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Activity and Structure of the Active-Site Mutants R386Y and R386F of *Escherichia coli* Aspartate Aminotransferase†

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ABSTRACT: Arginine-386, the active-site residue of *Escherichia coli* aspartate aminotransferase (EC 2.6.1.1) that binds the substrate α-carboxylate, was replaced with tyrosine and phenylalanine by site-directed mutagenesis. This experiment was undertaken to elucidate the roles of particular enzyme–substrate interactions in triggering the substrate-induced conformational change in the enzyme. The activity and crystal structure of the resulting mutants were examined. The apparent second-order rate constants of both of these mutants are reduced by more than 5 orders of magnitude as compared to that of wild-type enzyme, though R386Y is slightly more active than R386F. The 2.5-Å resolution structure of R386F in its native state was determined by using difference Fourier methods. The overall structure is very similar to that of the wild-type enzyme in the open conformation. The position of the Phe-386 side chain, however, appears to shift with respect to that of Arg-386 in the wild-type enzyme and to form new contacts with neighboring residues.

Aspartate aminotransferase plays a central role in amino acid metabolism by catalyzing the interconversion L-aspartate + α-ketoglutarate ⇄ L-glutamate + oxaloacetate. This enzyme is the most extensively studied of the aminotransferases and has been well characterized in terms of both physical and enzymatic properties. The reaction is catalyzed via a ping-pong Bi-Bi mechanism in which the essential cofactor converts between the pyridoxal phosphate and pyridoxamine phosphate form (Velick & Vavra, 1962). X-ray crystallographic studies have been carried out on L-aspartate aminotransferase (L-AspAT)[1] derived from various sources including chicken mitochondrion (Ford et al., 1980; Kirsch et al., 1984; Jansonius et al., 1985), chicken cytoplasm (Borisov et al., 1980), pig cytoplasm (Arnone et al., 1985), and *Escherichia coli* (Kamitori et al., 1988; Smith et al., 1989). The overall polypeptide fold has been found to be similar for all of these species, and all active-site residues are conserved (Kondo et al., 1987). The active enzyme is an α_2 dimer. As shown in Figure 1, each subunit consists of two domains. The large domain, which binds the PLP cofactor, encompasses residues 48–325. The small domain is defined as residues 15–47 and 326–410. Two independent active sites of the dimer are located at the cleft between the two domains, and each is comprised of residues from both subunits. High-resolution X-ray crystallographic studies (Kirsch et al., 1984; Arnone et al., 1985; Borisov et al., 1985; Harutyunyan et al., 1985; Smith et al., 1989) in conjunction with site-directed mutagenesis studies (Malcolm & Kirsch, 1985; Cronin & Kirsch, 1988; Toney & Kirsch, 1987; Hayashi et al., 1989; Inoue et al., 1989; Hayashi et al., 1990) have helped to elucidate the roles of several of the active-site residues. Specificity for dicarboxylic acids appears to be imparted by two active-site arginines. Arginine-386 in the small domain interacts with the α-carboxylate of the substrate. Arginine-292[2], (see footnote 2) located in the large domain of the other subunit, interacts with the side-chain carboxylate.

It has been observed in solution studies (Gehring & Christen, 1978; Pfister et al., 1978) as well as by X-ray crystallographic analyses (Kirsch et al., 1984; S. C. Almo and A. T. Danishefsky, unpublished results) that the enzyme undergoes a conformational change upon binding dicarboxylic acid substrates or inhibitors. This motion can be modeled, based on crystal structures of the open and closed forms, as a rigid-body rotation of the small domain with respect to the large domain, resulting in the closing of the enzyme around the substrate. Similar substrate-induced conformational changes have been observed in other enzyme systems, including

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[1] Abbreviations: L-AspAT, L-aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; rms, root-mean-square; $R_{sym} = \sum_n\sum_i|I_i - \bar{I}|/\sum_n\sum_i|\bar{I}|$, where I_i is the ith observation of the nth reflection and $\bar{I}$ is the mean of all observations of the nth reflection.

[2] The superscript 2 denotes residues located on the second subunit of the dimer.

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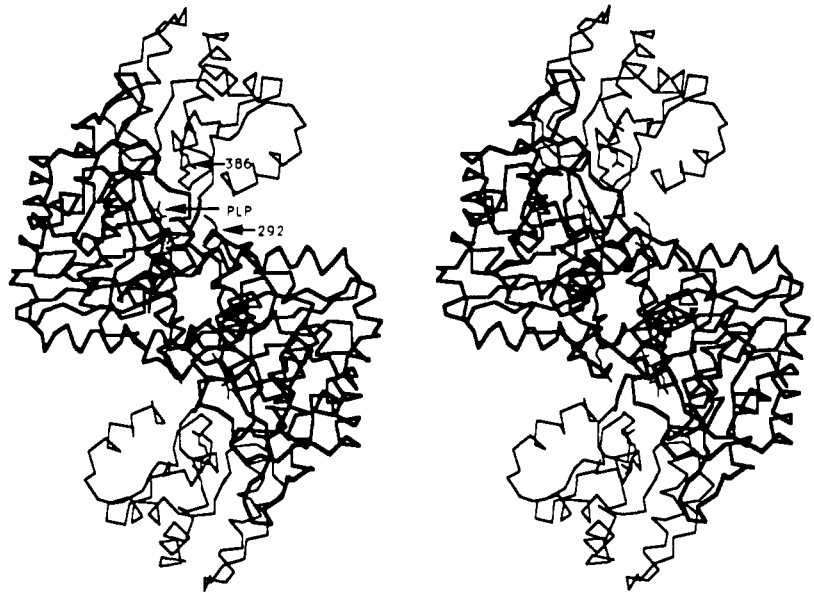

FIGURE 1: Stereoview of the α-carbon chain tracing of the wild-type *E. coli* L-AspAT dimer. The side chains of active-site residues, Arg-386 and Arg-292[2], and the pyridoxal phosphate cofactor are also shown. The large and small domains of each subunit are delineated by variations in line thickness. Interactions between the two monomers occur between the two large domains.

hexokinase (Bennett & Steitz, 1980), citrate synthase (Remington et al., 1982), and alcohol dehydrogenase (Eklund et al., 1981, 1982). One goal of our studies of L-AspAT is to try to understand the role that the conformational change of the enzyme plays in catalysis. This question might best be addressed if the equilibrium between the two conformational states could be perturbed. We are therefore interested in probing the specific enzyme–substrate interactions involved in causing the conformational change.

The bound substrate is anchored to the large domain by Schiff base formation with the cofactor as well as by formation of a salt bridge with Arg-292[2]. It is anchored to the small domain, in large part by salt-bridge formation with Arg-386. These enzyme–substrate interactions may effect the conformational change by pulling the two domains together. Alternatively, it has been suggested that, in the absence of substrate, repulsion between arginines-386 and -292[2] may contribute to favoring the open conformation of the enzyme (Kirsch et al., 1984; Jansonius & Vincent, 1987). If this is the case, then the role of substrate in effecting domain closure is simply to shield a repulsion between these two arginines. These two possibilities (which are not mutually exclusive) can be distinguished by site-directed mutagenesis experiments in the following way. If repulsion between Arg-386 and any region of the large domain is in fact responsible for holding the enzyme in the open conformation in the absence of substrate, then replacement of this residue with an uncharged one should cause the enzyme to adopt the closed conformation under these conditions.

In order to assess the effect of the electrostatic interactions of Arg-386 with the substrate carboxylates, and with residues in the large domain on the conformational equilibrium of this enzyme, we prepared and studied the mutants R386Y and R386F. In R386Y, the positive charge imparted by Arg-386 is removed, though a potential hydrogen bond donating group is retained at this position. Since the side chain of Tyr can extend almost as far as the side chain of Arg, we were interested to see if the hydroxyl group of Tyr-386 could interact with and orient the α-carboxylate group on the substrate. It was hoped that such a hydrogen bond would to some degree ameliorate the reduction in binding and catalysis expected from the removal of the Arg-386–substrate salt bridge and thereby facilitate structural study of the liganded enzyme. The same position was also mutated to a phenylalanine, which is sterically similar to tyrosine but lacks any hydrogen-bonding capacity. The activity of the mutant enzymes was then assayed with various substrates. The structure of R386F was also determined by X-ray crystallographic studies and compared with that of wild-type enzyme.

Materials and Methods

Preparation of L-AspAT Mutants. Site-directed mutagenesis of the *Asp C* gene (Gelfand & Rudo, 1977) which codes for *E. coli* L-AspAT was carried out by a modification of the method described earlier (J. Onnufer, unpublished results; Malcolm & Kirsch, 1985). Single-stranded DNA from selected clones was sequenced by the Sanger chain termination method (Sanger et al., 1977) using the Sequenase version 2.0 sequencing kit purchased from U.S. Biochemical Corp. The entire *Asp C* coding region was sequenced to verify that the desired mutation was the only change from the wild-type sequence.

The wild-type, R386Y, and R386F clones were then transformed into strain *MG204* [*his23(Am), proB trpA-605-(Am), lac13, lacZ118(Oc) gryA, rpsL, aspC::kanR, tyrB, RecA::tn10, ilvE*] (a gift from Ian Fotheringham, Nutrasweet Co.) for production of protein. This strain does not produce active L-AspAT. Cultures were grown to stationary phase in 2YT medium supplemented with 200 μg/mL ampicillin, 50 μg/mL kanamycin, and 15 μg/mL tetracycline.

The proteins were isolated and purified by pH titrations, gel filtration, and ion-exchange chromatography, using the methods described by Cronin and Kirsch (1988). The purity of the protein was 90–95% as judged from SDS gel electrophoresis. Minor impurities of molecular weight 55K and 85K were visible on gels stained with Coomassie Blue.

Measurement of Enzymatic Activity. Pseudo-first-order rate constants and pseudobimolecular rate constants were determined as described by Cronin and Kirsch (1988). Reactions were carried out, under single-turnover conditions, in 0.1 M TAPS buffer, pH 8.5, ionic strength adjusted to μ = 1.0 with KCl. Steady-state conditions were not used to determine kinetic constants of these relatively inactive mutants, in order to avoid the substantial error that results from even

Table I: Refinement Statistics for R386F

crystal data	
space group	$C222_1$
cell dimensions (Å)	a = 156.0
	b = 87.6
	c = 78.8
molecules/asymmetric unit	1
refinement results	
resolution (Å)	10–2.5
final R factor (%)	21.5
restraints (σ applied) rms deviations obsd	
bond length (0.020 Å)	0.011
bond angle (4°)	5°
deviation from planarity (0.070 Å)	0.075
overall B factor (Å^2) (not refined)	12.0

a minor amount of contaminating activity under these conditions. Substrate concentrations ranged from 0.5 to 10 mM for aspartate and from 20 mM to 0.2 M for glutamate, β-glutamate, and isoglutamine (4,5-diamino-5-oxopentanoic acid; Sigma Chemical Co.). Spectra were recorded with an HP8452 UV–visible spectrophotometer.

Inhibitor Binding. Binding constants of maleic acid (Sigma Chemical Co.) to wild-type enzyme, R386Y, and R386F were determined by monitoring the absorbance of the enzyme cofactor at 430 nm as a function of inhibitor concentration. Titrations were carried out in 20 mM potassium phosphate, pH 7.5. Data were fitted by linear regression to a double-reciprocal plot of absorbance as a function of substrate concentration. The extinction coefficient at 430 nm of the chromophoric coenzyme was assumed to be the same for the mutant as for the wild-type enzyme.

Crystallographic Studies. Crystals of the mutant R386F were grown by vapor diffusion against 50% saturated ammonium sulfate in 20 mM potassium phosphate buffer, pH 7.5, at a concentration of 5 mg/mL, similarly to those of wild-type enzyme (Smith et al., 1986). Unit cell dimensions were determined from 0-level precession photographs of the principal zones, using Buerger precession cameras mounted on a sealed-tube X-ray generator. Crystals displayed the symmetry of the space group $C222_1$ and had unit cell dimensions a = 156.0, b = 87.6, c = 78.8, $\alpha = \beta = \gamma = 90°$, similar to those of the wild-type enzyme. Diffraction data were collected to 2.5-Å resolution by using Cu Kα radiation from a rotating anode with a Xentronics area detector. Reduction of data was carried out by using standard software (Kabsch 1988a,b). R_{sym}[1] (I) over all reflections was 6.7%.

The structure of the mutant L-AspAT was determined by difference Fourier methods. Initial phases and structure factor amplitudes were calculated from the refined structure of the mutant K258A (Smith et al., 1989). A total of 10531 reflections with $I > \sigma(I)$, which corresponds to 71% of the unique data between 10 and 2.5 Å, were used in refinement of the structure. The first stage of refinement, which was carried out by using the molecular dynamics program XPLOR (Brunger, 1987), yielded an R factor of 23% for data from 8 to 2.5 Å. The protocol followed was very similar to that described by Brunger (1988) for the mutant K258A. Subsequent to refinement using XPLOR, the resulting model required only very minor amounts of manual refitting to an electron density map calculated by using $2F_{obs} - F_{calc}$ as coefficients in difference Fourier syntheses. Eleven cycles of the Hendrickson–Konnert restrained least-squares minimization (Hendrickson & Konnert, 1980) were then carried out in the resolution range of 10–2.5 Å yielding an R factor of 21.5%. The solvent correction described by Fraser (1978) was employed for the low-resolution terms. Refinement statistics are listed in Table I. Positions of residues 386 and 292 and one sulfate ion bound at the active site were reexamined by deleting the coordinates for these side chains from the model, carrying out several cycles of restrained least-squares refinement to reduce phase bias and using the resulting model in difference Fourier syntheses. Density corresponding to the positions of the residues in question was unaffected by this process.

Table II: Second-Order Rate Constants for L-AspAT and the Mutants R292D, R386Y, and R386F

	k_{cat}/K_m (M^{-1} s^{-1})			
substrate	wild type	R292D	R386Y	R386F
L-Asp	34500[a]	0.07[a]	1.0 ± 0.1	0.55 ± 0.05
L-Glu	4790[a]	0.01[a]	0.011 ± 0.001	0.0027 ± 0.0009
L-Arg	0.03[a]	0.43[a]		
L-Ala	0.61[a]	0.27[a]		
iso-Gln			0.001 ± 0.0001	<0.0005
β-Glu			<0.0005	<0.0005

[a] Cronin and Kirsch (1988).

Results

Enzyme Activity. The mutations at position 386 drastically reduced enzyme activity with respect to that of wild type, for the substrates L-Asp and L-Glu. Apparent second-order rate constants of the enzymic amino acid to keto acid half-reaction for the mutant enzymes are listed in Table II. These values represent a reduction of greater than 5 orders of magnitude as compared to those observed by Cronin and Kirsch (1988) with wild-type L-AspAT (35000 and 4790 M^{-1} s^{-1} with Asp and Glu, respectively, as substrates). The reduced levels of activities are similar to those observed for the wild-type enzyme with alanine as substrate (0.61 M^{-1} s^{-1}) and for the R292D mutant with arginine as substrate (0.43 M^{-1} s^{-1}) (Cronin & Kirsch, 1988). These two enzyme–substrate pairs have in common with the ones studied here the absence or alteration of one salt bridge between enzyme and substrate as compared to the wild-type enzyme with Asp and Glu as substrate.

Comparison of apparent second-order rate constants of R386Y with R386F (Table II) for the substrates Asp and Glu indicates that activity is 2–3-fold greater for R386Y than for R386F. One explanation for this modest enhancement is that it reflects the capability for formation of a weak hydrogen bond between Tyr-386 and the α-carboxylate on the substrate. Other explanations, such as differences in binding energies of the two mutants to the substrates, resulting from small differences in packing of these two residues within the active site, are also plausible.

It was of interest to determine if there are some substrates that could react more efficiently with R386Y than with the wild-type enzyme. Substrates which might form more favorable hydrogen bonds than L-Glu does with Tyr-386 were considered. For example, one might predict that the carboxylate moieties of β-glutamate could extend further toward the small domain and Tyr-386 than those of α-glutamate. Isoglutamine, which contains a neutral amide group in place of the α-carboxylate moiety of glutamate, might also be expected to bind more favorably than glutamate to R386Y, which contains no positively charged residue in a position to form a salt bridge with a substrate α-carboxylate. However, as indicated in Table II, apparent second-order rate constants for R386Y with both of these substrates are at least 10-fold lower than that with L-Glu. Perhaps interactions other than those with Arg-386 still contribute to the stabilization of the negative charge on the substrate α-carboxylate of L-Glu. It is also possible that the differing pKs of the amino groups of these three related molecules in their bound and unbound states affect the catalytic efficiencies.

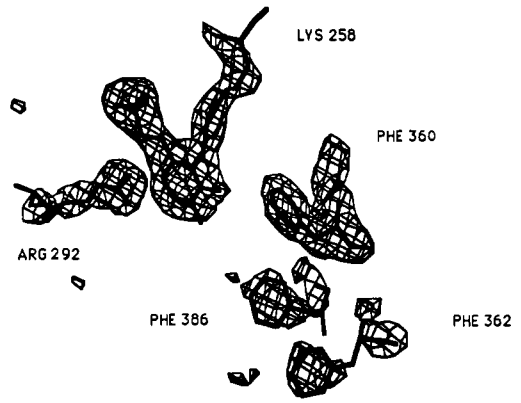

FIGURE 2: Model of active-site residues of R386F superimposed on the corresponding electron density map calculated by using $F_{obs} - F_{calc}$ as coefficients in the Fourier synthesis. Phases and F_{calc}'s were determined from the refined coordinates of R386F. Residues shown were deleted from the model coordinates. The electron density is contoured at 3σ.

It was further speculated that the mutant might now bind hydrophobic amino acids "backwards". That is, if the α-carboxylate interacts with Arg-292, the hydrophobic side chain could interact with position 386. This would place the amino group in the wrong orientation in the case of L-amino acids but in the proper orientation for D-amino acids. However, no activity was observed over the period of an hour in the presence of 20 mM D-Ala, D-Val, or D-His or 2 mM D-Tyr. This lack of activity may be rationalized by the fact that the extension (from the α-amino group) of the α-carboxylate on the substrate is one methylene shorter than that of the side-chain carboxylate. It is also possible that steric constraints imposed by the residues in the vicinity of position 386 impede binding of these substrates or that the mutated residue itself has reoriented so that substrate binding is unfavorable. Lastly, it should be considered that wild-type L-AspAT can accept L-Tyr as substrate with a relative activity approximately 2 orders of magnitude less than with L-Asp as substrate. A binding site for the L-Tyr side chain, proposed on the basis of modeling studies, may involve Trp-140 and Leu-18 (Seville et al., 1988). Binding of the D-Tyr side chain to such a native binding site could compete with binding in the region of Tyr-386.

Effect of the Mutation on Inhibitor Binding. In order to assess the loss in binding affinity of the enzyme to dicarboxylic acid substrate analogues resulting from removal of Arg-386, dissociation constants of maleate with wild-type enzyme and R386F/Y were determined. The dissociation constants for the inhibitor with wild-type enzyme, R386Y and R386F, are 0.008 (±0.001) M, 0.82 (±0.02), and 0.89 (±0.008) M, respectively, in 20 mM potassium phosphate buffer.

Crystal Structure of R386F. The 2.5-Å structure of R386F was solved by difference Fourier methods using the coordinates of the previously solved structure of the mutant K258A (Smith et al., 1989). The overall structure of R386F is very similar to that of the unliganded wild-type enzyme (S. C. Almo, unpublished results). The rms deviation in positions for the α-carbons between this mutant and the wild-type enzyme is 0.66 Å (0.53 Å for the large domain and 0.75 Å for the small domain). Notably, the enzyme is in the open conformation.

The integrity of the spatial relationships among the active-site residues is maintained in R386F, except for the mutated residue itself. Electron density, presumably corresponding to a sulfate anion, has previously been observed at the active site of the K258A mutant (Smith et al., 1989). We obsrved a similar feature in electron density maps of the R386F mutant. Part of the active-site region of an electron density map of this mutant is shown in Figure 2. The mutation does not seem to have long-range structural effects on the enzyme discernible at this resolution. However, the position of the side chain of Phe-386 is shifted from that of Arg-386. This point is illustrated in the comparison of the positions of some of the active-site residues for R386F and WT in Figure 3. In the wild-type enzyme, Arg-386 forms a hydrogen bond to Asn-194 and also comes in van der Waals contact with Phe-360. As shown in Figure 3, in R386F, Phe-386 shifts away from this position and toward Phe-362, though some van der Waals contact is still maintained with Phe-360.

Discussion

The effect of mutations at the position of Arg-386 was to drastically reduce activity of the enzyme (Table II). The degree of reduction in activity for R386F/Y with Asp as substrate was similar to that found for R292D with Arg as substrate and wild type with alanine as substrate (Cronin & Kirsch, 1988). Given these similarities, the decrease in activity observed most likely represents the contribution of one salt bridge between enzyme and substrate carboxylate to k_{cat}/K_m. Interestingly, in a study carried out by Inoue et al. (1989) on the R386K mutant, a much smaller reduction in activity was observed. The charged lysine group presumably accounts for this difference. In any of these cases, it is possible that the effect of the mutation on catalysis is more than just a local one. Specifically, if the interactions between the arginines and the substrate carboxylates are instrumental in triggering the conformational change, then the difference in activities between the wild type and mutant enzymes will reflect the resulting perturbation in the equilibrium between the open and closed conformations of the bound enzyme.

It is clear that R386F in its unliganded state exists in the open conformation. This result indicates that any repulsion that may exist between Arg-386 and the large domain cannot be solely responsible for favoring the open conformation of the enzyme in the absence of substrate. The mutant R292D, whose structure has previously been determined, likewise adopts the open conformation in the absence of substrate (S. C. Almo, unpublished results). The role of substrate binding in effecting the conformational change must then involve more than the neutralization of a repulsion between the two ac-

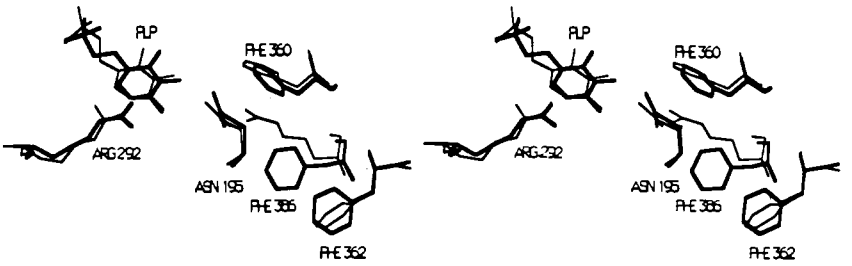

FIGURE 3: Stereoview of superpositions of the active-site residues of wild type and R386F. The best fit of the positions of the α-carbons of the two structures was used to superimpose them.

tive-site arginines. It is still unclear, however, whether or not the electrostatic attraction of substrate with either of these residues is essential in triggering the closed conformation.

Ideally, one would like to perturb the conformational equilibrium without affecting the chemistry of catalysis. Such an experiment was attempted by mutation of Met-326, a strictly conserved methionine at the putative hinge region of the enzyme (A. T. Danishefsky, unpublished results). This mutation had little effect on the activity of the enzyme in vitro. Though mutants of other residues at this hinge region have not been examined, it remains a possibility that the conformational change in L-AspAT is determined in most part by interactions of active-site residues with substrate and that perturbing the equilibrium of the conformational states cannot be accomplished without considerably altering the active site.

It was thought that R386Y might retain some capacity for hydrogen bonding to the α-carboxylate of substrate via the hydroxyl of Tyr-386. In fact, there is some increase in activity for R386Y as compared to R386F, but it is small. There are several reasons why a favorable hydrogen bond might not form between Tyr-386 and the substrate. The hydroxyl group may not extend as far toward the substrate as the guanidinium on the arginine of wild-type enzyme. In an idealized tyrosine, the oxygen of the hydroxyl extends 6.4 Å from the α-carbon. On the basis of coordinates from the wild-type L-AspAT structure, the nitrogens of the guanidinium group of Arg-386 extend approximately 6.4 and 6.8 Å from the α-carbon of this residue. However, on the basis of our structure of R386F, even if the side chain is equal to that of the arginine in length, it may be misoriented for optimal hydrogen bonding to substrate. In particular, it was noted in the wild-type structure that the two active-site arginines appear to interact with neighboring residues so that they will be oriented optimally to interact with substrate (S. C. Almo, unpublished results). For example, Arg-292 is sandwiched between Asn-142 and Asp-15. Arg-386 appears to be fixed in position by Phe-360 and by a hydrogen bond with the side chain of Asn-194. In R386F, Phe-386 can no longer be oriented in this way by Asn-194. Moreover, as illustrated in Figure 3, the position of Phe-386 appears to reorient with respect to that of Arg-386 in the wild-type enzyme. Perhaps aromatic–aromatic interactions of the type described by Burley and Petsko (1986) between Phe-386 and the surrounding aromatic residues, Phe-360 and Phe-362, are influencing the orientation of this side chain. If a similar change in position occurred for R386Y as well, then the hydroxyl group on Tyr-386 would not be oriented properly to hydrogen bond to the α-carboxylate on the substrate. These observations may be related to the absence of a larger increase in activity of R386Y as compared to R386F. In the absence of a crystal structure of R386Y, it cannot be known if the position of the Tyr is more similar to that of Arg-386 in wild type or to that of Phe-386 in R386F. Thus far, crystallization of R386Y has proved more difficult than that of R386F.

Acknowledgments

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Registry No. L-AspAT, 9000-97-9; L-Arg, 74-79-3; L-Tyr, 60-18-4; L-Phe, 63-91-2; L-Asp, 56-84-8; L-Glu, 56-86-0; iso-Gln, 636-65-7; β-Glu, 1948-48-7.

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