Kinetic Characterization of Methylthio-D-ribose-1-Phosphate Isomerase

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Abstract (max 250 words)

Methylthio-D-ribose-1-phosphate (MTR1P) isomerase (MtnA) catalyzes the reversible isomerization of the aldose MTR1P into the ketose methylthio-D-ribulose 1-phosphate. It serves as a member of the methionine salvage pathway that many organisms require for recycling methylthio-D-adenosine, a byproduct of *S*-adenosylmethionine metabolism, back to methionine. MtnA is of mechanistic interest because unlike most other aldose–ketose isomerases, its substrate exists as an anomeric phosphate ester and therefore cannot equilibrate with a ring-opened aldehyde that is otherwise required to promote isomerization. To investigate the mechanism of MtnA, it is necessary to establish reliable methods for determining the concentration of MTR1P and to measure enzyme activity in a continuous assay. This chapter describes several such protocols needed to perform steady-state kinetics measurements. It additionally outlines the preparation of [^{32}P]MTR1P, its use in radioactively labeling the enzyme, and the characterization of the resulting phosphoryl adduct.

Keywords

Methionine salvage; Isomerase; Methylthio-D-ribose 1-phosphate; Methylthio-D-ribose-1-phosphate isomerase; Kinetics; Covalent adduct; Radioactive labeling; *o*-Phenylenediamine

1. Introduction

Methionine is an essential amino acid used in a variety of biological processes outside its role as one of the twenty canonical amino acids in proteins. Once it is incorporated into *S*-adenosyl-L-methionine (SAM), the reactive sulfonium group enables several reactions including methylation of DNA, ribosomal RNA, and phospholipids; radical reactions catalyzed by iron-sulfur enzymes; and aminopropyl transfer as part of polyamine biosynthesis. This last process leaves the sulfide-containing 5′-methylthio-D-adenosine (MTA) as a byproduct. Because reduced sulfur species such as this are energetically costly to biosynthesize from inorganic sources (Auger et al., 2002; Kertesz, 2000; Leustek & Saito, 1999; Sekowska et al., 2000), many organisms depend on the methionine salvage pathway to recycle MTA back to methionine (Scheme 1).

[Insert Scheme 1 here]

Despite the ubiquity of the methionine salvage pathway across several kingdoms, there are several reactions for which mechanistic details are lacking. In particular, the interconversion of methylthio-D-ribose 1-phosphate (MTR1P) and methylthio-D-ribulose 1-phosphate (MTRu1P) catalyzed by MTR1P isomerase (MtnA) represents a unique aldose–ketose isomerization, which as the name implies is the interconversion of an aldose—a carbohydrate with an aldehyde group in its ring-opened form—and a ketose—a carbohydrate with a ketone group in its ring-opened form, with the carbonyl at the second carbon. This process formally involves reduction of the aldehyde and oxidation of the adjacent secondary alcohol.

Most enzymes that catalyze such an interconversion utilize one of two canonical mechanisms: (1) a proton-transfer mechanism or (2) a hydride-transfer mechanism (Scheme 2).

[Insert Scheme 2 here]

The proton-transfer mechanism, the more common of the two, features two successive acid–base reactions. In the first step, the presence of the carbonyl acidifies the proton on the α-carbon, such that it is removed by a neighboring Brønsted base, producing a resonance-stabilized *cis*-enediolate intermediate. In the second step, the proton is transferred to the other unsaturated carbon of the intermediate. In the hydride-transfer mechanism, a metal typically assists by coordinating the two neighboring oxygen atoms, which lowers the pK_a of the alcohol. After deprotonation, the metal then serves to increase the electrophilicity of the carbonyl, and together with the adjacent alkoxide, this promotes a [1,2] hydride shift.

A keen eye will notice that MTR1P contains a phosphate ester at the anomeric carbon. Despite its classification as an aldose, the absence of a hemiacetal precludes equilibration with an open-chain aldehyde, without which neither of the canonical mechanisms is possible. The fact that MtnA must utilize a distinct mechanism inspired our initial investigations into this enzyme, which involved characterization of its kinetic properties and evaluating the possibility of covalent catalysis via phosphoryl transfer to an active-site residue (Veeramachineni et al., 2022). The following sections describe several methods we explored for quantification of the substrate and the establishment of a continuous assay for measuring steady-state kinetics. Additionally detailed are the preparation of ^{32}P-labeled substrate, its covalent labeling of MtnA, and the investigation of the stability of the resulting adduct.

2. Quantification of MTR1P and steady-state kinetics of MtnA

2.1. Expression and purification of MtnA, MtnB, and MtnK

This section describes the preparation of *Bacillus subtilis* MtnA and two additional enzymes from the methionine salvage pathway that are fundamental to studying this enzyme. MtnK is

required for the last step of the synthesis of the substrate MTR1P (see Section 2.2.5), and MtnB serves as a coupling enzyme in two of the assays described in Section 2.3.

2.1.1. Materials and equipment

- pET15b plasmids containing the genes encoding N-terminal His_6-tagged *B. subtilis* MtnA, MtnB, and MtnK. The plasmids used in this protocol were obtained from Prof. John Gerlt (University of Illinois) (Imker et al., 2007), but they can also be constructed by cloning or gene synthesis.
- *Escherichia coli* BL21 (DE3) chemically competent cells (Invitrogen, One Shot)
- Luria–Bertani (LB) broth, Miller
- Ampicillin, 100 mg/mL stock solution
- Optional for preparing a glycerol stock:
  - 50% (v/v) aqueous glycerol (≥ 0.5 mL)
  - Cryovial or similar tube
  - Cryogen (dry ice/acetone or liquid nitrogen)
- Isopropyl β-thio-D-galactopyranoside (IPTG), 0.5 M stock solution
- 1 M Tris-HCl (pH 7.9) stock solution
- 500 mM $MgCl_2$ stock solution
- Protease inhibitor cocktail tablet (cOmplete, Roche Diagnostics)
- DNase I
- Binding buffer (40 mM Tris-HCl, 500 mM NaCl, 5 mM $MgCl_2$, adjusted to pH 7.8 at 25 ºC)

- Elution buffer (40 mM Tris-HCl, 500 mM imidazole, 500 mM NaCl, 5 mM $MgCl_2$, adjusted to pH 7.8 at 25 ºC)
- Incubator shaker
- Erlenmeyer flasks, 2 L
- Spectrophotometer
- Disposable cuvettes, 1 mL
- Centrifuge, large capacity (Sorvall RC-3C Plus or similar)
- Centrifuge, medium capacity (Eppendorf 5810 R or similar)
- Centrifuge bottles
- M-110L Microfluidizer processor (Microfluidics)
- Syringe filter, 0.45 μm (Corning)
- ÄKTA FPLC system (GE Healthcare)
- HisPrep FF 16/10 column (GE Healthcare)
- Stirred cell (Amicon 8400)
- Regenerated cellulose membrane discs, 10 kDa cutoff (Millipore Ultracel, part PLGC07610 or similar)
- Dialysis tubing (Fisherbrand 12–14 kDa cutoff range, part 21-152-15 or similar)
- Beaker (1 L)
- Storage buffer (50 mM Tris-HCl, 100 mM NaCl, adjusted to pH 7.8 at 25 ºC)
- Shallow Dewar flask
- Liquid nitrogen
- Cryovials or similar tubes

2.1.2. Protocol

(1) *E. coli* cells are transformed with each plasmid following the manufacturer's protocols.

(2) Overnight cultures are prepared by inoculating LB broth (10 mL per 1 L of expression culture) containing 100 μg/mL ampicillin with transformed cells and allowing to grow overnight at 37 ºC with shaking (150 rpm).

Optional: To save a glycerol stock, equal volumes (e.g., 500 μL) of overnight culture and 50% (v/v) glycerol are mixed in a cryovial. The glycerol stock is flash-frozen in a dry ice bath or liquid nitrogen and stored at –80 ºC.

(3) In each 2-L Erlenmeyer flask, 1 L of LB broth containing 100 mg ampicillin is prepared, and this is inoculated with 10 mL of overnight culture. Cultures are shaken (150 rpm) at 37 ºC and allowed to grow up to 4 h until OD_{600} reaches 0.6.

(4) The incubator is reduced to 25 ºC, and expression is induced overnight (~16 h) by adding IPTG to a final concentration of 0.5 mM.

(5) The cells are harvested by centrifugation at 4000×*g* for 45 min at 4 ºC.

Pause Point: The cell pellets may be stored in a –20 ºC freezer for brief (1–2 days) storage or a –80 ºC freezer for longer-term storage.

(6) The cell pellet is resuspended in a minimum volume of 20 mM Tris-HCl (pH 7.9) containing 5 mM $MgCl_2$, one protease inhibitor cocktail tablet (per 10 L of culture), and 5–10 mg Dnase I and lysed by passage through a M-100L Microfluidizer. Other devices or methods (e.g., French press, sonication) commonly used for lysing bacteria may be substituted.

(7) The cell lysate is centrifuged at ≥13,000×*g* for 45 min at 4 ºC.

(8) The supernatant is passed through a 45-μm syringe filter and applied to a Ni^{2+}-charged HisPrep FF column, previously equilibrated with binding buffer, using an FPLC system.

(9) The column is washed with 5 column volumes of 5% elution buffer, 95% binding buffer, then eluted with a linear gradient 5–100% elution buffer over 9 column volumes.

(10) The identity and purity of protein are confirmed by SDS-PAGE under reducing conditions.

(11) Fractions containing the desired pure protein are pooled and concentrated through a cellulose membrane within an Amicon ultrafiltration device.

(12) The concentrate is transferred to dialysis tubing and dialyzed against 1 L storage buffer following the manufacturer's protocol.

(13) The concentration of protein is measured spectrophotometrically using ε_{280} = 26,930, 39,880, and 27,960 M^{-1} cm^{-1} for MtnA, MtnK, and MtnB, respectively, as calculated using the ProtParam utility of Expasy (Wilkins et al., 1999).

(14) Protein is flash-frozen as ~50 μL balls by dropping from a pipette directly into liquid nitrogen, transferring to cryovials, and stored at –80 ºC.

2.2. Synthesis of MTR1P and [^{32}P]MTR1P

As of the time of the writing of this chapter, MTR1P has not been available commercially. The following procedures detail the chemical synthesis of the immediate precursor, methylthio-D-ribose (MTR), and the enzymatic phosphorylation that provides access to gram-scale quantities of MTR1P and to [^{32}P]MTR1P (Scheme 3).

[Insert Scheme 3]

2.2.1. Synthesis of methyl 2,3-*O*-isopropylidene-5-*O*-p-tolylsulfonyl-β-D-ribofuranoside (2)

2.2.1.1. Materials

- Pyridine
- Calcium hydride
- Molecular sieves
- *p*-Toulenesulfonyl chloride (ThermoFisher Scientific)
- Methyl 2,3-*O*-isopropylidene-β-D-ribofuranoside (**1**; Combi-Blocks)
- Petroleum ether
- Ethyl acetate

2.2.1.2. Protocol

Note: Familiarity with standard synthetic organic chemistry techniques, glassware, and apparatus is assumed. The synthesis is carried out with slight modifications to a previously known protocol (Ferreira et al., 2010; Levene & Stiller, 1933).

(1) Pyridine is dried by treating 150 mL pyridine with 5 g of calcium hydride overnight, followed by distillation under argon directly onto molecular sieves after discarding the first 10 mL.

(2) *p*-Toluenesulfonyl chloride (1.4 g, 7.50 mmol) is added in portions to a solution of methyl 2,3-*O*-isopropylidene-β-D-ribofuranoside (**1**; 1.0 g, 5.00 mmol) in dry pyridine (3 mL) at 0 ºC. The reaction is monitored by TLC (R_f = 0.25, petroleum ether/ethyl acetate, 9:1 by volume).

(3) After 3 h, the reaction mixture is poured onto crushed ice (10 mL) with vigorous stirring**.**

(4) The resultant white precipitate is filtered and washed with ice water. After air drying, the product is obtained as a white amorphous solid. Spectral data can be found in the literature (Ferreira et al., 2010).

2.2.2 Synthesis of methyl 2,3-*O*-isopropylidene-5-methylthio-β-D-ribofuranoside (3)

2.2.2.1 Materials

- Methyl 2,3-*O*-isopropylidene-5-*O*-p-tolylsulfonyl-β-D-ribofuranoside (**2**; see Section 2.3.1)
- Sodium thiomethoxide (Acros Organics)
- Dimethyl formamide (DMF), anhydrous
- Toluene
- Water
- Brine

2.2.2.2 Protocol

Note: The synthesis is carried out with slight modifications to a previously known protocol (Overend & Parker, 1951).

(1) The tosyl group on the starting material is displaced by thiomethoxide by refluxing methyl 2,3-*O*-isopropylidene-5-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside (**2**; 500 mg, 1.4 mmol) and 4 equivalents of sodium thiomethoxide (400 mg, 5.6 mmol) in DMF overnight.

(2) The reaction mixture is diluted with 10 volumes of toluene and extracted sequentially against equal volumes of water and brine.

(3) The toluene layer is dried over anhydrous sodium sulfate prior to rotary evaporation under reduced pressure. Spectral data can be found in the literature (Myers & Abeles, 1990).

2.2.3 Synthesis of 5-deoxy-5-methylthio-D-ribose (MTR)

2.2.3.1 Materials

- Methyl 2,3-*O*-isopropylidene-5-methylthio-β-D-ribofuranoside (**3**; see Section 2.3.2)
- 1 N sulfuric acid
- 1 M barium hydroxide
- Diatomaceous earth (standard super-Cel NF, Acros Organics)

2.2.3.2 Protocol

(1) Methyl 5-deoxy-2,3-*O*-isopropylidene-5-methylthio-β-D-ribofuranoside (**3**) is refluxed in 1 N H_2SO_4 (10 mL 1 N H_2SO_4 for 100 mg starting material) for 5 h and stirred overnight at room temperature.

(2) The reaction mixture is neutralized with 1 M barium hydroxide followed by centrifugation at 13,000 rpm for 25 min and filtration through a sintered glass funnel (fine porosity) containing a thin layer of acid-washed diatomaceous earth to remove barium sulfate.

(3) Rotary evaporation under reduced pressure yields MTR, which is used without additional purification in the next step. Spectral data can be found in the literature (Myers & Abeles, 1990).

2.2.4 Alternative synthesis of MTR

Ashida et al. prepared MTR by hydrolysis of *S*-adeonsylmethionine with HCl (Ashida et al., 2003). This method is much simpler than the combined steps described in Sections 2.3.2 and 2.3.3, but because of the high cost of the starting material (commonly over $1,000 per gram), it is best suited for milligram-scale preparations.

2.2.5 Synthesis of 5-deoxy-5-methylthio-D-ribose 1-phosphate (MTR1P)

2.2.5.1 Materials and Equipment

- MTR (see Section 2.3.3)
- MtnK (see Section 2.2)
- ATP
- 500 mM $MgCl_2$ stock solution
- 500 mM dithiothreitol (DTT) stock solution
- 1 M Tris-HCl (pH 7.9)
- 100 mM KOH
- 100 mM hydrochloric acid
- Diatomaceous earth (standard super-Cel NF, Acros Organics)
- Triethylamine
- Dry ice
- 1-L filter flask with rubber stopper
- 1-L beaker or graduated cylinder
- Glass Pasteur pipette
- Rubber tubing
- HPLC system

- Phenomenex Luna® 5 μm C18(2) LC column (250 × 21.2 mm)
- 50% (v/v) aqueous methanol
- Econo-Pac chromatography column (Bio-Rad)
- Amberlite IR-120 resin, Na^+ form

2.2.5.2 Protocol

Note: The enzymatic synthesis of MTR1P from MTR is carried out using MtnK and ATP according to Scheme 2 (Imker et al., 2007).

(1) To a reaction mixture containing 40 mM MTR, 45 mM ATP, 5 mM $MgCl_2$, 5 mM DTT, and 20 mM Tris-HCl (pH 7.9) is added MtnK (5 μM final). The reaction is allowed to proceed at 37 ºC, and the pH is periodically adjusted back to 7.9 using 100 mM KOH.

(2) After completion, the protein is precipitated by acidifying the medium to pH 4 using 100 mM HCl.

(3) The suspension is centrifuged, and the supernatant is vacuum filtered over acid-washed diatomaceous earth before being evaporated to dryness.

Pause point: The crude MTR1P can be stored briefly (1–2 days) at 4 ºC. Triethylammonium bicarbonate (TEAB) buffer needed in step 5 can be prepared during this time as follows.

(4) In a fume hood, 13.9 mL (100 mmol) of triethylamine is diluted with ~950 mL of water in a beaker or graduated cylinder equipped with a stir bar. Into one end of a suitable length of rubber tubing is inserted the broad end of a glass Pasteur pipette. The other end of tubing is attached to the side arm of a 1-L vacuum flask, which is charged with dry ice and sealed with a rubber stopper. The Pasteur pipette is

inserted into the stirring triethylamine/water mixture, and carbon dioxide is allowed to bubble until the buffer reaches pH 7.5. The TEAB buffer is diluted to 1.00 L with water and stored in a tightly closed bottle at 4 ºC for up to 2 months.

(5) The crude MTR1P is dissolved in 2 mL of 100 mM TEAB (pH 7.5) and purified by reversed-phase HPLC in 500-µL portions using a Phenomenex Luna® 5 µm C18(2) LC column (250 × 21.2 mm), equilibrated with 97% solvent A (100 mM TEAB) and 3% solvent B (50% aqueous methanol, v/v). After injection, a gradient program is applied as follows: 0–30 min, isocratic 3% B; 30–40 min, linear gradient 3–20% B; 40–50 min, linear gradient 20–50% B; 50–60 min, linear gradient 50–100% B; hold at 100% A. Fractions are collected at an interval of 1 min at 8 mL/min flow rate, and those containing MTR1P (see Section 2.4 for detection) are pooled and evaporated to dryness multiple times from water to remove excess TEAB, confirmed by ^{1}H NMR.

(6) The solid residue is redissolved in 1 mL of water, and the solution passed by gravity over an Econo-Pac chromatography column containing 10 mL of cation exchange resin (Amberlite IR-120 resin, Na^+ form) to exchange triethylammonium with Na^+ as the counterion. The column is washed with 5 mL of water, and the pooled aqueous eluent is concentrated to dryness by rotary evaporation.

(7) MTR1P is stored at –20 ºC either as a solid or as a stock solution of known concentration (see Section 2.4 for quantification).

2.3. Quantification of MTR1P

The determination of MTR1P concentration is important for enzyme kinetics and other quantitative applications. We investigated several methods, some acting indirectly by quantifying

the phosphate present in MTR1P, others functioning more specifically on the product of the MtnA-catalyzed reaction, MTRu1P, and one measuring MTR1P directly by ^{1}H-NMR spectroscopy. Each method has its advantages and disadvantages, which will be summarized at the end of this section.

2.3.1. Malachite green assay

This assay is best used as a qualitative test to detect the presence of MTR1P due to its high sensitivity and ease of preparation and detection. First introduced by Baykov et al., this procedure uses the complex formed among the basic dye malachite green, ammonium molybdate, and inorganic phosphate under acidic conditions (Baikov et al., 1988). The formation of the green molybdophosphoric acid complex measured at 630 nm is directly related to the free inorganic phosphate concentration. Whereas most phosphate esters are stable in the presence of the reagent, the phosphate group of MTR1P is attached by a glycosidic bond and is therefore readily hydrolyzed, liberating an equivalent of inorganic phosphate. Quantification is possible with the use of a calibration curve, yet this assay does not distinguish between phosphate obtained from MTR1P and inorganic phosphate that may have already been present in solution. Therefore, this assay is recommended for qualitative purposes (e.g., identification of MTR1P during purification).

2.3.1.1. Materials and equipment

- Sulfuric acid
- Malachite green
- Ammonium molybdate
- 11% (v/v) polysorbate 20 (Tween 20, BDH)
- 100 μM sodium phosphate, monobasic stock solution (if a standard curve is needed)

2.3.1.2. Protocol

(1) Concentrated sulfuric acid (60 mL) is added slowly to 300 mL of water. After cooling to room temperature, 0.44 g of malachite green is added, forming an orange solution.

Pause point: The orange dye solution can be stored at room temperature for at least one year. The following steps should be performed immediately before use.

(2) To 10 mL of dye solution is added 2.5 mL of 7.5% (w/v) ammonium molybdate and 0.2 mL of 11% polysorbate 20.

Optional: If the concentration of phosphate is to be quantified, a standard curve should be generated using a dilution series of phosphate standard covering the range of MTR1P concentrations to be tested (e.g., 1–10 μM).

(3) Mix one volume of color reagent with four volumes of each sample and allow to stand for 10 min. The presence of phosphate is indicated by a greenish blue color.

(4) For quantitative purposes, the absorbance is measured spectrophotometrically at 630 nm. The concentration of MTR1P is determined either by interpolation from the standard curve or from its slope (i.e., ε_{630}).

2.3.2. Purine nucleoside phosphate–xanthine oxidase (PNP-XO) assay

The purine nucleoside phosphorylase (PNP)-xanthine oxidase (XO) assay is a modification of the procedure by de Groot et al. (De Groot et al., 1985), which uses three enzymes to quantify phosphate esters such as MTR1P (Scheme 4). Alkaline phosphatase hydrolyzes MTR1P to liberate inorganic phosphate. PNP captures this phosphate by catalyzing the phosphorolysis of inosine to hypoxanthine and ribose 1-phosphate, which is effectively irreversible in the presence of excess inosine. Finally, XO oxidizes hypoxanthine to uric acid, which absorbs at 293 nm.

[Insert Scheme 4 here]

2.3.2.1. Materials and equipment

- Purine nucleoside phosphorylase, bacterial (MilliporeSigma) or human recombinant [expressed and purified as described in the literature (Murkin et al., 2007)]
- Xanthine oxidase, ammonium sulfate suspension (MilliporeSigma)
- Alkaline phosphatase, calf intestine (Roche Diagnostics)
- Alkaline phosphatase buffer (Roche Diagnostics)
- 1 M Tris-HCl (pH 7.5) stock solution
- 50 mM inosine stock solution
- 1 mM sodium phosphate, monobasic stock solution (if a standard curve is desired)
- Spectrophotometer
- Cuvettes

2.3.2.2. Protocol

(1) A known volume of MTR1P is hydrolyzed with alkaline phosphatase following the manufacturer's protocol. In brief, this involves dilution of MTR1P with the supplied alkaline phosphatase buffer followed by incubation with 1 unit of alkaline phosphatase at 37 ºC for 10 min.

(2) In a total volume of 1.00 mL in a cuvette, the phosphate-containing sample is mixed with 100 mM Tris-HCl (pH 7.5), 2.5 mM inosine, and 0.05 units of XO. A_{293} is recorded.

(3) PNP solution (0.5 units) is added, and once the absorbance reaches a plateau, A_{293} is again recorded. This absorbance is corrected for dilution by multiplying by the total volume in mL after addition of PNP.

(4) After calculating the change in A_{293}, the concentration of liberated phosphate is obtained by dividing by the extinction coefficient, ε_{293} = 12,900 M^{-1} cm^{-1} (Gordon & Ford, 1972).

2.3.3. Fuculose-1-phosphate aldolase (FucA) assay

The physiological reaction catalyzed by fuculose-1-phosphate aldolase (FucA) involves the aldol addition of dihydroxyacetone phosphate (DHAP) with L-lactate to yield L-fuculose 1-phosphate. The enzyme has been found to have broad enough specificity to accept a variety of aldehydes as alternatives to lactate that combine with DHAP to form carbohydrates with 3*R*,4*R* absolute configuration (i.e., D-erythro) (Fessner & Sinerius, 1994). One such product is D-ribulose 1-phosphate, which forms from glycolaldehyde and DHAP as substrates (Fessner et al., 1991). Based on this result, we had initially expected FucA to synthesize MTRu1P using DHAP and thioacetaldehyde. However, instead of favoring the synthetic direction as is typically the case for biological aldol reactions, the cleavage direction was favored. This prompted our investigation of FucA as a coupling enzyme to shuttle MTRu1P to DHAP, which could be reduced with NADH catalyzed by glycerol-3-phosphate dehydrogenase (Scheme 5).

[Insert Scheme 5 here]

2.3.3.1. Expression and purification of fuculose-1-phosphate aldolase

2.3.3.1.1. Materials and equipment

- Stab culture of *E. coli* strain HB101 cells transformed with pUC18-derived expression vector with gene encoding *E. coli* fuculose-1-phosphate aldolase (FucA) from Dr. Wolf-Dieter Fessner's laboratory (Techische Universitat, Darmstadt, Germany) (Fessner et al., 1991).
- Luria–Burtani (LB) Miller agar plates containing 100 µg/mL ampicillin

- Luria–Bertani (LB) broth, Miller
- Ampicillin, 100 mg/mL stock solution
- Isopropyl β-thio-D-galactopyranoside (IPTG), 0.5 M stock solution
- 1 M Tris-HCl (pH 7.9) stock solution
- 50 mM $ZnCl_2$ stock solution
- 500 mM β-mercaptoethanol (BME) stock solution
- Protease inhibitor cocktail tablet (cOmplete, Roche Diagnostics)
- Dnase I
- Binding buffer (20 mM Tris-HCl, 0.5 mM $ZnCl_2$, 10 mM BME, adjusted to pH 7.5 at 25 ºC)
- Elution buffer (20 mM Tris-HCl, 500 mM NaCl, 0.5 mM $ZnCl_2$, 10 mM β-BME, adjusted to pH 7.5 at 25 ºC)
- Incubator shaker
- Erlenmeyer flasks, 2 L
- Spectrophotometer
- Disposable cuvettes, 1 mL
- Centrifuge, large capacity (Sorvall RC-3C Plus or similar)
- Centrifuge, medium capacity (Eppendorf 5810 R or similar)
- Centrifuge bottles
- M-110L Microfluidizer processor (Microfluidics)
- Syringe filter, 0.45 μm (Corning)
- ÄKTA FPLC system (GE Healthcare)

- Q-Sepharose FF 26/13 column (GE Healthcare)
- HiLoad 26/600 Superdex 75 (GE Healthcare; column volume = 320 mL)
- Stirred cell (Amicon 8400)
- Regenerated cellulose membrane discs, 3 kDa cutoff (Millipore Ultracel or similar)
- Shallow Dewar flask
- Liquid nitrogen
- Cryovials or similar tubes

2.3.3.1.2. Protocol

(1) An LB agar plate containing 100 mg/L ampicillin is streaked with *E. coli* cells previously transformed with the plasmid encoding FucA and incubated overnight at 37 ºC.

(2) A single colony is used to inoculate a 100-mL sterile starter culture containing LB Miller broth and 100 μg/mL ampicillin. The culture is incubated overnight with shaking at 37 ºC.

(3) In each 2-L Erlenmeyer flask, 1 L of LB broth containing 100 mg ampicillin is prepared, and this is inoculated with 10 mL of overnight culture. Cultures are shaken (150 rpm) at 37 ºC and allowed to grow up to 4 h until OD_{600} reaches 0.6.

(4) Expression is induced at 37 ºC for 4 h by adding IPTG to a final concentration of 0.5 mM.

(5) The cells are harvested by centrifugation at 4000×*g* for 45 min at 4 ºC.

Pause Point: The cell pellets may be stored in a –20 ºC freezer for brief (1–2 days) storage or a –80 ºC freezer for longer-term storage.

(6) The cell pellet is resuspended in a minimum volume of binding buffer containing one protease inhibitor cocktail tablet (per 10 L of culture) and 5–10 mg Dnase I and lysed by passage through a M-100L Microfluidizer. Other devices or methods (e.g., French press, sonication) commonly used for lysing bacteria may be substituted.

(7) The cell lysate is centrifuged at ≥13,000×*g* for 45 min at 4 ºC.

(8) The supernatant is passed through a 45-µm syringe filter and applied to a Q-Sepharose FF 26/13 column, previously equilibrated with binding buffer, using an FPLC system.

(9) The column is washed with 2 column volumes (140 mL) of binding buffer followed by elution with a linear 0–60% gradient (350 mL; 5 column volumes) against elution buffer.

(10) The identity and purity of protein are confirmed by SDS-PAGE under reducing conditions.

(11) Fractions containing the desired pure protein (~24 kDa) are pooled and concentrated to 15 mL through a cellulose membrane (3 kDa cutoff) within an Amicon ultrafiltration device.

Pause point: The ion-exchange purified protein can be stored briefly (1–2 days) at 4 ºC before performing size-exclusion chromatography.

(12) The concentrated protein is applied to a HiLoad 26/600 Superdex 75 (column volume = 320 mL), previously equilibrated with binding buffer. The protein is eluted with binding buffer (1–2 mL/min) with monitoring at 280 nm.

(13) Fractions corresponding to the major peak are pooled. The protein concentration is determined spectrophotometrically using ε_{280} = 21,430 M^{-1} cm^{-1} as calculated using the ProtParam utility of Expasy (Wilkins et al., 1999).

(14) Protein is flash-frozen as ~50 µL balls by dropping from a pipette directly into liquid nitrogen, transferring to cryovials, and stored at –80 ºC.

2.3.3.2. Fuculose-1-phosphate aldolase (FucA) assay

2.3.3.2.1. Materials and equipment

- 1 M Tris-HCl (pH 7.5) stock solution
- 100 mM EDTA stock solution
- 500 mM DTT stock solution
- 20 mM NADH stock solution
- FucA (see Section 2.4.3.1)
- Glycerol-3-phosphate dehydrogenase, rabbit muscle (Roche) or human recombinant (gifted by Prof. John Richard, University at Buffalo) (He et al., 2018)

2.3.3.2.2. Protocol

(1) In a total volume of 1.00 mL in a cuvette, MTR1P (10–100 µM is recommended) is mixed with 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 200 µM NADH, 1 µM glycerol-3-phosphate dehydrogenase, and 1.5 µM FucA. A_{340} is monitored until a stable value is obtained, which is recorded.

(2) MtnA (0.5 μM final) is added, and once the absorbance stops decreasing, A_{340} is again recorded. This absorbance is corrected for dilution by multiplying by the total volume in mL after addition of MtnA. $|\Delta A_{340}|_{sample}$ is calculated as the absolute value of the difference between the A_{340} readings.

(3) $|\Delta A_{340}|_{bg}$, reflecting any background changes, is measured by repeating steps 1 and 2 for a control sample lacking MTR1P.

(4) The concentration of NADH consumed, which is equal to the concentration of MTR1P, is obtained by subtracting $|\Delta A_{340}|_{bg}$ from $|\Delta A_{340}|_{sample}$ and then dividing by the extinction coefficient, ε_{340} = 6,220 M^{-1} cm^{-1}.

2.3.4. *o*-Phenylenediamine (OPDA) assay

As part of the methionine salvage pathway, the product of MtnA-catalyzed isomerization, MTRu1P, is a substrate for MtnB, which catalyzes a dehydration reaction to form 2,3-diketo-5-methylthiopentyl 1-phosphate (DK-MTP 1-P) (Chen & Hartman, 1995). The diketone reacts with *o*-phenylenediamine (OPDA) to form a quinoxaline product, which has an absorbance maximum at 320 nm (Scheme 6) (Ashida et al., 2003).

[Insert Scheme 6 here]

2.3.4.1. Materials and equipment

- 10 μM MtnA intermediate dilution (see Section 2.2)
- MtnB (see Section 2.2)
- 200 mM *o*-phenylenediamine (OPDA) stock solution
- 1 M triethanolamine-HCl (pH 7.5) stock solution
- 500 mM $MgCl_2$ stock solution
- 500 mM DTT stock solution

- Spectrophotometer
- Cuvettes

2.3.4.2. Protocol

(1) In a total volume of 1.00 mL in a cuvette at 25 ºC, MTR1P (10–100 μM recommended) is mixed with 50 mM triethanolamine-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 20 mM OPDA, and 10 μM MtnB. The baseline A_{320} is recorded.

(2) The reaction is initiated by the addition of MtnA (100 nM final), and once the absorbance reaches a plateau, A_{320} is again recorded. This absorbance is corrected for dilution by multiplying by the total volume in mL after addition of MtnA. The change in A_{320} is then calculated.

(3) To determine ε_{320}, the concentration of a stock solution of MTR1P was measured by ^{1}H-NMR spectroscopy (see Section 2.4.5), and steps 1–2 were repeated for 0–100 μM MTR1P. The slope of the standard curve (Figure 1) was found to be 7,500 and 6,470 M^{-1} cm^{-1} from measurements by two independent researchers, giving an average ε_{320} of 7,000 M^{-1} cm^{-1}.

[Insert Figure 1 here]

2.3.5. ^{1}H-NMR spectroscopy

This method of determining concentration is based on comparison of integrated peaks of the substrate and a standard (Amyes & Richard, 2007). Imidazole is a convenient internal standard for aqueous samples because it exhibits two singlets that are usually well separated from the signals of the analyte.

2.3.5.1. Materials and equipment

- Imidazole

- Deuterium oxide

2.3.5.2. Protocol

(1) A stock solution (10–100 mM) of imidazole is prepared by dissolving an accurately weighed sample in D_2O.

(2) An approximately equimolar sample of imidazole and MTR1P is prepared by mixing accurately known volumes of the imidazole stock solution and of a concentrated stock (> 10 mM) of MTR1P. The sample is diluted to 700 µL with D_2O and transferred to an NMR tube.

(3) A ^{1}H-NMR spectrum (Figure 2) is acquired using a relaxation delay between pulses of 120 s (> 8× T_1 of slowest relaxing proton) (Wasylishen & Cohen, 1974).

[Insert Figure 2 here]

(4) The mole ratio is calculated by comparing the integral of the C-4,5 signal (δ7.1 ppm) from imidazole, divided by 2, with the sum of the integrals of all MTR1P signals, divided by 9. In the provided spectrum, the ratio is 0.69. The stock MTR1P concentration is calculated by multiplying the mole ratio by the concentration of imidazole in the sample and by the dilution factor for MTR1P.

2.3.6. Advantages and disadvantages

Assay	Advantages	Disadvantages
Malachite green	• Highest sensitivity (nanomolar range) • Inexpensive • Provides visible color change, making it convenient for qualitative identification.	• Cannot differentiate between free phosphate and acid-sensitive phosphate esters (e.g., MTR1P) • Requires a standard curve for quantitative applications • Active reagent must be prepared freshly
PNP-XO	• Mild conditions • Moderate sensitivity	• Requires three auxiliary enzymes

	• Can differentiate between free phosphate and MTR1P	• Requires hydrolysis of MTR1P before assay
FucA	• MTR1P can be assayed directly without pretreatment • Continuous measurements possible (best at high MTR1P concentrations)	• Requires two auxiliary enzymes • Lowest sensitivity among colorimetric assays • Considerable lag phase, so not recommended for kinetics with low MTR1P concentrations
OPDA	• Only one auxiliary enzyme required • Continuous measurements possible over a broad concentration range (best for kinetics)	• A small lag phase exists (~5 min), which could limit kinetics measurements at short timescales.
^{1}H NMR	• Minimal materials required • Accurate even in the presence of inorganic phosphate	• Lowest sensitivity (millimolar range) • Requires access to NMR spectrometer

2.4. Steady-state kinetics measurements

Of the MTR1P quantification methods described in Section 2.4, the FucA and OPDA assays are capable of functioning continuously and could be considered for measurement of MtnA activity. Although the FucA system was found to convert MTR1P stoichiometrically to glycerol 3-phosphate, it was complicated by an extended lag phase at low substrate concentrations, even in the presence of extremely high concentrations of FucA. This problem is presumably due to an unfavorable K_m for MTRu1P, which is not the natural substrate, requiring significant accumulation for FucA to achieve a steady state. Because this assay is ineffective at lower MTR1P concentrations, it is not suitable for general use in steady-state kinetics measurements.

Although the OPDA assay also exhibits a lag, it is much shorter (~5 min). The limiting factor is apparently the formation of the quinoxaline; considering that OPDA is already present at 20 mM, this reaction cannot easily be accelerated by increasing its concentration. Nevertheless, increasing

the concentration of the coupling enzyme MtnB and/or reducing the concentration of MtnA are effective strategies for reducing the effects of the lag phase if needed. Thus, the OPDA assay is recommended for all steady-state kinetics experiments. One note of caution is that in our experience, OPDA is not stable at elevated temperature, causing background absorption during assays at 37 ºC. This problem did not occur over the course of >60 min at 25 ºC.

3. Detection and characterization of phosphorylated protein

3.1 Synthesis of [^{32}P]MTR1P

When this protocol was being developed, the primary challenge was to transfer as much of the γ-phosphoryl group from [γ-^{32}P]ATP onto MTR as possible. This process requires the use of ATP carrier to generate enough material for ease of handling in downstream applications. The timing of addition of carrier was found to be crucial for obtaining high specific radioactivity. When both labeled and unlabeled ATP were added at the same time, it was observed that less than 30% of the label ended up on MTR1P, while almost 40% was lost in the form of free phosphate (hydrolysis). It is possible that phosphoryl transfer was inefficient due to product inhibition by the relatively high concentration of ADP under these conditions. In contrast, when [γ-^{32}P]ATP was allowed to react for 30 min prior to adding the ATP carrier, 60–65% incorporation of label was obtained, with loss to hydrolysis reduced to 25–30% and about 5% remaining as unreacted ATP.

3.1.1 Materials and Equipment

- MTR (see Section 2.3.3)
- MtnK (see Section 2.2)
- 20 mM ATP stock solution
- [γ-^{32}P]ATP (3000 Ci/mmol, PerkinElmer)
- Liquid scintillation counter

- 500 mM $MgCl_2$ stock solution
- 500 mM DTT stock solution
- 1 M Tris-HCl (pH 7.9) stock solution
- Centrifugal filters (modified PES, 10 kDa cutoff, VWR)
- 100 mM TEAB (pH 7.5) (see Section 2.3.4.2, step 4)
- HPLC system
- Waters Delta-Pak C18, 15 µm, 100 Å LC column (300 × 3.9 mm)
- 50% (v/v) aqueous methanol
- Unlabeled MTR1P (see Section 2.3.4)

3.1.2 Protocol

Safety caution: For all procedures requiring the handling of ^{32}P, it has been assumed that the user has received appropriate safety training, and safety measures beyond those described here may be necessary; always obey local or state regulations. All manipulations should be performed behind plastic shields designed for containing radiation from ^{32}P decay. After each manipulation, a Geiger counter should be used to check for contamination of gloves and instruments. Several practice runs should be performed with a trace amount of ^{32}P before attempting a synthesis at higher specific activity. The HPLC column that is used for purification of the final product will become contaminated with ^{32}P, so it should be used for no other purpose until radioactivity is no longer detectable.

(1) A reaction mixture (1 mL total volume) is prepared containing 100 µM MTR, 500–750 µCi [γ-^{32}P]ATP, 5 mM $MgCl_2$, 5 mM DTT, and 20 mM Tris-HCl (pH 7.9). To this is added MtnK (10 µM final), and the reaction is allowed to proceed at 37 ºC for 30 min.

(2) ATP carrier (200 μM final) is added, and the reaction is allowed to incubate for an additional 90 min.

(3) The protein is removed using centrifugal filters (10 kDa cutoff).

(4) The labeled MTR1P is purified (repeated ~150 μL injections) by reversed-phase HPLC on an analytical C18 column equilibrated with solvent A (50 mM TEAB) and eluted at 2 mL/min with a gradient protocol using solvent B (50% aqueous methanol), as follows: 0–9 min, 100% A; 9–18 min, linear gradient 0–5% B; 18–22 min, linear gradient 5–50% B; 22–24 min, hold at 50% B; 24–30 min, hold at 100% A. Fractions are collected at 1 min intervals and checked for radioactivity on a scintillation counter (Figure 3). Fractions containing [^{32}P]MTR1P are pooled and evaporated to dryness under reduced pressure multiple times with water.

[Insert Figure 3 here]

(5) The radioactive residue is redissolved in 500 μL of water, and the concentration of MTR1P is adjusted to 100 μM with MTR1P carrier, as analyzed using the FucA or OPDA assay (see Sections 2.4.3 and 2.4.4). The [^{32}P]MTR1P solution is stored at –80 ºC and should be used as soon as possible to minimize radioactive decay. The typical final specific radioactivity of [^{32}P]MTR1P ranged (8.5–11.2) × 10^6 cpm/nmol.

3.2 Detection and isolation of radiolabeled MtnA

3.2.1 Qualitative method

The following method uses electrophoresis to separate protein from unbound substrate. Coomassie staining confirms the presence of protein, while phosphor imaging confirms the presence of radioactivity. Thus, this procedure provides visual confirmation of the presence of enzyme that possesses a covalently attached ^{32}P label.

3.2.1.1 Materials and equipment

- 1 M triethanolamine-HCl (pH 7.8) stock solution
- MtnA (see Section 2.2)
- [^{32}P]MTR1P (see Section 3.1)
- Microcentrifuge tubes
- 10% (w/v) sodium dodecyl sulfate (SDS) stock solution
- Mini-PROTEAN TGX precast gels (Bio-Rad)
- 2X Laemmli sample buffer, no reducing agent added (Bio-Rad)
- Sequi-Blot PVDF Membrane (Bio-Rad)
- Mini-PROTEAN Tetra Cell (Bio-Rad)
- Electrophoresis power supply
- Tris-glycine buffer: 25 mM Tris, 192 mM glycine (pH 8.3)
- Phosphor screen (Molecular Dynamics)
- PharosFX Plus Molecular imager (Bio-Rad)
- Coomassie blue staining solution: 0.1% (w/v) Brilliant Blue R (Acros) in 10% (v/v) acetic acid, 50% (v/v) methanol, 40% (v/v) water
- Destaining solution: 40% (v/v) aqueous methanol

3.2.1.2 Protocol

(1) In a microcentrifuge tube containing a final concentration of 20 mM triethanolamine-HCl (pH 7.8), 80–91 μM [^{32}P]MTR1P and 39–44 μM MtnA are added, maintaining a molar ratio of protein to substrate at 1:2.1. The enzyme and the substrate are allowed to incubate at 25 ºC for 30 s before a final concentration

of 2% (w/v) of sodium dodecyl sulfate (SDS) is added to quench the reaction (1 volume of 10% (w/v) SDS per 4 volumes of reaction mixture).

(2) Quenched samples are mixed 1:1 (v/v) with 2X Laemmli sample buffer, and 4–5 μL (ca. 6–9 ng of MtnA) is loaded onto a Mini-PROTEAN TGX precast polyacrylamide gel.

(3) Nonreducing electrophoresis is performed in a Mini-PROTEAN Tetra Cell containing Tris-glycine buffer supplemented with 0.1% (w/v) SDS at 50 V for 15 min followed by 100 V for 90 min.

(4) The protein is electroblotted in Tris-glycine buffer containing only 0.01% (w/v) SDS for 15 min at 100 V onto a double layer of PVDF membrane (the extra layer prevents any pass-through of the protein).

(5) The membranes are exposed to a phosphor screen for 1 day before scanning on a phosphor imager.

(6) The PVDF membrane is stained in Coomassie blue staining solution for 30 s before destaining in three rounds of destaining solution for 5 min each.

3.2.2 Quantitative method

The following method uses size-exclusion chromatography to separate protein from unbound substrate and liquid scintillation counting to quantify the extent of protein radiolabeling. Labeling is performed using concentrations of protein higher than the concentration of labeled substrate. This process ensures higher concentrations of the enzyme–substrate complex, thereby resulting in greater yields of covalently modified protein.

3.2.2.1 Materials and equipment

- 1 M triethanolamine-HCl (pH 7.8) stock solution

- MtnA (see Section 2.2)
- [^{32}P]MTR1P (see Section 3.1)
- Microcentrifuge tubes
- 10% (w/v) sodium dodecyl sulfate (SDS) stock solution
- 5 mL HiTrap desalting columns (GE Healthcare)
- 500 mM ammonium bicarbonate (pH 7.8) stock solution
- NanoDrop 2000c spectrophotometer (ThermoFisher Scientific)
- TriCarb 2910 liquid scintillation counter (PerkinElmer)
- Scintillation cocktail
- Scintillation vials (20 mL)
- Liquid nitrogen
- Cryovials or similar tubes

3.2.2.2 Protocol

(1) In a microcentrifuge tube containing a final volume of 100 µL, 20 mM triethanolamine-HCl (pH 7.8), 30 µM [^{32}P]MTR1P (5.5×10^5 cpm/nmol), and 330 µM MtnA are added. The enzyme and the substrate are allowed to incubate at 25 ºC for 30 s before a final concentration of 2% (w/v) of sodium dodecyl sulfate (SDS) is added to quench the reaction.

(2) Three 5-mL HiTrap desalting columns are connected in series and equilibrated with 20 mM ammonium bicarbonate (pH 7.8).

(3) The quenched reaction mixture is injected into the HiTrap desalting columns, followed by 20 mM ammonium bicarbonate (pH 7.8), collecting fractions every 500 μL.

(4) Fractions are analyzed for the presence of protein by measuring A_{280} on a NanoDrop spectrophotometer and for ^{32}P using a liquid scintillation counter.

(5) The fractions containing radioactively labeled protein are pooled and step 4 is repeated to determine the total amount of protein (in nmol) and radioactivity (in cpm) bound.

(6) The pooled protein is flash-frozen in liquid nitrogen and stored at –80 ºC for use in additional experiments (see Sections 3.3.2 and 3.4.2).

3.2.3 Analysis and statistics

SDS-PAGE of labeled MtnA

[Insert Figure 4 here]

The presence of radioactivity on the electrophoretically separated enzyme after incubating it with [^{32}P]MTR1P (Figure 4) confirms that MtnA becomes covalently labeled with ^{32}P. Most protocols for SDS-PAGE contain a reducing agent such as BME, but this is not compatible with the adduct on MtnA. In the presence of 5% BME, only trace amounts of labeled protein could be detected. This result indicates that the phosphorylated protein is sensitive to the presence of nucleophiles, which is confirmed in Section 3.4 below.

Calculation of percentage of covalent adduct that exists in MTR1P-bound MtnA

When [^{32}P]MTR1P and excess MtnA are mixed, the substrate exists in free (S*) and bound (E•S*) forms, and the enzyme exists in free (E) and bound forms (E•S*). A fraction of the binary complex in turn exists as a covalently modified form (symbolized E–S*, though the chemical

nature of the modification is unknown). The fraction of the MTR1P-bound MtnA that exists as a covalent adduct is therefore [E–S*]/[E•S]. To determine this value, [E•S] can be estimated as 21 μM using equation 1,

$$[ES] = \frac{([E]_t+[S]_t+K_M)-\sqrt{([E]_t+[S]_t+K_M)^2-4[E]_t[S]_t}}{2} \quad (1)$$

where $[E]_t$ (330 μM) and $[S]_t$ (30 μM) are the total concentrations of enzyme and substrate, respectively, and K_M (124 μM at pH 7.8) is the Michaelis constant. After size-exclusion chromatography, the 23 nmol of MtnA recovered (70.5% of the total loaded) contained 2.6×10^4 cpm. Adjusting to 100% of the total loaded, this corresponds to 3.7×10^4 cpm, which is 0.067 nmol E–S* based on the specific activity of [^{32}P]MTR1P. Given that the total volume was 100 μL, [E–S*] = 0.67 μM. [E–S*]/[E•S] = (0.67 μM)/(21 μM) = 0.032. Thus, about 3.2% of the bound substrate existed as a covalent adduct.

3.3 Effect of pH on radiolabeled MtnA

The effect of pH on the phosphorylated enzyme provides insight into the identity of the amino acid involved. The various nucleophilic side chains form different phosphorylated functional groups that have characteristic stabilities to acidic and/or basic pH.

3.3.1 Qualitative method

The following procedure provides a visual indication of the relative stability of ^{32}P-labeled MtnA as a function of pH.

3.3.1.1 Materials and equipment

- Phosphor screen (Molecular Dynamics)
- PharosFX Plus Molecular imager (Bio-Rad)

- Coomassie blue staining solution: 0.1% (w/v) Brilliant Blue R (Acros) in 10% (v/v) acetic acid, 50% (v/v) methanol, 40% (v/v) water
- Destaining solution: 40% (v/v) aqueous methanol
- 0.1 M HCl/KCl (pH 1)
- 0.1 M citric acid/sodium citrate (pH 3)
- 0.1 M citric acid/sodium citrate (pH 5)
- 0.1 M KH_2PO_4/K_2HPO_4 (pH 7)
- 0.1 M $Na_2B_4O_7$/HCl (pH 9)
- 0.1 M $KHCO_3/K_2CO_3$ (pH 11)
- 0.2 M KOH/KCl (pH 13)
- 1 M KOH (pH 14)

3.3.1.2 Protocol

Safety caution: The following procedure involves some manipulation of PVDF membrane containing radioactive protein. Care should be taken with handling the membrane to ensure that the exposure of hands to uncovered radioactivity is limited to a few seconds.

(1) A PVDF membrane is prepared such that it contains one lane of immobilized ^{32}P-labeled protein for each pH to be tested (see Section 3.2.1).

(2) The PVDF membrane is stained with Coomassie blue staining solution for 30 s before destaining in three rounds of destaining solution for 5 min each.

(3) The membrane is cut into strips between lanes, and each strip is incubated for 30 min in one of the buffer solutions listed in 3.3.1.1 at 25 ºC.

(4) The strips are blotted dry, placed together in their original arrangement, and exposed to a phosphor screen for 1 day before scanning on a phosphor imager.

3.3.2 Quantitative method

The following method uses ultrafiltration to separate protein from unbound radioactivity released by hydrolysis at various pH values during incubation for 30 min. Quantification of free ^{32}P is performed by liquid scintillation counting. A control sample is prepared such that free radioactivity is immediately separated from protein prior to the incubation period. This control tests for radioactivity on the protein immediately after mixing and accounts for any loss of label during the ultrafiltration process. Labeling is performed using concentrations of protein higher than the concentration of labeled substrate to minimize the amount of substrate that remains unbound.

3.3.2.1 Materials and equipment

- 0.1 M HCl/KCl (pH 1)
- 0.1 M citric acid/sodium citrate (pH 3)
- 0.1 M citric acid/sodium citrate (pH 5)
- 0.1 M KH_2PO_4/K_2HPO_4 (pH 7)
- 0.1 M $Na_2B_4O_7$/HCl (pH 9)
- 0.1 M $KHCO_3/K_2CO_3$ (pH 11)
- 0.2 M KOH/KCl (pH 13)
- 1 M KOH (pH 14)
- Control buffer: 20 mM NH_4HCO_3 (pH 7.8)

- Centrifugal filters, modified polyethersulfone (PES), 10 kDa cutoff, 500 µL (VWR 82031-348)
- Microcentrifuge
- TriCarb 2910 liquid scintillation counter (PerkinElmer)
- Scintillation cocktail
- Scintillation vials (20 mL)
- Liquid nitrogen
- Cryovials or similar tubes

3.3.2.2 Protocol

(1) A master stock of phosphorylated MtnA purified by size-exclusion chromatography (see Section 3.2.2) is prepared or thawed (from step 6 of Section 3.2.2). Let n be the number of different pH values to be tested. The volume of labeled protein required is $\geq (n + 1) \times 10$ µL.

(2) Into each of n microcentrifuge tubes is pipetted 190 µL of one of the buffers listed above (3.3.2.1). As a control, 190 µL of 20 mM NH_4HCO_3 (pH 7.8) is pipetted into one additional tube.

(3) An aliquot (10 µL) of purified phosphorylated MtnA from step 1 is transferred into each of the tubes in step 2. While the test samples are left to incubate at 25 ºC for 30 min, the control sample is immediately passed through a centrifugal filter (10 kDa cutoff) not exceeding 14,000×g in a microcentrifuge.

(4) After incubation, the test samples of varying pH are centrifuged through centrifugal filters (10 kDa cutoff).

(5) The filtrate of each sample including the control is counted for radioactivity using a liquid scintillation counter.

3.3.3 Analysis and statistics

pH stability of phosphorylated enzyme by SDS-PAGE

[Insert Figure 5]

The protein on the PVDF membrane is analyzed after exposure to various pH conditions for 30 min (Figure 5). The Coomassie-stained bands reveal the stability of the protein, while the intensity of the bands of radioactivity shows the relative stability of the covalent adduct. For a semiquantitative evaluation, the intensities of the radioactive bands can be determined by imaging software. In the given example, by comparison to the band at pH 9, maximal losses of radioactivity occurred at pH 1 (60% relative loss) and at pH 13 and 14 (80% and 90%, respectively).

pH stability of phosphorylated enzyme by radioactive counting

The radioactivity recovered from the control sample provides the "leaky" radioactivity that elutes from the centrifugal filters independent of pH and incubation time. This amount is subtracted from the radioactivity measured in each filtrate after pH exposure. The difference is divided by the amount of radioactivity in each 10 μL aliquot of master stock, and the resulting fraction of intact radiolabeled adduct is plotted as a function of pH (Figure 6).

[Insert Figure 6 here]

The pH profiles can be compared with those from similar studies to identify the modified residue. Phosphoramidate linkages (from Lys, Arg, or His) are stable at basic pH (Besant et al., 2009), so these are not a candidate in MtnA. Phosphocysteine is known to be least stable at pH 2–4 but unusually stable at lower and higher pH (Guan & Dixon, 1991), which is also inconsistent

with MtnA's pH profile. Although phosphate esters (from Ser, Thr, or Tyr) are stable over a broad pH range, there are no such candidates in the active site of MtnA. Acyl phosphates (from Asp or Glu) are known to be unstable at both pH extremes; phosphoaspartate exhibits an inverted U-shaped pH profile similar to that observed for MtnA (Anthony & Spector, 1972). Based on these results and considering aspartate residues are present in the active site, it is the most likely candidate.

3.4 Effect of nucleophiles on radiolabeled MtnA

Phosphorylated amino acid residues have varying electrophilicity with respect to exogenous nucleophiles. In particular, the presence of the phosphoryl group in aspartate and glutamate residues converts the carbonyl from being electron-rich as a carboxylate to electron-deficient as an acyl phosphate. The carbon is therefore especially susceptible to nucleophilic attack, which is evaluated in the following procedures.

3.4.1 Qualitative method: BME and TCEP

The following procedure provides a visual indication of the relative stability of ^{32}P-labeled MtnA as a function of the concentration of two common biochemical reducing agents—BME and tris(2-carboxyethyl) phosphine (TCEP)—detected by SDS-PAGE.

3.4.1.1 Materials and equipment

- All materials and equipment in Section 3.2.1.1
- 100 mM BME stock solution
- 100 mM TCEP stock solution

3.4.1.2 Protocol

(1) Let n be the number of different concentrations of reducing agent (nucleophile) to be tested. A master mix of $\geq n \times 5.0$ μL [^{32}P]MTR1P/MtnA is prepared by following Step 1 of Section 3.2.1.2.

(2) Into n microcentrifuge tubes is added 10.0 μL of 2X Laemmli sample buffer, followed by 0–2.0 μL of 100 mM stock reducing agent and 5.0–3.0 μL water so that the sum of the two added volumes is 5.0 μL.

(3) A 5.0-μL aliquot of radiolabeled MtnA master mix is pipetted into each tube. This provides a final concentration range of 0–10 mM BME or TCEP.

(4) Each sample is loaded onto a Mini-PROTEAN TGX precast polyacrylamide gel.

(5) The protocol in Section 3.2.1.2 from Step 3 onward is followed to obtain Coomassie-stained PVDF membranes and corresponding autoradiograms.

3.4.2 Quantitative method: hydroxylamine and pyridine

Because hydroxylamine and pyridine are known to react with acyl phosphate groups at neutral pH (Anthony & Spector, 1972), they were also tested with the radioactively labeled MtnA adduct. The following procedure provides a quantitative evaluation of the percentage of ^{32}P-labeled MtnA remaining after 10 min of treatment with varying concentrations of each amine.

3.4.2.1 Materials and equipment

- 2X stock solutions of NH_2OH: 0.1, 0.2, 0.5, 1, and 2 M NH_2OH in 0.1 M Tris-acetate buffer (adjusted to pH 7 with acetic acid)
- 2X stock solutions of pyridine: 0.1, 0.2, 0.5, 1, and 2 M pyridine in 0.1 M Tris-acetate buffer (adjusted to pH 7 with acetic acid)
- *tert*-Butanol
- 10% (w/v) SDS stock solution

- Microcentrifuge
- Microcentrifuge tubes
- TriCarb 2910 liquid scintillation counter (PerkinElmer)
- Scintillation cocktail
- Scintillation vials (20 mL)

3.4.2.2 Protocol

(1) A master mix of phosphorylated MtnA purified by size-exclusion chromatography (see Section 3.2.2) is prepared or thawed (from step 6 of Section 3.2.2). Let n be the number of different concentrations of amine (nucleophile) to be tested. The volume of labeled protein required is $\geq n \times 10$ µL.

(2) Into n microcentrifuge tubes is added 10 µL of each stock solution of amine.

(3) A 10-µL aliquot of radiolabeled MtnA master mix is pipetted into each tube. This provides a final concentration range of 0–1 M NH_2OH or pyridine.

(4) After 10 min at 25 ºC, 180 µL of *tert*-butanol is added to precipitate the protein.

(5) The tubes are centrifuged at 13,000 rpm at 4 ºC until the supernatant is clear (~30 min). The supernatant is discarded to radioactive waste.

(6) The pellet is resuspended in 250 µL of *tert*-butanol and step 5 is repeated once.

(7) The pellet is dissolved in a minimum of 1% (w/v) SDS, and the solution is counted for radioactivity using a liquid scintillation counter.

3.4.3 Dephosphorylation kinetics

The following procedure measures the kinetics of ^{32}P loss from labeled MtnA in the presence of varying concentrations of hydroxylamine.

3.4.3.1 Materials and equipment

- 2X stock solutions of NH_2OH: 0, 0.1, 0.2, and 0.5 M NH_2OH in 0.1 M Tris-acetate buffer (adjusted to pH 7 with acetic acid)
- *tert*-Butanol
- 10% (w/v) SDS stock solution
- Microcentrifuge
- Microcentrifuge tubes
- Vortex mixer
- TriCarb 2910 liquid scintillation counter (PerkinElmer)
- Scintillation cocktail
- Scintillation vials (20 mL)

3.4.3.2 Protocol

(1) A master mix (> 240 μL) of phosphorylated MtnA purified by size-exclusion chromatography (see Section 3.2.2) is prepared or thawed (from step 6 of Section 3.2.2).

(2) Into four microcentrifuge tubes is added 60 μL of each stock solution of NH_2OH (including the negative control).

(3) Into 24 tubes (6 per concentration of NH_2OH) is added 180 μL of *tert*-butanol.

Note: A stopwatch should be available for the next steps. Aliquots will be removed from multiple samples at different times; those containing higher concentrations of NH_2OH will require sampling more frequently. Because this requires some guesswork, the entire experiment may need to be repeated with carefully chosen time intervals.

(4) A 60-µL aliquot of radiolabeled MtnA master mix is pipetted into each of the four tubes containing NH_2OH (including the negative control). This provides a final concentration range of 0–250 mM NH_2OH. Begin timing immediately after mixing.

(5) As soon as possible, 20 µL is transferred from each of the four tubes into a separate tube containing *tert*-butanol and mixed by vortexing; the time is recorded.

(6) Repeat step 5 at each of five additional timepoints, whose intervals may differ for each concentration of NH_2OH.

(7) The tubes are centrifuged at 13,000 rpm at 4 ºC until the supernatant is clear (~30 min). The supernatant is discarded to radioactive waste.

(8) Each pellet is resuspended in 250 µL of *tert*-butanol and step 7 is repeated once.

(9) Each pellet is dissolved in a minimum of 1% (w/v) SDS, and the solution is counted for radioactivity using a liquid scintillation counter.

3.4.4 Analysis and statistics

Nucleophile susceptibility of phosphorylated enzyme by SDS-PAGE

[Insert Figure 7 here]

The Coomassie-stained bands on the PVDF membrane indicate that the protein is unaffected by the presence of reducing agents up to 10 mM (Figure 7). The autoradiograph, on the other hand, reveals a concentration-dependent loss of radiolabel in the presence of both nucleophiles. Additionally, the absence of radioactive labeling when MtnA was first exposed to SDS (lane 1 in Figure 7) indicates that adduct formation requires proper protein folding.

Nucleophile susceptibility of phosphorylated enzyme by radioactive counting

[Insert Table 1 here]

The results from incubations with either NH_2OH or pyridine show a concentration-dependent increase in loss of radioactivity from MtnA (Table 1). NH_2OH acts as a better nucleophile than pyridine, leading to dephosphorylation of nearly half of the labeled protein at a concentration of 50 mM.

Kinetics of dephosphorylation by hydroxylamine

[Insert Figure 8 here]

The rates of dephosphorylation can be obtained for various concentrations of NH_2OH by measuring the loss of radioactive label at varying timepoints (Figure 8A). The dephosphorylation conforms to a first-order decay process that is faster as the concentration of NH_2OH is increased. The concentration dependence of the observed first-order rate constant, k_{obs}, conforms to equation 2,

$$k_{\mathrm{obs}} = \frac{k_{\mathrm{max}}[\mathrm{NH_2OH}]}{K+[\mathrm{NH_2OH}]} + k_{\mathrm{bg}} \quad (2)$$

where k_{max} is the maximal first-order rate constant above background, k_{bg} is the first-order rate constant for background loss of ^{32}P, and K is the concentration of NH_2OH that gives a half-maximal rate of dephosphorylation above background (Figure 8B). The graph is linear for $[NH_2OH] \leq 100$ mM (dashed line in Figure 8B) with a slope of 9.0×10^{-3} M^{-1} s^{-1}, which represents the second-order rate constant for dephosphorylation. From these results, it is clear the covalently modified protein is sensitive to nucleophilic attack, consistent with the adduct existing as an acyl phosphate.

4. Summary

We have presented five methods for quantifying MTR1P, each with advantages and disadvantages. While two of these methods could be used continuously to measure isomerization catalyzed by MtnA, the OPDA method stood out as the superior method for measuring steady-state kinetics and other experiments requiring continuous coupling of the product MTRu1P. Additionally, methods were presented for radioactive labeling of the enzyme using [^{32}P]MTR1P and for characterizing the nature of the covalent adduct including sensitivity to pH and nucleophiles. The approaches described here may assist in future investigations of methylthio-D-ribose-1-phosphate isomerase and related enzymes, as well as in the characterization of other proteins that are phosphorylated by their substrate.

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Tables

Table 1. Sensitivity of phosphorylated protein to hydroxylamine and pyridine.

Concentration of amine (mM)	Percentage of ^{32}P remaining [a]	
	Hydroxylamine	Pyridine
0	92	93
50	42	89
100	26	69
250	21	47
500	11	14
1000	7.5	7

[a] The percentage of radioactivity remaining after 10 min was normalized to total radioactivity on the protein in 100 mM Tris-acetate buffer (pH 7) at time 0.

Scheme Legends

Scheme 1. The methionine salvage pathway of *Bacillus subtilis*, featuring methylthio-D-ribose-1-phosphate isomerase (MtnA). MtnK, methylthio-D-ribose kinase; MtnB, methylthio-D-ribulose-1-phosphate dehydratase.

Scheme 2. Canonical mechanisms of enzyme-catalyzed isomerization of aldoses and ketoses. (A) Proton-transfer mechanism. (B) Hydride-transfer mechanism.

Scheme 3. Chemoenzymatic synthesis of MTR1P. MtnK, methylthio-D-ribose kinase.

Scheme 4. Purine nucleoside phosphorylase (PNP)–xanthine oxidase (XO) coupled assay for detecting the phosphate liberated from MTR1P by alkaline phosphatase. AlkP, alkaline phosphatase; Ino, inosine; R1P, ribose 1-phosphate; Hx, hypoxanthine. Extinction coefficient is in units of M^{-1} cm^{-1}.

Scheme 5. Fuculose-1-phosphate aldolase (FucA) coupled assay for detecting MTR1P. DHAP, dihydroxyacetone phosphate; G3PDH, glycerol-3-phosphate dehydrogenase; L-G3P, L-glycerol 3-phosphate; NADH, nicotinamide adenine dinucleotide, reduced form; NAD^+, nicotinamide adenine dinucleotide, oxidized form. Extinction coefficient is in units of M^{-1} cm^{-1}.

Scheme 6. *o*-Phenylenediamine (OPDA) assay for detecting MTR1P. DK-MTP 1-P, 2,3-diketo-5-methylthiopentyl 1-phosphate. Extinction coefficient is in units of M^{-1} cm^{-1}.

Figure Legends

Figure 1. Standard curve for the OPDA assay. The change in A_{320} associated with quinoxaline formation is plotted as a function of MTR1P concentration, as determined by the ^{1}H-NMR assay (see Section 2.4.5). Error bars represent standard deviations from triplicate samples.

Figure 2. ^{1}H-NMR assay for determining MTR1P concentration. The spectrum (D_2O) is from a sample containing a 1:0.69 mole ratio of imidazole standard to MTR1P. Integrals and peak assignments are labeled.

Figure 3. Reversed-phase HPLC purification of [^{32}P]MTR1P. After removal of protein by ultrafiltration, the reaction mixture was injected onto a Delta-Pak C18 column (Waters, 15 μm, 100 Å, 3.9 × 300 mm) at 2 mL/min using a gradient protocol between 50 mM TEAB and 50% (v/v) aqueous methanol. Fractions were collected every 1 min and counted in a liquid scintillation counter. (A) Chromatogram monitoring radioactivity as a function of fraction number. [^{32}P]MTR1P was identified in fractions 11–14 by the OPDA assay. (B) Chromatogram monitoring A_{260}, used for identification of ADP/ATP.

Figure 4. SDS-PAGE of ^{32}P-labeled MtnA in the absence (–) and presence (+) of 5% (v/v) β-mercaptoethanol prior to electrophoresis. The top row is the autoradiograph of the PVDF membrane, showing the presence of radioactivity. The bottom row is the PVDF membrane after Coomassie staining, showing the presence of protein.

Figure 5. Effect of pH on stability of ^{32}P-labeled MtnA. The Coomassie-stained PVDF membrane (top row) and autoradiograph (bottom row) after treatment at the indicated pH for 30 min. The browning at pH 14 suggests instability of Coomassie at this extreme pH.

Figure 6. The effect of pH on ^{32}P-labeled MtnA isolated by size-exclusion chromatography.

Figure 7. Effect of reducing agents on the stability of ^{32}P-labeled MtnA. MtnA was incubated with [^{32}P]MTR1P for 30 s before diluting with Laemmli buffer containing 0–10 mM BME or TCEP, followed by SDS-PAGE. The row on the top is the Coomassie-stained PVDF membrane, while the bottom row is the autoradiograph. The first lane (red box) contains MtnA that was denatured with SDS before incubation with [^{32}P]MTR1P.

Figure 8. Dephosphorylation kinetics of ^{32}P-labeled MtnA in the presence of hydroxylamine. (A) Size-exclusion purified ^{32}P-labeled MtnA was incubated with 0 (black circles), 50 mM (red diamonds), 100 mM (blue squares), or 250 mM (green triangles) NH_2OH in 50 mM Tris-acetate (pH 7). At each timepoint, the protein was recovered by precipitation with *tert*-butanol and counted for remaining radioactivity. The data were fit to a first-order decay function. (B) Replot of the observed first-order rate constant, k_{obs}, as a function of NH_2OH concentration. The solid black curve is a fit to hyperbolic equation 2, and the dashed line is a linear fit (slope = 9.0×10^{-3} M^{-1} s^{-1}) through the first three points.