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PAPER

Structural and biochemical analyses reveal how ornithine acetyl transferase binds acidic and basic amino acid substrates†

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Structural and biochemical analyses reveal how ornithine acetyl-transferases catalyse the reversible transfer of an acetyl-group from a basic (ornithine) to an acidic (glutamate) amino acid by employing a common mechanism involving an acetyl-enzyme intermediate but using different side chain binding modes.

Introduction

Ornithine acetyl transferases (OAT) catalyse the reversible transfer of an acetyl group from *N*-α-acetyl-L-ornithine (NAO) to L-glutamate (L-Glu) to give *N*-α-acetyl-L-glutamate (NAG) and L-ornithine (L-Orn) (Scheme 1). The proposed mechanism for OATs involves an acetyl-enzyme intermediate that is covalently linked to an *N*-terminal threonine residue, the α-amino group of which enables general base catalysis.[1–3] From a mechanistic perspective OATs are interesting because they catalyse a very simple acyl-transfer reaction; they can thus serve as a useful model for detailed studies on the stereoelectronics of catalysis *via* acyl-enzyme complexes, which occur in the catalytic cycles of many enzymes including proteases, transpeptidases and esterases. The binding of L-Glu/L-Orn to OAT is also interesting from the perspective of how an enzyme can bind amino acid substrates with either basic (ornithine) or acidic (glutamate) side chains that must react similarly with the acyl-enzyme intermediate.

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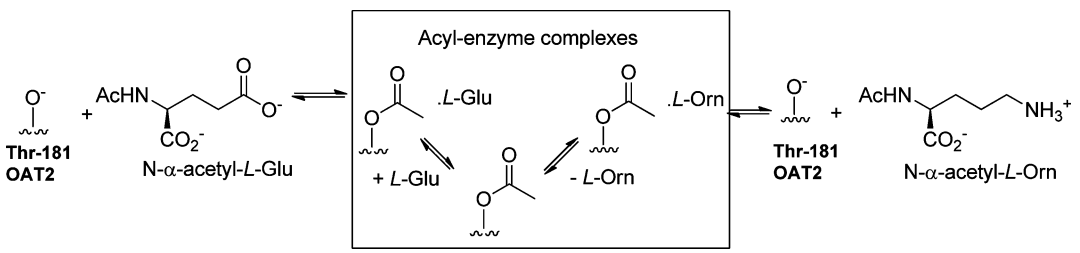
Crystal structures for an OAT in apo- and acetyl-enzyme complex forms from *Streptomyces clavuligerus* (OAT2) which is involved in clavulanic acid biosynthesis have been reported.[1,4] Recently, a structure of an OAT from *Mycobacterium tuberculosis* (OAT_M, 32% sequence identity with OAT2) has also been reported in apo-form (PDB ID: 3IT4) and in complex with L-Orn (PDB ID: 3IT6).[5] Here, we describe structural and biochemical analyses that provide insights into how OATs bind their acidic and basic substrates by employing different side chain binding modes whilst maintaining a common mechanism for *N*-acetyl transfer (Fig. 1–4).

Results and discussion

We worked initially to obtain crystal structures for the OAT2 acetyl-enzyme complex with L-Glu or L-Orn. Following crystallization attempts with NAG, we were able to obtain crystals of OAT2 in complex with L-Glu (Table S1†). Optimization of crystallization conditions led to a 2.7 Å resolution structure for OAT2 acylated at Thr-181 and in complex with L-Glu (referred to as the acetyl-OAT2-glutamate complex). OAT is a member of the extensive *N*-terminal nucleophile (Ntn) family of hydrolases. Identified Ntn hydrolases undergo autoproteolysis to yield two chains (α and β subunits) which fold to give the active protein.[6–8] As observed for wildtype OAT2 (PDB ID: 1VZ8) and OAT2-acetyl-enzyme (OAT2-AEC) (PDB ID: 2VZK)



Scheme 1 The ornithine acetyl transfer reaction showing the proposed acetyl-enzyme complex (AEC). The kinetic mechanism is ping-pong bi-bi.[3]

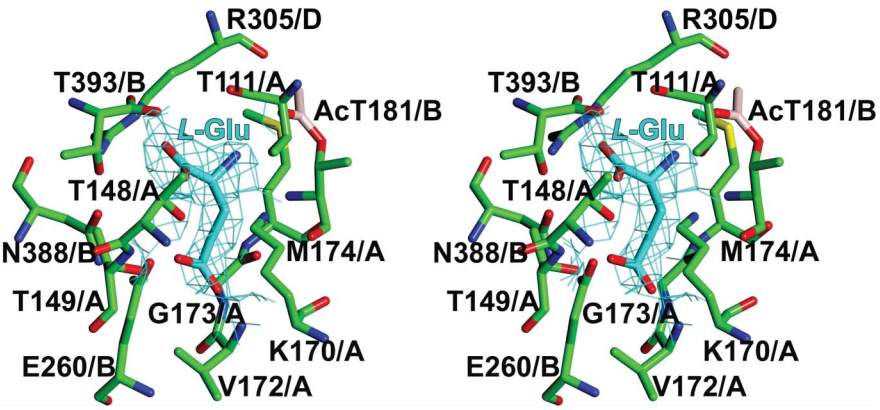


Fig. 1 Stereoviews derived from the the acetyl-OAT2-glutamate crystal structure (AB molecule) (PDB ID: 2YEP). The $2mF_o$-DF_c map in blue is contoured at 1σ.

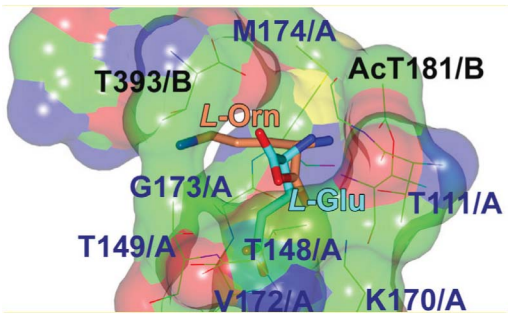


Fig. 2 Comparison of the observed binding modes for L-Orn and L-Glu. Note that the L-Orn structure is with apo-OATM whereas the L-Glu structure is with the OAT2 acetyl enzyme complex. Note that the position of the α-amino groups of L-Glu and L-Orn are similar, but that binding of their side chains is different.

structures, the overall structure of the acetyl-OAT2-glutamate complex (PDB ID: 2YEP) has the characteristic Ntn αββα fold[7] and forms an α4β4 heterotetramer as observed for many Ntn-enzymes (Fig. S1†). There are four αβ molecules in each asymmetric unit in the crystalline form of OAT2,[1] with a total of eight subunit chains: A(α), B(β), C(α), D(β), E(α), F(β), G(α) and H(β) in each asymmetric unit (Fig. S1†). The overall conformation of acetyl-OAT2-glutamate complex is similar to that of the apo-structure and the acetyl-OAT-glutamate complex (RMSD values for Cα backbone atoms 0.35 Å (PDB ID: 1VZ6) and 0.23 Å (PDB ID: 2VZK), respectively).

In the four heterodimeric α,β molecules in the asymmetric unit of the acetyl-OAT2-glutamate complex, different sized regions of positive electron density at the active sites were observed in mF_o-DF_c difference maps, which were not for protein-residues (Fig. 1 and Fig. S2†). Sfcheck[9] and OMIT map analyses[10] were used to reduce the possibility of model bias. In molecules AB, CD and GH, positive electron density was observed around γ-O of Thr-181 (Fig. 1 and Fig. S2†), as observed for the OAT2-AEC structure (PDB ID: 2VZK).[1] This positive electron density was modelled and refined as an *O*-acetyl group. Additional positive electron density was observed in the active sites of molecules AB and CD, which was too large to accommodate acetate, water or tris(hydroxymethyl) aminomethane (tris) molecules (from the crystallisation buffer). This density was modelled and refined as an L-Glu molecule (Fig. 1 and Fig. S2†).

There was no evidence for binding of L-Glu in molecules EF and GH. In molecule EF, no extra positive density was observed around Thr-181; however, positive density was observed in the active site which was refined as an acetate ion (as was done for molecule EF in the case of the reported OAT2-AEC structure).[1] In molecule GH, positive electron density around Thr-181 was modelled and refined as an acetyl group; further positive electron density was observed in the molecule GH active site which was refined as an acetate ion (Fig. S2d†). The positions of the acetate ions in EF and GH overlap with that of L-Glu in AB and CD. Subsequent descriptions refer to the AB molecule except where stated, *i.e.* to an acetyl-OAT2-glutamate complex.

Analysis of the position of L-Glu within the acetyl-OAT2-glutamate complex active site suggests its position may be relevant to a catalytically productive complex (Fig. 1 and Fig. 4). The Cα carboxylate group of L-Glu is positioned to interact with the side chain of Thr-148 (which is also involved in binding the side chain carboxylate of L-Glu) and that of Thr-393 (2.6 Å, from the *C*-terminal region of chain B of the same molecule). (In molecule CD, the *C*-terminal region is only resolved to Glu-390, explaining the lack of observation for such an interaction for this molecule.) A water molecule is positioned within hydrogen bonding distance of both the Cα amino and carboxylate groups (not shown in Fig. 1). The ester of the acetyl-enzyme complex is in the *cis*(*Z*)-conformation, as observed for the OAT2-AEC structure and for acyl-enzyme complexes in proteases[11] and penicillin binding proteins;[12,13] it has been proposed that the *cis*(*Z*)-conformation may reflect a stereochemically favoured catalytic pathway in protease catalysis.[14] In molecules AB (and CD) the α-amino group of L-Glu is close to the ester carbonyl of acetyl-Thr-181 (*N*α-Glu to carbonyl carbon of acyl-Thr-181: 3.4 Å). However, the angle between the L-Glu α-amino group and the carbonyl of the Thr-111 acetyl group is 88° (Fig. 1 and Fig. S2†) which is less than the optimal angle (~100°), for nucleophilic attack onto a carbonyl group,[15,16] this may reflect (in part) a non-productive protonation state (*i.e.* RNH_3^+) of the L-Glu α-amino group (see below). The L-Glu α-amino group is positioned close (2.8 Å) to the N-terminal α-amino group of Thr-181, consistent with the proposed role of the Thr-181 α-amino group in general acid–base catalysis during acetylation and deacetylation.

The carboxylate side chain of L-Glu is positioned to bind *via* one of its oxygens (Oε2) with the alcohols of Thr-148 (2.3 Å)

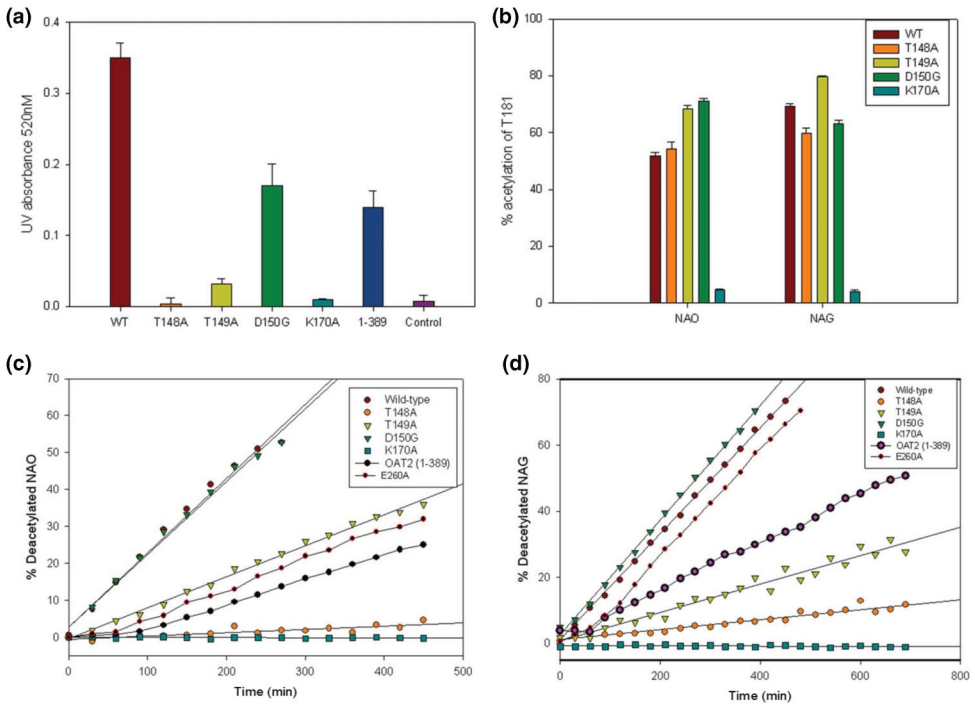


Fig. 3 OAT2 variant kinetics. a) UV-ninhydrin assays of wildtype OAT2 and variants showing the production of free ornithine (absorbance at 520 nm) in acetyl transfer reaction (NAO/L-Glu). b) MALDI/MS analyses showing the extent of acetylation of Thr-181 by OAT2 wild type and the variants, when incubated with NAG and NAO under standard conditions. c) and d) ^{1}H NMR kinetic analyses on wildtype and variants of OAT2, showing the rate of hydrolysis of a single substrate (NAG or NAO) to give the deacetylated amino acids.

and Thr-149 (2.5 Å); Oε1 of the carboxylate is positioned to bind with the backbone amide of Val-172 (2.2 Å) and Oε2 of Asp-150 (3.2 Å). Lys-170 forms one face of the side chain binding pocket, but at least in the acetyl-OAT2-glutamate complex structure is not positioned to directly interact with the L-Glu side chain carboxylate (4 Å) (Fig. 1 and Fig. S2†). However, modelling studies suggest that Lys-170 may form an electrostatic interaction with the L-Glu carboxylate side chain in solution and more generally that there may be more flexibility in side chain binding than implied by analysis of the crystal structures alone (see below).

Although we have not obtained a structure for OAT2 in complex with L-Orn, Sankaranarayanan *et al.* have reported a structure for an OAT from *Mycobacterium tuberculosis* (OAT_M) in complex with L-Orn.[5] In this structure the L-Orn α-amino group is bound in a similar position to that of L-Glu in our acetyl-OAT-glutamate structure (compare Fig. 2), suggesting a closely conserved mechanism for *N*-acetylation/deacetylation for L-Orn and L-Glu; Small differences in the positioning of the α-amino group may reflect the fact that that the L-Glu structure is for an acetyl-enzyme complex, while for the L-Orn structure, Thr-200$_{OATM}$ (Thr181$_{OAT2}$) is not acetylated at Oγ. In contrast to the positioning of the α-amino groups, the binding directions of the α-carboxylate and side chains are different in the L-Glu and L-Orn structures, with the C-α carboxylate of L-Orn occupying part of the same pocket that binds the L-Glu carboxylate side chain. This arrangement results in the α-hydrogens of L-Glu and L-Orn pointing in different directions (Fig. 2). The L-Orn C-α carboxylate is positioned to interact with the side chains of Thr-148$_{OAT2}$ (Thr-166$_{OATM}$, 2.7 Å) and Lys-170$_{OAT2}$ (Lys-189$_{OATM}$, 2.9 Å). The L-Orn side chain extends towards the outside of the active site, as predicted,[4] such that it is positioned to interact with the side chains of Glu-260$_{OAT2}$ (Glu-280$_{OATM}$, 2.8 Å), Asn-388$_{OAT2}$ (Asn-399$_{OATM}$, 2.7 Å and Thr-393$_{OAT2}$ (Ser-404$_{OATM}$, 2.7 Å).

Active site substitutions and a *C*-terminal truncation of OAT2 were then made to investigate the importance of selected residues that appear to be involved in substrate side-chain binding as observed in the crystal structure (Thr-148, Thr-149, Asp-150, Glu-260 and Lys-170). Initially, variants were made using standard techniques, and purifications attempted as described for wildtype OAT2.[1] Different levels of impaired autoproteolysis into α-, and β- subunits were observed for the variants (by SDS-PAGE analysis). The T148A, K170A variants and T148A/T149A doubly substituted variant resulted in a complete lack (<5%) of processing (Fig. S3†). OAT2 T149A gave a ~20 : 80 mixture and OAT2 D150G results in a ~50 : 50 mixture of unprocessed : processed proteins, respectively (Fig. S3†). Previous work has shown that substitution of the nucleophilic residue of Ntn hydrolases blocks autoprocessing.[17,18] Our results imply that additional active site residues involved in catalysis/substrate binding are also involved in autoprocessing. Because some substitutions in full length OAT2 hindered autoprocessing, we developed a co-expression procedure for the α- and β-subunits, wherein they were expressed using different plasmids (see Supplementary Methods†). Following development work with wildtype OAT2, the T148A, T149A, D150G, K170A, E260A and OAT2Δ1-389 OAT2 variants were successfully produced as $\alpha_2\beta_2$ heterotetramers (to >90% purity by SDS-PAGE).

The variants were then analysed by an assay employing spectrophotometric detection (520 nm) of L-Orn (produced by the acetyl transfer reaction of NAO/*L*-glu) after derivitisation with

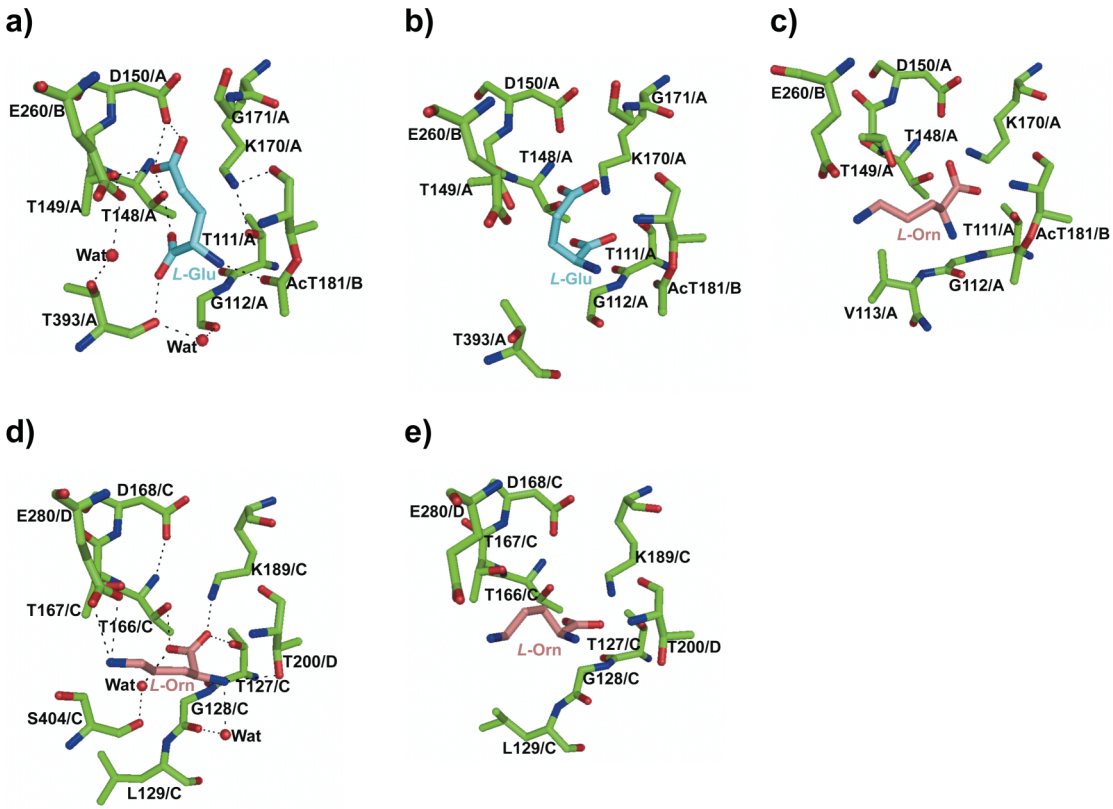


Fig. 4 Comparison of the active site conformations of the acetyl-OAT2-L-Glu (a and b) and OAT_M-L-Orn (d and e) complexes before (a and d) and after (b and e) molecular dynamics simulations (MDS). c) MDS studies on an acetyl-OAT2 model with L-Orn docked by GOLD (CCDC Software Limited). The side chain orientations of acetyl-Thr-181 and oxyanion hole residues, Thr-111 and Gly-112 remain similar after MDS. Note the different position of the Cα-carboxylate in (a) and (d). L-Glu and L-Orn are in cyan and salmon sticks, respectively. A, B, C and D refer to the A (α), B (β), C (α) and D (β) chains of the AB and CD molecules of $OAT2/OAT_M$.

Table 1 OAT2 variant kinetics

Protein	Autoprocessing to α-, β-subunits (~%)	Acetyl transferase activity (NAO/L-Glu) (~%)[a]	NAG hydrolysis (Initial rate)[b] μMmin^{-1}	NAO hydrolysis (Initial rate)[b] μMmin^{-1}	Acetylation of Thr-181 with NAG (~%)[d]	Acetylation of Thr-181 with NAO (~%)[d]
Wild type	100	100	3.20 ± 0.05[c]	4.02 ± 0.06[c]	70	50
T148A	0	2	0.32 ± 0.01[c]	0.18 ± 0.01[c]	60	55
T149A	80	10	0.86 ± 0.03[c]	1.67 ± 0.02[c]	80	70
D150G	50	50	3.44 ± 0.08[c]	3.93 ± 0.02[c]	65	75
K170A	0	5	<0.05	<0.05	<5	<5
OAT2Δ1–389	100	40	1.56 ± 0.09[c]	0.80 ± 0.01[c]	N.D.	N.D.
E260A	N.D.	40	2.26 ± 0.05[c]	1.25 ± 0.04[c]	N.D.	N.D.

[a] Production of Orn from NAO/L-Glu, as monitored by UV-ninhydrin detection of ornithine. [b] Production of L-Glu and L-Orn from NAG and NAO respectively as monitored by ^{1}H NMR. [c] Errors are shown as standard deviations of analyses in triplicate. [d] Monitored by MALDI/MS.% Values refer to wildtype OAT2 activities, except for (d). N.D., not determined.

ninhydrin.[19] The results for the variants showed that the OAT2Δ1-389, and E260A variants had ~40%, the D150G variant ~50%, and the T149A variant ~10% of the wild type activity under standard conditions. The T148A and K170A variants were almost inactive (Table 1 and Fig. 3a). In general, these results support the crystallographic analyses and indicate that the side chains of Lys-170, Thr-148, Thr-149, Glu-260, and the *C*-terminal region, but not Asp-150, are important for OAT2 activity.

In the absence of an acceptor substrate,[1] OAT2 catalyses the hydrolysis of NAG/NAO to L-Glu and L-Orn. To investigate the relative importance of different residues in the interactions with L-Orn and L-Glu, and OAT2, ^{1}H NMR assays were carried out wherein the OAT2 variants were incubated separately with NAG or NAO, to monitor L-Glu and L-Orn production (Fig. 3c, d). Consistent with the coupled assays, these assays revealed that the D150G variant did not affect NAG/NAO hydrolysis under standard conditions (Table 1), and that the K170A variant was near completely inactive (Table 1). Activities were reduced for all the other variants with both NAG and NAO. In each case the T148A variant was less active than the T149A variant, suggesting that Thr-148 is more important in the overall reaction (Table 1). The OAT2Δ1-389 and E260A variants were found to hydrolyse

NAG faster than NAO (Table 1), again supporting the structural analyses that the *C*-terminal region and Glu-260 play a more important role in binding NAO/L-Orn than they do for NAG/L-Glu.

Mass spectrometry (MS) was then employed to investigate whether an acetyl-enzyme intermediate is formed during OAT2 variant catalysis. Following incubation with an excess of NAO or NAG, the variant proteins were digested with trypsin, and the fragments then analysed by MALDI/MS. Except for K170A, in all cases, the variants were found to be acetylated on the peptide fragments bearing the *N*-terminal nucleophile Thr-181 (^{181}TLLTFFATDAR)[1] consistent with them operating *via* the same mechanism as the wildtype OAT2 (Fig. 3b and Table 1).

Molecular dynamics simulations (MDS) were then carried out to further investigate the role of individual residues in the apparently different binding modes of OAT2 to its substrates. MDS for the acetyl-OAT2-glutamate complex supported the overall structure observed crystallographically; However, movement of the *C*-terminus was observed (see below) (Fig. S4†). MDS on the OAT_M L-Orn complex structure[5] were also carried out and indicated that that the crystallographically observed conformation was stable.

Because crystal structures with L-Orn in complex with OAT2, or the OAT_M acetyl-enzyme complex, are not available, docking of L-Orn was carried out using the acetyl-OAT2-glutamate structure in absence of the L-Glu. With OAT2, two orientations of L-Orn were tested, one of them comparable to that observed for L-Glu in the OAT2 crystal-structure, and one where the binding direction at the α-carboxylate and side chains were as observed for the binding of L-Orn to OAT_M. Interestingly, of the two L-Orn orientations investigated by MDS, only the one analogous to the binding of L-Orn as observed in the OAT_M structure[5] was stable (Fig. 4c) supporting the proposal of different side chain binding modes for L-Orn and L-Glu.

MDS were carried out on the acetyl-OAT2-L-Glu/L-Orn complexes in all reasonable substrate protonation states and with the Nα amino group of Thr-181 either in protonated or neutral forms (see Table S2† for results with L-Glu). Only the protonation states in which the Cα carboxylate and side chains of substrates were ionised, the Nα-amino group of the substrate was neutral, and in which Nα amino group of acetyl-Thr-181 was protonated rendered systems with favourable organisation for nucleophilic attack of the α-amino groups of L-Glu/L-Orn onto the carbonyl group of the AEC for the duration of the MDS (Table S2†). These observations are consistent with our proposed mechanism, where a nucleophilic attack is not possible with a protonated Nα-amine.[1] Collectively these results support the proposal that OATs employ a common acetyl transfer mechanism for L-Glu and L-Orn in which both L-Glu and L-Orn α-amino groups bind similarly (see Table S3† for mean distances between the Nα amines of L-Glu and L-Orn and carbonyl carbon of acetyl-Thr-181 during the MDS), in which the L-Orn α-carboxylate and L-Glu carboxylate-bearing side chain occupy a common pocket (Fig. 4).

In the acetyl-OAT2-glutamate structure, the carbonyl group of acetyl-Thr-181 is bound in an oxyanion hole formed by the alcohol side chain of Thr-111 and backbone amide of Gly-112 (Fig. 4a, 5), as observed in the OAT2-AEC structure.[1] During MDS, the side chain of Thr-111 was observed to move out of the oxyanion hole to hydrogen bond with the backbone carbonyl of Thr-181, as observed for the acyl-OAT2 complex, and additionally to the Cα-carboxylate of either L-Orn/L-Glu.

In terms of the residues involved in side chain binding of L-Orn/L-Glu, the MDS studies in general support the crystallographic analyses (Fig. 4 and Fig. 5). They also support the role of the *C*-terminus in being more important in L-Orn than in L-Glu binding. However, during MDS for the acetyl-OAT2-glutamate complex employing the proposed catalytically productive protonation states, differences were observed in the orientation of L-Glu in its binding pocket relative to that adopted in the crystal structure for the same complex (Fig. 4a and Fig. 4b). The side chain carboxylate of L-Glu was observed to move during MDS and adopt a stable position in which it is positioned to interact with the hydroxyl of Thr-149, the side chain amine of Lys-170 and backbone amide of Thr-181 (Fig. 4b). In contrast, in the acetyl-OAT2-glutamate crystal structure the side chain carboxylate of L-Glu was observed to interact with side chain hydroxyls of Thr-148 and Thr-149, and the backbone amide of Val-172 (Fig. 4a). The MDS thus support the apparently essential role of the Lys-170 side chain in catalysis (Fig. 3 and Fig. 4).

In the MDS of the OAT_M L-Orn complex the side chain amino group of L-Orn was sometimes observed to move to interact with Ser-404 (OAT_M) of the *C*-terminus, and Glu-280 (OAT_M), and the carboxylate group of L-Orn to interact with Thr-166 and Lys-189 (OAT_M) (Fig. 4e, see below). Although the MDS results support the crystallographic analyses describing different side chain binding modes for L-Glu and L-Orn, they also imply there may be flexibility in side chain orientation of the residues involved in binding. Therefore, we cannot exclude the possibility that in solution the preferred binding modes may be somewhat different, or that there can be alternative modes to those observed crystallographically.

In MDS based on the acetyl-OAT2-glutamate complex, the *C*-terminal helix (residues 380 to 393) was observed to become unstructured and move away from the active site, providing sufficient room for L-Glu to be released. In contrast, in the MDS based on the acetyl-OAT2-ornithine complex, the *C*-terminal helix remained in place, with the interactions between Asn-388 and the L-Orn side chain being preserved (Fig. S4†). In MDS based on the OAT_M L-Orn complex,[5] Ser-404 (equivalent to Thr-393 (OAT2) remained in close proximity to L-Orn during the length of the MDS with its hydroxyl group positioned to interact with the L-Orn side chain. These observations are supportive of the present and previous crystallographic and solution studies suggesting that the *C*-terminal region is more important in binding L-Orn than L-Glu.

MDS were also carried out, using the proposed catalytically 'productive' protonation states, on the biochemically analysed OAT2 variants (data not shown). In support of the experimental results, in MDS of the D150G variant, both the acetyl-OAT2-glutamate/ornithine complexes were stable. In contrast, for the K170A variant, both L-Glu/Orn were immediately released from the active site, consistent with the lack of activity for this variant. For MDS on the T148A, T149A variants, both substrates remained in the active site, but the mean distance between the substrate Nα amino group and the carbonyl carbon of acetyl-Thr181 increased relative to that for the wild type complex (Table S3†). For the E260A variant, the acyl-OAT2 complex was less

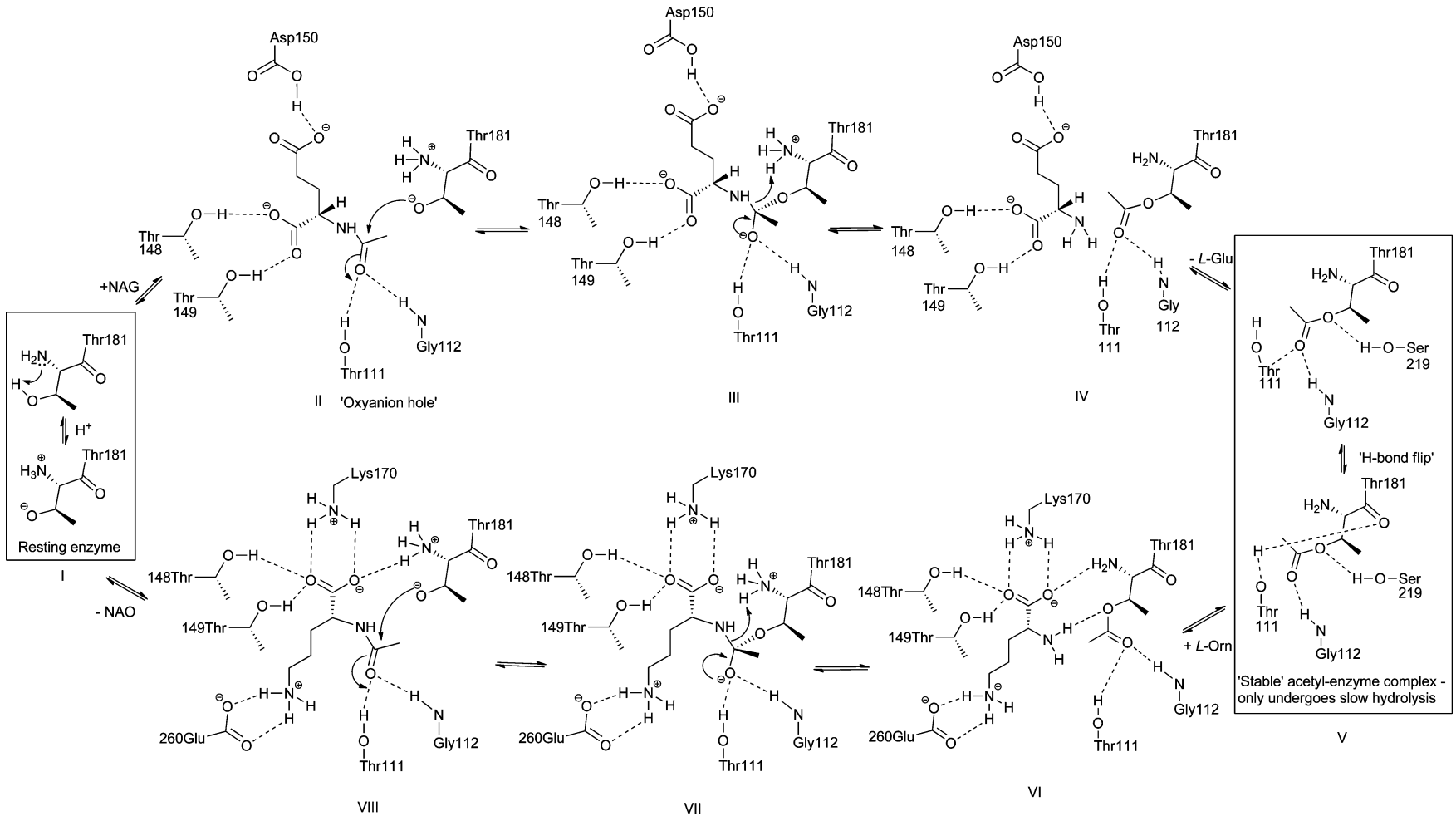


Fig. 5 Mechanistic proposal for ornithine acetyl transferases. The resting enzyme (I), L-Glu complexes (II–IV), 'stable' acetyl enzyme complex (V), and L-Orn complexes (VI–VIII) are shown. Note only selected residues proposed to be involved in substrate binding are shown and there is evidence for conformational changes in catalysis. See text for discussion. NAO = *N*-α-acetyl-L-ornithine; NAG = *N*-α-acetyl-L-glutamate.

stable with L-Orn than it was with L-Glu, again consistent with solution analyses (Table S3†).

In conclusion, the combined biophysical, solution, and MDS studies imply that the *N*-acetylation/deacetylation mechanisms for L-Orn/NAO and L-Glu/NAG follow closely related mechanistic pathways, in part because of the similar position of the α-amino group relative to the acetyl group of the acetylated Thr-181 (Fig. 5). The crystallographic studies, supported by MDS, reveal that the L-Glu carboxylate and L-Orn amino side chains likely bind in different orientations with the L-Orn α-carboxylate and L-Glu carboxylate-bearing side chains occupying the same pocket (Fig. 4 and Fig. 5). The MDS reveal that the side chain binding may be more dynamic than implied by the crystal structures, with the *C*-terminus playing a role, especially for NAO/L-Orn binding. Overall, it seems likely that for ornithine acetyl transferases, catalysis may be regarded as a property of the 'whole active site' and beyond, as proposed for other enzymes, including triosephosphate isomerase[20] and more recently proteases including elastase.[16,21] The combined studies also reveal that both neutral (*e.g.* Thr-148) and charged side chains (Lys-170 for both L-Glu and L-Orn, and Glu-260 for L-Orn) are involved in binding. The work should provide a basis for engineering OATs to further expand their substrate selectivity.

Experimental

Experimental details are given in the supporting information.†

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