

A peptide mimic of the chemotaxis inhibitory protein of *Staphylococcus aureus*: towards the development of novel anti-inflammatory compounds

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Abstract Complement factor C5a is one of the most powerful pro-inflammatory agents involved in recruitment of leukocytes, activation of phagocytes and other inflammatory responses. C5a triggers inflammatory responses by binding to its G-protein-coupled C5a-receptor (C5aR). Excessive or erroneous activation of the C5aR has been implicated in numerous inflammatory diseases. The C5aR is therefore a key target in the development of specific anti-inflammatory compounds. A very potent natural inhibitor of the C5aR is the 121-residue chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS). Although CHIPS effectively blocks C5aR activation by binding tightly to its extra-cellular N terminus, it is not suitable as a potential anti-inflammatory drug due to its immunogenic properties. As a first step in the development of an improved CHIPS mimic, we designed and synthesized a substantially shorter 50-residue adapted peptide, designated CHOPS. This peptide included all residues important for receptor binding as based on the recent structure of CHIPS in complex with the C5aR N terminus. Using isothermal titration calorimetry we demonstrate that CHOPS has micromolar affinity

for a model peptide comprising residues 7–28 of the C5aR N terminus including two *O*-sulfated tyrosine residues at positions 11 and 14. CD and NMR spectroscopy showed that CHOPS is unstructured free in solution. Upon addition of the doubly sulfated model peptide, however, the NMR and CD spectra reveal the formation of structural elements in CHOPS reminiscent of native CHIPS.

Keywords C5a receptor · CHIPS · Inflammation · Anaphylatoxin

Introduction

As part of the host defense system, the human complement cascade initiates inflammatory responses directed against invading infectious microorganisms, injury and other threatening conditions (Lee et al. 2008). Complement factor C5a is the most powerful pro-inflammatory agent generated during complement activation. C5a interacts with the membrane associated G-protein-coupled C5a receptor (C5aR) resulting in chemotaxis of specific white blood cells, activation of phagocytes, release of granule-based enzymes, and the generation of oxidants (Guo and Ward 2005). C5a is a 74-residue glycoprotein comprising a bundle of four anti-parallel α -helices stabilized by three disulfide bonds (PDB ID code: 1KJS). Binding and activation of the C5aR by C5a is considered a two-step process, in which residues in the region between 12 and 46 of C5a bind to a primary binding site located in the extra-cellular N terminus of the C5aR. Subsequently the C-terminal portion of C5a (residues 69–74) binds to the C5aR activation domain located inside the receptor core (Chen et al. 1998; Gerber et al. 2001). Together these two binding sites provide the complex of C5a and the C5aR

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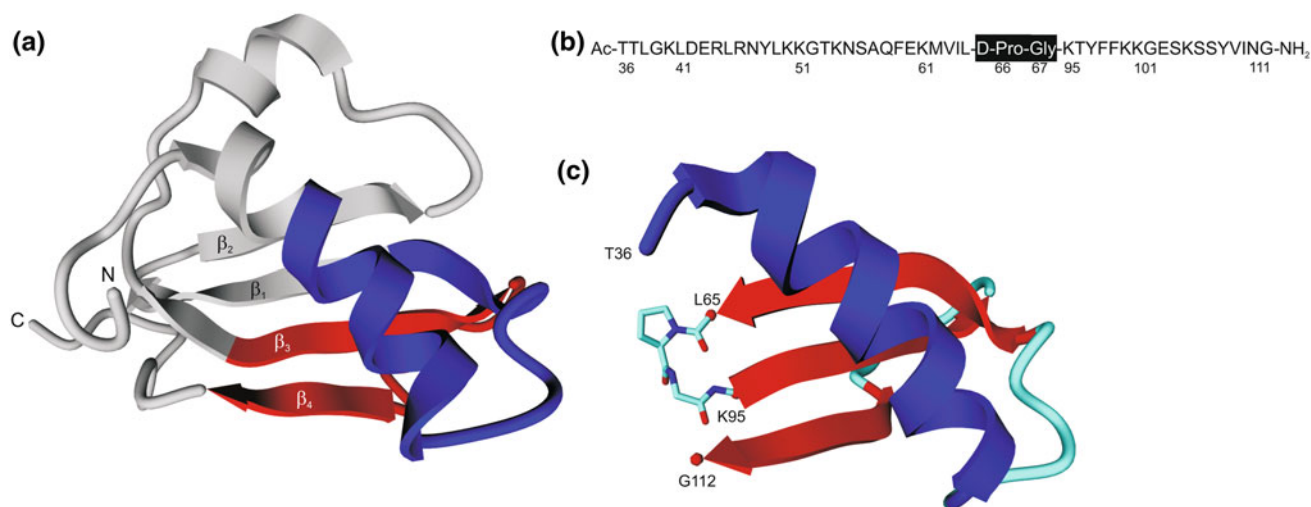


Fig. 1 Topology design of the CHOPS construct. **a** Cartoon representation of one of the NMR structures of CHIPS₃₁₋₁₂₁ (PDB ID code: 1XEE). The two regions interacting with the C5aR are indicated: residues 43–61 (α -helix and β_1) and residues 95–111 (β_3 and β_4). The N and C termini as well as the numbering of the β -strands are

indicated. **b** The amino acid sequence of CHOPS. The D-Pro-Gly linker is indicated in *black*. **c** Cartoon representation of CHOPS based on the structure of CHIPS₃₁₋₁₂₁. The D-Pro-Gly linker is shown in *stick representation*. The numbering of the terminal residues and of the residues flanking the linker is indicated

sub-nanomolar affinity ($K_d \approx 0.60$ nM; van Epps et al. 1993). Although under normal circumstances C5a-mediated C5aR activation is highly favorable, excessive levels of C5a can be deleterious to the host and have been related to numerous inflammatory and autoimmune diseases (e.g. rheumatoid arthritis, inflammatory bowel disease and reperfusion injury). Specific inhibition of C5aR activation is considered a promising strategy to treat such conditions (Allegretti et al. 2005). So far, the design and development of novel anti-inflammatory agents was primarily focussed on small organic compounds and short C5a peptide analogs, which bind with high affinity to the C5aR activation site (Allegretti et al. 2005; Monk et al. 2007). An alternative approach to inhibit C5aR activation was inspired by the discovery of the Chemotaxis Inhibitory Protein of *Staphylococcus aureus* abbreviated as CHIPS (Veldkamp et al. 2000). CHIPS is a 121-residue immune evasive protein excreted by *Staphylococcus aureus* bacteria in order to prevent host inflammatory responses triggered by formylated peptides and C5a. CHIPS binds to the formylated peptide receptor (FPR) and the C5aR with high affinity ($K_d = 35.4 \pm 7.7$ nM and $K_d = 1.1 \pm 0.2$ nM, respectively; Postma et al. 2004). Mutational studies revealed that the C5aR blocking activity of CHIPS is entirely conserved in a protein fragment lacking the first 30 residues. This truncated protein was designated CHIPS₃₁₋₁₂₁ and its structure was solved by Haas et al. (2005). CHIPS₃₁₋₁₂₁ has an entirely different folding topology compared to C5a and is composed of a single α -helix packed onto a four-stranded anti-parallel β -sheet. This particular topology is

present in several other *S. aureus* proteins with immune modulating properties (Haas et al. 2005).

In contrast to C5a, CHIPS binds exclusively to the C5aR N terminus (Postma et al. 2005). This part of the receptor is post-translationally modified by introduction of two sulfate groups on tyrosine residues at positions 11 and 14 (Farzan et al. 2001). Sulfation of these tyrosines appeared to be crucial for tight binding to CHIPS₃₁₋₁₂₁ as was concluded from ITC binding studies using several sulfated and unsulfated mimics of the C5aR N terminus (Bunschoten et al. 2009; Ippel et al. 2009). The highest affinity for CHIPS₃₁₋₁₂₁ ($K_d = 8.4 \pm 1.1$ nM) was observed for a peptide composed of residues 7–28 of the C5aR with both tyrosine residues sulfated (designated C5aR₇₋₂₈S₂; Ippel et al. 2009). This peptide binds almost as strong to CHIPS as the native C5aR. This implies that all moieties essential for the interactions between CHIPS and the C5aR are present within this peptide mimic. The free N terminus of the C5aR is virtually unstructured, which is also the case for the short receptor mimics. Upon binding to CHIPS, residues 10–24 of these C5aR mimics adopt a well-defined conformation (PDB ID code: 2K3U; Ippel et al. 2009). In the complex, residues 10–14 and 19–24 of C5aR₇₋₂₈S₂ form two short stretches of β -strand, which are hydrogen bonded in an anti-parallel fashion to strand β_4 and residues 104–107 of CHIPS₃₁₋₁₂₁, respectively. These two stretches are interconnected by a single turn comprising residues 15–18. Sulfated tyrosine 11 interacts mainly with residues in the α -helix of CHIPS₃₁₋₁₂₁, while sulfated tyrosine 14 is primarily accommodated by residues in the loop between

the α -helix and the first β -strand (residues 52–59; Fig. 1a). The sequence between residues T66 and Y94 of CHIPS_{31–121} does not contribute to interactions with the receptor, but is essential for its native structure (Ippel et al. 2009).

Despite its strong C5aR inhibitory potency, intact CHIPS itself is not amenable for use as an anti-inflammatory agent. Several immunogenic surface epitopes have been identified by Gustafsson et al. (2009). A recent study of Wright et al. (2007) showed the presence of anti-CHIPS antibodies in numerous serum samples of human donors. Therefore, administration of intact CHIPS protein can potentially lead to adverse immunogenic responses. Here, we describe the design, chemical synthesis, and analysis of a protein construct in which specific segments of CHIPS crucial for interactions with the C5aR have been incorporated while a number of non-interacting segments were omitted. We denote this protein construct with the acronym CHOPS, which stands for 'CHemotaxis inhibitory cOnstruct Protein of *Staphylococcus aureus*'. The ultimate goal is to obtain a CHOPS molecule, which is non-immunogenic, but has a high inhibitory potency for the C5aR.

Materials and methods

Materials

Peptide grade DiPEA, DCM, NMP, TFA, piperidine, and HPLC grade solvents were purchased from Biosolve B. V. (Valkenswaard, The Netherlands). Fmoc-protected amino acids were purchased from GL Biochem Ltd. (Shanghai, China). Side-chain protecting groups were chosen as: Boc for lysine, ^tBu for aspartic acid, glutamic acid, serine, threonine, and tyrosine, Trt for asparagine and glutamine, and Pbf for arginine. Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification.

Compound analysis

Analytical HPLC was performed using an automatic HPLC system (Shimadzu) with an analytical reversed-phase column and a UV detector operating at 214 nm with a flow rate of 0.75 mL/min. A Phenomenex Gemini C18 column (110 Å, 5 µm, 250 × 4.6 mm) was used. TFA buffers were used (buffer A: H₂O:MeOH, 80:20 v:v; buffer B: H₂O:MeOH, 5:95 v:v, both containing 0.1% TFA). Elution was effected with a linear gradient from 100% A to 100% B over 48 min. Preparative HPLC was performed using an automatic Prep LCMS-QP8000α HPLC system (Shimadzu) with a preparative reversed-phase column and a UV detector operating at 214 nm with a flow rate of 12.5 mL/min. A Reprosil-Pur C18-AQ column (120 Å, 10 µm,

250 × 22 mm) was used. TFA buffers were used (buffer A: H₂O:MeOH, 80:20 v:v; buffer B: H₂O:MeOH, 5:95 v:v, both containing 0.1% TFA). Elution was effected with a linear gradient from 100% A to 100% B over 100 min. The peptides were characterized using electrospray mass spectrometry (ESI-MS), which was performed on a Thermo Finnigan LCQ DECA XP MAX ion trap mass spectrometer.

Peptide synthesis

CHOPS was synthesized on a Rink Amide PEG resin (0.52 mmol/g) (Matrix Innovation Inc., Montreal, Canada) on a 0.25 mmol scale. The peptide was assembled using an automatic ABI 433A Peptide Synthesizer, equipped with a UV-monitoring system, which was used to monitor the Fmoc removal step, i.e., formation of the dibenzofulvene-piperidine adduct absorbing at 301 nm. ABI FastMoc 0.25 mmol protocols were applied, with the exception of a standard double coupling of 45 min. The synthesis was carried out on 0.48 g resin. The resin was washed with DCM and NMP (five times). Subsequently, 1 mmol of the appropriate amino acid was dissolved in NMP (2 mL), and HBTU/HOBt (1 mmol, 2.78 mL of 0.36 M) in NMP was added. To this mixture, DiPEA (1 mL, 2 M) in NMP was added, and the activated amino acid was then transferred to the reaction vessel. After 45 min, the reaction vessel was drained and the resin was washed with NMP (three times) followed by addition of another batch of pre-activated amino acid, which was allowed to couple for another 45 min. Next, any of the remaining free amino groups were acetylated with an acetic anhydride capping solution (0.5 M Ac₂O, 0.125 M DiPEA, and 0.015 M HOBt in NMP) for 15 min. After capping the Fmoc protective group was removed from the N terminus by treatment with 20% piperidine solution in NMP twice (3 and 7.6 min). The last coupling cycle was followed by removal of the Fmoc-group by a 20% piperidine solution, washing the resin with NMP, and acetylation of the N terminus by treatment with the acetic anhydride capping solution for 15 min. Finally, the resin was washed with NMP (five times) and DCM (six times), removed from the reaction vessel, washed with ether, and dried in vacuo over P₂O₅.

The anchored peptide obtained in this way was de-protected and cleaved from the solid support by treatment with TFA/H₂O/TIS (95/2.5/2.5, 25 mL) for 2 h at room temperature. The mixture was filtered and the residue washed thoroughly with TFA (two times 10 mL). The reaction mixture was concentrated in vacuo to a volume of approximately 10 mL and added dropwise to 90 mL MTBE/*n*-hexane (1/1 v/v) solution. The precipitate was collected by centrifugation (3,000 rpm, 10 min), the supernatant was decanted and the pellet was resuspended in MTBE/*n*-hexane (1/1 v/v) (100 mL) and centrifuged again.

This procedure was repeated twice. Afterwards, the pellets were dissolved in CH₃CN/water (1/1 v/v) (ca. 60 mL) and lyophilized to give 595 mg of the crude peptide as a white fluffy solid.

The crude peptide (400 mg) was dissolved in 30 mL buffer A, 10 mL buffer B and purified by preparative HPLC (Reprosil-Pur C18-AQ, TFA buffers) in eight runs. Pure fractions were pooled and lyophilized to give 29 mg of pure CHOPS. The CHOPS sample was characterized by analytical HPLC (Gemini C18, TFA buffers, R_t = 36.4 min, Purity > 98%) (Supplementary Figure 1) and by ESI-MS (Supplementary Figure 2; calculated average mass $[M + 5H]^{5+}$ for C₂₆₁H₄₂₂N₇₀O₇₄S: 1,152.34; found: 1,152.30).

The C5a receptor mimic peptides C5aR_{7–28} and C5aR_{7–28}S₂ were synthesized as has been described previously (Bunschoten et al. 2009).

ITC experiments

The binding affinities of the C5aR mimics C5aR_{7–28} and C5aR_{7–28}S₂ with CHOPS were measured using an ITC₂₀₀ Microcalorimeter (MicroCal) operating at 283 K. The measuring cell was filled with 208 µL of a 0.19 mM solution of CHOPS in a 20 mM sodium phosphate buffer at pH 6.5. The concentration of CHOPS was determined by OD₂₈₀ measurements. The syringe was loaded with 30 µL of a 2.5 mM solution of one of the C5aR-mimics in the same buffer system. After each incremental addition of the solution in the syringe, the integrated heat change due to binding was measured. The data were analyzed using the Microcal Origin software and fitted by non-linear regression analysis. Three independent experiments were carried out. The experimental errors were estimated by Monte Carlo simulations using the standard deviations of the individual experiments.

NMR spectroscopy

NMR samples of C5aR_{7–28}, C5aR_{7–28}S₂ and CHOPS were at concentrations of 0.8–1 mM in 10/90% (v/v) D₂O/H₂O sodium phosphate buffers (20 mM, pH 6.5). Spectra were recorded at 288 K on a Varian INOVA 500 and a Bruker Avance 750 MHz spectrometer equipped with an HCN triple-resonance pulsed field gradient probe. Sequential ¹H NMR assignments of CHOPS were accomplished using a combination of NOESY, TOCSY, and ¹³C-HSQC spectra.

CD measurements

CD spectra (190–260 nm) were recorded on an Olis RSM1000 spectrophotometer operating at 2 nm spectral resolution (slit size 1.24 nm). Samples of CHIPS_{31–121}

(43 µM) in 20 mM sodium phosphate buffer (pH 6.5), CHOPS (50 µM), C5aR_{7–28} and C5aR_{7–28}S₂ (50 µM) in 10 mM sodium phosphate buffer (pH 7.4) were measured at 298 K using a 0.5 mm cuvette. To gain a satisfactory S/N ratio five scans were summed, with data points averaged by three-point triangular smoothing.

Results and discussion

Design of CHOPS

The *Staphylococcal* protein CHIPS is one of the most potent inhibitors of C5a-induced inflammatory responses presently known. In contrast to the numerous agents developed to interact directly with the C5aR activation site located inside the receptor core (Proctor et al. 2006; Chen et al. 2010), CHIPS blocks activation by C5a by binding with high affinity to the flexible extra-cellular N-terminal portion of the C5aR (Postma et al. 2005). The interaction surface of CHIPS_{31–121} with the C5aR comprises ~20% of its solvent accessible surface and is not confined to a limited region of the protein. The interactions between CHIPS and the C5aR involve a substantial number of non-sequential amino acids optimally positioned in the inhibitory protein to provide tight binding. A successful mimic of CHIPS should not only include the amino acid residues (or mimics of these) crucial for C5aR binding, but also the amino acids responsible for the proper spatial arrangement dictated by the CHIPS folding topology. Our first approach to build such a structure is to leave out a limited number of residues which do not interact directly with the C5aR, but with the intention to leave the structural integrity of CHIPS_{31–121} intact. NMR titration studies revealed that two regions of CHIPS_{31–121} show relatively large perturbations in the backbone and Cβ chemical shifts (Ippel et al. 2009): the first region includes residues 43–61, which comprises part of the α-helix and the subsequent loop connecting strand β₁ (Fig. 1a). The second region is composed of residues 95–111 and comprises strands β₃ and β₄ of CHIPS_{31–121}. The non-interacting portion of CHIPS_{31–121} comprises strand β₂ and the long loop connecting β₂ with β₃ (Fig. 1a). This portion could potentially be left out by directly connecting strand β₁ with β₃ via a tight turn. Inspection of the NMR structural models of CHIPS_{31–121} reveals that residue L65 at the end of strand β₁ and residue K95 at the start of strand β₃ are proximal and offer an excellent opportunity to link the two fragments interacting with the C5aR together to form one short, contiguous sequence. Several β-hairpin inducing sequences have been described and reviewed in the literature (Blanco et al. 1998; Stotz and Topp 2004). One of the smallest peptide fragments, which induces a β-turn and facilitates the

formation of an anti-parallel β -sheet, is the dipeptide D-Pro-Gly (Haque and Gellman 1997). This fragment was chosen to link the N- and C-terminal segments of CHIPS, which interact with the C5aR, together. These segments were chosen to comprise the complete elements of secondary structure as present in native CHIPS (i.e. the α -helix and β -strands β_1 , β_3 , and β_4) in order to pursue structural integrity. The resulting construct consisted of the CHIPS amino acid sequences T36-L65 and K95-G112 interconnected by D-Pro-Gly (Fig. 1b). A number of residues suggested to be part of discontinuous immunogenic epitopes by Gustafsson et al. (2009) are not present in this construct (designated CHOPS). A model representation of CHOPS based on the structure of native CHIPS_{31–121} is presented in Fig. 1c.

Affinity of CHOPS for the C5aR N terminus

The affinity of the CHOPS fragment for the C5aR was determined using isothermal titration calorimetry (ITC). We synthesized two peptide mimics of the C5aR N terminus: unsulfated peptide C5aR_{7–28} representing residues 7–28 of the C5aR and peptide C5aR_{7–28}S₂, the same sequence with tyrosine residues 11 and 14 O-sulfated. We titrated a solution of CHOPS with these peptides and recorded the subsequent heat exchange upon formation of the complex. Two typical ITC experiments are shown in Supplementary Figure 3. Clearly, titration of the doubly sulfated peptide C5aR_{7–28}S₂ to CHOPS resulted in a substantial exothermic effect (Supplementary Figure 3a) while no significant response was detected in the ITC experiment with the unsulfated peptide C5aR_{7–28} (Supplementary Figure 3b). Gratifyingly, the affinity of CHOPS for C5aR_{7–28}S₂ was in the micromolar range ($K_d = 3.6 \pm 0.2 \mu\text{M}$; $n = 3$). The complete thermodynamic analysis of these ITC data plus the comparison with previous ITC studies of CHIPS_{31–121} and C5a peptide mimics is compiled in Supplementary Table 1.

NMR spectroscopy

Previous NMR studies revealed that the synthetic peptides C5aR_{7–28} and C5aR_{7–28}S₂, which mimic the N-terminal portion of the C5aR, were very flexible in solution and did not have detectable propensity for any preferred secondary structure. Although there is no detailed structure available for the intact C5aR, it is expected that its free extra-cellular N terminus (residues 1–35) is unstructured as well. The protein CHIPS_{31–121} does adopt a well-defined conformation with flexible regions at the termini and some disorder in the loop region between the α -helix and strand β_1 (Haas et al. 2005). As could be inferred from ¹⁵N relaxation studies this particular loop region adopts an ordered conformation in the complex with C5aR_{7–28}S₂ (Ippel et al.

2009). NMR spectra of the free CHOPS construct appear to be typical for a largely unstructured polypeptide chain (Supplementary Figure 4a). 2D NOE spectra of free CHOPS contain predominantly sequential NOEs, but a few long-range contacts could be identified. These non-sequential cross-peaks are indicative for an anti-parallel orientation of strands β_1 and β_3 , which are bridged by the β -hairpin inducing D-Pro-Gly sequence (Fig. 2).

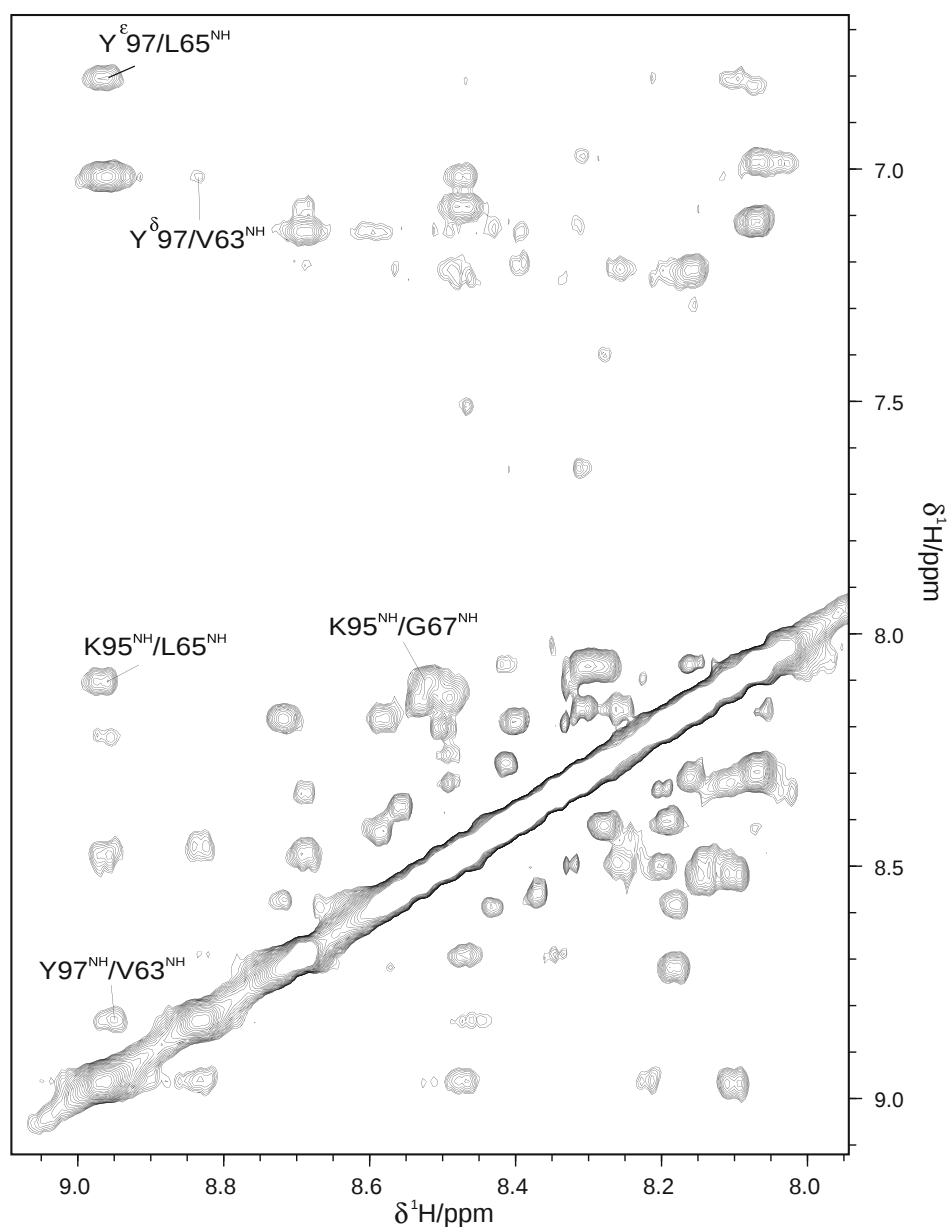
Titration of CHOPS with the unsulfated receptor mimic C5aR_{7–28} did not result in any changes in the ¹H-spectrum of the latter. In contrast, titration of CHOPS with the sulfated receptor mimic C5aR_{7–28}S₂ resulted in increased dispersion of resonance lines, which is characteristic for non-random coil behavior (Supplementary Figure 4b). The complex between CHOPS and C5aR_{7–28}S₂ is still flexible and the ¹H-spectra show a high degree of overlap. Nevertheless, we could assign some of the NMR signals as indicated in Supplementary Figure 4b. These new signals are at comparable positions as in spectra of the complex between CHIPS_{31–121} and C5aR_{7–28}S₂, and indicative for the formation of native-like structure. Similar features were observed in ¹H–¹³C HSQC spectra upon titration of C5aR_{7–28}S₂ to CHOPS (Fig. 3).

The NOESY spectra of the CHOPS:C5aR_{7–28}S₂ complex suffer from severe overlap, but still a limited number of long-range intra-molecular and inter-molecular NOE cross-peaks could be assigned. Inter-molecular NOEs were identified between the aromatic side-chain protons of sulfated tyrosine sY14 of C5aR_{7–28}S₂ and the side-chains of T53 (Fig. 4a) and Y108 (Fig. 4c) of CHOPS. H13 of C5aR_{7–28}S₂ has an NOE contact with V109 of CHOPS (Fig. 4b). Intra-molecular NOEs were identified between T53 and L49 and between Y97 and V109 (Fig. 4a). Similar peaks were observed in NOESY spectra of the complex between CHIPS_{31–121} and C5aR_{7–28}S₂. The position of these residues in the NMR structure of the CHIPS_{31–121}:C5aR_{7–28}S₂ complex is indicated in Fig. 4d, e.

CD spectroscopy

The presence of residual structure in the C5aR mimics and CHOPS was also monitored by CD spectroscopy. The CD spectra of the C5a-receptor mimics C5aR_{7–28} and C5aR_{7–28}S₂, and CHOPS show no structural features apart from a shallow minimum at 200 nm (Fig. 5). The CD spectrum of free CHIPS_{31–121} comprises a large positive signal around 190 nm and a minimum around 205 nm (Fig. 5). This spectrum does not change significantly upon binding of C5aR_{7–28}S₂ (data not shown). Titration of CHOPS with a stoichiometric amount of unsulfated receptor mimic C5aR_{7–28} resulted in an increase of the CD signal, although the shape of the spectrum did not change (Fig. 5a). Stoichiometric titration of CHOPS with sulfated

Fig. 2 Section of the NOESY spectrum of free CHOPS in solution. Several cross-peak assignments are shown indicative for the presence of a β -hairpin comprising strands β_1 and β_3

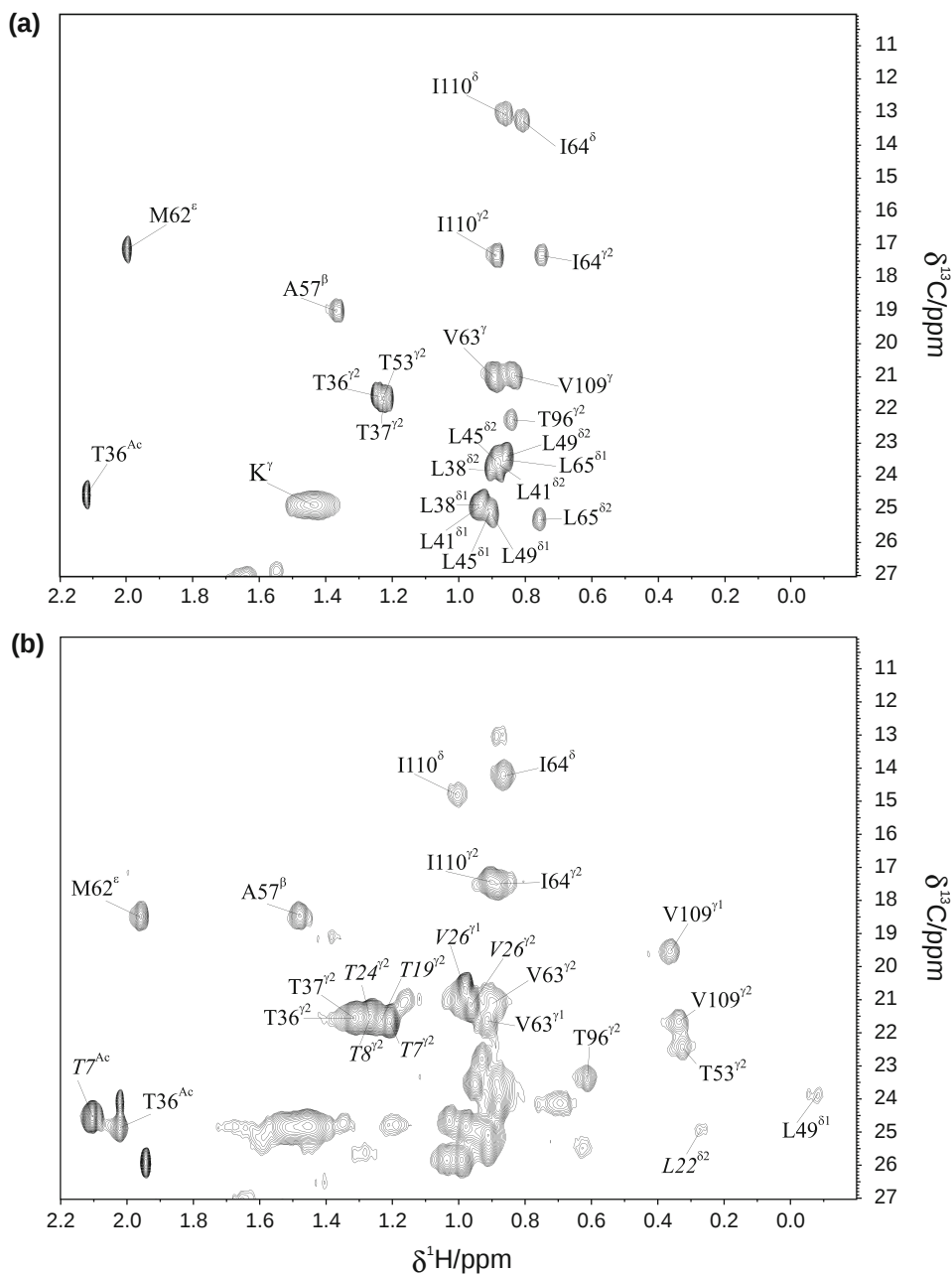


receptor mimic C5aR₇₋₂₈S₂, on the other hand yielded a clear change of the CD spectrum: an increase in the signal around 190 nm and a shift of the minimum from 200 to 205 nm (Fig. 5b). These changes shift the appearance of the CD spectrum towards that of CHIPS₃₁₋₁₂₁ although at lower intensities.

In this study we aimed to create a shorter version of the immune evasive protein CHIPS based on the NMR structure of the complex between CHIPS₃₁₋₁₂₁ and C5aR₇₋₂₈S₂. The construct we designed (CHOPS) comprises all portions of CHIPS₃₁₋₁₂₁ important in the interaction with the C5aR. Portions outside the binding region including strand β_2 and the connecting loop between β_2 and β_3 were discarded. This was accomplished by coupling strand β_1 and β_3 together via a D-Pro-Gly linker segment. The resulting

50-residue long peptide appeared to be largely unfolded apart from some residual structure around the β -hairpin inducing D-Pro-Gly linker sequence. ITC studies revealed that CHOPS binds to the doubly sulfated C5a-receptor mimic C5aR₇₋₂₈S₂ with micromolar affinity ($K_d = 3.6 \pm 0.2 \mu\text{M}$). Although the affinity of C5aR₇₋₂₈S₂ to CHOPS is three orders of magnitude lower compared to binding to CHIPS₃₁₋₁₂₁ ($K_d = 8.4 \pm 1.2 \text{ nM}$; Ippel et al. 2009), this is a very promising result for a first lead compound. No detectable affinity of CHOPS was observed in the ITC measurements using the unsulfated mimic C5aR₇₋₂₈. This is consistent with previous measurements of CHIPS₃₁₋₁₂₁ and C5aR₇₋₂₈, which revealed that the absence of the two sulfate moieties results in an almost 400-fold decrease in affinity.

Fig. 3 ^1H - ^{13}C HSQC spectra of CHOPS. **a** Methyl group region of the ^1H - ^{13}C HSQC spectrum free CHOPS. **b** Similar spectrum of CHOPS in complex with receptor mimic C5aR₇₋₂₈S₂. The assignments are indicated. Peaks originating from C5aR₇₋₂₈S₂ are shown in *italic font*



NMR spectroscopy confirmed the results obtained by ITC. Titration of CHOPS with unsulfated receptor mimic C5aR₇₋₂₈ resulted in the sum of its constituent ^1H -spectra, while titration of doubly sulfated peptide C5aR₇₋₂₈S₂ yielded a completely different ^1H -spectrum with signals shifted from their random coil position. The latter is indicative for the formation of defined structural elements. The NMR titration experiments using C5aR₇₋₂₈S₂ showed binding in a fast-exchange regime, compatible with the observed micromolar affinity by ITC (Cavanagh et al. 2007). Several characteristic features observed in spectra of the CHIPS₃₁₋₁₂₁:C5aR₇₋₂₈S₂ complex were

also present in spectra of CHOPS:C5aR₇₋₂₈S₂. Specific resonances of residues L49, T53, T96, Y97, Y108, V109 and N111 have chemical shifts comparable with their counterparts in the CHIPS₃₁₋₁₂₁:C5aR₇₋₂₈S₂ complex. We also observed NOE cross-peaks between residues sY14, T53, L49, and Y108 and between residues H13, V109, and Y97 in NOESY spectra of CHOPS:C5aR₇₋₂₈S₂. These peaks are reminiscent of NOE contacts observed in spectra of the complex between CHIPS₃₁₋₁₂₁ and C5aR₇₋₂₈S₂ and reveal that CHOPS, in the presence of sulfated peptide C5aR₇₋₂₈S₂, adopts similar structures as native CHIPS₃₁₋₁₂₁.

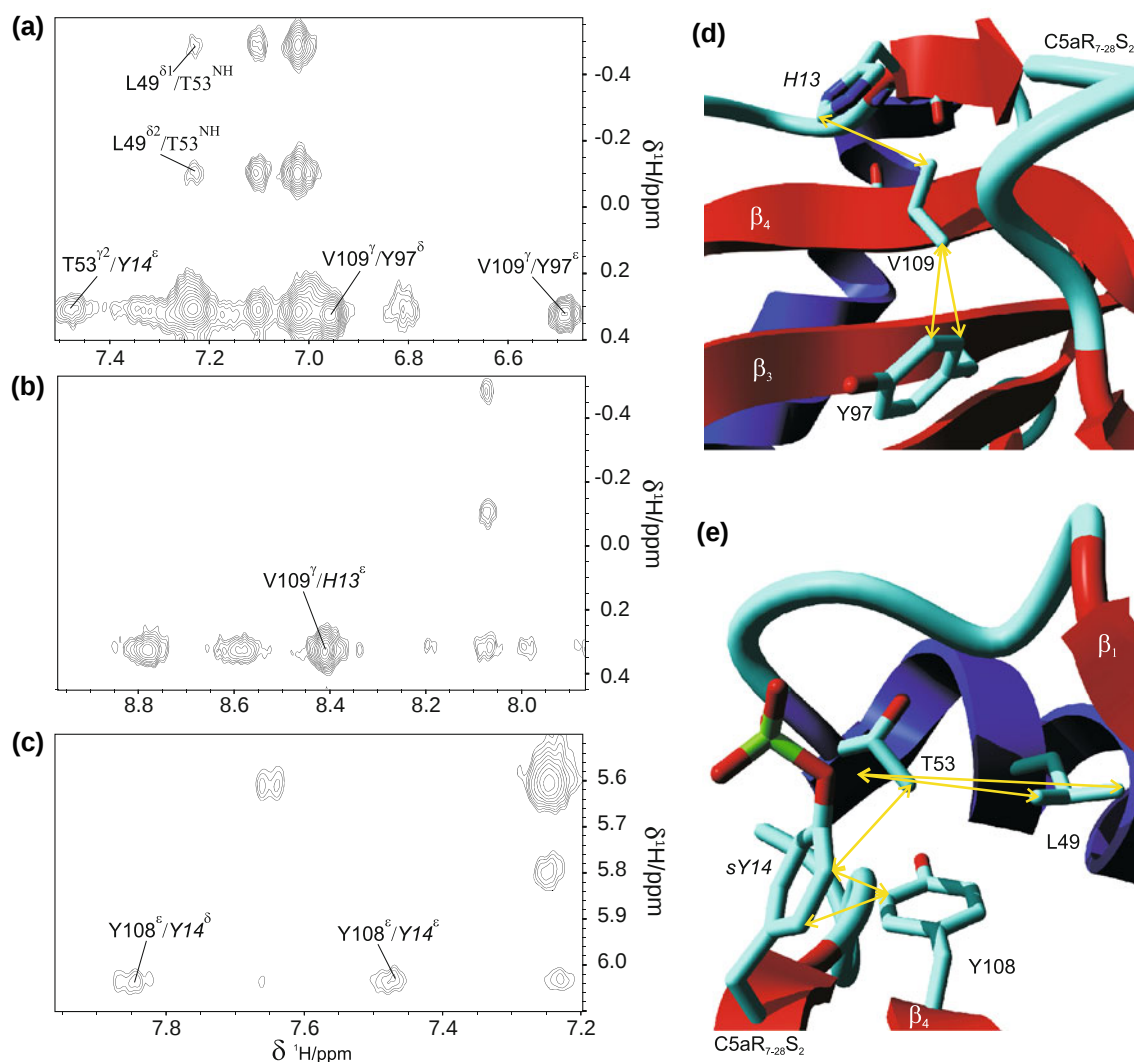


Fig. 4 Observed cross-peaks in the NOESY spectrum of the CHOPS:C5aR₇₋₂₈S₂ complex in relation to structural features of the CHIPS₃₁₋₁₂₁:C5aR₇₋₂₈S₂ complex (PDB ID code: 2K3U). **a–c** Different sections of the NOESY spectrum recorded at 288 K of a 1:1 mixture of CHOPS and C5aR₇₋₂₈S₂. Identified long-range intra-molecular and inter-molecular NOE cross-peaks are indicated

The structural characteristics of CHOPS, either free in solution or in complex with the C5aR mimics, were also studied by CD spectroscopy. The spectra of the separate peptides (CHOPS, C5aR₇₋₂₈ or C5aR₇₋₂₈S₂) did not show any significant absorption apart from a shallow minimum around 200 nm. Titration of CHOPS with receptor mimic C5aR₇₋₂₈ increased the amount of absorption, but not the position of its minimum. Titration of CHOPS with the doubly sulfated receptor mimic C5aR₇₋₂₈S₂ caused an increase in absorption around 190 nm as well as a shift of the absorption minimum from 200 to 205 nm. Although the maximum and minimum intensities are smaller, the overall shape of the CD spectrum of the CHOPS:C5aR₇₋₂₈S₂ complex resembles that of native CHIPS₃₁₋₁₂₁.

(*normal fonts* for residues belonging to CHOPS and *italic fonts* for residues belonging to C5aR₇₋₂₈S₂). **d–e** Cartoon representation of the structure of the CHIPS₃₁₋₁₂₁:C5aR₇₋₂₈S₂ complex. The side-chains of the residues identified in **a–c** are shown in stick representation. NOE cross-peaks observed in **a–c** are indicated by arrows in the structure

Conclusions

We have designed and synthesized a significantly reduced-size mimic of the protein CHIPS, which we coined CHOPS. This construct has a binding affinity of 3.6 μ M with respect to the sulfated receptor mimic C5aR₇₋₂₈S₂, but no affinity for the unsulfated receptor mimic C5aR₇₋₂₈. We conclude, based on NMR and CD studies, that CHOPS adopts structures comparable with native CHIPS₃₁₋₁₂₁ upon binding of C5aR₇₋₂₈S₂. The CHOPS:C5aR₇₋₂₈S₂ complex is, however, still flexible. We expect that improved affinity can be achieved by introduction of more rigid moieties, which will force this mimic more into the structure of native CHIPS.

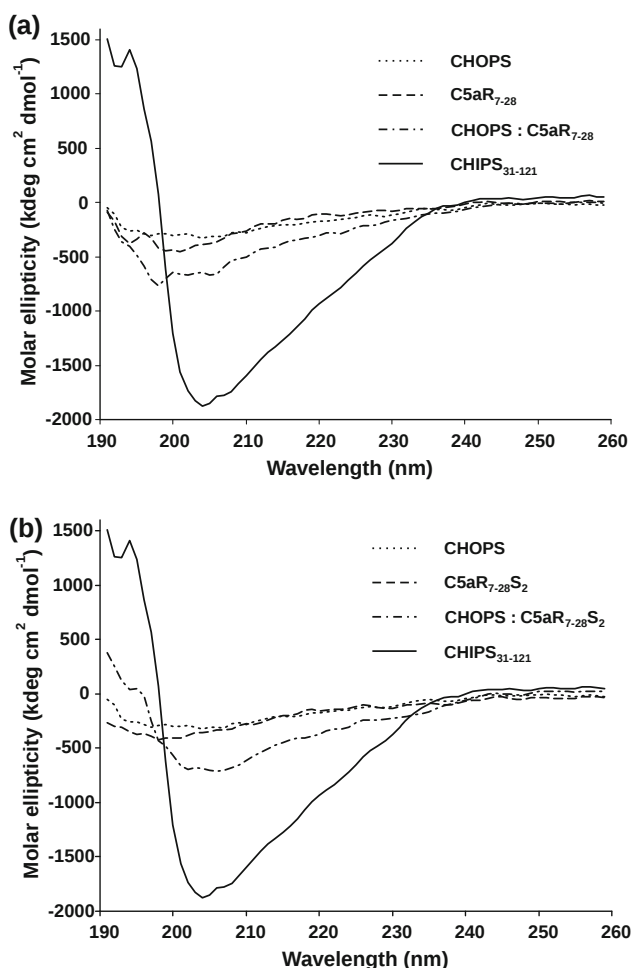


Fig. 5 Circular dichroism spectra (CD) of CHOPS, CHIPS₃₁₋₁₂₁ and C5aR mimics. **a** CD spectra of CHOPS, C5aR₇₋₂₈, CHOPS:C5aR₇₋₂₈, and CHIPS₃₁₋₁₂₁. **b** CD spectra of CHOPS, C5aR₇₋₂₈S₂, CHOPS:C5aR₇₋₂₈S₂, and CHIPS₃₁₋₁₂₁

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