Pergamon

Tetrahedron Letters 39 (1998) 4929–4932

TETRAHEDRON LETTERS

Synthesis of a designed transmembrane protein by thioether ligation of solubilised segments: Nα-haloacetylated peptides survived resin cleavage using TFA with EDT as scavenger

Darren R. Englebretsen[a]*, Christin T. Choma[b] and George T. Robillard[a]

[a] *Biochemistry Department, University of Groningen, Nijenborgh 4, Groningen 9747AG, The Netherlands*
[b] *Biochemsitry and Biophysics Department., University of Pennsylvania, Philadelphia, PA 19104-6059, USA.*

Received 17 April 1998; accepted 27 April 1998

Abstract: Nα-haloacetylated peptides made by Fmoc solid phase synthesis survived cleavage when EDT was used as a cleavage component. Two segments of a desgned transmembrane protein, one bromoacetylated, the other containing a cysteine, and each bearing a "solubilising tail" peptide, were synthesised by Fmoc SPPS. The two peptides were joined via thioether ligation. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: solid phase synthesis; peptides; cleavage reactions

In our work on the synthesis of designed transmembrane proteins chemoselective ligation[1] of peptide segments was a particularly attractive synthetic method. This is because it is usually easier to synthesise, purify and then ligate two smaller peptides (30-40 amino acids) rather than synthesise one longer one. In addition, chemical ligation methods may also allow incorporation of strategies for solubilising the peptide components.[2] Thus compatibility between peptide solubilising and chemical ligation strategies would be important. We studied the compatibility of thioether ligation with our peptide "solubilising tail" method by the synthesis of a designed transmembrane protein.

Thioether ligation makes use of the highly specific reaction between thiols and haloacetyl groups at neutral to mildly alkaline pH. The thiols may be from cysteine residues[3] or from peptides functionalised at the C-terminus with 2-mercaptoethylamido groups.[4-7] Ligation via a cysteine thiol results in a linkage incorporating a 1:1 substitution for the backbone atoms of two amino acid residues, while ligation via a 2-mercaptoethylamido group results in a linkage which is a surrogate for two glycine residues.[4,5,7] The success of the thioether ligation method relies on the ability to cleave resin-bound Nα-haloacetylated synthetic peptides without modification of the haloacetyl groups. This was first achieved by liquid hydrogen fluoride cleavage of Nα-haloacetylated peptides, made by Boc SPPS.[8-10] In such HF cleavages it was recommended that the use of sulphur-containing scavengers be avoided,[10] possibly to prevent reaction of the haloacetyl groups with sulphur-containing scavengers. Nα-haloacetylated peptides have also been cleaved after Fmoc SPPS using TFA,[11] TFA with anisole as scavenger,[12] or TMSBr.[13] To the best of our knowledge cleavage of Nα-haloacetylated peptides with mixtures containing TFA and thiol scavengers such as EDT has not been reported, despite the proven utility of EDT in suppressing side reactions during cleavage of peptides made by Fmoc SPPS.[14] Given the effectiveness of EDT as a scavenger to suppress side reactions during cleavage of peptides made by Fmoc SPPS, we were interested to study the cleavage of Nα-haloacetylated, solubilised peptides using cleavage mixtures containing EDT. We then studied thioether ligation of one of the resulting solubilised, haloacetylated peptides. The results of these studies are described below.

PII: S0040-4039(98)00898-3

Results and discussion

In a preliminary experiment, the predominant masses of Nα-chloroacetyl-(Gly-Lys)$_6$-Gly-amide and Nα-bromoacetyl-(Gly-Lys)$_6$-Gly-amide cleaved using reagent K[14] were 1261.7 Da and 1305.1 Da respectively (calculated masses: Nα-chloroacetyl-(GK)$_6$G-amide: 1261.9 Da, Nα-bromoacetyl-(GK)$_6$G-amide 1306.4 Da). MALDI-TOF analysis of the crude cleaved peptides showed that the Nα-chloro and Nα-bromo-acetyl groups had not reacted with EDT or thioanisole during cleavage to give measurable levels of by-products.

A complex Nα-bromoacetylated peptide, bearing at its C-terminus a solubilising peptide linked via a 4-hydroxymethylbenzoic acid [4-Hmb] derived ester, was synthesised. Nα-bromoacetyl-EAELENAVYLNAL VSLLNALVSLWTAKNPGAA-[4-Hmb]-(GK)$_6$G-amide (1) was cleaved with a mixture of TFA/EDT/water. An HPLC chromatogram of crude cleaved 1 is given as Figure 1a.

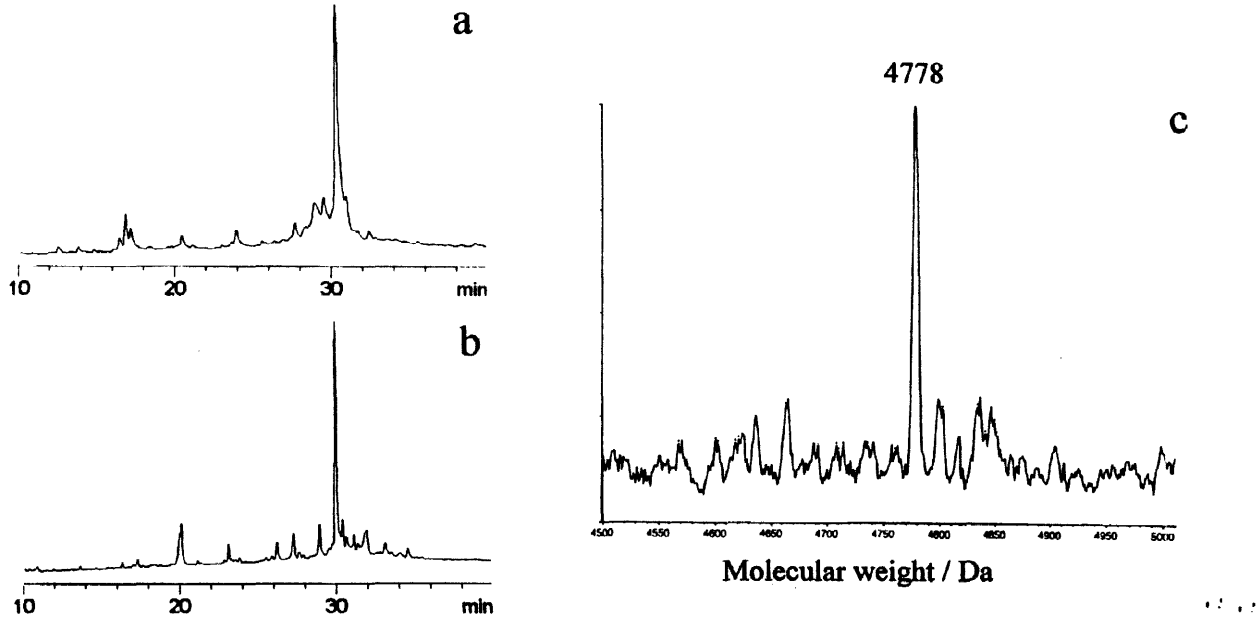

Figure 1. a) HPLC chromatogram of crude cleaved bromoacetylated peptide **1**. b) HPLC chromatogram of non-bromoacetylated **1**. HPLC conditions: Vydac C4, 0-80% B over 40 min at 1 ml/min, solvent A: 0.1% TFA, solvent B: 0.1% TFA, 10% water, 90% acetonitrile; monitored at 214 nm. c) Reconstructed ESMS spectrum of crude cleaved **1**.

Figure 1a shows that this long (46 aa) bromoacetylated peptide was obtained in good purity when cleaved using a mixture containing EDT. For comparison Figure 1b shows crude cleaved **1** which was without an Nα-bromoacetyl group. The major mass of the crude cleaved peptide mixture was found by electrospray mass spectrometry (ESMS) to be 4778 Da (calculated for **1**: 4778 Da). No EDT-peptide adduct (calculated mass 4791 Da) was seen in the reconstructed electrospray mass spectrum of crude cleaved **1** (Figure 1c).

Under the strongly acidic conditions of peptide cleavage we expected that there would be little reaction of thiol scavengers such as EDT with Nα-chloro or Nα-bromoacetyl-functionalised peptides, and this was indeed the case. However, a note of caution must be added. It has been found that in some cases certain bromoacetylated peptides reacted with the thiol of a cysteine even during cleavage with cold liquid HF.[10] Thus, the possibility exists that *sequence-related* reactions between thiol scavengers and peptide bromoacetyl groups may occur under acidic conditions, although we did not see evidence of such reactions in this study using TFA.

HPLC purified bromoacetylated peptide **1**, 4.93 mg, was ligated with 3.97 mg of the cysteine-bearing peptide Ac-C(acm)GGSPDQVWLNVLVSLLNVLVSLYTAQKAKNC-[4-Hmb]-(GK)$_6$G-amide (**2**), as shown in diagram form in Figure 2a. In this reaction both **1** and **2**, which were segments of a designed transmembrane protein, had "solubilising tail" peptides attached to their C-termini via 4-Hmb esters.

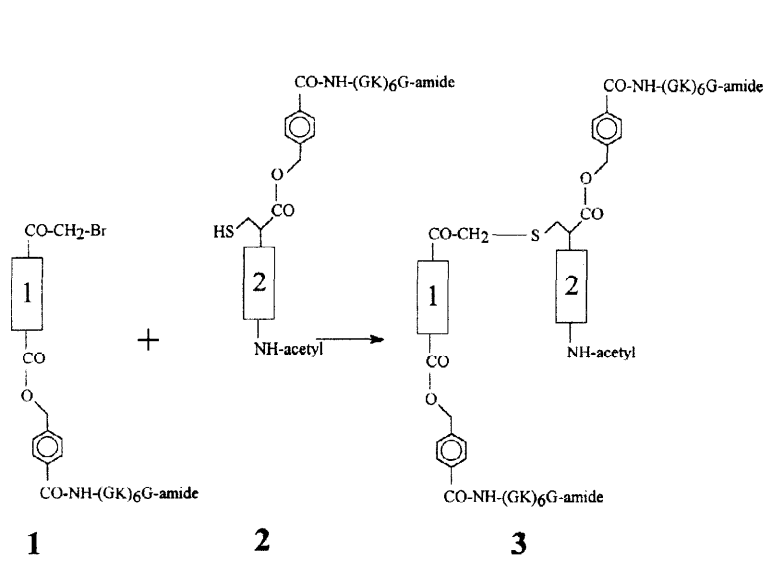

Figure 2. a) Thioether ligation of solubilised peptides **1** and **2**; b) HPLC chromatogram of the ligation of **1** and **2**; c) HPLC purified ligated peptide **3**. HPLC conditions as in Figure 2.

An HPLC chromatogram of the ligation mixture is given as Figure 2b. The mass of the major peak at 32.3 minutes was found by ESMS to be 9545 Da (calculated for thioether ligated product **3**: 9546 Da). The ligated peptide was purified by HPLC to yield 3.26 mg of **3** (Figure 2c).

In our work on the synthesis of designed membrane proteins thioether ligation was an attractive synthetic method because each segment could be made more soluble via an attached, selectively cleavable "solubilising tail" peptide.[2,15] The ligation reaction of Nα-bromoacetylated peptide **1** and cysteine-bearing peptide **2**, without loss of the solubilising peptides, showed that the neutral aqueous conditions used for thioether ligation were compatible with the 4-Hmb ester used to link hydrophobic and solubilising peptides. However, we have not determined whether thioether ligation is compatible with the glycolamide ester linkage used in the original Boc "solubilising tail" method.[15]

The methods described in this paper will be applicable to the synthesis and purification of other natural and designed membrane proteins. Thus a combination of the popular Fmoc method of SPPS, cleavage of haloacetylated peptides using TFA with efficient thiol scavengers, thioether ligation, and "solubilising tail" methods should simplify the synthesis and purification of these often difficult-to-purify proteins.[16]

Experimental

The peptide $(GK)_6G$-amide was synthesised using an ABI 433 automated peptide synthesiser, standard ABI Fastmoc SPPS protocols, and Novabiochem amide linker resin. The free N-terminal amine of the peptide was blocked by reaction of 1 mmole (8 eq) of the anhydride of either α-chloroacetic or α-bromoacetic acid for 30 minutes. The peptide-resins were washed with NMP followed by DCM, and dried under vacuum. Each peptide-resin was treated with 3 ml of premixed Reagent K (TFA:water:thioanisole:EDT:phenol 40:2:2:1:3 v/v/v/v/w) for one hour at room temperature.[14] The peptides were precipitated in 20 ml ether for 15 minutes, collected by filtration, washed extensively with ether, dissolved in 15% aqueous acetic acid, and then analysed by MALDI-TOF MS.

Peptides **1** and **2** were synthesised as above. Both of these peptides bore a "solubilising tail" linked to the peptide via a 4-Hmb ester linkage, as described elsewhere.[2] Peptides **1** and **2** were cleaved from the resin with a mixture of TFA:water:EDT (38:1:1 v/v/v) for one hour, and collected as above. Both **1** and **2** were

purified using standard HPLC techniques (Vydac C4, 25 x 250 mm, 0-70% B over 70 min at 8 ml/min, solvent A: 0.1% TFA; solvent B: 0.1% TFA, 10% water, 90% acetonitrile).

1 (4.93 mg) and **2** (3.97 mg) were ligated by dissolution in 2 ml of helium sparged buffer, 100 mM phosphate, pH 7, containing 6M urea. The course of the ligation reaction was followed by HPLC. After 24 hours the ligated peptide **3** was purified by HPLC (yield 3.26 mg) and characterised by HPLC and ESMS.

Acknowledgements

We thank Margot Jeronimus-Stratingh and Andries Bruins of the Mass Spectrometry section, Department of Pharmacy, Groningen University, for electrospray mass spectra.

References

1. Muir, T.W., Dawson, P.E., and Kent, S.B.H. Protein synthesis by chemical ligation of unprotected peptides in aqueous solution. In: G.B. Fields, editor. Methods in enzymology: Solid phase peptide synthesis. New York: Academic Press, **1997**, 289, 266-298 *and references therein.*
2. Choma, C.T., Robillard, G.T., and Englebretsen, D.R. *Tetrahedron Letts.*, **1998**, 39, 2417-2420.
3. Muir, T.W., Williams, M.J., Ginsberg, M.H., and Kent, S.B.H. *Biochemistry*, **1994**, 33, 7701-7708.
4. Englebretsen, D.R., Garnham, B.G., Bergman, D.A., and Alewood, P.F. *Tetrahedron Letts.*, **1995**, 48, 8871-8874.
5. Ramage, R., Biggin, G.W., Brown, A.R., Comer, A., Davison, A., Draffan, L., Jiang, Lu, Morton, G., Robertson, N., Shaw, K.T., Tennant, G., Urquhart, K., and Wilken, J. Methodology for chemical synthesis of proteins. In: R. Epton, editor. Innovations and perspectives in solid phase synthesis and combinatorial libraries. Birmingham: Mayflower Press, **1996**, 1-10.
6. Resins which give peptides functionalised with C-terminal 2-mercaptoethylamido functional groups (e.g. Cysteamine 4-methoxytrityl resn, Cysteamine 2-chlorotrityl resin) are commercially available. See, for example, Novabiochem catalog and peptide synthesis handbook, **1997/1998**.
7. Englebretsen, D.R., Bergman, D.A., Garnham, B.G., Brinkworth, R.I., Fairlie, D.P., and Alewood, P.F. Structure/function studies of the "flaps" region of HIV-1 protease by total chemical synthesis. In: P.T.P. Kaumaya and R.S.Hodges, editors. Peptides: Chemistry, structure and biology. England: Mayflower Scientific Ltd., **1996**, 589-590.
8. Lindner, W., and Robey, F.A. *Int. J. Peptide Protein Res.*, **1987**, 30, 794-800.
9. Robey, F.A., and Fields, R.L. *Analytical Biochemistry*, **1989**, 177, 373-377.
10. Robey, F.A. Bromoacetylated synthetic peptides. In: M.W. Pennington and B.M. Dunn, editors. Methods in Molecular Biology 35: Peptide synthesis protocols. Totowa, New Jersey: Humana Press, **1994**, 73-90.
11. Gibb, B.C., Mezo, A.R., and Sherman, J.C. Synthesis and characterization of a *de novo* four-helix bundle. In: P.T.P. Kaumaya and R.S. Hodges, editors. Peptides: Chemistry, structure and biology. England: Mayflower Scientific Ltd., **1996**, 585-586.
12. Morton, G. The chemical synthesis of proteins. University of Edinburgh, **1997**.
13. Futaki, S., Ishikawa, T., Niwa, M., Kitagawa, K., and Yagami, T. *Tetrahedron Letts.*, **1995**, 36, 5203-5206.
14. King, D.S., Fields, C.G., and Fields, G.B. *Int. J. Peptide Protein Res.*, **1990**, 36, 255-266.
15. Englebretsen, D.R., and Alewood, P.F. *Tetrahedron Letts.*, **1996**, 46, 8431-8434.
16. Olivera, E., Miranda, A., Albericio, F., Andreu, D., Paiva, A.C.M., Nakaie, C.R., and Tominga, M. *J. Peptide Res.*, **1997**, 49, 300-307.