Nonenzymatic gluconeogenesis-like formation of fructose 1,6-bisphosphate in ice

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The evolutionary origins of metabolism, in particular the emergence of the sugar phosphates that constitute glycolysis, the pentose phosphate pathway, and the RNA and DNA backbone, are largely unknown. In cells, a major source of glucose and the large sugar phosphates is gluconeogenesis. This ancient anabolic pathway (re-)builds carbon bonds as cleaved in glycolysis in an aldol condensation of the unstable catabolites glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, forming the much more stable fructose 1,6-bisphosphate. We here report the discovery of a nonenzymatic counterpart to this reaction. The in-ice nonenzymatic aldol addition leads to the continuous accumulation of fructose 1,6-bisphosphate in a permanently frozen solution as followed over months. Moreover, the in-ice reaction is accelerated by simple amino acids, in particular glycine and lysine. Revealing that gluconeogenesis may be of nonenzymatic origin, our results shed light on how glucose anabolism could have emerged in early life forms. Furthermore, the amino acid acceleration of a key cellular anabolic reaction may indicate a link between prebiotic chemistry and the nature of the first metabolic enzymes.

origin of metabolism | gluconeogenesis | metabolism | evolution | nonenzymatic reactions

The metabolic network is a large cellular system that generates life’s building blocks including amino acids, nucleotides, and lipids. Most of the metabolic pathways that participate in this network are well understood, but their evolutionary origin remains an unsolved problem. The network contains many reactive and unstable intermediates, yet its topological organization is widely conserved and considered ancient. It has therefore been extensively debated to what extent this structure could be the result of Darwinian selection and thus be of postgenetic origin or whether the metabolic network topology dates back to a nonenzymatic chemistry (1–4). A series of recently discovered chemical networks provides evidence for the latter. In the presence of simple inorganic ions as found in Archean sediment, and under conditions that are reminiscent of the chemistry that operates in cells, nonenzymatic reactions replicate essential topological elements of the most central metabolic pathways. Nonenzymatic chemical reactions currently replicate glycolysis (including the Embden–Meyerhof–Parnas pathway, the Entner–Doudoroff pathway, and their many variants), the pentose phosphate pathway, the catabolic reactions of the TCA cycle, and the formation of the methyl group donor S-adenosylmethionine (5–8). Moreover, reactions of glycolysis and the pentose phosphate pathway, as well as reactions replicating the oxidative TCA cycle, can each occur in distinct but unifying reaction milieus, respectively. This implies that simple inorganic catalysts were shaping the topological structure of the metabolic network. In other words, the reactions that sugar phosphates undergo in the presence of the highest concentrated transition metal in Archean sediment [Fe(II)] and the reactions that TCA intermediates undergo in the presence of sulfate radicals are reflected in the topological organization of the metabolic network (6, 8).

To support life, cellular metabolism depends on the parallel occurrence of anabolism and catabolism. Otherwise, metabolism would cease once the thermodynamic equilibrium, to which chemical networks evolve, is reached (typically the point where all available substrates are consumed). In enzyme-catalyzed metabolism, the simultaneous occurrence of catabolic and anabolic reactions is achieved by coupling the primary metabolic reaction to secondary reactions, coenzyme functions, or active membrane transport processes. As a consequence, metabolism—on the global scale—is not an energy-producing process, but instead an endergonic process that requires constant energetic input, provided mainly through photosynthesis. A key unresolved problem, however, is how the early precursors of metabolism could have escaped equilibrium before modern enzymes were in place (2, 9, 10). Although this problem is debated in the context of the origin of metabolism and is a key factor in defining the hypothetical environments for the origin of life, the parallel occurrence of anabolism and catabolism in the same cellular system constitutes so far an unsolved problem.

Gluconeogenesis produces hexose-phosphates, which form the substrates for glycolysis and the pentose phosphate pathway (and hence, the sugar phosphate that constitutes the RNA and DNA backbone) out of prebiotically plausible (11) but unstable (5) tri- to pentacarbon phosphates. Central carbon metabolism thus could escape equilibrium in a situation that allows a parallel occurrence of gluconeogenesis and glycolysis (9, 12). Such scenario, however, is missing so far. Gluconeogenesis rebuilds the C-C bonds cleaved in glycolysis in one particular reaction, the aldol addition of glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) forming the much more stable fructose 1,6-bisphosphate (F16BP).

Significance

It is still unknown how an early metabolism produced the sugar phosphates central for life. We provide evidence that gluconeogenesis, the anabolic counterpart to glycolysis, could have emerged nonenzymatically. We describe that the gluconeogenic carbon-bond–forming reaction has a nonenzymatic pendant that occurs in ice and that leads to the accumulation of fructose 1,6-bisphosphate as (re-)built from glycolytic catabolites. As a nonenzymatic glycolysis has been described previously, the discovery of this reaction could both help to explain the origin of the larger cellular sugar phosphates and provide a scenario in which an early metabolic system was able to escape equilibrium. The reaction further hints that the earliest anabolic enzymes could have been as simple as single amino acids.


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Enzymatically, this reaction is reversibly catalyzed by F16BP-aldolase, a highly conserved glycolytic enzyme. The coupling of the aldolase to the subsequent phosphatase reaction in the ancient Archaean enzyme parologue renders gluconeogenesis unidirectional, leading to the formation of the large sugar phosphates in parallel to the occurrence of glycolysis in the same organism. This enzyme therefore has been named the “pacemaker” for gluconeogenesis. It has further been suggested

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Fig. 1. Nonenzymatic formation of fructose 1,6-bisphosphate in ice. (A) The aldol reaction of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate to D-fructose 1,6-bisphosphate catalyzed by fructose 1,6-bisphosphate aldolase forms the C-C bonds in gluconeogenesis. (B) No formation of fructose 1,6-bisphosphate as measured by LC-MS/MS (shown is the SRM transition m/z 339 → m/z 97) after incubation of DL-glyceraldehyde and dihydroxyacetone phosphate in NaHCO₃ for different lengths of time at room temperature. At RT, the substrates G3P and DHAP degrade within days (Fig. S1). (C) Formation of fructose 1,6-bisphosphate as measured by LC-MS/MS (shown is the SRM transition m/z 339 → m/z 97) after incubation of DL-glyceraldehyde and dihydroxyacetone phosphate in NaHCO₃ for different lengths of time at −20 °C. (D) The in-ice aldol addition of DL-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in NaHCO₃ is accelerated by lysine and glycine. Samples were kept at −20 °C for different lengths of time over a period of 58 d, thawed at the same time, and measured by LC-MS/MS. Error bars indicate ±SD. (E) Samples containing DL-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, and glycine in NaHCO₃ were incubated at −80 °C, −20 °C, and room temperature and measured with 2D NMR (TOCSY). Furthermore, measurements of the −20 °C sample spiked with 20 μM F16BP (second panel from right) and of the F16BP standard chemical were recorded. The comprehensive 2D NMR spectra are given in Fig. S3. (F) Comparison of product formation (SRM transition: Q₁—m/z 339 → Q₃—m/z 97) in ice (−20 °C) of samples containing different quantities of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate and their combination. Samples were incubated for 14 d in 100 mM NaHCO₃ with 1 mM glycine. Error bars indicate ±SD. (G) Product formation (SRM transition m/z 339 → m/z 97) in samples containing DL-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, and glycine in NaHCO₃ that were dried and dissolved five times.
that the event of this enzyme could have enabled the evolutionary origin of gluconeogenesis (12). Indeed, although a nonenzymatic aldol reaction of aldehydes and ketones has been achieved in various contexts, or has provided nonmetabolic compounds as in the formose reaction (13), it has failed so far for the phosphorylated compounds that play a role in central carbon metabolism (14–20).

Here, we report the discovery of a nonenzymatic counterpart to the gluconeogenic aldol condensation. The reaction occurs in ice and forms fructose 1,6-bisphosphate by forming carbon bonds in a condensation of the glycolytic metabolites G3P and DHAP. We show that the product continues to accumulate over months in a permanently frozen solution and that the reaction is accelerated by single amino acids. Our data hence propose a nonenzymatic origin of gluconeogenesis.

Results

At elevated temperature, the F16BP-aldolase substrates G3P and DHAP are unstable and convert within minutes to pyruvate (4). Also at room temperature and under alkaline conditions, they decompose within days (Fig. S1A). Although this interconversion resembles glycolysis, no gluconeogenesis-like reaction is observed under the same conditions. Neither at 70 °C (5) nor at room temperature (Fig. 1B) do the metabolites undergo a nonenzymatic condensation because no products resembling fructose 1,6-bisphosphate were detected by targeted analytics using a highly sensitive, selective reaction monitoring (SRM) assay (Materials and Methods). The potential formation of F16BP was monitored for 14 d, which is much longer than the stability of G3P and DHAP would have permitted their condensation to occur (Fig. S1A). We discovered, however, that a nonenzymatic aldol condensation is enabled upon freezing the reaction mixture to −20 °C. In ice, G3P and DHAP were consumed at a much slower rate than at room temperature (Fig. S1B). Here, the consumption of the metabolites was associated with the appearance of an SRM signal corresponding to a hexose bisphosphate (Fig. 1C). The in-ice–formed metabolite represented F16BP according to chromatographic retention time, mass-to-charge ratio, SRM transitions, and matching fragmentation spectra (Fig. 1C and Fig. S1D). The reaction product was detected after 24 h. Then, we incubated and analyzed samples over a period of 81 d. The hexose bisphosphate continuously accumulated (Fig. 1D). The reaction did occur in the presence of various buffer substances, although it was fastest at pH values above 9 (Fig. S1B).

A nonenzymatic glycolysis is accelerated by transition metals (5), and Zn(II) ions are involved in the catalytic mechanism of class I aldolases (21, 22). Therefore, we screened for the effects of different metal ions. We found that several of them had a small impact on the reaction rate (Fig. S1F). The evolutionarily older class II aldolases instead use amino acid catalysis involving a catalytic lysine. Here, significant effects were detected for several amino acids (Fig. S1E). In two cases (lysine and glycine), a substantial acceleration, in particular in the early time points, was observed (Fig. 1D). As the reaction mechanism in both class I F16BP aldolase and the archaean F16BP aldolase (using amino acid catalysis) are based on a Schiff base as intermediate, it can be speculated that the in-ice acceleration by amino acids follows a similar reaction mechanism (Fig. S2C).

Next, we used 2D NMR as an orthogonal technique to LC-MS/MS to confirm that the metabolite formed in-ice in the presence of amino acids is indeed F16BP. The spectra of the in-ice (−20 °C) reaction mixture displayed features consistent with the presence of F16BP (Fig. 1E and Fig. S3 for the entire 2D-NMR spectra), as confirmed by the addition (spiking) of a low concentration (20 μM) of pure F16BP chemical standard (Fig. 1E and Fig. S3, Bottom panels). The spectra obtained from samples kept at −80 °C and room temperature (RT) are lacking these features. In particular, the sample kept at RT shows different patterns indicative of decomposition of the reactants rather than condensation reactions (Fig. S3). The 2D-NMR and LC-MS/MS therefore agree with the nonenzymatic formation of F16BP in ice.

It is unlikely that a nonenzymatic condensation reaction is fully specific. Likewise, a majority of metabolic enzymes are promiscuous, an essential feature for the evolutionary expansion of metabolism (23). We hence considered the formation of isomers to be a possibility. Indeed, the MS/MS spectrum revealed slight differences in the intensity of fragment ion transitions, an indicator of the presence of isomers to fructose 1,6-bisphosphate that coeluted chromatographically (Fig. S1D). One possible source of these isomers is a self-condensation of DHAP or G3P, respectively. Indeed, we observed both ketone–ketone and aldehyde–aldehyde self-condensations to occur at low rates (Fig. 1F). In particular, the G3P self-condensation was much slower than the aldehyde–ketone aldol addition. Therefore, the ice matrix enables condensation reactions with the ketone–aldehyde condensation as occurring in gluconeogenesis being preferred over self-condensation of the three-carbon phosphates (Fig. 1F and Fig. S2A for different concentrations).

Next, we speculated about the mechanism that enables the in-ice aldol condensation. We first tested the effect of freeze–thaw cycles. Freezing–thawing did not have a significant impact on the reaction rate (Fig. S2E), nor did a slow- or a fast-freezing procedure make a difference in product formation (Fig. S2D). Furthermore, it has previously been shown that ice increases concentration of the solute in the liquid phase of the eutectic mixture, which can accelerate bimolecular reactions (24, 25). Considering that a concentration effect could enable the aldol condensation, we exposed G3P and DHAP to repeated desiccation/rehydration cycles at room temperature. This procedure indeed formed fructose 1,6-bisphosphate as well (Fig. 1G). This result shows that a nonenzymatic gluconeogenesis-like aldol condensation also occurs at higher temperature under the appropriate conditions.

Discussion

The evolutionary origins of glucose and glucose metabolism are unknown. However, glycolysis and the pentose phosphate pathway, which exist in several variants, are evolutionarily ancient and their intermediates are of key importance for some of the most crucial processes that define life. Glycolytic metabolites are implicated in cellular energy metabolism as a main source of ATP, pentose phosphates constitute the RNA and DNA backbone, and both provide precursors for amino acid metabolism, lipid, and nucleotide biosynthesis. Because ATP generation, amino acid biosynthesis, and nucleotide biosynthesis all are essential for protein biosynthesis, it is intuitive that sugar phosphates were already implicated in the earliest forms of metabolism, at least those that predated protein biosynthesis (and hence the presence of modern enzymes). Although we have previously presented evidence for nonenzymatic glycolytic and PPPLike reactions (5, 6), so far there has been no evidence for a situation that could prevent such a chemical network from reaching equilibrium. In other words, no scenario for a nonenzymatic gluconeogenic pathway that could occur next to a nonenzymatic glycolysis has been suggested so far.

Here, we show that G3P and DHAP metabolites condense to form fructose 1,6-bisphosphate by rebuilding the C-C bond cleaved in glycolysis nonenzymatically. It is highly interesting that this reaction was observed in ice. First, the three-carbon phosphates that are essential for central metabolism are unstable at higher temperatures (5). Our results hence show that these phosphates are stabilized in ice not only by a lower temperature, but also as they convert nonenzymatically into the much more stable fructose 1,6-bisphosphate. Second, nonenzymatic catalytic reactions resembling glycolysis are accelerated at higher temperature (5). Freezing and thawing might thus have enabled the co-occurrence of anabolic and catabolic reactions, favoring the
ancestral spontaneous self-organization of metabolic cycles, as widely debated (26). Such freeze-and-thaw cycles are highly frequent in different environments (day-night, season cycles) and could have occurred in many places on the early Earth. Attractive environments for such scenarios are in particular hot springs and geysers, in which freezing and thawing occurs in close proximity and a huge variety of temperature gradients exist. In any case, ice matrices have been abundantly available throughout history, with several theories implying that the Earth’s surface might have been largely frozen several times (27, 28). Interestingly, the plausibility of ice as a matrix has also been discussed with respect to other aspects about the origin of metabolism. Ice may have helped to establish compartmentalization, essential for any form of metabolism, and it enables the synthesis of nucleobases (29–32) and supports a non-enzymatic polymerization and a template-directed mechanism for copying RNA (33–35). Freeze–thaw cycles thus could have helped to achieve both the copying of nucleic acids and nonenzymatic reactions that form the metabolic components of RNA and DNA. Whereas the evolution of the metabolic network becomes essential only with the existence of early life, our results further imply that at least some of the nonenzymatic reactions that shaped the structure of the metabolic network might have been important at the stage of its origins. A nonenzymatic gluconeogenesis could have helped in building up glycolytic carbohydrates that had already obtained their universal importance in the earliest organisms, enabled by freezing conditions over long timescales. Life certainly depends on higher temperatures. Our data show that it is also possible that the nonenzymatic formation of fructose 1,6-bisphosphate was enabled by mimicking the concentration effects of ice by repeated desiccation/rehydration cycles. Under favorable conditions, a nonenzymatic gluconeogenesis is hence plausible for mesophilic environments as well. Furthermore, these results are also compatible with an autotrophic origin of life. Cycles of anabolism and catabolism could have occurred without the preaccumulation of metabolites.

The results of this study further contribute to evidence (5, 6, 8) that mundane, moderate reaction conditions rather than rare, niche, or extreme environments were key to shaping the topology of the metabolic network. Indeed, the origin of complex multistep metabolic pathways is difficult to explain solely by Darwinian selection in the absence of a chemical template. This implies that metabolic pathways date back topologically to chemical networks (36, 37). A key aspect of the nonenzymatic formation of fructose 1,6-bisphosphate is the acceleration of the reaction by simple amino acids, in particular glycine, the formation of which has been reductively aminated in the earliest autopoietic prebiotic processes, as in the Miller–Urey experiment (38). Acting as a minimal enzyme, a gluconeogenesis accelerated by single amino acids could form a link between prebiotic chemistry and the origin of the first amino-acid–based enzymes.

In summary, we report the discovery of a nonenzymatic, in-ice aldol condensation that enables the formation of C-C bonds among catalytic three carbon phosphates and forms fructose 1,6-bisphosphate as in gluconeogenesis. Our results reveal a plausible scenario for the origin of glucose anabolism that can operate—accelerated by simple amino acids—in a similar environment to the previously described nonenzymatic glycolysis (5, 6). The parallel occurrence of glucose catabolism and anabolism as driven by a simple, nonenzymatic chemistry might be able to explain how central metabolism originated in the defined compartment of the cell and how it escaped equilibrium, thereby helping to understand why glucose metabolism became universally important for life.
NaHCO₃ with 1 mM glycine) were frozen and thawed eight times (incubated at −20 °C for 8 d with 1 h thawing at room temperature each day).

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Supporting Information
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Fig. S1. (A) Stability of three-carbon phosphates DL-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate at room temperature and the formation of fructose 1,6-bisphosphate (not observed at RT) in NaHCO$_3$ solution. Error bars indicate ±SD. (B) Stability of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in −20 °C ice with and without amino acids in NaHCO$_3$ solution. Error bars indicate ±SD. Both glycine and lysine accelerate the formation of F16BP (Fig. 1B) as well as the consumption of the three-carbon phosphates. (C) SRM transitions used to quantify (m/z 339 → m/z 97) and qualify F16BP (m/z 339 → m/z 79, m/z 339 → m/z 241) shown for a F16BP chemical standard. (D) Fragmentation spectra of the formed reaction product resulting from an aldol reaction of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in ice (Upper) compared with a commercial fructose 1,6-bisphosphate chemical standard (Lower). The MS/MS spectra overlap in the fragment ions, but the fragment ion intensities are not fully identical, which indicates a partial overlap of isomers as formed from self-condensation of G3P and DHAP (as demonstrated in Fig. S2A). (E) Formation of fructose 1,6-bisphosphate as measured by LC-MS/MS (shown is the SRM transition m/z 339 → m/z 97) after incubation of DL-glyceraldehyde and dihydroxyacetone phosphate with different amino acids (1 mM). Reactions were performed in NaHCO$_3$ for 14 d at −20 °C. (F) Formation of fructose 1,6-bisphosphate as measured by LC-MS/MS (shown is the SRM transition m/z 339 → m/z 97) after incubation of DL-glyceraldehyde and dihydroxyacetone phosphate with different salts (1 mM). Reactions were performed in NaHCO$_3$ for 14 d at −20 °C.
Fig. S2.  (A) Comparison of product formations (SRM transition: Q1—m/z 339 → Q3—m/z 97) in ice (−20 °C) at different time points of samples containing different quantities of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in 100 mM NaHCO₃ with 1 mM glycine. Error bars indicate ±SD. (B) The pH and buffer dependency of the in-ice reaction. Samples contained dihydroxyacetone phosphate, DL-glyceraldehyde phosphate, and glycine, and the pH was adjusted to different values by using different buffers [0.1 M HCl (1.5), 0.01 M HCl (2.5), no buffer (3.3), 0.1 M 90/10 CH₃COOH/NaCH₃COO (3.7), 0.1 M, 50/50 CH₃COOH/NaCH₃COO (4.7), 0.00122 M NaOH (5.2), 0.1 M 10/90 CH₃COOH/NaCH₃COO (5.6), 0.1 M, 90/10 NaH₂PO₄/Na₂HPO₄ (5.8), 0.00366 M NaOH (6.8), 0.1 M 10/90 NaH₂PO₄/Na₂HPO₄ (7.8), 0.011 M NaOH (8.9), 0.1 M NaHCO₃ (9.5), 0.1 M 90/10 NaHCO₃/Na₂CO₃ (9.6), 0.1 M 50/50, NaHCO₃/Na₂CO₃ (10.2), 0.1 M 10/90 NaHCO₃/Na₂CO₃ (10.7), 0.033 M NaOH (11), and 0.1 M NaOH (12.9)]. The reaction mixture was incubated for 14 d at −20 °C. For the quantitation, the SRM transition m/z 339 → m/z 241 was used as in the acidic samples, a partially coeluting compound showed a m/z 339 → m/z 97 (but not m/z 339 → m/z 241) signal. (C) Proposed mechanism for an aldol reaction of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate involving a Schiff base as intermediate. (D) A rapid-freezing process in which the samples were frozen quickly at −80 °C (in dry ice) before increasing the temperature to −20 °C was compared with a slow-freezing process in which the samples were directly incubated at −20 °C. Samples contained dihydroxyacetone phosphate, DL-glyceraldehyde phosphate, and glycine in NaHCO₃, and the incubation time at −20 °C was 14 d. (E) Samples with 0 thaw–freeze cycles (sample was kept at −20 °C for 8 d) were compared with samples that were frozen and thawed eight times (incubated at −20 °C for 8 d with 1 h thawing at room temperature each day). Samples contained dihydroxyacetone phosphate, DL-glyceraldehyde phosphate, and glycine in NaHCO₃.
Fig. S3. Samples containing DL-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, and glycine in NaHCO₃ were incubated at −80 °C, −20 °C, and room temperature and measured with 2D NMR. Furthermore, measurements of the −20 °C sample spiked with 20 μM F16BP (Lower, Left) and of the pure F16BP standard were recorded.