Helical Conformation at the Carboxy-Terminal Portion of Human C3a Is Required for Full Activity†

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ABSTRACT: Human C3a, a 77-residue fragment released during complement activation, is a potent spasmogen that contracts smooth muscle, enhances vascular permeability, and suppresses humoral immune responses. Studies with synthetic peptides have shown that the active site of this anaphylatoxin resides in the COOH-terminal portion of C3a; the minimal peptide structure capable of expressing activity contains residues 73–77, Leu-Gly-Leu-Ala-Arg (C3a-73–77). Longer synthetic C3a analogue peptides, e.g., C3a-57–77 containing the 21 COOH-terminal amino acids, exhibit activity nearly equivalent to that of intact C3a. Circular dichroism spectra of peptide C3a-57–77 in aqueous buffer containing 25% (v/v) trifluoroethanol indicated helical structure (41% helix), and analysis of the sequence suggested an amphipathic surface. We have synthesized several 21-residue peptide analogues of the natural C3a sequence containing residues 57–77 that were designed to enhance helix and to accentuate amphipathy. Syntheses were designed to include strategic placement of the helix-promoting residues 2-aminobutyric acid (β-methylalanine) and 2-aminoisobutyric acid (α-methylalanine). Two 21-residue C3a analogue peptides that were designed to enhance helical content were shown to exhibit greater biological activity than either the native factor C3a or C3a-57–77. Moreover, activity was abrogated by the appropriate placement of helix-breaking residues, e.g., proline, suggesting that a conformational requirement for activity is genuine. These observations suggest that a helical conformation is requisite for optimal C3a activity and that in intact C3a the NH_2-terminal portion (residues 1–21) and the disulfide-linked core (residues 22–57) function primarily to stabilize ordered conformation at the COOH-terminal region of the molecule.

Human anaphylatoxin C3a is a 77-residue fragment cleaved during activation of the third component of the serum complement system. It is as potent humoral effector of the inflammatory response (Hugli & Muller-Eberhard, 1978; Hugli, 1981, 1984) and recently has been associated with immunoregulatory activity (Morgan et al., 1982). Understanding the functional attributes of this factor has been enhanced by numerous structural studies. Following the amino acid sequence determination of human C3a in 1975 by Hugli, structure–function studies using synthetic peptides ensued (Hugli & Erickson, 1977; Caporale et al., 1980). These efforts gave rise to a series of peptides based on the COOH-terminal sequence of C3a that exhibit activity and specificity similar to those of the native polypeptide. The essential active site of the anaphylatoxin was shown to reside in the carboxy-terminal pentapeptide Leu-Gly-Leu-Ala-Arg (C3a-73–77), and increasing the length of the peptide increased activity.

In 1980, Huber *et al.* reported the X-ray structural analysis of crystalline human C3a and presented a molecular model of the polypeptide. Three features of the model are noteworthy: (1) residues 15–57 form a globular core consisting of three different helical stretches linked to one another by three disulfide bonds; (2) projecting from this central domain to the carboxy terminus is a peptide region consisting of 20 residues of which 15 assume a helical arrangement capped by the active site pentapeptide, residues 73–77; (3) in the crystals used, the amino-terminal residues 1–14 were unresolvable. We have portrayed two possible configurations for C3a in solution. Figure 1A shows a model with amino- and carboxyl-terminal portions extended; conversely, Figure 1B portrays a more compact molecule with residues 1–14 folded back and possibly interacting with the "core" portion of the molecule as recently proposed for human C5a by Greer (1985).

A 21-residue peptide corresponding to the C3a carboxy terminus, i.e., residues 57–77, has been synthesized and shown by Huey et al. (1984) and Lu et al. (1984) to be nearly equivalent in activity to the natural C3a molecule. Lu et al. (1984) also examined the structural and conformational properties of peptide C3a-57–77.[1] Circular dichroism data indicated that the synthetic C3a peptide assumed an ordered conformation only in aqueous phosphate buffer containing trifluoroethanol (TFE); in the presence of 25% TFE, a helical content of 41% was estimated. Additionally, an amphipathic character was realized when the peptide was analyzed by the helical wheel axial projection as described originally by Schiffer and Edmundson (1968).

Results of our work with synthetic peptide analogues to the natural 21-residue COOH-terminal peptide of human C3a are reported. These peptide analogues were designed to retain topological features required for amphiphilicity, as gleaned from computer graphic analysis of the human C3a crystal structure, and to assume a more stable helical conformation. The latter was achieved by incorporating helix-promoting amino acid residues 2-aminobutyric acid (Paterson & Leach, 1978) and 2-aminoisobutyric acid (Burgess & Leach, 1973; Paterson et al., 1981) at every fourth position in the sequence

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[1] Synthetic peptide nomenclature: C3a-57–77, naturally occurring COOH-terminal sequence of C3a; C3a-57–77-Ala,Ab, alanine at residue positions 57, 59, 61, 63, 67, and 71 and Ab at residue positions 60, 64, 68, and 72; C3a-57–77-Ala,Aib, alanine at residue positions 57, 59, 61, 67, and 71 and Aib at residue positions 60, 64, 68, and 72; C3a-57–77-Ala,Ab,Pro_2, same as C3a-57–77-Ala,Ab except proline at residue positions 62 and 70.

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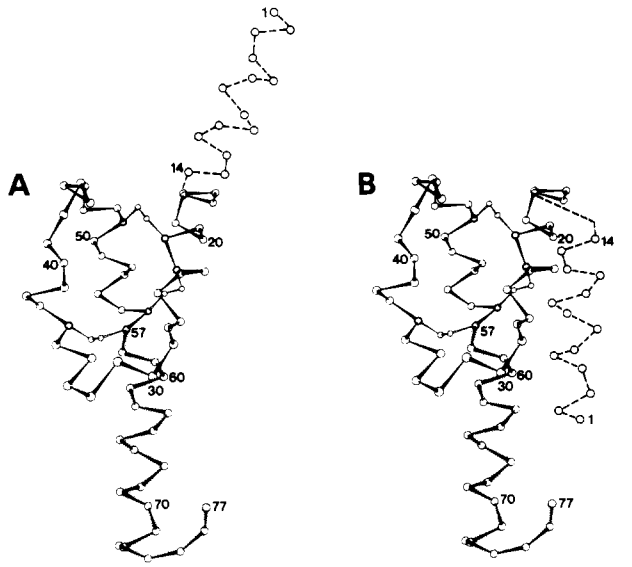

FIGURE 1: Molecular models portraying the backbone peptide structure of human C3a anaphylatoxin have been adapted from the X-ray crystallographic analysis of C3a by Huber et al. (1980). The amino-terminal residues 1–14 were not resolved by the X-ray studies and are indicated here by dashed lines. We propose two possible conformations for the folded C3a molecule in solution. Panel A portrays the amino terminus as a helix projecting away from the disulfide-linked core of the molecule. Panel B shows the amino-terminal region folded back onto the globular core and proximate to the carboxy-terminal region, which contains helix and the active center of the molecule at the terminus. In each model, residues 57–77 are highlighted.

starting from the carboxy end at position 72 (Table II). As the peptide folded, it was thought that the helix-stabilizing hydrogen bonds would most likely occur between the carbonyl oxygen and the amide proton of methylalanyl residues separated by three amino acids. By enhancement of helix formation and retainment of amphiphilic topology, a series of synthetic peptides were made that were approximately 2.5 times as active as native C3a and showed an estimated helical content of 49 ± 2% in aqueous trifluoroethanol mixtures.

Experimental Procedures

Solid-Phase Peptide Synthesis. Reagents used in peptide syntheses were purchased from the indicated vendor: Boc amino acids, Bachem and Vega; diisopropylcarbodiimide, 2-aminoisobutyric acid, and L-2-aminobutyric acid, Aldrich. The *tert*-butoxycarbonyl (Boc)[2] derivative of L-2-aminobutyric acid was prepared a described by Itoh et al. (1975) or purchased from Bachem Fine Chemicals, Torrance, CA. Modification of the procedure of Itoh et al. was necessary to prepare Boc-2-aminoisobutyric acid. Briefly, 10.3 g (0.1 mol) of 2-aminoisobutyric acid was added to a rapidly stirred solution of 2-methyl-2-propanol/water (3.5:1); addition of 4 g (0.1 mol) of di-*tert*-butyl dicarbonate (Bachem) over 30 min followed. The reaction mixture temperature rose to 45 °C and then cooled to ambient temperature as stirring continued overnight. The turbid reaction solution was diluted to 200 mL with water, and extracted 2 times with 30-mL portions of hexane. The aqueous phase was acidified to pH 3 by addition of solid citric acid and extracted 4 times with 30-mL portions of cold ethyl acetate. The pooled extracts were dried over anhydrous Na_2SO_4, and solvent was removed in vacuo to yield a yellowish oil that readily crystallized with the addition of hexane at 4 °C. Collected crystals were washed with hexane and recrystallized from ethyl acetate/hexane: 10.2 g (50.2%), mp 116–118 °C [lit. mp 117–118 °C (Jorgensen, 1981)]; NMR ($CDCl_3$) δ 1.34 [s, 9 H, $C(CH_3)_3$], 1.53 [s, 6 H, $C(CH_3)_2$].

All peptides were synthesized according to established Merrifield solid-phase synthetic procedures (Erickson & Merrifield, 1976; Stewart & Young, 1984). The first amino acid Boc-N^{δ}-tosyl-L-arginine, was esterified to chloromethylated polystyrene resin (Bio-Rad, 1% cross-linked, 1.37 milliequiv/g) as described previously (Hoeprich & Doolittle, 1983). Subsequent amino acids were double coupled, initially through a diisopropylcarbodiimide-mediated reaction and secondly by utilizing a preformed 1-hydroxybenzotriazole ester in dimethylformamide. In the case of Boc-2-aminoisobutyric acid, coupling was accomplished with only the performed 1-hydroxybenzotriazole ester in a 50% mixture of dimethylformamide and dimethyl sulfoxide for periods of time ranging from 3 h to overnight. In general, the Boc group was removed by treating the protected peptide resin for 25 min with 45% trifluoroacetic in dichloromethane (v/v), followed by 5-min neutralization with 5% diisopropylethylamine in dichloromethane (v/v). The resin was washed before and after each deprotection, neutralization, and coupling step with appropriate solvents. Boc group removal and completeness of coupling was monitored qualitatively by a ninhydrin color test (Kaiser et al., 1970). All peptides were cleaved from the resin, and simultaneous side-chain protecting group removal was attained by exposure to anhydrous hydrogen fluoride (HF) for 40 min at 0–4 °C in the presence of a 5-fold molar excess of anisole. After removal of HF by water aspiration, the resin was washed with anhydrous ethyl ether to remove anisole. The crude peptide was washed from the resin with 10% acetic acid (200 mL/g of resin); the combined washings were lyophilized.

Purification of the peptides was accomplished by reverse-phase high-performance liquid chromatography (HPLC). With a semipreparative column (0.9 × 50 cm, μBondapak, Waters), the desired peptide was eluted during gradient development of the chromatogram. The elution program ranged from 90% solvent A (0.1% trifluoroacetic acid/water) and 10% solvent B (0.03% trifluoroacetic acid/acetonitrile) to 50% A and 50% B over 65 min. Prior to experimental application, the purified peptides were desalted on reverse-phase Sep-Pak cartridges (Waters) as described by the manufacturer and lyophilized. Amino acid analyses were performed on a Spinco Model 121M analyzer (Beckman). A portion of each peptide was hydrolyzed in 5.7 N HCl at 110 °C for 24 h in evacuated sealed tubes. Amino acid sequence analysis of selected peptides was determined with a gas–liquid phase sequenator (Applied Biosystems, Model 470A).

Circular Dichroism Spectroscopy. CD spectra were recorded with a Jobin-Yvon Dicrographe III attached to a Nicolet (Model 535) signal averager. Silica cuvettes with light path length of 0.2 cm were used for recording far-UV spectra. The reported spectra are an average of at least two measurements; each spectrum was corrected for base-line absorbance. The mean residue ellipticity is expressed in units of deg cm^2/dmol. The mean residue weights used were 115 for C3a-57–77 and 102 for C3a-57–77-Ala,Ab or C3a-57–77-Ala,Aib and C3a-57–77-Ala,Ab,Pro_2. Peptides were solubilized in 0.1 M phosphate buffer, pH 7.2, at a final concentration of 1 mg/mL. An aliquot of each (100 μL) was diluted to 1 mL in a volumetric flask with water or with an appropriate TFE/water mixture. Each sample was allowed to incubate in the instrument at 25 °C for 30 min under nitrogen purge prior to recording the spectrum.

Computer Graphic Analysis. A tape of atomic coordinates for crystalline human C3a was kindly provided by Professor

[2] Abbreviations: Ab, 2-aminobutyric acid; Aib, 2-aminoisobutyric acid; Boc, *tert*-butyloxycarbonyl.

Table I: Amino Acid Composition of C3a-57–77 and Analogue Peptides[a]

amino acid	C3a-57–77	C3a-57–77-Ala,Ab	C3a-57–77-Ala,-Aib	C3a-57–77-Ala,-Aib,Pro_2
histidine	1.9 (2)			
arginine	4.2 (4)	3.3 (3)	3.1 (3)	3.0 (3)
aspartic acid	1.1 (1)	1.0 (1)	1.2 (1)	1.0 (1)
threonine	1.0 (1)			
serine	1.1 (1)			
glutamic acid	2.2 (2)	2.0 (2)	2.1 (2)	1.0 (1)
proline				2.4 (2)
glycine	1.0 (1)	0.9 (1)	1.1 (1)	1.0 (1)
alanine	2.5 (2)	8.2 (8)	8.1 (8)	6.6 (7)
cysteine	0.8 (1)			
aminobutyric acid		4.2 (4)[b]		4.4 (4)
aminoisobutyric acid			3.7 (4)[c]	
isoleucine	1.0 (1)			
leucine	3.2 (3)	2.5 (2)	2.4 (2)	2.0 (2)
tyrosine	0.8 (1)			

[a] Values are expressed as moles per mole of peptide and represents an average of three determinations. [b] Color factor used for α-aminobutyric acid = 7.61×10^{-5}. [c] Color factor used for α-aminoisobutyric acid = 2.24×10^{-4}.

R. Huber, Max-Planck Institute, Munich. This structure was analyzed by GRAMPS graphic language interpreter (O'Donnell & Olsen, 1981) augmented with GRANNY—a molecular modeling program designed to handle molecular surface features in an interactive real time manner (Connolly & Olsen, 1983).

Biological Activity. Guinea pig ileal assays were performed as described by Schultz (1910). Briefly, ileal strips were suspended in a 1.5-mL bath containing Tyrode's buffer at pH 7.4 and 37 °C with continuous oxygen purge. Concentrations of peptide or native factor were added at a minimal dose range ($n = 4$) to induce muscle contraction, i.e., full pen deflection.

Vascular permeability was measured in guinea pig skin following the procedure of Cochrane and Muller-Eberhard (1968). Samples were injected subcutaneously into the shaved back of a guinea pig that, 5–10 min previously, had been given 0.5 mL of a solution of Evan's blue dye (1% w/v) by cardiac puncture. After 30 min, the animal was sacrificed and skinned and the extent of dye infiltration assessed.

Results

Peptide Structure. The peptide corresponding to residues 57–77 of C3a and analogues to this 21-residue molecule were synthesized as described above. A small portion of each purified peptide was hydrolyzed and evaluated by amino acid analysis; in each case, the observed values were in excellent agreement with theoretical values (Table I). Additionally, a small portion (20–50 nmol) of material was sequenced with Edman chemistry as performed by a gas–liquid phase sequenator. The sequence analyses of C3a-57–77 and the analogue containing 2-aminobutyric acid confirmed the expected order of residues; as such, other peptide analogues were not sequenced, trusting in the fidelity of Merrifield chemistry (Table II).

Computer graphic analysis of the C3a structure, in particular the carboxy-terminal region, revealed a ridge of hydrophilic residues spanning one side of the helix. Contained

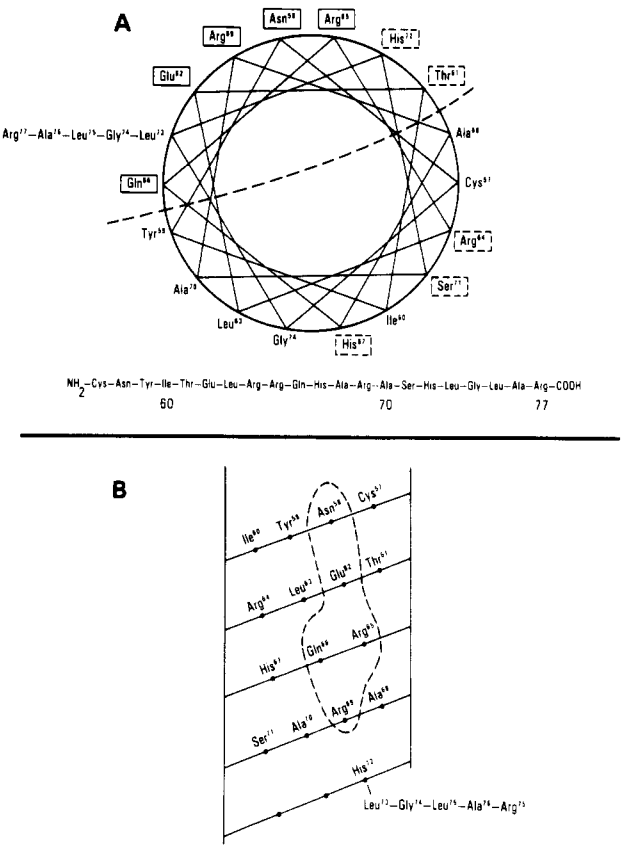

FIGURE 2: (A) Helical wheel projection of the human C3a-57–77 peptide. The upper left side of the projection contains residues with hydrophilic side chains. The boxed residues identified by solid lines are those that compose a hydrophilic area on the helix surface. Boxed residues identified by dashed lines are additional hydrophilic residues lying outside this cluster. (B) A cylinderical projection of the human C3a-57–77 peptide. This projection is analogous to a cylinder cut longitudinally and flattened. Outlined by the dashed line are those residues comprising a hydrophilic face on the surface of the putative helix. The hydrophilic face was defined by computer graphic analysis (see Experimental Procedures), which also revealed the amphipathic character of the helical surface.

in this stretch were residues Asn-58, Glu-62, Arg-65, Gln-66, and Arg-69. The results of computer graphic analysis are represented in a more conventional manner shown in Figures 2 and 3. The top portion of each figure shows an axial projection of residues about the helix and is based on the "helical wheel" analysis first described by Schiffer and Edmundson (1968). The section of hydrophilic residues identified above is readily apparent. The lower portion of Figure 2 contains a cylindrical projection of the C3a-57–77 peptide, and the prominent hydrophilic region is outlined. The hydrophilic face and the essential COOH-terminal pentapeptide were retained in analogues of C3a-57–77 that we designed and synthesized. Figure 3 shows projections of peptide analogues designed to enhance helix stability. Alanine was incorporated along with the 2-aminobutyric acid isomers because it has a high helix-forming potential (Chou & Fasman, 1978). The analogues designed for enhancing helix stability contained alanine and either 2-aminobutyric acid or 2-aminoisobutyric acid substitutions. Additionally, an analogue was synthesized

Table II: C3a-57–77 and Peptide Analogues

peptide	sequence	purpose of design
C3a-57–77	C-N-Y-I-T-E-L-R-R-Q-H-A-A-R-A-S-H-L-G-L-A-R	native factor sequence
C3a-57–77,Ala,Ab	A-N-A-Ab-A-E-A-Ab-R-Q-A-Ab-R-A-A-Ab-L-G-L-A-R	helix-enhanced sequence
C3a-57–77,Ala,Ab,Pro_2	A-N-A-Ab-A-P-A-Ab-R-Q-A-Ab-R-P-A-Ab-L-G-L-A-R	helix-disrupted sequence
C3a-57–77-Ala,Aib	A-N-A-Aib-A-E-A-Aib-R-Q-A-Aib-R-A-A-Aib-L-G-L-A-R	helix-enhanced sequence

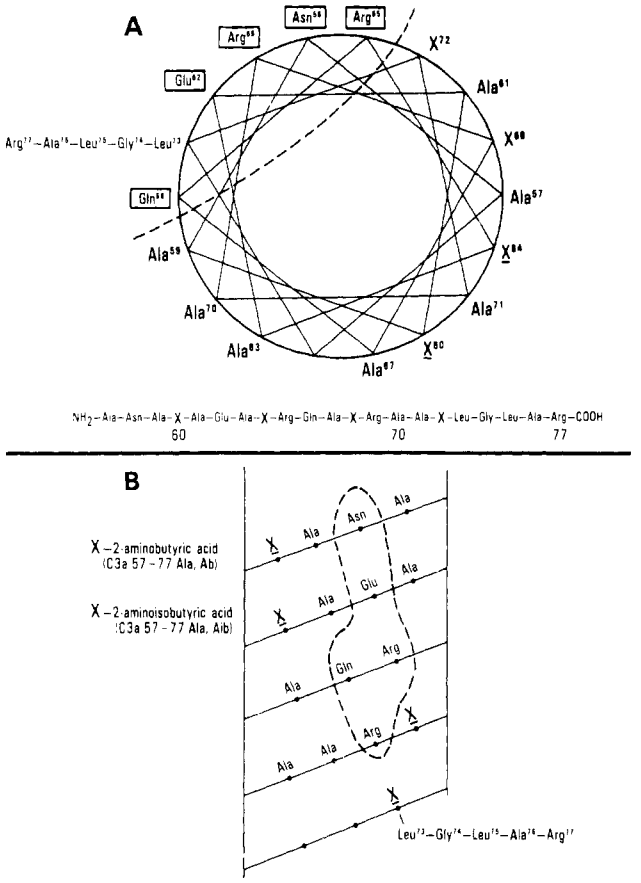

FIGURE 3: (A) Helical wheel projection of human C3a-57–77 peptide substituted by alanine and either 2-aminoisobutyric acid or 2-aminobutyric acid. The dashed line separates hydrophilic residues from the hydrophobic residues. Either 2-aminobutyric acid (Ab) or 2-aminoisobutyric acid (Aib) was substituted for X in an attempt to enhance or stabilize helical content. (B) A cylinderical projection of the substituted human C3a-57–77 peptide. This orientation shows features of the amphipathic helix that were maintained (i.e., the hydrophilic face outlined by the dashed line) and indicates where the helix-enhancing residues were incorporated to make the hydrophobic side of the helix more uniform.

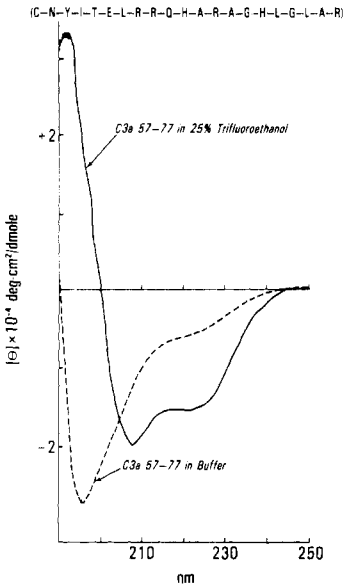

FIGURE 4: Circular dichroism spectra of the C3a-57–77 peptide (C-N-Y-I-T-E-L-R-R-Q-H-A-R-A-G-H-L-G-L-A-R) in the presence and absence of trifluoroethanol. The spectra were recorded as described under Experimental Procedures. Shape changes in the curve with minima at 208 and 222 nm are indicative of helical structure.

Table III: Summary of Circular Dichroism Spectroscopy on C3a-57–77 and Peptide Analogues

peptide	estimated % helix in buffer	estimated % helix in buffer + 25% TFE
C3a-57–77	17.2	41
C3a-57–77-Ala,Ab	23	47
C3a-57–77-Ala,Aib	26	51

that contained two prolyl residues substituted at positions 62 and 70 (Table II). Placement of helix-disrupting residues at these locations should maximize loss of helical content as predicted from Chou–Fasman secondary structure estimation calculations (Chou & Fasman, 1978). Proline substitutions in the C3a-57–77-Ala,Ab analogue were undertaken because of the difficulty encountered with coupling proline, a secondary amine, and 2-aminoisobutyric acid, an α,α'-dialkyl amino acid, in the same peptide.

Circular Dichroism Studies. The far-UV CD spectra of C3a-57–77 in 0.01 M phosphate at pH 7.2 with and without trifluoroethanol (TFE) added are shown in Figure 4. The spectrum in buffer alone is quite typical for a random coil structure, e.g., a minimum of 204–206 nm and a negative inflection between 220 and 230 nm with no significant absorbance around 200 nm (Manavolan & Johnson, 1983). A marked red shift of the negative extremum occurs with the addition of trifluoroethanol (25% by volume). The minima at 209 and 223 nm are quite typical for an α-helix as is the pronounced positive band at 201 nm. The helical content of the peptide in buffer alone was estimated to be only 17.2% and increased dramatically with the addition of trifluoroethanol to approximately 41% helix (Table III, based on molar ellipticity at 222 nm; 100% helix = 3.4×10^4 deg cm^2/dmol; Adler et al., 1974). The CD measurements of the various analogue peptides show the same changes in overall ordered structure when the molecule is solubilized in a TFE/buffer mixture. In addition to having more helical content in 25% TFE, the analogue peptides uniformally exhibit more order, i.e., helical content, in buffer alone than does the C3a-57–77 peptide. The peptide containing 2-aminoisobutyric acid is estimated to have the most helical content. This result was expected since incorporating an α,α'-dialkyl amino acid in a peptide bond constrains ϕ and χ torsional angles to values that encourage helical conformation (Burgess & Leach, 1973; Paterson et al., 1981). These observations suggest that designing peptides to be more helical, or perhaps just more readily induced into helix, was successful. Ability to assume a helix can be destroyed as seen in the spectra of proline-containing C3a-57–77-Ala,Ab. In 25% TFE/buffer, the spectrum of C3a-57–77-Ala,Ab,Pro_2 was essentially unchanged relative to that obtained in buffer alone (Figure 5).

Biological Activity. The results of measuring typical anaphylatoxin peptide activities are shown in Table IV. In the smooth muscle contraction assay using guinea pig ileum, all peptides were capable of contracting the muscle. Values reported in Table IV are the lowest concentration that gives a full response in the assay. The helix-enhanced analogues prove to be more active than either the natural factor C3a or C3a-57–77 peptide. Additionally, each peptide was tachyphylactic to itself and to the other peptides as well as to C3a. The proline-containing 21-residue peptide C3a-57–77-Ala,Ab,Pro_2 showed considerably diminished activity; it exhibits activity on the order of the carboxy-terminal active site pentapeptide C3a-73–77. These results suggest that helical conformation or a tendency to form helix is an important feature of C3a for expression of maximal activity.

Anaphylatoxins are also capable of enhancing vascular permeability (Cochrane & Muller-Eberhard, 1968). In this

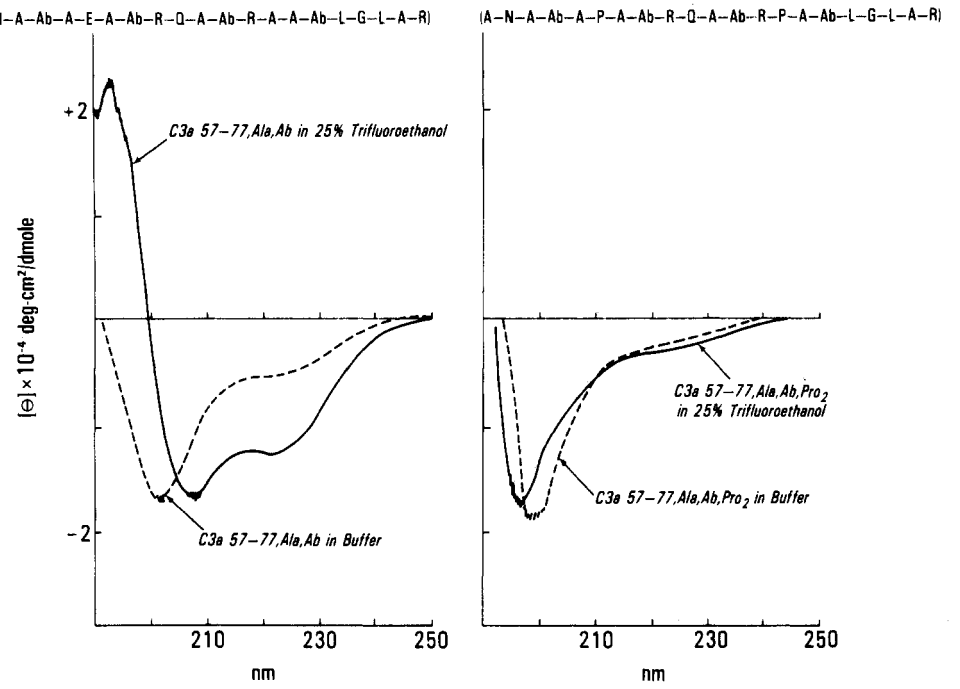

FIGURE 5: Circular dichroism spectra of two C3a-57–77 analogue peptides. The recordings on the left were obtained from the C3a-57–77 analogue containing 2-aminobutyric acid (Ab) either in buffer or in a buffer/trifluoroethanol solution (25% v/v). Helix enhancement is observed in the trifluoroethanol solution. The spectra on the right are from the C3a-57–77 analogue containing two prolyl residues (C3a-57–77,Ala,Ab,Pro_2) incorporated to disrupt helical conformation. All spectra were recorded under conditions described under Experimental Procedures.

Table IV: Biological Activity of C3a-57–77 and Peptide Analogues

	ileal assay[a]		vascular permeability assay[b]	
peptide	concn (M)	rel act. (%)	act.	concn (mol)
C3a	3×10^{-9}	100	ND	ND
C3a-57–77	2.9×10^{-9}	100	++	5×10^{-10}
C3a-57–77-Ala,Ab	1.2×10^{-9}	250	+++	5×10^{-10}
C3a-57–77-Ala,Aib	1.2×10^{-9}	250	+++	5×10^{-10}
C3a-57–77-Ala,Ab,Pro_2	9×10^{-7}	0.33		5×10^{-10}
Leu-Gly-Leu-Ala-Arg	5×10^{-6}[c]	0.20	ND	ND

[a] Guinea pig ileal strips (1.0 cm) suspended in 2.0 mL of tissue bath containing Tyrode's buffer at pH 7.4 and 37 °C with continuous oxygen purge. Concentrations indicate dose required to induce muscle contraction. [b] Anesthetized guinea pigs given 0.5 mL of solution of Evan's blue (1% w/v) via cardiac puncture; 50-μL aliquots of peptide solutions were injected subcutaneously. Thirty minutes later, the animals were sacrificed, and dye infiltration was qualitatively scored. [c] Assay done in 5.0 mL of tissue bath.

assay the amount of circulating dye infiltrating an area of skin injected with the factor is assessed. The results shown in Table IV were scored qualitatively. As observed with the guinea pig ileum contraction assay, the helix-enhanced peptides were more active on a molar basis than natural C3a, and the peptide with disrupted helical structure (e.g., proline-containing peptide) was essentially inactive relative to a normal saline control.

Discussion

The anaphylatoxin C3a is relatively unique in that detailed structure–function relationships can be investigated at a molecular level owing to a wealth of accumulated data from both natural and synthetic peptides (Hugli, 1981). For instance, chemical structures of C3a from human and other species have been elucidated on the basis of studies beginning 17 years ago with Cochrane and Muller-Eberhard (1968), who identified the activity of C3a as distinct from that of C5a. Early on, the fundamental significance of the carboxy-terminal region of C3a was appreciated, especially the essential nature of the carboxy-terminal arginine. Removal of this residue by a serum carboxypeptidase abolished C3a activity (Bokisch et al., 1969). Shortly after the completion of the primary structure of human C3a (Hugli, 1975), studies with synthetic peptides confirmed the notion that the active center was localized entirely at the carboxy-terminal end of the molecule (Erickson & Hugli, 1977).

Structural studies using circular dichroism spectroscopy indicated that 40–45% of the residues in C3a assume an α-helical conformation (Hugli et al., 1975). Ordered conformation at the carboxy-terminal region, which includes the essential arginine, emerged as a plausible hypothesis for explaining apparent secondary structural requirements about the active center of C3a. More recently, X-ray crystallographic analysis (Huber et al., 1980) has resulted in elucidation of a comprehensive molecular model that fits well with established structural and functional information (Figure 1). A prominent feature in this model is the α-helical carboxy-terminal portion of the molecule that extends from Asn-58 to His-72 and ends with an irregularly-folded pentapeptide sequence Leu-Gly-Leu-Ala-Arg (C3a-73–77). In this work, we examined biological activity of the anaphylatoxin as a function of the helical conformation at the carboxy terminus. Previous studies using synthetic peptides based on the sequence of C3a have shown that residues 73–77 constitute a minimal peptide capable of eliciting anaphylatoxin-like activity (Caporale et al., 1980). Moreover, this pentapeptide sequence is invariant in C3a molecules sequenced to date from various species, and synthetic analogues of the pentapeptide exhibit markedly reduced function (Unson et al., 1984). Recently, the peptide segment containing residues 57–77 (C3a-57–77) was synthesized, and on a molar basis, its activity is remarkably similar to that of the native C3a molecule (Huey et al., 1984). Conformational analysis of C3a-57–77 indicated a significant helical content when it was solubilized in helix-inducing trifluoroethanol/aqueous buffer mixtures (Lu et al., 1984). Furthermore, scrutiny by computer graphics and helical wheel projections of the topological arrangement about the helix revealed a

"surface" or cluster of hydrophilic amino acid side chains that suggested amphipathic helical structure (Figures 2 and 3). The synthetic peptide analogues of C3a-57–77 that were designed for this study have linked structural and conformation aspects of the carboxy-terminal region with an additional focus on maintaining amphiphilicity. Fundamental to this experimental approach was a strategic incorporation of the helix-promoting amino acids 2-aminobutyric acid (β-methylalanine) and 2-aminoisobutyric acid (α-methylalanine). Methylalanyl-containing analogues were selected because these residues, on the basis of energy minimization calculations, preferentially encourage helix formation by virtue of constrained ϕ and χ torsional angle rotation, especially in the case of 2-aminoisobutyric acid (Paterson & Leach, 1978; Paterson et al., 1981). Placement of these amino acids in every fourth position in the peptide should enhance helix formation and subsequent stabilization through hydrogen-bond formation between the carbonyl oxygen of one methylalanyl residue to an amide proton of another 13 atoms away, i.e., one complete helical turn. This concept proved to be correct from both our structural and our functional assessment of the putative helix-enhanced peptide analogues of C3a-57–77. The CD spectroscopic measurements indicate a higher degree of order or helix content for each of the substituted peptide analogues in both aqueous buffer and trifluoroethanol/aqueous buffer mixtures when compared to C3a-57–77 (Table III). Although the increased structural integrity was modest (15–18% more helix), the synthetic strategy and peptide design proved to be correct.

Biologic activity of the 2-aminobutyric acid and 2-aminoisobutyric acid substituted analogues was increased by a factor of 2.5 over that of C3a-57–77 and of the native factor C3a (Table IV). In the ileal contraction assay, all the peptides were tachyphylactic to themselves and to C3a. The augmentation of activity is significant and suggests that the stabilized helical conformation is responsible for improved functional performance by the peptide. Results that further support this notion were provided by incorporation of proline, a helix-disrupting residue, into the 21-residue peptide (Table IV). The biological activity of C3a-57–77-Ala,Ab,Pro_2 was reduced markedly compared to that of C3a-57–77, almost to the level of the C3a-73–77 pentapeptide alone. Coincident with reduced activity was the predicted loss of helical conformation as measured by CD spectroscopy (Figure 5). Overall, functional activity appears to be influenced by the conformational integrity of the carboxy-terminal portion of the C3a molecule. In particular, incorporating residues like the 2-aminobutyric acid isomers with sterically constrained bond angle rotation at position 72 may have fixed the active site pentapeptide (residues 73–77) in a more optimal configuration for receptor binding while the other substitutions promoted both appropriate pentapeptide orientation and a more stable helix throughout the 21-residue peptide analogue. Collectively, the effect is to induce conformation of the ligand that is more conducive to optimal receptor interaction. The concept that conformation of the carboxy-terminal portion of the C3a anaphylatoxin is responsible for the activity differentiated between C3a-73–77 and intact C3a has long been asserted and is now strongly supported by these studies. As suggested by Lu et al. (1984), other portions of the intact C3a molecule most likely serve to promote or stabilize regular secondary structure at the carboxy-terminal region of the molecule and thereby optimize activity. To some extent, we have successfully replaced these tertiary conformational requirements in C3a by designing a peptide segment that is capable of assuming a stabilized helix.

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Registry No. Aib, 62-57-7; Boc-Aib, 30992-29-1; C3a, 80295-42-7; C3a-57–77, 88389-51-9; C3a-57–77-Ala,Ab, 100790-40-7; C3a-57–77-Ala,Aib, 100790-41-8; C3a-57–77-Ala,Ab,Pro_2, 100811-95-8; Leu-Gly-Leu-Ala-Arg, 66157-46-8; di-*tert*-butyl dicarbonate, 24424-99-5.

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