Metabolite damage and repair in metabolic engineering design

Jiayi Sun^a, James G. Jeffryes^b, Christopher S. Henry^{b,c}, Steven D. Bruner^d, Andrew D. Hanson^{a,*}

^a *Horticultural Sciences Department, University of Florida, Gainesville, FL, USA*

^b *Mathematics and Computer Science Division, Argonne National Laboratory, Argonne, IL, USA*

^c *Computation Institute, The University of Chicago, Chicago, IL, USA*

^d *Department of Chemistry, University of Florida, Gainesville, FL, USA*

*Corresponding author: E-mail address adha@ufl.edu (A.D. Hanson)

Abbreviations: SynBio, synthetic biology; NAD(P)HX, NAD(P)H hydrates;

ABSTRACT

The necessarily sharp focus of metabolic engineering and metabolic synthetic biology on pathways and their fluxes has tended to divert attention from the damaging enzymatic and chemical side-reactions that pathway metabolites can undergo. Although historically overlooked and underappreciated, such metabolite damage reactions are now known to occur throughout metabolism and to generate (formerly enigmatic) peaks detected in metabolomics datasets. It is also now known that metabolite damage is often countered by dedicated repair enzymes that undo or prevent it. Metabolite damage and repair are highly relevant to engineered pathway design: metabolite damage reactions can reduce flux rates and product yields, and repair enzymes can provide robust, host-independent solutions. Herein, after introducing the core principles of metabolite damage and repair, we use case histories to document how damage and repair processes affect efficient operation of engineered pathways – particularly those that are heterologous, non-natural, or cell-free. We then review how metabolite damage reactions can be predicted, how repair reactions can be prospected, and how metabolite damage and repair can be built into genome-scale metabolic models. Lastly, we propose a versatile ‘plug and play’ set of well-characterized metabolite repair enzymes to solve metabolite damage problems known or likely to occur in metabolic engineering and synthetic biology projects.

Key words: Metabolic engineering, Synthetic biology, Metabolite damage, Metabolite repair, Side-reaction, Enzyme promiscuity

1. Introduction

Metabolic engineering and metabolic synthetic biology (SynBio) focus on pathways and the fluxes through them, as does metabolic biochemistry in general. While essential, this focus tends to lead to an idealized, ‘pathway-centric’ mindset that sees enzymes as perfectly specific and their intermediates and products as the only relevant compounds in chemical space (Danchin, 2017; Lerma-Ortiz et al., 2016; de Lorenzo et al., 2015). Excessively pathway-centric thinking is problematic for metabolic engineering and SynBio as well as for basic understanding of metabolic networks because it downplays metabolism’s dark underside (Golubev, 1996). In this neglected underside, metabolites continuously undergo damaging chemical and enzymatic side-reactions *in vivo*, and cells ceaselessly fight this damage with a suite of repair enzymes (Van Schaftingen et al., 2009). The products of metabolite damage are basically wasteful and often toxic. If allowed to accumulate, they can disrupt the function of both native and engineered pathways (Collard et al., 2016; Schwander et al., 2016).

After decades on the margins, metabolite damage reactions and the matching repair enzymes are now entering mainstream metabolic biochemistry, and examples of damage and repair are being uncovered at an increasing rate (reviewed by Hanson et al., 2016; Keller et al., 2015; Linster et al., 2013; Van Schaftingen et al., 2013). There is also a growing case list of metabolic engineering and metabolic SynBio projects whose design has taken metabolite damage and repair into account – with positive outcomes. To help consolidate the place of metabolite damage and repair in engineering project design, in this review we first summarize the core principles involved using engineering-relevant examples. We then illustrate these principles in action using a series of engineering case-histories, and describe how metabolite damage and repair reactions can be predicted and modeled. Lastly, we propose a set of repair enzymes that are likely to prove useful in diverse engineering projects. Fuller treatments of metabolite damage and repair – including the iconic oxygenase and other side-reactions of the photosynthetic CO₂-fixing enzyme RubisCO – are available in recent reviews (Erb and Zarzycki, 2016; Hanson et al., 2016; Linster et al., 2013).

2. Chemical and enzymatic damage to metabolites

As just noted, metabolite damage has two sources: spontaneous chemical reactions that take place under *in vivo* conditions, and enzyme errors. These errors involve an enzyme acting, at a low rate, on a substrate other than the physiological one (enzyme promiscuity or sloppiness), or catalysis of an abnormal (‘misfire’) reaction on the physiological substrate. Spontaneous reactions may involve only metabolites, i.e. small molecules, or attack by a metabolite on large molecules, i.e. proteins or nucleic acids. Whether of spontaneous or enzymatic origin, damage products are essentially a wasteful diversion of pathway flux, and are often inhibitory to enzymes or otherwise actively detri-

mental or functionally compromised (Linster et al., 2013). It is important to note at the outset that metabolite damage reactions are favored by high metabolic flux rates and large pool sizes (Ito et al., 2016; Rzem et al., 2007; Sullivan et al., 2013) because metabolic engineering approaches generally aim to achieve high fluxes and involve expanded pools. Engineering efforts thus often aggravate metabolite damage and must find ways to cope with it. While it is in principle possible to reduce damage from enzyme promiscuity or misfires by enzyme engineering, in practice this is not easy because of the trade-off between improved specificity and catalytic activity (Tawfik, 2014). Another potential way to reduce damage is by sequestering chemically reactive metabolites or problematic substrates for promiscuous enzymes within multienzyme complexes (metabolons) or special compartments, but as such approaches are in their infancy they are again not easy to implement (Singleton et al., 2014; Young et al., 2017). Figure 1 illustrates the various types of metabolite damage using examples from three core sectors of primary metabolism: cofactors, sugar phosphates and amino acids. Damage reactions like those in Figure 1 – chemical or enzymatic – occur in almost all cells almost all the time, and in at least some cases are accelerated by suboptimal conditions (Intlekofer et al., 2017; Piedrafita et al., 2015).

2.1. Cofactor damage

NADH and NADPH hydrates (Fig. 1A) are formed by a side-reaction of the core glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase and also spontaneously, particularly at high temperatures (Acheson et al., 1988; Marbaix et al., 2011; Oppenheimer and Kaplan, 1974). The hydrates (abbreviated NAD(P)HX) exist as *R* and *S* epimers; they inhibit various dehydrogenases (Prabhakar et al., 1998; Yoshida and Dave, 1975) and consequently cannot be allowed to accumulate.

5-Formyltetrahydrofolate (Fig. 1B) is formed from 5,10-methylenetetrahydrofolate by a misfire reaction of the near-ubiquitous folate-dependent enzyme serine hydroxymethyltransferase (Stover and Schirch, 1990) and is also formed spontaneously from 5,10-methylenetetrahydrofolate at low pH (Baggott, 2000). 5-Formyltetrahydrofolate is potent inhibitor of folate-dependent enzymes, and therefore must be removed to prevent its build-up (Stover and Schirch, 1993).

2.2. Sugar phosphate damage

Methylglyoxal, also called pyruvaldehyde (Fig. 1C), forms spontaneously from the triose phosphate intermediates of glycolysis – glyceraldehyde 3-phosphate and dihydroxyacetone phosphate – via an elimination reaction that is further accelerated by a side-activity of triose phosphate isomerase (Richard, 1993). Methylglyoxal, a reactive dicarbonyl compound, is a potent glycating agent that reacts spontaneously with the amino or thiol groups of other metabolites, proteins, and DNA (Fig.

1C) (Richarme et al., 2016; Richarme et al., 2017; Thornalley, 2008). The initial glycation products (aminocarinols or hemithioacetals) can undergo a series of dehydrations, oxidations, and rearrangements (Maillard reactions) that produce Schiff bases, Amadori products, advanced glycation end products, and cross-links between macromolecules (Richarme et al., 2015). There is thus a cascade effect in which the initial damage product (methylglyoxal) goes on to create further damage.

4-Phosphoerythronate and 2-phospho-L-lactate (Fig. 1D) are generated by side-reactions of two glycolytic enzymes (Collard et al., 2016). 4-Phosphoerythronate is formed as a consequence of glyceraldehyde 3-phosphate dehydrogenase acting on the pentose phosphate pathway intermediate erythrose 4-phosphate, which is itself a reactive glycation agent (Van Schaftingen et al., 2012). 2-Phospho-L-lactate is formed by pyruvate kinase acting on L-lactate. 4-Phosphoerythronate inhibits the pentose phosphate pathway enzyme 6-phosphogluconate dehydrogenase and 2-phospho-L-lactate inhibits phosphofructokinase, which makes the glycolytic activator fructose-2,6-bisphosphate.

2.3. Amino acid damage

Deaminated glutathione (Fig. 1E), in which a keto group replaces the α -amino group of glutathione's glutamyl moiety, is produced by a side-activity of many transaminases (Peracchi et al., 2017) and at least one decarboxylase (Novogrodsky and Meister, 1964). The ketoacid moiety of deaminated glutathione cyclizes spontaneously, giving two anomeric forms; this cyclization makes the transamination reaction that produces deaminated glutathione essentially irreversible. Deaminated glutathione may interfere with the activity of glutathione-dependent enzymes, and its formation can represent a major drain on the glutathione pool (Peracchi et al., 2017).

5-Oxoproline (also called pyroglutamate) is the lactam form of glutamate (Fig. 1F). It is formed via spontaneous cyclization of glutamine (Tritsch and Moore, 1962), glutamate (Park et al., 2001), and the proline biosynthesis intermediate γ -glutamyl phosphate (Orlowski and Meister, 1971). 5-Oxoproline forms very readily from glutamine; the conversion rate is 10% per day in physiological conditions (pH 7.2, moderate ionic strength, 37°C) (Tritsch and Moore, 1962). Although the mechanism is unclear, 5-oxoproline is widely toxic (Niehaus et al., 2017; Park et al., 2001). In addition to being a universal metabolite damage product, 5-oxoproline is a normal metabolite of glutathione in eukaryotes (Kumar and Bachhawat, 2012).

2.4. Damage products pervade metabolomics profiles

Chemically or enzymatically damaged metabolites are almost surely major contributors to the 'dark matter' of metabolomes, until now largely ignored (Fiehn et al., 2011; Showalter et al., 2017). All the

damage product examples above are detectable by targeted or untargeted GC-MS or LC-MS metabolomics analyses (Collard et al., 2016; Niehaus et al., 2014; Peracchi et al., 2017; Ringling and Rychlik, 2013; Shaheen et al., 2014; Thornalley and Rabbani, 2014; van der Werf et al., 2007). It follows that the same should be true of the plethora of other damage products that side-reactions can generate from the enormous chemical diversity of metabolites (Fiehn et al., 2011; Peracchi et al., 2017). A major obstacle to identifying such damage products is that – like most of the examples above – they are not in metabolite libraries and they cannot be purchased. In a later section (5.1) we show how computational chemistry can help overcome this obstacle by predicting the structures and properties of damage products for which no experimental data are yet available.

3. Damage repair enzymes

Damage repair enzymes, defined broadly, either undo damage by reconvertng damaged molecules to normal ones, or dispose safely of harmful damage products by converting them to harmless ones. These activities are also called metabolite proofreading and damage pre-emption, respectively (Linster et al., 2013; Van Schaftingen et al., 2013). We illustrate repair principles using the enzymes that repair the cofactor, sugar phosphate, and amino acid damage products described above (Fig. 1).

3.1. Cofactor repair

NADHX and NADPHX are reconverted to NADH and NADPH by the sequential action of NAD(P)HX epimerase and NAD(P)HX dehydratase, which both occur in all domains of life (Marbaix et al., 2011; Niehaus et al., 2014) (Fig. 1A). The dehydratase is specific for the *S* epimer of NAD(P)HX (Acheson et al., 1988) and the epimerase facilitates conversion of the *R* form to the *S* form used by the dehydratase. Epimerization also proceeds spontaneously, so that the epimerase may not always be needed (Acheson et al., 1988). The dehydratase is ATP- or ADP-dependent (Marbaix et al., 2011), illustrating the general principle that repair mechanisms often have an energetic cost (Linster et al. 2013).

5-Formyltetrahydrofolate is reconverted to the mainstream folate 5,10-methylenetetrahydrofolate by 5-formyltetrahydrofolate cyclo-ligase in an ATP-dependent reaction (Stover and Schirch, 1993) (Fig. 1B). This repair enzyme occurs in all domains of life. Certain prokaryotes instead use an enzyme that transfers the 5-formyl group to glutamate, yielding tetrahydrofolate (Jeanguenin et al., 2010).

3.2. Sugar phosphate repair

Methylglyoxal has long been known to be rendered harmless by conversion to D-lactate, via lactoyl-glutathione, by the near-ubiquitous enzymes glyoxalase I and II acting in tandem (Thornalley, 1990) (Fig. 1C). Recent work has uncovered another near-ubiquitous enzyme, DJ-1, that repairs methylgly-

oxal-glycated arginine, lysine, and cysteine residues in proteins by acting on the early glycation intermediates (aminocarbonyls or hemithioacetals) to release lactate, thereby forestalling the Maillard cascade (Richarme et al., 2015) (Fig. 1C). DJ-1 also repairs methylglyoxal-glycated guanine bases in nucleotides and nucleic acids, again releasing lactate (Richarme et al., 2017). Because DNA glycation raises mutation frequencies, DJ-1 helps maintain DNA integrity. DJ-1 can in addition repair glyoxal-glycated proteins and nucleic acids (Richarme et al., 2015; Richarme et al., 2017).

4-Phosphoerythronate and 2-phospho-L-lactate are both hydrolyzed by a widely conserved phosphatase (PGP in mammals, Pho13 in yeast), yielding innocuous products (Collard et al., 2016) (Fig. 1D). There are probably similar phosphatases in plants and bacteria (Beaudoin and Hanson, 2016).

3.3. Amino acid repair

Deaminated glutathione is safely hydrolyzed to the normal metabolites α -ketoglutarate and cysteinylglycine by the enzyme Nit1 (Fig. 1E), which occurs in eukaryotes and many prokaryotes (Peracchi et al., 2017). The cysteinylglycine product can be hydrolyzed by various peptidases.

5-Oxoproline is hydrolyzed to glutamate by ATP-dependent 5-oxoprolinases (Niehaus et al., 2017; Van der Werf et al., 1971) (Fig. 1F). The eukaryotic-type 5-oxoprolinase is a single gene product whereas the prokaryotic-type enzyme has three subunits encoded by different genes (Niehaus et al., 2017).

4. Engineering case histories

Designing an engineering solution depends on first diagnosing the problem. As stressed in the Introduction, metabolite damage has historically been underdiagnosed. Nevertheless, there are good examples from metabolic engineering and SynBio where metabolite damage problems were diagnosed correctly and solved. Four such cases are summarized below. Collectively, these cases illustrate three general principles: (i) Without the appropriate metabolite repair enzymes, an engineered metabolic pathway may not function efficiently, and can spark genome instability that leads to its own inactivation. (ii) Installing a non-native pathway in a platform organism always runs some risk of failure because the platform lacks sufficient repair capacities, and the more alien the pathway is to the platform, the higher the risk. (iii) This risk is even higher in synthetic pathways consisting only of purified pathway enzymes because such systems have no repair capacities at all.

4.1. 1,3-Propanediol production

Anaerobic fermentation of glycerol to 1,3-propanediol can be engineered in *Escherichia coli* using four *Klebsiella pneumoniae* enzymes: glycerol dehydratase and 3-hydroxypropionaldehyde reductase to produce 1,3-propanediol; and glycerol dehydrogenase and dihydroxyacetone kinase to provide the NADH needed and to yield dihydroxyacetone phosphate as a carbon and energy source for growth (Tong et al., 1991) (Fig. 2A). Growth and 1,3-propanediol production were found to be limited by methylglyoxal accumulation; the limitation was relieved by overexpressing glyoxalase I, which improved cell growth and raised 1,3-propanediol production by 50% (Zhu et al., 2001) (Fig. 2A). Glyoxalase I is considered the rate-limiting step in the glyoxalase pathway (Takasawa et al., 2011). This case shows how problematic engineering strategies that expand triose phosphate pools can be, due to the inevitable accompanying rise in methylglyoxal formation (see section 2.2). It is also a paradigmatic example of metabolite repair being incorporated into the metabolic engineering design cycle.

4.2. Mevalonate pathway

Operation of the yeast mevalonate pathway in *E. coli* can be compromised by buildup of its isopentenyl and dimethylallyl diphosphate intermediates, which are electrophilic damage agents that cause cells to cease growth or to mutate, silencing the pathway (Martin et al., 2003; Withers et al., 2007). Prospecting for a solution by screening a *Bacillus subtilis* genomic library for genes that overcome isopentenyl/dimethylallyl diphosphate toxicity identified two repair phosphatases, NudF and YhfR, that hydrolyze the diphosphates to harmless isopentenols (Fig. 2B) (Withers et al., 2007). NudF and the similar enzyme NudB were later creatively repurposed to build non-natural pathways to isopentenol biofuels (Chou and Keasling, 2012; Zheng et al., 2013). Besides demonstrating damage diagnosis and parts prospecting for repair enzymes, this case highlights how a non-native pathway can destabilize its own genes via metabolite damage-driven hypermutation (de Lorenzo, 2014).

4.3. 1,4-Butanediol production

A pathway from succinyl-CoA or α -ketoglutarate to the non-natural product 1,4-butanediol was engineered in *E. coli* by installing five heterologous enzymes (Yim et al., 2011) (Fig. 2C). One of several undesired by-products of this alien pathway was γ -butyrolactone, formed from the intermediate 4-hydroxybutyryl-CoA via a spontaneous damage reaction (Fig. 2C). The problem of γ -butyrolactone accumulation was solved by installing a heterologous γ -butyrolactonase (Chen et al., 2010) to hydrolyze the lactone to 4-hydroxybutyrate, thus recycling it back into the 1,4-butanediol synthesis pathway (Burgard et al., 2016) (Fig. 2C). This case illustrates how absence of a repair enzyme from the platform organism can degrade the efficiency of an alien pathway. As 4-hydroxybutyryl-CoA is not a native *E. coli* metabolite, *E. coli* cells would not be predicted to have a dedicated repair enzyme for γ -butyrolactone; the engineering outcomes confirmed that such an enzyme was indeed lacking.

4.4. Synthetic CO₂ fixation pathway

A twelve-step, non-natural CO₂ fixation cycle (the CETCH cycle) was built from purified recombinant enzymes originating from nine different organisms (Schwander et al., 2016). The cycle output was measured as malate production. To obtain effective cycle operation, it was necessary to deal with various side-reactions. One such side-reaction, mediated by the cycle enzyme β -methylmalyl-CoA lyase, was the formation of malyl-CoA from glyoxylate and acetyl-CoA (Fig. 2D) (Erb et al., 2010). Installation of a repair enzyme – malyl-CoA thioesterase from *Rhodobacter sphaeroides* (Erb et al., 2010) – prevented the buildup of malyl-CoA (Fig. 2D) (Schwander et al., 2016). This case emphasizes that a miscellany of enzymes used to assemble a non-natural pathway will by definition encounter metabolites to which they were never exposed in their native hosts, providing many opportunities for new side-reactions arising from enzyme promiscuity. Such promiscuous reactions can in principle be predicted at the design stage, as discussed below (section 5.1). Another issue raised indirectly by the CETCH cycle and other systems comprised of purified enzymes (e.g., Bujara et al., 2010; Opgenorth et al., 2016) is that carbonyl intermediates such as glyoxylate (Fig. 2D) – a highly reactive glycating agent (Dutta et al., 2007) – are virtually certain to react with enzymes over time, leading in some cases to their inactivation. The same is likely true of the CETCH cycle intermediate acrylyl-CoA, a very reactive electrophile that attacks sulfhydryl groups (Curson et al., 2014).

4.5. Chemistry of secondary pathways

The biosynthetic pathways to secondary metabolites produce a vast array of diverse and complex small molecules. The products commonly include therapeutics, making these pathways high-value targets for SynBio and metabolic engineering efforts. In contrast to primary metabolism, very little is known about metabolite repair enzymes in secondary metabolism, and metabolite repair has rarely been utilized in secondary pathway engineering. Indeed, secondary product biosynthesis genes, particularly in microbes, are often strictly clustered and the function of each of the gene products can be assigned, with no metabolite repair machinery apparently present. This suggests that repair may not be widespread in secondary pathways – which seems paradoxical, given the many chemically reactive intermediates in these pathways. One explanation may be metabolic tunneling (channeling) of highly reactive intermediates amongst pathway enzymes, e.g. the strictosidine aglycone common to monoterpene indole alkaloid pathways (Stavrinides et al., 2015). Also, secondary pathways typically operate in the native host with low efficiency, producing mixtures of products with relatively low yields of each. This low output presumably suffices for the biological need/use of the natural product and increased efficiency (via repair of intermediates) is not needed. In contrast, metabolic engineering and SynBio efforts aim for high flux and efficiency, so it is reasonable to think that incorporating

repair machinery into engineered systems will be useful. Somewhat similar considerations apply to the analogous area of engineering efficient pathways to degrade recalcitrant non-natural chemical structures that contaminate the environment (Dvořák et al., 2017). As with the biosynthetic intermediates of many therapeutics (some of which can become contaminants), the intermediates of degradation may be totally alien to engineered microbes, again making repair machinery useful.

One established example of repair in secondary metabolism is the occurrence of external (type II) thioesterases in biosynthetic pathways to polyketide (PK) and nonribosomal peptide (NRP) secondary metabolites (homologous enzymes function in fatty acid pathways) (Horsman et al., 2016). The enzymatic machinery to PKs and NRPs consists of large modular, multidomain synth(et)ase proteins in which the substrates and pathway intermediates are attached to small carrier domains via thioester linkages to a phosphopantetheine prosthetic group. Sources of errors or stalls in the pathway can be misloading of incorrect building blocks by pathway enzymes or by the promiscuous action of the post-translational modifying phosphopantetheine transferase (PPTase) (Fig. 2E) (Beld et al., 2014). In their canonical reaction, PPTases use coenzyme A (CoA) as substrate to add a thiol-containing phosphopantetheine arm to the carrier domain, but promiscuous activity can potentially use acetyl-CoA or other cellular acyl-CoAs instead, and so add an acylated phosphopantetheine. Either of these error pathways could stall the assembly line and/or produce aberrant products. Type II thioesterases can hydrolyze these aberrant intermediates, restoring the free holo-synth(et)ase. Proposals for mechanism include inherent specificity for incorrect versus correct thioesters or use of low level, non-specific activity acting on kinetically stable, stalled machinery (Heathcote et al., 2001). Indeed, a majority of PK and NPR biosynthetic gene clusters contain an external thioesterase and removing the gene significantly reduces natural product yield in several systems (Kotowska and Pawlik, 2014).

5. Predicting and modeling metabolite damage and repair

5.1. Predicting metabolite damage reactions

The diversity of potential metabolite damage reactions makes these reactions challenging to predict. Fortunately, numerous computational tools have been developed to systematically predict new potential reactions and metabolites in biological systems for the purpose of proposing novel metabolic pathways; these tools include BNICE (Hatzimanikatis et al., 2005), EnviPath (Wicker et al., 2016), Retropath (Fehér et al., 2014), and ReactPRED (Sivakumar et al., 2016). Starting from known biochemical reactions, they all derive generic reaction rules that define reactive chemical substructures and the corresponding substructure in the products. These reaction rules can then be applied to alternative substrates in order to predict new potential reactions and products in a manner similar to the “find and replace” function in a text editor. The rules can be derived from manual curation of

known reactions (e.g. BNICE, EnviPath) or generated algorithmically (Retropath, ReactPRED); both methods depend on having a source database of biochemical data such as MetaCyc (Caspi et al., 2016) and KEGG (Kanehisa et al., 2014) from which to derive the rules. The reaction rule approach has been used to predict enzyme promiscuity reactions and products by applying reaction rule sets to public databases such as KEGG and EcoCyc (Jeffryes et al., 2015). The resulting database of promiscuity products is particularly useful for proposing novel metabolites that correspond to unannotated features in untargeted metabolomics datasets (Allard et al., 2017).

This cheminformatic reaction rule approach was also recently extended to the prediction of potential damage chemistry (Lerma-Ortiz et al., 2016). Because spontaneous reactions are poorly represented in MetaCyc, KEGG, and similar databases (just 3% of reactions in MetaCyc are currently annotated as spontaneous), this effort required the development of a new database of known spontaneous reactions, merging the 398 spontaneous reactions from MetaCyc with 366 reactions gathered from the primary literature. A total of 106 reaction rules were derived from these spontaneous reactions, and were applied to a database of known metabolites to generate >72,000 predicted damage products. These predicted damage products and the reactions that form them comprise the Chemical Damage Metabolic *In silico* Network Expansion (or CD-MINE) (Lerma-Ortiz et al., 2016). The CD-MINE offers a rich set of damage possibilities that can be filtered with metabolomic or genomic evidence to identify potential damage problems and to design repair solutions.

5.2. Prospecting for repair enzymes

Repair enzymes can be prospected by classical or computational strategies. Classical approaches were recently reviewed (Hanson et al., 2016; Linster et al., 2013) so are only briefly outlined here, focusing on those helpful in engineering. Computational approaches are then covered in more detail.

Classical bioprospecting strategies for repair enzymes are essentially manual, require expert knowledge, and are of two types: biochemical and genomic (Linster et al., 2013). In both cases, it is necessary to first diagnose what the damage product is, and preferably what the damage reaction is. This information is mined from the literature or obtained by metabolome analysis. The biochemical strategy envisions potential repair pathways for the damage product, searches for the enzyme activities involved, and clones the corresponding enzymes. NAD(P)HX dehydratase (section 3.1), the PGP/Pho13 phosphatase (section 3.2), and ethylmalonyl-CoA decarboxylase (Table 1) were found in this way. The comparative genomic strategy rests on the 'guilt by association' principle (Hanson et al., 2009). This principle predicts that genes encoding repair enzymes will be clustered on prokaryotic chromosomes with, fused to, or co-expressed with (i) genes for enzymes whose promiscuous act-

ivities generate damage products, (ii) genes for synthesis or utilization of metabolites that undergo damage (chemical or enzymatic), and (iii) genes specifying other enzymes in the same repair pathway. NAD(P)HX epimerase and glutamate formyltransferase (section 3.1) and prokaryotic 5-oxoprolinase (section 3.3) were discovered by this type of approach.

In addition to these classical methods, the power of cheminformatics algorithms to predict enzymatic reactions can be readily applied to predict potential repair reactions for damaged metabolites. Figure 3 illustrates how the repair reactions (all hydrolytic) from the engineering case studies in Sections 4.1–4.4 can be retrospectively predicted by generalized reaction rules. Even if a specific repair reaction has already been hypothesized based on other lines of evidence, cheminformatics can be used to ensure that a more comprehensive set of reaction possibilities are considered. For example, each of the damaged metabolites from the above four case studies exists in the MINE database of potential novel enzyme reactions (Jeffryes et al., 2015), allowing mining of this database for other potential repair pathways.

Finally, once a repair reaction has been proposed, cheminformatics can help discover candidate enzymes that catalyze it. The EC-BLAST tool (Rahman et al., 2014) can compare a proposed reaction to known biochemical reactions from KEGG and output a list of reactions that are similar to the query. This similarity is based on either matching the functional groups of the reactants and products in the two reactions or on the bonds that are broken and formed in each case. Critically, not all enzymes that mediate similar reactions are sequence homologs. EC-BLAST can thus identify a more complete range of candidate enzymes with similar substrate range and function to the proposed repair reaction than simply searching within a single protein family.

5.3. Incorporating metabolite damage and repair in genome-scale metabolic models

The above considerations show how numerous potential damage reactions, and subsequent potential repair pathways, can be spawned just from the portion of the biochemical reaction space that is known and well understood. Many of these damage reactions will never cause problems in either a wild-type or a heavily engineered cell, but in certain cases an otherwise innocuous damage mechanism may become problematic when a heavily engineered cell begins operating in a state that is far removed from its natural homeostatic condition. Identifying which of the overwhelming number of potential damage reactions may fit in this category can be a great challenge. Fortunately, metabolic modeling algorithms are well equipped to address this challenge. Potential damage reactions, and associated predicted repair pathways, may be integrated into metabolic models using algorithms like gapfilling (Satish Kumar et al., 2007) and flux variability analysis (Mahadevan and Schilling, 2003) to

determine: (i) which potential damage and repair pathways are likely to be operational and relevant to a particular host organism; (ii) which damage and repair pathways are likely to function when a target pathway is overexpressed or transferred to a new host; (iii) the potential impact on product yields and growth rates when various amounts of flux pass through a damage and repair pathway; and (iv) potential biomarkers that may appear in transcriptomes or metabolomes when a particular damage or repair activity is taking place. Models can also help engineers to identify modifications to the cell that could mitigate problematic metabolite damage or induce a potential repair mechanism.

Given the potential for metabolic models to aid in the management of metabolite damage that may arise as a result of modifications made during metabolic engineering, it is no surprise that damage and repair pathways have begun to appear in the most sophisticated genome-scale metabolic models. Specifically, the iJO1366 *E. coli* model (Orth et al., 2011) includes 57 spontaneous reactions, many of which fit the criteria for metabolite damage. Twenty one of these reactions lead to products that are subsequently enzymatically converted to a more innocuous form, i.e. are handled by repair mechanisms. Despite these advances, *E. coli* and other platform or chassis organisms almost surely have many undiscovered metabolite damage reactions and repair pathways (Linster et al., 2013), which obviously cannot yet be included in models. Therefore much remains to be done in this area.

6. A damage repair enzyme kit

The above damage and repair examples and engineering case histories demonstrate that metabolite damage is a clear and present danger to all engineered pathway systems, especially non-natural or cell-free ones, and that enough is known to design solutions to at least the most obvious damage problems. The value of applying damage and repair principles in pathway design is gaining recognition (Erb et al., 2017), as captured by the statement: “the concept of metabolic proofreading has only rarely been considered for synthetic pathway design...We believe that this design principle should be included more systematically in metabolic engineering in the future” (Schwander et al., 2016).

To facilitate adoption of damage and repair principles, Table 1 lists damage reactions to a score of central metabolites and a matching set of repair enzymes, with references to their characterization and engineering applications. About half of these enzymes have already been covered in section 3 and are documented there; the others are briefly documented in Table 1 itself. Table 1 is not a full inventory of reported metabolite damage reactions and repair enzymes; there are many more (Hanson et al., 2016; Lerma-Ortiz et al., 2016; Linster et al., 2013). Rather, it focuses on enzymes that seem likely to be widely useful when engineering primary metabolism in diverse organisms or *in vitro*. Fluxes through the pools of the central metabolites subject to the damage reactions in Table 1

are generally high. This tends to favor side-reactions because, as noted in Section 2, the more the flux through a canonical enzymatic reaction, the more the flux through that enzyme's promiscuous side-reactions (Rzem et al., 2007). Central metabolites are thus the most likely ones to need repair. The enzymes in Table 1 are nothing like a panacea, but singly or in combination they can take care of various widespread damage problems and so constitute useful parts for engineering design.

Acknowledgements

This work was supported by the National Science Foundation [grant numbers MCB-1611711, MCB-1611952] and by an endowment from the C.V. Griffin Sr. Foundation.

References

- Acheson, S.A., Kirkman, H.N., Wolfenden, R., 1988. Equilibrium of 5,6-hydration of NADH and mechanism of ATP-dependent dehydration. *Biochemistry* 27(19), 7371–7375.
<http://dx.doi.org/10.1021/bi00419a030>.
- Aliprandi, P., Navarro, E., Raynaud, C., Bestel Corre, G., Soucaille, P., 2017. New microorganism and method for the production of 1,2-propanediol based on NADPH dependent acetol reductase and improved NADPH supply. United States Patent Application Publication No. US2017 0145446 A1
- Allard, P.-M., Genta-Jouve, G., Wolfender, J.-L., 2017. Deep metabolome annotation in natural products research: towards a virtuous cycle in metabolite identification, *Curr. Opin. Chem. Biol.* 36, 40–49. <http://dx.doi.org/10.1016/j.cbpa.2016.12.022>.
- Baggott, J.E., 2000. Hydrolysis of 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate at pH 2.5 to 4.5. *Biochemistry* 39(47), 14647–14653. <http://dx.doi.org/10.1021/bi001362m>.
- Beaudoin, G.A., Hanson, A.D., 2016. A guardian angel phosphatase for mainline carbon metabolism. *Trends Biochem. Sci.* 41, 893–894. <https://doi.org/10.1016/j.tibs.2016.08.005>.
- Beld, J., Sonnenschein, E.C., Vickery, C.R., Noel, J.P., Burkart, M.D., 2014. The phosphopantetheinyl transferases: catalysis of a post-translational modification crucial for life. *Nat. Prod. Rep.* 31(1) 61–108. <https://doi.org/10.1039/c3np70054b>.
- Bujara, M., Schümperli, M., Billerbeck, S., Heinemann, M., Panke, S., 2010. Exploiting cell-free systems: Implementation and debugging of a system of biotransformations. *Biotechnol. Bioeng.* 106(3), 376–389. <https://doi.org/10.1002/bit.22666>.
- Burgard, A., Burk, M.J., Osterhout, R., Van Dien, S., Yim, H., 2016. Development of a commercial scale process for production of 1,4-butanediol from sugar. *Curr. Opin. Biotechnol.* 42, 118–125.
<http://dx.doi.org/10.1016/j.copbio.2016.04.016>.

- Cai, H., Clarke, S., 1999. A novel methyltransferase catalyzes the methyl esterification of *trans*-aconitate in *Escherichia coli*. J. Biol. Chem. 274(19), 13470–13479.
<http://dx.doi.org/10.1074/jbc.274.19.13470>.
- Caspi, R., Billington, R., Ferrer, L., Foerster, H., Fulcher, C.A., Keseler, I.M., Kothari, A., Krumnacker, M., Latendresse, M., Mueller, L.A., Ong, Q., Paley, S., Subhraveti, P., Weaver, D.S., Karp, P.D., 2016. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res. 44(D1), D471–D480.
<http://dx.doi.org/10.1093/nar/gkv1164>.
- Chen, B., Fan, L.Q., Xu, J.H., Zhao, J., Zhang, X., Ouyang, L.M., 2010. Biocatalytic properties of a recombinant *Fusarium proliferatum* lactonase with significantly enhanced production by optimal expression in *Escherichia coli*. Appl. Biochem. Biotechnol. 162(3), 744–756.
<https://doi.org/10.1007/s12010-009-8819-1>.
- Chou, H.H., Keasling, J.D., 2012. Synthetic pathway for production of five-carbon alcohols from isopentenyl diphosphate. Appl. Environ. Microbiol. 78(22), 7849–7855.
<http://dx.doi.org/10.1128/AEM.01175-12>.
- Collard, F., Baldin, F., Gerin, I., Bolsée, J., Noël, G., Graff, J., Veiga-da-Cunha, M., Stroobant, V., Vertommen, D., Houddane, A., Rider, M.H., Linster, C.L., Van Schaftingen, E., Bommer, G.T., 2016. A conserved phosphatase destroys toxic glycolytic side products in mammals and yeast. Nat. Chem. Biol. 12(8), 601–607. <http://dx.doi.org/10.1038/nchembio.2104>.
- Curson, A.R., Burns, O.J., Voget, S., Daniel, R., Todd, J.D., McInnis, K., Wexler, M., Johnston, A.W., 2014. Screening of metagenomic and genomic libraries reveals three classes of bacterial enzymes that overcome the toxicity of acrylate. PLoS One 9(5), e97660.
<http://dx.doi.org/10.1371/journal.pone.0097660>.
- Danchin, A., 2017. Coping with inevitable accidents in metabolism. Microb. Biotechnol. 10(1), 57–72.
<http://dx.doi.org/10.1111/1751-7915.12461>.
- Delpierre, G., Rider, M.H., Collard, F., Stroobant, V., Vanstapel, F., Santos, H., Van Schaftingen, E., 2000. Identification, cloning, and heterologous expression of a mammalian fructosamine-3-kinase. Diabetes 49(10), 1627–1634. <https://doi.org/10.2337/diabetes.49.10.1627>.
- Dutta, U., Cohenford, M.A., Guha, M., Dain, J.A., 2007. Non-enzymatic interactions of glyoxylate with lysine, arginine, and glucosamine: a study of advanced non-enzymatic glycation like compounds. Bioorg. Chem. 35(1), 11–24. <https://doi.org/10.1016/j.bioorg.2006.07.005>.
- Dvořák, P., Nikel, P.I., Damborský, J., de Lorenzo V., 2017. Bioremediation 3.0: Engineering pollutant-removing bacteria in the times of systemic biology. Biotechnol. Adv. 35(7), 845–866.
<https://doi.org/10.1016/j.biotechadv.2017.08.001>.

- Erb, T.J., Frerichs-Revermann, L., Fuchs, G., Alber, B.E., 2010. The apparent malate synthase activity of *Rhodobacter sphaeroides* is due to two paralogous enzymes, (3S)-methylmalyl-coenzyme A (CoA)/ β -methylmalyl-CoA lyase and (3S)-methyl-CoA thioesterase. *J. Bacteriol.* 192(5), 1249–1258. <http://dx.doi.org/10.1128/JB.01267-09>.
- Erb, T.J., Jones, P.R., Bar-Even, A., 2017. Synthetic metabolism: metabolic engineering meets enzyme design. *Curr. Opin. Chem. Biol.* 37, 56–62. <http://dx.doi.org/10.1016/j.cbpa.2016.12.023>.
- Erb, T.J., Zarzycki, J., 2016. Biochemical and synthetic biology approaches to improve photosynthetic CO₂-fixation. *Curr. Opin. Chem. Biol.* 34, 72–79. <http://dx.doi.org/10.1016/j.cbpa.2016.06.026>.
- Fehér, T., Planson, A.G., Carbonell, P., Fernández-Castané, A., Grigoras, I., Dariy, E., Perret, A., Faulon, J.L., 2014. Validation of RetroPath, a computer-aided design tool for metabolic pathway engineering. *Biotechnol. J.* 9(11), 1446–1457. <http://dx.doi.org/10.1002/biot.201400055>.
- Fiehn, O., Barupal, D.K., Kind, T., 2011. Extending biochemical databases by metabolomic surveys. *J. Biol. Chem.* 286(27), 23637–23643. <https://doi.org/10.1074/jbc.R110.173617>.
- Fortpied, J., Gemayel, R., Stroobant, V., van Schaftingen, E., 2005. Plant ribulosamine/erythrosamine 3-kinase, a putative protein-repair enzyme. *Biochem. J.* 388(3), 795–802. <https://doi.org/10.1042/BJ20041976>.
- Gemayel, R., Fortpied, J., Rzem, R., Vertommen, D., Veiga-da-Cunha, M., Van Schaftingen, E., 2007. Many fructosamine 3-kinase homologues in bacteria are ribulosamine/erythrosamine 3-kinases potentially involved in protein deglycation. *FEBS J.* 274(17), 4360–4374. <https://doi.org/10.1111/j.1742-4658.2007.05948.x>.
- Golubev, A.G., 1996. The other side of metabolism: a review. *Biochemistry (Mosc.)* 61(11), 2018–2039.
- Hanson, A.D., Henry, C.S., Fiehn, O., de Crécy-Lagard, V., 2016. Metabolite damage and metabolite damage control in plants. *Annu. Rev. Plant Biol.* 67, 131–152. <http://dx.doi.org/10.1146/annurev-arplant-043015-111648>.
- Hanson, A.D., Pribat, A., Waller, J.C., de Crécy-Lagard, V., 2009. 'Unknown' proteins and 'orphan' enzymes: the missing half of the engineering parts list – and how to find it. *Biochem. J.* 425(1), 1–11. <http://dx.doi.org/10.1042/BJ20091328>.
- Hatzimanikatis, V., Li, C., Ionita, J.A., Henry, C.S., Jankowski, M.D., Broadbelt, L.J., 2005. Exploring the diversity of complex metabolic networks. *Bioinformatics* 21(8), 1603–1609. <https://doi.org/10.1093/bioinformatics/bti213>.

- Heathcote, M.L., Staunton, J., Leadlay, P.F., 2001. Role of type II thioesterases: evidence for removal of short acyl chains produced by aberrant decarboxylation of chain extender units. *Chem. Biol.* 8(2), 207–220. [https://doi.org/10.1016/S1074-5521\(01\)00002-3](https://doi.org/10.1016/S1074-5521(01)00002-3).
- Horsman, M.E., Hari, T.P., Boddy, C.N., 2016. Polyketide synthase and non-ribosomal peptide synthetase thioesterase selectivity: logic gate or a victim of fate? *Nat. Prod. Rep.* 33(2), 183–202. <http://dx.doi.org/10.1039/c4np00148f>.
- Intlekofer, A.M., Wang, B., Liu, H., Shah, H., Carmona-Fontaine, C., Rustenburg, A.S., Salah, S., Gunner, M.R., Chodera, J.D., Cross, J.R., Thompson, C.B., 2017. L-2-Hydroxyglutarate production arises from noncanonical enzyme function at acidic pH. *Nat. Chem. Biol.* 13(5), 494–500. <http://dx.doi.org/10.1038/nchembio.2307>.
- Ito, T., Yamauchi, A., Hemmi, H., Yoshimura, T., 2016. Ophthalmic acid accumulation in an *Escherichia coli* mutant lacking the conserved pyridoxal 5'-phosphate-binding protein YggS. *J. Biosci. Bioeng.* 122(6), 689–693. <http://dx.doi.org/10.1016/j.jbiosc.2016.06.010>.
- Jeanguenin, L., Lara-Núñez, A., Pribat, A., Mageroy, M.H., Gregory, J.F. 3rd, Rice, K.C., de Crécy-Lagard, V., Hanson, A.D., 2010. Moonlighting glutamate formiminotransferases can functionally replace 5-formyltetrahydrofolate cycloligase. *J. Biol. Chem.* 285(53), 41557–41566. <https://doi.org/10.1074/jbc.M110.190504>.
- Jeffries, J.G., Colastani, R.L., Elbadawi-Sidhu, M., Kind, T., Niehaus, T.D., Broadbelt, L.J., Hanson, A.D., Fiehn, O., Tyo, K.E., Henry, C.S., 2015. MINEs: open access databases of computationally predicted enzyme promiscuity products for untargeted metabolomics. *J. Cheminform.* 7, 44. <https://doi.org/10.1186/s13321-015-0087-1>.
- Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2014. Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res.* 42, D199–D205. <https://doi.org/10.1093/nar/gkt1076>.
- Keller, M.A., Piedrafita, G., Ralser, M., 2015. The widespread role of non-enzymatic reactions in cellular metabolism. *Curr. Opin. Biotechnol.* 34, 153–161. <http://dx.doi.org/10.1016/j.copbio.2014.12.020>.
- Kildegard, K.R., Hallström, B.M., Blicher, T.H., Sonnenschein, N., Jensen, N.B., Sherstyk, S., Harrison, S.J., Maury, J., Herrgård, M.J., Juncker, A.S., Forster, J., Nielsen, J., Borodina, I., 2014. Evolution reveals a glutathione-dependent mechanism of 3-hydroxypropionic acid tolerance. *Metab. Eng.* 26, 57–66. <http://dx.doi.org/10.1016/j.ymben.2014.09.004>.
- Kotowska, M., Pawlik, K. 2014. Roles of type II thioesterases and their application for secondary metabolite yield improvement. *Appl. Microbiol. Biotechnol.* 98(18) 7735–7746. <http://dx.doi.org/10.1007/s00253-014-5952-8>.

553 Krasnikov, B.F., Nostramo, R., Pinto, J.T., Cooper, A.J., 2009. Assay and purification of omega-
 554 amidase/Nit2, a ubiquitously expressed putative tumor suppressor, that catalyzes the deamid-
 555 ation of the α -keto acid analogues of glutamine and asparagine. *Anal. Biochem.* 391(2), 144–
 556 150. <http://dx.doi.org/10.1016/j.ab.2009.05.025>.
 557 Kumar, A., Bachhawat, A.K., 2012. Pyroglutamic acid: throwing light on a lightly studied metabolite.
 558 *Curr. Sci.* 102(2), 288–297. <http://www.jstor.org/stable/24083854>.
 559 Lee, B.C., Gladyshev, V.N., 2011. The biological significance of methionine sulfoxide stereochem-
 560 istry. *Free Radic. Biol. Med.* 50(2), 221–227.
 561 <http://dx.doi.org/10.1016/j.freeradbiomed.2010.11.008>.
 562 Lerma-Ortiz, C., Jeffries, J.G., Cooper, A.J., Niehaus, T.D., Thamm, A.M., Frelin, O., Aunins, T.,
 563 Fiehn, O., de Crécy-Lagard, V., Henry, C.S., Hanson, A.D., 2016. 'Nothing of chemistry dis-
 564 appears in biology': the Top 30 damage-prone endogenous metabolites. *Biochem. Soc. Trans.*
 565 44(3), 961–971. <http://dx.doi.org/10.1042/BST20160073>.
 566 Linster, C.L., Noël, G., Stroobant, V., Vertommen, D., Vincent, M.F., Bommer, G.T., Veiga-da-
 567 Cunha, M., Van Schaftingen, E., 2011. Ethylmalonyl-CoA decarboxylase, a new enzyme involv-
 568 ed in metabolite proofreading. *J. Biol. Chem.* 286(50), 42992–43003.
 569 <http://dx.doi.org/10.1074/jbc.M111.281527>.
 570 Linster, C.L., Van Schaftingen, E., Hanson, A.D., 2013. Metabolite damage and its repair or pre-
 571 emptation. *Nat. Chem. Biol.* 9(2), 72–80. <http://dx.doi.org/doi:10.1038/nchembio.1141>.
 572 de Lorenzo, V., 2014. From the selfish gene to selfish metabolism: revisiting the central dogma.
 573 *Bioessays* 36(3), 226–235. <http://dx.doi.org/10.1002/bies.201300153>.
 574 de Lorenzo, V., Sekowska, A., Danchin, A., 2015. Chemical reactivity drives spatiotemporal organis-
 575 ation of bacterial metabolism. *FEMS Microbiol. Rev.* 39(1), 96–119.
 576 <http://dx.doi.org/10.1111/1574-6976.12089>.
 577 Mahadevan, R., Schilling, C.H., 2003. The effects of alternate optimal solutions in constraint-based
 578 genome-scale metabolic models. *Metab. Eng.* 5(4), 264–276.
 579 <https://doi.org/10.1016/j.ymben.2003.09.002>.
 580 Marbaix, A.Y., Noël, G., Detroux, A.M., Vertommen, D., Van Schaftingen, E., Linster, C.L., 2011.
 581 Extremely conserved ATP- or ADP-dependent enzymatic system for nicotinamide nucleotide
 582 repair. *J. Biol. Chem.* 286(48), 41246–41252. <http://dx.doi.org/10.1074/jbc.C111.310847>.
 583 Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., Keasling, J.D., 2003. Engineering a mevalon-
 584 ate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* 21(7), 796–802.
 585 <http://dx.doi.org/10.1038/nbt833>.
 586 Niehaus, T.D., Elbadawi-Sidhu, M., de Crécy-Lagard, V., Fiehn, O., Hanson, A.D., 2017. Discovery
 587 of a widespread prokaryotic 5-oxoprolinase that was hiding in plain sight. *J. Biol. Chem.*

588 <http://dx.doi.org/10.1074/jbc.M117.805028>.

589 Niehaus, T.D., Gerdes, S., Hodge-Hanson, K., Zhukov, A., Cooper, A.J., ElBadawi-Sidhu, M., Fiehn,
590 O., Downs, D.M., Hanson, A.D., 2015. Genomic and experimental evidence for multiple meta-
591 bolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. BMC Genomics 16, 382.
592 <https://doi.org/10.1186/s12864-015-1584-3>.

593 Niehaus, T.D., Richardson, L.G., Gidda, S.K., ElBadawi-Sidhu, M., Meissen, J.K., Mullen, R.T.,
594 Fiehn, O., Hanson, A.D., 2014. Plants utilize a highly conserved system for repair of NADH and
595 NADPH hydrates. Plant Physiol. 165(1), 52–61. <https://doi.org/10.1104/pp.114.236539>.

596 Novogrodsky, A., Meister, A., 1964. Control of aspartate beta-decarboxylase activity by transamin-
597 ation. J Biol Chem 239(3), 879–888.

598 Opgenorth, P.H., Korman, T.P., Bowie, J.U., 2016. A synthetic biochemistry module for production of
599 bio-based chemicals from glucose. Nat. Chem. Biol. 12(6), 393–395.
600 <https://doi.org/10.1038/nchembio.2062>.

601 Oppenheimer, N.J., Kaplan, N.O., 1974. Glyceraldehyde-3-phosphate dehydrogenase catalyzed
602 hydration of the 5-6 double bond of reduced β -nicotinamide adenine dinucleotide (β -NADH).
603 Formation of β -6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide. Biochemistry
604 13(23), 4685–4694. <http://dx.doi.org/10.1021/bi00720a002>.

605 Orłowski, M., Meister, A., 1971. Partial reactions catalyzed by gamma-glutamylcysteine synthetase
606 and evidence for an activated glutamate intermediate. J. Biol. Chem. 246(23), 7095–7105.

607 Orth, J.D., Conrad, T.M., Na, J., Lerman, J.A., Nam, H., Feist, A.M., Palsson, B.Ø., 2011. A compre-
608 hensive genome-scale reconstruction of *Escherichia coli* metabolism – 2011. Mol. Syst. Biol. 7,
609 535. <http://dx.doi.org/10.1038/msb.2011.65>.

610 Park, C.B., Lee, S.B., Ryu, D.D., 2001. L-Pyroglutamate spontaneously formed from L-glutamate
611 inhibits growth of the hyperthermophilic archaeon *Sulfolobus solfataricus*. Appl. Environ.
612 Microbiol. 67(8), 3650–3654. <http://dx.doi.org/10.1128/AEM.67.8.3650-3654.2001>.

613 Peracchi, A., Veiga-da-Cunha, M., Kuhara, T., Ellens, K.W., Paczia, N., Stroobant, V., Seliga, A.K.,
614 Marlaire, S., Jaisson, S., Bommer, G.T., Sun, J., Huebner, K., Linster, C.L., Cooper, A.J.L., Van
615 Schaftingen, E., 2017. Nit1 is a metabolite repair enzyme that hydrolyzes deaminated gluta-
616 thione. Proc. Natl. Acad. Sci. USA 114(16), E3233–E3242.
617 <http://dx.doi.org/10.1073/pnas.1613736114>.

618 Piedrafita, G., Keller, M.A., Ralser, M., 2015. The impact of non-enzymatic reactions and enzyme
619 promiscuity on cellular metabolism during (oxidative) stress conditions. Biomolecules 5(3),
620 2101–2122. <http://dx.doi.org/10.3390/biom5032101>.

621 Prabhakar, P., Laboy, J.I., Wang, J., Budker, T., Din, Z.Z., Chobanian, M., Fahien, L.A., 1998. Effect
 622 of NADH-X on cytosolic glycerol-3-phosphate dehydrogenase. *Arch. Biochem. Biophys.* 360(2),
 623 195-205. <https://doi.org/10.1006/abbi.1998.0939>.

624 Rahman, S.A., Cuesta, S.M., Furnham, N., Holliday, G.L., Thornton, J.M., 2014. EC-BLAST: a tool
 625 to automatically search and compare enzyme reactions. *Nat. Methods* 11(2), 171–174.
 626 <http://dx.doi.org/10.1038/nmeth.2803>.

627 Richard, J.P., 1993. Mechanism for the formation of methylglyoxal from triosephosphates. *Biochem.*
 628 *Soc. Trans.* 21(2), 549-553. <http://dx.doi.org/10.1042/bst0210549>.

629 Richarme, G., Liu, C., Mihoub, M., Abdallah, J., Leger, T., Joly, N., Liebart, J.C., Jurkunas, U.V.,
 630 Nadal, M., Boulloc, P., Dairou, J., Lamouri, A., 2017. Guanine glycation repair by DJ-1/Park7
 631 and its bacterial homologs. *Science* 357(6347), 208–211.
 632 <http://dx.doi.org/10.1126/science.aag1095>.

633 Richarme, G., Marguet, E., Forterre, P., Ishino, S., Ishino, Y., 2016. DJ-1 family Maillard deglycases
 634 prevent acrylamide formation. *Biochem. Biophys. Res. Commun.* 478(3), 1111–1116.
 635 <http://dx.doi.org/10.1016/j.bbrc.2016.08.077>.

636 Richarme, G., Mihoub, M., Dairou, J., Bui, L.C., Leger, T., Lamouri, A., 2015. Parkinsonism-associated
 637 protein DJ-1/Park7 is a major protein deglycase that repairs methylglyoxal- and glyoxal-
 638 glycated cysteine, arginine, and lysine residues. *J. Biol. Chem.* 290(3), 1885–1897.
 639 <https://doi.org/10.1074/jbc.M114.597815>.

640 Ringling, C., Rychlik, M., 2013. Analysis of seven folates in food by LC–MS/MS to improve accuracy
 641 of total folate data. *Eur. Food Res. Technol.* 236(1), 17–28.
 642 <https://doi.org/10.1007/s00217-012-1849-x>.

643 Rzem, R., Van Schaftingen, E., Veiga-da-Cunha, M., 2006. The gene mutated in L-2-hydroxyglutaric
 644 aciduria encodes L-2-hydroxyglutarate dehydrogenase. *Biochimie* 88(1), 113–116.
 645 <https://doi.org/10.1016/j.biochi.2005.06.005>.

646 Rzem, R., Vincent, M.F., Van Schaftingen, E., Veiga-da-Cunha, M., 2007. L-2-hydroxyglutaric
 647 aciduria, a defect of metabolite repair. *J. Inherit. Metab. Dis.* 30(5), 681–689.
 648 <https://doi.org/10.1007/s10545-007-0487-0>.

649 Satish Kumar, V., Dasika, M.S., Maranas, C.D., 2007. Optimization based automated curation of
 650 metabolic reconstructions. *BMC Bioinformatics* 8, 212.
 651 <https://doi.org/10.1186/1471-2105-8-212>.

652 Schwander, T., Schada von Borzyskowski, L., Burgener, S., Cortina, N.S., Erb, T.J., 2016. A
 653 synthetic pathway for the fixation of carbon dioxide in vitro. *Science* 354(6314), 900–904.
 654 <http://dx.doi.org/10.1126/science.aah5237>.

655 Shaheen, F., Shmygol, A., Rabbani, N., Thornalley, P.J., 2014. A fluorogenic assay for methyl-
656 glyoxal. *Biochem. Soc. Trans.* 42(2), 548–555. <http://dx.doi.org/10.1042/BST20140028>.

657 Showalter, M.R., Cajka, T., Fiehn, O., 2017. Epimetabolites: discovering metabolism beyond building
658 and burning. *Curr. Opin. Chem. Biol.* <https://doi.org/10.1016/j.cbpa.2017.01.012>.

659 Singleton, C., Howard, T.P., Smirnoff, N., 2014. Synthetic metabolons for metabolic engineering. *J.*
660 *Exp. Bot.* 65(8), 1947–1954. <https://doi.org/10.1093/jxb/eru050>.

661 Sivakumar, T.V., Giri, V., Park, J.H., Kim, T.Y., Bhaduri, A., 2016. ReactPRED: a tool to predict and
662 analyze biochemical reactions. *Bioinformatics* 32(22), 3522–3524.
663 <https://doi.org/10.1093/bioinformatics/btw491>.

664 Stavrinides, A., Tatsis, E.C., Foureau, E., Caputi, L., Kellner, F., Courdavault, V., O'Connor, S.E.,
665 2015. Unlocking the diversity of alkaloids in *Catharanthus roseus*: nuclear localization suggests
666 metabolic channeling in secondary metabolism. *Chem. Biol.* 22(3), 336–341.
667 <https://doi.org/10.1016/j.chembiol.2015.02.006>.

668 Stover, P., Schirch, V., 1990. Serine hydroxymethyltransferase catalyzes the hydrolysis of 5,10-
669 methenyltetrahydrofolate to 5-formyltetrahydrofolate. *J. Biol. Chem.* 265(24), 14227–14233.

670 Stover, P., Schirch, V., 1993. The metabolic role of leucovorin. *Trends Biochem. Sci.* 18(3), 102–
671 106. [https://doi.org/10.1016/0968-0004\(93\)90162-G](https://doi.org/10.1016/0968-0004(93)90162-G).

672 Sullivan, L.B., Martinez-Garcia, E., Nguyen, H., Mullen, A.R., Dufour, E., Sudarshan, S., Licht, J.D.,
673 Deberardinis, R.J., Chandel, N.S., 2013. The proto-oncometabolite fumarate binds glutathione
674 to amplify ROS-dependent signaling. *Mol. Cell.* 51(2), 236–248.
675 <https://doi.org/10.1016/j.molcel.2013.05.003>.

676 Takasawa, R., Tao, A., Saeki, K., Shionozaki, N., Tanaka, R., Uchiro, H., Takahashi, S., Yoshimori,
677 A., Tanuma, S., 2011. Discovery of a new type inhibitor of human glyoxalase I by myricetin-bas-
678 ed 4-point pharmacophore. *Bioorg. Med. Chem. Lett.* 21(14), 4337–4342.
679 <https://doi.org/10.1016/j.bmcl.2011.05.046>.

680 Tawfik, D.S., 2014. Accuracy-rate tradeoffs: how do enzymes meet demands of selectivity and
681 catalytic efficiency? *Curr. Opin. Chem. Biol.* 21, 73–80.
682 <https://doi.org/10.1016/j.cbpa.2014.05.008>.

683 Thornalley, P.J., 2008. Protein and nucleotide damage by glyoxal and methylglyoxal in physiological
684 systems. Role in ageing and disease. *Drug metabol. Drug Interact.* 23(1-2), 125–150.
685 <https://doi.org/10.1515/DMDI.2008.23.1-2.125>.

686 Thornalley, P.J., 1990. The glyoxalase system: new developments towards functional characteriz-
687 ation of a metabolic pathway fundamental to biological life. *Biochem. J.* 269(1), 1-11.
688 <https://doi.org/10.1042/bj2690001>.

689 Thornalley, P.J., Rabbani, N., 2014. Assay of methylglyoxal and glyoxal and control of peroxidase
690 interference. *Biochem. Soc. Trans.* 42(2), 504–510. <https://doi.org/10.1042/BST20140009>.

691 Tong, I.T., Liao, H.H., Cameron, D.C., 1991. 1,3-Propanediol production by *Escherichia coli* express-
692 ing genes from the *Klebsiella pneumoniae dha* regulon. *Appl. Environ. Microbiol.* 57(12), 3541–
693 3546.

694 Tritsch, G.L., Moore, G.E., 1962. Spontaneous decomposition of glutamine in cell culture media.
695 *Exp. Cell. Res.* 28(2), 360–364. [https://doi.org/10.1016/0014-4827\(62\)90290-2](https://doi.org/10.1016/0014-4827(62)90290-2).

696 Van der Werf, M.J., Overkamp, K.M., Muilwijk, B., Coulter, L., Hankemeier, T., 2007. Microbial
697 metabolomics: toward a platform with full metabolome coverage. *Anal. Biochem.* 370(1), 17–25.
698 <https://doi.org/10.1016/j.ab.2007.07.022>.

699 Van der Werf, P., Orlowski, M., Meister, A., 1971. Enzymatic conversion of 5-oxo-L-proline (L-pyrroli-
700 done carboxylate) to L-glutamate coupled with cleavage of adenosine triphosphate to adenos-
701 ine diphosphate, a reaction in the γ -glutamyl cycle. *Proc. Natl. Acad. Sci. USA* 68(12), 2982–
702 2985.

703 Van Schaftingen, E., Collard, F., Wiame, E., Veiga-da-Cunha, M., 2012. Enzymatic repair of Ama-
704 dori products. *Amino Acids* 42(4), 1143–1150. <http://dx.doi.org/10.1007/s00726-010-0780-3>.

705 Van Schaftingen, E., Rzem, R., Marbaix, A., Collard, F., Veiga-da-Cunha, M., Linster, C.L., 2013.
706 Metabolite proofreading, a neglected aspect of intermediary metabolism. *J. Inherit. Metab. Dis.*
707 36(3), 427–434. <http://dx.doi.org/10.1007/s10545-012-9571-1>.

708 Van Schaftingen, E., Rzem, R., Veiga-da-Cunha, M., 2009. L-2-Hydroxyglutaric aciduria, a disorder
709 of metabolite repair. *J. Inherit. Metab. Dis.* 32(2), 135–142.
710 <http://dx.doi.org/10.1007/s10545-008-1042-3>.

711 Wicker, J., Lorschach, T., Gütlein, M., Schmid, E., Latino, D., Kramer, S., Fenner, K., 2016. envPath
712 – The environmental contaminant biotransformation pathway resource. *Nucleic Acids Res.*
713 44(D1), D502–D508. <https://doi.org/10.1093/nar/gkv1229>.

714 Wilson, S.M., Gleisten, M.P., Donohue, T.J., 2008. Identification of proteins involved in formaldehyde
715 metabolism by *Rhodobacter sphaeroides*. *Microbiology* 154(1), 296–305.
716 <http://dx.doi.org/10.1099/mic.0.2007/011346-0>.

717 Withers, S.T., Gottlieb, S.S., Lieu, B., Newman, J.D., Keasling, J.D., 2007. Identification of isopent-
718 enol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid pre-
719 cursor toxicity. *Appl. Environ. Microbiol.* 73(19), 6277–6283.
720 <http://dx.doi.org/10.1128/AEM.00861-07>.

721 Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., Khandurina, J., Trawick,
722 J.D., Osterhout, R.E., Stephen, R., Estadilla, J., Teisan, S., Schreyer, H.B., Andrae, S., Yang,

723 T.H., Lee, S.Y., Burk, M.J., Van Dien, S., 2011. Metabolic engineering of *Escherichia coli* for
 724 direct production of 1,4-butanediol. Nat. Chem. Biol. 7(7), 445–452.
 725 <https://doi.org/10.1038/nchembio.580>.

726 Yoshida, A., Dave, V., 1975. Inhibition of NADP-dependent dehydrogenases by modified products of
 727 NADPH. Arch. Biochem. Biophys. 169(1), 298–303.
 728 [https://doi.org/10.1016/0003-9861\(75\)90344-6](https://doi.org/10.1016/0003-9861(75)90344-6).

729 Young, E.J., Burton, R., Mahalik, J.P., Sumpter, B.G., Fuentes-Cabrera, M., Kerfeld, C.A., Ducat,
 730 D.C., 2017. Engineering the bacterial microcompartment domain for molecular scaffolding
 731 applications. Front. Microbiol. 8, 1441. <https://doi.org/10.3389/fmicb.2017.01441>.

732 Zheng, Y., Liu, Q., Li, L., Qin, W., Yang, J., Zhang, H., Jiang, X., Cheng, T., Liu, W., Xu, X., Xian, M.,
 733 2013. Metabolic engineering of *Escherichia coli* for high-specificity production of isoprenol and
 734 prenol as next generation of biofuels. Biotechnol. Biofuels. 6, 57.
 735 <http://dx.doi.org/10.1186/1754-6834-6-57>.

736 Zhu, M.M., Skraly, F.A., Cameron, D.C., 2001. Accumulation of methylglyoxal in anaerobically grown
 737 *Escherichia coli* and its detoxification by expression of the *Pseudomonas putida* glyoxalase I
 738 gene. Metab. Eng. 3(3), 218–225. <https://doi.org/10.1006/mben.2001.0186>.

Figure legends

Fig. 1. Examples of metabolite damage and repair reactions from central metabolism. Red arrows are damage reactions and blue arrows are repair reactions. (A) NAD(P)H damage and repair. NAD(P)HX dehydratase is ATP-dependent in eukaryotes (as shown) but ADP-dependent in prokaryotes. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; (B) 5,10-Methenyltetrahydrofolate (5,10-CH=THF) damage and repair. SHMT, serine hydroxymethyltransferase; FT, glutamate formyltransferase; 5-FCL, 5-formyltetrahydrofolate cyclo-ligase; 5-CHO-THF, 5-formyltetrahydrofolate; THF, tetrahydrofolate, pABG, *p*-aminobenzoylglutamate. The dashed blue arrow represents several reactions of one-carbon metabolism that can convert tetrahydrofolate to 5,10-methenyltetrahydrofolate. (C) Triose phosphate damage and repair. DHAP, dihydroxyacetone phosphate; TPI, triose phosphate isomerase; GSH, glutathione; GloI, glyoxalase I; GloII, glyoxalase II. (D) Damage to erythrose 4-phosphate and lactate, and repair by PGP phosphatase. The dashed red arrow represents the conversion of 1,4-bisphosphoerythronate (1,4-BP-erythronate) to 4-phosphoerythronate by phosphoglycerate kinase or acylphosphatase (Collard et al., 2016). **P, phosphate**. (E) Glutathione damage and repair. The cyclic form of deaminated glutathione predominates in solution (Peracchi et al., 2017). (F) Spontaneous damage reactions leading to 5-oxoproline, and repair by 5-oxoprolinase.

Fig. 2. Examples of the use of metabolite repair enzymes in metabolic engineering and SynBio projects. Red arrows are damage reactions and blue arrows are repair reactions. Dashed arrows represent more than one reaction. (A) Overexpression of glyoxalase I (boxed) boosted flux in an engineered anaerobic glycerol fermentation pathway to 1,3-propanediol in *E. coli* by preventing buildup of the triose phosphate damage product methylglyoxal. (B) Expression of the phosphatases NudF or YhfR relieved toxicity of an engineered mevalonate pathway in *E. coli* by hydrolyzing its isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) intermediates to the corresponding alcohols. IDP and DMADP are colored red to indicate that they are reactive electrophiles. (C) Installation of a lactonase to support an engineered 1,4-butanediol pathway in *E. coli* increased product yield by countering spontaneous formation of γ -butyrolactone from the pathway intermediate 4-hydroxybutyryl-CoA. (D) Addition of malyl-CoA thioesterase (MCT) to support the synthetic *in vitro* CETCH cycle of CO₂ fixation facilitated effective cycle operation by hydrolyzing malyl-CoA, a byproduct formed by a promiscuous reaction of the cycle enzyme β -methylmalyl-CoA lyase (MCL). (E) Inclusion of an external (type II) thioesterase (TE) in biosynthetic pathways to polyketides or non-ribosomal peptides prevents errors or stalls in the pathway caused by misloading of incorrect building blocks by pathway enzymes, or by the promiscuous action of phosphopantetheine transfer-

ase (PPTase). The thioesterase selectively hydrolyzes these aberrant intermediates, regenerating the free holo-synth(et)ase.

Fig. 3. Repair reactions from engineering case studies and the generalized reaction rules that predict them. The figure depicts the generalized reaction rules from BNICE that predict four repair reactions from the engineering case study section. For each rule a depiction was generated with ChemAxon MarvinSketch. An “A” in the image may be any non-hydrogen atom. The “W” near an atom (for wildcard) indicates that the atom may or may not be member of a ring while a “0” indicates that the atom is in no rings. Some atoms may have multiple valid bond patterns and these alternatives are indicated by the corresponding R#.

Table 1

A 'plug and play' set of repair enzymes with likely applications in engineering core metabolism

Damage reactions	Repair enzymes	Uses in engineering
<i>Central carbon metabolism</i>		
Triose-P ^a → methylglyoxylate [S, E]	Glyoxalase I and II (section 3.2)	(section 4.1)
Methylglyoxylate → glycations [S]	DJ-1 Deglycase (section 3.2)	
Erythrose 4-P, ribose 5-P → glycations [S]	Ribulosamine/erythrulosamine 3-kinases (deglycation) (Fortpied et al., 2005; Gemayel et al., 2007)	
Fructose 6-P → glycations [S]	Fructosamine 3-kinase (deglycation) (Delpierre et al., 2000)	
Erythrose 4-P → 4-P erythronate [E]	PGP/Pho13 phosphatase (section 3.2)	
Lactate → 2-P lactate [E]	PGP/Pho13 phosphatase (section 3.2)	
cis-Aconitate → trans-aconitate [S, E]	trans-Aconitate methyltransferase (Cai and Clarke, 1999)	
α-Ketoglutarate → L-α-hydroxyglutarate [E]	L-α-Hydroxyglutarate dehydrogenase (Rzem et al., 2006)	
Oxidations → formaldehyde, other aldehydes [S, E]	Formaldehyde-activating enzyme/formaldehyde dehydrogenase/ S-formylglutathione hydrolase (Wilson et al., 2008)	(Kildegaard et al., 2014)
Butyryl-CoA → ethylmalonyl-CoA [E]	Ethylmalonyl-CoA decarboxylase (Linster et al., 2011)	
<i>Amino acid metabolism</i>		
Glutathione → deaminated glutathione [E]	Nit1 (section 3.3)	
Glutamine/asparagine → α-ketoglutaramate/α-keto-succinamate [E]	Nit2 ω-amidase (Krasnikov et al., 2009)	
Free/bound methionine → methionine sulfoxides [S]	Methionine sulfoxide reductases (Lee and Gladyshev, 2011)	
Amino acids → imines/enamines [E]	RidA enamine deaminases (Niehaus et al., 2015)	
<i>Cofactor metabolism</i>		
NAD(P)H → NAD(P)HX [S, E]	NAD(P)HX dehydratase/NAD(P)HX epimerase (section 3.1)	(Aliprandi et al., 2017)
5,10-Methenyltetrahydrofolate → 5-formyltetrahydrofolate [S, E]	5-Formyltetrahydrofolate cyclo-ligase or glutamate formimino-transferase (section 3.1)	

^a Abbreviations: -P, phosphate; CoA, coenzyme A; S, spontaneous reaction; E, enzymatic side-reaction.

Engineered Pathway	Repair Enzyme	BNICE Reaction Rule	Reaction Rule Depiction
1,3-Propanediol production	Glyoxalase I	5.2.1.a
	Glyoxalase II	3.1.2.a
Mevalonate pathway	Diphosphatase	3.1.3.a
1,4-Butanediol production	γ -Butyrolactonase	3.1.1.a
Synthetic CO ₂ fixation	Maly-CoA thioesterase	3.1.2.a