SUPPORTING INFORMATION

Substrate Deconstruction and the non-Additivity of Enzyme Recognition

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Supplementary methods

**Protein expression and purification.**

*Cloning and purification of E. coli Adenosine deaminase (ADA).* ADA was cloned from E. coli K12 genomic DNA purchased from ATCC. The PCR product was amplified using the primer pair 5’-GGGACTTCCATATGATTGATACCACCCTGCATTAACCTG-3’ and 5’-CCCGAATTCTTACTCTGCAGGCAGCTTTTTTCTCG-3’. The restriction sites for NdeI and EcoRI were inserted into the forward and reverse primers, respectively. The PCR product was purified, digested with NdeI and EcoRI and ligated into a pET130a(+) vector, which was previously digested with the same two restriction enzymes. The cloned gene fragment was sequenced to verify fidelity of the PCR amplification.

Plasmid containing ADA was transformed into BL21-DE3 competent cells (Novagen). A single colony was used to inoculate a 5 mL overnight culture of LB medium containing 50 µg/mL kanamycin. Each overnight culture was used to inoculate 1.0 L of LB medium containing 50 µg/mL kanamycin. Liter cultures were grown at 37° C, supplemented with 1 mM ZnCl2 and 0.1 mM bipyridyl and induced with 50 µM isopropyl D1thiogalactopyranoside (IPTG) when A$_{600}$ of 0.6 was reached. At the time of induction, the temperature was turned to 20° C and allowed to shake for 18 hrs before cells were harvested at 8,000 rpm for 10 minutes.

The cells were resuspended in 50 mM HEPES buffer (pH 7.5) containing 0.1 mg/mL phenylmethylsulfonyl fluoride. Cells were lysed by sonication. Soluble protein was separated from cell debris by centrifugation, nucleic acids were removed by protamine sulfate drip, and protein partitioned by ammonium sulfate precipitation. The precipitated protein (35-70% ammonium sulfate saturation) was resuspended in 50 mM HEPES buffer (pH 7.5) and loaded onto a High Load 26/60 Superdex 200 prep grade gel filtration column (GE Healthcare).

*Cloning and purification of T. maritima MTA-SA H deaminase (SAHD).* SAHD was cloned into a pET30a vector and expressed in BL21 cells in 1L shaker flasks. Cells were induced by 1 mM IPTG. The expressing cells were resuspended in 50 mM HEPES, pH 7.5 and lysed via sonication. DNA was precipitated with protamine sulfate and removed with centrifugation. The protein was precipitated by increasing ammonium sulfated concentration to 70% saturation, spun down, then redissolved in 5 mL 50 mM HEPES, pH 7.5. The protein solution was filtered and loaded onto a GE HiLoad 26/200 Superdex 200 gel filtration column. Active fractions were collected, loaded onto a ResourceQ anion exchange column, and eluted with a 1 M gradient of NaCl.
Cloning and purification of V. cholerae Phosphoserine phosphatase (PSP). The gene for PSP was cloned into pSGX3 as described in 1. The PSP-pSGX3 vector was transformed into BL21(DE3) E. coli containing the pRIL plasmid (Stratagene) and used to inoculate an overnight culture containing 25ug/mL Kanamycin and 34ug/mL Chloramphenicol. The culture was allowed to grow overnight at 37° C in a shaking incubator. 1ml of the overnight culture was used to inoculate 1L of PASM-5052 auto-induction media containing 100ug/mL Kanamycin and 34ug/mL Chloramphenicol.2 The culture was placed in a LEX48 airlift fermenter and incubated at 37° C for four hours and then at 22° C overnight. The culture was harvested and pelleted by centrifugation and stored at -80° C.

The cells were resuspended in lysis buffer (20mM HEPES pH 7.5, 500mM NaCl, 20mM Imidazole, and 10% Glycerol) and lysed by sonication. The lysate was clarified by centrifugation at 35,000g for 30min. Clarified lysate was loaded onto an AKTAxpress FPLC (GE Healthcare). Lysate was loaded onto a 1mL HisTrap FF column (GE Healthcare), washed with 10 column volumes of lysis buffer, and eluted in buffer containing 20mM HEPES pH 7.5, 500mM NaCl, 500mM Imidazole, and 10% Glycerol. The purified sample was loaded onto a HiLoad S200 16/60 PR gel filtration column which was equilibrated with SECB buffer (20mM HEPES pH7.5, 150mM NaCl, 10% Glycerol, and 5mM DTT). Peak fractions were collected and allowed to incubate with 1mg of TEV-protease overnight at 4° C. EV-protease and uncleaved protein was removed by passing the over 1mL of Ni Sepharose High Performace (GE Healthcare).

Cloning and purification of B. thetaiotaomicron Flavin mononucleotide phosphatase (FMP). The gene for FMP was amplified from Bacteroides thetaiotaomicron Strain VPI-5482 genomic DNA using 5’TACTTCCAATCCATGAAAAAGTAAAGGAATTAAACCTTACTTTGATTAGG-3’ as the forward primer and 5’-TATCCACCTTTACTGTTATTTTTTTCGTGAAAAGATGGGCTCCAGTC-3’ as the reverse primer. PCR was preformed using KOD Hot Start DNA Polymerase (Novagen). The conditions were: 2 min at 95°C, followed by 40 cycles of 30 sec at 95° C, 30 sec at 66° C, and 30 sec at 72° C. The amplified fragment was cloned into the N-terminal TEV cleavable 6x-His-tag containing vector, pNIC28-Bsa4, by ligation-independent cloning.3,4

The FMP-pNIC28-Bsa4 vector was transformed into BL21(DE3) E. coli containing the pRIL plasmid (Stratagene) and used to inoculate an overnight culture containing 25ug/mL Kanamycin and 34ug/mL Chloramphenicol. The culture was allowed to grow overnight at 37° C in a shaking incubator. 1ml of the overnight culture was used to inoculate 1L of PASM-5052 auto-induction media containing 100ug/mL Kanamycin and 34ug/mL Chloramphenicol.2 The culture was placed in a LEX48 airlift fermenter and incubated at 37° C for four hours and then at 22° C overnight. The culture was harvested and pelleted by centrifugation and stored at -80° C.
The cells were resuspended in lysis buffer (20mM HEPES pH 7.5, 500mM NaCl, 20mM Imidazole, and 10% Glycerol) and lysed by sonication. The lysate was clarified by centrifugation at 35,000g for 30min. Clarified lysate was loaded onto an AKTAxpress FPLC (GE Healthcare). Lysate was loaded onto a 1mL HisTrap FF column (GE Healthcare), washed with 10 column volumes of lysis buffer, and eluted in buffer containing 20mM HEPES pH 7.5, 500mM NaCl, 500mM Imidazole, and 10% Glycerol. The purified sample was loaded onto a HiLoad S200 16/60 PR gel filtration column which was equilibrated with SEC buffer (20mM HEPES pH7.5, 150mM NaCl, 10% Glycerol, and 5mM DTT). Peak fractions were collected and allowed to incubate with 1mg of TEV-protease overnight at 4° C. EV-protease and uncleaved protein was removed by passing the over 1mL of Ni Sepharose High Performance (GE Healthcare).

**Enzymatic assays.**

*Adenosine deaminase (ADA).* The activity of ADA against adenosine ($\Delta \varepsilon_{263} = 6900$ M$^{-1}$ cm$^{-1}$), adenine ($\Delta \varepsilon_{262} = 4600$ M$^{-1}$ cm$^{-1}$), 9-methyladenine ($\Delta \varepsilon_{263} = 4340$ M$^{-1}$ cm$^{-1}$), and 9-hydroxymethyladenine ($\Delta \varepsilon_{263} = 4800$ M$^{-1}$ cm$^{-1}$) was measured in a direct assay. Deamination was monitored directly. The reaction was run at pH 7.5 in 25 mM HEPES. Inhibition was determined by monitoring the changes in the rate of adenosine deamination in the absence and in the presence of the potential inhibitors.

*MTA-SAHD deaminase (SAHD).* Activity screen assays for SAHD were performed in a 96-well quartz plate with a path length of 0.64 cm in a Spectramax 384 Plus UV-vis spectrophotometer over a region of 240nm – 300nm. Adenine, 9-methyladenine and 9-hydroxymethyladenine (100 µM) were incubated for 12 hours in absence and in presence of 1 µM SAHD in 50 mM HEPES, pH 7.7. Inhibition assays were performed in a 96-well quartz plate with a path length of 0.64 cm in a Spectramax 384 Plus UV-vis spectrophotometer at 263 nm. The rate for 5-thiomethyladenosine deamination was measured in absence and in presence of the potential inhibitors. The reaction was run in 50 mM HEPES at pH 7.7, and potential inhibitors were used at 5 mM.

*Isoaspartyl dipeptidase (IAD).* IAD catalyzed hydrolysis of β-asp-leu was measured spectrophotometrically in the presence and absence of 2 mM of the following amino acids: L-alanine, L-glutamate, L-phenylalanine, glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-methionine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tryptophan and L-tyrosine. The release of the product L-aspartate was measured with a coupling system as previously described.5

*Phosphoserine phosphatase (PSP).* The rate of PSP catalyzed hydrolysis of phosphate substrates was measured spectrophotometrically by using the Enzchek phosphate assay kit (Invitrogen) to continuously monitor phosphate formation ($\Delta \varepsilon_{360} = 11000$ M$^{-1}$ cm$^{-1}$). The 200 µL reaction solutions
contained 25 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl2, 0.05-5 µM PSP, 0.2 mM MESG, 1 U purine nucleoside phosphorylase and various concentrations of substrate. Inhibition assays were performed spectrophotometrically by monitoring the change in the rate of phosphoserine hydrolysis with increasing inhibitor concentration.

**Flavin mononucleotide phosphatase (FMP).** The rate of FMP catalyzed hydrolysis of Flavin mononucleotide was monitored using BIOMOL Green (Enzo Life Sciences) to measure the concentration of inorganic phosphate formed in the reaction solutions. The 20 µL reaction solutions contained 25 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl2, 2 µM FMP and various concentrations of substrate. The reaction was stopped by adding 20 µL of BIOMOL Green reagent. In parallel, the background level of phosphate release was measured using a control reaction mixture, which excluded FMP. Inhibition assays were performed by monitoring the change in the rate of Flavin mononucleotide hydrolysis with increasing inhibitor concentration. The rate of FMP catalyzed hydrolysis of mannose-1-phosphate (38), glucose-1-phosphate (39) and ribose-5-phosphate (40) was measured spectrophotometrically by using the Enzchek phosphate assay kit (Invitrogen). To investigate a possible synergistic effect of lumichrome (fragment 41), we tried assessing the activity of 38, 39 and 40 in presence of 41. Because the 41 is a fluorophore, we switched from the Enzchek assay to BIOMOL Green. Unfortunately, the background phosphate for these substrates is outside of the range for that assay (> 10 nanomoles), making it impossible to measure enzyme activity.

**AmpC β-lactamase (AMPC).** The hydrolysis of 7-aminocephalosporanic acid N-formyl by AMPC was monitored at 265 nm. Enzyme concentration was 2 nM and the assay was run in 50 mM sodium cacodylate, pH 6.5 and 0.01% Triton. The reaction was performed in 300 µL cuvettes (0.1 cm pathway) and initiated by addition of AMPC. AMPC inhibition assays were performed spectrophotometrically by monitoring the change in rate of CENTA hydrolysis (60 µM) at 405 nm with increasing inhibitor concentration. CENTA was purchased from Tydock Pharma (Modena, Italy). All assays were run in 50 mM sodium cacodylate (pH 6.5), with 0.01% Triton to prevent non-specific inhibition via aggregation. Reactions were performed in 1 mL cuvettes and initiated by addition of AMPC.

**Crystal growth and structure determination.**

Apo crystals of AMPC were grown by hanging drop vapor diffusion, equilibrated over 1.7 M potassium phosphate buffer (pH 8.6-8.8). Drops were set up by mixing 2 µL of protein (4 mg/mL) with 2 µL of well solution. 1 µL of microseeding solution was added to promote crystal growth. Crystals appeared after 2-7 days of equilibration at 20 ºC. Compounds 45 and 46 were soaked for 5-10 minutes into apo AMPC crystals at 100 mM and at saturated concentration, respectively, in 25% sucrose, 1.7 M
potassium phosphate buffer (pH 8.6-8.8) before flash-cooling the crystals in liquid nitrogen. For compound 48, the crystal was soaked in 50 mM 48 in 1.7 M potassium phosphate buffer (pH 8.6), then more 48 powder was sprinkled on the drop. Following the soaks, the crystal was immersed in a cryoprotectant solution of saturated 48, 25% sucrose, and 1.7 M potassium phosphate (pH 8.6) before flash-cooling in liquid nitrogen.

Diffraction was measured at Beamline 8.3.1 of the Advance Light Source (ALS, Lawrence Berkeley National Lab, CA). Reflections were indexed, integrated and scaled using the XDS package in the space group C2 with two molecules in the asymmetric unit. Structure refinement was carried out using Phenix. Coot was used for model building and the PRODRG server was used to generate coordinates and ligand restraints. Some of the inhibitor bond lengths and angles were modified according to values observed in the CSD.

The initial phasing model was an apo AMPC model (PDB entry 1KE4), with water molecules and ions removed. PHENIX was used for all steps of structure refinement. The model was positioned initially by rigid body refinement and subjected to one round of simulated annealing to reduce model bias. This was followed by multiple cycles of individual coordinate refinement and B-factor refinement. At resolution better than 1.6 Å, B-factors were refined anisotropically for all atoms, except for water oxygen atoms. For each structure the geometry was assessed using Molprobity. There were no outliers in the Ramachandran statistics and >98 % of all residues were in favored regions.
Supplementary tables

**Supplementary Table S1.** Activity of Iso-aspartyl dipeptidase against β-aspartyl-leucine in absence and in presence of 18 L-amino acids (all amino acids are fragments of the β-aspartyl dipeptide substrates).

| Structure | $K_i$ | Structure | $K_i$ |
|-----------|-------|-----------|-------|
| 16 | $1 \times 10^5$ M$^{-1}$s$^{-1}$ ($k_{cat}/k_{M}$) | 25 | > 4 mM |
| 17 | > 4 mM | 26 | > 4 mM |
| 18 | > 4 mM | 27 | > 4 mM |
| 19 | > 4 mM | 28 | > 4 mM |
| 20 | > 4 mM | 29 | > 4 mM |
| 21 | > 4 mM | 30 | > 4 mM |
| 22 | > 4 mM | 31 | > 4 mM |
| 23 | > 4 mM | 32 | > 4 mM |
| 24 | > 4 mM | 33 | > 4 mM |
| 15 | > 4 mM |
**Supplementary Table S2.** Data collection and refinement statistics.

One crystal was used for each structure. a Values in parentheses represent highest resolution shells. b Calculated for both molecules in asymmetric unit.

|                | AmpC/45 | AmpC/46 | AmpC/48 |
|----------------|---------|---------|---------|
| **PDB code**   | 4OLD    | 4OKP    | 4OLG    |
| **Data collection** |         |         |         |
| Space group    | C2      | C2      | C2      |
| Cell dimensions |         |         |         |
| a, b, c (Å)    | 118.45, 76.42, 97.88 | 118.12, 76.49, 97.62 | 118.66, 77.15, 98.14 |
| α, β, χ (º)    | 90.00, 115.95, 90.00 | 90.00, 115.85, 90.00 | 90.00, 115.95, 90.00 |
| Resolution (Å) | 30-1.48 (1.52–1.48) | 30-1.37 (1.41–1.37) | 30-1.71 (1.76–1.71) |
| R<sub>merge</sub> (%) | 2.5 (34.4) | 3.3 (46.9) | 3.0 (26.0) |
| Completeness (%) | 99.1 (98.0) | 98.7 (95.6) | 99.4 (97.6) |
| I/Sigma        | 26.2 (3.6) | 23.6 (3.3) | 28.3 (4.8) |
| Redundancy     | 3.2 (3.1) | 4.2 (4.0) | 4.0 (3.9) |
| **Refinement** |         |         |         |
| Resolution (Å) | 29.34 – 1.48 | 29.28 – 1.37 | 29.41 – 1.71 |
| No. of reflections (test set) | 124458 | 161759 | 82245 |
| R<sub>work</sub> / R<sub>free</sub> (%) | 16.4 / 18.8 | 14.3 / 16.5 | 16.6 – 20.0 |
| No. atoms      |         |         |         |
| Protein<sup>a</sup> | 5712 | 5734 | 5641 |
| Ligand<sup>b</sup> | 42 | 15 | 40 |
| Water          | 624     | 812     | 423     |
| B-factors (Å<sup>2</sup>) |         |         |         |
| Protein<sup>a</sup> | 18.1 | 16.28 | 28.2 |
| Ligand<sup>b</sup> | 43.0 | 32.0 | 55.7 |
| Water          | 27.9    | 30.0    | 35.5    |
| R.m.s. deviations |         |         |         |
| bond lengths (Å) | 0.028 | 0.005 | 0.022 |
| bond angles (º) | 2.65 | 1.13 | 2.06 |
Supplementary figures

**Supplementary Figure S1.** Activity of AmpC β-lactamase against N-formyl 7-aminocephalosporanic acid (48). The hydrolysis of N-formyl 7-aminocephalosporanic acid was monitored at 265 nm, in presence of 2 nM enzyme.
**Supplementary Figure S2.** Stereoviews of the active site electron density for each AMPC complexed structure. (A) 2Fo-Fc electron density map (green) for 48 and the catalytic Serine 64 highlights the covalent nature of the complex. (B) 2Fo-Fc electron density map for 45 shows multiple binding modes for the ligand, bound away from the catalytic Serine 64. (C) Fo-Fc electron density map for 46, bound to a pocket that lies more than 17 Å away from the active site.
Supplementary references

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