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# Crystal Structure of Human Ornithine Transcarbamylase Complexed With Carbamoyl Phosphate and L-Norvaline at 1.9 Å Resolution

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***ABSTRACT*** **The crystal structure of human ornithine transcarbamylase (OTCase) complexed with carbamoyl phosphate (CP) and L-norvaline (NOR) has been determined to 1.9-Å resolution. There are significant differences in the interactions of CP with the protein, compared with the interactions of the CP moiety of the bisubstrate analogue *N*-(phosphonoacetyl)-L-ornithine (PALO). The carbonyl plane of CP rotates about 60° compared with the equivalent plane in PALO complexed with OTCase. This positions the side chain of NOR optimally to interact with the carbonyl carbon of CP. The mixed-anhydride oxygen of CP, which is analogous to the methylene group in PALO, interacts with the guanidinium group of Arg-92; the primary carbamoyl nitrogen interacts with the main-chain carbonyl oxygens of Cys-303 and Leu-304, the side chain carbonyl oxygen of Gln-171, and the side chain of Arg-330. The residues that interact with NOR are similar to the residues that interact with the ornithine (ORN) moiety of PALO. The side chain of NOR is well defined and close to the side chain of Cys-303 with the side chains of Leu-163, Leu-200, Met-268, and Pro-305 forming a hydrophobic wall. C-δ of NOR is close to the carbonyl oxygen of Leu-304 (3.56 Å), S-γ atom of Cys-303 (4.19 Å), and carbonyl carbon of CP (3.28 Å). Even though the N-ε atom of ornithine is absent in this structure, the side chain of NOR is positioned to enable the N-ε of ornithine to donate a hydrogen to the S-γ atom of Cys-303 along the reaction pathway. Binding of CP and NOR promotes domain closure to the same degree as PALO, and the active site structure of CP-NOR-enzyme complex is similar to that of the PALO-enzyme complex. The structures of the active sites in the complexes of aspartate transcarbamylase (ATCase) with various substrates or inhibitors are similar to this OTCase structure, consistent with their common evolutionary origin. Proteins 2000; 39:271–277.** © **2000 Wiley-Liss, Inc.**

**Key words: protein crystallography; ornithine transcarbamylase; urea cycle; ornithine transcarbamylase deficiency**

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## INTRODUCTION

Human ornithine transcarbamylase (OTCase) catalyzes the formation of citrulline from carbamoyl phosphate (CP) and L-ornithine (ORN) in the urea cycle (Fig. 1).[1] Deleterious mutations in the human OTCase gene produce clinical hyperammonia and subsequent neurological symptoms or even death, a condition known as ornithine transcarbamylase deficiency (OTCD).[2]

The crystal structures of unliganded catabolic *Pseudomonas aeruginosa* OTCase,[3] unliganded *Escherichia coli* OTCase,[4] unliganded anabolic *Pyrococcus furiosus* OTCase,[5] PALO-liganded *E. coli* OTCase,[6] and PALO-liganded human OTCase[7] have recently been determined. Anabolic human and *E. coli* OTCases are trimeric, whereas anabolic *P. furiosus* and catabolic *P. aeruginosa* OTCases are dodecameric. The trimer is the smallest oligomer with enzymatic activity, with larger aggregates having additional features such as allosteric regulation or thermal stabilization. The trimers of OTCases are homologous to the catalytic trimer of *E. coli* ATCase, which has been studied for more than 30 years as an archetypal allosteric system.[8]

We have previously reported the crystal structure of human and *E. coli* OTCase complexed with the bisubstrate analogue PALO.[6,7] However, although PALO mimics well the phosphate and carbonyl groups of CP and the carboxylate and α-amino groups of ORN, PALO is a poor analogue of the tetrahedral intermediate. Hence, it is important to determine the structure of ternary enzyme complexes with a substrate and substrate analogue bound. Here, we have determined the structure of the ternary complex of human OTCase with CP and NOR, an inhibitor of ORN. The structure has been determined and refined at 1.9-Å resolution, enabling the substrate, CP, and inhibitor, NOR, to be positioned unambiguously.

*Abbreviations:* OTCase, ornithine transcarbamylase; ATCase, aspartate transcarbamylase; CP, carbamoyl phosphate; NOR, L-norvaline; ORN, L-ornithine; PALO, *N*-phosphonacetyl-L-ornithine; PALA, *N*-phosphonacetyl-L-aspartate; rmsd, root mean square deviation.

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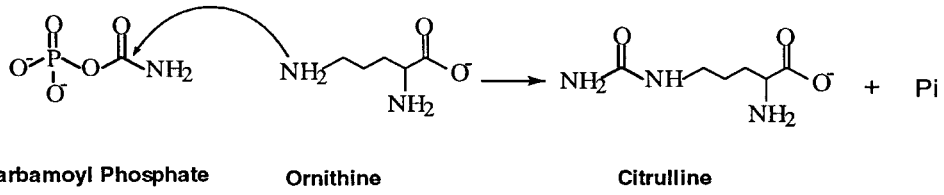


Fig. 1. Schematic drawing of the reaction catalyzed by OTCase.

## MATERIALS AND METHODS

### Crystallization, Data Collection, and Processing

Human wild-type recombinant OTCase was prepared, purified, and stored as previously described[9]; PALO was synthesized by Drs. G. Barany and D. Venugopal (Department of Chemistry, University of Minnesota) as described by Morizono et al.[9] Because carbamoyl phosphate has a short half-life in solution at room temperature, the usual methods for growing diffraction quality crystals cannot be used. Instead, CP- and NOR- complexed crystals were prepared by soaking PALO-liganded crystals grown as previously described[7] with 100 mM CP and NOR, which were dissolved in the mother liquid containing 2% polyethylene glycol 400, 2 M ammonium sulfate, 0.1 M sodium Hepes at pH 7.5. Ligand exchange was accomplished by transferring the crystals with a loop to a fresh solution of CP and NOR every 12 h for seven times. PALO is a reversible inhibitor that can be displaced by CP as demonstrated when affinity chromatography is used to purify the enzyme, in which the enzyme is first bound to a PALO affinity column and then eluted with 10 mM CP.[9] Given their relative binding constants of $1.1 \times 10^{-4}$ M (CP) and $1.4 \times 10^{-7}$ M (PALO) and the $10^{17}$-fold dilution we have performed, we are confident that almost all of the PALO was replaced by CP and NOR. A similar replacement has been successfully carried out with ATCase.[10]

X-ray data collected at 90°K at beamline X12B at the National Light Source at Brookhaven National Laboratories using 1.0702-Å wavelength radiation were processed with DENZO and SCALEPACK.[11] Crystals were soaked in a solution containing ≈30% glycerol for about 1 min and cryocooled with liquid nitrogen before mounting. Intensities were measured with a CCD detector; a total of 90 frames covering 90° of the $\phi$ axis were collected. The agreement factor was 5.9% for the 799,411 observations of 52,412 unique reflections from 35.0 to 1.9 Å. Data collection statistics are given in Table I.

Unit cell dimensions for this crystal (space group $P2_13$, $a = b = c$ =125.41 Å) are isomorphous to PALO-liganded crystals. Fifteen cycles of rigid body refinement using the native PALO-complexed structure with PALO and all solvent omitted as the starting model brought the residual $R$ to 30.9% ($R_{free}$ =32.1%) for the 8.0–3.0-Å resolution shell. Because the electron density for CP and NOR was clearly visualized from the $|F_o| - |F_c|$ and $2|F_o| - |F_c|$ maps (Fig. 2), the carbamoyl plane of CP was well defined, and ligands were built into the structure by using the graphics program O.[12] The resulting model was refined with the program XPLOR,[13] and higher resolution data were gradually added until all reflections to 1.9-Å resolution were included. Low-resolution data to 35.0 Å were included after the bulk solvent correction was applied.[14] Individual isotropic $B$ values were refined, and solvent molecules were added when the phases had been extended to 1.9-Å resolution. Water molecules were identified as peaks of well-defined electron density in $|Fo| - |Fc|$ ($> 3\sigma$) and $2|Fo| - |Fc|$ maps ($> 1\sigma$) by using the CCP4 programs PEAKMAX[15] and O.[12] All waters formed at least one hydrogen bond to protein or well-established water molecules. In total, 223 waters were included in the final model. Final values were 0.186 for the working $R$ factor and 0.206 for $R_{free}$, for reflections greater than $2\sigma$ between 35 and 1.9 Å (Table I).

**TABLE I. Crystallographic Data and Refinement Statistics**

| | |
|---|---|
| Space group | $P2_13$ |
| Cell dimensions (Å) | a = b = c = 125.41 |
| Resolution limits (Å) | 35-1.9 Å |
| Number of reflections (unique) | 799,411 (52,412) |
| Rmerge | 5.9% (14.9%)[a] |
| $<I>/\sigma$ | 53.4 (21.2)[a] |
| Number of atoms in final model | 2772 |
| Number of reflections involved in refinement | 51,666 (5,755)[a] |
| R value | 18.6% (24.7%)[a] |
| $R_{free}$ value | 20.6% (25.6%)[a] |
| rmsd ideal bond length (Å) | 0.012 |
| rmsd ideal bond angle (°) | 2.46 |
| rmsd ideal dihedral angle (°) | 23.96 |

[a]Statistics in brackets are for the highest resolution shell (1.99–1.90 Å).

During the refinement, the same randomly selected set of 10% reflections from various resolution bins, which were not included in the refinement, was used to calculated $R_{free}$ to monitor the progress of refinement.[16] The program PROCHECK[17] was used to confirm the accuracy of structure. The $\phi$ and $\psi$ values of 92.7% residues are located in the most favored regions in the Ramachandran plot, as expected for a good quality model. The unusual torsional angles of two outliers, Leu-163 and Leu-304, have been rationalized in terms of their structural and functional roles.[7] The only energetically unfavorable *cis*-peptide bond is found between Leu-304 and Pro-305, which is a part of the conserved -His-Cys-Leu-Pro- motif and is functionally important. The conformation of this motif is maintained by the extensive hydrogen bond network, especially by the interactions with the side chain of His-302. The rms deviation from ideality for the bond lengths and bond angles were 0.012 Å and 2.46°, respectively. Average temperature factors for backbone, side chain, ligands, and

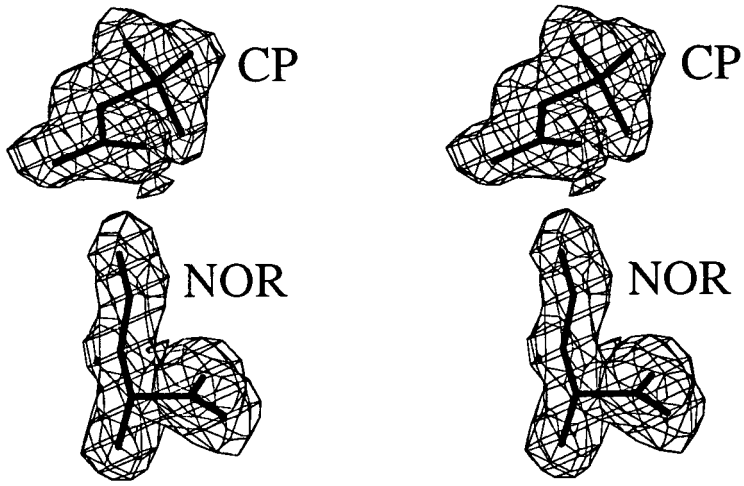


Fig. 2. 2| Fo| -| Fc| electron density map (contour at 1σ ) around the ligands. The conformation of carbamoyl phosphate (CP) and L-norvaline (NOR) are unambiguously defined.

223 solvent atoms are 18.26, 20.29, 14.81, and 29.57 Å$^2$, respectively. Refined coordinates have been deposited into the Protein Data Bank (I.D. code 1C9Y).

## RESULTS AND DISCUSSION

### Overview of the Structure

The enzyme is a trimer of exact threefold symmetry with one active site per monomer (Fig. 3). Each monomer has two structural domains, linked by two interdomain helices, and each domain has a central parallel β -sheet surrounded by α-helices and loops with α/β topology. The active site is located in the cleft between the two domains and shared by adjacent monomers. The overall structure of the CP-NOR-liganded enzyme is very similar to that of PALO-liganded OTCase with an rmsd of 0.15 Å for 321 equivalent C$^\alpha$ positions. The structure indicates that the magnitude of domain closure induced by CP and NOR is very similar to that induced by PALO. This result is consistent with fluorescence spectroscopy experiments in which the fluorescence signal change induced by adding CP and NOR is the same as that produced by adding PALO.[18]

### Carbamoyl Phosphate-Binding Site

The residues that are involved in binding CP are shown in Figure 4 and listed in Table II. The most important features of this structure are interactions between the primary nitrogen of CP and the main chain carbonyl oxygens of Cys-303 and Leu-304 and the side chains of Gln-171 and Arg-330, which are not observed in the PALO-liganded structure. These interactions are important in understanding the catalytic mechanism.

Although we cannot distinguish an oxygen and a nitrogen from the electron density map, we can confidently identify the orientation of the carbamoyl group based on its hydrogen-bonding environment and by comparing it with the PALO-liganded structure. The amide plane of CP rotates about 60° relative to the equivalent plane of PALO when the carbonyl oxygen is used as its apex (Fig. 5). Although the carbonyl carbon of CP shifts about 0.8 Å from that of the carbonyl carbon of PALO, the 0.5 Å shift of the carbonyl oxygen of CP is not sufficient to disrupt its interactions with Thr-93, Arg-141, His-168, and Arg-330 seen in the PALO-liganded structure. The side chain of Arg-330, which is unique to OTCase and invariant, is positioned above the amide plane of CP, in close contact with the carbonyl carbon of CP (2.94 Å). The equivalent residue in ATCase is Gly-292, which is also invariant. The function of Arg-330 seems to be to position CP and polarize the carbonyl bond to enable the δ amino group to attack the carbonyl carbon more readily. In ATCase the side chain of Arg-296, which is conserved in ATCases, occupies a position similar to that of Arg-330 in OTCase. Although the side chain of Arg-296 in ATCase is slightly farther from CP than Arg-330 is in OTCase, it may have a function similar to Arg-330.

Most of the residues that are involved in binding CP are the same as those that interact with analogous groups of PALO, and the conformations of the active site residues are also similar to those in the PALO-liganded complex. However, because the mixed anhydride oxygen of CP replaces the methylene carbon of PALO, it forms a new interaction with the guanidinium group of Arg-92. As a result, the position of the side chain of Arg-92 shifts 0.6 Å toward the substrate. As in PALO-liganded OTCase, Arg-92 also interacts with the phosphate oxygen of CP and forms a salt bridge to Glu-122 from the adjacent subunit. The function of Arg-92 seems to be unique. When Arg-92 was mutated to glycine, leucine, and histidine, catalytic activity was dramatically reduced.[19–21] It has been suggested that the positive charge of this residue is important to facilitate formation of the binary enzyme-CP complex, and elimination of the positive charge results in an enzyme that binds its substrates in a random order.[20] Catalytic activity can be rescued by adding exogenous guanidines, although binding of substrates is still random.[21] The NOR-CP-OTCase structure indicates that Arg-92 is not involved in binding NOR, but mutation of this residue affects L-norvaline affinity, implying that this residue affects the conformational changes that accompanies CP binding. The residue equivalent to Arg-92 was also investigated

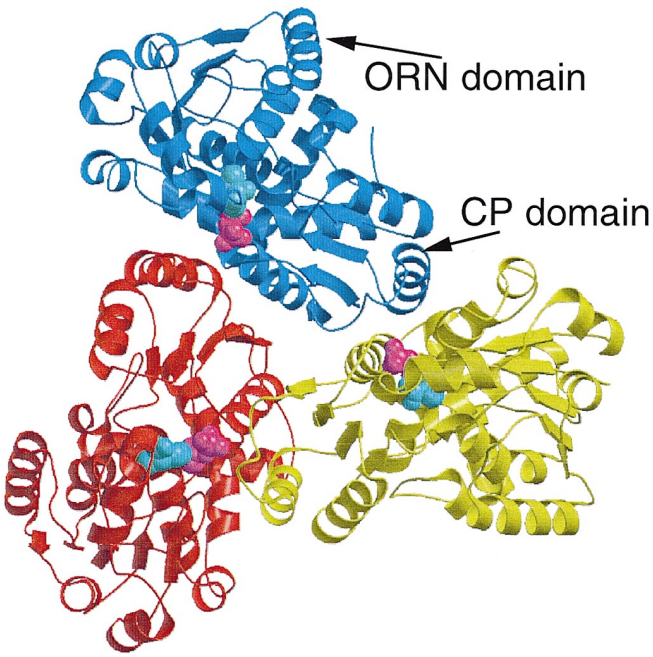


Fig. 3. Ribbon representation of the OTCase trimer based on the ternary structure. The three monomers are colored blue, red, and yellow, respectively. The CP and ORN are shown as space filling model colored pink and light blue, respectively. The view is down the molecular threefold axis from the concave face. The ribbon trace was generated with the program MOLSCRIPT[29] and Raster 3D.[30,31]

in *E. coli* ATCase.[22] Replacing this residue with Ala causes 17,000-fold loss of activity but does not affect the binding of substrates or substrate analogues. It was proposed that the side chain of this arginine is essential for proper ionization of the phosphate group.

### Norvaline-Binding Site

As shown in Figure 2, the conformation of NOR can be unambiguously determined. When the structure of PALO-liganded OTCase is superimposed on that of the ternary complex, the position and orientation of NOR is very similar to the ORN moiety of PALO, with a difference of 0.8 Å for the C-δ atom. As in the PALO-liganded structure, the α-amino group interacts with the side chains of Asp-263, Ser-267, and Asn-199, whereas the carboxyl group interacts with the side chain of Asn-199 and the side chain of Lys-88 via a water and main chain nitrogen of Met-268 and two waters (Table II). The side chain of NOR is surrounded by the hydrophobic side chains of Leu-163, Leu-200, Met-268, and Pro-305 and is also close to the S-γ atom of Cys-303, supporting the previous proposal that Cys-303 functions as a general base in the catalytic reaction to accept one hydrogen from the δ-amino group of ornithine.[7] Because the position and conformation of NOR are so similar to those of the ornithine moiety of PALO, NOR appears to represent well the binding of ornithine before bond formation between the δ-amino group of ornithine and the carbonyl carbon of CP.

### Catalytic Mechanism

A possible catalytic mechanism has been proposed on the basis of the structure of human OTCase complexed with the bisubstrate analogue PALO.[7] However, because one nitrogen atom is missing in PALO, PALO is not a good analogue for the tetrahedral intermediate. Furthermore, because a bond links the CP and ornithine moieties together in PALO, the position and conformation of bound substrates before bond formation cannot be derived clearly from the PALO-complexed structure. The NOR-CP-OTCase structure provides a new insight into an intermediate step in the catalytic reaction and enables a simple model to be developed. If we assume that ornithine binds to the enzyme in the same position and conformation as NOR, we can superimpose ornithine onto the crystallographically determined structure of NOR bound to enzyme. By changing the dihedral angles of the N-ϵ, C-δ, C-γ, and C-β atoms, the N-ϵ atom of ornithine can be modeled in a position that enables it to form hydrogen bonds to the S-γ atom of Cys-303 (≈2.7 Å) and the carbonyl oxygen of Leu-304 (≈2.8 Å) to be ≈3.0 Å from the carbonyl carbon of CP.

The proposed mechanism based on this structure is schematically shown in Figure 6. During the catalytic reaction, CP binds to the enzyme first because it is in the deeper pocket. When the second substrate, ORN, binds to the enzyme, its N-ϵ atom will interact with the S-γ atom of Cys-303 and the carbonyl oxygen of Leu-304. This mechanism supports the proposal that Cys-303, which is conserved in a -His-Cys-Leu-Pro- motif, is directly involved in the catalytic reaction as a general base. Cys-303 has been shown to be involved in binding ornithine by chemical modification[23] and site-directed mutagenesis.[19] Its basicity is increased by a hydrogen bond to the side chain of Asp-263, which also interacts with the α-amino group of L-ornithine. Human OTCase has a pH optimum between

**TABLE II. Interactions Between the Protein and Substrates**

| Substrate atoms | Protein atoms (distance Å) |
|---|---|
| CP O1P | Arg141 NH1 (2.74), His117 NE2 (2.85)[a] |
| CP O2P | Thr93 N (2.79), Thr93 OG1 (2.87), Ser90 OG (2.69), Arg141 NH2 (3.21) |
| CP O3P | Thr91 N (2.77), Arg92 N (2.97), Arg92 NE (3.18), Arg92 NH2 (2.90) |
| CP OA | Arg92 NE (2.79) |
| CP O1 | Thr93 OG1 (3.08), His168 NE2(2.90), Arg141 NH2 (3.04), Arg330 NH1 (2.94) |
| CP NP | Gln171 OE1 (2.69), Cys303 O (2.93), Leu304 O (3.56), Arg330 NH1 (3.23) |
| NOR CE | Leu163 CD1 (3.66), Cys303 SG (4.19), Leu304 O (3.56) |
| NOR CD | Met268 CE (4.29) |
| NOR CB | Leu163 CD1 (3.81), Ile200 CD1 (4.04) |
| NOR N | Asn199 OD1 (2.79), Asp263 OD2 (2.67), Ser267 OG (2.91) |
| NOR OT1 | Met268 N (2.88), Wat387 O1 (2.74) |
| NOR OT2 | Asn199 ND2 (2.93), Wat383 O1 (2.73) |

[a]His117 is from an adjacent polypeptide subunit in the catalytic trimer.

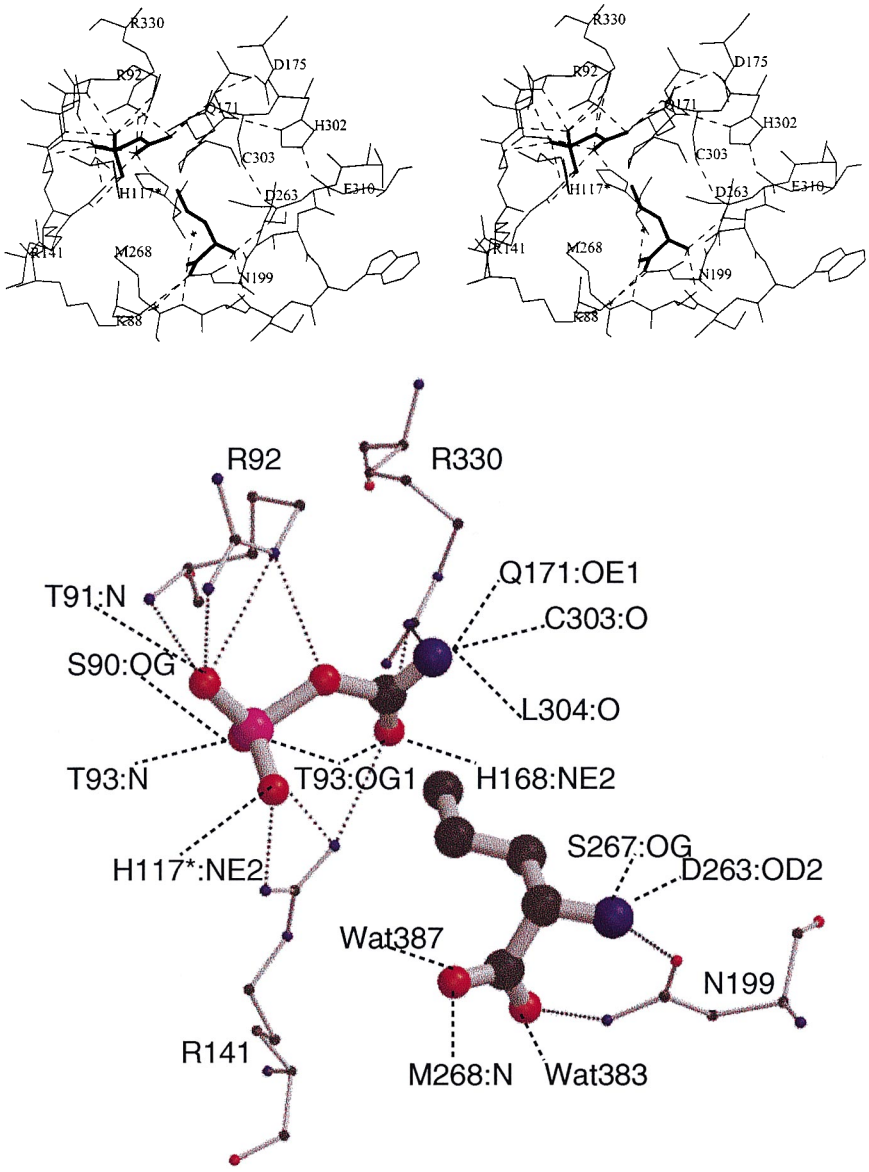


Fig. 4. Stereoview (**top**) and schematic (**bottom**) drawing showing the interactions of the carbamoyl phosphate and L-norvaline with the active site residues. Ligands are shown in bold. The residue indicated with * is from an adjacent subunit.

7.5 and 8.0, lower than the pKa of a free sulfhydryl, consistent with the suggested role of Cys-303. The model predicts that the chirality of the tetrahedral intermediate will be the *S* configuration and that the partially negatively charged carbonyl oxygen of CP in the tetrahedral intermediate will be stabilized by the positive charged side chains of His-168, Arg-141, and Arg-330. The positive charged ϵ nitrogen of ORN will be neutralized by transferring a proton to the phosphate group via His-168 or to waters, to facilitate release of products as proposed in ATCase.[22]

### Comparison With Aspartate Transcarbamylase

Several crystal structures of ATCase complexed with substrates or substrate analogues have been solved or

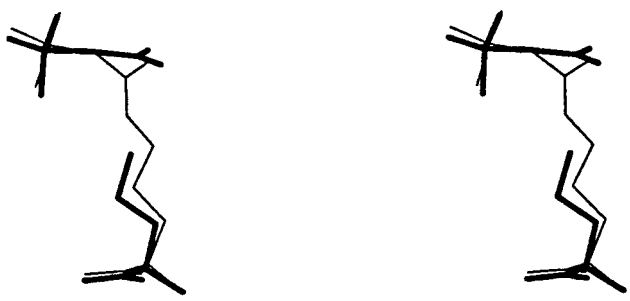

Fig. 5. The position of carbamoyl phosphate and L-norvaline (thick line) compared with the position of PALO (thin line) when superposition of the CP-NOR-OTCase structure on the PALO-liganded structure.

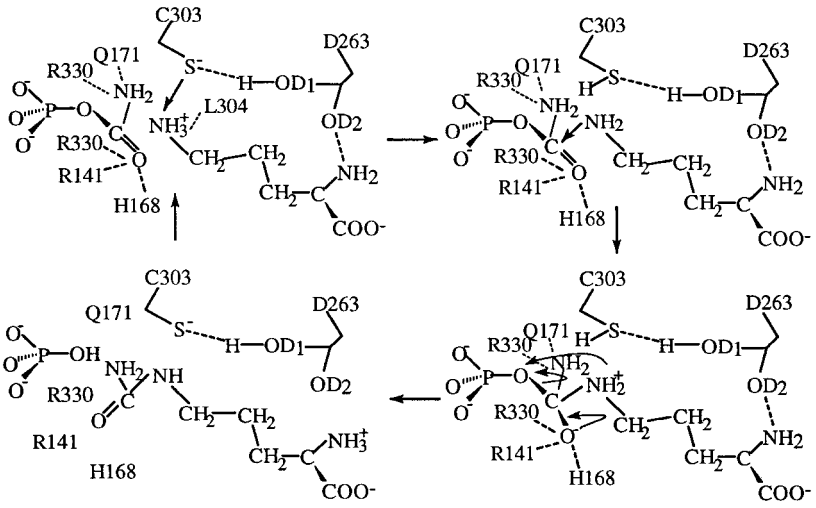


Fig. 6. Schematic drawing of the proposed catalytic mechanism.

modeled.[8,24–26] The most closely related is the crystal structure of ATCase complexed with CP and succinate.[8] The results reported here confirm that CP binding in OTCase is very similar to that in ATCase. Because of the limited resolution, the conformation of ligands in these structures, especially the L-Asp analogues substrate malonate or succinate, are not unambiguously determined. Our results indicate that L-norvaline represents well the ORN moiety of PALO, in contrast to the CP-succinate-ATCase complex, where succinate seems to be in a different conformation from the L-Asp moiety of N-phosphonacetyl-L-aspartate (PALA). One possible reason for the difference is that the phosphate group and amide groups of PALA draw the α carbon of the amino group further into the active site.[8] However, the CP-NOR-OTCase structure indicates that the difference seems to be intrinsic to succinate rather than to PALA. Loss of chirality of succinate makes it more conformationally flexible than L-norvaline, consistent with L-norvaline being a better inhibitor for ORN ($K$i = 9.1 × $10^{-6}$ M at pH 7.7[27] than succinate is to L-aspartate ($K$i = 4.4 × $10^{-4}$ M at pH 6.0.[28]

Although the second substrate is different for OTCase and ATCase, the reactive amino group points to the same CP plane in OTCase and ATCase, implying that the attacking mode of the deprotonated δ-amino group of ornithine in OTCase is very similar to that of the α-amino group of aspartate in ATCase. The results reported here demonstrate that the two enzymes, OTCase and ATCase, not only have a similar primary and tertiary structures but also have a similar catalytic reaction mechanism.

## ACKNOWLEDGMENTS

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