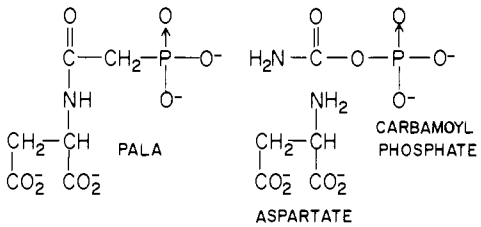
Structure of the Inhibitor of Aspartate Transcarbamylase *N*-(Phosphonacetyl)-L-aspartate

Giuseppe Zanotti,[†] Hugo L. Monaco,*[†] and Jefferson Foote[‡]

Contribution from the Istituto di Chimica Organica, Universita di Padova, Via Marzolo 1, Padova, 35100 Italy, and Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received April 30, 1984*

Abstract: The crystal structure of the tricyclohexylammonium salt of *N*-(phosphonacetyl)-L-aspartate was determined from X-ray diffraction data. The crystals are orthohombic, space group $P2_12_12_1$, with $a = 23.431$ Å, $b = 6.749$ Å and $c = 19.237$ Å. The structure was solved by a combination of direct methods and Fourier differences and refined to a weighted R factor of 7.3% and an unweighted R factor of 9.0%. Since the compound is a potent inhibitor of the allosteric enzyme aspartate carbamoyltransferase, it was considered of interest to compare its structure with those of the substrates L-aspartate and carbamoyl-L-aspartate. As the structure of the latter is not known, we crystallized the analogous salt dicyclohexylammonium carbamoyl-L-aspartate and solved its structure. These crystals are monoclinic, space group $C2$, with $a = 26.238$ Å, $b = 5.889$ Å, and $c = 15.416$ Å and $\beta = 116.1°$. The structure was solved by the same method used for the *N*-phosphonacetyl-L-aspartate crystals and refined to an unweighted R factor of 10.0%. Virtually all the bond distances found in the two compounds give values which agree reasonably well with those reported for analogous compounds with the same bonds. A comparison of carbamoyl-L-aspartate and L-aspartate shows that in the solid state the two ions adopt an almost identical conformation for the entire moiety they have in common. The *N*-phosphonacetyl-L-aspartate anion, on the other hand, presents a different disposition of the carboxylate groups, which are displaced by the phosphonacetyl group into a conformation which is not found in many related structures.

The compound *N*-phosphonacetyl-L-aspartate (PALA) was originally designed to embrace within one molecule the structures of the two substrates of aspartate transcarbamylase (ATCase), L-Aspartate and carbamoyl phosphate.[1] PALA has proven to



be the most potent reversible inhibitor of this enzyme known, binding to *E. coli* ATCase with a dissociation constant of 10^{-8} M. As such, it is central to two current areas of investigation. By virtue of blocking the de novo pyrimidine biosynthetic pathway, of which ATCase catalyzes the first step, PALA is effective in reversing the proliferation of some varieties of slow-growing solid tumor.[2] PALA also has seen wide use in studies of the catalytic and allosteric mechanisms of *E. coli* ATCase.[3] There is substantial evidence from biochemical studies,[4] solution X-ray scattering,[5] and X-ray crystallography[6] that ATCase undergoes a notable conformational change upon binding of PALA. The conformation adopted by the enzyme bound to PALA is thought to be the R state in the two-state allosteric model,[7] and the complex of PALA with ATCase is the subject of a crystallographic structure determination.[6]

The claim that PALA is a transition-state analogue has been criticized,[8] because the dissociation constant cited above is no lower than the product of the dissociation constants of the two substrates. Desirable would be a comparison of the geometry of PALA with the geometries of a substrate pair, in part to identify any structural features which make PALA an unfaithful replica of the two substrates. To address this concern, crystallographic structure determinations were undertaken of PALA and *N*-carbamoyl-L-aspartate (CLA).

Experimental Procedures

Synthesis and Crystallization of PALA. The free acid form of PALA was synthesized as described.[9] This product was dissolved in water, neutralized with distilled cyclohexylamine, and dried. Tricyclohexylammonium–PALA was recrystallized three times from 95% ethanol/acetone to a constant melting point of 193–195 °C. Purity was verified by proton NMR and elemental analysis. Anal. ($C_{24}H_{49}N_4O_5P$) C, H, N, P. Subjecting a 1% solution of tricyclohexylammonium–PALA in 95% ethanol to vapor diffusion vs. acetone gave single crystals suitable for diffraction studies.

Table I. Crystal Data

	PALA	CLA
stoichiometry	$C_6H_7NO_8P\cdot3(C_6H_{14}N)$	$C_5H_7N_2O_5\cdot2(C_6H_{14}N)$
molecular weight	552.65	375.49
space group	$P2_12_12_1$	C2
Z	4	4
a, Å	23.431 (9)	26.238 (9)
b, Å	6.749 (3)	5.889 (3)
c, Å	19.237 (8)	15.416 (7)
β, deg		116.1 (2)
v, Å^3	3042	2139
ρ(calcd), g cm^{-3}	1.207	1.166

Synthesis and Crystallization of CLA. Reaction of aspartate and KCNO in slightly basic solution gave CLA.[10] The product was converted to the free acid by passage through Dowex 50W-X8, H^+ form.

[†] Padova.
[‡] Berkeley.

(1) Collins, K. D.; Stark, G. R. *J. Biol. Chem.* **1971**, *246*, 6599–6605.
(2) (a) Ehrlichman, C.; Donehower, R. C.; Klecker, R.; Chabner, B. A. *Proc. Am. Soc. Cancer Res.* **1981**, *22*, 366. (b) Johnson, R. K.; Swyryd, E. A.; Stark, G. R. *Cancer Res.* **1978**, *38*, 371–378.
(3) (a) Jacobson, G. R.; Stark, G. R. In "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1973; Vol. IX, pp 225–308. (b) Kantrowitz, E. R.; Pastra-Landis, S. C.; Lipscomb, W. N. *Trends Biochem. Sci.* **1980**, *5*, 124–128. (c) Kantrowitz, E. R.; Pastra-Landis, S. C.; Lipscomb, W. N. *Trends Biochem. Sci.* **1980**, *5*, 150–153. (d) Schachman, H. K. *Harvey Lect.* **1974**, *68*, 67–113.
(4) (a) Blackburn, M. N.; Schachman, H. K. *Biochemistry* **1977**, *16*, 5084–5091. (b) Howlett, G. J.; Schachman, H. K. *Biochemistry* **1977**, *16*, 5077–5083.
(5) Moody, M. F.; Vachette, P.; Foote, A. M. *J. Mol. Biol.* **1979**, *133*, 517–532.
(6) Ladner, J. E.; Kitchell, J. P.; Honzatko, R. B.; Ke, H. M.; Volz, K. W.; Kalb (Gilboa), A. J.; Ladner, R. C.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3125–3128.
(7) Monod, J.; Wyman, J.; Changeux, J.-P. *J. Mol. Biol.* **1977**, *12*, 88–118.
(8) Fersht, A. "Enzyme Structure and Mechanism"; Freeman: San Francisco, 1977; pp 252–253.
(9) Kempe, T. D.; Seaver, E. A.; Bruist, M.; Stark, G. R. *Cell.* **1976**, *9*, 541–550.
(10) Nyc, J. F.; Mitchell, H. K. *J. Am. Chem. Soc.* **1947**, *69*, 1382–1384.

0002-7863/84/1506-7900$01.50/0

Table II. PALA Fractional Non-Hydrogen-Atom Coordinates

atom	x/a	y/b	z/c
	PALA		
P	0.8011 (2)	0.1495 (8)	0.3200 (2)
O(1P)	0.7997 (5)	0.331 (2)	0.3622 (5)
O(2P)	0.7894 (4)	−0.034 (2)	0.3605 (5)
O(3P)	0.8588 (4)	0.128 (2)	0.2787 (4)
C(1P)	0.7465 (6)	0.161 (2)	0.2517 (6)
C(1)	0.7561 (6)	0.337 (3)	0.2059 (8)
N(2)	0.7846 (5)	0.311 (2)	0.1483 (6)
C(2)	0.7984 (7)	0.471 (2)	0.1035 (7)
C(3)	0.8531 (6)	0.582 (2)	0.1257 (7)
C(4)	0.8037 (8)	0.403 (2)	0.0298 (8)
C(5)	0.8474 (6)	0.778 (2)	0.1619 (7)
O(1)	0.7344 (4)	0.497 (2)	0.2227 (5)
O(2)	0.8090 (5)	0.534 (2)	−0.0146 (5)
O(3)	0.8036 (6)	0.228 (2)	0.0163 (6)
O(4)	0.8116 (4)	0.895 (1)	0.1341 (5)
O(5)	0.8772 (3)	0.818 (2)	0.2146 (5)
	Cyclohexylammonium **1**		
N_1	0.7066 (4)	0.659 (2)	0.3539 (5)
$C_1(1)$	0.6085 (7)	0.568 (3)	0.3280 (9)
$C_1(2)$	0.5507 (8)	0.658 (4)	0.3003 (9)
$C_1(3)$	0.5279 (7)	0.805 (3)	0.3429 (9)
$C_1(4)$	0.5700 (7)	0.992 (3)	0.3481 (8)
$C_1(5)$	0.6283 (7)	0.903 (3)	0.3767 (8)
$C_1(6)$	0.6499 (7)	0.745 (3)	0.3279 (8)
	Cyclohexylammonium **2**		
N_2	0.7432 (4)	0.882 (2)	−0.0004 (5)
$C_2(1)$	0.6581 (7)	0.685 (3)	0.0344 (8)
$C_2(2)$	0.6105 (8)	0.655 (4)	0.0832 (9)
$C_2(3)$	0.5719 (7)	0.858 (4)	0.0879 (9)
$C_2(4)$	0.6085 (8)	1.024 (3)	0.1044 (9)
$C_2(5)$	0.6597 (8)	1.050 (3)	0.0548 (9)
$C_2(6)$	0.6941 (6)	0.858 (3)	0.0519 (7)
	Cyclohexylammonium **3**		
N_3	0.8690 (4)	0.650 (2)	0.3415 (5)
$C_3(1)$	0.9568 (7)	0.463 (3)	0.3605 (8)
$C_3(2)$	1.0075 (8)	0.436 (3)	0.409 (1)
$C_3(3)$	1.0428 (7)	0.648 (4)	0.4080 (9)
$C_3(4)$	1.0055 (8)	0.816 (4)	0.438 (1)
$C_3(5)$	0.9520 (6)	0.831 (3)	0.3865 (8)
$C_3(6)$	0.9189 (5)	0.637 (3)	0.3905 (7)

Table III. CLA Fractional Non-Hydrogen-Atom Coordinates

atom	x/a	y/b	z/c
	CLA		
N(1)	0.2841 (4)	0.034 (2)	0.1143 (7)
N(2)	0.2247 (3)	−0.156 (1)	0.1641 (5)
C(1)	0.2659 (5)	−0.165 (2)	0.1351 (8)
C(2)	0.2004 (4)	−0.370 (2)	0.1757 (7)
C(3)	0.1578 (5)	−0.310 (2)	0.2159 (8)
C(4)	0.1720 (5)	−0.503 (2)	0.0855 (8)
C(5)	0.1856 (6)	−0.199 (3)	0.3123 (9)
O(1)	0.2902 (4)	−0.343 (1)	0.1365 (6)
O(2)	0.1715 (4)	−0.711 (1)	0.0944 (6)
O(3)	0.1509 (3)	−0.410 (1)	0.0038 (4)
O(4)	0.2205 (4)	−0.298 (2)	0.3813 (5)
O(5)	0.1729 (4)	0.0	0.3170 (6)
	Cyclohexylammonium **1**		
N_1	0.1314 (3)	−0.969 (2)	−0.0689 (6)
$C_1(1)$	0.0720 (6)	−0.962 (4)	−0.145 (1)
$C_1(2)$	0.0357 (9)	−0.984 (5)	−0.100 (1)
$C_1(3)$	−0.030 (1)	−0.854 (5)	−0.175 (2)
$C_1(4)$	−0.030 (1)	−0.724 (5)	−0.231 (2)
	−0.040 (1)	−0.852 (8)	−0.277 (2)
$C_1(5)$	0.0003 (9)	−0.718 (5)	−0.273 (1)
$C_1(6)$	0.0611 (8)	−0.733 (4)	−0.201 (1)
	Cyclohexylammonium **2**		
N_2	0.2626 (4)	−0.724 (2)	0.4298 (6)
$C_2(1)$	0.332 (2)	−0.752 (7)	0.449 (3)
	0.308 (1)	−0.782 (6)	0.402 (2)
$C_2(2)$	0.332 (2)	−1.001 (8)	0.444 (3)
	0.349 (1)	−0.565 (8)	0.423 (3)
$C_2(3)$	0.414 (2)	−1.041 (9)	0.490 (3)
	0.403 (2)	−0.595 (9)	0.400 (3)
$C_2(4)$	0.389 (2)	−1.023 (8)	0.398 (3)
	0.423 (1)	−0.861 (7)	0.432 (3)
$C_2(5)$	0.385 (2)	−0.741 (8)	0.351 (3)
	0.351 (2)	−0.978 (9)	0.494 (3)
$C_2(6)$	0.323 (1)	−0.741 (5)	0.352 (2)

The free acid was neutralized with cyclohexylamine and concentrated, and the dicyclohexylammonium salt was crystallized by addition of acetone. Anal. ($C_{17}H_{34}N_4O_5$) C, H, N. The melting point was 189–191 °C. Single crystals for diffraction work were obtained by vapor diffusion of a 1 M aqueous solution of dicyclohexylammonium–CLA vs. acetone.

Crystal Data Collection and Reduction. Single crystals of both compounds were used for space group determination and data collection. Unit cell parameters and other pertinent data are listed in Table I. The approximate dimensions of the crystal of the PALA salt were 0.5 × 0.08 × 0.05 mm. Those of the analogous CLA compound were 1.2 × 0.5 × 0.15 mm. Intensity data were collected on a Philips PW 1100 four-circle diffractometer by use of the $\theta/2\theta$ scanning technique. The scanning speed was 0.60 deg/min and the background was measured for 20 s. The scan width was 1.0°. During the data collection, three reference reflections which were monitored every 180 s did not show any significant fluctuations. With use of Mo Kα radiation monochromatized by a graphite crystal, 2477 independent reflections (up to θ = 23°) were measured for the PALA crystal and 2102 independent reflections (up to θ = 25°) for the CLA crystal. Of these, 1170 reflections in the first case and 1325 reflections in the second were found to have $I \geq 3\sigma(I)$, σ being evaluated from the counting statistics. The experimental absorption correction[11] and the standard corrections for Lorentz and polarization effects were used. The data were put on an absolute scale by Wilson's method.

Structure Determination of the Tricyclohexylammonium Salt of PALA. The structure was solved by direct methods using the 250 highest normalized structure factors in the phasing program MULTAN 80.[12] The E map showed several fragments of the molecule, and the remaining parts were subsequently revealed by Fourier difference maps. Refinement was carried out by block-matrix least-squares, allowing the non-hydrogen atoms to vibrate anisotropically. The scattering factors used were taken from the "International Tables for X-ray Crystallography".[13] The hydrogen atoms of the PALA ion were localized in the difference Fourier maps, but they were included in the refinement in idealized positions and varied by using the group refinement procedure. The hydrogens of the cyclohexylammonium rings were introduced in calculated positions and subsequently refined in separate cycles. The quantity minimized was

$$w(|F_o| - |F_c|)^2 \text{ with } w = 4.7(\sigma^2(F) + 0.0001|F|^2)^{-1}$$

The final conventional unweighted R factor was 0.090 and the R factor weighted by w was 0.073.

Structure Determination of the Dicyclohexylammonium Salt of CLA. The method used to solve this structure was analogous to that described for the PALA salt. In this case the Fourier difference maps showed disorder in the cyclohexylammonium rings, and therefore two different conformations with fractional occupancies were used in the refinement. The block-matrix least-squares refinement was carried out by allowing the non-hydrogen atoms to vibrate anisotropically. In the case of the disordered cyclohexylammonium ions, thermal parameters and occupancies of the atoms for which a second position was evident were refined in alternate cycles. The quantity minimized was the same which was defined for the PALA structure, but in this case the weight w was made equal to 1. The final conventional R factor was equal to 0.100.

With the exception of MULTAN 80, all the programs used in the computations belonged to the system of crystallographic programs SHELX76.[15]

Results

Atomic coordinates with estimated standard deviations are listed in Table II for the tricyclohexylammonium salt of PALA and Table III for the dicyclohexylammonium salt of CLA. Thermal parameters and fractional hydrogen coordinates are available as

(11) North, A. C. T.; Phillips, D. C.; Mathews, F. S. *Acta Crystallogr., Sect. A* **1968**, *A24*, 351–359.

(12) Germain, G.; Main, P.; Woolfson, M. M. *Acta Crystallogr., Sect. A* **1971**, *A27*, 368–376.

(13) "International Tables for X-ray Crystallography", 3rd ed.; Kynoch Press: Birmingham, England, 1974.

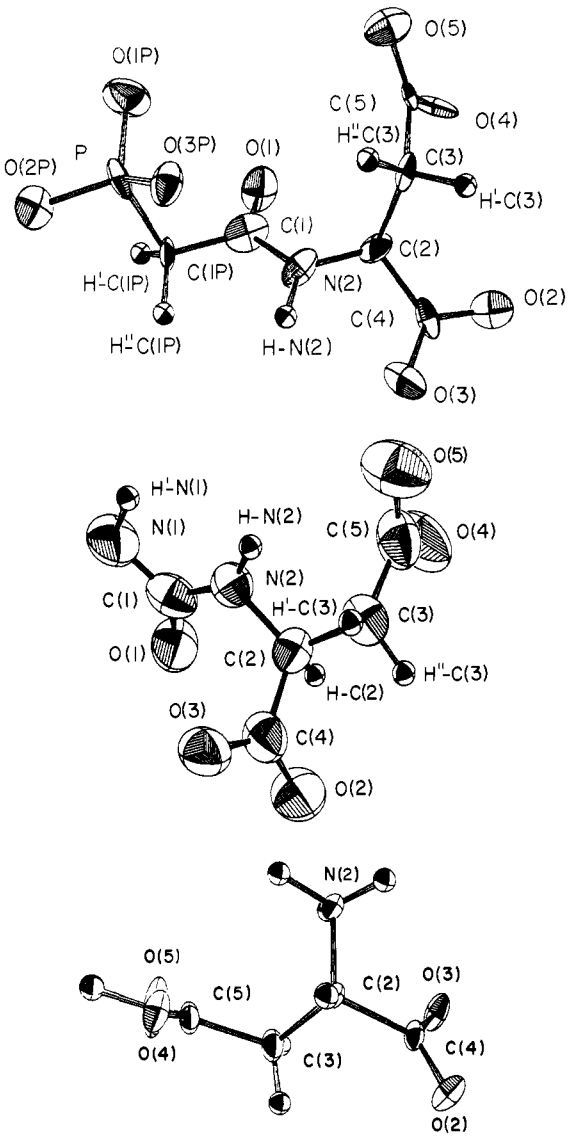


Figure 1. Structures of PALA (top), CLA (middle), and L-aspartic acid[16] (bottom). Structures were plotted with use of the program ORTEP-II.[14] Hydrogen atoms in each structure are represented by spheres of 0.12-Å radius. The numbering system of aspartic acid is chosen to correspond to the aspartate moieties of PALA and CLA.

Table IV. PALA Bond Distances (Å) and Valence Angles (deg)

P-O(1P)	1.47 (1)	C(2)-C(3)	1.54 (2)
P-O(2P)	1.49 (1)	C(2)-C(4)	1.50 (2)
P-O(3P)	1.58 (1)	C(3)-C(5)	1.50 (2)
P-C(1P)	1.83 (1)	C(4)-O(2)	1.24 (2)
C(1P)-C(1)	1.50 (2)	C(4)-O(3)	1.21 (2)
C(1)-O(1)	1.24 (2)	C(5)-O(4)	1.27 (2)
C(1)-N(2)	1.31 (2)	C(5)-O(5)	1.26 (2)
N(2)-C(2)	1.42 (2)		
O(2P)-P-O(1P)	113.6 (6)	O(1)-C(1)-N(2)	123 (2)
O(2P)-P-O(3P)	110.2 (6)	C(1)-N(2)-C(2)	122 (2)
O(1P)-P-O(3P)	112.0 (7)	N(2)-C(2)-C(4)	111 (1)
C(1P)-P-O(3P)	103.9 (6)	N(2)-C(2)-C(3)	113 (1)
C(1P)-P-O(2P)	106.4 (7)	C(3)-C(2)-C(4)	110 (1)
C(1P)-P-O(1P)	110.2 (7)	C(2)-C(3)-C(5)	119 (1)
P-C(1P)-C(1)	110 (1)	C(3)-C(5)-O(4)	114 (1)
C(1P)-C(1)-O(1)	119 (1)	C(3)-C(5)-O(5)	121 (1)
C(1P)-C(1)-N(2)	118 (2)	C(2)-C(4)-O(2)	116 (1)
		C(2)-C(4)-O(3)	120 (1)

part of the supplementary material. The conformations of the two anions are shown in Figure 1, in which the structure of L-aspartic acid[16] is included for comparison. The same figure

Table V. CLA Bond Distances (Å) and Valence Angles (deg)

C(1)-O(1)	1.22 (2)	C(3)-C(5)	1.49 (2)
C(1)-N(1)	1.36 (2)	C(4)-O(2)	1.23 (2)
C(1)-N(2)	1.34 (2)	C(4)-O(3)	1.26 (2)
N(2)-C(2)	1.46 (2)	C(5)-O(4)	1.20 (2)
C(2)-C(3)	1.54 (2)	C(5)-O(5)	1.23 (2)
C(2)-C(4)	1.48 (2)		
O(1)-C(1)-N(1)	121 (1)	C(2)-C(4)-O(2)	117 (1)
O(1)-C(1)-N(2)	121 (1)	C(2)-C(4)-O(3)	122 (1)
N(1)-C(1)-N(2)	118 (1)	O(2)-C(4)-O(3)	122 (1)
C(1)-N(2)-C(2)	118 (1)	C(3)-C(5)-O(4)	121 (1)
N(2)-C(2)-C(3)	107 (1)	C(3)-C(5)-O(5)	117 (1)
N(2)-C(2)-C(4)	114 (1)	O(4)-C(5)-O(5)	122 (1)
C(2)-C(3)-C(5)	112 (1)		

Table VI. Torsion Angles of PALA Compared with Those of Related Molecules

torsion angle,[a] deg	PALA	CLA	D,L-Asp[19]	L-Asp[16]	succinate[20]
C(4)-C(2)-C(3)-C(5)	132	174	174	178	180
C(2)-N(2)-C(1)-O(1)		-14			
C(2)-N(2)-C(1)-N(1)		173			
C(2)-N(2)-C(1)-C(1P)	-177				
C(1)-N(2)-C(2)-C(4)	-152	-62			
C(1)-N(2)-C(2)-C(3)	84	175			
N(2)-C(2)-C(4)-O(2)		151	171	145	
N(2)-C(2)-C(4)-O(3)	-9	-26	-7	-38	
N(2)-C(2)-C(3)-C(5)	-103	-61	-62	-62	
C(2)-C(3)-C(5)-O(4)	-45	-65	-176	-51	
C(2)-C(3)-C(5)-O(5)	136	114	3	131	
O(1P)-P-C(1P)-C(1)	59				
P-C(1P)-C(1)-N(2)	96				
P-C(1P)-C(1)-O(1)	88				

[a] The values are given in degrees. The torsion angle $W(I,J,K,L)$ is defined as the angle between the vector JI and the vector KL viewed down JK. The sign of W is positive if JI is to be rotated clockwise into KL.

defines the numbering system used. The bond lengths and valence angles of the PALA ion are given with their standard deviations in Table IV.

In the PALA ion, the phosphorus atom is found at the center of a largely distorted tetrahedron, with bond angles ranging in value from 103.9 (6)° to 113.6 (6)°. (Standard deviations are expressed here and elsewhere in this report as a parenthetical coefficient whose magnitude is the same as that of the last significant digit of the preceding number.) The oxygen–phosphorus distances fall clearly into two categories: two short ones, P–O(1P) (1.47 (1) Å) and P–O(2P) (1.49 (1) Å), and a long one, P–O(3P) (1.58 (1) Å). The first two distances represent P–O bonds, whereas the third is indicative of a P–OH bond, as found previously in several other compounds:[17] β-aminoethylphosphonic acid, P–O = 1.50 Å and P–OH = 1.57 Å; β-ciliatine, P–O = 1.50 Å and P–OH = 1.57 Å; and phosphoethanolamine, P–O = 1.50 Å and P–OH = 1.56 Å. The phosphorus–carbon distance, which is 1.83 (1) Å, also agrees well with the values found in these compounds: 1.82 Å in β-aminoethylphosphonic acid and 1.80 Å in β-ciliatine. The C(1)–O(1) and C(1)–N(2) bond distances are 1.24 and 1.31 Å, in excellent agreement with the values reported for a standard peptide unit:[18] 1.24 and 1.32 Å. A small anomaly is found in the distance C(2)–N(2), which being 1.42 Å is slightly shorter than expected for a standard C–N value, 1.45 Å, and also shorter

(14) Johnson, C. K. ORTEP-II: A Fortran Thermal-Ellipsoid Plot Program for Crystal Structure Illustrations, Oak Ridge National Laboratory, 1976.
(15) Sheldrick, G. M. SHELX76: A Program for Crystal Structure Determination, University of Cambridge, 1976.
(16) Derissen, J. L.; Endeman, H. J.; Peerdeman, A. F. *Acta Crystallogr., Sect. B* **1968**, *B24*, 1349–1354.
(17) (a) Darriet, M.; Darriet, J.; Cassaigne, A.; Neuzil, E. *Acta Crystallogr., Sect. B* **1975**, *B31*, 469–471. (b) Okaya, Y. *Acta Crystallogr.* **1966**, *20*, 712–715. (c) Kraut, J. *Acta Crystallogr.* **1961**, *14*, 1152–1154.
(18) Pauling, L. Corey, R. B.; Branson, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1951**, *37*, 205–211.

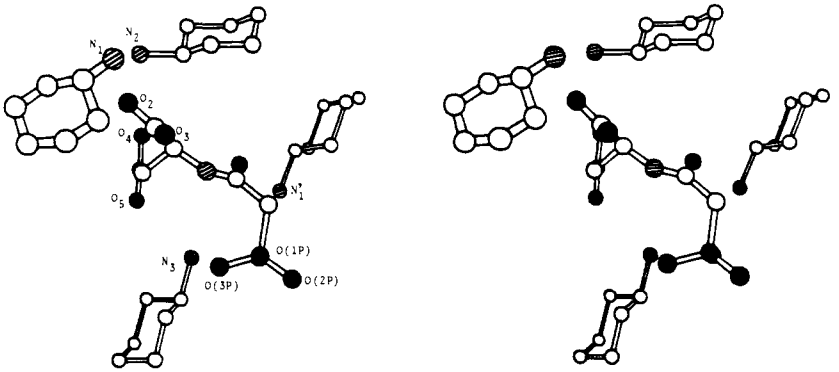


Figure 2. PALA crystal environment. The PALA anion is shown in this stereoview, surrounded by cyclohexylammonium ions. The cyclohexylammonium marked with a prime (′) symbol is from a different asymmetric unit. The distances between the positively charged nitrogens and negatively charged oxygens are as follows: N_1···O(2), 2.87 Å; N_1···O(3), 3.22 Å; N_2···O(2), 2.82 Å; N_2···O(4), 3.04 Å; N_3···O(5), 2.70 Å; N_3···O(1P), 2.72 Å; N_1'···O(1P), 3.11 Å; N_2'···O(2P), 2.96 Å.

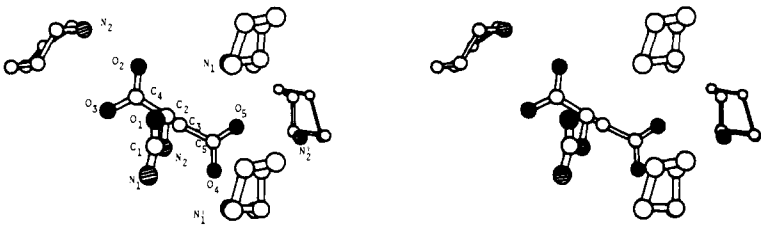


Figure 3. CLA crystal environment. This figure shows a stereoview of CLA with cyclohexylammonium counterions. As in the PALA structure, CLA is charge neutralized in part by cyclohexylammonium cations from a different asymmetric unit. As in Figure 2, these molecules are marked by a prime symbol (′). Some oxygen–nitrogen interatomic distances are the following: N_2···O(2), 2.72 Å; N_1···O(4), 2.71 Å; N_2'···O(4), 2.76 Å; N_1'···O(5), 2.76 Å.

than the distance found in D,L-aspartic acid, 1.49 Å.[19] The rest of the aspartic moiety has bond distances which compare reasonably well with the values found in the crystal structures of aspartic acid.[16,19] In the carbonyl groups, the four oxygen–carbon bond lengths appear to be quite similar, near 1.25 Å, and in agreement with the values expected for fully ionized acid groups.

The bond distances and valence angles of the CLA anion which are given in Table V present slightly larger standard deviations, owing to the disorder present in the cyclohexylammonium ions, which increases the level of imprecision of the entire structure. The bond lengths compare well with those of the common part of the PALA structure, with the exception of the C(2)–N(2) distance, which in this case is 1.46 Å, and therefore closer to the value of 1.49 Å found in D,L-aspartic acid.

The torsion angles of the two compounds are given in Table VI, in which some other related compounds have been included for comparison. A strong similarity is evident in the conformation of the common parts of CLA, D,L-aspartic acid, L-aspartic acid, and succinate, particularly in the C(4)–C(2)–C(3)–C(5) torsion angle relating the position of the two carboxyl groups. The conformation of the PALA anion, on the other hand, appears significantly different from the others. Thus the C(4)–C(2)–C(3)–C(5) torsion angle, which in all other compounds has values ranging from 174° to 180°, is in this case 132°. Another very different torsion angle is C(1)–N(2)–C(2)–C(4), which is −62° in CLA and −152° in PALA. The difference in the value of the first angle is a reflection of the fact that in the PALA anion the relative position of the two carboxyl groups is very different from that found in the other molecules chosen for comparison. The second angle shows the relative position of the different groups attached to the nitrogen of aspartic acid in the two compounds discussed. Whereas in CLA the small carbamoyl group does not induce a very significant change in the relative position of the two carboxyl groups, in the PALA anion the displacement of the bulky phosphate-containing moiety forces the carboxyls into a position which is not found in any of the other related structures. The configuration of the peptide-like bond in PALA and CLA is in both cases trans.

The environment of the PALA and CLA ions in the unit cell of the crystals is shown in Figures 2 and 3. The figure legends give the shortest interatomic distances between positively charged nitrogens and negatively charged oxygens. The figures include the cyclohexylammonium rings which, though belonging to a different asymmetric unit, interact with the anion shown. These rings have been labeled with the prime (′) symbol. In neither structure is there a one-to-one charge neutralization between positive ammonium groups and negative oxygens. Both the positively charged nitrogens of the cyclohexylammonium rings and the negatively charged oxygens of the central anions interact with more than one atom of the opposite charge. Also very different are the charge distributions around the central ion in the two cases discussed. Whereas in the CLA structure there are no two positively charged ammonium groups at a distance shorter than 7 Å, in the PALA structure there are two positively charged nitrogens at the very short distance of 3.81 Å from one another. These two groups are found interacting with the negatively charged phosphate group.

Discussion

Crystal-structure information on the substrates of ATCase has consisted of reports on the structures of phosphate and aspartate, elucidated many years ago.[16,21] Presentation of the structures of PALA and CLA in this report expands this information. The separate geometries of a complete reactant pair, CLA and phosphate, are now known. The PALA structure must to some extent resemble the transition state of the carbamoyl transfer reaction. We expect these small-molecule structures to be useful for modeling in conjunction with crystallographic studies on ATCase. The PALA and CLA structures may also be significant in their own right; several aspects of their geometry may have a relevance to the enzymic reaction.

The conformation of aspartate when bound to the active site of ATCase has long been a curiosity. The observation that maleate, with carboxylates constrained to a cis configuration by a double bond, was an excellent inhibitor (K_i = 4 mM), whereas

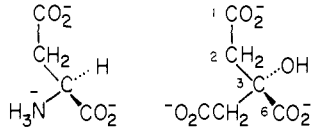
(19) Rao, S. T. *Acta Crystallogr., Sect. B* **1973**, *B29*, 1718–1720.

(20) McAdam, A.; Currie, M.; Speakman, S. C. *J. Chem. Soc. A* **1971**, 1994–1997.

(21) Hassel, O. *Z. Elektrochem.* **1925**, *31*, 523–529.

fumarate, the trans isomer, was a poor inhibitor (K_i = 145 mM)[22] was compelling evidence that aspartate must bind to the enzyme with its carboxylates in approximately a syn-periplanar conformation. However, an NMR study showed the most stable conformation of aspartate in solution to have its two carboxylates anti-periplanar.[23] The anti-periplanar conformation is also seen in four aspartate X-ray structures and in the X-ray structures of many close analogues: ammonium hydrogen malate, chlorosuccinic acid, two methyl succinates, and numerous succinate structures.[16,19,20,24] The CLA structure described in this report also falls into this pattern. The mechanism for selecting the less stable syn-periplanar conformation is unknown, as the structure of a PALA–ATCase complex has been solved only to low resolution,[6] and an attempted phosphate–CLA difference Fourier showed difference density only for phosphate.[25] A complex between PALA and the catalytic subunit of ATCase has been crystallized, but no solution has been reported.[26] A number of positively charged residues have been implicated in substrate binding,[27] and suitable positioning of these could account for binding of aspartate with a syn-periplanar torsion angle. Yet, a rationale for why a relatively rare rotamer of aspartate is selected has been lacking. No account has been made of this apparent waste of binding free energy.

In the PALA structure described in this report, the torsion angle between the two carboxylates is 132°, considerably removed from the anti-periplanar conformation. A reasonable question is whether this atypical angle arises from a packing artifact or is a manifestation of an innate tendency of the molecule to disfavor the anti-periplanar conformation. Considerable evidence supports the latter explanation. The substituted succinates cited above, favoring anti-periplanar disposition of carboxylates, all possess relatively small α-substituents, whereas the α-substituent in PALA, the phosphonacetamide moiety, is more massive than succinate itself, and negatively charged. When a different set of substituted succinates is examined, those with bulkier, charged substituents more aptly analogous to PALA, the anti-periplanar disposition of carboxylates is found to be an uncommon conformation. A syn-clinal conformation is seen in many citrate structures.[28] (The common regions of PALA and citrate are compared below.) The



same conformation is seen in the structures of potassium dihydrogen isocitrate, ethylenediamine 2*S*- and 4*S*-hydroxycitrate, rubidium ammonium hydrogen fluorocitrate, and *N*,*N*′-ethylenediaminedisuccinic acid.[29] In addition, an NMR study of the conformation of isocitrate in solution indicated that the anti-periplanar rotamer constituted only 20% of the population.[30]

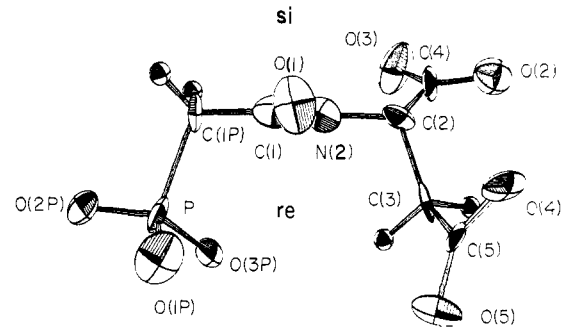


Figure 4. Structure of PALA, viewed down the carbonyl. The *si* and *re* faces of the carbonyl C(1)–O(1) are labeled. In this orientation the curl the PALA ion adopts is easily seen.

The structures likely to occur during enzymic catalysis are a Michaelis complex of aspartate abutting the carbonyl of carbamoyl phosphate (or phosphate abutting the carbonyl of CLA), a tetrahedral intermediate, and a transition state with partial formation of an N–C bond. All of these can be considered substituted succinates. The intramolecular forces disfavoring anti-periplanar carboxylates in the class of succinates with bulky, charged α-substituents also seem to distort the anti-periplanar rotamer of PALA. If the same holds for the enzyme-bound structures, the lowest energy conformation may have an acute angle, rather than the ~180° torsion angle common in the free substrates. As an acute angle would likely be further stabilized by suitable positioning of cationic active site residues, this design explains the failure of fumarate to inhibit, and the potency of maleate as an inhibitor.

A second feature of the PALA structure may pertain to catalysis. The phosphonomethyl and succinate moieties extend outward from the *re* face of the carbonyl C(1)–O(1), giving the molecule a sharp curl, best seen in the orientation of Figure 4. Analogy to the handedness of this curl suggests that nucleophilic attack on the *re* face of CLA and carbamoyl phosphate may be a lower energy pathway than *si* attack. However, considerable rotation of the phosphonomethyl and succinate moieties is possible about the two sp^2–sp^3 bonds. The stereochemistry of the enzymic reaction is unknown, and no other evidence exists to confirm or disprove this.

Acknowledgment. We are grateful to Dr. Kim Collins for suggesting the crystallization method which in the end proved successful. We also thank Dr. Stephen Holbrook for assistance in using the computer graphics system. This research was supported by a grant from the National Research Council (Italy) and by grants to Dr. Howard K. Schachman from the National Institute of General Medical Sciences (GM 12159) and the National Science Foundation (PCM80-23012). Some of the computer-generated drawings in this paper were drawn at the computer facility of the Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, supported by the United States Department of Energy.

Registry No. PALA tricyclohexylammonium salt, 92900-65-7; CLA dicyclohexylammonium salt, 92900-66-8; aspartate transcarbamylase, 9012-49-1.

Supplementary Material Available: A listing of observed and calculated structure factor amplitudes, thermal parameters of non-hydrogen atoms, and coordinates and thermal parameters of hydrogens (19 pages). Ordering information is given on any current masthead page.

(22) Porter, R. W.; Modebe, M. O.; Stark, G. R. *J. Biol. Chem.* **1969**, *244*, 1846–1859.
(23) Kainosho, M.; Ajisaka, K. *J. Am. Chem. Soc.* **1975**, *97*, 5630–5631.
(24) (a) Bhat, T. N.; Vijayan, M. *Acta Crystallogr., Sect. B* **1976**, *B32*, 891. (b) Dawson, B. *Acta Crystallogr., Sect. B* **1977**, *B33*, 882–884. (c) Versichel, W.; Van de Mieroop, W.; Lenstra, A. T. H. *Acta Crystallogr., Sect. B* **1978**, *B34*, 2643–2645. (d) Kryger, L.; Rasmussen, S. E.; Danielsen, J. *Acta Chem. Scand.* **1972**, *26*, 2339–2348. (e) Schouwstra, Y. *Acta Crystallogr., Sect. B* **1973**, *B29*, 1636–1641.
(25) Honzatko, R. B.; Lipscomb, W. N. *J. Mol. Biol.* **1982**, *160*, 265–286.
(26) Foote, A. M.; Winkler, F. K.; Moody, M. F. *J. Mol. Biol.* **1981**, *146*, 389–391.
(27) (a) Greenwell, P.; Jewett, S. L.; Stark, G. R. *J. Biol. Chem.* **1973**, *248*, 5994–6001. (b) Kantrowitz, E. R.; Lipscomb, W. N. *J. Biol. Chem.* **1976**, *251*, 2688–2695. (c) Lauritzen, A. M.; Lipscomb, W. N. *J. Biol. Chem.* **1982**, *257*, 1312–1319.
(28) Glusker, J. P. *Acc. Chem. Res.* **1980**, *13*, 345–352.
(29) (a) Van der Helm, D.; Glusker, J. P.; Johnson, C.; Minkin, J.; Burrow, N.; Patterson, A. L. *Acta Crystallogr., Sect. B* **1968**, *B24*, 578–592. (b) Stallings, W.; Blount, J.; Srere, P.; Glusker, J. P. *Arch. Biochem. Biophys.* **1979**, *193*, 431–438. (c) Carrell, H. L.; Glusker, J. P. *Acta Crystallogr., Sect. B* **1973**, *B29*, 674–682. (d) Scarbrough, F.; Voet, D. *Acta Crystallogr., Sect. B* **1976**, *B32*, 2715–2717.
(30) Bastian, E. J.; Martin, R. B. *J. Phys. Chem.* **1972**, *76*, 3073–3076.