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A Possible Receptor-Binding Function for the N-Terminus of Connective Tissue Activating Peptide III†

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ABSTRACT: Connective tissue activating peptide III (CTAP-III) is an 85-residue peptide which has been purified from platelets and shown to possess mitogenic activity toward a variety of fibroblastic cell lines. β-Thromboglobulin (βTG) is an 81-residue peptide which is derived from CTAP-III by cleavage of the N-terminal tetrapeptide Asn-Leu-Ala-Lys which results in the loss of mitogenic activity. The near-UV CD spectra for the two proteins indicated that the conformations as well as the electronic environments of the two disulfide bonds, and also of the single aromatic tyrosine residue, were similar in CTAP-III and βTG. However, differences in the far-UV CD spectra of these proteins indicated a substantial decrease in α-helical content for βTG (29%) as compared to CTAP-III (38%). Structure prediction analysis also suggested that the longer N-terminal segment of CTAP-III may form an α-helix. The N-terminal region of βTG, which lacks this tetrapeptide, was predicted to be in an unordered, or possibly a turn, conformation. This predicted structural difference appears to be due to the high helix-forming potential of the N-terminal tetrapeptide Asn-Leu-Ala-Lys in CTAP-III. These results suggest a possible structural role for the N-terminal region of CTAP-III in the expression of the biologic activities of this protein. On the basis of these studies, a reasonable hypothesis to account for the difference in mitogenic activity between βTG and CTAP-III is that the N-terminal region must be helical for receptor binding to occur.

Connective tissue activating peptide III (CTAP-III)[1] (also known as low-affinity platelet activating factor), β-thromboglobulin (βTG), platelet basic protein (PBP), and platelet factor 4 (PF4) are structurally related proteins located in the α-granules of human platelets and released by a number of factors that stimulate platelet activation (Paul et al., 1980). The amino acid sequence of CTAP-III is known (Castor et al., 1983), and its molecular weight determined from the primary sequence (9278) agrees with that estimated by SDS–PAGE (Castor et al., 1977). PBP is a probable precursor to CTAP-III and contains nine additional amino-terminal residues (Holt et al., 1986), while βTG is probably a product of CTAP-III since it lacks the amino-terminal sequence Asn-Leu-Ala-Lys found in CTAP-III but is otherwise identical in sequence with this peptide (Castor et al., 1983; Niewiarowski et al., 1980). The conversion of PBP to CTAP-III to βTG may be due to a platelet-derived heat-labile protease(s) (Niewiarowski et al., 1979). CTAP-III exhibits about 50% amino acid sequence homology with PF4 (Lawler, 1981; Niewiarowski & Levine, 1979), which is a high-affinity heparin-binding protein whose three-dimensional structure has recently been determined (St. Charles et al., 1989).

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[1] Abbreviations: CTAP-III, connective tissue activating peptide III; βTG, β-thromboglobulin; PF4, platelet factor 4; CD, circular dichroism.

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CTAP-III is a major mitogenic component of human serum that stimulates the in vitro synthesis and growth of human synovial, dermal, and cartilage-derived fibroblastic cell lines (Castor et al., 1979, 1981) and of several nonhuman cell strains (Paul et al., 1980; Castor et al., 1979). CTAP-III also stimulates glycolysis (glucose uptake and lactate formation) and glycosaminoglycan synthesis in these same cell lines (Castor et al., 1977, 1979; Castor & Whitney, 1979). CTAP-III-mediated stimulation requires both RNA and protein synthesis and appears to involve second messengers, such as cyclic AMP and prostaglandins (Castor, 1974, 1975). CTAP-III may also play a role in the formation of matrix ground substance and in the connective tissue proliferative responses associated with wound healing as well as with acute and chronic inflammation (Castor et al., 1983; Castor, 1981). With the use of antibody reagents that are cross-reactive with CTAP-III and βTG, elevated plasma levels of these peptides have been associated with many disease conditions including arthritis (Niewiarowski & Levine, 1979; MacCarter et al., 1981; Myers et al., 1980).

βTG lacks the biologic activities of CTAP-III in spite of its nearly identical sequence (Niewiarowski & Levine, 1979). Since the only chemical difference between βTG and CTAP-III is the slightly longer N-terminus of the latter, it is apparent that the N-terminal sequence, Asn-Leu-Ala-Lys, must exert an important influence on the function of CTAP-III. Biologic activity is not intrinsic to the tetrapeptide alone, since earlier studies demonstrated that a synthetic tetrapeptide with the sequence Asn-Leu-Ala-Lys does not stimulate DNA or glycosaminoglycan synthesis in fibroblastic cell lines nor does it potentiate or interfere with the mitogenic activity of CTAP-III (Castor, 1983). These results suggest that the structural environment of the tetrapeptide in addition to the tetrapeptide itself is critical for expression of CTAP-III activity. In this report, we have investigated the secondary and tertiary structures of CTAP-III and βTG by circular dichroism (CD) and also by structure prediction methods. We wished to ascertain whether structural differences between these two polypeptides could be observed, and whether such differences could be attributed to the N-terminal tetrapeptide and thereby might account for the differences in biological function for these two closely related proteins.

Materials and Methods

CTAP-III was prepared from an acetone precipitate of an acid/ethanol extract of outdated blood bank platelets by gel filtration and ion-exchange chromatography and was judged to be homogeneous by SDS–PAGE.

βTG was kindly provided by Dr. D. S. Pepper (Regional Center, Royal Infirmary, Edinburgh, Scotland) (Moore & Pepper, 1980). The amino-terminal sequences of the CTAP-III and βTG preparations were determined by automated phenyl isothiocyanate degradation with a Beckman 890C sequencer as described (Castor, 1983; Walz et al., 1977). Purity of the protein preparations was also estimated by an enzyme-linked immunoassay using a fluorophore end-point signal and an antibody preparation specific for the amino-terminal tetrapeptide sequence of CTAP-III (Davis et al., 1985). The protein concentrations of βTG and CTAP-III dissolved in distilled water were estimated from their absorbances at 215 and 225 nm (Waddell, 1956).

For CD measurements, the proteins were adjusted to equivalent concentrations based on superposing UV absorbance spectra in the range 205–240 nm. CD spectra were recorded on a JASCO 500A recording spectropolarimeter. Molar ellipticities for the near-UV CD were computed by assuming molecular weights of 9278 for CTAP-III (Castor et al., 1983) and 8851 for βTG (Niewiarowski et al., 1980). For the far-UV CD, molar ellipticities were calculated by assuming a mean residue molecular mass of 109 g/mol based on the amino acid composition (Castor et al., 1983; Niewiarowski et al., 1980). The program CONTIN (Provencher & Glockner, 1981) was used to analyze secondary structures from the far-UV CD spectra. The method described by Argos (1985) was used to predict protein secondary structures from the amino acid sequences of CTAP-III and βTG.

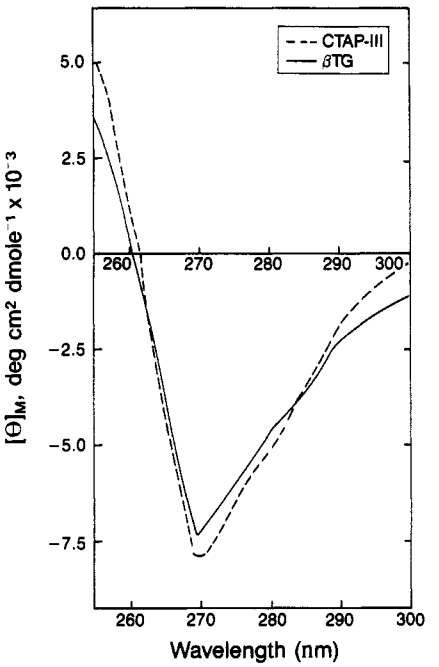

FIGURE 1: Near-UV CD spectra for CTAP-III and βTG. Lyophilized samples of CTAP-III and βTG were dissolved at 0.5 mg/mL in a buffer composed of 0.005 M sodium phosphate/0.75 M NaCl, pH 7.2. Spectra were measured in a 2-mm quartz cell at 22 °C. Thirty-two scans were recorded with a scan rate of 50 nm/min and a time constant of 0.25 s. Spectra were averaged, and the base line was subtracted.

Results and Discussion

The amino-terminal sequence of the βTG preparation was determined to be Gly-Lys-Glu-Glu-(Ser)-Leu in agreement with the published sequence (Niewarski et al., 1980; Castor et al., 1983). No other amino acids were detected. Using an enzyme-linked immunoassay and antibody specific for the CTAP-III amino-terminal tetrapeptide, no CTAP-III reactivity was detected for the βTG preparation. On the basis of the above assays, the βTG preparation was judged to contain less than 1% contamination with CTAP-III. The CTAP-III preparation gave 15% of the same sequence as βTG and 85% of a sequence beginning with Asn-Leu-Ala-Lys-Gly-Lys on the sequencer. Thus, the CTAP-III preparation contained approximately 15% of the βTG peptide product.

The near-UV CD spectra of proteins and peptides reflect the electronic environments around the aromatic amino acids and disulfides and are thus a sensitive monitor to changes in tertiary structure (Strickland, 1974). The near-UV CD spectra for βTG and CTAP-III adjusted to equivalent protein concentrations were nearly identical and exhibited rather broad minima ($[\theta]_m$ ~7500 deg·cm^2/dmol) near 270 nm (Figure 1). The intensity of the CD spectra and also their lack of fine structure suggest that the near-UV CD of CTAP-III and βTG are dominated by the two disulfides (Strickland, 1974). The equivalence of the two spectra indicates that the conformations and environments of the disulfides and also of the single

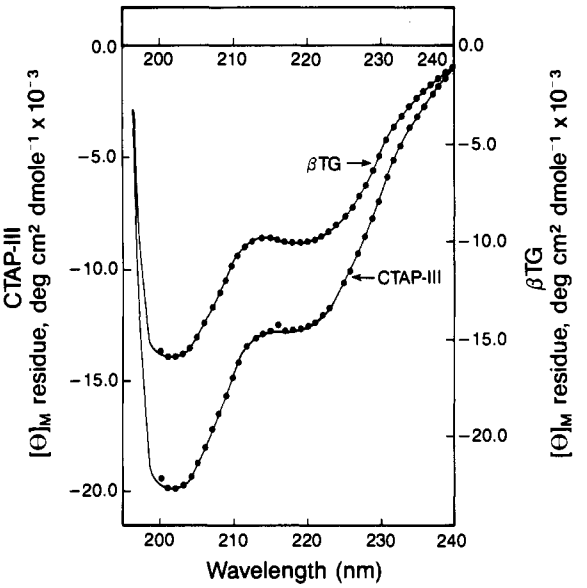

FIGURE 2: Far-UV CD spectra of CTAP-III and βTG. Solution conditions: 0.001 M sodium phosphate buffer/0.15 M NaCl, pH 7.2, 22 °C. Protein concentrations: 0.066 mg/mL for CTAP-III; 0.058 mg/mL for βTG. Thirty-two spectra were recorded at 20 nm/min with a time constant of 1 s. Solid circles indicate averaged, baseline-corrected spectra. Solid lines represent the computed spectra based on a linear combination of CD spectra of 16 proteins whose secondary structures are known accurately from X-ray crystallography (Provencher & Glockner, 1981).

Table I: Secondary Structure Estimates for CTAP-III and βTG[a]

	βTG		CTAP-III	
	CD	prediction	CD	prediction
α-helix	29	32	39	41
β-sheet	19	12	13	12
remainder	52	56	47	47

[a] All values are expressed as percent. Secondary structure analysis of CD spectra was performed by the method of Provencher and Glockner (1981). Secondary structure estimates for CTAP-III have been corrected for the presence of 15% βTG in the sample. The resulting estimates all lie within one percentage point of the uncorrected values (38%, 14%, and 48% for α-helix, β-sheet, and remainder, respectively). Secondary structure predictions based on amino acid sequence data were performed by the method of Argos (1985). Amino acid conformational preference parameters (Argos et al., 1983) were computationally smoothed over successive groups of three residues; three smoothing cycles were carried out. The values for the predicted remainder structure are given as the sum of the predicted turn and coil structures (shown in Figure 3) in order to facilitate comparison with the CD analysis, which does not distinguish between coil and turn.

aromatic tyrosine side chain are similar for both peptides.

The far-UV CD spectra for βTG and CTAP-III, on the other hand, differed substantially (Figure 2). While both spectra were similar in overall shape, with minima around 202 and 220 nm, the spectrum for CTAP-III exhibited a higher degree of ellipticity. Both spectra were subjected to secondary structure analysis which attempts to fit an observed CD spectrum to a linear sum of experimental CD spectra obtained for 16 proteins of known three-dimensional structure (Provencher & Glockner, 1981). Good agreement between calculated and observed spectra was obtained for both CTAP-III and βTG by this analysis. On the basis of the resulting secondary structure estimates from this analysis (Table I), CTAP-III was predicted to contain a substantially higher α-helix content (38%) than βTG (29%).

Secondary structure predictions of α-helix content, based on the amino acid sequences for both proteins, agreed with the experimentally derived quantities obtained from the CD

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          -4   1        10        20        30        40
CTAP-III  NLAK GKEESLDSDL YAELRCMCIK TTSGIHPKNI QSLEVIGKGT
          aaaa aaaaatttta aaaaaaaaaa ttttttttt aaaaactttt

βTG       GKEESLDSDL YAELRCMCIK TTSGIHPKNI QSLEVIGKGT
          ttttttttta aaaaaaaaaa ttttttttt aaaaactttt

                  50        60        70        80
          HCNQVEVIAT LKDGRKICLD PDAPRIKKIV QKKLAGDESAD
          aaaaabbbbb ctttcccctt ttttbbbbb aaaaatttttt
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FIGURE 3: Secondary structure predictions for CTAP-III and βTG. The two sequences are aligned with numbering based on the βTG sequence. Only the N-terminal portion of the CTAP-III sequence is shown since the C-terminal region predicts identically with βTG. The predicted secondary structures are annotated a (helix), b (strand), t (turn), and c (coil). Predictions were done by using the procedure described by Argos (1985).

analysis (Table I). The predicted disposition of secondary structure elements for CTAP-III and βTG suggested that the secondary structures of the N-termini of these proteins are substantially different (Figure 3). The first nine residues of CTAP-III predict α-helix; this region encompasses the first five N-terminal residues of βTG which are predicted to be in an unordered conformation in the latter protein. The difference in predicted structure for the N-termini of CTAP-III and βTG is a consequence of the strong helix-forming potential of the tetrapeptide Asn-Leu-Ala-Lys present only in CTAP-III. These results suggest that removal of the N-terminal tetrapeptide during the proteolytic conversion of CTAP-III to βTG results in conversion of the N-terminus from a helical to a disordered, or possibly a turn, structure and that this conversion has little structural effect on the remainder of the peptide. These conclusions are supported by both the near- and far-UV CD results. The latter indicated a relative increase of 9% in α-helix content (equivalent to about eight residues, or about 10% of the structure) for CTAP-III compared to βTG. Such a marked difference in secondary structure would also be expected to elicit changes in the near-UV CD if there are any optically active residues associated with or nearby these secondary structure elements. The only residues which would be expected to give rise to near-UV CD for both proteins are Tyr11 and the disulfides formed by Cys16–Cys42 and Cys18–Cys58 (βTG numbering is used). Since the near-UV CD spectra in βTG and CTAP-III were nearly identical, the secondary structure differences should be confined to regions near the termini and away from the interior sequences, consistent with the structure prediction results. Moreover, it would be difficult to ascribe the differences in the far-UV CD spectra to structures other than the N-termini since the two proteins are otherwise identical in sequence.

The proposed difference in secondary structure may be the basis for the functional differences of CTAP-III and βTG. The synthetic N-terminal tetrapeptide Asn-Leu-Ala-Lys does not exert any of the biological activities of CTAP-III nor does it seem to be able to compete with CTAP-III for receptor binding (Castor, 1983). The analysis presented here suggests that the tetrapeptide at the N-terminus of CTAP-III may serve to nucleate a helix which could span eight or nine residues. This helix would encompass the first four to five residues of the βTG sequence which are predicted to be nonhelical in this protein. This is an example of a context-dependent structure—a sequence, in this case the residues Gly-Lys-Glu-Glu-(Ser), which can assume two alternative secondary structures depending on the presence or absence of the upstream tetrapeptide Asn-Leu-Ala-Lys. Possibly it is the N-terminal helix which confers biologic activity to CTAP-III by presenting the N-terminal residues in a conformation required for receptor binding. This hypothesis could explain the biologic inactivity of βTG; it is inert because it lacks the appropriate structure

at its N-terminus. Whether this recognition structure is comprised only of the tetrapeptide Asn-Leu-Ala-Lys or whether it includes additional residues in common with the N-terminal segments of both proteins cannot be ascertained from this analysis.

The predicted structural polymorphism for the N-terminal Gly-Lys-Glu-Gly-(Ser) segment in βTG and CTAP-III suggests that this segment is relatively flexible or malleable. Although direct experimental evidence for the secondary structure of a given region of a protein or a polypeptide can best be provided by X-ray or NMR crystallography, CD spectroscopy is a very sensitive technique for assessing secondary and tertiary structural differences. The application of CD to the present example was particularly powerful since any CD spectral differences should readily be attributable to the N-terminal regions—the only regions in the two proteins under study which differ chemically. The recently determined three-dimensional structure of PF4 (St. Charles et al., 1989) indicates that this closely related protein is structurally disordered in the region which is homologous in sequence with the N-termini of CTAP-III and βTG. Crystallographic disorder is often due to structural flexibility. In conclusion, the sequence data, near and far-UV CD spectra, and secondary structure prediction analyses altogether provide the basis for an internally consistent hypothesis for a role for the tetrapeptide, Asn-Leu-Ala-Lys, as a helix-nucleating agent in CTAP-III. The resulting tendency of the N-termini of CTAP-III and βTG to form different secondary structures may account for the functional differences, such as receptor binding, exhibited by these two closely related proteins.

Added in Proof

Castor et al. (1989) have recently shown that the des-1–13 and des-1–15 isoforms of CTAP-III exhibit some of the biological activities characteristic of the parent peptide. Thus, regions of CTAP-III in addition to the amino-terminal tetrapeptide are likely to play a role in modulating the receptor binding and biological functions of this molecule. Their results also suggest that the sequence immediately downstream of the tetrapeptide may play a role in masking the activity of βTG but not of CTAP-III in which we find that this sequence is probably involved in a helical structure.

Registry No. Connective tissue activating peptide III, 69344-77-0.

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