Aspartate aminotransferase: Investigation of the active sites

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An investigation of the crystal structure of cytosolic pig-heart aspartate aminotransferase (AAT, E.C.2.6.1.1) was carried out to determine the structural requirements for ligand recognition by the active site. Structural differences were observed between the two active sites of the AAT dimer. The natural ligand, L-aspartate, was docked into both active sites using various methods. However, due to structural differences, the ligand was able to form all the necessary interactions for initial binding in only one of the active sites. The program GRID (P. J. Goodford, J. Med. Chem. *1985, 28, 849–857) was used to predict favorable binding sites for the functional groups of the aspartate ligand. These binding sites corresponded to the position of the docked aspartate ligand, indicating that substrate recognition takes place before any major conformational changes occur within the enzyme.*

Keywords: aspartate aminotransferase, ligand docking, binding site predictions, computer-graphic modeling

AAT is an enzyme involved in the metabolism of aspartic acid in both the peripheral and central nervous systems, where aspartic acid acts as an excitatory amino acid involved in synaptic transmission.[1–5] AAT is a pyridoxal-5-phosphate (PLP) dependent enzyme which occurs as a dimer. It has been isolated and crystallized, both with and without inhibitors bound in the active site.[6–18] The enzyme has been shown to undergo some conformational changes around the active site, accompanied by a hinged movement of the small domain on binding of a substrate.[6,10–15] The catalysis of the reversible transamination reaction L-aspartate + 2-oxoglutarate $\rightleftharpoons$ oxaloacetate + L-glutamate has been shown to occur via a stepwise mechanism involving covalent bonding of the coenzyme PLP to the substrate to form an aldimine as the first of the series of spectroscopically identifiable intermediates.[10,12–14] Both the binding sites appear to have similar affinity for L-aspartate in solution, but in the crystal structure one binding site retains full catalytic activity, while the other is inactive.[6,11–14]

In this work the solid-state geometries of the two active sites are compared by a variety of methods to determine the requirements for recognition of aspartic acid (the substrate) by the catalytically active subunit.

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METHODS

The crystal structure of cytosolic AAT from pig heart was kindly supplied by A. Arnone and C. Hyde. The resolution of the structure is 2.25 Å, with an *R* factor of 22.5%; however, the active-site regions are well defined. The Cα coordinates of cytosolic AAT from chicken heart, with a resolution of 3.2 Å,[17] were obtained from the Brookhaven Protein Data Bank[19] and used for comparison of the overall tertiary structure. This structure is reported to have 2-oxoglutarate bound to the active site. The structures were examined and compared using the program HYDRA.[20] Docking of aspartic acid was done by three methods: manual docking with energy monitoring in HYDRA; visual docking in SHOW (written and developed in our laboratories); and using a simplex docking routine in MACROMODEL.[21–23] Ligand conformations were studied using the multiconformer option in MACROMODEL and the structures optimized using the AMBER parameters as supplied in the program and by the use of potential energy maps plotted from data generated in MACROMODEL. Favorable conformations of zwitterionic, neutral, and the single, negatively charged species were ascertained by a global search involving the rotation of all variable torsion angles. Docking used the minimum energy conformation found in these searches.

The active sites were also studied by use of the program GRID, which determines likely interaction sites between the enzyme surface and probes with the characteristics of particular functional groups. A grid spacing of 1 Å was used, because it was found to be adequate to detect these areas. The energy contours obtained were displayed in the program SHOW.

SHOW sums the noncovalent interaction energy between a putative inhibitor and the enzyme. The electrostatic, van der Waals, and hydrogen-bonding energies are precalculated at grid points (using GRID) and the positions of the inhibitor atoms used to interpolate between grid points. (If there is significant hydrogen bonding, the van der Waals energy is ignored.) This summation is fast, and allows the GRID dielectric treatment to be used as the inhibitor is moved around manually with the dials. Individual probe-atom en-

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ergy contours can also be displayed as an overall visual guide for docking.

The programs MACROMODEL and GRID were run on a VAX 11/750 computer, and HYDRA and SHOW were run on an IRIS 3130 workstation.

RESULTS AND DISCUSSION

Superimposition of pig-heart and chicken-heart cytosolic AAT

The AAT dimer consists of two subunits, each having one active site. Both can be divided into what have previously been described as the large and small domains.[6,10–14] The large domain contains seven α helices and seven β strands, while the small domain is less compact and more flexible. The active site is located in a pocket between the two domains.

The tertiary structure of cytosolic pig-heart AAT was compared with that of the cytosolic enzyme from chicken heart. It was expected that differences observed would help in modeling conformational changes that take place after the binding of aspartic acid. The α-carbon backbones of both enzymes were superimposed using the least-squares algorithm in HYDRA. The root-mean-square (RMS) of the superimposition was 1.09 Å and the maximum displacement 2.32 Å. As seen in Color Plate 1, both cytosolic sources of AAT have very similar tertiary structures. Larger differences were expected in the superimposition, since the cytosolic chicken-heart AAT was reported to have the ligand, 2-oxoglutarate, bound to the active site. The differences between the Cα positions of the active site residues were all less than 1.76 Å. This indicates that either there is very little conformational change in cytosolic chicken-heart AAT on the binding of 2-oxoglutarate, or perhaps there is no ligand bound in this crystal structure.

The structural similarity between the two ligand-free cytosolic sources of AAT is expected, since they show a high level (83%) of sequence homology. Similarities in tertiary structure have previously been observed between mitochondrial chicken-heart AAT and cytosolic pig-heart AAT.[6,8,11,14,24] The crystal structure of cytosolic chicken-heart AAT, with a resolution of 3.5 Å, has also been reported to have many of the tertiary structural features observed in cytosolic pig-heart AAT.[11] The superimposition of the cytosolic sources of AAT clearly shows this to be the case.

The active sites

Residues from both subunits of the AAT dimer participate in each active site (the subunits will be referred to as subunit 1 and subunit 2). In the active site from subunit 1, these are Ser-107, Gly-108, Thr-109, Trp-140, His-143, His-189, Asp-194, Asp-222, Arg-224, Tyr-225, Ser-255, Phe-256, Ser-257, Lys-258, Phe-360, and Arg-386; from subunit 2 they are Arg-292*, Tyr-70*, Asn-297*, and Ser-296* (subunit 2 residues are denoted by *). The coenzyme PLP is covalently linked to Lys-258, and its orientation is governed by an extensive hydrogen-bonding network and by numerous residues (including Trp-140, His-143, Asp-222, Tyr-225), which form a pocket in which PLP is situated. The phosphate group of the coenzyme is held firmly in position by ionic interactions and polar contacts with surrounding residues (e.g., Arg-266, Ser-255, Ser-257, Thr-109, Tyr-70*[6,11–14]). The active site of subunit 1 is shown in Color Plate 2; for clarity only four residues plus the coenzyme PLP have been distinguished.

Previous experimental and modeling results obtained by other workers indicate that the guanidinium groups of Arg-386 and Arg-292* interact through hydrogen bonds and ionic interactions with the α- and β-carboxylate groups, respectively, of L-aspartate, the substrate.[6,11–14,25] The amino group of L-aspartate points toward the coenzyme but makes no direct van der Waals contact. The substrate's β-carboxylate group also forms hydrogen bonds to Trp-140. Once the substrate is in this orientation, transamination can take place.

The docking of the substrate was carried out using favorable conformations of L-aspartate. The substrate was docked into the active site of both subunits: In subunit 1, the interaction energy was monitored throughout the docking procedure and a minimum-energy value was observed when the substrate was positioned in the orientation previously described for initial binding (Color Plate 3). This was the case for all three docking methods. However, in subunit 2, the β-carboxylate group of L-aspartate was unable to interact with the guanidinium group of Arg-292 while maintaining the other necessary interactions. The orientation of the Arg-292 in subunit 2 is markedly different from that in subunit 1. The guanidinium group of Arg-292 is pointing out of the active site in subunit 2, whereas in subunit 1 Arg-292* points into the active site toward the coenzyme. The two active sites were superimposed via residues 257 and 258 to identify any other structural differences (Color Plate 4). The majority of the active-site residues superimpose quite well, with an average displacement of approximately 0.3 Å. The superimposition of the two arginine residues, 386 and 292*, is poor; the distance between the Arg-292 and Arg-292* CZ atoms is 3.78 Å, while the distance between the Arg-386 and Arg-386* CZ atoms is 1.11 Å. These differences are due to rotation of the side chains, rather than the large conformational changes known to occur when a ligand is bound. The rotation of both arginine residues was monitored in HYDRA, and indicates substantial barriers to rotation. Two regions of favorable conformation occur for Arg-292* in the active site of subunit 1. These are the conformations with the torsion angle CA-CB-CG-CD at approximately $-60°$, close to the original conformation, and the other pointing out into the channel leading to the active site at $-160°$. In the active site of subunit 2, the two regions of favorable conformation for CA-CB-CG-CD of Arg-292 are at approximately 160° and 34°. The barriers to rotation of Arg-292 were substantially greater than for Arg-292* due to the increased number of intramolecular contacts.

Differences in the position of Arg-386 and Arg-292* make it unlikely that subunit 2 could bind L-aspartate for transamination to take place without reorientation of the side chains; hence, subunit 2 is inactive in the solid state. This supports previous studies by other workers,[6,11–14] which reported a functional asymmetry of the AAT dimer in the solid state. Solution studies have shown the two active sites to be equivalent and independent,[6,11–14] indicating that rotation of these side chains is possible in solution, so that

the asymmetry has been attributed to packing forces in the crystal lattice.[6,11-14]

GRID results

Both active sites of the cytosolic pig-heart AAT dimer were investigated using GRID. A wide variety of probes were used to locate potential ligand binding sites including water, various nitrogen and oxygen probes, halogens, methyl, and phosphorus and sulfur probes. Interaction energies were displayed as three-dimensional contour surfaces, together with the active site and the docked aspartic acid ligand, using SHOW. A negative energy level depicts regions that should be favored for ligand binding.[26] Any interactions that were predicted in a solvent-inaccessible region were disregarded completely.

Subunit 1 Because it is known that the α- and β-carboxylate groups of aspartic acid interact with the guanidinium groups of Arg-386 and Arg-292*, respectively, we would expect to see carboxylate "hotspots" (favorable interaction sites) corresponding to where the docked carboxylate groups lie. A carboxylate probe with properties similar to an aspartic acid oxygen does, indeed, have hotspots corresponding to the α- and β-carboxylate groups of the docked aspartic acid (Color Plates 5*a* and *b*) when contoured at an energy level of -4 kcal/mol. The low-energy conformation of aspartic acid shown in Color Plates 5*a* and *b* was docked into the active site using the simplex method in MACROMODEL. Manual docking of the same low-energy conformer of aspartic acid in HYDRA did not correspond as well to the predicted hotspots. Other general oxygen probes, such as carbonyl and hydroxyl, also predict similar hotspots, corresponding to the locations of the docked α- and β-carboxylate groups, although in the case of the carbonyl probe, the interaction is weaker.

All the nitrogen probes (ammonium, amine, amide) correctly predict the position of the docked aspartic nitrogen. The hotspots for an ammonium probe with properties similar to an aspartic acid nitrogen, contoured at an energy level of -5 kcal/mol, are shown in Color Plates 5*a* and *b*. All probes run on subunit 1 had common hotspots situated in the channel leading into the active site when contoured at an energy level of -5 kcal/mol. There was one other region in the active site where GRID had predicted favorable interactions with all the probes (except for hydrophobic probes). This region was roughly parallel to both Trp-140 and the pyridine ring of PLP at a distance of approximately 3.5–4.0 Å from the PLP pyridine ring. This region was solvent-accessible and could also be reached by suitable substitution on the β-carbon of the aspartic acid ligand.

The program GRAB[26] was used to perform subtractions of the various GRID maps to produce difference maps, which display regions around the active site where the interactions of one probe are favored relative to another (i.e., the common interactions have been removed). The difference map between a carboxylate probe and an ammonium probe is given in Color Plates 6*a* and *b*. Where the carboxylate probe is favored (yellow) the hotspots correspond to the positions of the α- and β-carboxylate groups of the docked aspartate ligand. Where the ammonium probe is favored (blue) there is a hotspot corresponding to the position of the docked aspartate nitrogen. The difference map clearly describes the orientation of the aspartate ligand upon initial binding. There are only a few hotspots in the channel leading into the active site where either the carboxylate or ammonium probe is favored. The individual probes have strong interactions in this region; these interactions must therefore be common to both probes. Similar difference maps are obtained when carboxyl, carbonyl, and hydroxyl probes are subtracted from amide or ammonium probes.

Difference maps between various oxygen probes show that (1) a carboxyl probe predicts the interaction between the β-carboxylate of aspartic acid and Arg-292* more accurately than a carbonyl probe, and (2) there is little difference between an hydroxyl and a carbonyl probe.

Both an ammonium probe and an amide probe predict hotspots corresponding to the position of the docked aspartate nitrogen. However, a difference map between an ammonium probe and an amide probe indicates that there are regions in the active site where one probe is favored over the other. Where the ammonium probe is favored, the hotspots are parallel to PLP and Trp-140 at approximately 3.5 Å from the PLP pyridine ring, whereas when the amide probe is favored, the hotspots are situated in the channel leading into the active site.

Subunit 2 The structural differences between the active sites of the two subunits were illustrated by the differences in the hotspot sites predicted for the same probes in the two active sites. A carboxylate probe at an energy level of -4 kcal/mol predicts hotspots corresponding to the position of the α-carboxylate group of the docked aspartate ligand. However, the hotspots predicted between the carboxylate probe and Arg-292 lie away from the active site and cannot be accessed by the β-carboxylate group of the docked aspartate ligand if the contacts between the ligand and Arg-386* and Trp-140* are to be maintained. Also, the strong hotspots leading into the active site of subunit 1 are absent in the active site of subunit 2. This is also the case for the other oxygen probes.

The various nitrogen probes all predict favorable interactions leading into the active site, but none has any hotspots corresponding to the position of the docked aspartate amino group. In other words, the interaction between the amino group of the aspartate ligand and Trp-140* is not predicted as a favorable interaction in this subunit. Thus, it is not possible for a ligand to bind to this active site while maintaining the necessary contacts between Arg-386*, Trp-140*, and Arg-292, and so be in the necessary orientation for transamination to take place.

CONCLUSION

The superimposition of cytosolic pig- and chicken-heart AAT crystal structures shows clearly that the overall tertiary structure is very similar for both sources of cytosolic enzyme. Docking of the natural ligand aspartic acid into the two active sites of crystalline cytosolic pig-heart AAT was carried out. In the active site of subunit 1, the ligand was able to form all the necessary interactions for initial binding at a minimum energy level. This was not the case, however, for the active site in subunit 2, where the ligand was unable to maintain interactions with Arg-386* and Trp-140* if it

was also to interact with Arg-292. The interaction between Arg-292 and the β-carboxylate group of the aspartate ligand has been shown by site-directed mutagenesis studies[25,27–38] to be necessary for the transamination of aspartic acid.

Superimposition of the two active sites of cytosolic pig-heart AAT displayed structural differences, particularly in the orientation of Arg-292*, which accounts for differences in activity between the two subunits in the crystalline state.

In subunit 1, the GRID results predicted favorable binding sites for the α- and β-carboxylate groups of the aspartate ligand and also for the aspartate amino group. These predicted binding sites correspond to the positions of the docked ligand's functional groups. Hence, it appears that substrate recognition must occur first, and then the small domain movement accompanying transamination takes place. Results from both GRID and docking for subunit 2 indicate that in the enzyme's solid-state conformation, it would be unlikely for a ligand to bind into this active site in a way that would allow transamination to occur.

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