Disulfide bond polymerization of a cyclic peptide derived from the surface loop 4 of class 1 OMP of *Neisseria meningitidis*

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Summary

Two peptides derived from the surface loop 4 of class 1 Outer Membrane Protein (OMP) of *Neisseria meningitidis* were synthesized on solid phase using the Boc/Bzl strategy: one containing the entire loop 4 cyclized and the other representing the polymerized cyclic loop 4. To test a more efficient cyclic peptide presentation, in the present study a strategy was developed to obtain polymers of cyclic peptides. In order to obtain the polymeric cyclic peptide, two protecting groups for cysteine were used – Acm and Mob. The Cys(Acm)-protected cyclic peptide was obtained after removing the Mob group. The polymerization reaction was carried out by simultaneous deprotection/oxidation of S-Acm with iodine. Analysis of the polymeric cyclic peptide in Tris-tricine-SDS-PAGE showed different bands with molecular weights higher than expected for the corresponding monomeric cyclic peptide. Both peptides were used in immunization of four different mouse strains. The antisera raised against the peptides were evaluated by ELISA and Western blotting vs. OMP preparation of *N. meningitidis*. The titers raised against the polymerized cyclic peptide were higher than the ones raised against the cyclic peptide. The antisera elicited did not show bactericidal activity. Nevertheless, the antisera elicited against the polymeric cyclic peptide in the CBA/J mouse strain showed opsonic activity. The antibodies raised against the polymeric cyclic peptide were successfully used as probes in Western blotting experiments to verify the display of loop 4 peptide on the surface of filamentous phage M13.

Abbreviations: Boc, *t*-butyloxycarbonyl; Bzl, benzyl; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DIPCDI, *N,N*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; DMS, dimethylsulfide; DMSO, dimethylsulfoxide; DTT, dithiotreitol; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; HAc, acetic acid; HF, hydrogen fluoride; HPLC, high-performance liquid chromatography; MBHA, 4-methylbenzylamine; TFA, trifluoroacetic acid; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; FAB, fast-atom bombardment; DHB, dihydroxybenzoic acid.

Introduction

Neisseria meningitidis possesses class 1 outer membrane proteins (OMPs) which are responsible for meningococcal subtype specificity [1] and are interesting targets for vaccine development. Bactericidal antibodies

against class 1 OMP play a major role in protection against meningococcal disease [2].

A two-dimensional model of class 1 OMP predicts the presence of eight cell surface-exposed loops in the protein. The bactericidal antibodies are directed against the predicted apices of variable loops 1 and 4 [3].

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Immunization with linear peptides derived from variable loops of class 1 protein, corresponding to different subtypes conjugated to tetanus toxoid, produced high antipeptide titers in mice, but, unfortunately, the antibodies generated recognized the homologous class 1 protein (and the whole bacteria) only poorly, and induce no complement-mediated bactericidal activity [3].

In order to mimic the surface loops of class 1 OMP of *N. meningitidis*, it is necessary to decrease the conformational freedom of the synthetic peptides derived from these loops. Immunization of rabbits with a 36-mer unconjugated cyclic peptide, consisting of the entire surface-exposed loop 4 of class 1 protein from meningococcal strain MC58 (subtype P1.16b), produced antisera that promoted complement-mediated bactericidal activity [4]. Taking into account these results, our first interest was to synthesize the cyclic peptide, derived from the surface loop 4 of class 1 OMP from the meningococcal strain B385 (subtype P1.15) to study its ability to induce antisera that promote complement-mediated bactericidal activity in mice, when it was used unconjugated. The meningococcal strain B385 is the most frequent clinical isolate in our country. Our second goal was to design and synthesize a construction where the cyclized peptide could be presented as a polymeric form to compare the response against each type of peptide presentation.

Most peptides in solution are flexible and acquire many conformations. The biologically relevant conformation can be achieved by disulfide cyclization [5] or lactam formation [6]. The combination of different cysteine-protecting groups makes it possible to prepare peptides containing multiple disulfide bridges [7, 8]. The AcM protecting group [9] is essentially stable at acidic and basic pH and therefore compatible with both Boc and Fmoc chemistries. The combination of Mob and AcM groups in synthesis permits achieving regioselective disulfide bonds. In our work, we combined these protecting groups to obtain the polymeric cyclic peptide.

Experimental procedure

All Boc-amino acids and the MBHA resin were obtained from Bachem (Switzerland). Solvents were obtained from Caledon (Canada) or Merck (Germany).

Peptide synthesis

The peptides were synthesized using the Boc/Bzl strategy and the 'tea bag method' [10] on 300 mg of MBHA resin (substitution level 0.38 mmol/g) each. The Boc group was used for N $^{\alpha}$ -protection. The side-chain protecting groups used were acetamidomethyl (AcM) and 4-methoxybenzyl (Mob) for Cys; benzyl (Bzl) for Ser, Asp and Thr; tosyl (Tos) for Arg; 2,4-dinitrophenyl (Dnp) for His; dichlorobenzyl (Cl₂-Bzl) for Tyr; and chlorobenzyloxycarbonyl (Cl-Z) for Lys. Cleavage of the Boc group was carried out with 37.5% TFA in DCM during 30 min. The TFA salt was neutralized with 5% DIEA in DCM three times for 2 min each. The amino acids were coupled using DIPCDI and the completion of the reaction was monitored by the ninhydrin test [11]. Side-chain deprotection [except for Cys (AcM)] and cleavage from the resin was performed following the 'low-high' HF procedure [12] with HF-DMS-*p*-cresol (25:65:10) during 2 h at 0 °C and with HF-DMS-anisole-thiocresol (79.8:10:10:0.2) during 1 h at 0 °C, respectively. The peptides were extracted with 30% HAc in water and lyophilized.

Purification and characterization

The reduced peptides were purified by HPLC (Pharmacia-LKB), using a RP-C18 column (25 × 250 mm, Vydac). A linear acetonitrile/water gradient from 15 to 45% B during 50 min and a flow rate of 4 mL/min were used. The crude and purified peptides were analyzed using an analytical RP-C18 column (4.6 × 100 mm, Vydac) and a linear gradient from 0 to 60% B in 40 min, the flow rate was 0.8 mL/min. The absorbance was monitored at 226 nm.

The cyclic peptides were characterized by FAB-MS and MALDI-MS, respectively.

Mass spectrometry

The FAB mass spectrum was obtained in a double-focusing two-sector JMS HX-110HF (JEOL, Japan) equipped with a FAB gun, a collision cell and a JEOL DMA-5000 data analysis system. The conditions for the measurements were the same as reported previously [13].

The MALDI-MS spectrum was performed using a Voyager Elite XL time-of-flight mass spectrometer equipped with a reflector and a delayed extraction system (PerSeptive Biosystems). An aqueous solution of C9-1 (0.5 μ L) and 0.5 μ L of a saturated solution of dihydroxybenzoic acid (DHB) containing 0.1% of TFA were mixed on the sample plate and dried at room temperature. The sample was ionized with a N₂ laser

(337 nm) and the positive ions were accelerated at -20 kV. A delayed extraction of 100 ns was used for the measurement. A mixture of angiotensin-I and insulin was used for the calibration of the mass spectrometer.

Intramolecular disulfide bond formation

The peptides were reduced with 100 mM DTT in 6M guanidine hydrochloride, 100 mM Tris at pH 8.2 during 2 h at 37°C and purified using a RP-C18 column (particle size, $40\text{--}63\ \mu\text{m}$). The reduced peptides were eluted with an isocratic gradient of TFA-acetonitrile- H_2O (1:49.5:49.5). The oxidation of thiol groups was carried out with 20% DMSO in water [14]. The reduced peptides were dissolved in HAc- H_2O (1:19) at 0.2 mM and the pH was adjusted to 6 with ammonium hydroxide solution (25% in water). DMSO was added to achieve the desired concentration. The completion of the oxidation reaction was monitored by an Ellman test [15].

Intermolecular oxidation (polymerization reaction)

The cyclic peptide (C9-1) was polymerized by simultaneous deprotection/oxidation of *S*-Acm with I_2 [16]. The peptide was dissolved at 32 mM in HAc- H_2O (4:1) and 3 equiv of HCl were added. Afterward, 10 equiv of I_2 in methanol were added and mixed during 15 min. The reaction was stopped with a solution of 10 equiv of ascorbic acid in water. The mixture was purified by gel filtration on a PD10 column (Pharmacia) with 150 mM ammonium acetate solution.

Tris-tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris-tricine-SDS-PAGE) [17] was used to determine the size of the polymeric cyclic peptide. 10% T, 3% C gel was used as a uniform separating gel, only overlaid by a 4% T, 3% C stacking gel. The molecular weight standards were incubated in a reducing sample buffer and the cyclic peptide and polymeric cyclic peptide were incubated in a non-reducing sample buffer for 30 min at 40°C .

Antisera

Balb/C, CBA/J, C57BL/6 and DBA/2 mice were housed under standard conditions of temperature and humidity with a 12 h lighting cycle and with food and water available *ad libitum*. Five mice per strain were immunized subcutaneously with $20\ \mu\text{g}$ of cyclic peptide or polymeric cyclic peptide, adsorbed to 2 mg of aluminium hydroxide on days 1, 15, and 30. Ten days after the third immunization the mice were bled and sera were stored at -20°C .

ELISAs

Flat-bottomed microtiter plates were coated overnight at 4°C with $5\ \mu\text{g/mL}$ of outer membrane protein vesicles (OMPV) of *N. meningitidis* strain B385 in 0.05 M sodium carbonate buffer at pH 9.6. Test antisera were serially diluted in PBS containing tween-20 (0.05%) (PBST). The antibody binding was detected using horseradish peroxidase-conjugate anti-mouse IgG (Amersham) with *o*-phenylenediamine and H_2O_2 as enzyme substrates. Absorbance was measured at 492 nm.

SDS-PAGE and Western blotting

Outer membrane protein preparations of strain B385 or 60 μg of filamentous phage were suspended in Laemmli sample buffer and separated by 10% SDS-PAGE as described by Laemmli [18]. The proteins were transferred to nitrocellulose paper as described [19]. In the case of the outer membrane protein detection, the nitrocellulose sheet was stripped and the strips were incubated for 1 h at RT with antisera pools at a dilution of 1:100. As positive control we used the mAb 5E8 at $4\ \mu\text{g/mL}$. This mAb recognizes the sequence HYTRQNNADVFP that is present in the meningococcal subtype P1.15 (data pending publication, kindly provided by Consuelo Nazábal Gálvez from CIGB, Cuba). For the detection of the loop 4 peptide exposition on the surface of phage M13, the nitrocellulose sheet was incubated with the antibodies raised in C57BL/6 mice against the polymerized cyclic peptide at a dilution 1:100.

Bactericidal assay

The bactericidal assay was done as described previously [20]. Twofold dilutions of the sera were assayed with inoculum of 80–100 colony-forming units per well, in the presence of 25% human complement.

Opsonization and phagocytosis measurement

Meningococci strain B385 killed by 70% ethanol were labeled with FITC [21]. As effector cells, we used human polymorphonuclear leukocytes (PMN) prepared by ammonium chloride lysis of heparinized blood. The opsonization assay was performed as reported previously [22]. The results are given as percent positive effector cells (fluorescent cells) within the gate, i.e. fluorescence above the negative control consisting of meningococci, complement source and effector cells.

Figure 1. Outline of the synthesis of the polymeric cyclic peptide derived from the surface loop 4 class 1 OMP from meningococcal strain B385.

Phage display of loop 4 peptide sequence

The loop 4 of class 1 protein from strain B385 was amplified by polymerase chain reaction under the following conditions: 30 cycles at 95 °C for 1 min, 65 °C for 1 min and 72 °C for 30 s. The reaction components were 1 µg of chromosomal DNA from the strain B385; 50 pmol of each primer (TCCCATGGCCTGCCCCGATCCAAAACAGCAAG (sense) and TAGTCGACCTAGCAGCATCCGGGCTTGCCGAC (antisense)); 200 µM of each deoxynucleoside triphosphate (dNTP); PCR buffer (10 mM KCl; 20 mM Tris-HCl pH 8.8; 10 mM (NH₄)₂SO₄; 2 mM MgSO₄; 0.1% Triton X-100), doubly distilled water up to a final volume of 75 µl, and 1 U per reaction of *Thermus aquaticus* DNA polymerase. The amplified DNA fragment was SalI/NcoI digested and cloned into the phagemid vector pEXmide4 [23], previously digested with the same enzymes.

Cells of *E. coli* strain TG-1 [F' *traD36 lacI^q* Δ(*lacZ*)M15 *proA⁺B⁺/supE* Δ(*hsdM-mcrB*)5(*rK⁻-mK⁻McrB⁻*) *thi* Δ(*lac-proAB*)] were transformed with the recombinant phagemid vector and infected with the M13KO7 wild-type filamentous phage. Western blotting analysis confirmed the display of the peptide.

Routine recombinant DNA and phage methodologies were carried out as described [24].

Figure 2. Analytical RP-HPLC profiles of C9-1 peptide. (a) Linear peptide; (b) cyclic peptide. Column: RP-C18 (4.6 × 100 mm, Vydac). Gradient: from 0 to 60% B during 40 min. Flow: 0.8 mL/min. λ = 226 nm.

Results and discussion

Peptide synthesis, oxidation and polymerization

The sequences of the peptides derived from the surface loop 4 of class 1 OMP from *N. meningitidis* strain B385 are shown in Table 1.

The peptide (C9-2) was synthesized adding one Cys residue at the N- and C-termini of the sequence to obtain the cyclic peptide. In this case the Boc-Cys(Mob)-OH derivative was used.

In order to obtain the polymerized cyclic peptide (C9-1), we combined two different cysteine-protecting groups: Boc-Cys(Mob)-OH and Boc-Cys(Acm)-OH. The Cys(Mob) was added to the N- and C-termini in the peptide, followed by three Gly and Cys(Acm) at each side of the sequence. Figure 1 shows the general strategy used to obtain the polymeric cyclic peptide.

The intramolecular and intermolecular oxidation reactions were carried out in solution. The intramolecular disulfide bond was obtained by oxidation of the free thiol groups of cysteine. The intermolecular disulfide bond was obtained by simultaneous deprotection/oxidation of the S-Acm groups.

Table 1. Sequences of the peptides derived from the surface loop 4 of class 1 OMP from *N. meningitidis* strain B385

Sequence	Peptide
$\text{CGGGCPIQNSKSAYTPAHYTRQNNADVFPVAVVGKPGSCGGGC-NH}_2$ Acm Acm	C9-1
$\text{AcCPIQNSKSAYTPAHYTRQNNADVFPVAVVGKPGSC-NH}_2$	C9-2

Figure 3. Analytical RP-HPLC profiles of C9-2 peptide. (a) Linear peptide; (b) cyclic peptide. Column: RP-C18 (4.6 × 100 mm, Vydac). Gradient: from 0 to 60% B during 40 min. Flow: 0.8 mL/min. $\lambda = 226$ nm.

Figures 2 and 3 show the RP-HPLC profile of peptides C9-1 and C9-2, respectively. Profile 'a' in each figure corresponds to the linear peptide. Both linear peptides had a significant undesirable polymeric population obtained during the cleavage reaction. Therefore, it was necessary to reduce the crude material previous to oxidation. The peptides were reduced with 100 mM DTT in a denaturing solution and purified on an RP-HPLC system. Profile 'b' in each figure corresponds to the cyclic peptide. The reduced and purified peptides were cyclized with 20% DMSO in water during three days. The oxidation reaction was stopped

when the Ellman test was negative. The cyclic peptide elutes at lower retention time than the linear peptide in both cases.

The cyclic peptides C9-1 and C9-2 were characterized by MALDI-MS and FAB-MS, respectively (Figure 4). The molecular weights determined experimentally showed good agreement with the theoretical average masses calculated for each peptide, taking into account the modifications contained within their structures.

The peptide C9-1 was designed to obtain the polymerized cyclic loop 4. After obtaining the intramolecular disulfide bond, the cyclic peptide was polymerized at 32 mM by simultaneous deprotection/oxidation of the S-Acm group with iodine. The acid medium was used to mitigate some side reactions that take place under the conditions used in the intermolecular oxidation reaction.

Figure 5 shows the Tricine-SDS-PAGE of the polymerized cyclic peptide (C9-1). Lane 1, in the gel, corresponds to the molecular weight standard. Lane 2 corresponds to the cyclic peptide (4.4 kDa molecular weight), and lane 3 shows bands of different sizes higher than the monomer size, corresponding to the polymeric cyclic peptide population.

Immunogenicity of synthetic peptides

All attempts to obtain protective antibodies using linear peptides derived from the surface loops of class 1 outer membrane proteins have been unsuccessful [3, 4].

Christodoulides et al. [4] have shown that rabbits immunized with the unconjugated 36-mer cyclic peptide, derived from the surface loop 4 of class 1 OMP from meningococcal strain MC58, which represents the subtype P1.16b, produced antiserum that promoted complement-mediated bactericidal activity. Following this previous report, the equivalent unconjugated cyclic peptide, derived from meningococcal strain B385,

Figure 4. (A) MALDI-MS and (B) FAB-MS spectra of the cyclic peptides C9-1 and C9-2, respectively. The theoretical masses of the peptides are indicated in parentheses in each spectrum.

Figure 5. Tricine-SDS-PAGE. Separating gel (10% T, 3% C). Lane 1, molecular weight standards incubated with reducing sample buffer (indicated in kDa); lane 2, monomer peptide incubated with non-reducing buffer sample; lane 3, polymeric cyclic peptide incubated with non-reducing buffer sample. The samples were visualized by silver staining.

which represents the subtype P1.15, was used for immunization of mice in order to study its ability to mimic the conformation in the native protein and to promote complement-mediated bactericidal activity.

In a first attempt, the cyclic peptide was used for immunizing Balb/C mice and the antibodies elicited were able to recognize the native protein only poorly. The immune response of laboratory animals to synthetic peptides may vary even among strains within species. Therefore, our next step was to immunize different mouse strains to discard the genetic background as the cause of the unsatisfactory results achieved with Balb/C strain.

Borrás-Cuesta et al. [25] demonstrated that immunogenicity of linear synthetic peptides can be enhanced by polymerization. To test a more efficient cyclic peptide presentation, in the present paper we developed a strategy to obtain a polymer of cyclic peptides. This approach could be useful in the synthetic vaccine field.

Four mouse strains: Balb/C (H-2^d), CBA/J (H-2^k), C57BL/6 (H-2^b) and DBA/2 (H-2^d) were immunized with the C9-2 and polymerized C9-1 peptides (Table 1) containing the loop 4 of class 1 OMP from meningococcal strain B385.

To determine the ability of anti-peptide antisera to recognize native class 1 protein, they were assayed with OMPV from strain B385 in ELISA (Figure 6). The highest levels of antibodies were elicited against the polymeric cyclic peptide, independently of the mouse strain used.

Figure 6. Reactivity of the murine anti-cyclic and anti-polymeric cyclic peptides polyclonal antibodies against meningococcal outer membrane vesicles from strain B385, measured by ELISA. The titers are expressed as the maximal dilution that gave an optical density at 492 nm twice greater than preimmune sera after 15 min of incubation with substrate.

Figure 7. Western blots of antisera obtained by immunization with synthetic peptides. Line 1, control with anti-class 1 protein P1.15 mAb; line 2, antisera anti-B385 OMPv; line 3, antisera anti-polymeric cyclic peptide in CBA/J; line 4, antisera anti-polymeric cyclic peptide in C57BL/6; line 5, antisera anti-cyclic peptide in C57BL/6; line 6, antisera anti-polymeric cyclic peptide anti-DBA/2; line 7, antisera anti-polymeric cyclic peptide in Balb/C; line 8, antisera anti-cyclic peptide in Balb/C; line 9, antisera anti-cyclic peptide in CBA/J; line 10, antisera anti-cyclic peptide in DBA/2; line 11, pre-immune sera. Antisera pools were tested against meningococcal outer membrane vesicles.

The anti-peptide antisera were also assayed by Western blotting against outer membrane preparations from meningococcal strain B385 (Figure 7). The antibodies recognized the natural protein. The strongest signal was obtained with the pCBA/J and pC57BL/6 antisera. The results obtained by ELISA correlated with those from Western blotting. The results obtained by immunization with polymer of the cyclic peptide loop 4 demonstrate that the polymeric presentation of cyclic peptide enhances the immune response.

The antisera elicited against cyclic and polymeric cyclic peptides did not show bactericidal activity. Nevertheless, when the test for the measurement of the opsonic activity was performed, the antisera elicited against the polymeric cyclic peptide in CBA/J mice showed the highest percentage of phagocytized bacteria (data not shown). This fact indicates that the

polymeric presentation of cyclic peptides partially supplies the conformational requirements of this epitope, permitting the antiserum to recognize the native protein. Although, in this study, the peptide was subjected to cyclization and polymerization in an attempt to restrict its conformation to more closely resemble the native loop structure, it induced antibodies in mice that presumably contain a large proportion of non-functional and conformationally irrelevant antibodies. From a theoretical point of view, this could happen due to the incorrect presentation of the epitope to the immune system of mice. On the other hand, it is possible that the T-cell epitope presented in this peptide is not effective for the specific haplotypes of mouse strain used, and therefore the T-cell help provided is poor. It is also possible that this peptide does not have epitopes recognized by T cells of the mouse strain used.

Figure 8. Analysis by Western blotting of 60 μ g of filamentous phages, tested with the sera elicited in the strain C57BL/6 against the polymeric cyclic peptide. Lane 1, 10 μ g of meningococcal outer membrane vesicles; lanes 2–3, recombinant filamentous phage displaying the loop 4 of class 1 from strain B385; lane 4, filamentous phage packing the pEXmide4 native phagemid vector; lane 5, wild type filamentous phage M13. The molecular weight markers are indicated on the left in kDa.

Hoogerhout et al. [26] have shown that peptides derived from class 1 protein elicited functional response in mice when conjugated. At present, our research group is doing experiments to test the immunogenicity in mice of these peptides conjugated to a carrier protein.

Finally, the antibodies were evaluated for their ability to recognize the peptide representing the loop 4 from class 1 protein of meningococcal strain B385 when exposed on the surface of filamentous phage M13. The Western blotting analysis of Figure 8 shows that the sera elicited in the strain C57BL/6 against the polymeric cyclic peptide recognized two recombinant filamentous phage expressing the former peptide fused to the PIII protein of M13 [23]. The sera did not recognize the control phage used. Similar results were obtained when the phages were immunoassayed with the sera elicited against the polymeric cyclic peptide in the other mouse strains.

Conclusions

In this paper we have described a synthetic methodology to obtain polymers of cyclic peptides combining the *S*-Mob and the *S*-Acm protection for cysteine. The peptide derived from the surface loop 4 of class 1 OMP from *N. meningitidis* strain B385 was polymerized following this strategy. The cyclic and polymeric cyclic peptides were used in immunological studies. The titers, measured by ELISA, raised against the polymeric cyclic peptide were higher than the ones raised against the cyclic peptide, and the strong signal in Western blotting was obtained using the antisera raised in CBA/J and C57BL/6 strains. The antisera elicited did not show bactericidal activity. Nevertheless, the antiserum elicited against the polymeric cyclic peptide in CBA/J mice showed opsonic activity. The antibodies raised against the polymeric cyclic peptide were successfully used as probes in Western blotting experiments to verify the display of the loop 4 peptide at the surface of filamentous phage M13.

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