Selective and facile cyclization of N-chloroacetylated peptides from the C4 domain of HIV Gp120 in LiCl/DMF solvent systems

Key words: cyclization; HIV C4 peptide; LiCl/DMF solvent system; N-chloroacetyl peptide; unprotected peptides

Abstract: Lithium salts have been reported to mediate the solubilization of peptides in organic solvents in 1989 (Seebach, D., Thaler, A. & Beck, A. K. Helv. Chim. Acta 1989; 72, 857–867). The use of Li salts in an organic solvent to influence cyclization of a reactive peptide that only polymerizes in an aqueous solvent, has not been reported. Here, the selective and facile cyclization of N-chloroacetylated, C-cysteine amide peptides from the C4 domain of HIV-1 gp120 in LiCl/DMF solvent systems is demonstrated. The addition of stoichiometric amounts of Tris base to 1 mg/mL peptide in LiCl/DMF solutions was sufficient to drive the cyclization to completion within 3 h at ambient temperatures. Cyclic peptides were the only detectable reaction products and these were confirmed using reversed-phase HPLC and mass spectrometric analyses of the final products. In aqueous solutions at pH 7.4, only polymers were obtained as judged by HPLC and SDS–PAGE. The method of using Li salts in an organic solvent to enhance the cyclization of unprotected amphipathic peptides may be useful in many situations beyond those described here.

Abbreviations: DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; S-CMC, S-carboxymethylcysteine; THF, tetrahydrofuran.

Lithium salts were reported to mediate the solubilization of peptides in organic solvents in 1989 [1]. As a nonconventional method in the synthesis of peptide compounds, Li-mediated solubilization of peptides would be most useful for carrying out reactions on specific amino acids that require aprotic, nonpolar and non-nucleophilic solvents. Although
it was demonstrated using NMR that ions influence peptide conformational equilibria in an organic solvent [1], the use of Li salts in an organic solvent to influence the cyclization of a reactive peptide that only polymerizes in an aqueous solvent, has not been reported.

In 1987, chloroacetyl moieties, placed at the N-terminus of a fully protected synthetic peptide, were shown to remain intact following normal HF deprotection procedures [2]. Deprotected peptides, N-terminally labeled with either chloroacetyl or bromoacetyl groups, are now routinely synthesized in high yield. Such reactive leaving groups as the haloacetyl moieties would then be used to specifically cyclize a peptide that was N-terminally haloacetylated and contained a thiol in another position in the peptide [2–7]. N-terminal haloacetylated resin-bound partially protected peptides are now used as starting materials to make libraries of cyclic peptides [8,9].

Previously, we showed that the N-chloroacetylated, C-cysteine amide form of the 18-amino acid C4 peptide from HIV-1 gp120 would polymerize, in a head-to-tail fashion, in an aqueous environment when the pH of the solution was brought to between 7 and 8 [10]. To mimic physiological ionic strength and pH, the chloroacetylated C4 peptide could be reacted in phosphate-buffered saline, pH 7.4 and, under these conditions, only the peptide polymer (peptomer) formed, with no indication of the monomeric cyclic peptide being present, as judged by reversed-phase HPLC. The C4 peptomer displayed a prominent β-helical conformation and, in this form, induced the formation of rabbit antibodies that recognized native and recombinant gp120 from HIV-1 [10].

A recent publication suggested that the C4 domain might be in a β-sheet–loop–β-sheet conformation when bound to recombinant CD4 [11] and this added urgency to our efforts in constructing peptides with appropriate conformations to realistically mimic the C4 domain in the intact protein gp120. To synthesize a ‘loop’ component of C4, the research effort was redirected to avoid making peptomers of C4 with emphasis on the monomeric form of cyclized C4. Here, the selective and facile cyclization of N-chloroacetylated [2–5], C-cysteine amide peptides from the C4 domain of HIV-1 gp120 in LiCl/DMF solvent systems is demonstrated.

Materials and Methods

All the reagents used here, including DMF, LiCl, Tris base, 5,5'-dithiobis (2-nitrobenzoic acid) and Ellman’s reagent, were purchased from Aldrich Chemical Co. The N-chloroacetyl, C-cysteine amide C4 peptides described here were custom-made by Biosynthesis, Inc. (Lewisville, TX, USA).

Unprotected N-chloroacetyl, C-cysteine amide peptides were cyclized on a 1-mg/mL scale. For 10 mg N-ClAc-RIKQIINMWQEVGKAMYAC-NH₂, suspended in 10 mL DMF, the following procedure was employed: solid LiCl in 250 mg portions was added with stirring to the peptide suspension until all the peptide had dissolved. For the full-length 18-mer given above, the addition of 1.5 g LiCl was sufficient to dissolve 10 mg in 10 mL DMF and this took 15 min. Following the complete dissolution of the peptide, the reaction of the C-terminal thiol with the N-terminal chloroacetyl moiety was tested and initiated by the addition of solid Tris. The reaction was followed using Ellman’s reagent [12], which monitored the disappearance of the free thiol. The reaction was complete in 1.5 h but was allowed to proceed for an additional 1.5 h at room temperature.

The cyclic peptide was isolated in pure form using the following procedure on a 10-mg/10-mL scale. Upon completion of the cyclization reaction [3 h, 25°C], 20 mL deionized water was added to the reaction mixture and the solution was frozen and lyophilized overnight to remove the DMF and H₂O. The resulting solid was redissolved in 20 mL H₂O and passed through a tC2 Sep-Pak® Plus cartridge (Waters Associates, Milford, MA, USA). The cartridge was washed extensively with deionized water containing 0.1% trifluoroacetic acid to remove the Li salts and residual DMF and the cyclic peptide was eluted with a 5-mL bolus of 70% aq. CH3CN. The eluant was dried overnight and a ≥ 90% yield of the cyclic C4 peptides were stored dessicated in the dark at ambient temperature.

Analytical procedures are given in the individual figure legends with the exception that amino acid analyses were performed as described previously using the Picotag® system which was used to confirm the presence of S-carboxymethylcysteine (S-CMC) in acid hydrolsates of the cyclic peptides used here. The S-CMC confirms the presence of the thioether that was formed when the peptides were cyclized N-terminus to C-terminus [13].

Results

The C4-derived peptides cyclized using the approach given in this report are listed in Table 1.

In order to learn more about the behavior of the C4 peptide in solvents that are less polar than water, several organic solvents were tested for dissolving the 18-amino acid N-
Table 1. Peptides cyclized in LiCl/DMF

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>Ac-G-G-Q-O-Q-I-I-N-M-W-Q-E-V-G-K-C-NH₂</td>
<td>Cyclized in LiCl/DMF</td>
</tr>
<tr>
<td>Ac-Q-I-I-N-M-A-W-Q-E-V-G-K-C-NH₂</td>
<td>Cyclized in LiCl/DMF</td>
</tr>
<tr>
<td>Ac-Q-I-I-N-M-S-Q-E-V-G-K-C-NH₂</td>
<td>Cyclized in LiCl/DMF</td>
</tr>
<tr>
<td>Ac-N-M-W-Q-E-V-G-K-C-NH₂</td>
<td>Cyclized in LiCl/DMF</td>
</tr>
</tbody>
</table>

In summary, complete conversion of the linear N-chloroacetylated, C-cysteine amide C₄ peptides to the cyclic form occurs within 3 h at ambient temperatures at a concentration of 1 mg/mL peptide in DMF with sufficient amounts of LiCl.

Discussion

This paper describes one alternative approach that may be taken to cyclize synthetic peptides that would only polymerize in aqueous solutions. Because many peptides cyclize in water, it may not be necessary to use the method described here. However, there may be isolated cases in which cyclization is quite desirable but other solvents and/or approaches were unsuccessful for cyclizing a particular peptide, as demonstrated here for the C₄ domain of gp120.

Proof that the chloroacetylated, cysteine amide-containing peptides cyclized exclusively in LiCl/DMF was given by a combination of observations. First, the disappearance of the free sulfhydryl groups in the N-chloroacetylated, C-cysteine amide-containing peptides in solution after the addition of Tris was followed. Sulfhydryl disappearance was followed using Ellman’s reagent [12] on 50 μL aliquotes of the reaction solution. This indicated that the C-terminal sulfhydryl group was being consumed. Second, a shift in the elution time of the peptide on reversed-phase HPLC to times earlier than the linear, noncyclized starting peptide provided proof that a change in the starting peptide had occurred and the change involved the formation of a new molecule with less available hydrophobic surface. Third, the stoichiometric amounts of S-CMC [13] in acid hydrolysates of the cyclized peptides can be formed only by the reaction of the C-terminal cysteine with the N-chloroacetylated moiety. Finally, mass spectrometry confirmed that the isolated product was of a mass consistent with a cyclized form of the linear starting material.

The exclusive cyclization of the C₄ peptides used here was an unexpected surprise insofar as the intent at the outset was to synthesize peptomers having molecular masses in excess of those obtained previously in aqueous solutions [10]. In addition, from a basic chemistry viewpoint, this peptide is amphipathic and often difficult to handle in organic solvents or above certain concentrations in salt-containing aqueous solutions (unpublished data). However, there are possible explanations for the exclusive cyclization of these peptides in LiCl/DMF solutions.

Peptide bonds contain strong π-character and this causes linear peptides to assume extended conformations. Cycliza-
tion is thus often unfavorable since the C- and N-termini are kept apart in remote positions (14). However, Li-salts are believed to influence the π-character of the peptide carbonyl and the hydrogen bonds involving the carbonyl oxygens (1). Therefore, in the presence of Li, unnatural conformations can be generated.

In addition, depending on the amino acid sequence of a peptide, it is reasonable to hypothesize that various side chains in a peptide could interact to fold a peptide intramolecularly when Li is present to destroy or move the π bonds involving the carbonyl oxygens. Thus, the N- and C-termini of the peptide in LiCl/DMF could end up in close proximity to each other and reside in an ideal location to allow for cyclization when the appropriate reactive moieties are present at both termini. Such is the case here with haloacetyl groups at the N-terminus and sulphydryl groups at the C-terminus. Further experimental work would have to be carried out to confirm the influences Li has on peptide bond π-characteristics and intramolecular side-chain interactions.

The C4 peptide and its fragments are of tremendous importance and interest because they constitutes a functionally vital part of the envelope protein, gp120, on HIV-1 that binds to CD4 on the surface of T cells (15). In other studies, similar amphipathic C4 peptides from gp120 were shown to shift conformations based on solvent polarity (16–18), and these results, in hindsight, may have been prophetic for the result obtained here: In the complex of gp120 bound to CD4, the C4 domain was shown to be present in a β-sheet–loop–β-sheet configuration (11) and the cyclic form of the C4 peptide reported here may be a better mimic of the loop portion than the C4 α-helix reported previously (10). Thus, analogues of the cyclic peptide described here should be more suitable immunogens for being components in an anti-HIV vaccine than the helical peptide described previously (10). The immunology and biology of the cyclic C4 peptides are the topics of communications elsewhere.

Several approaches have been taken to cyclize peptides (19–21), the most popular at the present time being via

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**Figure 1.** Reaction scheme showing the exclusive cyclization or polymerization of the N-chloroacetyl amphipathic C4 peptide, depending on the solvent system used. Aqueous solvents yield only head-to-tail polymers, whereas in LiCl/DMF only the cyclic peptide is formed at concentrations of 1 mg/mL as used in this study. The N-chloroacetyl, C-cysteine amide C4 peptides described here were purchased from BioSynthesis, Inc. (Lewisville, TX, USA).

**Figure 2.** Reversed-phase HPLC chromatograms of the linear N-chloroacetyl, C-cysteine amide C4 [broken lines] vs. the cyclic C4 peptide [solid line]. Column: Vydac C4 column, 4.6×250 mm; mobile phase: A, 0.1% TFA, B: 1% TFA in CH3CN; gradient, linear 0–100% B in 20 min; flow rate 1.0 mL/min; detection: UV, 280 nm.

**C4 Peptomer**
disulfide bond formation between two cysteine residues, both placed at various positions within the peptide chain [19]. For the methods described here, use of oxidizing agents, including DMSO as a solvent, are omitted in order to maintain the integrity of the free sulfhydryls so that the sulfhydryl is available for reaction with the N-chloroacetyl moiety. A key advantage of the thioether formed here over disulfides is that the thioether bond is stable in cell extracts, whereas disulfide-linked cyclic peptides are not [7]. In addition, thioether linkages were recently shown to be nonimmunogenic, a biological property that may be distinctively advantageous over other types of bond used to attach segments in cyclic peptides [22]. In contrast, the use of Li salts in DMSO with peptides having sulfhydryl groups at the N- and C-termini may be quite beneficial for making disulfide-linked cyclic peptides.

The method of using Li salts in an organic solvent to enhance the cyclization of unprotected peptides will probably be useful in many situations involving syntheses of cyclic peptides beyond those described here.

References


