

Three-dimensional structure of carbamoyl phosphate and succinate bound to aspartate carbamoyltransferase

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ABSTRACT The three-dimensional structure of the ternary complex of carbamoyl phosphate, succinate, and aspartate carbamoyltransferase (EC 2.1.3.2) has been determined to 2.6-Å resolution. The binding of the phosphate of carbamoyl phosphate is similar to the binding of the phosphonate of *N*-(phosphonoacetyl)-L-aspartate (PALA); interacting with the carboxylates of succinate are some of the same residues that interact with the carboxylates of PALA. The amino group of carbamoyl phosphate donates hydrogen bonds to the main-chain carbonyls of residues Pro-266 and Leu-267 and the side-chain carbonyl of Gln-137. In comparing the structure of the active sites in the PALA-enzyme complex to the active sites in the carbamoyl phosphate-succinate-enzyme complex, we find that they are similar.

Aspartate carbamoyltransferase (also called aspartate transcarbamylase, ATCase; EC 2.1.3.2) from *Escherichia coli* catalyzes the reaction between L-aspartate and carbamoyl phosphate to yield *N*-carbamoyl-L-aspartate and phosphate (1). The dodecameric enzyme consists of two catalytic trimers ($2C_3$) and three regulatory dimers ($3R_2$) that spontaneously assemble to form the holoenzyme (C_6R_6) (2, 3). As the paradigmatic regulatory enzyme, ATCase has been studied for more than 30 years not only in an effort to understand its allosteric properties but also because it catalyzes a reaction that is requisite for cell division (4, 5). Consequently, ATCase has been a target for antineoplastic agents (6, 7). However, there remain many questions concerning the specific conformational changes that aspartate and carbamoyl phosphate produce, inducing the homotropic allosteric transition. One important answer to these questions came in the solution of the structure of the R state of ATCase complexed with the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA; $K_i = 2 \times 10^{-8}$ M) (8, 9). To address the numerous remaining structural questions, we have embarked upon a number of crystallographic studies in which the enzyme was complexed with various combinations of substrates and substrate analogues. Regrettably, the substrates carbamoyl phosphate and aspartate react too rapidly for x-ray diffraction studies under usual conditions (although it might be possible to study the reaction in real time by using the technique of Laue diffraction). Hence, we have chosen to determine the structure of the ternary complex of enzyme, carbamoyl phosphate, and succinate, an excellent competitive inhibitor with respect to aspartate ($K_i = 4.4 \times 10^{-4}$ M at pH 6.0) (10).

MATERIALS AND METHODS

The half-life of carbamoyl phosphate is short [about 11 hr at 20°C in the crystallization buffer, as shown by ^{31}P NMR (J.E.G., unpublished results)] compared to the time required to grow diffraction-quality crystals. Consequently, the car-

bamoyl phosphate- and succinate-ligated crystals were prepared by soaking crystals grown in the presence of 1.0 mM PALA (9) with saturating amounts of carbamoyl phosphate and succinate. The ligand exchange was accomplished by placing the crystals in a buffer containing 20 mM carbamoyl phosphate, 30 mM succinate, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, and 20% polyethylene glycol (molecular weight 8000) at pH 5.9 and 20°C. Every 12 hr the buffer was replaced by a fresh solution; the total crystal soaking time was 60 hr. Since the buffer was changed five times and diluted by a factor of 100 each time, the total dilution of the original buffer was at least 1×10^{10} . Considering the binding constants for carbamoyl phosphate (2×10^{-5} M) and succinate, the fact that PALA is a reversible inhibitor, and the dilution of the original PALA buffer, we are confident that essentially all of the PALA was displaced.

Only one crystal, with dimensions $1.0 \times 1.0 \times 1.8$ mm, was used for data collection. This crystal was mounted by floating it into a 1.5-mm silanized glass capillary filled with freshly prepared buffer. Immediately after the crystal was dried and the capillary was sealed, the orientation of the crystal was determined and data collection commenced. The unit-cell dimensions for this crystal of space group $P321$ were $a = b = 122.3$ Å and $c = 156.0$ Å. Contained in the asymmetric unit were two catalytic and regulatory pairs (2 CR), related by an approximate molecular twofold axis. This crystal was isomorphous to PALA-ligated crystals. Diffraction maxima were measured by using multiwire proportion chambers at the University of Virginia (11). The crystal was irradiated with graphite-monochromatized Cu $K\alpha$ x-rays produced by a Rigaku RU-200 operating at 45 kV and 200 mA. After the two detectors were set to the desired 2θ values, the asymmetric unit of reciprocal space was collected over a period of 14 hr by using ω scans (0.07° step size) at various settings of χ and ϕ . The intensities were integrated and then corrected for background and for Lorentz and polarization factors. The data were scaled to a data set collected at the University of California, San Diego, on the PALA-ligated enzyme (9) in 5° ranges of ω by using only a linear scale factor according to the method of Fox and Holmes (12), yielding an agreement factor $R_{\text{merge}} (= \sum_{hkl} |I - \bar{I}| / \sum_{hkl} I)$ of 5.2% for the 61,884 observations of 27,223 independent reflections from 8.0- to 2.6-Å resolution.

The electron density for carbamoyl phosphate and succinate (hereafter referred to as CS) was clearly identified from a map calculated by using $|F_o - F_c|$ as coefficients and phases and F_c values calculated from the native R-state structure (PALA and all solvent omitted). Initial concerns that the density assigned to carbamoyl phosphate might actually correspond to phosphate (formed from the uncatalyzed decomposition of carbamoyl phosphate) and a water molecule were allayed by inspecting maps drawn at successively higher contour levels. These maps showed that the density was always contiguous and not separated into two unequal

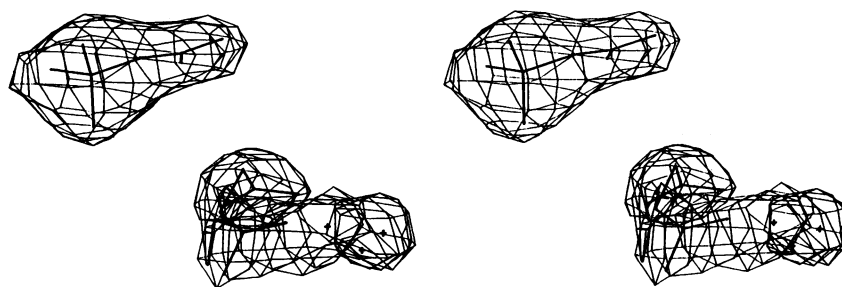


FIG. 1. Section of a $|F_o - F_c|$ map calculated as discussed in the text and contoured at 2.2σ . Although there are only data to 2.6-Å resolution, the plane of the carbamoyl group is nonetheless readily defined.

portions even at higher contour levels; a distinct separation would be expected if, in fact, phosphate and a water molecule were bound. The ligands and 145 water molecules were then built into the structure by the program FRODO (13) running on an Evans and Sutherland PS300 interfaced to a VAX 11/780. The resulting model was refined by the program XPLOR (14), running on a Cray X/MP at the Pittsburgh Supercomputing Center, to a crystallographic R of 19.4%. [$R = \sum_{hkl} (||F_o| - |F_c||) / |F_o|$. The rms deviations from ideality for the bond lengths and bond angles were 0.019 Å and 3.8°, respectively.] In order to ensure that the differences observed between the CS- and PALA-ligated structures were not artifacts of the refinement protocol, we re-refined the PALA-ligated structure against the data collected at the University of California, San Diego (9), using a protocol similar to that used to refine the CS-ligated structure. A thorough description of these refinements will be the subject of a future publication.

RESULTS AND DISCUSSION

From the results of difference sedimentation (15, 16), difference spectroscopy (8, 17), and low-angle x-ray scattering (18) experiments, it is known that either PALA or carbamoyl phosphate combined with succinate induces similar conformational changes in ATCase. However, PALA exhibits noncompetitive inhibition with respect to aspartate, whereas succinate shows competitive inhibition (8, 10). Indeed, there have been questions as to how well PALA defines the aspartate binding site (19). The results of the present study indicate that the residues that bind the phosphate group of carbamoyl phosphate and the carboxylates of succinate are the same as those that bind to the analogous groups of PALA.

Surprisingly, the active-site residues are in similar conformations in both structures; the estimated experimental error in the coordinates is about 0.4 Å based on relationships derived by Luzzati (20). It is possible to regard succinate and carbamoyl phosphate as excellent analogues of PALA on the basis that the two ATCase structures compared here share many similar interactions between the enzyme and ligands and also because the positions of the active-site residues are approximately identical.

Still, there are several new interactions between carbamoyl phosphate and succinate and the enzyme. For example, the mixed-anhydride oxygen of carbamoyl phosphate—analogue to the methylene group in PALA—interacts with the guanidinium group of Arg-54. Another network of interactions not present in the PALA-ligated complex occurs between the primary carbamoyl nitrogen and the main-chain carbonyls of Pro-266 and Leu-267 and side-chain carbonyl of Gln-137. Interacting with the carbonyl of carbamoyl phosphate are Thr-55, Arg-105, and His-134, residues that also bind to the carbonyl of PALA. However, in comparing the interactions that succinate makes with the active-site residues to the binding of the dicarboxylic acid portion of PALA, we find that succinate is not positioned to make as many hydrogen bonds and salt links. The carboxylates of succinate interact primarily with Arg-167 and Arg-229. The orientation of the carboxylate occupying the position analogous to the β carboxylate of PALA precludes it from binding to Lys-84. These results might be inherent to the binding of succinate or they could be due to the low pH at which this experiment was conducted. In the vicinity of the active site, the water structure is like that in the PALA-ligated structure except for the absence of two water molecules near succinate's carboxylates; these waters, which exhibit strong density in the

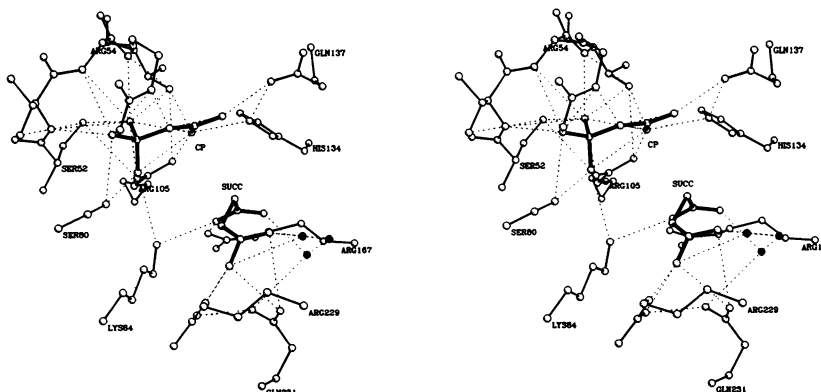


FIG. 2. Stereo drawing illustrating the binding of carbamoyl phosphate (CP) and succinate (SUCC) to the active site residues. Potential hydrogen bonds and salt links are depicted as dotted lines. Three water molecules close to the carboxylates are represented by filled circles. Residues Pro-266 and Leu-267 are omitted for clarity.

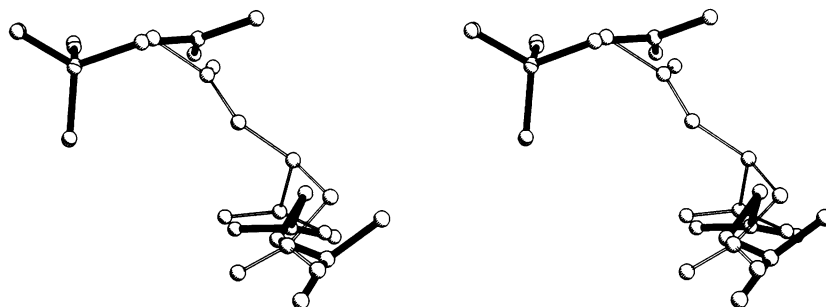


FIG. 3. The position of succinate relative to carbamoyl phosphate (thick lines) compared to the orientation of the dicarboxylic acid moiety of PALA to its phosphonoacetyl group (thin double lines) in the A active site. We superimposed the phosphonate bond of PALA onto the phosphate bond of carbamoyl phosphate.

PALA structure, show no density in the CS structure. This might be the result of succinate's weaker binding and more rapid exchange into the solvent, causing the missing waters to have such low occupancies and/or high temperature factors as to render them unobservable.

Most importantly, the multitude of interactions—seven hydrogen bonds—between the carbamoyl group and the active-site residues unambiguously defines its orientation as shown in Fig. 1. Although we cannot distinguish between an oxygen and a nitrogen from the electron density, we can confidently identify the orientation of the carbamoyl group based on the predicted hydrogen-bonding environment: the nitrogen binding site is composed exclusively of hydrogen-bond acceptors, while the carbonyl oxygen site includes primarily hydrogen-bond donors. These interactions are likely to be of paramount importance for orienting the carbamoyl group for nucleophilic reaction by the amino group of aspartate and for stabilizing the subsequent transition state. Our definition of the nitrogen binding site also explains why ATCase binds carbamoyl phosphate more tightly than acetyl phosphate (10). A methyl group would not interact as favorably with the hydrogen-bond acceptors as would an amino group. The enzyme might also use the interactions to the carbamoyl moiety to distort the amino and carbonyl groups from planarity as a mechanism to increase the electrophilicity of the carbonyl carbon.

It is difficult to predict whether or not the position of succinate, as determined from this experiment (Fig. 2), might represent the binding of aspartate before the bond between aspartate and carbamoyl phosphate is formed, or whether it represents the binding at some later stage of the reaction. When we compare the conformations of the succinate molecules bound to the upper (label A) and lower (label C)

catalytic chains, we find that they are different. For the succinate bound to the A chain, the carboxylates are close to a trans orientation, with a dihedral angle of about 160° ; in the C chain, the dihedral angle is approximately 120° . Compared to the conformation of PALA (the dihedral angle is about 70° in both the A and C active sites), the succinates are significantly more extended (Figs. 3 and 4). Although the density for succinate does not unambiguously define its conformation and it might be possible to build succinate in a different conformation, we believe that the density has been accurately fit. We suggest that the phosphonate and amide groups of PALA draw the carbon α to the amino group farther into the active site, thus forcing the carboxylates into a more eclipsed conformation. This would allow the α carboxylate to bind to both Arg-105 and Arg-167 and the β carboxylate to interact with Lys-84, Arg-229, and Gln-231. There is evidence that maleic acid binds more tightly than fumaric acid; however, succinate binds better than maleic acid, implying that the optimal carboxylate conformation is closer to cis than trans (21). The similarity between the position and conformation of succinate in the C active site and that of the dicarboxylic acid entity of PALA implies that this succinate might represent the binding of aspartate at some intermediate stage of the bond formation between the amino group of aspartate and the carbonyl carbon of carbamoyl phosphate. Energy minimization and molecular dynamics calculations might provide additional information on this question.

Nevertheless, we have developed a simple model of a mechanism by superimposing aspartate onto the crystallographically determined structure of succinate bound to the C chain. When in this position, the amino group of aspartate is 2.1 Å from the carbonyl carbon of carbamoyl phosphate. This model predicts that the chirality of the putative tetrahedral

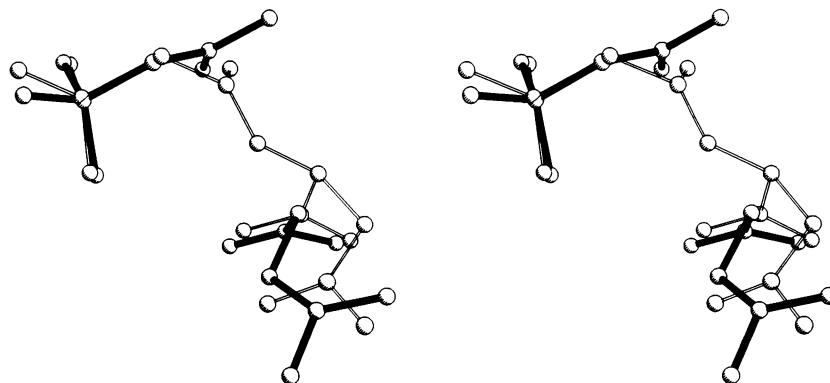


FIG. 4. As in Fig. 3 except that this is the C active site.

intermediate will be *S*, in agreement with previous results (22) and relevant to the design of tetrahedral transition-state analogues (23, 24). The proximity of the anhydride oxygen to the amino group (2.8 Å) lends credibility to the previously proposed four-center proton-transfer mechanism (8). Although 4.3 Å from the amino group of the model, His-134 might act as a general base and could deprotonate aspartate's amino group after the formation of the tetrahedral intermediate. To facilitate the breakdown of the tetrahedral intermediate, His-134 could also transfer a proton directly, or via a water molecule, to a phosphate oxygen, thereby making it a better leaving group. Which oxygen would be the most favorable one to protonate? Here, it is helpful to consider the mechanism of the reverse reaction: the reaction between phosphate and *N*-carbamoyl-L-aspartate. Under the conditions of optimum enzyme activity (pH 8.3), the predominant phosphate species in solution will be the dianion, although within the confines of the enzyme's active site the state of protonation is not known and the reactive species might well be the trianion. If we assume that the dianion is the reactive species, we would expect one of the oxygens *not* bearing a proton to act as the nucleophile, since the more basic atom will be the better nucleophile. Consequently, in the forward reaction, we would predict that the mixed-anhydride oxygen should *not* be protonated in favor of one of the other three, based on the principle of microscopic reversibility. These hypotheses could be tested by molecular dynamics trajectories and molecular orbital calculations.

In summary, we find that the terminal oxygens of the phosphate of carbamoyl phosphate and the terminal oxygens of the phosphate of PALA are bound similarly. We also find that the carboxylate groups of succinate and those of PALA are bound similarly to ATCase. We suggest mechanisms based on these structures as outlined above.

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