Systems Biocatalysis: An Artificial Metabolism for Interconversion of Functional Groups

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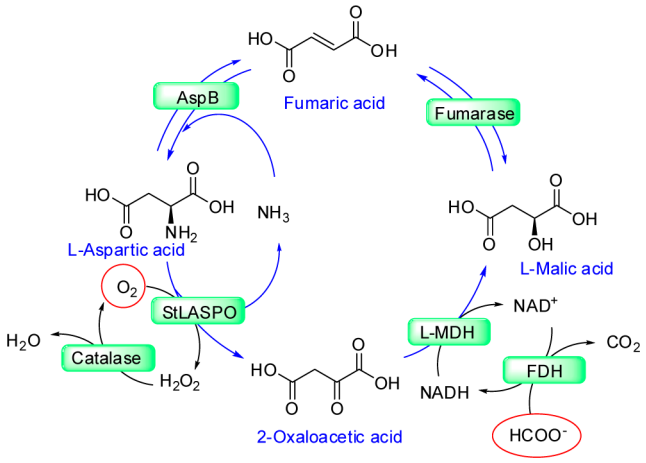
The synthetic utility of reactions catalyzed by isolated enzymes in vitro is continuously improving as a result of the progress of protein engineering: enzymes with higher stability, improved specific activity, wider substrate specificity have been made available. Moreover, the efficiency reached in cofactor regeneration and the advancement in reaction engineering unceasingly allow catalyzation of new reactions and processes.[1] Biocatalysts can be integrated in synthetic processes in combination with chemical processes or in cascade reactions in which a series of enzymes catalyze successive synthetic steps. This sequence of reactions catalyzed by enzymes in vitro can be considered as equivalent to an artificial metabolic pathway[2] and can, in principle, be organized by assembling the required enzymes and cofactors in a one-pot arrangement.[3] One such system can compete with metabolic engineering, a fast growing green methodology for the production of simple and complex molecules at the cellular level.[2a,4] This latter approach is often hampered by the presence of deviating consumptive cellular metabolism for energy generation and growth and by the frequent issues of enzymes' inhibition or regulation. Indeed, the optimization of single-step reactions and of enzyme solubility in a complex environment as well as the generation of a steady flux are not straightforward.[5]

Systems biocatalysis is a novel approach that aims at building artificial metabolic pathways in vitro for the production of useful products as an alternative to metabolic engineering or synthetic biology. The goal of the construction of a metabolic pathway using enzymes in a sequence not having an equivalent in Nature would be the obtainment of a final product or, in general, the transformation of functional groups one into the other. To pursue this task in a one-pot system, it is necessary to build up a coherent library of modular enzymatic activities to be applied in predictable functional group transformations on a series of interconnecting steps, which is a long-sought objective in applied biocatalysis. This goal can be approached by applying a combination of protein discovery and engineering techniques in the adaptive modification of enzymes to tune their catalytic ability.

In this view, we have devised a nonnatural pathway realizing an open cycle in which it is possible to enter/exit at different levels, thus determining the direction and the output of this artificial metabolism. The cyclic character of the pathway reported in Scheme 1 is of particular interest when, in the presence of all six enzymes, starting from one of the substrates, a steady state is reached, mimicking a "futile cycle" similar to the ones observed in natural, complex metabolic paths.[6] Compared with a natural metabolic cycle, what is evident is the reduced complexity of the artificial one, not depending on

Scheme 1. Proposed Synthetic Metabolic Cycle[a]



[a]The enzymes are highlighted in green boxes, the compounds consumed when the full cycle is active are enclosed in red circles.

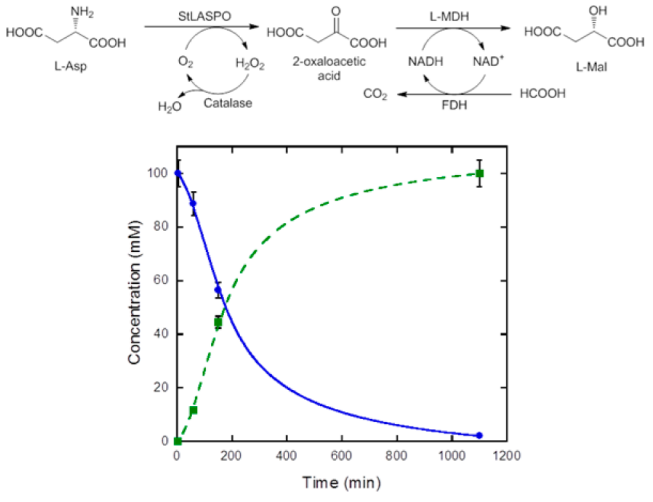


Figure 1. Time course of conversion of L-aspartate (blue) into L-malate (green) by the two-step enzymatic process catalyzed by StLASPO and L-malate dehydrogenase (see Supporting Information for details).

cell requirements, in which regulation and shift of equilibrium can be easily controlled by adding the catalyst and playing with the reagents' concentrations and in which there is a theoretical opportunity to produce any of the intermediate compounds from any of the other metabolites.

The artificial metabolic cycle depicted in Scheme 1 has been made possible by the availability of six enzymatic activities. Three of them are well-known, robust enzymes: aspartase (AspB, EC 4.3.1.1 from *Bacillus* sp.);[7] fumarase (EC 4.2.1.2 from porcine heart); L-malate dehydrogenase (L-MDH, EC 1.1.1.37 from bovine heart); and two commercial ancillary enzymes, catalase (EC 1.11.1.6) and formate dehydrogenase (FDH, EC 1.2.1.2) from microbial sources. The sixth enzymatic activity (L-amino acid oxidase), in contrast, although present in several organisms, has found few applications in biocatalysis for the scarce availability of recombinant enzyme forms and, often, its low specific activity.[8] Recently, however, an L-aspartate oxidase (EC 1.4.3.16) from the thermophilic bacteria *Sulfolobus tokodaii* (StLASPO) has been applied in a demonstrative kinetic resolution showing a remarkable thermal stability and is available for lab scale applications.[9] As indicated in Scheme 1, the proposed wheel links together an alkenoic acid with an amino acid, a ketoacid, a hydroxyacid, and again the starting alkenoic acid, thus closing the cycle. This wheel turns counterclockwise only because the StLASPO-catalyzed step is not reversible; nevertheless, the reverse direction may be, in principle, triggered by using different catalysts (such as an amino acid dehydrogenase or an amino transferase) able to reversibly transform 2-oxalacetate into aspartate. This also parallels what happens in cells to avoid the establishment of a futile cycle.

We analyzed the feasibility of the proposed cycle by investigating each single step and their connections. The oxidation of L-Asp catalyzed by StLASPO yields the corresponding imine as an intermediate, which in turn spontaneously hydrolyzes to oxaloacetate. The rapid conversion of the latter to L-malate, catalyzed by L-MDH with NADH as hydrogen donor, minimizes pyruvate formation, which would originate from the spontaneous decarboxylation of 2-oxaloacetate (Figure 1). Thus, to a solution of 100 mM L-aspartate and 250 mM formate at pH 8.0 in the presence of ≈7 mM NADH were added FDH, L-MDH and catalase, and StLASPO as the "limiting" enzyme (0.32 U). Although NADH was used in a substoichiometric amount, the flux was not regulated or limited by the reduced form of the cofactor, which is efficiently regenerated by the system formate/carbon dioxide in the presence of FDH. The course of the reaction was followed by HPLC, and the transformation was complete after 20 h (Figure 1), then a cascade reaction involving five enzymes could be established when the previous reaction configuration was added with fumarate and AspB.

The transformation of fumarate into L-malate through L-Asp and 2-oxalacetate required the combined catalytic action of AspB, StLASPO, L-MDH, catalase, and FDH in the presence of ammonia, formic acid, and NADH (the latter in a substoichiometric amount). In addition, in this case, the multistep reaction was monitored by HPLC where it was possible to follow in a time-dependent manner the evolution of the reaction profile from the presence of the peaks corresponding to fumarate, aspartate, and malate (Figure 2). In the reaction conditions indicated in the experimental section, the flux comes to an end in 5 h.

Fumarate can undergo both water and ammonia addition with appropriate catalysis. Thus, in the presence of fumarase, the equilibrium with L-malate establishes a loop in which at equilibrium, all the concentrations of the involved species remain constant: a steady state. With an NADH recycling system, the net result is the oxidation of formate to carbon dioxide by molecular oxygen, as explained in Scheme 1. The evidence of a dynamic steady state in which intermediates convert each other while their concentration remains constant is based on a number of experimental results (other than HPLC analysis), see below.

In the presence of NADH as the limiting reagent, the decrease in the absorbance at 340 nm (corresponding to NADH maximum absorption) upon addition of L-Mal is due to oxidation of the reduced form of nicotinamide cofactor and indicates that the cycle is active. In fact, the cofactor delivers a hydride ion to 2-oxalacetate, which is the product of the three preceding reaction steps. Thus, ammonia, StLASPO, fumarase, AspB, catalase, and L-MDH were assembled at pH 8.0 and 37 °C in the presence of 0.18 mM NADH (i.e., in the absence of the formic acid/FDH system required to recycle NADH).

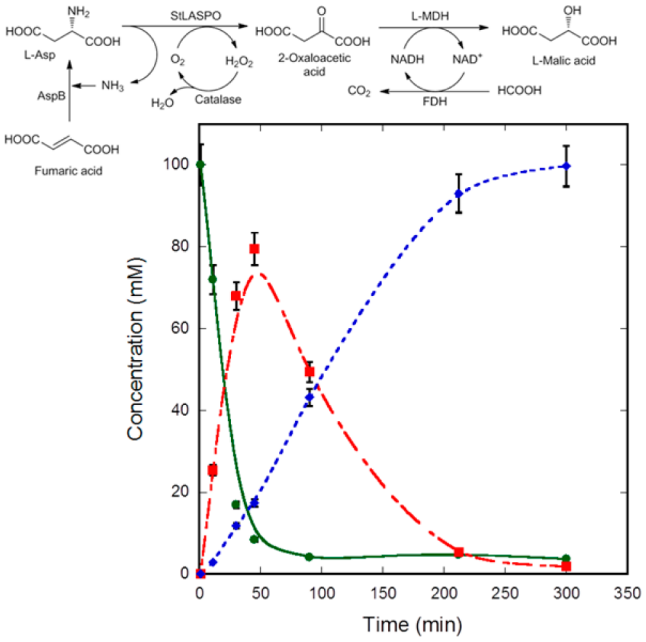


Figure 2. Time course of conversion of fumarate (green) into L-malate (blue) by the three-step enzymatic process catalyzed by AspB, StLASPO, and L-MDH (see Supporting Information for details). The amount of the intermediate L-aspartate is also shown (red).

When L-malate (10 mM, in large excess) was added, the absorbance at 340 nm diminished up to the complete consumption of the cofactor. Successive additions of NADH aliquots restored the cycle, as proved by the renewed decrease in 340 nm absorption (Figure 3).

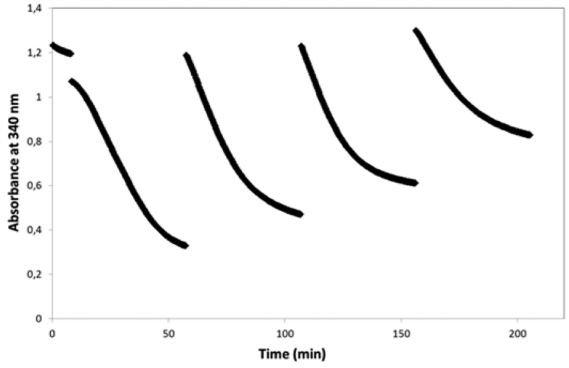


Figure 3. Time course of cyclic conversion of L-malate to L-malate by the four-step enzymatic process catalyzed by fumarase, AspB, StLASPO, and L-MDH (see Supporting Information for details), followed as absorbance of NADH at 340 nm. Each curve represents the addition of 168 μmol of NADH (the limiting substrate).

To assess the actual existence of a continuous catalytic cycle, we built up a system in which malate was used in stoichiometric excess (4-fold higher) compared with NADH, which was discontinuously added cycle after cycle. In the absence of the NADH/NAD^+ recycle (by formate–FDH), if the system was a simple stoichiometric cascade, the decrease in absorption at 340 nm should stop after malate complete consumption. If, conversely, a real cycle was active, malate should be continuously regenerated and any added amount of NADH eventually completely depleted so that the net reaction would be the complete oxidation of the nicotinamide cofactor with simultaneous consumption of dioxygen. As shown in Figure 4, this is the case: successive additions of aliquots of the cofactor

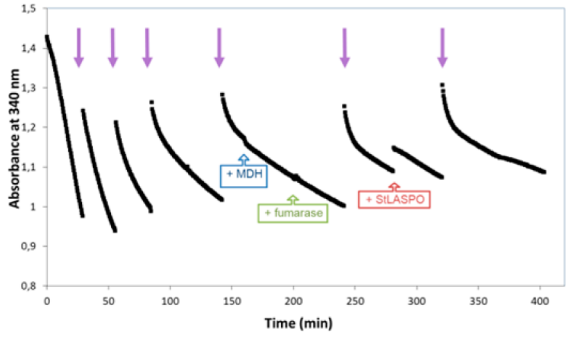


Figure 4. Time course of cyclic conversion of L-malate to L-malate by the four-step enzymatic process catalyzed by fumarase, AspB, StLASPO, and L-MDH (see Supporting Information for details) followed as absorbance of NADH at 340 nm. The reaction resembles the one reported in Figure 3, but with a significant excess of L-malate (0.5 μmol), as compared with NADH (0.12 μmol), and in the absence of the formate–FDH system required to recycle the NADH cofactor. Purple arrows (↓) indicate the addition of 0.12 μmol of NADH: the cycle is controlled by the amount of NADH added to the reaction mixture. Boxes indicate the addition of further aliquots of the enzymes.

(reaching a 1.5-fold molar excess over L-malate) show again NADH consumption corresponding to the flux continuing to run. Under these conditions, the series of reactions constitutes a futile cycle, resulting in the conversion of NADH to NAD^+ by molecular oxygen consumption and using a substoichiometric amount of L-malate (Scheme 1). Intriguingly, NADH works as the substrate of the overall cyclic pathway (it is the limiting factor), and malate is continuously restored (as normally happens for a cofactor). Under these conditions, we can provocatively state that the cycle works as NADH oxidase.

The time course reported in Figure 4 shows that, cycle after cycle, the rate of NADH consumption slows down: addition of aliquots of fresh enzymes does not restore the initial rate, indicating that this deceleration should not be attributed to the inactivation of any of the enzymes. Conversely, the addition of lactate dehydrogenase, which acts on pyruvate (a possible byproduct arising from the spontaneous decarboxylation of 2-oxalacetate) gave a significant effect; in fact, additional NADH is rapidly depleted from the reaction mixture following lactate dehydrogenase addition (data not shown). Altogether, the previous experiment shows that the spectrophotometric assay based on NADH absorption suffers from two main limitations: (i) The interval of linearity of the absorbance at 340 nm is restricted to 0.0–1.0, and the signal saturation is observed at 1.5; moreover, the baseline increases cycle after cycle as a result of the accumulation of NAD^+, which has an absorption tail at 340 nm. Thus, the sensitivity decreases with time. (ii) The assay method is based on the accumulation of an unstable intermediate (2-oxaloacetate) at the end of each cycle. Its partial decomposition to pyruvate alters the cycle kinetics slowing down the total reaction.

Taking into consideration that, during the steady state with recycling of the cofactor, the concentrations of all involved species should remain constant except for formate (which is oxidized to carbon dioxide) and for molecular oxygen (which is reduced to water) and that, among those two species, it is the oxygen concentration that can be easily measured in a continuous way with high precision by employing an

oxygraph,[10] we shifted to a novel assay system to detect the cycle activity. In particular, we took advantage of a Clark-type electrode for monitoring in real time the oxygen depletion from the solution. In such a configuration, 2-oxaloacetate does not accumulate because it is immediately turned into L-malate so that its decomposition does not occur to a significant extent and does not hamper the cycle rate. To sum up, in the presence of L-aspartate, oxygen is continuously consumed; its continuous addition keeps the cycle active. If L-aspartate becomes the limiting factor, oxygen consumption stops and can be restored by addition of a fresh aliquot of the substrate (Figure 5). The reaction comes to an end when all the L-aspartate in the reaction mixture has been consumed. Upon addition of additional substrate, the O_2 consumption starts again.

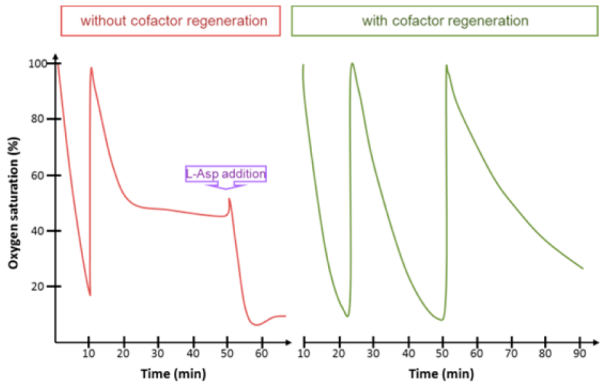


Figure 5. Time course of cyclic conversion of L-aspartate to L-aspartate by the four-step enzymatic process catalyzed by fumarase, AspB, and StLASPO (see Supporting Information for details) followed as oxygen consumption. Red, reaction in the absence of L-MDH; green, the same reaction in the presence of L-MDH.

Interestingly, this metabolic-like pathway can be defined as *nonnatural*, being the reverse of the part of the urea cycle in which fumarate is obtained from L-Asp by the action of argininosuccinate lyase (at the expense of an ATP molecule) and is successively transformed into malate by the enzyme fumarase. Malate is then used by malate dehydrogenase, becoming oxaloacetate and producing a molecule of NADH. Oxaloacetate is eventually recycled to aspartate by the glutamate−aspartate aminotransferase, so the possibility to have the wheel in Scheme 1 turning clockwise is offered by Nature.[11]

The cyclic features of this enzyme-catalyzed reaction sequence offer the opportunity to enter the cycle at any level as well as to produce any of the cycle intermediates. We analyzed this chance by starting the conversion by feeding fumarate or L-malate (10 mM final concentrations). In both cases, the time course of conversion resembled the one reported in Figure 5 (not shown).

Moreover, the enzyme catalyzed reaction sequence can be finalized to the generation of a useful product. In fact, starting from L-malate (or from fumarate), nonnatural D-malate can be obtained. When starting from L-malate and replacing L-MDH with a commercial D-MDH (EC 1.1.1.83), the reaction proceeded with the net formation of D-malate starting from L-malate (Figure 6) so that the net result is a stereoinversion. This event was followed by the depletion of NADH when present in limiting conditions and in the absence of cofactor recycling. When fumarate was the substrate, D-malic acid

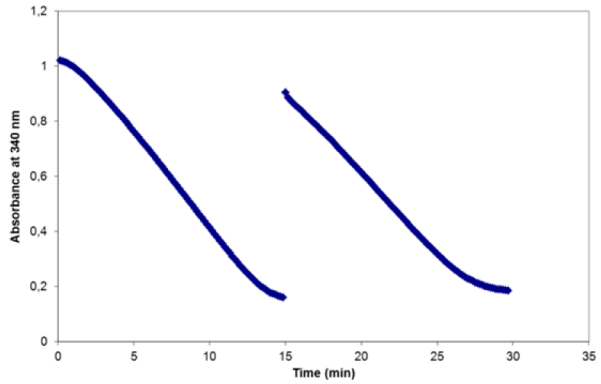


Figure 6. Time course of stereoinversion of L-malate into D-malate by the four-step enzymatic process catalyzed by fumarase, AspB, StLASPO, and D-MDH (see Supporting Information for details), followed as absorbance of NADH at 340 nm. Each curve represents the addition of 168 μmol of NADH (the limiting substrate).

formation in the presence of NADH recycling could be detected by HPLC (data not shown).

In conclusion, we have assembled a series of six enzymes building a cycle involving four components, each characterized by a different functional group. When employing all the six enzymatic catalysts in one pot, the addition of any of the substrates as starting material establishes a steady state in which concentrations of all four components remain unchanged: a futile cycle, mimicking a natural metabolic pathway. Although it can be of general interest to establish and regulate a new metabolic-like futile cycle, this can become productive by stopping the wheel at the desired stage: each of the four compounds can be the initiator of the enzyme-catalyzed sequence. Indeed, it is possible just by changing a single enzyme in the sequence to produce D-malate out of L-malate.

Thermodynamic limitations are overcome both in the closed and open cycle by introducing elements of irreversibility, thus shifting the equilibria toward the desired direction. This system constitutes a proof-of-concept of *systems biocatalysis,* which is a novel approach consisting of organizing enzymes in vitro to generate an artificial metabolism for synthetic purposes. The interconversion of functional groups is the main objective of biocatalysis, and systems organizing a series of enzymes to achieve multistep reactions represent the advanced target of enzymatic catalysis for organic synthesis. The replication of such a system in a structure-independent transformation requires the development of generalistic enzyme catalysts or of alternative/exchangeable enzymes with different substrate preferences.

ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website.

Materials, enzymes and employed devices; analytical procedures; experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

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