Discovery of a Substrate Selectivity Motif in Amino Acid Decarboxylases Unveils a Taurine Biosynthesis Pathway in Prokaryotes

Giulia Agnello[†], Leslie L. Chang[‡], Candice M. Lamb[‡], George Georgiou,[‡,§,†,*] and Everett M. Stone[‡,*]

[‡]Departments of Biomedical and Chemical Engineering, [§]Section of Molecular Genetics and Microbiology and [†]Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas 78712.

SUPPORTING INFORMATION

Materials and Methods

Reagents, strains and media: Oligonucleotides and gBlocks were purchased from Integrated DNA Technologies (Coralville, IA). Restriction enzymes, *Phusion* high fidelity DNA polymerase, T4 DNA ligase, dNTPs, NiCo21(DE3) Competent *E. coli* and chitin beads were from New England Biolabs (Ipswich, MA). *O*-phthalaldehyde (OPA) reagent was from Agilent Technologies (Santa Clara, CA). LB and TB growth media was from BD Difco. L-cysteine sulfinic acid monohydrate, L-cysteic acid monohydrate, L-glutamate, L-aspartate, chloramphenicol, pyridoxal 5′-phosphate hydrate and 2-mercaptoethanol (BME) were from Sigma-Aldrich (St. Louis, MO). The Gateway human open reading frame (ORF) collection was from Thermo. Kanamycin was from Fisher. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was from OmniPur. BCA Protein Assay Kit was from Thermo Scientific. Nickel-nitrilotriacetic acid (Ni^{2+}-NTA) resin was from Qiagen.

Molecular Biology methods: Genes encoding the *Polaribacter irgensii 23-P* and *Synechococcus sp. PCC 7335* CSAD-like proteins were assembled synthetically with inclusion of coding sequence of an N-terminal hexahistidine affinity tag using a set of 3-4 gene-optimized overlapping gBlocks fragments designed by DNA Works [1]. Fragments were assembled by the Gibson method [2] using a PCR-amplified pET28-a vector (Novagen) containing overlaps to the first and last gBlocks fragments and transformed in *E.coli* MC1061 electrocompetent cells. The coding regions of the resulting plasmids pPi-CSAD and pSs-CSAD were sequenced

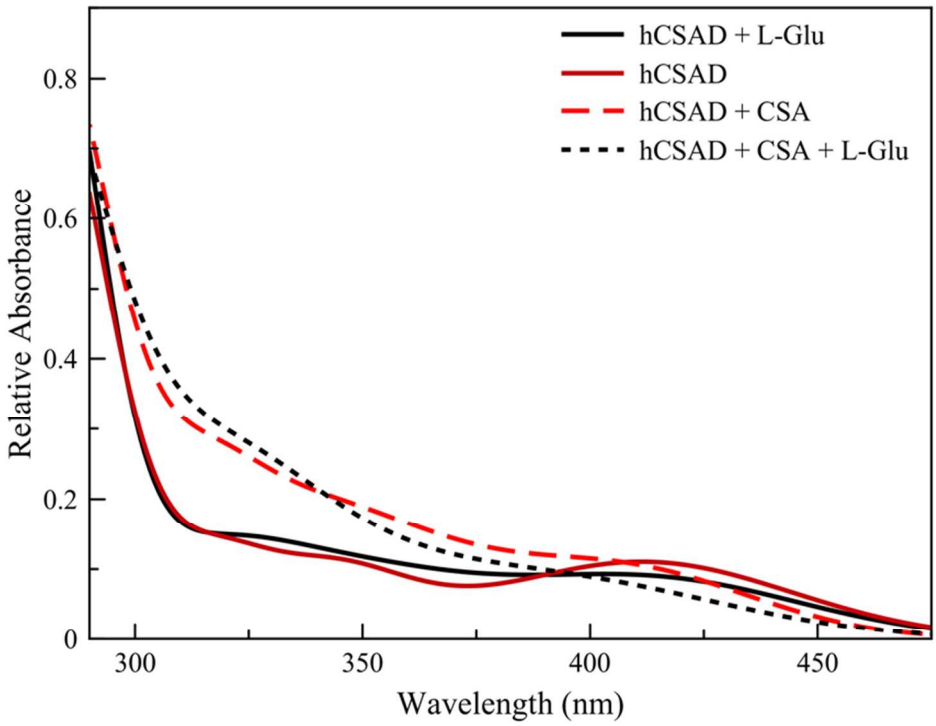
to verify there were no undesired mutations and transformed into *E.coli* BL21(DE3) cells for subsequent expression.

Expression and Purification: E.coli Rosetta (DE3) cells containing pCSAD plasmid were cultured overnight at 37°C in LB medium supplemented with 50µg/mL kanamycin and 34µg/mL chloramphenicol. *E. coli* NiCo21(DE3) cells containing pGAD and *E.coli* BL21(DE3) cells containing pSsCSAD were cultured overnight at 37°C in LB medium supplemented with 50µg/mL kanamycin. *E. coli* BL21(DE3) cells containing pPiCSAD were cultured overnight at 37°C in TB medium supplemented with 50µg/mL kanamycin. The overnight cultures were used to inoculate fresh medium (1:200) and were grown until the optical density at 600 nm (A_{600}) reached a value of 0.7-0.8. The cultures were cooled to 25°C (in the case of pCSAD, pGAD and pSsCSAD) and to 16°C (in the case of pPiCSAD) and supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5mM to induce protein expression. After incubation overnight, the cells were harvested by centrifugation at 3500rpm for 20min at 4°C. The cell pellets were re-suspended in cold lysis buffer [10mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, (pH 8.0)] and kept on ice. The cell suspensions were lysed by two passes through a French pressure cell, filtered using 0.45µm pore membranes, and subsequently pelleted at 14000rpm for 20 min at 4°C. All subsequent purification steps were performed at 4°C. The resulting supernatant (soluble fraction) was applied to 0.5mL of nickel-nitrilotriacetic acid (Ni2+-NTA) resin pre-equilibrated with lysis buffer. The resin was washed with 30 bed volumes of lysis buffer and 15 bed volumes of wash buffer [10mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole, (pH 8.0)]. The resin was incubated with 6 bed volumes of elution buffer [50mM NaH_2PO_4, 300mM NaCl, 250mM Imidazole, (pH 8.0)] for 10 minutes, after which the eluate was collected dropwise. In the case of hGAD-67 kDa, the elution fraction was applied to 2mL of chitin beads, incubated for 10 minutes and then eluted with additional 4mL of elution buffer. The elution fraction was applied to an Amicon Ultra 30K MWCO filter, buffer exchanged against activity buffer [100mM NaH_2PO_4 buffer, 10µM PLP (pH 7.4)] 5-6 times, mixed with glycerol (10% final concentration) and finally snap-frozen with liquid nitrogen and stored at -80°C. An identical protocol was followed for expression of hCSAD and hGAD-67 kDa variants. Protein concentrations of hCSAD and hCSAD mutants were determined using a calculated extinction coefficient of 69,822 M^{-1} cm^{-1} [3]. Protein

concentrations of hGAD-67 kDa and hGAD-67 kDa variants were determined using the BCA assay [4]. All the proteins purified were analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4 to 20% precast Tris-Glycine gel (NuSep Ltd.) run under reducing conditions (2.5% BME) and stained with GelCode Blue (Thermo Scientific). When necessary, proteins that displayed partially purity (e.g. PiCSAD) were assessed by SDS-PAGE gel band intensity using a densitometry imaging program (ImageQuant, Amersham Biosciences) to estimate concentration. The expression of hCSAD, hGAD-67 kDA, PiCSAD and SsCSAD was confirmed by Western Blotting using mouse monoclonal anti-polyhistidine peroxidase.

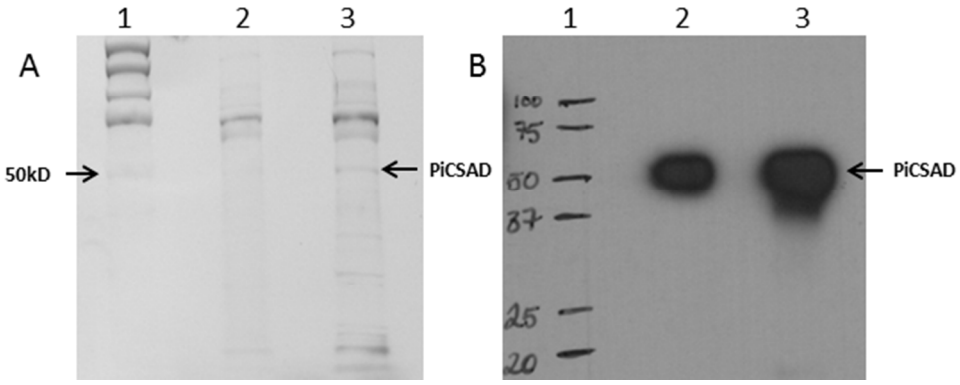
SI Figure 1.

UV-Vis absorption scans of hCSAD holoenzyme (red solid line), hCSAD plus L-Glu (black solid line), hCSAD plus CSA (red dashed line) and hCSAD plus CSA and L-Glu (black dashed line).



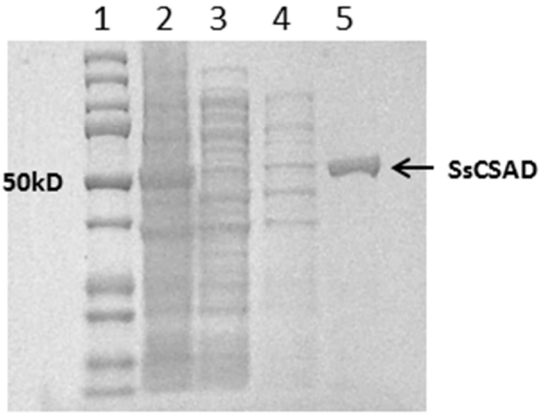
SI Figure 2.

A) SDS-PAGE gel stained with coomassie blue and **B)** western blot probed with anti-polyhistidine peroxidase of partially purified recombinant *N*-term His_6-tagged *Polaribacter irgensii 23-P* CSAD (PiCSAD) expressed in *E.coli*. Lane 1: molecular weight ladder, lanes 2 and 3: separate purification preparations.



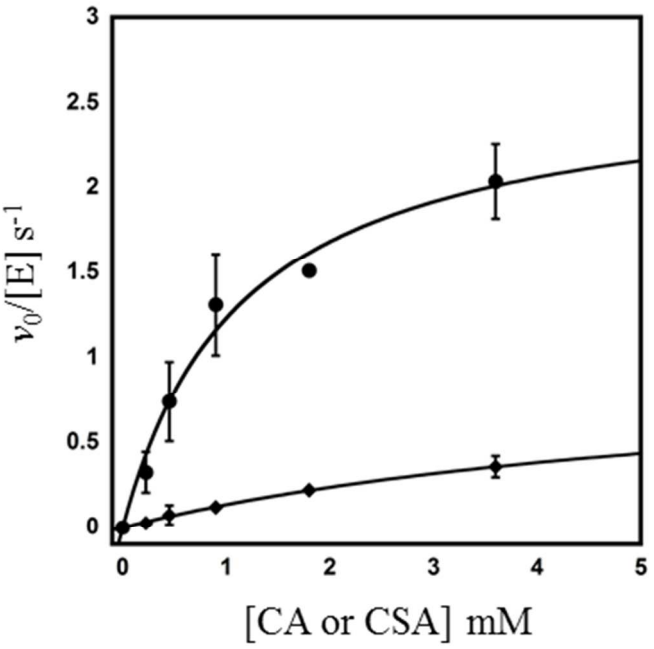
SI Figure 3.

SDS-PAGE gel stained with coomassie blue of recombinant *N*-term His_6-tagged *Synechococcus sp. PCC 7335* CSAD_(SsCSAD) expressed in *E.coli.* Lane 1: molecular weight ladder, lane 2: whole cell lysate, lane 3: soluble fraction, lane 4: flow though, and lane 5: final purified protein.

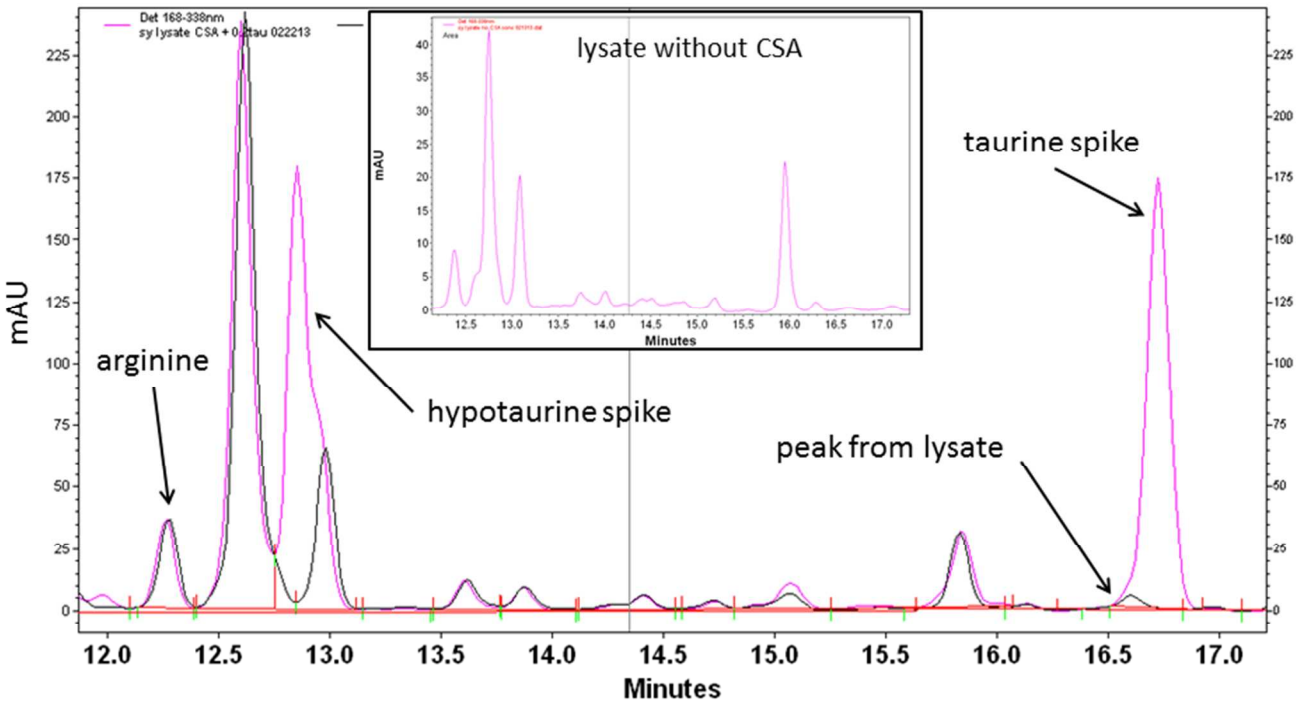


SI Figure 4.

Plot of the initial rates of product formation from recombinant SsCSAD incubated with CA (♦) or CSA (●).



SI Figure 5. High performance liquid chromatography (HPLC) traces of a representative lysate from *Synechococcus sp. PCC 7335* cultured with CSA and an overlay with the same lysate spiked with 0.2 mM hypotaurine/taurine. Inset: chromatogram of lysate from *Synechococcus sp. PCC 7335* cultured without CSA.



SI Table 1. Oligonucleotides used for cloning and construction of hGAD-67 kDa and hCSAD genes.

Variant	Primer	Sequence
hCSAD WT	hCSAD-F hCSAD-R	5'-atagtagctagcatggctgactcagaagcactcccctccc-3' 5'-gctattgaattctcacaggtcctggcctagccgctccagctc-3'
hCSAD F94S	hCSAD-F94S-F hCSAD-F94S-R	5'-cggttcttcaaccagctctcctctgggttggatccc-3' 5'-gggatccaacccagaggagagctggttgaagaaccg-3'
hCSAD S114N	hCSAD-S114N-F hCSAD-S114N-R	5'-cgcattatcactgagagcctcaacaccaaccagtacacatatgaaatc-3' 5'-gatttcatatgtgtactggttggtgttgaggctctcagtgataatgcg-3'
hCSAD Y116F	hCSAD-Y116F-F hCSAD-Y116F-R	5'-ctcaacaccagccagtttacatatgaaatcgcccccgtgtttgtg-3' 5'-cacaaacacgggggcgatttcatatgtaaactggctggtgttgag-3'
hCSAD S114N Y116F	hCSAD-S114N-Y116F-F hCSAD-S114N-Y116F-R	5'gctctggccgggcgcattatcactgagagcctcaacaccaaccagttcacatatgaaatc-3' 5'gatttcatatgtgaactggttggtgttgaggctctcagtgataatgcgcccggccagagc-3'
hCSAD F94S S114N Y116F	hCSAD-F94S-S114N-Y116F-F hCSAD-F94S-S114N-Y116F-R	For this variant we used the primers hCSAD-S114N–Y116F-F and –R and hCSAD-F94S as a template
hGAD WT	hGAD-F hGAD-R	5'-ctcgcggtagaattcatggcgtcttcgaccccatc-3' 5'-attcttatgcggccgcttacagatcctggcccagtctttctatc-3'
hGAD S192F	hGAD-S192F-F hGAD-S192F-R	5'-cctcgatttttcaaccagctcttcactggattggatattattggc-3' 5'-gccaataatatccaatccagtgaagagctggttgaaaaatcgagg-3'
hGAD N212S	hGAD-N212S-F hGAD-N212S-R	5'-ctgacatcaacggccaataccagcatgtttacatatgaaattgcaccagtg-3' 5'-cactggtgcaatttcatatgtaaacatgctggtattggccgttgatgtcag-3'
hGAD F214Y	hGAD-F214Y-F hGAD-F214Y-R	5'-gccaataccaacatgtacacatatgaaattgcaccagtgtttgtc-3' 5'-gacaaacactggtgcaatttcatatgtgtacatgttggtattggc-3'
hGAD N212S F214Y	hGAD-N212S-F214Y-F hGAD-N212S-F214Y-R	5'ggagaatggctgacatcaacggccaataccagcatgtacacatatgaaattgcaccagtg-3' 5'cactggtgcaatttcatatgtgtacatgctggtattggccgttgatgtcagccattctcc-3'
hGAD S192F N212S F214Y	hGAD-S192F-N212S-F214Y-F hGAD-S192F-N212S-F214Y-R	For this variant we used the primers hGAD-N212S–F214Y-F and –R and hGAD-S192F as a template

SI References

1. Hoover DM, Lubkowski J (2002) DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis. Nucleic acids research 30: e43-e43.
2. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods 6: 343-345.
3. Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. Analytical biochemistry 182: 319-326.
4. Wiechelman KJ, Braun RD, Fitzpatrick JD (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Analytical biochemistry 175: 231-237.