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Pyridoxal 5′-Phosphate-Dependent α,β-Elimination Reactions: Mechanism of *O*-Acetylserine Sulfhydrylase

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ABSTRACT

O-Acetylserine sulfhydrylase catalyzes the replacement of the β-acetoxy group of *O*-acetyl-L-serine with sulfide to generate L-cysteine. The reaction represents the final step in the biosynthesis of L-cysteine in enteric bacteria and plants. A quinonoid intermediate has not been detected using a variety of kinetic and spectroscopic probes for the wild-type or mutant enzymes, ruling out an E_1 mechanism. The structure of the external Schiff base intermediate indicates an anti elimination. *O*-Acetylserine sulfhydrylase is the only known pyridoxal 5′-phosphate-dependent enzyme that catalyzes a β-elimination reaction to have an anti E_2 mechanism.

In 1966, Dunathan[1] put forth a unified theory to explain the multifunctional nature of pyridoxal 5′-phosphate (PLP) as a cofactor of enzymes involved in the metabolism of amino acids. Dunathan suggested that it is the arrangement of functional groups around C_α in the external aldimine intermediate that determines the lability of the bond, Figure 1. For maximum lability, the functional group should be orthogonal to the PLP ring to maximize $\sigma-\pi$ overlap to produce the new π bond. In Figure 1A, the C−X bond is subject to cleavage. In the case of α,β-elimination reactions, an additional constraint is the position of the group to be eliminated at the β-carbon. To facilitate elimination, the leaving group at the β-carbon must be out of the $C_\alpha-C_\beta$ plane, R in Figure 1B. Research from numerous investigators over the past three and a half decades has borne out the hypothesis put forth by Dunathan. In the present overview, α,β-elimination reactions will first be considered in general, and this will be followed by an overview of the reaction catalyzed by the PLP-dependent enzyme *O*-acetylserine sulfhydrylase (OASS), with emphasis on the α,β-elimination of acetic acid from *O*-acetyl-L-serine. Finally, α,β-elimination reactions catalyzed by other PLP-dependent enzymes will be considered and compared to the OASS reaction.

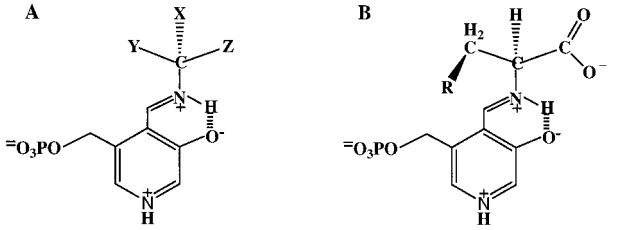

FIGURE 1. Structures illustrating that lability of a bond about the α-carbon depends on its ability to be orthogonal to the plane of the pyridine ring. (A) X is labile. (B) Structure for an anti α,β-elimination reaction, with the new π-bond to be formed parallel to the plane of the pyridine ring.

α,β-Elimination Reactions

α,β-Elimination may generally proceed, dependent on the leaving group in the β-position, via an E_1 or E_2 reaction. If protonation of the leaving group is required, the base that removes the α-proton likely carries out a proton transfer to the β-position, and the reaction will be E_1. If the leaving group is poor, even if there is no protonation required and/or the α-proton is not acidic, the reaction will likely proceed in an E_1 or E_1cB fashion. If the leaving group is good, and there is no protonation required, the reaction likely proceeds in an E_2 fashion.[4] However, it is also possible to have an E_1 reaction under these conditions with a carbonium ion intermediate, e.g., for compounds with a good leaving group that does not require protonation and an electron-withdrawing substituent such as F or aryl on the α-carbon. There are numerous examples from the literature of concerted elimination reactions. It is instructive to point out a few of them here and contrast them to similar reactions that proceed in a stepwise fashion. Mayer et al.[2] studied the elimination from a β-acetoxy ketone and compared it to the elimination from a β-acyloxy ketone to form an enone. In the β-acyloxy ketone, the acyloxy group is a five-membered lactam, which is strained. Although neither reaction requires a proton transfer to the leaving group, elimination of the acetoxy exhibits a rate-limiting proton transfer and proceeds via an E_1cB reaction. The internal strain of the lactam makes acyloxy a better leaving group, and elimination of the acyloxy proceeds via an E_2 reaction. A comparison of the elimination from *S*-aryl-*S*-(2-cyanoethyl)-*S*-methylsulfonium tetrafluoroborate salts and *S*-aryl-*S*-2-cyanoethanes gave similar results.[3] Elimination from the activated sulfonium to give the thioether product proceeded via an E_2 reaction, while elimination of the poorer thiophenoxide leaving group proceeds via an E_1cB mech-

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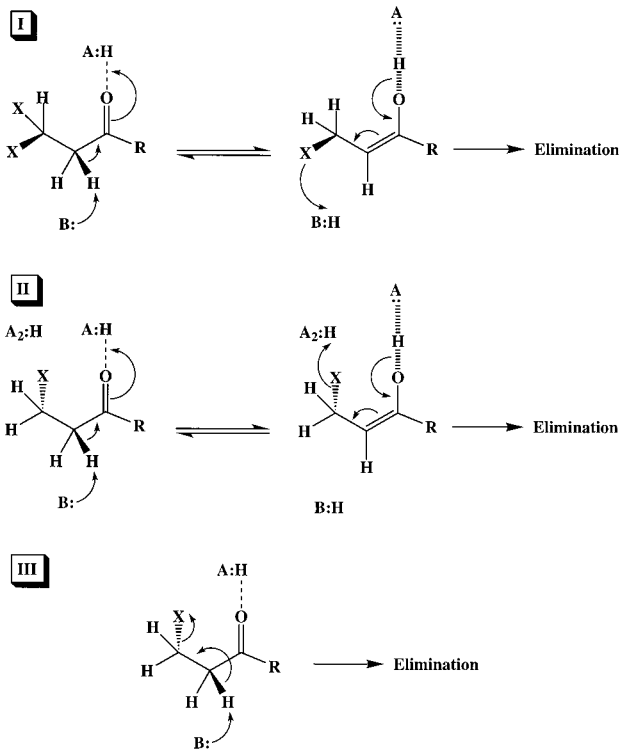

FIGURE 2. Possible pathways for an elimination reaction α to a carbonyl. (I) General-base-catalyzed α-proton abstraction with a general acid assist via protonation of the carbonyl oxygen. The general base also protonates the leaving group in the syn E_1 reaction. (II) A second general acid protonates the leaving group in the anti E_1 reaction. (III) No protonation of the leaving group is required in the anti E_2 reaction.

anism. Generally, the E_2 reaction is favored in cases where there is a good leaving group and the α-proton is acidic.[5]

α,β-Elimination reactions from an aldehyde, ketone, carboxylic acid, or thioester have been discussed previously.[5−7] It has been suggested that the difference in pK_a values between the α-carbon acids listed above and the enzymic general base that accepts the proton is minimized, in the case of enzyme-catalyzed reactions, as a result of a concerted proton transfer (general acid catalysis) to the carbonyl oxygen, generating an enol intermediate, Figure 2 (**I**, **II**). There is also a difference in the pK_a for the carbonyl oxygen and the enzymic general acid, but this difference also tends to zero as the reaction proceeds and the carbonyl oxygen becomes more enolate-like. Thus, the driving force for the initial proton abstraction is the electrophilic catalysis by the general acid, Figure 2. In **I**, the reaction proceeds via protonation of the leaving group by the conjugate acid of the general base that accepted the α-proton initially. Because of the suprafacial proton transfer, this type of reaction requires a syn elimination. The reaction could also proceed with the assistance of another, general acid residue, as shown in **II**, and this reaction tends to favor the energetically more favorable anti elimination. There is the added possibility that the hydrogen bond formed with the general acid is of the low-barrier type, a higher energy bond that would facilitate formation of the enol-like intermediate.[5−7] Another possibility, not shown, makes use of some other electrophile, such as a metal ion, that could serve to aid in acidifying the α-proton, as proposed for the mandelate racemase reaction.[8] As suggested above, an E_2 reaction is expected where no protonation of a good leaving group is required.

Data for elimination of HBr from 2-bromobutane by an E_2 reaction to give 2,3-butene indicate the anti elimination is preferred by 3:1.[9] Nonbonding interactions between the methyl groups in the activation complex are thought to lead to a higher activation energy for the syn, compared to the anti elimination pathway. On the other hand, stereoelectronic considerations, i.e., allowance for maximum orbital overlap in the formation of the new π-bond, favor the anti elimination. All things being equal, the anti elimination is slightly favored energetically compared to the syn elimination, especially when there is no requirement for a proton transfer from the general base to the leaving group.

In the case of the PLP-dependent α,β-elimination reactions, the electrophilic catalyst is provided by the PLP cofactor, Figure 1A, via the protonated imine alone or with the assistance of the pyridine ring. Thus, the need for a general acid catalyst is negated. The PLP-dependent α,β-elimination/addition reactions can be carried out via four distinct pathways that can be distinguished by differences in stereochemistry, Figure 3. The pathway on the left in Figure 3 shows an anti elimination to generate an α-aminoacrylate Schiff base intermediate, followed by either an anti (left) or syn (right) addition to generate a new β-substituted amino acid. The anti elimination/addition (left) will result in overall retention of configuration at the β-carbon, while the anti elimination/syn addition will result in overall inversion of configuration. The same is true for the right pathway, which begins with a syn elimination. Syn addition and anti addition will result in overall retention and inversion of configuration, respectively, at the β-carbon. In all of the pathways shown in Figure 3, elimination can occur via an E_1 (stepwise) or an E_2 (concerted) reaction, Figure 4. In the E_1 reaction, the intermediate quinonoid results from stabilizing the carbanion formed upon C_α proton abstraction by delocalizing the electron pair on C_α to N1 of the pyridine ring.

The quinonoid intermediate absorbs at wavelengths around 500 nm and can thus be easily detected if it builds up in the pre-steady state.[10] To form the quinonoid intermediate, the pyridinium nitrogen must either be protonated or become protonated by or ion-paired to an enzyme side chain as the intermediate forms, Figure 5. The most common enzyme residue that interacts with the pyridinium nitrogen (**I** in Figure 5) is an aspartate anion, as found in the aminotransferase class, with D222 in aspartate aminotransferase as the prototypical example.[11] However, essentially any lone pair could serve the same function (**II** in Figure 5), as exemplified by S377 in the β-subunit of tryptophan synthase.[12] In the case where N1 of the pyridine ring is unprotonated, residues such as arginine, as exemplified by alanine racemase,[13] or a serine residue could serve to generate the transient hydrogen bond. The hydrogen bond formed could certainly be of

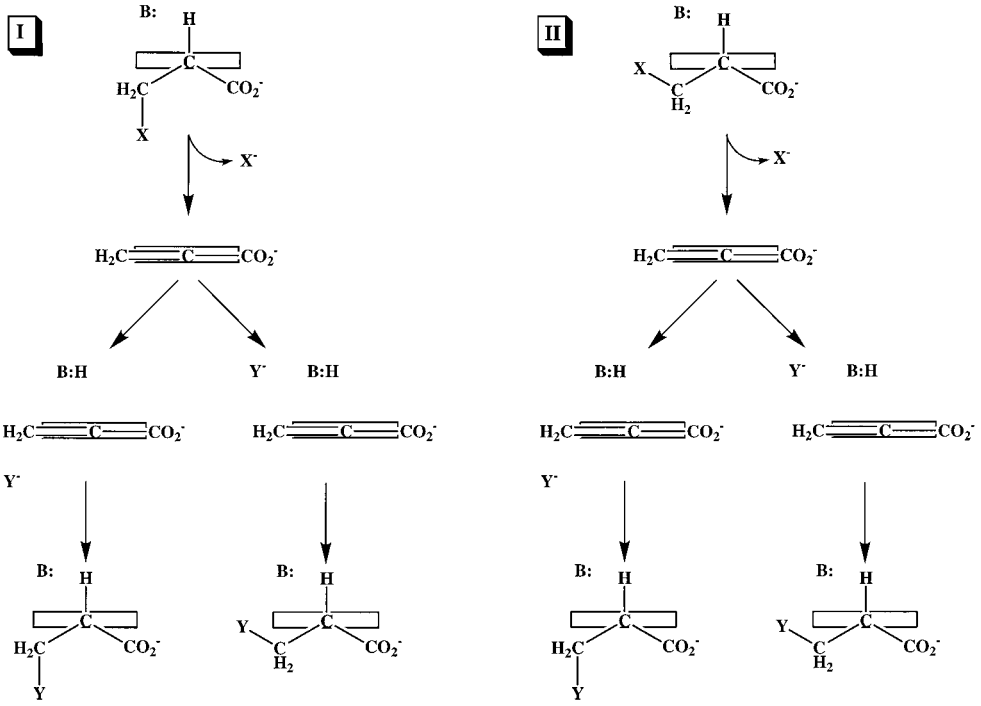

FIGURE 3. Stereochemical courses for an α,β-elimination/addition reaction in PLP-dependent enzymes. The PLP is viewed from the C4′ carbon and is represented by a rectangle. In pathway I, the elimination is anti, while in II the elimination is syn. Addition to the aminoacrylate intermediate can then be anti (left path in I and II) or syn (right path in I and II).

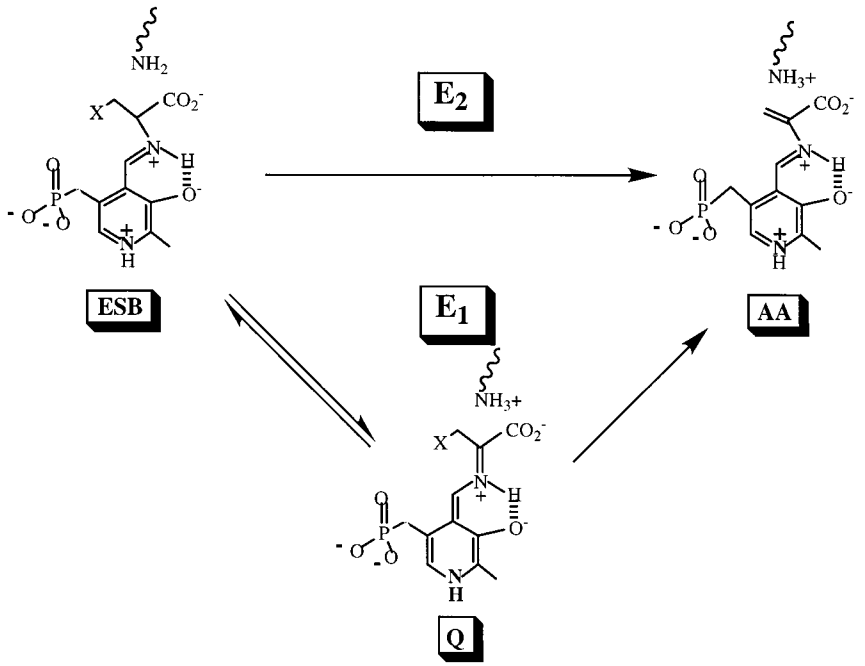

FIGURE 4. Pathway for an E_2 and E_1 α,β-elimination for PLP-dependent enzymes. Note the presence of the resonance-stabilized carbanion (quinonoid) as an intermediate in the E_1 pathway.

the low-barrier type to stabilize the resulting quinonoid intermediate. Considering PLP-dependent β-elimination reactions, Dunathan states, "There is every reason to believe that all PLP-dependent enzymatic reactions proceed via the (external) Schiff base to the anion or quinonoid form." [1] However, in the same review he also suggests that the reaction could follow the E_2 path when X is a particularly good leaving group. Further, based on the reactions studied at the time the review was written, Dunathan hypothesized that all bond-breaking would take place on only one face of the substrate−cofactor external Schiff base.

O-Acetylserine Sulfhydrylase

Cysteine biosynthesis in enteric bacteria proceeds via a two-step enzymatic pathway.[14] The first step is catalyzed by serine acetyltransferase, which is responsible for

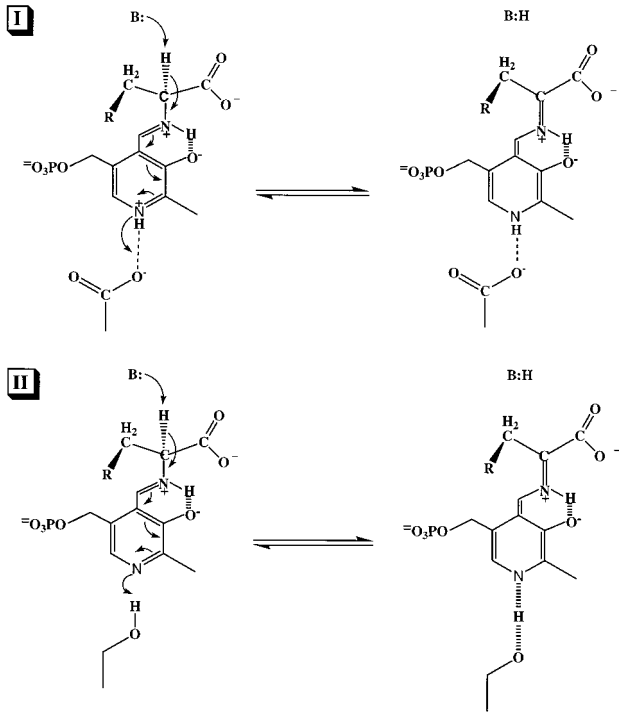

FIGURE 5. Stabilization of the quinonoid intermediate. (I) Formation of the quinonoid is facilitated by an ionized carboxylate ion-paired to the protonated nitrogen of the pyridinium ring. (II) The quinonoid is stabiized by donation of a hydrogen bond from a serine hydroxyl as the quinonoid forms. The hydrogen bond could be of the low-barrier type.

converting acetyl CoA and L-serine to *O*-acetyl-L-serine (OAS), and the second step is catalyzed by *O*-acetylserine sulfhydrylase, which converts OAS and sulfide to L-cysteine and acetate. *O*-Acetylserine sulfhydrylase is a pyridoxal 5′-phosphate (PLP)-dependent enzyme with one PLP per subunit.[14] The PLP is covalently bound in Schiff base linkage to lysine-41 of OASS, the internal aldimine.[15] There are two isozymes of OASS, A and B, that are thought to be expressed under aerobic and anaerobic conditions, respectively. The A isozyme is dimeric, with a molecular weight of 68 900.[16]

Steady-state kinetic studies indicate that the kinetic mechanism of OASS-A is ping-pong or double displacement,[17,18] Figure 6. Ultraviolet spectral studies indicate the internal Schiff base nitrogen is protonated and that the predominant tautomer is the ketoeneamine.[17,19,20] Thus, the first half of the reaction results in the conversion of the internal Schiff base and OAS to acetate and an external Schiff base with α-aminoacrylate. The second half of the reaction involves the reaction of the α-aminoacrylate intermediate with inorganic sulfide to regenerate the internal Schiff base and give the second product, L-cysteine. (The protonation state of N1 will be considered in more detail below.)

The structure of crystalline orthorhombic OASS-A was solved to 2.2 Å resolution using the technique of multiple isomorphous replacement.[21] A monomer of the OASS dimer is composed of an N-terminal (residues 1−145) domain and a C-terminal (residues 146−315) domain. Both domains consist of a central β-sheet structure surrounded by α-helices. One stretch of the N-terminal domain (residues 13−34) "crosses over" into the C-terminal domain, forming the first two strands of its central β-sheet. The overall structure of OASS-A is similar to that of the β-subunit of tryptophan synthase (β-TRPS).[12] Figure 7 shows a ribbon diagram of a dimer of OASS-A, with the PLP molecules represented with a stick-and-ball diagram to highlight their location. The perspective in Figure 7 is from solvent looking into the active-site cleft. The *re* face of the internal Schiff base is exposed.

The PLP in OASS-A is located at the interface between the two structural domains, deeply buried within the protein. A schematic view from the same perspective as in Figure 7, but as a closeup, is shown in Figure 8. The 5′-phosphate of PLP, shown in magenta and red, acts as an anchor and is hydrogen-bonded to a glycine/threonine-rich loop, part of which is shown.[21] ^{31}P NMR studies of OASS-A[19,20,22] give a chemical shift of 5.2 ppm with a line width of 20.5 Hz, consistent with the motion of the bound phosphate restricted to that of the protein. The phosphate is dianionic as bound to the protein. The PLP C4′ is in Schiff base linkage with the ϵ-amine of K41 in OASS-A[15] and the imine nitrogen is within hydrogen-bonding distance to O3′ of PLP, which is also hydrogen-bonded to N71. The pyridinium nitrogen of PLP is within hydrogen-bonding distance of S272, located at the N-terminal end of α-helix 10.

A chemical mechanism has been proposed for the sulfhydrylase, Figure 9.[23] The internal Schiff base, **I**, is protonated to begin the reaction, and OAS binds as the monoanion (α-amine pK = 7.7).[23] An enzyme residue with a pK of about 7 must be protonated for optimum catalysis and binding. The function of this residue is unclear, but the residue is most likely involved in the conformational changes the protein undergoes as the reaction proceeds. It is speculated that Ser272 donates a hydrogen bond to N1, as suggested by the proximity of a helix dipole to it, and thus N1 is unprotonated. The nucleophilic α-amine of the amino acid substrate attacks C4′ of the imine orthogonal to the *re* face, which is exposed at the bottom of the active-site cleft. The OAS external Schiff base, **IV**, is formed presumably via the intermediacy of *gem*-diamine intermediates, **II** and **III**, as shown in Figure 9. Formation of **III** is likely catalyzed by O3′ of the PLP cofactor, which hydrogen bonds to the lysine NH, facilitating the intramolecular proton transfer to the ϵ-amino nitrogen. The binding site for OAS is defined by the K41A mutant, which is isolated in a closed conformation as an external Schiff base with free methionine (an analogue of OAS) from the growth medium.[24] The mutant protein provides some indication of the location of the side chain of OAS. The side chain of Met extends away from the PLP cofactor toward the entrance to the active site, Figure 10. The PLP is viewed edge-on, with PLP from the internal Schiff base shown in cyan and superimposed on the cofactor for the external Schiff base in green. The internal Schiff base lysine is shown on the left, while the side chain of Met is shown on the right (S is in red). The active-site entrance is to the right. Very similar structures have been

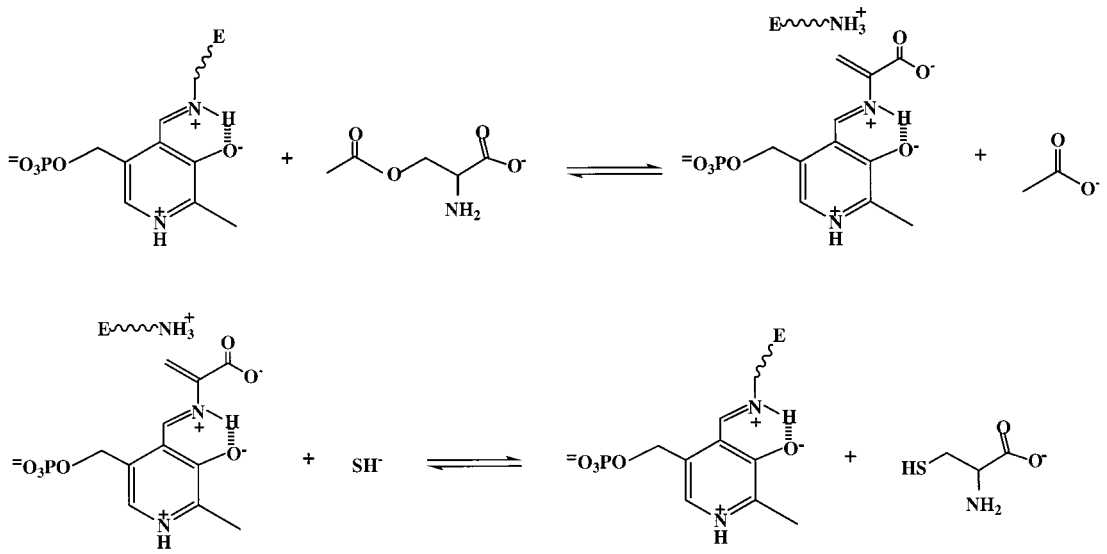

FIGURE 6. Double displacement mechanism for OASS. In the first half-reaction, the internal Schiff base and *O*-acetyl-L-serine are converted to an α-aminoacrylate Schiff base and acetate. In the second half-reaction, sulfide is added to generate the L-cysteine product and regenerate the internal Schiff base.

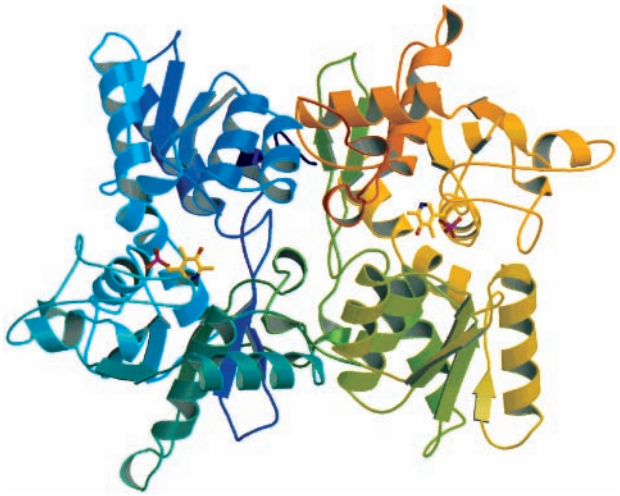

FIGURE 7. Ribbon diagram of a dimer of OASS-A. The monomer–dimer interface is in the center, with the left monomer in blue to blue-green and the right monomer in yellow-green to rust. The view is from solvent looking into the active site. The PLP moieties are shown in a stick diagram at the bottom of the active-site cleft.

published for the K87T mutant of β-TRPS with serine and tryptophan bound. A similar rigid-body rotation is observed upon formation of the external Schiff base, but the positioning of the substrate side chain is quite different in β-TRPS, leading to changes in the orientation of the bound cofactor (see below).[25] The ϵ-amine of K41, the internal Schiff base lysine, is displaced upon formation of the external Schiff base. The lysine is unprotonated (p*K* = 8.2)[19,23] and acts as a general base to accept the α-proton of OAS to generate the α-aminoacrylate intermediate, **VI**, via the possible intermediacy of a quinonoid intermediate, **V**. If **V** is formed, a proton must be donated, at least transiently, to N1. The only way this could feasibly happen is if a low-barrier hydrogen bond could be formed as shown. However, since the p*K*'s of the serine and N1 are not matched, the quinonoid does not form. In the reactions catalyzed by the aminotransferases, the p*K*'s of the aspartate and N1 are much better matched and the hydrogen bond can form, stabilizing the quinonoid intermediate. Proof that K41 is the general base comes from site-directed mutagenesis studies of OASS-A in which the lysine was changed to alanine.[15] The K41A mutant has lost its ability to catalyze the normal reaction and shows the buildup of the OAS external Schiff base, **IV**. Whether the α,β-elimination reaction that results in formation of **VI** proceeds via a quinonoid intermediate, **V**, that is, an E_1 reaction, will be discussed below. The second half of the reaction proceeds via a reversal of steps **I**–**VI**, with SH^- as the nucleophile that adds to **VI**.[23] As the pH increases in the absence of a nucleophile, the α-aminoacrylate intermediate, **V**, is transiminated to regenerate **I** and free α-aminoacrylate, which decomposes to pyruvate and ammonia.[19] The abortive β-elimination reaction of OASS-A is very slow, 10^{-4}-fold compared to the β-substitution reaction, 240 s^{-1}. When the α-aminoacrylate intermediate is generated at pH 7, K41 is protonated,[26] while increasing the pH results in a deprotonation of K41 and an enhancement of the abortive transimination reaction. Thus, one of the ways OASS-A stabilizes the reactive α-aminoacrylate Schiff base for nucleophilic attack by sulfide is by maintaining K41 protonated.

Pre-steady-state kinetic studies and primary deuterium isotope effects indicate that the first half-reaction is slow overall, and the slowest step in the first half-reaction is abstraction of the α-proton.[26,27] Rapid-scanning stopped-flow data show the rapid formation of the OAS external Schiff base prior to a rate-limiting (400 s^{-1}) formation of the α-aminoacrylate intermediate.[26] The second half-reaction is very rapid (1000 s^{-1}) and has a higher equilibrium constant than the first half-reaction. Of the intermediates shown in Figure 9, only the external Schiff base, **III**, and the α-aminoacrylate external Schiff base, **V**, are observed in pre-steady-state studies.[26]

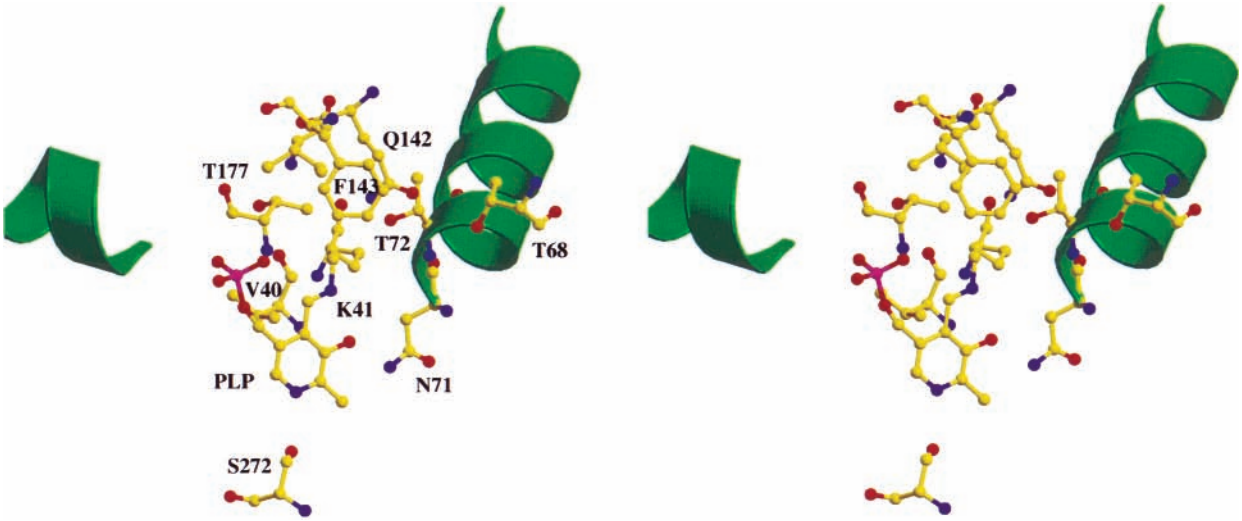

FIGURE 8. View of the active site from the perspective of solvent, showing interactions with the cofactor in the internal Schiff base. The *re* face of the internal Schiff base between PLP and K41 is exposed. Serine 272 is within hydrogen-bonding distance of N1 of the cofactor, while N71 is within hydrogen-bonding distance of O3′ of the cofactor. The 5′-phosphate is in magenta, with an interaction to T177 shown.

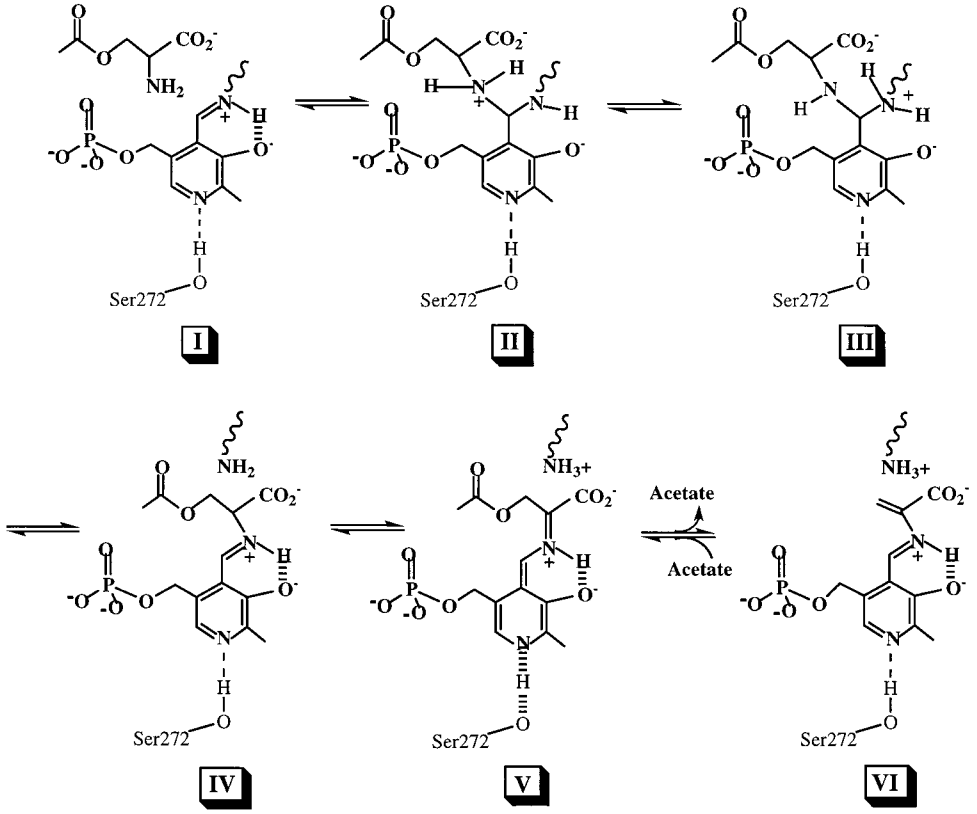

FIGURE 9. Possible chemical mechanism for the first half of the OASS reaction. The internal Schiff base (I), shown with N1 unprotonated, reacts with the monoanionic form of OAS to generate the external Schiff base (III) via *gem*-diamine intermediates (e.g., II). If IV is formed, a proton must be donated to N1, and elimination of acetate results in formation of the α-aminoacrylate Schiff base (V).

Observation of a quinonoid intermediate, **V**, in a β-elimination reaction is consistent with an E_1 mechanism. As discussed above, formation of the quinonoid intermediate requires the pyridinium nitrogen to be protonated or to become protonated as the intermediate forms. The quinonoid intermediate has been observed for a number of PLP-dependent enzymes that catalyze a β-elimination reaction. Tryptophanase catalyzes the elimination of indole from L-tryptophan and will also catalyze the exchange of the α-proton in L-alanine.[28] Addition of L-alanine to tryptophanase results in the appearance of a new absorption band at 500 nm, due to the formation of **V**. Similar absorption bands have been observed for the tyrosine phenol-lyase- and tryptophan synthase-catalyzed exchange of the α-proton of L-alanine.[29] The intermediate, **V**, is detected in transient kinetic studies of the elimination of indole from L-tryptophan catalyzed by tryptophanase,[30] the elimination of phenol from L-tyrosine catalyzed

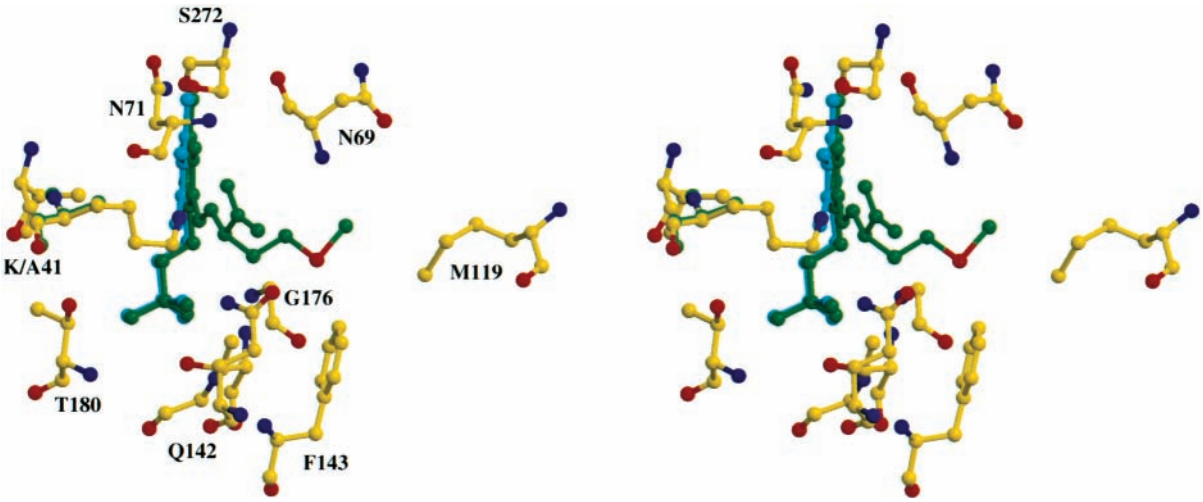

FIGURE 10. Overlay of the active-site structures of the native and K41A mutant enzymes. Note that the methyl of A41 superimposes on the β-methylene of K41. The PLP is viewed perpendicular to the plane of the paper, with the *re* face to the right. The side chain of methionine, in Schiff base linkage to PLP, is shown in green, with S in red.

by tyrosine phenol-lyase,[31] and the replacement of the β-hydroxyl of L-serine with indole catalyzed by tryptophan synthase.[32−34,51] It should be noted that quinonoid species were detected transiently in the β-TRPS reaction using either L-serine ($\lambda_{max} \approx 460$ nm) or L-tryptophan ($\lambda_{max} \approx 476$ nm).[34,51] In both cases, the quinonoid absorbs at higher energy compared to those observed for other PLP-dependent enzymes (see above).

Even though a quinonoid intermediate, **V**, has been observed in the closely related β-TRPS reaction,[33,35] the intermediate has not been observed in the OASS-A reaction. Transient kinetic studies of the OASS-A reaction in the direction of L-cysteine synthesis exhibit no detectable levels of **V**.[26] The rate-limiting step in the formation of **VI** by OASS-A is abstraction of the α-proton,[27] so it is not surprising that **V** is not observed in this reaction direction. However, if **VI** is preformed and pushed against acetate in the stopped-flow, **V** is not detected in H_2O or in D_2O, despite an observed solvent deuterium isotope effect of 2.5, as expected for rate-limiting protonation of the α-carbon to form **IV**, Figure 11 (unpublished work of C.-H. Tai in this laboratory).

Recently, a site-directed mutant of β-TRPS has been prepared in which S377, which is hydrogen-bonded to N1 of the pyridinium ring, is relaced by D or E.[36] The mutant protein exhibits a pronounced absorption band for **V** when L-serine is added to the enzyme under equilibrium conditions, as expected on the basis of an increased stabilization of **V** by the ionic interaction between N1 and D or E. A similar mutant enzyme has been prepared for OASS-A (unpublished results of C.-H. Tai in this laboratory), but no **V** is detected even transiently in the direction of formation of **VI** from OAS and enzyme, or in the direction of formation of **I** from **VI** and acetate in the presence or absence of D_2O.

Finally, viewing the three-dimensional structures of β-TRPS (not shown) and OASS-A, there is a significant difference in the vicinity of the pyridinium nitrogen. In the OASS-A structure, S272 is at the N-terminal end of

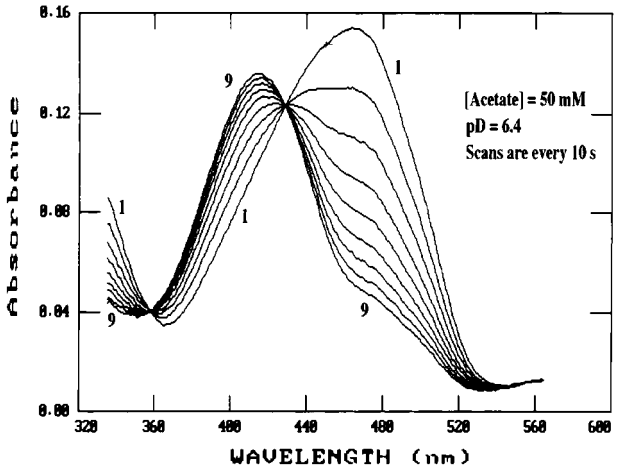

FIGURE 11. Rapid-scanning stopped-flow spectra of the OASS reaction in the direction of formation of OAS in D_2O at pD 6.4. The α-aminoacrylate intermediate (15 μM), which absorbs maximally at 470 nm, was preformed and reacted with acetate (50 mM). The spectrum was then recorded as a function of time as indicated. Note that a clean isosbestic point is observed as the α-aminoacrylate intermediate decays and the internal Schiff base ($\lambda_{max} = 412$ nm) is generated. No quinonoid intermediate ($\lambda_{max} \approx 490$ nm) is observed.

helix 10 and is within hydrogen-bonding distance to N1.[21] The close proximity of the helix dipole would be contraindicated if N1 were to be protonated, and thus the formation of **V** would not be expected. In the β-TRPS structure,[12] S377 is not homologous to S272 and is not at the N-terminal end of a helix, and thus N1 could be protonated. Structural data are consistent with formation of **V** in the β-TRPS reaction, as discussed above. Taken together, there is no evidence for the formation of **V** in the OASS-A reaction.

Knowledge of the stereochemistry of a reaction is imperative in determining the overall reaction pathway. The stereochemistry at C-3 of the amino acid for the overall reaction catalyzed by OASS-A was determined by Floss et al.[37] These authors showed that the reaction from OAS to L-cysteine proceeds with retention of configuration

Table 1. Enzymes and Overall Stereochemistry of β-Elimination and β-Replacement Reactions Involving PLP

enzyme	reaction	stereochemistry at C-3	ref
	β-Replacement Reactions		
O-acetylserine sulfhydrylase	*O*-acetyl-L-serine + H_2S ⇌ L-cysteine + acetate	retention	43
cystathionine synthase	L-serine + L-homocysteine ⇌ cystathionine + H_2O	retention	43
β-cyanoalanine synthase	L-cysteine + HCN ⇌ *β*-cyanoalanine + H_2S	retention	44
tyrosine phenol-lyase	L-serine + phenol ⇌ L-tyrosine + H_2O	retention	45
tyrosine phenol-lyase	L-tyrosine + resorcinol ⇌ L-dopa + phenol	retention	46
Tryptophanase	L-serine + indole ⇌ L-tryptophan + H_2O	retention	41, 42
tryptophan synthase	L-serine + indole ⇌ L-tryptophan + H_2O	retention	37, 47, 50
	β-Elimination Reactions		
tryptophanase	L-serine → pyruvate + NH_3 + H_2O	retention	41, 42
tryptophanase	L-tryptophan → pyruvate + NH_3 + indole	retention	41, 42
S-alkylcysteine lyase	L-cystine → pyruvate + NH_3 + *S*-thio-L-cysteine	retention	44
D-serine dehydratase	D-threonine → α-ketobutyrate + NH_3 + H_2O	retention	48
D-serine dehydratase	D-threonine → α-ketobutyrate + NH_3 + H_2O	retention	49

[a] In all cases, the starting amino acid was synthesized with a chiral *β*-carbon with known stereochemistry at C-3 by stereospecific addition of D and/or T. The product analyzed in the case of the *β*-replacement reactions was the product amino acid, and in the case of the *β*-elimination reactions the α-keto acid was analyzed.

at C-3, Table 1, indicating that sulfide adds to the same face of **VI** from which acetate departed. Data in Table 1 do serve to eliminate two of the possible pathways outlined in Figure 3. The anti elimination/addition and syn elimination/addition pathways will give an overall retention of configuration, while the other two pathways will not. Whether the elimination proceeds as an anti or a syn reaction is discussed below.

Figure 10 shows a view of the active site of the K41A mutant (with the PLP perpendicular to the plane of the paper) from the 3′-hydroxyl side of the cofactor. The figure represents an overlay of the K41A and free enzyme structures and shows K41 directed toward the *si* face of the PLP cofactor, with A41 of the mutant aligned with the *β*-methylene of K41. Based on the L-configuration of the methionine in aldimine linkage with the active-site PLP, the *β*-proton is directed from the *si* face of the cofactor, which is appropriate since K41 is the general base that must accept this proton in the elimination reaction.[15] The side chain of Met is perpendicular to the plane of the aldimine linkage and directed away from the *re* face of the cofactor toward the entrance to the active site. It would thus appear that the α-proton and the *β*-substituent will leave at approximately 180° from one another, and data are consistent with an anti elimination of the elements of acetic acid to generate the α-aminoacrylate intermediate. The absence of a quinonoid intermediate further indicates a concerted or E_2 anti elimination. The conclusion is the expected result since the concerted anti elimination is favored, as discussed above, under conditions where proton transfer is not required for a good leaving group, as is true for the OASS reaction. The mechanism is not consistent with the hypothesis of Dunathan that all bond-breaking steps take place on the same face of the external Schiff base.

The anti E_2 mechanism for the α,*β*-elimination reaction catalyzed by OASS allows an estimate of the transition state structure for the elimination based on kinetic deuterium isotope effects.[27] The primary kinetic deuterium isotope effect is dependent on pH, with a maximum value of 2.8 observed at pH 5.5. A value identical to the value obtained from steady-state measurements is obtained

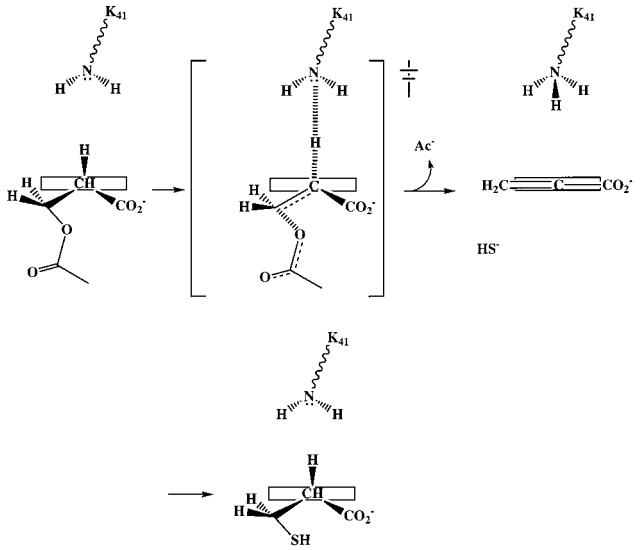

FIGURE 12. Mechanism of the α,*β*-elimination of acetic acid from the OAS external Schiff base. The PLP is shown as in Figure 3. An estimate of the transition state for the anti E_2 reaction is shown as early for C—H and C—O bond cleavage, with the latter lagging behind C—H bond cleavage.

from stopped-flow measurements of the first-order rate constant for formation of the α-aminoacrylate intermediate with OAS and OAS-2-*d*, indicating that the value of 2.8 is the intrinsic isotope effect on α-proton abstraction.[26] The maximum value of the primary isotope effect based on semiclassical considerations is 6−8,[38] with the value reaching a maximum when the proton is symmetrically placed between the donor carbon acid and the general base acceptor.[39] To determine whether the transition state for the elimination reaction is early or late, secondary kinetic deuterium isotope effects were measured by comparing the rate with OAS and OAS-3,3-d_2. The minimum value expected for the secondary deuterium kinetic isotope effect is 1, while a maximum value equal to the secondary equilibrium isotope effect is predicted.[40] The secondary deuterium equilibrium isotope effect was measured to be 1.8 by comparing the equililibrium constant for the first half-reaction with those for OAS and OAS-3,3-d_2.[27] The *β*-secondary deuterium kinetic isotope

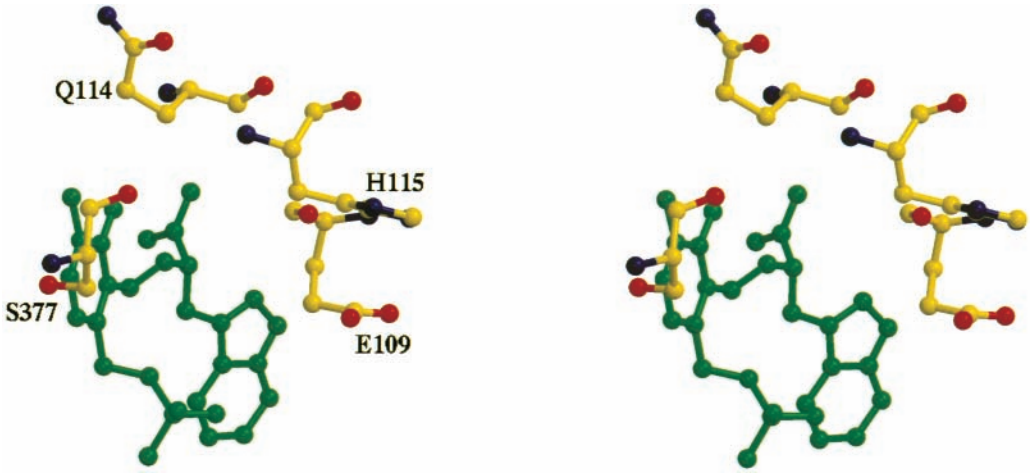

FIGURE 13. Active-site structure of the K87T mutant of the β-subunit of tryptophan synthase. The external Schiff base is shown in green. The PLP is viewed on the *si* face with the 5′phosphate side chain extending from the bottom and C4′ with the Schiff base linkage to the right. The α-carbon has the α-carboxylate and the β-carbon pointing toward the *re* face, and the indole substituent on the β-carbon pointing back toward the *si* face along with the α-proton. Serine 377 is shown in hydrogen-bonding distance to N1 of the pyridine ring, and glutamate 109 is thought to participate as an acid−base catalyst, facilitating elimination of the indole side chain. T87, which replaces the internal Schiff base lysine, K87 (not shown), would be positioned near H115 and directed toward the α-proton on the *si* face of the cofactor-substrate adduct.

effect measured at pH 5.5 is 1.1, quite small in comparison to the value of 1.8.[27] Thus, data suggest a concerted α,β-elimination that is substrate-like with little C_α−H or C−O bond cleavage in the transition state, that is, an early transition state. A schematic representation of the transition state is shown in Figure 12.

To corroborate the proposed E_2 mechanism, the ^{18}O kinetic isotope effect on C−O bond cleavage at the β-position will be measured with H or D substituted on C_α. Since there appears to be no commitments in the OASS reaction, the predicted result from this experiment is no change in the ^{18}O kinetic isotope effect whether H or D is present on the α-carbon, indicating C−H and C−O bond cleavage take place in the same transition state.

Other Enzymes

The stereochemistries of the reactions catalyzed by a number of enzymes that catalyze β-elimination reactions that involve PLP are given in Table 1. Note that whether β-replacement reactions or β-elimination reactions are monitored, retention of configuration at C-3 is observed. Thus, the face of the external Schiff base from which the β-substitutent is eliminated is the same in all cases as that to which either the new nucleophile or a proton is added.

The β-subunit of tryptophan synthase is the best studied of the enzymes that caytalyze a β-replacement reaction. As for OASS, the abstraction of the α-proton is catalyzed by the internal Schiff base lysine (K87).[25] The synthesis of tryptophan catalyzed by tryptophan synthase shown in Table 1[41] was studied in H_2O and in D_2O. In D_2O, the α-proton is transferred to the indole product, indicating a suprafacial α-proton abstraction and its addition to indole. In addition, as suggested above, a quinonoid intermediate is observed as a transient in the replacement of the β-hydroxyl of L-serine by indole.[32−34] Taken together, data indicate an E_1 syn elimination reaction.[41] Since retention of configuration is observed for the synthesis of tryptophan from L-serine and indole, Table 1, the hydroxyl of L-serine must also be eliminated from the same face as that to which the indole side chain is added. In agreement with the experimental data, the structure of the K87T mutant with tryptophan bound in an external Schiff base linkage to the active-site PLP, Figure 13, show the α-proton and the indole side chain directed toward the *si* face of the substrate−cofactor plane.[25] These results are diametrically opposed to those obtained for OASS but expected for the elimination of the poor leaving groups, hydroxide and indole, in this reaction, both of which require protonation to assist in their elimination.

Two reactions catalyzed by tryptophanase have also been studied with respect to the stereochemical course, viz., the β-elimination of indole from L-tryptophan[41] and the synthesis of L-tryptophan from L-serine and indole.[42] In the β-elimination reaction, the α-proton is transferred stereospecifically to the methyl group of the pyruvate formed,[41] while the α-proton is transferred to the indole product in the β-replacement reaction.[42] As is the case with tryptophan synthase, the data indicate a suprafacial proton transfer. Taken together with the observation of a quinonoid intermediate in the tryptophanase reaction,[30] and the retention of configuration at C-3, the mechanism of the reactions catalyzed must take place via an E_1 syn elimination.

The stereochemical course of the elimination reactions catalyzed by the remaining reactions listed in Table 1 has not been elucidated, but a generalization can be made. With the exception of the acetate leaving group in the OASS reaction, the leaving group in all of the other reactions is poor and likely will require protonation. As a result, it is expected that all of the remaining enzymes will catalyze their reactions via an E_1 syn elimination.

Conclusions

The OASS-catalyzed α,β-elimination of acetic acid from *O*-acetyl-L-serine proceeds via an anti E_2 reaction, based on the absence of a quinonoid intermediate along the reaction pathway and the structure of the external Schiff base. Based on primary and secondary kinetic deuterium isotope effects, the transition state for the E_2 reaction is early and asynchronous with C_β−O bond cleavage lagging behind C_α−H bond cleavage.

The OASS-catalyzed elimination reaction is unique in the class of PLP-dependent enzymes that catalyze β-replacement reactions in that it is likely the only one that catalyzes an anti E_2 reaction. All other enzymes in the class likely catalyze a syn E_1 reaction, based on previous stereochemical studies and/or the nature of the leaving group in the β-position. Generally, however, the PLP-dependent β-eliminases follow the expected reaction course, based on what has generally been found experimentally for E_1 vs E_2 and syn vs anti reactions (see above).

Finally, Dunathan's hypothesis that all bond-breaking steps will take place on the same face of the cofactor−substrate external Schiff base is not borne out by the results obtained with OASS. Cleavage of the C_α−H bond takes place on the *si* face of the Schiff base, while C_β−O bond cleavage takes place on the *re* face of the Schiff base.

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