Refinement and Comparisons of the Crystal Structures of Pig Cytosolic Aspartate Aminotransferase and Its Complex with 2-Methylaspartate*

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Two high resolution crystal structures of cytosolic aspartate aminotransferase from pig heart provide additional insights into the stereochemical mechanism for ligand-induced conformational changes in this enzyme. Structures of the homodimeric native structure and its complex with the substrate analog 2-methylaspartate have been refined, respectively, with 1.74-Å x-ray diffraction data to an R value of 0.170, and with 1.6-Å data to an R value of 0.173. In the presence of 2-methylaspartate, one of the subunits (subunit 1) shows a ligand-induced conformational change that involves a large movement of the small domain (residues 12–49 and 327–412) to produce a “closed” conformation. No such transition is observed in the other subunit (subunit 2), because crystal lattice contacts lock it in an “open” conformation like that adopted by subunit 1 in the absence of substrate. By comparing the open and closed forms of cAspAT, we propose a stereochemical mechanism for the open-to-closed transition that involves the electrostatic neutralization of two active site arginine residues by the negative charges of the incoming substrate, a large change in the backbone (\(\phi,\psi\)) conformational angles of two key glycine residues, and the entropy-driven burial of a stretch of hydrophobic residues on the N-terminal helix. The calculated free energy for the burial of this “hydrophobic plug” appears to be sufficient to serve as the driving force for domain closure.

It frequently has been observed that an enzyme will undergo a large conformational change to bring catalytic groups into functionally active orientations in response to substrate binding at the active site. This type of ligand-induced conformational change was proposed by Koshland in his “induced fit” model (1) and reflects the flexible nature of large proteins (2). X-ray crystallographic methods have been used to directly reveal ligand-induced structural changes in several enzymes (3–

4); e.g. aspartate aminotransferase (see below), hexokinase (5), alcohol dehydrogenase (6), and citrate synthase (7).

Aspartate aminotransferase (AspAT) is one of the key enzymes in amino acid metabolism. The enzyme is responsible for the following reversible transamination reaction.

\[
\text{L-Aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{L-glutamate} + \text{oxaloacetate}
\]

**Reaction 1**

Oxaloacetate produced in this way in the cytosol can be converted to glucose via the gluconeogenesis pathway, or it can be used to indirectly transport NADH into the mitochondria via the malate-aspartate shuttle (8). Since its discovery in 1937, AspAT has been studied extensively, and its functional, mechanistic, and structural properties have been reviewed in great detail (9–13). In early studies, the ligand-induced conformational changes of AspAT during catalysis were suggested by changes in the reactivity of a cysteine residue (14, 15). More recently, direct evidence for conformational changes upon ligand binding has been obtained from the x-ray crystallographic studies of AspAT from several sources: chicken mAspAT (13, 16), chicken cAspAT (17, 18), Escherichia coli AspAT (19, 20) and pig cAspAT (21). In all cases, a similar open-to-closed transition has been observed. However, many aspects of the mechanism of the substrate-induced domain movement, such as the identification of all the residues that have an essential role in domain closure and the characterization of the driving force for domain movement, have yet to be fully resolved.

Pig heart cAspAT is a dimeric enzyme of identical 412-residue subunits (molecular mass of 92,700 Da). Each subunit consists of a large and a small domain, and the active site is located at the interface between the two domains. At the active site, one molecule of the coenzyme pyridoxal 5′-phosphate is covalently linked to the e-amino group of lysine 256 in the large domain through an aldimine linkage. Two different conformations for the small domain were characterized previously from a 2.7-Å resolution structure of native cAspAT and a 3.2-Å difference Fourier map of a cAspAT-substrate analog complex (21). In the absence of substrates, the two identical subunits are spatially related by 2-fold symmetry, adopting the so-called “open conformation.” The binding of substrate in the active site of one of the subunits (referred to as subunit 1) induces the small domain of that subunit to shift toward the active site, forming the “closed conformation.” In contrast to solution studies, which show that both subunits are reactive and independent (22, 23), only subunit 1 in crystalline cAspAT shows a

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The atomic coordinates (codes I1ajfs and I1ajs) and structure factors (codes I1ajrf and I1ajsf) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: AspAT, aspartate aminotransferase; cAspAT, cytosolic AspAT; mAspAT, mitochondrial AspAT; MeAsp, di-2-methylaspartate; PEG, polyethylene glycol; r.m.s., root mean square.
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conformational change on binding substrate because crystal lattice contacts lock the other subunit (subunit 2) in the open conformation (Fig. 1).

Here we present the 1.74-Å structure of cAspAT and the 1.6-Å structure of cAspAT complexed with MeAsp. Analysis of these two structures reveals important elements of the stereochemical mechanism for domain movement in AspAT.

EXPERIMENTAL PROCEDURES

Crystallization—Highly purified cytosolic aspartate aminotransferase from pig heart was prepared as described by Yang and Metzler (24). Prior to crystallization, the native enzyme was dialyzed and concentrated against 40 mM sodium acetate at pH 5.4 (25) and then increased in size with a seeding technique that has been described previously (26). The plate-shaped crystals have orthorhombic space group symmetry (space group P2(1)2(1)2(1), with unit cell dimensions a = 125.0 Å, b = 130.8 Å, c = 55.8 Å), and each asymmetric unit consists of one dimer. This set of crystallization conditions will be referred to as the “standard crystallization solution” to distinguish it from other soaking solutions that change the ionic environment and the structure of the enzyme. Specifically, we have determined that under the standard crystallization conditions two bound acetate anions from the buffer induce the structural transition of the small domain in subunit 1 from the open to the closed conformation (27, 28). A Rigaku AFC6R diffractometer fitted with a San Diego Multiwire Systems area detector was used to collect this data set. The data set from two cAspAT crystals soaked in 40 mM sodium formate (91,745 reflections out of 444,811 measurements) contains 95.9% of the possible reflections to a resolution of 1.74 Å. The R<sub>sym</sub> value for this data set is 5.4%. All diffraction data were scaled and merged according to the procedure of Howard et al. (29).

The initial x-ray diffraction data sets for the complex with MeAsp were collected to a resolution of 2.5 Å with an Enraf-Nonius CAD4 diffractometer. Later, a 1.6-Å resolution data set was collected on a single cAspAT-MeAsp crystal grown in 300 mM sodium formate using a Rigaku AFC6R diffractometer fitted with a San Diego Multiwire Systems area detector. A total of 114,162 unique reflections (476,759 measurements) were collected to a resolution of 1.6 Å. At this point, the effect of 40 mM acetate on cAspAT structure was not recognized, and this data set was collected using cAspAT crystals grown under the standard crystallization conditions. Subsequently, high resolution diffraction data were collected on cAspAT crystals soaked in 40 mM sodium formate, eliminating the acetate-induced movement of the small domain in subunit 1 (27, 28). A Rigaku AFC6R diffractometer fitted with a San Diego Multiwire Systems area detector was used to collect this data set. The data set from two cAspAT crystals soaked in 40 mM sodium formate (91,745 reflections out of 444,811 measurements) contains 95.9% of the possible reflections to a resolution of 1.74 Å. The R<sub>sym</sub> value for this data set is 5.4%. All diffraction data were scaled and merged according to the procedure of Howard et al. (29).

The initial x-ray diffraction data sets for native cAspAT were collected to a resolution of 2.4 Å with an Enraf-Nonius CAD4 diffractometer. Later, a 1.6-Å resolution data set was collected on a single cAspAT-MeAsp crystal grown in 300 mM sodium formate using a Rigaku AFC6R diffractometer fitted with a San Diego Multiwire Systems area detector. A total of 114,162 unique reflections (476,759 measurements) were collected to a resolution of 1.6 Å. At this point, the effect of 40 mM acetate on cAspAT structure was not recognized, and this data set was collected using cAspAT crystals grown under the standard crystallization conditions. Subsequently, high resolution diffraction data were collected on cAspAT crystals soaked in 40 mM sodium formate, eliminating the acetate-induced movement of the small domain in subunit 1 (27, 28). A Rigaku AFC6R diffractometer fitted with a San Diego Multiwire Systems area detector was used to collect this data set. The data set from two cAspAT crystals soaked in 40 mM sodium formate (91,745 reflections out of 444,811 measurements) contains 95.9% of the possible reflections to a resolution of 1.74 Å. The R<sub>sym</sub> value for this data set is 5.4%. All diffraction data were scaled and merged according to the procedure of Howard et al. (29).

Refinement of cAspAT—Table I contains a summary of the final refinement statistics for the native cAspAT structure and the cAspAT-MeAsp complex as reported in the restrained least squares refinement program PROLSQ (30, 31).

The 2.7-Å resolution atomic model of Hyde et al. (32) was used as the starting point for subsequent refinement of the native enzyme. This unrefined model had an R value of 34.2% at 3.5-Å resolution and relatively poor stereochemistry. A global temperature factor of 30 Å<sup>2</sup> initially was applied to all atoms. In subsequent refinement cycles, individual atomic temperature factors were refined, and a 2-fold noncrystallographic symmetry restraint was employed. The resolution was gradually increased during the refinement process so that all of the data between 20.0- and 2.4-Å resolution with magnitudes greater than 4 σ were included. After the entire model was rebuilt twice using 2Fo − Fc and Fo − Fc “omit” maps (i.e. maps with the atoms of the section being viewed deleted), the crystallographic R value decreased to 22.5% at 2.4-Å resolution. At this stage, the restraint on 2-fold noncrystallo-

FIG. 1. Stereo diagram (drawn with MOLSCRIPT (57)) showing an α-carbon tracing of the cAspAT dimer and the ligand-induced movement of the small domain that takes place when MeAsp binds to the active site of subunit 1 (solid thin lines). Crystal lattice contacts prevent the binding of MeAsp and small domain movement in subunit 2 (solid thick lines). Dashed lines show the position of the small domain of subunit 1 in the cAspAT-MeAsp complex. The positions of selected residues having structural or functional roles are labeled.
ligands. However, subunit 2 is always locked in the open conformation due to lattice contacts that inhibit its motion.

Secondary Structure—The secondary structure assignments shown in Fig. 3 are based on the stereochemical rules defined by Kabsch and Sander (34) as implemented in the program DSSP. With the exception of Ser296, the cAspAT and the cAspAT-MeAsp complex backbone torsion angles (the \( \phi \) and \( \psi \) angles of all of the nonglycine amino acids) fall in the stereochemically allowed region of the Ramachandran plot as produced by the program PROCHECK (35). Ser296 is located in the middle of an eight-residue loop that connects helix 11 and helix 12 (see Fig. 3), and its position is well defined in the cAspAT electron density map (with individual atomic temperature factors ranging from 7 to 12 Å\(^2\)). From a survey of well-refined high resolution protein structures, Herzberg and Moult (36) found that nonglycine residues that fall in the prohibited regions of the Ramachandran plot are usually associated with some aspect of protein function. This is clearly the case for Ser296, since it is part of the coenzyme binding site. Specifically, in both subunit 1 and subunit 2 the side chain hydroxyl group of Ser296 interacts via a water molecule with the phosphate group of the coenzyme on the opposite subunit. The same strained conformation has been observed for Ser296 in chicken mAspAT (16), chicken cAspAT (18), and \( E.\ coli \) cAspAT (20, 37). However, in the case of the mitochondrial isoenzyme, the authors also listed the following residues as falling in a disallowed region of the Ramachandran plot: Ser107, Ser167, Lys183, Tyr263, Ser296, since it is part of the coenzyme binding site.

Effects of Acetate and Formate Anions on the Structure of cAspAT—If the atoms of an atomic model are assumed to behave as isotropic harmonic oscillators, then the crystallographic atom temperature factor (B) is related to the average displacement (\( \mu \)) of an atom by the relationship \( B = 8\pi^2\mu^2 \). While the isotropic harmonic oscillator assumption is frequently inaccurate, crystallographic B values are, nevertheless, an empirical measure of atomic mobility (4, 33). In the case where groups of atoms move between two low energy conformations, the temperature factors can also be a measure of atomic occupancies.

In Fig. 2 the temperature factors of the backbone atoms of two cAspAT atomic models are compared; one is the result of refinement against diffraction data collected on crystals equilibrated with 40 mM acetate at pH 5.4, and the other is the result of refinement against data collected on crystals equilibrated with 40 mM formate at pH 5.4. In both atomic models the small domain of each subunit was positioned in the open conformation. The large differences in temperature factors between the two models are isolated to the residues of the small domain of subunit 1, and they reflect the acetate-induced disorder of the small domain of subunit 1. More detailed studies have shown that the open and closed conformations in subunit 1 are present at about equal occupancy in 40 mM acetate, whereas this equilibrium shifts almost completely to the open conformation in 40 mM formate\(^2\) (27, 28). Full occupancy of the closed conformation in subunit 1 can be achieved with very high concentrations (~300 mM) of acetate or dicarboxylate ligands. However, subunit 2 is always locked in the open conformation due to lattice contacts that inhibit its motion.

### Table I

|                | cAspAT | cAspAT-MeAsp |
|----------------|--------|--------------|
| R factor (%)   | 17.0   | 17.3         |
| Resolution range (Å) | 8.0–1.74 | 8.0–1.6 |
| Number of reflections | 88,868 | 108,895 |
| Number of water molecules | 348    | 325          |
| Average B factors (Å\(^2\)) | 24.1   | 22.8         |

### Deviations from ideal geometry

- Bonding distance (Å): 0.011 (0.010), 0.013 (0.010)
- Angle (1–3 distance): 0.026 (0.015), 0.028 (0.015)
- Planar (1–4 distance): 0.041 (0.030), 0.044 (0.030)
- Planar groups (σ): 0.013 (0.010), 0.014 (0.010)
- Chiral centers (Å\(^2\)) | 0.145 (0.080), 0.156 (0.080)
- Nonbonded distances (Å): 0.168 (0.20), 0.170 (0.20)
- Multiple torsion (Å): 0.171 (0.20), 0.168 (0.20)
- Hydrogen bond (Å): 0.163 (0.20), 0.156 (0.20)
- Torsion angles (degrees): 4.3 (5.0), 4.4 (5.0)
- Staggered (Å): 18.3 (15.0), 18.2 (15.0)
- Transverse (Å): 28.8 (25.0), 28.7 (25.0)
- Thermal factors (Å\(^2\)): 2.5 (2.0), 2.5 (2.0)
- Side chain (1–2 atoms): 3.8 (3.0), 3.8 (3.0)
- Side chain (1–2 atoms): 7.2 (4.0), 7.6 (4.0)
- Side chain (1–2 atoms): 10.8 (6.0), 11.2 (6.0)

*Numbers in parentheses are the target values used in PROLSQ (30).*

The 1.6-Å resolution data next were included in the refinement, and after several rounds of refinement and manual rebuilding the model included 408 water molecules and two acetate ions, and the R value decreased to 17.6% for the 2 σ data between 8.0- and 1.6-Å resolution. At this resolution the two acetate ions were observed very clearly in the active site of subunit 1 (but not in subunit 2) at the positions of the carboxyl groups of a bound substrate molecule. It is now clear that carboxyl can mimic the binding of substrate and induce the movement of the small domain in subunit 1. Detailed analysis has shown that under our standard crystallization conditions (i.e., in 40 mM acetate), both the open and closed conformations are present at approximately equal occupancy in subunit 1. Therefore, subsequent refinement was carried out using the 1.74-Å diffraction data set collected on cAspAT crystals soaked in 40 mM formate. The last stages of refinement included moving the two acetate ions in subunit 1 and rebuilding two alternative conformations of the side chain of Arg296 in subunit 2 (see below). Water molecules were retained in the model only if their temperature factors were less than 50 Å\(^2\).
Tertiary Structural Changes between cAspAT and the cAspAT-MeAsp Complex—The sieve-fitting procedures described by Gerstein and Chothia (39) and Lesk (40) were used to characterize structural differences between cAspAT and cAspAT-MeAsp complex. Specifically, the backbone atomic coordinates of subunit 1 of the native enzyme and of the cAspAT-MeAsp complex were superimposed with the least-squares method of Kabsch (41) as implemented in the program BMFIT (42). This superposition included all 412 residues and had an overall r.m.s. deviation of 1.24 Å. Of these, 160 backbone atoms (40 residues) of the two structures had deviations of 2.0 Å or more and were not included in the next superposition cycle. Several more iterations of superposition were carried out with progressively smaller threshold values until no backbone atoms showed a deviation of more than 0.4 Å, leaving a total of 1088 backbone atoms in each of the two structures.

This sieve-fitting protocol identified the following residues as structurally isomorphous in the native enzyme and the cAspAT-MeAsp complex: Val5–Gln11, Leu 50–Thr139, His 143–His193, Thr 198–Ala224, Glu 234–Asp312, and Glu 318–Met326. Thus, the small domain is defined as the N-terminal residues Ala12–Val49 and C-terminal residues Ala 327–Gln412, and the large domain as residues Leu 50–Met326. The N-terminal peptide, residues Ala1–Gln11, was not assigned to either domain because it extends away from each subunit to interact with the adjacent subunit (see Fig. 1).

Gerstein and Chothia (39) defined the “static core” of lactate dehydrogenase as those “residues that are both part of secondary structure and that remained after sieve-fitting.” Using this definition, the static core of the large domain consists of 632 backbone atoms that include the residues of all of the helices except H13 (i.e. residues 51–61, 77–88, 93–96, 107–122, 143–149, 170–179, 202–215, 234–245, 277–292, and 301–311) and all of the strands (residues 100–106, 133–137, 155–159, 161–162, 167–168, 185–189, 218–223, 250–255, and 267–273). The r.m.s. deviation between the backbone atoms of the large domain static core in the open and closed conformations is 0.14 Å, indicating that these residues are in fact motionless with respect to substrate binding within the error limits usually associated with high resolution structures.

After the large domain static cores of subunit 1 of the native enzyme and the cAspAT-MeAsp complex are superimposed, positional differences in the small domain of as much as 5 Å are observed (Fig. 4A). While the largest movements occur in small domain residues, large domain residues Gly227, Phe228, and Ala229 (which are located in the domain-domain interface) also show positional changes of as much as 2.5 Å.

When the small domains of subunit 1 in cAspAT and the cAspAT-MeAsp complex are superimposed, the r.m.s. residual of the backbone atoms is 0.86 Å. When compared with the corresponding residual of 0.29 Å for the large domain, this indicates that the tertiary structure of the small domain is
clearly altered between the open and closed conformations. To determine which parts of the small domain change, the sieving fitting analysis was carried out using the same criteria that were applied to the large domain. This allowed a static core for the small domain to be defined as most of the residues of four out of five helices (H14, H15, H16, and the second half of H13) and three β-strands (B1, B2, and B4). Since superposition the backbone atoms of these static core residues results in an r.m.s. deviation of only 0.23 Å, it follows that the residues outside the static core undergo significant tertiary structural changes as a result of the open-to-closed transition. In particular, residues in helix 1 (Leu16–Glu26) and the loop residues 36–49 at the N terminus of the small domain show the largest positional differences relative to those of the static core residues (see Fig. 4B).

The Active Sites of cAspAT and cAspAT-MeAsp—By forming an array of noncovalent interactions with the coenzyme-MeAsp adduct, active site residues Gly258, Lys258, Arg292, and Arg292 stabilize the external aldime of the cAspAT-MeAsp complex (Fig. 5). In particular, the α-carboxyl group of the substrate interacts with both Gly238 and Arg292. The interaction of Gly238 is brought about by a large shift in the loop containing Gly238 that positions the backbone nitrogen atom of Gly238 within hydrogen bonding distance (about 3.0 Å) of the α-carboxyl group. In the case of Arg292, its α-carbon shifts by about 1.2 Å toward the active site, and its side chain shifts by as much as 2.5 Å so that an ionic interaction neutralizes α-carboxyl group. The environment around Arg292 remains relatively unchanged in both open and closed conformations due to the coordinated movement of Gly238 and other small domain residues.

The substrate-Arg292 interaction involves a shift in the position of the side chain of Arg292 on the neighboring subunit. In the absence of substrate, two different conformations of the Arg292 side chain are observed, but only one conformation exists in the active site of the cAspAT-MeAsp complex, where a strong (3.0 Å) electrostatic interaction forms between the β-carboxyl group of MeAsp and the Arg292 guanidinium cation. The strength of this interaction greatly reduces the mobility of the side chain of Arg292 (its atomic temperature factors range from 8 to 15 Å²). Unlike Arg292, the substrate-induced movement of Arg292 does not involve a change in the position of its backbone atoms. The movement of the Arg292 guanidinium group by as much as 7.9 Å is accomplished by changes in two side chain dihedral angles (Δχ3 = 130°, and Δχ3 = 165°).

Transaldimination from the internal to the external aldime liberates the side chain of Lys258. The free ε-amino group of Lys258 has been proposed as the base that accepts a proton from the α-carbon atom of the substrate and as the acid that donates the proton to the formyl carbon to form the ketimine intermediate (43). Consistent with this proposal is the observation that the α-amino group of Lys258 in the MeAsp complex is only 2.9 Å from the C-4 atom of the external aldime. Because of the importance of this interaction, bias in positioning the side chain of Lys258 was reduced by omitting it in the penultimate stage of least squares refinement. Then the side chain of Lys258 was carefully fit into a 2Fo – Fc electron density map before its atomic coordinates and temperature factors were refined in the final series of least squares refinement cycles.

The Interface between the Large and Small Domains—Although the ligand-induced movement of the small domain is large, many of the interactions between the large and small domains in the MeAsp complex are the same as those of native cAspAT. In particular, the number of hydrogen bonds (14 and 13 for the open and closed forms, respectively, including the direct and water-mediated hydrogen bonds) and the number of van der Waals contacts (16 and 14 for the open and closed forms, respectively) do not change much in the two structures. In addition, little change is seen in the number of water molecules associated with the domain interface (10 in the open conformation versus 11 in the closed conformation), and the buried surface area of the interface remains constant in the two structures. This high degree of structural isomorphism in the domain-domain interface suggests that there is only a small energy barrier between the open and closed conformations, which is consistent with the estimated minimum value of 2–3 kcal/mol estimated by Pfister et al. (44).

Most of the domain-domain interface interactions are formed by residues that are elements of loop structure. Two of these loop regions come from the small domain (residues 48–49 and 356–360), and three come from the large domain (residues 192–199, 225–229, and 258–263). In particular, small domain residues 356–360 and large domain residues 192–199 form an extensive set of interactions in both the open and closed conformations, creating the major framework of the interface. It is also interesting to note that unlike other residues of the large domain, the peptide composed of residues Phe228, Ala229, and Ser230 (residues that are not part of the active site) has two distinct conformations that depend on the ligation stage (see Fig. 4A). The structural basis for this ligand-induced movement is unclear at present.

DISCUSSION

Rigid Body Rotation of the Small Domain—To a first approximation, domain closure in cAspAT can be described by a rigid body rotation of the small domain relative to the large domain. This rigid body movement of the small domain core, expressed...
as a screw rotation about a unique axis, was calculated after the large domain static core residues of subunit 1 in the native enzyme and the MeAsp complex were superimposed (Fig. 6). The components of the screw rotation amount to a rotation of 10.3° and a translation of 0.4 Å. The screw rotation axis is almost parallel to the molecular dyad (it actually makes an angle of 2° with the dyad), and it passes through the domain-domain interface near Met326 in helix 13 and residues from two turns (residues 357–360 and 193–196). Since the very long helix 13 bridges between the large and small domains, the location of the screw axis next to helix 13 allows domain closure to take place without a large (energetically costly) distortion of the helix. Instead, the open-to-closed movement of the small domain takes requires only a small bend in helix 13 at Met326 (Fig. 6).

The ligand-induced movement of the small domain is not a pure rigid body motion, however. Superposition of the open and closed forms of the small domain shows that the movement of the loop from Gly36 to Val42 is not completely linked to the motion of the core residues of the small domain (Fig. 4B). This deviation from pure rigid body motion has its origin in large changes of the conformational angles of two residues, Gly36 and Gly38.

The Importance of Gly36 and Gly38 in Small Domain Movement—There are many examples of ligand-induced domain closure (e.g., adenylate kinase (45) where the open-to-closed transition includes significant changes in the backbone φ,ψ angles of “joint residues” between the mobile domain and the remainder of the protein (46)). To detect joint residues in cAspAT, the magnitudes of the changes in backbone torsion angles between the open and closed conformations were calculated as a “distance” in φ,ψ space (φ,ψ distance = (φ² + ψ²)½) and plotted versus residue number (Fig. 7). By far the largest changes in φ,ψ angles were observed for two glycine residues, Gly36 (Δφ = 8.4°, Δψ = 94.3°) and Gly38 (Δφ = 44.7°, Δψ = 60.3°). The φ,ψ angles of Gly36 change from the allowed region for nonglycine residues in the Ramachandran plot (φ = −95.3°, ψ = −11.7°) to the forbidden region for nonglycine residues (φ = −86.9°, ψ = −106°) in response to ligand binding, while the corresponding large φ,ψ changes for Gly38 take place within the allowed region for nonglycine residues (φ = −125.5°, ψ = 28.1° to φ = −80.8°, ψ = 88.4°). All other residues, including small domain residues at the domain-domain interface (i.e. residues 49–50 and 326–327), showed much smaller or no significant changes in backbone torsion angles. Similar backbone conformational changes in Gly36 and Gly38 have been observed in E. coli AspAT (37) and mAspAT (47).

These observations and the fact that residues 36 and 38 have been conserved as glycine in all AspATs sequenced (38) indicate that the Gly36-Val37-Gly38 peptide is a very interesting target for mutagenesis experiments. To date, studies have been carried out with the site-directed mutants G36A, V37A, G38A, and G38S (48, 49). Relative to wild-type cAspAT, the G38A and G38S mutations, respectively, decrease $k_{cat}$ from 230 s⁻¹ to 11.6 s⁻¹ and 1.44 s⁻¹ and increase $K_m$ for aspartate from 1.85 mM to 7.9 mM and 50 mM (48). Thus, the catalytic efficiency of the enzyme ($k_{cat}/K_m$) decreases by a factor of 0.012 for G38A, and by 0.00023 for G38S, confirming the predicted requirement for glycine at this site. X-ray diffraction studies of G38A and G38S (48) showed that these mutations do not perturb the structure of the unliganded enzyme in the open conformation, implying that the altered kinetic parameters reflect impairment of the substrate-induced closure of the small domain and/or the interactions that stabilize the enzyme-substrate complexes. In fact, x-ray studies of G38A and G38S crystals soaked in 300 mM MeAsp (28) have shown that the open-to-closed equilibrium of the small domain is shifted greatly toward the open conformation relative to the MeAsp complex of the wild-type enzyme under the same conditions. Furthermore, modeling the G38A-MeAsp and G38S-MeAsp complexes with the small domain in the closed conformation indicates that the α-carbon of Ala38 or Ser38 would be only 3.0 Å from the MeAsp 2-methyl group. Thus, the prevention of direct steric conflict with substrate-pyridoxal 5’-phosphate intermediate complexes is very likely to be the reason residue 38 has been conserved as glycine.

In the case of G36A, $k_{cat}$ is decreased 11-fold, but the $K_m$ for aspartate is increased only by a factor of 1.4. Although crystallographic data are not yet available for G36A, analysis of the wild-type enzyme (see above) predicts that an alanine mutation should inhibit transition to the closed conformation because residue 36 normally assumes φ,ψ angles in the closed conformation that only are appropriate for glycine. Therefore, inhibiting the open-to-closed transition in this way has a large impact on the enzyme’s catalytic efficiency.

In contrast to the Gly36 and Gly38 mutations, the V37A mutation has no measurable effect on substrate turnover as measured by $k_{cat}$ (48), suggesting that the stereochemical mechanism for domain closure has not been compromised. The crystallographic data are consistent with this interpretation of an unaltered $k_{cat}$ value in that the structure of the unliganded enzyme in the open conformation is not perturbed (48), and MeAsp induces closure of the small domain in V37A as it does in the wild-type enzyme (28). However, the V37A mutation...
does increase the $K_m$ for aspartate by a factor of 6.81, and the $K_d$ for MeAsp is increased 4.1-fold (48). This may reflect an increase in local dielectric constant (Val37 is 3.8 Å from the MeAsp α-carboxyl group) that weakens polar and ionic interactions between the α-carboxyl group of the incoming substrate molecule and Gly38 and Arg386.

Differences in Small Domain Movement in AspAT Molecules from Different Sources—Different values for the magnitude of movement of the small domain have been reported for the various forms of AspAT that have been studied crystallographically. In chicken mAspAT, a ligand-induced rotation of 13.6° was observed (16), while in the case of E. coli AspAT the binding of MeAsp is accompanied by only a 5–6° rotation of the small domain (20, 37, 50). Smith et al. (50) and Okamoto et al. (37) concluded that the basis for this difference is that the unliganded small domain of E. coli AspAT is not opened as much as it is in mAspAT. On the other hand, Malashkevich et al. (18) compared the closed conformations of cAspAT and mAspAT from chicken and concluded that ligand binding induces the small domain of mAspAT to close more fully than it does in cAspAT, with the movement of N-terminal helix 1 showing the biggest difference between the two isoenzymes. McPhalen et al. (47) also noticed that helix 1 in mAspAT moves differently relative to the rest of the small domain residues, and they showed that helix 1 is rotated further by 10° relative to the core of the small domain. Our analysis of pig cAspAT indicates that helix 1 rotates only an additional 3.0° relative to the static core of the small domain. Differences in the movement of helix 1 between isoenzymes may to be due to a sequence difference at position 15 (i.e., Asp15 in mAspAT versus Val15 in cAspAT), a residue in the small domain located just before the helix. In the closed conformation of mAspAT, the side chain carboxyl group of Asp15 maintains an ionic interaction with the guanidinium group of the buried residue Arg292 in the active site. This interaction could stabilize helix 1 of mAspAT in a more closed position.
Ligand-induced Conformational Changes in cAspAT

Coenzyme Movement in AspAT—During transaldimination the coenzyme undergoes a large conformational change in cAspAT (Fig. 5 and Table II). Specifically, the $\chi$ and $\phi$ torsional angles (as defined in Fig. 8) vary, respectively, from values of $+36^\circ$ and $+41^\circ$ in the internal aldimine (the average of three values as described in Table II) to $-27^\circ$ and $+68^\circ$ in the external aldimine (subunit 1 of the cAspAT-MeAsp structure). The internal-to-external aldimine transition, however, does not result in large changes of the $\psi$ and $\theta$ angles (Table II). The $\chi$ rotation (i.e. the apparent rotation about the C-4’--C-4 bond) is simply the result of the exchange of the Lys258 $N$-$\epsilon$--C-4’ internal aldimine bond for the new external aldimine MeAsp N--C-4’ bond. On the other hand, the change in $\phi$ reflects a true rotation about the C-5–C-5’ bond. As first predicted by Karpeisky and Ivanov (11), it is this rotation that shifts the position of the C-5’ carbon of the coenzyme about 2 Å from a noncovalent N . . . C-4’ distance in the enzyme-substrate Michaelis complex to the covalent N--C-4’ bond in the external aldimine intermediate.

The conformations of the internal and external aldimines as described above are significantly different from the corresponding coenzyme conformations described in earlier reports that were based on lower resolution x-ray studies of cAspAT and the cAspAT-MeAsp complex (21, 51, 52). In particular, an unrefined 2.7-Å electron density image of the internal aldimine was fit to an atomic model with a 2.7-Å electron density image of the internal aldimine was fit to the cAspAT-MeAsp complex (21, 51, 52). In particular, an unrefined coenzyme conformations described in an earlier report that involved 120° rotations about the O-5--P bond (51). How-ever, since the much higher resolution images of cAspAT and the cAspAT-MeAsp complex now show that the low resolution analysis of the internal aldimine conformation was incorrect, there is no need to invoke the more elaborate phosphate rotor mechanism for the internal-to-external aldimine transition. The simpler mechanism of Karpeisky and Ivanov (11) seems more likely in the case of the cytosolic isoenzyme.

This issue is still not completely resolved, however. High resolution structures of the mAspAT-maleate complex and the mAspAT-MeAsp complex indicate that in the mitochondrial isoenzyme $\phi$ varies from $-16^\circ$ to $+85^\circ$ during the internal-to-external aldimine transition, thus requiring the C-4’--H group to pass through an intermediate eclipsed position with O-5’ if the Karpeisky and Ivanov mechanism is assumed (13, 20). In the case of E. coli enzyme, one high resolution (1.8-Å) study indicates that the internal-to-external aldimine transition involves a $\phi$ rotation of $-16^\circ$ to $+110^\circ$ (37), whereas another study at 2.5-Å resolution (under slightly difference crystallization conditions) documents a corresponding $\phi$ rotation of $+46^\circ$ to $+73^\circ$ (20). Assuming all of the current, highly refined AspAT structures are reasonably accurate, the diversity of coenzyme conformations implies that a range of conformations are energetically accessible in the native enzyme. If one assumes the Karpeisky and Ivanov mechanism is valid in all forms of AspAT, then the movement of the C-4’--H group through an intermediate eclipsed position with O-5’ may not be energetically prohibitive. Alternatively, the phosphate rotor mechanism may be operative in some cases.

Hydrophobic Free Energy as the Driving Force in Domain Closure—The changes in solvent-accessible surface area between cAspAT and cAspAT-MeAsp are displayed in Fig. 9. Although the ligand-induced conformational changes are large, with movements of more than 5 Å in subunit 1, less than a 2% increase in buried surface area occurs as a result of the open-to-closed transition (i.e. a change from 15,300 Å$^2$ in the open conformation to 15,030 Å$^2$ in the closed conformation). Most residues show very small, insignificant differences in buried surface area, with even the mobile surface residues displaying changes of less than ± 20 Å$^2$ for the most part. As expected from the above discussion of domain-domain interactions, the interdomain residues do not show any noticeable differences in buried surface area, confirming that these residues maintain a closely packed interface in both the open and closed conformations.

However, the open-to-closed transition does result in some large localized changes in buried surface area for a few residues of subunit 1 as well as for a pair of residues (Tyr$^{79}$ and Arg$^{282}$) in the large domain of subunit 2. With the exception of Arg$^{166}$ in subunit 1, all of these large changes are the result of increases in buried surface area that are coupled to the binding of substrate and the movement of small domain residues over the active site. (Arg$^{166}$, a surface residue of the large domain, is more exposed to solvent after the open-to-closed conformational change, and its side chain torsion angles change significantly.)

**Fig. 7. Differences in the backbone conformational angles of subunit 1 of the native enzyme versus those in subunit 1 of the enzyme-MeAsp complex.** The differences is calculated as the "distance" in $\phi,\psi$ space ($\phi,\psi$ distance = $(\phi^2 + \psi^2)^1/2$).

| Angle | Atoms | cAspAT | cAspAT-MeAsp |
|-------|-------|--------|--------------|
|       |       | Subunit 1 | Subunit 2 | Subunit 1 | Subunit 2 | Internal aldimine$^b$ |
| $\chi$ | N--C-4’--C-4--C-3 | 42° | 30° | -27° | 37° | 36 (6) |
| $\phi$ | C-4--C-5--C-5’--O-5’ | 38° | 48° | 68° | 36° | 41 (6) |
| $\psi$ | C-5--C-5’--O-5’--P | -173° | -168° | 176° | -172° | -171° (3) |
| $\theta$ | C-5’--O-5’--P--OP | 109° | 92° | 110° | 110° | 104 (10) |

$^a$ Angles averaged over subunits where the coenzyme exists as the internal aldimine (i.e. cAspAT subunit 1, cAspAT subunit 2, and cAspAT-MeAsp subunit 2).

$^b$ S.D. value is shown in parentheses.
molecules will form around the apolar atoms. On the other hand, without the compensating negative charges of the substrate carboxylic groups, the positively charged arginines 386 and 292 require the open conformation in order to have access to water and counterions in the solvent. Thus, Arg\(^{386}\) and Arg\(^{292}\) are not buried in the open conformation, but this is at the expense of exposing the apolar side chains of the hydrophobic plug residues to solvent. The binding of substrate shifts this equilibrium by electrostatically neutralizing the positively charged side chains of Arg\(^{386}\) and Arg\(^{292}\), allowing them to be buried by the hydrophobic plug as it closes over the active site.

Hydrophobic forces are thought to be the dominant forces in protein folding and in protein-ligand binding, and the free energy associated with the burial of hydrophobic atoms has been estimated to be approximately 24 cal/(mol Å\(^2\)) (55, 56). In the case of domain closure in cAspAT, the calculated free energy for burying the 222 Å\(^2\) of the hydrophobic plug amounts to 5.3 kcal/mol. This is more than enough energy to drive the conformational change, which has been estimated to require 2–3 kcal/mol subunit (44). It is important to also note that the apolar character of the hydrophobic plug is well conserved (except for one residue) for the 11 known vertebrate AspAT sequences as well as for E. coli AspAT (38). The sequence of the hydrophobic plug is of the form H-X-H-H-H, where H represents a hydrophobic residue, and X corresponds to Val in cAspAT or Asp in mAspAT and E. coli AspAT. In 4 out of 5 cytotoxic enzymes, the sequence Pro\(^{14}\)-Val\(^{15}\)-Leu\(^{16}\)-Val\(^{17}\)-Phe\(^{18}\) is found, with alanine replacing Leu\(^{16}\) in the chicken enzyme.

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![Fig. 8. Atom labeling convention and definition of torsional angles for the internal alldine.](image)

![Fig. 9. Changes in the solvent-accessible surface area of subunit 1 residues (A) and subunit 2 residues (B) that are associated with the open-to-closed transition in subunit 1.](image)
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