Mechanism of Inactivation of Ornithine Transcarbamoylase by
N⁶-(N'-Sulfodiaminophosphinyl)-l-ornithine, a True Transition State Analogue?

CRYSTAL STRUCTURE AND IMPLICATIONS FOR CATALYTIC MECHANISM

The crystal structure is reported at 1.8 Å resolution of Escherichia coli ornithine transcarbamoylase in complex with the active derivative of phaseolotoxin from Pseudomonas syringae pv. phaseolicola, N⁶-(N'-sulfodiaminophosphinyl)-l-ornithine. Electron density reveals that the complex is not a covalent adduct as previously thought. Kinetic data confirm that N⁶-(N'-sulfodiaminophosphinyl)-l-ornithine exhibits reversible inhibition with a half-life in the order of ~22 h and a dissociation constant of \( K_D = 1.6 \times 10^{-13} \) M at 37 °C and pH 8.0. Observed hydrogen bonding about the chiral tetrahedral phosphorus of the inhibitor is consistent only with the presence of the R enantiomer. A strong interaction is also observed between Arg⁵⁷ N and the P-N-S bridging nitrogen indicating that imino tautomers of N⁶-(N'-sulfodiaminophosphinyl)-l-ornithine are present in the bound state. An imino tautomer of N⁶-(N'-sulfodiaminophosphinyl)-l-ornithine is structurally analogous to the proposed reaction transition state. Hence, we propose that N⁶-(N'-sulfodiaminophosphinyl)-l-ornithine, with its unique three N-P bonds, represents a true transition state analogue for ornithine transcarbamoylases, consistent with the tight binding kinetics observed.

Ornithine transcarbamoylase (OTCase, ornithine carbamoyltransferase; E.C. 2.1.3.3) catalyzes the reaction between carbamoyl phosphate (CP) and l-ornithine (Orn) to form l-citrulline and phosphate (Fig. 1a). In plants and microbes OTCase is involved in arginine biosynthesis, whereas in mammals it is located in the mitochondria and is part of the urea cycle. Although not thermodynamically favored, the reverse reaction is efficiently catalyzed by a specialized catabolic OTCase (1). This activity is found in microbes that possess the arginine deiminase pathway, which enables the generation of ATP under anaerobic conditions (2). OTCase is closely related to some other carbamoyltransferases, most notably aspartate transcarbamoylase (ATCase, aspartate carbamoyltransferase; E.C. 2.1.3.2) (3), the key allosteric pyrimidine biosynthetic enzyme. The catalytic subunits of transcarbamoylases are composed of two domains: a CP-binding domain and an amino acid-binding domain. Each of the two discrete substrate-binding domains (SBDs) have an α/β topology with a central β-sheet embedded in a alpha-helical structure. The basic quaternary structure is a trimer, with active sites located at the interface between the protein monomers (4, 5). Anabolic OTCase is the simplest form of the enzyme, comprising a single homotrimeric unit (1), whereas the catabolic OTCase is a dodecamer of four trimers in a tetrahedral arrangement (5). ATCase is also a dodecamer with two trimers and three regulatory dimers (4). Amino acid sequences and resultant structures of the CP-binding domains are very closely related within this transcarbamoylase family. Conservation of key substrate-binding residues suggests that these transcarbamoylases share a common chemical mechanism.

OTCase has a compulsory ordered mechanism with CP the first substrate to bind and phosphate the last product released (6–9). The enzyme from Escherichia coli W follows a Theorell-Chance mechanism where the concentration of the ternary complex is kinetically insignificant (8). pH studies indicate that only the Orn zwiterion with an uncharged δ-amino group binds productively to OTCase (9). The chemical mechanism involves a nucleophilic attack from the electron pair of the δ-amino group of Orn on the carbonyl group of CP. The transition state (TS) is postulated to involve an oxyanion tetrahedral intermediate (Fig. 1a) (10, 11). The roles of active site residues in stabilizing an oxyanion intermediate are unknown, but two conserved arginine residues contribute positive charges to the active site. Whereas Arg⁹⁶ is thought to interact with the carbonyl oxygen of CP, Arg⁵⁷ is also implicated in catalysis. Mutation of Arg⁵⁷ to Gly results in poor catalytic efficiency with \( k_{cat} \) reduced by ~10⁴ (12).

Two OTCase crystal structures have been solved with the bisubstrate analogue N⁶-(phosphonoacetyl)-l-ornithine (PALO) (13) (Fig. 1b) bound to the active site (11, 14) and are related to the crystal structure of ATCase in complex with the inhibitor N⁶-(phosphonoacetyl)-l-aspartate (PALA) (15, 16). Comparison

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The atomic coordinates and structure factors (code 1DUV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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† The abbreviations used are: OTCase, ornithine transcarbamoylase; ATCase, aspartate transcarbamoylase; CP, carbamoyl phosphate; NCS, noncrystallographic symmetry; Orn, L-ornithine; PALA, N⁶-(phosphonoacetyl)-l-aspartate; PALO, N⁶-(phosphonoacetyl)-l-ornithine; PSOrn, N⁶-(N'-sulfodiaminophosphinyl)-l-ornithine; RMS, root mean squared; SBD, substrate-binding domain; TAPS, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TS, transition state.
OTCase/liter of culture were obtained. The bisubstrate analogue for OTCase, PALO, was synthesized as described by de Martina et al. (26) and linked to epoxy-activated Sepharose 6B (Amersham Pharmacia Biotech). OTCase was purified to homogeneity in a one-step affinity procedure as described by Templeton et al. (27), except that the extraction buffer was 50 mM TAPS, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. OTCase was assayed in 50 mM TAPS, pH 8.0, 5 mM CP and 3 mM Orn, using colorimetric determination of citrulline (27). Purified enzyme had a specific activity between 1250 and 1450 units/mg protein under these conditions. The mass of the purified OTCase monomer was determined as 36,775.2 (± 1.0) Da (calculated 36,775.65) using a LC-Q electrospray mass spectrometer (Finnigan, San Jose, CA).

**Purification of PSOrn—** Unlabeled PSOrn was prepared as previously reported (23). [14C]PSOrn was purified from an 150-ml culture filtrate of P. syringae pv. phaseolicola ICMP 4419 grown on minimal medium at 18 °C. After 2.5 days of growth 20 μCi of [U-14C]-L-Orn (Amersham Pharmacia Biotech) was added, and the culture was harvested 12 h later. [14C]-Labeled phaseolotoxin was purified by chromatography on quaternary aminoethyl- and LH-20 Sephadex (Amersham Pharmacia Biotech). Approximately 2% of the PSOrn was radiolabeled. [14C]Phaseolotoxin (0.75 mg) was reacted with 4 units of leucine aminopeptidase (Sigma) in 200 μl of 25 mM ammonium bicarbonate buffer, pH 7.8, for 16 h. Completion of the reaction was confirmed by two-dimensional thin layer chromatography. The reaction mixture was loaded onto a quaternary aminoethyl-Sephadex column, and [14C]PSOrn was eluted with a 0.05–0.5 M ammonium bicarbonate gradient. [14C]PSOrn Exchange Measurements—OTCase (20 nmol) was inactivated with [14C]PSOrn (25 nmol) in 50 mM TAPS, pH 8.0. The inactivated OTCase was desalted on a PD-10 column (Amersham Pharmacia Biotech), filter sterilized, and incubated with a 200-fold excess of unlabeled PSOrn in 50 mM TAPS, pH 8.0, at 37 °C. Samples were removed at the appropriate times and desalted on a PD-10 column to remove free [14C]PSOrn, and the 14C in the void volume was measured. 14C samples were counted for 10 min in an LKB 1214 Rackbeta liquid scintillation counter (Amersham Pharmacia Biotech).

**Calculation of Observed Rate Constant for PSOrn Binding—** Equimolar concentrations of OTCase and PSOrn (1 nmol) were incubated in 1 ml of 50 mM TAPS, pH 8.0, at 37 °C. Portions (50 μl) were removed at 20-s intervals and assayed for OTCase activity. Results were plotted as 1/e – 1/e0 against time, where e0 is the initial concentration of enzyme and e is the concentration of active enzyme at time t. The slope of this line equals the observed second-order rate constant (28, 29) for the binding of PSOrn to OTCase.

**Cryocatalysis of Inactivated OTCase—** Purified OTCase (5–10 mg) was incubated with a 50% molar excess of PSOrn for 30 min at 0 °C. Enzyme activity was inhibited by >99% under these conditions. Inactivated enzyme was desalted on a PD-10 column equilibrated in 20 mM HEPES, pH 7.5, containing 1 mM dithiothreitol and frozen. The screening of conditions for crystal growth was conducted using the hanging drop vapor diffusion technique under normal oxidizing conditions at room temperature. Crystal forms most suitable for structure determination were obtained when equal volumes of protein (8 mg/ml) were combined with an aqueous solution containing polyethylene glycol 8000 17.8% (w/v) and polyethylene glycol 1000 2.2% (w/v) at pH 5.5 (molar liquor). After 1–2 weeks pyramidal crystal forms were observed. Such large irregular crystals were prepared for cryo conditions by equilibration (5–10 min) in a solution containing 21% (w/v) polyethylene glycol (8000:1000 as detailed for the mother liquor), with 20% (v/v) 2-methyl-2,4-pentanediol as cryoprotectant.

Cryoprotected crystals were flash frozen in a stream of nitrogen at −160 °C. The x-ray diffraction intensities were recorded with an R-Axis II detector (MRC, The Woodlands, TX) to a resolution of 1.7 Å. The data were reducible with Rmerge = 4.6% (23.5%) and 86% (35%) completeness with I/σ(I) = 18.7 (2.9) (values in parentheses for the 1.76–1.70 Å shell) in a primitive orthorhombic cell (a = 86.7, b = 134.2, c = 109.3 Å). Systematic absences for (h 0 k) and (0 h 0) but not for (0 0 l) reflections indicated that the space group was probably P212121. This space group was confirmed by translation function results detailed below. The asymmetric unit of the observed cell contains one OTCase trimer, and the solvent content is ~50% occupied by solvent.

**Structure Solution and Refinement—** The primitive orthorhombic data were probed by molecular replacement using the program Amore (30) with trimeric search models derived (excluding PALO and solvent) from the 2.8 Å resolution crystal structures of PALO-liganded E. coli OTCase (14) and the apo enzyme (18) (Protein Data Bank entries 2OTC and 1AKM, respectively). A significant peak (more than twice the next largest peak) was observed in the cross-rotation functions calculated

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of OTCase—** The argI coding sequence from E. coli K12 was polymerase chain reaction-amplified from the plasmid pAIL102 (25) and cloned into the expression vector pKK223–3 (Amersham Pharmacia Biotech). Expression levels of up to 100 mg of

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**Fig. 1. Kekulé representation of proposed OTCase mechanism and inhibitors.** a, a proposed reaction mechanism for OTCase. The side chain amino group of Orn undergoes a nucleophilic attack upon the carbonyl carbon of CP (left) to form a tetrahedral transition state (middle). The Orn swittetion with an uncharged amino group is shown. Charge rearrangement releases citrulline and phosphate (right). b, amino (left) and imino (middle) tautomers of PSOrn compared with PALO (right).

of these structures with unliganded enzyme structures (17, 18) demonstrate that OTCase and ATCase undergo a conformational change upon substrate binding, bringing substrates together and facilitating condensation (14, 19). Specifically, in OTCases, the binding of substrates and/or inhibitors induces a conformational change in the 240s loop2 to form a closed conformation.

N3-(N'-Sulfodiaminophosphinyl)-L-ornithine (PSOrn; Fig. 1b) is an extremely potent inhibitor of OTCases from a variety of sources (20, 21). PSOrn is produced by aminopeptidase cleavage of phaseolotoxin, the tripeptide phytotoxin produced by the bean pathogen pv. phaseolicola (22, 23). PSOrn has an unusual structure involving three N-P bonds (24) but has structural similarities to PALO and to the substrates of OTCase. OTCase is inactivated by PSOrn stoichiometrically, and it was suggested that this interaction might be via a covalent linkage (21). To characterize the nature of this interaction, we have crystallized the OTCase-PSOrn complex and refined the structure at high resolution. Implications for the chemical mechanism of transcarbamoylas case are discussed.
from 8–4 Å resolution with both search models using a 40 Å sphere radius. Translation searches were conducted with data from 8 to 4 Å resolution in space groups P2_12_12 and P2_12_12, and a unique solution was identified in P2_12_12. The PALO-ligated E. coli OTCase search model gave an optimal fit with a correlation coefficient of 0.57 against backgrounds of maxima at 0.50. Rigid body refinement of the search model with data to 2.3 Å increased the correlation coefficient to 0.63 (r = 36% and R_{exp} = 37%). A SigmaA (31) weighted F_o – F_c map revealed a pair of dominant peaks (>1σ) at each of the three CP-binding domains in the trimer matching the phosphorus and sulfur atoms of bound PSOrn.

Refinement of atomic positions and B-factors was carried out using Crystallography and NMR System, Version 0.5 (32). The structural model output from Amore was subjected to 600 steps of torsion angle molecular dynamics with a starting temperature of 2500 K. This resulted in r = 28% and R_{exp} = 32% using all data to 2.3 Å. From these coordinates one monomer was selected and the noncrystallographic symmetry (NCS) operators required to generate the other two monomers were calculated. Strict NCS constraints were then applied. The phosphorus and sulfur atoms previously identified were included in the model with r = 31% and R_{exp} = 32% at 2.3 Å resolution. Subsequent refinement and inclusion of data to 2.0 Å resulted in r = 32% and R_{exp} = 33%. The trimer was generated, and NCS restraints were applied. Energy minimization and B-refinement resulted in r = 28% and R_{exp} = 31% at 2.0 Å resolution. At this stage water molecules were gradually incorporated into the model. An electron density feature was modeled as a water molecule if it was >4 σ (3.5 σ in the final stages) in F_o – F_c maps and made at least one hydrogen bonding contact in the range 2.5–3.5 Å. Subsequent refinement using the data to 1.8 Å resolution reduced R and R_{free} to 25 and 27%, respectively, and resulted in F_o – F_c electron density around the binding site as shown in Fig. 2. PSOrn was modeled into this density. A search of the Cambridge Structural Data Base using various fragments of the PSOrn structure resulted in one related compound, bis(di-isopropylamino)-phenyl(trifluoro-methylsulfonyl)amino/phosphine oxide. Values for P-N, P=O, N=S, and S=O bond lengths, as well as relevant bond angles, were obtained from this crystal structure (33). The compounds and used as initial refinement restraints in Crystallography and NMR System Version 0.5. During the final stages of refinement, these restraints were removed, and the bond lengths and angles were restrained to be identical in each of the three PSOrn molecules in the trimer of OTCase. Further rounds of refinement including manual model adjustments, removal of NCS restraints, and addition of more water molecules resulted in r = 19.2% and R_{exp} = 22.1% using all data to 1.7 Å. For the last cycles the reflections used for R_{exp} were included in the refinement resulting in r = 19.4%. The final model contains three OTCase monomers, three PSOrn molecules, 735 water molecules, and one molecule of 2-methyl-2,4-pentanediol. The RMS deviations from dictionary bond lengths and bond angles are 0.012 Å and 1.5°, respectively.

RESULTS

Subunit Structure—The OTCase-PSOrn monomer conformation closely resembles that observed in the 2.8 Å crystal structure of the E. coli OTCase-PALO complex (14). In both structures the relative orientations of the SBDs are observed to be almost equivalent with OTCase in the “closed” subunit conformation (Fig. 3). Superposing the individual SBDs of the PALO complex with the SBDs of subunits G, H, and I of the PSOrn structure leaves RMS differences of 0.4, 0.4, and 0.3 Å, respectively for the 133 Ca atoms comprising the CP-binding domain, and RMS differences of 0.4, 0.4, and 0.4 Å for the 185 Ca atoms comprising the Orn-binding domain. These RMS differences are comparable with those obtained when the entire subunits of the two tertiary structures are superposed, with RMS differences of 0.4, 0.4, and 0.4 Å for 318 Ca atomic positions, indicating the general equivalence of the relative subunit conformations.

Oligomeric Structure—Some small but significant differences are observed in the quaternary arrangement of the three subunits of the OTCase-PSOrn trimer when compared with the strictly NCS averaged E. coli OTCase-PALO homotrimer (14). Although some deviation from exact three fold symmetry is evident at high resolution in the enzyme-PSOrn trimer, each of the three subunits are still closely related, superposing with an RMS difference of 0.3 Å for 332 Ca atoms. Two of the three subunits (H and I) can be superposed by a rotation of 120°, whereas the third subunit (G) is oriented away from the approximate 3-fold axis and is related to the other two subunits by rotations of 120° and 119.5°, respectively.

A detailed comparison of the structures of the three independent subunits G, H, and I reveals some small differences. As reported in other transcarbamoylase structures, the 80-s loop from a neighboring subunit of the trimer (Fig. 3, shown in red), protrudes into each of the three CP-binding sites to form an intersubunit active site (4). However, in the crystals analyzed here there are indications that the sulfur atom of Met236, which interacts with the neighboring subunits 80s loop (Fig. 3), is oxidized to either a sulfoxide or sulfone. The varying levels of residual difference electron densities within 2 Å of the sulfur atoms of Met236 suggest that the extent of oxidation is not equivalent in each of the three subunits, with G > I > H. The electron density for the 80s loop in the I subunit (interacting with Met236 of the G subunit) is weak and is consistent with the higher temperature factors (>50 Å^2) as compared with corresponding loops in subunits G and H. Only the side chain of
The observed electron densities and refined temperature factors for almost all of the amino acid side chains surrounding the three crystallographically independent PSOrn molecules are consistent with single conformations (other than the 80s loop of subunit I discussed above). Pairwise superposition of 13 Ca atoms adjacent (5 Å) to bound PSOrn molecules allows a precise (RMS difference of 0.2 Å for 121 atoms) comparison of the substrate-binding sites in each of the three subunits (not shown). In superposing the active sites, the largest and most significant difference (>0.2 Å) is observed in the CP-binding site of subunit G, whose guanidinium of Arg57, in comparison with its position in the other subunits, is displaced laterally with respect to PSOrn by 0.4 Å. The carbohydrate conformation of the nearby Glu87 (1 subunit) is also altered to preserve an intersubunit (I-G) salt bridge. Despite these small differences, the same set of interactions with identically placed and structured PSOrn molecules are observed in each of the three independent active sites (Table 1).

Structure of the PSOrn-binding Site—PSOrn occupies the adjacent Orn- and CP-binding sites found in the closed conformation of each of the three subunits. Electron density clearly defines the positions of all nonhydrogen atoms in each PSOrn molecule (Fig. 2), and there is no evidence of covalent attachment to the enzyme as previously postulated (21). Instead, PSOrn interacts noncovalently with substrate-binding residues, consistent with kinetic experiments, which show that PSOrn competes for the CP-binding site of OCTase (21). Around the active site, the E. coli PSOrn-OCTase structure closely parallels that reported at 1.85 Å resolution for PALO bound to human OCTase (Fig. 4a) (11). Glu82 of E. coli OCTase is not conserved in OCTases, and the intersubunit interactions of its side chain are substituted by similar interactions of the imidazole of His817 in human OCTase. Additionally, the conformation of the side chain amide of Gln136 is inverted when compared with the conformation reported for Gln171 in the human enzyme (11). The Gln136 side chain conformation is fixed by hydrogen bonding with the proton-accepting carbonyl of Asp240 (not shown). Other side chain positions in the PSOrn bound complex, which are not equivalent to those in the PALO complex, include Arg57 (see below), Met236, and Cys273 (Fig. 4a).

Not surprisingly, the Orn components of both PSOrn and PALO interact with the Orn-binding domain in a similar fashion (Fig. 4a). Briefly, the side chains of Asn167, Asp231, and Ser235, and the peptide nitrogen of Met236 combine with two ordered water molecules to form a tight hydrogen bonding network surrounding the N3-amino (equivalent to the α-amino of Orn; see Fig. 5 for atom designation) and carboxylate groups of PSOrn (Figs. 4b and 5). Local hydrogen bonding networks suggest that the Oy of Ser235 is indeed a hydrogen bond acceptor in its interaction with the PSOrn N3-amino group. A tetrahedral arrangement of three hydrogen bond acceptors around the N3-amino group is consistent with this amino group being protonated, analogous to that proposed for the productive binding of Orn (9). Adjacent to this site of recognition, the side chains of Leu128, His133, Met236, and Cys273 form a hydrophobic cleft in which the hydrocarbon stem (C2, C3, and C4; Figs. 4b and 5) of PSOrn lies, and the other face of which is solvent exposed.

Amino acid conformations at the CP-binding site are also remarkably similar despite distinct differences in the chemical structures of the inhibitors. The sulfamyl group of PSOrn substitutes for the phosphate group in PALO (Figs. 1b and 4a). The carbonyl oxygen of PALO could be mimicked by either the phosphonyl oxygen (O3, Fig. 5) or amide (N) of PSOrn, depending on the chirality of the PSOrn phosphorus. Although the electron density of PSOrn alone does not allow discrimination between these possibilities, the former case is shown in Figs. 2 and 4 for reasons discussed below.

A significant difference noted when comparing the structures of bound inhibitors concerns the relative position of the nitrogen in Fig. 5 (N1). In PSOrn, N1 is shifted toward one of the tetrahedrally arranged sulfamyl oxygens (Figs. 4a and 5, O1). However, despite this sulfamyl oxygen being only 3.1 Å from PSOrn nitrogen N1, it is oriented and positioned poorly to form a strong hydrogen bond (Fig. 4b, shown as a green line). Similarly, despite N1 being 3.0 Å from the carbonyl oxygen of Leu274, its orientation makes it unlikely to contribute to a
strong hydrogen bonding interaction. This is in contrast to the equivalent nitrogen within the OTCase-PALO (11, 14) and ATCase-PALA (16) complexes, which form hydrogen bonds with their corresponding leucines. Apart from this N1 nitrogen, the other two nitrogens within the unique phosphotriamide of PSOrn (Fig. 5, N1 and N2) do not have counterparts in PALO and PALA and consequently form novel interactions with the CP-binding domain.

Chirality of PSOrn Phosphorus—Electron density alone did not allow discrimination to be made between lone oxygen and nitrogen atoms bonded to the tetrahedral phosphorus of PSOrn (the lobes of electron density appear similar, Fig. 2). Discrimination was achieved by examination of the hydrogen bonding networks encompassing the inhibitor (Figs. 4b and 5). One of these undefined atoms interacts (at a distance of 2.6–2.8 Å) with the main chain carbonyl of Cys273 and with the carbonyl of the side chain of Gln136, which are both positioned as hydrogen bond acceptors (Fig. 4b). The carbonyl of Leu274 and the guanidinium of Arg106 are also in close contact (Fig. 4b, green lines). This arrangement of hydrogen bond acceptor oxygens would interact strongly with a P-amino group but is inconsistent with the presence of the alternative P–O oxygen occupying this position within the PSOrn molecule.

The other undefined atom bound to the tetrahedral phosphorus forms hydrogen bonds (2.7–3.1 Å) with three likely hydrogen bond donors; these being the guanidinium group of Arg106, the imidazole of His133, and the hydroxyl of Thr58 (Figs. 4b and 5). Although the guanidinium of Arg106 is ideally orientated to donate a proton in a hydrogen bonding interaction (at a distance of 2.7–2.8 Å), the roles of His133 and Thr58 are readily deduced by examination of surrounding hydrogen bonding networks. The pattern of hydrogen bonds implies that a Ne protonated tautomer of the imidazole of His133 is present, enabling it to donate a proton to the undefined PSOrn atom, and that a single rotamer of the side chain hydroxyl of Thr58 is predominant. This rotamer positions the proton of the hydroxyl to donate in a bifurcated hydrogen bonding interaction with both the undefined PSOrn atom and a sulfamyl oxygen (Fig. 5). A similar situation involving the phosphonacetyl group of PALO is also evident when the high resolution human OTCase-PALO structure is examined (Ref. 11; Protein Data Bank entry 1OTH). Although the guanidinium of Arg319 is also in close proximity to this second undefined PSOrn atom (2.9–3.1 Å), a strong hydrogen bond (not shown) with Thr58 places the side chain in an inappropriate orientation for a strong hydrogen bonding interaction. In fact, the charged guanidinium of Arg319 is approximately equidistant from both undefined atom positions about the tetrahedral phosphorus. Hence, the manner in which Arg319 contributes to the binding of PSOrn is likely to be a weaker electrostatic interaction (Figs. 4b and 5, green lines). Although conserved in OTCases, Arg319 is not conserved in ATCases and hence is not thought to participate directly in catalysis. However, it has been suggested to be functionally equivalent to Arg296 of E. coli ATCase (conserved in ATCases).
and may similarly polarize the active site (36). Combined observations lead to the conclusion that the P=O oxygen is located at this second undefined position and that any negative charge on this oxygen is likely to be stabilized by the nearby positively charged groups.

Given the diametrically different environments at two atomic positions around the phosphorotriamide, it is concluded that the chiral phosphorus of bound PSOrn is present in the R configuration displayed in Figs. 2 and 4. Because the electron densities indicate unit occupancy for the inhibitor bound in the crystal, it is apparent that the R enantiomer is the biologically active configuration of the toxin. Given that binding of PSOrn to OTCase appears to be stoichiometric, it is likely that the phaseolotoxin, from which PSOrn is prepared, is also synthesized exclusively as the R enantiomer.

**P-N-S Bridging Nitrogen of PSOrn**—The P-N-S bridging nitrogen is unique in PSOrn. In PALO and PALA it is replaced by a CH₂ group, which attaches a phosphonate group to a carbonyl oxygen (Fig. 1b). A particularly prominent difference in active site amino acid conformation between the PSOrn, PALO, and PALA complexes concerns the side chain of Arg⁵⁷ (Fig. 4a). The Nε of Arg⁵⁷ is 2.8 Å from the bridging nitrogen of PSOrn compared with the observed distances of 3.4 Å to the bridging CH₂ carbons of PALO (Protein Data Bank entries 2OTC and 1OTH) and PALA (subunit C of Protein Data Bank entry 1D09). In the PALO and PALA crystal structures, the equivalent of Arg⁵⁷ stabilizes the binding of a phosphonate group. In the PSOrn structure, a similar interaction is observed whereby the guanidinium of Arg⁵⁷ hydrogen bonds a sulfamyl oxygen. These same sulfamyl/phosphonate oxygens form a second hydrogen bond with the main chain amides of the same respective arginines (Figs. 4b and 5). Uniquely, in the PSOrn structure, the Nε of Arg⁵⁷ makes a third hydrogen bond, acting as a donor to the P-N-S bridging nitrogen of the inhibitor. The nature of this third hydrogen bond can be gleaned with some certainty. Firstly, the Nε of this arginine is almost certainly protonated as the guanidinium side chain of arginine is the most polar of all amino acid side chains (37). Secondly, the Nε hydrogen position is defined accurately by known bond length (0.94 Å) and by the observed plane of the nonhydrogen atomic positions of the guanidinium group. The orientation of this guanidinium group is such that the position of the Nε hydrogen is coplanar with the P-N-S bridge (with bridging nitrogen ~1.9 Å distant). In this context, the P-N-S nitrogen of PSOrn can only be a hydrogen bond acceptor if bound PSOrn is present as an imino tautomer, consistent with the inferred polarization of the N⁶-(N'-sulfodiaminophosphinyl) moiety (Fig. 1b). With the exception of these novel interactions, all of the hydrogen bonding interactions that are observed in the bound PSOrn complex are most similar to those reported for the allied PALO complexes.

**Determination of Rate Constants for Formation of the OTCase-PSOrn Complex**—The observation that PSOrn is bound covalently to the OTCase active site required re-evaluation of previous experiments, which indicated otherwise (21). The OTCase-PSOrn complex could not be dissociated by ethanol precipitation, extensive dialysis, or gel filtration in the presence of 10 mM CP (Ref. 21 and data not shown). However, when [¹⁴C]PSOrn-OTCase was challenged with a 200-fold excess of unlabeled PSOrn in 50 mM TAPS pH 8.0 at 37 °C, Samples were removed at 0, 2, 4, 6, 8, 10, 12, and 24 h, and the ¹⁴C remaining bound was measured (b) as compared with a control experiment with no unlabeled PSOrn (a). Each time point was measured in triplicate, and the slope was calculated by linear regression (r² = 0.9945, p < 0.0001). The initial rate of dissociation was determined for CP (8, 21) and ~10⁵-fold lower than the dissociation constant for the bisubstrate inhibitor PALO (8.0 × 10⁻⁶ M) (38). The extremely low dissociation constant estimated for the OTCase-PSOrn complex warrants PSOrn to be included as a member of the tight binding class of enzyme inhibitors (39).

**DISCUSSION**

The OTCase reaction mechanism is thought to proceed through a tetrahedral TS. This mechanism (Fig. 1a) is based on structural data and isotopic experiments with OTCase and by analogy to that proposed for ATCase (7, 9, 10, 11, 19, 40, 41). We propose that PSOrn acts as a TS analogue (42) because it binds tightly yet reversibly to OTCase and has close structural homology with the proposed OTCase reaction mechanism TS.

As observed in OTCase-PALO complexes (11, 14), the binding of PSOrn induces a compression of SBDs with the enzyme closing around the inhibitor. Like PALO, PSOrn interacts with...
residues of the CP domain that are generally conserved in the transcarbamoylase family comprising OTCase and ATCase. Additional enzyme-inhibitor interactions observed in the OTCase-PSOrn complex are relevant to our understanding of the detail of the chemical mechanism. Particularly important are interactions about the chiral phosphorus of PSOrn. A TS in which a chiral tetrahedral carbon is obligatory would be analogous to the binding of a particular enantiomer of PSOrn with respect to its chiral phosphorus. A high energy state or any intermediate on the reaction pathway that approximates an R configuration could of course be stabilized by the very same set of hydrogen bonding interactions made by PSOrn with OTCase. An R configuration in the TS would result from a stereospecific nucleophilic attack. In this scenario, prepositioning may be accompanied by enzyme-mediated distortion of the planar carbonyl group of CP toward the final tetrahedral R configuration, sterically assisting nucleophilic attack by the lone electron pair from the δ-nitrogen of Orn. Alternatively, this could be considered to be a preorganization of CP binding by the enzyme in the enzyme-substrate complex. Although a chiral tetrahedral carbon in the TS is not a steric requirement of the overall reaction, it is not uncommon that a catalytic reaction pathway may include chiral structures.

Neither the predominant tautomer of PSOrn in solution (24) nor the pK_a value(s) of the chemical groups of PSOrn bound to the active site of OTCase are known. The orientation and hydrogen bonding interactions of the Orn component of PSOrn are consistent with the presence of a zwitterion with a protonated α-amino group (N3, Fig. 5). The protonation and charged state of the PSOrn groups occupying the CP-binding site are less certain. With three positively charged guanidinium groups nearby, it is possible that deprotonated forms of PSOrn are the predominant bound species in these crystals. The observation of a strong interaction involving the Ne of Arg^77 indicates that the guanidinium group donates a hydrogen to the lone pair of electrons of an sp2 hybridized imino nitrogen (Figs. 4b and 5, N2). We conclude that were the P-N-S nitrogen protonated, steric hindrance would shift the guanidinium group of Arg^77 back toward a position in space similar to that observed in the PALO and PALA complexes. Additional negative charge resulting from deprotonation of the bridging P-N-S nitrogen could be stabilized by nearby guanidinium groups and by delocalization between the sulfamyl and/or P=O oxygens. Of significance to the catalytic mechanism, should Arg^77 adopt a similar conformation about CP, it would be acting to polarize the bridging C=O oxygen on the pathway toward phosphate release (Fig. 1a).

Although the Orn and sulfamyl groups of PSOrn bind in orientations and positions that approximate those observed for their counterparts in complexes of PALO (Orn and phospho- nate, respectively), the position of the N1 nitrogen of the Orn component differs in the two complexes (Figs. 4a and 5). N1 could interact with the main chain carbonyl of Leu^274 and/or a sulfamyl oxygen (Fig. 5). If mirrored in the TS this proximity of N1 to the sulfamyl oxygen would be consistent with concerted intramolecular proton transfer between the Orn δ-amino group and a terminal phosphate oxygen of CP (40), as shown in Fig. 1a.

The strength of the OTCase-PSOrn interaction predicted from the structural data is consistent with the low dissociation constant estimated (1.6 × 10^{-12} M). It is worth noting that the k_{on} observed for PSOrn (5.8 × 10^6 M^{-1} s^{-1}) is similar to the equivalent rate constant for CP (6.5 × 10^6 M^{-1} s^{-1}) (8). Hence, although PSOrn competes equally with CP for free enzyme, the slow rate of dissociation (9 × 10^{-6} s^{-1}) means that PSOrn effectively irreversibly inhibits OTCase. Classical TS theory, which proposes that the enzymatic rate enhancement (typically 10^{10}–10^{18}) (43) is principally due to stabilization of an otherwise unlikely intermediate, would predict that these TS-like inhibitors resemble the activated substrate(s) in the TS as closely as is chemically feasible. Although the OTCase catalytic rate of enhancement is unknown, the high affinity of PSOrn relative to substrate (−10^6) implies that there exists a substrate-like bound state for which the Gibbs free energy is very much lower than the enzyme-substrate complex. This further supports the hypothesis that a structural state analogous to the OTCase-PSOrn complex forms part of the enzymatic reaction pathway. Subtle differences in structure between the enzyme-inhibitor complex and the TS may account for TS binding affinities that are even higher than that reported here for PSOrn.

This study of the mode of action of PSOrn has conclusively identified it as a tight-binding TS-like inhibitor of OTCase. The low dissociation constant for PSOrn accounts for the potency of phaseolotoxin as a phytotoxin and inhibitor of microbial growth (44). Given that the unique features of the OTCase-PSOrn interaction are confined to the conserved CP domain, it is likely that these observations are relevant to the transcarbamoylase family in general. In particular, the CP-binding sites of ATCases are almost structurally identical to those of OTCases. Poor OTCase inhibition by phosphinylphosphinate bisubstrate mimics, which contain a nonchiral tetrahedral phosphate (45), confirms the importance of the TS-like configuration of PSOrn. This suggests that inhibitors with the appropriate enantiomer of an sulfamidinophosphosphoryl group attached to the appropriate amino group of the relevant amino acid could be potent inhibitors of other transcarbamoylase enzymes.

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Transition State Analogue of OTCase
Mechanism of Inactivation of Ornithine Transcarbamoylase by $N^\delta$-($N'$-Sulfodiaminophosphinyl)-l-ornithine, a True Transition State Analogue?:
CRYSTAL STRUCTURE AND IMPLICATIONS FOR CATALYTIC MECHANISM
David B. Langley, Matthew D. Templeton, Barry A. Fields, Robin E. Mitchell and Charles A. Collyer

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Folylpoly-γ-glutamate carboxypeptidase from pig jejum. Molecular characterization and relation to glutamate carboxypeptidase II.

Charles H. Halsted, Erh-hsin Ling, Ruth Luthi-Carter, Jesus A. Villanueva, John M. Gardner, and Joseph T. Coyle

Page 20422: The units on the vertical axis of Fig. 3 should read “FGCP (pmol·mg·min).” The units for $V_{\text{max}}$ in Table II should read “pmol·mg·min.”

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Mechanism of inactivation of ornithine transcarbamoylase by $N\delta$-(N'-sulfodiaminophosphinyl)-L-ornithine, a true transition state analogue? Crystal structure and implications for catalytic mechanism.

David B. Langley, Matthew D. Templeton, Barry A. Fields, Robin E. Mitchell, and Charles A. Collyer

The structural designation of the chirality of the tetrahedral carbon in the putative TS is incorrectly stated to be R. The chirality of the tetrahedral phosphorus in PSOrn was shown to be R, but because the P-N-S bridging nitrogen (in PSOrn) is structurally homologous to oxygen in TS then the order of priority of the substituents is consequently different. The observation of an R phosphorus in PSOrn actually implies a tetrahedral S carbon in TS.

Page 20018, left column, lines 9–18 should read: A high energy state or any intermediate on the reaction pathway that approximates an S configuration could of course be stabilized by the very same set of hydrogen bonding interactions made by PSOrn with OTCase. An S configuration in the TS would result from a stereospecific nucleophilic attack. In this scenario, prepositioning may be accompanied by enzyme-mediated distortion of the planar carbonyl group of CP toward the final tetrahedral S configuration, sterically assisting nucleophilic attack by the lone electron pair from the nitrogen of Orn.

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