Protein Immobilization

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Membrane-Bound Stable Glycosyltransferases: Highly Oriented Protein Immobilization by a C-Terminal Cationic Amphipathic Peptide**

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Pathogenic Gram-negative bacteria produce glycolipid antigens called lipopolysaccharides on their surfaces, many of which mimic host cell surface carbohydrate structures to mask the pathogen from host immune surveillance.[1] The human gastric pathogen *Helicobacter pylori* can express both type 1 and type 2 Lewis blood-group antigens[2] that also are found in gastric epithelial cell surface carbohydrate structures.[3] It is well documented that α1,3/α1,4-fucosyltransferases [(α1,3/α1,4)-FucTs] are crucial enzymes responsible for the synthesis of Lewis-type antigens. Although molecular cloning and expression of the *H. pylori* α1,3/α1,4-FucTs gene have been reported,[4] production of full-length FucTs from *H. pylori* has not been achieved because of the insolubility caused by the C-terminal sequence that has two to ten repeats of seven amino acids, known as heptad repeats, followed by a highly conserved region rich in cationic and hydrophobic residues.[5]

It was suggested that the heptad repeat region contains a leucine zipperlike motif responsible for dimerization, which might be essential for enzyme function.[4] On the other hand, it is considered that the two putative amphipathic α helices might function as a membrane anchor with the hydrophobic face embedded in the membrane and the positive charges interacting with negatively charged phospholipid head groups.[6] It seems likely that C-terminal amphipathic α helices and the preceding heptad repeat region in *H. pylori* α1,3/α1,4-FucTs may be functionally equivalent to the N-terminal transmembrane domain and the stem region of mammalian counterparts known as typical Golgi-resident type II membrane glycosyltransferases.[7]

Taylor et al. revealed that removal of the C-terminal putative amphipathic α helices dramatically increased both the expression level and solubility of *H. pylori* (α1,3/α1,4)-FucTs without significant loss of the specific enzyme activity.[8] It was also reported that the poor solubility of this enzyme can be improved by systematic deletion of the C terminus involving heptad repeats,[9] and large quantities of these soluble truncated *H. pylori* α1,3-FucTs allow for the investigation of crystal structure and insight into the catalytic mechanism.[10] We postulated the existence of a specific mechanism for controlling the affinity of the C-terminal region with bacterial membrane phospholipids, to prevent the formation of undesirable insoluble aggregates during biosynthetic processes of intact and full-length FucTs as naturally occurring bacterial membrane-bound enzymes. Our attention was directed toward a sequence similarity of this amphipathic C-terminal tail with a unique class of short and linear cationic peptides showing antimicrobial activity.[11]

Herein, we show direct evidence of the specific functions of this unique C-terminal peptide of bacterial membrane-bound glycosyltransferases. The findings also lend this mechanism to a novel and general concept allowing for highly oriented immobilization of engineered enzymes on membrane-mimetic artificial solid surfaces.

To assess the importance of the conformational changes in a putative secondary structure of the C-terminal amphipathic peptide of *H. pylori* α1,3/α1,4-FucTs, we synthesized a model peptide containing 24 C-terminal amino acid residues, GGG*FKIYRKAYQKSLPLLRTIRRWVKK* (G3-capped C-terminal tail). The circular dichroism (CD) spectra of this synthetic model revealed that formation of an α-helical structure is induced by interaction with *n*-dodecylphosphocholine (DPC) micelles at both pH 7.0 and pH 10.0, while this peptide does not form any specific secondary structures as indicated by the random-coil patterns in the absence of DPC micelles (Figure 1 and Figure S1 in the Supporting Information).

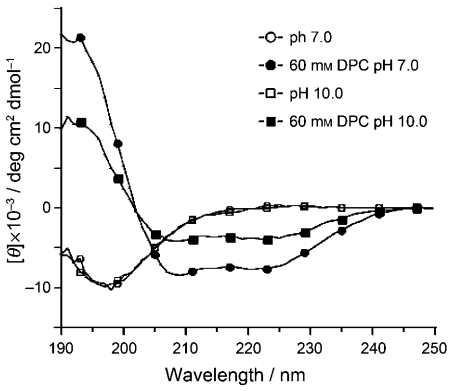

Figure 1. CD spectra of a synthetic model peptide of the C-terminal amphipathic tail of *H. pylori* α1,3-FucT (100 μM), in 5 mM phosphate buffer (pH 7.0) or 5 mM glycine buffer (pH 10.0) in the presence or absence of 60 mM DPC micelles.

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This exciting finding prompted us to challenge the expression of full-length *H. pylori* α1,3-FucT as a soluble and highly active form by controlling the mode of interaction between the positively charged lysine residues in the C-terminal peptide and the phospholipid membrane. We hypothesized that insoluble aggregation of recombinant full-length enzymes generated in *Escherichia coli* during a common expression/purification procedure in Tris–HCl buffer (pH 7.5) might be prevented by performing this process at a higher pH region. This should accelerate the deprotonation of ε-amino groups of lysine residues (pK_a = 10.8) resulting in the reduced affinity of this amphipathic region for negative head groups of phospholipids.

In addition, we also demonstrated that LgtA, one of the important bacterial glycosyltransferases responsible for the biosynthesis of lipopolysaccharides in *Neisseria meningitidis*, can be used as a versatile biocatalyst in transferring GlcNAc to Gal terminals at a broad pH region (pH 6.0–11.0).[12] Taking this unexpected stability of LgtA in strong basic media into consideration, we decided to express a full-length recombinant *H. pylori* α1,3-FucT (J99 strain)[5] in *E. coli*, in which all procedures for purification after chromatography using a HisTrapQ column are carried out in 25 mM glycine/NaOH buffer solution (pH 10.0). It was demonstrated that the fractions of full-length recombinant protein did not show any significant loss in activity and the purified "membrane-free" enzyme solution could be concentrated to 13 mg mL^{-1} corresponding to 16 U mL^{-1} of activity; this solution can be stored for 12 months with satisfactory activity. The full-length recombinant *H. pylori* α1,3-FucT exhibited excellent characteristics such as a broad range of optimal pH (pH 6.5–10.5) and temperature (20–45 °C), and highly improved solubility under general neutral conditions (Figure S2 in the Supporting Information).

As shown in Figure 2, we proposed a specific structural feature of full-length *H. pylori* α1,3-FucT in the presence of a large excess of phospholipid micelles, in which α helices of the C-terminal amphipathic tail at pH 7 altered significantly into a less structured form at pH 10. In fact, this reversible conformational alteration at the C-terminal region made full-length expression of unstable membrane-bound glycosyltransferase possible. It is clear that the deprotonation of five lysine residues at pH 10 influences the affinity of this region with membrane phospholipids and depresses undesired precipitation in extracts of the bacterial organism. As a result, the purified full-length *H. pylori* α1,3-FucT exhibited dramatically improved solubility without loss of the enzymatic activity.

The unique structural characteristics of *H. pylori* α1,3-FucT encouraged us to apply this concept to direct anchoring of recombinant enzymes on the surface of membrane-mimetic magnetic beads, as represented in Figure 2. We thought that the phospholipid membrane-induced α helix at the C terminus would allow a general method to immobilize unstable membrane-bound proteins on artificial phospholipid-like materials in a highly oriented manner. In addition, it seems that the strong resistance of the zwitterionic phosphorylcholine motif to nonspecific protein adsorption[13] is greatly beneficial for practical use of self-assembled enzymes to achieve high specificity, efficiency, and reproducibility.

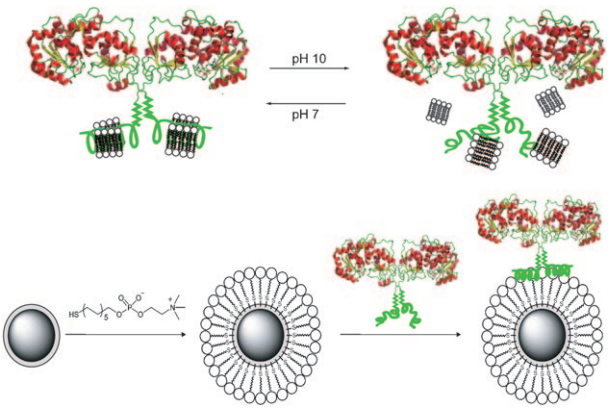


Figure 2. Proposed mechanism of the DPC micelle-induced formation of an α-helical structure in the C-terminal amphipathic tail of *H. pylori* α1,3-FucT. Top: The pH-dependent interaction between the C-terminal tail and DPC micelles induces reversible conformational change. Bottom: Application to the self-immobilization of recombinant full-length *H. pylori* α1,3-FucT on a membrane-mimetic magnetic bead.

Thus, phospholipid-free recombinant full-length *H. pylori* α1,3-FucT (376 mU) was incubated with magnetic beads (1 mg) coated with 11-mercaptoundecylphosphorylcholine[14] in Tris–HCl (100 mM, pH 7.5) at 4 °C for 2 h. The potential of the beads carrying full-length *H. pylori* α1,3-FucT was compared with that of the free soluble enzyme. The relative

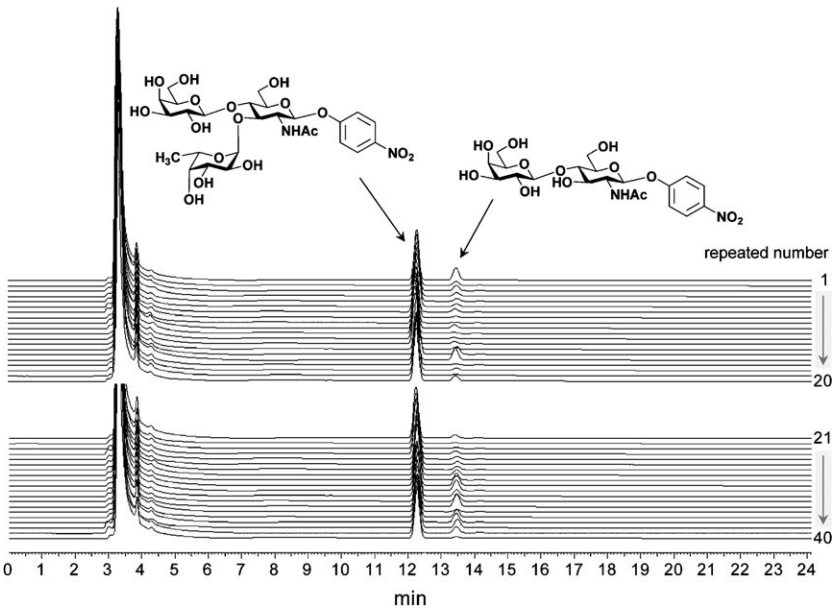
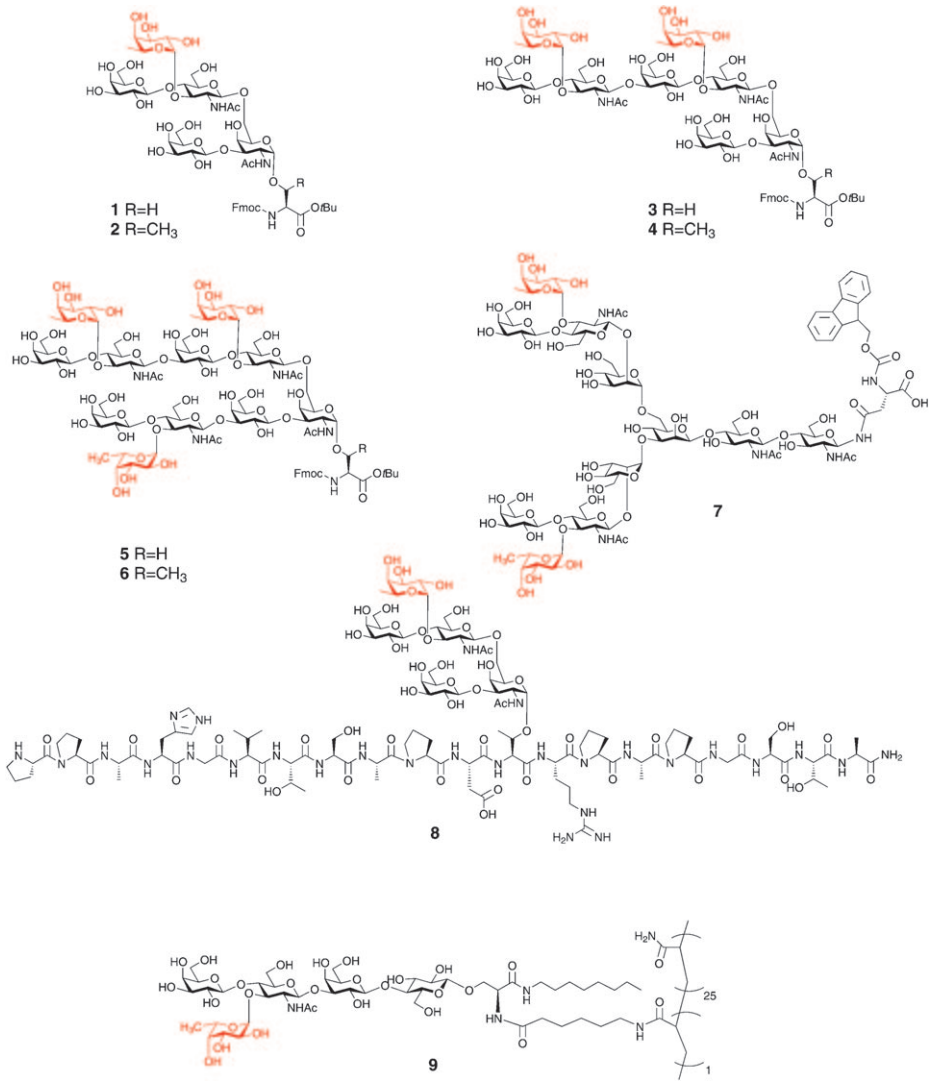


Figure 3. Recyclability in the 40 times repeated use of full-length recombinant *H. pylori* α1,3-FucT–magnetic beads for the preparation of a Lewis X trisaccharide derivative.

activity of the immobilized enzyme was almost identical to that of the free enzyme (Figure S3 in the Supporting Information), which indicated that anchoring of the full-length enzyme on the membrane-mimetic surface by the C-terminal α-helix tail did not influence the native quaternary structure and catalytic functions. It was also demonstrated that the full-length enzyme can be dissociated from the beads by treatment with glycine/NaOH buffer (100 mM, pH 10.0), while 0.4% Triton X-100 did not disturb this interaction (Figure S3 in the Supporting Information).

One of the most important features of immobilized biocatalysts is their stability during repeated use. It was revealed that the immobilized *H. pylori* α1,3-FucT (1 mg, 376 mU) in Tris–HCl buffer (50 mM, pH 7.5) shows satisfactory stability in repeated use when the fucosylation of *p*-nitrophenyl LacNAc (1 mg) was tested in the presence of excess GDP-Fuc (15 equiv) at room temperature for 3 h. Surprisingly, no significant loss of activity was detected during trials repeated 40 times over two weeks (four uses/day), which indicated that the large-scale synthesis (1–100 mg) of Lewis X derivatives can be performed by this system (Figure 3). It is clear that reactions proceeded smoothly to afford various Lewis antigenic derivatives, such as Fmoc-Thr/Ser with *O*-glycans (**1**–**6**), Fmoc-Asn with *N*-glycan (**7**), mucin glycopeptide (**8**), and a glycosphingolipid derivative (**9**), in high yields (96–100%) as shown in Scheme 1 (see also Figures S4–S13 and Tables S1–S6 in the Supporting Information). It is noteworthy that various enzymes of the cytoplasm side of *H. pylori* responsible for the synthesis of outer lipopolysaccharides involve an amphipathic cationic peptide sequence in the C-terminal region (Table S7 in the Supporting Information).[4,15–18] This might suggest the general importance of putative C-terminal peptides for topological display of membrane-bound bacterial proteins. Our interest was focused on the feasibility of this method for displaying engineered proteins on phospholipid self-assembled monolayers (SAMs)[14] in a site-specific and highly oriented manner.



Scheme 1. Various Lewis antigenic structures synthesized by means of immobilized *H. pylori* α1,3-FucT. Fmoc = 9-fluorenylmethoxycarbonyl.

To demonstrate this concept we selected tentatively a recombinant human β1,4-galactosyltransferase (rhβ1.4-GalT) fusion bearing the C-terminal amphipathic tail of *H. pylori* α1,3-FucT. The rhβ1.4-GalT fusion with a signal peptide LPETG followed by the His_6-tag[19] was employed as an acyl donor substrate for sortase A-mediated conjugation with the peptide GGG*FKIYRKAYQKSLPLLRTIRRWVKK* (a model peptide used for CD analysis), an acyl acceptor. As anticipated, rhβ1.4-GalT modified with a membrane-anchoring C-terminal tail of *H. pylori* α1,3-FucT was immobilized automatically on the surface of phosphorylcholine SAMs on magnetic beads, and exhibited high potential as a practically available catalyst without any loss of enzymatic activity (Figure 4 and Figure S14 in the Supporting Information).

In conclusion, we have revealed the structural basis and functional role of the C-terminal amphipathic tail of *H. pylori* α1,3-FucTs in the construction of highly oriented membrane-bound enzymes at the cytoplasmic face of the bacterial inner membrane. The mechanism in the conformational alteration of this C-terminal tail enabled full-length expression of highly active recombinant enzyme. Considering the fact that various bacterial glycosyltransferases have such a putative C-terminal cationic and amphipathic tail,[20] the mechanism appears to be

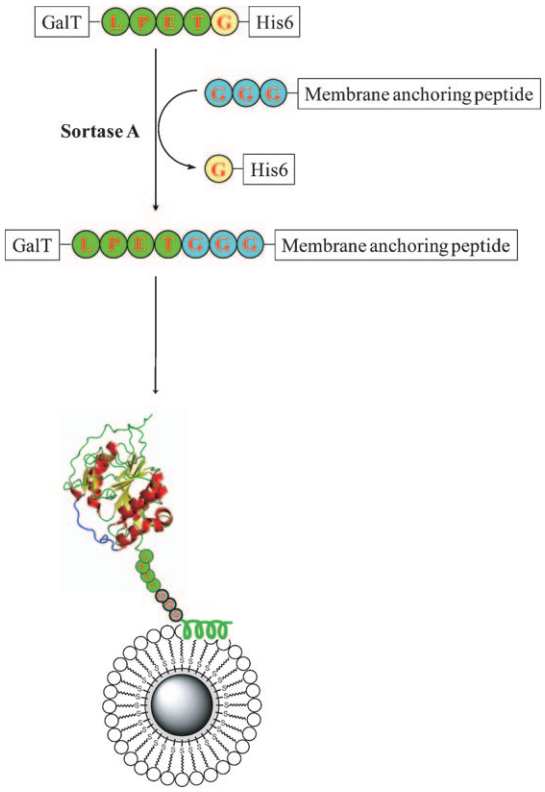


Figure 4. Immobilization of recombinant human β1.4-GalT through the membrane-anchoring C-terminal tail of *H. pylori* α1,3-FucT.

a general process to form complicated systems to synthesize outer-membrane lipopolysaccharides. More importantly, we demonstrated that attachment of this C-terminal amphipathic peptide of *H. pylori* FucTs allowed for site-specific immobilization of an engineered human glycosyltransferase on SAMs without significant loss of enzymatic activity.

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