Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Structural and Biochemical Analyses on the Binding of Acidic and Basic Substrates to Ornithine Acetyl Transferase

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Supplementary Information

Supplementary experimental section

Cloning, expression and purification of OAT2 variants

Expression and purification of wildtype OAT2 was carried out as described.[1] The preparation of OAT2 variants by subunit coexpression involved insertion of a stop codon (TGA) after the codon encoding for Ala-180 in the vector *orf6*/pTYB12 (ampicillin resistant) resulting in a construct $orf6_{1\text{-}180}$/pTYB12 which enabled production of the OAT2 α-subunit with an N-terminal intein tag. The gene encoding for the OAT2 β-subunit was amplified from *orf6*/pTYB12 using primers and subcloned into the pET24a vector (kanamycin resistant) to give the construct $orf6_{181\text{-}393}$/pET24a. Site directed mutagenesis was carried out on the *orf6*/pTYB12 and $orf6_{1\text{-}180}$/pTYB12 plasmids to introduce the required point mutations. The truncated OAT2 *C*-terminal mutant ($orf6_{1\text{-}389}$/pTYB12) was prepared by inserting a stop codon after the codon encoding Asn-388. The two vectors ($orf6_{1\text{-}180}$/pTYB12 (α); $orf6_{181\text{-}393}$/pET24 (β)) (with the appropriate mutations on the β-subunit) were transformed in *E.coli* BL21 (DE3) cells, and expression was carried out at 28 ˚C by induction with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Protein purification was carried out as for wildtype OAT2.[1]

Enzyme assays

For the ^{1}H NMR NAG/NAO hydrolysis experiments, the assay mixture contained Tris-HCl (50 mM, pH 7.5), OAT2 solution (50 μM), NAO/NAG (2 mM) and D_2O in a final volume of 100 μl. Scans were taken every 30 minutes over a period of 12 hours on a Bruker Ultrashield 500 MHz spectrometer at 298 K. The UV-ninhydrin assays and MALDI/MS were carried out as described.[1,2]

Crystallization, data collection and structure solution

OAT2 was prepared as reported.[2] Screening for OAT2 crystals in the presence of NAG was as described.[1] The optimized crystal growth conditions, with incubation at 17 ºC consisted of OAT2 (12 mg/mL) in ammonium sulphate (1.4 M), lithium sulphate (0.12 M), Tris-HCl pH 8.5 (0.1 M), and *N*-α-acetyl-*L*-glutamate (0.15 M). These conditions produced single prism-shaped crystals, of approximate dimensions 0.25 x 0.20 x 0.30 mm (after 4 days).

Data were collected using an Xcalibur Nova instrument (Oxford Diffraction). Intensities were integrated and scaled with the program CrysAlis (Aligent Technologies). Subsequent data reduction and model refinement used the CCP4 suite.[3] Four monomers derived from the A chain of wildtype OAT2 (PDB id:1vz6) were identified using PHASER.[4] The model was refined using REFMAC[5] and adjusted using O[6] and COOT.[7] NCS restraints were removed in the final stages of refinement with CNS.[8] The quality and stereochemistry of the structure was monitored after the refinement with WHATCHECK[9] and PROCHECK.[10] Serial soaking with NAG solutions, flash-cooling of these crystals in liquid nitrogen resulted in structures with partial occupancy of NAG in one OAT2 molecule and also scattered, unresolved regions of electron density (data not shown). The atomic coordinates and structure factors for the acyl-OAT2 complex (PDB ID: 2YEP) have been deposited at the Protein Database in Europe (PDBe; http://www.ebi.ac.uk/pdbe).

Molecular dynamics simulations

Molecular dynamics simulations (MDS) were performed using the acyl-OAT2-glutamate (PDB ID: 2YEP) and OAT_M-*L*-Orn (PDB ID: 3it6) complex crystal structures. Residues with missing atoms in the crystal structures were built using psfgen of NAMD2.[11] Throughout MDS studies, the amino group of the Thr-181 was acetylated; and the *C*-termini of Ala-180 and Thr-393 were in their carboxylate form. Default protonation states were used for all the

residues except for Thr-181 and the substrates. It is proposed that *L*-Glu/*L*-Orn bind initially with their α-amino nitrogens in a protonated state. Concomitant with binding to the enzyme, this proton is possibly transferred to the *N*-terminal amino group acetyl-Thr-181 or to a water molecule. Therefore, in the MDS involving the unmodified enzyme, the protonation state of the α-amino group of Thr-181, and the two ligands (*L*-Glu and *L*-Orn) were varied to check whether the protein and the ligands were capable of forming stable complexes under different protonation states. Histidines were protonated at the delta-position. Different protonation states of both the substrates and the acetylated residue Thr-181 were investigated. *L*-Orn was docked in molecule CD using GOLD.[12,13] Two orientations of *L*-Orn were tested, one of them comparable to that of the *L*-Glu molecule in the crystal, and a second configuration where the binding direction at the α-carboxylate and side chains were reversed to that of glutamate. The atomic models were solvated using the Solvate plug-in of VMD[14] using a box padding of 14x14x15 $Å^3$ dimensions. Waters from the crystal structure were included in the models. Sodium ions were added to neutralize the systems. The total system sizes were ~102,000 atoms. MDS on the crystal structure of OAT_M *L*-Orn complex as reported by Sankaranarayanan *et al.*[15] were carried out following the same protocol. Different variants were analyzed in terms of their potential impact on the flexibility of catalytic residues and their effect on the substrate binding. Variant structures were generated with the VMD program.

Each system was first minimized over 1,000 steps using a conjugate gradient procedure, followed by 200 ps of unconstrained dynamics at 100 K and 200 K respectively. Unrestrained MDS for 6 ns were performed at 298 K. The CHARMM27 force field with CMAP correction[16] was used, together with TIP3P model for water molecules.[17] Default CHARMM parameters were used for ions in bulk solution. Periodic boundary conditions were adopted and the particle mesh Ewald algorithm was used to treat the electrostatics interactions.[18] Van der Waals forces were smoothly switched off at 10-12 Å. Bonds with hydrogen atoms were restrained by the SETTLE algorithm,[19] in order to use a 2 fs time step. The multi time step algorithm r-RESPA[19] was used to integrate the equation of motion. Non-bonded short-range forces were computed every time step, while electrostatic forces were updated every 2 time steps. MDS were performed using the NPT ensemble; the pressure was kept at 1 atm using the Nose-Hoover Langevin piston method,[20] with a damping time constant of 100 ps and a period of 200 ps. The temperature maintained by coupling to a Langevin thermostat, with a damping coefficient of 5 ps^{-1}. Calculations were performed using version 2.6 of NAMD.[11,21]

Supplementary figures and tables

Fig. S1: **View from the acyl-OAT2-glutamate crystal structure showing the four subunits/eight chains of OAT2 acyl-enzyme complex (PDB ID: 2YEP).** The AB, CD, EF and GH molecules are shown in different colors corresponding to the eight different chains. Thr-181, glutamate and acetate are in red sticks.

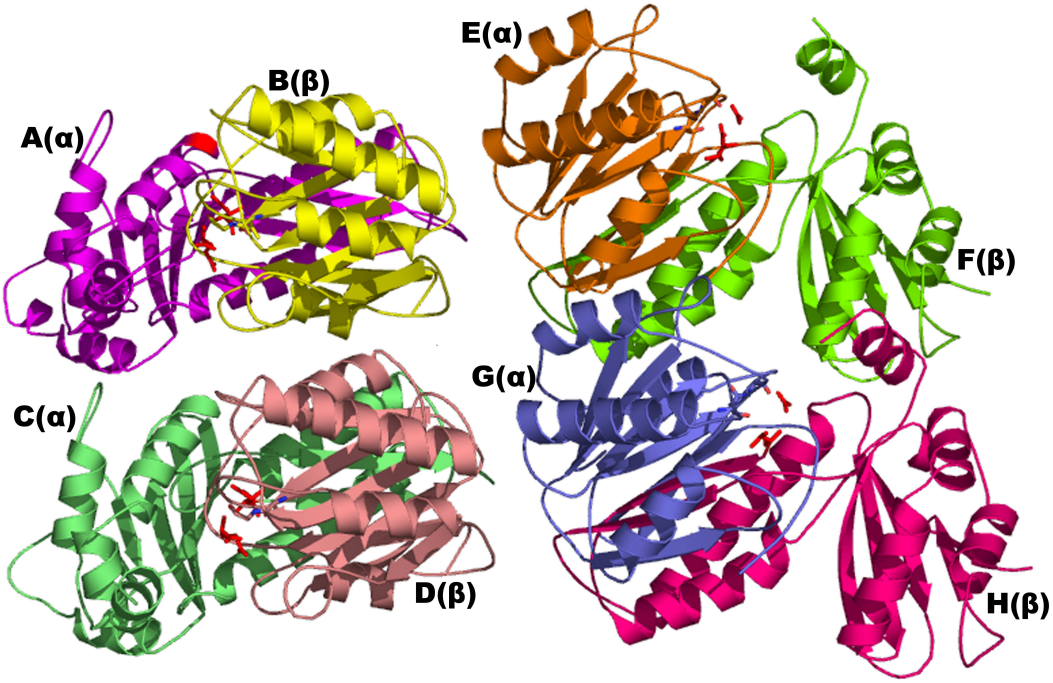
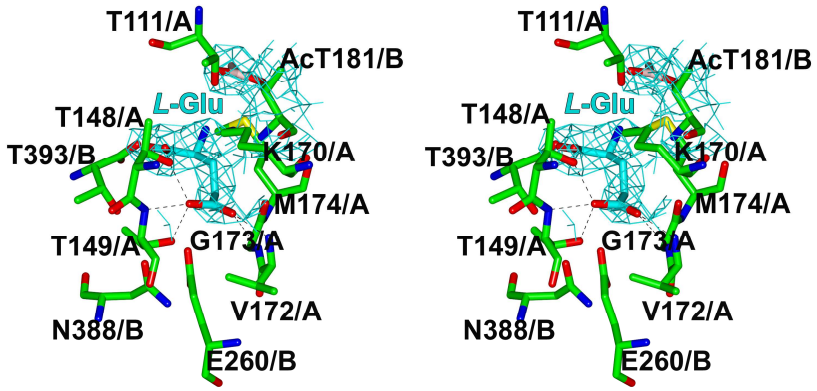
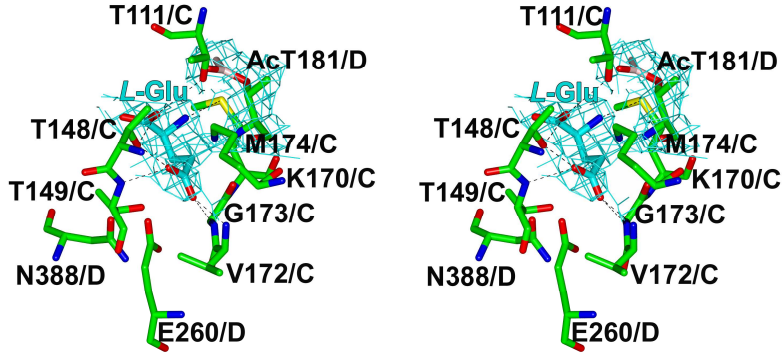


Fig. S2: Stereoviews derived from the the acetyl-OAT2-glutamate crystal structure (PDB ID: 2YEP). AB, CD, EF and GH molecules are in panels a, b, c and d, respectively. The 2mF_o-DF_c map in blue is contoured at 1σ.

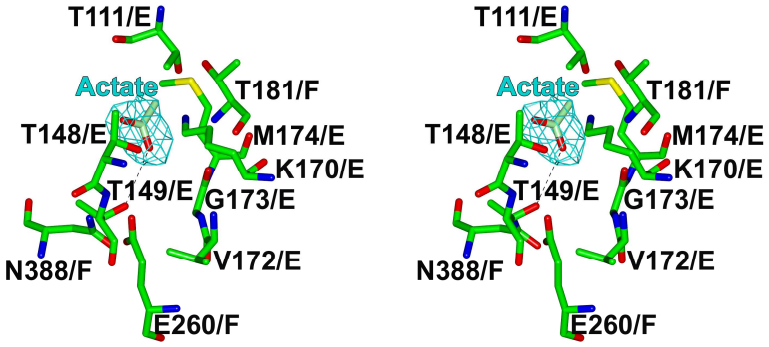
a



b



c



d

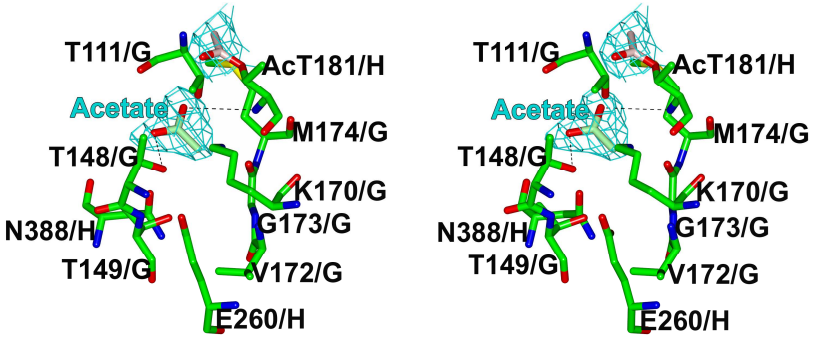


Fig. S3: **SDS-PAGE gel of the purified OAT2 variants**: T148A, T149A, D150G, and K170A showing the different levels of autoprocessing that occur under standard expression conditions.

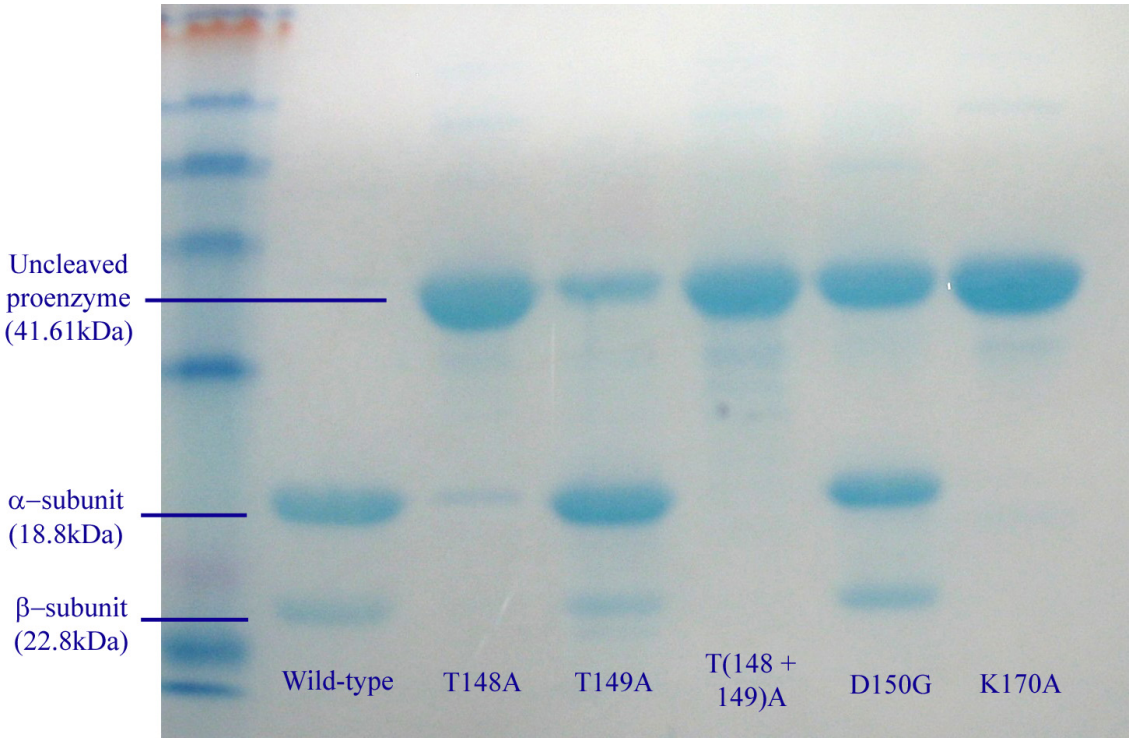


Fig. S4: **Backbone root mean square deviations (RMSD) of the α-helical component of OAT2 as a function of time.** Values associated with the simulation starting with the crystal structure (PDB ID: 2YEP), where *L*-Glu is present are shown in black. Values for the same system when the contribution of the *C*-terminus residues (Asp-379 to Thr-393) is not taken into account are shown in red. Values for the system where *L*-Orn was docked are shown in pink (whole α-helical component) and blue (in the absence of the *C*-terminus).

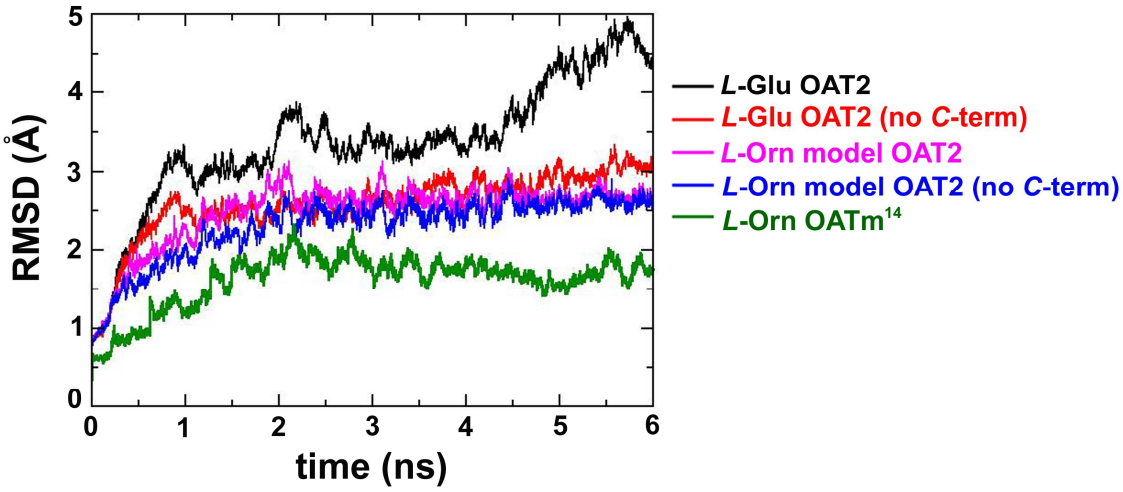


Table S1: X-Ray data collection and refinement statistics.

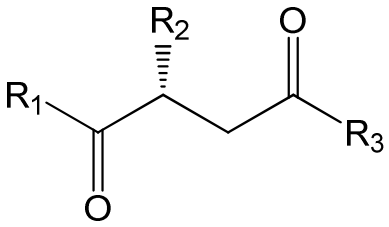
X-ray source		Xcalibur Nova	
Wavelength (Å)		CuK_α (1.5418)	
PDB Acquisition Code		2YEP	
Space group		$P2_1$	
Unit Cell Dimensions (*a* Å, *b* Å, *c* Å, β °)		61.22, 73.42, 172.33, 93.26	
Resolution shell (Å)		61.12 – 2.70	2.77 – 2.70
Number of Reflections		40637	2916
Average $I/\sigma(I)$		7.9	3.0
Completeness (%)		96.0	95.7
R_{cryst} (%)*		21.8‡	
R_{free} (%) (based on 5.4% of reflections)		23.6‡	
RMS deviation§		0.015 (1.7)	
B factors (Å^2)‖			
	From Wilson Plot	14.5	
	Chain A	9.3/10.7	
	Chain B	7.4/8.9	
	Chain C	8.6/9.8	
	Chain D	8.4/9.2	
	Chain E	14.9/16.0	
	Chain F	14.4/15.5	
	Chain G	17.2/18.8	
	Chain H	17.3/18.3	
Number of Water Molecules		419	
Ramachandran favoured/outliers (%)		94.6/0.1	

* $R_{cryst} = \sum| |F_{obs}| - |F_{calc}| | / \sum |F_{obs}| \times 100$

§ RMS deviation from ideality for bonds (followed by the value for angles).

‖ Average *B* factors in order: main chain, side chain (by chains)

Table S2: Protonation states of *L*-Glu, and *N*-α amino group of acetyl-Thr-181 that were investigated by MDS on the acetyl-OAT2 glutamate complex. The observed distances are given as the mean between the *N*-α atom of the substrate and the carbon of the CO ester group of acetyl-Thr-181 over the time course of the MDS; the angles given are formed by the *N*-α of the substrate and the CO atoms of acetyl-Thr-181 observed over the time course of the MDS.



Set	Protonation states of L-Glu	Protonated Acetyl-Thr-181		Deprotonated Acetyl-Thr-181	
		Distance(Å) Nα-CO	Angle (°) Nα-CO	Distance (Å) Nα-CO	Angle (°) Nα-CO
1G	R_1=R_3=O^- R_2=NH_3^+	5.1 ± 0.5	98 ± 11	9.2 ± 1.0	107 ± 15
2G	R_1=R_3=OH R_2=NH_3^+	10.3 ± 1.4	96 ± 17	10.9 ± 3.3	106 ± 16
3G	R_1=O^- R_3=OH R_2=NH_3^+	5.9 ± 0.5	94 ± 11	8.8 ± 1.7	82 ± 24
4G	R_1=OH R_3=O^- R_2=NH_3^+	7.8 ± 0.7	99 ± 12	6.9 ± 0.3	97 ± 13
5G	R_1=R_3=O^- R_2=NH_2	3.8 ± 0.3	88±7 (0-1.5 ns) 118±11 (1.5-6ns)	4.8 ± 0.7	103 ± 19
6G	R_1=R_3=OH R_2=NH_2	7.9 ± 3.1	86 ± 12	6.0 ± 1.5	102 ± 15
7G	R_1=O^- R_3=OH R_2=NH_2	4.0 ± 0.3	116 ± 9	6.2 ± 1.5	100 ± 12
8G	R_1=OH R_3=OO^- R_2=NH_2	6.5 ± 1.0	98 ± 12	6.9 ± 2.7	96 ± 33

Table S3: Computed mean distances (Å) and angles (degrees) (with standard deviations), during the time course of the MDS on OAT2 variants (in the case of K170A, a stable complex was not formed). Distances are between the Nα atom of the substrate and the CO of acetyl-Thr-181; angles are formed by the Nα of the substrate and the CO atoms of acetyl-Thr-181.

Protein/Variant	**Distance** (Nα-Substrate---CO Acetyl-Thr-181) Å		**Angle** (Nα-Substrate---CO Acetyl-Thr-181) °	
Acyl-OAT2-glutamate/ornithine	L-Glu	L-Orn	L-Glu	L-Orn
wild type	3.8 ± 0.3	3.3 ± 0.1	88 ± 7 (0-1.5ns) 118 ± 11 (1.5-6ns)	73 ± 9
D150G	3.6 ± .3	3.9 ± 0.4	72 ± 19	120 ± 14
K170A	>10	>10	N.A.	N.A.
T148A	7.7 ± 1.0	6.0 ± 0.6	107 ± 14	63 ± 9
T149A	4.4 ± 1.0	4.5 ± 0.6	62 ± 22	84 ± 11

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