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Recreating ancient metabolic pathways before enzymes

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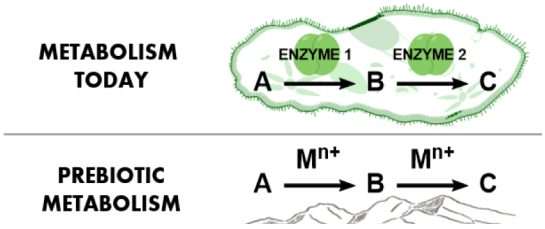

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Recreating ancient metabolic pathways before enzymes

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ABSTRACT

The biochemistry of all living organisms uses complex, enzyme-catalyzed metabolic reaction networks. Yet, at life's origins, enzymes had not yet evolved. Therefore, it has been postulated that non-enzymatic metabolic pathways predated their enzymatic counterparts. In this account article, we describe our recent work to evaluate whether two ancient carbon fixation pathways, the rTCA (reductive tricarboxylic acid) cycle and the reductive AcCoA (Wood-Ljungdahl) pathway, could have operated without enzymes and therefore have originated as prebiotic chemistry. We also describe the discovery of an Fe^{2+}-promoted complex reaction network that may represent a prebiotic predecessor to the TCA and glyoxylate cycles. The collective results support the idea that most central metabolic pathways could have roots in prebiotic chemistry.

1. Introduction

Metabolic function is one of the most critical features of living systems, on par with replication and information transfer. For this reason, the emergence of metabolism represents an important milestone in the transition from chemistry to biochemistry.[1] As far as we know, anabolism (build-up) and catabolism (breakdown) have functioned together since life's origins, forming dynamic reaction networks that operate through a small number of "core" organic intermediates.[2,3] The traditional approach of the organic chemist towards understanding the origins of biochemistry has been a "bottom-up" methodology to search for potentially prebiotic syntheses of biomolecules.[4-6] More recently, a "systems" approach has been made to uncover the possible common origins of several fundamental classes of biomolecules such as sugars, lipids, amino acids, and intermediary metabolites.[7-11] While this new approach is welcomed, some important caveats to the results so far obtained are: 1) that they often require sequential reactions that are not mutually compatible and are therefore unlikely to occur without human intervention;[12] 2) they employ reagents foreign to biochemistry; and 3) the reactions used and the intermediates and pathways followed are very different from biological metabolism. Several authors have noted that this set of traits make it unlikely for evolution to emerge and act on such chemistry to produce the biochemistry we know today and have questioned its relevance to abiogenesis.[13-17] Counterpoised to this is a "top-down" approach to prebiotic chemistry in which ancestral core metabolic pathways are inferred from qualitative or quantitative network analysis of metabolism or from phylogenetic reconstructions.[4,18] In this way, two ancient CO_2-fixation pathways, the reductive AcCoA pathway (also known as the Wood-Ljungdahl pathway)[19] together with the reductive tricarboxylic acid (rTCA or reverse Krebs) cycle (in whole[2] or in part[20]) have been proposed as candidates for what biochemistry may have looked like before enzymes, dating back to prebiotic chemistry. In opposition, some authors have argued that billions of years of evolution would have erased traces of prebiotic chemistry in the biological record and that prebiotic chemistry was likely radically different from biochemistry.[5] In our view, the idea that prebiotic chemistry bore no resemblance to biochemistry seems unlikely. First, chemical networks can evolve to a limited extent in the absence of Darwinian selection.[21] Therefore, if such a drastic change to life's chemistry ever occurred, it had to have happened after the onset of genetics. A proto-metabolic system of a complexity sufficient to get life all the way to that point would have been very difficult to replace. Evolution tends to settle for solutions that are easy to discover and "good enough", rather than the ideal solution. Why then would life not simply have optimized existing metabolic networks, rather than re-written them completely? We felt that the absence of evidence for a non-enzymatic proto-metabolism resembling biochemistry was simply due to the lack of sustained experimental efforts. Indeed, until 2017 little chemical evidence for non-enzymatic metabolic pathways existed, besides an accidentally discovered non-enzymatic glycolysis-like pathway catalyzed by Fe^{2+}.[22]

In an attempt to unify "top-down" and "bottom-up" approaches, researchers in our laboratory set out to experimentally reconstruct the ancient metabolic pathways identified by the top-down approach without enzymes using simple inorganic catalysts and reagents that would have been found on a lifeless planet, such as metals, metal ions and minerals. Reproducing ancestral metabolic chemistry non-enzymatically in a chemical laboratory is rooted in bioorganic chemistry and catalysis, but requires inputs from various other disciplines including biochemistry, evolutionary biology and geology. Thus, it may produce compelling examples

of prebiotic chemistry that would have been likely to operate under early Earth's conditions while explaining the origins of the biochemistry we know today.[1,22] In the following sections, we report on the recent endeavors of our team to achieve this goal.

2. Reductive AcCoA (Wood-Ljungdahl) pathway

The Wood-Ljungdahl pathway (Figure 1) is used to obtain activated acetate by both bacteria and archaea (in the latter case it does not have a net dependence on ATP).[20] It is often considered to be the most ancient of the six known biological CO_2 fixation pathways on the basis of phylogenetic reconstructions and network analysis.[17- 20, 22] The Wood-Ljungdhl pathway is short, linear, heavily reliant on transition metals and is therefore hypothesized to have initially arisen as prebiotic chemistry.[19,25-27] On the other hand, how could it have started before enzymes when the propensity of CO_2 to form C-C bonds in water is quite poor?[5] Some studies tackle this problem by using C1 building blocks that are more reactive than CO_2 (carbon monoxide and methyl thiol),[28] by applying very high temperature and pressure to neat formic acid (250 °C, 2000 bar),[29] or by using highly reducing (-1.1 V) electrochemical potentials on greigite ($Fe^{(II)}Fe^{(III)}{}_2S_4$) electrodes.[30] It has also been shown that fresh iron nanoparticles can reduce CO_2 to acetate (minor product) at 200 °C under 40 bar CO_2 pressure.[31] These reports, however, are either hard to support geochemically in the context of early Earth's environment or seem incompatible with other organic chemistry that would be required for a primitive metabolism.

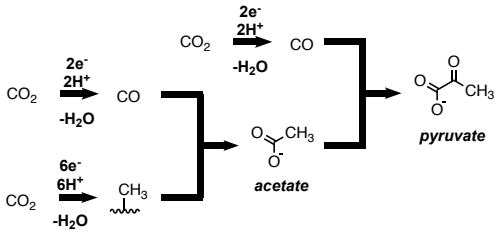

Figure 1. A simplified depiction of the reductive AcCoA pathway.

Inspired by the fact that enzymes and cofactors of the reductive AcCoA pathway are replete with transition metals (Fe, Ni, Co, Mo or W), we suspected the potential reactivity of such metals in non-enzymatic carbon fixation processes.[32] After a screen of various reduced metals in a KCl-water solution at 100 °C under 35 bar of CO_2 pressure, we found that Mo, Fe, Ni, Co, Mn and W all promoted formate and acetate production in appreciable concentrations (up to 0.28 ± 0.01 mM acetate). Furthermore, Fe, Ni and Co were also able to extend CO_2 fixation to furnish the C3 product pyruvate in up to 0.03 ± 0.01 mM concentrations at 100 °C under 35 bar of CO_2, with Fe^0 producing the best results. In most cases, a basic workup was required to liberate surface-bound species. This, together with control experiments in which Wood-Ljungdahl pathway intermediates in solution were shown not to react further when submitted to the reaction conditions, indicate that the CO_2-fixing reactions are likely occurring on the surface of the metal particle. What is clear is that iron-promoted CO_2 fixation in water selectively produces the same intermediates and end-products of the biological Wood-Ljungdahl pathway (formate, acetate and pyruvate). Further studies are ongoing in our laboratory to better understand the reaction mechanism, but our current understanding of the process roughly resembles the biological pathway. This experimentally trivial but mechanistically complex reaction may constitute a prebiotic precursor to the biological reductive AcCoA pathway.

Could metallic iron have been a prebiotic reagent on the early Earth? Metallic iron constitutes 80% of the Earth's core but is relatively rare in the Earth's crust.[33] Native iron is found in meteorites[34] and is generated transiently in the Earth's mantle,[35] but it is not clear whether it or a similar reduced mineral might be produced closer to the Earth's surface, such as in hydrothermal vents. Alternatively, two recent models developed to account for the surprisingly high concentration of iron-loving elements in the Earths' crust have proposed their presence resulted from a collision between Earth and a moon-sized object about 4.51 billion years ago.[36,37] In one computer simulation consistent with physical evidence from zircons, the collision fractures the impactor to its core, causing a quantity of metallic Fe equivalent to three times the Earth's oceans to rain down to the young planet's surface for many millions of years.[36] Although it remains to be seen whether this hypothesis will survive further scrutiny, at this stage the potential of metallic iron for prebiotic chemistry should not be ruled out.

3. Reductive Tricarboxylic Acid cycle

Another of the most evolutionarily preserved biochemical pathways is the rTCA cycle (Figure 2).[25] It is used by both bacteria and archaea in both its complete[2] and incomplete versions.[20] Five intermediates of this hypothetical hybrid pathway (acetate, pyruvate, oxaloacetate, succinate and α-ketoglutarate) constitute the universal feedstocks and precursors for the syntheses of amino acids, lipids, nucleic acids, sugars or cofactors.[1,24] The rTCA cycle contains eleven intermediates, but only five different types of reactions: reductive carboxylations, redox-neutral carboxylations, reductions, de/-hydrations and a retro-aldol cleavage. In living organisms, these reactions are promoted by enzymes and cofactors that often contain metal ions – iron as FeS clusters in particular – in their active sites. Six of the eleven reactions do not depend on ATP, while five do. Specifically, two carboxylations, one reductive carboxylation and the retro-aldol reaction require energy from ATP.[25]

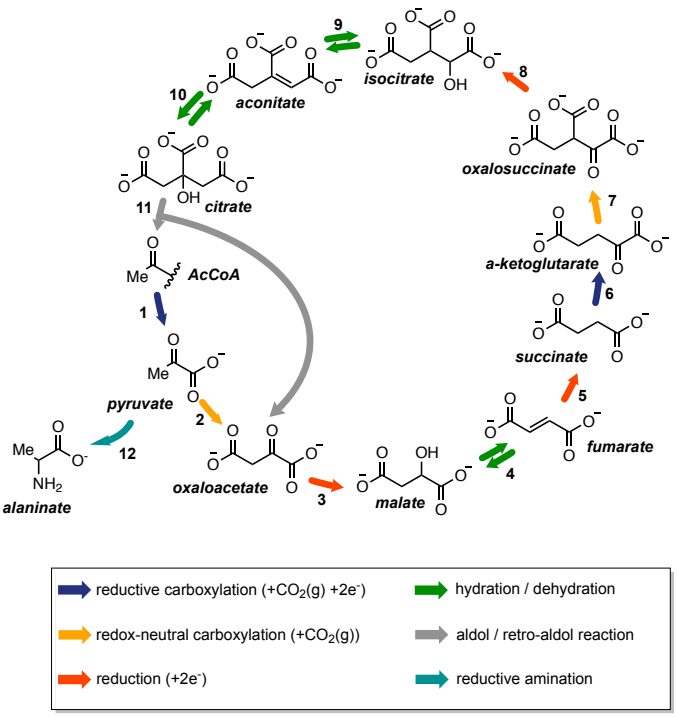

Figure 2. The reductive tricarboxylic acid cycle, additionally showing the reductive amination of pyruvate to give alanine.

One of the attractive features of the rTCA cycle as a model for prebiotic chemistry is that it possesses an autocatalytic topology. In the full rTCA cycle, the retro-aldol splitting of citrate creates an

autocatalytic feedback loop, meaning it has the theoretical potential to double the number of its intermediates with every turn of the cycle. Orgel has noted that a prebiotic rTCA cycle would be unlikely to self-sustain despite its autocatalytic nature due to likely parasitic reactions leading off-cycle.[38] On the other hand, it has been argued that in a hypothetical prebiotic reaction network composed of a primitive rTCA cycle and AcCoA pathway, the autocatalytic property of the rTCA cycle combined with a sufficient influx of acetyl from the AcCoA pathway could ensure the network's sustainability and self-amplification even in the face of low yields.[1] An alternative suggestion for a prebiotic version of the rTCA cycle is the linear "horseshoe" version of the rTCA pathway, which includes only the first six steps, starting from acetate and leading to α-ketoglutarate.[20] This shorter truncated version, though not autocatalytic, still contains all five universal metabolic precursors and might therefore be more attainable on the early Earth in the absence of enzymes.

Despite many hypothesis papers on a prebiotic rTCA cycle, reports on non-enzymatic catalysis of its reactions remained limited. Cody and coworkers had examined the retro-aldol cleavage of citrate under metal sulfide catalysis at temperatures approaching 200 °C, at which temperature many competing decomposition reactions occur.[39] Zhang and Martin reported that two of the reduction reactions could be promoted by mineral UV photocatalysis.[40] Further investigations on these reactions were made by Guzman and coworkers.[41,42] The S^0/H_2S redox couple was also found to promote some reductive reactions of the rTCA cycle.[43] Notably lacking in these works was the ability to perform multiple sequential reactions under a single set of experimental conditions.

It should also be noted that efforts have been made to uncover conditions where the cycle runs without enzymes in the *oxidative* direction (the Krebs or TCA cycle). Ralser and coworkers reported a sulfate radical mediated system, based on the combination of FeS and $S_2O_8^{2-}$, where many of the TCA cycle reactions could be performed in high efficiencies in one pot.[44] The described network could have been a prebiotic way to break down already existing intermediates to simpler intermediates en route to CO_2. This is in principle a key function of the Krebs cycle in modern organisms. However, it should be noted that the described network did not encompass two other key aspects of the TCA cycle: 1) its ability to build up, not just break down, molecules through the C-C bond-forming aldol reaction and 2) the TCA cycle's ability to harvest the energy of oxidative decarboxylation in the form of high-energy thioesters. Prebiotic chemistry using such a partial non-enzymatic TCA cycle would equally need some process to build up those complex starting molecules in the first place.

Prompted by the presence of metals in the active sites of enzymes, we hypothesized that if the rTCA cycle was prebiotically plausible, promoting its reactions in water using transition metals or metal salts (without the enzymatic scaffold) should be possible at least to some extent. Our investigation was focused on the reduction, hydration and dehydration reactions of the rTCA cycle: steps 3-5 and 8-10 (Figure 2), which in biology do not require ATP. The results of this recent report[45] are summarized below. Two-electron reductions (reactions 3, 5, and 8) were the most straightforward, easily accomplished with several reducing agents (including Fe^0, Ni^0, Zn^0) under acidic conditions and temperatures between 25-140 °C. Following extensive screening of transition metals and their salts, we found that reduction and dehydration reactions of the bottom arc of the rTCA cycle (reactions 3-5) can be effected in a one-pot sequence by Fe^0 and Zn^{2+} in 1 M HCl. The upper arc of the cycle (reactions 8-10) proved more challenging due to the reversible aconitate hydration reaction (reaction 10), which was promoted uniquely by Cr^{3+} under acidic conditions at 140 °C. Eventually, we demonstrated that reactions 8-9-10, as a one-pot sequence, can be mediated by a mixture of Fe^0, Zn^{2+} and Cr^{3+} in 1 M HCl at 140 °C. Those conditions were verified to promote the lower arc (reactions 3-5), resulting in a single set of conditions that promotes six of the eleven steps of the rTCA cycle.

Amino acid synthesis was achieved via reductive amination of α-ketoacids under the same set of acidic conditions used to promote parts of the rTCA cycle, by using hydrazine (an intermediate and product of both biological and non-biological reduction of N_2[46,47]) as nitrogen source and Fe^0 as reductant. In this way, alanine was obtained from pyruvate. Unfortunately, the synthesis of other possible amino acids (aspartate from oxaloacetate; glutamate from α-ketoglutarate) was unsuccessful under these conditions.[45]

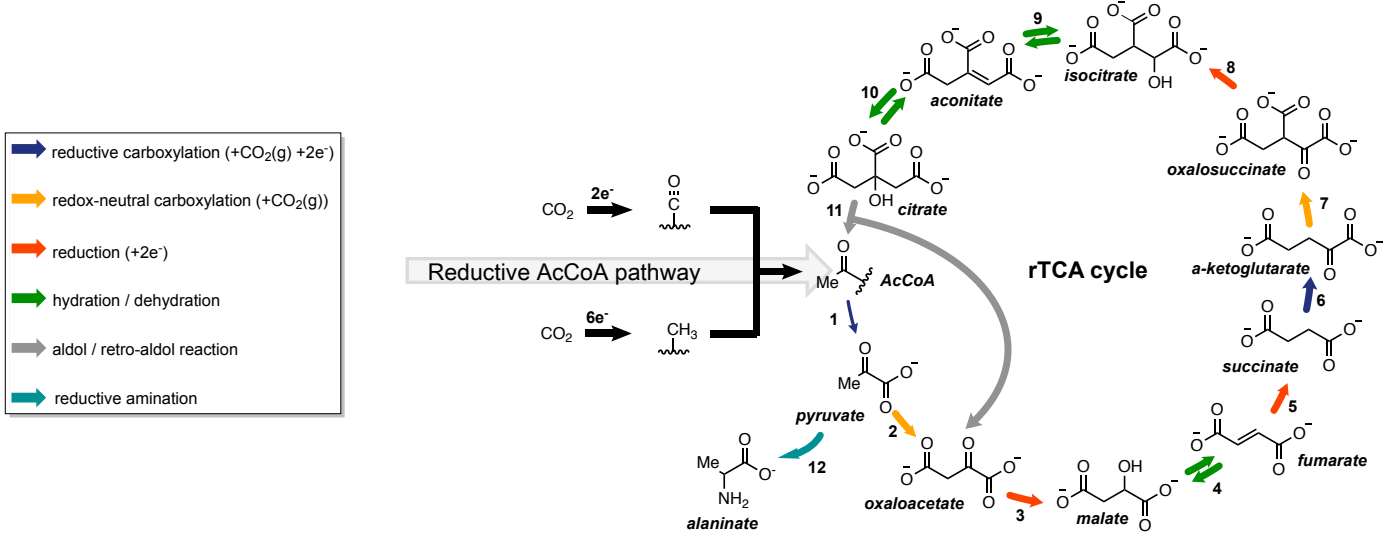

Figure 3. A hypothetical network comprising the reductive AcCoA pathway and the rTCA cycle.

Intriguingly, the efficiencies of the reaction sequences we reported may be improved, and reaction temperatures lowered, with the use of micelles which form nano-sized compartments in water.[48] For example, the lower arc of the rTCA cycle (reactions 3-5) occurs at room temperature in the presence of Fe^0 and Zn^{2+} and micelles, whereas it otherwise requires a temperature of at least 80 ºC. This proof-of-concept experiment points to the role of compartmentalization in prebiotic processes, which may have been provided by naturally occurring micelles[49] or by micropores of naturally occurring minerals, such as those found in hydrothermal vents.[20] Coincidentally, a hot, acidic, metal-rich aqueous medium is currently found in some hydrothermal vents, hypothesized by some to be the cradle of life.[44]

What should be made of this study? Should the rTCA cycle be considered prebiotic? Even if not functional as a full pathway, sequences of the rTCA cycle could have primed the subsequent evolution of a functional enzymatic pathway by providing the intermediates. Although our experiments show that most of the reactions of the rTCA cycle can be carried out without enzymes under a common set of conditions, two important limitations exist. First, the strongly acidic conditions employed limit the compatibility of these reactions with others that are probably necessary on the path to life, such as amino acid polymerization to form peptides. In response to this criticism, we showed that many of the reactions of the rTCA cycle can be run under the milder acidic conditions used for non-enzymatic AcCoA pathway-like CO_2 fixation described in the last section (140 ºC, water under 35 bar CO_2), albeit in lower yields.[32] However, the only non-enzymatic reaction in the rTCA cycle that require such forcing conditions is the hydration of aconitate to citrate. All others occur at 100 ºC in CO_2-saturated water. One interpretation of this observation could be that aconitate hydration to give citrate, and therefore the emergence of the autocatalytic form of the rTCA cycle from a prior linear form, was a later development.

A second, more important, criticism is that none of the four ATP-dependent steps of the rTCA cycle were found to work in the absence of enzymes, including all of the critical C-C bond forming reactions. Of course, our lack of success in this endeavor does not mean it is impossible, but numerous attempts by different individuals in our lab using various catalysts, conditions and ATP-mimicking reagents over a period of three years never returned a single positive result.

Contrasting those failures with the relative ease with which we uncovered all six of the ATP-independent steps of the rTCA cycle, we have become swayed by suggestions that the functional role of ATP and therefore reactions that depend on ATP, at least in the context of core carbon metabolism, are later products of chemical evolution.[17] In other words, our current opinion is that although the rTCA cycle may potentially still be prebiotic, it probably did not emerge at the very earliest stage of prebiotic chemistry.

4. A metabolism-like Fe^{2+}-promoted complex reaction network: synthesis and breakdown of the universal metabolic precursors

To bypass the bottleneck to C-C bond formation posed by ATP-dependent reactions, we turned to the recent theoretical work of Segrè and coworkers.[17] Starting from a network of all metabolic reactions in the KEGG database, they found a large and interconnected subset of the metabolic network that does not depend on phosphorus either as a starting material, product or co-factor. The hypothetical network was highly dependent on Fe-S enzymes and thioester intermediates. Of the CHO compounds in that network, pyruvate and glyoxylate were the most well-connected, indicating that if a phosphate-free metabolism once existed, those two compounds would have played key roles.[17]

Less than a year later, Springsteen, Krishnamurthy and co-workers reported a bicyclic non-enzymatic reaction network driven by H_2O_2-mediated oxidation and oxidative decarboxylation.[51] Key C-C bond-forming reactions in the network were the aldol reactions of glyoxylate with pyruvate, oxaloacetate, and malonate.

Building on the "top-down" clues from the phosphate-free metabolic network,[17] as well as from the "bottom-up" study of glyoxylate-based linked cycles,[51] we performed a high-throughput reaction screen of glyoxylate and pyruvate with various transition metal salts under various prebiotically plausible conditions.[52] We found that Fe^{2+} salts in 70 ºC water can trigger the formation of a complex reaction network from pyruvate and glyoxylate (Figure 4). Similar to Ralser's non-enzymatic network driven by strong oxidants,[44] the observed reaction network spans most of the reactions and intermediates of TCA and glyoxylate cycle, including all five universal metabolic precursors (acetate, pyruvate, oxaloacetate, succinate and α-ketoglutarate; compare with Figure 2). Reactions within the network were of the same classes found in the TCA and rTCA cycles. C-C bonds were formed via aldol reactions and broken through oxidative decarboxylation, decarboxylation and retro-aldol reactions. Redox reactions, hydration/dehydrations were also found to be involved.

Upon the addition of hydroxylamine (an intermediate in biological nitrogen cycles[53] and a prebiotically plausible nitrogen source[54,55]) and metallic iron (as reductant), the system could be extended towards four biological amino acids (glycine, alanine, aspartic acid and glutamic acid) via reductive amination of their precursor ketoacids in one pot and without modifying the reaction temperature. The system reached its maximum complexity after 24 h, with sixteen biomolecules detected in solution: only two TCA cycle intermediates (oxalosuccinate and citrate) and one glyoxylate cycle intermediate (citrate) were missing at that stage.[52]

The observation that much of the TCA and glyoxylate cycles are contained within this network may point to their roots as prebiotic chemistry. However, there are a couple of key differences between this reaction network and previously reported chemistry that mimicked the TCA cycle.[44] First, the current network features C-C bond-forming reactions, providing a potential explanation for how molecular complexity could be built before being broken down. Second, the current network does not rely on a strong oxidant to be driven forward. The main chemical driving force is the reactivity of the high energy ketoacid functional group in the starting materials and intermediates. Nucleophilic attack on glyoxylate is highly favorable and many intermediates of the reaction network are also ketoacids, which are prone to entropically favored oxidative decarboxylation. The lack of a strong directional redox driving force might mean that parts of this network could be coaxed to proceed in either redox direction under the right environmental conditions. In other words, it might be a prebiotic precursor to both the TCA/glyoxylate and rTCA cycles.[18,56]

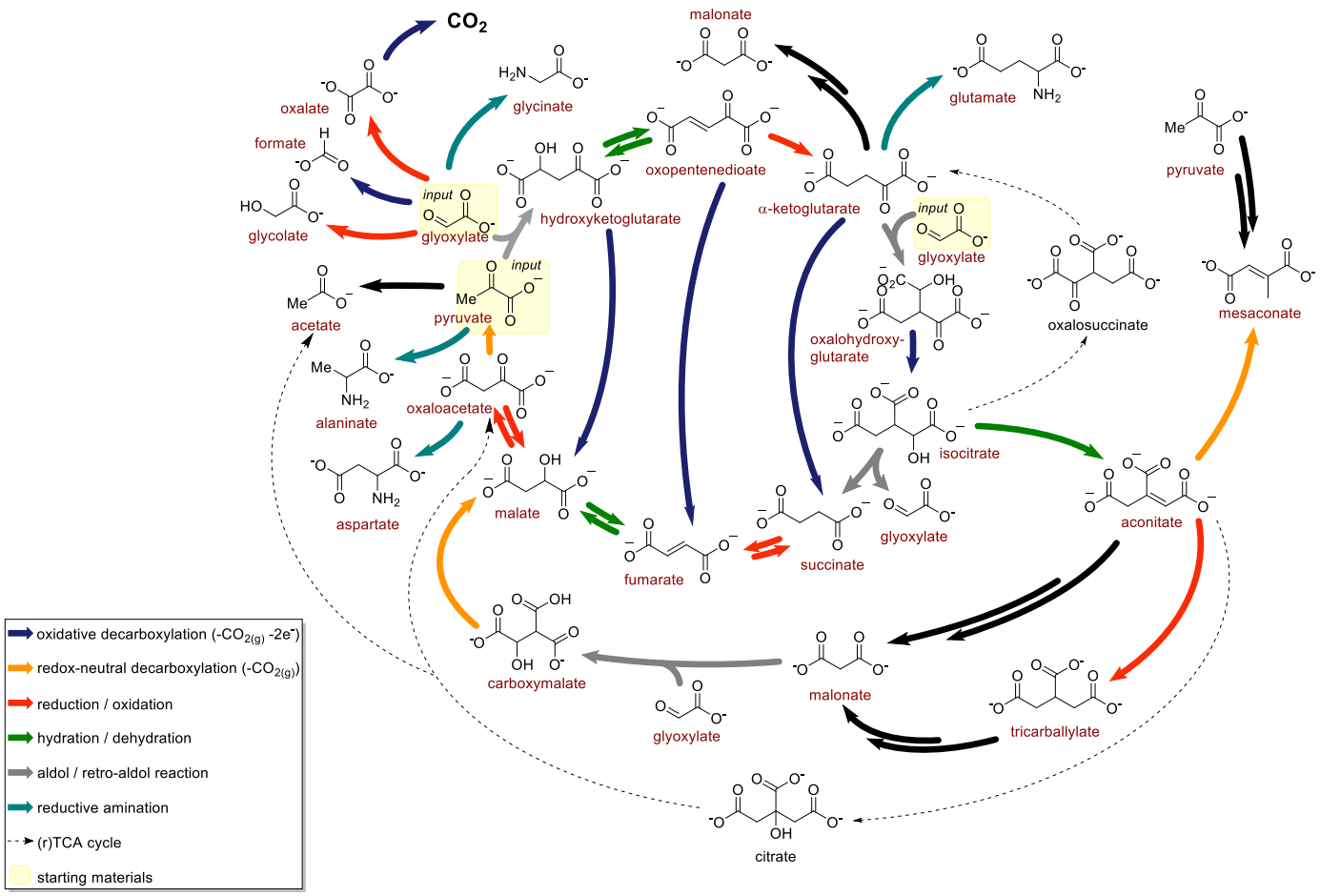

Figure 4. Reaction network arising from pyruvate and glyoxylate, promoted by Fe^{2+} in water at 70 °C. (r)TCA cycle reactions not detected within the network are shown as dotted arrows.

5. Conclusions

Many additions to metabolism must have happened between prebiotic chemistry and the emergence of the Last Universal Common Ancestor from which all known life on Earth arose, and neither were all prebiotic reactions preserved by today's life.[57] However, the experimental findings from our group summarized here, as well as from others,[13] support the idea that certain enzyme-catalyzed biological reactions, and even entire biochemical pathways, may have had fully operational prebiotic precursors relying on non-enzymatic catalysts. A non-enzymatic origin for core biochemistry offers a satisfying explanation for why life uses the reactions, intermediates and pathways that it does – because it started that way from the very beginning.

Although important details remain to be defined, the path from a non-enzymatic metabolism to an enzymatic one can be roughly sketched out. The molecular diversity of the reaction network increases as it comes in contact with additional important bio-elements such as S and P. Next, the products of the network, perhaps organic co-factors or small peptides, enable ligand-accelerated catalysis of the metals, functioning as non-coded proto-enzymes until the emergence of a primitive genetic code. Once Darwinian selection emerged, metabolic pathways could continue to evolve through previously proposed mechanisms,[58] either in the forward direction (Granick's hypothesis[59]) or the reverse direction (Horowitz's hypothesis[60]). However, core metabolism is replete with intermediates that serve no other function than being stepping stones to useful products further downstream in the pathway.[13] Newly discovered reactions leading to functionless intermediates would only serve as dead ends, leaching away valuable functional compounds and exerting a negative selection pressure. Therefore, the Darwinian evolution of metabolism was likely limited to cases where the newly discovered metabolic chemistry bridged an existing functional product to a new functional product in the pathway. The consequence is that Darwinian evolution would mostly discover reactions or sequences of reactions that emerge at once to create new functional products, highlighting the importance of enzymatic promiscuity.

This is an exciting time for research into the origins of biological metabolism, but much work remains to be done. Efforts in our lab are already underway to recapitulate more of core biochemistry without enzymes, including the bioenergetic role of sulfur chemistry.[61]

Acknowledgments

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