Minireview

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Structure and Mechanism of *O*-Acetylserine Sulfhydrylase*

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The *O*-acetylserine sulfhydrylase (OASS) *from Salmonella typhimurium* catalyzes a β-replacement reaction in which the β-acetoxy group of *O*-acetyl-L-serine (OAS) is replaced by bisulfide to give L-cysteine and acetate. The kinetic mechanism of OASS is ping-pong with a stable α-aminoacrylate intermediate. The enzyme is a homodimer with one pyridoxal 5′-phosphate (PLP) bound per subunit deep within the protein in a cleft between the N- and C-terminal domains of each of the monomers. All of the active site residues are contributed by a single subunit. The enzyme cycles through open and closed conformations as it catalyzes its reaction with structural changes largely limited to a subdomain of the N-terminal domain. The elimination of acetic acid from OAS is thought to proceed via an *anti*-E2 mechanism, and the only catalytic group identified to date is lysine 41, which originally participates in Schiff base linkage to PLP. The transition state for the elimination of acetic acid is thought to be asynchronous and earlier for Cβ–O bond cleavage than for Cα–H bond cleavage.

The biosynthesis of L-cysteine in enteric bacteria, such as *Salmonella typhimurium* and *Escherichia coli*, and in plants proceeds via a two-step pathway (Fig. 1). The amino acid precursor of L-cysteine is L-serine, which undergoes a substitution of its β-hydroxyl with a thiol in two steps. Serine acetyltransferase (EC 2.3.1.30) catalyzes the acetylation (by acetyl-CoA) of the β-hydroxyl of L-serine to give *O*-acetyl-L-serine (OAS)[1] (1). The final step, the α,β-elimination of acetate from OAS and the addition of H_2S to give L-cysteine is then catalyzed by *O*-acetylserine sulfhydrylase (OASS; EC 4.2.99.8). In enteric bacteria, two isozymes of OASS, A and B, are produced under aerobic and anaerobic growth conditions, respectively (2). The A and B isozymes are homodimeric with M_r of about 68,900 and 64,000, respectively, and each has one tightly bound pyridoxal 5′-phosphate (PLP) per subunit. The structure and mechanism of the A isozyme of OASS from *S. typhimurium* will be the focus of this minireview. For a more comprehensive review, see Ref. 3. It has recently been shown that OASS-A is negatively regulated by small anions binding to an allosteric site at the dimer interface (4), but because of space limitations this aspect will not be discussed in this article.

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[1] The abbreviations used are: OAS, *O*-acetyl-L-serine; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5′-phosphate; TNB, 5-thio-2-nitrobenzoate.

Kinetic Mechanism

The kinetic mechanism of OASS is Ping Pong Bi Bi (Fig. 2) as shown by initial velocity studies in the absence and presence of products and dead end inhibitors, isotope exchange at equilibrium, and equilibrium spectral studies (5, 6). *O*-Acetyl-L-serine binds to the internal aldimine form of the enzyme (*E*), and acetate is released as the first product. Bisulfide then adds as the second substrate to the α-aminoacrylate intermediate form of the enzyme (*F*), and L-cysteine is released as the final product. The initial velocity pattern obtained in the absence of added inhibitors exhibits competitive inhibition by both substrates, which is normally diagnostic for a ping-pong mechanism, resulting from substrates binding to the incorrect enzyme form (OAS to F and bisulfide to E). In the present case, however, the inhibition results from the binding of substrates to an inhibitory allosteric site (7).

The turnover number of the enzyme is 280 s^{-1}, and the first half-reaction limits the overall reaction. The second half-reaction is very fast and likely diffusion limited with a first-order rate constant of >1000 s^{-1} measured at 5 μM bisulfide at pH 6.5 (8). In a classical one-site ping-pong mechanism, the individual half-reactions are independent of the concentration of the other substrate. This is not the case for OASS, and the V/K_{OAS} values were not identical when bisulfide (10^5 M^{-1} s^{-1}) or 5-thio-2-nitrobenzoate (TNB; 37 M^{-1} s^{-1}) was the nucleophilic substrate (11). The *V/K* values for the second half-reaction were within a factor of 2 of one another when different amino acid substrates were used. These data are reconciled as a result of a decrease in activity of the enzyme dependent on occupancy of the allosteric anion-binding site (see below). The rate with TNB is 10^4-fold lower than that obtained with bisulfide. With TNB as the second substrate, one-third of the α-aminoacrylate intermediate is converted to the amino acid product, *S*-(3-carboxy-4-nitrophenyl)-L-cysteine, and two-thirds are converted to pyruvate and ammonia.

Structure of OASS-A

The three-dimensional structure of OASS-A without ligands bound has been solved to 2.2-Å resolution (9). The enzyme is a homodimer of 315 amino acid residues per subunit. The dimer is arranged such that entry to the two active sites is on the same side (Fig. 3). The subunits interact with one another only at the dimer interface, and each of the active sites is made up of amino acids from a single subunit. The recently identified allosteric anion-binding site, located at the dimer interface, provides a reason for the overall dimeric structure other than stability (4).

Each subunit is composed of two domains, an N-terminal domain, comprised predominantly of residues 1–145, and a C-terminal domain, comprised predominantly of residues 146–315. A portion of the N-terminal domain sequence, residues 13–34, crosses over into the C-terminal domain, forming the first two strands of its central β-sheet. Each domain is composed of an α/β-fold with a central twisted β-sheet surrounded by α-helices (Fig. 3). The active site is located at the interface of the two domains deep within the protein.

The PLP cofactor in the active site cleft is in Schiff base linkage with the ε-amino group of Lys-41. The pyridine ring fits into a shallow crevice of the active site and is supported at the back by Val-40. The 5′-phosphate of PLP is anchored by eight hydrogen bonds contributed by main chain and side chain

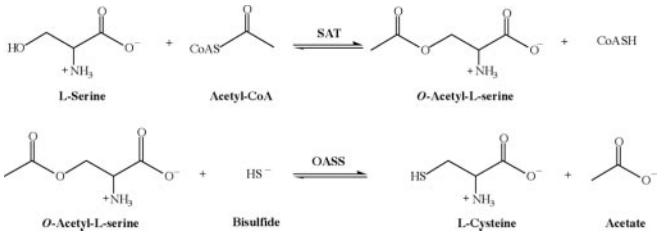

FIG. 1. **Pathway for the biosynthesis of L-cysteine from its L-serine precursor.** Cysteine synthesis occurs in two steps catalyzed by serine acetyltransferase (*SAT*), which is responsible for acetylation of serine, and OASS, which is responsible for the substitution of acetate with sulfide.

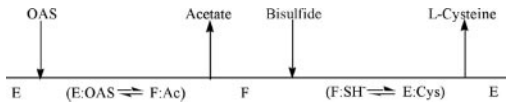

FIG. 2. **Schematic depiction of the ping-pong kinetic mechanism of OASS.** In the mechanism, *E* represents the internal Schiff base, and *F* represents the α-aminoacrylate external Schiff base. In the first half of the reaction OAS is converted to the α-aminoacrylate external Schiff base and the first product, acetate, is released, and in the second half of the reaction bisulfide is added to give the final product cysteine.

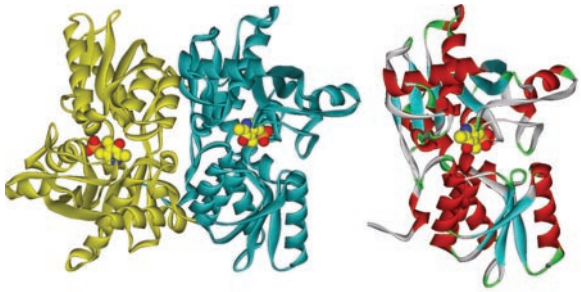

FIG. 3. **Ribbon representation of the structure of OASS-A.** *Left*, the dimer of OASS-A with PLP in space-filling representation. A molecular 2-fold axis runs from the *lower left* to the *upper right* of the molecule. *Right*, a monomer of OASS-A is shown. The two-domain nature of the structure is shown with the central, twisted β-sheet in both domains surrounded by helices. The entry to the active site is on the *left* of the monomer. The figure was prepared using DS Viewer 5.0 from Accelrys.

functional groups in the Gly-176–Thr-180 loop (Fig. 4) (9). The 5′-phosphate of the cofactor is dianionic as suggested by ^{31}P NMR (10, 11), and its charge is partially neutralized by the dipole of helix 7 in the C-terminal domain, one of three helix dipoles directed toward the active site. The phenolic O-3′ of the cofactor is within hydrogen-bonding distance of the amide nitrogen of the Asn-71 side chain and the Schiff base nitrogen. Residue 71 is part of a loop (residues 68–72) called the asparagine loop, and along with Gln-142, it binds the substrate carboxylate in the external Schiff base and subsequent intermediates along the reaction pathway. A second helix dipole, contributed by helix 2, is directed at the asparagine loop and likely contributes to charge neutralization when substrate is bound. Finally, the pyridine nitrogen of the cofactor is within hydrogen-bonding distance to Ser-272 and the dipole of helix 10. On the basis of the proximity of the helix dipole, it is unlikely that N-1 of the PLP is protonated. A view looking into the active site indicates the *re* face of the PLP imine is exposed to the incoming amino acid.

Replacement of the Schiff base lysine, Lys-41, with Ala resulted in isolation of a yellow enzyme suggesting PLP was bound in Schiff base linkage with a free amino acid, and this was shown to be methionine (12). Unlike the free enzyme, which is instantaneously reduced by $NaBH_4$, the K41A mutant enzyme was resistant to reduction, suggesting the enzyme was in a closed conformation. The structure of the K41A mutant enzyme of OASS-A revealed an external Schiff base between the enzyme and L-methionine (13). An overlay of the overall structure of the free enzyme and that of the K41A mutant enzyme shows a large conformational change in a subdomain of the N-terminal domain in which the central β-sheet has relaxed, closing the active site. The closed form has the methionine substrate analog buried within the protein, and the active site cleft is no longer accessible to bulk solvent with only a narrow channel remaining to allow the passage of small molecules such as acetate (the first product) or bisulfide (the second substrate). In the open form, the C terminus is less ordered than the N terminus, but it becomes more ordered in the closed form of the enzyme.

Formation of the external Schiff base with methionine results in a rotation of the cofactor and subsequent movements of the pyridine nitrogen toward the protein interior and the external Schiff linkage closer to the active site entrance (Fig. 5). The α-carboxylate group of the amino acid substrate is in a strong hydrogen-bonding network with residues of the asparagine loop (residues 68–72). Asp-69 moves 7 Å compared with its position in the open conformation to make two new hydrogen bonds, one with the α-carboxylate of amino acid substrate and the other with the O-3′ of PLP. The interaction between the asparagine loop and the α-carboxylate is the trigger that causes the movement of the subdomain consisting of β-strands 4 and 5 and α-helices 3 and 4, reducing the twist of the central β-sheet and closing the site. Active site closure serves to expel bulk solvent and to properly position functional groups for catalysis. The cofactor pyridine ring, the aldimine linkage, and the α-carboxylate group are all co-planar although Cα of the amino acid substrate lies outside this plane. Modeling in the Schiff base lysine, Lys-41, shows alignment of its β-methylene with Ala-41 of the mutant enzyme. The lysine points toward the *si* face of the cofactor. The methionine side chain is directed toward the active site entrance and away from the *re* face of the cofactor, *i.e.* the α-proton and side chain of methionine are *anti*.

Chemical Mechanism

The ping-pong kinetic mechanism elucidated for the OASS-A indicates its reaction is catalyzed in two steps as an elimination-addition reaction to give the overall β-substitution reaction. Ultraviolet-visible spectral studies are consistent with an internal Schiff base (λ_{max} = 412 nm) present as the resting form of the enzyme and the α-aminoacrylate external Schiff base (λ_{max} = 330, 470 nm) present after elimination of the β-acetoxy group (5, 10, 11, 14).

The pH dependence of V/K_{OAS}, which reflects free enzyme and the free amino acid substrate, exhibits a p*K* of 7 on the acidic side, whereas values of 6.7 on the acid side and 8.3 on the base side were measured with β-chloro-L-alanine (BCA) as the amino acid substrate (15). The pH dependence of V/K_{TNB}, which reflects the α-aminoacrylate external Schiff base and free TNB, shows p*K* values of 6.9–7.1 on the acidic side and 8.3 on the basic side. There are no ionizable groups in the active site of OASS-A that could be responsible for the p*K* of 7 (Fig. 4), and there is no need for a general base in the elimination of acetate or chloride, both stable leaving groups. The basic p*K* observed in the V/K_{BCA} profile is that of the α-amine of the amino acid substrate required to be unprotonated for nucleophilic attack on C-4 of the PLP Schiff base. The basic p*K* observed in the V/K_{TNB} profile is attributed to the ϵ-amino group of Lys-41, which must act as a general acid to donate a proton to Cα as the product external Schiff base is formed.

The enzyme also exhibits a β-elimination reaction in the absence of the nucleophilic substrate in which transimination

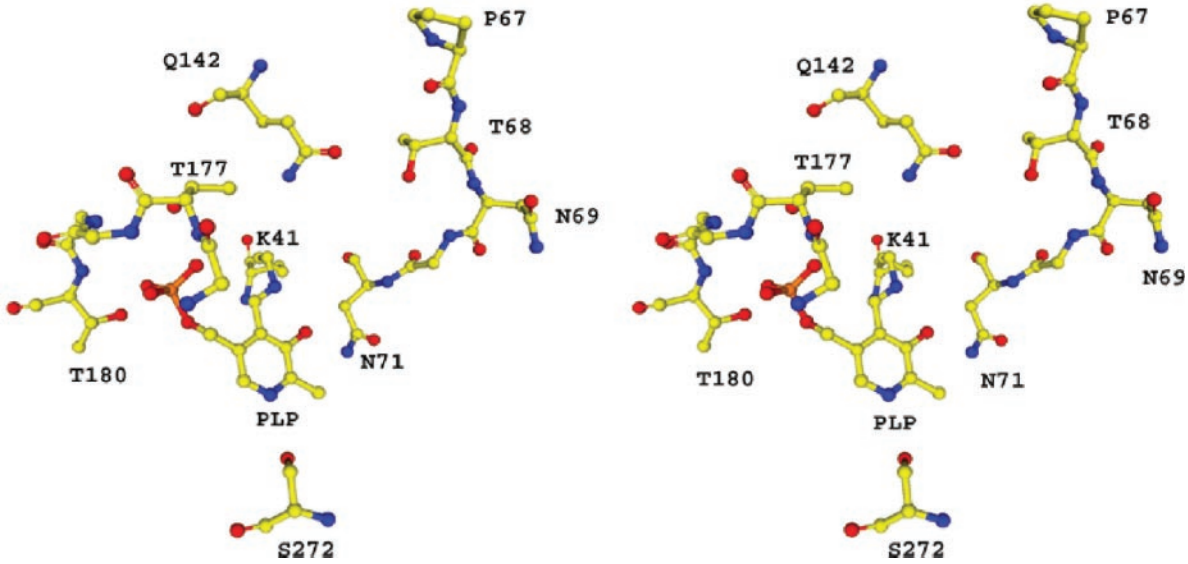

FIG. 4. **Stereo view looking into the active site of OASS-A.** The *re* face of the cofactor is visible at the bottom of the active site cleft. The Schiff base linkage is shown between C-4′ of PLP and Lys-41. Ser-272 is within hydrogen bonding distance to N-1 of the pyridine ring. The asparagine loop is shown on the *right*, and the threonine loop, which interacts with the 5′-phosphate, is on the *left*. The side chain of Asn-71 interacts with O-3′ of the cofactor. The figure was prepared using DS Viewer 5.0 from Accelrys.

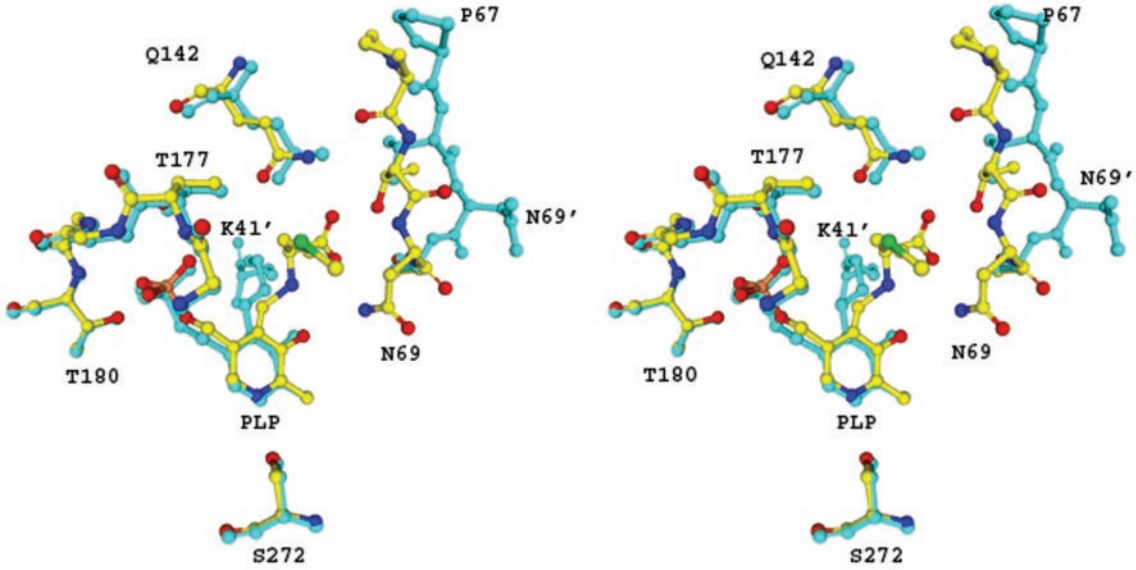

FIG. 5. **Overlay of the active sites of the wild type free enzyme (*aquamarine*) and the methionine external Schiff base of the K41A mutant enzyme (*multicolored*).** Note the change in position of Asn-69 of the asparagine loop as it moves to interact with the substrate carboxylate and O-3′ of the coenzyme. The side chain of methionine is directed out of the figure toward the entrance to the active site. The *prime* indicates the open form of the enzyme. The figure was prepared using DS Viewer 5.0 from Accelrys.

of the α-aminoacrylate external Schiff base occurs giving free enzyme and free α-aminoacrylate, which decomposes in solution to pyruvate and ammonia (10). The pH dependence of the first order rate constant for disappearance of the α-aminoacrylate intermediate gives a pK of 8.2, which reflects the ϵ-amino group of Lys-41. The value agrees well with the base side pK in the V/K_{TNB} profile for the overall β-replacement reaction (see above).

The group with a pK of about 7 observed in both half-reactions reflects the same enzyme group that must be unprotonated for optimum activity. The lack of a need for additional acid-base or nucleophilic catalysis and the absence of an ionizable group in the active site suggest the group is required to be unprotonated to stabilize the optimum catalytic conformation of the enzyme. In agreement with this suggestion a pK of about 7 is observed in the following processes. Excitation of OASS-A at 280 nm gives two emission maxima, one at 340 nm due to Trp emission and a second at 500 nm resulting from triplet-singlet energy transfer between Trp-51 and the PLP, and the latter thus reflects the relative orientation of the two fluorophores. The pH dependence of enhancement of long wavelength fluorescence by acetate gives a pK of 7 (16). Phosphorescence (17) and time-resolved fluorescence (18–20) studies have identified two conformers, and their interconversion exhibits a pK of 7. The proposed pH-dependent conformational change may be the one required to close the active site prior to chemistry.

On the basis of the above information, the following general mechanism is proposed for OASS using the natural substrates OAS and bisulfide (Fig. 6). The resting enzyme is in the open conformation, and above pH 7 it can undergo the conformational change to the closed conformation. The amino acid substrate, OAS, binds as the monoanion with its α-amine unprotonated to carry out a nucleophilic attack on C-4′ of the internal Schiff base (**I**). As the external Schiff base (**III**, observed tran-

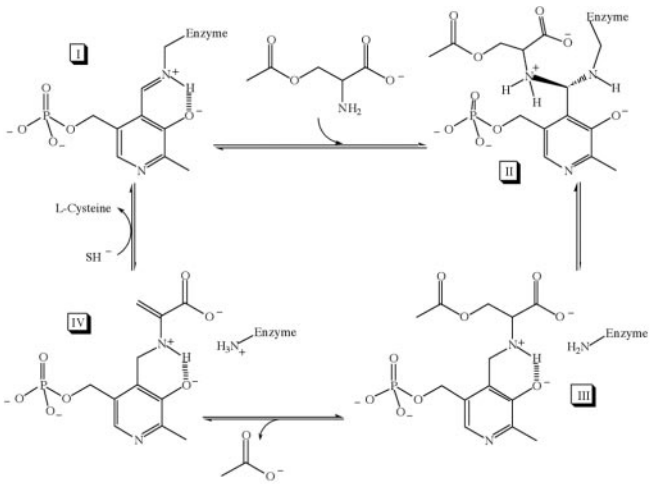

FIG. 6. **Minimal chemical mechanism of OASS.** The reaction begins with binding of OAS to the internal aldimine (**I**) (λ_{max} = 412 nm) and proceeds to the α-aminoacrylate intermediate (**IV**) via a geminal diamine (**II**) (λ_{max} = 340 nm) and external Schiff base (**III**) (λ_{max} = 418 nm) intermediates.

siently in the pre-steady state with OAS as the substrate (21)) is formed via *geminal*-diamine intermediates (one of these is shown as **II**) the active site closes triggered by the interaction of substrate α-carboxylate with the asparagine loop. Lysine-41, initially in the internal Schiff base linkage with PLP, serves as a general base to deprotonate Cα (**III**) in the β-elimination reaction responsible for the release of acetate to end the first half-reaction. Acetate diffuses away from the active site as it opens partially to release product and allow entry of bisulfide. The difference in conformation of the external Schiff base (**III**) and α-aminoacrylate external Schiff base (**IV**) forms of the enzyme is clearly shown by differences in their ^{31}P NMR chemical shifts (22). At the beginning of the second half-reaction (**IV**) Lys-41 is protonated and bisulfide diffuses into the active site and attacks Cβ of α-aminoacrylate to give the cysteine external Schiff base. Transimination then occurs to expel the cysteine product.

On the basis of the external Schiff base structure (Fig. 5) the α-proton is directed away from the *si* face of the cofactor, whereas the methionine side chain is directed away from the *re* face toward the entrance to the active site in anticipation of its expulsion. The structure is consistent with *anti*-elimination, and an *E*2 reaction is preferred compared with an *E*1 reaction in the case where no protonation of the leaving group is required as is the case for the OASS reaction (23). In an *E*1 reaction, a quinonoid intermediate should be produced as the α-proton is abstracted by Lys-41 acting as a general base. Because α-proton abstraction is rate-limiting in the direction of formation of the α-aminoacrylate external Schiff base (see above), the quinonoid intermediate would not be observed in the direction of its formation but should be in the direction of formation of the OAS external Schiff base from the α-aminoacrylate external Schiff base and acetate. Consistent with an *E*2 reaction, no quinonoid intermediate is observed under these conditions, even in the presence of D_2O, which slows down protonation at Cα (23, 24). In addition, as suggested above, the dipole of helix 10 is in close proximity to and directed toward the pyridine nitrogen of the cofactor making it unlikely that N-1 is protonated. Finally, changing Ser-272 to Ala, (which eliminates any possibility of hydrogen bonding to N-1, or aspartate, which should stabilize a quinonoid intermediate) has no effect on V/K_{OAS} (25). Taken together, data are consistent with an *anti-E*2 reaction or concerted elimination of the α-proton and the acetoxy group. Measurement of the primary ^{18}O kinetic isotope effect on acetoxy elimination in the absence and presence of deuterium at Cα is planned. If the ^{18}O kinetic isotope effect measured with OAS remains the same or increases when measured with OAS-2D, results will support a concerted elimination.

The primary deuterium isotope effect measured using OAS-2D in the steady state is pH-dependent, increasing from a value of 1.7 at neutral pH to a limiting value of 2.8 at low pH (8). Data indicate that OAS is a sticky substrate with a stickiness factor of 1.5, that is once OAS binds to enzyme it goes on to produce the α-aminoacrylate intermediate 1.5 times faster than it dissociates from enzyme. An isotope effect within error identical to the value of 2.8 obtained in the steady state is also measured monitoring the appearance of the α-aminoacrylate intermediate at 470 nm in the pre-steady state. The value of 2.8 is thus the intrinsic isotope effect on α-proton abstraction. The frame of reference for primary kinetic deuterium isotope effects on the basis of semiclassical considerations is 6–8 for a symmetric transition state, and the value of 2.8 thus suggests an early or late transition state (26). Substitution of deuterium at C-2 of OAS allows measurement of a secondary kinetic deuterium isotope effect. The frame of reference for secondary kinetic isotope effects in the OASS reaction is the secondary isotope effect on the equilibrium constant for formation of the α-aminoacrylate intermediate (27). This secondary equilibrium isotope effect is 1.8 as measured experimentally, whereas the value for the secondary kinetic isotope effect is quite modest with a measured value of 1.1, suggesting very little hybridization change at Cβ in the transition state. Assuming an *anti-E*2 reaction, data are consistent with an asynchronous transition state in which little bond formation has occurred between the ϵ-amino of Lys-41 and the α-proton of the OAS external Schiff base, and even less bond cleavage has occurred between Cβ and the acetoxy oxygen.

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