Pyridoxal-5′-phosphate (PLP, the active form of vitamin B₆) is an essential cofactor that is used for various reactions including α/β eliminations, retro-aldol cleavages, transaminations and racemizations (Toney 2011). Two pathways for PLP biosynthesis have been described. The 1-deoxy-D-xylulose-5-phosphate (DXP)-independent pathway is found in most bacteria, archaea and eukaryotes, while a DXP-dependent pathway is found in some bacteria including the proteobacteria, firmicutes, chlorobi, cyanobacteria and aquificae (Tanaka et al. 2005; Fitzpatrick et al. 2007; Mukherjee et al. 2011; Rosenberg et al. 2017). The DXP-dependent pathway synthesizes PLP from erythrose-4-phosphate, glyceraldehyde-3-phosphate and pyruvate in a series of seven enzymatic steps.

Formation of the pyridine heterocyclic ring in this pathway is catalyzed by the pyridoxine-5′-phosphate synthase (E.C. 2.6.99.2), which is encoded by pdxJ in Escherichia coli (Figure 1). In contrast, the DXP-independent pathway uses two enzymes to create the heterocyclic pyridine ring of PLP from glutamine, glyceraldehyde-3-phosphate and ribose-5-phosphate (Mukherjee et al. 2011) (Figure 1). Salvage of B₆ vitamers requires enzymes that are conserved across organisms. Notably, pyridoxine phosphate oxidase (PNPO, PdxH; E.C.:1.4.3.5) is required for de novo synthesis in the DXP-dependent pathway and is required for salvage in organisms using either pathway.

The two enzymes unique to the DXP-independent pathway to PLP form a complex comprised of a glutaminase and a PLP synthase subunit (EC 4.3.3.6) (Burns et al. 2005; Raschle et al. 2005; Strohmeier et al. 2006). In general, the glutaminase subunit liberates ammonia from glutamine and delivers it to the PLP synthase subunit, where it combines with glyceraldehyde-3-phosphate and ribose-5-phosphate to form PLP (Burns et al. 2005; Raschle et al. 2005; Strohmeier et al. 2006). Similar ammonia tunneling is a feature of several multi-subunit synthase enzymes that use glutamine as a source of ammonia including, carbamoyl-phosphate synthetase (E.C. 6.3.5.5), anthranilate synthase (E.C. 4.1.3.27), amino-deoxychiorominate synthase (E.C. 2.6.1.85) and imidazole glycerol...
phosphate synthase (E.C. 4.3.2.-) (Makoff and Radford 1978; Klem and Davison 1993; Romero et al. 1995; Viswanathan et al. 1995). In cases where it has been tested, the glutaminase subunit is dispensable both in vitro and in vivo when there are high levels of ammonia (Huang et al. 2001; Belitsky 2004).

Several PLP synthase enzymes from bacteria, archaea, yeast and plants, have been characterized biochemically with and without the associated glutaminase (Dong et al. 2004; Burns et al. 2005; Raschle et al. 2005; Gengenbacher et al. 2006; Strohmeier et al. 2006; Zein et al. 2006; Raschle et al. 2007; Hanes et al. 2008; Neuwirth et al. 2009; Raschle et al. 2009; Moccand et al. 2011; Robinson et al. 2016; Rodrigues et al. 2017). A variety of names have been used for the genes encoding PLP synthase and glutaminase enzymes, e.g., SNZ/pdxS/pdx1, SNO/pdxT/pdx2, respectively. The varied nomenclature has complicated the analyses and comparison of these enzymes across organisms. For simplicity the names for the glutaminase SNZ1 and synthase SNO1 in vivo and SNO2/pdxT/pdx2 in vitro have been used.

SNZ1 encodes a PLP synthase that uses glyceraldehyde-3-phosphate, ribose-5-phosphate, but the need for exogenous pyridoxine, and showed that SNZ2/3 could have a role in thiamine synthesis and/or metabolism, although to our knowledge this idea was not directly tested experimentally.

A long-time interest in metabolic network structure and robustness, and redundancy in vitamin biosynthesis, with a focus on thiamine (reviewed in (Downs 2006; Koenigschnie and Downs 2010)), prompted us to explore the functional roles of the Sno/Snz proteins in S. cerevisiae. Herein we assimilate the genetic and biochemical characterization of the SNZ paralogs to lay the groundwork for future work on the integration of the biosynthesis of two essential cofactors, thiamine and PLP, in Salmonella enterica and S. cerevisiae. The data presented confirmed that SNZ3 encodes a PLP synthase, quantified the transcriptional regulation of SNZ2 and SNZ3 by thiamine, and showed that strains lacking SNZ2 and SNZ3 required thiamine supplementation for growth. Together these data provide the first report of a functional role for these genes in vivo.

### MATERIALS AND METHODS

#### Strains, Media and Chemicals

**Yeast:** *S. cerevisiae* strains used in this work were derived from YJF153 (MATa HO:dsdAMX4, a haploid derivative of YPS163) (Li and Fay 2017), and the relevant genotypes are listed in Table 1. *S. cerevisiae* strains were routinely grown on rich medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose and 20 g/L agar (YPD). Two variations on defined medium were used to monitor vitamin requirements. Synthetic defined media (SD, SG) contained 1.7 g/L yeast nitrogen base without amino acids or nitrogen (YNB, Sunrise Science catalog no. 1500-100) or the respective drop-out as indicated (YNB-Pyridoxine, YNB-Thiamine, YNB-Pyridoxine-Thiamine) (Sunrise Science), 5 g/L ammonium sulfate, 20 g/L agar and either 20 g/L dextrose or 30 g/L glycerol as carbon source.

A minimal medium (Minimal Vitamin Dextrose; MVD) contained 1.7 g/L YNB-Vitamins (Sunrise Science), biotin (0.002 mg/L), and D-pantothenic acid hemicalcium salt (0.4 mg/L), 5 g/L ammonium sulfate, 20 g/L agar and 20 g/L dextrose as carbon source. Thiamine (0.4 mg/L) and/or pyridoxine (0.4 mg/L) were added as indicated. Antibiotics used for deletion marker selection were added to the
following final concentrations in YPD: 400 mg/L geneticin (G-418 sulfate), 200 mg/L Hygromycin B, and 100 mg/L nourseothricin sulfate (clonNAT) (Gold Biotechnology). A lower concentration of 200 mg/L geneticin was used for maintenance of strains with G-418 resistance. 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) was purchased from LabSeeker, Inc.

Bacteria: Media for bacterial growth were Nutrient Broth (NB) containing 8 g/L Difco Nutrient broth and 5 g/L NaCl, lysogeny broth (LB), or superbroth (SB; 32 g/L tryptone (Fisher Scientific), 20 g/L yeast extract (Fisher Scientific), 5 g/L NaCl with 0.05 N NaOH). Solid media contained 1.5% agar. Antibiotics were added at the following concentrations in rich media, unless otherwise indicated: kanamycin (Kn), 50 mg/L; chloramphenicol (Cm), 20 mg/L; ampicillin (Ap), 100 mg/L. Minimal media was no-carbon E medium (NCE) (Vogel and Bonner 1956) with 1 mM MgSO_4, 0.1x trace minerals (Balch et al. 1979), with either glucose (11 mM) or glycerol (22 mM) (Fisher Scientific) as a sole carbon source. Minimal medium with low nitrogen was no-carbon and nitrogen (NCN) (Davis et al. 1980) with 1 mM glutamine and glucose or glycerol as sole carbon source. All strains of *S. enterica* are derived from strain LT2 and their relevant genotypes are described in Table 1. Chemicals were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise indicated.

Genetic Techniques

In-frame deletions of genes in *S. enterica* were created with Lambda-Red recombineering as described (Datsenko and Wanner 2000). Insertions were reconstructed by transduction into DM7080 with the high-frequency generalized transducing mutant of bacteriophage P22 (HT105/1, int-201) (Schmieger 1972). Primers used to generate these deletions are listed in Table 1.

### Table 1 – Strains, Plasmids and Primers

| Strain Number | Genotype          | Saccharomyces cerevisiae          |
|---------------|-------------------|-----------------------------------|
| YJF153        | WT                |                                   |
| DMy49         | snz2::kanMX-loxP  |                                   |
| DMy51         | snz2::kanMX-loxP  |                                   |
| DMy52         | snz2::natMX-loxP  | snz2::hphMX-loxP                   |
| DMy53         | snz2::kanMX-loxP  |                                   |
| DMy54         | snz2::kanMX-loxP  | snz3::hphMX-loxP                   |
| DMy55         | snz1::kanMX-loxP  |                                   |
| DMy56         | snz1::kanMX-loxP  | snz2::hphMX-loxP                   |
| DMy57         | snz2::kanMX-loxP  |                                   |
| S. enterica   |                   |                                   |
| DM7080        | ΔaraCBAD          |                                   |
| DM15839       | ΔaraCBAD pdxJ662::Kn | pSU18                         |
| DM15840       | ΔaraCBAD pdxJ662::Kn | pDM1595                        |
| DM15843       | ΔaraCBAD pdxJ662::Kn | pDM1595 pBAD24                |
| DM15844       | ΔaraCBAD pdxJ662::Kn | pDM1595 pDM1596               |

**Plasmid Name**

- pSU18
- pBAD24
- pDM1595
- pDM1596

**Primer Name**

- CM
- AGP3R
- RPDR3
- pdxJ F
- pdxJ R
- 5’ SNZ2 Saci
- 3’ SNZ2 XbaI
- SNZ1 F
- SNZ1 R
- SNZ2 F
- SNZ2 R
- SNO1 F
- SNO1 R
- 5’ SNO2 Ncol 2
- 3’ SNO2 NsiI 2
- 5’ SNO2 NheI
- 3’ SNZ NcoI
- ALG9 qRT-PCR F
- ALG9 qRT-PCR R
- UBC6 qRT-PCR F
- UBC6 qRT-PCR R
- SNZ2/3 qRT-PCR F
- SNZ2/3 qRT-PCR R

Underlining identifies an added ribosome binding site (RBS), bold letters represent start codon.
Gene disruptions in S. cerevisiae were made using a described gene replacement method (Hegemann and Heick 2011). Antibiotic cassettes were amplified from the appropriate plasmid using primers listed in Table 1. Five μg of purified DNA was transformed into S. cerevisiae by incubating cells suspended in a mixture of 33% polyethylene glycol 3350 (PEG 3350), 100 mM lithium acetate, and 0.28 mg/mL salmon sperm DNA at 42°C for 90 min. The transformed cells were recovered in YPD for 3 hr with shaking at 30°C and plated to YPD with the appropriate antibiotic. Colonies that grew on the plates after three days were streaked onto selective media and insertions were confirmed by colony PCR. Insertions in SNZ2 and SNZ3 were distinguished by PCR using gDNA as a template and AGP3R, RPD3R, and CM primers listed in Table 1.

Molecular Techniques

Plasmids were constructed using standard molecular techniques. Plasmid DNA was isolated using the PureYield Plasmid MiniPrep System (Promega, Madison, WI). Q5 DNA polymerase (New England Biolabs, Ipswich, MA) was used to amplify DNA with primers synthesized by Integrated DNA Technologies, Coralville, IA or Eton Bioscience, Inc., Research Triangle Park, NC. PCR products were purified using the PCR purification kit (Qiagen, Venlo, Limburg, The Netherlands). Restriction endonucleases were purchased from New England Biolabs, Ipswich, MA, and ligase was purchased from ThermoScientific, Waltham, MA.

Growth Analysis

Bacteria: Growth of S. enterica strains were monitored at OD600 in 96 well plates with a BioTek ELx808 plate reader. Strains were grown overnight in NB with Cm or Ap as indicated and inoculated at 1% into 96 well plates with a BioTek ELx808 plate reader. Strains were grown for 24 hr in SD with shaking at 30°C and plated to SD with shaking (200 rpm) to an OD600 of 0.6. The temperature was lowered to 30°C, arabinose added to a final concentration of 0.2% and cells were incubated for 19 hr prior to harvesting by centrifugation. The cell pellet was resuspended in Buffer A (50 mM HEPES, 300 mM NaCl, 20 mM Imidazole, pH 7.5 at 4°C) with DNase (0.025 mg/mL), lysozyme (1 mg/mL) and phenylmethylsulfonyl fluoride (0.1 mg/mL) and kept on ice for one hour. The cell suspension was lysed at 20 kpsi using a Constant Systems Limited One Shot (United Kingdom), and cell lysate was cleared at 48,000 x g for 50 min at 4°C. The cell-free extract was passed through a 0.45 μm PVDF filter (Millipore) and injected onto a precolumnated 5 mL HisTrap HP Ni-sepharose column. The column was washed with 5 column volumes of Buffer A, followed by 5 column volumes of 4% Buffer B (50 mM HEPES, 300 mM NaCl, 500 mM Imidazole, pH 7.5 at 4°C) and finally a gradient of Buffer B from 4 to 100% over 10 column volumes. Fractions containing Snz3p were concentrated, rTEV protease was added at a 50:1 protein to rTEV ratio and the mixture set for 3.5 hr at room temperature before it was dialyzed into Buffer A overnight at 4°C with 3 buffer changes. The tagless protein was separated from His6-Snz3p and His6-rTEV by gravity column chromatography with HisPur Ni-NTA resin. Snz3p was concentrated by centrifugation using a 10 kD filter (Millipore), exchanged into a 50 mM HEPES buffer, pH 7.5, with 10% glycerol using a PD10 column (GE Healthcare), flash-frozen in liquid nitrogen and stored at -80°C until use. Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce) with bovine serum albumin as a standard. The Snz3p preparation was >98% pure based on densitometry.

PLP synthase assay

Snz3p was thawed and dialyzed into assay buffer (50 mM Tris-HCl, pH 8.0). Reactions were performed in a buffer of 50 mM Tris-HCl, pH 8.0 at 37°C with ammonium sulfate (10 mM), D/L glyceraldehyde-3-phosphate, ribose-5-phosphate and Snz3p (Raschle et al. 2005; Neuwirth et al. 2009).
The lack of any auxotrophy indicated the strain had the functional wild type allele of the transcriptional regulator S. cerevisiae (S288C) has a mutant allele of abhorrent expression of the thiamine regulon (Brion et al., 2014). The former point is relevant in that the status of the YJF153 strain of S. cerevisiae was chosen for use because it i) has a wild type allele of the transcriptional regulator THI3, and ii) has no auxotrophy. The former point is relevant in that the status of THI3 impacts expression of genes in the thiamine regulon, which includes SNZ2 and SNZ3, due to its activity as a co-activator with Thi2p (Rodriguez-Navarro et al., 2002; Nosaka et al., 2005; Mojzita and Hohmann, 2006; Brion et al., 2014). The standard laboratory strain of S. cerevisiae (S288C) has a mutant allele of THI3 which results in abhorrent expression of the thiamine regulon (Brion et al., 2014). The lack of any auxotrophy indicated the strain had the functional metabolic network needed to dissect metabolic interactions and detect often subtle connections between pathways.

Eight strains (single, double and triple mutants) were constructed to query the role of the SNZ paralogs. Standard yeast drop-out media with glucose or glycerol were used and the data for seven of the mutants are shown in Figure 2. Growth of an snz2 strain was indistinguishable from the snz2 strain under these conditions (data not shown). Consistent with previous observations, SNZ1 was required for growth in dextrose medium lacking only pyridoxine (SD-PN). However, when thiamine was also excluded (SD-PN-Thiamine), a single functional copy of any of the SNZ paralogs allowed growth. These data were consistent with a model where each PLP synthase had the capacity to generate sufficient PLP for growth, but the regulation of SNZ1,2,3 by thiamine prevented them from contributing to PLP synthesis in its presence. The expression of SNZ2 or SNZ3 in trans had been shown to complement an snz1 mutant, but this is the first demonstration that at chromosomal levels, either SNZ2 or SNZ3 was sufficient for PLP synthesis that allowed optimal growth.

A similar analysis was done on media with glycerol rather than dextrose to compare fermentative vs. respiratory lifestyles (Figure 2B). The data were generally similar, with two differences noted. First, deletion of SNZ1 severely decreased, but did not eliminate growth on SG-PN medium. These data suggested that either the PLP requirement was lower during glycerol respiration, or the repression of SNZ2,3 in the presence of thiamine was weaker on glycerol. Second, we noted that the snz2,3 double mutant grew poorly on SD-PN-Thiamine, while it showed robust growth on SG-PN-thiamine, suggesting SNZ1 was not always sufficient for PLP synthesis.

**RESULTS AND DISCUSSION**

**SNZ1, 2, 3 paralogs have distinguishable roles in vivo**

Mutants of S. cerevisiae YJF153 lacking one or more of the SNZ paralogs were constructed to evaluate the role of these proteins in vivo (Table 1). The YJF153 strain of S. cerevisiae was chosen for use because it i) has a wild type allele of the transcriptional regulator THI3, and ii) has no auxotrophy. The former point is relevant in that the status of THI3 impacts expression of genes in the thiamine regulon, which includes SNZ2 and SNZ3, due to its activity as a co-activator with Thi2p (Rodriguez-Navarro et al., 2002; Nosaka et al., 2005; Mojzita and Hohmann, 2006; Brion et al., 2014). The standard laboratory strain of S. cerevisiae (S288C) has a mutant allele of THI3 which results in abhorrent expression of the thiamine regulon (Brion et al., 2014). The lack of any auxotrophy indicated the strain had the functional metabolic network needed to dissect metabolic interactions and detect often subtle connections between pathways.

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**Minimal vitamin medium clarifies the role of SNZ2 and SNZ3:** It was formally possible that additional nutrients in the drop out medium were complicating the interpretation of the nutritional phenotypes due to unanticipated metabolic interactions or regulation. The results above were readressed using medium with supplementation, rather than drop-out. Control experiments showed that S. cerevisiae strain YJF153...
grew well on synthetic dextrose medium with YNB-vitamins if both biotin and pantothenate provided. This medium was designated as Minimal Vitamin Dextrose medium (MVD) and used in the subsequent experiments. The growth of the eight strains described above was quantified in liquid MVD, with pyridoxine and/or thiamine added exogenously (Figure 3). Several points were taken from the resulting data. The parental wild type strain (YJF153), DMy53 (snz2), and DMy57 (snz3) strains had full growth on each medium (Figure 3A,B,C), while the triple mutant (DMy52) failed to grow in the absence of PN (Figure 3D). The other three strains that lacked SNZ1 grew in MVD, supporting the conclusion that either isozyme was sufficient for PLP synthesis in the absence of repression by thiamine. However, the strains that depended on SNZ2 and/or SNZ3 for PLP synthesis, failed to grow if thiamine was present in the medium (Figure 3 E,F,G). To verify the explanation that the lack of growth was due to transcriptional repression caused by thiamine, transcript levels of SNZ2 and SNZ3 were determined by qRT-PCR. The relative expression level of SNZ2/3 in YJF153 grown in MVD compared to MVD containing thiamine, PN, or both was measured. The data in Figure 4 showed that the addition of thiamine repressed transcription of SNZ2/3 approximately 10-fold. The presence of PN did not affect this repression and had no detectable effect by itself. These data validated the model that the conditional auxotrophy of strains lacking SNZ1 in the presence of thiamine is due to the transcriptional repression of SNZ2 and SNZ3.

The data in Figure 3 shed new light on the role and limitation of SNZ1 function that was only hinted at by the data in Figure 2. The snz2,3 double mutant failed to grow on MVD medium (Figure 3H). These data showed the PLP synthase encoded by SNZ1 was not able to provide sufficient PLP synthesis for growth. Growth was restored by the addition of either thiamine or pyridoxine. The growth behavior of the snz2,3 double mutant was consistent with a scenario in which thiamine synthesis required PLP and Snz1p alone could not synthesize sufficient PLP to satisfy this requirement. In fact, in S. cerevisiae, synthesis of the 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) moiety of thiamine involves use of PLP as a substrate by a poorly characterized HMP-P synthase enzyme, Thi5p (Wightman and Meacock 2003). Consistently, exogenous addition of HMP (but not the thiazole moiety) restored growth to snz2,3 double mutant. Together, these data suggest the synthesis of thiamine, potentially via Thi5p, has unique PLP requirements which could help explain the presence of multiple SNZ/SNO paralogs in S. cerevisiae.

SNO1 is required for PLP synthesis when ammonia is limiting in vivo

In S. cerevisiae each SNZ paralog has a corresponding SNO glutaminase subunit (Rodríguez-Navarro et al. 2002). The need for the glutaminase activity in PLP synthesis has not been clearly demonstrated in vivo (Rodríguez-Navarro et al. 2002; Stolz and Vielreicher 2003). The role of SNO1 on MVD was tested (Figure 5). The strain lacking SNO1 had a small but reproducible growth defect when thiamine was added, that was corrected with the addition of PN. This result suggested that SNO1 was required for optimal PLP synthesis. This interpretation was complicated by the presence of other SNO paralogs, despite the assumption their expression was completely repressed by the thiamine (Rodríguez-Navarro et al. 2002).
Figure 4Expression of SNZ2/3 is repressed by thiamine. Expression of SNZ2 and SNZ3 was determined by qRT-PCR when YJF153 was grown for twelve hours in MVD medium with various additions. Fold change represents the ratio of expression on MVD supplemented with thiamine (0.4 mg/L), pyridoxine (0.4 mg/L), or both (as indicated) compared to the expression on MVD with no supplements. Error bars indicate the 95% confidence interval of three independent biological replicates.

The SNZ/SNO pathway for PLP synthesis is less complex than the multi enzyme DXP-dependent pathway used by S. enterica and other organisms. If functional, introduction of these enzymes into S. enterica would i) allow characterization of single paralogs, and ii) provide a heterologous system that could be used to probe S. enterica with a simplified metabolic network. A plasmid expressing SNZ3 from the lac promoter on pSU18 (pDM1595) was introduced into a S. enterica strain lacking the pyridoxine-5-phosphate (PNP) synthase (E.C. 2.6.99.20) encoded by pdxJ (Figure 1). SNZ3 provided in trans supported growth of the resulting strain in minimal (NCE) media with glyceraldehyde-3-phosphate as a carbon source. These data showed that SNZ3 was necessary and sufficient to synthesize PLP in the pdxJ mutant of S. enterica (Figure 6A), and were generally consistent with the ability of SNZ1 from Cercospora nicotianae to complement a pdxJ strain of E. coli (Wetzel et al. 2004).

Figure 5SNO1 contributes to PLP biosynthesis in S. cerevisiae. A sno1 mutant was grown on minimal vitamin dextrose medium (open circles), with addition of thiamine (open squares), and with addition of thiamine and pyridoxine (filled squares). Error bars indicate the standard deviation of three independent biological replicates.

Figure 6SNZ3 and SNO3 can synthesize PLP in Salmonella enterica. Growth of pdxJ mutant of S. enterica with various plasmids was monitored for growth. In panel (A) the pdxJ mutant carried an empty vector (pSU18) (circles) or a plasmid expressing SNZ3 (squares). Each of these two strains were grown on NCE (i.e., high ammonia) minimal medium with glyceraldehyde-3-phosphate as substrates was used to define basic kinetic parameters of Snz3p. The K_m for ribose-5-phosphate was 0.09 ± 0.02 mM while the K_m for glyceraldehyde-3-phosphate was 0.29 ± 0.03 mM (Figure 7). The K_cat for ribose-5-phosphate was 0.044 min⁻¹ while the K_cat for glyceraldehyde-3-phosphate was 0.042 min⁻¹. These data were similar to those reported for Snz1p (K_m = 0.11 mM and 0.33 mM, K_cat = 0.036 min⁻¹ and 0.039 min⁻¹ for ribose-5-phosphate and glyceraldehyde-3-phosphate, respectively).
when assayed in the absence of Sno1p (Neuwirth et al. 2009). Kinetic constants available for the Bacillus subtilis PLP synthase in the absence of the glutaminase subunit demonstrate that while this synthase has a higher affinity for ribose-5-phosphate and glyceraldehyde-3-phosphate (K_m = 0.068 mM and 0.077 mM, respectively), its catalytic turnover is similar (K_cat = 0.02 min^-1) (Raschle et al. 2005).

**Conclusions**

SNZ3 encodes a functional PLP synthase that uses glyceraldehyde-3-phosphate, ribose-5-phosphate, and ammonia as substrates. Despite the near identical kinetic constants of Snz1p and Snz3p, results here demonstrate the two isozymes have different roles in vivo. The data showed that SNZ2 or SNZ3, but not SNZ1, was sufficient to generate PLP for growth on MVD medium (i.e., in the absence exogenous PN or Thi). SNZ1 supported growth only when PN and/or thiamine were provided. This result suggested that SNZ1 was unable to satisfy the PLP requirement for thiamine synthesis. The finding that SNZ2 and/or SNZ3 are important for thiamine, specifically HMP, synthesis supports a connection between SNZ2/3 and the Thi5p family of enzymes. The poorly characterized Thi5p enzymes use PLP as a substrate rather than a co-factor to generate the HMP-P moiety used for thiamine synthesis (Lai et al. 2012; Coquille et al. 2012). The finding that the lack of a specific SNZ paralog impacts thiamine biosynthesis, out of many metabolic pathways that use PLP as a cofactor, suggests that there are unique requirements for PLP in this pathway, likely involving the Thi5p family of enzymes.

To our knowledge these data provided the first evidence of distinct roles for the SNZ paralogs in vivo that was not due to regulation of gene expression. It is worth noting that the phenotypes key to the above conclusions were not obvious from past studies using dropout media (Rodriguez-Navarro et al. 2002; Stolz and Vielreicher 2003). In fact, the previous studies led to the conclusion that SNZ1 encoded the primary PLP synthase, a conclusion that the results herein bring into doubt. Although not conclusive from the results with S. cerevisiae, studies with the heterologous host S. enterica showed that the glutaminase subunit SNO3 is dispensable for PLP synthesis in the presence of excess ammonia. These data further showed that the DXP-dependent pathway for PLP synthesis could be replaced by a single gene (SNZ3) in S. enterica, and defined a heterologous system that will be valuable in studies to probe network structure with a simplified B6 metabolism.

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**Figure 7** Snz3p is a PLP synthase. Saturation curves for Snz3p were determined by measuring the initial rate of PLP formation vs. ribose-5-phosphate (A) or D/L-glyceraldehyde-3-phosphate (B) as a substrate. Reactions were performed in 50 mM Tris pH 8.0 at 37 °C, containing 85 μM Snz3p. When ribose-5-P was titrated, the reaction mix contained 2 mM D/L-glyceraldehyde-3-phosphate and 20 mM NH4. When D/L-glyceraldehyde-3-phosphate was titrated, the reaction mix contained 1 mM ribose-5-phosphate and 20 mM NH4. All reactions were performed in triplicate, and error bars are shown.
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