The tRNA synthetase paralog PoxA modifies elongation factor-P with (*R*)-β-lysine

Hervé Roy[1,5], S. Betty Zou[4], Tammy J. Bullwinkle[1], Benjamin S. Wolfe[2], Marla S. Gilreath[3], Craig J. Forsyth[2], William W. Navarre[4] and Michael Ibba*[1,3,]

[1]Department of Microbiology, Center for RNA Biology, [2]Department of Chemistry, and [3]Ohio State Biochemistry Program, Ohio State University, Columbus, Ohio 43210, USA.

[4]Department of Molecular Genetics, University of Toronto, Toronto, ON, M5S 1A8, Canada.

[5]Present address: Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 12722 Research Parkway, Orlando, Florida 32826, USA.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Strain, plasmids and general methods. *E. coli* K-12 BW25113 and the isogenic mutants JW4106-2 (*yjek::kan*) and JW4116-1 (*poxA::kan*) were obtained from the Keio collection [1] via the Yale Genetic Stock Center. PoxA from *Salmonella enterica* serovar Typhimurium was expressed in *E. coli* and purified as described earlier[2]. *poxA* and *efp* from *E. coli* were cloned into the expression plasmid pTYB11 (NEB) and expressed in the strain BL21 (DE3) to allow protein production and purification as described before[3]. EF-P over-produced in this manner, containing an intein tag that was subsequently removed, did not contain any detectable post-translational modifications, as assessed by mass spectrometry. Proteins were dialyzed against 25 mM Tris-HCl (pH 8.1), 150 mM NaCl, 4 mM 2-mercaptoethanol, and 50% glycerol and stored at -20°C. Wild-type *Salmonella enterica* serovar Typhimurium 14028s, the *poxA* null mutant strain WN353, *poxA yjeK* double null mutant WN356, the *poxA* complementing plasmid (pPoxA), and the enzymatically dead PoxA plasmid (pPoxA$_{(E251A)}$) were previously described [2]. The pPoxA plasmid was used as a template for site directed mutagenesis using PCR amplification and DpnI digestion. The following primers were used to generate the A298G mutation in pPoxA: 5' GGATTGTTCCGGCGTGGGACTGGGTGTTGATCG 3' and 5' CGATCAACACCCAGT CCCACGCCGGAACAATCC 3'. *E. coli* LysRS was prepared as previously described[4]. (*R*)-β-lysine was synthesized as described in the Supplementary Method online.

ATP/PPi exchange. Reactions were carried out at 37 °C in 100 mM glycine-NaOH (pH 9.0), 18 mM KCl, 10 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 3 mM β-mercaptoethanol, 2 mM [^{32}P]-PPi (1 cpm/pmol), 10 mM amino acid, and 2 μM PoxA or 20 nM LysRS. After 0 to 15 min, 25 μl aliquots were removed and added to a solution containing 1% charcoal, 5.6% $HClO_4$, and 75 mM PPi, filtered through a 3MM Whatman filter disk under vacuum and washed 3 times with 5 mL of water and once with 5 mL of ethanol. The filters were dried, and the radiolabeled ATP bound to the charcoal quantified by liquid scintillation counting (Ultima Gold, Packard Instrument Co.). K_M and k_{cat} values were determined with varying amounts of amino acid (2 - 100 mM) or ATP (50 μM - 2 mM). The optimal pH was determined using three buffer systems ranging from pH 7.2 - 10 with data fitted to the following equation:

$$k_{cat(obs)}=k_{cat}/(1+10^{(pKa1-pH)}+10^{(pH-pKa2)}).$$

EF-P aminoacylation. Aminoacylation was performed at 37 °C in 100 mM glycine-NaOH (pH 9.0), 18 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 3 mM β-mercaptoethanol, 50 μM [^{14}C]-lysine, 5 μM PoxA and 20 - 100 μM EF-P. At various times, 10 μl aliquots were removed and quenched with 3 μl of protein loading dye and analyzed on a 10% SDS-PAGE. After migration, the gel was dried, stained and the radioactivity quantified by phosphorimaging. The amount of [^{14}C]-lysylated EF-P was calculated by comparison of the band intensities with those of known amounts of [^{14}C]-lysine. The concentration of active EF-P was determined by measuring the plateau of aminoacylation of the protein.

Detection of aminoacylated EF-P. Aminoacylation was performed at 37 ºC in 100 mM glycine-NaOH (pH 9.0), 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, 20μM lysine variant, 1.2 µM PoxA and 5µM EF-P (or K34A variant). After incubation for 10 minutes, reactions were diluted 5 fold in ice cold loading buffer (10mM Tris-HCl pH6.8, 25% glycerol) and loaded onto pH 5-8 isoelectric focusing (IEF) gels (BioRad). IEF gels were incubated in 0.7% acetic acid for 10 minutes and EF-P was transferred to Hybond™-C Extra nitrocellulose membrane (Amersham) at 43mA for 1 hour at 4 ˚C using SemiPhor semidry transfer apparatus (Hoefer). Membrane was blocked with 20% skim milk powder in TTBS (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween20) for 1 hour. Primary rabbit antibodies to a C-term peptide of EF-P (ProSci) were diluted 1:1250 in TTBS + 3% BSA and hybridized to membrane-bound EF-P for 1 hour. After several washes in TTBS, the membrane was incubated in a 1:5000 dilution of horseradish peroxidase-linked ECL™-anti-rabbit IgG from donkey (GE Healthcare) in TTBS + 20% milk powder for 1 hour. Free antibodies were removed by several washes with TTBS + milk powder, TTBS, and TTBS (minus Tween20). Western blots were developed using ECL™ Plus Lumigen™ detection kit (GE Healthcare) for 5 minutes and imaged with ChemiDoc™-XRS+ (BioRad).

ATP consumption assay. Hydrolysis of ATP by PoxA was monitored in 100 mM glycine-NaOH (pH 9.0), 18 mM KCl, 10 mM $MgCl_2$, 3 mM β-mercaptoethanol, 200 µM ATP, 0.5 µCi [α 32P]-ATP, 2 µM PoxA, and saturating amounts of lysine (80-100 mM) or metabolite extract as determined by ATP/PPi exchange assay. After various time of incubation at 37 °C, the reaction was quenched by mixing equal volume (2 µl) of the reaction with acetic acid. 0.4 µl of the latter mixture were analyzed by thin layer chromatography on PEI cellulose plates (Sigma)

developed in acetic acid:1 M NH_4Cl:water (2.5:5:42.5). [α^{32}P]-ATP and [α^{32}P]-AMP were visualized and quantified by phosphorimaging.

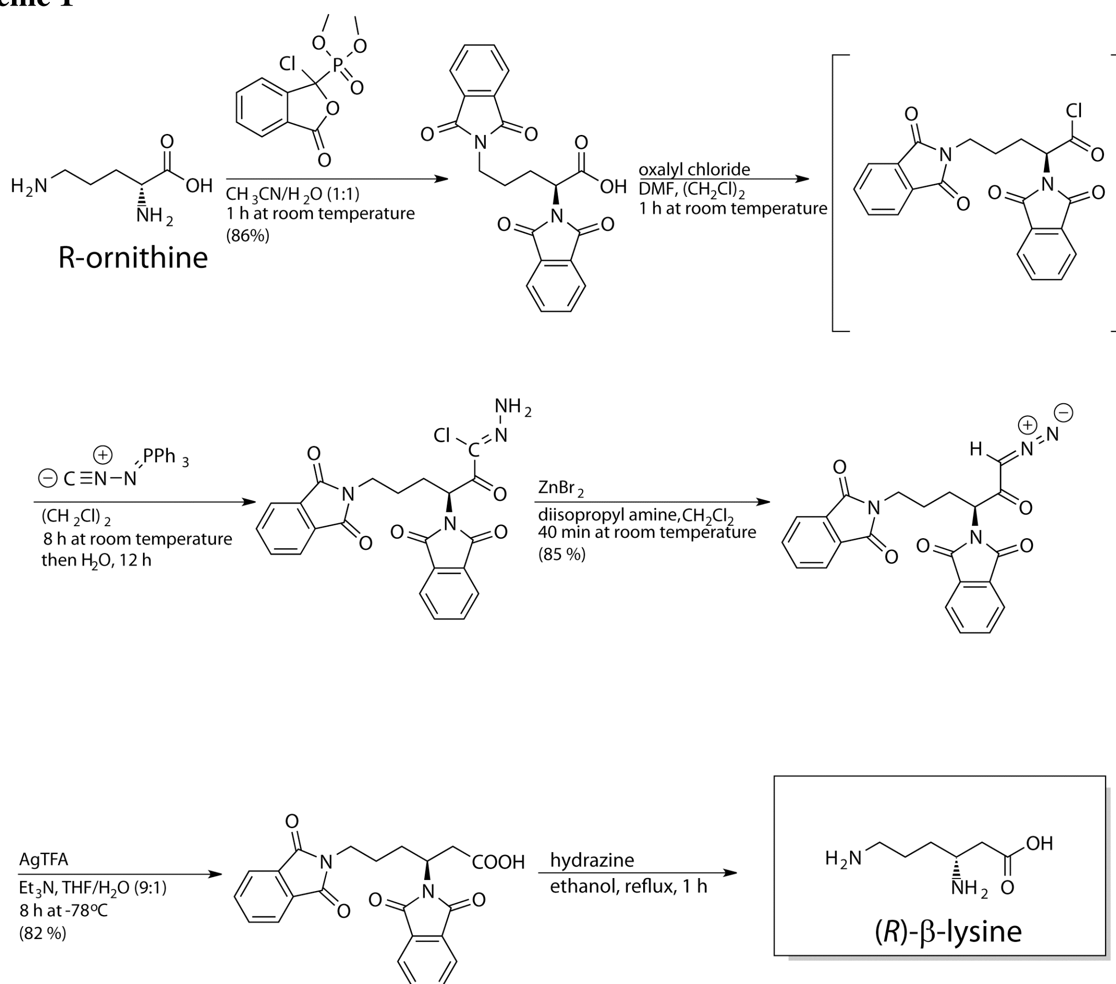
Metabolite extraction and separation. *E. coli* K-12 BW25113 was used in order to allow use of a homologous model system where metabolite extracts could be prepared from a non-pathogenic microbe. *E. coli* strains were grown at 37 °C in flasks containing 2 L of LB medium under agitation. The biomass was harvested by centrifugation, washed once with 100 mM Tris-HCl (pH 8.0), and once with water. Cells were resuspended in 140 ml of ice cold 50% methanol and frozen on dry ice. The suspension was thawed on ice and the cells were pelleted by centrifugation. The biomass was submitted to two additional freeze-thaw cycles, all the supernatants were collected, pooled, and filtrated on an Amicon centrifugal filter unit (Ultracel-3K). The metabolite extract was evaporated and resuspended in 1.6 ml of water. Metabolites were separated by analytical TLC on silica gel HLF (Analtech) using Dioxane:26% Ammonia:water (6:1:6) as a mobile phase. Metabolites were separated by flash chromatography (75 ml) using silica gel (mesh 220-440, Alfa Aesar) as stationary phase and the solvent described above as mobile phase. The presence of lysine or PoxA substrate in flash chromatography fractions was tested on a dried aliquot by measuring the ATP/PPi exchange activity in the presence of *E. coli* LysRS or PoxA, respectively.

Synthesis of β-Lysine

General description. (*R*)-β-lysine was prepared from (*R*)-2,5-diaminopentanoic acid [(*R*)-ornithine] via an Arndt-Eisert homologation process[5] (scheme 1). This involved bis-phthaloyl protection of (*R*)-ornithine using 3-chloro-3-(dimethoxyphosphoryl)-isobenzofuran-1(3*H*)-one[6].

The bis-phthalimide derivative of (*R*)-ornithine was converted into a diazomethylketone using *N*-isocyanoiminotriphenylphosphorane[7] via the intermediacy of the acid chloride. The diazomethylketone was then treated with silver trifluoroacetate to induce Wolff rearrangement to the beta amino acid. Finally the phthaloyl groups were removed using hydrazine to give (*R*)- β-lysine.

Scheme 1



*Diphthaloyl ornithine***.** To a solution of 3-chloro-3-(dimethoxyphosphoryl)-isobenzofuran-1(3*H*)-one (4.35 g, 15.75 mmol) and D or L-ornithine (0.885 g, 5.25 mmol) in MeCN/H2O (1:1 (v/v), 25 mL) was added *i*-Pr_2NEt (5 mL, 29 mmol) and the mixture stirred at 24°C for 1 h. The

mixture was concentrated *in vacuo* to a small volume then acidified with 2 M HCl, and extracted with ethyl acetate (4 x 50 mL). The product was purified by silica gel column chromatography using dichloromethane/diethyl ether (14:1 to 8:1, v/v) to yield diphthaloyl ornithine as a white solid. HRMS-ESI (*m/z*) [M+Na+]: calcd: 415.0901, found: 415.0892; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.80 (s, 1H), 7.88-7.77 (m, 4H), 7.76-7.64 (m, 4H), 4.99 (dd, *J* = 10.4, 5.2 Hz, 1H), 3.82-3.54 (m, 2H), 2.43-2.18 (m, 2H), 1.83-1.58 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.30, 168.37, 167.52, 134.29, 133.96, 131.99, 131.66, 123.67, 123.30, 77.38, 77.06, 76.74, 51.28, 36.97, 25.77, 25.42.

Diazoketone. To a solution of D-*N,N*-diphthaloyl ornithine (2.475 g, 33.5 mmol) in 1,2-dichloroethane (35 mL) was added a catalytic amount of *N,N*-dimethylformamide followed by oxalyl chloride (0.811 mL, 9.46 mmol). After 1 h the solution was concentrated *in vacuo*. The residue was dissolved in 1,2-dichloroethane (80 mL) and the solution was concentrated to 25 mL then added drop-wise over 10 min to a suspension of *N*- isocyanoimino-triphenylphosphorane (2.86 g, 9.47 mmol) in 1,2-dichloroethane (15 mL). After 2 h, water (8 mL) was added and the mixture was stirred for 12 h. The phases were separated, and the organic phase was dried over Na_2SO_4, filtered and concentrated to an oil. This was dissolved in CH_2Cl_2 (20 mL), and anhydrous $ZnBr_2$ (0.3 g, 1.3 mmol) and diisopropylamine (1.1 mL) were added. The mixture was stirred for 40 min before being filtered through silica and concentrated. Flash chromatography of the residue on silica gel eluting with hexanes/ethyl acetate (1:1) gave the diazoketone as a yellow solid (2.246 g, 85 % yield). HRMS-ESI (*m/z*) [M+Na+]: calcd: 439.1013, found: 439.1015; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92–7.81 (m, 4H), 7.81-7.70 (m, 4H), 5.44 (s, 1H), 4.94 (dd, *J* = 11.2, 4.6 Hz, 1H), 3.82-3.67 (m, 2H), 2.41 (dddd, *J* = 14.3, 11.2,

8.6, 5.8 Hz, 1H), 2.22-2.11 (m, 1H), 1.78-1.64 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.31, 168.37, 167.80, 134.38, 133.98, 132.05, 131.65, 123.70, 123.28, 56.56, 54.05, 36.86, 25.44, 25.18.

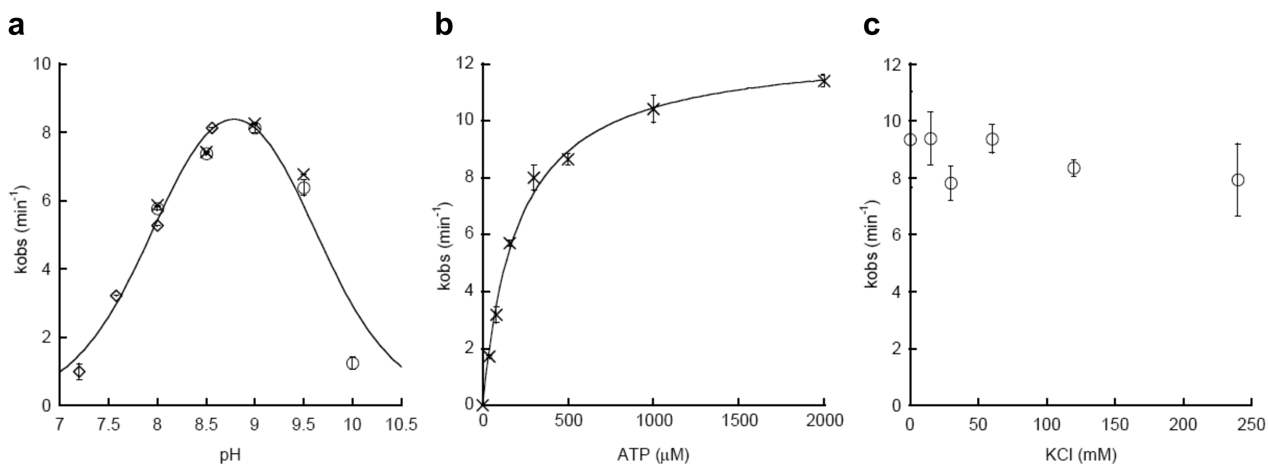
N,N-Diphthaloyl-3,4-diaminohexanoic acid. The diazoketone (2.246 g, 5.39 mmol) was dissolved in a mixture of THF/water (9:1, v/v) and cooled to -78 °C. A solution of silver trifluoroacetate (0.131 g) in triethylamine (2.11 mL) was added in the dark and the mixture was stirred vigorously as the mixture was allowed to warm to room temperature. The mixture was stirred for 8 h at rt then acidified with 10 % HCl. The suspension was extracted with ethyl acetate (4 x 50 mL). The combined organic phases were dried, filtered, concentrated *in vacuo* and the residue purified by column chromatography eluting with hexanes/ethyl acetate (2:3) to give *N,N*-diphthaloyl-3,4-diaminohexanoic acid as a tan foam (82 %). HRMS-ESI (*m/z*) [M+Na^+]: calcd: 429.1057, found: 429.1053; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.58 (s, 1H), 7.79 – 7.70 (m, 4H), 7.65 (ddd, *J* = 5.2, 3.0, 1.5 Hz, 4H), 4.65 (tt, *J* = 10.1, 5.1 Hz, 1H), 3.71 – 3.55 (m, 2H), 3.18 (dd, *J* = 16.7, 9.3 Hz, 1H), 2.78 (dd, *J* = 16.7, 5.4 Hz, 1H), 2.08 (dtd, *J* = 15.2, 10.1, 5.2 Hz, 1H), 1.81 – 1.70 (m, 1H), 1.68 – 1.52 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.82, 168.31, 168.21, 134.03, 133.94, 131.93, 131.58, 123.33, 123.20, 47.18, 37.23, 36.47, 29.64, 25.40.

3,4-Diaminohexanoic acid. To a solution of *N,N*-diphthaloyl-3,4-diaminohexanoic acid (0.238 g) in 95 % ethanol (2.5 mL) was added a 1 M solution of hydrazine in ethanol (1.5 mL). The resultant mixture was heated at reflux for 1 h. The mixture was allowed to cool to room temperature and the solvent was removed *in vacuo*. To the residue was added 5 % aqueous HCl

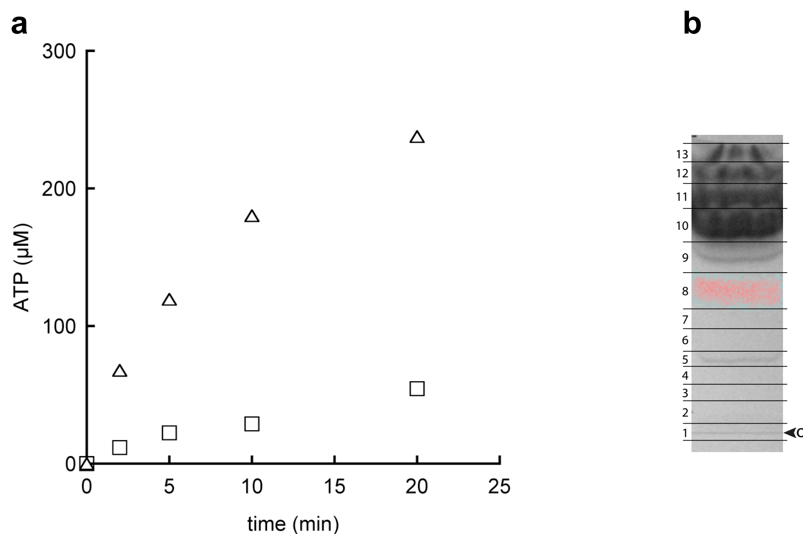
(10 mL) and the mixture was stirred for 10 min. The suspension was then filtered and the filtrate concentrated to yield crude 3,4-diaminohexanoic acid. This was suspended in methanol and filtered. The filtrate was concentrated to give a mixture of 3,4-diaminohexanoic acid and methyl 3,4-diaminohexanoate. The mixture was dissolved in 5 mL of 5 % aqueous HCl and heated at reflux for 1 h. The solvent was removed under a gentle stream of air to yield 3,4-diaminohexanoic acid bis-hydrolchloride.as a yellow solid. Spectroscopic data for this compound were consistent with the previously reported literature values[8].

SUPPLEMENTARY RESULTS

Supplementary Figure 1. Determination of optimal reaction conditions for α-lysine dependent ATP/PPi exchange activity of PoxA. **(a)** Optimal pH determination. 0.1 M of Tris-HCl (crosses), or glycine-NaOH (circles), or HEPES-NaOH (diamonds) were used to cover pH ranging from 7.2 to 10 (pKa1=8.0, pKa2=9.6, pHo=8.8). (**b**) Determination of saturating amount of ATP. Michaelis-Menten kinetic parameters were determined (K_M= 206 μM, k_{cat} = 12 min^{-1}). (**c**) Effect of KCl concentration on PoxA activity. Rates were measured in the presence of 40 mM L-lysine.

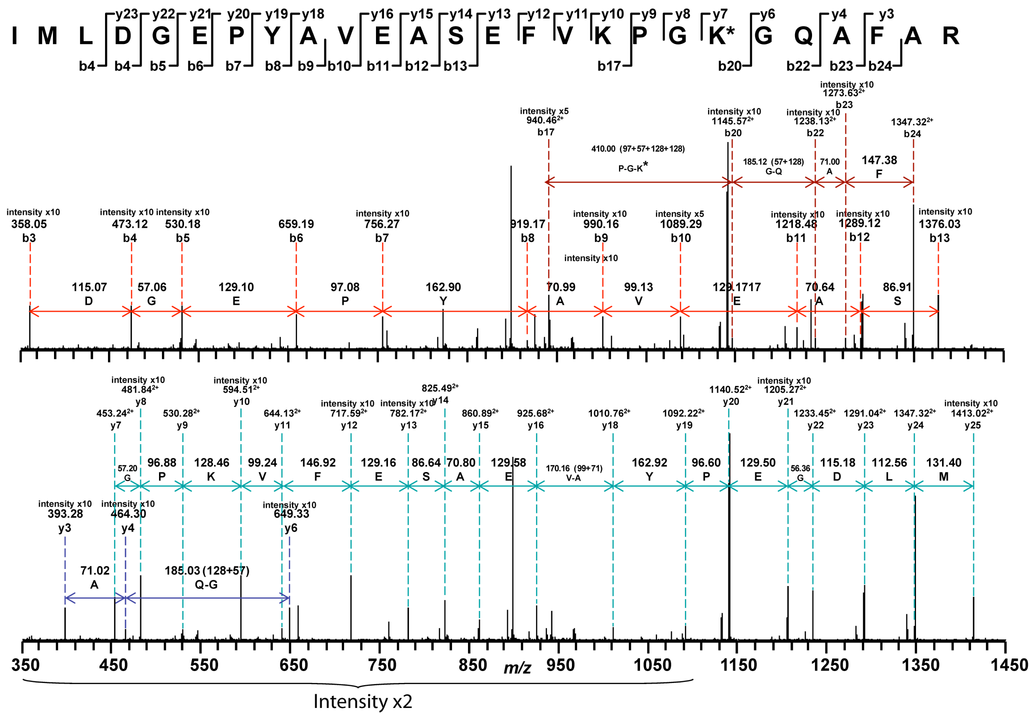


Supplmentary Figure 2. **Amino acid recognition by PoxA.** (**a**) Stimulation of PoxA-catalyzed ATP/PPi exchange by lysine or a metabolite extract. Formation of ATP was monitored in the presence of 3 mM lysine (□) or in the presence of a metabolite extract obtained from *E. coli* (△). The concentration of lysine within the extract was 380 μM as determined by measuring ATP/PPi exchange activities of *E. coli* LysRS in presence of various amounts of extract, and using a K_M value for lysine of 43 μM[25]. (**b**) Metabolite fractionation by TLC. Metabolites were visualized under UV and migration of lysine was revealed by co-migration of a small amount of [^{14}C]-Lys and by phosphorimaging (red band). Metabolites were extracted from the plates and each fraction (numbers) was tested as a substrate in an ATP/PPi exchange assay with PoxA; only fractions 6 and 7 stimulated PoxA activity.

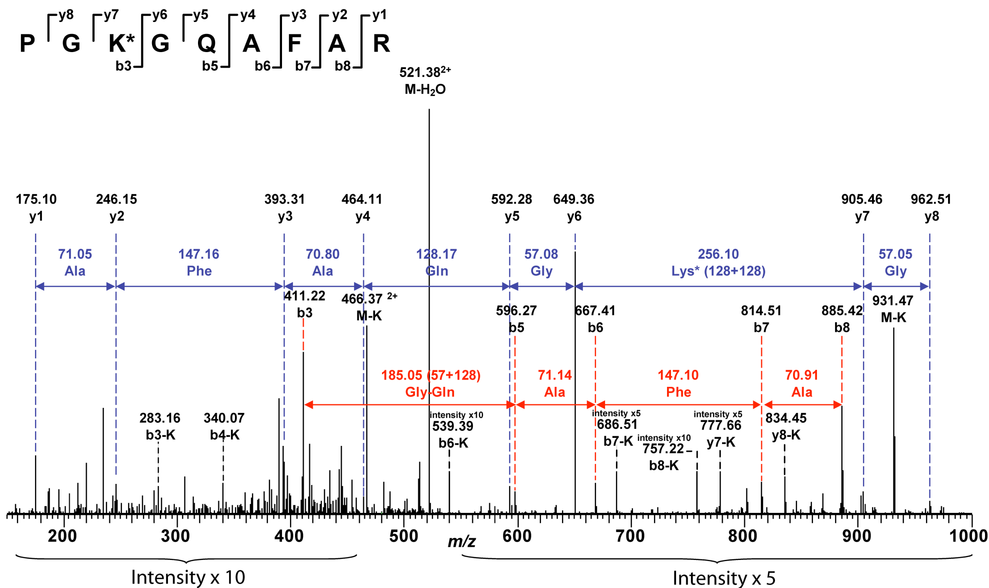


Supplementary Figure 3. **Determination of the mass of modified EF-P.** The mass of EF-P immunopurified from cell-free extracts (**a**) or modified in vitro using PoxA and cell-free extracts (**b**) was determined. Peak intensities of indicated regions of the spectra were scaled down for display.

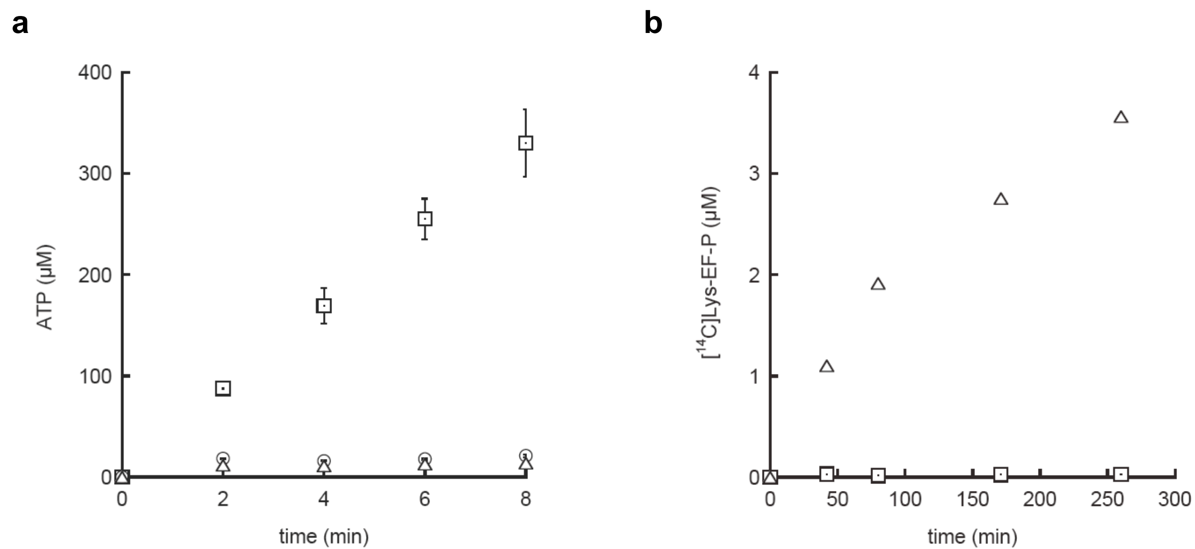
a



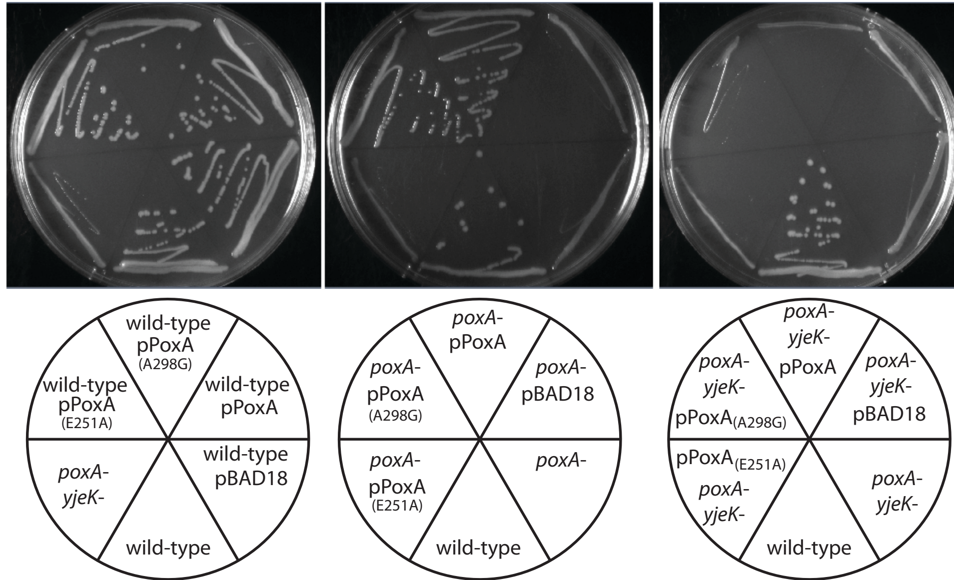
b



Supplementary Figure 4 **Amino acid specificity of PoxA and LysRS.** (**a**) ATP/PPi exchange activity of LysRS in the presence of α-lysine (□), (*S*)-β-lysine (○) or (*R*)-β-lysine (Δ). Exchange activity was monitored with 100 nM LysRS and 5 mM amino acid. (**b**) Lysylation of EF-P in the presence or absence of (*R*)-β-lysine. Posttranslational modification of EF-P was conducted in the presence of 2 μM PoxA, 40 μM EF-P, 100 μM [^{14}C]-lysine and in the absence (Δ) or presence of 100 μM (*R*)-β-lysine (□). It should be noted that lysylation of EF-P with [^{14}C] α-lysine cannot be detected using the reaction conditions applied in Figure 3a.



Supplementary Figure 5. Growth of *Salmonella* mutants on AB2. Growth on Difco Antibiotic Medium 2 plates (AB2 plates; BD Diagnostics, Sparks, MD) after incubation for 16 hours at 37 °C.



Growth of *Salmonella poxA* mutants was completely rescued by either pPoxA or $\mathrm{pPoxA}_{\mathrm{A298G}}$, indicating that PoxA A298G (equivalent to *E. coli* A294G, Table 1) retains function *in vivo*. Phenotypic rescue was not achieved with an inactive E251A variant[2] of PoxA. Next, we sought to determine whether the enhanced α-lysine activation activity of the PoxA A298G variant could compensate for function in strains lacking the enzyme required to synthesize β-lysine, 2,3-β-lysine aminomutase. These data suggest that α-lysine cannot functionally substitute for β-lysine, although the four-fold lower catalytic efficiency (k_{cat} / K_M) for the α- compared to the β-amino acid for the corresponding *E. coli* PoxA variant suggests that reduced activity could also contribute to the lack of a complementation phenotype.

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