Enzymatic Synthesis and Regeneration of 3′-Phosphoadenosine 5′-Phosphosulfate (PAPS) for Regioselective Sulfation of Oligosaccharides

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Sulfated biomolecules play important roles in many biological processes.[1] The sulfated Le^a tetra- and pentasaccharides,[1b] for example, are potent inhibitors of the cell adhesion molecule P-selectin, and sialyl Lewis x with a sulfate group at the 6-position of galactose is a ligand for L-selectin.[1c] Many glycosaminoglycans such as heparin and dermatan sulfate are also sulfated and are involved in numerous cellular functions.[1d-f] In addition, the sulfation of hydroxysteroids provides hydrophilic forms for excretion.[2]

In the course of sulfation, inorganic sulfate is activated first and then transferred to the acceptor.[3] The two key enzymes involved in this activation process are ATP sulfurylase (EC 2.7.7.4) and adenosine-5′-phosphosulfate (APS) kinase (EC 2.7.1.25)[4] (Scheme 1). 3′-Phosphoadenosine 5′-phosphosulfate (PAPS) is the product generated in these two enzymatic reactions and is a substrate and cofactor for the enzymatic sulfation. The high cost and instability of PAPS[5] and the problem of product inhibition caused by 3′-phosphoadenosine 5′-phosphate (PAP)[6] require regeneration of PAPS *in situ* for enzymatic sulfation on large scales.

Several groups have reported the multistep synthesis of PAPS[7,8] on nanomole to micromole scales using chemical methods or biological processes based on isolated enzymes. As the genes in *Escherichia coli* coding for ATP sulfurylase and APS kinase are known,[9] we have developed overexpression systems for the overproduction of ATP sulfurylase and APS kinase[10] that are used in conjunction with regeneration of ATP from ADP in the synthesis of PAPS. This new process avoids the problem of substrate and product inhibition[11] and allows the synthesis of PAPS on >100 mg scales under an optimized condition.[12] In addition, PAPS was used in the enzymatic sulfation of *N,N′*-diacetylchitobiose catalyzed by a Nod factor sulfotransferase (NodST),[13] cloned and overexpressed in this laboratory,[14] coupled with regeneration of PAPS (Scheme 2). The PAPS regeneration is made possible using 3′-nucleotidase (EC 3.1.3.6), to selectively hydrolyze PAP to AMP, which is then converted *in situ* to PAPS.

For the synthesis of PAPS (Scheme 1), a solution of Tris-HCl buffer (50 mM, pH 8.0, 100 mL) containing 20 mM KCl, 40 mM Na_2SO_4, 5 mM $MgCl_2$, 2 mM ATP, 2 mM PEP, 20 units of ATP sulfurylase, 560 units of APS kinase, 400 units of inorganic pyrophosphatase, and 2000 units of pyruvate kinase was incubated at room temperature under N_2. After 8 h, the reaction solution was filtered to remove proteins and insoluble precipitates by using 10 000 NMWL regenerated cellulose membrane (Millipore Co., Bedford, MA). The filtrate was chromatographed through a Mono-Q anion exchange column, eluted with a linear gradient of NH_4HCO_3 from 20 to 800 mM, and the desired fractions were collected and treated with Dowex 50W-X8 (H^+). The resulting neutral solution was lyophilized to give the desired product as white powder (78 mg, 73% overall yield). The TLC, FPLC, ^{1}H NMR, and ^{31}P NMR data were identical with those of an authetic sample (Sigma Co.); ^{31}P NMR (HOD) δ 3.12, −10.65; negative electrospray unit mass calcd for $C_{10}H_{11}N_5O_{13}Li_3P_2S$ (M - 2Li$^+$ + H$^+$) 518, found 518; positive electrospray unit mass calcd for $C_{10}H_{12}N_5O_{13}Li_4P_2S$ (M + H$^+$) 532, found 532.

For the synthesis of *N,N′*-diacetylchitobiose 6-sulfate catalyzed by NodST coupled with regeneration of PAPS, the reaction was carried out at 25 °C in 9.4 mL of Tris-HCl (100 mM, pH 7.5) containing 40 mM Na_2SO_4, 20 mM $MgCl_2$, 3 units of ATP sulfurylase, 2.5 units of APS kinase, 12 units of inorganic pyrophosphatase, 600 units of pyruvate kinase, 300

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(4) Robbins, P. W.; Lipmann, F. *J. Biol. Chem.* **1958**, *233*, 686.

(5) PAPS is available from Sigma Co. ($36/mg). The half-life of PAPS in aqueous solution at pH 8.0 (50 mM, Tris-HCl) is approximately 20 h.

(6) The K_i value was determined to be 14 μM for the hydroxysteroid sulfotransferase. See: Marcus, C. J.; Sekura, R. D.; Jakoby, W. B. *Anal. Biochem.* **1980**, *107*, 296.

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(9) Leyh, T. S.; Taylor, J. C.; Markham, G. D. *J. Biol. Chem.* **1988**, *263*, 2409.

(10) The two primers cys DN5 (5′ATATT*GAGCTC*GATCAAATACGACTTACTCACCTG) and cys DN3 (5′GCGC*AAGCTT*TTATTATTTATCCCCCAGCAAATC) were designed to specifically complement the C-terminal and N-terminal gene sequences of ATP sulfurylase (cys D and cys N genes). The other two primers, cys C5 (5′ATATT*GAGCTC*GCGCTGCATGACGAAAAC) and cys C3 (5′GCGC*AAGCTT*TTATTAGGATCTGATAATATCGTT), were designed to specifically complement with the N- and C-terminal sequences of APS kinase (cys C gene). The primers cys DN5 and cys C5 contained a *Sac*I restriction site and the N-terminal six amino acid sequences of the genes. The primer cys DN3 and cys C3 contained a *Hin*dIII restriction site, stop codons, and the C-terminal six amino acid sequences of the genes. After ligation of the digested PTrcHis A vector and inserts, the DNA was transformed into *E. coli* XL1-Blue MRF′, and the cloned genes were grown on LB medium containing 150 μg/mL ampicillin and induced at 25 °C with 0.5 mM IPTG. Approximately 430 units/L ATP sulfurylase and 560 units/L APS kinase were obtained. The cells were disrupted with French Press, centrifuged to remove cell debris, fractionated with 45% ammonium sulfate, and purified with DEAE Sepharose 6B-Cl column using a KCl gradient (0−1.0 M). ATP sulfurylase is also commercially available from Sigma Co. ($70 for 50 units).

(11) Renosto, F.; Seubert, P. A.; Segel, I. H. *J. Biol. Chem.* **1984**, *259*, 2113. APS is an inhibitor of APS kinase (K_i = 23 μM) and of ATP sulfurylase (K_i = 0.25 μM). ADP is an inhibitor of APS kinase (K_i = 0.2 μM): see ref 8a.

(12) High yields (80−85%) of PAPS were obtained with excess sulfate (40 mM) and a low concentration of ATP (2 mM) and phosphoenolpyruvate (2 mM) at pH 8.0. Perhaps under this condition the equilibrium is shifted toward PAPS[4,8a] (Renosto, F.; Martin, R. L.; Segel, I. H. *J. Biol. Chem.* **1989**, *264*, 9433).

(13) Nod factor sulfotransferases were described, but none of the enzymes or genes were isolated. Fisher, R. F.; Long, S. R. *Nature* **1992**, *357*, 655; Roche, P.; Debelle, F.; Maillet, F.; Lerouge, P.; Faucher, C.; Truchet, G.; Denarie, J.; Prome, J.-C. *Cell* **1991**, *67*, 1131. Lerouge, P.; Roche, P.; Faucher, C.; Maillet, F.; Truchet, G.; Prome, J.-C.; Denarie, J. *Nature* **1990**, *344*, 781. Faucher, C.; Camut, S.; Denarie, J.; Truchet, G. *Mol. Plant-Microbe Interact.* **1989**, *2*, 291. Faucher, C.; Maillet, F.; Vasse, J.; Rosenberg, C.; van Brussel, A. A. N.; Truchet, G.; Denarie, J. *J. Bacteriol.* **1988**, *170*, 5489. Atkinson, E. M.; Palcic, M. M.; Hindsgaul, O.; Long, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8418. Schwedock, J.; Truchet, G. *Mol. Plant-Microbe Interact.* **1989**, *2*, 291. Cevantes, E.; Sharma, S. B.; Maillet, F.; Vasse, J.; Truchet, G.; Rosenberg, C. *Mol. Microbiol.* **1989**, *3*, 745.

(14) The genes from *Rizobium meliloti* were amplified with the designed primers NodHST5′(5′ATATT*GAATTC*ATTTTCATGACCCATTCCA) and NodHST3′(5′GGCGC*GGATCC*TTAGTCGTTAGCAAGCTC), ligated into the plasmid pKEN2 via the *Bam*HI and *Eco*RI restriction sites, and transformed into *E. coli* BL21 (DE3). The cells were selected and grown in the presence of 250 μg/mL ampicillin and 0.5 mM IPTG at 30 °C. Approximately 610 units/L of the enzyme after cell lysis by French Press was obtained.

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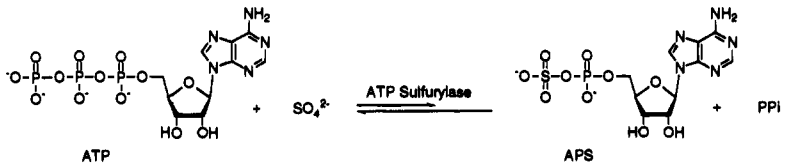
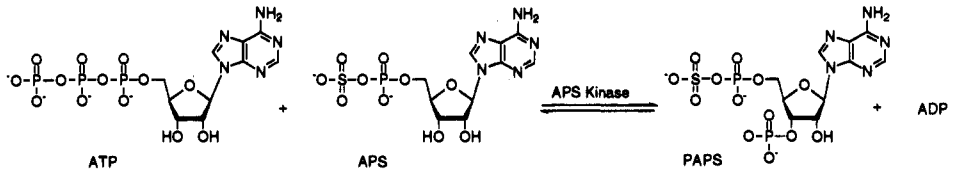
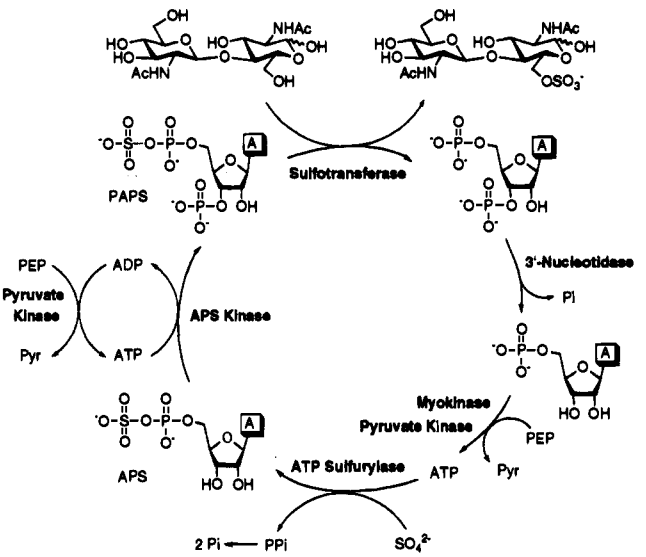
Scheme 1. Enzymatic Synthesis of PAPS





Scheme 2. Regeneration of 3′-Phosphoadenosine 5′-Phosphosulfate (PAPS) for the Synthesis of *N,N′*-Diacetylchitobiose 6-Sulfate Catalyzed by a Recombinant Nod Factor Sulfotransferase[a]



[a] Boxed A: adenosine. PEP: phospho(enol)pyruvate. Pyr: pyruvate. PPi: pyrophosphate. ADP: adenosine 5′-diphosphate.

units of myokinase, 2.4 mL of NodST, 0.36 units of 3′-nucleotidase,[15] 1.5 mM ATP (7.8 mg, 0.0141 mmol), 15 mM phospho(enol)pyruvate (36.4 mg, 0.155 mol), and 5 mM *N,N′*-diacetylchitobiose (20 mg, 0.047 mmol).

The product formation was monitored by TLC with $CHCl_3$/MeOH/AcOH/H_2O (25:15:2:1) and the *p*-anisaldehyde stain solution. After 2 days, the reaction solution was centrifuged to remove the precipitate and the supernatant was concentrated and passed through Sephadex-G25 twice with water to obtain the desired product (20 mg, 84% yield).[16] The ^1H NMR spectrum of the product shows the downfield shifts of H_5, H_{6A}, and H_{6B} of the reducing-end sugar [δ −4.031 (H-α5), 3.979 (H-β5), 4.154 (H-α6A), 4.102 (H-β6B), 4.100 (H-α6B), 4.071 (H-β6B)]. Additionally, the DEPT experiment showed a downfield shift for one of the two primary carbons, and the other remained the same.

As demonstrated in this communication, a practical synthesis of PAPS and its application to the regioselective sulfation of an oligosaccharide coupled with regeneration of PAPS have been established. This enzymatic process should be useful for the large-scale preparation of various bioactive oligosaccharide sulfates.[17,18]

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Supporting Information Available: Procedures for the overexpression of enzymes (ATP sulfurylase, APS kinase and the Nod factor sulfotransferase) and their preparation for synthesis (22 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(15) This enzyme is highly selective for PAP. Although PAPS is also slowly hydrolyzed, the product APS is converted to PAPS in the system.

(16) ^1H NMR (HOD): δ 5.086 (1H, d, J = 2.5 Hz, H-α1), 4.612 (1H, d, J = 8.0 Hz, H-β1[a]), 4.529 (1H, d, J = 8.5 Hz, H-α1′), 4.523 (1H, d, J = 8.5 Hz, H-β1′[a]), 4.154 (1H, d, J = 11.0 Hz, H-α6A), 4.102 (1H, dd, J = 2.0, 11.0 Hz, H-β6A), 4.100 (1H, m, H-α6B), 4.071 (1H, dd, J = 3.5, 11.0 Hz, H-β6B), 4.031 (1H, m, H-α5), 3.979 (1H, m, H-β5), 1.989 (6H, s, 2 × *N*-acetyl), 1.931 (6H, s, 2 × *N*-acetyl). ^{13}C NMR (HOD): 175.1 (CH_3CO), 175.1 (CH_3CO), 174.8 (CH_3CO), 174.8 (CH_3CO), 101.6, 101.5, 95.3, 90.9, 90.8, 79.4, 78.9, 76.2, 73.9, 73.8, 72.6, 70.0, 69.5, 68.2, 66.6, 65.2, 60.7, 55.8, 53.8, 49.2, 23.6 (CH_3CO), 22.5 (CH_3CO), 22.5 (CH_3CO), 22.2 (CH_3CO). Electrospray mass: calcd for $C_{16}H_{27}N_2O_{14}S$ ($M - H^+$) 503, found 503.

(17) The *E. coli* strains for the three recombinant enzymes will be deposited with American Type Culture Collection (ATCC).

(18) NodST has a broad substrate specificity with the following relative rates: GlcNAcβ1,4GlcNAc (1), GlcNAcβ1,4GlcNAcβ1,4GlcNAc (1.4), (GlcNAcβ1,4GlcNAc)$_2$ (1.9), Galβ1,4GlcNAc (0.4), Galβ1,3GlcNAc (0.3), Glcβ1,4Glc (0.3), GlcNAcβ1,4GlcNAcβ1,NAsn (0.4).