Enhanced Catalysis by Active-Site Mutagenesis at Aspartic Acid 153 in *Escherichia coli* Alkaline Phosphatase†

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ABSTRACT: Bacterial alkaline phosphatase catalyzes the hydrolysis and transphosphorylation of phosphate monoesters. Site-directed mutagenesis was used to change the active-site residue Asp-153 to Ala and Asn. In the wild-type enzyme Asp-153 forms a second-sphere complex with Mg^{2+}. The activity of mutant enzymes D153N and D153A is dependent on the inclusion of Mg^{2+} in the assay buffer. The steady-state kinetic parameters of the D153N mutant display small enhancements, relative to wild type, in buffers containing 10 mM Mg^{2+}. In contrast, the D153A mutation gives rise to a 6.3-fold increase in k_{cat}, a 13.7-fold increase in k_{cat}/K_m (50 mM Tris, pH 8), and a 159-fold increase in K_i for P_i (1 M Tris, pH 8). In addition, the activity of D153A increases 25-fold as the pH is increased from 7 to 9. D153A hydrolyzes substrates with widely differing pK_a's of their phenolic leaving groups (PNPP and DNPP), at similar rates. As with wild type, the rate-determining step takes place after the initial nucleophilic displacement (k_2). The increase in k_{cat} for the D153A mutant indicates that the rate of release of phosphate from the enzyme product complex (k_4) has been enhanced.

Bacterial alkaline phosphatase (BAP)[1] is a periplasmic enzyme that catalyzes the hydrolysis of phosphate monoesters (McComb et al., 1979). The catalytic mechanism has been the subject of numerous kinetic (Coleman & Gettins, 1983a,b) and structural studies (Sowadski et al., 1985). The generally accepted catalytic mechanism involves four steps, including the transient formation of a covalent phosphoryl-enzyme intermediate (E–P_i) with Ser-102 (Hull et al., 1976):

$$E + ROP_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*ROP_i \underset{k_{-2}[ROH]}{\overset{k_2}{\rightleftharpoons}} E\text{–}P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E^*P_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_i \quad (1)$$

Subsequently, water (hydrolysis), or an alternative nucleophilic acceptor such as an alcohol (transphosphorylation), dephosphorylates the phosphoryl-enzyme intermediate (Barrett et al., 1969). The wild-type enzyme exhibits maximal activity (k_{cat}/K_m) around pH 8 where the rate-limiting step is release of the tightly bound phosphate (k_4), P_i, from the enzyme product complex.

The dimeric enzyme consists of 449 amino acids per monomer (Bradshaw et al., 1981) with a metal cluster (2 zincs and 1 magnesium) located at the active site in each monomer. Recently, the crystal structure of the enzyme–phosphate complex, E^*P_i, has been solved at 2.0-Å resolution (Kim & Wyckoff, 1989, 1991). This structure now permits a detailed examination of the constellation of interactions in the active site. Over the past 5 years, several groups have used site-directed mutagenesis to probe the structural and functional requirements in the active site. In general, these studies have shown that the active site can tolerate a variety of mutations and still retain significant activity. For example, replacement of the key nucleophilic Ser-102 with Cys produces a mutant enzyme with 25% the activity of wild type (Butler et al., 1989; Ghosh et al., 1986), while remarkably replacement with the nonnucleophilic residue Leu gives rise to a functional phosphatase, albeit with greatly diminished activity (0.5% of the activity of wild type) (Butler-Ransohoff et al., 1992). Stereochemical studies with the S102C mutant show that the overall sequence of steps in the catalytic mechanism is conserved, but the rate-determining step is altered from release of phosphate (k_4) to phosphorylation of the enzyme (k_2) (Butler-Ransohoff et al., 1988a). Substitutions for Arg-166 support the crystallographic assessment of this residue as contributing to the strong binding of phosphate but show that Arg-166 is not essential for activity. Replacement of Arg-166 with Ser, Ala, Gln, and Lys decreases the k_{cat} values by about an order of magnitude while increasing the K_m and K_i (phosphate) by 3–10-fold (Butler-Ransohoff et al., 1988b; Chaidaroglou et al., 1988). Examination of the crystal structure shows that Ser-102 and Arg-166 directly interact with the noncovalently bound phosphate (Figure 1). The positively charged guanidium side chain of Arg-166 forms a salt bridge with two of the oxygens of phosphate in the noncovalent enzyme–product complex. This interaction is presumably important for the enzyme–substrate complex as well. These results are in sharp contrast with mutagenesis studies on staphylococcal nuclease. Replacement of either of two active-site Arg residues (which are hydrogen bonded with the phosphodiester in the enzyme–substrate complex) with Gly essentially abolishes activity (Serpersu et al., 1987). The mutagenesis and crystallographic studies with BAP suggest that the active-site zincs play a major role in binding substrate, stabilizing reaction intermediates (and transition states), and

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[1] Abbreviations: BAP, bacterial alkaline phosphatase; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; PNPP, 4-nitrophenyl phosphate; DNPP, 2,4-dinitrophenyl phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid. Mutations are specified by the following notation: the naturally occurring amino acid residue, residue number, and new amino acid residue. Single-letter amino acid symbols are used.

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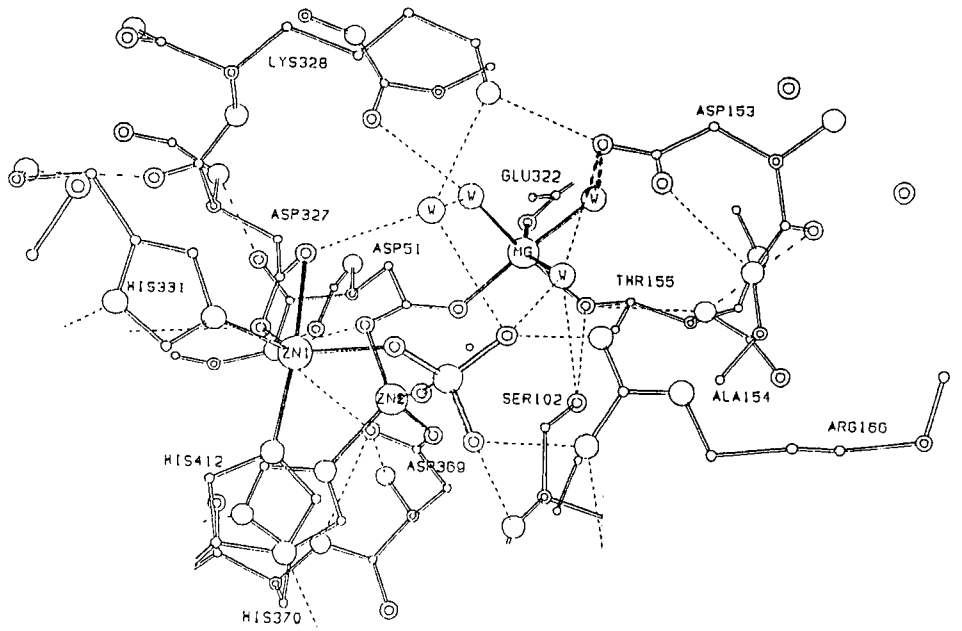

FIGURE 1: Active site of the bacterial alkaline phosphatase–inorganic phosphate complex at 2.0-Å resolution. Water molecules are indicated by an encircled W, and hydrogen bonds are shown as dashed lines. One of the carboxylate oxygens of Glu-322 has been omitted for clarity. Reprinted with permission from Kim and Wyckoff (1991). Copyright 1991 Academic Press Limited.

activating the nucleophiles Ser-102 and water for the double in-line displacement.

Recent studies have demonstrated that substitution at active-site residues that are not directly involved in the reaction pathway can have profound effects on the catalytic event. Mandecki has recently screened 180 mutants of active-site residues 99–107 and found 8 enzymes with increased phosphatase activity (Mandecki et al., 1991). A 35-fold increase in specific activity (at pH 10) was observed for the D101S mutation. Earlier studies have shown that alanine substitutions at Asp-101 (Chaidaroglou & Kantrowitz, 1989) and Lys-328 (Xu & Kantrowitz 1991) produce mutant enzymes with increased k_{cat} values in the presence of the transphosphorylating buffer, Tris. In both cases the mutations (D101A and K328A) appear to be increasing the rate of transphosphorylation. Chaidaroglou and Kantrowitz have suggested that the increase in activity of the D101A mutant (3-fold at pH 9.4) is caused by greater accessibility in the active site for the sterically demanding alternative phosphate acceptor, Tris. The L328A mutation is 14 times more active than wild type at pH 10.3. Examination of the crystal structure reveals that Lys-328 is hydrogen bonded to a water, which is in turn hydrogen bonded to enzyme-bound phosphate (E^*P_i). In this case the rate increase is achieved by an increase in the transphosphorylation process and a decrease in affinity of inorganic phosphate (increase in k_4/k_{-4}).

The recent results with the Lys-328 mutation prompt us to report our mutagenesis studies with the neighboring residue Asp-153. The 2.0-Å structure shows an intricate network of hydrogen bonding throughout the active site (Figure 1). The carboxylate of Asp-153 is hydrogen bonded to two of the three water molecules that are ligands to the active-site magnesium and has a water-mediated salt link to one of the oxygens of enzyme-bound phosphate, E^*P_i. In addition, Asp-153 is hydrogen bonded to the ammonium side chain of Lys-328. We report that replacement of Asp-153 produces mutant enzymes that are sensitive to the presence of exogenous magnesium and show greatly increased activity.

MATERIALS AND METHODS

Bacterial Strains and Media. Escherichia coli strain AW1043 (Δ*lac gal*U *gal*K Δ(*leu-ara*) *pho*A-E15 *pro*C::Tn5) was used as the host cell for all experiments except site-directed mutagenesis, in which the male derivative AW1043F′ Tet [Δ*lac gal*U *gal*K Δ(*leu-ara*) *pho*A-E15 *pro*C::Tn5 F′ Tet] was used (Inouye et al. 1981). Standard LB medium was used for general propagation of cells and for mutagenesis. For enzyme production and isolation, the host cells were grown in low phosphate (0.1 mM KH_2PO_4)-containing MOPS medium (Neidhardt et al., 1974) or a low-phosphate Tris medium (Neu & Heppel 1965) maintained at ~pH 7.8. All media contained ampicillin (250 mg/mL) and kanamycin (50 mg/mL).

Mutagenesis. The mutagenesis was performed using an M13 vector, as previously described (Butler-Ransohoff et al., 1988b). Specifically designed oligonucleotides (21-mers) were synthesized using the phosphoramidite method (Beaucage & Caruthers, 1981) to produce the desired mutations. The double primer method of Zoller and Smith was used to construct the heteroduplex DNA (Zoller & Smith, 1984). Mutant plasmids were identified by colony screening with ^{32}P-labeled oligomer encoding the mutant sequence. The mutant gene was then subcloned into pBR322 between the *Bam*HI and *Hin*dIII sites. Sanger dideoxy DNA sequencing of the double-stranded plasmid verified the presence of the desired mutation.

Production and Purification of the Mutant Enzymes. Transformed AW1043 cells containing the appropriate plasmid were grown in 0.5 L of either low-phosphate MOPS or low-phosphate Tris media (with ampicillin and kanamycin, vide supra) at 37 °C until the OD at 600 nm reached ~2. The wild-type and D153N mutant enzymes were isolated from the cells following cold osmotic shock by ion-exchange chromatography on DEAE-cellulose (10 mM Tris, pH 8, NaCl gradient, 10–200 mM) as previously described. After isolating the D153A mutant by cold osmotic shock, heat-sensitive protein impurities were removed by heating the cold water wash at 75-80 °C for 10 min. The precipitated proteins were

Table I: Michaelis–Menten Parameters of the Mutant Alkaline Phosphatases D153N and D153A with PNPP as Substrate

enzyme	buffer[a]	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
wild type	1 M Tris	31.0 ± 0.5	19.5 ± 0.2	1.6×10^6
D153N	1 M Tris	30.0 ± 1.0	20.4 ± 5.0	1.5×10^6
D153A	1 M Tris	214 ± 21	142 ± 15	1.5×10^6
wild type	50 mM Tris	7.0 ± 0.4	16.4 ± 0.9	4.3×10^5
D153N	50 mM Tris	10.4 ± 1.1	12.2 ± 2.0	8.5×10^5
D153A	50 mM Tris	44.0 ± 2.0	7.8 ± 0.9	59.0×10^5

[a] All assay buffers were 10 mM $MgCl_2$ and 50 μM $ZnSO_4$, pH 8.0, 25 °C.

removed by simple filtration, and the filtrate was purified by ion-exchange chromatography as described for D153N. The activity of the D153A enzyme did not change upon successive heat treatments. The electrophoretic mobility of all mutants were identical to that of wild type (10% SDS/polyacrylamide gel electrophoresis). Protein concentrations were measured from the absorbance at 278 nm (ϵ = 0.71 cm^2/mg) (Plocke & Vallee, 1962).

Assays. The velocity of the enzyme-catalyzed reaction was followed spectrophotometrically using PNPP as substrate and measuring the appearance of 4-nitrophenolate ($\epsilon_{410} = 1.6 \times 10^4$, pH 8.0, $pK_a = 7.16$). Molar absorptivities were confirmed at different pH values after complete hydrolysis. All measurements were at 25 ± 0.1 °C in Tris·HCl buffer containing 10 mM $MgCl_2$ and 50 μM $ZnCl_2$ (or 50 μM $ZnSO_4$) using either a Perkin-Elmer Lambda 5 spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer. We did not notice any appreciable difference in the steady-state kinetics when $ZnSO_4$ was used in place of $ZnCl_2$ in the assay buffers.[2] Values of k_{cat} and K_m were obtained from the Lineweaver–Burk plots of six to ten points or by direct fitting of the rate data using Marquardt analysis. All points were determined in at least triplicate.

RESULTS

Asparagine (D153N) and alanine (D153A) substitutions were made for the aspartate residue at position 153 using standard M13 mutagenesis techniques (Butler-Ransohoff et al., 1988b; Zoller & Smith, 1984). Table I displays the Michaelis–Menten kinetic data at pH 8 in 1 M and 50 mM Tris using PNPP as substrate. Under these assay conditions the conservative D153N mutation displays activity that is comparable to wild type in 1 M Tris and a 2-fold increase in k_{cat}/K_m in 50 mM Tris. In sharp contrast, the D153A mutation shows a 6.9-fold increase in k_{cat} and a 7.3-fold increase in K_m, leaving k_{cat}/K_m relatively unchanged in 1 M Tris. In 50 mM Tris the k_{cat} and k_{cat}/K_m for the D153A mutant are increased, respectively, by factors of 6.3 and 13.7, relative to wild type. All of the enzymes are sensitive to the concentration of the Tris buffer. Comparison of the rates in high Tris (1 M) versus low Tris (50 mM) reveals that the D153A mutant increases 4.9 times in the high-Tris buffer while the wild type increases by a factor of 4.4 and the D153N mutant by a factor of 2.9. This behavior is consistent with the involvement of Tris in the transphosphorylation pathway.

At alkaline pH, release of phosphate is rate determining in the wild-type enzyme and phosphate is a competitive inhibitor with a K_i of 5.6 ± 1.1 μM (PNPP substrate, 10 mM $MgCl_2$, 50 μM $ZnSO_4$, 1 M Tris, pH 8). Under the same assay conditions, the D153N mutation results in a small 1.75-fold increase in the K_i to 9.8 ± 0.2 μM. In contrast, the nonpolar D153A mutation gives rise to a dramatic ~160-fold increase in the K_i to 890 ± 60 μM.

[2] For a list of inhibitors of BAP see McComb et al. (1979), pp 269–275.

Table II: Activity Dependence of AP's on the Presence of $ZnSO_4$ and $MgCl_2$ in the Assay Buffer[a]

		velocity [$\times 10^{-7}$, $M/(s \cdot \mu g)$]		
[$MgCl_2$] (mM)	[$ZnSO_4$] (μM)	wild type	D153N	D153A
10	50	0.72	1.93	18.9
10		0.69	1.95	16.2
	50	0.67	0.45	1.1
		0.71	0.29	0.99

[a] Assay conditions: 1.0 mM PNPP and 1 M Tris, pH 8, 25 °C.

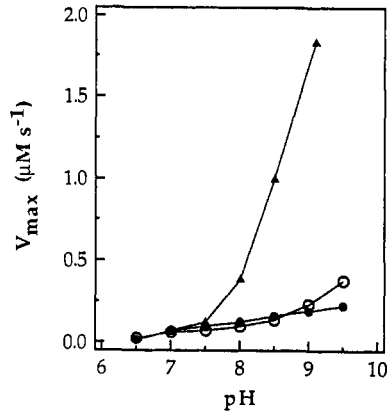

FIGURE 2: pH dependence of the maximal velocities of wild type (●), D153N (○), and d153A (▲) [5 mM PNPP, 50 mM Tris, ionic strength 200 mM (adjusted with NaCl), 25 °C].

The wild-type enzyme strongly binds Zn^{2+} and Mg^{2+} cations in the active site and shows little sensitivity to the inclusion of 50 μM $ZnSO_4$ and/or 10 mM $MgCl_2$ in the assay buffer (Table II). In contrast, both mutants were highly dependent on the inclusion of Mg^{2+}. The D153A mutant showed an ~17-fold increase and the D153N mutant showed a 6.7-fold increase in activity in the Mg^{2+}-containing buffer. The mutants showed only a weak sensitivity to Zn^{2+}. It has previously been shown that the apoenzyme is completely inactive. Addition of 4 equiv of Zn^{2+} per equivalent of enzyme dimers restores ~70% of the activity (Bosron et al., 1977). Mg^{2+} alone has no effect on the apoenzyme; however, full activity can only be achieved when 4 equiv of Zn^{2+} and 2 equiv of Mg^{2+} are bound per equivalent of enzyme dimers. The data with the mutant enzymes suggest that the binding of Zn^{2+} is unperturbed and the affinity for Mg^{2+} is reduced.

Figure 2 compares the variation in V_{max} with pH for the mutant and wild-type enzymes. The Asn mutation causes little departure from the wild-type pH profile. V_{max} for the wild-type increases by a factor of 2.8 from pH 7 to 9, while the D153N increases by a factor of 3.8. The D153A mutation shows a 25-fold increase over this same range, revealing the ionization of a kinetically important species.

DISCUSSION

Although Asp-153 does not play a direct role in the catalysis, i.e., it does not participate in bond formation or rupture with the substrate nor directly hydrogen bond with the substrate or product complexes, the mutagenesis studies clearly reveal the importance of this residue in the catalytic action of the enzyme. Replacement of the acidic aspartate residue at position 153 with the neutral alanine produces a more active enzyme. Except for a marked dependence on exogenous Mg^{2+}, replacement of aspartate with asparagine does not alter the

activity of BAP. Kinetic characterization of the D153A mutant with DNPP [k_{cat} 13 s^{-1}, K_m 1.5 μM, 50 mM Tris, 200 mM IS (NaCl), pH 7.5] as a substrate shows Michaelis parameters similar to those found with PNPP [k_{cat} 14.9 s^{-1}, K_m 2.2 μM, 50 mM Tris, 200 mM IS (NaCl), pH 7.5]. Although these are only two points, the leaving groups have widely different pK_a's (*p*-nitrophenol 7.2 and 2,4-dinitrophenol 4.0) and provide evidence that the rate-determining step occurs after loss of the phenoxide. The mutant enzymes also show a dependence on the Tris concentration, suggesting that the general double displacement mechanism is still operating. The 6–7-fold increase in k_{cat} observed with the D153A mutant requires that the release of P_i (k_4) is facilitated relative to wild type. Pre-steady-state studies on the wild type show that k_3 is an order of magnitude greater than k_4 in 50 mM Tris at pH 8.3. Therefore, although k_4 must be increased, release of P_i may still be the rate-determining step. However, the ~160-fold increase in K_i of P_i indicates that the binding of phosphate is greatly weakened. This suggests that k_4 may be enhanced enough that it is now greater than k_3. Examination of the pre-steady-state kinetics should resolve this point.

Examination of the crystal structure of the E*P_i complex reveals several modes in which Asp-153 indirectly interacts with the reaction centers. The carboxylate side chain of Asp-153 is involved in three types of interactions: hydrogen bonding with three waters, salt bridge formation with the positively charged Lys-328 side chain, and hydrogen bonding with the Ala-154 amido hydrogen. Two of the water molecules involved with Asp-153 are also coordinated to Mg^{2+}, making Asp-153 a second-sphere ligand. The interaction of Asp-153 with the two waters coordinated to Mg^{2+} should contribute to the binding of Mg^{2+} by increasing the strength of the Mg–O bonds. The salt bridge with Lys-328 positions the conformationally flexible side chains of both residues. In turn, Lys-328 is aligned to hydrogen bond with a water that is hydrogen bonded to the noncovalently bound P_i. Replacement of Asp-153 with asparagine is a conservative mutation even though charge is changed, the steric bulk is largely unaltered, and the electronic perturbations should modify but still permit the hydrogen-bonding scheme of the wild type to exist. In contrast, the 5 hydrogen-bonding interactions with the β-carboxy group of Asp-153 will be absent in the D153A mutation. These changes result in an active-site architecture that decreases the binding affinity of substrate and product under the high-Tris conditions. The loss of the second-sphere coordination to Mg^{2+} should reduce the stability of the coordinated waters. The activity dependence on exogenous Mg^{2+} in the buffer mixture suggests that the binding of Mg^{2+} may be perturbed in the mutant enzymes. In addition, the presence or absence of a negative charge at position 153 will affect the local ionic environment and modulate the acidities of the waters coordinated to Mg^{2+}.

The pH dependence of the D153A mutant markedly departs from the behavior of the wild type as the rate rapidly increases above pH 7.5. Assuming k_4 is rate determining, the increase in rate may be caused by a loss of a stabilizing hydrogen bond to phosphate and/or electrostatic repulsion of the anionic phosphate by the ionization of an active-site species. A potential candidate for this interaction is one of the waters (Wat 454)[3] that is coordinated to Mg^{2+}, which in the wild type is also hydrogen bonded to Asp-153 and the noncovalently bound phosphate. The alanine mutation may permit the water to ionize to form a Mg^{2+}-bound hydroxyl which may destabilize the noncovalent binding of phosphate. Alternatively, if dephosphorylation of the enzyme (k_3) is rate limiting, the pH profile may reflect the ionization of the Zn^{2+}-bound water to form the more nucleophilic Zn^{2+}-bound hydroxyl. Model studies with zinc–macrocyclic polyamine complexes show pK_a's in the range of 7.3–8.7 (Koike & Kimura, 1991).

It is interesting to compare our results with mutagenesis at Arg-166. Replacement of Arg-166 with Ala increases the K_i of P_i 47-fold (10 mM Tris, pH 8) while decreasing k_{cat} by a factor of 41. Other mutations at 166 are consistent with the idea that P_i is more weakly bound in all mutations (Ser, Glu, Lys) and release of phosphate from the enzyme product complex is no longer rate limiting. Further studies with different substrates and pre-steady-state studies give evidence for rate-determining dephosphorylation of E–P_i (k_3). Examination of the crystal structure shows that the guanidine side chain forms two strong hydrogen bonds by way of a four-center interaction with two oxygens of the bound product phosphate. The primary function of Arg-166 is to bind the phosphate/phosphate head group and stabilize some of the increase in charge density around the phosphorus during the bond breaking and forming processes. Consequently, removal of Arg-166 reduces the binding affinity of P_i (increase in K_i) and decreases the rate of dephosphorylation (k_3) and k_{cat}. In contrast, substitution at 153 increases both K_i and k_{cat}. The D153A mutation decreases the binding of P_i without destabilizing the intermediates and transition states involved in the chemical steps.

The activity exhibited by the D153A mutation is similar to that recently reported by Xu and Kantrowitz for mutations at Lys-328 (Xu & Kantrowitz, 1991). Both D153A and L328A mutations show substantial increases in k_{cat}, K_m, and K_i. In the wild type, Lys-328 forms a "water-mediated salt bridge" with the active site P_i. In addition, Lys-328 forms a salt bridge with Asp-153 which may serve to position the side chains of both residues as part of the intricate hydrogen-bonding network. The effects observed by mutating one of these residues may contain contributions resulting from the greater conformational flexibility engendered at the other site.

Although Asp-153 does not directly contact the noncovalently bound P_i and presumably has no direct interactions with the covalent phosphoryl-enzyme intermediate, it plays an important role in the catalytic activity of the enzyme. The presence of a full negative charge at position 153 does not seem to be important to maintain the activity of the wild type. The increase in k_{cat} for the D153A mutant indicates that the rate of release of phosphate from the enzyme–product complex (k_4) has been enhanced. At present, it is not clear if the rate-determining step has been altered. Additional kinetic studies are in progress to address this question and to increase our understanding of these mutant enzymes.

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[3] The numbering is taken from the crystal structure by Kim and Wyckoff (1991).

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