Determinants of Substrate Specificity in ω-Aminotransferases*

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Ornithine aminotransferase and 4-aminobutyrate aminotransferase are related pyridoxal phosphate-dependent enzymes having different substrate specificities. The atomic structures of these enzymes have shown (i) that active site differences are limited to the steric positions occupied by two tyrosine residues in ornithine aminotransferase and (ii) that, uniquely among related, structurally characterized aminotransferases, the conserved arginine that binds the α-carboxylate of α-amino acids interacts tightly with a glutamate residue. To determine the contribution of these residues to the specificities of the enzymes, we analyzed site-directed mutants of ornithine aminotransferase by rapid reaction kinetics, x-ray crystallography, and 13C NMR spectroscopy. Mutation of one tyrosine (Tyr-85) to isoleucine, as found in aminobutyrate aminotransferase, decreased the rate of the reaction of the enzyme with ornithine (Tyr-85) to isoleucine, as found in aminobutyrate aminotransferase, decreased the rate of the reaction of the enzyme with ornithine 1000-fold and increased that with 4-aminobutyrate 16-fold, indicating that Tyr-85 is a major determinant of specificity toward ornithine. Unexpectedly, the limiting rate of the second half of the reaction, conversion of ketoglutarate to glutamate, was greatly increased, although the kinetics of the reverse reaction were unaffected. A mutant in which the glutamate (Glu-235) that interacts with the conserved arginine was replaced by alanine retained its regiospecificity for the ω-amino group of ornithine, but the glutamate reaction was enhanced 650-fold, whereas only a 5-fold enhancement of the ketoglutarate reaction rate resulted. A model is proposed in which conversion of the enzyme to its pyridoxamine phosphate form disrupts the internal glutamate-arginine interaction, thus enabling ketoglutarate but not glutamate to be a good substrate.

Ornithine aminotransferase (Orn-AT) and 4-aminobutyrate aminotransferase (GABA-AT) are members of a large family of pyridoxal 5′-phosphate (PLP)-dependent enzymes that catalyze a wide range of reactions on amino acids (1). Each enzyme operates by a mechanism, common to all aminotransferases (Fig. 1), in which the cofactor shuttles between pyridoxaldehyde and pyridoxamine forms by means of two coupled half-reactions (2, 3). The half-reaction converting ketoglutarate to glutamate is the same for both enzymes as well as for the majority of other aminotransferases. In this half-reaction, the chemical changes occur at the α-carbon. The substrate specificity of the enzymes thus arises from the other half-reaction that transfers an amino group distant from the α-carbon. For this reason, the enzymes are known as “ω-aminotransferases” (4). In the case of GABA-AT, the amino group transferred is the only amino group in the substrate molecule, whereas Orn-AT specifically selects the ω-amino group of ornithine despite the presence of a second amino group on Ca with the same configuration as that in glutamate (3).

The three-dimensional structures of both enzymes have been solved in the unliganded form as well as in complex with various substrate analogues (5–7). The enzymes have the same basic fold as the best studied member of the largest family of PLP-dependent enzymes, namely aspartate aminotransferase (8). The root mean square deviation of Ca positions in Orn-AT and GABA-AT is relatively large at 2.1 Å, and the enzymes have only 17% sequence identity (6). Nevertheless, the arrangements of residues at the active sites of the two enzymes are strikingly similar (Fig. 2). All of the direct contacts between cofactor and enzyme protein are conserved. Sterically, there are only two significant differences in the active site regions. One is a straightforward substitution in which Tyr-85 in Orn-AT is replaced by isoleucine in GABA-AT.4 The other is an exchange of Tyr-55 in Orn-AT for Phe-351* in GABA-AT.5 Despite arising from completely different parts of the molecule, the Ce atoms of the aromatic rings of these last named residues occupy the same steric position, but the planes of their aromatic rings are perpendicular to each other. The necessary space for the aromatic ring of Tyr-55 in Orn-AT exists because residue 351 is glycine. Conversely, space for the Phe-351* aromatic ring is available because the homologue of residue 55 in GABA-AT is alanine. One of the aims of this study was to determine the extent to which Orn-AT can be converted to GABA-AT by mutating the relevant residues together as well as individually.

A further aim of the study was to test a hypothesis advanced to explain the ability of these enzymes to act at ω- and α-amino groups in alternate half-reactions (5). We addressed this particular aspect of specificity in the case of Orn-AT because ornithine has both an α- and an ω-amino group. The “Glu-235 switch” hypothesis was inspired by the observation that, uniquely in Orn-AT and GABA-AT, a conserved arginine (Arg-413) is engaged in tight H-bond/salt bridge interactions with the side chain carboxylate of Glu-235 (5). This arginine is one of only four residues that are conserved in all the aminotransferases from the same family (9). The crystal structure of

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4 In this study, residues occupying homologous sequence positions will be numbered as in Orn-AT.
5 The asterisk indicates that the residue is from the neighboring subunit.
Orn-AT bound to an ornithine analogue shows that Arg-413 remains masked by Glu-235 and that the \( \alpha \)-carboxylate of ornithine is bound by Arg-180 with the non-reacting \( \alpha \)-amino group held between the OH groups of Tyr-55 and Tyr-85 (5). These observations led to the proposal that the interaction between Glu-235 and Arg-413 is important in preventing binding of ornithine in an orientation that would lead to transamination of the \( \alpha \)-amino group of this substrate. It was further proposed that in the second half-reaction, Glu-235 switches its position and uncovers Arg-413 so that ketoglutarate and glutamate can bind productively through their respective \( \alpha \)-carboxylate groups (5). To assess the contribution of the proposed Glu-235 switch in determining Orn-AT substrate specificity, we produced Orn-AT E235S and E235A mutants and analyzed their kinetic properties.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis, Expression, Preparation, and Assay of Enzymes—* A DNA fragment encoding a fusion protein consisting of residues 26–439 of wild-type Orn-AT precursor protein coupled to maltose-binding protein in the expression vector pMal-c2 (New England Biolabs) was transformed into XL1 Escherichia coli cells and used for expression of the wild-type enzyme (5). The same vector was used for mutagenesis with the QuikChange™ site-directed mutagenesis system (Stratagene). Expressed proteins were purified according to the standard protocol described in the manual provided with the pMal™ expression and purification system except that ketoglutarate (2 mM) and pyridoxal phosphate (20 \( \mu \)M) were included in the solution used to lyse the bacteria. In this solution and in solutions used for affinity and DE-Sepharose col-
umns, 20 mM Tris/HCl, pH 7.6, was replaced by 20 mM EPPS, pH 7.6. This purification resulted in enzyme that was completely in the EL form. For experiments requiring the EM form of the enzyme, the EL form (0.5 ml, 100 μM) was treated with 5 μl of 100 mM l-ornithine and separated on Sephadex G-25 equilibrated in 20 mM EPPS, pH 7.6. Orn-AT and GABA-AT were assayed by the methods of Peraino et al. (10) and Silverman and Levy (11), respectively.

**Acquisition and Analysis of Kinetic Data**—Experiments analyzing the half-reactions of Eₐ and Eₐₚ with single substrates were carried out on a stopped-flow spectrophotometer (Model SF-1, Hi-Tech, Salisbury, UK). All the reactions were carried out at 25 °C in 20 mM EPPS brought to pH 7.6 with NaOH. Absorbance was measured at 420 nm and was displayed using the IS-2 software supplied with the instrument. All of the stopped-flow analyses of single reactions fitted well to single exponential processes. At least three experiments were carried out at each substrate concentration. Amplitudes and apparent first-order rate constants (kₐₚ) were determined using the non-linear regression software package provided with the instrument. Equation 1 was used to analyze the dependence of kₐₚ on substrate concentration for the reactions of ornithine and glutamate with EL forms of the enzyme and of ketoglutarate with EM forms.

\[
k_{\text{obs}} = k_{\text{lim}} K_s [S] + [S]
\]

(Eq. 1)

Equation 2 was used to analyze the dependence of kₐₚ on [GABA] in its reaction with Eₐ forms

\[
k_{\text{obs}} = k_{\text{lim}} [S] + [S] + k_b
\]

(Eq. 2)

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**Results and Discussion**

**Determination of Kinetic Properties**—To determine the kinetic parameters controlling the separate half-reactions with different substrates, we conducted single turnover experiments in which we followed changes in coenzyme absorbance as the enzyme converts between its 420-nm-absorbing pyridoxalimine form (EL) and its 330-nm-absorbing pyridoxamine form (EM). An analysis of the kinetics of the separate half-reactions of wild-type rat kidney Orn-AT with ornithine, glutamate, and ketoglutarate has been reported earlier (3). In the present work, similar analyses were conducted on recombinant wild-type human Orn-AT to provide a reliable basis for determining the effects of the mutations. A study of the half-reaction of wild-type Orn-AT with GABA was also carried out. Changes in absorbance from all the half-reactions fitted well to single exponential processes, and the observed first-order rate constants (kₐₚ) showed hyperbolic dependence on substrate concentration (Equations 1 and 2, "Experimental Procedures"). The results of all these kinetic analyses are reported in TABLES ONE through FOUR.

**Properties of the Wild-type Enzyme**—The kₐₚ/Kᵣ values found for the wild-type enzyme indicated that it is 450 times more effective at catalyzing the reaction of EL with ornithine than that with glutamate. The
difference between the kinetic behavior toward the two substrates was entirely in the $K_s$ term, an observation that accords well with the hypothesis that Glu-235 must disengage from its interaction with Arg-413 before glutamate can bind productively to EL. We expected that this disengagement would also be necessary for ketoglutarate to bind the EM form of the enzyme. However, we found that the reaction of EM with ketoglutarate is the fastest of the half-reactions of the wild-type enzyme tested in this study and has a $K_s$ value lower than that for ornithine in its reaction with EL. This observation suggested that, in the case of EM, Arg-413 is readily available for binding ketoglutarate, possibly because the interaction between Arg-413 and Glu-235 is weakened. Further observations, which will be presented later in this study, supported this proposal.

Properties of Mutant Forms of Orn-AT—The structure of Orn-AT bound to ornithine analogues shows that the $\alpha$-amino group of ornithine is held between the OH groups of Tyr-55 and Tyr-85 (5). Mutation of either residue resulted in a similar lowering of $k_{lim}/K_s$: 400-fold for Tyr-55 and 1000-fold for Tyr-85. Thus, the contribution of each of these interactions to decreasing the activation energy for the reaction is relatively high (14–16 kJ/mole), demonstrating that both of these tyrosines are major contributors to the productive binding of ornithine. The fact that Y55A and Y55G mutations led to a relatively small decrease (8-fold maximum) of $k_{lim}/K_s$ for the reactions with glutamate and ketoglutarate suggests that the cavity created by excision of the Tyr-55 side chain has not significantly modified the structure of the active site.

When the half-reaction with GABA was analyzed, the only mutant to show a substantial increase in reactivity toward GABA was Orn-AT-FIGURE 3. Reaction of GABA with EL form of Orn-AT-Y85I. The mutant enzyme (6 μM, E₁ form) was mixed with [GABA] in the following concentrations: 0.125 mM (a), 0.25 mM (b), 0.5 mM (c), and 5 mM (d).
determined because $k_{\text{obs}}$ increased linearly with ketoglutarate concentration until the reaction became too fast to be measured since it was complete within the 2-ms mixing time of the instrument. The value of $k_{\text{obs}}$ was greater than 2000 s$^{-1}$ at ketoglutarate concentrations above 12.5 mM. Thus, this mutation increased the value of $k_{\text{lim}}$ for the EM:ketoglutarate reaction by at least 12-fold and the value of $K_s$ by at least 48-fold. We were surprised that the Y85I mutation so greatly affected the ketoglutarate reaction but had no effect on the glutamate reaction. This observation would be explained if the reaction of $E_i$ with glutamate includes an extra step in which Glu-235 is displaced from Arg-413 by a conformational change that is slow enough to make a major contribution to $k_{\text{obs}}$ for the reaction in this direction.

Structure of Orn-AT-Y85I—We have solved the structure of Orn-AT-Y85I to a resolution of 3 Å. Apart from the mutation itself, there were no significant differences between wild-type and mutant structures. Furthermore, the backbone and Cβ, Cγ1, and C8 atoms of Tyr-85 in wild-type Orn-AT and Ile-85 in Y85I mutant Orn-AT are in almost identical positions (Fig. 5a). Two small differences between the structures of the mutant and wild-type enzymes should be noted. First, in the mutant, the methyl group Cγ2 of Ile-85 fitted into a pocket between the Lys-292-PLP aldimine and the amide of Asn-266. This pocket was vacant in the wild-type enzyme. Second, in the mutant, the substrate binding cavity was substantially enlarged because of the absence of the atoms of the tyrosine side chain (Fig. 5a). These differences, together with the loss of Van der Waals contacts between Tyr-85 side chain and the substrate (5), may explain the significant decrease in reactivity toward ornithine observed for Orn-AT-Y85I.

However, the 19-fold increase in $k_{\text{lim}}/K_s$ toward GABA also contributed significantly to the changed specificity. Our kinetic data indicated that Ile-85 does not have a major effect on substrate binding as shown by the relatively small change in $K_s$. Instead, the 19-fold increase in $k_{\text{lim}}/K_s$ was clearly due to a 60-fold increase in $k_{\text{lim}}$. Superimposition of the structures of GABA-AT and Orn-AT-Y85I (Fig. 5a) shows that Ile-85 is shifted by about 2 Å with respect to the homologous Ile-72 of GABA-AT. Together with this positional shift, the two isoleucines displayed different rotamer conformations because GABA-AT Ile-72 makes contact with Phe-351*, which is not present in the Orn-AT structure. Assuming that GABA binds to the active site as in GABA-AT with its carboxylate bound to Arg-180 (6), it seems unlikely that the side chain of Tyr-85 would hinder binding, although the 3-fold increased $K_s$ term indicates that binding to form the external aldimine is made slightly more difficult by the Y85I mutation. We propose that the 60-fold increase in $k_{\text{lim}}$ arises because Ile-85 constrains the orientation of GABA substrate so that its Cγ–H bond is held more nearly perpendicular to the cofactor imine plane, thereby accelerating the proton abstraction that is central to catalysis (18). Aspartate aminotransferase (Asp-AT) has Val-37 in a position similar to that of Ile-85 in Orn-AT-Y85I (8). One of the valine Cγ atoms is seen to make contact with Cβ of the substrate analogue α-methyl aspartate in the way we propose for Ile-85 and GABA in Orn-AT-Y85I.

Finally, the major difference in the kinetic behavior of WT-Orn-AT and the Y85I mutant concerned the large increases of both $k_{\text{lim}}$ and $K_s$ for the reaction of $E_i$ with ketoglutarate. These were probably due to the introduction of Cγ2 of Ile-85 into a position close to Lys-292, which mediates the proton transfer, and also close to the carbon atoms (C4’ of the cofactor and Cγ of GABA) between which the proton is transferred. Conceivably, the C4’–H bond of the cofactor was also constrained to be more nearly orthogonal to the cofactor ring.

Kinetic and Structural Properties of Orn-AT-Y85I-Y55A-G320F—We were unable to detect any reaction between the $E_i$ form of this mutant

![FIGURE 4. Reactions of Orn-AT-Y85I with ketoglutarate. The mutant enzyme (6 μM) in the $E_a$ form was mixed with ketoglutarate (kG). a, course of reaction observed at 0.5 mM ketoglutarate. b, course of reactions observed at 2.5, 5, and 12.5 mM ketoglutarate. c, dependence of observed first-order rate constant on ketoglutarate concentration.](image-url)
(33 μM) and ornithine at concentrations up to 50 mM and times up to 30 min. The absorption spectrum (A_{420} = 0.22, A_{280} = 1.84) remained constant throughout the experiment. However, slow reactions between the EL form of this mutant were observed with both glutamate and GABA. In both cases, A_{420} decreased exponentially and k_{obs} increased linearly with substrate concentration up to the highest concentration examined (0.1 M). At this concentration of glutamate, some precipitation of the protein was observed. Although independent values of k_{lim} and K_s could not be determined for these reactions, the slope of the line relating k_{obs} with substrate concentration is equal to k_{lim}/K_s. The value of this constant was very much lower for both substrates than for the wild-type enzyme and all other mutants tested. The reaction of the EM form of Orn-AT-Y85I:Y55A:G320F with ketoglutarate was more than 100 times faster than the reaction of the EL form with either glutamate or GABA, and the dependence on ketoglutarate concentration was hyperbolic. However, the value of k_{lim}/K_s was more than 15,000 times lower than for the wild-type enzyme and all other mutants tested. The reaction of the E_M form of Orn-AT-Y85I:Y55A:G320F with ketoglutarate was more than 100 times faster than the reaction of the E_L form with either glutamate or GABA, and the dependence on ketoglutarate concentration was hyperbolic. However, the value of k_{lim}/K_s was more than 15,000 times lower than for the wild-type enzyme with both k_{lim} and K_s being adversely affected. Comparison of the structures of the active sites of GABA-AT and the triple mutant Orn-AT-Y85I:Y55A:G320F (Fig. 5b) shows that the newly introduced Phe-320* side chain introduced into Orn-AT does not occupy the same steric position as Phe-351* in GABA-AT, although the backbone atoms of the residues are in almost identical positions.

The complete loss of reactivity with ornithine was not surprising. Single mutations of the tyrosine residues that bind the α-amino group of ornithine (Tyr-85 and Tyr-55) made the reaction with ornithine 1000 and 400 times slower, respectively. Not only were both these tyrosines missing, but the ring of Phe-320* occupied the space taken up by the α- and β-carbons of ornithine when it was bound to the wild-type enzyme. It seemed likely that the major loss of activity toward glutamate, GABA, and ketoglutarate is caused by obstruction of the carboxylate substrate binding site by Phe-320*.

A possible explanation for the observation that the Phe-320* side chain does not occupy the space vacated by the Tyr-55 side chain and so does not adopt a similar orientation to that of Phe-351* in GABA-AT is that in GABA-AT, Phe-351* makes contact with the ring of Tyr-348*, a residue arising from a turn at a conserved proline residue (Phe-347 in GABA-AT, Phe-316 in Orn-AT). The Orn-AT residue corresponding to Tyr-348* in GABA-AT was glycine. It seemed that, in the absence of the potential Van der Waals and hydrophobic interactions with the homologue of Tyr-348*, Phe-320* takes up a position in which it makes these interactions with Phe-177.

**Reactions of Glu-235 Mutants**—The kinetics of the half-reactions of the E_L forms of Orn-AT-E235S and Orn-AT-E235A with ornithine were also adversely affected: 8- and 1600-fold decreases, respectively. The structure of Orn-AT-WT complexed with 5-fluoromethylornithine showed that the Cγ and Cδ of Glu-235 make Van der Waals contact with the Ca and Cδ of the ornithine analogue. It seemed likely that these contacts contribute both to tighter binding and to proper orientation of this substrate. Both E235S and E235A mutations led to a lowering of k_{lim} and an increase in K_s, although the relatively minor effects of the serine mutation suggest that the hydroxyl group of this residue remains H-bonded to Arg-413, leaving the atoms of its side chain still able to make some productive contact with ornithine.

**FIGURE 5. Stereo image of superimposed active sites.** a, wild-type GABA-AT (carbon atoms in light blue) and Orn-AT-Y85I (carbon atoms in orange). The carbon atoms of Tyr-85 in wild-type Orn-AT are yellow. Mutated residues are denoted by an asterisk. b, GABA-AT (carbon atoms in light blue), Orn-AT Y85I/Y55A/G320F (carbon atoms in orange), and side chains of Gly-320, Tyr-55, and Tyr-85 in wild-type Orn-AT (carbon atoms in yellow).
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The E235S mutation produced the same minor effect on \(k_{\text{lim}}\) for the reaction of \(E_\text{w}\) with glutamate as for the reaction with ornithine. However, whereas \(K_e\) for the ornithine reaction was increased 5-fold, \(K_e\) for glutamate was decreased 10-fold. The E235A mutation produced qualitatively similar results, but quantitatively, the effects were much greater. For glutamate, the values of \(k_{\text{lim}}\) and \(K_e\) were lowered 28- and 18,000-fold, respectively, with the result that \(k_{\text{lim}}/K_e\) was increased 650-fold. Comparison of the relevant \(k_{\text{lim}}/K_e\) values showed that mutation of Glu-235 to serine produced a switch in specificity away from ornithine and toward glutamate of 50-fold, whereas mutation of this residue to alanine produced a switch in the same direction by a factor of 10^6. These observations suggested that a major function of the interaction between the side chains of Arg-418 and Glu-235 is to limit reaction with glutamate, and, to a lesser extent, to promote reaction with ornithine. The further observations that the Glu-235 mutations had little effect on the reaction of \(E_\text{M}\) with ketoglutarate added support to the proposal, made earlier in this work, that Glu-235 interacts weakly (or not at all) with Arg-418 in the \(E_\text{M}\) form of the enzyme.

Effect of E235A Mutation on the Regiospecificity of the Reaction with Ornithine—We conducted experiments to test the proposal that masking of Arg-418 by Glu-235 prevents Arg-418 from binding the \(\alpha\)-carboxylate of ornithine, thus ensuring that transamination occurs exclusively at the \(\omega\)-amino group of this substrate. The transamination mechanism requires proton abstraction from the carbon atom bearing the transferred amino group and, if a single half-reaction is carried out in D\(_2\)O, the relevant proton is exchanged (19). This exchange occurs because the half-reaction between amino substrate and oxo product continues to run in both directions after equilibration is reached, although there is no net change in substrate and product concentrations. It has previously been shown by proton NMR spectroscopy that rat kidney Orn-AT exclusively replaces the pro-5 proton on C\(\delta\) of ornithine (3). In this work, we confirmed this observation by demonstrating that the area under the peak attributed to the two protons on C\(\delta\) of ornithine (30 mM) was reduced to half after 50 min in the presence of recombinant human Orn-AT (0.3 mM). We also used \(^{13}\)C NMR analysis of the same deuterated ornithine sample to demonstrate that the exchange at C\(\delta\) was stereospecific. Proton broad band decoupled \(^{13}\)C NMR was a particularly appropriate technique for detecting and quantifying replacement of \(^1\)H by \(^2\)H at identifiable carbon atoms because all the \(^1\)C peaks appeared as sharp singlets until \(^1\)H was replaced by \(^2\)H. Fig. 6 shows the \(^{13}\)C NMR spectrum of ornithine before and after treatment with Orn-AT in D\(_2\)O. The peak due to C\(\delta\) was a singlet before treatment and a 1:1:1 triplet after. This demonstrated that only one of the \(^1\)H atoms on C\(\delta\) has been replaced (replacement of both \(^1\)H atoms would have resulted in a quintuplet). The peak attributed to the C\(\alpha\) proton was a singlet in both samples. We experienced some difficulty in conducting the same experiment with the mutant Orn-AT-E235A. When we used the same enzyme concentration as in the corresponding experiment with the wild-type enzyme (0.3 mM), the solution became cloudy immediately after adding ornithine and, after 16 h, a dense precipitate had formed. After separation of precipitated protein by centrifugation and soluble protein by centrifugal ultrafiltration, \(^{13}\)C NMR showed a small amount of replacement at C\(\delta\) (data not shown). A similar experiment carried out at a lower enzyme concentration (0.1 mM) for 48 h resulted in substantially less precipitation and more extensive replacement (Fig. 5c).

The signal due to C\(\delta\) was now composed of a singlet and a triplet. This showed that some of one of the protons on C\(\delta\) had been replaced by \(^2\)H to produce the triplet. The singlet was attributed to C\(\delta\), which still had two \(^1\)H atoms. The peak due to C\(\alpha\) remained as a sharp singlet, showing that no exchange had taken place at C\(\alpha\). Measurement of the relative heights of the residual \(^{13}\)C singlet and the \(^{13}\)C triplet suggested the fraction of \(^1\)H exchanged to be estimated as \(\approx 50\%\). Thus, after unmasking Arg-418 by mutating Glu-235, Orn-AT retained its ability to transaminate the \(\omega\)-amino group of ornithine in preference to the \(\alpha\)-amino group, demonstrating that the Arg-418/\(\text{Glu-235}\) interaction is not responsible for the regiospecificity of the wild-type enzyme in its reaction with ornithine.

Binding of Dicarboxylate Ligands—Our kinetic analysis yielded the following important observations. Wild-type Orn-AT \(E_\text{w}\) enzyme reacted better with ornithine than with glutamate because of a higher affinity for ornithine. This preference was reversed when Glu-235 was mutated either to serine or to alanine. Indeed, both mutations resulted in lower \(K_e\) values for glutamate. However, this was not what we observed when considering the second half-reaction catalyzed by the enzyme, i.e. that between \(E_\text{M}\) and ketoglutarate. Mutation of Glu-235 had little effect on that reaction. E235S had virtually no effect, and E235A increased \(k_{\text{lim}}/K_e\) but 100 times less than that of the \(E_\text{w}\)/glutamate reaction. To understand this different behavior toward the two dicarboxylate substrates, we must keep in mind that in the \(E_\text{w}\) form that reacts with glutamate, the cofactor is bound as a protonated aldimine with Lys-292, whereas in the \(E_\text{M}\) form that reacts with ketoglutarate, the coenzyme is pyridoxal phosphate and is not covalently bound to the enzyme. The crystallographic structure of the enzyme, solved using the \(E_\text{M}\) form (6, 7), shows a network of H-bonds connecting Glu-235 and Arg-413 to O’ of the cofactor via the side chain amide of Gln-266. It is possible that when the cofactor is pyridoxal phosphate, this H-bond network is affected so as to weaken the interaction between Arg-413 and Glu-235. We thus propose a system in which the Glu-235 switch is closed in the \(E_\text{w}\) form, thereby allowing productive binding of ornithine but impeding binding of glutamate and open in the \(E_\text{M}\) form, making Arg-413 available to bind ketoglutarate. This model fits well with the abundant evidence, demonstrating that, in vivo, Orn-AT operates in the direction in which ornithine and ketoglutarate are the substrates (4). Moreover, the...
hypothesis is supported by the recently published structure (20) of a mutant form of GABA-AT (I50Q) that is partially in the \( E_M \) form and in which the homologue of Glu-235 (E211) is seen to be rotated away from the homologous conserved arginine. Furthermore, the structure of the homologue of Orn-AT-E235S (GABA-AT-E211S), in which the switch must be permanently open, shows the enzyme to be entirely in the \( E_M \) form (20), reinforcing the proposal that the status of the cofactor and the Glu-235 switch are linked.

REFERENCES
1. Mehta, P., and Christen, P. (2000) Adv. Enzymol. Relat. Areas Mol. Biol. 74, 129–184
2. John, R. A., and Fowler, L. J. (1976) Biochem. J. 155, 645–651
3. Williams, J. A., Bridge, G., Fowler, L. J., and John, R. A. (1982) Biochem. J. 201, 221–225
4. John, R. A., and Fowler, L. J. (1985) in Transaminases (Christen, P., and Metzler, D., eds), pp. 413–421, John Wiley & Sons, Inc., New York
5. Storici, P., Capitani, G., Müller, R., Schirmer, T., and Jansonius, J. N. (1999) J. Mol. Biol. 285, 297–309
6. Storici, P., Capitani, G, De Biase, D., Moser, M., John, R. A., Jansonius, J. N., and Schirmer, T. (1999) Biochemistry 38, 8628–8634
7. Liu, W., Peterson, P. E., Carter, R. J., Zhou, X., Langston, J. A., Fisher, A. J., and Toney, M. J. (1994) Biochemistry 33, 10896–10905
8. Ford, G. C., Eichele, G., and Jansonius, J. N. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2559–2563
9. Mehta, P. K., Hale, T. I., and Christen, P. (1993) Eur. J. Biochem. 214, 549–561
10. Peraino, C., Bunville, L. G., and Tahmisian, T. N. (1969) J. Biol. Chem. 255, 10284–10289
11. Silverman, R. B., and Levy, M. A. (1981) Biochemistry 20, 1197–1203
12. Shen, B. W., Henning, M., Hohenester, E., Jansonius, J. N., and Schirmer, T. (1998) J. Mol. Biol. 277, 81–102
13. CCP4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760
14. Lamzin, V. S., and Wilson, K. S. (1993) Acta Crystallogr. Sect. D Biol. Crystallogr. 49, 129–147
15. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
16. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
17. Philippsen, A. (1998). DINO: a visualization system for structural data, Version 0.5, Biozentrum, University of Basel, Switzerland
18. Dunathan, H. C. (1966) Proc. Natl. Acad. Sci. U. S. A. 55, 712–716
19. Banks, E. C., Bell, M. P., Lawrence, A. J., and Vernon, C. A. (1966) Pyridoxal Catalysis: Enzymes and Model Systems (Snell, E. E., Braunstein, A. E., Severin, E. S., and Torcinski eds) pp. 191–202, Wiley Interscience, New York
20. Liu, W. S., Peterson, P. E., Langston, J. A., Jin, X.G., Zhou, X. Z., Fisher, A. J., and Toney, M. D. (2005) Biochemistry 44, 2982–2992
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