AcsD catalyzes enantioselective citrate desymmetrization in siderophore biosynthesis

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Fig. S1: Alignment of type A, B and C NIS synthetases. Highlighted in red: residues binding ATP in AcsD that are absolutely conserved. Highlighted in blue: residues binding ATP in AcsD not totally conserved. *: AcsD residues mutated in this study.

Protein sequences and Genbank references used in constructing the alignment

Type A AcsD - Pectobacterium chrysanthemi gi|16118732; PvsD - vibrioferrin biosynthesis protein *Vibrio parahaemolyticus* gi|23307118; IucA - IucA protein *E. coli* CFT073 gi|26109870. Type B AcsA - AcsA *P. chrysanthemi* gi|16118729; SbnC - SbnC *Staphylococcus aureus* gi|32441989; PvsB - vibrioferrin biosynthesis protein PvsB *V. parahaemolyticus* gi|23307116. Type C IucC - IucC *E. fergusonii* gi|22034306; DesD - hypothetical protein SCO2785 *Streptomyces coelicolor* A3(2) gi|21221236; AcsC - AcsC *P. chrysanthemi* gi|16118727.
**Fig. S2:** AcsD activity assays to identify substrates. (a) Hydroxylamine acts as a surrogate nucleophilic substrate allowing probing of the carboxylic acid substrate specificity of AcsD by monitoring at 540 nm. (b) Time dependent change in absorbance at 540 nm following incubation of citric acid, hydroxylamine, ATP, MgCl$_2$ and His$_6$-AcsD (black ■), and with a control of heat-inactivated His$_6$-AcsD (open ○). (c) Relative cpm obtained for the release of $[^{32}$P] from $[^{32}$P]-ATP with time, for incubation mixtures with Citric acid, L-serine, $[^{32}$P]-ATP, MgCl$_2$ and His$_6$-AcsD relative to those experiments carried out with His$_6$-AcsD boiled for inactivation (d + e) Cpm values obtained after incubation of Citric acid or α-Ketoglutaric acid, $[^{32}$P]-ATP, MgCl$_2$, Tris buffer (50 mM, pH8) and with one of the following solutions: 2,4-diaminobutyric acid (DAB) or ethanolamine (Eth) or D-serine (D-Ser) or L-serine (L-Ser). The reaction was initiated by addition of His$_6$-AcsD (■). The $[^{32}$P] released in the supernatant (d) from the $[^{32}$P]-ATP, during the reaction, and the $[^{32}$P]-ATP binding the charcoal (e) were measured by scintillator counter. The controls solutions were carried out with His$_6$-AcsD inactivated 10 min at 373 K (†). (f) Raw data for the decrease in fluorescence from NADH with time in coupled assays for AMP production with AcsD, citric acid, ATP, Mg$^{2+}$ and different nucleophilic substrates. L-serine (black □) is the preferred substrate compared to D-serine (orange ▲) or ethanolamine (olive ◇). Hydroxylamine (grey ○) as second smallest nucleophiles (water would be the smallest) accelerates the reaction much faster as reactions where any nucleophiles was omitted (blue ○). Reactivity of all other tested substrates (L-alanine (purple △), β-alanine (dark yellow □) and glycine (dark cyan ▼)) fell in the same range as those observed for reactions lacking any nucleophiles (blue ○). This rest activity can be attributed to water acting as the nucleophile. Reactions lacking His$_6$-AcsD (wt) but containing L-serine (red ▼) showed no detectable activity. (g) Stereo selectivity of AcsD, time course of NADH consumption with D-serine (4 repeats), L-serine (2 repeats) and control reactions (no AscD). The plots are not corrected for background rate. (h) The rate of NADH consumption (corrected for background) with a serine racemate and D-serine resulted in only ~90 % and ~22 % of the rate using L-serine. This indicates that AcsD is stereo-selective for L-serine. (i) NADH consumption in the modified AcsD activity assay using His$_6$-AcsD to demonstrate AMP (and by implication PP$_i$) formation by AcsD. Omitting myokinase (orange circles) resulted in no detectable activity. Thus AcsD cannot itself form ADP (and generate P$_i$), as ADP is the substrate of pyruvate kinase (the next enzyme in coupled assay) and its creation by AcsD would have been detected. Myokinase converts AMP and ATP into ADP. ATP is present in the reaction mixture and AMP must therefore be produced by AcsD.

(a) ![Chemical Structure](image)

(b) ![Diagram](image)
Fig. S3: Analysis and characterization of N-citryl-L-serine isolated from the incubation of citric acid and L-serine with recombinant AcsD, ATP, and Mg^{2+}. (a) ESI-TOF-MS spectroscopic analysis. Top panel: measured spectrum. Bottom panel: simulated spectrum. (b) ¹H NMR spectrum (D₂O, 700 MHz), (c) ¹³C NMR spectrum (D₂O, 500 MHz), (d) COSY spectrum (D₂O, 700 MHz), (e) HSQC spectrum (D₂O, 700 MHz) and (f) HMBC spectrum (D₂O, 700 MHz) of N-citryl-L-serine isolated from the incubation of citric acid and L-serine with recombinant AcsD, ATP, and Mg^{2+}.
**Fig. S4**: Time course of the AcsD-catalyzed condensation of citric acid with L-serine. MS/MS analyses indicate that the concentration of O-citryl-L-serine decreases relative to the concentration of N-citryl-L-serine with increasing incubation time. These data suggest that O-citryl-L-serine is rearranging to N-citryl-L-serine in the incubation.
1 hour 30 minutes

-MS2(278.0), 0.5-0.5min #(28-30)

-MS2(278.0), 0.7-0.8min #(35-38)

m/z

110.9 156.5 197.7 259.7

Intens. x10^4

0 0.5 1.0 1.5 2.0 2.5 3.0
**Fig. S5:** AcsD kinetic assays (a) Calibration of the fluorescence based AcsD activity assay. At high NADH concentrations (>1 mM) fluorescence intensity starts decreasing due to fluorescence quenching effects. (b) Even at lower concentrations of NADH the conversion from fluorescence intensity to concentration is not precisely linear. This effect occurs in water (black) as well in reaction buffer (minus AcsD) (blue). The standard curve was measured every day in buffer for each set of experiments and used to derive NADH concentration from fluorescence readings. (c) The AcsD activity assay measures production of AMP in coupled assay. A control with 2 µM AMP (pink □) shows a ~6.5 fold faster reaction than 2 µM His<sub>6</sub>-AcsD (wt, black □) indicating AcsD is rate limiting. A reaction without L-serine (orange ⭐) shows residual activity, suggesting water molecules can act as a nucleophile. No activity was observed in reaction where ATP (blue ◊) or myokinase (purple ○) or citrate (green ▽) or AcsD (red ∆) was omitted. (d) AcsD activity assay for wt AcsD (black △), H444A (blue ◊), H444N (green ▽) and the control with no AcsD (red □). (e) Data for the R305 mutants. (f) Background subtracted turnover rates for mutants. (g) Enzyme kinetics for citrate, fitted with Michaelis Menten equation (dotted lines), corrected for background rate. (h) Derived apparent kinetic parameters for citrate. The values were corrected by subtracting the background rate.

![Graph a](image-a)

![Graph b](image-b)
| MgCl₂ | K_M (mM) | V_max (µM/min) | k_cat (s⁻¹) | k_cat/K_M (M⁻¹ s⁻¹) |
|-------|----------|----------------|-------------|---------------------|
| 15 mM | 5.96 ± 0.86 | 71.44 ± 4.06 | 2.14 x 10⁴ | 3.6 x 10⁷ |
| 30 mM | 14.67 ± 0.02 | 73.24 ± 0.046 | 2.20 x 10⁴ | 1.5 x 10⁵ |

Fig. S6: Additional structural biology. (a) Surface representation of the water filled cavity adjacent to the ATP binding pocket. We suggest that PP_i is displaced into this pocket during catalysis. In this Figure, AcsD is rotated 180° compared to the view in Fig. 2. The atoms of AcsD are colored as Fig. 2. (b) 2F_o-F_c map of ATP contoured at 1.0 σ (c) F_o-F_c map of ATP contoured at 2.5 σ. (d) 2F_o-F_c map of adenosine, sulfate and citrate contoured at 1.0 σ (e) F_o-F_c map of adenosine, sulfate and citrate contoured at 2.5 σ. (f) Adenosine (carbon colored green) and sulfate (highlighted in blue) from the citrate adenosine AcsD complex overlap with ATP (carbon colored yellow) from the ATP AcsD complex (based on superposition of protein atoms). (g) List of interactions between AcsD – B and citrate. (h) List of interactions between AcsD and ATP.
| Citrate atom | AcsD residue and atom | Distance Å | Domain |
|--------------|-----------------------|------------|--------|
| OA 1/0       | Thr 282OG1             | 3.2        | 2 - loop5 |
| OA 1/0       | Arg 369NH1             | 3.7        | 2 – β 12 |
| OA 1/0       | Lys 293CE              | 3.5        | 2 - β 10 |
| OA 2/0       | Arg 369NH1             | 3.57       | 2 – β 12 |
| CAC /0       | Asp 464OD2             | 3.54       | 3 – loop 10 |
| CA /0        | Asp 464OD2             | 3.18       | 3 – loop 10 |
| CA /0        | Ser 279CB              | 3.68       | 2 - loop5 |
| CA /0        | Water O/42             | 3.33       |         |
| CB /0        | Asp 464OD2             | 3.22       | 3 – loop 10 |
| CBC /0       | Arg 281NH2             | 3.54       | 2 - loop5 |
| CBC /0       | Arg 281NE              | 3.37       | 2 - loop5 |
| CBC /0       | Ser 279CB              | 3.57       | 2 - loop5 |
| OB 2/0       | Ser 279CB              | 3.24       | 2 - loop5 |
| OB 2/0       | Ser 279O               | 3.45       | 2 - loop5 |
| OB 2/0       | Water O/222             | 2.48       |         |
| OB 2/0       | Arg 281NE              | 3.36       | 2 - loop5 |
| OB 1/0       | Lys 293CE              | 3.19       | 2 – β 10 |
| OB 1/0       | Arg 281CZ              | 3.26       | 2 - loop5 |
| OB 1/0       | Arg 281NE              | 2.80       | 2 - loop5 |
| OB 1/0       | Arg 281NH2             | 3.09       | 2 - loop5 |
| OHB /0       | Glu 466OE1             | 2.99       | 3 – loop 10 |
| OHB /0       | Asp 464OD1             | 3.44       | 3 – loop 10 |
| OHB /0       | Asp 464CG              | 3.28       | 3 – loop 10 |
| OHB /0       | Asp 464OD2             | 2.45       | 3 – loop 10 |
| OGC /0       | Asp 464OD2             | 3.64       | 3 – loop 10 |
| OG 1/0       | His 44NE2              | 3.65       | 3 – loop9 |
| OG 2/0       | Arg 305 NH2            | 2.93       | 2 – loop7 |
| ATP atom | P   | AcSD residue and atom | Distance Å | Domain |
|----------|-----|-----------------------|------------|--------|
| O1G/1    | γ   | Gln 446 NE2            | 3.17       | 3 – loop9 |
| O1G/1    | γ   | Gln 446 OE1            | 3.20       | 3 – loop9 |
| O1G/1    | γ   | Mg ion                 | 2.04       |        |
| O1G/1    | γ   | Asp 464 OD2            | 3.22       | 3 – loop 10 |
| O2G/1    | γ   | Arg 369 NH2            | 2.65       | 2 – β 12 |
| O2G/1    | γ   | Lys 293 NZ             | 2.94       | 2 – β 10 |
| O2G/1    | γ   | Asp 464 OD1            | 2.63       | 3 – loop 10 |
| O2G/1    | γ   | Asp 464 OD2            | 3.36       | 3 – loop 10 |
| O3G/1    | γ   | Arg 369 NH1            | 3.03       | 2 – β 12 |
| O3G/1    | γ   | Ser 279 OG             | 2.90       | 2 – loop 5 |
| O3G/1    | γ   | Thr 282 OG1            | 2.74       | 2 – loop 5 |
| O3G/1    | γ   | HOH 123                | 3.02       |        |
| O3B/1    | γ   | Ser 279 OG             | 3.26       | 2 – loop 5 |
| O1B/1    | β   | Arg 281 NH2            | 3.24       | 2 – loop 5 |
| O1B/1    | β   | Arg 281 NE             | 2.76       | 2 – loop 5 |
| O1B/1    | β   | HOH 239                | 2.91       | 2        |
| O1B/1    | β   | Asp 464 OD2            | 3.39       | 3 – loop 10 |
| O2B/1    | β   | Asp 464 OD2            | 2.91       | 3 – loop 10 |
| O2B/1    | β   | Asp 464 OD1            | 3.27       | 3 – loop 10 |
| O2B/1    | β   | Lys 293 NZ             | 2.90       | 2 – β 10 |
| O2B/1    | β   | Glu 466 OE1            | 3.47       | 3 – loop 10 |
| O3A/1    | β   | Asp 464 OD2            | 3.47       | 3 – loop 10 |
| O3A/1    | β   | HOH 139                | 2.51       |        |
| O1A/1    | α   | Arg 305 NH2            | 3.04       | 2 – loop 7 |
| O3A/1    | α   | HOH 140                | 3.02       |        |
| O1A/1    | α   | Asp 464 OD2            | 3.39       | 3 – loop 10 |
| O2A/1    | α   | His 444 NE2            | 2.92       | 3 – loop 9 |
| O2A/1    | α   | Mg ion                 | 2.45       |        |
| O5/1     | α   | Arg 305 NH2            | 3.01       | 2 – loop 7 |
| O5/1     | α   | HOH 139                | 3.07       |        |
| O4/1     | ribose | Gin 466 CB            | 3.23       | 3 – loop 9 |
| O2/1     | ribose | Thr 301 OG1           | 2.85       | 2 – loop 7 |
| O2/1     | ribose | Thr 301 CG2           | 3.04       | 2 – loop 7 |
| O2/1     | ribose | HOH 6                 | 3.08       |        |
| N1/1     | base | Asn 509 ND2           | 3.11       | 3 – α 13 |
| N1/1     | base | Asn 509 ND1           | 3.48       | 3 – α 13 |
| C2/1     | base | His 444 ND1           | 3.31       | 3 – loop 9 |
| C2/1     | base | His 444 CE1           | 3.49       | 3 – loop 9 |
| N6/1     | base | Asn 509 OD1           | 3.45       | 3 – α 13 |
| N6/1     | base | HOH 56                | 2.52       |        |
| N6/1     | base | Leu 445 CD1           | 3.47       | 3 – loop 9 |
| N7/1     | base | HOH 56                | 2.76       |        |
| N7/1     | base | His 170 CA            | 3.41       | 2 – loop 4 |
Fig. S7: Similarity between the cAPK fold and AcsD suggests a chemical link between the enzyme families. In each structure the position of ATP is shown in magenta (1E8X a cAPK kinase) or yellow (AcsD). In orange, the glycine rich loop, L5, in green, key catalytic residues on L9 and L10 and in red conserved residues are shown, making important interactions with ATP or Mg$^{2+}$. (a) AcsD is shown in ribbon with ATP from AcsD and 1E8X (superposed) bound, 1E8X which is shown in black matches in part the secondary structure. (b) Close up of the matching region of secondary structure (c) The similar structural region of AcsD (left) compared with 1E8X (right) showing ATP bound in different orientation with key residues. (d) The AcsD protein structure is shown in grey space fill, ATP (colored in yellow) binds in large pocket. Superimposed is the ATP from 18EX (colored in magenta). This binding conformation is not possible in AcsD as it penetrates into the structure. (e) Schematic illustration of the ATP orientation in AcsD (in red) and in kinases (in blue). The key electrophilic phosphate $\alpha$ in AcsD and $\gamma$ in kinases superimpose.
**Fig. S8:** Overexpression of *acsD* in *E. coli*, purification and analysis of recombinant His<sub>6</sub>-AcsD. (a) 8 % SDS-PAGE analysis of AcsD overproduction and purification. Lane 1 = molecular weight standards (kDa). Lane 2 = soluble protein fraction after elution from Ni-NTA column. Lane 3 = AcsD after tag removal. (b) Chromatogram from gel filtration analysis of AcsD, Lane 3 in (a). The observed retention volume indicates that it is a dimer. The peak marked 4 when analyzed did not contain AcsD (or any protein).
### Table S1: Data collection and refinement statistics.

|                  | Apo              | SAD (Se Met)    | Citrate (overlaps with ATP) | ATP              | Adenosine–citrate – SO₄<sup>2-</sup> |
|------------------|------------------|----------------|-----------------------------|------------------|---------------------------------------|
| **Data collection** |                  |                |                             |                  |                                       |
| Space group      | P2₁,₂,₂₁        | P2₁,₂,₂₁       | P2₁,₂,₂₁                    | P2₁,₂,₂₁         | P1                                    |
| Cell dimensions  | a = 80.3,       | a = 80.4,      | a = 79.9,                   | a = 57.6,        |                                       |
| (Å), (°)         | b = 95.7,       | b = 95.6,      | b = 94.5,                   | b = 69.10,       |                                       |
|                  | c = 161.1       | c = 160.5      | c = 160.3                   | c = 57.8,        |                                       |
| Resolution (Å)* | 50.0-2.25       | 50.0-2.63      | 81.6-2.8                    | 81.1-2.2         | 45.9 - 2.95                           |
| (high resolution)| 2.38-2.25       | 2.95-2.8       | 11.4 (38.1)                 | 14.3 (44.5)      |                                       |
|                  | 9.8 (40.2)      | 15.7 (40.7)    | 15.7 (5.9)                  | 6.2 (1.9)        |                                       |
|                  | 94.4 (91.5)     | 99.9 (99.9)    | 98.2 (98.5)                 |                  |                                       |
|                  | 3.0 (3.0)       | 4.2 (4.3)      | 10.1 (7.3)                  | 2.0 (2.0)        |                                       |
|                  | 2.2             | 2.2            | 2.1                         | 2.6              |                                       |
| **Refinement**   |                  |                |                             |                  |                                       |
| Unique reflections| 56200           | 37598          | 30736                       | 61355            | 29165                                 |
| R<sub>work</sub> / R<sub>free</sub> | 18.6 / 24.1 | 20.2 / 28.0 | 21.6 / 27.7                | 20.2 / 26.7      |                                       |
| No. atoms        |                  |                |                             |                  |                                       |
| Protein          | 9250            | 9215           | 9252                        | 9361             |                                       |
| Water            | 505             | 130            | 277                         | 73               |                                       |
| Citrate          | 0               | 13             | 0                           | 13               |                                       |
| ATP              | 0               | 0              | 62                          | 0                |                                       |
| Adenosine        | 0               | 0              | 0                           | 38               |                                       |
| L-serine         | 0               | 0              | 7                           | 0                |                                       |
| Mg               | 0               | 0              | 2                           | 0                |                                       |
| SO₄<sup>2-</sup>| 0               | 0              | 10                          |                  |                                       |
| B-factors (Å<sup>2</sup>) |           |                |                             |                  |                                       |
| Protein          | 17.0            | 12             | 12                          | 20               |                                       |
| Water            | 25.3            | 12             | 12                          | 9                |                                       |
| Citrate          | 0               | 4              | 9                           | 27               |                                       |
| ATP              | 0               | 11             | 12                          | 0                |                                       |
| Mg               | 0               | 0              | 23                          | 0                |                                       |
| L-serine         | 0               | 0              | 13                          | 0                |                                       |
| Adenosine        | 0               | 0              | 0                           | 39               |                                       |
| SO₄<sup>2-</sup> | 0               | 0              | 0                           | 23               |                                       |
| Rmsd bonds (Å)   | 0.008 / 1.099   | 0.013 / 1.461  | 0.01 / 1.33                 | 0.016 / 1.747    |                                       |
| / angles (°)     |                  |                |                             |                  |                                       |
| B-factor deviation |                |                |                             |                  |                                       |
| Bond/angle (Å<sup>2</sup>) |         |                |                             |                  |                                       |
| Main chain       | 0.657 / 0.75    | 0.451 / 0.783  | 0.495 / 0.823               | 0.568 / 1.031    |                                       |
| Side chain       | 1.313 / 2.033   | 1.096 / 1.823  | 1.278 / 1.939               | 1.539 / 2.561    |                                       |
| PDB code         | 3FFE            | 2WO2           | 2WO4                        | 2WO3             |                                       |

* Values in parentheses refer to the highest resolution shell.
SUPPORTING METHODS

Cloning and overexpression of *acsD* in *E. coli*.
The *acsD* gene was amplified by PCR from cosmid pL9G1 containing Sau3A fragment of *Pectobacterium chrysanthemi* (strain 3937) genomic DNA (kindly provided by Dominique Expert, INRA/INA, Paris) as the template. A CACC sequence was introduced before the natural start codon, to allow the directional TOPO® cloning of blunt-end PCR products into pET151/D-Topo®. The forward primer (5'- CACCCTCGAG-ATGAACAACGAAATCATGA -3') had an *Xho*I site (underlined) between the CACC sequence and the start codon. The reverse primer (5'- GCACGCGGATCC-TTAACCATGCTGCACCTCAC -3') had a *Bam*HI restriction site (underlined) after the stop codon at the 3' end of the gene. The PCR mixture (50 μL) contained the pL9G1 cosmid as template (50 ng), 2 μM of each primer, 0.1 mM of each dNTP (Fermentas), 5 % dimethyl sulfoxide and 3.5 U Expand high fidelity DNA polymerase (Roche) in 1X Expand reaction buffer with MgCl₂. Reaction conditions consisted of an initial denaturation step of 368 K for 5 min followed by 30 cycles of 368 K for 45 s, 333 K for 45 s, and 345 K for 1 min. The 1863-bp PCR product containing the 1834-bp *acsD* gene was separated on a 1 % agarose gel (SeaKem® LE agarose, Rockland, USA), visualized by staining with 0.2 μg/mL ethidium bromide and extracted from the gel using a QIAquick Gel Extraction Kit (Qiagen).

The purified PCR product (25 ng) was mixed with the expression vector (pET151/D Topo® vector) (15-20 ng), using the Champion™ pET Directional Topo Expression Kit (Invitrogen) and used to transform One Shot® Topo10 chemically competent *E. coli* cells. The transformation mix was plated on LB plates supplemented with ampicillin (50 μg/mL). The sequence of the cloned gene in the pET151/D-Topo® expression vector, was confirmed by DNA sequencing (Molecular Biology Service, Department of Biological Sciences, University of Warwick). One correct clone (pNK004) was used to transform *E. coli* BL21Star (DE3) (Invitrogen) for expression of the full-length *acsD* gene as an N-terminal His₆ in-frame fusion.
For overexpression of *acsD*, 400 mL of LB medium supplemented with ampicillin (50μg/mL) was inoculated with a 2 mL overnight culture of *E. coli* BL21Star (DE3) / pNK004 and incubated with shaking at 200 rpm and 310 K. Incubation continued until the optical density at 600 nm of the culture reached 0.7-0.8, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression of *acsD*. The culture was then incubated overnight at 180 rpm and 288 K.

**Purification of His₆-AcsD for chemical studies**

Cells were harvested by centrifugation, the pellet was resuspended in 10 mL of 20 mM Tris buffer-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole and 10 % glycerol, and lysed in the presence of 1mM phenylmethylsulphonyl fluoride (PMSF) using a French Press (17,000 psi internal cell pressure) followed by sonication for 2 min (ultrasonic processor, Jencons). After removal of cellular debris by centrifugation (18,000 x g for 20 min, at 277 K), the supernatant was applied to a 1mL HiTrap™ HP affinity column (Nickel Sepharose High Performance, GE Healthcare) equilibrated with a solution containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, and 10 % glycerol. Unbound proteins were removed by washing with 20 mL of equilibration buffer and His₆-AcsD was eluted with 4 mL of elution buffer (20 mM Tris-HCl, pH8.0, 100 mM NaCl, 300 mM imidazole, 10 % glycerol).

Fractions were analyzed by electrophoresis (8 % SDS-PAGE), and those containing His₆- AcsD were pooled, washed and concentrated to 1 mL using Amicon® Ultra filtration with a 30,000 MWCO membrane (Millipore) in buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 10 % glycerol. His₆- AcsD was then aliquoted and frozen at 193 K. The proteins were used for all subsequent experiments without further purification, unless otherwise noted.
Confirmation of His6- AcsD identity and analysis of native oligomerization state

Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. The molecular weight of native His6- AcsD was determined by gel filtration on a 110 mL superose 12 prep grade gel filtration resin poured in a XK 16/50 column (Amersham Biosciences), equilibrated with a solution containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 10 % glycerol, at a flow rate of 0.75 mL/min. The column was calibrated with the kit for molecular weights 12,000-200,000 Da (Sigma), consisting of cytochrome c (12,400 Da), carbonic anhydrase (29,000 Da), bovine serum albumin (66,000 Da), alcohol dehydrogenase (150,000 Da), β-amylase (200,000 Da), and blue dextran (2,000,000 Da). This shows the His6-AcsD to be a dimer, we performed a similar analysis with the tag-cleaved protein (vide infra) and also see a dimer (Fig. S8b).

To confirm the identity of purified His6-AcsD, electrospray ionization mass spectrometry (ESI-MS) was performed on tryptic digests of the proteins (The Biological Mass Spectrometry and Proteomics Facility in the Department of Biological Sciences, University of Warwick). 7 of the predicted tryptic fragments were identified by this analysis for recombinant AcsD from *P. chrysanthemi*.

Overproduction and purification of “tag cleaved” AcsD and mutated derivatives for crystallization

Cultures were launched from *E. coli* BL21 (DE3) (transformed with pET151/D-TOPO AcsD) in LB /Amp overnight at 310 K. These cells were harvested diluted into the large scale cultures. 10 L of cells were grown in LB media with ampicillin (final concentration 100 µg/mL) at 310 K. When the cells reached an A600 = 0.6, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the temperature was lowered to 288 K. High temperatures led to insoluble over-expression. After a further 18 hour incubation period, cells were harvested by centrifugation at 2500 g, 277 K for 30 min, and resuspended and homogenized in 100-200 mL binding buffer (20 mM sodium phosphate, 40 mM imidazole, 500 mM NaCl, 10 % glycerol), 1 mg/mL lysozyme and 40 µg/mL DNaseI. Cell lysis was done either by cell disrupter (Constant Systems Ltd) or by sonication (6-8 cycles of 30 sec...
and 1 min break). Cell debris was separated from protein solution by centrifugation at 20,000 rpm (rotor SS-34) for 30 min.

The first purification step of His$_6$-AcsD was performed by immobilized metal affinity chromatography (IMAC) with Ni-beads. Unbound protein was washed with 50-100 column volumes (CV) of binding buffer (20 mM sodium phosphate, 40 mM imidazole, 500 mM NaCl, 10 % glycerol). His$_6$-AcsD was eluted three times with 2-3 CV elution buffer (20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, 10 % glycerol) and eluates were desalted using a HiPrep 26/10 desalting column (Amersham Bioscience) with 20 mM Tris pH 7.5, 500 mM NaCl, 10 % glycerol. The His$_6$-tag was cleaved with a 1:50 dilution of 1 mg/mL TEV-protease (O/N at RT) and removed by a 5 mL HisTrap HP column (Amersham Bioscience) and finally purified by gel filtration (GF) using a Superdex 200 or Sephacryl 200 column (GE Healthcare) with GF buffer (50 mM Tris pH 7.5, 500 mM NaCl, 10 % glycerol). Protein was concentrated to 6 or 9 mg/mL using protein concentrating devices (50kDa Vivaspin), frozen in liquid nitrogen and stored at 193 K. The identity and integrity of protein was confirmed by mass spectrometry.

**Overproduction, purification and crystallization of selenomethione labeled non-tagged AcsD**

Start cultures of *E. coli* BL21 (DE3) (Novagen) with pET151/D-TOPO AcsD were launched in LB /Amp overnight at 310 K. These cells were harvested at 293 K and gently washed twice in PBS before being resuspended in minimal culture media and diluted into the large scale cultures. 10 L of cells were grown in minimal culture media (111 mM glucose, 42.3 mM Na$_2$HPO$_4$.7H$_2$O, 22.1 mM KH$_2$PO$_4$, 18.8 mM NH$_4$Cl, 2.5 mM Mg SO$_4$, 25 µM thiamine, 30 µM Fe$_2$(SO$_4$)$_3$, pH 7.4) with ampicillin (final concentration 100 µg/mL) and 50 mg L$^{-1}$ L-Se-Met at 310 K. When the cells reached an A$_{600}$ = 0.6, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the temperature was lowered to 288 K. High temperatures led to insoluble over-expression. After a further 18 hour incubation period, cells were harvested by centrifugation at 2500g, 277 K for 30 min, and resuspended in phosphate buffer saline (PBS). The mixture was centrifuged as before, and
cell pellets were stored at 193 K. Purification of the labeled protein was carried out as described for the native non-tagged protein.

**Structural Biology**

Native protein was concentrated to 5 – 6 mg mL$^{-1}$ in pH 7.5 10 mM Tris HCl 150 mM NaCl 10 % glycerol for crystallization. Full experimental details for the native protein have been reported elsewhere $^2$. Protein for co-crystallization was concentrated to 5-9 mg mL$^{-1}$ in pH 7.5 50 mM Tris HCl 500 mM NaCl 10 % glycerol. A 2.63 Å data set was collected on ID14-4 of the European Synchrotron Radiation Facility (ESRF) at 100 K on a single cryoprotected selenomethionine crystal at $\lambda = 0.979$ Å using ADSC Q315r detector. Data were indexed and scaled with HKL2000 and selenium sites located using SHLEXC/D/E $^3$. Selenium sites were refined in SOLVE $^4$ to 3.7 Å before solvent flattening and phase extension to 3.2 Å in RESOLVE $^5$ and to 2.7 Å with DM $^6$. At this stage maps were interpretable and automated model building using BUCCANEER $^7$ traced 980 residues. The model was substantially re-built by hand using XFIT $^8$ at 2.7 Å. Phases for the apo structure were extended to 2.25 Å using this model and the native data set. The final model consists of 7 – 587 in both monomers with small breaks in both chains at residues 97 – 100 and 572 – 577. REFMAC5 $^9$ was used to refine the structure (5 % of the data were omitted to validate refinement protocol) with appropriate NCS restraints applied. TLS $^{10}$ parameters were refined for each monomer (7 domains). COOT $^{11}$ was used for manual model manipulation and solvent molecules were added with ARP/wARP. The final apo model was assessed in Molprobity $^{12}$. The data from citrate (100K, BM14 ESRF, $\lambda = 0.873$ Å, MAR225) and ATP (100K, $\lambda = 0.933$ Å, ADSC Q4) structures were indexed and scaled with $^{13}$ and with MOSFLM $^{14}$/ SCALA $^{15}$ as implemented in CCP4 $^{16}$ for the citrate complex. The final native structure (without water molecules) was employed as the molecular replacement model using AMoRe $^{17}$ for both complexes. Both complex structures were refined using the same protocol outlined above. ATP is bound in both monomers in the
complex structure but citrate is only found in monomer B of its complex. Full statistics are given in Table S1.

In our earlier attempts to obtain a citrate complex, we observed that at high citrate concentrations, we can bind citrate in the triphosphate portion of the ATP binding site (100K, BM14 ESRF, $\lambda = 0.873$ Å, MAR225). The three carboxylate groups of citrate superimpose with the three phosphate groups of ATP.

Superimposing ATP of all 250 in the PDB deposited ATP co-complex structures to the AcsD bound ATP revealed that only 7 structures show an $\alpha$ and $\gamma$ ATP phosphate coordination with Mg. These seven structures are mostly kinases (1CSN, 1PHK, 1QMZ, 1QL6) 18-21 including two cAMP-kinases (1ATP, 1RDQ) 22,23 and one ribonucleotide transformylase which is involved in the purine biosynthesis (1KJ9) 24. A similarity domain search with DALI 25 and SSM 26 showed that the central two domains (palm and fingers) do have structural matches to several cyclic AMP dependant protein kinases (cAPK) which bind NTP including 1E8X27, 2GQR 28 and 1CJA29. ATP in AcsD is an inverted orientation relative to the phosphates in the phosphoinositide 3-kinase 1E8X27 (Fig. 6f and S7).

**AcsD activity assay**

For detecting the activity of AcsD and its mutants a modified coupled enzyme assay was used 30. The assay is based on coupling the AcsD dependent formation of AMP to the lactate dehydrogenase oxidation of NADH. Decrease in NADH levels was monitored in real time by fluorescence using an excitation of 376 nm and an emission of 462 nm. The assay was carried out using a Cary Eclipse spectrometer (Varian) and Perkin-Elmer 1 mL cuvettes. In order to acquire the appropriate concentration regime for accurate NADH detection, reactions lacking AcsD were measured with NADH concentrations up to 2 mM (Fig. S5a). Above 1 mM NADH fluorescence intensities decreased with increasing NADH concentrations, presumably due to by self quenching. The concentration range we employed from 50 to 300 µM NADH (in both reaction buffer and in water)
shows a non-linear relationship (Fig. S5b). If one assumes a linear relationship the fit of kinetic curves is acceptable but shows deviations from the normal shape. We therefore chose to convert the fluorescence intensity to NADH concentration by use of a calibration curve. The regression is exponential and the fitting function obtained by Origin was used to re-calculate and convert the raw fluorescent intensity data to NADH concentration with Excel (Fig. S5b). Standard calibration graphs were obtained on a daily basis before each set of experiments to ensure reliable conversion. When these derived values for NADH concentration, the fit to standard kinetic curves was improved. Data for kinetics where fitted with the Michaelis Menten equation \( v = \frac{v_{\text{max}} [S]}{[S] + K_M} \) using Origin software with the background rate (no AcsD) subtracted. The assay was shown to be rate limiting for AcsD and the rate to double or half in response to the corresponding change in AcsD concentration. Addition of exogenous 2 μM AMP to the reaction results in a rate of NADH consumption over six times faster than His6-AcsD (Fig. 4). This shows that the other enzymes in the coupled assay are not rate limiting even at artificially high concentrations of AMP. Addition of exogenous AMP to a control reaction lacking AcsD also shows activity. We confirmed that the kinetics of the His6-AcsD and “tag cleaved” AcsD were identical within error.

**AcsD activity with \(^{32}\text{P}\)-ATP.**

50 mM tris buffer (pH8), 12 mM MgCl₂, 2.25 mM ATP, 3 mM citric acid or α-ketoglutaric acid aqueous solution, 25 mM L-serine or D-serine or ethanolamine or L-2,4-diaminobutyric acid were incubated with 8.3 μCi \(^{32}\text{P}\)-ATP for a total reaction volume of 200 μL. The reaction was initiated by addition of 6.1 μM His6-AcsD and the mixture incubated 1 hr at 310 K. The reaction was stopped by addition of 700 μL of a solution of EDTA (50mM) in phosphate buffer (5 mM) buffered at pH5. A solution of 5 % of aqueous charcoal (400 μL) was then added. The mixture was incubated 15 min at RT and occasionally vortexed. After centrifugation 5 min at 4000 rpm, the pellet was washed twice with water and 9 mL of scintillator fluid was added. 0.9 mL of supernatant was centrifuged 5 min and the pellet discarded. 0.5 mL of the supernatant was added to 9 mL of
scintillator fluid. The $[^{32}\text{P}]$ released in the supernatant from the $[^{32}\text{P}]-\text{ATP}$, during the reaction, and the $[^{32}\text{P}]-\text{ATP}$ binding the charcoal were checked by scintillator counter. Having identified the correct partners, the increase in $[^{32}\text{P}]$ released in the supernatant was monitored with time. A control reaction was carried out using boiled His$_6$-AcsD (Fig. S2c).

**Time dependent incubation of His$_6$-AcsD with various electrophiles and hydroxylamine.**

The increase in absorbance at 540 nm was monitored according with time for the solutions of citric acid (3 mM) and hydroxylamine (200 mM). The control reaction was carried out using boiled His$_6$-AcsD (Fig. S2b). No optical density changes were detected after 30 minutes incubation at 310 K, without MgCl$_2$, or with CTP, GTP or TTP instead of ATP, or without citric acid or hydroxylamine.

**Small scale characterization of product of incubation of His$_6$-AcsD with citric acid and L-serine**

The solutions of citric acid, and L-serine were prepared in Tris buffer (25 mM) and the pH was adjusted to 8, and 8.3 mM citric acid was incubated with 37.5 mM L-serine, 2.25 mM ATP, 15 mM MgCl$_2$, 25 mM Tris-HCl (pH 8.0) and 3 $\mu$M His$_6$-AcsD (after Ni-NTA purification and desalting) in a final volume of 200 $\mu$L for 2 h at 310 K. The reaction was initiated by addition of the enzyme and was stopped by addition of 10 $\mu$L of an aqueous solution of TCA (7 %). The precipitated enzyme was then discarded by centrifugation 10 min at 4,000 rpm. No products could be detected in control incubations with enzyme inactivated by boiling for 10 minutes. LC-MS analysis of the reaction mixture was carried out using an Eclipse XDB-C18 column (150 x 4.6mm, 5$\mu$m, Agilent) connected to an Agilent 1100 HPLC instrument. The outflow was connected via a splitter (10 % flow to MS, 90 % flow to waste) to a Bruker HCT+ mass spectrometer fitted with an electrospray source operating in positive ion mode. Absorbance was monitored at 210 nm. The compounds were eluted using an isocratic solution of water + TFA (0.1 %), with a 1 mL/min flow rate. No products
were detected by LC-MS, for incubations with citric acid and L-serine without ATP or L-serine without MgCl₂.

**Incubation and purification of His₆-AcsD with [1,2]-¹³C₂-citric acid and L-serine.**

To prepare [1,2]-¹³C₂-citric acid, 4.5 mM [1,2]-¹³C₂ sodium acetate, 5.1 mM coenzyme A, 4.8 mM ATP, 12 mM MgCl₂, 25 mM Tris-HCl (pH 8) were incubated with 3.9 U of acetyl coenzyme A synthetase, during 1 hour at 310 K. 5 mM oxaloacetic acid buffered solution (pH 7) and 10 mM Tris buffer (pH 8) and 66 U of si-citrate synthase were added. The reaction mixture was incubated at 310 K. After 1 h, the mixture was centrifuged (10 min at 4000 rpm) and the supernatant was recovered. 8 mM Tris buffer (pH 8), 4 mM ATP, 4 mM MgCl₂, 30 mM L-serine buffered solution (pH 8) and 10 µM of His₆-AcsD were added. The reaction was incubated at 310 K, overnight.

The reaction was stopped with 0.2 mL of 10 % aqueous trichloroacetic acid. The precipitated enzyme was removed by centrifugation (10 min at 4000 rpm) and the supernatant was analyzed by LC-MS, under the conditions described previously. A major compound eluted after 3.1 minutes was detected with m/z=282 [(M+H)⁺] corresponding to doubly-labeled N-citryl-L-serine derived from [1,2-¹³C₂]-citric acid.

The compound was purified as described in the methods section and fractions were collected and analyzed by ESI-MS. Those containing the compound with m/z=282 (retention time ~7 min on the C18 column and ~ 5 min on the Synergy Fusion column) were combined and freeze dried.

The isolated compound was analyzed by ESI-MS/MS (Bruker HCT+ spectrometer equipped with electrospray sources operating in positive ion mode) and ¹H and ¹³C NMR spectroscopy (Bruker AV700 spectrometer equipped with TCI cryoprobe, Bruker AV500 spectrometer equipped with DUI cryoprobe, D₂O pH 7-7.5).
MS/MS analyses of pure N-citryl-L-serine and the products of the AcsD-catalyzed condensation of citric acid with L-serine

Pure N-citryl-L-serine was dissolved in water and introduced by direct injection into a Bruker HCT+ spectrometer fitted with an electrospray source operating in negative ion mode. Settings were as follows: CID amplitude: 1.5; skim 1: 14 V, isolation window: ± 1 Da; scan range: 50-500 Da.

The enzymatic reaction was carried out in Tris buffer (25 mM, pH 8) containing 15 mM MgCl₂, 2.25 mM ATP, 3 mM citrate and 25 mM L-serine (each prepared in Tris buffer (20 mM) and buffered at pH 8) in a total volume of 1 mL. The mixture was incubated at 37°C and the reaction was initiated by addition of 6.4 μM purified recombinant His₆-AcsD. 200 μL aliquots were removed after 10, 30, 60 and 90 minutes and passed through a filter (Alltech) by centrifugation at 6,000 rpm for 1 minute. Each sample was analyzed by direct injection ESI-MS/MS as described above.
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