Supporting Information

Towards the conversion of CO_2 into optically pure N-carbamoyl-*L*-aspartate and orotate by an *in vitro* multi-enzyme cascade

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Supplemental Figure S1

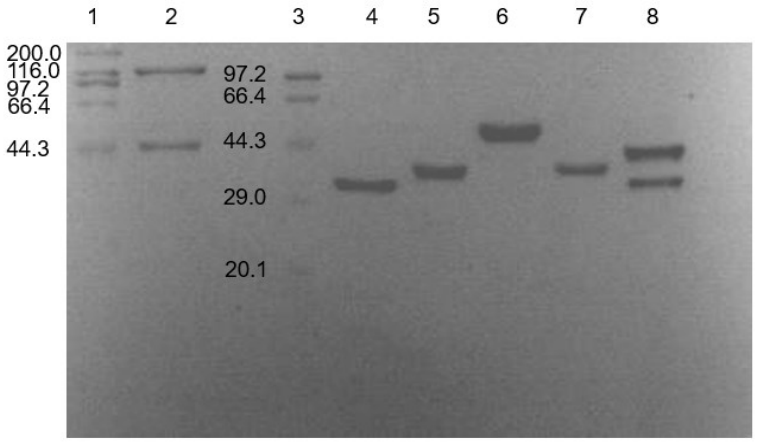

Figure S1. SDS-PAGE analysis of the enzymes used in this study. The codon-optimized genes of all enzymes were synthesized and overexpressed in *E. coli* Rosetta (DE3). Enzymes were purified based on the binding of the His-tag onto nickelcharged resin. 1: protein maker (high); 2: CPS; 3: protein maker (low); 4: *Ec*ATCase; 5: *Aa*ATCase; 6: DHOase; 7: DHOD; 8: PPK. CPS from *E. coli* is a heterodimer with a 41 kDa small subunit carA catalyze the hydrolysis of glutamine and a 118 kDa large subunit carB catalyze the synthesis of carbamoyl phosphate.[1] PPK belongs to PA0141 family. Genomes of this family generally encode 2 or 3 PPK paralogs, which frequently contain a single PPK2 domain.[2] PPK used in this study could not utilize P_3 as a phosphate donor but is capable of regenerating 3 M ATP from 1 M P_6. DHOase from *A. aeolicus* completely lacked catalytic activity unless forms a 1:1 stoichiometric complex with ATCase from the same organism.[3] Thus we overexpressed and purified the *Aa*ATCase. It should be noted that, *Aa*ATCase was only used to "wake up" DHOase, not for the first stage of producing *L*-CAA, which was realized by *Ec*ATCase.

Supplemental Figure S2

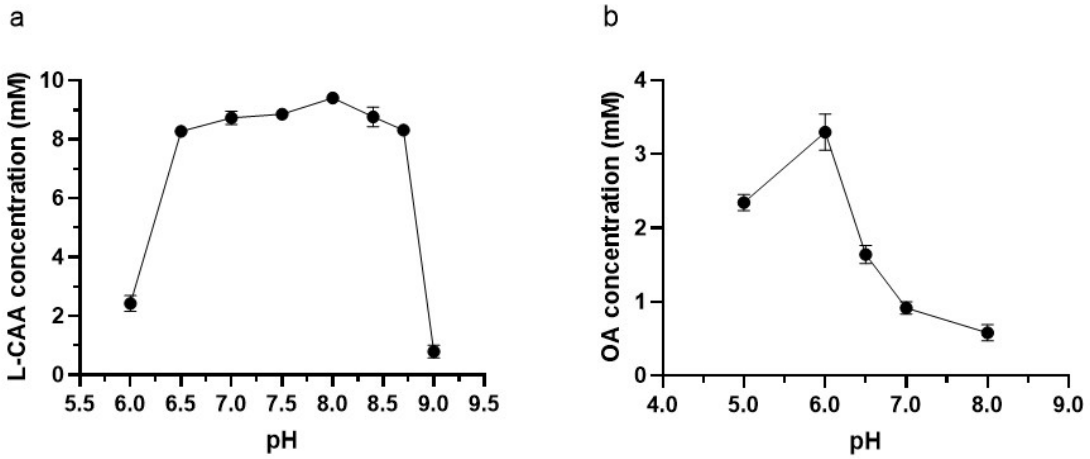

Figure S2. Effect of pH on the reaction. (a) The effect of pH on *L*-CAA synthesis was performed in a 1.5 mL EP tube containing 100 mM KCl, 2 mM ADP, 10 mM L-Asp, 150 mM NH_4HCO_3, 20 mM polyP, 40 mM $MgCl_2$, 3.0 U/mL CPS, 1.0 U/mL ATCase, and 0.5 U/mL PPK. The total volume of the reaction system was 1.0 mL and was performed at 45 °C (in a metal bath) for 30 min. The results showed that when the pH was in the range of 6.5 to 8.7, the generation of *L*-CAA remained above 8 mM. When the pH was greater than 9.0, the precipitation of Mg^{2+} might affect the reaction. The highest *L*-CAA titers were obtained at pH 8.0. **(b)** The effect of pH on OA synthesis was performed in a 1.5 mL EP tube containing 5 mM *L*-CAA (prepared from the first stage), 10 mM fumarate, 0.1 mM $CoCl_2$, 0.1 mM FMN, 1.0 U/mL DHOase (molar ratio 1:1 bound to *Aa*ATCase), and 2.0 U/mL DHOD. The total volume of the reaction system was 1.0 mL and was performed at 65 °C (in a metal bath) for 30 min. The highest OA titers were obtained at pH 6.0. Therefore, the first stage was performed at pH 8.0, and the second stage was conducted at pH 6.0. All measurements were carried out in triplicates.

Supplemental Figure S3

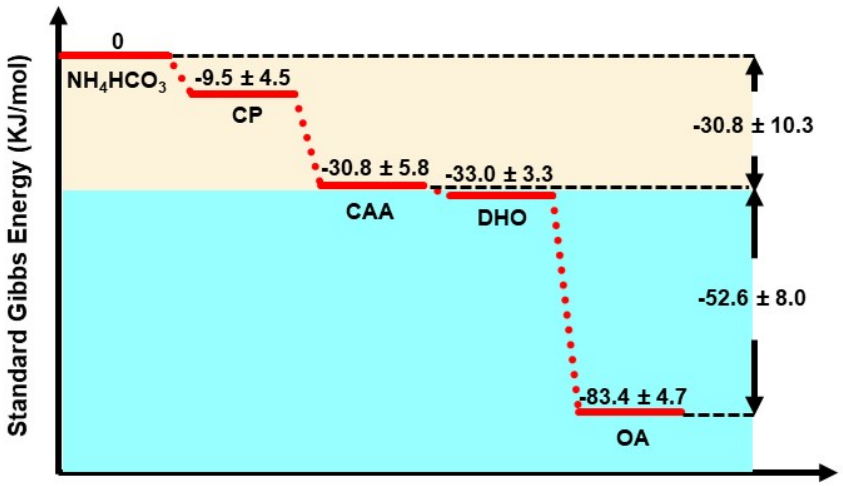

Figure S3. The standard Gibbs energy changes (ΔG'°) of reactions. The first stage was calculated at pH 8.0 while the second stage was calculated at pH 6.0. Data from http://equilibrator.weizmann.ac.il/.

Supplemental Figure S4

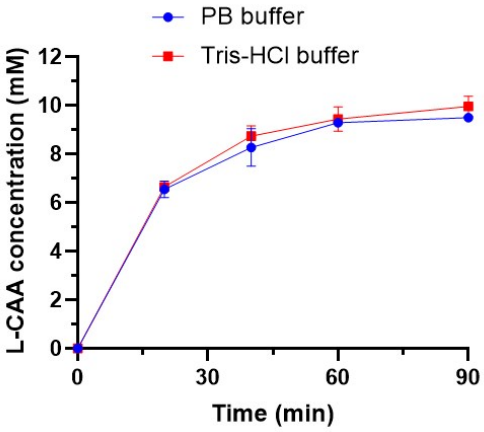

Figure S4. Effects of different buffers on *L*-CAA synthesis in the first stage. The reaction was performed in a 1.5 mL EP tube containing 100 mM KCl, 150 mM NH_4HCO_3, 2 mM ADP, 20 mM polyP, 40 mM $MgCl_2$, 10 mM L-Asp, 3.0 U/mL CPS, 1.0 U/mL ATCase, and 0.5 U/mL PPK in 50 mM PB buffer or 100 mM Tris-HCl buffer, respectively. The results showed that the buffer just had little effect on *L*-CAA production. All measurements were carried out in triplicates.

Supplemental Figure S5

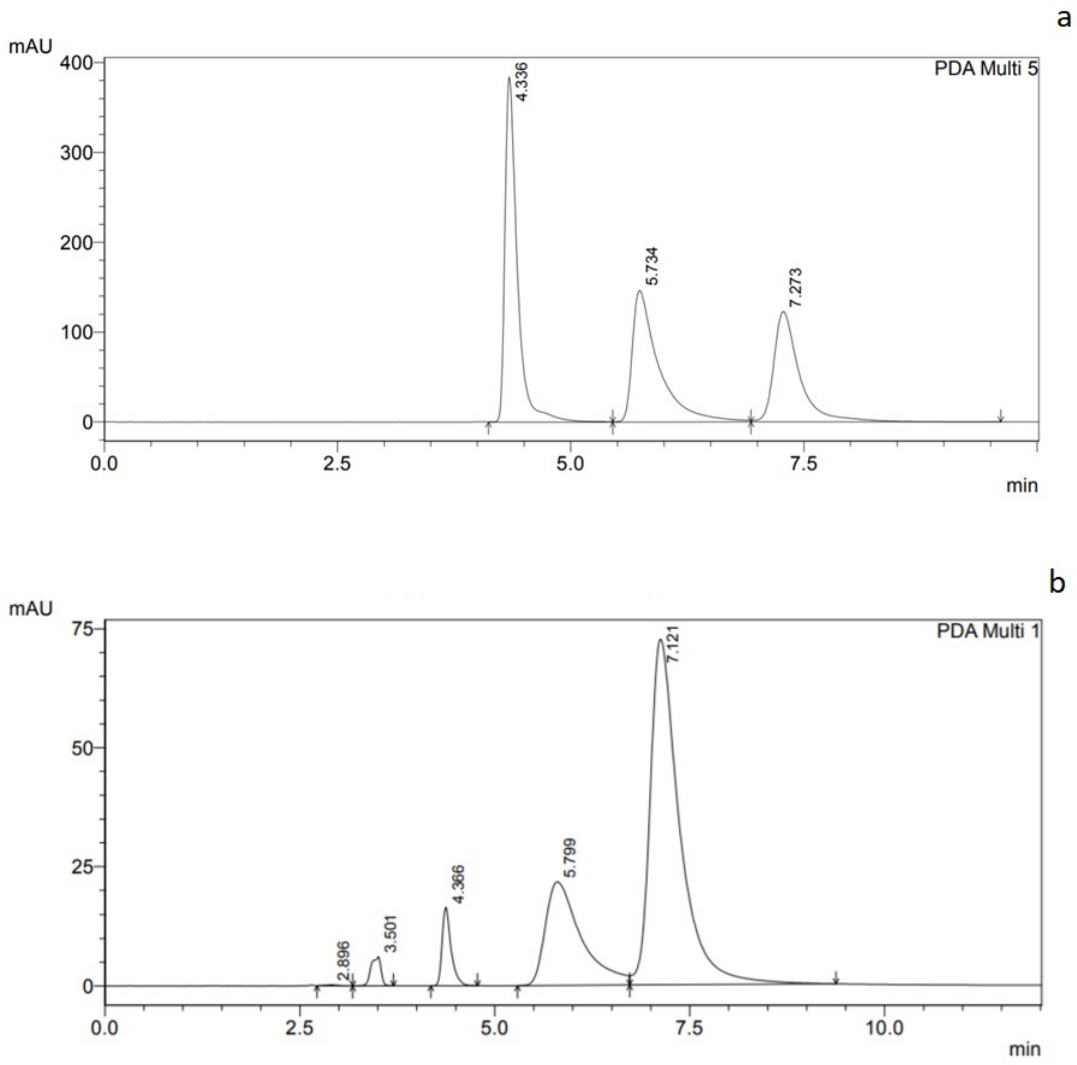

Figure S5. (a) The HPLC profile of the OA, ATP, and ADP standard mixture. OA exhibited a retention time of approximately 4.3 min while ATP was near 5.7 min and ADP was near 7.2 min. **(b) The HPLC analysis of the second stage reaction.** Fumarate exhibited a retention time of approximately 3.5 min.

Supplemental Figure S6

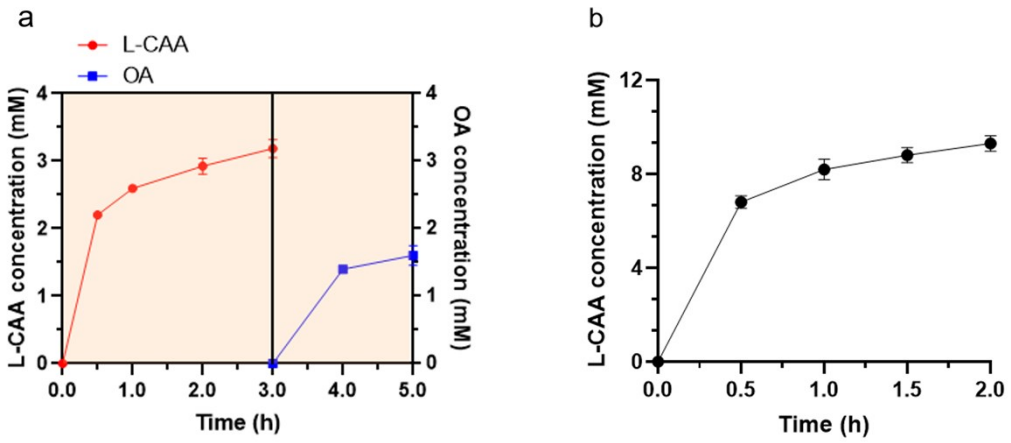

Figure S6. The proof-of-concept biosynthesis of *L*-CAA and OA. (a) In the first stage, *L*-CAA production was performed in 50 mM PB buffer (pH 8.0) containing 100 mM KCl, 50 mM NH_4HCO_3, 2 mM ADP, 20 mM polyP, 40 mM $MgCl_2$, 10 mM Asp, 3.0 U/mL CPS, 1.0 U/mL ATCase, and 0.5 U/mL PPK. In the second stage, OA was produced in *L*-CAA solution (pH 6.0) by adding 0.1 mM $CoCl_2$, 0.1 mM FMN, 10 mM fumarate, 1.0 U/mL DHOase (1:1 stoichiometric complex with *Aa*ATCase) and 1.0 U/mL DHOD. Both reactions were performed at 45 °C. **(b)** *L*-CAA production was performed in 50 mM PB buffer (pH 8.0) containing 100 mM KCl, 10 mM $NaHCO_3$, 10 mM glutamine, 2 mM ADP, 20 mM polyP, 40 mM $MgCl_2$, 10 mM Asp, 3.0 U/mL CPS, 1.0 U/mL ATCase, and 0.5 U/mL PPK. The reaction was performed at 45 °C. All measurements were carried out in triplicates.

Supplemental Figure S7

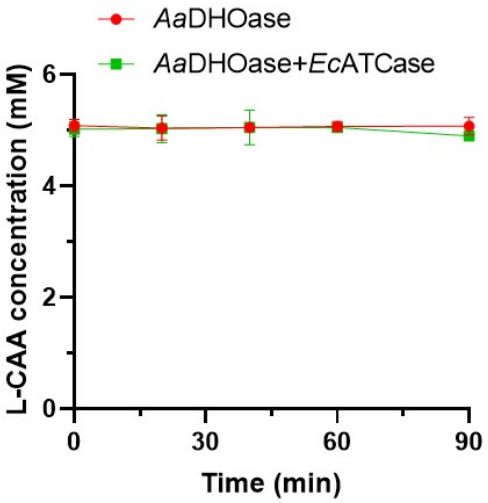

Figure S7. Verifying that DHOase completely lacked catalytic activity. The reaction was performed in a 1.5 mL EP tube containing 5 mM *L*-CAA, 0.1 mM $CoCl_2$, and 1.0 U/mL DHOase. The total volume of the reaction system was 1.0 mL and was performed at 65 °C (in a metal bath). Samples were taken at different point-in-time to monitor the remaining *L*-CAA. After forming a 1:1 stoichiometric complex with *Ec*ATCase, DHOase still completely lacked catalytic activity. All measurements were carried out in triplicates.

Supplemental Table S1

Table S1. The DNA sequences of all enzymes used in this study.

Enzymes	Plasmid	Sequence
CPS	pET28a-*carAB*	ttgattaagtcagcgctattggttctggaagacggaacccagtttcacggtcgggccat aggggcaacaggttcggcggttggggaagtcgttttcaatacttcaatgaccggttat caagaaatcctcactgatccttcctattctcgtcaaatcgttactcttacttatccccatatt ggcaatgtcggcaccaatgacgccgatgaagaatcttctcaggtacatgcacaaggt ctggtgattcgcgacctgccgctgattgccagcaacttccgtaataccgaagacctct cttcttacctgaaacgccataacatcgtggcgattgccgatatcgatacccgtaagctg acgcgtttactgcgcgagaaaggcgcacagaatggctgcattatcgcgggcgataa cccggatgcggcgctggcgttagaaaaagcccgcgcgttcccaggtctgaatggca tggatctggcaaaagaagtgaccaccgcagaagcctatagctggacacaagggag ctggacgttgaccggtggcctgccagaagcgaaaaaagaagacgagctgccgttc cacgtcgtggcttatgattttggtgccaagcgcaacatcctgcggatgctggtggata gaggctgtcgcctgaccatcgttccggcgcaaacttctgcggaagatgtgctgaaaa tgaatccagacggcatcttcctctccaacggtcctggcgacccggccccgtgcgatt acgccattaccgccatccagaaattcctcgaaaccgatattccggtattcggcatctgt ctcggtcatcagctgctggcgctggcgagcggtgcgaagactgtcaaaatgaaattt ggtcaccacggcggcaaccatccggttaaagatgtggagaaaaacgtggtaatgat caccgcccagaaccacggttttgcggtggacgaagcaacattacctgcaaacctgc gtgtcacgcataaatccctgttcgacggtacgttacagggcattcatcgcaccgataa accggcattcagcttccaggggcaccctgaagccagccctggtccacacgacgcc gcgccgttgttcgaccactttatcgagttaattgagcagtaccgtaaaaccgctaagta atcaggagtaaaagagccatgccaaaacgtacagatataaaaagtatcctgattctgg gtgcgggcccgattgttatcggtcaggcgtgtgagtttgactactctggcgcgcaagc gtgtaaagccctgcgtgaagagggttaccgcgtcattctggtgaactccaacccggc gaccatcatgaccgacccggaaatggctgatgcaacctacatcgagccgattcactg ggaagttgtacgcaagattattgaaaaagagcgcccggacgcggtgctgccaacga tgggcggtcagacggcgctgaactgcgcgctggagctggaacgtcagggcgtgtt ggaagagttcggtgtcaccatgattggtgccactgccgatgcgattgataaagcaga agaccgccgtcgtttcgacgtagcgatgaagaaaattggtctggaaaccgcgcgttc cggtatcgcacacacgatggaagaagcgctggcggttgccgctgacgtgggcttcc cgtgcattattcgcccatcctttaccatgggcggtagcggcggcggtatcgcttataac cgtgaagagtttgaagaaatttgcgcccgcggtctggatctctctccgaccaaagagt tgctgattgatgagtcgctgatcggctggaaagagtacgagatggaagtggtgcgtg ataaaaacgacaactgcatcatcgtctgctctatcgaaaacttcgatgcgatgggcatc cacaccggtgactccatcactgtcgcgccagcccaaacgctgaccgacaaagaata tcaaatcatgcgtaacgcctcgatggcggtgctgcgtgaaatcggcgttgaaaccgg tggttccaacgttcagtttgcggtgaacccgaaaaacggtcgtctgattgttatcgaaat gaacccacgcgtgtcccgttcttcggcgctggcgtcgaaagcgaccggtttcccgat tgctaaagtggcggcgaaactggcggtgggttacaccctcgacgaactgatgaacg acatcactggcggacgtactccggcctccttcgagccgtccatcgactatgtggttact aaaattcctcgcttcaacttcgaaaaattcgccggtgctaacgaccgtctgaccactca

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Table S2. The prices of the main substrates and products in the system.

Substance	**Price (RMB/ton)***
polyP	6000
NH_4HCO_3	1300
L-Asp	15000
Orotate	295000
$NaHCO_3$**	1800
Glutamine**	60000

* Data from http://china.chemnet.com/.

** $NaHCO_3$ and Glutamine were the old substrates, which had been replaced with NH_4HCO_3 in our system.

References

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