Modeling the Three-Dimensional Structures of Bacterial Aminotransferases†

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ABSTRACT: The refined crystallographic structure of the "closed" conformation of chicken mitochondrial aspartate aminotransferase has been used as a template for the construction of models of the two *Escherichia coli* aminotransferases encoded by the *tyrB* and *aspC* genes. The main results are as follows: (1) Only minor changes are required in the coordinates of the backbone atoms to accommodate the large number of substituted side chains. (2) All deletions and insertions required to allow maximum primary sequence alignment are on the solvent-accessible surface. (3) Charged residues are all located on the surface, in contact with solvent, except for certain conserved active site residues. (4) The close packing within the hydrophobic core is maintained. (5) The interactions between the subunits are maintained. (6) Modeling of tyrosine as an external aldimine into the active sites points to several residues that could be involved in determining the substrate specificities of these aminotransferases.

The metabolically important interconversion of amino acids and their corresponding keto acids is accomplished by the family of aminotransferases (EC 2.6.1.*x*). On the basis of criteria such as substrate specificity, primary sequences, subunit molecular weights, and quaternary structures, over 60 distinct enzymes of this family have been identified from both eukaryotic and prokaryotic sources [see Christen and Metzler (1985) for a recent collection of reviews on various aminotransferases].

The most thoroughly studied of these are the mitochondrial and cytosolic aspartate aminotransferase isozymes (mAAT[1] and cAAT, respectively) from higher vertebrates (EC 2.6.1.1). Both of these enzymes are composed of two identical subunits containing around 400 amino acid residues. There are two noninteracting active sites per dimer which contain residues from both of the subunits (Jansonius & Vincent, 1987). Each subunit contains a large and a small domain. On the basis of several crystallographic structures of chicken mAAT containing various substrate analogues and inhibitors a detailed mechanism of catalysis has been proposed for dicarboxylic acid substrates (Kirsch et al., 1984; Jansonius & Vincent, 1987). Briefly, this involves orientation of the substrate in the active site by interaction of the carboxyl functions with two arginine residues. This results in a movement of the small domain which effectively closes off access to the active site and generates the "closed" form of the enzyme. The PLP coenzyme forms an external aldimine with the substrate prior to the transfer of the substrate amino function to the coenzyme and subsequent release of the keto acid. The release of product requires the return of the small domain to its original position, generating the "open" form of the enzyme. A crucial step in the catalytic pathway is the 1,3 proton transfer. Lysine-258 functions as proton acceptor and donor during this process.

Recently, the cDNA sequences coding for several aminotransferases have been determined. These include eukaryotic cDNAs coding for mAAT (Jaussi et al., 1985; Joh et al., 1985; Obaru et al., 1986), ornithine AT (Mueckler & Pitot, 1985), and tyrosine AT (Grange et al., 1985) and the *Escherichia coli* genes coding for branched-chain amino acid AT (*ilvE*) (Kuramitsu et al., 1985a), phosphoserine AT (*serC*) (Duncan & Coggins, 1986), aspartate AT (*aspC*), and tyrosine AT (*tyrB*) (Fotheringham et al., 1986; Kuramitsu et al., 1985b,c).

From a comparison of the available primary sequences it is evident that four of these enzymes show 40–50% identity with each other. These are mAAT, cAAT, and the *aspC* and *tyrB* gene products. (The latter two enzymes will subsequently be referred to as eAAT and eTAT, respectively.) The other aminotransferases show no obvious identity either with this group or with each other.

The four homologous enzymes show remarkable differences in their catalytic properties: cAAT and mAAT catalyze the transamination of aromatic substrates very poorly, eTAT can use aromatic compounds very efficiently, and eAAT possesses intermediate properties (Mavrides & Orr, 1975; Mavrides & Christen, 1978; Powell & Morrison, 1978).

The availability of the cloned genes makes it possible to probe structure–function relationships of these enzymes by using the technique of site-directed mutagenesis. The rational design of mutation experiments requires the availability of a high-resolution three-dimensional structure. Unfortunately, the only crystallographic structures for which accurate coordinates are available at present are those of m- and cAAT (Ford et al., 1980; Jansonius et al., 1985; Arnone et al., 1985). However, a crystallographic structure determination of eAAT is under way (Smith et al., 1986).

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[1] Abbreviations: AT, aminotransferase; mAAT and cAAT, chicken mitochondrial and pig cytosolic aspartate aminotransferases; eAAT and eTAT, *Escherichia coli* aspartate and tyrosine aminotransferases; PLP, pyridoxal 5′-phosphate; PPL-iodotyrosine, *N*-(5′-phosphopyridoxyl)-3-iodo-L-tyrosine.

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We have modeled the three-dimensional structures of eAAT and eTAT using the backbone fold of chicken mAAT in its closed conformation as the initial template. This approach has been applied to several other proteins. [See Ripka (1986) and Blundell et al. (1987) for reviews.]

Our results show that, even though the primary structure identity between the aminotransferases is only around 40%, it is possible to build stereochemically acceptable structures for both bacterial enzymes with minimal changes to the backbone fold. From the models we are able to identify several residues that could be involved in determining the substrate specificities of the enzymes. The involvement of these residues is not apparent from an alignment of the primary sequences.

Materials and Methods

The model building was performed on an Evans and Sutherland PS300 graphics system interfaced to a VAX 11/730 using a Rice University version of the program FRODO (Jones, 1978) and an Iris 3130 graphics system by Silicon Graphics using the program MENDYL by Tripos Associates.

The template used for the constructions was that of chicken heart mAAT–maleate complex. This crystallizes in the orthorhombic space group $C222_1$, with one subunit per asymmetric unit. The structure is in the so-called closed conformation (Jansonius et al., 1985) and has been refined to an R factor of 15.9% at 2.3-Å resolution (M. Vincent, unpublished results).

The alignment of the amino acid sequences was taken from Fotheringham et al. (1986). Stereochemical regularization of the structures was performed with the restrained least-squares refinement program PROLSQ of Hendrickson and Konnert (1980) as modified by Vincent and Priestle (1985). The residue numbering scheme used below follows that of the pig cytosolic enzyme, in line with common praxis (Christen & Metzler, 1985). The use of the symbol "*" indicates that the residue in question is from the neighboring subunit. The described orientation of the molecule, and in particular that of the active site, is the same as that used in Kirsch et al. (1984).

The procedure followed in building the models of both eAAT and eTAT was as follows: (i) The primary sequences of the bacterial enzymes were aligned with that of mAAT (Fotheringham et al., 1986). (ii) Where necessary, the mAAT residues were replaced by the appropriate residues to generate the required bacterial protein primary sequence. This involved 236 and 235 substitutions to generate eAAT and eTAT, respectively. The spacial environment of each new residue was checked visually for close contacts or overlap with neighboring residues. The main problems that were encountered involved new arginine, methionine, and lysine residues which were originally inserted into the structure in an extended conformation. However, in each case it was possible to remove unacceptable van der Waals contacts and to model the side chains into stereochemically acceptable conformations by rotations around one or more side-chain bonds. (iii) During step ii the mAAT backbone fold used in the model proteins was not altered in any way. The optimal alignment of the primary sequences, however, required deletions at positions 3–4, 129–130, and 232 in both eAAT and eTAT plus an insertion at position 64 in the case of eTAT. It was found that all these sites were on the surface of the molecules and that the necessary interruptions of the existing path of the polypeptide chain could be accommodated with only minor manipulation of the backbone torsion angles in the immediate vicinity of the changes (less than three residues away). Finally, several residues were deleted at both the N- and C-termini.

Table I: A Comparison of the Residues in mAAT, eAAT, and eTAT in the Subunit Interface above the Active Site

residue no.	mAAT	eAAT	eTAT	residue no.	mAAT	eAAT	eTAT
41	R	K	Y	68*	K	K	S
46	K	K	I	69*	E	N	L
67*	D	T	A				

(iv) The final step involved subjecting the models to several cycles of stereochemical regularization, by use of the program PROLSQ (Hendrickson & Konnert, 1980). Following this the structures were examined individually and compared with that of the parental mAAT.

The entire procedure was carried out on an individual subunit. The dimer was generated with the appropriate 2-fold symmetry operator, and the subunit–subunit contacts were then checked visually. Only minor changes to side-chain torsion angles were needed to relieve bad contacts.

The modeling of substrates in the active sites was achieved by using the coordinates of the external aldimine complex formed between PLP and 2-methylaspartate (Jansonius & Vincent, 1987) as an initial template. This complex is isomorphous with the enzyme–maleate complex and has recently been refined at 2.3-Å resolution to an R factor of 14.7% (M. Vincent, unpublished results). The aspartate side chains was replaced by that of tyrosine. Subsequently, the side chains of both this tyrosine and neighboring active site residues were rearranged to relieve bad contacts.

Results and Discussion

Overall Polypeptide Chain Folding. Inspection of either of the model protein backbones when superimposed upon that of mAAT clearly shows that only very minor changes in the atomic coordinates are required to accommodate the large number of substituted residues within the core. The superimposed α-carbon skeletons of the mAAT and either bacterial enzyme structure are essentially indistinguishable in all regions of the enzyme except in the regions of deletion and insertion. Slightly larger displacements are observed here (on the order of 2–3 Å). However, all these distortions are very localized and extent no more than three residues from the particular site. As mentioned above, all these regions are at the surface of the protein where it can be expected that distortions in the backbone fold are unlikely to disrupt the overall packing. This was found to be the case. Furthermore, none of these sites are in functionally significant regions such as the active site or subunit–subunit interface.

The root mean square deviation for all the α-carbon atoms relative to mAAT is 0.35 Å for eTAT and 0.47 Å for eAAT. These values include regions of deletion and insertion where 2–3-Å shifts of backbone atoms occur.

Charged Residues. The distribution of charged residues in the mitochondrial and bacterial enzymes is very different. An analysis of the primary structures shows that there are between 60 and 70 positions at which charged and uncharged residues are interchanged between mAAT and either bacterial enzyme. In spite of this large reorganization of the charge distribution we find that the charged residues are still all located on the solvent-accessible surface of the model proteins (except for the totally conserved Asp-222, Arg-292*, and Arg-386, which are all important in catalysis).

Worthy of note is the different electrostatic potential in the region above the active sites of mAAT and eTAT. This involves residues 41 and 46 from the subunit which contributes the majority of the active site residues and residues 67*, 68*, and 69* from the other subunit. As shown in Table I, this

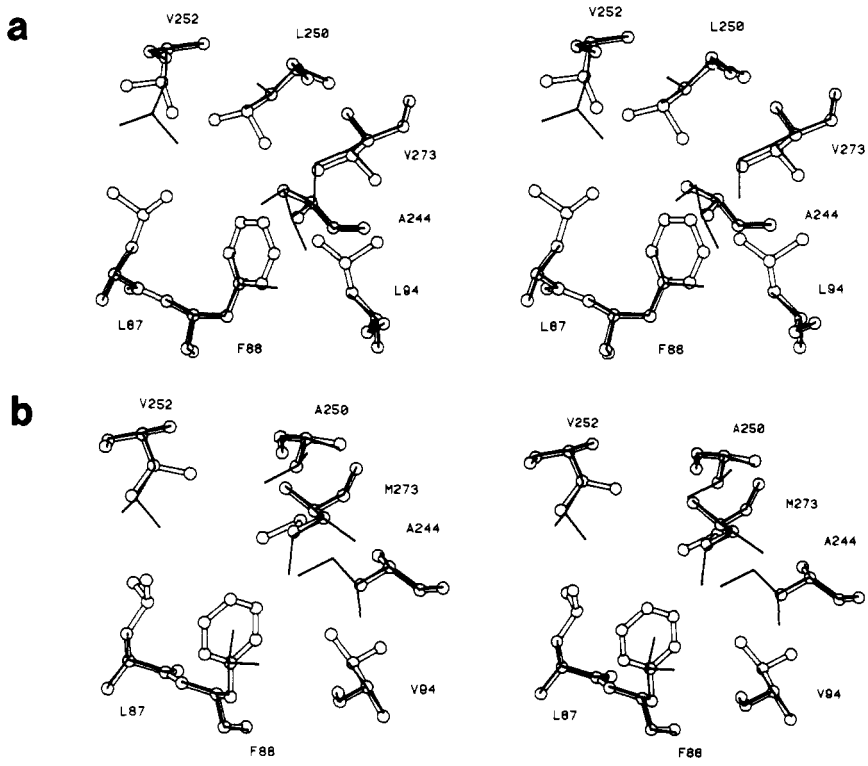

FIGURE 1: Stereo drawing of some core residues in the models of (a) eAAT and (b) eTAT superimposed upon those in the mAAT structure. In both cases the hydrophobic packing is maintained, in spite of the changes in the residues. The residues of the bacterial enzymes are depicted with open bonds and are labeled in single-letter code; those of mAAT (unlabeled) are superimposed with solid bonds.

Table II: A Comparison of Some Residues in mAAT, eAAT, and eTAT, the Side Chains of Which Pack Together[a]

residue no.	mAAT	eAAT	eTAT	residue no.	mAAT	eAAT	eTAT
87	A	L	L	250	V	L	A
88	L	F	F	252	L	V	V
94	A	L	V	273	I	V	M
244	I	A	A				

[a]The spatial relationships between these residues is illustrated in Figure 1.

region, which is highly charged in mAAT and eAAT, is occupied by uncharged residues in eTAT. It is too far from the active site to be involved directly in substrate binding or catalysis, but a long-range effect, such as electrostatic steering of charged substrates (Getzoff et al., 1983), is a possibility.

Another interesting feature is the presence of additional charged residues in eTAT, namely, Glu-141 and Arg-293*, which are accessible to solvent but situated within the active site region. The other two proteins have no charged residues in these positions. This phenomenon will be discussed under The Active Sites.

Buried Residues. Perhaps the strongest indication that the models we have constructed must reflect the real structures reasonably accurately comes from the observation of "compensatory residue changes". These involve two or more residues, juxtaposed in space, that are replaced by other residues in such a way that the packing remains compact with acceptable van der Waals distances.

As an example, consider the seven residues listed in Table II and illustrated in Figure 1. This group of residues is buried in the large domain (Jansonius et al., 1985). Neither eAAT nor eTAT has the same residues as mAAT in any of the listed positions. It can be expected that any unfavorably close contacts in this region would result in a structural rearrangement. However, from an inspection of Figure 1 it can be seen that the replacements are highly complementary: the existence of a bulkier side chain in one position (e.g., Leu-88 → Phe for mAAT → eAAT) is compensated by smaller side chains at another (e.g., Leu-252 → Val, Ile-273 → Val, and Ile-244 → Ala). In this way, stabilizing van der Waals contacts are maintained without any distortion to the polypeptide chain fold. Large distortions would have had unpredictable ramifications throughout the rest of the protein and would have disqualified mAAT as a template.

The Subunit–Subunit Interface. The residues at the subunit–subunit interface are also discussed under Charged Residues and The Active Sites. An examination of the interface shows that the contacts found in mAAT are maintained in the bacterial enzymes. Thus, for example, consider the interaction of the N-terminal arm of each subunit with the adjacent subunit. This interaction surface contributes at least as much to the integrity of the dimer as the interface area near the 2-fold axis (Jansonius et al., 1985). In mAAT this surface mainly involves Trp-5, Trp-6, and Val-9 in the N-terminal arm and Phe-122*, Phe-123*, Val-251*, Val-272*, and Val-283* in the adjacent subunit. In both of the bacterial enzymes Trp-5 and Trp-6 are replaced by Met and Phe residues. In the adjacent subunit there are compensating bulkier hydrophobic residues at positions 251* and 272* (Ile and Leu in eAAT and Leu and Val in eTAT) which pack closely with Met-5 and Phe-6. Val-9 in mAAT is replaced by Ile in eAAT. There is a compensatory change in the adjacent subunit where Val-283* in mAAT is replaced by Ala in eAAT.

The Active Sites. The most striking difference between the three enzymes resides is in their substrate specificities. We have presented evidence above that the fold of the polypeptide backbone is essentially identical in all three species. Therefore, the explanation for the distinct catalytic properties of the enzymes must lie in the actual chemical and physical properties of the individual side chains within the active site. The relevant residues are listed in Table III. Out of the 28 residues listed,

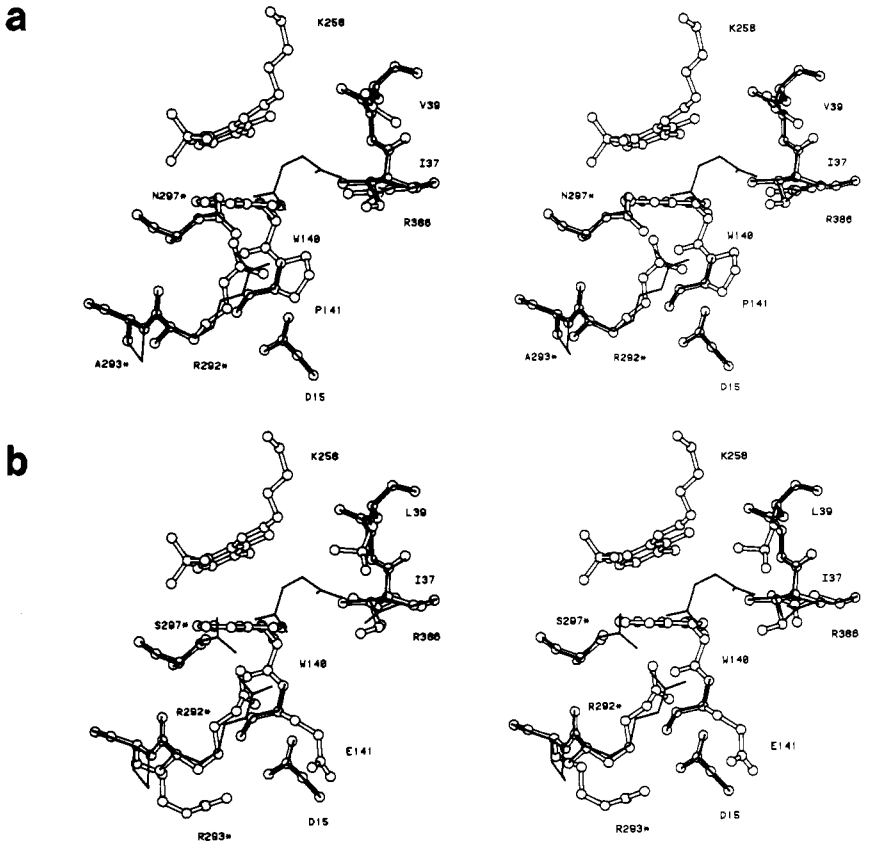

FIGURE 2: Stereo drawing of the active site regions in the models of (a) eAAT and (b) eTAT (open bonds) superimposed upon that in the mAAT structure (solid bonds) to illustrate the main differences between the substrate binding sites. The eAAT and eTAT residues are labeled in single-letter code. PLP is shown as the internal aldimine with Lys-258. The inhibitor maleate in the mAAT active site is also shown, hydrogen-bonded/salt-bridged to Arg-386 and Arg-292*. The positions of Trp-140 and the internal aldimine in the models are identical with those in the mAAT structure and have, in the latter, been omitted for clarity. The completely conserved residues Asp-15, Gly-38, Arg-386, and Arg-292* are included. All the other residues shown, namely, 37, 39, 141, 293*, and 297, are not the same in all three enzymes.

Table III: A Comparison of the Residues in mAAT, eAAT, and eTAT Which Form the Active Site[a]

residue no.	mAAT	eAAT	eTAT	residue no.	mAAT	eAAT	eTAT
15	D	D	D	193	H	H	N
17	I	I	I	194	N	N	N
18	L	L	L	222	D	D	D
37	V	I	I	224	A	A	A
38	G	G	G	225	Y	Y	Y
39	A	V	L	255	S	S	S
70*	Y	Y	Y	258	K	K	K
107	S	G	G	266	R	R	R
108	G	G	G	292*	R	R	R
109	T	T	S	293*	P	A	R
140	W	W	W	296*	S	S	S
141	G	P	E	297*	N	N	S
142	N	N	N	360	F	F	F
143	H	H	H	386	R	R	R

[a] Those positions at which different residues are found are underlined.

differences are found at only six positions between mAAT and eAAT and at eight positions between mAAT and eTAT. The high degree of similarity of the active sites must reflect the underlying similarity of the transaminase reactions catalyzed by the three enzymes.

How could the few dissimilar residues bring about the very different substrate specificities? The variable residues at positions 107, 109, and 257 interact with the coenzyme but not with the substrate. Therefore, it is unlikely that these residues are significant modulators of substrate specificity. The variable residues that line the substrate binding pocket can be divided into three groups: (i) the hydrophobic pair at positions 37 and 39 which lie at the top right-hand side of the pocket; (ii) the pair at positions 141 and 293* which lie to the bottom and left of the pocket; (iii) residue 297* which lies to the left of the pocket. This last residue is too distant to be a major contributor to the binding of aromatic substrates. The discussion below, therefore, deals only with the residues in categories i and ii. Figure 2 emphasizes the different residues in the substrate binding region in the active sites in the eAAT and eTAT models relative to mAAT.

Modeling a Catalytic Intermediate. The role of the above residues was further explored by fitting the Schiff base of PLP and tyrosine [external aldimine (Braunstein, 1973)] into the active site. During this procedure the PLP moiety was held in a position identical with that found in the 2-methylaspartate external aldimine complex of mAAT (i.e., the closed form), and the imine linkage was fixed in a conformation that is coplanar with the coenzyme pyridinium ring.

A crystallographic structure of apo-mAAT with PPL-iodotyrosine bound to it has been solved previously at 2.8-Å resolution (Eichele, 1980). The enzyme exists in the *open* form, and the hydroxyphenyl group is oriented toward residues 37–39. The α-carboxyl group interacts with Arg-292* (rather than Arg-386), and the α-proton points *away* from the proton-transferring ϵ-amino group of Lys-258. This is obviously a nonproductive binding mode. The existence of the bulkier side chains at positions 37 and 39 in eAAT and eTAT (Table III) makes it sterically impossible to fit the substrate aromatic side chain into the same position as in mAAT. It would, therefore, appear the bulkier replacements at positions 37 and 39 may play a role in preventing aromatic substrates from

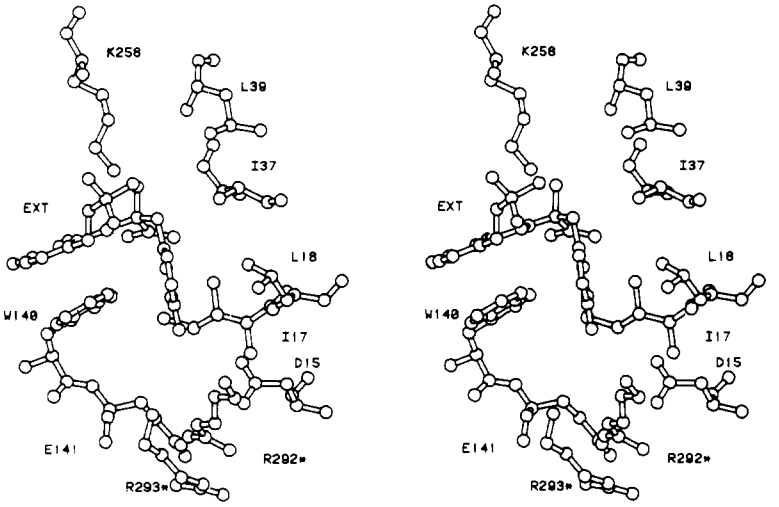

FIGURE 3: Stereo drawing of the proposed conformation of the PLP-tyrosine external aldimine intermediate in the active site of eTAT. The phenolic ring is sandwiched between Trp-140 and Leu-18. There is a methyl group attached to the α-carbon atom of the substrate to emphasize that the α-proton would be in the correct position to be transferred to Lys-258.

binding in the nonproductive orientation.

Is it possible to fit the aromatic substrate complex into the active site in a productive mode? This involves the α-proton pointing *toward* Lys-258 and, therefore, being available for transfer, and the α-carboxylate toward Arg-386, thus inducing domain closure. One possible orientation of the tyrosine Schiff base is shown in Figure 3. In this complex the aromatic ring is "sandwiched" between residues Trp-140 and Leu-18 such that the centroid–centroid separation of Trp-140 and the phenolic ring is 4.4 Å. The angle between the rings is approximately 90°. Similar interactions between aromatic residues of proteins have been described previously and shown to be energetically favorable (Burley & Petsko 1985, 1986). This is the first possible example of such an interaction between a protein and substrate. Leu-18 provides a hydrophobic "wall" that restricts the movement of the substrate side chain and thereby facilitates the aromatic–aromatic interaction.

Both residues Trp-140 and Leu-18 are conserved in mAAT, eAAT, and eTAT. Therefore, the binding of a tyrosine substrate in the described position is possible in all three species, in agreement with the observation that all are capable of transaminating aromatic substrates to a greater or lesser extent.

Interestingly, cAAT contains a phenylalanine at position 18. Model building shows that, in the closed structure, this residue would block the proposed aromatic substrate binding site. This could explain the even poorer ability of the cytosolic, relative to the mitochondrial, enzyme to transaminate aromatic substrates (Mavrides & Christen, 1978).

The other region that appears to play a role in substrate specificity includes residues 15, 141, 292*, and 293*, which are located near the bottom of the active site. From Table III it can be seen that eAAT and mAAT both contain a pair of oppositely charged residues: Asp-15 and Arg-292*. cAAT has Glu-141 and Arg-292* in this region. However, eTAT contains *two* such pairs: Asp-15 and Glu-141, the totally conserved Arg-292*, plus an additional arginine at 293*. It is difficult to explain the existence of an increased charge density within the active site of the enzyme, which utilizes aromatic substrates most efficiently. However, it should be pointed out that eTAT must utilize both aromatic and dicarboxylic acid substrates (glutamate and 2-oxoglutarate) with high efficiency (Powell & Morrison, 1978). The network of charges may facilitate rotation of Arg-292* out of the active site, a movement that is required to accommodate the bulky aromatic side chain. Movement back into the active site is required for the correct binding of the dicarboxylic acid substrates (Jansonius et al., 1985). This proposal is supported by the observation that the side chain of Arg-292* can have different orientations in mAAT (Eichele, 1980).

This work has established that modeling of the three-dimensional structures of both eAAT and eTAT on the basis of the known structure of mAAT can be accomplished without any significant changes in the backbone fold of the polypeptide chain. All ionizable residues remain on the surface of the resulting structures and the van der Waals interactions within the hydrophobic cores remain intact. How good our model of eAAT is in detail will become apparent when the coordinates of the crystal structure become available.

Of most interest is the question of how substrate specificity is brought about. The model-building study reported here identifies tentatively those residues that determine enzyme specificity. No single residue seems to determine by itself the different specificities of the three enzymes with respect to dicarboxylic acid and aromatic substrates. Rather, it appears that these specificities result from a cooperation of several residues in different parts of the active site.

Our proposals concerning substrate specificities are now being tested by site-directed mutagenesis. A series of mutant enzymes, which contain "hybrid" active sites, is being constructed. This approach involves replacing one or more of the key residues identified as being involved in substrate specificity in one enzyme (e.g., eAAT) by the corresponding residues in another (e.g., eTAT). Then the effect of this replacement on the steady-state kinetic parameters of the enzyme is measured. In this way, the residues responsible for the remarkable differences in specificity of the three enzymes will hopefully be detected.

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