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Native chemical ligation of hydrophobic peptides in organic solvents‡

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The application of chemistry to hydrophobic peptides and membrane-spanning helices is hampered by the fact that they are only poorly soluble in aqueous buffers and that they have a tendency for aggregation. These properties lead to difficulties when purifying them after chemical synthesis and particularly interfere with native chemical ligation. Here, we describe native chemical ligation of model peptides in the organic solvent dimethylformamide (DMF) under anhydrous conditions. Best results concerning yields and complete solubility are obtained if thiophenole is used in the presence of LiCl. These conditions might be applicable also for the ligation of transmembrane helices. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ligation in organic solvents; transmembrane proteins; peptide synthesis; hydrophobic peptides

Introduction

Cellular membranes harbour an important class of proteins which encompass diverse families like receptors, channels, transporters or proteins involved in energy transduction. About one-third of the human genome encodes for these membrane proteins [1], but despite their significance for cellular functions and medicinal applications the structures of only a few membrane proteins have been solved so far. The reason for this discrepancy is the fact that they are embedded in membranes, resulting in difficulties in expressing the proteins heterologously and solubilising them in an active state. Consequently, chemical studies and approaches to the total chemical synthesis of membrane proteins have been scarce up to now.

The total chemical synthesis of integral membrane-spanning helices would offer unique atom-by-atom control over the covalent structure of these proteins, which would be quite important for elucidating their function by biochemical and biophysical means [2]. Methods like solid-phase peptide synthesis (SPPS) [3], native chemical ligation (NCL) [4] and expressed protein ligation (EPL) [5] have been developed into tools that, nowadays, routinely allow the assembly of soluble proteins of up to 200 amino acids and the incorporation of non-natural amino acids, chromophores, isotopic labels or modifications for the protein immobilization on surfaces [2]. In contrast to soluble proteins, membrane proteins contain sequences of amino acids that reside within the lipid bilayer, and are consequently highly hydrophobic. This inherent hydrophobicity causes numerous experimental difficulties associated with the synthesis, purification and characterization of hydrophobic peptides and proteins [6,7]. Different strategies were developed to improve the handling properties of transmembrane peptides. Modifications to the HPLC mobile phase were successful in some cases, but there is no evidence that they are generally applicable [8–10]. A further improvement in the preparation of membrane-spanning peptides was introduced by Kent and co-workers who attached an arginine tag to the *C*-terminus via a thioester [6]. It can be removed by hydrolysis or is removed in the course of the ligation reaction.

It appears that smaller membrane-spanning peptides can readily be synthesized and purified. However, having purified the peptides, their NCL constitutes the next major challenge. The main problems are again connected to poor solubility and the tendency of hydrophobic peptides to aggregate under standard ligation conditions. To overcome these obstacles, ligations have been carried out, e.g. by adding organic solvents and/or detergents such as DDM, SDS and DPC [10–13]. Approaches which take advantage of the native environment for transmembrane domains such as ligation reactions in a lipid environment [14] or lipidic cubic phase [15] have also been described. However, reaction conditions in pure organic solvents have not been carried out so far.

Here, we present data on native chemical ligation in organic solvents. The rationale for using water-free reaction conditions was guided by two considerations: extreme hydrophobic peptides might be readily soluble in appropriate organic solvents and reaction times could be prolonged because the peptide thioester cannot be hydrolysed. As a model system, small hydrophobic peptides were chosen for analysing different ligation scenarios. The sequences are derived from transmembrane-spanning helices of

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the archaeal transducer protein *Np*HtrII. *Np*HtrII consists of two transmembrane *N*-terminal α-helices followed by a large cytoplasmic coiled coil domain [16,17]. *Np*HtrII mediates – in complex with sensory rhodopsin II (*Np*SRII), a seven-transmembrane α-helical protein – negative phototaxis in *Natronomonas pharaonis* [18].

Materials and Methods

General

Boc-L-Leu-OCH_2Pam resin was obtained from NeoMPS (Strasbourg, France). Boc-protected amino acids and O-benzotriazole-*N*,*N*,*N*,*N*-tetramethyluroniumhexafluorophosphate (HBTU) were purchased from Merck Chemicals (Nottingham, United Kingdom). Dichloromethane and dimethylformamide were obtained from Applied Biosystems (Darmstadt, Germany). Trifluoroacetic acid was purchased from Roth (Karlsruhe, Germany). All other chemicals were obtained from Sigma Aldrich (Taufkirchen, Germany) in the highest purity available.

Synthesized peptides were analysed by reverse phase (RP) HPLC on a Beckmann (Krefeld, Germany; System Gold; modules 126, 168, 508) dual-pump high-pressure mixing system with 214-nm UV and 280-nm detection using a C18 column (Bischoff, 250 × 4.6 mm, 5 µm). The molecular masses of the peptides were determined by electrospray ionization mass spectrometry (ESI-MS) on an ESI-MS LCQ Advantage MAX (Finnigan). The preparative purification was carried out on a C18 column (Bischoff, 250 × 20 mm, 5 µm) using a Waters System (Eschborn; Germany; Controller 600, Dual λ Absorbance Detector 2487) and a Waters fraction collector.

Peptide synthesis of *Np*HtrII55-59-S(Leu) and *Np*HtrIIA60C-65

*Np*HtrII55-59-S(Leu) and *Np*HtrIIA60C-65 were synthesized manually using Boc-chemistry, *in situ* neutralization and HBTU activation protocols on a 0.2-mmol synthesis scale [19]. The *N*-terminal peptide *Np*HtrII55-59-S(Leu) was synthesized on a resin that generates a *C*-terminal thioester after HF-cleavage [20]. The peptides were deprotected and cleaved from the resin with water-free HF and *p*-cresol as scavenger for 1 h at 0 °C. Peptides were purified by HPLC on a preparative C18 column using linear gradients from buffer A (0.1% (v/v) TFA in water) to B (0.08% (v/v) TFA in acetonitrile). Fractions were analysed by ESI-MS.

*Np*HtrII55-59-S(Leu) was obtained in a yield of 54% (based on the synthesis scale) after HPLC purification (ESI-MS: found 704.4 Da, calculated for [M + H] 704.8 Da). *Np*HtrIIA60C-65 was obtained in a yield of 43% (based on the synthesis scale) after HPLC (ESI-MS: found 605.5 Da, calculated for [M + H] 605.7 Da).

Ligation experiments in organic solvents

Ligations of the test peptides *Np*HtrII55-59-S(Leu) and *Np*HtrIIA60C-65 were carried out in four different water-free organic solvents (TFE, DMF, NMP and DMSO) at 40 °C. The peptides were dissolved in the desired solvent (~3.5 mM) and a thiol additive was added. We used either a combination of 3% (v/v) thiophenol and 3% (v/v) benzyl mercaptan or (4-carboxylmethyl) thiophenol (MPAA) (~5 mM). To facilitate the nucleophilic reaction of thiols with the *C*-terminal thioester, triethylamine was added to a final concentration of 10 mM. The reaction mixtures were stirred at 40 °C and were monitored by ESI-MS without prior purification. Data were taken at different time points.

To counteract aggregation of the peptides during ligation experiments, different additives were tested. α-cyclodextrin was added to the reaction mixture in a concentration of 1% (w/v). Different salts were used in concentrations as described in the section on Results and Discussion. The reaction mixtures were stirred at 40 °C, at each time point an aliquot was withdrawn and the reaction quenched by adding 20 µl of 1 : 1 acetonitrile : water 0.1% TFA. After removing the solvent under reduced pressure and reduction of S–S bridges by TCEP (*tris*(2-carboxyethyl) phosphine HCl), the crude product mixture was analysed with HPLC on a C18 column. The concentration of adducts, ligation product or thioester exchange product was determined by the integration of peak areas using 32 Karat Software (Beckman Coulter) with detection at 214 nm. Yields were determined by dividing the area of product by the total area of adducts and products. It should be noted that this representation leads to lower calculated yields if adducts have different concentrations.

Results and Discussion

Native chemical ligations in organic solvents

In order to test the efficiency of NCL in organic solvents, two short peptides, which contain a possible ligation site for the synthesis of the transmembrane model system *Np*HtrII, were synthesized (Figure 1). In a first set of experiments, the progress of the ligation reaction in an organic solvent was followed by ESI-MS without prior purification.

Four different organic solvents and the effect of added base were tested. DMF, NMP and DMSO are polar aprotic solvents which are able to dissolve transmembrane peptides [21]. TFE had already been used in an NCL reaction albeit as additive to aqueous buffers [11]. Without the addition of base, no product was generated, no matter which solvent was used. In the next set of experiments, we investigated the influence of the base triethylamine on the reaction kinetics in DMF, NMP, DMSO and TFE. Generally, product formation was observed when triethylamine in a concentration of 10 mM in DMF or NMP and 100 mM in TFE was added. In DMSO, no product was formed even after the addition of 10 mM triethylamine. The fastest reaction occurred in DMF (Figure 2) immediately after mixing adducts, and product formation could be observed (peak at 1103.7 Da in Figure 2a). After 2 h the reaction had progressed considerably (Figure 2b). As expected, there was no hydrolysis of the thioester observed or other by-products formed.

It should be noted that the addition of base might lead to racemisation, especially at the *C*-terminal thioester carrying alanine residue. Further experiments are needed to clarify if racemisation occurs and if milder bases or lower concentrations can be used without leading to racemisation.

In order to get more information about the reaction rate and the role of the base in the ligation, solvent conditions were further analysed. After quenching the reaction with 1 : 1 acetonitrile : water 0.1% TFA at different time points, subsequent removal of the

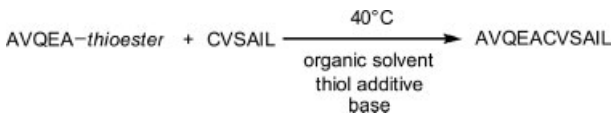

Figure 1. General scheme of NCL reactions in organic solvents (thioester =~ SCH_2COLeu). Reactions were carried out in four organic solvents (DMF, NMP, DMSO and TFE), using benzyl mercaptan, thiophenol or MPAA as thiol additive and triethylamine as base.

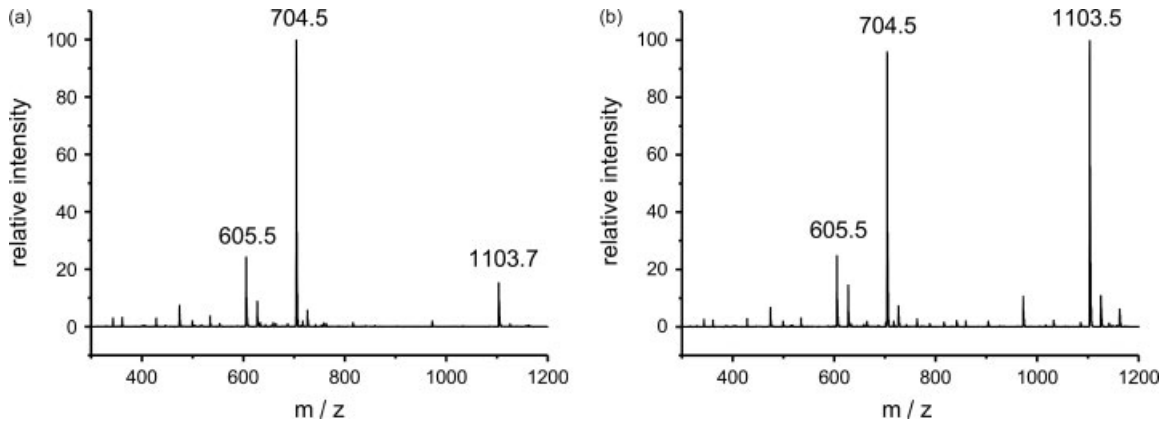

Figure 2. Native chemical ligation in DMF/10 mM TEA. (a) ESI-MS after 0 h (handling time ~10 min). (b) ESI-MS after 2 h. Theoretical masses: *Np*HtrIIA60C-65 (604.7 Da), *Np*HtrII55-59-S(Leu) (703.8 Da) and *Np*HtrII55-65 (1103.3 Da).

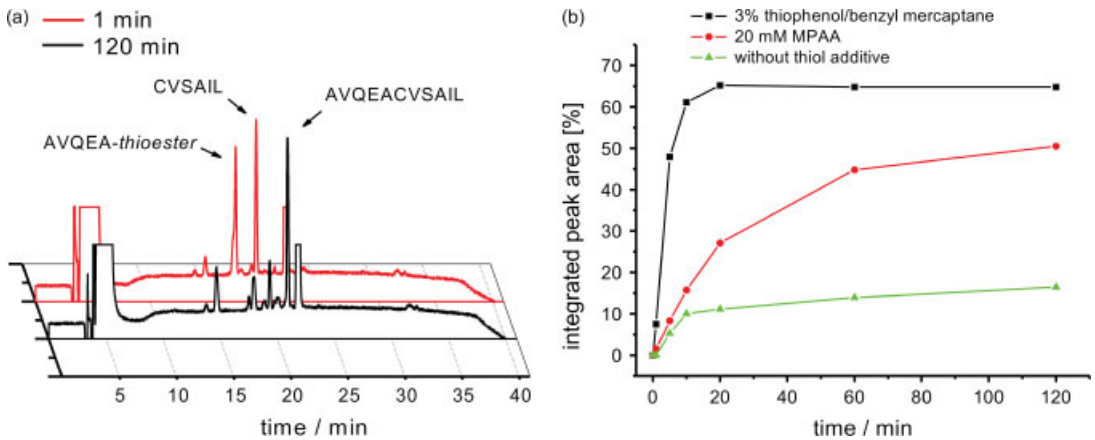

Figure 3. (a) NCL in organic solvents (R =~ SCH_2COLeu; reaction conditions: 3.5 mM of each peptide, 20 mM triethylamine and 20 mM MPAA in DMF, 40 °C). HPLC analysis of the reaction mixture after 1 min (red curve) and 120 min (black curve). The peaks at 5 and 22 min due to solvent and thiol catalysts are truncated for clarity. The emerging peak at 20.9 min was analysed with ESI-MS and assigned to the ligation product. (b) Effect of the thiol additive on the ligation efficiency (reaction conditions: 3.5 mM AVQEA-thioester, 3.5 mM CVSAIL and 20 mM triethylamine in DMF, 40 °C).

organic solvent under reduced pressure and reduction of possible S–S bonds by TCEP (*tris*(2-carboxyethyl)phosphine HCl), the crude product mixture was analysed by HPLC on a C18 column. The amounts of adducts, ligation product or thiol exchange product were determined by integration of the corresponding peak areas.

As an example, HPLC traces from a typically ligation experiment are shown in Figure 3. The reaction was carried out using 3.5 mM for each peptide, 20 mM TEA and 20 mM MPAA dissolved in DMF. According to analysis by ESI-MS, the adducts *Np*HtrII55-59-S(Leu) and *Np*HtrIIA60-65 could be assigned to the peaks at 17.5 and 19.3 min, respectively. The product eluted at 20.9 min. It is evident that at the first time point itself product formation is observed (Figure 3a, red trace). After 120 min only small amounts of adducts are left (Figure 3a, black trace), concomitant with a substantial increase in product formation.

We examined also the role of the thiol additive in ligations carried out in DMF. Two common thiol catalysts were used: either a mixture of thiophenol/benzyl mercaptan or MPAA [22]. From the kinetic course (Figure 3b), it is apparent that the ligation reaction is quite fast if thiophenol/benzylmercaptan is used as additive. After a few minutes, the product appears and after 20 min (half life ~5 min) the reaction is completed with high yields. If MPAA is added the reaction is much slower, whereas without additives almost no product is observed.

As exemplified above, reaction conditions using thiophenol/benzyl mercaptan showed the fastest reaction rate. However, due to the high concentration of thiophenol and benzyl mercaptan, HPLC analysis became difficult because these additives could not readily be removed quantitatively prior to chromatography. To obtain a better quality of the HPLC chromatogram, MPAA – although MPAA showed slower reaction rates – was used throughout the further experiments.

The results of the NCL experiments are summarised in Table 1. As was already evident from the previous experiments the highest yield was obtained with thiophenol/benzyl mercaptan, whereas without additive only 14% product was generated after 60 min. Altering the concentration of the base TEA leads to an optimum at about 10–20 mM. At 100 mM TEA the yield decreases by almost a factor of 2. On increasing the concentration of the peptides from 3.5 to 14 mM, no change in yields was observed. Decreasing the temperature from 40 to 21 °C lowers the ligation yield to 19%. Whether the decrease in yields is due to reduced solubility of peptides or due to reduced reaction rates has to be further analysed. Summarizing the results, the best reaction conditions are obtained for 20 mM TEA and 20 mM MPAA. If thiophenol/benzyl mercaptan is used, the yields can be increased to 65%.

Table 1 also contains data reflecting the fact that under all reaction conditions a precipitate is formed after prolonged

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Table 1. Ligation yields in dependence of reaction conditions

Reaction conditions	Yield after 60 min	Precipitation (12 h)
3.5 mM of each peptide, 20 mM TEA, 40 °C	14%	Yes
3.5 mM of each peptide, 20 mM TEA, 20 mM MPAA, 40 °C	45%	Yes
3.5 mM of each peptide, 100 mM TEA, 5 mM MPAA, 40 °C	26%	Yes
14 mM of each peptide, 10 mM TEA, 5 mM MPAA, 40 °C	42%	Yes
14 mM of each peptide, 10 mM TEA, 5 mM MPAA, 21 °C	19%	Yes
3.5 mM of each peptide, 20 mM TEA, 3% (v/v) thiophenol, 3% (v/v) benzyl mercaptan, 40 °C	65%	Yes

reaction times (12 h). The precipitate consisted of remaining reactants and predominantly ligation product. Because reactants were also detected in the precipitate, reaction conditions had to be found which avoided the formation of precipitates. In order to increase the solubility of the hydrophobic peptides, different additional additives were tested (α-cyclodextrin or higher order cyclodextrins, lithium chloride and sodium trifluoroacetate). Cyclodextrins are known to be able to harbour hydrophobic compounds in their cavity [23]. Lithium salts have been reported to mediate the solubilization of peptides in organic solvents [24,25] and TFA facilitates the dissociation of peptides or protein aggregates [26].

Table 2 summarises the results. The solubility of the peptides in DMF could not be increased by the use of α-cyclodextrin. Cyclodextrins with larger cavities might be more effective and have to be tested in future experiments. An addition of sodium trifluoroacetate or lithium chloride increased the solubility of the reactants in DMF; however, these reagents strongly decreased the reaction rate. For sodium trifluoroacetate, the yield can be increased if higher concentrations of the peptides are used. The supplementation with LiCl seems to be quite concentration dependent. While no reaction was observed with 2.0 M LiCl, 12% product was formed after 60 min on reducing the molarity of LiCl by about a factor of 10. In this case also no precipitation occurred. The exchange of MPAA by thiophenol/benzyl mercaptan did not lead to an increase of the reaction yield after 60 min, but did lead to a yield of 60% after a 12-h reaction. It should be noticed that a conversion of the *N*-terminal peptide to the benzyl mercaptan exchange product can be observed (data not shown). Obviously this thioester is quite stable under these conditions. Taking this data into account, we tested thiophenol as thiol additive without the addition of benzyl mercaptan. The reaction yield after 1 h increased to 39% and after 12 h we observed a yield of 64%. Importantly, no precipitation was detected in either case. If in the presence of LiCl thiol additives are omitted, the yields are slightly better than without the addition of salt (20% *vs* 14%; see above). Importantly, the reaction does not stop and no precipitation is observed (data not shown).

Table 2. The effect of cyclodextrin and salt additives on yield and peptide precipitation

Reaction conditions	Yield after 60 min	Precipitation (12 h)[a]
14 mM of each peptide, 10 mM TEA, 5 mM MPAA, 1% (w/v) α-cyclodextrin	40%	Yes
3.5 mM of each peptide, 20 mM TEA, 3% (v/v) thiophenol, 3% (v/v) benzyl mercaptan, 1% (w/v) α-cyclodextrin	57%	Yes
3.5 mM of each peptide, 10 mM TEA, 5 mM MPAA, 0.17 M CF_3COONa	2%	Minor
14 mM of each peptide, 10 mM TEA, 5 mM MPAA, 0.17 M CF_3COONa	17%	Minor
7 mM of each peptide, 10 mM TEA, 10 mM MPAA, 2.0 M LiCl	No reaction	n.d.
3.5 mM of each peptide, 20 mM TEA, 20 mM MPAA, 0.18 M LiCl	12%	No
3.5 mM of each peptide, 20 mM TEA, 20 mM MPAA, 0.09 M LiCl	20%	Minor
3.5 mM of each peptide, 20 mM TEA, 0.18 M LiCl, 3% (v/v) thiophenol, 3% (v/v) benzyl mercaptan	14% (60%)[b]	No
3.5 mM of each peptide, 20 mM TEA, 0.18 M LiCl, 3% (v/v) thiophenol	39% (64%)[b]	No

[a] Precipitation of reactants was determined by HPLC. Yes: almost quantitative precipitation of all reactants; minor: partial precipitation of *Np*HtrIIA60C-65 and the ligation product; no: no precipitation observed; n.d.: not determined.
[b] In brackets: yields after 12 h.

The results described so far showed that NCL of small hydrophobic peptides can be carried out in organic solvents with high yields. Best results were obtained by using dry DMF in the presence of triethylamine. The aggregation problem might be overcome by using sodium trifluoroacetate or lithium chloride, although other salt additives that are soluble in organic solvents like $Ti(OCHMe_2)_4$ or $ZnCl_2$ [24] might also be suitable. However, in any case, a possible racemization has to be checked even here. It is also worth noting that best reaction conditions were identified without solubilizing additives. The short reaction time observed in this case might also reduce the opportunity for racemization.

Concluding Remarks

In this work, we present data on NCL reactions in organic solvents. As a model system, small hydrophobic peptides were synthesized for analysing different ligation scenarios. We have shown that DMF is a suitable solvent for fast and efficient ligation of small hydrophobic peptides if a base like triethylamine is used and thiol additives are added. The problem of precipitation of these peptides and their ligation product can be overcome by the addition of salts like LiCl or sodium trifluoroacetate. Thus, native chemical ligation in organic solvents could be a suitable tool

for the synthesis of hydrophobic peptides and might surmount the problems of solubility and the tendency to aggregate under standard aqueous conditions.

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