Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1417–1420

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis of a Disulfide-Linked Octameric Peptide Construct Carrying Three Different Antigenic Determinants

Goran Kragol,[a,*] Laszlo Otvos, Jr.,[a] JingQi Feng,[a] Walter Gerhard[a] and John D. Wade[b]

[a]*The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA*
[b]*Howard Florey Institute, University of Melbourne, Victoria 3010, Australia*

Received 14 February 2001; accepted 30 March 2001

Abstract—In an effort to develop peptide vaccines against the influenza virus, we have successfully synthesized a disulfide-linked octameric homodimer that bears four copies of the influenza virus M2 protein ectodomain as well as two copies each of T-helper cell hemagglutinin epitopes, the I-E^d restricted S1 and the I-A^d restricted S2 fragments. Peptide attachment was via intermolecular disulfide formation from free sulfhydryl-bearing cysteine derivatives in solution. This reaction was efficient only when the amino-group of the cysteine was Fmoc-protected.

As part of an on-going endeavor to develop peptide vaccines against influenza virus, we recently reported the synthesis of branched peptide vaccine prototypes carrying single copies of 24-amino acid residue ectodomain of the M2 protein, the I-E^d major histocompatibility complex (MHC) restricted S1 and the I-A^d restricted S2 fragments as well as four mannose moieties for improved delivery via mannose-receptors of the antigen presenting cells.[1] In contrast to influenza vaccine candidates that utilize the antigenic properties of the viral glycoprotein, the ectodomain of the transmembrane ion-channel protein M2 has the potential advantage of providing highly crossreactive protection.[2,3] As polyvalency, which permits crosslinking of antigen-specific immunoglobulin receptors on B-cells,[4] and presence of strong covalently linked helper T-cell determinants are important features of potent B-cell immunogens, we envisioned a vaccine prototype carrying four copies of the M2 ectodomain B-cell epitope and two copies each of the S1 and S2 T-helper cell epitopes. Nevertheless, the assembly of such highly complex and well-defined multimeric peptide constructs that are useful for subunit vaccine development requires the use of state-of-the-art solid-phase/solution synthesis.

Since four-branched peptide constructs tend to be of higher purity than eight-branched,[5] we decided to assemble tetrameric construct **2** with two copies of M2 and one copy each of the S1 and S2 epitopes. Subsequent intermolecular disulfide-bond connection of two terminal cysteine residues would then generate high purity homodimer **7** having the desired octameric structure (Scheme 1). This strategy would also allow a comparison of the immunogenicity of the constructs **2** and **7**. Disulfide bridges usually stabilize the three-dimensional structure of extracellular proteins by bridging a single chain or by linking different polypeptide chains, such as occurs with relaxin and insulin.[6] Intermolecular disulfide linkages have also been successfully used for the conjugation of peptides to carrier proteins or for the preparation of proteins with full enzymatic activity.[7,8] However, because of the high molecular weight of the construct **2** and thus an anticipated high steric hindrance around its backbone that may prevent effective disulfide formation, we assumed that connection of two terminal cysteines would be a crucial step in the final yield of the desired octameric construct **7**. This communication reports the successful oxidation of this highly complex artificial protein.[9]

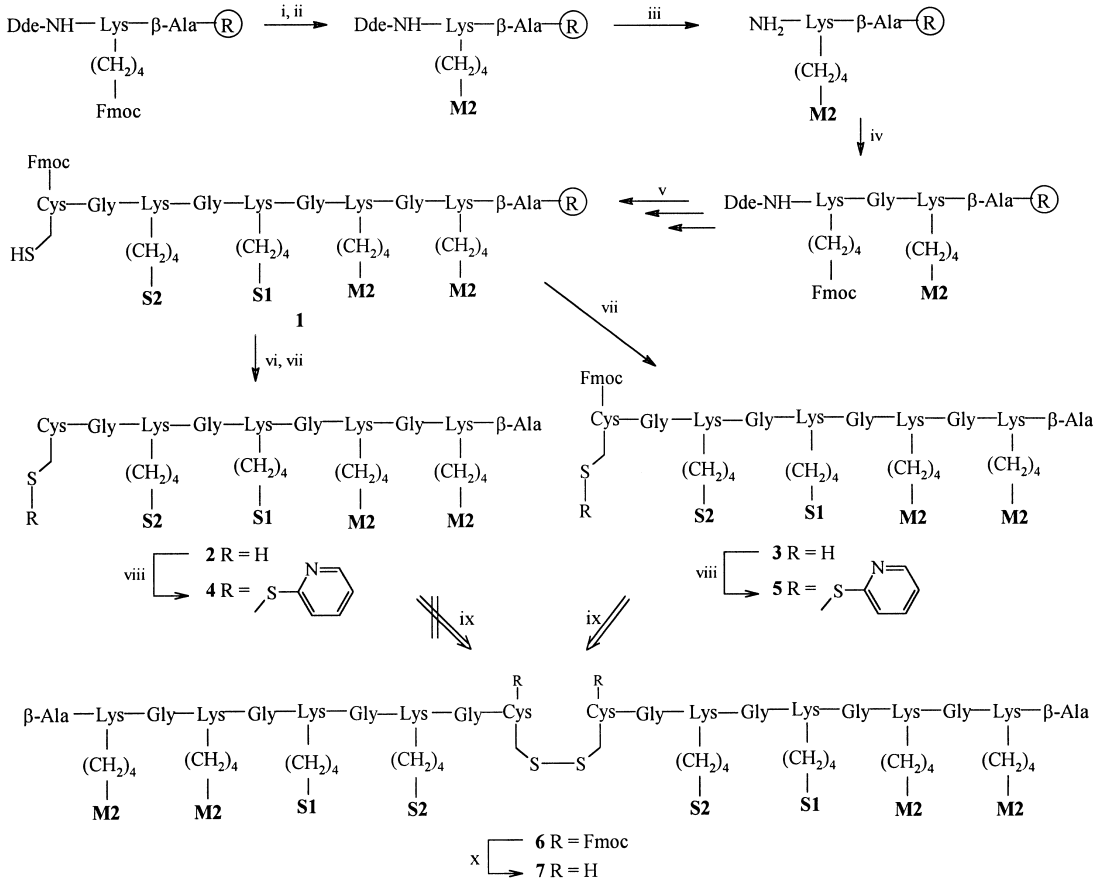
For the assembly of the resin-bound construct **1**, we used a strategy that includes the sequential and regioselective synthesis of M2, S1, and S2 epitopes on the side chains of ε-amino groups of the poly(lysine-glycine) backbone during the course of backbone extension on the solid support (Scheme 1).[10] Assembly was accomplished using continuous flow synthesizer (MilliGen 9050) and Fmoc-PAL-polyethyleneglycol-polystyrene

*Corresponding author. Fax: +1-215-898-5821; e-mail: kragol@wistar.upenn.edu

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00252-9

resin with low initial capacity (0.15 mmol g^{-1}, 0.25 g). A 4-fold molar excess of each amino acid was activated with HATU and coupled to the growing peptide chain for 1–3 h, depending upon the individual coupling difficulties predicted by Peptide Companion algorithm (Windowchem, Fairfield, CA, USA). The terminal Fmoc group was removed from one-half of the resin bound construct **1** using the end deprotection cycle on the synthesizer. Peptide construct **2** was cleaved from the resin by a 4-h treatment with reagent K (TFA/water/phenol/thioanisole/ethanedithiol 82.5:5:5:5:2.5, v/v/v/v/v) mixture. Analytical RP-HPLC showed one main peak with retention time of 37.1 min, and the expected molecular weight of 8994 Da (Table 1). Preparative RP-HPLC purification yielded 7.9 mg of pure construct **2**.

Among the numerous methods for disulfide bond formation, we chose to use the simplest yet still efficient, that of oxidation of free sulfhydryl-bearing cysteine derivatives in solution.[12] Two methods were initially examined: oxidation in the presence of atmospheric oxygen in 0.5 mM Tris–HCl (pH 8.0),[13] and dimethyl sulfoxide (DMSO)-mediated oxidation in 5% aqueous acetic acid/20% DMSO solution (pH 5.5).[14] In order to monitor the dimerization rate, a combination of RP-HPLC, MALDI-MS and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used. However, none of these analytical techniques detected the presence of the target homodimer **7** after either 24 or 72 h at room temperature. The rate of disulfide bond formation during conventional air-oxidation reaction has been reported to be greatly enhanced by the addition of 2,2′-dithiodipyridine solution in methanol.[15] However, in the case of construct **2** this modification also failed to produce the dimer **7**. Since 2,2′-dithiodipyridine is often used for the formation of disulfide-linked heterodimers,[8] we prepared and purified the activated construct **4** (Scheme 1). When activated construct **4** was added in excess to the solution of **2** in a 0.1 M sodium acetate (pH 5.0),[8,13] we could only observe disappearance of the peak corresponding to the activated construct **4** on RP-HPLC. Although an increase of the area under the peak corresponding to construct **2** was also detected, after purification of the later peak MALDI-MS and SDS-PAGE showed that



Scheme 1. . **M2** = SLLTEVETPIRNEWGSRSNDSSDP; **S1** = SFERFEIFPKE; **S2** = HNTNGVTAASSHE. (i) 20% piperidine/DMF; (ii) standard Fmoc-solid-phase synthesis of M2 with terminal Boc-protected serine; (iii) manual Dde cleavage using 2% hydrazine/DMF (3×3 min, 10 mL/0.5 g of resin); (iv) Fmoc standard coupling of Fmoc-Gly-OH and Dde-Lys(Fmoc)-OH; (v) addition of M2, S1, and S2 and final coupling of Fmoc-Gly-OH and Fmoc-Cys(Trt)-OH; (vi) 20% piperidine/DMF; (vii) cleavage from the resin using reagent K, 2.5 mL/0.125 g of resin-bound construct; (viii) 2.0 mg of 2,2′-dithiodipyridine dissolved in 100 μL of HPLC solvents A/B (1:9) was added to the solution of 0.5 mg of **2** or **3** in 100 μL of HPLC solvents A/B (1:1), after 1 h at room temperature preparative RP-HPLC purification yielded ~0.4 mg (80%) of activated constructs **4** and **5**; (ix) (1) 1.0 mg of **2** or **3** in 200 μL of 0.5 M Tris–HCl (pH 8) or (2) 1.0 mg of **2** or **3** in 200 μL of 5% acetic acid/20% DMSO solution in water (pH 5.5); or (3) same conditions as in 1, only 50 μL of 1 mM solution of 2,2′-dithiodipyridine in methanol was added or (4) 0.4 mg of **2** or **3** and 0.6 mg of activated **4** and **5**, respectively, in 200 μL of 0.1 M sodium acetate (pH 5); reactants were mixed in a glass vial and allowed to stay at rt for 24 h; (x) 20% piperidine/DMF, 1 h.

the target homodimer **7** was not present in the reaction mixture.

Our conclusion was that terminal cysteine residues were too sterically hindered to allow disulfide formation. Consequently, it was decided to try homodimerization of the Fmoc-amino protected cysteine construct **3**. Dimerization of such derivatives may have the advantage of allowing better HPLC resolution because of the hydrophobicity of the Fmoc-group causing a significant delay of the retention time. As a potentional drawback of this approach, the bulky Fmoc-group could well further increase the steric hindrance around the N-terminal end of the peptide backbone. Nevertheless, the same modification, as became evident later, may change the overall conformation of the construct **3** allowing the sulfhydryl group to be better exposed for the desired disulfide formation. The Fmoc-protected construct **3** was cleaved from the second half of the resin and purified in a same way as construct **2** yielding 6.5 mg of pure product. Construct **3**, in comparison with construct **2**, had an almost 2-min delayed retention time (Table 1). This suggests that the Fmoc-group was well exposed to the reversed-phase packing material of the chromatography column. On that basis and in complete contrast to the previous, failed dimerization attempts of construct **2**, both dimerization methods that utilized the Fmoc-protected construct **3** or corresponding activated construct **5** were successful. However, the presence of the dimer was not obvious based solely on chromatographic data. After the first attempt of dimerization using a mixture of constructs **3** and **5** no new peaks were detected by RP-HPLC. As in the case of the constructs **2** and **4**, we could only detect the disappearance of the construct **5** and an increase of the area under the peak of the construct **3**. Nevertheless, MALDI-MS and SDS-PAGE after 24 h reaction time unambiguously showed that both target homodimer **6** and monomer **3** were present in the reaction mixture but they could not be separated by RP-HPLC. It became clear that shielding of the Fmoc groups by surrounding epitopes decreases its influence on the hydrophobicity of the homodimer **6**. In addition, laser-induced rupture of disulfide bonds yielded MALDI-MS spectra that consistently showed the presence of only small amounts of the dimer and mostly the monomer. However, SDS-PAGE of the purified monomer/dimer peak verified the dominance of the homodimer **6** following every oxidation method that we tried (Fig. 1).

Table 1. Analytical data of the synthetic peptide constructs[11]

Construct	RP-HPLC retention time (min)[a]	MALDI-MS $[(M+H)^+]$	
		Calculated	Observed
2	37.1	8994	8994
3	38.9	9217	9218
4	37.6	9105	—
5	39.4	9328	—
6	38.9	18,434	—
7	37.6	17,988	17,993[b]

[a]5 min isocratic elution of 5% B followed by linear gradient from 5 to 65% B for 45 min.
[b]17,987 by ESI-MS.

Since new methods for the Fmoc group removal in solution[16,17] could cause disulfide-bond cleavage, in this instance the Fmoc groups were removed by dissolving homodimer **6** in 20% piperidine/dimethylformamide (DMF) solution which is preferentially used in solid-phase synthesis.[18] After 2 h of reaction time, RP-HPLC showed one main peak with the highest retention time and some smaller peaks (Fig. 2). SDS-PAGE of the purified products identified the main peak as deprotected homodimer **7** while a small, less hydrophobic peak was shown to be monomer **2**. By this approach, it was observed that the smallest amount of monomer **2** was obtained using DMSO mediated oxidation or the reaction between **3** and **5**.

In order to avoid unnecessary purifications and subsequent product loss, DMSO-mediated dimerization was optimized in the following manner. Construct **3** (0.8 mg) was dissolved in 50 μL of 5% acetic acid/20% DMSO solution. Aliquots of 1 μL were taken every hour, diluted with 25 μL of 20% DMF/piperidine and after 1 h the rate of oxidation was assessed by analytical RP-HPLC. After 4 h the reaction reached the point where no further dimerization could be detected but there was still some construct **3** present in the mixture. The reaction mixture was then diluted with 0.5 mL of 20% DMF/pyridine solution. After 1 h, the mixture was further diluted with 0.5 mL of RP-HPLC solvent A and purified directly on the preparative RP-HPLC column.

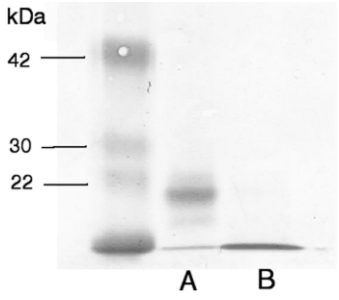


Figure 1. SDS-PAGE of: (A) purified mixture after DMSO mediated oxidation of **3**; (B) construct **3**.

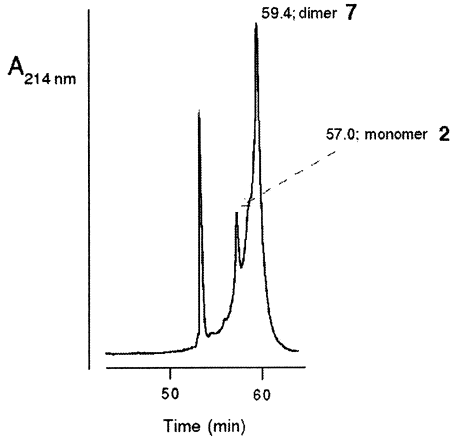


Figure 2. RP-HPLC of the crude mixture after Fmoc deprotection of the purified monomer **3**/dimer **6** mixture (5 min isocratic elution of 5% B followed by linear gradient from 5 to 65% B for 90 min).

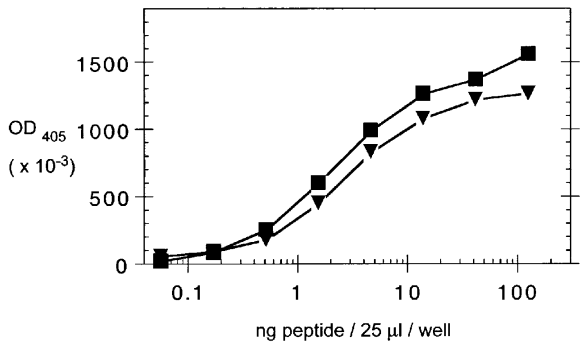


Figure 3. ELISA testing of monomer **2** (triangles) and dimer **7** (squares) for the presence of antigenically active M2 determinants.

This method yielded 0.275 mg (68%) of the homodimer **7**. When analyzed by ESI-MS, a relatively complex spectrum was obtained although clear series of multiply charged ions were observed indicating a mass of approximately 17,987 Da.

Monomer **2** and homodimer **7** were tested by ELISA for the presence of antigenically active M2 determinants. Both peptides could readily be detected at concentrations of less than 1 ng/well by the M2-specific monoclonal antibody 14C2[19] (Fig. 3). Preliminary studies have shown that both peptides induce an M2-specific antibody response upon immunization of mice (3 µg of the peptide constructs in PBS given intranasally, adjuvanted with a CpG oligonucleotide and cholera toxin)[20] and that homodimer **7** is slightly more effective in this regard than monomer **2**.

Taken together, this small-scale synthesis of the disulfide-linked homodimer **7** can provide an efficient means of preparation of similar constructs. It can enable preparation of adequate quantities of the desired products in good purity for immunological studies. However, for the larger-scale synthesis of successful vaccine candidates the method should be further optimized.

Acknowledgements

This work was supported by NIH grants GM45011 and AI46457.

References and Notes

1. Kragol, G.; Otvos, L., Jr. *Tetrahedron* **2001**, *57*, 957.
2. Neirynck, S.; Deroo, T.; Saelens, X.; Vanlandschoot, P.; Jou, W. M.; Fiers, W. *Nat. Med.* **1999**, *5*, 1157.
3. Frace, A. M.; Klimov, A. I.; Rowe, T.; Black, R. A.; Katz, J. M. *Vaccine* **1999**, *17*, 2237.
4. The minimal spacing between B-cell epitopes required for crosslinking is not known.
5. Grant, G. A.; Crankshaw, M. W.; Gorka, J. *Meth. Enzymol.* **1997**, *289*, 395.
6. Wade, J. D.; Tregear, G. W. *Meth. Enzymol.* **1997**, *289*, 637.
7. Mezö, G.; Mihala, N.; Andreu, D.; Hudecz, F. *Bioconjugate Chem.* **2000**, *11*, 484.
8. Baca, M.; Muir, T. W.; Schnölzer, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 1881.
9. Cysteine residues originally present in the M2 ectodomain and S2 epitope were replaced with serines. See for example: Chen, W.; Yewdell, J. W.; Levine, R. L.; Bennink, J. R. *J. Exp. Med.* **1999**, *189*, 1757
10. In comparison to conventional MAP design (Tam. J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5409) this strategy has the advantage of increased control over the spacing of antigenic determinants.
11. All synthetic compounds were analyzed and purified by RP-HPLC using a Waters low pressure mixing gradient system and Phenomenex analytical and preparative Jupiter C_{18} columns. Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. MALDI-MS was undertaken at the Wistar Institute Protein Microchemistry Laboratory. SDS-PAGE, was processed at rt at 110 V, transferred to poly(vinylidene difluoride) membrane overnight at 10 V and stained with amidoblack. ESI-MS was performed at the M-Scan Inc., West Chester, PA.
12. Annis, J.; Hargitai, B.; Barany, G. *Meth. Enzymol.* **1997**, *289*, 198.
13. Hoffmann, R.; Dawson, N. F.; Wade, J. D.; Otvos, L., Jr. *J. Peptide Res.* **1997**, *50*, 132.
14. Tam, J. P.; Dong, X. C.; Wu, C. R. *Lett. Pept. Sci.* **1999**, *6*, 265.
15. Maruyama, K.; Nagasawa, H.; Suzuki, A. *Peptides* **1999**, *20*, 881.
16. Sheppeck, J. E., II.; Kar, H.; Hong, H. *Tetrahedron Lett.* **2000**, *41*, 5329.
17. Leggio, A.; Liguori, A.; Napoli, A.; Siciliano, C.; Sindona, G. *Eur. J. Org. Chem.* **2000**, 573.
18. Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161.
19. Zebedee, S. L.; Lamb, R. A. *J. Virol.* **1988**, *62*, 2762.
20. McCluskie, M. J.; Davis, H. L. *Vaccine* **1999**, *18*, 231. Although this immunization protocol is optimal for our animal model it may not be applicable for humans.