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Lysine Biosynthesis in *Saccharomyces cerevisiae:* Mechanism of α-Aminoadipate Reductase (Lys2) Involves Posttranslational Phosphopantetheinylation by Lys5†

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ABSTRACT: A key step in fungal biosynthesis of lysine, enzymatic reduction of α-aminoadipate at C_6 to the semialdehyde, requires two gene products in *Saccharomyces cerevisiae*, Lys2 and Lys5. Here, we show that the 31-kDa Lys5 is a specific posttranslational modification catalyst, using coenzyme A (CoASH) as a cosubstrate to phosphopantetheinylate Ser_{880} of the 155-kDa Lys2 and activate it for catalysis. Lys2 was subcloned from *S. cerevisiae* and expressed in and purified from *Escherichia coli* as a full-length 155-kDa enzyme, as a 105-kDa adenylation/peptidyl carrier protein (A/PCP) fragment (residues 1−924), and as a 14-kDa PCP fragment (residues 809−924). The apo-PCP fragment was covalently modified to phosphopantetheinylated holo-PCP by pure Lys5 and CoASH with a K_m of 1 μM and k_{cat} of 3 min^{-1} for both the PCP and CoASH substrates. The adenylation domain of the A/PCP fragment activated *S*-carboxymethyl-L-cysteine (k_{cat}/K_m = 840 mM^{-1} min^{-1}) at 16% the efficiency of L-α-aminoadipate in [^{32}P]PP_i/ATP exchange assays. The holo form of the A/PCP 105-kDa fragment of Lys2 covalently aminoacylated itself with [^{35}S]*S*-carboxymethyl-L-cysteine. Addition of NADPH discharged the covalent acyl-S-PCP Lys2, consistent with a reductive cleavage of the acyl-S-enzyme intermediate. These results identify the Lys5/Lys2 pair as a two-component system in which Lys5 covalently primes Lys2, allowing α-aminoadipate reductase activity by holo-Lys2 with catalytic cycles of autoaminoacylation and reductive cleavage. This is a novel mechanism for a fungal enzyme essential for amino acid metabolism.

The essential amino acid lysine is biosynthesized by two completely distinct pathways in prokaryotes and plants versus lower eukaryotes. In bacteria (including cephamycin-producing actinomycetes), this six-carbon dibasic amino acid is elaborated via the diaminopimelate (DAP)[1] pathway with decarboxylation of DAP drawing off the lysine final product. This pathway has drawn substantial attention because DAP is a key component in bacterial peptidoglycan assembly and transpeptidative cross-linking (*1*). The DAP pathway enzymes are therefore targets for new antibiotic development (*2*).

In higher fungi, including *Saccharomyces cerevisiae* and *β*-lactam antibiotic producers such as *Penicillium chrysogenum and Acremonium chrysogenum* (*3*), the lysine biosynthetic pathway is routed from α-ketoglutarate through homocitrate, homoaconitate, and homoisocitrate (*4*). From homoisocitrate, oxidative decarboxylation to 2-ketoadipate and then transamination introduce what becomes the C_2 amino group of lysine in α-aminoadipate. α-Aminoadipate, with analogy to DAP in the bacterial biosynthetic pathway, is a branch point metabolite, acting both as an essential precursor for penicillins in the ACV (L-*δ*-(α-aminoadipoyl)-cysteine-D-valine) tripeptide synthetase step (*5*) and as a progenitor of the essential amino acid lysine. The lysine route requires a reduction of the C_6 carboxylate of α-aminoadipate as well as an amination step. The reduction occurs via the action of α-aminoadipate reductase, the product of the *LYS2/LYS5* genes, to yield α-aminoadipate semialdehyde. α-Aminoadipate semialdehyde, in turn, condenses with glutamate to form saccharopine, which is hydrolyzed to the final products lysine and α-ketoglutarate (Figure 1).

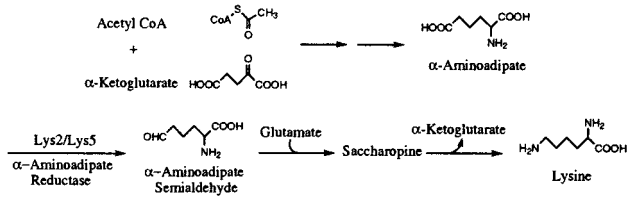

FIGURE 1: Schematic of the lysine biosynthetic pathway in fungi (except certain Phycomycetes, including *S. cerevisiae*). α-Aminoadipate reductase, product of the *LYS2* and *LYS5* genes, reduces α-aminoadipate to α-aminoadipate semialdehyde.

The *LYS2* gene has been cloned from *S. cerevisiae* (*6*) and more recently from *Schizosaccharomyces pombe* (*7*), the penicillin producer *P. chrysogenum* (*3*), and the opportunistic pathogen *Candida albicans* (*8*). The *S. cerevisiae* Lys2 has 1392 amino acids and a molecular weight of 155 kDa, with homologies to non-ribosomal peptide synthetases in the amino two-thirds of the protein (*3*, *7*, *8*) and nicotinamide-

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[1] Abbreviations: DAP, diaminopimelate; ACV, L-*δ*-(α-aminoadipoyl)-cysteine-D-valine; PPTase, phosphopantetheinyl transferase; CoASH, coenzyme A; ACP, acyl carrier protein; PKS, polyketide synthase; PCP, peptidyl carrier protein; NRPS, non-ribosomal peptide synthetase; Ppant, phosphopantetheine; A/PCP, adenylation/peptidyl carrier protein; S-CMCys, *S*-carboxymethyl-L-cysteine.

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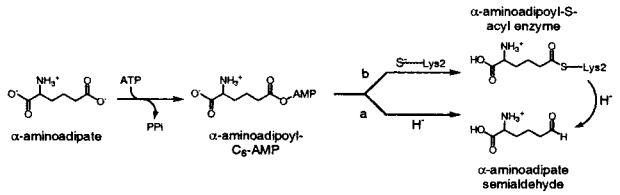
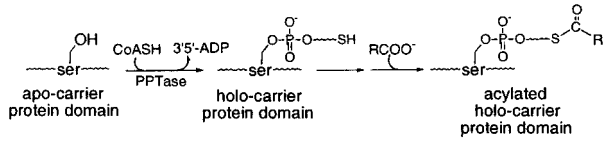
Scheme 1



Scheme 2



dependent dehydrogenases in the last third of the protein (*9*). It has been known from early enzymatic studies (*10, 11*) that the reduction of α-aminoadipate to the C_6 semialdehyde involves concomitant stoichiometric cleavage of ATP to AMP and pyrophosphate and that the reaction most likely involves an α-aminoadipoyl-C_6-AMP mixed anhydride. This enzyme-bound species was proposed to be reduced by NADPH to release AMP and the C_6 semialdehyde (Scheme 1, path a).

In *S. cerevisiae*, it is also known that the *LYS5* gene product is required for α-aminoadipate reduction (*12, 13*). Proposals have been advanced that this small (272 amino acid, 31 kDa) Lys5 protein is a required subunit in a Lys2/Lys5 heterodimer, although no direct evidence has been presented for such a two-subunit organization (*11, 14*).

During the course of studies on posttranslational priming of the apo forms of non-ribosomal peptide synthetases such as enterobactin synthetase (*15*), we recently described a newly identified family of enzymes, the phosphopantetheinyl transferases (PPTases) (*16*). PPTases add the phosphopantetheinyl moiety from coenzyme A (CoASH) to a conserved serine side chain in the acyl carrier protein (ACP) domain of polyketide synthases (PKS) and the peptidyl carrier protein (PCP) domain of non-ribosomal peptide synthetases (NRPS) (Scheme 2). This posttranslational priming converts inactive apo forms of both PKS and NRPS into active holo forms that can now covalently load activated monomers onto the phosphopantetheine (Ppant) thiol groups. Acyl chain elongation then proceeds from upstream to downstream carrier protein domains (*17*). Homology searching indicated that the 31-kDa Lys5 protein of *S. cerevisiae* could be a PPTase priming catalyst (*16*). Furthermore, Lys2 possessed a 120-amino acid stretch (residues 809−924, see Figure 2) that had the hallmarks of a consensus apo-PCP domain.

In this work we have overproduced and purified *S. cerevisiae* Lys5, fragments of Lys2, and full-length Lys2 in *E. coli* and employed them to validate that Lys5 is a specific PPTase for apo-Lys2. We reformulate the mechanism of α-aminoadipate reductase, Lys2, as the hydride-mediated reductive decomposition of a covalent α-aminoadipoyl-S-PCP acyl enzyme intermediate (Scheme 1, path b).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Materials. Competent cells of *E. coli* strains DH5α and BL21(DE3) were purchased from Gibco BRL. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. The expression vectors pET22b and pET28b were purchased from Novagen. [^{3}H]CoASH (200 Ci/mol with 70% of radioactivity in Ppant of CoASH) was prepared by DuPont New England Nuclear and purified by previously described methods (*16*). [^{35}S]*S*-Carboxymethyl-L-cysteine (1000 Ci/mmol) was custom-synthesized by DuPont New England Nuclear. Unlabeled CoASH, L-α-aminoadipate, D-α-aminoadipate, *S*-carboxymethyl-L-cysteine, DL-α,ϵ-diaminopimelate, adipate, L-glutamate, NADPH, o-aminobenzaldehyde, tris(2-carboxyethyl)phosphine (TCEP), and bacterial protease inhibitor cocktail were all purchased from Sigma.

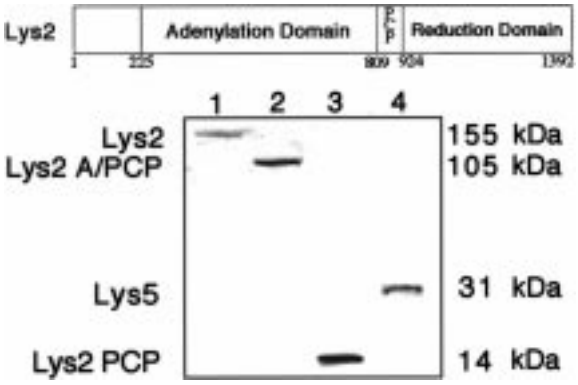


FIGURE 2: Schematic of the domain organization of Lys2 proteins (above) and 4−20% gradient SDS−PAGE gel (below) of *S. cerevisiae* Lys2, fragments of Lys2, and Lys5 overexpressed in and purified from *E. coli:* (lane 1) 155-kDa full-length Lys2, (lane 2) 105-kDa Lys2 fragment, amino acids 1−924, containing the A and PCP domains, (lane 3) 14-kDa Lys2 fragment, amino acids 809−924, containing the PCP domain, (lane 4) 31-kDa Lys5 protein.

Recombinant DNA Methods. Recombinant DNA techniques were performed as previously described (*18*). Purification of DNA fragments amplified by polymerase chain reaction (PCR), gel purification of DNA fragments, and plasmid DNA preparation were performed using QIAquick, QIAEX II, and QIAprep kits, respectively (QIAGEN). PCRs were carried out using *Pfu* DNA polymerase as described by the enzyme suppliers (Stratagene). DNA sequencing was performed by the Dana Farber Molecular Biology Core Facility (Boston, MA). DNA primers were obtained from Integrated DNA Technologies (Coralville, IA) *S. cerevisiae* (wild-type strain S288C) genomic DNA was purchased from Novagen. DNA of the *S. cerevisiae* open reading frame YBR115c (*LYS2*) was obtained from Research Genetics (Huntsville, AL).

Overproduction and Purification of Lys5, Lys2 PCP, Lys2 A/PCP, and Lys2. The gene encoding Lys5 was amplified from *S. cerevisiae* genomic DNA using the primer pairs: 5′-GAATTCCATATGGTTAAAACCACTGAAGTAGTAA-GCGAA-3′ and 5′-CCCAAGCTTTTATAAACCATCATTTT-CGATGAAATAATC-3′. The first primer introduced a *Nde*I restriction site (underlined), while the second primer introduced a *Hin*dIII restriction site into the PCR product. The *Nde*I/*Hin*dIII-digested PCR product was cloned into pET22b to give pET22b-Lys5 and this plasmid employed to transform *E. coli* BL21(DE3) cells.

Cultures of *E. coli* pET22b-Lys5 BL21(DE3) [2 L, 2x TY media with 50 μg/mL ampicillin] were grown at 25 °C

to an OD_{600} of 0.6. Cultures were induced with 1 mM isopropyl 1-thio-β-D-galactoside (IPTG) and grown an additional 3 h. Cells were resuspended in buffer A [50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT)] and lysed by two passages through a French pressure cell at 15 000 psi. After clarification by centrifugation (17000*g*), the lysate was fractionated by ammonium sulfate precipitation. The 30−50% fraction was loaded onto a Sephacryl S-100 column (2.5 × 115 cm) at a flow rate of 1 mL/min of buffer A. Fractions were analyzed by SDS−PAGE, and protein concentration was determined using the colorimetric Biorad protein assay.

The DNA encoding the PCP and A/PCP fragments of Lys2 was amplified using PCR methods from the *S. cerevisiae* ORF YBR115c (*LYS2*) using the following primers: (1) 5′-GAATTCCATATGGATAAACTACCATTG-3′, (2) 5′-CGGGAGCTCTCCACCCGATGATTTAATTCTGTCAATTTC-3′, and (3) 5′-GCGACTCACATATGACTACGAAAAGGTCTGGA-3′ (*Nde*I and *Xho*I sites underlined). Cloning into the *Xho*I site of pET22b adds a C-terminal hexahistidine tag to the overexpressed protein, appending the amino acid sequence LEHHHHHH. The PCR product obtained with primers 1 and 2 was cloned into pET22b to give pET22b-Lys2PCP. The PCR product obtained with primers 2 and 3 was cloned into pET22b to give pET22b-Lys2 A/PCP. Sequencing of this plasmid revealed two point mutations, which were converted back to wild type by the PCR technique splicing by overlap extension (*19*) employing the primer pairs: 5′-CCTGATCCAACTAAGAACTTGGGCT-3′, 5′-GCCCAAGTTCTTAGTTGGATCAGG-3′ and 5′-AAGTCCCGTTCTTTCACTTATCGC-3′, 5′-GCGATAAGTGAAAGAACGGGACTT-3′.

Cultures of *E. coli* BL21(DE3) transformants for the overexpression of Lys2 PCP and Lys2 A/PCP were grown, resuspended, and lysed as described above, except buffer B [5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9)] was used. Cell lysates were clarified by centrifugation and loaded onto charged 5-mL His·Bind columns (Novagen) at flow rates of 1.0 mL/min. The columns were then washed with 50 mL of buffer B followed by 30 mL of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl at pH 7.9). The proteins were eluted from the columns by addition of 0.5 M imidazole, 0.25 M NaCl, 10 mM Tris-HCl at pH 7.9. Fractions containing the proteins as analyzed by SDS−PAGE were pooled and dialyzed twice against 2 L of 50 mM Tris-HCl (pH 8.0), 2 mM DTT, and 5% glycerol. Protein concentrations were determined by the Biorad protein assay and from the calculated extinction coefficients at 280 nm of 6 970 M^{-1} for Lys2 PCP and 103 830 M^{-1} for Lys2 A/PCP (*20*).

The DNA encoding full-length Lys2 was amplified using PCR methods from the *S. cerevisiae* ORF YBR115c (*LYS2*) using the primers: 5′-GCGACTCACATATGACTAACGAAAAGGTCTGGA-3′ and 5′-CCCAAGCTTTTAAGCTGCTGCGGAGCTTCCACGAGCACC-3′ (*Hin*dIII site underlined). This PCR product was digested with *Nco*I/*Hin*dIII and cloned into pET28b to produce pET28b-Lys2end. pET22b-Lys2 A/PCP was digested with *Xba*I/*Sac*II and the 2880-base pair piece ligated into *Xba*I/*Sac*II-cut pET28b-Lys2end to yield pET28b-Lys2.

Cultures for the overexpression of Lys2 were prepared as described above for Lys5 except 1.7 mL of bacterial protease inhibitor cocktail (Sigma) was added to the 7 g cells resuspended in 35 mL of buffer A. After lysis and ammonium sulfate precipitation, the 30−50% fraction was dialyzed overnight against buffer C [25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT]. This sample was loaded onto a Q-Sepharose column (2.5 × 10 mL) at 1.0 mL/min. The column was washed with 150 mL of buffer C, and the following series of gradients were applied: 50 mL of 0 to 0.2 M KCl (in buffer C), 250 mL of 0.2 to 0.4 M KCl, and 50 mL of 0.4 to 1 M KCl. Lys2 eluted at approximately 0.3 M KCl. Fractions containing Lys2, identified by α-aminoadipate-dependent ATP-pyrophosphate exchange activity, were pooled and concentrated, and the buffer was exchanged for buffer C + 5% glycerol. This sample was loaded onto a Sephacryl S-300 column (2.5 × 115 cm) at 1.0 mL/min. Fractions containing Lys2 were pooled and concentrated. Protein concentrations were determined by the Biorad protein assay and from the calculated extinction coefficient at 280 nm of 169 380 M^{-1} for Lys2.

Assay for Apoprotein to Holoprotein Conversion by Transfer of [^{3}H]Phosphopantetheine. The radioassay for determination of phosphopantetheinyl transferase activity was performed as described previously (*16*). In a final volume of 100 μL, substrate (Lys2 PCP or A/PCP) was incubated with 75 mM MES-acetate (pH 5.5), 10 mM $MgCl_2$, 5 mM DTT, 104 μM [^{3}H]CoASH, and Lys5. Reactions were initiated by addition of 20 nM Lys5, incubated at 37 °C for the specified time, and quenched with 0.8 mL of 10% trichloroacetic acid (TCA) with bovine serum albumin (BSA) (375 μg) added as a carrier. Precipitated proteins were centrifuged and washed three times with 10% TCA. The protein pellet was dissolved in 150 μL of 1 M Tris base and the amount of incorporated [^{3}H]Ppant quantified by liquid scintillation counting.

The assay for determination of the K_m of Lys5 for Lys2 PCP was performed with 20 nM Lys5 for 20 min. Reactions to determine the K_m of Lys5 for CoASH were performed with 20 nM Lys5 and 20 μM Lys2 PCP for 20 min.

For the autoradiograph depicted in Figure 4, 100-μL reactions were prepared as described above with 175 nM Lys5, 20 μM Lys2 PCP, or 10 μM Lys2 A/PCP and 75 μM [^{3}H]CoASH (200 Ci/mol) for 30 min at 37 °C. Reactions were quenched with 10% TCA without BSA and washed, and precipitated protein was resuspended in 25 μL of SDS sample buffer with 3 μL of 1 M Tris base. Samples were loaded onto a 4−20% gradient polyacrylamide gel, electrophoresed, Coomassie-stained, destained, and dried. The dried gel was exposed to a Fuji BAS-IP TR 2040 phosphoimager plate overnight and visualized with a Fujix BAS 1000 phosphoimager.

Preparation of Samples for Mass Spectrometry. Reactions for analysis by mass spectrometry were prepared as described above using final concentrations of 500 nM Lys5, 50 μM Lys2 PCP, and 100 μM unlabeled CoASH. MALDI-TOF mass spectrometry was performed at the Howard Hughes Medical Institute Biopolymers Facility at Harvard Medical School.

ATP/[^{32}P]PP_i Exchange Activity. ATP-pyrophosphate exchange was assayed as described previously (*21*) with minor modifications. Reactions (100 μL) contained 75 mM Tris-HCl (pH 8.8), 10 mM $MgCl_2$, 5 mM DTT, 5 mM ATP, and enough Lys2 A/PCP to maintain linear initial velocity

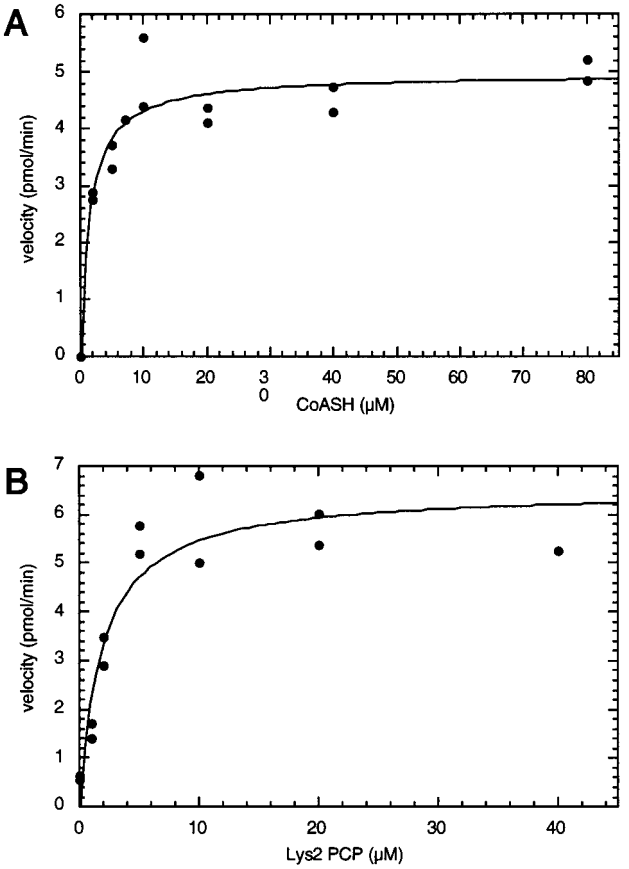


FIGURE 3: Velocity versus substrate concentration plots for the reaction of Lys5 with varying amounts of (A) CoASH with Lys2 PCP at 20 μM and (B) Lys2 PCP with CoASH at 104 μM. Both sets of reactions were carried out at pH 5.5 with 20 nM Lys5 for 20 min as described in Materials and Methods. Data shown is the average of two experiments. Lys5 exhibited a K_m of 1 μM and k_{cat} of 3 min^{-1} toward Lys2 PCP and a K_m of 1 μM and k_{cat} of 3 min^{-1} toward CoASH.

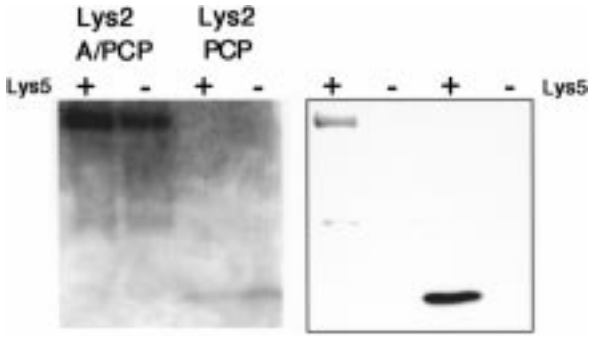


FIGURE 4: 4−20% gradient SDS−PAGE gel (left) and autoradiogram (right) demonstrating phosphopantetheinylation with [^{3}H]-CoASH of Lys2 PCP and Lys2 A/PCP by Lys5. Reactions were carried out at pH 7.5 for 30 min with 175 nM Lys5, 75 μM [^{3}H]-CoASH (200 Ci/mol), and either 20 μM Lys2 PCP or 10 μM Lys2 A/PCP.

conditions (for L-α-aminoadipate 19.2 nM Lys2 A/PCP for 5 min, for D-α-aminoadipate 860 nM Lys2 A/PCP for 5 min, for *S*-carboxymethyl-L-cysteine 172 nM Lys2 A/PCP for 5 min, and for adipate 8.6 μM Lys2 A/PCP for 10 min). Reactions were initiated with 1 mM sodium [^{32}P]pyrophosphate (3.9 Ci/mol), quenched with 0.5 mL of a charcoal suspension (1.6% w/v activated charcoal, 0.1 M tetrasodium pyrophosphate, 0.35 M perchloric acid), and centrifuged. The charcoal pellet was washed twice with 0.8 mL of water and resuspended in 0.5 mL of water, and ^{32}P incorporation into ATP was quantified by liquid scintillation counting.

Radioassay for the Detection of Covalent Incorporation of [^{35}S]S-Carboxymethylcysteine into Lys2. A TCA precipitation assay was employed to detect loading of [^{35}S]*S*-carboxymethylcysteine onto Lys2. Reactions (100 μL) contained 75 mM MES (pH 7.5), 10 mM $MgCl_2$, 3 mM TCEP, 2 mM ATP, 0.5 mM CoASH, 400 μM [^{35}S]*S*-carboxymethylcysteine (380 Ci/mol), 14 nM Lys5, and 77 nM Lys2. Reactions were preincubated for 30 min at 37 °C to allow phosphopantetheinylation of Lys2 before initiation by addition of ATP and [^{35}S]*S*-carboxymethylcysteine. Reactions were quenched and counted as described above for the assay for apo to holo conversion.

Spectrophotometric Assay for the Formation of α*-Aminoadipate Semialdehyde*. α-Aminoadipate semialdehyde production was detected as described previously (*11*, *22*), where the cyclized form of α-aminoadipate semialdehyde, Δ^1-piperidine carboxylate, is trapped as the dihydroquinazolinium salt (λ_{max} = 460 nm, ϵ = 925 M^{-1} cm^{-1}). Reactions (200 μL) containing 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2.5 mM DTT, 5 mM L-α-aminoadipate, 500 μM CoASH, 15 nM Lys5, and 35 nM Lys2 were preincubated for 30 min at 37 °C to allow phosphopantetheinylation. Assays were initiated by addition of 2.5 mM ATP, 5 mM *o*-aminobenzaldehyde, and varying concentrations of NADPH and monitored for 3 min by absorbance at 460 nm in a Perkin-Elmer Lambda 6 UV−vis spectrophotometer.

RESULTS

Purification of Lys5 PPTase, Lys2 Fragments, and Lys2. Analysis of the 272-amino acid sequence of Lys5 revealed the [GXD...(F/W),XXKE(S/A/C)XXK] conserved residues characteristic of the PPTase enzyme family (*16*). Furthermore, in the 1392-amino acid sequence of *S. cerevisiae* Lys2, we noted the conserved residues of an amino acid-AMP ligase (adenylation domain), phosphopantetheinylation site (PCP domain), and NADPH binding motif (*6*, *7*, *9*, *23*). To examine these activities, Lys5, the PCP domain of Lys2 (amino acids 809−924), the adenylation and PCP domains of Lys2 (amino acids 1−924), and full-length Lys2 were cloned from *S. cerevisiae* DNA, overexpressed in *E. coli*, and purified (Figure 2). Lys5 and Lys2 were purified in their native forms, while the Lys2 PCP domain and Lys2 A/PCP double-domain fragment were expressed as C-terminal hexahistidine fusions and purified by nickel affinity chromatography. Lys5 (31 kDa) and the full-length Lys2 (155 kDa) were obtained in high purity in quantities of 0.2 and 0.7 mg/L of culture, respectively, from induced *E. coli* cells. Likewise, the PCP and A/PCP fragments of Lys2 were obtained in yields of 14 and 45 mg/L of culture.

Lys5 Catalyzes Phosphopantetheinylation of Lys2 PCP and Lys2 A/PCP. Covalent posttranslational phosphopantetheinylation of Lys2 fragments containing PCP domains was detected using [^{3}H]CoASH as a cosubstrate in a TCA precipitation assay. Lys5 demonstrated PPTase activity toward the 14-kDa Lys2 PCP fragment, with a K_m of 1 μM and a k_{cat} of 3 min^{-1}. Toward CoASH, Lys5 also exhibited a K_m of 1 μM and a k_{cat} of 3 min^{-1} (Figure 3).

The PPTase activity of Lys5 was also verified by autoradiography. The autoradiograph in Figure 4 demonstrates

Table 1: Kinetic Parameters for Amino Acid-Dependent ATP/[^{32}P]PP$_i$ Exchange Catalyzed by Lys2 A/PCP[a]

	K_m mM	k_{cat} min^{-1}	k_{cat}/K_m mM^{-1} min^{-1}
L-α-aminoadipate	0.47	2500	5300
D-α-aminoadipate	2.7	38	14
S-carboxymethyl-L-cysteine	0.43	360	840
adipate	10	0.27	0.027
(L,D)-diaminopimelate	> 30	ND	< 2 x 10^{-3}
L-glutamate	> 30	ND	< 2 x 10^{-3}

[a] Reactions were carried out in duplicate as described in Materials and Methods.

the ability of Lys5 to covalently label both the 14-kDa Lys2 PCP fragment and the 105-kDa Lys2 A/PCP fragment with the [^{3}H]phosphopantetheine moiety of [^{3}H]CoASH.

Conversion from apo to holo form was further validated by MALDI-TOF mass spectral analysis of Lys2 PCP (data not shown). The apo-Lys2 PCP fragment exhibited a mass of 14 381 Da (calculated mass 14 380 Da). After incubation with Lys5, the holo-PCP fragment displayed a mass of 14 738 Da (calculated 14 720 Da), having gained, within error, the expected 340 mass units of Ppant.

Adenylation Activity of Lys2 A/PCP. The 105-kDa Lys2 fragment containing the proposed N-terminal adenylation and PCP domains was employed to assess adenylation activity via amino acid-dependent ATP/[^{32}P]PP$_i$ exchange. As expected, L-α-aminoadipate was an efficient substrate for adenylation by Lys2 A/PCP (Table 1), but D-α-aminoadipate was competent at only about 1/400 the catalytic efficiency. The 4-thio analogue of L-α-aminoadipate, *S*-carboxymethyl-L-cysteine (S-CMCys), also stimulated ATP/PP$_i$ exchange, at 15% of the catalytic efficiency of the natural substrate, α-aminoadipate. The substantial activity of S-CMCys to support reversible aminoacyl-AMP formation suggested its utility as a radioactive substrate in the absence of readily available radiolabeled L-α-aminoadipate. DL-α,ϵ-diaminopimelate, adipate, and L-glutamate were extremely poor substrates for Lys2 A/PCP-catalyzed adenylation.

Lys2 A/PCP Is Covalently Loaded with [^{35}S]S-Carboxymethylcysteine. The above demonstration that Lys2 A/PCP is both active for α-aminoadipate-AMP formation and covalently primable with phosphopantetheine on its PCP domain led us to the next experimental test of mechanism. After Lys2 has been phosphopantetheinylated, we propose

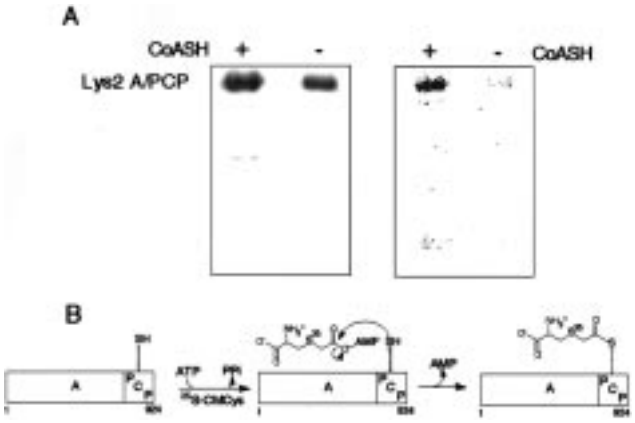


FIGURE 5: (A) 4−20% gradient SDS−PAGE gel (left) and autoradiogram (right) depicting CoASH-dependent covalent loading of Lys2 A/PCP with [^{35}S]*S*-carboxymethylcysteine. Reactions were carried out at pH 7.5 for 7 min and contained 145 nM Lys5, 8.6 μM Lys2 A/PCP, 100 μM [^{35}S]*S*-carboxymethylcysteine (230 Ci/mol), and, when present, 500 μM CoASH. (B) Schematic of aminoacylation reaction to form a thioester linkage of an amino acid, in this case [^{35}S]*S*-carboxymethylcysteine, to the phosphopantetheine tether of the Lys2 A/PCP PCP domain.

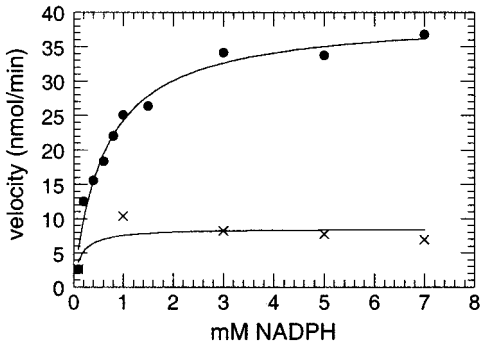


FIGURE 6: Velocity versus substrate concentration plot for reactions of Lys2 with NADPH and α-aminoadipate to form α-aminoadipate semialdehyde. Reactions were carried out at pH 7.5 with 35 nM Lys2, 15 nM Lys5, and, when present, 500 μM CoASH: (●) Lys2, Lys5, with CoASH; (×) Lys2, Lys5, without CoASH. Lys2 exhibited a K_m of 620 μM toward NADPH and k_{cat} of 670 min^{-1}.

that the Ppant terminal thiol attacks the aminoacyl adenylate, loading the amino acid onto Lys2 via a covalent thioester linkage to the holo-PCP domain (Figure 5B). The L-α-aminoadipate analogue [^{35}S]*S*-carboxymethyl-L-cysteine, prepared by custom synthesis, was employed to detect this loading in a TCA precipitation assay. Loading was observed to be proportional to Lys2 A/PCP concentration and saturable over time (data not shown). In addition, aminoacylation was demonstrated to be CoASH-dependent, as visualized by SDS gel electrophoresis and autoradiography (Figure 5A), demonstrating radioactive 105-kDa Lys2 A/PCP double-domain-labeled with the [^{35}S]S-CMCys.

Holo-Lys2 Produces α-Aminoadipate Semialdehyde. The formation of the product of reduction of α-aminoadipate, α-aminoadipate semialdehyde, can be monitored spectrophotometrically. From this assay, the K_m of Lys2 for NADPH was determined to be 620 μM and the k_{cat} for α-aminoadipate semialdehyde production to be 670 min^{-1} (Figure 6). Under identical conditions, but without CoASH, a low level of product was formed, presumably resulting from a small fraction of holo-Lys2 phosphopantetheinylated *in vivo* during overexpression in *E. coli*.

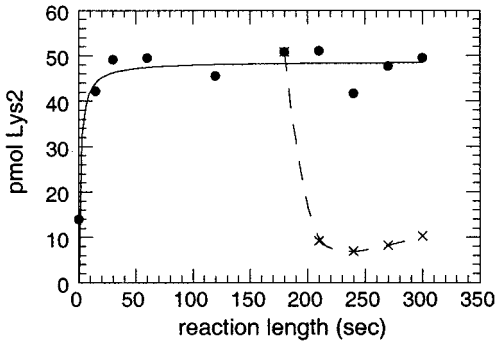
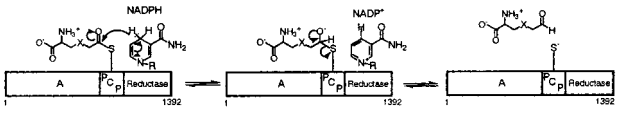


FIGURE 7: Loading of Lys2 with [^{35}S]*S*-carboxymethylcysteine and cleavage upon addition of NADPH. Data shown is the average of two experiments. Reactions were carried out with 14 nM Lys5, 77 nM Lys2, 400 μM [^{35}S]*S*-carboxymethylcysteine (380 Ci/mol), and 500 μM CoASH: (●) Lys2, Lys5, with CoASH, without NADPH; (×) Lys2, Lys5, with CoASH, addition of NADPH to 5 mM after 180 s.

Scheme 3



NADPH Addition Causes Cleavage of the [^{35}S]S-Carboxymethylcysteine-S-Lys2 Acyl Enzyme. If the aminoacyl-S-PCP acyl enzyme (Figure 5B) is an intermediate in catalytic turnover, then it should be reductively released by the cosubstrate NADPH (Scheme 3). A TCA precipitation assay was employed to assess whether NADPH can reductively release thioesterified [^{35}S]S-CMCys from Lys2. After preincubation with Lys5, holo-Lys2 rapidly accumulated the [^{35}S]S-CMCys acyl-S-PCP intermediate in the absence of NADPH, as detectable by TCA precipitation (Figure 7). On addition of NADPH at the 180-s time point and TCA precipitation at 300 s, the ^{35}S label was discharged, consistent with the reductive cleavage of the radioactive acyl-S-PCP form of Lys2 in the presence of NADPH.

DISCUSSION

This study reveals the molecular logic of the two-protein system of *S. cerevisiae* Lys2 and Lys5, found in *S. cerevisiae* and other fungi that produce lysine via the fungal homocitrate/aminoadipate route. The large size of Lys2 (155 kDa) is a reflection of at least three and possibly four component domains (Figure 2). Residues 1−224 are presently unassigned for function, while residues 225−808 constitute a typical 60-kDa adenylation (A) domain for aminoacyl-AMP formation. Residues 809−924 comprise the apo-PCP domain and 925−1392 the NADPH-utilizing reductase domain. In this work, we have assessed elements of function of the A domain, PCP domain, and complete Lys2 enzyme.

The ability to express and overproduce the 14-kDa PCP domain in *E. coli* provided the initial substrate to assay and validate the catalytic posttranslational modification function of *S. cerevisiae* Lys5. Lys5, also overproduced in and purified from *E. coli*, displayed strong homology to PPTases, which load an apo-PCP domain with the phosphopantetheinyl moiety of CoASH (*16*). Both covalent incorporation of tritiated phosphopantetheine catalyzed by Lys5 and mass spectrometric analysis confirmed holo-Lys2 PCP domain formation. These results established Lys5 as priming enzyme for Lys2 and were then corroborated using the larger, two-domain fragment, Lys2 A/PCP.

Lys5 appears to exhibit specificity toward apo-PCP domain substrates (data not shown). While we have not assessed the ability of Lys5 to prime the apo-ACP domains of either the yeast cytoplasmic type I fatty acid synthase (FAS) or the mitochondrial apo-ACP of type II FAS, Stuible et al. have recently noted that knockouts of FAS2, PPT2, and Lys5 have specific and nonoverlapping phenotypes, consistent with partner protein-specific priming by these three yeast PPTases (*24*).

In turn, the primed, holo form of Lys2 A/PCP was then a proper substrate to evaluate the next step in the proposed mechanism, activation of the amino acid α-aminoadipate and transfer onto the phosphopantetheinyl terminal thiol as a covalent acyl-S-PCP enzyme intermediate. Because radiolabeled aminoadipate was not commercially available, to demonstrate covalent attachment to Lys2 A/PCP we sought a surrogate amino acid that was both available in radiolabeled form and a substrate for the A domain. The A domain is conveniently assayed by amino acid-dependent [^{32}P]PP_i/ATP exchange which revealed that *S*-carboxymethyl-L-cysteine, an analogue of aminoadipate with C_4 replaced by sulfur, sustained a robust isotope exchange (Table 1). We then demonstrated formation of the acyl enzyme CMCys-S-Lys2 A/PCP using [^{35}S]S-CMCys as a substrate, dependent on prior phosphopantetheinylation with Lys5.

We next expressed the full-length 155-kDa *S. cerevisiae* Lys2 in *E. coli*, purified it to homogeneity, and demonstrated its activity, formation of α-aminoadipate semialdehyde (k_{cat} = 11 s^{-1}). Finally, we validated that the radiolabeled CMCys-S-enzyme (and correspondingly the α-aminoadipoyl-S-enzyme in normal turnover) is reductively cleaved by NADPH. Thus the catalytic logic for Lys2 to produce the C_6 aldehyde from C_6 α-aminoadipate is to reduce not the free acid nor the acyl-AMP but rather an acyl-thioester, covalently tethered at the PCP domain (Scheme 1b). This has clear formal mechanistic analogy to the reverse direction of the well-known glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*25*). In the case of GAPDH, the three-carbon aldehyde substrate reacts with an active site thiol, provided by a cysteine side chain, to reversibly form the thiohemiacetal tetrahedral adduct. Now, oxidation yields the acyl enzyme thioester intermediate (Scheme 3). The acyl group is phosphorylated by P_i in the GAPDH case, by AMP in the Lys2 back reaction.

With regard to catalytic strategy of Lys2, why convert the acyl-AMP intermediate to the acyl-S-enzyme intermediate before reduction, or for that matter why spend an ATP and not just reduce the free acid? For both Lys2 and GAPDH, from a thermodynamic perspective, thiohemiacetals are much easier to oxidize than free aldehydes and also to reduce than free acids. The input of energy then is required to convert the resonance-stabilized COO^- of substrates to activated derivatives (RCOO-AMP, RCOO-PO_3) to accumulate to high mole fraction the RCOS-enzyme that reacts with hydride from reduced nicotinamide coenzyme.

The organization of Lys2 is unusual for a non-ribosomal peptide synthetase, with a reductase domain fused downstream of the prototypic A/PCP domain pair. In a formal sense, the reductase domain is the element catalyzing release of the covalent acyl-enzyme intermediate. In most NRPS and

PKS multimodular enzyme systems, acyl chain release is believed to be catalyzed by a thioesterase (TE) domain (*26*, *27*). The chain release is then generally hydrolytic with either water or an electron-rich internal group (e.g., a side chain −OH) in the acyl chain acting as the kinetically competent nucleophile to release the completed natural product as a free acid (e.g., vancomycin, ACV) (*28*, *29*), cyclic lactone (e.g., erythromycin) (*27*), or cyclic lactam (e.g., bacitracin) (*26*). In the Lys2 case, hydrolytic release of the C_6 aminoadipoyl-S-PCP-enzyme would be an energy-wasting futile cycle and hydrolysis is probably scrupulously avoided. Reductive release rather than hydrolytic release generates aldehyde rather than acid product. One does not expect reductive release to be the favored acyl enzyme chain cleavage route in the termination steps of most NRPS and PKS catalysts because, in general, free aldehydes are more thermodynamically activated and act as uncontrollably reactive carbonyl groups in biological milieus. In the Lys2 case, the α-aminoadipate semialdehyde cyclizes rapidly and essentially quantitatively to the cyclic imine, Δ^1-piperidine carboxylate, suppressing adventitious reactivity.

Homology searches for other NRPS catalysts with reductase domains fused downstream of A/PCP paired domains turn up two additional examples, the saframycin biosynthetic system (*9*) and the *nrp* cluster in the *Mycobacterium tuberculosis* genome (*30*). The substrates and products of the mycobacterial *nrp* are as yet unknown, but in saframycin biosynthesis an Ala-Gly-Tyr-Tyr-CHO is likely to be reductively released by this route prior to intramolecular cyclization to a six-ring hemiaminal structure in the antitumor antibiotic (*30*).

A second unusual feature of Lys2 as a non-ribosomal peptide synthetase type catalyst is its regiospecific activation of its amino acid substrate. Activation of L-α-aminoadipate not at the α (C_2) carboxylate but rather at the distal ϵ (C_6) carboxylate results in a relaxed specificity toward the α carbon chirality, since D-α-aminoadipate is reversibly activated as the aminoacyl-AMP (Table 1) as is the desamino substrate adipate. In this regard, the adenylation domain of Lys2 behaves more like a fatty acid-activating domain than a typical amino acid-activating domain.

An analogous fungal α-aminoadipate-activating domain with specificity for AMP attachment at substrate C_6 is ACV synthetase (*5*), which produces the penicillin precursor tripeptide L-*δ*-(α-aminoadipoyl)-cysteine-D-valine. As in Lys2, in ACV synthetase, the adenylation domain for L-α-aminoadipate is at the N-terminus of the first module and also activates *S*-carboxymethylcysteine. It is conceivable that the A domains of ACV synthetase and Lys2 are exchangeable. The A domain of Lys2 may be prototypic for activation and incorporation of nonproteinogenic *β* and *γ* amino acids by many NRPSs to create nonstandard peptide connectivity, for example, the *β*-alanyl residues in the antitumor antibiotic bleomycin (*31*).

With the molecular logic of Lys2 revealed as an NRPS with a reductase domain fused in frame and of Lys5 as a priming PPTase, this information may be useful for design of inhibitors as antifungal agents since Lys2 and Lys5 are essential for fungal growth (*12*). Indeed, the recent sequencing of the *LYS2* homologue from the human pathogen *C. albicans* (*8*) reveals it has the same domain organization as *S. cerevisiae* Lys2.

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