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Implantation of Post-translational Tyrosylprotein Sulfation into a Prokaryotic Expression System

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Tyrosine O-sulfation is a common post-translational modification of proteins. A previous study estimates that up to approximately 1% of tyrosine residues in all proteins in an organism are in a sulfated form, and researchers have confirmed tyrosine O-sulfation in about 350 proteins.[1] However, the literature contains only a few reports in this area since the discovery of protein tyrosine sulfation in 1954.[2] Tyrosine O-sulfation occurs in the *trans*-Golgi network, and the targets belong to classes of secretory, plasma membrane, and lysosomal proteins, which reflect their specific intracellular locations.[3] Tyrosylprotein sulfotransferases (TPSTs, EC 2.8.2.20) are type-II membrane proteins, with a single α-helical transmembrane segment that exposes the catalytic domain in Golgi lumen. They are responsible for catalyzing the transfer of a sulfuryl group from the universal sulfuryl group donor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), onto a specific tyrosine residue within target proteins.[4]

Sulfation of tyrosine residues located within specific peptide sequences enhances the strength of protein–protein interactions and further mediates numerous crucial physiological events, such as anticoagulation, inflammation, and HIV infection.[1b,5] The sulfated proteins have great potential for medical use. An application includes sulfated hirudin, an anticoagulant secreted from the medicinal leech (*Hirudo medicinalis*), which is a potent thrombin inhibitor.[6] The sulfated antibody helps defend against HIV infection by binding to the HIV-1 gp120 CCR5-interactive region.[7] However, these recombinant proteins expressed in *Escherichia coli* or yeast are non-sulfated, due to the absence of *TPST* gene in these organisms.[3] Researchers have developed a delicate process for the expression of tyrosine-sulfated protein in *E. coli*, by introducing the amber nonsense codon to encode a sulfotyrosine residue.[8] Despite the ability to introduce a sulfotyrosine residue during protein translation, the geometric interference of multiple sulfotyrosine clusters, leaky amber suppression, and poor uptake of sulfotyrosine into expression host are bottlenecks to overcome.[8a] These days, most sulfated proteins and peptides are available through chemical synthesis and TPST catalysis; however, the cumbersome procedure, unsatisfactory yield, and the impurity of TPST sources, from either tissue extracts or cell cultures, obstruct the process.[4b–c,9] Alternatively, a TPST-catalyzed reaction provides high specificity for tyrosine-sulfated proteins, although the extremely low catalytic efficiency of TPSTs (the turnover number (k_{cat}) for most known TPSTs is approximately 10^{-5} s^{-1}) might cause low yield and incomplete tyrosine sulfation.[10] The unavailability of homogeneous and ample quantities of TPST might also be part of the reason for the lack of research activity in this important area. In addition, TPST is labile and difficult to purify by using routine purification methods.[9d]

In this report, we demonstrate an ultra-efficient system for the in vitro synthesis of tyrosine-sulfated proteins. Incorporating this system into a bacterial host produced sulfated tyrosine residue within the specific domain of a target protein. Prokaryotic expression thus provided a constant source of active and homogeneous TPST in large quantities. To prevent interference from the TPST hydrophobic region, we removed the N-terminal transmembrane domain of human TPST isoform 2 (*h*TPST-2). Topological analysis of the *h*TPST-2 primary sequence is shown in Figure S1 in the Supporting Information. The NusA fusion tag with *h*TPST-2 (NusA-*h*TPST-2) was purified to near homogeneity (Figure S2). According to previous reports, TPST catalytic efficiency (k_{cat}/K_m) is too low (ca. 1 M^{-1} s^{-1} by using PSGL-1 as substrate) for analysis by using general spectroscopic methods. Instead, we need a much more sensitive radioactive probe (^{35}S) to monitor TPST activity.[11] Traditional TPST assay uses commercial PAPS, which are usually contaminated with significant amounts of 3′-phosphoadenosine-5′-phosphate (PAP).[12] PAP is a potent inhibitor for many sulfotransferases and its presence might be the cause of TPST's low catalytic efficiency as previously reported.

In Scheme 1, we propose to continuously produce fresh PAPS and avoid the drawbacks of instability, contamination, and the expense of commercial PAPS. We prepared PAPS from the reaction catalyzed by PAPS synthetase (PAPSS), a bifunctional enzyme composed of ATP sulfurylase and adenosine 5′-phosphosulfate (APS) kinase, in two sequential reactions.[13] The coupling of human PAPSS isoform 1 (*h*PAPSS-1) catalysis as delineated in Scheme 1, allowed the continuous generation of in situ PAPS from inorganic sulfate and ATP, and provided a supply of freshly synthesized PAPS to the TPST-catalyzed reaction. It is possible to generate PAPS from adequate amounts of *h*PAPSS-1 (Figure S3). Figure 1A shows that the productive rate of PSGL-1 sulfation increases dramatically after replacing commercial PAPS with PAPSS-generated PAPS. Sulfated PSGL-1 was undetectable by Western blotting when using commercial PAPS as sulfuryl group donor. The kinetics of PSGL-1 sulfation by using PAPSS-generated [^{35}S]PAPS (synthesized from ATP and $^{35}SO_4^{2-}$) is shown in Figure 1B. The apparent K_m value obtained in this study (24 μM) is in good agreement with that determined with commercial PAPS as sulfuryl group donor (27 μM).[11a] The apparent V_{max} observed in this study, however, was nearly 45-fold higher than that previously reported (3.2 vs.

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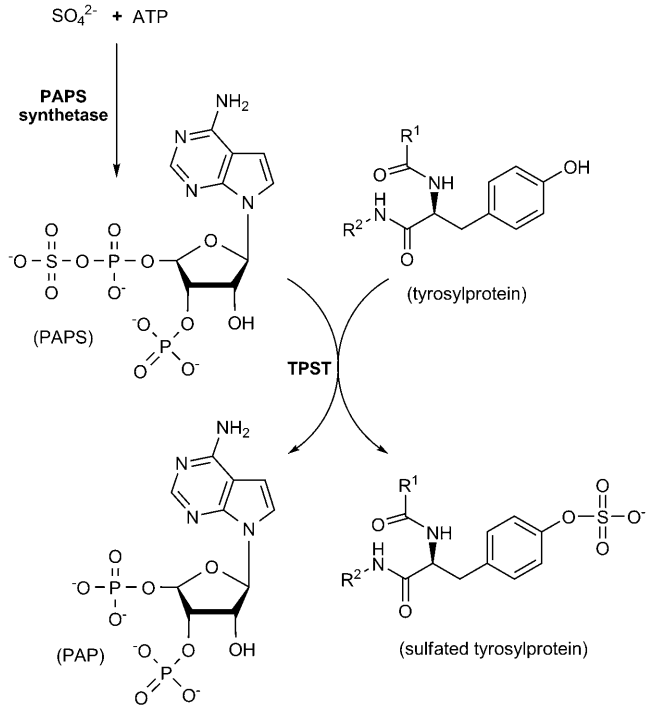

Scheme 1. PAPSS-coupled TPST catalytic reaction. TPST transfers sulfuryl group from PAPS, which is generated from inorganic sulfate and ATP in PAPSS catalysis, to the tyrosine residues within specific domain in proteins and peptides.

0.07 nmol min^{-1} mg^{-1}). We attribute this observation to the absence of initial PAP inhibition that is so common with commercially available PAPS. PAP might also be a noncompetitive inhibitor for PSGL-1 sulfation in the TPST catalyzed reaction (Scheme 1), and consequently, the use of freshly generated PAPS produced a K_m value in agreement with literature values for commercial PAPS, whereas V_{max} increased (Figure 1 B). Moreover, the recombinant NusA–*h*TPST-2 remained relatively stable at 30 °C or lower (Figure S4). In addition to the fresh and ample supply of PAPS, other possible reasons for the high catalytic efficiency of PSGL-1 sulfation might be due to a lack of initial PAP, other inhibitor, contamination, and the improved stability of NusA-TPST fusion protein (Figure S4).

We used a similar strategy to produce tyrosine-sulfated proteins in vivo, by incorporation of suitable genes by using bacterial cultivation. Figure 2 A depicts the layout of the "built-in" chemoenzymatic machinery. In brief, *hPAPSS-1* and *hTPST-2* genes were both subcloned into identical expression vector (pACYCDuet-1) with separate open-reading frames. The target gene (*PSGL-1*) was subcloned to another expression vector (pGEX-4T1) with distinct antibiotic resistance. By co-expressing the two expression vectors, the bacterial colony was simultaneously able to accommodate the genes of *hPAPSS-1*, *hTPST-2*, and *PSGL-1* (Figure 2 B). Tyrosine-sulfated PSGL-1, extracted from the bacteria containing both plasmids, was detectable by Western blotting (upper panel of Figure 2 C). The control experiment (the bacterial host without *hTPST-2* gene-containing plasmid) also produced a large amount of GST-fused PSGL-1 protein (lower panel of Figure 2 C) but was free of sulfated GST-PSGL-1 according to the Western blot analysis. Our results strongly indicate that the in vivo chemoenzymatic machinery system was responsible for the highly efficient post-translational tyrosine sulfation within the bacterial cell samples. This system can greatly facilitate the development and production of sulfated proteins for therapeutic and medical applications.

In conclusion, we have demonstrated that the chemoenzymatic machinery for protein tyrosine sulfation is inexpensive, and offers savings in time and effort. The complete system, from production to isolation and detection of sulfated proteins, can be integrated with high efficiency and high confidence. We were also able to show that protein tyrosine sulfation in *Escherichia coli* BL21(DE3) provides an excellent niche to specifically produce tyrosine-sulfated products. At present, few literature reports contribute to the understanding of either TPST, or sulfated proteins/peptides at a biochemical level. The successful combination of our PAPS-generating system (by PAPSS), and sulfated-protein production (by TPST) provides a

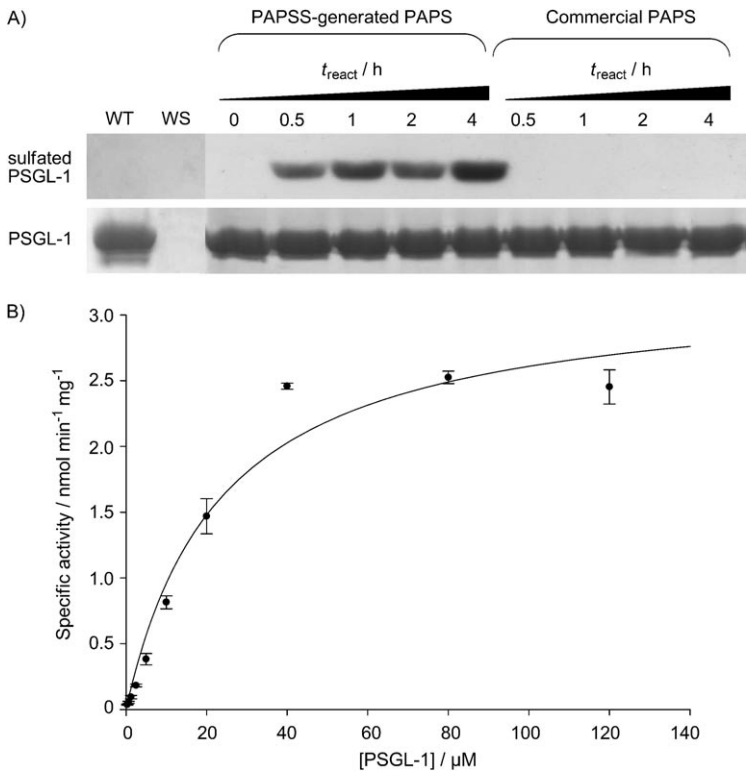

Figure 1. Characterization of chemoenzymatic machinery for protein tyrosine sulfation. A) Sulfation of PSGL-1 with PAPSS-generated PAPS and commercial PAPS. Sulfated PSGL-1 was probed by anti-sulfotyrosine monoclonal antibody (upper panel). The SDS-PAGE of total PSGL-1 stained by Coomassie blue is shown in the lower panel as an internal control. WT and WS were the control experiments, which represented the full catalytic reaction without *h*TPST-2 and substrate (PSGL-1), respectively. B) Michalis–Menten plot of NusA–*h*TPST-2 kinetics. The apparent K_m and V_{max} values were determined as 24 μM and 3.2 nmol min^{-1} mg^{-1}, respectively. The *h*TPST-2 catalyzed reactions were determined under standard condition as described in the Experimental Section.

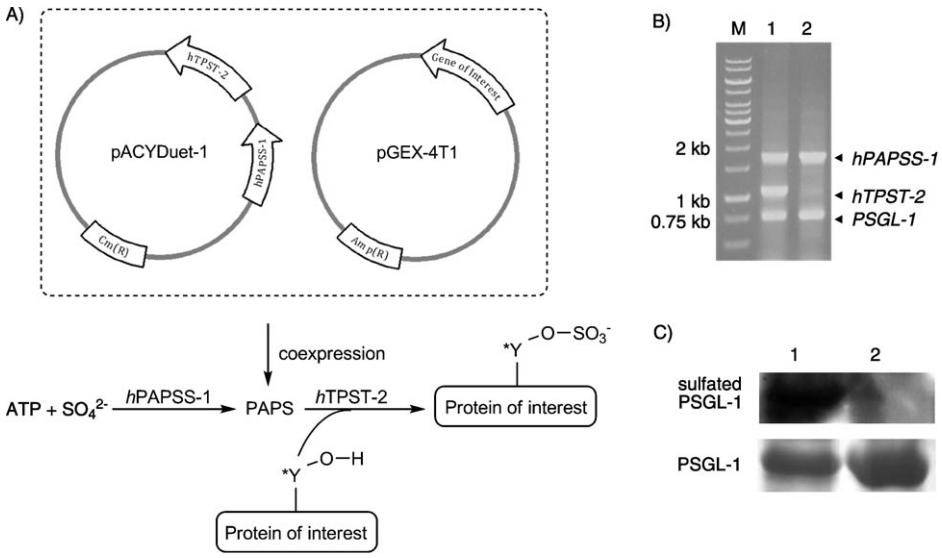

Figure 2. Implantation of chemoenzymatic machinery in vivo for protein tyrosine sulfation. A) Scheme for in vivo protein tyrosine sulfation. Plasmids that contained *hPAPSS-1/hTPST-2* and the target gene (*PSGL-1*), respectively, were coexpressed in a bacterial host. The gene products were expected to produce a series of reagents for protein tyrosine sulfation in bacteria. The tyrosine residue (Y) within the specific domain of target protein is labeled with asterisk. B) Expression of *hPAPSS-1*, *hTPST-2*, and *PSGL-1* genes in bacterial host. Lane 1 indicates the PCR products from the bacterium coexpressed with plasmids containing *PSGL-1* and *hPAPSS-1/hTPST-2* genes. Lane 2 is the control experiment obtained from the bacterium incorporated with both clones of *PSGL-1* and *hPAPSS-1*. C) In vivo production of sulfated PSGL-1. Lane 1 was obtained from bacterium containing *PSGL-1* and *hPAPSS-1/hTPST-2* genes. Sulfated PSGL-1 was probed by anti-sulfotyrosine monoclonal antibody (upper panel). The SDS-PAGE of total PSGL-1 stained by Coomassie blue was shown in the lower panel as internal control (lower panel). The bacterium of the control experiment (lane 2) was in the absence of *hTPST-2* in the protein sulfation system.

tool for both fundamental research and industrial applications in protein tyrosine sulfation. Moreover, it would be interesting if we were to implant this tyrosine-sulfation machinery in eukaryotes, which intrinsically possess endogenous TPST and PAPSS, because more substrates and protein-tyrosine sulfation functions would be uncovered.

Experimental Section

The standard PAPSS-coupled TPST catalysis composed of 50 mM MES at pH 6.5, 5 mM β-mercaptoethanol, 4 mM Na_2SO_4, 1 mM $MgCl_2$, 1 mM ATP, 20 μM PSGL-1 peptide (ATEYEYLDYDFL), 1 μg *h*PAPSS-1, 1 U pyrophosphatase, and 4 μg *h*TPST-2 for 45-min incubation at 37 °C in a final volume of 20 μL. The TPST-catalyzed reaction using commercial PAPS was conducted under similar conditions except that 20 μM PAPS was used to replace the PAPSS system. In vivo protein tyrosine sulfation was demonstrated by coexpressing both plasmids of *hPAPSS-1/hTPST-2* (pACYCDuet-1) and *PSGL-1* peptide (ATEYEYLDYDFL) (pGEX-4T1) in BL21(DE3) with ampicillin (50 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}) as antibiotics. A single colony was cultivated in LB broth at 37 °C until the OD_{A600} reached to 0.8–1 and then induced with 1 mM IPTG for 16 h at 20 °C. The cultures were harvested and extracted in lysis buffer (50 mM Tris–HCl at pH 8.0, 150 mM NaCl, and 10% glycerol) for further analysis.

Detailed protocols for enzyme cloning, expression, purification, PAPSS assay, TPST kinetics assay, thermal stability, in vivo protein tyrosine sulfation, and immunoblotting are described in the Supporting Information.

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