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Lysine 238 Is an Essential Residue for α,β-Elimination Catalyzed by *Treponema denticola* Cystalysin*

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Treponema denticola **cystalysin is a pyridoxal 5′-phosphate (PLP) enzyme that catalyzes the α,β-elimination of L-cysteine to pyruvate, ammonia, and H_2S. Similar to other PLP enzymes, an active site Lys residue (Lys-238) forms an internal Schiff base with PLP. The mechanistic role of this residue has been studied by an analysis of the mutant enzymes in which Lys-238 has been replaced by Ala (K238A) and Arg (K238R). Both apomutants reconstituted with PLP bind noncovalently ~50% of the normal complement of the cofactor and have a lower affinity for the coenzyme than that of wild-type. Kinetic analyses of the reactions of K238A and K238R mutants with glycine compared with that of wild-type demonstrate the decrease of the rate of Schiff base formation by 10^3- and 7.5×10^4-fold, respectively, and, to a lesser extent, a decrease of the rate of Schiff base hydrolysis. Thus, a role of Lys-238 is to facilitate formation of external aldimine by transimination. Kinetic data reveal that the K238A mutant is inactive in the α,β-elimination of L-cysteine and β-chloro-L-alanine, whereas K238R retains 0.3% of the wild-type activity. These data, together with those derived from a spectral analysis of the reaction of Lys-238 mutants with unproductive substrate analogues, indicate that Lys-238 is an essential catalytic residue, possibly participating as a general base abstracting the Cα-proton from the substrate and possibly as a general acid protonating the β-leaving group.**

Cystalysin utilizes pyridoxal 5′-phosphate (PLP)[1] as its coenzyme and is categorized as a member of the α subfamily of PLP-dependent enzymes that include aspartate aminotransferase. The cDNA from *Treponema denticola* has been heterologously cloned in *Escherichia coli*, the recombinant enzyme has been crystallized, and the three-dimensional structure solved (1). The PLP cofactor in the active site forms a Schiff base with the ε-amino group of Lys-238 of cystalysin (2). The spectrum of cystalysin exhibits absorption maxima at 418 and 320 nm, in addition to the protein band at 281 nm. The 418-nm band is due to the protonated internal aldimine, whereas the 320-nm band is due to a substituted aldamine. The apparent pK_{spec} of this spectral transition is ~8.4 (2).

Cystalysin catalyzes the α,β-elimination of L-cysteine to generate pyruvate, ammonia, and H_2S (3). A catalytic mechanism has been suggested in which, after transaldimination, the released Lys-238 abstracts the Cα proton from the substrate with its deprotonated ε-amino group, producing a carbanionic intermediate stabilized as the characteristic quinonoid intermediate. Then, after reaching an optimal position within hydrogen bonding distance to Sγ, Lys-238 protonates the sulfur atom, and the resulting thiol is released. Finally, reverse transaldimination of the PLP aminoacrylate takes place; Lys-238 attacks the C4′ atom, converting the aminoacrylate into an iminoproprionate, which is released and hydrolyzed to pyruvate and ammonia (1). Recently, substrate specificity studies reveal that cystalysin displays catalytic features similar to those of cyste(i)ne desulfhydrase (C-DES). Indeed, several sulfur- and non-sulfur-containing amino acids as well as disulfidic amino acids serve as substrates for cystalysin, with L-djenkolic acid and L-cystine being better substrates than L-cysteine (2). The pH dependence of the kinetic parameters for α,β-elimination indicates that a single ionizing group with a pK value of ~ 6.6 must be unprotonated to achieve maximum velocity. This pK has been tentatively associated with the ionization of Lys-238 (2).

Lysine residues involved in internal aldimines in PLP-dependent enzymes have been proposed to have a multitude of roles. They have been shown to increase the reactivity of the PLP 4′-aldehyde group, facilitating the formation of an external aldimine between the substrate and the PLP cofactor (3–9). In addition, lysine residues have also been shown to be involved in catalysis (4–7, 10, 11), the formation of enzyme-substrate intermediates and product release (5, 8, 9), and cofactor binding (4, 5, 12).

Although progress in the spectroscopic and kinetic features of *T. denticola* cystalysin has been made recently, the catalytic mechanism and the individual residues essential for enzyme activity remain to be elucidated. To understand the functional contribution of the active site lysine residue (Lys-238) to stages of the reaction catalyzed by cystalysin, we have changed Lys-238 of cystalysin to Ala or Arg and have studied the kinetic and spectroscopic properties of the mutants. Evidence is provided that Lys-238, in addition to increasing the PLP binding affinity and facilitating the formation of external aldimine, plays an essential catalytic role in α,β-elimination.

EXPERIMENTAL PROCEDURES

Materials—PLP, pyridoxamine 5′-phosphate (PMP), L-cystine, β-chloro-L-alanine, glycine, L-homoserine, L-methionine, NADH, pyruvate, rabbit muscle L-lactic dehydrogenase, methylamine, ethanolamine, ammonium chloride, and isopropyl β-D-thiogalactoside were from

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[1] The abbreviations used are: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PNP, pyridoxine 5′-phosphate; HPLC, high pressure liquid chromatography.

Sigma. L-cysteine and L-serine were from Fluka. All other chemicals were of highest grade commercially available.

Site-directed Mutagenesis—The K238A and K238R mutant forms of cystalysin were made on the wild-type construct pUC18:*hly* (13) using the QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). The kit employs double-stranded DNA as a template, two complementary oligonucleotide primers containing the desired mutation, and *Dpn*I endonuclease to digest the parental DNA template. Oligonucleotides were synthesized by MWG-Biotech AG (Anzinger, Germany). The K238A and K238R mutants were produced using as primers 5′-GCTCCGTCTGCAACATTTAATATAGCAGGAATGGGC-3′, 5′-GCTCCGTCTAGAACATTTAATATAGCAGGAATGGGC-3′, and their complementary oligonucleotides, respectively. The coding regions of the mutated *hly* genes were sequenced to confirm the mutations. *E. coli* strain DH5α cells were transformed and used for expression.

Expression and Purification of K238A and K238R Mutants—The conditions used for expression of the K238A and K238R mutants 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 following modifications of the procedure described previously (2). After the DEAE-FF-Sepharose XK 26/60 step, the enzymatic solution was concentrated to ~6 ml and rechromatographed on the same resin (1.6 × 13-cm column) equilibrated in 10 mM potassium phosphate buffer, pH 7.6. The enzyme was eluted with a 225-ml linear gradient whose final concentration of potassium phosphate was 50 mM, pH 7.6. Active fractions were pooled and concentrated to ~3 ml by Centriplus (Amicon).

The protein concentration in all cystalysin samples was determined by absorbance spectroscopy using a previously determined extinction coefficient of 12.77×10^4 M^{-1} cm^{-1} at 281 nm (2). PLP content of wild-type and mutant enzymes was determined by releasing the coenzyme in 0.1 M NaOH and using $\epsilon = 6600$ M^{-1} cm^{-1} at 388 nm.

Preparation and Reconstitution of Apo-K238A and Apo-K238R Mutants—Apoenzymes K238A and K238R were prepared by incubation of enzyme (~1 mg/ml) with 0.5 M hydroxylamine in 0.5 M phosphate buffer, pH 6.9, for 3 h at 25 °C; this was followed by gel filtration on a desalting Hi-Prep column 26/10 equilibrated with 0.5 M phosphate buffer, pH 6.9. For reconstitution, a 10-fold molar excess of PLP was added, and, after 1 h, the solution was loaded on the above column previously equilibrated with 20 mM phosphate buffer, pH 7.4. The enzyme was then concentrated in microconcentrators. The apparent equilibrium constant for dissociation of PLP from K238A, K_d, was determined by measuring the molar fraction of enzyme-bound PLP, calculated as $([PLP]_{total} - [PLP]_{free})/[K238A]_{total}$ in the presence of PLP at a concentration varying from 20 to 950 nM. $[PLP]_{total}$ and $[PLP]_{free}$ were experimentally determined by HPLC in combination with ultrafiltration as described previously (14). The K_d value for dissociation of PLP from K238R was determined by measuring enzyme activity of the apoenzyme (2.7 μM) in the presence of PLP ranging from 0.05 to 20 μM.

Enzyme Activity Assay—α,β-eliminase activity was measured by an spectrophotometric assay coupled with lactic dehydrogenase as reported previously (2). To determine the kinetic parameters of the catalysis, the assays were performed as indicated above using a fixed amount of enzyme, whereas L-cysteine or β-chloro-L-alanine concentration was varied from 0.15 to 10 mM. The experimental data were fit into the Michaelis-Menten equation to determine K_m and k_{cat} values.

Spectrophotometric 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 the small amount of precipitate. Fluorescence spectra were taken with a FP750 Jasco spectrofluorometer using 5-nm excitation and emission bandwidths at a protein concentration varying from 1 to 8 μM. Spectra of blanks, *i.e.* samples containing all components except cystalysin, were taken immediately before the measurements of samples containing protein. Blank spectra were subtracted from the spectra containing the enzyme. CD spectra were obtained using a Jasco V-710 spectrophotometer with a thermostatically controlled compartment at 25 °C. For near-UV and visible wavelengths, protein concentration varied from 0.8 to 1 mg/ml in a cuvette with a 1-cm path length. Routinely, three spectra were recorded at a scan speed of 50 nm/min with a bandwidth of 2 nm and averaged automatically, except where indicated. For far-UV measurements, the protein concentration was 0.1 mg/ml with a 0.1-cm path length.

Pre-steady-state Kinetic Analysis by UV-Vis Stopped-flow Spectroscopy—Wild-type cystalysin (7 μM) was mixed with glycine in 20 mM potassium phosphate, pH 7.4. Reactions proceeded at 25 °C, and coenzyme absorbance changes were monitored using a Biologic SFM3 mixer with a TC-100 (1-cm path length) quartz cell coupled to a BioKine PMS-60. The dead time was 3.6 ms at a flow velocity of 12 ml/s. Absorbance scans (500) from 300 to 550 nm were collected on a logarithmic time scale with a J&M Tidas 16 256 diode array detector (Molecular Kinetics, Pullman, WA) and analyzed using either SPECFIT (Spectrum Software Associates, Chapel Hill, NC) or Biokine 4.01 (Biologic, Claix, France) to determine the observed rate constants.

Curve-fitting Analysis—Binding of ligands (L) to wild-type and active site lysine mutants was monitored by pre-steady-state and steady-state analysis, respectively. The data were fitted to a single exponential process (Equation 1, shown below) to obtain k_{obs}.

The binding of ligands to cystalysin, either wild-type or mutant, was followed by measuring the change in the absorbance and/or emission fluorescence intensity with time upon the addition of ligand. The kinetic measurements were performed at several ligand concentrations using an excess of ligand over enzyme concentration. For each ligand concentration, the time course was recorded, and the absorbance and/or fluorescence readings at each ligand concentration were fitted to a pseudo-first-order kinetic model, shown here in Equation 1,

$$A_t = A_{eq} - (A_{eq} - A_0)e^{-k_{obs}t} \qquad \text{(Eq. 1)}$$

where A_0 is the absorbance and/or emission fluorescence reading prior to ligand addition, A_t is the reading at the time t, and A_{eq} is the reading at equilibrium

For ligands where the dependence of k_{obs} was linear, the data were fitted to Equation 2, shown here,

$$E + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E\,L \qquad k_{obs} = k_{on}[L] + k_{off} \qquad \text{(Eq. 2)}$$

which describes a single-step binding model to determine k_{on} and k_{off}. k_{on} is the apparent second-order rate constant for the formation of the Schiff base from free enzyme and ligand, and k_{off} is the first-order rate constant for the decay of the Schiff base species to free enzyme and ligand.

For ligands where k_{obs} had a hyperbolic dependence on ligand concentration, the k_{obs} data were fitted to Equation 3, shown here,

$$E + L \underset{}{\overset{K_{EL'}}{\rightleftharpoons}} EL' \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EL \qquad k_{obs} = k_{+2}\frac{[L]}{K_{EL'} + [L]} + k_{-2} \qquad \text{(Eq. 3)}$$

which describes a two-step binding model, assuming that the first step is rapid, wherein $K_{EL'}$ is the dissociation constant for the intermediate formed prior to the formation of the Schiff base species, and k_{+2} and k_{-2} are first-order rate constants for the interconversion between the intermediate (EL′) and the final Schiff base species (EL). The parameters in Equation 3 are related to those in Equation 2 as follows: $k_{on} = k_{+2}/K_{EL'}$ and $k_{off} = k_{-2}$. Amplitude data ($\Delta A = A_{eq}$-A_0) were fitted to Equation 4, as shown,

$$\Delta A = \Delta\varepsilon_{420nm}\frac{[E][Gly]}{(K_d + [Gly])} \qquad \text{(Eq. 4)}$$

to determine the dissociation constant (K_d) for Schiff base formation between glycine and wild-type cystalysin, where [E] represents the total concentration of the enzyme.

As shown in Equation 5,

$$Y = Y_{max}\frac{[E]_t + [PLP]_t + K_d - \sqrt{([E]_t + [PLP]_t + K_d)^2 - 4[E]_t[PLP]_t}}{2[E]_t} \qquad \text{(Eq. 5)}$$

the K_d value of the mutant-coenzyme complex was obtained using a tight binding hypothesis.

All data analysis to determine model-derived kinetic parameters was performed by nonlinear curve fitting using KaleidaGraph 3.52 (Synergy Software, Reading, PA).

RESULTS

To define the functional role(s) of the lysine residue that forms an internal aldimine with PLP in the active site of cystalysin, we compared some spectroscopic and catalytic properties of the wild-type enzyme and mutants in which Lys-238 is replaced by alanine or arginine. The yield of K238A and K238R mutants after purification was ~70–80% that of the wild-type

protein. The purified mutant proteins were homogeneous, as indicated by a single band on SDS/PAGE.

Spectroscopic Properties of K238A and K238R Mutants—Wild-type cystalysin displays characteristic absorption spectra with maxima at 418 and 320 nm, attributable to the ketoenamine form of the Schiff base and a substituted aldamine, respectively (2). The spectral properties of the PLP aldehyde are known to differ significantly from the internal aldimine and to give rise to maxima at 290, 390, and/or 335 nm (15). As isolated, the K238A mutant displays an absorption maximum at 412 nm, whereas the K238R mutant exhibits two peaks, one centered at 390–400 nm and the other at 320 nm. K238A and K238R mutants bind 1 and 0.37 mol of PLP per mol of dimer, respectively. The ratio $A_{320}/A_{390-400}$ in the K238R mutant is ~3. HPLC analysis of the supernatants, obtained after reduction of the mutants with $NaBH_4$ followed by denaturation and centrifugation, indicates the presence of two peaks. On the basis of its retention time, one peak, corresponding to ~43 and 78% of the original coenzyme content of K238A and K238R mutants, respectively, was identified as pyridoxine 5′-phosphate (PNP). The other peak does not migrate with the same retention time as any of the controls (PLP, PMP, PNP) but elutes with a retention time similar to those of PLP adducts with amine or amino acids and most likely arises from the adduct of PLP with an uncharacterized compound. The reconstituted holoenzymes K238A and K238R (obtained by complete removal of the cofactor followed by treatment with 10-fold molar excess of PLP and removal of unbound coenzyme (described under "Experimental Procedures")) contained 1.0 and 1.1 mol of PLP per dimer, respectively, as judged by NaOH treatment and HPLC analysis. As shown in Fig. 1*A*, whereas the reconstituted K238A mutant exhibits an absorption band at 400 nm, the reconstituted K238R mutant shows two absorbance maxima at 390 and 320 nm. Absorption bands at 388 and 330 nm are present in the spectra of free PLP. However, in PLP free in solution, the 388-nm chromophore is the most abundant species, whereas it is the minor component of the spectrum of K238R mutant. The ratio of A_{280}/A_{400} is 15 for the reconstituted K238A mutant compared with an A_{280}/A_{418} value of 10 for wild-type cystalysin; for reconstituted mutant K238R, the A_{280}/A_{390} and A_{280}/A_{320} ratios are ~40 and 15, respectively. After reduction of the reconstituted mutants with sodium borohydride followed by denaturation with trichloroacetic acid, HPLC analysis of the supernatant reveals that the original PLP content was completely converted into PNP. When the wild-type enzyme was treated with $NaCNBH_3$, which does not readily reduce a free aldehyde group but does reduce a Schiff base (16), the absorption at 418 nm was diminished in parallel with an increase in the absorption at 330 nm. This result is consistent with reduction of the internal Schiff base formed between the aldehyde group of PLP and the ϵ-amino group of Lys-238 in the wild type enzyme. In contrast, treatment of both reconstituted mutants with $NaCNBH_3$ did not alter the spectra. Altogether, these data indicate that reconstitution of both mutants with PLP gives rise to proteins in which PLP binds as the free aldehyde. Thus, these reconstituted forms of K238A and K238R mutants were used for the following kinetic and spectral analysis.

The absorption spectra of the internal aldimine of the wild-type depend on pH; titration of the enzyme-bound absorbance over the pH range 6–9.7 has shown that the 320-nm band increases at high pH values, whereas the 418-nm band decreases (2). In contrast, no changes in absorption spectra of apomutants K238A and K238R reconstituted with PLP were observed between pH 6 and 9.7.

Fig. 1*B* shows CD spectra of wild-type and reconstituted

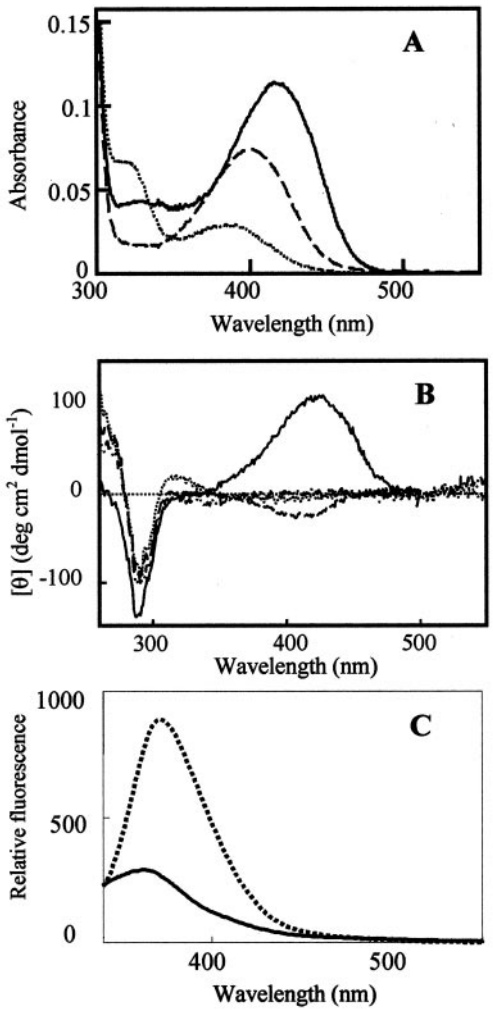

FIG. 1. **Absorption and CD spectra of wild-type and the K238A and K238R mutants of cystalysin.** *A*, absorption of wild-type (—), K238A (— — —), and K238R (···) in 20 mM potassium phosphate, pH 7.4, at a concentration of 9.1 μM. *B*, CD spectra; symbols for wild-type, K238A, and K238R are the same as for *panel A* along with apo-wild-type cystalysin (–··–··–). *C*, fluorescence emission spectra upon excitation at 320 nm; the symbols for wild-type and K238R are the same as for *panel A*. No emission was observed upon excitation at 320 nm of K238A.

K238A and K238R enzymes in the visible and near-UV region. PLP bound to the wild-type exhibited a positive circular dichroism band at 412 nm, as reported previously (2). In contrast, whereas the K238A mutant shows a negative Cotton effect with λ_{max} at 407 nm and a molar ellipticity lower than that of the positive Cotton effect of the wild-type enzyme, the K238R mutant displays two modest signals, a negative band in the 400-nm region and a positive dichroic signal at 320 nm. With respect to wild-type, both mutants also display a decrease of negative dichroic bands in the aromatic region of 288–296 nm and an increase of the positive band at 275 nm. Spectra in the near-UV region of both mutants are reminiscent of that of wild-type apocystalysin, as shown in Fig. 1*B*. The circular dichroism spectra in the range 190–240 nm of both mutants and the wild-type enzyme appeared almost indistinguishable; they display minima at 222 and 208 nm, which are characteristic of a protein with a high content of α-helical structure (data not shown). These data suggest that, although small conformational changes may occur in the region immediately surrounding the site of the mutation, no appreciable change in the secondary or tertiary structure occurs upon the substitution of alanine or arginine for Lys-238.

When excited at 281 nm, the fluorescence emission spectrum of wild-type cystalysin shows two maxima at 337 nm and ~500 nm; the ratio of F_{337}/F_{500} is ~86 (2). The band at the shorter wavelength is a result of intrinsic tryptophan fluorescence, whereas the band at the longer wavelength is due to the delayed Schiff base fluorescence. Excitation at 281 nm of both reconstituted K238A and K238R mutants shows an emission spectrum with a maximum at only 337 nm whose intensity is 1.5–1.6-fold higher than that of the wild-type. Excitation of the wild-type enzyme at 418 nm results in a faint emission band at 504 nm (2). Upon excitation at 400 nm, whereas reconstituted K238A mutant emitted at 499 nm with an emission intensity 2-fold higher that that of the wild-type, reconstituted K238R shows no emission at ~500 nm. Excitation at 320 nm of reconstituted K238R mutant shows an ~3-fold increased emission fluorescence intensity when compared with that of the wild-type with a maximum at 371 nm (red-shifted 4 nm with respect to the maximum of the wild-type) (Fig. 1*C*).

Binding Affinity of K238A and K238R Mutants for PLP Cofactor—The data for reconstitution to K238A or K238R holoenzymes *versus* PLP concentration have been collected by measuring the molar fraction of enzyme-bound PLP or the recovery of enzyme activity, respectively (see "Experimental Procedures"). Titration analyses of both apomutants with PLP fitted to Equation 5 yielded K_d values for PLP-K238A and PLP-K238R complexes equal to 70 ± 16 nM and 0.9 ± 0.1 μM, respectively (data not shown). The apparent K_d for dissociation of PLP from wild-type has been found to be 6.6 ± 1 nM (2). Thus, substitution by alanine or arginine, in addition to resulting in a significant loss of the bound coenzyme, apparently causes a ~ 10- and 150-fold decrease in PLP-binding affinity, respectively.

Kinetic Properties of K238A and K238R Mutants—Apomutant K238A, reconstituted with PLP, was essentially inactive under the standard assay conditions in which the k_{cat} values for the wild-type toward L-cysteine or β-chloro-L-alanine were ~11 s^{-1} or 60 s^{-1} at 25 °C, respectively (2). On the contrary, the reconstituted K238R mutant displays a detectable eliminase activity. Steady-state kinetics data for the reaction of this mutant were collected for a range of L-cysteine or β-chloro-L-alanine concentrations by measurement of pyruvate formation. Although the K_m values (0.6 ± 0.1 mM for L-cysteine, 1.9 ± 0.2 mM for β-chloro-L-alanine) are not significantly altered by mutation, the k_{cat} values for the reaction are 1.12 ± 0.05 min^{-1} for L-cysteine and 6.8 ± 0.3 min^{-1} for β-chloro-L-alanine, ~ 500-fold lower than that for the wild-type (2). Thus, per mole of cofactor, the k_{cat} of this mutant is 0.3% of that of the wild-type.

Formation of External Aldimine—Glycine behaves as a non-productive analogue of wild-type cystalysin. The addition of glycine to cystalysin causes the immediate appearance of an absorption band at 429 nm attributable to the formation of the external aldimine (2). Thus, the interaction between cystalysin and glycine represents a useful model for the analysis of formation and hydrolysis of the external Schiff base, and the comparison between the reactions of wild-type and Lys-238 mutants with this ligand could provide insight into the role of active site lysine in a step that is on the catalytic pathway of cystalysin.

The kinetics of glycine binding to wild-type cystalysin is too rapid to be measured with a conventional spectrophotometer. Thus, the reaction of wild-type with glycine was followed by multiwavelength (300–550 nm) stopped-flow spectroscopy under pre-steady-state conditions. Upon mixing cystalysin with glycine, the spectrum of the initial species is similar to that of free enzyme, except that the absorption maximum at 418 nm is red-shifted 11 nm, indicating that a fast process is complete within the dead time of the stopped flow. This is

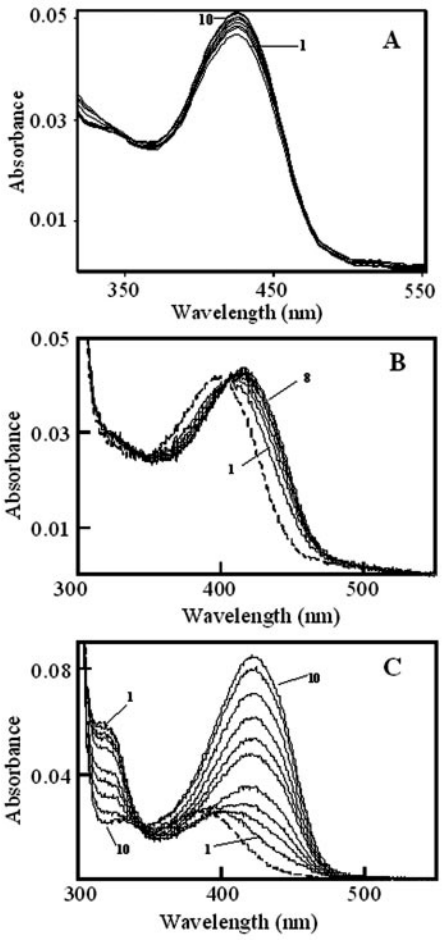

FIG. 2. **Time-dependent spectral changes occurring upon addition of glycine to wild-type and the K238A and K238R mutants with glycine.** *A*, multiwavelength kinetic analysis of the reaction of 3.2 μM wild-type with 10 mM glycine in 20 mM potassium phosphate buffer, pH 7.4. Representative spectra at selected time points are displayed between *lines 1* (3 ms) and *10* (6 s) (the *lines* between these two, from *bottom* to *top*, represent 10, 20, 50, 100, 200, 500 ms, and 1 and 2 s, respectively) after mixing the enzyme with glycine. *B*, absorption spectrum of a 5.7 μM K238A mutant in 20 mM potassium phosphate buffer (pH 7.4) is displayed (– – –)along with the absorption spectra of the mutant plus 5 mM glycine (—) between *lines 1* (0.5 min) and *8* (18.5 min) (the *lines* between these two, from *bottom* to *top*, represent 2.5, 4.5, 6.5, 8,5, 11.5, and 13.5 min, respectively). *C*, absorption spectra of a 7.7 μM K238R mutant in 20 mM potassium phosphate buffer (pH 7.4) is displayed (– – –) along with the absorption spectra of the mutant plus 100 mM glycine (—) between *lines 1* (0.5 min) and 10 (239.5 min) (the *lines* between these two, from *bottom* to *top*, represent 1.4, 3.5, 7.5, 20.5, 28.5, 39.5, 65.5, and 121.5 min, respectively).

followed by a monophasic process in which A_{429} increases (Fig 2*A*). The apparent first-order rate constant, k_{obs}, shows a hyperbolic dependence on glycine concentration in the range 0.5–50 mM. A two-step model is required to describe Schiff base formation, providing kinetic evidence that an intermediate (EL′) is formed prior to the Schiff base species (EL). Almost the same concentration of glycine is required to saturate the kinetic intermediate ($K_{EL'}$ = 1.4 mM) and the Schiff base species (K_d = 1.0 ± 0.2 mM, a value in good agreement with that previously determined under steady-state conditions; Ref. 2). The ratio of k_{+2}/K_{EL-} provides a measure of k_{on} (25 mM^{-1} min^{-1}; Table I).

Like the wild-type enzyme, reconstituted K238A and K238R mutants exhibit absorbance changes upon the addition of glycine consisting in a time-dependent appearance of an absorbance band centered at 420 and 422 nm, respectively (Fig. 2, *B* and *C*). When the K238A and K238R mutants were excited at

TABLE I
Formation of external aldimine of wild-type and K238A and K238R mutants with glycine

	Wild-type	K238A	K238R
$K_{EL'}$ (mM)	1.4 ± 0.2	n.a.[a]	n.a.[a]
k_{+2} (min^{-1})	35 ± 1	n.a.[a]	n.a.[a]
k_{-2} (k_{off}) (min^{-1})	0.6 ± 0.1	0.031 ± 0.004	0.0048 ± 0.0003
k_{on} ($mM^{-1}min^{-1}$)	25 ± 3	0.0265 ± 0.0003	0.00033 ± 0.00001
K_d (mM)	1.2 ± 0.1	1.2 ± 0.1	14.5 ± 0.1

[a] Not applicable, as a linear dependence of k_{obs} on glycine concentration was observed.

420 and 422 nm in the presence of glycine, an increase in emission florescence with time at 505 nm was observed. The rates of the changes are similar to those obtained from UV/visible spectrophotometry. The observed rate constant for these changes, k_{obs}, measured by the use of the conventional mixing in the absorbance and/or fluorescence spectroscopy, has a linear dependence on glycine concentration, and Schiff base formation was modeled by a single-step process. Table I lists the association and dissociation constants (k_{on} and k_{off}, calculated as described under "Experimental Procedures") as well as the dissociation equilibrium constants (K_d, calculated as the ratio of k_{off}/k_{on}) for the reaction of glycine with reconstituted K238A and K238R mutants. Likewise, although formation of external aldimine by wild-type with L-serine occurs during the mixing time, it is complete in 15 min upon the addition of 5 mM L-serine to the K238A mutant and 140 min upon addition of 50 mM L-serine to the K238R mutant.

Formation of the Quinonoid Intermediate—L-Homoserine and L-methionine are also found to be unproductive substrate analogues of cystalysin, and their binding to the wild-type enzyme produces immediately an equilibrating mixture of external aldimine and quinonoid species. This entails that L-homoserine and L-methionine stop at the quinonoid intermediate (2). To determine whether the quinonoid step has been affected by the active site lysine mutations, the interaction of Lys-238 mutants with these ligands has been studied. Changes, consisting of a slow appearance of a peak at 417 nm and a small absorbance at 506 nm, could be observed upon the addition of L-homoserine to the reconstituted K238R mutant (Fig. 3*A*). L-Methionine produces similar changes over a time period of 150 min (data not shown). Both spectra with L-homoserine and L-methionine reflect an equilibrium mixture of external aldimine and quinonoid species dominated by the external aldimine base intermediate. On the contrary, the addition of L-homoserine (Fig. 3*B*) or L-methionine to a reconstituted K238A mutant leads to a time-dependent shift in the λ_{max} of the 400-nm band to 424 nm. No species absorbing at or near 500 nm, corresponding to the quinonoid intermediate, could be seen even over a period of 20 h. The intermediates formed between reconstituted K238A and K238R mutants and the above ligands are very stable because the absorption spectra were essentially unchanged after gel filtration.

CD Changes of Wild-type, K238A and K238R Mutants upon Addition of Ligands—As reported previously, after the addition of glycine and L-serine to the wild-type cystalysin, a negative dichroic signal at ~460 nm appears, whereas after the addition of L-homoserine and L-methionine, a decrease in the intensity of the 422-nm dichroic band was observed (2) (Fig. 4, *inset*). As shown in Fig. 4, a negative dichroic band in the 425–440 nm region, even if with a different intensity, characterizes the CD spectra of the K238A mutant complexed with either glycine, L-serine, L-homoserine, or L-methionine. In contrast, the Lys-238R mutant enzyme shows a negative ellipticity band that is quite modest in the 400-nm region in the presence of either L-homoserine or L-methionine, more definite at 430 nm in the presence of L-serine, and remarkable at 425 nm in the presence of glycine. It should be noted that, although the CD changes induced by the above ligands on the wild-type occur during the

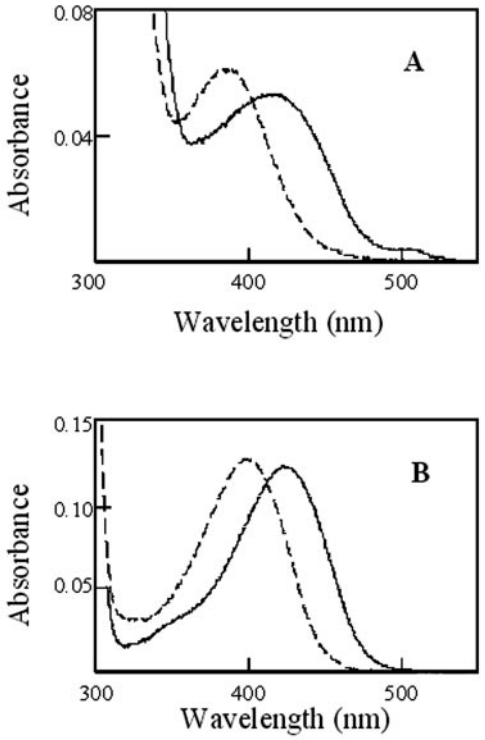

FIG. 3. **Spectral changes occurring upon the addition of L-homoserine to the K238R and K238A mutants.** *A*, absorption spectra of a 20 μM K238R mutant (– – –) and the mutant 4.5 h after the addition of 50 mM L-homoserine in 20 mM potassium phosphate buffer, pH 7.4 (—). *B*, absorption spectra of a 20 μM K238A mutant (– – –) and the mutant 30 min after the addition of 50 mM L-homoserine in 20 mM potassium phosphate buffer, pH 7.4 (—).

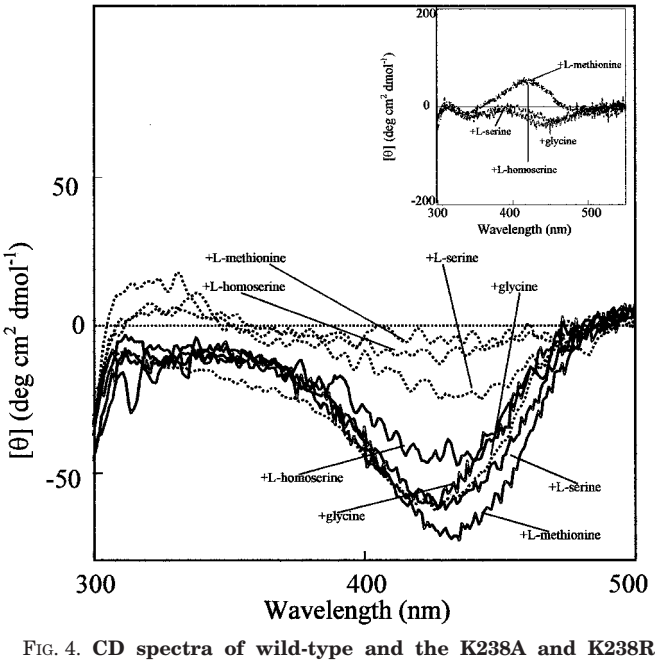

FIG. 4. **CD spectra of wild-type and the K238A and K238R mutants of cystalysin in the presence of glycine, L-serine, L-homoserine, and L-methionine.** CD spectra of K238A (—) and K238R (···) in the presence of saturating concentrations of glycine, L-serine, L-homoserine, and L-methionine recorded after reaching a steady-state equilibrium. *Inset,* CD spectra of wild-type in the presence of glycine, L-serine, L-homoserine, and L-methionine.

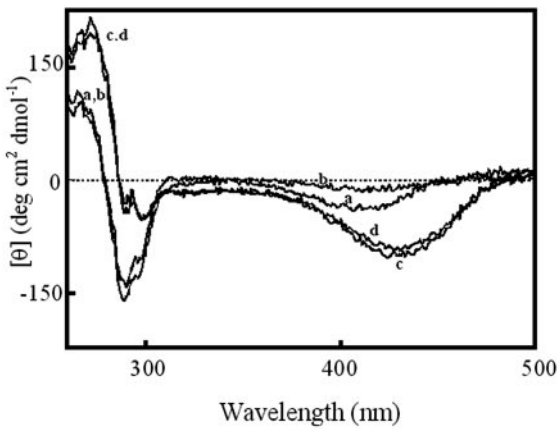

FIG. 5. **Effect of methylamine on CD spectra of K238A mutant.** CD spectra of 8 μM K238A mutant (*a*) and the mutant 40 min after the addition of 0.2 M methylamine (*b*) and 25 min after the further addition of 50 mM L-serine (*c*). *d* represents the CD spectrum of K238A 25 min after the addition of 50 mM L-serine. Spectra were taken in 20 mM potassium phosphate buffer, pH 7.4.

mixing time, they are complete in minutes or hours after the addition of these ligands to mutants.

Effect of Exogenous Amines on Catalytic and Spectroscopic Properties of Mutant L238A Reconstituted with PLP—In the active site lysine mutant of aspartate aminotransferase (10), D-amino acid transaminase (4), tryptophan synthase (5), *O*-acetylserine sulfhydrylase (6), and alanine racemase (7), it was observed that exogenous amines can partially or totally substitute for the catalytic role of the active site lysine. To study the effect of exogenous amines on the apomutant Lys-238A reconstituted with PLP, various amines were added to the reaction mixture. When the mutant was assayed with a lactate dehydrogenase assay system toward L-cysteine either at pH 7.4 or 9 in the presence of either 0.2 M methylamine, 0.2 M ethanolamine, or 1 M ammonium chloride, pyruvate production was undetectable.

The addition of methylamine, ethanolamine, or ammonium chloride to the apomutant Lys-238A reconstituted with PLP at pH 7.4 results in an absorbance shift from 400 to 408 nm, suggesting the formation of a Schiff base. The rate constant of the buildup of the 408-nm species in the presence of 0.2 M methylamine is 0.31 ± 0.03 min^{-1}. A decrease of the negative dichroic band shifted to 403 nm was observed upon the addition of 0.2 M methylamine to the mutant. The CD spectrum of the methylamine-mutant complex appears similar to that of the apomutant Lys-238A reconstituted with PLP, but is remarkably different from that of the wild-type cystalysin (Fig. 5). This suggests that methylamine forms a Schiff base with the C4′ of PLP in a different manner with respect to Lys-238 in the wild-type cystalysin.

The addition of glycine at varying concentrations (1–20 mM) to the complex K238A-methylamine results in spectral changes consisting of a time-dependent absorbance shift to 420 nm. This event is due to the transaldimination with glycine. The reactions follow pseudo-first-order kinetics with association and dissociation rate constant values of 0.020 ± 0.001 mM^{-1}min^{-1} and 0.045 ± 0.002 min^{-1}, respectively. The apparent resulting K_d was found to be 2.2 ± 0.1 mM. As shown in Fig. 5, upon the addition of L-serine to the methylamine-mutant complex, a negative dichroic at 430 nm is generated identical to that formed after addition of L-serine to the reconstituted mutant.

DISCUSSION

All PLP-dependent enzymes bind the carbonyl group of coenzyme through an internal aldimine with the ϵ-amino group of a lysine residue. The lysine residue has been found to serve one or more roles in the enzymatic reactions, *i.e.* cofactor binding (4, 5, 12), formation of enzyme-substrate intermediate (4–9), catalysis (4–7, 10, 11), or product release (5, 8, 9). Although the catalytic role of Lys has been studied in many PLP enzymes, the role of the Lys residue has not been extensively studied for any of the PLP eliminases. To gain insights into the functions of the Schiff base lysine, it was changed to Ala or Arg by using site-directed mutagenesis.

The mutations introduced by site-directed mutagenesis did not cause a significant conformational change as verified by far-UV CD, and both of the reconstituted mutants with PLP contain ~50% of the normal complement of the cofactor. Our findings that mutation of Lys-238 to Ala or Arg increases the apparent dissociation constant for PLP 10- and 150-fold, respectively, provide evidence that Lys-238 plays a role in cofactor binding. The observation that these mutants are able to form stable holoenzymes despite the absence of the active site lysine residue shows that non-covalent interactions between protein and cofactor are sufficient to ensure tight binding. Studies with several other PLP enzymes have already shown that chemical modification of the active site lysine (17–19) or amino acid replacement (4, 19–24) does not prevent PLP binding. The 418-nm absorption peak of the wild-type cystalysin derived from the internal aldimine with Lys-238 was shifted to a slightly shorter wavelength (400 nm) by replacement of Lys-238 by Ala, whereas it results in two absorbance bands at 390 and 320 nm by replacement by Arg. Treatment with $NaBH_4$ and $NaCNBH_3$ provides evidence that PLP is bound in these mutants as free aldehyde. Although the absorbing species at 390–400 nm can be easily attributed to the free aldehyde form of the enzyme-bound PLP, the 320-nm species is probably from the hydrated form of the coenzyme, as previously suggested for active site lysine mutants of D-amino acid transaminase (4), ornithine decarboxylase (8), aminolevulinate synthase (11), and glutamate decarboxylase (25). The emission band at 371 nm following excitation at 320 nm of the reconstituted K238R mutant, together with the finding that, unlike wild-type, K238R does not undergo changes in the absorption spectra of the cofactor depending on pH, is consistent with the attribution of the 320-nm species to a PLP-hydrated form. The fact that the Lys-238 mutants present a visible CD spectrum distinct from that of wild-type provides further evidence that their cofactor binding mode is different from the wild-type arrangement. These data demonstrate the importance of the internal aldimine for a correct rigid or asymmetric binding of PLP. The finding that the dichroic signals of Lys-238 mutants in the near-UV region are similar to those of the apo-wild-type but different to those of the holo-wild-type is consistent with the lack of the delayed Schiff base fluorescence upon excitation at 281 nm of the mutants. This implies that the lack of an internal aldimine has altered the asymmetry of certain aromatic amino acids, most probably those associated with the active site.

In comparison to the wild-type enzyme, the rate of formation and decay of the Schiff base species has been significantly decreased by mutation of Lys-238 to Ala or Arg, demonstrating that a major role of Lys-238 is to accelerate these steps. This result has been mainly obtained by a detailed analysis of the interaction of either wild-type or Lys-238 mutants with glycine, a non-productive substrate analogue that binds to the enzyme stopping at the external aldimine step. We estimate that mutation of Lys-238 to Ala or Arg in cystalysin decreases k_{on} for glycine by at least 10^3- and 7.5×10^4-fold, respectively. A decrease in the rate of formation of an external Schiff base between cystalysin and the substrate, L-serine, has also been observed. Similar effects on Schiff base formation have been

reported for many PLP enzymes (4–8) upon mutation of the catalytic Lys in these enzymes. The results support an early proposal that formation of internal aldimines facilitates transimination (3). The k_{off} for decay of Schiff base of glycine with reconstituted K238A and K238R mutants is decreased by about 20- and 125-fold, respectively, with respect to that of the wild-type. This is consistent with the stability of the external Schiff bases that Lys-238 mutants form with glycine and the other ligands examined. Thus, the replacement of active site lysine in cystalysin by Ala or Arg has a larger effect on Schiff base formation than Schiff base hydrolysis. It was not expected that substitution of Lys-238 by Arg would affect the rate of formation and decay of the external Schiff base more than substitution by Ala. It can be envisaged that the mispositioning of the PLP aldehyde function could be more relevant in K238R than in K238A because of a steric interactions between PLP and the arginine side chain. Alternatively, the reduction in reactivity of the K238R-bound PLP in external aldimine formation could be due to the extensive hydration of the predominant 320-nm chromophore, which is absent in the K238A mutant. Nevertheless, it should be noted that whereas the K238A mutant is inactive in the α,β-elimination, K238R retains 0.3% of the wild-type activity. This could mean that, unlike Ala, Arg in position 238 partially substitutes for Lys in the catalytic role, thus suggesting that the bulkier Arg residue in position 238 can be structurally and functionally tolerated in the active site of cystalysin. Substitution of active site lysine by Arg in other PLP enzymes also has been found to lead to less active enzymes that turn over substrates slowly (8, 21). For many PLP-dependent enzymes, the active site lysine residue plays an essential role as the catalytic base to abstract the α-proton in the first chemical step of the reaction. For example, mutation of K258 with Ala in aspartate aminotransferase decreased the rate of this step by 10^6-10^8-fold (10). Following this view, we have analyzed the reaction of Lys-238 mutants with L-homoserine and L-methionine, unproductive substrate analogues whose binding to wild-type gives rise to an equilibrium mixture of external aldimine and quinonoid species (2). The external aldimine but not the quinonoid species is detectable during the reaction of K238A mutant with these ligands. Instead, in addition to the external aldimine, K238R shows the accumulation of a band at ~504 nm in the presence of the above ligands. These results could indicate that mutation of the active site lysine to Arg does not prevent Cα-hydrogen abstraction from the external Schiff base. On the other hand, mutation of Lys-238 to Ala seems to block the reaction at the step of the external Schiff base. These data are consistent with the idea that Lys-238 is an essential catalytic residue and possibly responsible for α-proton removal. Nevertheless, quinonoid formation catalyzed by K238R needs consideration. It can be advanced that, if the guanidino group of Arg in position 238 is positively charged and thus unable to act as a general base catalyst, α-hydrogen abstraction could be accomplished by a water molecule located at the active site. This proposal, however, is in contrast with the lack of quinonoid detection for the K238A mutant. Alternatively, the free base form of Arg-238 might directly abstract the Cα proton. The pH dependence of k_{cat} and k_{cat}/K_m values could indicate that the pK_a of the Lys-238 ϵ-amino group in the external aldimine complex of wild-type cystalysin is ~6.6 (2), although the ϵ-amino group of free lysine has a pK_a value of 10.5. If we assume that the Arg-238 guanidino group (pK_a = 12.5 for free arginine) has a similar environmentally ~4 unit decrease in pK_a, at pH 7.4 (the pH employed) there will be roughly 80 times less Arg in the reactive free base form than Lys. This would mean that there is much less neutral Arg present to abstract the hydrogen in the mutant as compared with Lys. Perhaps this pK_a difference could account for most of the reduced activity of the Arg mutant. This is only an attempt to explain enzymatic activity data of K238R with respect to that of the wild-type. The mechanism of direct proton abstraction by Arg, far from being proved, is favored because of the similarity to the mechanism already proposed for the wild-type reaction (1). Therefore, the mutant enzyme K238R could be considered as an attenuated enzyme that has lost some of its catalytic power but retains enough to be a good model for revealing intermediate states of the α,β-elimination.

Although the quinonoid species has been postulated to be an obligatory intermediate in the α,β-elimination reactions, at present there is no evidence that the quinonoid species observed upon binding of L-homoserine and L-methionine to wild-type cystalysin and the K238R mutant is directly on the reaction pathway. Another aspect to be considered is that that the accumulation of the quinonoid intermediate by the K238A mutant can be undetectable, because the relative rates of formation and breakdown of this intermediate could have been altered. Altogether, these considerations make it doubtful that Lys-238 might play the catalytic role in the α-proton abstraction. Therefore, the possibility can be taken into account that the catalytic role of Lys-238 could be the protonation of the β-leaving group. β-chloro-L-alanine is a highly reactive amino acid that is converted to HCl, pyruvate, and ammonia slowly in the absence of enzyme or more rapidly in model reactions with PLP (26–29). Abeles and co-worker (30) have suggested that β-elimination of a weak leaving group requires protonation, whereas β-elimination of the strong chloride leaving group of β-chloro-L-alanine does not. Kinetic data collected with Lys-238 mutants in the presence of β-chloro-L-alanine show that, as in the presence of L-cysteine, K238A is inactive, whereas K238R retains 0.3% of the wild-type activity. This reinforces the idea that Lys-238 is involved in the α-hydrogen abstraction even if it does not rule out the possibility of the involvement of Lys-238 in the protonation of the β-leaving group. The dissection of the mechanistic pathway of cystalysin along with the detection and identification of reaction intermediates is required to resolve this issue.

Circular dichroism signals of the wild-type-ligand and Lys-238 mutant-ligand complexes reveal how replacement of Lys-238 by Ala or Arg alters the cofactor binding mode in the reaction intermediates. The observation that formation of the external aldimines of K238A with either glycine L-serine, L-homoserine, or L-methionine increases, even to a varying degree, the weak intensity of the coenzyme ellipticity band could indicate that PLP becomes more rigidly oriented when its carbonyl group forms a covalent bond with the bound ligand. Similar effects could be observed upon binding of glycine, and to a minor extent, L-serine to the K238R mutant. The only exceptions are seen in the reaction intermediates of K238R with L-homoserine and L-methionine, suggesting that the double bond and the PLP ring have relaxed in these external aldimines. A negative CD maximum around 425 nm, reflecting a similar asymmetric orientation of the bound PLP, is only displayed by external aldimine formed between glycine and either wild-type or Lys-238 mutants. In contrast, each of the other ligands (L-serine, L-homoserine, and L-methionine) gives rise to different dichroic signals upon binding either K238A or K238R or wild-type. It should be noted that, whereas glycine binds to wild-type cystalysin, stopping at the external aldimine step, the other ligands are able to go beyond this step (2). Thus, Lys-238 in cystalysin appears to play a role in the achievement in the external aldimine of the proper orientation of PLP, which is an essential prerequisite for favorable orientation of the labile Cα-H aldimine bond perpendicular to the pyridine ring.

It has been demonstrated for many PLP enzymes that primary amines functionally replace the PLP binding Lys (4–7, 10). Methylamine is able to form a Schiff base with C4′ of PLP bound of K238A, absorbing at 408 nm. However, the CD features of the K238A-methylamine uncomplexed or complexed with L-serine are different from the corresponding signals of the wild-type, but they are almost similar to those of K238A. This could explain why methylamine has no effect on eliminase activity. Furthermore, although exogenous amines have been found to facilitate the formation of external aldimine by many active site lysine mutants (4, 7, 10, 17), the addition of glycine to the methylamine-K238A complex does not appear to have significant effect on the rate of formation and decay of the external aldimine. A similar effect has been already reported for the mutant $\alpha_2\beta_2$ tryptophan synthase complex having the β-subunit lysine 87 replaced by threonine (5).

In conclusion, lysine 238 of cystalysin apparently fulfills a triple role, *i.e.* it strengthens the PLP binding and enhances the formation and dissociation of the enzyme and ligand Schiff bases, allowing more facile transimination. It also has a catalytic essential role, possibly participating as general base abstracting the Cα proton from the substrate and a general acid protonating the β-leaving group.

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