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Efficient chemical synthesis of human complement protein C3a†

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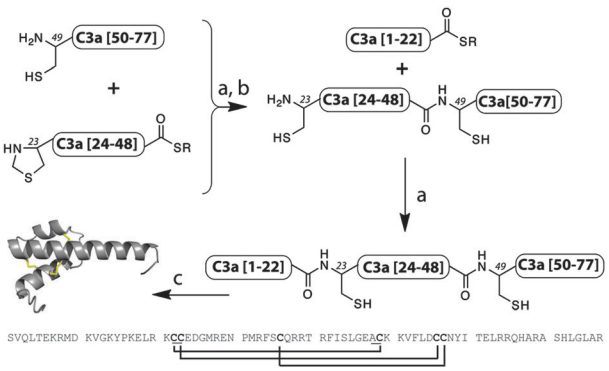
We report the total chemical synthesis of human C3a by one-pot native chemical ligation of three unprotected peptide segments, followed by efficient *in vitro* folding that yielded the anaphylatoxin C3a in high yield and excellent purity. Synthetic C3a was fully active and its crystal structure at 2.1 Å resolution showed 3 helices and a C-terminal turn motif.

The anaphylatoxins C3a and C5a are key mediators of the complement system, which is a key part of innate immunity involved in recognition and elimination of microbial pathogens and infected cells.[1] They selectively bind to their respective G protein-coupled receptors (C3aR and C5aR) triggering a variety of pro-inflammatory processes and have consistently been linked to a number of infectious, inflammatory, neurodegenerative and autoimmune diseases.[1,2] Currently, C3a is commercially produced in relatively low yields by biological means (recombinant expression or purification from plasma)[3,4] resulting in a market value of approximately USD 5000 per mg of protein. In addition, these approaches often require protein purification tags and additional steps for their removal and suffer from the inherent lability of C3a in biological fluids. C3a is rapidly inactivated within seconds by carboxypeptidase-mediated cleavage of a single C-terminal arginine residue (Arg^{77}).[5,6] The resulting protein, C3a-desArg (also termed acylation-stimulating protein, ASP),[7] does not bind C3aR, lacks any pro-inflammatory activity but instead has been shown to stimulate triglyceride synthesis and glucose uptake in adipose tissue.[8–10] To circumvent the problems associated with the isolation of C3a from biological sources we sought a total chemical synthesis approach enabling preparation of homogenous full length C3a.



Scheme 1 Chemical synthesis of human C3a. a: Native chemical ligation; b: Thz → Cys conversion; c: folding and disulfide formation. The amino acid sequence is given in the lower section with cysteines in bold, ligation sites underlined and connectivity of the three disulfides indicated.

Human C3a is a 77 residue protein containing three intramolecular disulfide bonds between C^{22}–C^{49}, C^{23}–C^{56}, and C^{36}–C^{57}.[11,12] Because polypeptides of this size are difficult to obtain in high purity by standard stepwise solid phase peptide synthesis (SPPS),[13] we envisioned a fragment ligation approach by employing Kent's native chemical ligation (NCL).[14] The 77 amino acid polypeptide chain is retro-synthetically split into three peptide segments of about similar length and the three polypeptides are then joined consecutively in the C- to N-terminal direction by NCL as shown in Scheme 1. Temporary protection of Cys^{23} was realized by substitution with thiazolidine-4-carboxylic acid (Thz).[15] The individual fragments C3a[1–22]-α-thioester, C3a[23–48]-α-thioester and C3a[49–77] were prepared by highly optimized *in situ* neutralization *tert*-butyloxycarbonyl (Boc) SPPS in high yield and purity.[16] Both ligations and the intermediate conversion of Thz^{23} to Cys^{23} were carried out in one-pot fashion.[15] The first NCL was carried out in 6 M guanidine HCl, 200 mM Na_2HPO_4, 50 mM tris(2-carboxyethyl)phosphine·HCl (TCEP) and 50 mM 4-mercaptophenylacetic acid (MPAA), pH 7.1. The reaction was complete after 6 h, then methoxyamine HCl was added and the pH was adjusted to 4.0. After completion of the deprotection, the

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† Electronic supplementary information (ESI) available: Full details on the synthesis, pharmacological assays and structure determination. Atomic coordinates and structure factors for C3a have been deposited in the PDB (ID: 4I6O). See DOI: 10.1039/c3cc40537k

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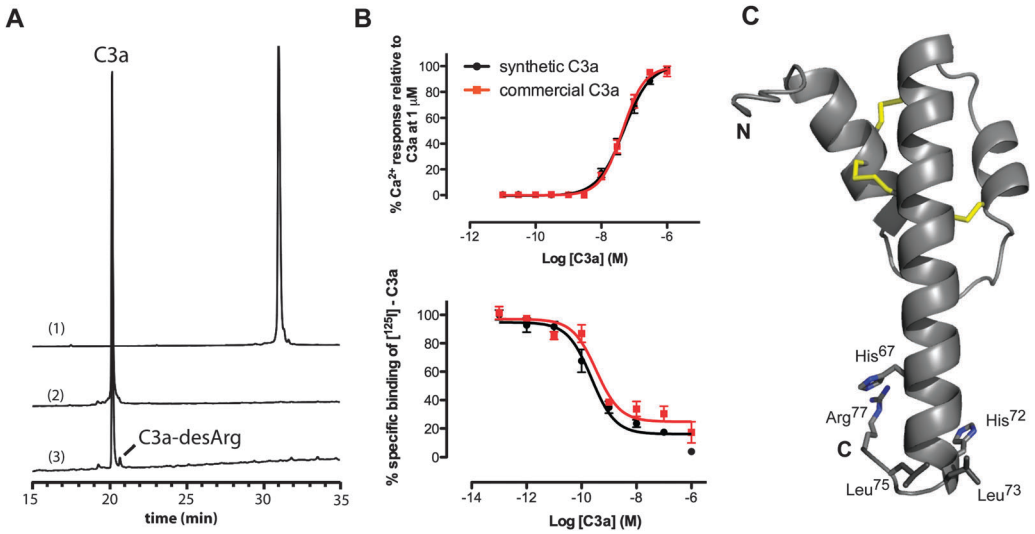

Fig. 1 (A) RP-HPLC analysis of fully reduced C3a (1), the crude folding mixture after 2 h (2) and commercially available material (3). (B) Concentration–response curves for synthetic (●) and commercially available (■) C3a as determined by a fluorescence Ca^{2+} release assay (upper panel) and by competitive radioligand binding assay using 80 pM [^{125}I]-C3a binding to HMDM (lower panel). (C) Ribbon representation of the structure of synthetic C3a at 2.1 Å. Pharmacophore residues are shown in stick representation.

mixture was readjusted to pH 7.1 and the second ligation initiated by adding C3a[1–22]-α-thioester (complete after 6 h). This one-pot approach afforded fully reduced C3a[1–77] without purification of intermediate products in a short time (~24 h), good yield (41%) and with high purity (Fig. S2, ESI†).

The final folding of a cysteine-rich polypeptide chain into its 3D structure can often be problematic because it tends to yield multiple disulfide isomers that are difficult to separate and results in lower overall yield. This often necessitates optimization of folding conditions on a case-by-case basis. We found that folding and correct formation of the three disulfide bonds of C3a was most effective in 50 mM Na_2HPO_4, pH 7.5 supplemented with a redox system of 8 mM reduced glutathione, 1 mM oxidized glutathione, being quantitative after 2 h at room temperature (Fig. 1A). Native C3a was recovered in excellent yield after preparative RP-HPLC purification (61%). MS analysis suggested formation of all three disulfide bonds [observed MW: 9088.71 ± 0.2 Da; calculated MW: 9088.65 Da (average isotope composition)]. HPLC analysis indicated that synthetic C3a was homogenous and devoid of truncated side-products, unlike commercial material from biological sources (Fig. 1A).

To confirm that C3a was folded in the correct biologically active conformation, intracellular Ca^{2+} release and [^{125}I]-C3a radioligand binding assays were performed on human monocyte-derived macrophages (HMDM) expressing C3aR (Fig. 1B). Synthetic and commercial C3a were equipotent agonists (EC_{50} 48 ± 10 nM *vs.* 43 ± 7 nM, respectively) as measured by intracellular Ca^{2+} release, with comparable binding affinities for C3aR (IC_{50} 0.2 ± 0.1 nM (synthetic) *vs.* 0.3 ± 0.1 nM (commercial) against 80 pM [^{125}I]-C3a) in a competitive radioligand binding assay. These values are in excellent agreement with those reported[17] and indicate that synthetic material is indistinguishable from plasma-derived C3a. Furthermore, studies conducted here with the C3aR-selective antagonist SB290157,[18] as well as receptor desensitization experiments, also demonstrated that synthetic C3a was selective for C3aR (Fig. S6 and S7, ESI†).

To confirm the covalent structure of the synthetic material, we determined the 3D structure of synthetic full-length C3a by X-ray crystallography. Rhomboid-shaped crystals were obtained by the hanging-drop vapor diffusion method using 49–51% (v/v) 2-methyl-2,4-pentanediol (MPD) as the precipitant, crystals appearing within 6 days. The structure of C3a was determined by molecular replacement using the structure of C5a-desArg[19] as a search model and refined to a resolution of 2.1 Å, with a final *R* factor of 20.8% and an R_{free} of 26.7%. Peptide backbone and amino acid side chains are generally well defined, except for residues 1–9, whose electron densities were not observed due to high degree of flexibility at the N-terminus. Overall, this highly refined C3a structure is similar to a 3.2 Å structure of C3a-desArg described by Huber and Deisenhofer (Fig. 1C), except that the 3 helices are better resolved and the C-terminus is not disordered, rather there is a clear C-terminal turn motif between residues 73–77.[12]

In conclusion, we have demonstrated that total chemical synthesis is an efficient alternative for obtaining access to multi-milligram quantities of homogenous C3a. This approach gives complete control over the structure and will enable generation of unique chemical analogues of C3a (such as protease-resistant or fluorescently labeled variants) that will prove useful in future complement research. Based on our experience,[20] the one-pot NCL approach developed by Bang and Kent[15] is the most promising strategy for chemically accessing small and medium sized proteins up to 100 amino acids in length.

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