Analyses of variants of the Ser/Thr dehydratase IlvA provide insight into 2-aminoacrylate metabolism in Salmonella enterica

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RidA is a conserved and broadly distributed protein that has enamine deaminase activity. In a variety of organisms tested thus far, lack of RidA results in the accumulation of the reactive metabolite 2-aminoacrylate (2AA), an obligate intermediate in the catalytic mechanism of several pyridoxal 5′-phosphate (PLP)-dependent enzymes. This study reports the characterization of variants of the biosynthetic serine/threonine dehydratase (EC 4.3.1.19; IlvA), which is a significant generator of 2AA in the bacteria Salmonella enterica, Escherichia coli, and Pseudomonas aeruginosa and the yeast Saccharomyces cerevisiae. Two previously identified mutations, ilvA3210 and ilvA3211, suppressed the phenotypic growth consequences of 2AA accumulation in S. enterica. Characterization of the respective protein variants suggested that they affect 2AA metabolism in vivo by two different catalytic mechanisms, both leading to an overall reduction in serine dehydratase activity. To emphasize the physiological relevance of the in vitro enzyme characterization, we sought to explain in vivo phenotypes using these data. A simple mathematical model describing the impact these catalytic deficiencies had on 2AA production was generally supported by our data. However, caveats arose when kinetic parameters, determined in vitro, were used to predict formation of the isoleucine precursor 2-ketobutyrate and model in vivo (growth) behaviors. Altogether, our data support the need for a holistic approach, including in vivo and in vitro analyses, to generate data used in understanding and modeling metabolism.

Microbial metabolic networks are robust systems able to absorb perturbations caused by internal and external stress (1–4). Metabolic robustness provides an evolutionary advantage to an organism exposed to sudden and frequent environmental shifts, but the integration of pathways can complicate the ability to predict metabolic potential or model flux distribution based solely upon genomic cataloging (5–7). The extensive integration of metabolic pathways within the network can lead to unanticipated solutions to metabolic perturbations that are often uncovered by genetic analyses (8–10). This point is exemplified by the observable differences between Salmonella enterica and Escherichia coli with respect to 2-aminoacrylate (2AA) stress and thiamine biosynthesis, despite the conservation of all the component enzymes in each (8, 11). The observation that organisms containing the same components may alter the configuration of those parts indicates the need for genetic and biochemical approaches to supplement genomic cataloging in efforts to model cellular metabolism (5, 12).

A number of pyridoxal 5′-phosphate (PLP)-dependent β-eliminase enzymes produce and release the reactive molecule 2AA. 2AA then inactivates target PLP enzymes through covalent modification of active-site bound PLP (13–16). RidA subfamily members, belonging to the broader Rid (YigF/YER057c/UK114) protein family, are enamine/imine deaminases that reduce 2AA accumulation (13, 14, 17, 18). Strains of S. enterica lacking RidA do not grow on minimal pyruvate medium or minimal glucose medium containing serine. These growth defects are due to 2AA generated by the biosynthetic serine/threonine dehydratase (IlvA; EC 4.3.1.19) from serine. S. enterica ridA mutants show a mild growth defect in minimal glucose medium, attributable to 2AA generated from endogenously generated serine. Inclusion of serine increases endogenous serine pools and results in greater 2AA production. A ridA mutant fails to grow because of the increased 2AA-dependent damage to multiple enzymes (19–22). This global stress is best prevented by mechanisms that reduce 2AA production (e.g. allosteric inhibition of IlvA by isoleucine) (14, 23–27). Lack of growth on minimal pyruvate medium has been attributed to 2AA-dependent damage of a single enzyme, the branched-chain amino acid aminotransferase (IlvE; EC 2.6.1.42) (21). WT S. enterica, grown on pyruvate, experiences a flux bias away from isoleucine biosynthesis and toward valine, although sufficient isoleucine is still produced for growth (28). However, ridA mutants undergo 2AA-dependent damage of IlvE, the penultimate enzyme in the isoleucine synthesis pathway, and experience an isoleucine limitation (14, 21). An overview of the role of RidA in the context of branched-chain amino acid biosynthesis is depicted in Fig. 1.

Suppressor mutations that restored growth to a ridA mutant on minimal pyruvate mapped to ilvA and encoded variant IlvA proteins (26). The suppressor alleles of ilvA, ilvA3210 and ilvA3211 were used to predict formation of the isoleucine biosynthesis and toward valine, although sufficient isoleucine is still produced for growth (28). However, ridA mutants undergo 2AA-dependent damage of IlvE, the penultimate enzyme in the isoleucine synthesis pathway, and experience an isoleucine limitation (14, 21). An overview of the role of RidA in the context of branched-chain amino acid biosynthesis is depicted in Fig. 1.

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2 The abbreviations used are: 2AA, 2-aminoacylrate; PLP, pyridoxal 5′-phosphate; KMV, 2-keto-3-methylvalerate; DNPH, 2,4-dinitrophenylhydrazine; 2-KB, 2-ketobutyrate; 2AC, 2-aminoacylrate; NB, nutrient broth.
Refining the 2-aminoacylate metabolic model

Figure 1. 2-Aminoacylate in the context of branched-chain amino acid metabolism. The pathways for branched-chain amino acid biosynthesis and 2-aminoacylate hydrolysis are shown schematically. Enzymes are indicated next to the step they catalyze. IlvA catalyzes the first committed step in isoleucine biosynthesis using threonine as a substrate. IlvA can also dehydrate serine to form 2-aminoacylate. RidA activity increases the rate of hydrolysis for both enamine products of IlvA. Elimination of RidA causes accumulation of 2-aminoacylate, which can damage IlvE. (2AC would also accumulate but is not known to have deleterious effects.) Importantly, due to AvtA, absence of active IlvE prevents isoleucine but not valine biosynthesis. IlvA is sensitive to feedback inhibition by isoleucine, as indicated in the schematic. Intermediates in isoleucine and valine biosynthesis compete for enzymes IlvC, IlvD, and IlvE. Abbreviations: AL, 2-acetolactate; AHB, 2-aceto-3-hydroxybutyrate.

IlvA3211, restored WT levels of IlvE activity and allowed isoleucine biosynthesis sufficient for growth. Isolation and initial analysis of these suppressors was prior to the elucidation of RidA function and the role of 2AA in causing the phenotypes of a ridA mutant. Notably, the suppressor mutations were unable to restore growth of a ridA mutant on medium containing serine, indicating the IlvA variants still produce 2AA when serine levels are elevated.

Based on the current understanding of the RidA paradigm, two scenarios could explain how the IlvA variants restored growth of a ridA mutant on pyruvate medium but not in the presence of serine. First, the IlvA variants could have decreased affinity for the 2AA precursor, L-serine. Second, the catalytic turnover of the variants could be reduced, maintaining sufficient 2-aminocrotonate production for isoleucine biosynthesis but minimizing 2AA formation. In either scenario, 2AA production by the variants would be reduced but not eliminated.

The goal of this work was to biochemically characterize the IlvA variants and better understand the synthesis and impact of 2AA formation by IlvA in the metabolic context of branched-chain amino acid synthesis. We further addressed the feasibility of using kinetic and metabolite concentration data to model the factors controlling IlvA-dependent 2AA metabolism and its impact in vivo.

Results

IlvA3210 and IlvA3211 alleles suppress one 2AA-dependent growth defect

Growth of strains with WT ilvA, ilvA3210, and ilvA3211 was quantified, and the data are shown in Fig. 2. The ridA mutant failed to grow with pyruvate as a sole carbon source (Fig. 2, A and B) or when minimal glucose medium included serine (Fig. 2, C and D). As reported previously, the ilvA3210 and ilvA3211 alleles significantly improved growth of the ridA mutant on pyruvate but not in minimal glucose with serine (21). On pyruvate medium, (i) growth of the ridA ilvA3210 and ridA ilvA3211 mutants was not fully restored to WT levels, and (ii) the ilvA3210 allele caused a growth defect in an otherwise WT strain. Both of these phenotypes were corrected by the addition of isoleucine (Fig. 2). In glucose medium containing 5 mM serine, the ridA ilvA3210 and ridA ilvA3211 strains grew only slightly better than a ridA mutant (Fig. 2, C and D). In total, these data were consistent with the following hypotheses: (i) IlvA142T and IlvA191S have reduced dehydratase activity; (ii) the reduced serine dehydratase activity of the IlvA variants decreases 2AA available to damage cellular targets; and (iii) ilvA3210 decreases flux enough to cause a starvation for isoleucine on pyruvate, even in the absence of a ridA mutation.

IlvA alleles restore branched-chain amino acid aminotransferase activity

Threonine dehydratase (IlvA) and branched-chain amino acid aminotransferase (IlvE) activity was assayed in the crude extracts of strains containing four different ilvA alleles (Table 1, white shading) and strains carrying the same ilvA alleles in addition to a ridA mutation (Table 1, gray shading). Analysis of the strains with a WT ridA provides insight into the impact of the IlvA variants on the biosynthetic pathway. Each of the ilvA alleles caused a decrease in total threonine dehydratase activity, with ilvA3210 (IlvA142T, DM15898) and ilvA3211 (IlvA191S, DM15936) causing an ~10-fold decrease. Despite the decrease in threonine dehydratase activity, the strains had WT growth level on minimal glucose medium, emphasizing the excess capacity the cells have to synthesize isoleucine. IlvA and IlvE are encoded in an operon (ilvGMEDA), and the ratio of their activity suggests the status of isoleucine synthesis and/or starvation in the cell (29). IlvE activity was significantly increased in the ilvA3210 strain, suggesting that the decreased activity of IlvA142T caused a limitation of isoleucine, which resulted in induction of the operon. Consistent with this model, previous work showed by Western blotting that more IlvE protein accumulates in the ilvA3210 strain than in the WT strain (21). These data correlate with the growth defect of DM15898 (ilvA3210) on pyruvate, where flux through the isoleucine synthesis pathway is less than on glucose medium. Although threonine dehydratase activity measured in crude lysates was slightly higher for IlvA142T than IlvA191S, assuming a similar induction
across the ilv operon, the data suggest the specific activity of IlvAA_{A142T} in vivo is no greater than that of IlvAG_{G191S}. In total, the results show that the capacity of the biosynthetic pathway exceeds what is needed to satisfy growth and supports a hypothesis that allosteric regulation is a major driver controlling metabolic efficiency.

The impact of eliminating RidA in the strains described above is shown by the gray shading in Table 1. IlvE is irreversibly damaged by 2AA and can be used as a proxy for the amount of this metabolite that accumulates in vivo (14). A ridA mutant (DM3480) accumulates 2AA, which results in the ~50% reduction in IlvE activity, compared with WT (DM9404) (Table 1).
Refining the 2-aminoacrylate metabolic model

Considering the WT protein, the values obtained for the Michaelis-Menten constant ($K_m$) and catalytic rate constant ($k_{cat}$) with serine or threonine were consistent with values previously observed for *S. enterica* IlvA (31). The IlvA$_{L447F}$ variant had a lower $K_m$ value for serine and a higher $k_{cat}$ value with both threonine and serine substrates. Compared with WT enzyme, the IlvA$_{A142T}$ variant had significantly higher $K_m$ and lower $k_{cat}$ values for both substrates, whereas the IlvA$_{G191S}$ variant had significantly lower $K_m$ and $k_{cat}$ values for both substrates. The specificity constant ($k_{cat}/K_m$) of an enzyme can be used to assess preference for different substrates. The $k_{cat}/K_m$ values showed that all four IlvA proteins were more effective at using threonine than serine (Fig. 3). However, the ratio of specificity constants (threonine $k_{cat}/K_m$ versus serine $k_{cat}/K_m$) increases from 5 for IlvA to 8 and 9 for IlvA$_{A142T}$ and IlvA$_{G191S}$, respectively. This suggests that IlvA$_{A142T}$ and IlvA$_{G191S}$ have a slightly stronger preference for threonine over serine than WT enzyme.

The relative *in vitro* stability of each enzyme variant was determined by measuring enzyme activity over 8 h in the presence of 200 mM serine. IlvA$_{A142T}$ was the least stable and had an 80% reduction in activity after 8 h (40 versus 184 nmol of pyruvate min$^{-1}$). IlvA$_{G191S}$ was moderately stable with a 40% reduction in activity after 8 h (18 versus 30 nmol of pyruvate min$^{-1}$). In contrast, WT IlvA (716 versus 777 nmol of pyruvate min$^{-1}$) and IlvA$_{L447F}$ (1,043 versus 1,162 nmol pyruvate min$^{-1}$) showed only a 10% loss in activity over the same time.

**Modeling the competing synthesis of 2AA and 2AC in vivo**

Use of threonine and serine by IlvA generates two significant metabolites, 2-aminoacrylate (2AC) and 2-aminoacrylate, respectively. 2AC, which is not known to be detrimental if it accumulates, is deaminated to 2-ketobutyrate, a precursor of isoleucine. In contrast, 2AA is detrimental if it accumulates before its conversion to pyruvate, as described above. Thus, the two products of IlvA have opposing impacts on the growth phenotypes, and therefore differences in how the IlvA variants impacted production of both 2AC and 2AA *in vivo* was significant. The kinetic information of each of the four IlvA enzymes (Fig. 3) was used to explore the relationship between formation of 2AA and 2AC in cells with different *ilvA* alleles. Reported concentrations of endogenous metabolites in *E. coli* were used to estimate the concentration of L-serine (67 μM) and L-threonine (180 μM) (32). The concentration of IlvA (20 μM) was based on the concentration of other PLP-dependent enzymes in *E. coli* (33). For simplicity, known cooperative and allosteric behaviors of IlvA were excluded from the calculations (34, 35). Equation 1 predicts product formation for a specified substrate (serine or threonine), accounting for competitive inhibition by the alternative substrate (36).

$$V_{prod} = \frac{k_{cat}(\text{Sub})}{K_m(\text{Sub}) + [\text{Sub}]}$$

*V$_{prod}$* is the rate of product production ($\mu$mol/s); $k_{cat}(\text{Sub})$ is the turnover number for the substrate (s$^{-1}$); $[\text{Sub}]$ is IlvA active-site concentration (μM); $[\text{CompSub}]$ is substrate concentration (μM); $K_m(\text{Sub})$ is the Michaelis-Menten constant for the substrate (μM); and $K_m(\text{CompSub})$ is the Michaelis-Menten constant for the competing substrate (μM).

### Table 1

| Genotype          | Strain      | IlvA Activity$^*$ | % IlvA Activity | $K_m$ | $k_{cat}$ |
|-------------------|-------------|------------------|----------------|-------|----------|
| wild-type         | DM9404      | 67 ± 14          | 100            | 31 ± 3| 52 ± 1   |
| ridt              | DM1480      | 51 ± 3           | 76             | 53 ± 1| 36       |
| ilvA219 (ilvA$_{L447F}$) | DM6947      | 28 ± 1           | 42             | 103 ± 2| 26       |
| ilvA219 ridt      | DM6946      | 20 ± 3           | 30             | 27 ± 1| 36       |
| ilvA3210 (ilvA$_{A142T}$) | DM15898    | 7 ± 2            | 10             | 162 ± 16| 84       |
| ilvA3210 ridt     | DM15899     | 3 ± 1            | 4              | 136 ± 7| 118      |
| ilvA3211 (ilvA$_{G191S}$) | DM15936    | 4 ± 3            | 6              | 97 ± 7 | 118      |
| ilvA3211 ridt     | DM15937     | 14 ± 2           | 21             | 114 ± 4| 118      |

$^*$ Indicated strains were grown to stationary phase in minimal glucose medium containing 0.67 mM glycine.

Because IlvA uses serine and threonine as competing substrates, it was possible that differential substrate specificities influenced the *in vivo* effects described above. Kinetic parameters were determined from purified preparations of the four IlvA protein variants, using either serine or threonine substrate (Fig. 3). The threonine saturation curves for all four proteins were fit using the equation by Michaelis-Menten. The saturation curves using serine substrate showed mild positive cooperativity, with Hill coefficients ($h$) greater than 1. Because the fit of the curves was close using the equation from Michaelis-Menten, the slight cooperative effects were not considered in determining the kinetic values of the proteins. This tactic simplified the comparisons between the results with serine and threonine substrates.

**Characterization of purified IlvA variants**

Use of threonine and serine by IlvA generates two significant metabolites, 2-aminoacrylate (2AC) and 2-aminoacrylate, respectively. 2AC, which is not known to be detrimental if it accumulates, is deaminated to 2-ketobutyrate, a precursor of isoleucine. In contrast, 2AA is detrimental if it accumulates before its conversion to pyruvate, as described above. Thus, the two products of IlvA have opposing impacts on the growth phenotypes, and therefore differences in how the IlvA variants impacted production of both 2AC and 2AA *in vivo* was significant. The kinetic information of each of the four IlvA enzymes (Fig. 3) was used to explore the relationship between formation of 2AA and 2AC in cells with different *ilvA* alleles. Reported concentrations of endogenous metabolites in *E. coli* were used to estimate the concentration of L-serine (67 μM) and L-threonine (180 μM) (32). The concentration of IlvA (20 μM) was based on the concentration of other PLP-dependent enzymes in *E. coli* (33). For simplicity, known cooperative and allosteric behaviors of IlvA were excluded from the calculations (34, 35). Equation 1 predicts product formation for a specified substrate (serine or threonine), accounting for competitive inhibition by the alternative substrate (36).

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| ilvA3211 ridt     | DM15937     | 14 ± 2           | 21             | 114 ± 4| 118      |

$^*$ Indicated strains were grown to stationary phase in minimal glucose medium containing 0.67 mM glycine.
Using Equation 1, the amount of 2AC and 2AA generated during steady-state growth was estimated for each of the IlvA variants (Table 2). Consistent with the conclusion from the data in Fig. 3, Equation 1 predicts each IlvA enzyme generates more 2AC than 2AA. Furthermore, the variants that suppressed the ridA mutant phenotype on pyruvate produced dramatically less 2AA and 2AC than the WT protein. The growth of strains with these variants on minimal medium indicated that these variants produced sufficient 2AC to satisfy the isoleucine requirement of the strain. These data suggest that despite being simplified, Equation 1 does an adequate job of accounting for the effect of the IlvA variants in vivo and suggests the mechanism they use to suppress the ridA phenotype on pyruvate. The ilvA alleles that restored growth to a ridA mutant failed to restore growth when exogenous serine was added, indicating detrimental levels of 2AA still accumulated under these conditions. Equation 1 was used to calculate the impact of 5 mM serine on the products of IlvA. Assuming endogenous serine matched the external concentration, the data showed a significant increase in 2AA production in all strains (Table 2). The strains carrying ilvA3210 and ilvA3211 were calculated to have 2AA levels well above what accumulated in a WT strain without serine addition (85 and 29 μM/s for ilvA3210 and ilvA3211, respectively). Interestingly, the amount of 2AC was essentially unchanged in this condition.

A hallmark of the RidA paradigm is that the effects are prevented by the addition of isoleucine, which inhibits IlvA and prevents formation of the toxic 2AA. Addition of threonine was similarly shown to reduce the formation of 2AA, a result that was previously suggested to be due to competitive inhibition of serine catalysis by IlvA. However, for threonine to cause a 10-fold decrease in 2AA production by competitive inhibition, 55 mM L-threonine would be required (Equation 1). In contrast,
addition of 1 mM L-threonine eliminated the growth defect of a ridA mutant on minimal glucose medium (Fig. 4A) (26). Modeling of this condition with Equation 1 showed that 1 mM threonine did not affect the level of 2AA, but rather it significantly increased the production of 2AC. Based on this analysis, we hypothesized that the exogenous threonine increased isoleucine biosynthesis, which allosterically inhibited IlvA, decreasing 2AA production (Fig. 1). A ridA mutant expressing the isoleucine feedback-resistant variant has a growth defect when grown in minimal glucose medium due to the increased 2AA formed. In this strain, L-threonine did not restore growth on minimal medium, a result that is consistent with threonine acting to increase isoleucine synthesis and resulting in the allosteric inhibition of IlvA to allow growth (Fig. 4).

Discussion

Strains lacking ridA in S. enterica have several metabolic defects that result from the accumulation of 2AA in the absence of RidA deaminase activity. This study sought to use the biochemical characteristics of IlvA variants to better understand the mechanism by which they reversed one defect, growth with pyruvate as a carbon source. Analyzing the role of IlvA in ridA phenotypes is complicated by, among other things, the fact that the enzyme generates both 2AA (from serine) and 2AC (from threonine). The former is potentially damaging, and the latter is a metabolite necessary for synthesis of isoleucine. Therefore, variants that minimize 2AA formation cannot decrease 2AC so much that isoleucine synthesis is compromised. Kinetic analysis of four IlvA proteins, two of which suppressed a ridA growth defect, provided data to address the impact of 2AA and 2AC on growth. Based on these data and a simplified equation to characterize product formation in vivo, several conclusions were made.

IlvA variants able to suppress ridA phenotypes decrease 2AA production

Strains with IlvA<sub>A142T</sub> or IlvA<sub>G191S</sub> had significantly less threonine deaminase activity than WT in crude extracts. Equation 1 used data from Fig. 3 to estimate that 2AA production from either IlvA<sub>A142T</sub> or IlvA<sub>G191S</sub> was reduced 10-fold from WT IlvA (Table 2). IlvE activity in ridA ilvA<sub>3210</sub> and ridA ilvA<sub>3211</sub> strains was not statistically significantly different from the isogenic parents (ilvA<sub>3210</sub> and ilvA<sub>3211</sub>, respectively), allowing the conclusion that a 10-fold reduction in 2AA eliminated detectable damage to the target IlvE. Exogenous serine (5 mM) in the growth media could still lead to significant 2AA production (Table 2), which then eliminates growth (Fig. 2). The fact that the IlvA variants with reduced 2AA production also had decreased 2AC production highlights the difficulty in altering enzymes to impact a single substrate.

IlvA<sub>A142T</sub> and IlvA<sub>G191S</sub> were compromised catalytically and had increased preference for threonine substrate

An expanded two-state model accounting for cooperative interactions within E. coli IlvA has been described previously (34, 35). Because the saturation curves using threonine or serine substrate could be fit using the hyperbolic equation by Michaelis-Menten, the majority of enzyme active sites (in the absence of isoleucine) exist in the active R-state (35). This simplified determination and comparison of <i>K<sub>m</sub></i> and <i>k<sub>cat</sub></i> values for each enzyme. Increased <i>K<sub>m</sub></i> and lower <i>k<sub>cat</sub></i> values suggested that IlvA<sub>A142T</sub> bound each substrate less well than the WT enzyme and generated both products more slowly. Alternatively, IlvA<sub>G191S</sub> bound both substrates more readily (lower <i>K<sub>m</sub></i>) but was compromised in dehydratase activity (lower <i>k<sub>cat</sub></i>). Thus, both enzyme variants were defective in generation of 2KB and pyruvate, but by different mechanisms. These findings highlight two features of enzymes that could impact 2AA produc-
tion and stress in different organisms. Furthermore, the ratio of $k_{cat}/K_m$ values for threonine versus serine indicated that IlvA$_{A142T}$ and IlvA$_{G191S}$ had a greater preference for threonine over serine than WT enzyme. PLP enzymes, such as IlvA, are well known for their substrate and reaction promiscuity (37–39). This work shows how changes that result in greater specificity toward a single substrate can be undesirable because they can also hamper the maximal rate of catalysis.

Exogenous threonine increases flux toward isoleucine, indirectly controlling 2AA production

A previous report found that mutations increasing threonine biosynthesis could suppress the growth defect of a ridA mutant in the presence of serine (26). Restoration of IlvE activity suggested that threonine competed with serine for the active site of IlvA and thus reduced the amount of 2AA produced (26). Implementation of Equation 1 suggested this was an unlikely explanation for the mechanism of threonine, because the amount of threonine required to reduce 2AA generation 10-fold would be unrealistically high (55 mM). The data supported a model in which isoleucine synthesis was increased when threonine was provided (i.e. increased AC production and no change in 2AA). Analysis of the impact of threonine on the growth data, crude extract assays, and activity measurements using pure protein seemed inconsistent. First, purified IlvA$_{A142T}$ was not significantly different in catalytic capacity from the WT protein, yet in crude extract, it produced half the activity. Second, data from crude extracts confirmed the activity. Despite its simplicity, Equation 1 proved useful in predicting the physiological consequence of threonine addition on 2AA metabolism and thus refined a previous model. In total, the data and discussion herein emphasize the need for a holistic approach for understanding and modeling metabolism, as we seek to define subtle and integrated responses of the cell to metabolic stress and cellular perturbation.

Kinetic models can complement in vivo analyses

Assays of IlvA activity in crude extracts were the starting point for understanding how the IlvA variants impacted ridA phenotypes. Unexpectedly, there were several instances where the growth data, crude extract assays, and activity measurements using pure protein seemed inconsistent. First, purified IlvA$_{A142T}$ was not significantly different in catalytic capacity from the WT protein, yet in crude extract, it produced half the activity. Second, data from crude extracts confirmed the growth observation that strains expressing the IlvA$_{A142T}$ variant were starved for isoleucine, based on the apparent induction of the ilv operon (i.e. increased IlvE activity). However, the total activity of IlvA$_{A142T}$ in crude extracts was no lower than that of IlvA$_{G191S}$. This point was of interest because strains with IlvA$_{G191S}$ have sufficient isoleucine synthesis for growth. The disparity in isoleucine requirement between ilvA3211 and ilvA3210 strains underscores technical limitations and physiological complexity. Despite its simplicity, Equation 1 proved useful in predicting the physiological consequence of threonine addition on 2AA metabolism and thus refined a previous model. In total, the data and discussion herein emphasize the need for a holistic approach for understanding and modeling metabolism, as we seek to define subtle and integrated responses of the cell to metabolic stress and cellular perturbation.

Experimental procedures

Bacterial strains, media, and chemicals

Strains used in this work are provided in Table 3 and are all derivatives of Salmonella enterica serovar Typhimurium LT2. Minimal medium was no-carbon E supplemented with 1 mM MgSO$_4$ (41), trace minerals (42), and 11 mM glucose or 50 mM pyruvate as the sole carbon source. Difco nutrient broth (NB) (8 g/liter) containing NaCl (5 g/liter) was used as rich medium. Difco BiTek agar (15 g/liter) was added for solid medium.

| Bacterial strains, plasmids, and primers | Sequence |
|----------------------------------------|----------|
| ilvA$_{BbpQ}$/LT2$_{F}$ | 5'-GGGCTTCAGCAAGAAGCAAGAC-3' |
| ilvA$_{BbpQ}$/LT2$_{R}$ | 5'-GGGCTTCAGCAAGAAGCAAGCA-3' |
| ilvA$_{NdeI}$/LT2$_{F}$ | 5'-GGAGATTATGGCGGAGATGTCGAC-3' |
| ilvA$_{NdeI}$/LT2$_{R}$ | 5'-GGAGATTATGGCGGAGATGTCGAC-3' |

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Genetic techniques

Strains were constructed via transductional crosses using the high-frequency general transducing mutant of bacteriophage P22 (HT105/1, int-201) (43). Methods for performing trans-
ductions, isolation of cells from phage contamination, and identifying phage-free recombinants were previously described (44, 45). Briefly, recipient cells (~10^9 CFU) and transducing phage (~10^9 PFU) were preincubated for 1 h at 37 °C prior to plating on selective media. Transductants were purified by isolation using nonselective green indicator plates, and putative phage-free clones were identified as light green colonies. These clones were checked for phage sensitivity by cross-streaking with phage P22. Transductional crosses in E. coli were performed using bacteriophage P1vir, as described previously (8). E. coli BL21-AI ΔilvA823 was constructed by transduction of the ilvA213::kan locus from the Keio collection into the BL21-AI strain background (46). The kanamycin cassette was then resolved using flippase (FLP) recombinase, as previously described by Datsenko and Wanner (47), to create E. coli BL21-AI ΔilvA823.

**Molecular techniques**

The ilvA locus was amplified from LT2 strains DM7608 (ilvA3211) and DM7610 (ilvA3210) by PCR with Q5 high-fidelity DNA polymerase (New England Biolabs) using primers IlvA_BspQI_LT2_F and IlvA_BspQI_LT2_R (Table 3). The PCR product was purified, digested with BspQI, and ligated into the modified pET28b, pSAPKO-CH, as described by Galloway et al. (48). The resulting plasmids were named pDM1461 and pDM1543, which encoded IlvA_A142T and IlvA_G191S fused to a C-terminal penta-histidine tag, respectively. IlvA219 was amplified from LT2 strain DM6947 by PCR with Q5 high-fidelity DNA polymerase (New England Biolabs) using primers IlvA_NdeI_LT2_F and IlvA_XhoI_LT2_R (Table 1). The PCR product was purified, digested with NdeI and XhoI (New England Biolabs), and ligated into predigested pET20b (Novagen), digested with the same enzymes. The resulting plasmid was named pDM1420 and encoded IlvA_L447F fused to a C-terminal hexa-histidine tag. Constructs were transformed into E. coli DH5α, and transformants were screened for vectors containing the correct insert. Inserts were confirmed through sequence analysis, performed by Eton Biosciences.

**Protein purification**

The three plasmids generated above and pDM1578, which encoded S. enterica IlvA fused to a C-terminal hexa-histidine tag and was generated previously (13), were each inserted into an E. coli BL21-AI strain (Invitrogen) lacking the native ilvA locus. The resulting strains were inoculated into 2 ml of LB containing either ampicillin (pET20b-ilvA and pET20b-ilvA219) or kanamycin (pSapKOCH-ilvA3210 and pSapKOCH-ilvA3211) and allowed to reach full density. The resulting strains were inoculated into 2 ml of minimal medium containing 0.67 mM glycerate, 10 mM potassium phosphate, pH 8 (2018) 293(50) 19240 –19249 J. Biol. Chem.

**Quantification of growth**

A 2-μl aliquot of cells from an overnight NB culture was used to inoculate 198 μl of growth medium. 96-Well plates were incubated at 37 °C in a microplate reader (model EL808; BioTek Instruments) with shaking, and growth was monitored as the change in optical density at 650 nm (OD650) over time. Unless stated, growth experiments were performed in biological triplicate. Results were plotted using GraphPad Prism 7.0d, with curves representing averages and standard error of the means for the replicates.

**Dehydratase (IlvA) assays**

**Crude assay**—Full-density NB cultures were inoculated (1:100) into 100 ml minimal medium containing 0.67 mM glycerate and grown at 37 °C shaking to stationary phase (12 h) before being pelleted (10,000 g, 10 min) and stored as pellets at −80 °C. Cell pellets were resuspended in 2 ml of buffer (50 mM KPO4, pH 8.0, 0.4 mM DTT) and filtered through a 0.45-μm filter. Filtered lysates were bound to 5-ml HisTrap™ HP column and purified using the manufacturer’s protocol (GE Healthcare). The column was washed using resuspension buffer (100 mM KPO4, pH 8, 100 mM NaCl, 20 mM imidazole, and 10% glycerol) and then eluted with elution buffer (100 mM KPO4, pH 8, 100 mM NaCl, 500 mM imidazole, and 10% glycerol), in a linear gradient over the course of 10 column volumes. The fractions were assessed for purity, pooled, and concentrated using a 30,000 molecular weight cut-off Ultra-15 centrifugal filter (Amicon). Protein concentration was determined by the BCA assay (Pierce). Protein was frozen in liquid nitrogen and stored at −80 °C.

**Purified enzyme assay**—Pyruvate and 2-ketobutyrate formation was assayed by tracking the absorbance at 230 nm (13, 51) at room temperature (25 °C) for 2 mins. Assays were carried out in triplicate, using 100 mM potassium phosphate buffer, pH 8.0. To obtain values within the range of detection for the instrument, reactions used 100 nM of IlvA, IlvA_L447F, and IlvA_A142T for threonine assays and 200 nM of IlvA, IlvA_L447F, and IlvA_A142T for serine assays; 1 μM IlvA_G191S was used for both assays. The indicated concentrations of serine and threo-
nine were used to start each 200 μl reaction, which were assayed continuously in a quartz 96-well plate at room temperature by a SpectraMax Plus (Molecular Devices) supporting SoftMax Pro 7.0 software.

**Protein stability**—Pyruvate formation with purified protein was assayed as described above. Assays were carried out every 2 h, for a total of 8 h with 200 mM L-serine used to start each reaction. Each measurement was performed in duplicate, using 200 nM IlvA, IlvA_{L447F}, and IlvA_{A142T} and 1 μM IlvA_{G191S}. Enzyme preparations were kept on ice between measurements.

### Branched-chain amino acid aminotransferase (IlvE) assays

Full-density NB cultures were inoculated (1:100) into 5 ml of minimal medium containing 0.67 mM glucose, grown at 37 °C shaking to stationary phase (12 h) before being pelleted (10,000 × g, 2 min), and stored as pellets at −20 °C. IlvE activity was assayed according to previously described methods (27).

Cells were permeabilized in buffer (50 mM KPO₄, pH 8, 50 μM PLP, 10 mM 2-ketogluartate) containing 10% PopCulture Reagent (Novagen). The reaction was started with 20 mM L-isoleucine. The product, KMV, was derivatized by 2,4-dinitrophenylhydrazine (DNPH), producing a chromophore with absorbance of 540 nm. KMV was quantified using a standard curve from known quantities of KMV derivatized with DNPH and normalized to total protein content (50).

### Kinetic data analysis

Initial velocity calculations were estimated over the course of 2-min reactions tracking ΔmOD₂₃₀ min⁻¹ for various concentrations of L-threonine or L-serine. Standard curves for absorbance at 230 nm for known amounts of 2-ketobutyrate and pyruvate were used to transform ΔmOD₂₃₀ min⁻¹ to μmol of 2-KB min⁻¹ and μmol of pyruvate min⁻¹. A nonlinear regression fit (Equation 2) to a graph of the substrate concentration versus initial velocity was used to fit the data.

\[
v = \frac{V_{max}[S]^{h}}{K_{n/2} + [S]^{h}} \quad \text{(Eq. 2)}
\]

In the absence of cooperative effects (h = 1), this reduced to the equation by Michaelis-Menten (Equation 3), from which each IlvA variant’s Michaelis-Menten constant (Kₘ) and maximal velocity (Vₘₐₓ) values for each substrate could be determined. Calculations were performed using GraphPad Prism 7.0d.

\[
v = \frac{V_{max}[S]}{K_{M} + [S]} \quad \text{(Eq. 3)}
\]

The turnover number (kₜₐₜ) was obtained using the active-site concentration (Eₐ) (Equation 4).

\[
k_{cat} = \frac{V_{max}}{E_{a}} \quad \text{(Eq. 4)}
\]

Because IlvA is a dimer of dimers, containing two active sites per enzyme, Eₐ is equal to half of the enzyme concentration used in the assay (52, 53).

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**References**

1. Koenigsknecht, M. J., and Downs, D. M. (2010) Thiamine biosynthesis can be used to dissect metabolic integration. *Trends Microbiol.* 18, 240–247 CrossRef Medline.
2. Koenigsknecht, M. J., Lambrecht, J. A., Fenlon, L. A., and Downs, D. M. (2012) Perturbations in histidine biosynthesis uncover robustness in the metabolic network of *Salmonella enterica*. *PLoS ONE* 7, e48207 CrossRef Medline.
3. Albert, R., Jeong, H., and Barabasi, A. L. (2000) Error and attack tolerance of complex networks. *Nature* 406, 378–382 CrossRef Medline.
4. Bazurto, J. V., and Downs, D. M. (2011) Plasticity in the purine-thiamine metabolic network of *Salmonella*. *Genetics* 187, 623–631 CrossRef Medline.
5. Bazurto, J. V., and Downs, D. M. (2016) Metabolic network structure and function in bacteria goes beyond conserved enzyme components. *Microb. Cell* 3, 260–262 CrossRef Medline.
6. Cardinaline, S., and Arkin, A. P. (2012) Contextualizing context for synthetic biology—identifying causes of failure of synthetic biological systems. *Biotechnol. J.* 7, 856–866 CrossRef Medline.
7. Kittleson, J. T., Wu, G. C., and Anderson, J. C. (2012) Successes and failures in modular genetic engineering. *Curr. Opin. Chem. Biol.* 16, 329–336 CrossRef Medline.
8. Bazurto, J. V., Farley, K. R., and Downs, D. M. (2016) An unexpected route to an essential cofactor: *Escherichia coli* relies on threonine for thiamine biosynthesis. *MBio* 7, e01840–15 Medline.
9. Mehta, V., Athar, M., Jha, P. C., Panchal, M., Modi, K., and Jain, V. K. (2016) Efficiently functionalized oxalacetal[4]arenes: synthesis, characterization and exploration of their biological profile as novel HDAC inhibitors. *Bioorg. Med. Chem. Lett.* 26, 1005–1010 CrossRef Medline.
10. Kim, J., Kershner, J. P., Novikov, Y., Shoemaker, R. K., and Copley, S. D. (2013) Three serendipitous pathways in *E. coli* can bypass a block in pyridoxal-5′-phosphate synthesis. *Mol. Syst. Biol.* 6, 436 Medline.
11. Borchert, A. I., and Downs, D. M. (2017) The response to 2-aminoacrylate differs in *Escherichia coli* and *Salmonella enterica*, despite shared metabolic components. *J. Bacteriol*. pii: e00140–17 CrossRef Medline.
12. Downs, D. M., Bazurto, J. V., Gupta, A., Fonseca, L. L., and Voit, E. O. (2018) The three-legged stool of understanding metabolism: integrating metabolomics with biochemical genetics and computational modeling. *AIMS Microbiol.* 4, 289–303 CrossRef
13. Lambrecht, J. A., Flynn, J. M., and Downs, D. M. (2012) Conserved YigF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5′-phosphate (PLP)-dependent enzyme reactions. *J. Biol. Chem.* 287, 3454–3461 CrossRef Medline.
14. Lambrecht, J. A., Schmitz, G. E., and Downs, D. M. (2013) RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *MBio* 4, e00033–13 Medline.
15. Flynn, J. M., Christopherson, M. R., and Downs, D. M. (2013) Decreased coenzyme A levels in *ridA* mutant strains of *Salmonella enterica* result from inactivated serine hydroxymethyltransferase. *Mol. Microbiol.* 89, 751–759 CrossRef Medline.
16. Flynn, J. M., and Downs, D. M. (2013) In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5′-phosphate cofactor. *J. Bacteriol.* 195, 3603–3609 CrossRef Medline.
17. Hodge-Hanson, K. M., and Downs, D. M. (2017) Members of the RidA protein family have broad imine deaminase activity and can accelerate the *Pseudomonas aeruginosa* d-arginine dehydrogenase (DauA) reaction in vitro. *PLoS ONE* 12, e0185544 CrossRef Medline.
18. Downs, D. M., and Ernst, D. C. (2015) From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Mol. Microbiol.* **96**, 211–219 CrossRef Medline

19.ERNST, D. C., ANDERSON, M. E., AND DOWNS, D. M. (2016) L-2,3-diaminopropionate generates diverse metabolic stresses in *Salmonella enterica*. *Mol. Microbiol.* **101**, 210–223 CrossRef Medline

20. Lambrecht, J. A., Browne, B. A., and Downs, D. M. (2010) Members of the YigF/YER057c/UK114 family of proteins inhibit phosphoribosylamine synthesis in *vitro*. *J. Biol. Chem.* **285**, 34401–34407 CrossRef Medline

21. Christopherson, M. R., Schmitz, G. E., and Downs, D. M. (2008) YigF is required for isoleucine biosynthesis when *Salmonella enterica* is grown on pyruvate medium. *J. Bacteriol.* **190**, 3057–3062 CrossRef Medline

22. Enos-Berlage, J. L., Langendorf, M. I., and Downs, D. M. (1998) Complex metabolic phenotypes caused by a mutation in yigF, encoding a member of the highly conserved YER057c/YigF family of proteins. *J. Bacteriol.* **180**, 6519–6528 Medline

23. ERSN, D. C., AND DOWNS, D. M. (2016) 2-Aminoacrylate stress induces a context-dependent glycine requirement in *ridA* strains of *Salmonella enterica*. *J. Bacteriol.* **198**, 536–543 ERSN Medline

24. ERNST, D. C., AND DOWNS, D. M. (2018) Mmf1p couples amino acid metabolism to mitochondrial DNA maintenance in *Saccharomyces cerevisiae*. MBio **9**, e00084-18 Medline

25. Hodge-Hanson, K. M., Zoino, A., and Downs, D. M. (2018) Expression of PLP-independent racemases can reduce 2-aminoacrylate stress in *Salmo nella enterica*. *J. Bacteriol.* **200**, e00751-17 Medline

26. Christopherson, M. R., Lambrecht, J. A., Downs, D. M., and Downs, D. M. (2012) Suppressor analyses identify threonine as a modulator of *ridA* mutant phenotypes in *Salmonella enterica*. *PLoS* ONE **7**, e43082 CrossRef Medline

27. Schmitz, G., and Downs, D. M. (2004) Reduced transaminase B (IlvE) activity caused by the lack of yigF is dependent on the status of threonine deaminase (IlvA) in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **186**, 803–810 Medline

28. Barak, Z., Chipman, D. M., and Gollop, N. (1987) Physiological implications of the specificity of acetohydroxy acid synthase isozymes of enteric bacteria. *J. Bacteriol.* **169**, 3750–3756 CrossRef Medline

29. Chen, J. W., Bennett, D. C., and Umbarger, H. E. (1991) Specificity of attenuation control in the *ilvGMED* operon of *Escherichia coli* K-12. *J. Bacteriol.* **173**, 2328–2340 CrossRef Medline

30. LaRossa, R. A., and Van Dyk, T. K. (1987) Metabolic mayhem caused by 2-ketocacid imbalances. *Bioessays* **7**, 125–130 CrossRef Medline

31. Hoﬄer, J. G., and Burns, R. O. (1978) Threonine deaminase from *Salmonella typhimurium*. Effect of regulatory ligands on the binding of substrate to the active sites and the differentiation of the activator and inhibitor sites from the active sites. *J. Biol. Chem.* **253**, 1245–1251 Medline

32. Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J., and Rabinowitz, J. D. (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* **5**, 593–599 CrossRef Medline

33. Albe, K. R., Butler, M. H., and Wright, B. E. (1990) Cellular concentrations of enzymes and their substrates. *J. Theor. Biol.* **143**, 163–195 CrossRef Medline

34. Eisenstein, E. (1995) Allosteric regulation of biosynthetic threonine deaminase from *Escherichia coli*: effects of isoleucine and valine on active-site ligand binding and catalysis. *Arch. Biochem. Biophys.* **316**, 311–318 CrossRef Medline

35. Gallagher, D. T., Chinchilla, D., Lau, H., and Eisenstein, E. (2004) Local and global control mechanisms in allosteric threonine deaminase. *Methods Enzymol.* **380**, 85–106 CrossRef Medline

36. Segel, I. H., and Segel, L. D. (1992) An alternative substrate is not the same as a dead end inhibitor. *Biochem. Educ.* **20**, 155–157 CrossRef Medline

37. Lal, P. B., Schneider, B. L., Vu, K., and Reitzer, L. (2014) The redundant aminotransferases in lysine and arginine synthesis and the extent of aminotransferase redundancy in *Escherichia coli*. *Mol. Microbiol.* **94**, 843–856 CrossRef Medline

38. Toney, M. D. (2005) Reaction specificity in pyridoxal phosphate enzymes. *Arch. Biochem. Biophys.* **433**, 279–287 CrossRef Medline

39. Toney, M. D. (2011) Controlling reaction specificity in pyridoxal phosphate enzymes. *Biochim. Biophys. Acta* **1814**, 1407–1418 CrossRef Medline

40. Kuznetsova, I. M., Turoverov, K. K., and Uversky, V. N. (2014) What macromolecular crowding can do to a protein. *Int. J. Mol. Sci.* **15**, 23090–23140 CrossRef Medline

41. Vogel, H., and Bonner, D. M. (1956) Acetylornithase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **218**, 97–106 Medline

42. Balch, W. E., and Wolfe, R. S. (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32**, 781–791 Medline

43. Schmiewer, H. (1972) Phage P22 mutants with increased or decreased transduction abilities. *Mol. Gen. Genet.* **179**, 75–88 CrossRef Medline

44. Chan, R. K., Botstein, D., Watanabe, T., and Ogata, Y. (1972) Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II Properties of a high transducing lysate. *Virology* **50**, 883–898 CrossRef Medline

45. Downs, D. M., and Petersen, L. (1994) *apbA*, a new genetic locus involved in thiamine biosynthesis in *Salmonella typhimurium*. *J. Bacteriol.* **176**, 4858–4864 CrossRef Medline

46. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.2, 2006.0008 CrossRef Medline

47. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6640–6645 CrossRef Medline

48. Galloway, N. R., Tontkoushian, H., Nune, M., Bose, N., and Moomay, C. (2013) Rapid cloning for protein crystallography using type IIIS restriction enzymes. *Cryst. Growth Des.* **13**, 2833–2839 CrossRef Medline

49. Burns, R. O. (1971) 1-Threonine deaminase-biosynthetic (Salmonella typhimurium). *Methods Enzymol.* **17**, 555–560 CrossRef Medline

50. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 CrossRef Medline

51. Davis, L. (1965) A spectrophotometric method for the assay of threonine dehydratase. *Anal. Biochem.* **12**, 36–40 CrossRef Medline

52. Burns, R. O., and Zarleno, M. H. (1968) Threonine deaminase from *Salmonella typhimurium*. I. Purification and properties. *J. Biol. Chem.* **243**, 178–185 Medline

53. Zarleno, M. H., Robinson, G. W., and Burns, R. O. (1968) Threonine deaminase from *Salmonella typhimurium*. II. The subunit structure. *J. Biol. Chem.* **243**, 186–191 Medline