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Bacterial lipid modification *in vitro*: Synthetic peptide substrate for phosphatidylglycerol—Prolipoprotein diacylglyceryl transferase

A Tamil Selvan and K Sankaran*

Centre for Biotechnology, Anna University, Chennai 600 025, India

Lipid modification is an emerging protein-engineering tool for providing hydrophobic anchor to hydrophilic proteins for immobilizing them onto a variety of man-made and biological surfaces. In this respect, N-acyl S-diacylglyceryl modification of N-terminal cysteine, called bacterial lipid modification has many advantages including the fact that it can be achieved through popular prokaryotic expression of proteins. The modification proceeds in a three-step cascade reaction in which three inner membrane enzymes, Phosphatidylglycerol:Prolipoprotein diacylglyceryl transferase, signal peptidase II and apolipoprotein N-acyl transferase, participate. Bacterial expression has limitations including the necessity of expensive downstream processing. However, *in vitro* modification has not been possible so far because the enzymology of this pathway is not well studied due to difficulty in assaying these enzymes. As a first step to overcome this problem we have designed the peptide substrate, MKATKSAVGSTLAGCSSHHHHHH, for *in vitro* lipid modification specifically the first enzyme, Phosphatidylglycerol:Prolipoprotein diacylglyceryl transferase, which catalyzes the committed step. The design was based on bioinformatics analysis of more than 1000 bacterial lipoprotein precursors. This synthetic peptide substrate was soluble and contained other built-in features useful in easier handling and purification. The designed substrate exhibited expected properties and *in vitro* diacylglyceryl modification was confirmed by Tricine SDS PAGE based mobility shift assay.

Keywords: bacterial lipid modification, signal sequence, peptide substrate, assay
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Introduction

Covalent attachment of lipid to protein was first identified and demonstrated with major outer membrane lipoprotein of *Escherichia coli*, called Braun's lipoprotein[1]. Detailed biochemical analysis revealed that this protein contained an N-acyl S-diacylglyceryl modified cysteine group at its N-terminal[2]. Research on lipoprotein biosynthesis was started with the discovery that Braun's lipoprotein is synthesized as prolipoprotein with a signal sequence, which gets cleaved during its maturation[3]. The correct lipoprotein biosynthetic pathway was proposed by Sankaran and Wu in 1994[4], according to which, prolipoprotein is first modified by Phosphatidyl-glycerol:Prolipoprotein diacylglyceryl transferase (LGT) followed by cleavage by lipoprotein-specific signal peptidase (LSP) and then N-acylation of amino group by Apolipoprotein N-acyl transferase (LNT).

The characteristic signal sequence for lipid modification was identified as a consensus sequence of L[AS][GA]C at the C-terminal end of signal peptide and termed as lipobox[5]. This was in place of X-Ala-X normally seen in non-lipoprotein signal sequences[6]. Detailed mutational analysis including deletions, alterations and displacement of lipobox confirmed its essential role for recognition by modification and processing enzymes in the pathway[7]. Apart from the lipobox, lipoproteins signal sequence has the common tripartite structure with n-region (N-terminal region), h-region (hydrophobic region) and c-region (cleavage region). Detailed bioinformatics analysis of more than 1000 bacterial lipoproteins performed recently by us revealed that normally n-region contains one or more positively charged amino acids with a mean length of 5-7 amino acids. h-Region cannot tolerate any charged amino acid and the length can be from 7 to 22 amino acids with a modal value of 12. In the c-region containing lipobox, the Cys that gets modified is invariant at +1 position; -1 position can tolerate only Gly Ala and Ser; -2 position accommodates a variety of uncharged amino acids and -3 position is predominantly occupied by bulkier hydrophobic amino acids, in 75% of the cases it is Leu[8]. This analysis was very useful in designing the peptide substrate for lipid modification.

*Author for correspondence:
Tel: 91-44-22350772, Fax: 91-44-22350299
E-mail: ksankaran@annauniv.edu

All the three enzymes involved in lipoprotein maturation pathway have been identified. Their genes have been cloned, sequenced and studied by site-directed mutagenesis[9-11]. Among these enzymes LSP is well characterized[12]. The first enzyme of the pathway (LGT) that carries out bulk of the lipid modification was identified[9] most recently after the isolation of temperature sensitive mutant of *Salmonella typhimurium* defective in diacylglyceryl modification[13]. Biochemical characterization of LGT was performed using a synthetic peptide substrate corresponding to the first 24 amino acids of Braun's prolipoprotein using a radioactive assay[4]. This method was not only cumbersome but required extraordinary care associated with radioactive substances, clearly not suited for commercial proposition. Moreover, handling and purification of the peptide was difficult due to high degree of hydrophobicity and low solubility. These limitations prompted us to develop a simpler assay with a more soluble peptide, designed purely based on consensus sequence derived from previous experience and bioinformatics analysis. Electrophoretic mobility shift in Tricine SDS-PAGE has been a highly reliable laboratory analytical tool for monitoring lipid modification and therefore, diacylglyceryl modification of the new peptide substrate was confirmed by such a procedure. This is the first time a newly designed peptide has been lipid-modified *in vitro* using bacterial lipid modification enzyme.

Materials and Methods

Novel Design and Synthesis of Peptide Substrate for LGT

MKATKSAVGSTLAGCSSHHHHHH is a newly designed 23 amino acid synthetic peptide substrate for bacterial lipid modification. It was designed on the basis of understanding of bacterial lipid modification and a detailed bioinformatics analysis of more than 1000 bacterial prolipoproteins. It was synthesized using Applied Biosystems peptide synthesizer. Apart from consensus lipobox sequence of –LAGC-, it has a fairly hydrophilic h-region rendering the cationic peptide water-soluble. Since, C-terminal sequence beyond the first two amino acids following Cys residue is immaterial for recognition by biosynthetic enzymes, a poly His region has been included for increasing solubility, converting it into highly basic peptide and for easy purification using metal affinity chromatography. This peptide was routinely stored as 5 mg of peptide dissolved in 1 mL of 0.1 *M* HCl containing 1*M* guanidine hydrochloride.

Binding Character of the Peptide to IMAC

Immobilized metal affinity chromatography was performed using chelating sepharose matrix (chelating sepharose fast flow, Amersham Biosciences). Nickel was immobilized in the chelating sepharose matrix as per standard method[14]. 5 μg of peptide in the binding buffer (20 m*M* Tris HCL, *p*H 8.0, 0.5 *M* NaCl) was added to 5μL of matrix. After binding, the matrix was washed with bed volumes of binding buffer. Elution was performed with two bed volumes of elution buffer with various concentrations of imidazole (20 m*M* Tris HCl, *p*H 8.0, 0.5 *M* NaCl, Imidazole concentrations from 200 m*M* to 1*M*). Suitable amount of peptide was then fluorescent derivatized with Dansyl chloride and analyzed by Tricine SDS PAGE.

Preparation of Inverted Vesicles from LGT Hyper-expressing Clones as Enzyme Source

This enzyme is present in the inner side of the inner membrane facing cytoplasm and therefore inverted vesicles are the preferred source of the enzyme. *E. coli* strain BL-21 (DE3) (T7GT)[9]hyper expressing clone for LGT, BL-21 (DE3) (pRSET-B), vector (two colonies each) were grown in 400 ml of LB medium containing 100 μg/mL of ampicillin to log phase (0.6 OD at 600 nm) and induced with 1 m*M* IPTG for 2 h. Cells were harvested using centrifugation (6000 g, 5 min) and washed once with 10 mL of Tris-buffered-saline (TBS, 20 m*M* Tris HCl, *p*H 8.0, 0.9% NaCl). The washed cells were suspended in 10 mL of 20 m*M* Tris-HCl, *p*H 8.0-10 m*M* EDTA, *p*H 8.0 (TED) buffer containing 0.5 *M* NaCl and passed twice through French press at 15,000 psi. Lysed cells were centrifuged at 5000 g for 10 min to remove the debris. The supernatant was then centrifuged at 40,000 g for 1 h and the inverted vesicle pellet was suspended in 1mL TED and stored as 50 μL aliquots.

Diacylglyceryl Modification of Peptide Substrate

Assay was performed in 25 μL of 20 m*M* Tris-HCl (*p*H 8.0) containing 5 m*M* EDTA, 4 m*M* DTT, 100 m*M* guanidine hydrochloride, 100 μ*M* peptide, 250 μ*M* phosphatidyl glycerol, 0.08% octyl glucoside, inverted membranes from 10^9 cells. The reaction was allowed to proceed at 37°C for 30 minutes. After the reaction, the mix was frozen to stop the reaction.

Fluorescent labeling of Peptide using Dansyl Derivatisation

To the reaction mix, equal volume (25 μL) of 0.1 *M* Na_2CO_3, and 2 molar excess of Dansyl chloride with respect to the amino groups present in the peptide was added. This mix was incubated at 37°C for 30 min. 50 μL SDS-PAGE sample loading buffer was added and 10 μL was analyzed by Tricine SDS-PAGE.

Mobility Shift Analysis by Tricine SDS PAGE

Tricine SDS-PAGE was performed as described in the literature[16] using a mini set-up at constant potential of 100V. Electrophoresis was stopped when bromophenol blue dye reached the bottom of the gel. A typical run used to take about 3 h. The fluorescent peptide was viewed under UV and the profile was documented using gel documentation system. Upon lipid modification, the modified-peptide is retarded compared to native peptide by the highly cross-linked poly-acrylamide matrix and the extent of modification can be quantified using gel documentation system.

Other Methods

Phosphatidylglycerol (PG) from SIGMA was solubilized in TED containing 1% octylglucoside at a concentration of 4 m*M*. Substrate mix was prepared prior to the assay by adding 1 μL of the stock peptide solution, 2 μL of PG solution and 7 μL of TED buffer and sonicating in a bath sonicator.

Results and Discussion

Features of Newly Designed Peptide

The newly designed peptide was based on the prior knowledge of working with more hydrophobic peptide substrate for lipid modification and the detailed bioinformatics analysis done on more than 1000 prolipoprotein sequences reported in the literature and protein databases, mainly from bacterial genome sequences. The peptide substrate was designed for better solubility and easy purification using affinity system. Though the tripartite structure was maintained, major modification was carried out within the highly flexible hydrophobic region of the peptide, as this contributes to insolubility of signal peptides. This region was kept to minimum of 6 amino acids excluding large hydrophobic amino acids like leucine and isoleucine and retaining only valine. Serine was introduced to replace the bulky hydrophobic amino acids to make the region polar but uncharged. The consensus lipobox sequence, LAGC, seen in about 75% of prolipoproteins was retained. Another major modification was done in the region after the lipid modifiable cysteine residue. The first two amino acids of this region were retained as Ser as small sized amino acids following the lipobox has a role in modification. A histidine tag at the C terminal was introduced to facilitate purification of the peptide through immobilized metal affinity chromatography (Fig. 1).

Physico-chemical Properties of Newly Designed Peptide

Solubility of the substrate was found to be more than 5 mg per mL, as reflected by the primary sequence character. To keep the peptide in the reduced state, it was kept in acidic condition. Chromatography using nickel affinity matrix demonstrated higher affinity and avidity of the peptide in binding the matrix. Imidazole concentration of more than 1*M* was required to elute the peptide completely (Fig. 2). The thiol group in the peptide was prone to air-oxidation and was found to form dimer readily under neutral conditions. From the high concentrations of reducing reagents, both thiol based (4 m*M* dithiothreitol) and non thiol [5 m*M* Tris-(carboxyethyl) phosphine] based, required to keep the peptide at its reduced state. It appeared that the propensity to form a stable dimer was high and, therefore, a high reducing atmosphere has to be maintained during the assay to prevent the disulphide formation. Accordingly 4 m*M* DTT was used in the assay.

Diacylglyceryl Modification of Designed Peptide

When the peptide in its reduced state was subjected to lipid modification using synthetic PG as the lipid donor and inverted vesicle from clones hyper expressing LGT, the diacylglyceryl group from PG was expected to be transferred to the peptide. However, it was essential to label them in order to view the lipid-modified peptide and native peptide. Dansyl chloride was chosen to fluorescently label the peptide after the assay prior to loading onto Tricine-SDS-PAGE (Fig. 3). The successful enzymatic modification was apparent from hyper expressing clone Vs vector control and inactivated control. From the fluorescent intensity associated with the retarded modified form, it appeared that 100% modification can be achieved under the assay conditions. Heat-inactivated enzyme source or the vector control that has only basal level of the enzyme could not show modification as expected. Partial modification was obtained with the overnight stored enzyme source,

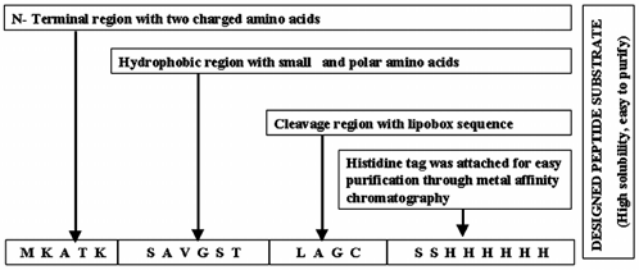

Fig 1—Design features of the synthetic peptide substrate

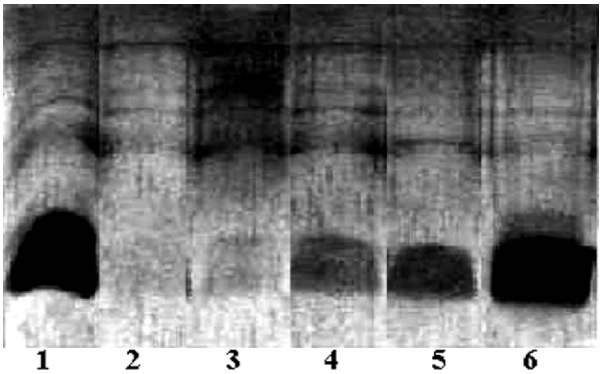

Fig 2—Affinity behaviour exhibited by newly designed peptide using IMAC matrix. Lane 1-Native peptide; Lane-2, 200 m*M* imidazole eluate; Lane-3, 400 m*M* imidazole eluate; Lane-4, 600 m*M* imidazole eluate; Lane-5, 800 m*M* imidazole eluate and Lane-6, 1*M* imidazole eluate.

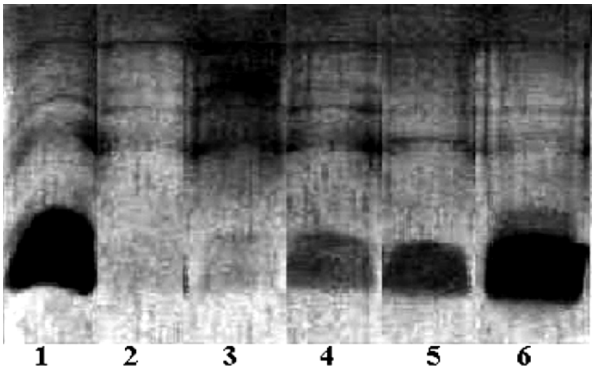

Fig 3—Tricine SDS-PAGE based assay for LGT. Lane-1-Enzyme control; Lane-2, Assay with inactivated enzyme source; Lane-3, Assay colony 1 of T7GT, fresh inverted vesicles; Lane-4, Assay colony 2 of T7GT, fresh inverted vesicles; Lane-5, Native peptide; Lane-6, Assay colony 1 from pRSETB fresh inverted vesicles; Lane-7, Assay colony old enzyme source and Lane-8, Vector control.

revealing the labile nature of the enzyme, which was known from the previous studies[5] using radioactive assay.

Significance of the Present Study

Bacterial lipid modification is essential for bacteria to carry out a variety of vital functions such as transport of nutrients and metabolites across membrane-aqueous interface. Bacterial lipid modification is gaining importance both in commercial as well biological applications because of its effectiveness in providing a comprehensive lipid modification at one end of a protein without affecting the folding or conformation[16]. Recently, we demonstrated it using a heterologous non-lipoprotein enzyme engineered into a lipoprotein using *E. coli* expression system[17]. This potent protein engineering biotechnological tool could be exploited for many purposes such as ELISA, biosensor, surface display of proteins, vaccine development, and liposomal integration for targeted drug delivery.

Though *in vivo* strategies for lipid modification of non-lipoproteins were successfully demonstrated in a few cases, but there are limitations to such a system, as it would involve purification of modified proteins and difficult to handle peptides. However, there is no cell-free system reported to easily address these concerns. For cell-free system, lipid modification enzymes are needed in their purified state with well-understood enzymology. Current radioactive assays cannot help the cause due to inherent difficulties of such procedures and the available peptide substrate has many disadvantages as explained above. The newly designed peptide seems to address these issues and has the attributes to develop an *in vitro* lipid modification system.

The His-tag can be used to trap the diacylglyceryl-modified peptide and for further cleavage and N-acylation using appropriate enzyme sources. Alternatively, a preparation that has all three-enzyme activities can be utilized to modify the immobilized peptide, which can be easily released and analyzed for the complete modification. Optimizing parameters using such systems will help to develop enzymatic reactors for *in vitro* lipid modifications as another viable alternative to the *in vivo* system. The new peptide along with standardized assay conditions will also aid in the understanding of the biochemical functioning of the enzymes in the pathway, an area not adequately probed.

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References

1 Braun V & Rehn K, Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall: The specific effect of trypsin on the membrane structure, *Eur J Biochem,* 10 (1969) 426-438.

2 Hantke K & Braun V, Covalent binding of lipid to protein: Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the *Escherichia coli* outer membrane, *Eur J Biochem*, 34 (1973) 284-296.

3 Inouye H, Wang S, Sekizawa J, Halegoua S & Inouye M, Amino acid sequence for the peptide extension on the prolipoprotein of *Escherichia coli* outer membrane, *Proc Natl Acad Sci USA,* 74 (1977) 1004-1008.

4 Sankaran K & Wu H C, Lipid modification of bacterial prolipoprotein, *J Biol Chem,* 269 (1994) 19701-19706.

5 Hayashi S & Wu H C, Lipoproteins in bacteria, *J Bioenerg Biomembr,* 22 (1990) 451-471.

6 Braun V & Wu H C, Bacterial cell wall, in *New comprehensive biochemistry*, Vol 27, edited by J M Ghuysen & R Hackenbeck (Elsevier Scince Puplishers, Amsterdam) 1993, 319-342.

7 Sankaran K & Wu H C, Bacterial lipoproteins, in *Lipid modification of proteins,* edited by M J Schlesinger (CRC Press, Boca Raton, FL) 1993, 163-181.

8 Madan Babu M & Sankaran K, DOLOP-database of bacterial lipoproteins, *Bioinformatics,* 18 (2002) 641-643.

9 Gan K, Sankaran K, Williams M G, Aldea M, Rudd K *et al*, The *umpA* gene of *Escherichia coli* encodes Phosphatidyl-glycerol:Prolipoprotein Diacylglyceryl Transferase and regulates the Thymidate Synthase level through translational coupling, *J Bacteriol,* 177 (1995) 1879-1882.

10 Yamagata H, Daishima K & Mizushima S, Cloning and expression of a gene coding for the prolipoprotein signal peptidase of *Escherichia coli, FEBS Lett,* 158 (1983) 301-304.

11 Gupta S D & Wu H C, Identification and subcellular localisation of Apolipoprotein N-acyltransferase in *E. coli*, *FEMS Microbiol Lett*, 78 (1991) 37-41.

12 Sankaran K & Wu H C, Bacterial lipoprotein signal peptidase, *Methods Enzymol*, 248 (1995) 169-179.

13 Gan K, Gupta S D, Sankaran K, Schmitt M B & Wu H C, Isolation of a temperature sensitive mutant of *Salmonella typhimurium* defective in prolipoprotein modification, *J Biol Chem,* 268 (1993) 16544-16559.

14 *Affinity chromatography* (Amersham Pharmacia Biotech) 2001, 46-50.

15 Schagger H & von Jagow G, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal Biochem,* 166 (1987) 368-379.

16 Kamalakkannan S, Murugan V & Sankaran K, Bacterial lipid modification of proteins and its potential applications, *BIOforum Europe*, 3 (2005).

17 Kamalakkannan S, Murugan V, Jagannadham M V, Nagaraj R & Sankaran K, Bacterial lipid modification of proteins for novel protein engineering application, *Protein Eng Design & Selection,* 17 (2004) 721-729.