	0.20	0.75
C$\bar{1}$s–C$\bar{1}$-esterase-inhibitor complex	0.24	0.26	0.50
C$\bar{1}$s–C$\bar{1}$-esterase-inhibitor complex, calculated	0.21	0.24	0.55

shoulders around 278nm can most probably be assigned to tyrosine residues, which may also contribute to the 285nm bands (Horwitz *et al.*, 1970; Horwitz & Strickland, 1971), whereas some of the fine structure of the spectra seen below 270nm (extremes at 262 and 268nm) can be assigned to phenylalanine residues (Horwitz *et al.*, 1969). The contributions from S–S bridges are generally more unspecific (Breslow & Weis, 1972; Strickland & Mercola, 1976; Bewley, 1977). Therefore the decrease of the ellipticity as a result of the reduction and *S*-carboxymethylation might be due either to the cleavage of the S–S bridges, or, alternatively, that the change of the polypeptide backbone to more unordered forms brought about an exposure of the aromatic side chains to the water medium. The changes seen in the near-u.v. region of the tetra-*S*-carboxymethylated inhibitor were also obtained with the unmodified inhibitor, if 10mM-dithioerythritol was added to the buffer.

C.d. studies on C$\bar{1}$s and its interaction with C$\bar{1}$-esterase inhibitor

The far- and near-u.v. spectra of C$\bar{1}$s are shown in Figs. 5(*a*) and 5(*b*) respectively. The mean-residue-weight ellipticity was low, indicating that the amount of α-helical structure is low. This also means that the estimation of the content of ordered structure by the method of Chen *et al.* (1974) will yield uncertain values that may be seriously affected by side-chain contributions and the length of the α-helices. The values given for C$\bar{1}$s in Table 1 should therefore be interpreted with caution. The c.d. spectrum in the near-u.v. region (Fig. 5*b*) is dominated by the tryptophan contribution around 296nm.

In Figs. 6(*a*) and 6(*b*), the far- and near-u.v. spectra of the stable complex between C$\bar{1}$s and C$\bar{1}$-esterase inhibitor are compared with the theoretical curves, representing the sum of the spectra of the individual components. Below 225nm, the

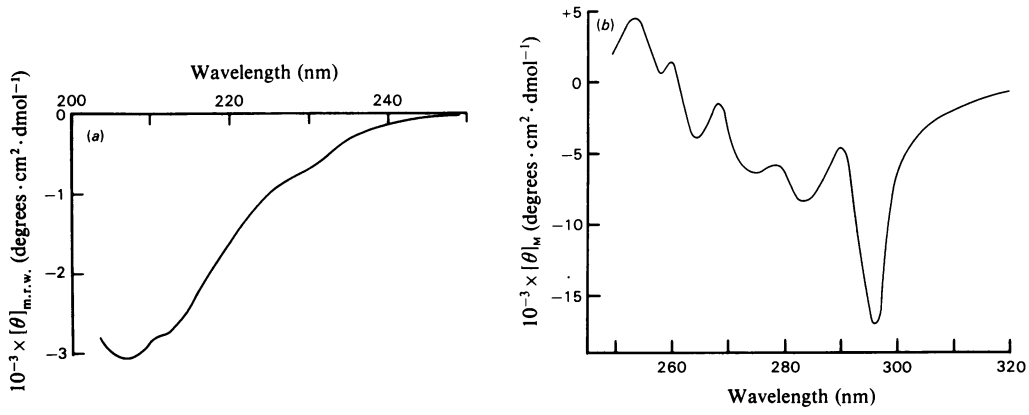

Fig. 5. *C.d. spectra of C1̄s in the far- (a) and near- (b) u.v. regions*

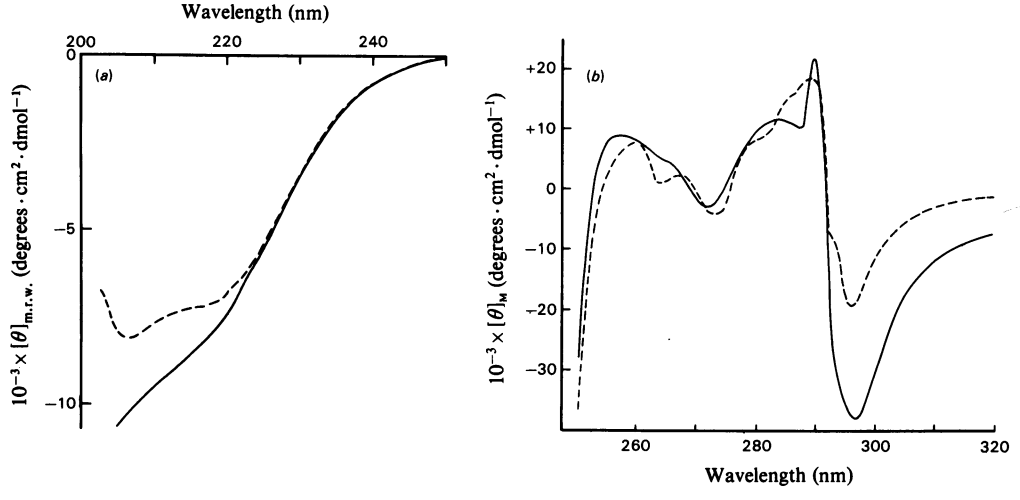

Fig. 6. *C.d. spectra of C1̄s–C1̄-esterase-inhibitor complex in the far- (a) and near- (b) u.v. regions*
——, Experimental curves; – – – –, theoretical curves obtained by summation of the contributions from the individual components.

ellipticity was more negative than expected, suggesting that the fraction of ordered structures may be somewhat larger in the complex than in the individual components. However, owing to the small fraction of α-helix in C1̄s, the quantitative evaluation of the spectra (Table 1) is uncertain. In the near-u.v. region (Fig. 6*b*) complex-formation resulted in a marked increase in negative ellipticity around 297 nm and a decrease of positive ellipticity around 285 nm, reflecting changes in tryptophan transitions.

Discussion

The reactions between many of the major proteinase inhibitors in plasma with proteolytic enzymes have been extensively studied during the last few years. Regarding α_2-antiplasmin (Wiman & Collen, 1979; Nilsson & Wiman, 1982*b*), antithrombin III (Fish *et al.*, 1979) and α_1-antitrypsin (Morii *et al.*, 1979), it has been suggested that formation of the very stable complexes with proteinases involves peptide-bond cleavage. C1̄-esterase inhibitor has

been shown to form a stable stoichiometric 1:1 complex with $C\bar{1}s$, but no peptide-bond cleavage was reported (Harpel & Cooper, 1975). In view of the results with the other inhibitors we therefore decided to investigate the $C\bar{1}s$–$C\bar{1}$-esterase-inhibitor reaction in more detail. Indeed, we were able to demonstrate the occurrence of a peptide with a molecular weight of about 5000 in the $C\bar{1}s$–$C\bar{1}$-esterase-inhibitor complex that was not present in the parent molecules. The peptide was demonstrated even before reduction, indicating that it is not disulphide-bonded to the rest of the complex. This is very similar to the results for the plasmin–α_2-antiplasmin reaction, where a peptide of about 8000 mol.wt. is released from the *C*-terminal portion of the inhibitor (Wiman & Collen, 1979; Nilsson & Wiman, 1982*b*). Furthermore, the results on the $C\bar{1}s$–$C\bar{1}$-esterase-inhibitor reaction are very similar, independently of whether excess inhibitor or enzyme was used, demonstrating the narrow specificity of $C\bar{1}s$ as a proteinase. On *N*-terminal amino acid analysis of the $C\bar{1}s$–$C\bar{1}$-esterase-inhibitor complex, all expected amino acids were found at about 1 mol/mol of complex, and in addition we were able to demonstrate a newly formed threonine residue by positive identification. These results suggest that the reactive-site peptide bond in the $C\bar{1}$-esterase inhibitor is cleaved in its stable complex with $C\bar{1}s$, and that the stability thus might be due to an ester bond with the active-site serine residue of $C\bar{1}s$, in agreement with a recent suggestion (Chesne *et al.*, 1982).

Recently we have shown that two of the three disulphide bridges in α_2-antiplasmin (Nilsson *et al.*, 1982) or in the $C\bar{1}$-esterase inhibitor (Nilsson & Wiman, 1982*c*) can be easily reduced without loss of the inhibitory activity, whereas reduction of the third disulphide bridge results in loss of the activity. In the present study we demonstrate that cleavage of the disulphide bridges in $C\bar{1}$-esterase inhibitor results in discrete changes of the c.d. spectra in both the near- and the far-u.v. regions. The data suggest a gradual decrease in ordered secondary structures.

The complex-formation between $C\bar{1}s$ and $C\bar{1}$-esterase inhibitor is also accompanied by characteristic changes in the c.d. spectra. In the near-u.v. region (Fig. 6*b*), a 2-fold increase in negative ellipticity is obtained, which is maximal around 297 nm, reflecting a tryptophan transition (Strickland *et al.*, 1969, 1971). Thus tryptophan residues are involved in the interaction between $C\bar{1}s$ and $C\bar{1}$-esterase inhibitor, either as a part of the interacting surfaces, or indirectly by secondary conformational changes. Changes in the c.d. spectra as a result of complex-formation have been detected previously in the interaction between chymotrypsin and α_1-antitrypsin (Bloom & Hunter, 1978). In addition, we were recently able clearly to demonstrate conformational changes in the formation of the plasmin–α_2-antiplasmin complex by a combination of c.d. studies (Nilsson *et al.*, 1982) and immunochemical studies (Wiman *et al.*, 1983).

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