Trends in Biochemical Sciences

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Review

Metabolite Repair Enzymes Control Metabolic Damage in Glycolysis

Guido T. Bommer,[1,*] Emile Van Schaftingen,[1,2,*] and Maria Veiga-da-Cunha[1,*]

Hundreds of metabolic enzymes work together smoothly in a cell. These enzymes are highly specific. Nevertheless, under physiological conditions, many perform side-reactions at low rates, producing potentially toxic side-products. An increasing number of metabolite repair enzymes are being discovered that serve to eliminate these noncanonical metabolites. Some of these enzymes are extraordinarily conserved, and their deficiency can lead to diseases in humans or embryonic lethality in mice, indicating their central role in cellular metabolism. We discuss how metabolite repair enzymes eliminate glycolytic side-products and prevent negative interference within and beyond this core metabolic pathway. Extrapolating from the number of metabolite repair enzymes involved in glycolysis, hundreds more likely remain to be discovered that protect a wide range of metabolic pathways.

Highlights

Many noncanonical metabolites are produced under physiological conditions by side-activities of enzymes and by spontaneous reactions.

Many of these noncanonical metabolites are repaired or reconverted to useful metabolites by metabolite repair enzymes.

A single repair enzyme may catalyze several repair reactions.

Metabolite repair reactions are sometimes catalyzed by a side-activity of a canonical enzyme (i.e., FBP1/2, PGK).

Major pathways may require multiple metabolite repair enzymes. In the case of glycolysis, 10 canonical glycolytic enzymes are assisted by 10 repair enzymes (G6PC3, PGP, ACYP1, NAXD, NAXE, L2HGDH, GLO1, GLO2, FN3K, MDP-1) and a transporter (G6PT).

Deficiencies in several of the metabolite repair enzymes can cause disease in humans or are lethal in higher vertebrates.

Side-Reactions Are Pervasive and Inevitable in Cellular Metabolism

Enzymologists have known for a long time that highly purified enzymes are not 100% specific. Beyond their physiological reactions, they can act with low efficiency on intracellular metabolites that resemble (but are not) their true substrate **(substrate promiscuity,** see Glossary and Box 1) (e.g., the side-activity of L-malate dehydrogenase on α-ketoglutarate [1,2]). In addition, they sometimes can catalyze an incorrect reaction on their physiological substrate **(catalytic promiscuity)** (e.g., the hydration of NADH catalyzed by glyceraldehyde-3-phosphate dehydrogenase, GAPDH [2,3]). Nevertheless, it was tacitly assumed that these side-reactions were irrelevant for cellular metabolism.

Sensitive metabolomic techniques have revealed a huge diversity of metabolites, some of which are **side-products** of canonical enzymes (Figure 1 and Box 1). Several lines of evidence indicate that the formation of noncanonical side-products is not only pervasive, but is also inevitable in cellular metabolism [2,4–6]. First, the ability of an enzyme to discriminate between a physiological substrate and a close structural analog of this substrate is limited. Proteins are not completely rigid, and show some flexibility. Thus, they can often accommodate substrates that differ slightly in size owing to the presence or absence of chemical functions such as a hydroxyl or methyl group [2,6]. Furthermore, during evolution, increased catalytic efficiency rather than perfect accuracy might have been the driving force [6]. Last but not least, some **noncanonical metabolites** are formed through unavoidable nonenzymatic reactions of unstable intermediary metabolites [7].

Enzyme promiscuity often leads to the formation of noncanonical metabolites that are not part of a known metabolic pathway. This causes several problems. First, these metabolites may accumulate in cells because they are inadequate substrates for canonical metabolic enzymes [8,9]. Second, these metabolites may inhibit canonical metabolic enzymes because they resemble classical metabolites and/or reaction intermediates [10,11]. In fact, it has been suggested that the separation of metabolic pathways into distinct compartments facilitates the organization of complex metabolic pathways that would otherwise succumb to autoinhibition [12] (Box 2).

[1]de Duve Institute, Université Catholique de Louvain (UCLouvain), Avenue Hippocrate 75, 1200 Bruxelles, Belgium
[2]Walloon Excellence in Life Sciences and Biotechnology (WELBIO), UCLouvain, Avenue Hippocrate 75, 1200 Bruxelles, Belgium

*Correspondence:
guido.bommer@uclouvain.be (G.T. Bommer),
emile.vanschaftingen@uclouvain.be (E. Van Schaftingen), and
maria.veiga@uclouvain.be (M. Veiga-da-Cunha).

Box 1. Side-Reactions of Enzymes: Irrelevant, Dangerous, Useful, or Regulatory?

The physiological function of an enzyme is to catalyze a reaction that fulfills a purpose in the biochemical network of a cell, most often to make a useful product. In most cases this is the reaction with the highest catalytic efficiency (K_{cat}/K_m). Nevertheless, most enzymes catalyze additional reactions (side-reactions) with a much lower (usually <1%) catalytic efficiency. These side-reactions may be the same type of reaction as the main reaction, but on a substrate that is different from the main physiological substrate (substrate promiscuity). Alternatively, the enzyme may catalyze a different reaction on its physiological substrate (catalytic promiscuity). Some of the resulting side-products are not part of known metabolic pathways (noncanonical metabolites). However, owing to their resemblance to physiological metabolites, some noncanonical metabolites can inhibit other enzymes and cause profound metabolic effects.

The classification of a metabolite as useful or not can be problematic in some cases. For example, a metabolite that is toxic in one organism might be part of a metabolic pathway in another organism (e.g., L-2-OH-glutarate [111,112] and 4P-erythronate [113]). Furthermore, in some cases we might not be aware of the entire metabolic network of the organism under investigation. In fact, enzyme promiscuity might increase the flexibility of metabolic pathways and open them up for future expansion or alterations in response to selective pressure [5,114,115].

In some cases, metabolic side-products are extremely potent **effectors** of other enzymes. Thus, they can affect the activity of biochemical pathways (e.g., 4P-erythronate, 2P-lactate, L-2-OH-glutarate [10,61]) and could be considered as regulatory molecules. However, this interpretation can be problematic because the formation of side-products in most cases cannot be controlled independently. Furthermore, their elimination by metabolite repair enzymes, in most cases, does not seem to be regulated and aims to reduce the concentration of the damaging compounds to a level below the concentration range where they can act on a 'target'. This is in stark contrast to regulatory molecules (e.g., fructose 2,6-bisphosphate, cAMP, acetyl-glutamate) which are formed and degraded by dedicated, highly regulated enzymes [116–119]. Although this line of reasoning cautions us to be careful regarding proposed regulatory functions of side-products, we should not exclude that present-day messy metabolites in the evolutionary future might become regulatory metabolites or *bona fide* intermediates in so far undeveloped pathways [115].

Last, accumulation of high concentrations of side-products can effectively withdraw valuable metabolites from cellular metabolism [3,13,14].

In this review we discuss a class of enzymes, metabolite repair enzymes, that destroy noncanonical metabolites or convert them into physiological metabolites. We then explore in depth the role of metabolic repair enzymes in the core metabolic pathway **glycolysis**.

Metabolite Repair Enzymes Eliminate Dangerous Side-Products and Play an Important Role in Core Metabolic Pathways

In the past 15 years >15 dedicated human enzymes have been discovered that destroy noncanonical metabolites or convert them into physiological metabolites [15]. This can alleviate metabolic perturbations and/or allow metabolite recovery [8,9,15]. Collectively, these enzymes have been termed 'metabolite repair enzymes' or 'clean-up enzymes'. In addition, other enzymes can pre-empt damage by limiting the abundance of highly reactive, physiologic metabolites [16–18] or by limiting the concentrations of substrates for side-activities (see comments below on lactate dehydrogenase, LDH). Of note, in some cases regulatory circuits might also serve to limit the concentrations of reactive metabolites. For example, accumulation of the metabolite phosphoenolpyruvate (P-enolpyruvate) in mammalian cells is limited by its feedback inhibition of the enzyme that produces the glycolytic activator fructose-2,6-bisphosphate (fructose-2,6BP) [19,20]. In addition, the stimulation of several forms of pyruvate kinase by fructose-1,6-bisphosphate (fructose-1,6BP) allows increased glycolytic flux with little increase in reactive triose phosphates [21,22] (see below).

Metabolite repair enzymes play an important role in the organization of mammalian intermediary metabolism because deficiency in several of them leads to human disease or to embryonic lethality in mice [1,11,23–26]. Furthermore, some metabolite repair enzymes are found in both prokaryotes and eukaryotes, indicating that they are evolutionarily conserved and might be essential for metabolic efficiency in all forms of life [3,18].

Glossary

Catalytic promiscuity: an enzyme acts on its physiological substrate to catalyze a reaction different from the physiological reaction (e.g., hydration instead of dehydrogenation).
Effector: for an enzyme, a molecule that stimulates or reduces its activity.
Gluconeogenesis: metabolic pathway that forms glucose from noncarbohydrate substrates such as pyruvate and lactate.
Glycolysis: metabolic pathway that converts glucose to pyruvate.
Multispecific (enzyme): an enzyme that acts on several structurally similar substrates in physiologically relevant reactions (e.g., aldolase B, acting on fructose-1P and fructose-1,6BP).
Noncanonical metabolites: metabolites that do not belong to classical metabolic pathways and result from enzyme side-reactions or from spontaneous (noncatalyzed) reactions.
Pentose phosphate pathway: metabolic pathway allowing the interconversion of glycolytic intermediates (with 3 or 6 carbons) with phosphorylated sugars containing 4, 5, or 7 carbons.
Side-product: enzymatic product resulting from substrate or catalytic promiscuity.
Substrate promiscuity: an enzyme acts with low efficiency on metabolites that resemble its physiological and usually best substrate.
Tricarboxylic acid cycle: also known as the Krebs cycle, a cyclic metabolic pathway allowing the oxidation the acetyl group of acetyl-CoA.

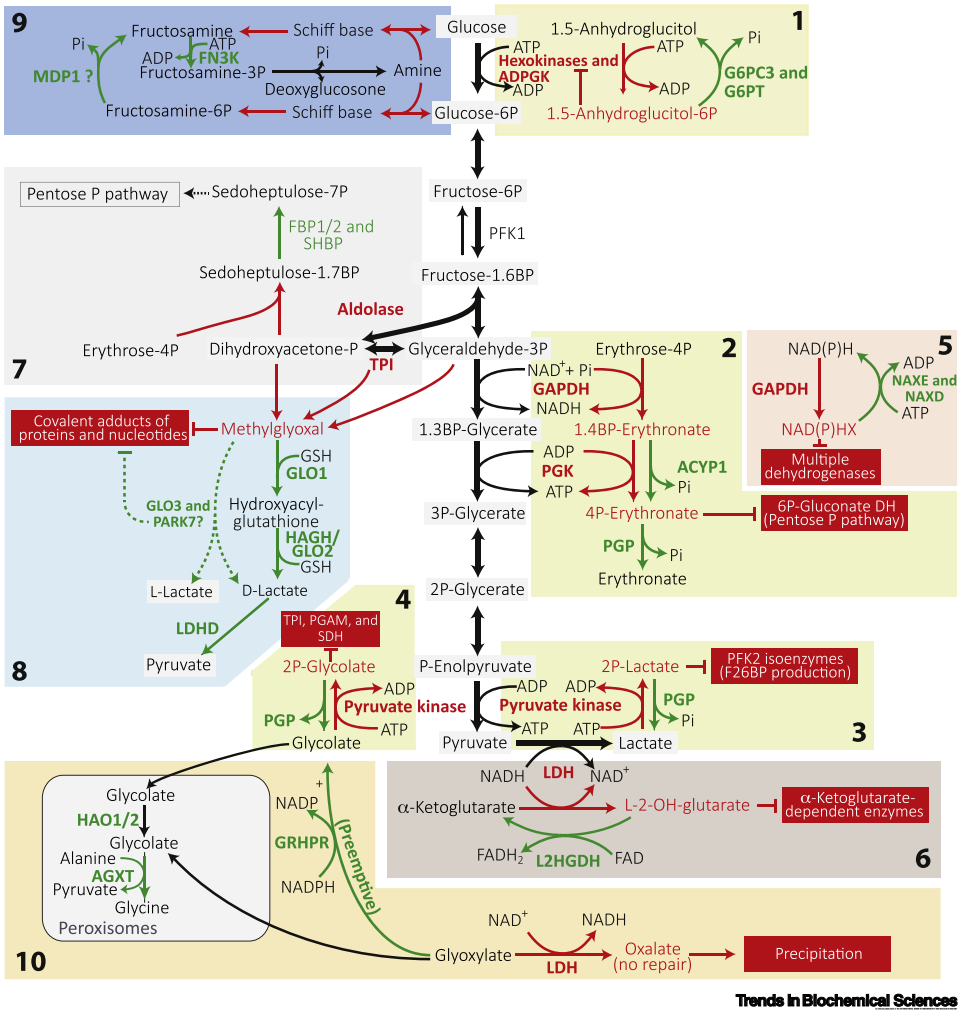

Figure 1. Metabolite Repair Enzymes in Glycolysis. Schematic representation of metabolite repair processes involved in glycolysis. Black indicates physiological reactions, red indicates side-reactions, and green indicates metabolite repair processes. (1) Repair of 1,5-anhydroglucitol-6P by glucose-6P translocase (G6PT) and glucose-6-phosphatase catalytic subunit 3 (G6PC3). (2–4) Elimination of 4P-erythronate, 2P-lactate, and 2P-glycolate by phosphoglycolate phosphatase (PGP). (5) Repair of hydrated pyridine nucleotides by NAD(P)HX dehydratase (NAXD) and NAD(P)HX epimerase (NAXE). (6) Repair of L-2-OH-glutarate by L-2-OH-glutarate dehydrogenase (L2HGDH). (7) Repair function or canonical metabolic function for sedoheptulose-1,7BP phosphatase (SHBP). (8) Repair of methylglyoxal by glutathione-dependent glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2 = HAGH, hydroxyacylglutathione hydrolase), and D-lactate dehydrogenase (LDHD). Unclear contribution of glutathione-independent glyoxalase 3 (GLO3) and PARK7. (9) Repair of spontaneous glucose and glucose-6P adducts on amino groups of proteins and metabolites by fructosamine-3-kinase (FN3K) and magnesium-dependent phosphatase 1 (MDP1). (10) Prevention of oxalate formation by maintaining low glyoxylate concentrations via the action of glyoxylate-reductase/hydroxypyruvate-reductase (GRHPR) and alanine glyoxylate-transaminase (AGXT). Abbreviations: ACYP1, acylphosphatase 1; ADPGK, ADP-dependent glucokinase; BP, bisphosphate; F26BP, glycolytic activator fructose 2,6-bisphosphate; FBP1/2, fructose 1,6-bisphosphatases 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAO1/2 hydroxy acid oxidases 1 and 2; LDH, L-lactate dehydrogenase; P, phosphate; PFK1, phosphofructokinase 1; PFK2, phosphofructokinase 2; PGK, phosphoglycerate kinase; Pi, inorganic phosphate.

The Identification of Metabolite Repair Enzymes Is Not Straightforward

Noncanonical metabolites may result from an extreme diversity of reactions, and identifying their structure is sometimes challenging (e.g., NADHX [27] and the substrate of Nit1 [14]). Furthermore, most are present in healthy cells at only very low concentrations and only become detectable when the relevant metabolite repair enzyme is inactivated. In many cases they are formed

Box 2. The Importance of Metabolite Damage Control in Higher Vertebrates

It is striking that defects in comparable enzymes have dramatic effects in mammals, but much milder effects in simpler organisms such as yeasts and *Escherichia coli*. Several factors are likely to contribute to this.

First, higher animals are composed of hundreds of different cell types, with different and often very specialized metabolic setups. Even if only one of these cell types (i.e., neutrophils in G6PC3 deficiency [11,34,35] or brain cells in L2-hydroxyglutaric aciduria [120] and NADHX repair deficiencies [23,24,64]) is sensitive to the deficiency, a clear disease phenotype may arise. Second, for a multicellular organism it is much more difficult to eliminate abnormal metabolites than for a monocellular organism, where only one membrane needs to be crossed (the outer membrane in Gram-negative bacteria is freely permeable for molecules with a molecular weight of ≤600–800 Da) [121]. Last but not least, the long lifetime of cells in a multicellular organism might make these cells more susceptible to the accumulation of toxic side-products.

To overcome these limitations while maintaining exceedingly complex metabolic circuits, higher organisms such as vertebrates may have developed strategies to avoid interference. In eukaryotes, separation of metabolite pathways into different subcellular compartments likely helps to prevent the production of side-products and avoid inhibitory interferences [12]. Furthermore, the elimination of many biosynthetic pathways for essential amino acids (~50 reactions saved) and vitamins (>25 reactions simply for vitamin B12) strongly decreases the chances of promiscuous reactions by decreasing the number of enzymes and intermediates. This line of reasoning is supported by the finding that introducing a new pathway for pyridoxine synthesis in *E. coli* was found to cause strong interferences by metabolites of the new pathway with already existing pathways and, reciprocally, interferences of metabolites from the existing network with enzymes of the new pathway [122]. Finally, some scanty evidence suggests that at least some mammalian enzymes are more specific than their bacterial counterparts (e.g., *E. coli* L-malate dehydrogenase [123] is much less specific than the mammalian enzyme [1]), but this has not been investigated systematically.

very slowly by side-activities that represent less than 1/1000 of the physiological enzyme activity [1,3,9,10]. Accordingly, even modest repair activities, in some cases, can prevent the accumulation of noncanonical metabolites [3,10]. An additional difficulty is that a functional phenotype will only be observed when the noncanonical metabolite interferes with a crucial metabolic pathway [11]. In this context, we need to mention that a relatively modest accumulation of some metabolic side-products can have strong effects on canonical metabolic pathways, and this might distract from the discovery of the underlying metabolite repair defect [10].

Like all enzymes, metabolite repair enzymes are also expected to have side-activities. In some cases, these activities might appear to catalyze novel physiological reactions, albeit at a rate that is far too low to influence the metabolism of the specific metabolites [28,29]. In other cases, activities may be observed with metabolites that are absent from cells [30]. To complicate matters even further, some metabolite repair enzymes have been shown to be **multispecific**, in other words they act on several different noncanonical metabolites, but they remarkably avoid acting on structurally similar physiological metabolites (see comments below on phosphoglycolate phosphatase, PGP) [10].

All these factors make the discovery of metabolite repair processes difficult. Furthermore, they suggest that some metabolite repair enzymes might be annotated with an incorrect function.

Metabolite Repair Enzymes in Glycolysis

To illustrate the importance of these mechanisms, we assembled our current knowledge on how metabolite repair enzymes take care of side-products made by glycolytic enzymes or generated by spontaneous reactions of glycolytic intermediates (Figure 1 and Table 1).

G6PC3 and G6PT Eliminate a Side-Product of Hexokinases That Can Lead to Neutropenia

Hexokinases, such as hexokinases 1–3 (HK1–3) and ADP-dependent glucokinase (ADPGK), perform the first step of glycolysis, the phosphorylation of glucose to glucose-6-phosphate (glucose-6P). Beyond their physiological substrate glucose, they also phosphorylate other sugars and, with much lower efficiency, the glucose analog 1,5-anhydroglucitol (also called

1-deoxyglucose), generating 1,5-anhydroglucitol-6P (Figure 1, pathway 1; Figure 2A) [31]. It had been known for many years that 1,5-anhydroglucitol-6P is a powerful inhibitor of HKs [32], but it was not considered that, because of considerable 1,5-anhydroglucitol concentrations in the plasma (about 100 μM) [33], 1,5-anhydroglucitol-6P was continuously formed in the cytosol of cells and that it therefore needs to be eliminated. More recently, however, it has been found that the accumulation of 1,5-anhydroglucitol-6P causes hereditary neutropenia in patients with deficiencies in proteins that serve to eliminate this side-product, the glucose-6P transporter (G6PT) and a phosphatase of so far unclear function that carries the 'misleading' name glucose-6-phosphatase catalytic subunit 3 (G6PC3) [11].

It turns out that 1,5-anhydroglucitol-6P is transported by G6PT into the lumen of the endoplasmic reticulum, where it is dephosphorylated by G6PC3 [11] (Figure 2B). G6PC3 is a homolog of glucose-6-phosphatase (G6PC1), the enzyme that produces glucose from glucose-6P in liver and kidneys [11]. It is a transmembrane protein present in the endoplasmic reticulum of virtually all mammalian tissues and has very low glucose-6-phosphatase activity compared with G6PC1 [11]. Unlike G6PC1 deficiency, which causes glycogen storage disease type Ia, G6PC3 deficiency does not cause metabolic symptoms, but leads to severe neutropenia, resulting in susceptibility to infections [34,35].

As stated, deficiency in G6PC3 or in G6PT has been shown to lead to the accumulation of 1,5-anhydroglucitol-6P in several tissues and cells types. Neutrophil granulocytes are particularly sensitive to this accumulation and to the inhibition of HKs because they have almost no mitochondria and rely largely on glycolysis for energy provision [36,37]. This explains why their number and their functionality are reduced both in humans and mice that are deficient in G6PC3 [34] or in G6PT [38]. Of note, these insights might be of clinical relevance because an antidiabetic drug (empagliflozin) that decreases the level of 1,5-anhydroglucitol in the blood increases neutrophil granulocyte counts in G6PC3-deficient mice, whereas administration of 1,5-anhydroglucitol further reduces them [11]. These findings underline the metabolite repair role of G6PC3 and G6PT *in vivo*.

About 90% of the 1,5-anhydroglucitol present in the human body derives from food, where it is widely distributed [39]. 1,5-Anhydroglucitol results from the enzymatic reduction of 1,5-anhydrofructose [40,41], which is produced during the breakdown of glycans by glycan lyases and by a side-activity of some glycosidases [42,43]. The latter enzymes catalyze a hydrolysis reaction that typically yields glucose, but some of them (e.g., the endoplasmic reticulum glycosidase II) also catalyze, at a low rate, an elimination reaction that releases 1,5-anhydrofructose [42,44]. 1,5-Anhydroglucitol is actively absorbed in the intestine [39,45] and is also actively reabsorbed in the proximal tubules of the kidneys by the Na^+-dependent hexose transporter SGLT4 [46,47]. This explains why the mammalian internal milieu is particularly rich in this glucose analog, which is not catabolized [39] and has, so far, no known physiological function.

PGP, a Multispecific Phosphatase, Eliminates Side-Products of GAPDH and Pyruvate Kinase

The **pentose phosphate pathway** intermediate erythrose-4-phosphate (erythrose-4P) resembles the glycolysis pathway intermediate glyceraldehyde-3-phosphate (glyceraldehyde-3P) (Figure 1, pathway 2; Figure 2C,D). It is therefore not surprising that the abundant glycolytic enzyme glyceraldehyde-3P dehydrogenase (GAPDH) also acts on this substrate [10,48]. The problem with this side-activity is that it leads to the formation of 4-phosphoerythronate (4P-erythronate), which is a very strong inhibitor of 6-phosphogluconate dehydrogenase (6P-gluconate DH, 6PGDH; K_i <1 μM), an enzyme of the pentose phosphate pathway [10,49] (Figure 2D). Therefore, we hypothesized that cells must contain a phosphatase that efficiently

Table 1. Formation of Noncanonical Metabolites and the Corresponding Repair Enzymes That Prevent Their Accumulation in the Context of Glycolysis

Formation		Product	Repair enzyme(s)	Mode of action	Loss-of-function phenotype *in vivo*	Refs
Mechanism	Substrate(s)					
Spontaneous	Glucose + lysine (free and in proteins)	ε-Fructoselysine	Fructosamine 3-kinase (FN3K)	Covalent protein modification	Increased free and protein-bound fructose-lysines in knockout mice	[95,96]
Spontaneous	Glucose-6-phosphate + lysine (free and in proteins)	ε-Fructoselysine-6-phosphate	Magnesium-dependent phosphatase 1 (MDP1)	Covalent protein modification	?	[97]
Hexokinases (HKs) or ADP-dependent glucokinase (ADPGK)	1,5-Anhydroglucitol	1,5-Anhydroglucitol 6-phosphate	Collaboration between glucose-6-phosphatase catalytic subunit 3 (G6PC3) and glucose-6-phosphate translocase (G6PT/SLC37A4)	Inhibition of low K_m hexokinases	Neutropenia in humans and mice with G6PC3 and G6PT deficiency	[11,31]
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NADH	*S* and *R* NADHX and NADPHX	NAD(P)HX dehydratase (NAXD) and NAD(P)HX epimerase (NAXE)	Inhibition of many dehydrogenases	Deficiency of dehydratase or epimerase causes encephalopathy in humans	[3,13,23,24,60,63–65]
Spontaneous	NADH and NADPH					
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Erythrose 4-phosphate	1,4-Bisphospho-erythronate	Acylphosphatase 1 (ACYP1) and phosphoglycerate kinase (PGK)	Unknown	?	[10]
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and acylphosphatase 1 (ACYP1)	Erythrose-4-phosphate	4-Phospho-erythronate	Phosphoglycolate phosphatase (PGP)	Strong inhibition of 6-phosphogluconate dehydrogenase	Embryonic lethality in mice	[10,26]
Triose-phosphate isomerase (TPI)	Triose phosphates: glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP)	Methylglyoxal	Glyoxalase 1 (GLO1), glyoxalase 2-(HAGH), and D-lactate dehydrogenase (LDHD)	Covalent modification	?	[82–85,94]
Spontaneous						

Pyruvate kinase	L-lactate	L-2-phospholactate	Phosphoglycolate phosphatase (PGP)	Inhibitor of several 6-phosphofructokinase 2 isoenzymes; reduced fructose 2,6-bisphosphate	Embryonic lethality in mice	[10,26]
Pyruvate kinase	Glycolate	2-Phosphoglycolate	Phosphoglycolate phosphatase (PGP)	Inhibitor of triose phosphate isomerase (TPI), succinate dehydrogenase (SDH), and phospho-glycerate mutase (PGAM)	Embryonic lethality in mice	[10,26]
L-lactate dehydrogenase (LDH) L-malate dehydrogenase	α-Ketoglutarate	L-2-hydroxyglutarate	L-2-hydroxyglutarate dehydrogenase (L2HGDH)	Inhibition of several α-ketoglutarate--dependent enzymes	L-2-hydroxyglutaric aciduria: neurological disorder with very high accumulation in tissues and body fluids	[1,25,61,68]
L-lactate dehydrogenase (LDH)	Glyoxalate	Oxalate	Enzymes reducing cytoplasmic [glyoxylate]: glyoxylate reductase/ hydroxypyruvate reductase (GRHPR) forms glycolate; alanine glyoxylate transaminase (AGXT1) forms glycine	Formation of insoluble precipitates	Primary hyperoxalic aciduria: kidney stones, failure, and calcifications	[98–101]

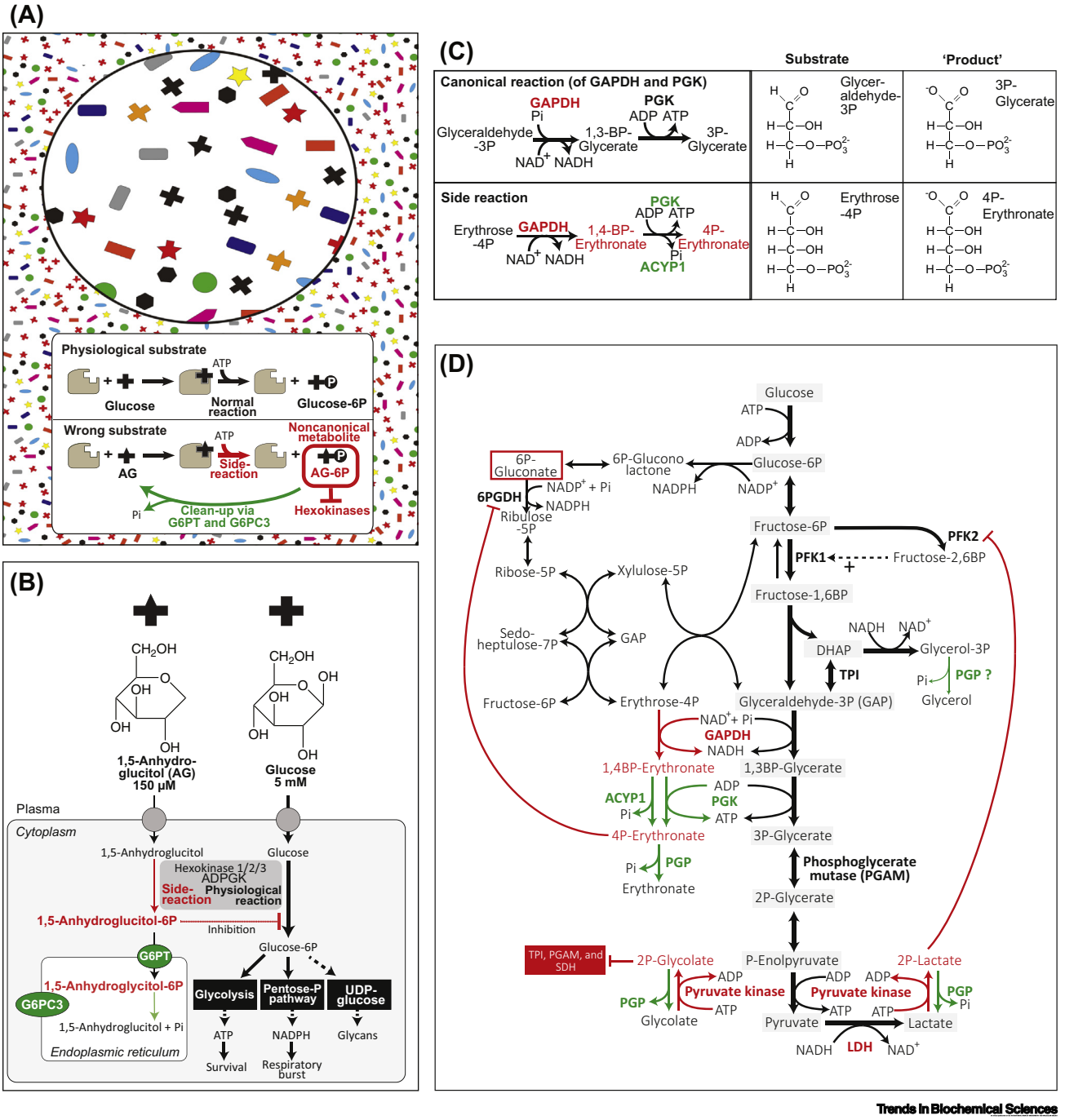

Figure 2. Repairing Errors of Hexokinases, Glyceraldehyde-3-Phosphate Dehydrogenase, and Pyruvate Kinase. Black indicates physiological reactions, red indicates side-reactions, and green indicates metabolite repair processes. (A,B) Illustration of the metabolite repair concept using the example of 1,5-anhydroglucitol 6P elimination. Though highly specific, metabolic enzymes work under very difficult conditions and are exposed to a large variety of metabolites at vastly different concentrations. The primary physiological substrate for hexokinases is glucose, although with low efficiency they can also act on the glucose-analog 1,5-anhydroglucitol (AG) to produce the side-product 1,5-anhydroglucitol 6P (AG-6P). ADP-dependent glucokinase (ADPGK) can also perform this reaction. AG-6P inhibits hexokinases and thereby glycolysis. Cells eliminate AG-6P in two steps. First, it is transported into the endoplasmic reticulum via glucose 6-phosphate translocase

(Figure legend continued at the bottom of the next page.)

destroys 4P-erythronate. This indeed proved to be the case. We purified 4P-erythronate phosphatase, identified its gene (*PGP*), and knocked it out in the human colorectal cancer cell line HCT116, the human osteosarcoma cell line U2OS, and in immortalized human fibroblasts. The PGP-deficient cell lines not only showed a marked increase (5–20-fold) in concentrations of 4P-erythronate but also even stronger increases in 6P-gluconate [10,30], the substrate of the enzyme that is inhibited by 4P-erythronate (Figure 2D) [10,50].

In HCT116 cells, PGP was shown to have additional metabolite repair functions, such as destroying L-2-phospholactate (2P-lactate) [10,51], which can be formed when pyruvate kinase erroneously acts on L-lactate (Figure 1, pathway 3; Figure 2D). Of note, the thermodynamic equilibrium of this reaction is poised towards the production of 2P-lactate [52]. Knocking out PGP in HCT116 cells caused a marked increase in the concentration of 2P-lactate, which in turn perturbed the regulation of glycolysis by inhibiting the synthesis of the glycolytic regulator fructose-2,6BP [10].

The name PGP relates to the fact that this enzyme hydrolyses a third noncanonical metabolite, 2-phosphoglycolate (2P-glycolate), which can be formed when pyruvate kinase erroneously acts on glycolate [30,52] (Figure 1, pathway 4; Figure 2D). Recent work indicates that HCT116 cells produce very little 2P-glycolate unless they are artificially provided with its biochemical precursor glycolate [30]. Lack of destruction of 2P-glycolate under these conditions has several metabolic consequences, including functional inactivation of phosphoglycerate mutase (PGAM) [30], as well as inhibition of succinate dehydrogenase (SDH) [30] and potentially triose phosphate isomerase (TPI) [53]. To what extent accumulation of 2P-glycolate might contribute to the phenotype of PGP loss in normal mammalian cells is still subject to investigation [26,30].

The metabolite repair function of PGP is remarkable because it eliminates three different glycolytic side-products. At first sight, this might be mistaken as lack of specificity. Nevertheless, although PGP acts with comparable efficiency on three noncanonical (and toxic) metabolites, it acts at least 100-fold less efficiently on most intermediates of glycolysis [10]. Remarkably, 3-phosphoglycerate (3P-glycerate) is a poorer substrate (more than fivefold lower activity at 1 mM) than 2P-glycolate and 4P-erythronate, although it is structurally very similar and intermediate in size between these two metabolites [10]. PGP should therefore be considered as a multispecific rather than a nonspecific phosphatase.

Of note, in the case of PGP, it has been pointed out that this enzyme also acts on the canonical metabolite glycerol-3P, a biochemical precursor of complex lipids and triacylglycerides (albeit with a catalytic efficiency that is 100-fold lower than for its other substrates) [29] (Figure 2D). Some data obtained by the analysis of PGP knockout murine embryonic fibroblasts and partial knockdown approaches *in vivo* and *in vitro* indicate that PGP loss alters cellular lipid metabolism [26,29,54]. However, given the very low catalytic efficiency of PGP on glycerol-3P, it is still unclear whether the observed effects are directly due to loss of PGP glycerol-3-phosphatase activity or

(SLC37A4/G6PT) and it is then dephosphorylated by glucose-6-phosphatase catalytic subunit 3 (G6PC3). (C) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acts with high efficiency on its substrate, glyceraldehyde-3P. It also acts on a structural analog, erythrose-4P, belonging to the pentose phosphate pathway. The resulting intermediate, 1,4BP-erythronate, is converted into 4P-erythronate using acylphosphatase 1 (ACYP1) and/or phosphoglycerate kinase (PGK). (D) Phosphoglycolate phosphatase (PGP) eliminates three glycolytic side-products. First, it dephosphorylates 4P-erythronate, which is a very strong inhibitor of 6-phosphogluconate dehydrogenase (6PGDH). Second, it dephosphorylates 2P-lactate, which is formed when pyruvate kinase erroneously acts on L-lactate, and is an inhibitor of several isoforms of the enzymes that produce the glycolytic activator, fructose 2,6BP. Last, it dephosphorylates 2P-glycolate, which can be formed when pyruvate kinase acts on glycolate (a metabolite that is largely absent from mammalian cells but is abundant in yeasts and plants) and inhibits succinate dehydrogenase, phosphoglycerate mutase, and possibly triose phosphate isomerase. Some evidence has been presented that PGP also acts on glycerol-3P, albeit with a 100-fold lower catalytic efficiency.

indirectly due to the deficiency of its metabolite repair functions. At this point, we cannot exclude that PGP (like G6PT) might serve both a metabolite repair and a canonical metabolic function.

Several lines of evidence indicate that PGP and its metabolite repair functions, which have been observed in two cancer cell lines and in immortalized human fibroblasts [10,30], play major roles in cellular metabolism. First of all, genetic inactivation of PGP in mice is embryonically lethal [26] (Box 2). Furthermore, the metabolite repair function of PGP is conserved throughout evolution. For example, the yeast PGP homolog pho13 has a similar substrate profile to mammalian PGP: its two best substrates are 2P-glycolate and 4P-erythronate. Genetic inactivation of pho13 leads to accumulation of 4P-erythronate and 2P-glycolate [10], which can be made from glycolate indirectly obtained from the glyoxylate cycle [55]. In comparison with mammalian cells, the increase in 6P-gluconate in pho13 knockout yeast cells is less dramatic (i.e., only five-fold), most likely due to compensatory upregulation of pentose phosphate pathway enzymes including 6PGDH [10,56].

ACYP1 Can Eliminate Side-Products of GAPDH

In the same way as GAPDH forms 1,3BP-glycerate from glyceraldehyde-3P, it converts erythrose-4P to 1,4BP-erythronate (Figure 1, pathway 2; Figure 2C,D). Because 3P-glycerate kinase (PGK) is not very active in dephosphorylating 1,4BP-erythronate [10], it is thought that dephosphorylation of 1,4BP-erythronate to 4P-erythronate is catalyzed by acyl-phosphatase 1 (ACYP1), a cytosolic enzyme with no well-defined function [57]. This is supported by *in vitro* data comparing the capacities of purified human ACYP1 and human PGK to dephosphorylate 1,4BP-erythronate. *Saccharomyces cerevisiae* does not have any acyl-phosphatase, but PGK is an extremely abundant protein (>1 % of total protein) in this organism [58]. Calculations indicate that its side-activity on 1,4BP-erythronate is sufficient to account for the formation of 4P-erythronate in this organism [10].

NAXD and NAXE, Two Extremely Well Conserved Enzymes, Repair Hydrated Forms of Pyridine Nucleotides

One of the two oldest known metabolite repair mechanisms allows hydrated forms of NADH and NADPH to be reconverted to regular NAD(P)H. An ATP-dependent dehydratase activity was shown in 1956 to repair a damaged form of NADH, called NADHX [59], that is formed by a side-reaction of GAPDH [27,60] (Figure 1, pathway 5; Figure 3). Later it was found that NAD(P)HX is a hydrated form of NAD(P)H in which the hydroxyl group on C6 of the nicotinamide ring is in the *S* or the *R* orientation [27]. It was also found that NADPH is spontaneously converted to NADPHX at the impressive rate of ~10% per hour at 37°C [3].

The molecular identification of the NAD(P)HX dehydratase (NAXD) showed that it is an extremely conserved protein which forms a bifunctional protein with an NAD(P)HX epimerase (NAXE) in most bacteria [3]. The dehydratase specifically acts on the *S* isomer, and the epimerase (a separate protein in most eukaryotes) facilitates the repair of the *R* isomer by converting it into the *S* isomer [61]. In eukaryotes, multiple forms of these enzymes are targeted to different cellular compartments containing pyridine nucleotides, including the cytosol, the mitochondria, the chloroplasts in plants, and the endoplasmic reticulum in mammals [62,63]. This, together with the wide species distribution, indicates that this enzyme pair is present in virtually all locations where pyridine nucleotides are present.

Deficiency of the dehydratase and the epimerase lead to the accumulation of NADHX and NADPHX, as well as a cyclic derivative of these nucleotides. The physiological consequences of these deficiencies are subtle in yeast, *E. coli*, and plants, but dramatic in humans [13,63].

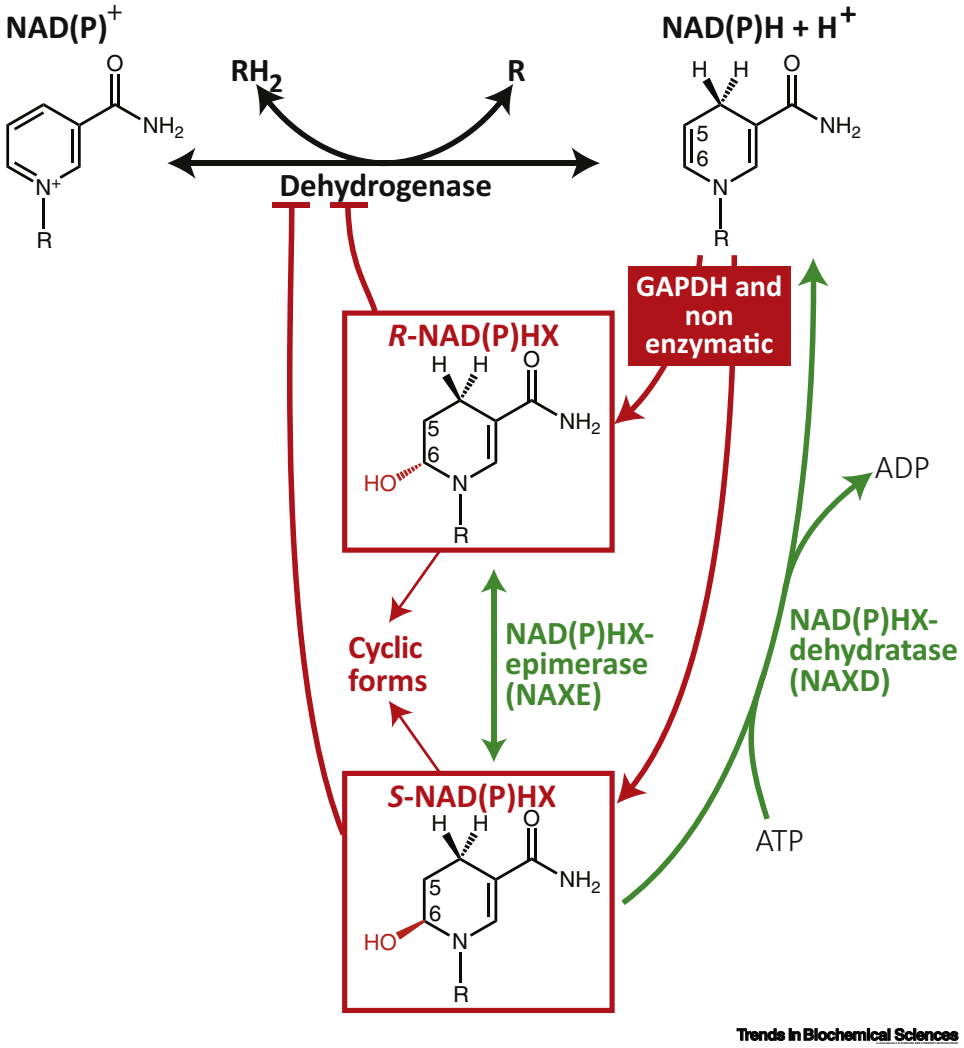

Figure 3. Two Extremely Well Conserved Proteins Repair Damaged Pyridine Nucleotides. Black indicates physiological reactions, red indicates side-reactions, and green indicates metabolite repair processes. Pyridine nucleotides ($NADP^+$/NADPH or NAD^+/NADH) are cofactors of numerous dehydrogenases and reductases. At a low rate, their reduced forms (NADPH or NADH) can be hydrated to form two stereoisomers of NADPHX and NADHX. This reaction proceeds spontaneously and, in the case of NADH, is facilitated by glyceraldehyde 3-phosphate dehydrogenase (GADPH). NADPHX and NADHX interfere with a large variety of dehydrogenases and must therefore be eliminated. The *S* stereoisomers of NADPHX and NADHX are repaired by the ATP-dependent NAD(P)X dehydratase (NAXD), whereas the *R* stereoisomers must first be converted into the *S* stereoisomers by NAD(P)X epimerase (NAXE). Failure to repair NAD(P)HX leads to lethal encephalopathy in patients with mutations in NAXD or NAXE.

Inactivating mutations of the *NAXD* or the *NAXE* genes lead to severe encephalopathy and death in early childhood after a fever episode [23,24,64]. It has been shown that NAD(P)HX inhibits several dehydrogenases [65] and, in yeast, a transhydrogenase involved in serine biosynthesis [13]. This suggests that NAXD and NAXE collaborate to protect several different metabolic pathways.

L2HGDH Corrects Mistakes of Lactate and Malate Dehydrogenases

The first description of L-2-hydroxyglutarate dehydrogenase (L2HGDH) dates back to 1937, before the **tricarboxylic acid cycle** was described, and it took >60 years to understand its

metabolic significance [1,25,66,67]. L2HGDH is a FAD-linked dehydrogenase that catalyzes the irreversible conversion of L-2-hydroxyglutarate (L-2-OH-glutarate) to α-ketoglutarate (Figure 1, pathway 6). In mammals, L-2-OH-glutarate is produced by side-reactions of LDH and malate dehydrogenase [1,68]. Although modest compared with their main activities, they lead to the formation of substantial amounts of L-2-OH-glutarate. In patients or mice with L2HGDH deficiency, L-2-OH-glutarate accumulates in body fluids and tissues, reaching millimolar concentrations in the brain [61,69]. The resulting disease, L-2-OH-glutaric aciduria, is characterized by a progressively debilitating neurological disorder and a marked increase in the frequency of brain tumors [70,71]. These observations clearly suggest a metabolite repair function of L2HGDH. Beyond this, it has recently been suggested that L-2-OH-glutarate plays an important role in metabolic adaptation to hypoxia [68,72]. Nonetheless, one must be careful not to overinterpret products of side-reactions as new signaling molecules [9,73] (Box 1).

Sedoheptulose-1,7BP Phosphatase: Repair or Metabolic Pathway?

Aldolases catalyze a reversible reaction required in both glycolysis and **gluconeogenesis**. Physiologically, aldolases condense dihydroxyacetone-P with glyceraldehyde-3P. However, their specificity is relatively wide and they can also act on aldehydes other than glyceraldehyde-3P [74,75]. Using erythrose-4P and dihydroxyacetone-P, aldolase can form sedoheptulose-1,7-bisphosphate (sedoheptulose-1,7BP) [75] (Figure 1, pathway 7). In mammalian cells, the latter can be dephosphorylated by fructose-1,6-bisphosphatase (FBP1/2) [76]. In addition, in yeast, a dedicated phosphatase has been described that dephosphorylates sedoheptulose-1,7BP (SHBP) [28]. It has been suggested that this represents part of a thermodynamically favorable pathway for the synthesis of ribose-5P, an intermediate of the pentose phosphate pathway. However, inactivation of this phosphatase causes a marked increase in the concentration of sedoheptulose-1,7BP, but does not affect ribose-5P concentration [28]. Hence, it is tempting to conclude that, under physiological conditions, the function of this enzyme might be to limit accumulation of sedoheptulose-1,7BP.

Glyoxalases Eliminate the TPI Side-Product Methylglyoxal

Methylglyoxal is mostly formed from triose phosphates, either by a spontaneous reaction or by a side-activity of TPI [77]. It is estimated that about 0.5 μmol/g of this compound is formed per day in skeletal muscle [78]. Owing to its two carbonyl groups, methylglyoxal readily reacts with amino groups (particularly of arginine residues) of proteins to form imidazolones and other advanced glycation end-products [79]. This is believed to play a role in diverse contexts such as diabetes, aging, and cancer [80–83].

The main pathway of methylglyoxal detoxification is the glyoxalase system, which consists of glyoxalases 1 (GLO1) and 2 (GLO2), and glutathione (Figure 1, pathway 8) [84]. The end-product is D-lactate, which is metabolized by the mitochondrial enzyme D-LDH (LDHD) [85]. Some organisms also have a glyoxalase 3 (GLO3), which converts methylglyoxal to lactate without the need for glutathione [86,87]. It has been reported that human PARK7 (also called DJ1), a protein of unknown function mutated in a recessive form of Parkinson's disease, has GLO3 activity [88]. However, in the presence of the highly active and ubiquitous GLO1 and 2 system, it is difficult to envisage an important role of this weak activity in methylglyoxal detoxification.

Several groups have also reported that PARK7 can actively deglycate methylglyoxal adducts of metabolites, proteins, and nucleotides [82,89,90]. Nevertheless, these claims have been disputed, and increased protein glycation was not consistently found in DJ1 knockout cell lines and animals (unpublished data and [91,92]).

There are presently no known human genetic diseases caused by GLO1 or 2 deficiency, and knockout mice are phenotypically normal, possibly due to compensatory upregulation of alternative pathways of methylglyoxal detoxification, such as several aldose/ketose reductases [93]. By contrast, two patients with massive urinary D-lactate excretion and accumulation of D-lactate in plasma caused by inactivating mutations in the *LDHD* gene have recently been reported [94]. A large part of the D-lactate in these patients likely originates from endogenous metabolism (i.e., methylglyoxal metabolism) because antibiotic treatment to eradicate the intestinal microbiome did not affect D-lactate concentrations.

FN3K Repairs Products of Spontaneous Reactions of Glucose with Amines

Glucose spontaneously reacts with primary amines to form Schiff bases, which undergo slow and spontaneous rearrangement to become fructosamines [95] (Figure 1, pathway 9). This type of reaction occurs with all amines and is particularly well known for the ε-amino groups of lysines and the N-terminal α-amino group of proteins. To repair this type of damage, fructosamine-3-kinase (FN3K) phosphorylates the third carbon of the fructose moiety, causing its destabilization and the release of a free amine, 3-deoxyglucosone, and inorganic phosphate [95]. Genetic inactivation of FN3K in mice leads to marked increases in protein glycation and in the urinary excretion of fructose-ε-lysine [96]. Thus FN3K seems to repair damage inflicted both to low-molecular weight compounds and to proteins.

Similarly to glucose, but at higher rates, glucose-6P reacts with amines to form fructosamine-6Ps, which are substrates for magnesium-dependent phosphatase 1 (MDP1) [97]. Future studies will be necessary to reveal whether this enzyme plays a role in limiting fructosamine-6P in cells or animals.

Maintaining Low Concentrations of Glyoxyate Prevents the Formation of Oxalate, a Side-Product of LDH

In mammals, oxalate precipitates can lead to considerable tissue damage, kidney stones, and kidney failure [98]. Oxalate is formed by the irreversible oxidation of glyoxylate by LDH in the cytosol [99,100] (Figure 1, pathway 10). To avoid this, several enzymes collaborate to keep cytosolic glyoxylate concentrations low, representing a striking pre-emptive mechanism to reduce oxalate formation.

In mitochondria and peroxisomes, glyoxylate can be detoxified to glycine by alanine glyoxylate aminotransferase (AGXT) in an almost irreversible reaction [101,102]. Nevertheless, to prevent formation of oxalate in the cytoplasm (and mitochondria), glyoxylate is converted to glycolate by glyoxylate/hydroxypyruvate reductase (GRHPR) in a NADPH-dependent reaction [103]. The resulting glycolate can then be reconverted into glyoxylate by glycolate oxidase (hydroxyacid oxidase, HAO1/2) in peroxisomes, where it is then detoxified into glycine [101]

When either AGXT or GRHPR is mutated, cells cannot maintain low glyoxylate concentrations in mitochondria, peroxisomes, or the cytoplasm. Instead, an increased amount of glyoxylate is converted to oxalate by LDH in the cytoplasm, and affected patients suffer from hyperoxaluria type I and type II [98]. Strikingly, mutations leading to mislocalization of AGXT in such a way that it is hypoactive in peroxisomes are sufficient to cause hyperoxaluria [104,105]. This stresses the importance of the presence of the detoxifying transaminase in this subcellular compartment.

In view of the extensive number of repair enzymes, it is perhaps surprising that mammals do not have one of the oxalate-destroying enzymes that are present in other organisms (e.g., oxalate oxidase [106], oxalate decarboxylase [107], oxalyl-CoA decarboxylase [108]). This might indicate that low concentrations of oxalate could be needed to fulfill some so far unidentified functions.

Concluding Remarks and Future Perspectives

One of the important messages of this review is that intermediary metabolism is not correctly summarized by the extremely complex metabolic maps that usually hang in the corridors of biochemistry laboratories. Numerous repair enzymes are necessary to allow smooth operation of the ensemble of reactions. Some of these enzymes are extremely conserved and play a key role in cellular metabolism. This means that they should be considered as an integral part of the architecture of metabolic networks.

In fact, their inclusion not only improves our understanding of how cellular metabolism works but may also give new insights into human diseases and aging, as well as facilitating process optimization in biotechnology (see Outstanding Questions) [109]. It is extremely likely that many more metabolite repair enzymes will be discovered [110]. It is therefore important to keep potential metabolite repair functions in mind when we study enzymes of still unknown or likely misannotated function, some of which are mutated in human diseases. We believe that this approach might lead to important insights into the pathogenesis of human diseases and reveal surprising new aspects of cellular metabolism.

Outstanding Questions

Humans and mice are more sensitive to defects in metabolite repair enzymes than microorganisms (Box 2). Do they need a larger variety of repair enzymes? Are mammalian (vertebrate) enzymes generally more specific than enzymes from lower organisms?

We have described how mutations in metabolite repair enzymes related to glycolysis result in diseases. Are other metabolite repair enzymes among enzyme-like proteins that are mutated in human diseases?

Noncanonical side-products can inhibit the activity of canonical enzymes, thereby affecting metabolic pathways. Does the accumulation of damaged metabolites contribute to aging and the development of (neuro)degenerative diseases?

Cancer cells show hyperactive cellular metabolism. Are metabolic repair enzymes particularly important to maintain this high metabolic activity? If so, metabolite repair enzymes might represent interesting targets for the development of therapeutic agents in the treatment of cancer.

Can we use metabolite repair enzymes for process optimization in biotechnology? For example, malyl-CoA thioesterase has been shown to destroy an unwanted side-product of β-methylmalyl-CoA lyase in the design of a new pathway for CO_2 fixation.

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