

Non-enzymatic gluconeogenesis-like formation of Fructose 1,6-bisphosphate in ice

Short title: In-ice formation of Fructose 1,6-bisphosphate

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Abstract

The evolutionary origins of metabolism, in particular the emergence of the sugar phosphates which constitute glycolysis, the pentose phosphate pathway as well as 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 (G3P) and dihydroxyacetone phosphate (DHAP), forming the much more stable fructose 1,6-bisphosphate. We here report the discovery of a non-enzymatic counterpart to this reaction. The in-ice non-enzymatic 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 non-enzymatic 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 provide a link between prebiotic chemistry and the nature of first metabolic enzymes.

Significant statement

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

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Introduction

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 organisation is widely conserved and considered ancient. It has hence been extensively debated to what extent this structure could be the result of Darwinian selection and thus be of post-genetic origin, or whether the metabolic network topology dates back to a non-enzymatic chemistry (1–4). A series of recently discovered chemical reactions provide 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, non-enzymatic reactions replicate essential topological elements of the most central metabolic pathways. These include 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 the ones replicating the oxidative TCA cycle can occur each 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 organisation of the metabolic network (6, 8).

In order 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, mainly provided through photosynthesis. A key unsolved problem is, however, 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 constitute the RNA and DNA backbone) out of prebiotically plausible (11) but unstable (5) three-carbon phosphates. Central carbon metabolism could hence escape equilibrium in a situation that allows a parallel occurrence of gluconeogenesis next to glycolysis (9, 12). Such scenario is however missing so far. Gluconeogenesis re-builds the C-C bonds cleaved in glycolysis in one particular reaction, the aldol addition of D-glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), that forms the much more stable fructose 1,6-bisphosphate (F16BP) (Figure 1A). Enzymatically, this reaction is reversibly catalysed by F16BP-aldolase, a highly conserved glycolytic enzyme. The coupling of the aldolase to the subsequent phosphatase reaction in the ancient Archaeal enzyme paralogue 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 has, therefore, been named the 'pacemaker' for gluconeogenesis. It has further been suggested that the event of this enzyme could have enabled the evolutionary origin of gluconeogenesis (12). Indeed, while a non-enzymatic aldol reaction of aldehydes and ketones have been achieved in various context, or have provided non-metabolic compounds as in the formose reaction (13), they have failed so far for the phosphorylated compounds that play a role in central carbon metabolism (14–20).

Here we report the discovery of a non-enzymatic 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 catabolites 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 proposes a non-enzymatic 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 (Figure S1A). While this interconversion reaction resembles glycolysis, no gluconeogenesis-like interconversion is observed under the same conditions. Neither at 70 °C (5) nor at room temperature (Figure 1B), the metabolites undergo a non-enzymatic condensation, and no products resembling fructose 1,6-bisphosphate were detected by targeted analytics using a highly sensitive, selective reaction monitoring (SRM) assay (Methods). The potential formation of F16BP was monitored for 14 days, which is much longer as the stability of G3P and DHAP would have permitted their condensation to occur (Figure S1A). We discovered, however, that a non-enzymatic aldol condensation is enabled upon freezing the reaction mixture to -20 °C. In ice, G3P and DHAP were consumed at a much slower rate as at room temperature (Figure S1B). Here, the consumption of the metabolites was associated with the appearance of an SRM signal corresponding to a six-carbon bisphosphate (Figure 1C). The in-ice formed metabolite represented fructose 1,6-bisphosphate (F16BP) according to chromatographic retention time, mass-to-charge ratio, SRM transitions and matching fragmentation spectra (Figure 1C, Figure S1D). The reaction product was detected after 24hrs. We then incubated and analysed samples over a period of 81 days. The six-carbon bisphosphate continuously accumulated (Figure 1D). The reaction

did occur in the presence of various buffer substances, was however fastest under alkaline conditions (Figure S1B).

A non-enzymatic 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 (Figure S1E). The evolutionary older class II aldolases instead use amino acid catalysis involving a catalytic lysine. Here, significant effects were detected for several amino acids (Figure S1F). In two cases (Lysine and Glycine) a substantial acceleration, in particular in the early time-points, was observed (Figure 1D). As the reaction mechanism in class I F16BP aldolase and the archeal F16BP aldolase (using amino acid catalysis) are both based on a Schiff base as intermediate, it can be speculated that the in-ice acceleration by amino acids follows a similar reaction mechanism (Figure 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 (Figure 1E, Figure S3 for the entire 2D-NMR spectra), as confirmed by the addition (spiking) of a low concentration (20 µM) of pure F16BP chemical standard (Figure 1E, Figure S3, bottom panels). The spectra obtained from samples kept at -80°C and room temperature (RT) are lacking these features, while showing different patterns in particular at RT indicative of decomposition of the reactants rather than condensation reactions (Figure S3). 2D-NMR and LC-MS/MS hence agree with the non-enzymatic formation of F16BP in ice.

It is unlikely that a non-enzymatic 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 co-eluted chromatographically (Figure S1D). One possible source of these isomers were a reaction of DHAP or G3P, respectively, with themselves. Indeed, we observed both ketone-ketone and aldehyde-aldehyde self-condensations to occur at low rates (Figure 1F). In particular the G3P self-condensation was much slower than the aldehyde-ketone aldol addition. Hence, 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 (Figure 1F, Figure S2A for different concentrations).

Next, we speculated about the mechanism that enable the in-ice aldol condensation. We first tested the effect of freezing-thawing cycles. Neither had freezing-thawing a significant impact on the reaction rate (Figure S2E), nor made a slow or a fast freezing procedure a difference to product formation (Figure S2D). Further, 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 (Figure 1G). As G3P and DHAP are stable at room temperature only for a short time (Figure S1A), this reaction is perhaps less plausible for the origin of metabolism. However, this result shows that a non-enzymatic gluconeogenesis-like aldol condensation also occurs at higher temperature under the appropriate conditions.

Discussion

The evolutionary origins of glucose and glucose metabolism are unknown so far. However,

glycolysis and the pentose phosphate pathway, that 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. As all, ATP generation, amino acid biosynthesis and nucleotide biosynthesis 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). While we have previously presented evidence for non-enzymatic glycolytic and PPP- like reactions (5, 6), there was so far however no evidence for situation that could prevent such a chemical network to reach equilibrium. In other words, there was no scenario for a non-enzymatic gluconeogenic pathway that could occur in next to a non-enzymatic glycolysis.

Here, we show that G3P and DHAP metabolites condense to form fructose 1,6-bisphosphate by re-building the C-C bond cleaved in glycolysis, non-enzymatically. 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 temperature (5) Our results hence show that they are not only stabilized in ice by lower temperature, but also as they convert non-enzymatically into the much more stable fructose-1,6 bisphosphate. Second, non-enzymatic catabolic reactions resembling glycolysis are accelerated at higher temperature (5). Freezing and thawing might thus have enabled the co-occurrence of anabolic and catabolic reactions, favouring 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 can occur on many places on the early Earth. Attractive environment for such a scenarios are in particular hot springs and geysers, in which freezing and thawing occurs in close proximity and a huge variety of temperature gradients establish. In any case, ice matrices were 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, it enables the synthesis of nucleobases (29–32) and supports a non-enzymatic polymerisation and a template-directed mechanism for copying RNA (33–35). Freeze-thaw cycles could hence both helped to achieve both the copying of nucleic acids as well as non-enzymatic reactions that form the metabolic components of RNA and DNA. Perhaps, our results further imply that at least some of the non-enzymatic reactions that shaped the structure of the metabolic network were already important at the stage of the origins of life. A non-enzymatic gluconeogenesis could have helped in building up glycolytic carbohydrates that obtained their universal importance already in earliest organisms, and enabled by freezing conditions, over long timescales. Life certainly depends on higher temperatures. Our data shows that also this is possible; the non enzymatic formation of fructose-1,6-bisphosphate was enabled by mimicking the concentration effects of ice by repeated desiccation/rehydration cycles. Under favourable conditions, a non-enzymatic gluconeogenesis is hence plausible for mesophilic environments as well.

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 non-enzymatic formation of fructose 1,6-bisphosphate is the acceleration of the reaction by simple amino acids, in particular glycine, whose formation has

been repeatedly reported even in the earliest attempts of simulating 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 first amino-acid based enzymes.

In summary, we report the discovery of a non-enzymatic, in-ice aldol condensation that enables the formation of C-C bonds among catabolic 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 non-enzymatic glycolysis (5, 6). The parallel occurrence of glucose catabolism and anabolism as driven by a simple, non-enzymatic chemistry, might be able to explain how central metabolism originated in the defined compartment of the cell, how it escaped equilibrium, and hence, help to understand why glucose metabolism became universally important for life.

Materials and Methods

DL-Glyceraldehyde 3-phosphate solution (45-55 mg/ml G5251), Dihydroxyacetone phosphate lithium salt ($\geq 95\%$ 37442), Fructose 1,6-bisphosphate trisodium salt hydrate ($\geq 98\%$, F6803), Fructose 6-phosphate disodium salt hydrate ($\geq 98\%$, F3627), Sodium bicarbonate, Octylamine (99%, O5802), Acetic acid (49199), Glycine ($\geq 99\%$, G8898), L-Lysine ($\geq 98\%$, L5501), L-Alanine ($\geq 98\%$, A7627), L-Aspartic acid ($\geq 98\%$, A9256), L-Arginine ($\geq 98\%$, A5006), L-Asparagine ($\geq 98\%$, A0884), L-Cystein ($\geq 98\%$, 30089), L-Glutamic acid ($\geq 98\%$, G1251), L-Glutamine ($\geq 98\%$, G3126), L-Histidine ($\geq 98\%$, H8000), L-Isoleucine ($\geq 98\%$, I2752), L-Leucine ($\geq 98\%$, L8000), L-Methionine ($\geq 98\%$, M9625), L-Phenylalanine ($\geq 98\%$, P2126), L-Proline ($\geq 98\%$, P0380), L-Serine ($\geq 98\%$, S4500), L-Threonine ($\geq 98\%$, T8625), L-Tryptophane ($\geq 98\%$, T0254), L-Tyrosine ($\geq 98\%$, T3754), L-Valine ($\geq 98\%$, V0500), Calcium chloride ($\geq 96\%$ C5670), Copper(II) chloride (99 % 751944), Iron(II) chloride (98 % 372870), Iron(III) chloride ($>99.99\%$, 451649), Magnesium sulfate (Bioreagent, M2643), Manganese(II) chloride ($>99\%$, 244589), Zinc(II) chloride ($>99.995\%$, 429430), Nickel(II) chloride (98%, 339350), Sodium hydroxide (1.06462), Acetic acid (49199), Sodium acetate (S2889), sodium phosphate monobasic ($\geq 99\%$, S8282) and sodium phosphate dibasic ($\geq 99\%$, S7907) were purchased from Sigma-Aldrich. Water (ULC-MS grade, 23214125), Acetonitrile (ULC-MS grade, Bio-012041) were purchased from Greyhound Chromatography.

Samples were prepared in Agilent vials (5183-2069, 5190-1599) with glass inserts (5181-1270). The reactions in ice were conducted in a freezer (Hotpoint RZA36) at $-20^{\circ}\text{C} \pm$

3°C. All reactions were stopped by transferring the samples to -80°C (Panasonic MDF-U55V freezer). Reactions were conducted in triplicates. If not explicitly specified the samples (30 ul) were composed as follows: 2mM dihydroxyacetone phosphate lithium salt and 2mM DL-glyceraldehyde 3-phosphate in 100mM NaHCO₃ w/ or w/o 1 mM glycine (pH =9.5).

Metabolites were quantified similar as previously described (5, 39) using an online coupled HPLC system (Agilent 1290 Infinity) and a triple-quadrupole mass analyser (Agilent 6470) operating in SRM mode (metabolite-optimized transitions were elaborate previously(39), and are for fructose 1,6-bisphosphate: m/z 339 -> m/z 97; glyceraldehyde 3-phosphate/dihydroxyacetone phosphate: m/z 169 -> m/z 97). F16BP was additionally qualified by the transitions m/z 339-> m/z 241 and m/z 339 -> m/z 79 (Figure S1C). For the chromatographic separation a C₈ column (Zorbax SB-C₈ Rapid Resolution HD, 2.1 × 50 mm, 1.8 um (Agilent)) was used. Mobile phase A and B contained 750 mg/l octylammonium acetate as ion-pairing reagent and consisted of 10% and 50% acetonitrile, respectively. A flow rate of 0.6 ml/min was applied and the elution was isocratic with 0% B for 1.5 min, followed by a gradient ramp to 70% B within 1.7 min.

Absolute quantities were evaluated by peak integration and external calibration using a 1:5 dilution series. Analytes were identified by SRM transitions as well as matching retention times with standards. Peak integration as well as evaluation of absolute quantities was performed with the Agilent MassHunter Workstation Software. Further analysis was done in R (R Core Team, <http://www.R-project.org>)

NMR spectra were acquired using a Bruker Avance IIIHD 800 MHz NMR spectrometer equipped with a TCI Cryoprobe. Excitation sculpting was used for water suppression in 1D (40) and 2D TOCSY (41) spectra (Bruker pulse sequences *zgesgp* and *dipsi2esgpph*, respectively). Spectra were acquired at a sample temperature of 25 °C. For the TOCSY spectra the mixing time was 60 ms; the relaxation delay 1.5 s; non-uniform sampling was employed in the indirect dimension; the total acquisition time was 2 h 22m per TOCSY spectrum. The samples measured contained 2mM DL-glyceraldehyde 3-phosphate, 2mM dihydroxyacetone phosphate and 1mM glycine in 100 mM NaHCO₃ and were incubated at -80°C (30 days), -20°C (30 days) and room temperature (4 days)

The pH measurements were performed with an InLab micro electrode (Mettler Toledo). In order to measure the pH dependency of the reaction, the pH of the samples (2mM dihydroxyacetone phosphate lithium salt and 2mM DL-glyceraldehyde 3-phosphate in 100mM NaHCO₃ with 1 mM glycine) were adjusted with the following buffers: 0.1M HCl (1.5), 0.01M HCl (2.5), no buffer (3.3), 0.1M 90/10 CH₃COOH/NaCH₃COO (3.7), 0.1M, 50/50 CH₃COOH/NaCH₃COO (4.7), 0.00122M NaOH (5.2), 0.1M 10/90 CH₃COOH/NaCH₃COO (5.6), 0.1M, 90/10 NaH₂PO₄/Na₂HPO₄ (5.8), 0.00366M NaOH (6.8), 0.1M 10/90 NaH₂PO₄/Na₂HPO₄ (7.8), 0.011M NaOH (8.9), 0.1M NaHCO₃ (9.5), 0.1M 90/10 NaHCO₃/Na₂CO₃ (9.6), 0.1M 50/50, NaHCO₃/Na₂CO₃ (10.2), 0.1M 10/90 NaHCO₃/Na₂CO₃(10.7), 0.033M NaOH (11), 0.1M NaOH (12.9)

For the desiccation/rehydration experiments, 300 ul samples (2mM dihydroxyacetone phosphate lithium salt and 2mM DL-glyceraldehyde 3-phosphate in 100mM NaHCO₃ with 1 mM glycine) were dried down with a vacuum concentrator (Eppendorf Concentrator plus) and the remaining powder dissolved in 50 µl deionised water. This step was repeated 5 times. In the final step the powder was dissolved in 300 ul water prior to LC MS/MS analysis.

Freeze/thaw experiment: Samples (2mM dihydroxyacetone phosphate lithium salt and 2mM DL-glyceraldehyde 3-phosphate in 100mM NaHCO₃ with 1 mM glycine) were

frozen and thawed 8 times (incubated at -20°C for 8 days with 1 hour thawing at room temperature each day).

Acknowledgements

This work was supported by a University of Cambridge/Wellcome Trust Interdisciplinary fellowship awarded to CM. Further support was provided by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001134), the UK Medical Research Council (FC001134), and the Wellcome Trust (FC001134). The NMR data were acquired at the Medical Research Council Biomedical NMR Centre at the Francis Crick Institute.

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Figure Legends

Figure 1: Non-enzymatic 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 as catalysed 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 \rightarrow m/z 97) after incubation of DL-glyceraldehyde and dihydroxyacetone phosphate for different lengths of time in aqueous solution at room temperature. At RT, the substrates G3P and DHAP degrade within days (Figure S1)

C) Formation of fructose 1,6-bisphosphate as measured by LC-MS/MS (shown is the SRM transition m/z 339 \rightarrow m/z 97) after incubation of DL-glyceraldehyde and dihydroxyacetone phosphate in NaHCO_3 for different lengths of time at -20°C .

D) The in-ice aldol addition of DL-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in NaHCO_3 is accelerated by lysine and glycine. Samples were kept at -20°C for different lengths of time over a period of 58 days, thawed at the same time and measured by LC-MS/MS. Error bars indicate \pm standard deviation

E) Samples containing DL-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and glycine in NaHCO_3 were incubated at -80°C , -20°C and room temperature and measured with 2D NMR. Furthermore, measurements of the -20°C sample spiked with $20\mu\text{M}$ F16BP (second panel from the right) and of the F16BP standard chemical were recorded. The comprehensive 2D-NMR spectra are given in Figure S3.

F) Comparison of product formation (SRM transition: Q1: m/z 339 \rightarrow 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 days in 100mM NaHCO_3 with 1mM glycine. Error bars indicate \pm standard deviation.

G) Product formation (SRM transition m/z 339 \rightarrow m/z 97) in samples containing DL-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and glycine in NaHCO_3 which were dried and dissolved 5 times.