Supporting Information

One Step Synthesis of Labeled Sugar Nucleotides for Protein *O*-GlcNAc Modification studies by Chemical Function Analysis of an Archaeal Protein

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Materials. Enzymes and reagents used for the molecular biology procedures, DNA ladders, and deoxynucleotide triphosphates (dNTPs) were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Oligonucleotides for DNA amplification were synthesized by Sigma Genosys (Woodland, TX). Thermostable inorganic pyrophosphatase (IPP) from *Thermococcus litoralis* (EC 3.6.1.1, M0296S) was purchased from New England Biolabs as a 2000 U/mL 50% glycerol solution in Tris buffer (pH 8.0). Isopropylthiogalactoside (IPTG) was obtained from Labscientific, Livingston, NJ. Protein molecular weight standards were obtained from BioRad (Hercules, CA). The QIAquick gel extraction kit was obtained from Qiagen (Valencia, CA) and the Zero Blunt PCR cloning kit was purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Bacterial Strains and Growth Conditions. Genomic DNA from *Pyrococcus furiosus* (ATCC 43587D), obtained from the American Type Culture Collection (Manassas, VA), was used as the source for the cloning experiments described herein. Oneshot Top10 competent cells (Invitrogen, Carlsbad, CA), *Escherichia coli* XL-10Blue cells (Stratagene, La Jolla, CA), and PCR-Blunt vectors (Invitrogen, Carlsbad, CA) were used for direct cloning of PCR products. *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, CA) was used in combination with the T7 expression system (pET21a vector; Novagen, Madison, WI.) for expression of the sugar nucleotidyltransferase gene. *E. coli* cells were grown on Luria-Bertani (LB, Sigma, St. Louis, MO) medium at 37 °C on an incubator shaker at 225 rpm. When required, antibiotics were added at the following concentrations to make the selective media: carbenicillin 50 μg/mL, kanamycin 50 μg/mL, chloramphenicol 25 μg/mL.

General Methods. Standard procedures to manipulate DNA, including plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation of *E. coli*, were performed by conventional methods (Sambrook 1989). The PCR was carried out in an Eppendorf Mastercycler gradient thermocycler (Eppendorf Scientific Inc. Westbury, NY). Protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Tris-HCl 10-20% gradients, Bio-Rad Laboratories, Hercules, CA). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined with the Bio-Rad protein assay kit according to the method of Bradford (Bradford 1976) using bovine serum albumin as the standard.

PCR Amplification, Cloning, Expression and Purification of *P. furiosus* Enzyme. Genomic DNA of *P. furiosus* (ATCC 43587D) was amplified by PCR synthesis using two oligonucleotide primers. The primers were designed in order to construct the G1P-TT expression plasmid. The forward primer, 5'- AAAC**CATATG**AAAGCTATAATACTTGCCGCTG -3', contains an *Nde*I restriction site (in bold) and the reverse primer, 5'-AAA**CTCGAG**TCATTTCTCAATCACCTT TTTCTCC-3', contains a XhoI restriction site (in bold) were synthesized from the putative glucose-1-phosphate deoxythymidylyltransferase gene of *P. furiosus*. The amplification reaction mixture contained standard *Pfu*DNA polymerase buffer, 375μM of dNTPs, 3 ng of each primer, 4 ng of total genomic DNA and 2.5 units of *Pfu* DNA polymerase. The cycling parameters of 94 °C for 2 min 40 sec followed by 30 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 2 min 15 s, with a final elongation step of 72 °C for 15 min. The amplified DNA, after agarose gel electrophoresis (1%) was purified using QIAquick

Gel Extraction kit and subcloned into a ZeroBlunt vector using ZeroBlunt PCR cloning kit, and was transformed into Oneshot Top10 and *E. coli* XL10 competent cells to check the correct insert. The resulting construct was then digested with *Nde*I and XhoI and was ligated to a pET21a vector containing a C-terminal histidine tag sequence (Novagen, Madison, WI) and previously digested with the same restriction enzymes. Aliquots of the ligation mixture were transformed into competent *E. coli* BL21 (DE3) cells. Transformants were selected at 37 °C grown on LB medium supplemented with carbenicillin.

The freshly transformed cells containing the desired plasmid were grown in LB until the optical density at 600 nm of the cell culture reached 0.6-0.8. Enzyme production was initiated by the addition of IPTG (1 mM) and the culture was incubated at 37 °C for the additional 4 h. Cells were harvested by centrifugation at 3600 x g for 10 min at 25 °C. The enzyme was purified essentially at 4 °C unless otherwise stated. The cells were disrupted by sonication (Fisher model 100 Sonic Dismembranator, Fisher Scientific, Pittsburgh, PA), after which unbroken cells and debris were removed by centrifugation (30 min at 10,000 x g). The supernatant was then heated at 100 °C for 5 min and the precipitated portion was removed by centrifugation at 12,000 x g for 20 min. The cleared lysate was then purified by metal chelate chromatography by following the recommended procedures provided by Novagen. The purified protein was concentrated and dialyzed into the Tris-HCl buffer (50 mM, pH 7.5) using a Microcon Centrifugal Filter Device, MWCO 10 kDa (Millipore, Billerica, MA). The protein was analyzed by SDS-PAGE analysis.

Mass Spectrometry. A Shimadzu LCMS 2010 quadrupole mass spectrometer (Shimadzu Scientific Instruments, Columbia, MD) equipped with an electrospray ionization (ESI) source was used in negative ion mode. The capillary temperature and the spray voltage were kept at 220 °C and 4.5 kV, respectively. The instrument was calibrated by direct infusion of polyethylene glycol (PEG) 200, 600, 1000, (1.5 µL/L, 2µL/L, and 15 µL/L, respectively) and raffinose (50 mg/L) in water/methanol (1:1, v:v) containing ammonium acetate (0.19 mM), 0.1% formic acid and 0.1% acetonitrile. For sample analysis the solvent acetonitrile/water/triethylamine, (35/65/0.2) was constantly infused into the ion source at 250 µL/min by the attached Shimadzu HPLC pump and the samples were injected (30 µL) via the auto sampler adapted to fit two 96-well plates. A preliminary MS chromatogram was obtained by scanning from 50-700m/z. To increase the signal to noise ratio, the instrument was set for selected ion monitoring (SIM) mode and all relevant m/z ions were monitored for further analysis of the enzymatic reactions. Inclusion of an Agilent Extend C18 column (2.1 x 50 mm, Agilent, Palo Alto, CA) in the system further increased the signal to noise ratio and decreased the appearance of sodium ion adducts by 10-15% without separation of reaction components. Postrun software (LCMS Postrun version 2.02, Shimadzu Scientific Instruments, Columbia, MD) was used to analyze the data from the ESI-MS chromatogram. Peaks were integrated to determine the relative intensity of each ion species monitored as compared to an internal standard.

Enzyme Assay. The nucleotidyltransferase activity of the bifunctional *P. furiosus* enzyme was determined by the consumption of glucose 1-phosphate (Glc1P) in the direction of dTDP-glucose biosynthesis. The enzymatic reaction was initiated by the addition of glucose 1-phosphate (5 mM) to a reaction mixture of 50 µL containing Tris-HCl buffer (25 mM, pH 7.5), inorganic pyrophosphatase (IPP, 0.2 U), purified enzyme solution (10 µL), $MgCl_2$ (5 mM), and dTTP (5 mM). Before adding Glc1P the reaction components were incubated at 80 °C for 5 min. Acetyltransferase activity of the enzyme was also monitored by the ESI-MS based assay as

described above. The reaction was initiated by the addition of glucosamine-1-phosphate (GlcN1P) to a reaction mixture of 50 μL containing Tirs-HCl buffer (25 mM, pH 7.5), Mg^{2+} (5 mM) and purified enzyme. Both the reactions were carried out at 80 °C for 5 min and 15 μL of the reaction mixture was quenched by the addition of 30 μL of 70% methanol/water containing AMP (3 mM) as an internal standard. The quenched solutions were centrifuged 10 minutes at 10,000 x g to precipitate the protein. Aliquots of the reaction mixtures were diluted with 135 μL of acetonitrile (acetonitrile/water/triethylamine (35/65/0.2). These samples (5 μL) were subjected to analysis via ESI-MS to determine the amount consumed of Glc1P for the nucleotidyltransferase reaction and GlcN1P for the acetyltransferase reaction compared to a blank containing no enzyme.

Optimal Activity Determination of the Enzyme. The optimal activity for the *P. furiosus* enzyme was measured at 80 °C between pH 4.0 to pH 9.6 using 50mM acetate, phosphate, and Tris-HCl buffer. The optimal temperature was measured at pH 7.5 between 30 °C to 110 °C. Relative acceptance of the enzyme to a number of sugar-1-phosphates and NTPs (UTP, dTTP, GTP and ATP), effects of divalent cations on catalytic conversion and Mg^{2+} ion concentrations were determined in Tris buffer (25 mM, pH 7.5) and at 80 °C.

Kinetic analysis. Calibration curves for determining the initial activities for nucleotidyltransferase activity of the enzyme were developed following the methods described previously (Zea 2004). In order to determine the kinetic parameters for the acetyltransferase reaction of the enzyme calibration curves were constructed for *N*-acetylglucosamine-1-phosphate (GlcNAc1P) and *N*-propylglucosamine-1-phosphate (GlcNPr1P). Calibration curve for GlcNAc1P was run in triplicate containing GlcNAc1P (5-200 μL), Tris buffer (pH 7.5, 25 mM) and Mg^{2+} (5 mM) in a final volume of 50 μL. The samples (30 μL) were diluted with 30 μL of 70% methanol/water containing AMP (6 μM) as an internal standard and centrifuged and diluted as described above. Calibration curve for GlcNPr1P was constructed by allowing the reaction components containing propylCoA (500 μM), limiting amount of GlcN1P (10-50μM), Tris buffer (pH7.5), Mg^{2+} (5 mM) and the enzyme (0.02 U). Reaction were carried out at 80 °C for 1h, as determined by the complete disappearance of the GlcN1P by ESI-MS and was quenched and centrifuged as described above. Fifteen μL of the mixtures were diluted with 135 μL of acetonitrile/water/triethylamine (35/65/0.2). These samples (30 μL) were subjected to analysis by ESI-MS to determine the amount of NPropGlcN1P (*m/z* 314) as compared to internal standard, AMP (*m/z* 346). A plot of the relative intensity of the product was plotted against the concentration of GlcN1P.

The values for K_M and *V*max were derived from enzymatic reactions run in triplicate and determined from the initial rates of UDP-glucose and dTDP-glucose formation using ESI-MS. The enzymatic reaction was initiated by the addition of glucose-1-phosphate (2-80 μM) to obtain a reaction mixture containing Tris buffer (25 mM, pH 7.5), inorganic pyrophosphatase (0.2 U), sugar nucleotidylyltransferase (5.5 x 10^{-4} U), and UTP/dTTP (400 μM) with a final volume of 50 μL. The kinetic values for acetyltransferase activity were also derived from enzymatic reactions run in triplicate and determined from the initial rates of NAcGlcN1P, NAcetylgalactosamine-1-phosphate (GalNAc1P) and GlcNPr1P for various substrates. Kinetics for GlcN1P (5-200 μM), GalN1P (5-200 μM), AcCoA **7** (5-200 μM), nPropCoA (5-200 μM), and *N,S*-diacetylcysteamine **8** (5-200 μM) were run in a 50 μL reaction volume containing enzyme (5.5 x 10^{-4} U) with fixed concentration of AcCoA (400 μM) for GlcN1P and GalN1P and fixed concentration of GlcN1P

(400 μM) for the other three substrates. Reactions were carried out at 80 °C for 5 min and 30 μL of reaction mixture was quenched by addition of 30 μL of 70% methanol/water containing AMP (6 μM) as an internal standard. The quenched solution was centrifuged and diluted as described above. These samples (30 μL) were subjected to analysis via ESI-MS to determine the amount of UDP/dTDP-sugars formed by the nucleotidyltransferase activity and GlcNAc1P, GlcNPr1P and GalNAc1P formed by the acetyltransferase reaction. All kinetic data was fitted with the non-linear regression algorithm in GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Error bars on the graphs represent the standard deviation of three independent averaged data points. In order to determine the kinetic mechanism of the enzyme, the nucleotidyltransferase reaction experiments were designed to provide data by varying the concentration of Glc1P at several fixed concentrations of dTTP and also varying the concentration of dTTP at several fixed concentrations of Glc1P according to the previously described approach (Cleland 1967)

Multiple Sequence Alignment. A multiple sequence alignment was performed using BLAST (www.ncbi.nih.gov). Motifs were defined as the regions with at least 11 strictly conserved residues among 30 consecutive positions (Bork 1996).

Analysis of Alternate Substrates. The enzyme (0.02 U) was incubated in a total volume of 50 μL reaction mixture containing 5 mM sugar-1-phosphates, 5 mM UTP/dTTP, 3 mM $MgCl_2$, 25 mM Tris buffer (pH 7.5), and 0.2 U IPP at 80 °C for 60 min. Acetyltransferase efficiency of the enzyme was also monitored after incubating the enzyme with a number of substrates (AcCoA **7**, nProCoA, *N,S*-diACcyst **8**, ClAcCyst **9** and Propcyst **10**) with or without UTP in a 50 μL reaction mixture for 40 min. The reactions were quenched and the formation of products were analyzed by ESI-MS. Percent conversion was calculated as the amount of sugar phosphate remaining in the reaction after incubation at specified time divided by the amount of substrate used in the reaction multiplied by 100. Each reaction was run in duplicate and the mean percent conversion was used. In order to check for nonenzymatic conversions of sugar-1-phosphates at the high temperature, the reaction mixture containing all the components except the sugar nucleotidyltransferase was also used as a control reaction for each set of reactions. Only the alkyne substrate **10,** but not **8** and **9**, reacted with the glucosamine-1-phosphate at the elevated temperatures in the absence of enzyme.

Table S1. Relative catalytic efficiency of the enzyme with various sugar-1-phosphates.

Sugar-1-phosphate[a]	*UTP conversion (%)*[b]	*dTTP conversion (%)*[b]
Glc1P	88±4	86±5
Man1P	80±6	50±6
Gal1P	0	0
Fuc1P	14±2	16±2
GlcN1P	16±2	20±1
GalN1P	0	0
NAcGlcN1P	70±5	80±7

[a] sugar-1-phosphates were incubated in a 50 µL reaction volume containing UTP/dTTP and enzyme along with other reaction components for 60 min.

[b] % conversion was calculated as the amount of sugar-1-phosphate remaining over the amount of sugar-1-phosphate used up after specified time multiply by 100.

Table S2. Relative catalytic efficiency of the acetyltransferase activity of the enzyme.

Substrate[a]	% conversion[a] (GlcN1P)	% conversion[b] (GalN1P)	% conversion[b] (GlcN1P-UTP)
7	96±3	80±4	100
nPropCoA	83±5	60±3	75±6
8	94±4	71±5	92±3
9	78±6	40±6	45±10
10[c]	95±3	66±3	56±7

[a] incubated with GlcN1P/GalN1P/GlcN1P-UTP in a final volume of 50 μL containing enzyme and other reaction components for 40 min.

[b] % conversion was calculated as the amount of starting substrate (GlcN1P/GalN1P) remaining over the amount of substrate consumed after the specified time multiplied by 100.

[c] a higher degree of nonenzymatic catalysis to form the intermediary product was observed in the presence of both GlcN1P and GalN1P at 80 °C, which were then used to make UDP-products in a one-pot reaction.

Table S3. Effects of divalent cation on the activity of the *P. furiosus* enzyme.

Divalent cation	*Relative activity (%)*
None[a]	0
Mg^{2+}	100
Ca^{2+}	48
Cu^{2+}	30
Mn^{2+}	28
Co^{2+}	6
Zn^{2+}	2

[a] All of the reaction components were incubated without any divalent cation at 80 °C.

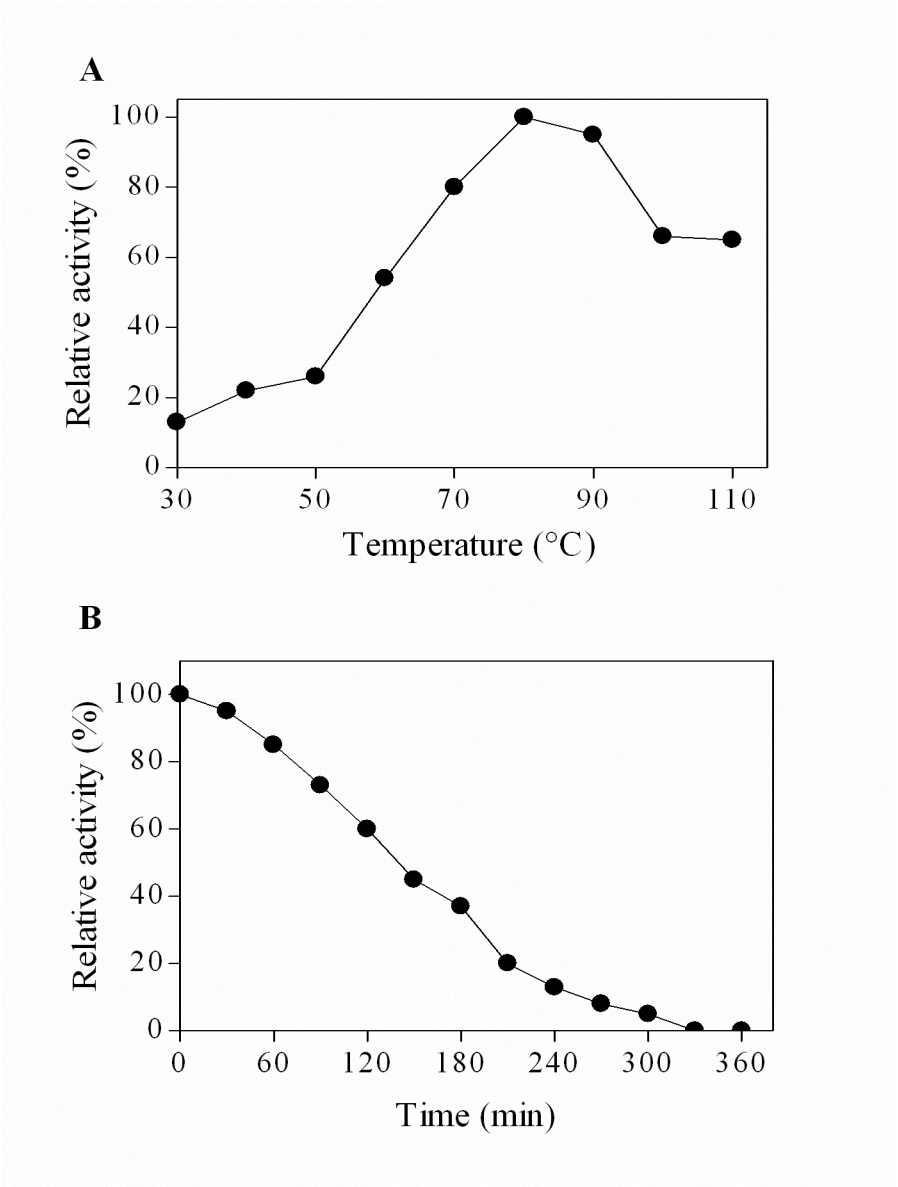

Figure S1. Effects of temperature on activity (A) and stability (B) of the enzyme. To check activity at various temperatures, the enzyme (0.005 units) was incubated with standard assay components for 10 min at 80 °C. The dTDP-glucose formed was analyzed by an ESI-MS based assay and expressed as the percentage of maximum activity. To check stability of the enzyme, the purified enzyme in Tris buffer without any other reaction components (200 μL total volume) was incubated at 80 °C. Several ten μL aliquots were removed at specified time intervals and the activity of the enzyme was determined at 80 °C after addition of all the other reaction components.

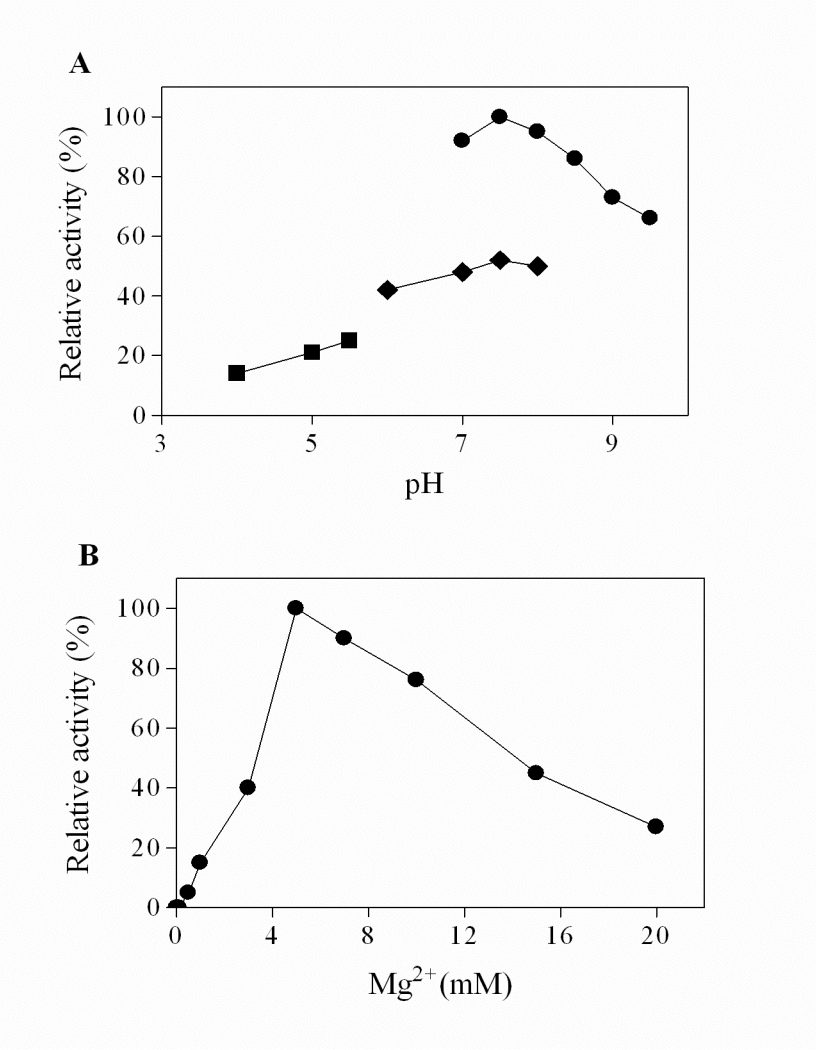

Figure S2. Effects of pH (symbol: square, acetate buffer; diamond, phosphate buffer; circle, Tris buffer) (A) and Mg^{2+} ion concentration on the activity of the enzyme.

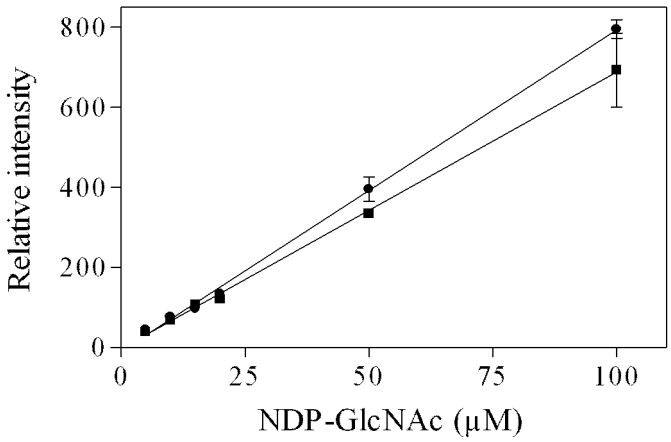

Figure S3. Calibration curves for the relative intensity of NDP-GlcNAc1P (*m/z* = 604 for dTDP-GlcNAc1P and 606 for UDP-GlcNAc1P). Symbol: circle, dTDP-GlcNAc1P; square, UDP-GlcNAc1P.

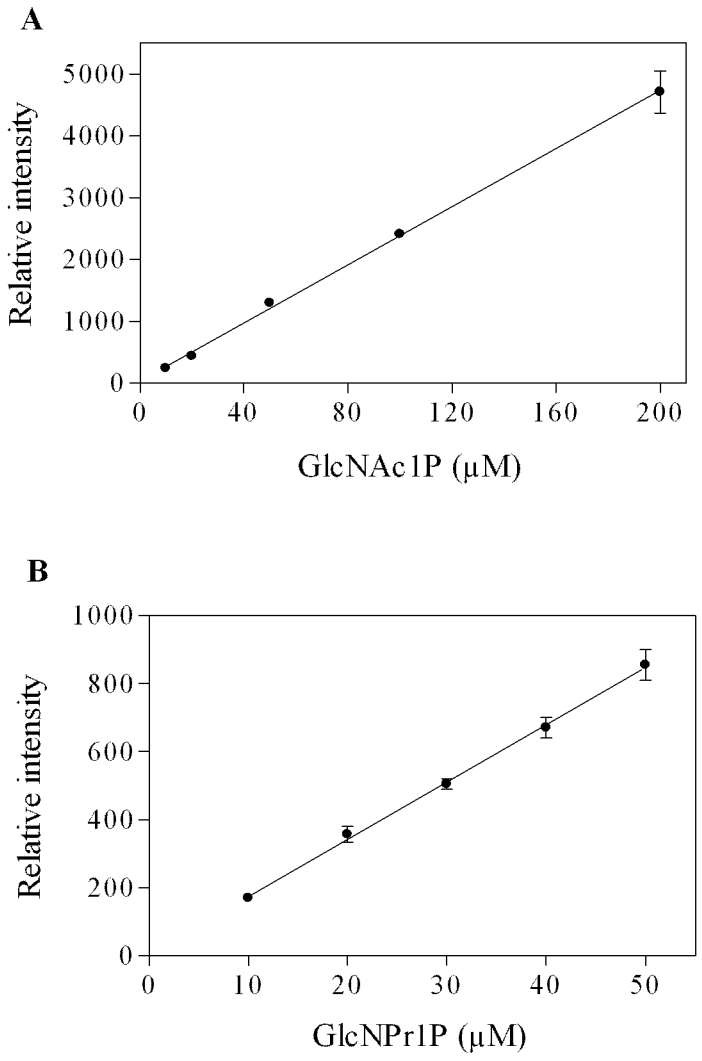

Figure S4. Calibration curves for the relative intensity of (A) GlcNAc1P (*m/z* = 300) and (B) GlcNPr1P (*m/z* = 314).

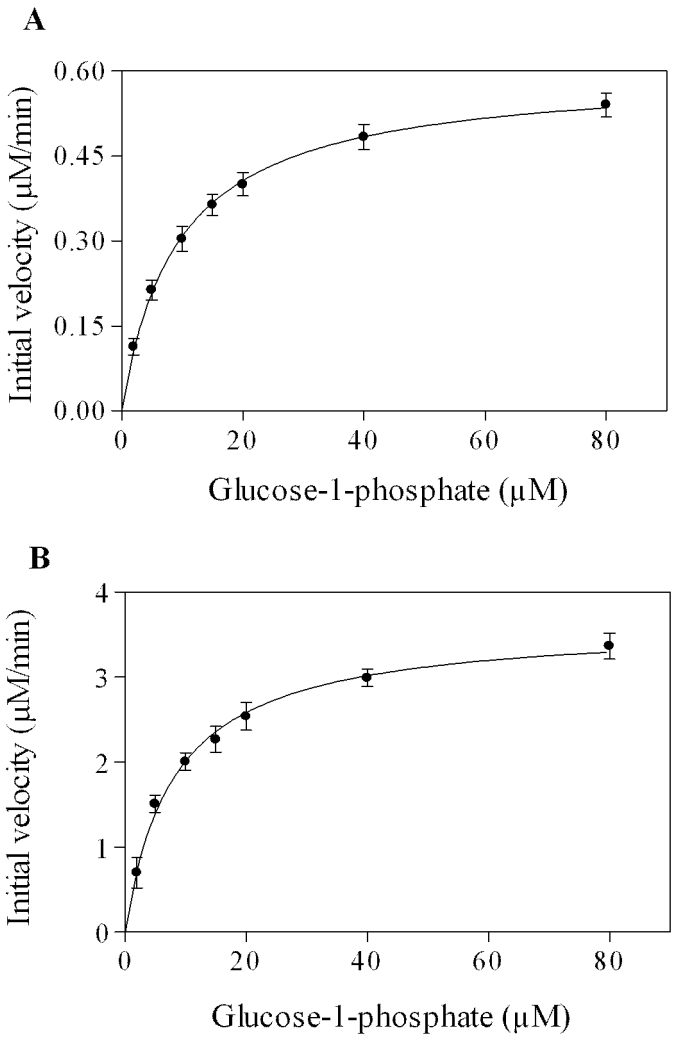

Figure S5. Michealis-Menten plots for recombinant *P. furiosus* enzyme in the direction of NDP-sugar synthesis for Glc1P with UTP (A) and with dTTP (B). Error bars on the graphs represent the standard deviation of three independent averaged data points.

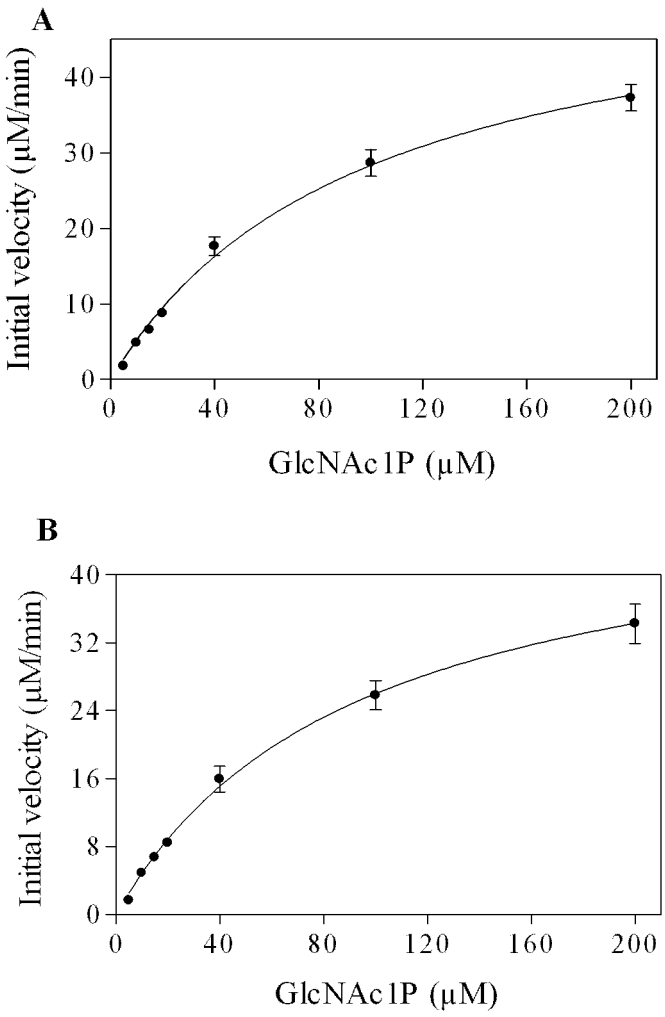

Figure S6. Michealis-Menten plots for recombinant *P. furiosus* enzyme in the direction of NDP-sugar synthesis for GlcNAc1P with UTP (A) and dTTP (B). Error bars on the graphs represent the standard deviation of three independent averaged data points.

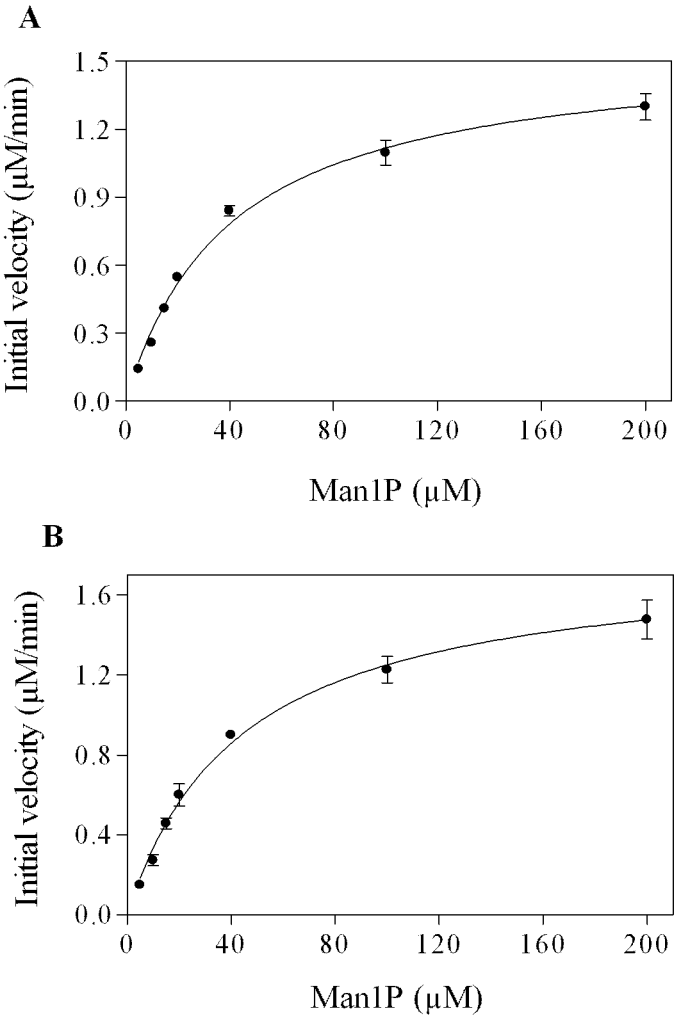

Figure S7. Michealis-Menten plots for recombinant *P. furiosus* enzyme in the direction of NDP-sugar synthesis for Man1P with UTP (A) and dTTP (B). Error bars on the graphs represent the standard deviation of three independent averaged data points.

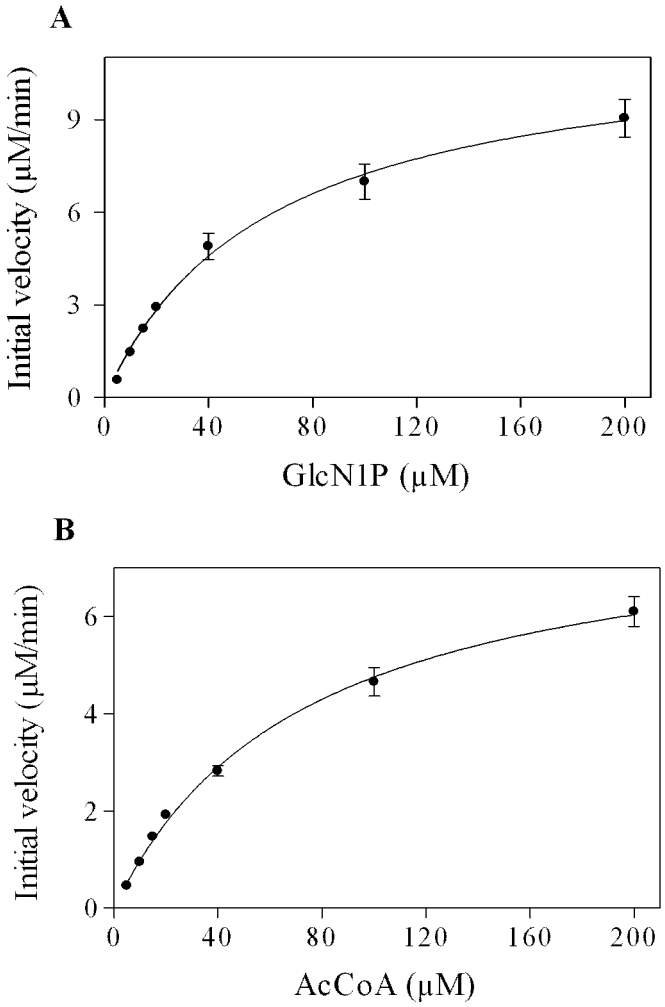

Figure S8. Michealis-Menten plots for recombinant *P. furiosus* enzyme in the direction of GlcNAc1P synthesis for GlcN1P (A) and AcCoA (B). Error bars on the graphs represent the standard deviation of three independent averaged data points.

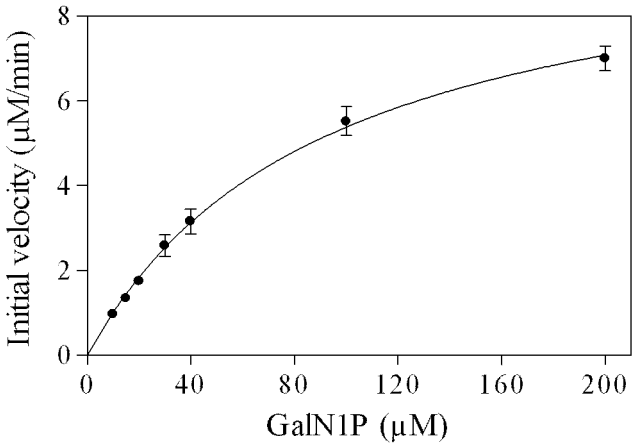

Figure S9. Michealis-Menten plots for recombinant *P. furiosus* enzyme in the direction of GalNAc1P synthesis. Error bars on the graphs represent the standard deviation of three independent averaged data points.

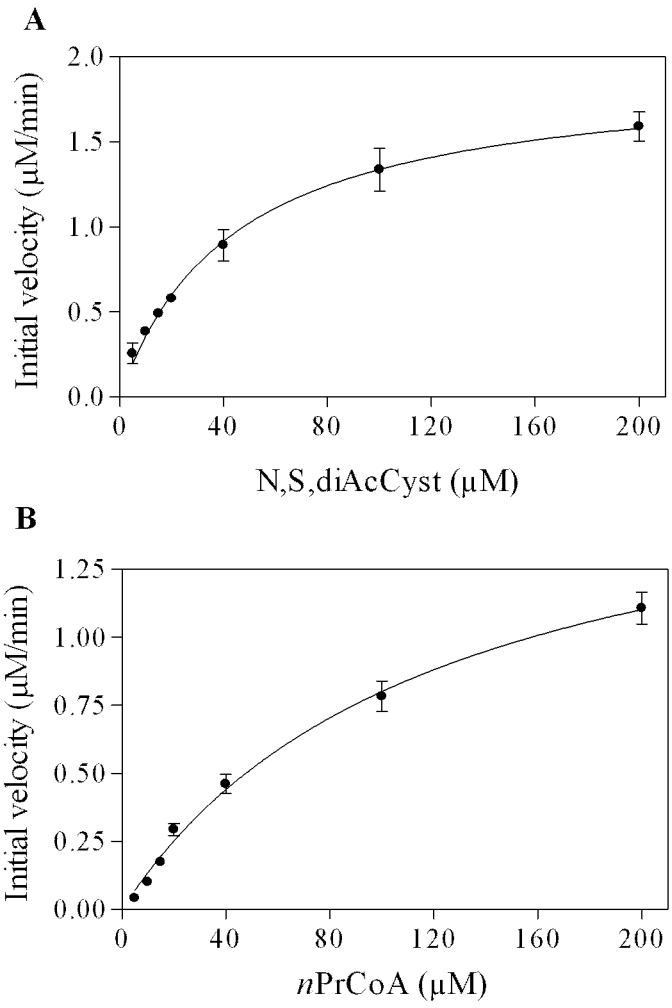

Figure S10. Michealis-Menten plots for recombinant *P. furiosus* enzyme for **8** (A) and *n*PrCoA (B). Error bars on the graphs represent the standard deviation of three independent averaged data points.

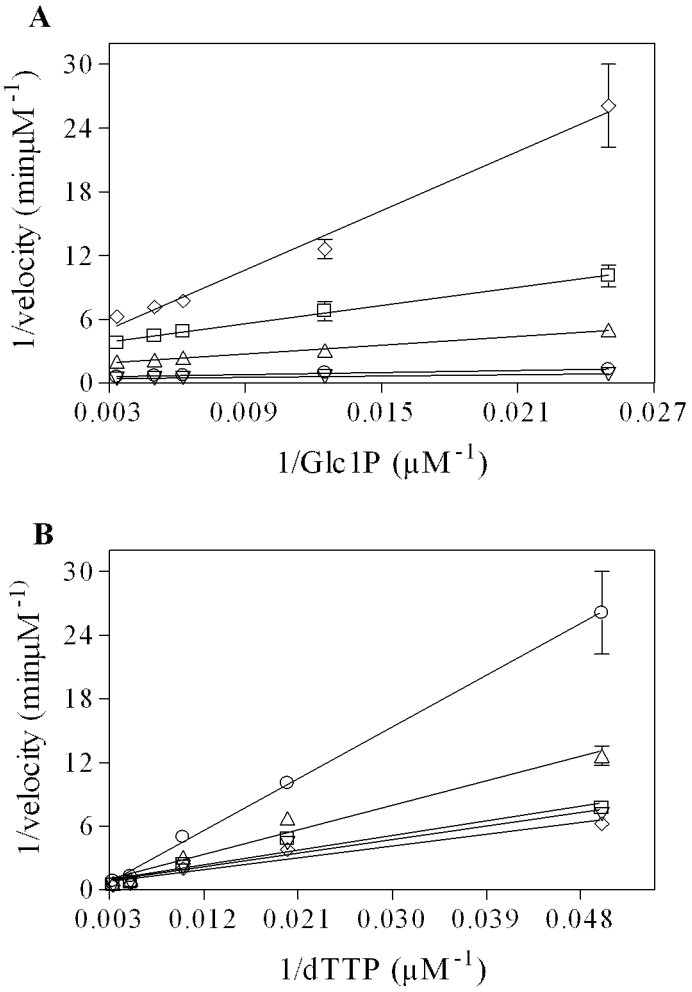

Figure S11. Steady state kinetic parameters of the enzyme from *P. furiosus*. (A) The double reciprocal plots are from assays (done in triplicate) with varying Glc1P concentration as a function of dTTP (μM): 20 (diamond), 50 (square), 100 (triangle), 200 (circle) and 300 (triangle-down faced). (B) The double reciprocal plots are from assays (done in triplicate) with varying UTP as a function of Glc1P concentration (μM): 5 (circle), 10 (triangle), 20 (square), 40 (triangle-down faced) and 80 (diamond).

Figure S12. Amino acid sequence alignment of glucose-1-phosphate thymidylyltransfeases. Amino acids conserved in every enzymes are bold marked and those are conserved in at least three enzymes are bold marked and italicized. Pfu: *Pyrococcus furiosus* (NC_003413); Sal: *Salmonella typhimurium* LT2 (AE008792.1); Psd: *Pseudomonas aeruginosa* PAO1 (NC_002516.1); Eco: *Escherichia coli* (AF529080.1); Sbd: *Shigella boydii* (AY529126.1)

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Pfu      1 --MKAIILAAGKGERLRpltDDRPKVVLKIANKPIISYVLENLDPF-VDEFIIVVKYMKE 57
Spn      1 MSNFAIILAAGKGTRMK---SDLPKVLHKVAGISMLEHVFRSVGAIQPEKTVTVVGHKAE 57
Bap      4 QEIIIVILAAGKGTRMK---SNHPKVLHFLGGKTILEHVIETAQSIKPKKIILVYSDQKK 60
Fnu      1 --MKSIIMAAGKGTRMK---SDLPKVVHLAHGKPMIVRIIDALNTLDVEENILILGHKRE 55
Hpy      1 -MLSVIILAAGKGTRMR---SSLPKTLHTICGEPMLFYILETAFS-ISDDVHLILHHQQE 55
Pmu      4 KALSIVILAAGKGTRMY---SDLPKVLHKIAGKPMVKHVIDTVKSIHAKNIHLVYGHGGE 60
Aae      1 --MRAVILAAGLGTRFK---SEKPKVLHEILGKPMLWYVITNVRNGRIDDIAVVVGHKAQ 55
Lin      2 SKRYAVVLAAGQGTRMK---SKLYKVLHPVCGKPMVEHVVDQISTLDVDKVVTIVGHGAE 58

Pfu     58 KVIDLLGDEFrgkPITYVEQGE--EEGTAAAVYSVKEFIESNEEF--FVVNGDL-YFEPD 112
Spn     58 LVEEVLAGQ-----TEFVTQSE--QLGTGHAVMMTEPIL-EGLSGHTLVIAGDTPLITGE 109
Bap     61 PVLSNIYNIp----IQWIIQKK--PQGTGHAILLAIKKIsDNTE--ILVLYGDVPFISPV 112
Fnu     56 KVLEVLGND-----VSYVVQEE--QLGTGHAVKQAIPKI-KDYDGDVLIINGDIPLIRKQ 107
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Aae     56 EVMEAFKNEn----LKFFIQENp-KGGTADAVLAAKDFF-SSYEGYVLIINGDSPLVSGE 109
Lin     59 KVQEHLAGK-----SEFVKQDE--QLGTAHAVLQAKSEL-AGKDGVTLVVCGDTPLIEAS 110

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Bap    113 SIKk------LQKSKK--QSKISLLTAKVKNPNGYGRILRKKG--KVISIIEDQDASNEQ 162
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Aae    110 TIRnmqqfiHMVRTYEGIKLGGVVLTTHLPDPTGYGRIIKEEGtDRIIRIVEEKDATPEE 169
Lin    111 TME------ALLKYHHEKRAKATILTTVIEDPTGYGRIIRDDL-GIVEKIVEHKDATEKE 163
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Bap   163 KNIKEIYSGIFIAQSKDLTRWLKKIDKKNEKQEFYATDIIALAHLEGSFIKTIEPLNYEE 222
Fnu   160 KKVKEINAGVYIFKAQALVKALEKINNNNEKGEYYITDVIEILSND-KKVISYSLEDSME 218
Hpy   161 KEIKSVNAGVYGFERDFLEKYLPKLHDQNAQKEYYLTDLIALGINENETIDAIFLK-EEC 219
Pmu   163 LKIQEINTGLLVADGKSLKKWLSQLTNNNAQGEYYITDVIALANQDGCQVVAVQASDFME 222
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Pfu   218 DIG--RPWDLLEVNEyILKTKLKHE---------------IRG--------VVEEGATII 252
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Fnu   219 IQGVNSKVELALVSK-VLRERKNTALMEDGVILIDPATTYIDDEVKIGRDTTIYPNVTLQ 277
Hpy   220 FLGVNSQTERAKAEE-IMLERLRKNAMDLGVVMQLPNSIYLEKGVSFKGECVLEQGVRLI 278
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Hpy   279 GN----------CL-------IE-NAHIKAySVIEESQIV-NSSVGPFAHARPKSVICN- 318
Pmu   282 GKVSLGHRVKIGAGCVLKNCQIGDDVEIKPySVLEEAIVGQAAQIGPFSRLRPGTALADN 341
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Pfu   357 ledsGRRKLGAIIGHNVKVGINVTIYPGRKIGSNSFIGPGVIVDKNIPQNVLVTVKQEKK 416
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Bap   389 ----GANKFKTIIGDNVLVGSNTQLIAPIKIAKNTTIAAGTTVTKDVNTPCLVYNTKEQK 444
Fnu   384 ----GKNKFKTEIGKDVFIGSDTMLVAPVNIGDNSLIGAGSVITKDVPSDSLSVERSKQI 439
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Pfu   417 VIEk---------- 420
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Pmu   445 HIQGWQRPtKQTKK 458
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Lin   445 NKLGYAKH-LNHGK 457
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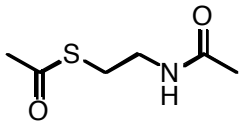
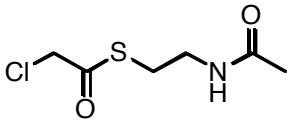
Figure S13. Amino acid sequence alignment of the *P. furiosus* bifunctional enzyme with other related enzymes. Pfu, *Pyrococcus furiosus* (NC_003413); Spn, *Streptococcus pneumoniae* (Accession no. 1G97_A); Bap, *Buchnera aphidicola* (Accession no. P57139); Fnu, *Fusobacterium nucleatatum* (NC_003454.1); Hpy, *Helicobacter pylori* (NC_000915.1); Pmu, *Pasteurella multocida* (NC_ 002663.1); Aae, *Aquifax aeolicus* (NC_000918.1); Lin, *Listeria innocua* (NC_003212.1). Amino acids are conserved in at least five organisms or more are bold marked.

General synthetic methods. All reagents were purchased from Aldrich (Milwaukee, WI) and used as received. Dichloromethane was filtered over alumin in a solvent purification system to eliminate water. Unless otherwise indicated, reactions were carried out under nitrogen atmosphere in oven-dried glassware. Reactions were monitored by thin layer chromatography (TLC) on EM Science 250 μm precoated silica gel plates (60 F_{254}). TLC plates were visualized with potassium permanganate stain. Purification of products was performed by flash column chromatography on Selecto Scientific silica gel (32-63) and HPLC grade solvents. ^{1}H and ^{13}C NMR were taken on a Varian VXR-300 instrument.



8

N,S-Diacetylcysteamine is commercially available from Aldrich.



9

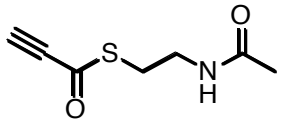
Synthesis of N-acetyl-*S*-chloroacetylcysteamine.

N-acetylcysteamine (342 mg, 2.87 mmol) and chloroacetic acid (247 mg, 2.61 mmol) were dissolved in dichloromethane (4 mL) and cooled to 0 °C. *N*,*N*-Dimethylaminopyridine (DMAP, 70 mg, 0.57 mmol) was added to the above solution followed by 1,3-dicyclohexylcarbodiimide (DCC, 591 mg, 2.87 mmol) and the reaction was allowed to warm to ambient temperature and stirred for 10 h. Hexane (10 mL) was added to the reaction mixture and the mixture was filtered over Celite. The solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel using 40% ethylacetate/hexane to yield **9** as a white solid (440 mg, 2.25 mmol, 86%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.94 (s, 3H), 3.07 (t, 2H, J = 6.3 Hz), 3.41 (m, 2H), 4.18 (s, 2H), 6.23 (br s, 1H).

^{13}C NMR (300 MHz, $CDCl_3$): δ 23.4, 29.4, 39.2, 48.2, 170.8, 194.6.

EIMS (70 eV) *m/z*: M^+ 196.



10

Synthesis of *N*-acetyl-*S*-propionylcysteamine.

N-acetylcysteamine (375 mg, 3.15 mmol) and propiolic acid (200 mg, 2.86 mmol) were dissolved in dichloromethane (6 mL) and cooled to 0 °C. DMAP (35 mg, 0.286 mmol) was added to the above solution followed by DCC (649 mg, 3.15 mmol) and the reaction was allowed to warm to ambient temperature and stirred for 12 h. Hexane (10 mL) was added to the reaction mixture and filtered over Celite. The solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel using 40% ethylacetate-hexane to yield **10** as a yellow oil (352 mg, 2.06, 72%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.97 (s, 3H), 3.14 (t, 2H, J = 6.3 Hz), 3.40 (s, 1H), 3.45 (m, 2H), 5.95 (br s, 1H).

^{13}C NMR (300 MHz, $CDCl_3$): δ 23.5, 29.7, 39.3, 79.4, 80.5, 170.6, 176.2.

EIMS (70 eV) *m/z*: M^+ 171.

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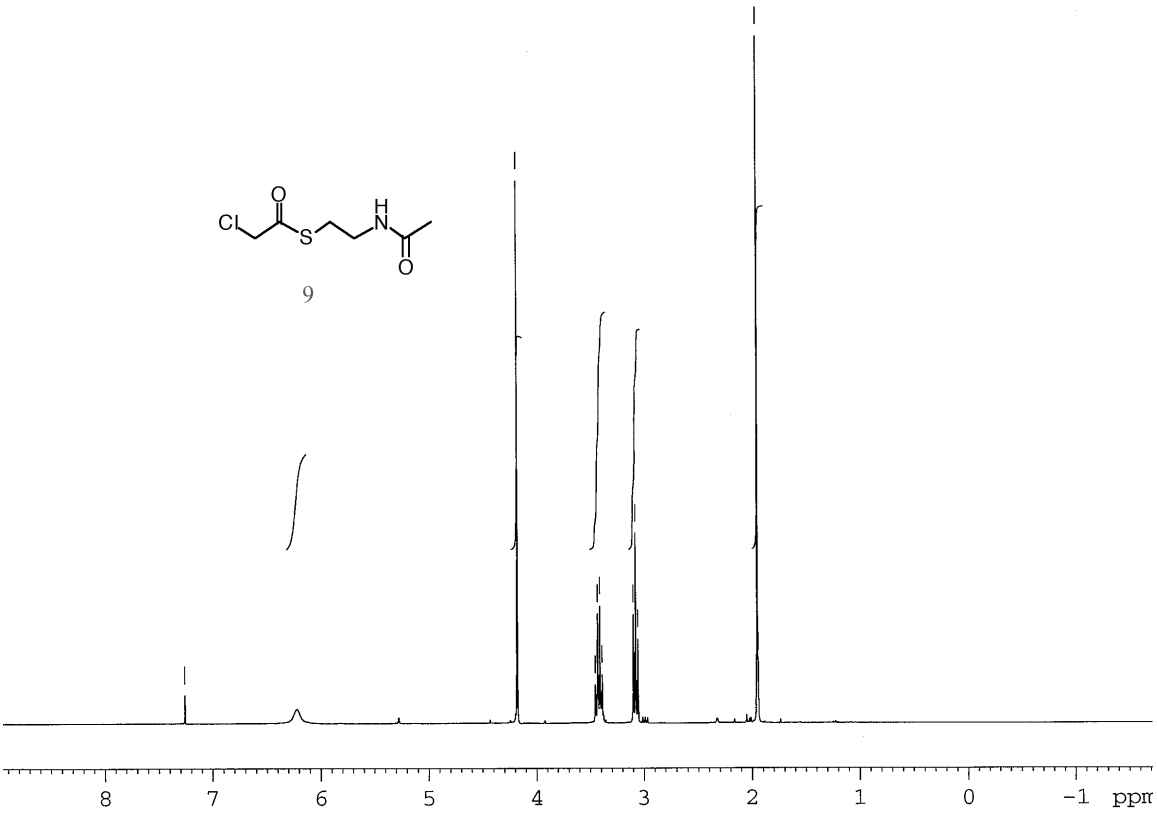
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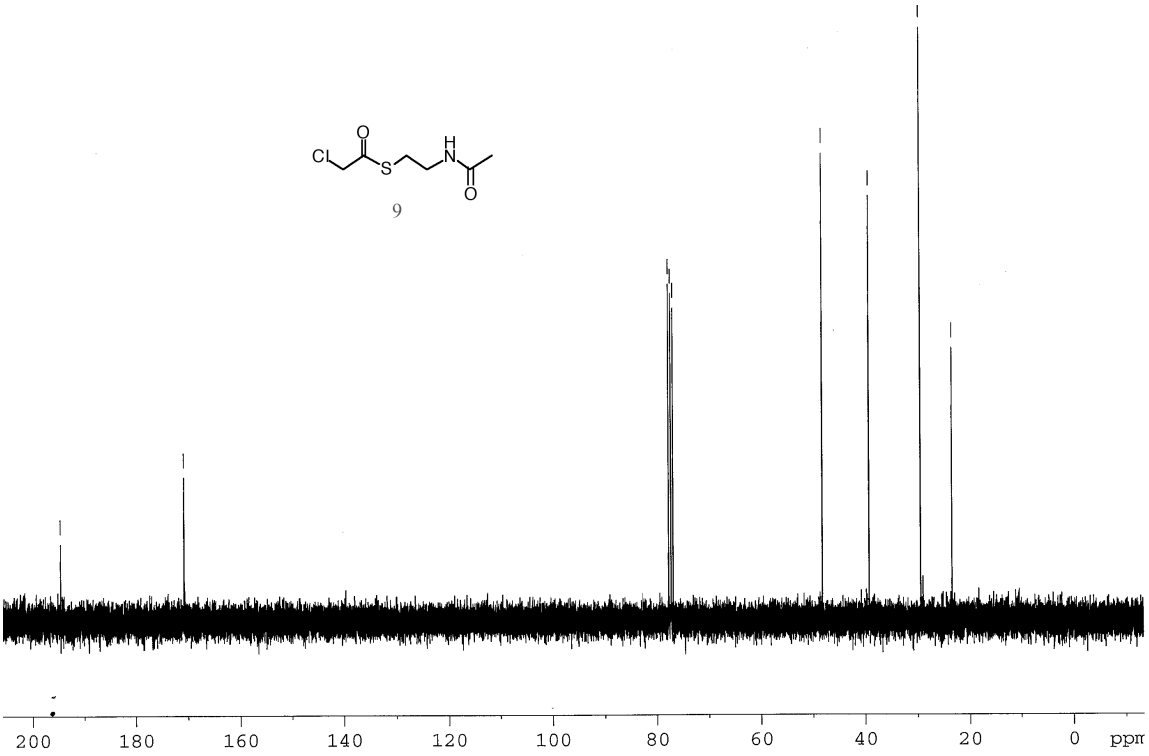
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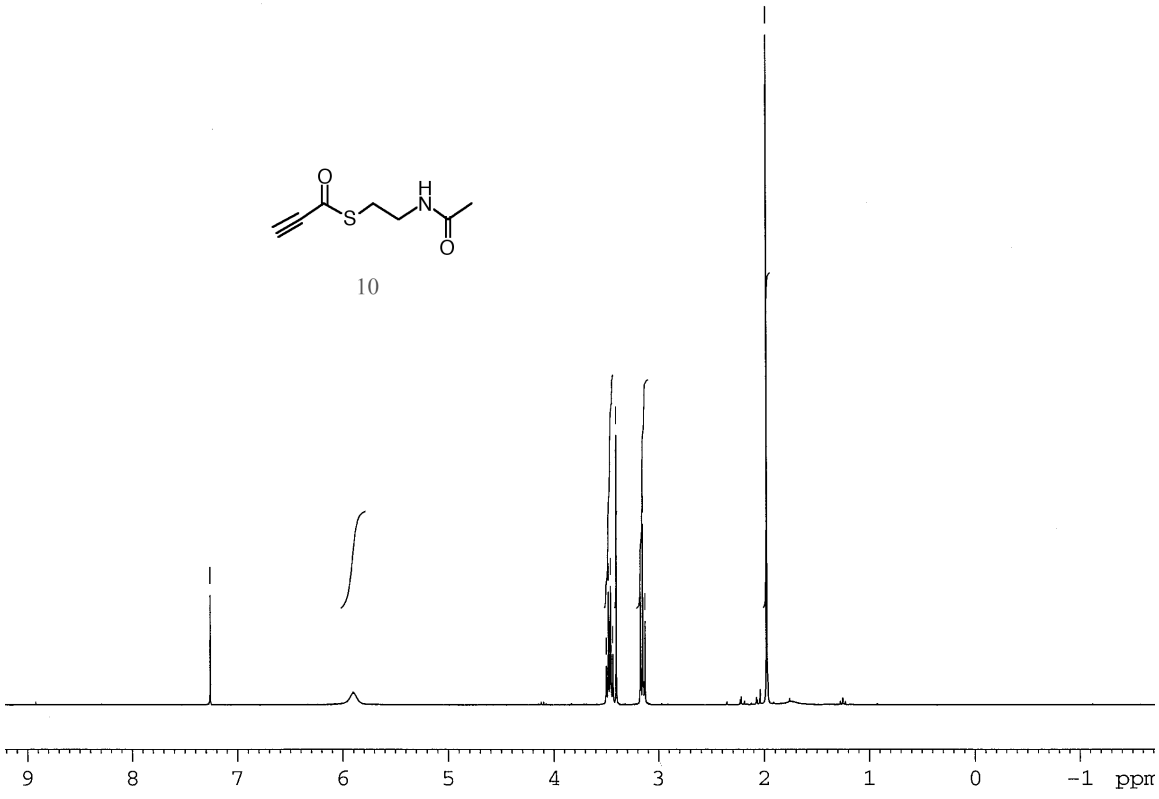
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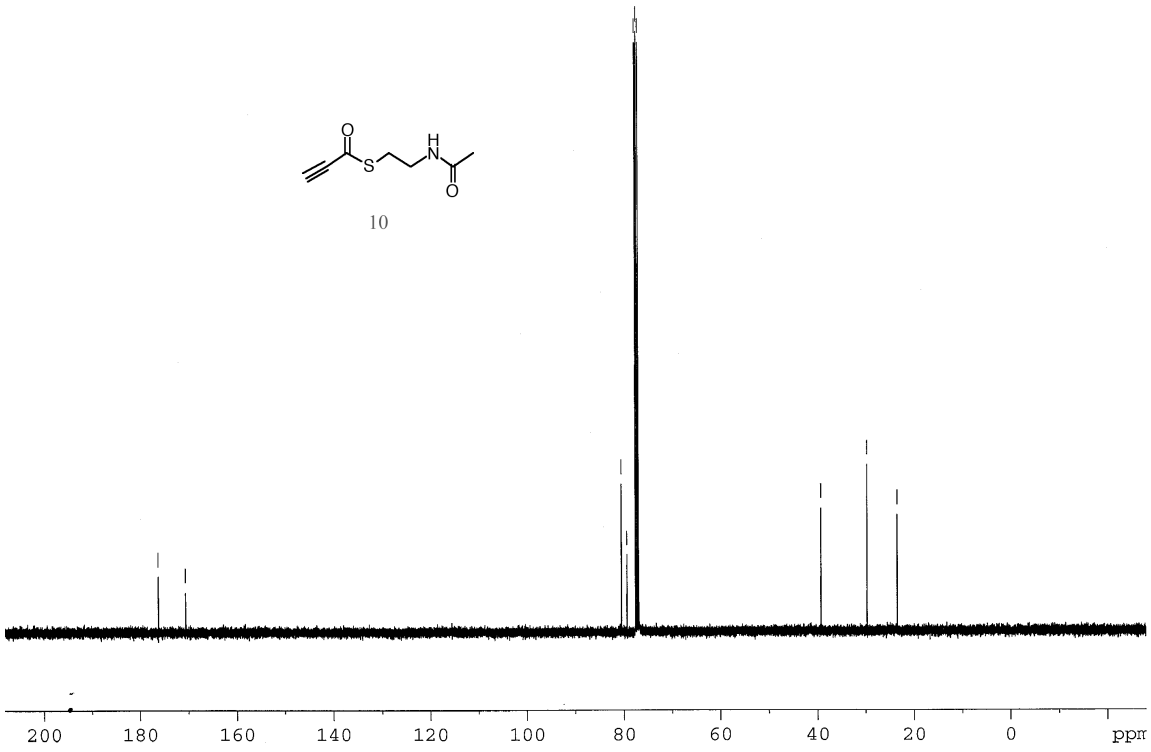
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