The crystal structure of human ornithine transcarbamoylase complexed with the bisubstrate analog N-phosphonacetyl-L-ornithine has been solved at 1.85-Å resolution by molecular replacement. Deleterious mutations produce clinical hyperammonemia that, if untreated, results in neurological symptoms or death (ornithine transcarbamylase deficiency). The holoenzyme is trimeric, and as in other transcarbamoylases, each subunit contains an N-terminal domain that binds carbamoyl phosphate and a C-terminal domain that binds L-ornithine. The active site is located in the cleft between domains and contains additional residues from an adjacent subunit. Binding of N-phosphonacetyl-L-ornithine promotes domain closure. The resolution of the structure enables the role of active site residues in the catalytic mechanism to be critically examined. The side chain of Cys-303 is positioned so as to be able to interact with the δ-amino group of L-ornithine which attacks the carbonyl carbon of carbamoyl phosphate in the enzyme-catalyzed reaction. This sulfhydryl group forms a covalent bond with the carbonyl carbon of carbamoyl phosphate in the enzyme-substrate complex. This sulfhydryl group is one of several active site residues conserved among all transcarbamoylases and is critical for activity.

Since ornithine transcarbamoylase (OTCase) catalyzes the formation of citrulline from carbamoyl phosphate (CP) and L-ornithine (ORN) in the urea cycle (1), deleterious mutations in the human OTCase gene produce hyperammonemia, with subsequent neurological symptoms or even death (ornithine transcarbamylase deficiency; OTCD). Approximately 140 mutations that give rise to OTCD have been identified (2), and several mutant enzymes have been purified and characterized biochemically (3, 4). Establishing the structural basis of their deleterious effects is of considerable clinical importance, as well as biochemical interest.

OTCase is homologous in structure and function to the catalytic trimer of Escherichia coli aspartate transcarbamoylase (ATCase), a widely studied allosteric enzyme (5). We have previously developed a homology model of human OTCase (6) based on the ATCase structure (7) which allowed the functional relationships for the human enzyme with certainty, its structural basis to be predicted. The crystal structures of unliganded catalytic Pseudomonas aeruginosa OTCase (8), unliganded E. coli OTCase (9), and unliganded anabolic Pseudomonas furiosus OTCase (11) confirmed many of these predictions. However, all the OTCase structures available to date are of bacterial enzymes and at only moderate resolution. All have significant sequence differences from the human enzyme (Fig. 1), and some function anabolically to synthesize arginine. To establish structure-function relationships for the human enzyme with certainty, its crystal structure is required.

We report herein the structure of human OTCase complexed with the bisubstrate analog N-phosphonacetyl-L-ornithine (PALO) at 1.8-Å resolution, the highest resolution OTCase crystal structure reported to date. 215 bound solvent molecules, some of which form clusters at the active site, can be identified. The resolution of the structure enables the function of active site residues to be examined with precision and a model of the catalytic mechanism to be proposed. We compare this structure with those of other transcarbamoylases, discuss possible functions for its unique regions, and describe the structural basis of the clinical effects of some common and severe mutations that produce OTCD.

**EXPERIMENTAL PROCEDURES**

**Crystallization**—Human wild type recombinant OTCase was prepared, purified, and stored as described previously (3); PALO was synthesized by Drs. G. Barany and D. Venugopal (Department of Chemistry, University of Minnesota) as described by Morizono et al. (3). Crystals were grown by vapor diffusion and the “hanging drop” method. 5 μl of a filtered solution of OTCase (20 mg/ml) and 4 mM PALO in 20 mM Tris acetate, 2 mM EDTA, 20 mM KCl, pH 7.4, were mixed with 2 μl of well solution containing 2% polyethylene glycol 400, 2 mM ammonium sulfate, 0.1 mM sodium Hepes, pH 7.5, and equilibrated with the well solution at 18 °C. Crystals belonging to space group P2_1_3 with approximate dimensions of 1.2 × 1.2 × 0.3 mm and cell dimensions a = b = c =...
125.9 Å appear within 2 weeks. The asymmetric unit contains one monomer and 73% solvent.

Data Collection—Crystals were soaked in a solution containing 30% glycerol for about 1 min and cryo-cooled with liquid nitrogen before mounting. X-ray data were collected at 120 K at beamline X12C at the National Light Source at Brookhaven National Laboratories using 1.10-Å wavelength radiation and processed with DENZO and SCALEPACK (12). Data collection statistics are given in Table I.

Structure Determination—The structure was solved by molecular replacement with PALO-liganded E. coli OTCase (10) as the search probe using the program Amore (13). The cross-rotation function was calculated with data from 15.0 to 4.0 Å and an integration radius in Patterson space of 25.0 Å. The cross-rotation function search gave a significant peak that was subsequently shown to be the correct solution with a correlation coefficient of 12.4%, 20% higher than the second noise peak. The highest peak in the translation function was also the correct solution, with a correlation coefficient of 45.5% and a residual of 46.8%, compared with the highest noise peak (correlation coefficient 20.6%, residual 55.4%). The 2Fo-2Fc maps computed with the molecular replacement phases were readily interpretable.

The polyalanine model from the molecular replacement solution after 15 cycle rigid body refinement was used as the starting model. Fourier maps were computed with weighted coefficients and model phases. After building the polyalanine model to the electron density map, 90% of side chain atoms could be identified, and PALO was readily visualized. A library derived from well refined structures in the Protein Data Bank was used extensively to model the loop segments with deletions or insertions. All residues except the two N-terminal residues were ultimately built into the electron density maps.

Refinement commenced at 3.0-Å resolution with an overall B value of 20 Å². 200 cycles of conventional positional refinement with XPLOR (14) brought the residual R to 26% (Rfree = 32%). PALO was included in the refinement at this stage, and higher resolution data were gradually added until all reflections to 1.85-Å resolution with F/s(F) were included. Low resolution data to 40.0 Å were included after the bulk solvent correction was applied (15). Individual isotropic B values were refined, and solvent molecules were added when the phases had been extended to 1.85-Å resolution. The model was adjusted with the computer graphics suite in the program O (16) after each refinement. The same randomly selected set of 10% of the reflections from various resolution bins was used to calculate Rfree at each stage of the refinement (17). Frequent references were made to Ramachandran plots to locate potential errors in the conformation of specific amino acid residues. PROCHECK (18) was used to confirm the accuracy of the model. Water molecules were identified as peaks of well defined electron density in uFo2u2uFc2u (u.3) and 2uFo2u2uFc2u maps (u.1) using the CCP4 program PEAKMAX (19) and the program O (16). All waters formed at least one hydrogen bond to protein or well established water molecules.
RESULTS AND DISCUSSION

Quality of the Model—Refinement statistics are given in Table I, and the quality of the final electron density map is illustrated in Fig. 2. The resolution of the structure enables each atom of PALO to be identified unambiguously. Final R values were 0.179 for the working R factor and 0.201 for R_free, for reflections with intensities greater than 2σ between 40 and 1.85 Å (Table I).

There are two outliers in the Ramchandran plot, Leu-163 and Leu-304, whose unusual torsion angles can be rationalized in terms of their structural and functional roles. Leu-163, located at one end of β-strand B5, lies to one side of the active site with its backbone nitrogen forming a strong hydrogen bond with the side chain of Asn-161. Both residues are conserved in all known OTCase sequences (11), indicating that this hydrogen bond is important for maintaining the backbone conformation between residues 161 and 163. The second outlier, Leu-304, is part of a conserved HCLP motif at the active site, and its carbonyl oxygen forms a strong hydrogen bond with the N-δ of PALO. Both residues are also outliers in the crystal structure of E. coli OTCase (10).

Tertiary and Quaternary Structure—As shown in Fig. 3, the tertiary structure of the three subunits is similar to those found in other transcarbamoylases (5–6, 8–11). The overall topology of each subunit is α/β, with 14 α-helices and 9 β-sheets. Each subunit is composed of two domains as follows: a CP binding or polar domain (residues 34–168 and 345–354) and an ORN binding or equatorial domain (residues 183–322). The extended C terminus (residues 345–354), found only in mammalian OT-

FIG. 2. 2Fo – Fc electron density map (contour 1σ) at the active site. The electron density is shown as a blue mesh. PALO is shown in bold; carbon and phosphorus atoms are shown in yellow, oxygen in red, and nitrogen in blue. The position and orientation of each atom in PALO can be determined unambiguously.

FIG. 3. Ribbon diagram of human OTCase liganded with the bisubstrate analog PALO. α-Helices are shown in red, β-strands in blue, and random coils in yellow. The C-terminal extension (residues 345–354), near helix H1, is shown in green. Helices H11 and H5 are inter-domain helices that link the CP and ORN domains. The bisubstrate analog, PALO, is shown as a space-filling model. Generated with MOLSCRIPT (30).

FIG. 4. Ribbon diagram of the human OTCase catalytic trimer. The identical subunits are colored pink, blue, and yellow. The bisubstrate analog PALO, shown in red, interacts with residues from two adjacent subunits. Helices H1, H5, and H11 are shown in brighter colors. The C-terminal extensions, shown in green, are exposed on the convex face of the enzyme. The view is down the molecular 3-fold axis from the convex face. Generated with MOLSCRIPT (30).

TABLE I

Crystallographic data and refinement statistics

| Property                                           | Value                                                                 |
|----------------------------------------------------|----------------------------------------------------------------------|
| Space group                                        | P2,3                                                                |
| Cell dimensions (Å)                                | a = b = c = 125.9                                                   |
| Resolution limits (Å)                              | 40–1.85 Å                                                           |
| Number of reflections (unique)                     | 1,393,080 (51,424)                                                  |
| Rmerge                                            | 9.3% (27.0%)                                                        |
| <1/Rmerge>                                         | 12.2 (3.4)                                                          |
| Completeness                                       | 86.4% (63.3%)                                                       |
| Number of atoms in final model                     | 2764                                                                |
| Number of reflections used in refinement           | 48218 (3249)                                                        |
| R value                                           | 17.9% (24.3%)                                                       |
| R_free value                                       | 20.1% (25.1%)                                                       |
| r.m.s.d. ideal bond length (Å)                     | 0.011                                                               |
| r.m.s.d. ideal bond angle (°)                      | 2.41                                                                |
| r.m.s.d. ideal dihedral angle (°)                  | 23.9                                                                |

* Statistics in parentheses are for the highest resolution shell (1.93–1.85 Å).
Cases, is part of the CP domain and adjacent to helix H1. Helices H5 (residues 169–182) and H11 (residues 322–344) connect the two domains with a hydrophilic cluster consisting of Lys-64, Lys-68, Glu-181, Glu-328, and Asn-329 holding H1, H5, and H11 together. These residues are strongly conserved in all known OTCase sequences.

The structure of the catalytically active trimer, shown in Fig. 4, is similar to those of other OTCases (8–11) and the catalytic trimer of ATCase (5, 7). The CP binding domains are located in the interior of the protein, and the binding domains for the second ligand are external. The interface between the subunits is formed primarily by residues from the CP binding domains, residues 89–94 from one subunit and 110–122 from an adjacent subunit. The salt bridge between Arg-92 and Glu-122, residues that are conserved in all known OTCases, and the hydrogen bond between the amide nitrogen of Ser-90 and the carbonyl oxygen of Asp-115 are likely to be important functionally since they appear to position His-117 to interact with phosphonate oxygen O1P of PALO.

**Active Site**—The structure of the active site is shown in Fig. 5, and residues that interact directly with PALO are listed in Table II. With two exceptions, these residues are conserved in the 33 known OTCase sequences (11). Thr-91 is replaced by Gly in an OTCase from *Pseudomonas syringae pv. phaseolicola* (20), whereas residue 117 may be His, Asn, or Arg. All ureotelic OTCases have a His at this position linking the phosphate

![Figure 5](https://example.com/fig5.png)

**Figure 5.** Stereo view (upper panel) and schematic (lower panel) showing the interaction of the bisubstrate analog PALO with active site residues. PALO is shown in bold. The residue indicated with * is from an adjacent subunit.

| PALO atoms | Protein atoms (distance Å) |
|------------|---------------------------|
| O1P        | Arg-141 NH₂ (2.64), His-117 NE₂ (2.88)* |
| O2P        | Thr-93 N (2.82), Thr-93 OG₁ (2.89), Ser-90 OG (2.82) |
| O3P        | Thr-91 N (2.92), Arg-92 N (2.87), Arg-92 NE (2.98), Arg-92 NH₂ (2.92) |
| O₁         | Thr-93 OG₁ (3.33), His-168 NE₂ (2.86), Arg-141 NH₂ (3.48), Arg-330 NH₂ (2.96) |
| NE         | Leu-304 O (2.85) |
| N          | Asn-199 OD₁ (2.94), Asp-263 OD₂ (2.70), Ser-267 OG (3.06) |
| OT₁        | Met-268 N (2.92), Wat-385 O₁ (2.87) |
| OT₂        | Asn-199 ND₂ (2.92), Met-268 O (2.92), Wat-381 O₁ (2.83) |

* His-117 is from an adjacent polypeptide subunit in the catalytic trimer.
oxygen of CP to Asn-121 in a potential pathway for transferring a proton between the solvent and CP. Residues in E. coli ATCase equivalent to Ser-90, Thr-91, Arg-92, Thr-93, Arg-141, His-168, and Leu-304 also interact with CP (Fig. 1). In addition, the carbonyl oxygen of Leu-304 in the HCLP motif is hydrogen-bonded to the N-δ of PALO. Although this motif is replaced by HPLP in E. coli ATCase, this hydrogen bond and a similar main chain conformation are maintained.

In contrast to the highly conserved binding site for CP, the ORN-binding site is quite different from the L-Asp-binding site in ATCase, as expected, since they bind different ligands. The principal residues that interact with ORN are Asn-199, Asp-263, Ser-267, and Met-268. Asn-199 is involved in domain closure; its carbonyl oxygen forms a hydrogen bond to the α-amino group of PALO (2.8 Å) and backbone nitrogen of Ile-200 (3.1 Å), whereas its amide nitrogen forms a hydrogen bond with the carboxyl oxygen OT2 of PALO (3.0 Å) and backbone oxygen of Ser-164 (2.9 Å). Asp-263 which links the α-amino group of PALO and the sulfhydryl group of Cys-303 is important in the catalytic reaction, as discussed below.

Seventeen water molecules near the active site form a complex hydrogen-bonded network. Two water molecules, Wat-381 and Wat-385, are directly involved in binding PALO. Wat-381 links OT2 of PALO to NZ of Lys-88 and the carbonyl oxygen of Met-268 and may play a role in domain closure. Wat-380 hydrogen bonds to the sulfur atom of Cys-303 and interacts with Asp-175 and Glu-326 via another water molecule, Wat-382. This network may act as a proton transfer pathway in the catalytic mechanism. Most of the remaining water molecules near the active site appear to provide structural stabilization; for example, Wat-394, which forms a hydrogen bond with the backbone nitrogen of Asp-196 and the backbone oxygen of Asp-263 and Thr-264, appears to help to position the loop between residues 263–286 and the B5-H5 loop so as to allow Asn-199 and Asp-263 to interact with ORN. Since the loop between residues 263 and 286 contains a conserved SMG sequence, it will referred to as the SMG loop.

Catalytic Mechanism—Cys-303, which is positioned so as to form the proton transfer pathway, is involved in the catalytic reaction. Cys-303, which is positioned so as to form the proton transfer pathway, is involved in the catalytic reaction.

**FIG. 6. Schematic drawing of a possible catalytic mechanism.**

**FIG. 7. Superposition of human OTCase (thick line) onto E. coli OTCase (thin line, upper panel) and P. aeruginosa OTCase (thin line, lower panel).** The extra loop in bacterial OTCases (residues 278–297 in E. coli OTCase) between helix H10 and β-strand B10 in human OTCase is indicated by *. The r.m.s.d. between human OTCase and E. coli OTCase is 1.29 Å for 303 equivalent C-α atoms. Superposition between human OTCase and P. aeruginosa OTCase was performed using 148 equivalent atoms in the CP domain only since the ORN domain is rotated significantly in PALO-liganded OTCase. The r.m.s.d. between human OTCase and P. aeruginosa OTCase for 148 equivalent C-α atoms in the CP domain is 1.39 Å.
be able to interact with the reactive δ-amino group of ORN, appears to play a central role in the catalytic mechanism and has been proposed to be involved in binding ornithine based on chemical modification (21) and site-directed mutagenesis (22). A catalytic triad, observed in the crystal structure of unliganded *E. coli* OTCase, consisting of residues equivalent to Cys-303, His-302, and Glu-310 was proposed to stabilize the thiolate form of Cys-303, enabling the enzyme to bind L-ornithine in the correct ionization state for catalysis (9). However, the distance between Cys-303 sulfur and ND1 of His-302 is 4.3 Å in PALO-ligated human OTCase and the other OTCase structures in the Protein Data Bank, too long for a strong interaction. Instead, the sulfur atom of Cys-303 forms a hydrogen bond with OD1 of Asp-263 (3.2 Å), which also forms a hydrogen bond to the α-amino group of PALO, whereas ND1 of His-302 forms a strong hydrogen bond to the backbone nitrogen of Leu-304 (2.9 Å). His-302 and Glu-310 are highly conserved and are also found in *E. coli* ATCase where they form a similar hydrogen-bonded network; however, their role may be primarily structural rather catalytic.

The interaction between the side chains of Cys-303 and Asp-263 would be expected to stabilize the thiolate form of the sulfhydryl group, enabling it to accept a proton from the δ-amino group of ORN and thus facilitating nucleophilic attack on the carbonyl carbon of carbamoyl phosphate. Replacing this Cys by Ser in rat OTCase increases the pH optimum of the catalytic reaction from 8.6 to 9.6 (22), consistent with the proposed mechanism. The correct orientation of the side chain of ORN relative to Cys-303 is maintained in part by a hydrophobic interaction between the side chains of Leu-163, Met-268, and the methylene groups of ORN (closest distance 4.2 Å). Gln-171, which is 4.0 Å from the carbonyl carbon of the CP moiety, is positioned so as to be able to stabilize the tetrahedral intermediate, as proposed for *E. coli* ATCase (23).
The high density of charged residues at the active site of OTCase may result in several ionizable residues having perturbed pK values and makes assignment of pK values and functions to specific residues from the pH dependence of enzymatic activity difficult. Previous determinations of the pH dependence of the catalytic parameters have suggested that the catalytically active form of the enzyme binds only the L-ornithine zwitterion, and both the α- and δ-amino groups have been suggested to be unprotonated (1, 24). The intrinsic pK values of the α- and δ-amino groups, the configuration of charged residues at the active site, and the interaction of Asp-263 with the α-amino group of ORN suggest that the α-amino group is more likely to be unprotonated. The environment around the δ-amino group lacks positively charged groups that would discriminate against species with the δ-amino group protonated, whereas deprotonation of the α-amino group would favor formation of the thiolate form of Cys-303.

A possible model of the catalytic mechanism is shown in Fig. 6. As in ATCase, the reaction mechanism is likely to involve an $S_{N}2$ displacement attack by the reactive amino group on the carbonyl carbon atom of CP (23). In ATCase the reactive α-amino group of aspartate is neutral and a sufficiently good nucleophile that a catalytic base is not required. In OTCase, the reactive δ-nitrogen of ORN is expected to be positively charged, as discussed above, and a base is required. The $S_{N}$ of Cys is positioned so as to be able to play this role, and its interaction with Asp-263 will stabilize the thiolate form. Attack by the deprotonated δ-amino group on the carbonyl carbon atom of CP will result in a positive charge developing on N-δ of ORN and a negative charge developing on the carbonyl oxygen of CP. The tetrahedral intermediate will be stabilized both by Gln-171, which is positioned so as to be able to form a hydrogen bond to the positive N-δ of ORN, and by Arg-141, Arg-330, and His-168, which form hydrogen bonds to the negative carbonyl oxygen of CP. When the tetrahedral intermediate breaks down to form citrulline and inorganic phosphate, the proton from the N-δ of ORN may be transferred to the inorganic phosphate ion generated in the reaction or to His-168. The proton that is picked up by Cys-303 may leave the active site via Wat-380 and Wat-382 or be transferred to the α-amino group of ORN, via Asp-263, to restore the active site structure for the next reaction cycle.

Comparison with Other OTCase Structures—Human OTCase is shown superimposed on PALO-liganded E. coli OTCase and unliganded P. aeruginosa catalytic OTCase in Fig. 7. Since there is a flexible hinge between the CP and ORN domains, the CP domains were superimposed. The orientation of the ORN binding domains relative to the CP domain in PALO-liganded E. coli and human OTCase is very similar and different from its orientation in unliganded E. coli and P. aeruginosa catalytic OTCase. The ORN domain of human OTCase is rotated −5° and 9°, respectively, relative to its orientation in the two unliganded structures. This motion results in domain closure, the enclosing of bound substrate analogs by the two domains of each subunit, a phenomenon also observed in E. coli ATCase (5). The hinges for this motion are the C terminus of H5 and the N terminus of H11, with the relative positions of H1, H5, and H11 fixed by an extensive hydrogen bond network consisting of conserved residues Lys-64, Glu-181, Glu-328, and Asn-329. The region of the protein that undergoes the largest motion is the SMG loop which corresponds to the 240s loops in E. coli OTCase and ATCase.

Bacterial OTCases have an additional loop of ~20 residues (residues 278–297 in E. coli OTCase) between helix H10 and β-strand B10, whereas ureotelic OTCases have ~10-residue extension at the C terminus. These sequence differences have little effect on the overall protein fold or the structure of the active site. Instead, they are more likely to play a role in regulating protein-protein interactions in vivo. Although the extra loop in bacterial OTCases is close to the SMG loop, its interaction with the SMG loop appears to be weak, since the latter is disordered in unliganded E. coli OTCase (9). However, as noted by Ha et al. (10), this extra loop would prevent OTCase from binding to the regulatory chain of ATCase in prokaryotes. This loop would not be required in eukaryotes, where OTCase is located in the mitochondria while ATCase is cytoplasmic.

The C-terminal extension in ureotelic OTCases is unique. Unlike bacterial transcarbamoylasases, in which the N and C termini are close to each other in the CP domain, the C terminus of human OTCase falls back to the ORN domain and forms a ridge on the convex face of the trimer (Fig. 4). Its conformation is maintained by hydrogen bonds between Tyr-345 OH−Asp-62 OD2 (2.7 Å), Tyr-345 OH−Arg-66 NH1 (2.8 Å), Leu-349 O−Trp-58 NE2 (2.9 Å), Lys-353 O−Gln-180 NE2 (2.6 Å), Phe-354 O−Leu-186 N (2.7 Å) and Phe-354 O−Lys-187 N (2.8 Å) (Fig. 8). Trp-58, Asp-62, Gln-180, Leu-186, and Lys-187, which help to maintain the conformation of the C-terminal extension, are conserved only in ureotelic OTCases and are quite variable in bacterial OTCases.

The C-terminal extension described above has an intriguing homology with several bacterial, nuclear, and mitochondrial membrane-associated proteins. Some examples of mitochondrial membrane proteins with sequence homology are shown in Fig. 9a. They include two yeast mitochondrial inner membrane carrier proteins: ymc1 and ymc2 (25, 26). In models of these carrier proteins, this sequence occurs in the loop between transmembrane helices 5 and 6 (Fig. 9b), a region that has been shown to be important in carrier-specific transport (27). Both OTCase and l-ornithine must be transported across the inner membrane, and electron microscopy indicates that OTCase is associated with the mitochondrial membrane. Since the C terminus is exposed on the convex face of the enzyme and has a sequence motif characteristic of membrane-associated proteins, it may be involved in mediating interactions with the membrane. Since the sequences are homologous, it clearly can-
not interact directly with the carrier protein; however, this sequence may enable both to interact with a third molecule. Whether this motif has a common function in all membrane proteins in which it occurs remains to be investigated.

Implications for Inherited OTCD—This structure indicates that a broad spectrum of structural phenomena gives rise to the clinical effects of the deleterious mutations that have been identified in human OTCase (Fig. 10). Many OTCD mutations are at or near active site. They include K88N, R92Q, T93A, H117L, R141Q/R141P, N161S, G195R, and R277W/R277Q. These mutations directly interfere with substrate binding and/or catalysis. Several mutations (L45P, A174P, P225L/P225T) involve a substitution either to or from proline, a well-known helix disrupter. Some mutations, for example, I172M, S192Q, G195R, and Q216E, are located in the core of the protein and would interfere with folding or destabilize the folded protein. Mutations such as T178M and E181G at the inter-domain interface would interfere with domain closure. Some OTCD mutations occur at the intersubunit interface, for example G79E, A102E, and D126G. These mutations alter the charge of the subunit surface and would affect assembly of the trimer. Others, such as R40H, Q180H/Q180T, and F354C, which are located on the convex face of the trimer, may affect the conformation of the C terminus, interfering with its interaction with other proteins or the membrane. These mutants warrant further investigation in vivo and in vitro.

Acknowledgments—We thank Dr. R. Sweet for assistance during data collection at beamline X12C in the National Synchrotron Light Source at Brookhaven National Laboratory. This facility is supported by the U.S. Department of Energy Offices of Health and Environmental Research and of Basic Energy Sciences and by the National Science Foundation. We also thank Drs. L. Bansazak and D. Ohlendorf for facilitating our use of equipment in the Kahlert Center for Structure Biology at the University of Minnesota. Some calculations were carried out on the Basic Science Computing Laboratory at the Minnesota Supercomputer Center. We thank Drs. G. Barany and D. Venugopal in the Department of Chemistry at the University of Minnesota for synthesizing PALO.

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Dashuang Shi, Hiroki Morizono, Ya Ha, Mika Aoyagi, Mendel Tuchman and Norma M. Allewell

J. Biol. Chem. 1998, 273:34247-34254.  
doi: 10.1074/jbc.273.51.34247

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