Supplementary Materials for:

Ground State Electronic Destabilization via Hyperconjugation in Aspartate Aminotransferase

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Table S1. Data Collection and Refinement Statistics for the K258A/DeazaPLP/L-Aspartate External Aldimine Structure

unit cell dimensions	
a,b,c (Å)	84.29, 155.25, 77.96
space group	$C222_1$
no. of monomers per asymmetric unit	1
resolution range (Å)	28.6-1.50 (1.54-1.50)
R_{sym}[a] (%)	3.8 (54.3)
$\langle I \rangle / \sigma \langle I \rangle$	15.7 (2.03)
no. of reflections	343,209 (15,708)
no. of unique reflections	80,890 (5,738)
redundancy	4.2 (2.7)
completeness (%)	98.7 (93.9)
R_{factor}[b] (%)	16.9
R_{free}[c] (%)	19.3
no. of protein atoms	3065
no. of cofactor atoms	24
no. of water atoms	450
no. of sulfate atoms	20
no. of ethylene glycol atoms	4
rmsd from ideality	
bond distance (Å)	0.016
bond angle (deg)	1.61
Average Isotropic B factor	
Protein	28
Solvent	42

[a] $R_{merge} = [\sum h \sum i |\boldsymbol{I}_h - \boldsymbol{I}_{hi}| / \sum h \sum i \boldsymbol{I}_{hi}]$ where $\boldsymbol{I}_h$ is the mean of $\boldsymbol{I}_{hi}$ observations of reflection *h*. Numbers in parenthesis represent highest resolution shell.

[b] *R*-Factor and [c] $R_{free} = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$ x 100 for 95% of recorded data (*R*-Factor) or 5% data (R_{free})

Experimental Procedures

Pyridoxal 5'-phosphate (PLP), L-aspartic acid, triethanolamine (TEA), and sodium hydroxide were from Sigma-Aldrich. [2,3,3-^{2}H]-L-aspartic acid was from Cambridge Isotope Laboratories. HPLC grade methanol, ammonium sulfate, ethylene glycol, and potassium chloride were from Fischer. Protein concentrators (30 kDalton cut-off, 500 µL volume) were from Millipore. DeazaPLP was synthesized previously.[1]

Enzyme Expression and Purification. A pUC119 plasmid containing the gene code for *E. coli* AAT[2] was transformed into *E. coli* MG204 cells by electroporation. Transformed cells, grown on LB agar plates containing 100 µg/mL carbenicillin, were transferred to 2YT media containing 100 µg/mL carbenicillin and grown with shaking at 225 rpm for 36 hours at 37 °C. Cells were collected by centrifugation at 5,000 × g for 30 min at 4 °C and were resuspended in lysis buffer: 20 mM potassium phosphate buffer, pH 7.0, 200 µM PLP, and 0.5 mg/mL lysozyme. The mixture was stirred on ice for 30 minutes then subjected to sonication consisting of 5 one-minute pulses performed for each 10 grams of cell paste. The lysed cells were centrifuged at 18,000 × g at 4 °C for 1 hour. Sodium acetate was added to the supernatant to a final concentration of 20 mM and the pH lowered to 4.9 by addition of dilute acetic acid. The resulting solution was cooled on ice for ten minutes followed by centrifugation at 18,000 × g for 30 min at 4 °C. The pellet, containing precipitated protein, was discarded and the supernatant was dialyzed against 3 changes of 20 mM sodium acetate pH 4.9, 20 µM PLP. The dialysate was centrifuged at 12,000 × g for 30 min at 4 °C then loaded at 2 mL/min onto a 50 mL Q-Sepharose Fast Flow anion exchange column and washed with 10 bed volumes of wash buffer (20 mM sodium acetate pH 4.9, 20 µM PLP) at a rate of 4 mL/minute. This was followed by a linear gradient elution from 100% wash buffer to 100% elution buffer (20 mM sodium acetate pH 4.9, 300 mM NaCl, 20 µM

PLP) over 1 L at a flow rate of 2 mL/minute. Fractions containing AAT, as judged by the absorbance at 430 nm, were analyzed by SDS-PAGE to assess purity. Pure fractions were combined, dialyzed against 3 changes of 20 mM HEPES, pH 7.5, and concentrated to 10 mg/mL. Aliquoted samples were flash frozen in liquid nitrogen and stored at -80 °C.

The apoenzyme was prepared as previously described[2] and reconstituted by overnight incubation with a 10-fold excess of deazaPLP. Dialysis was performed against 50 mm TEA pH 7.5, 100 mM KCl, 2 mM DTT to achieve a final free deazaPLP concentration of ~10 μM followed by concentration of the enzyme to ~15-20 mg/mL.

Crystallization, Data Collection, Structure Solution, and Refinement. Crystals were obtained by the hanging drop method. A 2 μL of drop of protein solution (15-20 mg/ml, 50 mM TEA pH 7.5, 100 mM KCl, 2 mM DTT, 10 μM deazaPLP) was mixed with 2 μL of the reservoir buffer containing 53-60% saturated ammonium sulfate and 50 mM TEA pH 7.5. L-Aspartate was added to a final concentration of 50 mM to the hanging drop to obtain the external aldimine. Large yellow crystals formed under the 54%-56% saturated ammonium sulfate conditions within 3-5 days. Crystals were mounted and stored in liquid nitrogen using 23% ethylene glycol in mother liquor for the cryoprotectant. Data were collected on beamline 9-2 at the Stanford Synchrotron Radiation Lightsource (SSRL). Diffraction data were processed with XDS. Crystal parameters, data collection, and refinement statistics are given in Table S1.

The structure was solved by molecular replacement using *E. coli* AAT (PDB ID 1ASE) as the search model. Model building was performed with COOT. Refinement, including TLS parameters, was accomplished with REFMAC followed by Phenix. Individual isotropic B-factors were refined for all atoms. Group occupancies were refined for residues with multiple

conformations, as well as waters, sulfates, and ethylene glycol. TLS refinement used groups determined by Phenix.

Binding Isotope Effect. The ratio of protiated to [2,3,3-^{2}H]-L-aspartate was determined on an Agilent 1100 ESI MSD mass spectrometer in negative ion mode. Plots of $\ln(D/H)_{free}$ versus ln(1-fractional reaction) were fitted to Equation 1, where $\ln(D/H)_0$ is the initial ratio of the isotopic peaks.[3]

$$\ln\left(\frac{D}{H}\right)_{\text{free}} = \ln\left(\frac{D}{H}\right)_0 + \left(\frac{1-BIE}{BIE}\right)\ln(1 - fract.rxn) \quad (1)$$

Three fully independent determinations were made, with the different fractional conversion points analyzed in triplicate. Stock solutions of protiated and deuterated L-aspartate in 5 mM TEA, pH 7.5 were prepared such that the final concentration of total L-aspartate was 10 mM. K258A was dialyzed against 10 mM TEA, pH 7.5. Typical final enzyme concentrations were in the 110-130 μm range. Substrate was added such that the fractional conversion (*i.e.*, [enzyme]/[total L-aspartate]) fell into the range of 0.65-0.25. Final volumes were 200 μL.

Substrate and enzyme solutions were mixed by pipetting and allowed to incubate 1 minute for K258A/PLP and 45 minutes for K258A/deazaPLP to allow external aldimine formation to equilibrate. Solutions were then transferred to a 30 kD cut-off protein concentrator and centrifuged at the maximum recommended speed for 5 min. The flow-through, containing free substrate, was diluted 1:1 with HPLC grade methanol. Solutions were transferred to a 250 μL syringe and injected into the mass spectrometer at a flow rate of 8.5 μL/minute. The m/z range for detection of protiated and deuterated aspartate was set to 130-137. Typically, data were collected for 8-10 minutes and the mass spectrum representing the average of ~500 scans was exported as a text file and imported into PeakFit v.4.11 to obtain the peak areas for protiated and

deuterated aspartate, respectively. These areas were used directly to determine the D/H ratios of substrate. Finally, plots of ln(1-fraction reaction) against $\ln(D/H_{remaining})$ were fitted to equation 1 to obtain the binding isotope effect.

References

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3. Cook, P. F., *Enzyme Mechanism from Isotope Effects*. CRC press: 1991.