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Rethinking glycolysis: on the biochemical logic of metabolic pathways

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Metabolic pathways may seem arbitrary and unnecessarily complex. In many cases, a chemist might devise a simpler route for the biochemical transformation, so why has nature chosen such complex solutions? In this review, we distill lessons from a century of metabolic research and introduce new observations suggesting that the intricate structure of metabolic pathways can be explained by a small set of biochemical principles. Using glycolysis as an example, we demonstrate how three key biochemical constraints—thermodynamic favorability, availability of enzymatic mechanisms and the physicochemical properties of pathway intermediates—eliminate otherwise plausible metabolic strategies. Considering these constraints, glycolysis contains no unnecessary steps and represents one of the very few pathway structures that meet cellular demands. The analysis presented here can be applied to metabolic engineering efforts for the rational design of pathways that produce a desired product while satisfying biochemical constraints.

The study of metabolism has been at the center of biological research since the nineteenth century[1]. Yet, with the advent of molecular biology and genetic research, some scientists have misguidedly treated metabolism as a field 'to be mastered and then put aside'[2]. In recent years, however, the scientific community has witnessed a renaissance in metabolic research born of a new understanding that metabolism is central to many biological phenomena[3–7]. Metabolic research, for example, has become vital in deciphering and treating various pathologies including cancer[8–11]. Recent research has also revealed unique metabolic capabilities supporting microbial growth in surprising conditions and environmental niches[12–14], deepening the understanding of the biosphere. Furthermore, many current research efforts attempt to address emerging challenges in sustainable energy, green chemistry and pharmaceuticals by tinkering with central and secondary metabolism[15–18]. Deep understanding is essential for all of these efforts: to study, manipulate or redesign metabolism, one must gain a solid grasp on the biochemical principles governing it.

In this review, we distill lessons from a century of metabolic research and introduce systematic observations that account for the structure of metabolic pathways. We apply these lessons to offer a fresh perspective on the most intensely investigated metabolic pathway: Embden-Meyerhof-Parnas glycolysis[19–27] (**Fig. 1**). Glycolysis serves two main metabolic functions. When terminal electron acceptors are not available, glycolysis supplies all of the ATP molecules required for cellular activity. Moreover, glycolytic intermediates are direct precursors of many cellular building blocks[28]. Our premise is that metabolic pathways such as glycolysis can be analyzed as evolutionary optimization problems. As such, we presume that the structure of glycolysis can be explained by understanding the selection pressures and constraints imposed on it during evolution. Indeed, we show that the natural glycolytic reaction sequence is simple and logical given the diverse biochemical constraints imposed on it. **Figure 1** serves as a guide to the metabolic aims and biochemical constraints shaping the glycolytic reaction sequence, which we discuss in detail below.

This review is intended to serve as a metabolic tutorial, not a discussion of current topics in metabolic research. As such, we focus on illuminating central principles and explaining observed structures rather than discussing, for example, the evolutionary emergence of pathways, control of metabolic activity or measurement and modeling of metabolic flux. Though many of the principles we discuss are well known, we integrate them to explain the logic of the structure of a central metabolic pathway. The principles discussed may not apply to all pathways and circumstances. Yet, we believe that by thinking carefully about the principles governing a metabolic pathway, the pathway can be transformed from a random assortment of reactions into a goal-driven, rationalizable process. With the recent focus on the engineering of synthetic pathways, the time is ripe to bring these ideas to center stage: good engineers must know the principles that make their machines tick.

The thermodynamic basis of metabolic pathways

As the laws of thermodynamics govern the feasibility of biochemical reactions, thermodynamics has a strong influence on the structure of metabolic pathways. To explain this effect, we address the energetic profile of general biochemical transformations. For the sake of simplicity, we focus here on aliphatic compounds composed of only carbon, hydrogen and oxygen. **Figure 2** shows the reduction potential (which quantifies how much a compound tends to accept electrons) of functional groups containing only carbon, hydrogen and oxygen. Although the exact reduction potential of each group depends on its molecular environment, the general trend is quite clear: more reduced carbons have higher reduction potentials—that is, they have a greater tendency to accept electrons[29,30]. Hence, the reduction potential of functional groups follows the general order of hydroxycarbons (CHOH) > carbonyls (COH) > carboxyls (COOH) (**Fig. 2**). This trend determines the energetics of simple redox reactions in which electrons are transferred from a donor molecule to an acceptor molecule (oxidoreductase reactions, enzyme classification 1.X.X.X). Moreover, this trend determines the energetics of more complex reactions, including, for example, the formation (or cleavage) of a carbon-carbon bond: when a carbon-carbon bond breaks, one carbon takes an electron from the other, which then becomes oxidized. The energetics of such reactions are determined primarily by the oxidation state of the carbon being oxidized[30].

The simple trend presented in **Figure 2** also accounts for the general structure and resource demands of many natural pathways. Reductive processes, for example, which use NAD(P)H as electron donor, are expected to be energetically constrained in the reduction

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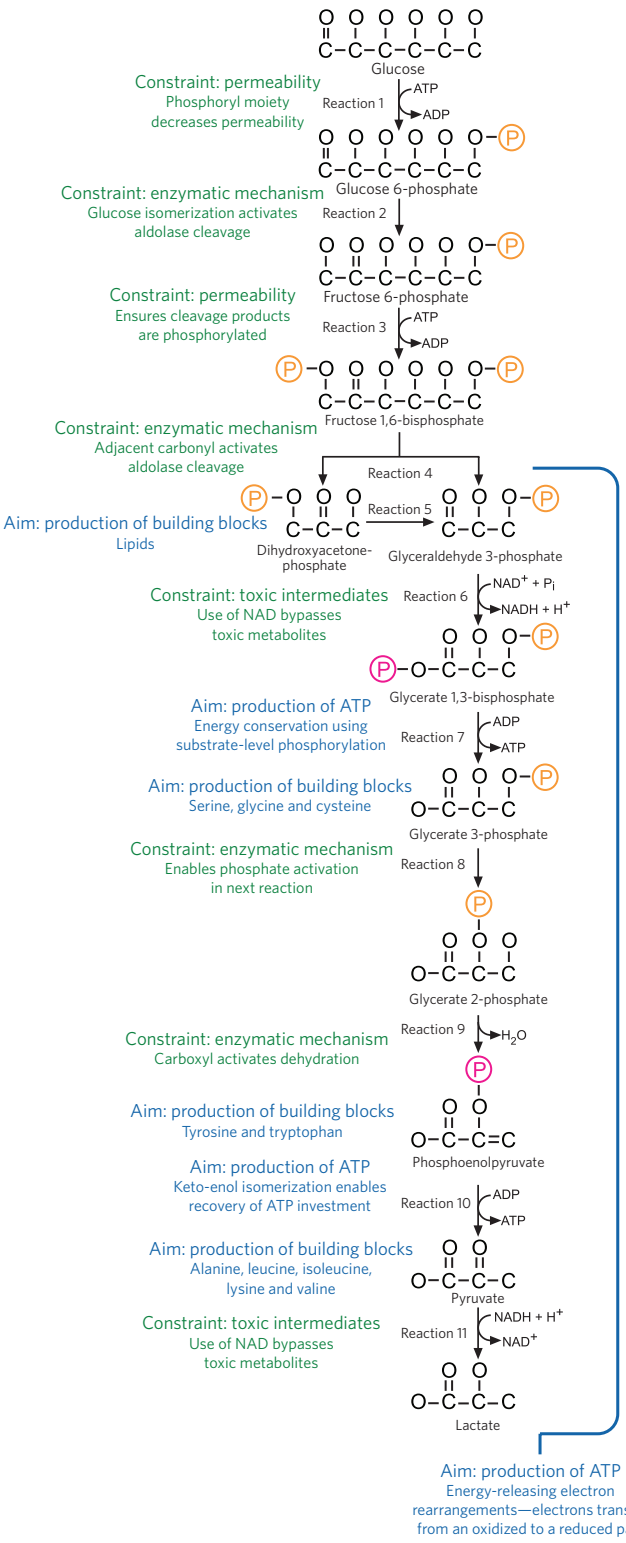

Figure 1 | The Embden-Meyerhof-Parnas glycolytic pathway. Blue indicates metabolic aims, and green corresponds to biochemical constraints, as discussed in detail in the main text. For clarity, sugars are drawn as linear chains and hydrogens are omitted. 'P_i' represents inorganic phosphate, and P represents a phosphate group (phosphoric acid, PO_4^{2-}) attached to a metabolite. Phosphate groups are colored according to the energy of hydrolysis of the bond between the compound and the phosphate moiety (explained in the text): pink corresponds to a bond with a high energy of hydrolysis, and orange represents a bond with a low energy of hydrolysis.

of carboxyls to carbonyls (as the reduction potential of carboxyls is substantially lower than that of NADPH; **Fig. 2**, gray to pink transition). Indeed, reductive pathways (for example, carbon fixation pathways) couple these reactions directly or indirectly to exergonic reactions (transformed Gibbs energy of reaction, Δ_rG', << 0) such as ATP hydrolysis. Similarly, oxidative processes, which use $NADP^+$ as electron acceptor, cannot oxidize hydrocarbons to hydroxycarbons without an energy investment (**Fig. 2**, blue to gray transition). The tricarboxylic acid (TCA) cycle, for example, which oxidizes succinate to malate (via fumarate), sidesteps this problem by using ubiquinone, which has a high reduction potential (transformed reduction potential under standard conditions, E'^{o}, > 0 mV), as an electron acceptor instead of NAD^+.

Glycolysis is a redox-neutral process: the terminal electron acceptor is the metabolic product of the molecule that originally donated the electrons. Hence, the net fermentation process effectively transfers electrons within a molecule. We refer to this internal oxidation-reduction process as an electron rearrangement. Energy is released during such a rearrangement if electrons are drawn from a part of the molecule with low reduction potential and received by another part of the molecule with higher reduction potential.

As more reduced carbons have higher reduction potential (**Fig. 2**), coupling the oxidation of an oxidized carbon with the reduction of a more reduced carbon within the same molecule results in a decrease in the Gibbs energy ($\Delta_rG' < 0$). When the difference in reduction potentials is large enough, some of the free energy released can be conserved as ATP (**Fig. 2**, black bar). Because electrons are transferred mostly in pairs and the energy released by ATP hydrolysis is ~50 kJ mol^{-1} (ref. 31), the minimal reduction potential difference that provides enough energy to produce one ATP molecule is ~250 mV ($\Delta_rG' = nF \times \Delta_rE' = 2F \times 0.25$ ~50 kJ mol^{-1}, with F being the Faraday constant). As illustrated in **Figure 2**, transferring two electrons from any of the oxidation states to a more reduced state can, in principle, be coupled to ATP production.

Now we can address the specific case of sugar fermentation. It is considerably simpler to begin by discussing electron rearrangement within glyceraldehyde ($C_3H_6O_3$) as it is half the size of glucose. Glyceraldehyde, like glucose, is a sugar: an organic molecule composed of hydroxycarbons and only one carbonyl group. Assuming that each electron rearrangement is carried to completion before the next one begins, all possible energy-releasing electron rearrangements within glyceraldehyde are shown in **Figure 3a**. There are two possible end products, lactate and 3-hydroxypropionate, both of which can be reached through two disjoint reaction sequences (**Fig. 3a**). Regardless of the exact path, energy equivalent to the formation of two ATP molecules is released during these rearrangements (**Fig. 2**, pink to blue transition).

Feasibility of reaction mechanisms

Energetic considerations are not the only factor determining whether or not a metabolic conversion can take place. Not all energetically favorable reactions can be catalyzed enzymatically. For example, the condensation of two ethanol molecules to *n*-butanol would be extremely useful for the biofuel industry[32]. Though this reaction would be highly exergonic (Δ_rG' ~ −30 kJ mol^{-1}), there is no known enzymatic mechanism that can catalyze it. Indeed, enzymes use only a limited number of reaction mechanisms[33,34]. Importantly, most biochemical reaction mechanisms require an activating group positioned at a specific location on the substrate to help stabilize the transition state of the reaction[33,34]. Common activating groups include carbonyls, carboxyls, thioesters and amines. Metabolic pathways achieve their functional goal using available enzymatic mechanisms that can impose restrictions on their operation. For example, many enzymes that use radical-based mechanisms are sensitive to molecular oxygen, limiting the pathway to anaerobic environments[35,36].

Glycolysis offers several examples of instances where the availability of enzymatic mechanisms restricts the structure of the pathway. For example, we can ask which of the energy-releasing electron rearrangements shown in **Figure 3a** is possible from a mechanistic point of view. Notably, each of the four possible initial electron rearrangements (A1, A3, B1 or B3) requires the reduction of a hydroxycarbon to a hydrocarbon. Mechanistically, this reduction cannot proceed directly but instead requires two steps: the hydroxyl is first dehydrated to form a double bond, which is then reduced to a hydrocarbon (**Fig. 3b**). **Figure 3c–e** shows all of the possible dehydrations that can take place in glyceraldehyde. It is noteworthy that each of these dehydrations involves the formation of an unstable intermediate that undergoes a spontaneous rearrangement.

The dehydrations shown in **Figure 3c,d** result in an enol, a double bond for which one carbon carries a hydroxyl group. Enols are unstable and spontaneously undergo isomerization to a carbonyl through keto-enol isomerization[37,38]. The result of the keto-enol isomerization corresponds to either the A1 or B1 electron rearrangement (**Fig. 3c,d**, respectively): one hydroxycarbon is oxidized to a carbonyl, and the other is reduced to a hydrocarbon. The dehydration shown in **Figure 3e** results in a ketene, a double bond in which one carbon is a carbonyl. Ketenes are very susceptible to nucleophilic attack. When water serves as the attacking nucleophile, the ketene is readily transformed to a carboxylic acid. The result of this reaction sequence corresponds to electron rearrangement B3 (**Fig. 3e**): a carbonyl is oxidized to a carboxyl, and a hydroxycarbon is reduced to a hydrocarbon. The A3 electron rearrangement is therefore infeasible because there is no mechanism that can support it: as shown in **Figure 3c**, the dehydration of the terminal hydroxycarbon of glyceraldehyde leads directly to electron rearrangement A1, instead. This is an example of how mechanistic constraints can eliminate an otherwise energetically feasible approach to a metabolic task.

The physicochemical properties of intermediates

Many metabolites are produced as a consequence of a pathway's activity. These metabolites may be pathway intermediates (that is, part of the reaction sequence) or the outcome of promiscuous enzyme activity ('underground metabolism')[39]. The properties of these metabolites also restrict the structure of a pathway. As it is difficult to predict what the underground metabolites associated with a pathway might be, we discuss only the constraints imposed by pathway intermediates. The major biochemical properties that should be considered in this context are toxicity, stability, permeability and affinity. Highly reactive compounds can damage the cell by spontaneously modifying important cellular components. Other compounds are unstable, degrading quickly ($t_{½}$ < 1 min) under physiological conditions and requiring costly systems to support their continuous renewal[40]. Excluding designated secretion products, leakage of metabolites through the lipid membrane is deleterious as it results in the loss of carbon and energy[41]. Additionally, the physicochemical properties of a compound affect the affinity of the enzymes using it[42]. It is preferable to use intermediates associated with high affinity that facilitate better kinetics.

One way to address these challenges is to operate the entire pathway, or some of its reactions, within a specialized compartment[43] or a protein complex wherein the intermediates are channeled between different enzymes[44,45]. Organisms that consume ethanolamine, for example, metabolize it within a microcompartment to minimize the toxicity of the intermediate acetaldehyde[43]. In another example, the unstable iminoaspartate, an intermediate in the biosynthesis pathway of NADH, is channeled between aspartate oxidase and quinolinate synthase to avoid its being hydrolyzed quickly[46]. However, channeling and separation into microcompartments are only known to occur in very specific cases. Moreover, as metabolites can leak from microcompartments and enzyme complexes, a highly toxic intermediate can still damage the cell.

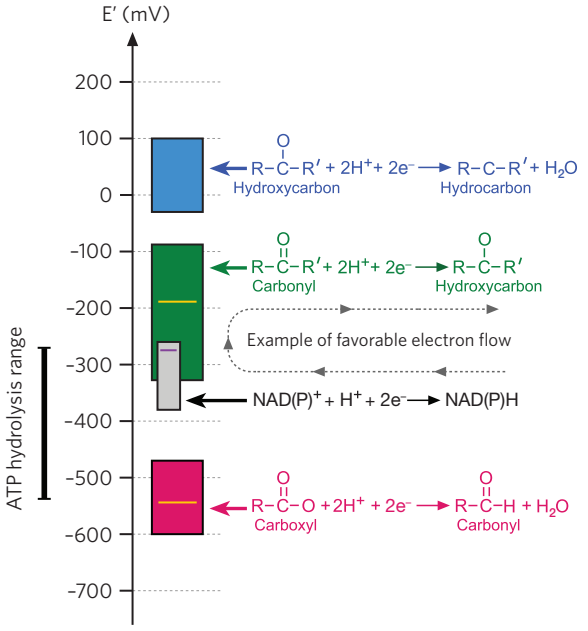

Figure 2 | The reduction potentials, *E′*, of half-reactions between functional groups composed of only carbon, oxygen and hydrogen. We collected available thermodynamic data on organic redox reactions and grouped them according to the specific functional group that is reduced. The blue, green and pink ranges represent the highest and lowest reduction potentials for each of the generalized half-reactions. The yellow line within the pink zone corresponds to the reduction potential of glycerate to glyceraldehyde (R = CH_2OH-CH(OH)), and the yellow line in the green zone corresponds to the reduction potential of pyruvate to lactate (R = CH_2; R′ = COO). The gray range corresponds to the reduction potential of NAD(P)H, assuming that [NAD(P)H]/[NAD(P)$^+$] ranges between 0.01 and 100. The purple line represents the reduction potential of NAD^+ under the physiological [NAD^+]/[NADH] in *Escherichia coli*[63]. The black vertical bar represents the difference in reduction potentials corresponding to the energy required to synthesize ATP from ADP and P_i given physiological concentrations[63] ($\Delta_r G'$ ~ –50 kJ mol^{-1}) and assuming transfer of two electrons.

We use glycolysis as an example to discuss the effect of metabolic intermediates on a pathway's structure, presuming that microcompartments and channeling are not present. We begin by discussing the effect of toxic metabolites. The effects of permeability, affinity and stability are discussed in later sections and in **Box 1**.

The dehydrations shown in **Figure 3c,d** are problematic as their products, methylglyoxal and malondialdehyde, are reactive and toxic. Generally speaking, all carbonyls (aldehydes and ketones) are reactive toward macromolecules[47,48]. Specifically, they spontaneously crosslink proteins, inactivate enzymes and mutagenize DNA[47,48]. However, the reactivity—and, therefore, the toxicity—of carbonyls varies greatly. Glucose, for example, can lead to protein inactivation, production of various cytotoxins and the development of pathologies[49,50]. However, the toxicity of glucose is relatively low; even after days of incubation with glucose, enzymes lose only a small fraction of their activity[48]. Glyceraldehyde, dihydroxyacetone and fructose are somewhat more toxic but are still relatively benign[48]. In contrast to these sugars, molecules that have a second carbonyl in the α- or β-position with respect to the first carbonyl, such as methylglyoxal and malondialdehyde, are highly reactive; they spontaneously modify macromolecules even at very low concentrations[47,51–56] and can completely deactivate enzymes within hours[48]. As such, these metabolites are a problematic choice for a central metabolic pathway.

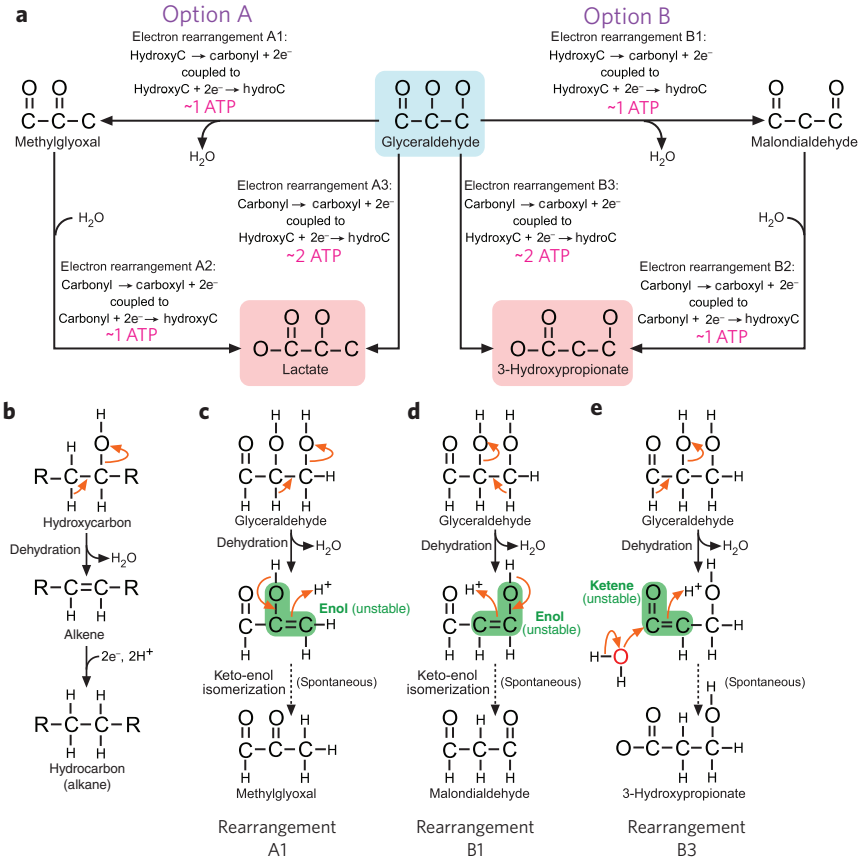

Figure 3 | Sequential assembly of the lower glycolytic reaction sequence according to biochemical constraints: the basic energetic and mechanistic constraints. (**a**) All possible electron rearrangements within glyceraldehyde that release energy, assuming that each electron rearrangement is carried to completion before the next one begins. The number of ATP molecules that can potentially be produced during each electron rearrangement is given in pink. HydroxyC, hydroxycarbon; hydroC, hydrocarbon. (**b**) The reduction of a hydroxycarbon to a hydrocarbon involves two steps: dehydration of the hydroxycarbon to a double bond and reduction of the double bond to a hydrocarbon. Curved orange arrows correspond to the movement of a pair of electrons. (**c**–**e**) All of the possible dehydration reactions glyceraldehyde can undergo. Dashed lines represent spontaneous reactions. (**c**,**d**) Dehydrations forming an enol—a double bond for which one carbon carries a hydroxyl group. The enol undergoes spontaneous isomerization to a carbonyl, a process known as keto-enol isomerization. Depending on the dehydration, the isomerization can result in methylglyoxal (**c**) or malondialdehyde (**d**). (**e**) A dehydration forming a ketene, a double bond in which one carbon is a carbonyl. Ketenes spontaneously react with water to form a carboxylic acid.

Box 1 | Natural variants of glycolysis

The existence of several natural alternatives to the Embden-Meyerhof-Parnas pathway suggests that there is a complex interplay between the constraints and metabolic goals that we present; that is, different organisms adapted to different environments may treat some goals and constraints as more important than others. Nonetheless, the natural glycolytic alternatives that are known to support growth satisfy most of the biochemical constraints discussed.

In our discussion, we have assumed a tradeoff between ATP yield and the chemical motive force[25] and suggested that producing two ATPs during glycolysis leaves sufficient chemical motive force for pathway reactions. However, some organisms occupy energy-poor environments or face little competition for resources and therefore use alternative pathways with higher ATP yield (at the expense of a lower metabolic rate). One such alternative recycles electrons through the production of molecular hydrogen rather than by reducing pyruvate[12,84,85]. This pathway enables the production of two extra ATP molecules per glucose via pyruvate oxidation to acetyl-CoA and substrate-level phosphorylation, which converts the thioester bond into the phosphoanhydride bond of ATP. However, this pathway is thermodynamically challenging and depends on hydrogen-consuming organisms that keep the concentration of hydrogen low[12,84,85]. A similar alternative—known as mixed-acid fermentation—enables the production of three ATPs per glucose through the cleavage of pyruvate to acetyl-CoA and formate and subsequent excretion of several compounds[86,87].

Other organisms rely on pathways with lower ATP yield. For example, the Entner-Doudoroff pathway is a common alternative to the Embden-Meyerhof-Parnas pathway that satisfies all of the constraints discussed. Though it produces only one ATP molecule per glucose, this pathway may sustain higher metabolic flux (owing to higher chemical motive force of intermediate reactions) and supports the assimilation of a wider range of carbon sources[88,89]. Remarkably, organisms that do not depend on the degradation of organic compounds for energy, such as phototrophs, may forfeit glycolytic ATP production altogether. These organisms substantially increase the chemical motive force of the pathway reactions by bypassing the substrate-level phosphorylation step and using glycolysis solely for the production of carbon skeletons[90,91].

Toxicity can differ between organisms, and so physiology may affect the choice of fermentation pathway. For example, many organisms produce ethanol instead of lactate. As both lactate and ethanol are toxic, the choice of one over the other mostly depends on the cell's tolerance for each compound. In other cases, toxicity is secondary to other concerns. When the concentration of inorganic phosphate is low, some organisms shift to a metabolic bypass that uses the toxic intermediate methylglyoxal and omits substrate-level phosphorylation[51,92,93]. These organisms cope with the toxicity of methylglyoxal rather than stop fermentation altogether because of phosphate deprivation.

Finally, the stability of intermediate metabolites is an important concern for thermophilic and hyperthermophilic organisms. At elevated temperatures, many phosphorylated metabolites become unstable and undergo spontaneous dephosphorylation. Prokaryotes operating in such conditions often do not phosphorylate glycolytic metabolites[94,95].

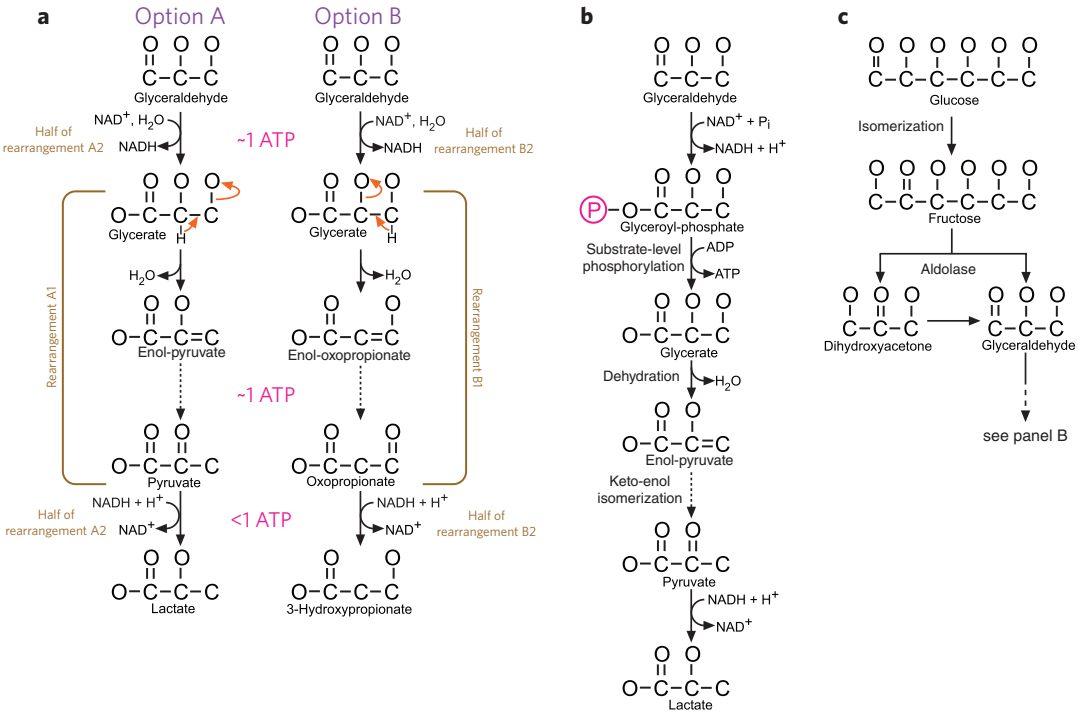

Figure 4 | Sequential assembly of the glycolytic reaction sequence according to biochemical constraints: redox carriers, feasible mechanisms and energy conservation. (**a**) Two energy-releasing electron rearrangements of glyceraldehyde that use NAD^+ to temporarily store electrons, thereby decoupling the electron-releasing and electron-consuming half-reactions of an electron rearrangement. Option A shows the pathway that occurs naturally. Option B does not occur naturally as it requires an oxygen-sensitive mechanism to catalyze the unactivated dehydration of glycerate. (**b**) Only one of the energy-releasing electron rearrangements in glyceraldehyde fermentation is coupled to ATP production via substrate-level phosphorylation. (**c**) Glucose fermentation via cleavage into two glyceraldehyde molecules: glucose is isomerized to fructose, enabling an aldolase cleavage reaction that produces glyceraldehyde and dihydroxyacetone. Dihydroxyacetone is then isomerized back to glyceraldehyde.

The dehydration shown in **Figure 3e** presents a similar difficulty. The formation of ketenes is extremely rare in biochemistry as they are highly reactive and can easily lead to the modification of macromolecules, resulting, for example, in irreversible enzyme inactivation[57,58]. Indeed, there are no known ketene-containing metabolites, and there are only a few enzymes whose mechanism has been proposed to involve a ketene intermediate[59,60].

Splitting electron rearrangements

If electron rearrangement A3 cannot take place from a mechanistic point of view and rearrangements A1, B1 and B3 give rise to toxic intermediates, how can fermentation proceed? The solution is to reorder the steps of an electron rearrangement to avoid reactive intermediates. If we break electron rearrangement 2 (A2 or B2) into two steps—reduction and oxidation—and perform the oxidative step before beginning rearrangement 1 (A1 or B1), no reactive two-carbonyl molecules are formed (**Fig. 4a**, options A and B). The oxidation and reduction halves of rearrangement 2 can be decoupled by using the electron carrier NAD to temporarily store the electrons, as shown in the first and last steps of **Figure 4a**.

There are two parallel pathway designs that could realize this general reaction sequence (marked as options A and B in **Fig. 4a**). Option B, however, is mechanistically challenging. In most dehydration reactions, the negative charge that develops on the deprotonated carbon (**Fig. 3b**) is stabilized by an electron-withdrawing group such as a carbonyl, carboxyl or thioester directly adjacent to the deprotonated carbon[61]. Whereas the dehydration in option A is activated in this way, in option B the carbonyl is too far removed. Such dehydrations are complex and require an oxygen-sensitive radical mechanism[61,62]. As glycolysis operates also in aerobic conditions in many organisms, an oxygen-sensitive dehydration mechanism is not a preferable alternative. Option A, which enables energy extraction while using available reaction mechanisms and nontoxic intermediates, is therefore the solution found in nature.

Sustaining high ATP production rate

Several factors govern the rate of a pathway. Among them are the kinetic parameters of pathway enzymes, the concentration of the intermediates and the energetic profile of the pathway reactions. As enzyme kinetic parameters and metabolite concentrations are discussed in detail elsewhere[41,42,63], here we focus on the effect of the pathway's energetic profile on the flux it can sustain. The chemical motive force associated with a reaction ($\Delta_r G'$) determines what fraction of the enzymatic machinery catalyzes the forward reaction[64,65]. High chemical motive force means that a large fraction of the enzymatic machinery catalyzes the forward reaction, resulting in a high metabolic conversion rate (per enzyme, all other factors being equal)[22,27]. For instance, a $\Delta_r G'$ of 6 kJ mol^{-1} corresponds to ~90% of the enzyme units catalyzing the forward direction (and ~10% catalyzing the reverse). Hence, assuming substrate saturation and similar turnover number (k_{cat}) values for the forward and backward reactions, the reaction proceeds at ~80% of its maximal rate.

Returning to glycolysis, two ATP molecules can theoretically be produced through the electron rearrangements of glyceraldehyde (**Fig. 4a**). Yet, producing two ATP molecules would require nearly all of the energy released (more than 100 kJ mol^{-1} out of ~120 kJ mol^{-1} (ref. 66)). The remaining motive force (10–20 kJ mol^{-1}) must be divided among several pathway reactions, leaving a very small motive force per enzyme. Therefore, to support high flux, only one net ATP molecule is produced during glyceraldehyde fermentation.

Box 2 | Biochemical principles applied to metabolic engineering: the example of 3-hydroxypropionate production

3-Hydroxypropionate is an attractive metabolic engineering target as it can be used to synthesize useful polymers (for example, poly(3-hydroxypropionic acid))[96]. **Figure 5** presents several proposed pathways for the production of this compound from pyruvate[96]. As these pathways have not yet been implemented, it might be helpful to apply the principles discussed here to highlight the challenges associated with each of these designs. Energetic considerations (**Fig. 2**) suggest that the oxidation of propionyl-CoA to acryloyl-CoA (**Fig. 5**, pathway A) using NAD as an electron acceptor is infeasible: the reduction potential of NAD^+ is ~−300 mV (**Fig. 2**), whereas that of acryloyl-CoA is well above 0 mV[97]. The dehydration of lactoyl-CoA to acryloyl-CoA in pathway B is not activated by an electron-withdrawing group and hence necessitates an oxygen-sensitive radical mechanism[61]. The conversion of alanine to β-alanine by an aminomutase is also performed via an oxygen-sensitive radical mechanism[98], limiting the operation of pathway D. Finally, the intermediates malonate-semialdehyde (pathways C, D and E) and acryloyl-CoA (pathways A and B) are moderately toxic as they can react with cellular macromolecules.

In choosing a particular pathway to implement, one must therefore choose which of these challenges to tackle given the application and expertise. For example, the energetically infeasible oxidation of propionyl-CoA to acryloyl-CoA using NADH (pathway A) can be bypassed by using an electron carrier with a high reduction potential instead, although this will require enzyme evolution. Alternatively, the oxygen sensitivity of pathways B and D can be sidestepped by cultivating the engineered cells anaerobically. Finally, the toxicity of malonate-semialdehyde or acryloyl-CoA can be overcome if their concentrations are kept low by overexpressing the enzymes that consume them.

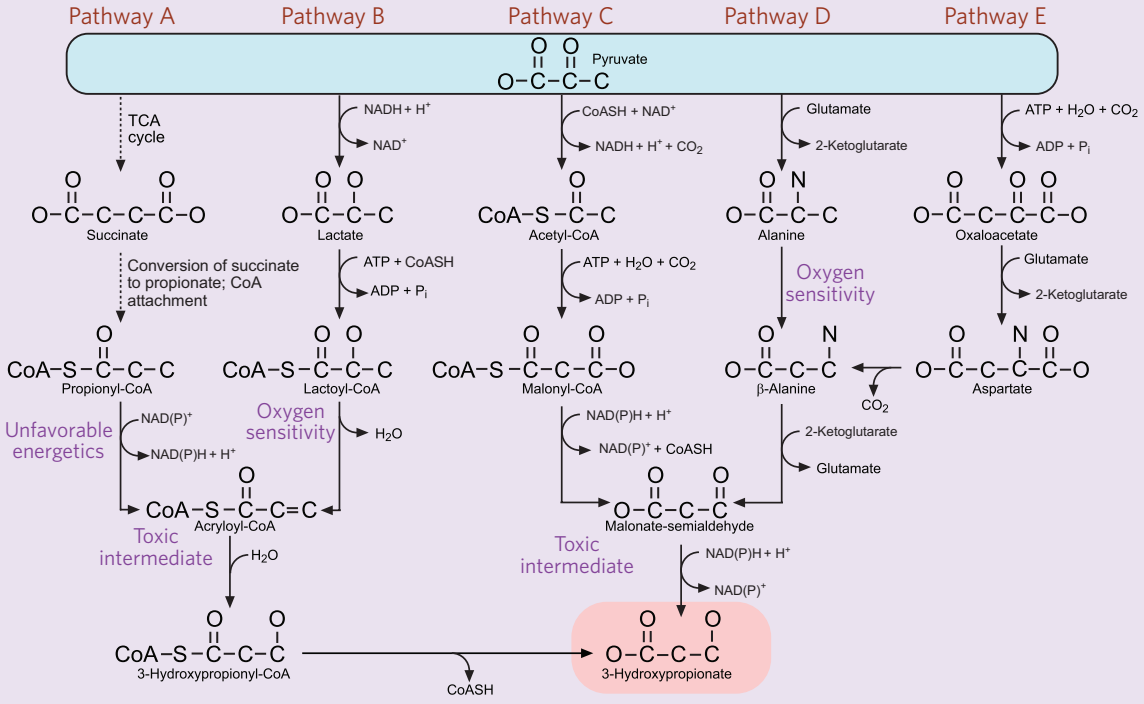

Figure 5 | Proposed pathways for the production of 3-hydroxypropionate from pyruvate. Energetic considerations suggest that the oxidation of propionyl-CoA to acryloyl-CoA (pathway A) is unfavorable. Known mechanisms that support the dehydration of lactoyl-CoA to acryloyl-CoA (pathway B) and the amine transfer within alanine to form β-alanine (pathway D) are oxygen sensitive. The intermediates malonate-semialdehyde (pathways C, D and E) and acryloyl-CoA (pathways A and B) are moderately toxic. Hydrogens omitted for clarity.

A substantial part of the remaining energy is dissipated, providing the chemical motive force required to maintain high flux.

Here again, an energetic consideration is not enough; a mechanism for the conservation of energy in the form of ATP is required. Apart from oxidative phosphorylation, which couples electron transport with the formation of a proton (or sodium) gradient across the membrane and ATP synthesis via ATPase, there is only one major mechanism enabling the conservation of energy as ATP: substrate-level phosphorylation. In this process, the energy released by the transfer of electrons from a carbonyl to NAD^+ is used to create a phosphoanhydride bond between the newly formed carboxyl and a phosphate ion (in some cases a thioester is formed, which is then replaced by a phosphoanhydride). Phosphoanhydride bonds have a high energy of hydrolysis—their hydrolysis releases a substantial amount of Gibbs energy ($\Delta_r G' << 0$)—as in ATP. Thus, these two phosphoanhydride bonds are interchangeable: the phosphate group is readily transferred from the carboxyl to ADP, forming ATP. The only reaction in **Figure 4a** in which an aldehyde is oxidized to a carboxyl is the oxidation of glyceraldehyde to glycerate. Indeed, this is how substrate-level phosphorylation takes place in glycolysis (**Fig. 4b**).

Fermenting glucose via glyceraldehyde

Glucose can be regarded as two glyceraldehyde molecules joined together. In fact, the cleavage of glucose is yet another electron rearrangement, converting a hydroxycarbon into a carbonyl and resulting in the formation of two glyceraldehydes. Consequently, one way to produce ATP while rearranging the electrons in glucose is

to break glucose into two glyceraldehydes and follow the process we described above. Alternatively, one might imagine many possible electron rearrangements within glucose itself. However, though several natural alternatives to glycolysis exist (**Box 1**), most of them proceed by cleaving glucose to glyceraldehyde. One important reason to ferment glucose through glyceraldehyde is that glyceraldehyde and downstream three-carbon metabolites are required for the production of many essential cellular components[28]. Another benefit of this approach is that it provides a simple metabolic connection between sugar catabolism and the TCA cycle (pyruvate is readily converted to the TCA cycle intermediates oxaloacetate and acetyl-CoA). Further, cleavage of glucose into two identical pieces may represent an economical strategy in terms of the number of enzymes required for fermentation[28,67,68].

However, the direct cleavage of glucose into two glyceraldehydes is mechanistically infeasible. Aldol cleavage reactions are activated by a carbonyl group on the carbon adjacent to the cleavage site[30,69,70]. Therefore, glucose is isomerized to fructose, enabling aldol cleavage to glyceraldehyde and dihydroxyacetone. The carbonyl on dihydroxyacetone is then isomerized 'back' to a terminal position, yielding a second glyceraldehyde (**Fig. 4c**).

Keeping intermediates phosphorylated

The picture of glycolysis we have painted so far (**Fig. 4b**,**c**) is quite close to that of the natural reaction sequence (**Fig. 1**). However, we have not yet addressed two properties of metabolites that can restrict the pathway's structure: permeability and affinity. Charged compounds, such as glycerate, pyruvate and lactate, cannot easily pass through the hydrophobic lipid membrane[71–73]. However, uncharged molecules, such as glucose, fructose, dihydroxyacetone and glyceraldehyde, can diffuse more freely through the membrane[73–75]. One way to substantially reduce their escape rate from the cell is to attach them to a charged moiety. A phosphate group serves this purpose well[76,77]. Hence, when glucose is transported into the cell, it is phosphorylated on its sixth carbon.

Glyceraldehyde and dihydroxyacetone are much smaller than glucose and have considerably higher membrane permeability. In fact, the permeability of glyceraldehyde and dihydroxyacetone is so high (similar to that of glycerol, estimated at ~10^{-7}–10^{-6} cm s^{-1} (refs. 78,79)) that a large fraction of the intracellular pool can leak out of a (prokaryotic) cell within seconds. Fructose 6-phosphate is thus phosphorylated once more (reaction 3 in **Fig. 1**), and, as fructose 1,6-bisphosphate has phosphate groups on either side of the molecule, both aldol cleavage products are guaranteed to contain a phosphate group (**Fig. 1**).

The attachment of a phosphate group not only is a solution to the permeability problem but also can increase enzyme affinity. Generally speaking, small substrates such as glyceraldehyde limit the affinity of the enzymes using them[42]. However, it was found that enzymes using substrates substituted with phosphate, CoA or other large modifiers have considerably higher affinity to their substrates than enzymes using the unsubstituted substrates[42]. Substitution with phosphate, for example, increases the affinity toward a substrate by a median factor of 4 (ref. 42). Hence, pathways using phosphorylated intermediates will most likely have increased affinity toward those intermediates and, therefore, sustain higher catalytic rates.

Recouping invested ATP

Glyceraldehyde 3-phosphate is chemically similar to glyceraldehyde and can be fermented in a similar manner. Indeed, the phosphate group located at the third carbon does not interfere with substrate-level phosphorylation on the first carbon (reaction 6 in **Fig. 1**). However, the phosphorylated hydroxyl on the third carbon of glyceraldehyde 3-phosphate presents one last energetic challenge.

Although in substrate-level phosphorylation the phosphate is bound to a carboxylic acid, the phosphate in glyceraldehyde 3-phosphate is bound to a hydroxyl group. Unlike a phosphoanhydride bond, a phosphoester between a phosphoric acid and a hydroxyl is a bond with a low energy of hydrolysis. Therefore, when a phosphate group is transferred from ATP to a hydroxyl group of glucose (reactions 1 and 3 in **Fig. 1**), energy is dissipated (20–30 kJ mol^{-1} under physiological conditions[66]), making the process irreversible. Nonetheless, the ATP invested in this irreversible phosphorylation must be recovered. Otherwise, the ATP formed by substrate-level phosphorylation will only recoup the initial ATP investment, and glycolysis will result in no net ATP gain.

To recover an ATP from the phosphoester bond, some of the energy released by the electron rearrangement of glycerate 3-phosphate to pyruvate (rearrangement A1 in **Fig. 4a**) is used. Rather than being dissipated to maintain high chemical motive force (described above), some of this energy is used to activate the phosphoester bond. First, the phosphate group on the terminal hydroxycarbon is transferred to the central hydroxycarbon by a mutase enzyme (reaction 8 in **Fig. 1**). Now, when the terminal hydroxycarbon is dehydrated, a phosphoenol group is formed (reaction 9 in **Fig. 1**). As mentioned above, enol groups have a strong preference to isomerize to a keto group. However, the phosphate group prevents this isomerization. The enol group's preference for isomerization 'pushes' the phosphate group out; that is, it energizes the phosphate. The activated phosphate is now transferred to an ADP (reaction 10 in **Fig. 1**), forming ATP and freeing the enol to isomerize to a carbonyl[80].

To summarize the logic leading to the glycolytic pathway function (**Fig. 1**): in upper glycolysis, glucose is phosphorylated and isomerized to enable its cleavage into two trioses. In lower glycolysis, glyceraldehyde 3-phosphate undergoes two major energy-releasing electron rearrangements. The first is coupled to net ATP production via substrate-level phosphorylation. The second recovers the ATP invested in the phosphorylation of glucose and provides chemical motive force for the pathway.

Implications and concluding remarks

In this review, we asked whether the logic of the structure of glycolysis could be explained using basic biochemical principles. Although glycolysis may at first seem complex, we demonstrated that it represents one of the simplest approaches that satisfies all biochemical constraints. However, the existence of several natural alternatives to the Embden-Meyerhof-Parnas pathway suggests that not all organisms are equally limited by these constraints. We discuss the salient features of these natural variants in **Box 1**.

In addition to helping explain the structure of natural pathways, we suggest that the biochemical constraints presented here will be useful in designing new biosynthetic pathways. An example of the application of these principles to a metabolic engineering project is given in **Box 2** and **Figure 5**.

In designing pathways, one must be aware of the tradeoffs between satisfying different constraints. For example, many enzymatic mechanisms require an activating carbonyl group[56]. However, as discussed, adding a carbonyl to a compound increases its reactivity and, in turn, its toxicity and instability[56]. Metabolite hydrophobicity offers another example. Enzymes have higher affinity toward substrates containing hydrophobic regions[42]. However, hydrophobic regions increase the substrate's membrane permeability and hence limit its cellular concentration[41].

Another challenge in implementing the guidelines presented is data availability. For some of the properties discussed here—thermodynamics and kinetics, for example—dedicated databases are currently available[66,81–83]. However, for other properties, data are scarce. Of special importance is the use of high-throughput techniques to measure the reactivity, stability and permeability of many metabolites simultaneously, thus providing reliable databases to support pathway design.

It is difficult to test experimentally the forces that shape the structure of metabolic pathways; we can only draw conclusions from pathways that exist in nature. Nevertheless, with the advent of synthetic biology, we are now in a position to analyze the principles governing metabolism by implementing and comparing synthetic pathways. We believe this approach will help us make sense of the biochemical logic of metabolism. Such systematic analyses of natural and engineered metabolism will help explain why some organisms use alternate pathways and will surely enable us to engineer metabolism with greater confidence.

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