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Site-directed Mutagenesis Provides Insight into Racemization and Transamination of Alanine Catalyzed by *Treponema denticola* Cystalysin*

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In addition to α, β-elimination of L-cysteine, *Treponema denticola* cystalysin catalyzes the racemization of both enantiomers of alanine accompanied by an overall transamination. Lys-238 and Tyr-123 or a water molecule located on the *si* and *re* face of the cofactor, respectively, have been proposed to act as the acid/base catalysts in the proton abstraction/donation at Cα/C4′ of the external aldimine. In this investigation, two site-directed mutants, K238A and Y123F, have been characterized. The Lys → Ala mutation results in the complete loss of either lyase activity or racemase activity in both directions or transaminase activity toward L-alanine. However, the K238A mutant is able to catalyze the overall transamination of D-alanine, and only D-alanine is the product of the reverse transamination. For Y123F the k_{cat}/K_m is reduced 3.5-fold for α,β-elimination, whereas it is reduced 300–400-fold for racemization. Y123F has ~18% of wild type transaminase activity with L-alanine and an extremely low transaminase activity with D-alanine. Moreover, the catalytic properties of the Y124F and Y123F/Y124F mutants rule out the possibility that the residual racemase and transaminase activities displayed by Y123F are due to Tyr-124. All these data, together with computational results, indicate a two-base racemization mechanism for cystalysin in which Lys-238 has been unequivocally identified as the catalyst acting on the *si* face of the cofactor. Moreover, this study highlights the importance of the interaction of Tyr-123 with water molecules for efficient proton abstraction/donation function on the *re* face.

Treponema denticola cystalysin catalyzes the pyridoxal 5′-phosphate (PLP)[1]-dependent α,β-elimination of L-cysteine to generate pyruvate, ammonia, and H_2S (1). It has been shown that several sulfur- and non-sulfur-containing amino acids as well as disulfidic amino acids also serve as substrates for this reaction (2). The protein is composed of two identical subunits, each of which comprises 399 amino acid residues. The three-dimensional structure of the enzyme has been solved to 1.9 Å (3). The PLP cofactor in the active site forms a Schiff base with the ε-amino group of Lys-238 of cystalysin (3). Through site-directed mutagenesis, spectroscopic, and kinetic studies, we have shown recently that Lys-238 is an essential residue for α,β-elimination catalyzed by the enzyme cystalysin. In addition to strengthening the coenzyme binding and facilitating transimination, this residue seems to participate as a general base abstracting the Cα proton from the substrate and possibly as a general acid, protonating the β-leaving group (4).

In a recent paper we have shown that, in addition to the lyase activity, the enzyme exhibits an alanine racemase activity accompanied by an overall transaminase activity toward both enantiomers of alanine (5). Considering that racemization is a side reaction of cystalysin, it occurs with a significant k_{cat} (~1 s^{-1}), even if it is ~1000-fold lower than that of alanine racemase (6). The structure of cystalysin with aminoethoxyvinylglycine has revealed that Lys-238 and Tyr-123, located on the *si* and *re* face of the cofactor, respectively, are structurally well positioned to be general acid/base catalysts in a two-base racemization mechanism (3). A similar active site architecture has been reported for the crystal structure of alanine racemase from *Bacillus stearothermophilus* with an alanine phosphonate in which PLP is bound such that Tyr-265′ faces the *si* side of the cofactor and Lys-39 the *re* side (7). For alanine racemase a two-base racemization mechanism involving Tyr-265′ and Lys-39 has been demonstrated by site-directed mutagenesis studies (8, 9). On the basis of structural and kinetic data of cystalysin, we have proposed that racemization occurs by a two-base mechanism. Lys-238 and Tyr-123 have been suggested as possible candidates for general acid-base catalysis, even though the possibility has not been excluded that the crystallographic water molecule located on the *re* side could serve as the acid/base catalyst (5). According to the generally accepted mechanism as well as on the basis of our studies, alanine racemase reaction is proposed to proceed in the following manner as shown in Scheme 1: (i) transaldimination between Lys-238 bound with PLP (I) and the α-amino group of alanine to produce an external aldimine II; (ii) abstraction of the α-hydrogen from alanine to produce a resonance-stabilized quinonoid intermediate III; (iii) reprotonation at the α-carbon of the quinonoid intermediate III on the side opposite to that where the α-hydrogen was abstracted; and (iv) the second transaldimination between IV and Lys-238 to release the product enantiomer of alanine. An equivalent route can be delineated for D-alanine. The transamination catalyzed by cystalysin is probably attained through the sequences I → II → III → VI or V → IV → III → VI for L-and D-alanine, respectively.

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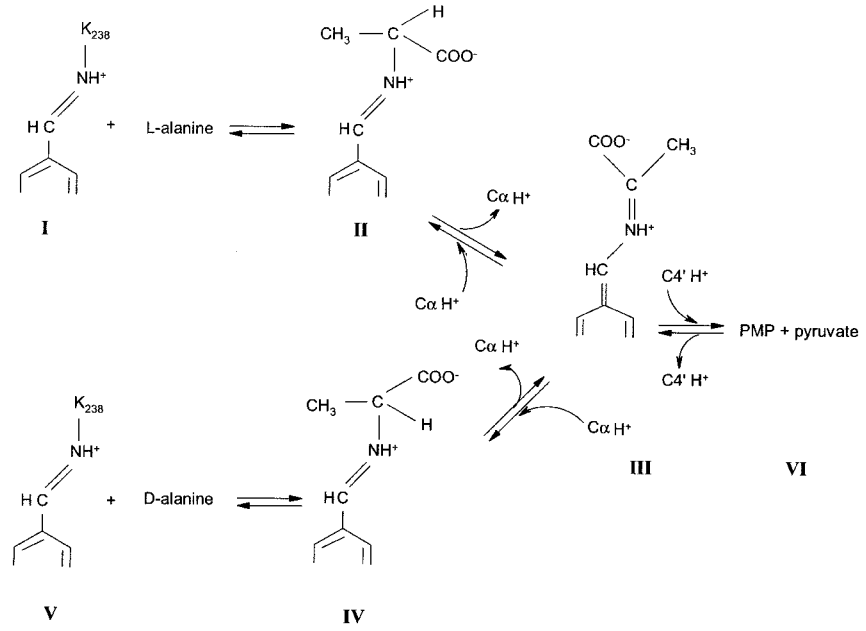
[1] The abbreviations used are: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.



SCHEME 1. **Reaction mechanism for the racemization and transamination of cystalysin.**

As a first step in testing the hypothetical function of Lys-238 and Tyr-123 in the catalysis of cystalysin, the enzymatic activities of the K238A and Y123F mutants were determined. The results provide evidence in support of our previously proposal (5) of a two-base racemization mechanism of both of the enantiomers of alanine. However, only the PLP binding lysine, Lys-238, has been unequivocally identified as the catalyst acting on the *si* face of the cofactor. In fact, the Y123F mutant exhibits poor racemase and transaminase activities. A previous modeling study of the binding modes of the enantiomers of alanine to wild-type cystalysin has indicated that Tyr-124 is too far from the α-hydrogen of the substrate (4.1 Å) for the α-proton abstraction (5). However, it cannot be excluded that the geometry of the active site could be altered upon replacement of Tyr-123 by phenylalanine. Thus, the possibility that the catalytic function of Tyr-123 could be replaced by Tyr-124 has been addressed by the spectroscopic and kinetic characterization of the Y124F and Y123F/Y124F mutants. These studies show that Tyr-124 cannot replace Tyr-123. Therefore, the role of Tyr-123 and/or water molecules in performing the proton abstraction/donation function on the *re* face of the cofactor is discussed.

EXPERIMENTAL PROCEDURES

Materials—PLP, pyridoxamine 5′-phosphate (PMP), β-chloro-L-alanine, L- and D-alanine, NAD^+, NADH, pyruvate, rabbit muscle L-lactic dehydrogenase, alanine dehydrogenase in 50% glycerol, D-amino acid oxidase, and isopropyl β-D-thiogalactopyranoside were from Sigma. All other chemicals were of the highest grade commercially available.

Site-directed Mutagenesis—All mutant forms of cystalysin were made on the wild-type construct pUC18:*hly* (10) using the QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). The kit employs double-stranded DNA as the template, two complementary oligonucleotide primers containing the desired mutation, and DpnI endonuclease to digest the parental DNA template. Oligonucleotides were synthesized by MWG-Biotech AG (Anzinger, Germany). The Y123F and Y124F mutants and the double mutant (Y123F/Y124F) of cystalysin were produced using as primers 5′-CATTATCATCACACCGGTTTTTTTATCCTTTCTTTAT-3′, 5′-CATTATCATCACACCGGTTTATTTTTCCTTTCTTTATGGC-3′, 5′-CATTATCATCACACCGGTTTTTTTTCCTTTCTTTAT-3′, and their complementary oligonucleotides, respectively. The mutated bases are underlined.

The coding regions of the mutated *hly* gene were sequenced to confirm the mutations. *Escherichia coli* strain DH5α cells were transformed and used for expression.

Expression and Purification of Mutant Cystalysin—The conditions used for expression of the mutant proteins in *E. coli* were as described for the wild-type enzyme (2). The wild-type and mutant forms of cystalysin were purified to homogeneity with the procedure described previously (4). The K238A mutant was obtained as reported previously (4). The protein concentration in all cystalysin samples was determined by absorbance spectroscopy using a previously determined extinction coefficient of 1.27×10^5 M^{-1} cm^{-1} at 280 nm (2). The PLP content of wild-type and mutant enzymes was determined by releasing the coenzyme in 0.1 M NaOH and by using $\epsilon = 6600$ M^{-1} cm^{-1} at 388 nm (11).

Enzyme Activity Assays—The α,β-eliminase activity of cystalysin was measured by the spectrophotometric assay coupled with lactate dehydrogenase as described previously (2). The conversion of alanine in the L → D or D → L direction was measured using D-amino acid oxidase and lactate dehydrogenase or L-alanine dehydrogenase as the coupling enzymes, respectively, as reported previously (5). The detection and quantification of PLP and PMP content were performed using the high pressure liquid chromatography procedure described previously (12). To determine the kinetic parameters of the catalytic activities, the assays were performed as indicated above using a fixed amount of enzyme, whereas the substrate concentration was varied from 0.02 to 10 mM and from 0.15 to 500 mM for eliminase and racemase or transaminase activities, respectively. The experimental data were fit into the Michaelis-Menten equation to determine K_m and k_{cat} values.

Binding Affinity of the Y123F, Y124F, and Y123F/Y124F Mutants for the PLP Cofactor—The apparent equilibrium constant for the dissociation of PLP from Y123F, Y124F, and Y123F/Y124F was determined by measuring the α,β-eliminase activity of the apoenzymes (0.6 μM) in the presence of PLP ranging from 0.01 to 9 μM. The K_d value of the enzyme-coenzyme complex was obtained using a tight binding hypothesis according to Equation 1, shown below,

$$Y = Y_{max}\frac{[E]_t + [PLP]_t + K_d - \sqrt{([E]_t + [PLP]_t + K_d)^2 - 4[E]_t[PLP]_t}}{2[E]_t} \quad \text{(Eq. 1)}$$

where $[E]_t$ and $[PLP]_t$ represent the total concentrations of the cystalysin mutant dimer and PLP, respectively, Y refers to the enzymatic activity change at various PLP concentrations, and Y_{max} refers to the enzymatic activity when all enzyme molecules are complexed with a coenzyme.

Molecular Modeling—The BUILDER package from Insight II (version 2000; MSI, Los Angeles, CA) was used to model the D-alanine-PLP and L-alanine-PLP conjugates, using the PLP-serine complex present in the crystal structure of serine hydroxymethyltransferase from *B. stearothermophilus* (13) as template. Tyr-123 and Tyr-124 from the crystal structure of cystalysin (Protein Data Bank code 1C7O) in its dimeric form were then mutated to phenylalanine to obtain the single Y123F and the double Y123F/Y124F mutants using the BIOPOLYMER package from Insight II. Both complexes were positioned into the modified active sites following the binding mode of aminoethoxyvinylglycine (3).

All minimizations were carried out using the Cff91 force field as implemented in Discover 2.9 and the Analysis package of Insight II. Active site solvent molecules, as present in the crystal structure, were considered. Non-bond terms were truncated at 40 Å (smoothing from 36 Å) with a switching function for van der Waals and electrostatic terms. Because the other water molecules were not explicitly included, a distance-dependent dielectric was used throughout the minimizations. The minimization protocol is described elsewhere (2). Briefly, after an initial minimization performed on the whole system to allow added hydrogens to adjust to the crystallographically defined environment, a gradually decreasing tethering force using the steepest descents and conjugated gradients was applied to the whole system until the system was totally relaxed. The maximum derivative achieved was 0.0001 kcal·mol^{-1}Å^{-1}. Only the PLP-Ala complex and main chain and side chains of each residue within 20 Å from the external aldimine were free to move.

Spectral Measurements—Absorption measurements were made with a Jasco V-550 spectrophotometer. The enzyme solution was drawn through a 0.2-μm filter to reduce light scattering from a small amount of precipitate. Fluorescence spectra were taken with a FP-750 Jasco spectrofluorometer using 5-nm excitation and emission bandwidths at a protein concentration varying from 0.9 to 2.2 μM. Spectra of blanks, *i.e.* of samples containing all components except cystalysin, were taken immediately before the measurements of samples containing protein. The blank spectra were subtracted from the spectra containing the enzyme. CD spectra were obtained using a Jasco J-710 spectropolarimeter with a thermostatically controlled compartment at 25 °C. For near-UV and visible wavelengths the protein concentration varied from 0.6 to 1 mg/ml in a cuvette with a 1-cm path length. Routinely, four spectra were recorded at a scan speed of 50 nm/min with a bandwidth of 2 nm and were averaged automatically except where indicated. For far-UV measurements the protein concentration was 0.1 mg/ml with a 0.1-cm path length.

Curve Fitting analysis—All data analysis for determining the model-derived kinetic parameters was performed by nonlinear curve fitting using MicroCal Origin 3.01 (MicroCal Software, Inc., Northamton, MA).

RESULTS

We used site-directed mutagenesis to prepare several variants of cystalysin with single and double replacements at putative active site residues. The purified variants were homogeneous as indicated by a single band on SDS-PAGE with a mobility identical to that of the corresponding wild-type protein. Yields of the mutant enzymes after the standard purification were ~ 70% with respect to that of the corresponding wild-type enzyme. The effects of amino acid substitution on the catalytic properties were determined to evaluate the function of these residues and gain insight into the mechanism of catalysis. Because the reactions catalyzed by cystalysin proceed through chromophoric intermediates formed between PLP and ligands, we have also examined the effects of amino acid replacement on the spectra of enzyme-ligand intermediates.

To test the structural integrity of the cystalysin mutants, we measured their circular dichroism spectra in the far-UV region. No differences have been observed between the spectra of the mutated and the wild-type forms of cystalysin, which indicates that the mutations do not affect the overall secondary structure of cystalysin (data not shown).

Spectroscopic Properties of Tyr-123 and Tyr-124 Mutants—As with wild-type, the Y123F, Y124F, and Y123F/Y124F variants bind 2 mol of PLP per dimer. In contrast, apo K238A reconstituted with PLP binds 1 mol of coenzyme per dimer, as reported previously (4). As shown in Fig. 1, *A* and *B*, the absorbance and CD spectra of Tyr-123 and Tyr-124 mutants in the UV-visible region are largely similar to those of the wild-type enzyme. The wild-type, Y123F, Y124F, and Y123F/Y124F mutant enzymes display optical activity (millidegrees per absorbance unit) values of 70, 79, 70, and 61 millidegrees/A_{418}, respectively. This suggests that the microenvironment of the internal aldimine in the Tyr-123 mutant enzymes is slightly different from that in the wild-type enzyme. For comparison, the absorbance and CD spectra of the apo K238A reconstituted with PLP reported previously (4) are also included in Fig. 1, *A*

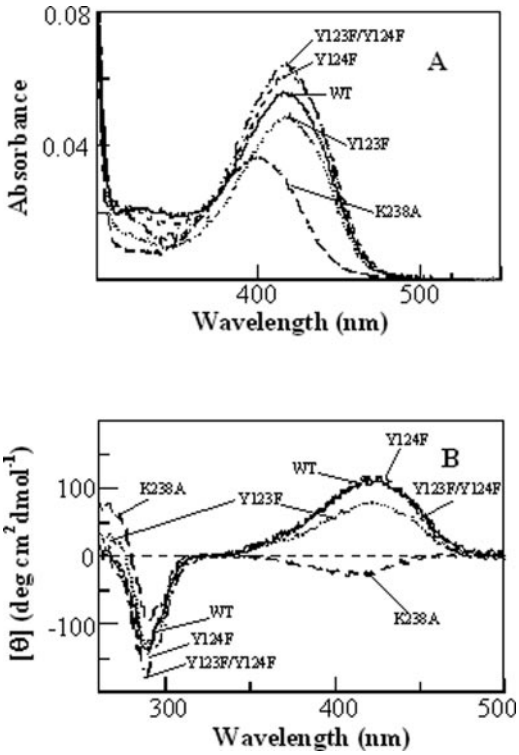


FIG. 1. **Absorbance and CD spectra of wild-type and mutants of cystalysin.** *A*, absorption of wild-type (*WT*) (——), Y123F (· · · ·), Y124F (-----), Y123F/Y124F (·–·–·–), and K238A (– – –) in 20 mM potassium phosphate buffer, pH 7.4, at a concentration of 4.5 μM. *B*, CD spectra; the symbols for wild-type, Y123F, Y124F, Y123F/Y124F, and K238A are the same as for *panel A*.

and *B*, respectively. The fluorescence of Tyr-123 and Tyr-124 mutants is similar to that of the wild-type. It is of interest that the intensity of the PLP emission fluorescence (excitation at 418 nm) of the Y123F and Y123F/Y124F mutants is 10-fold higher than that of the wild-type cystalysin (data not shown).

Enzyme Activities of Lys-238, Tyr-123, and Tyr-124 Mutants—Steady-state kinetic studies of the primary reaction (α,β-elimination) and of alanine racemase and transaminase activities were performed on the mutant enzymes and compared with those of wild-type. Although as reported previously (4) the K238A mutant is inactive in the α,β-elimination of β-chloro-L-alanine, Y123F, Y124F, and Y123F/Y124F mutants display, to varying extents, significant eliminase activity. A summary of the resulting steady-state parameters obtained for the α,β-elimination reaction toward β-chloro-L-alanine is listed in Table I. For both Y123F and Y123F/Y124F mutants, the K_m values were ~10-fold lower with respect to those of the wild-type. Furthermore, the observed k_{cat} values decreased by 25- and 40-fold for Y123F and Y123F/Y124F, respectively, compared with those of the wild-type enzyme, resulting in a ~ 3.5-fold reduction of the catalytic efficiency (k_{cat}/K_m). As seen in Table I, a slight increase was observed in the K_m and k_{cat} values for Y124F, leading to a 50% reduction of the catalytic efficiency with respect to wild-type. The results indicate that, unlike the Lys-238 residue, the Tyr-123 and Tyr-124 residues are not critical for the α,β-eliminase activity catalyzed by cystalysin.

The kinetic parameters determined for alanine racemase activity reveal that, although alteration of Lys-238 causes a complete loss of racemase activity, the mutation of Tyr-123 results in a 200-fold reduction of k_{cat}. These effects are similar of those observed for the primary reaction with the K238A variant but are significantly more than those observed for the primary reaction with the Y123F variant, where only a 25-fold

TABLE I
Steady-state kinetic parameters for α,β-elimination of β-chloro-L-alanine in 20 mM potassium phosphate buffer, pH 7.4, at 25 °C

	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	mM	$mM^{-1}\ s^{-1}$
Wild-type[a]	59.9 ± 2.3	1.21 ± 0.15	49.5 ± 6.4
K238A[b]			
Y123F	2.41 ± 0.08	0.17 ± 0.03	14.2 ± 2.5
Y124F	74 ± 2	3.2 ± 0.20	23.1 ± 1.6
Y123F/Y124F	1.44 ± 0.04	0.10 ± 0.02	14.4 ± 2.9

[a] From Ref. 2.
[b] From Ref. 4.

decrease in k_{cat} is produced. On the contrary, the kinetic parameters for alanine racemase activity are not significantly altered in the Y124F mutant, analogous to what was observed for the α,β-elimination reaction. For the Y123F/Y124F variant the K_m values and the k_{cat} for L-and D-alanine were ~ 10- and 200-fold lower, respectively, with respect to the corresponding values for wild-type (Table II).

Each Lys-238, Tyr-123, and Tyr-124 variant was also screened for the ability to catalyze the transamination of L- and D-alanine. Y124F mutant catalyzes transamination of L- and D-alanine with k_{cat} values of 0.0177 ± 0.0003 and 0.0035 ± 0.0004 min^{-1}, respectively, about half the value corresponding to wild-type (5). Both Y123F and Y123F/Y124F mutants catalyze the transamination of L-alanine with k_{cat} values 5- and 10-fold lower, respectively, than that of wild-type. On the other hand, under conditions of relatively high enzyme concentration (30 μM), normally saturating substrate concentrations (500 mM), and extensive time courses (2 h), the reaction of these mutants with D-alanine produces 0.51 μM PMP. Considering that under these conditions L-alanine is produced in amounts less than 0.1 K_m, it can be assessed that most of the PMP produced is due to the transamination of D-alanine rather than the transamination of L-alanine formed by racemization of D-alanine. It is noteworthy that the K238A mutant does not show detectable transaminase activity toward L-alanine, whereas it is able to catalyze the transamination of D-alanine. Initial velocity data for this reaction were fitted to Michaelis-Menten equation and yielded the kinetic parameters K_m = 24.5 ± 3.5 mM and k_{cat} = 0.125 ± 0.004 min^{-1}, resulting in a catalytic efficiency ~ 8-fold higher than that of wild-type for D-alanine (5).

As with wild-type, K238A and Y123F apomutants are able to convert the PMP form of the enzyme back into the PLP form in the presence of pyruvate. The kinetic parameters for this reaction are listed in Table III. The k_{cat}/K_m value of Y123F is reduced by only 2.5-fold as compared with that of wild-type enzyme. This reduction is due to approximately equal magnitude changes in the k_{cat} and K_m values. On the contrary, the k_{cat}/K_m of the K238A variant is increased by 25-fold as compared with that of the wild-type. The increase in the catalytic efficiency of this mutant is driven by the decrease in K_m (30-fold), as the k_{cat} is not appreciably affected. It is also of interest to note that only alanine in the D-form is generated by the K238A variant, whereas L-alanine is formed in amounts ~2-fold higher than those of D-alanine by the Y123F mutant. The amounts of L- and D-alanine produced from Y123F have been evaluated after 25 min of reaction of 100 μM variant enzyme in the presence of 200 μM PMP and 0.3 mM pyruvate. Thus, on the basis of the k_{cat} values of racemization and reverse transamination of the Y123F variant, it can be inferred that the production of D-alanine is mainly due to transamination rather than the conversion of L- into D-alanine. It should be noted that L- and D-alanine are produced in nearly equivalent amounts when apo wild-type reacts with pyruvate in the presence of PMP. However, given the higher racemization rate in this case, it was not possible to determine whether both enantiomers of alanine are formed by racemization rather than by transamination (5).

Binding Affinity of Y123F and Y124F Mutants for the PLP Cofactor—When wild-type or Y124F was mixed with PLP, the regain of eliminase activity occurred within the time required for the manual mixing of PLP and apoenzyme. In contrast, under the same experimental conditions the regain of activity upon the addition of a coenzyme to either apo Y123F or Y123F/Y124F as a function of time reached a near saturation value within 1 h. Titration analysis of the apomutants Y123F, Y124F, and Y123F/Y124F with PLP fitted to the appropriate equation yielded K_d values for the PLP-Y123F, PLP-Y124F, and PLP-Y123F/Y124F complexes equal to 295 ± 60, 164 ± 26, and 137 ± 30 nM, respectively (data not shown). The apparent K_d for the dissociation of PLP from the wild-type and the K238A mutant has been found previously to be 6.6 ± 0.1 (2) and 70 ± 16 nM (4), respectively.

Absorption and CD Spectral Changes of the K238A, Y123F, and Y124F Mutants with L- and D-Alanine—As reported previously, the binding of both alanine enantiomers to wild-type cystalysin leads to the appearance of an external aldimine absorbing at 429 nm and a band absorbing at 498 nm, indicative of a quinonoid species (5). The 429- and 498-nm absorbance bands decrease with time with the concomitant increase in the absorbance band at 325 nm. These events have been attributed to a half-transamination (5). As reported in Fig. 2*A*, whereas the addition of L-alanine to the K238A variant causes the appearance of an absorbing band at 420 nm that remains unchanged with time, the addition of D-alanine results in the immediate appearance of an absorbance band centered at 423 nm that decreases with time with the concomitant increase in the absorbance band at 325 nm. Either L- or D-alanine added to the wild-type results in the appearance of two negative dichroic bands at 441 and 340 nm (5). A negative dichroic band at 440 nm, although of lower intensity with respect to that corresponding to wild-type, characterizes the CD spectra of K238A with D-alanine. A negative ellipticity band centered at ~430 nm is observed instead in the presence of L-alanine (Fig. 2*B*).

The binding of L-alanine to Y123F results in mixtures of external aldimine and quinonoid species absorbing at 421 and 499 nm, respectively. The 421-nm band decreases with time and, concomitantly, an absorbance band at 325 nm increases, whereas the absorbance at 499 nm does not show appreciable changes (Fig. 3*A*). On the other hand, upon the addition of D-alanine to Y123F an absorbance band at 422 nm appears (Fig. 3*A*). No detectable changes could be observed during 7 h of reaction; this result is consistent with the extremely low conversion of PLP-bound into PMP (see above). Unlike the wild-type enzyme, the Y123F variant shows a positive CD band at 420 nm upon the interaction with both isomers of alanine (Fig. 3*B*).

When the reaction of the Y123F/Y124F variant with L- or D-alanine was examined, the spectral changes observed were qualitatively identical to those of Y123F. As with the wild-type, the Y124F variant exhibits absorbance changes upon the addition of both enantiomers of alanine consisting of an immediate appearance of absorbing bands at 429 and 500 nm that decrease with time along with the concomitant increase of an absorbance band at 325 nm (data not shown).

Molecular Modeling—To gain some insights into the binding modes of L-alanine and D-alanine, we decided to model their positions in the active site of cystalysin modified so that either Tyr-123 only or both Tyr-123 and Tyr-124 were mutated to phenylalanine. Substrate molecules were preoriented as the external aldimine with its α-carboxylate group pointing toward Arg-369 as observed in the complex between cystalysin and

TABLE II
Steady-state kinetic parameters for racemization of L- and D-alanine in 20 mM potassium phosphate buffer, pH 7.4, at 25 °C

	k_{cat}		K_m		k_{cat}/K_m	
	L-Ala	D-Ala	L-Ala	D-Ala	L-Ala	D-Ala
	s^{-1}		mM		$mM^{-1}\ s^{-1}$	
Wild-type	1.05 ± 0.03	1.4 ± 0.1	10 ± 1	10 ± 1	0.10 ± 0.01	0.14 ± 0.02
K238A	N.D.[a]	N.D.[a]				
Y123F	0.0058 ± 0.0002	0.0069 ± 0.0003	14.3 ± 1.8	22.4 ± 3.6	$4 \times 10^{-4} \pm 5 \times 10^{-5}$	$3 \times 10^{-4} \pm 5 \times 10^{-5}$
Y124F	1.96 ± 0.08	3.16 ± 0.03	20 ± 3	25.3 ± 0.7	0.1 ± 0.01	0.125 ± 0.004
Y123F/Y124F	0.0047 ± 0.0002	0.0042 ± 0.0001	1 ± 0.2	0.76 ± 0.08	0.0047 ± 0.0009	0.0056 ± 0.0006

[a] N.D., not detectable.

TABLE III
Steady-state kinetic parameters for reverse transamination of wild-type, K238A, and Y123F mutants in 20 mM potassium phosphate buffer, pH 7.4, at 25 °C

	k_{cat}	K_m	k_{cat}/K_m
	min^{-1}	mM	$mM^{-1}\ min^{-1}$
Wild-type	0.108 ± 0.005	0.19 ± 0.03	0.57 ± 0.09
K238A	0.086 ± 0.005	0.006 ± 0.001	14 ± 2
Y123F	0.083 ± 0.001	0.391 ± 0.032	0.21 ± 0.02

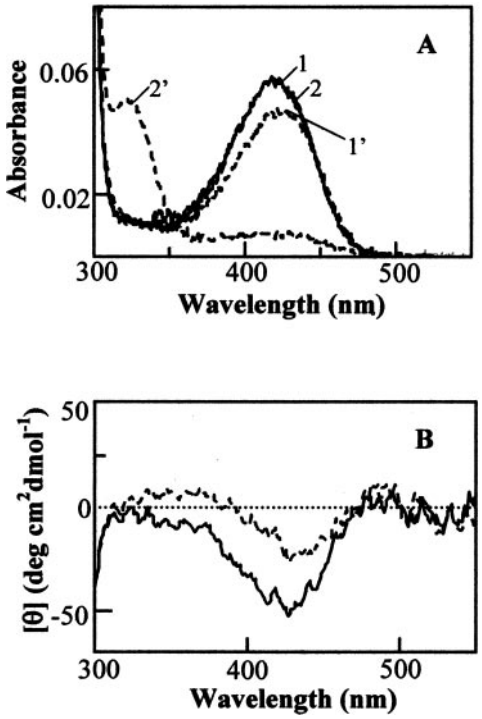


FIG. 2. **Absorbance and CD spectra of K238A in the presence of L- or D-alanine.** *A*, absorption spectra recorded immediately (*1*) or after 350 min (*2*) upon the addition of 500 mM L-alanine (——) to K238A and absorption spectra recorded immediately (*1′*) or after 60 min (*2′*) upon addition of 500 mM D-alanine (– – –) to K238A. The enzyme concentration was 6.3 μM in each case. *B*, CD spectra of K238A recorded immediately after the addition of 500 mM L-alanine (——) or D-alanine (– – –).

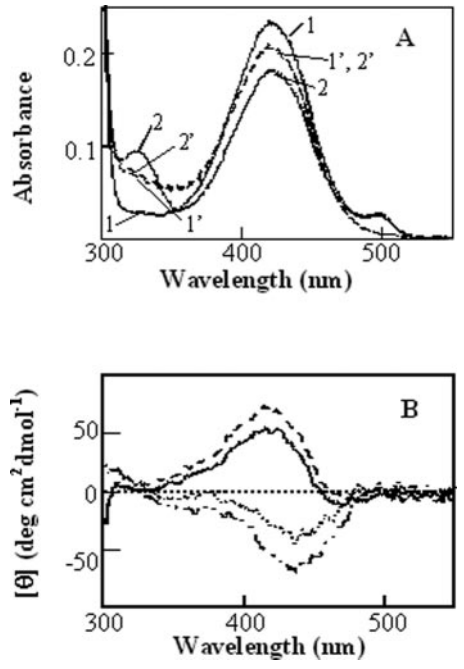


FIG. 3. **Absorbance and CD spectra of wild-type and Y123F in the presence of L- or D-alanine.** *A*, absorption spectra recorded immediately (*1*) or after 430 min (*2*) upon the addition of 500 mM L-alanine (——) to Y123F and absorption spectrum recorded immediately (*1′*) or after 420 min (*2′*) upon the addition of 500 mM D-alanine (– – –) to Y123F. The enzyme concentration was 20 μM in each case. *B*, CD spectra of Y123F recorded immediately after the addition of 500 mM L-alanine (——) or D-alanine (– – –). To allow a comparison, CD spectra of wild-type recorded immediately after the addition of 500 mM L-alanine (——) or D-alanine (·–·–·–) are included.

aminoethoxyvinylglycine, and the most severe steric clashes were manually removed. Overall, after the minimization of both complexes there were no major structural changes regarding the position of the mutated tyrosines and the interaction between PLP and the active site residues as compared with its position modeled inside the wild-type enzyme (5). The α-carboxylate group of alanine interacts through hydrogen bonds with the guanidinium group of Arg-369, and additional hydrogen bonds are formed with Asn-175 and with the main chain amide of Ala-39.

For the L-alanine docked into the active site of the single mutant enzyme Y123F, the ϵ-amino group of Lys-238 is still located within a suitable distance from the Cα hydrogen of the substrate (2.7 Å) to act as a catalytic base. However, when comparing the interaction energies between the substrate and cystalysin in its wild-type (5) and mutated form, two major differences are revealed as follows. 1) The α-methylic group is better accommodated inside the hydrophobic cleft formed by Phe-123; this observation is supported by the better interaction energy between the substrate and the phenylalanine residue (-3.9 kcal·mol^{-1}). The same value, measured for Tyr-123, was -1.72 kcal·mol^{-1}. 2) W733H, the crystallographic water molecule thought to act as a catalytic base on the *re* side of the cofactor (5), is displaced from its original position (5.2 Å from Phe-123 in the single mutant) because it is no longer stabilized by the interaction with the hydroxyl group of Tyr-123 as occurs in the wild-type enzyme. The latter data could also be explained by the less favorable hydrophobic environment contributed by the Phe-123 residue (Fig. 4*A*).

For D-alanine, the interaction energy with Phe-123 is still more favorable as compared with the wild-type enzyme (-3.4 kcal·mol^{-1} for the Y123F variant and -1.1 kcal·mol^{-1} for the wild-type enzyme). Again, W733H shifts from its original position; nevertheless, the extent of this displacement is lesser as

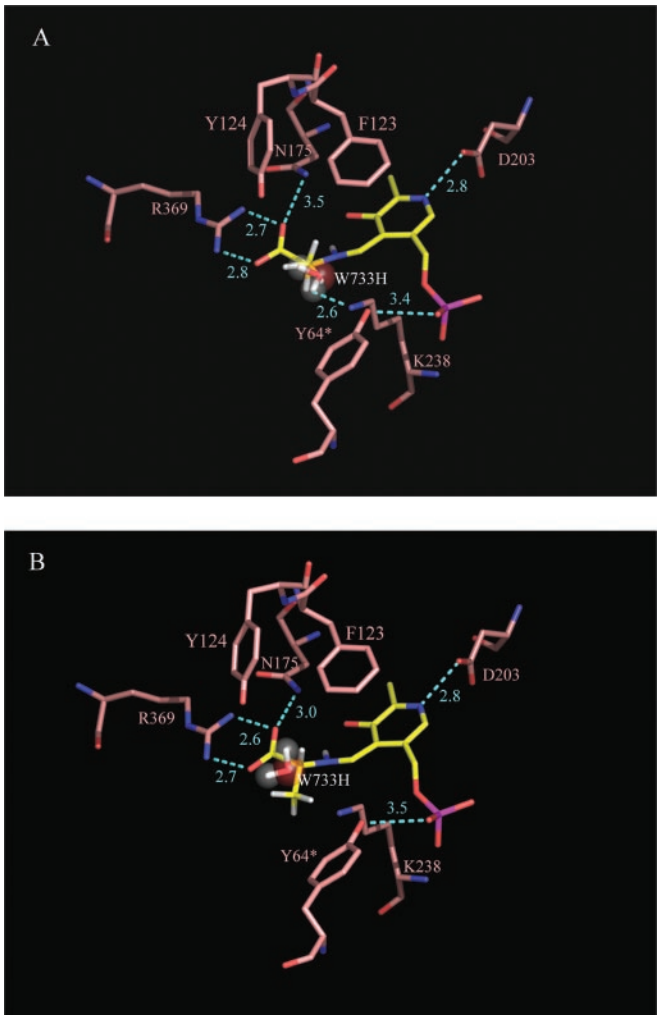


FIG. 4. **Modeling of the binding modes of L-alanine (*A*) and D-alanine (*B*) inside the active site of the Y123F mutant of cystalysin.** The Y123F variant of the *T. denticola* cystalysin active site residues that are involved in substrate and cofactor binding and catalysis are illustrated and labeled according to sequence position together with the alanine-PLP conjugate (represented as *yellow*) sticks. Oxygen atoms are colored *red*, nitrogen atoms are *blue*, and phosphorus is *purple*. Hydrogen bonds and interatomic distances (Å) are shown in *cyan*. An *asterisk* indicates a residue contributed from the other monomer. This figure was rendered using PyMOL (23). Single letter amino acid abbreviations are used with position numbers.

compared with the modeled interaction with L-alanine (4.9 Å from Phe-123) (Fig. 4*B*).

It appears that Tyr-124 plays a minor role as compared with Tyr123 in stabilizing the water molecule and the α-methylic group of the L-alanine for the following reasons. 1) The position of W733H, observed in the double mutant active site, is the same (5.2 Å from Phe-123) as compared with the single mutant enzyme; and 2) the interaction of Phe-124 with the α-methylic group of L-alanine is still more favorable as compared with the wild-type enzyme (-1.75 kcal·mol^{-1} for Phe124 and -1.16 kcal·mol^{-1} for Tyr124) but to a lesser extent with respect to Phe-123. When docked with D-alanine, the double mutant Y123F/Y124F displays no major differences compared with the single mutant enzyme (the interaction energy with the substrate is -1.42 kcal·mol^{-1}; W733H is 4.8 Å away from Phe-123).

DISCUSSION

Site-directed mutagenesis studies have been employed to gain insight into the mechanism of racemization and transamination of both enantiomers of alanine catalyzed by cystalysin. Kinetic data corroborated by molecular modeling studies have suggested a two-base mechanism involving Lys-238, the PLP-binding lysine, and Tyr-123 or a water molecule acting as acid-base catalysts (5). As a first step, Lys-238 and Tyr-123 were selected as targets for mutagenesis, and the kinetic and spectroscopic properties of the active-site mutants K238A and Y123F were analyzed to probe the hypothetical role of the mutated residues in racemase and transaminase activities.

Upon mixing either L- or D-alanine with K238A, a shift in the visible absorbance from 400 to 420 and 423 nm, respectively, was observed. This shift is consistent with the formation of an external aldimine absorbing at a lower wavelength with respect to those formed by wild-type with both of the enantiomers of alanine absorbing at 429 nm (5). Absorption and CD spectra of K238A in the presence of substrate analogs have indicated that Lys → Ala substitution does not impair Schiff base formation. However, kinetic analysis of this event has revealed that the replacement of Lys-238 by alanine decreases the rate of Schiff base formation and, to a lesser extent, the rate of Schiff base hydrolysis (4). The binding of D-alanine to K238A causes an increase in the intensity of the coenzyme ellipticity at 440 nm. A similar effect has been observed upon the addition of L- or D-alanine to the wild-type enzyme (5). On the other hand, L-alanine gives rise to a slightly different dichroic signal upon binding to the K238A mutant. This suggests that the external aldimine with L-alanine in the mutant could have a different microenvironment. As shown in Table II, the K238A variant does not show detectable racemase activity in both directions. Moreover, transaminase activity could not be observed for L-alanine. This result is consistent with the finding that, in the presence of L-alanine, the external aldimine but not the quinonoid species is detected and that the external aldimine remains unchanged with time. All of these results are compatible with the hypothesis that Lys-238 is the base located on the *si* face specifically abstracting an α-hydrogen from L-alanine. An identical role has already been proposed from studying the reaction of this mutant with the substrates undergoing α,β-elimination (4). On the other hand, the K238A variant has been found to catalyze transamination of D-alanine with a catalytic efficiency ~8-fold higher than that catalyzed by the wild-type enzyme. This finding is in line with the spectral changes observed for the variant-D-alanine complex consisting of a decrease of the absorbance band absorbing at 423 nm with the concomitant appearance of an absorbance band at 325 nm. The lack of detectable levels of the quinonoid intermediate upon the addition of D-alanine to the K238A variant is consistent with the possibility that the formation of the quinonoid intermediate is slower than reprotonation for the mutant protein. As with the apo wild-type enzyme, the apo K238A variant is able to catalyze the reverse transamination in the presence of PMP and pyruvate. This activity exhibits an ~25-fold increase in k_{cat}/K_m with respect to wild-type. This increase, which is the result of a tighter binding of pyruvate, could be the consequence of the variant enzyme-substrate complex being in an energy well deeper than the one in the wild-type enzyme-substrate complex. Thus, it can be suggested that Lys-238 plays a role in the stabilization of the ketimine. It is relevant that only D-alanine is formed by the apo K238A mutant under the same experimental conditions where apo wild-type cystalysin produces a racemic mixture of alanine by reverse transamination (5). The overall transamination of D-alanine catalyzed by the K238A variant strongly suggests that an acid-base catalyst could be on the *re* side of the cofactor. The role of this catalyst in the forward reaction consists of proton abstraction from the D-alanine-PLP external aldimine complex and reprotonation at the C4′ of the generated carbanionic intermediate to give pyruvate and PMP. In the reverse reaction, the same catalyst transfers a proton from

C4′ of the pyruvate-PMP ketimine intermediate to the Cα of the quinonoid to regenerate D-alanine.

On the basis of these data and those reported previously (5), we decided to check Tyr-123 as a possible acid-base catalyst. The active-site mutant Y123F has been constructed, expressed, and purified. Although the mutation introduced does not appreciably change either the PLP content or its absorption spectral features, it significantly reduces the kinetics of reconstitution to the holo form, increases the apparent equilibrium dissociation constant for PLP by ~45-fold, and slightly changes the cofactor microenvironment. Because Tyr-123 is the pyridoxal sandwiching residue (3), it can be envisaged that the Tyr → Phe substitution could loosen the base stacking with the pyridine cofactor moiety. The replacement of Tyr-123 by phenylalanine reduces the k_{cat}/K_m for the α,β-elimination of β-chloro-L-alanine by only ~ 3.5-fold. Thus, it is evident that the hydroxyl group of Tyr-123 does not play an essential role in this catalytic process. It should be noted that the affinity of this substrate for mutants in which Tyr-123 has been replaced (Y123F and Y123F/Y124F) has increased a few fold in comparison with wild-type. This could suggest that the proximity of the polar OH of Tyr-123 to the substrate results in a ground state destabilization that is used by the enzyme to promote substrate activation (3). Notably, the mutation of Tyr-123 to phenylalanine results in an enzyme with an ~300–400-fold reduced catalytic efficiency of racemase activity. The different extent in reducing lyase and racemase activities can be explained by considering that the α,β-elimination reaction differs markedly from the racemization in that the reprotonation step at the α-position of the deprotonated intermediate is not involved. The accumulation of the quinonoid intermediate observed in the L → D direction could be interpreted by considering that if Tyr-123 is compromised as a catalyst in the mutant, then the forward progress from the quinonoid via protonation by Tyr-123 is expected to be slowed. Moreover, Y123F exhibits an extremely low but measurable transaminase activity toward D-alanine. On the contrary, this mutant retains a significant transaminase activity toward L-alanine (18% in comparison with wild-type), which indicates that Tyr-123 is not essential for transamination of the L-enantiomer and that the catalytically uncompromised Lys-238 is operative on the *si* face. Additionally, Y123F is also able to catalyze the reverse transamination of pyruvate in the presence of PMP. It is noteworthy that this reaction leads to the formation of both L- and D-alanine, with the D- enantiomer being due mainly to transamination rather than racemization.

To verify the possibility that the residual racemase and transaminase activities displayed by Y123F could be ascribed to the neighboring Tyr-124 residue, the variant Y124F and Y123F/Y124F mutants have been characterized. The substitution of Tyr by Phe causes some minor structural changes resulting in decreased binding affinities of the mutants for PLP. Kinetic data for Y124F clearly demonstrate that this residue is not critical either for the primary or the secondary reactions catalyzed by cystalysin. In fact, the catalytic efficiencies of these reactions are weakly altered by this mutation. When the double mutant was screened for eliminase, racemase, and transaminase activities, evidence was provided for its ability to catalyze all these reactions with k_{cat} values comparable with the corresponding k_{cat} values of the Y123F variant. Thus, Tyr-124 does not replace Tyr-123 as the acid-base catalyst in the racemization and transamination pathways. Interestingly, the K_m values of the Y123F/Y124F variant for both enantiomers of alanine are decreased by ~10-fold as compared with that of wild-type. This finding agrees with the modeling studies that reveal that the more hydrophobic environment introduced by the substitution of both polar tyrosines with the apolar phenylalanines allows the methyl group of the substrate's side chain to be better accommodated.

On the basis of the above results and considerations, the attribution of the functional role as a acid-base catalyst to a group on the *re* face of the cofactor remains problematic in that either Tyr-123 or water molecules represent possible candidates. The x-ray structure of alanine racemase shows that two active site residues are well positioned to act as acid-base catalysts. These are Tyr-265′ and Lys-39, which lie on opposite faces of the coenzyme and are close to the Cα in the external aldimine from alanine phosphonate (7) and in the two isomeric phosphopyridoxylalanine structures (14). It has been demonstrated by mutagenesis that these are the two acid-base catalysts in a two-base racemization mechanism (8, 9). The Y265A variant has been found to be 1000-fold less active than the wild-type enzyme, whereas the K39A variant was shown to be completely inactive. The reduced racemase activity displayed by the Y265A variant has been ascribed to the substitution of the hydroxide ion in solvent water for an appropriate proton transfer catalyst of the enzyme.

In the case of cystalysin, the mutation of Lys-238 results in the complete loss of racemase activity, whereas the mutation of Tyr-123 causes a 200-fold reduction of this activity. Furthermore, the transaminase activities displayed by these mutants provide evidence for the presence of two acid-base catalysts on the opposite faces of the cofactor. The K_m values of Tyr-123 for both L- and D-alanine indicate that the binding of isomers of alanine is not significantly compromised by the mutation of Tyr-123 with phenylalanine. However, although the addition of saturating concentrations of either L- or D-alanine causes a similar absorbance change for both wild-type and the Y123F variant, it results in distinctly different changes in their dichroic bands. This suggests that the binding of both enantiomers of alanine to the mutant causes changes in the orientation of the coenzyme with respect to the neighboring residues that are different from those changes caused by the same ligands binding to the wild-type enzyme. Thus, it is reasonable to propose that the conformations of the external aldimines of the mutant enzyme with enantiomers of alanine differ from those of the wild-type. To gain some insight into the structural origin of this difference, the model of the Y123F variant complexed with either L- or D-alanine has been compared with that of wild-type. As reported previously for wild-type cystalysin, Tyr-123 is well positioned to interact with the Cα-proton (3 Å) and C4′(3.1 Å), and a water molecule is located within hydrogen-bonding distance to Tyr-123 (2.8 Å), the Cα-hydrogen (2.8 Å), and, to a lesser extent, Tyr-124 (3.5 Å) (5). Phenylalanine at position 123 causes a loosening of the interaction with the water molecule and the displacement of the water molecule from its original position. Thus, Tyr-123 seems to play an important role in stabilizing the interaction with the water molecule. This interaction could be crucial to the orientation of the pyridine ring of the PLP-alanine complex, which is an essential prerequisite for an external aldimine competent for catalysis. On the basis of our results, the possibility that the water molecule held in place by Tyr-123 and Tyr-124 may function as the catalyst on the *re* face of the cofactor should be taken into account. If this were true, the reduced racemase activity of Y123F could be due to a mispositioning of the water molecule following the replacement of Tyr-123 by phenylalanine. The existence of water molecules in the vicinity of or at the active site in PLP enzymes has already been asserted. In alanine racemase, a structural water molecule was identified to be in contact with both substrate and inhibitor in both active sites with the possible function of bridging residues in both active sites (15). In a second case, a

water molecule was determined as sitting on the putative substrate binding site of D-amino acid aminotransferase (16). In aromatic amino acid aminotransferase, water molecules are present to form a hydrogen bond network (17). However, in all of these enzymes water molecules are believed to play no catalytic role; rather, they seem to contribute to the binding of the substrate. On the other hand, for the K258H variant of aspartate aminotransferase (18) it was suggested that the 1,3-prototropic shift catalyzed by this mutant protein is mediated by a water molecule. Water molecules have also been suggested as representing excellent candidates for the protonation of external aldimine of threonine aldolase with glycine (19). A water molecule has been already proposed to lie on the *re* side of the coenzyme-substrate adduct and to serve as the acid-base catalyst during the racemization catalyzed by aspartate aminotransferase (20). In this regard it is noteworthy that racemization of cystalysin and aspartate aminotransferase occurs with k_{cat} values lower than the k_{cat} value of alanine racemase by $\sim 10^3$-fold and 5×10^7-fold, respectively. These data could suggest that when hydroxide ion in solvent water is functionally substituted for an appropriate proton transfer catalyst of the enzyme, racemization takes place with a lower efficiency. Therefore, we advance the idea that a water molecule could be more likely implicated in racemizations catalyzed by PLP enzymes as side reactions rather than in alanine racemases.

Another possibility to be considered is that Tyr-123 might play a direct role in the proton abstraction/donation at Cα/C4′ on the *re* face of the cofactor in the wild-type cystalysin and that it could be replaced by a water molecule in Tyr-123 mutants.

At present, because solid conclusions about the role of Tyr-123 and the water molecule in catalysis cannot be drawn, it is reasonable to propose that water molecules and their hydrogen bond interactions with Tyr-123 in the active site of cystalysin would be required to perform efficiently the proton abstraction/donation function on the *re* face of the cofactor. Nevertheless, all these data support a two-base racemization mechanism, regardless of the exact identity of the *re* face catalyst. To our knowledge, this represents the first evidence for the occurrence of proton removal and addition by a two-base mechanism in a PLP enzyme belonging to fold type I. In fact, up to now a single base "swinging door mechanism" has been demonstrated to occur for the alanine racemase reaction catalyzed by serine hydroxymethyltransferase (21) and tyrosine phenol-lyase (22).

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