Treponema denticola cystalysin exhibits significant alanine racemase activity accompanied by transamination: mechanistic implications[1]

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To obtain information on the reaction specificity of cystalysin from the spirochaete bacterium *Treponema denticola*, the interaction with L- and D-alanine has been investigated. Binding of both alanine enantiomers leads to the appearance of an external aldimine absorbing at 429 nm and of a band absorbing at 498 nm, indicative of a quinonoid species. Racemization and transamination reactions were observed to occur with both alanine isomers as substrates. The steady-state kinetic parameters for racemization, k_{cat} and K_m, for L-alanine are 1.05 ± 0.03 s^{-1} and 10 ± 1 mM respectively, whereas those for D-alanine are 1.4 ± 0.1 s^{-1} and 10 ± 1 mM. During the reaction of cystalysin with L- or D-alanine, a time-dependent loss of β-elimination activity occurs concomitantly with the conversion of the pyridoxal 5′-phosphate (PLP) coenzyme into pyridoxamine 5′-phosphate (PMP). The catalytic efficiency of the half-transamination of L-alanine is found to be 5.3×10^{-5} mM$^{-1} \cdot$ s^{-1}, 5-fold higher when compared with that of D-alanine. The partition ratio between racemization and half-transamination reactions is 2.3×10^3 for L-alanine and 1.4×10^4 for D-alanine. The pH dependence of the kinetic parameters for both the reactions shows that the enzyme possesses a single ionizing residue with pK values of 6.5–6.6, which must be unprotonated for catalysis. Addition of pyruvate converts the PMP form of the enzyme back into the PLP form and causes the concomitant recovery of β-elimination activity. In contrast with other PLP enzymes studied so far, but similar to alanine racemases, the apoform of the enzyme abstracted tritium from C4′ of both (4′*S*)- and (4′*R*)-[4′-^{3}H]PMP in the presence of pyruvate. Together with molecular modelling of the putative binding sites of L- and D-alanine at the active site of the enzyme, the implications of these studies for the mechanisms of the side reactions catalysed by cystalysin are discussed.

Key words: C-S lyase, pyridoxal 5′-phosphate, side reaction.

INTRODUCTION

Cystalysin from the spirochaete bacterium *Treponema denticola* is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyses the cleavage of L-cysteine to pyruvate, ammonia and H_2S [1]. The enzyme displays characteristic absorption spectra with maxima at 418 and 320 nm, attributed to the ketoenamine form of the Schiff base and to a substituted aldamine respectively. 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. The apparent pK_a value of 8.4 of the spectral transition seems to originate not in the ionization of Lys^{238} in the internal aldimine but in the ionization of an unidentified enzymic group, influencing the equilibrium between the species absorbing at 418 and 320 nm [2].

Cystalysin is a member of the α-family of PLP-dependent enzymes [3] and belongs to the fold type I family [4]. Although its active site seems to be optimized for catalysing α,β-elimination, it cannot be ruled out that it contains some structural elements required to catalyse other PLP reactions. In fact, side reactions are commonly observed in PLP enzymes and often provide interesting mechanistic and stereochemical information about the enzyme active-site structure.

As shown in Figure 1, the three-dimensional structure of cystalysin complexed with aminoethoxyvinylglycine reveals that Lys^{238} is located on the *si* face of the cofactor, whereas Tyr^{123} and Tyr^{124} lie on the *re* face [5] (Figure 1). These active-site residues could be properly positioned to act as base catalysts for the *pro-S* and *pro-R* proton abstraction from an appropriate substrate. A similar active-site architecture has been reported for the crystal structure of the complex of alanine racemase from *Bacillus stearothermophilus* with alanine phosphonate [6]. However, in this enzyme, a member of the alanine racemase family [7], PLP, is bound such that Tyr^{265}* faces the *si* side of the cofactor and Lys^{39} the *re* side (Figure 1). Kinetic and site-directed mutagenesis experiments, together with computational results, have provided evidence for a two-base racemization mechanism involving these residues as catalytic bases abstracting the α-hydrogen of substrates [8–12]. It is noteworthy that, in addition to racemization, alanine racemase catalyses transamination of both enantiomers of alanine [13] and α,β-elimination of both enantiomers of β-chloroalanine [14,15].

Investigation on possible side reactions catalysed by cystalysin has been undertaken. On the basis of the above information, as a first step of this analysis, we decided to study the interaction of cystalysin with L- and D-alanine. Although these compounds do not contain a suitable leaving group on the β-carbon and therefore cannot be substrates for α,β-elimination, they might bind to the enzyme and undergo reactions typical of PLP enzymes.

In the present study, we present kinetic evidence for an alanine racemase reaction catalysed by cystalysin with turnover times measured in seconds. We also report the spectroscopic and kinetic characterization of a minor reaction catalysed by the

Abbreviations used: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PNP, pyridoxine 5′-phosphate.

[1] Dedicated to the memory of Eraldo Antonini, eminent biochemist, prematurely deceased twenty years ago, on 19 March 1983.

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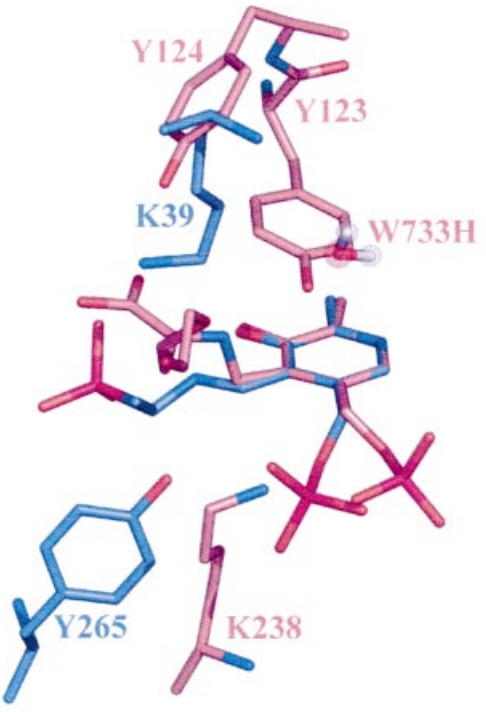


Figure 1 Comparison of the arrangement of potential acid–base catalysts in the active sites of cystalysin and alanine racemase

The complex between alanine racemase and alanine phosphonate (PDB code 1BD0; represented as blue sticks) and the complex between cystalysin and aminoethoxyvinylglycine (PDB code 1C7O; represented as pink sticks) are shown. Oxygen atoms are coloured red, nitrogen atoms blue and phosphorus atoms purple. The position of the water molecule W733H is also shown. This Figure was obtained using pyMOL [39].

enzyme consisting of the overall transamination of both alanine isomers. We have also investigated the pH dependence of these side reactions. Moreover, we provide the first evidence, for an enzyme belonging to the α-family of PLP enzymes, that hydrogen removal from C4′ of pyridoxamine 5′-phosphate (PMP) occurs randomly on both faces of the substrate–cofactor imine plane during half-transamination. These results, together with molecular modelling of the putative binding sites of L- and D-alanine at the active site of the enzyme, are interpreted in terms of a two-base mechanism.

EXPERIMENTAL

Materials

PLP, PMP, L- and D-alanine, L-alanine methyl ester, NAD^+, NADH, pyruvate, rabbit muscle L-lactate dehydrogenase, alanine dehydrogenase in 50% (v/v) glycerol, D-amino acid oxidase, bacterial branched-chain amino acid transaminase and isopropyl β-D-thiogalactoside were obtained from Sigma. L-Cysteine and L-alanine were obtained from Fluka. *Escherichia coli* aspartate aminotransferase was purified from cell extracts of an over-expressing clone kindly provided by Dr Roberto Contestabile (University of Rome 'La Sapienza', Italy). *E. coli* 4′-pyridoxine 5′-phosphate (PNP)/PMP oxidase was purified from *E. coli* clones as described previously [16]. [^{3}H]PNP was synthesized from unlabelled PLP and NaB[^{3}H]$_4$ (Amersham Pharmacia Biotech, Cologno Monzese, Italy) by the method of Yang and Schirch [17]. All other chemicals were of the highest grade commercially available. 1,3-Bis[tris(hydroxylmethyl)methylamino]-propane at 20 mM final concentration was used over the pH range 6–10, and the ionic strength was maintained constant by the addition of KCl.

Enzyme preparation

Cystalysin from *T. denticola* was expressed in *E. coli* and purified as described previously [2]. The enzyme concentration was determined using the molar absorbance coefficient ϵ_M of $12.77 \times 10^4\ M^{-1} \cdot cm^{-1}$ at 281 nm. The PLP content of holocystalysin was determined by releasing the coenzyme in 0.1 M NaOH and by using ϵ_M of $6600\ M^{-1} \cdot cm^{-1}$ at 388 nm.

Enzymic assays

Desulphydration

Desulphydrase activity was assayed by coupling the pyruvate produced from L-cysteine with the NADH-dependent lactate dehydrogenase-catalysed reaction. Assays were performed at 25 °C on a Jasco V-550 spectrophotometer at 340 nm. A 0.25 ml reaction mixture contained 2 mM L-cysteine, 300 μM NADH and 200 μg of lactate dehydrogenase in 20 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 1–10 μg of cystalysin. One unit of activity was defined as the amount of enzyme needed to convert 1 nmol of NADH into NAD/min at 25 °C; for NADH, ϵ_M $6220\ M^{-1} \cdot cm^{-1}$ at 340 nm was used.

Racemization

Racemase activity was determined using the following procedures. For the L → D direction, D-amino acid oxidase and lactate dehydrogenase were used as the coupling enzymes. The reaction was studied at 340 nm. For the D → L direction, the reaction was followed by the formation of NADH at 340 nm in a coupled reaction with L-alanine dehydrogenase. All experiments were performed at 25 °C. The reaction mixtures were prepared as follows: 6 μM cystalysin was incubated with different concentrations of L-alanine or L-alanine methyl ester or D-alanine in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C. Samples of 100 μl were withdrawn at various times and heated for 2 min at 100 °C. After removal of the precipitated protein by centrifugation, aliquots (10–40 μl) of the supernatants were subjected to the racemase assays. Reactions for the L → D direction were performed by adding the aliquot to a solution containing 0.2 mM NADH, D-amino acid oxidase (0.2 unit), lactate dehydrogenase (170 units) and 0.1 M 2-(*N*-cyclohexylamino)ethanesulphonic acid (pH 9) in a final volume of 250 μl. In contrast, reactions for the D → L direction were performed by adding the aliquot to a solution containing 10 mM NAD^+, alanine dehydrogenase (0.5 unit) and 0.1 M 2-(*N*-cyclohexylamino)ethanesulphonic acid (pH 9) in a final volume of 250 μl. The kinetic parameters of the enzyme in the L → D and D → L reactions were determined by fitting the initial-rate data to the following equation:

$$\frac{v}{E_t} = \frac{k_{cat}A}{(K_m + A)} \quad (1)$$

where E_t is the total enzyme concentration, k_{cat} the maximum velocity, A the alanine or alanine methyl ester concentration and K_m the apparent Michaelis–Menten constant.

The parameters k_{cat} and k_{cat}/K_m were plotted against pH on a logarithmic scale and fitted to the following equation:

$$\log Y = \log \frac{C}{1 + H/K_a} \quad (2)$$

where K_a represents the ionization constant for the enzyme or a reactant functional group, Y is the value of the parameter observed as a function of pH and C the pH-independent value of Y.

Transamination

Aminotransferase activity was measured as follows. Cystalysin (4 μM) was reacted with 5–200 mM L- or D-alanine in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C. Apoenzyme (4 μM) was reacted in the presence of 8 μM PMP with 0.1–2 mM pyruvate in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C. Absorbances A at 429 nm and 325 or 418 nm were taken as a function of time and fitted to a first-order kinetic equation.

The detection and quantification of PLP and PMP during the reactions were performed with the HPLC procedure described previously [18]. The rate constants (k_{PLP} and k_{PMP}) of decrease and increase in coenzyme (PLP or PMP) were obtained by fitting the coenzyme-concentration-versus-time curve to appropriate first-order kinetic equations.

Kinetic parameters for transamination and the pH dependence of the rate constant of PMP formation were obtained by fitting data to eqns (1) and (2) respectively.

Inactivation and reactivation experiments

Inactivation of cystalysin by L-alanine was measured as follows: the incubation mixture contained cystalysin (4.7 μM) and L- or D-alanine (200 mM) at 25 °C in 20 mM potassium phosphate buffer (pH 7.4). At various time intervals, aliquots were removed and assayed for residual desulphydrase activity as described above. The rate constant for inactivation ($k_{inactivation}$) was obtained by fitting the activity versus time curve to a single exponential model with an offset.

Reactivation of apocystalysin was followed by incubating 2.75 μM enzyme in the presence of 5.5 μM PMP with 1 mM pyruvate in 20 mM potassium phosphate (pH 7.4) at 25 °C. At various times, aliquots were withdrawn and subjected to the desulphydrase assay with L-cysteine as substrate. The rate constant for reactivation ($k_{reactivation}$) was obtained by fitting the activity-versus-time curve to a first-order kinetic equation.

Kinetic data were fitted to the appropriate equations using the Sigma Plot program (SPSS, San Rafael, CA, U.S.A.).

Preparation of apoenzymes

Apo-cystalysin, apo-(aspartate aminotransferase) and apo-(branched-chain amino acid transaminase) were prepared as described in [2,19,20].

Preparation of (4′*S*)- and (4′*R*)-[4′^{3}H]PMP

(4′*R*)-[^{3}H]PMP was prepared as described previously [19]. (4′*S*)-[^{3}H]PMP was prepared as follows. A solution containing 150 nmol of apobranched-chain amino acid transaminase, 3 nmol of PNP oxidase, 140 nmol of 4′-[^{3}H]PNP in 2 ml of 50 mM sodium *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid and 1 mM dithiothreitol (pH 7.6) was incubated at 37 °C for 5 h. The reaction mixture was concentrated to 250 μl by centrifugation in a Centricon-30 filter. It was then loaded on to a G-25 Sephadex column (1 cm × 18 cm) equilibrated previously with 25 mM potassium phosphate buffer, 25 mM L-glutamate, 1 mM dithiothreitol (pH 7.2), and eluted with the same buffer. Fractions (0.5 ml) were collected, and those containing branched-chain amino acid transaminase were pooled and concentrated by centrifugation in a Centricon-30 filter. The concentrated fraction in the Centricon-30 filter was diluted with 1 ml of 20 mM potassium phosphate buffer containing 1 mM dithiothreitol (pH 7.2) and again concentrated. This washing was repeated three times. The (4′*S*)-[^{3}H]PMP radioactive fraction appears to be in the Centricon filtrate. The filtrate was freeze-dried, and resuspended in 0.6 ml of water.

(4′*R*)- and (4′*S*)-[^{3}H]PMP were purified by reverse-phase column chromatography (Supelco Discovery C18 column) with a Jasco HPLC system equipped with a UV-1570 detector by the method of Bertoldi and Borri Voltattorni [18]. The specific radioactivities of the prepared (4′*R*)- and (4′*S*)-[^{3}H]PMP were 5.5×10^4 and 1.6×10^4 c.p.m./nmol respectively.

Abstraction of C4′ hydrogen of PMP during transamination

The reaction mixture (50 μl) contained 100 nmol of pyruvate, 0.2 nmol of (4′*S*)- or (4′*R*)-[4′-^{3}H]PMP and 0.2 nmol of apocystalysin in 50 mM potassium phosphate buffer (pH 7.4). The reaction was performed at 25 °C for 1 h and terminated by the addition of 10 μl of 3 M HCl. The mixture was immediately frozen in liquid nitrogen and dried with a Speed Vac concentrator. The residue was dissolved in 60 μl of water and subjected to a radioactivity assay. The tritium released from PMP was expressed as volatile radioactivity, which was estimated by subtraction of the radioactivity finally remaining from the radioactivity initially added to the reaction mixture. Radioactivity was determined by a Beckman Instruments LS 1801 liquid-scintillation counter with Beckman Ready Gel as a scintillator.

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 an FP750 Jasco spectrofluorimeter using 5 nm bandwidths on both sides 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 measurement of samples containing protein. Blank spectra were subtracted from the spectra of samples containing the enzyme. CD spectra were obtained using a Jasco V-710 spectropolarimeter with a thermostatically controlled cell compartment at 25 °C. For near-UV and visible wavelengths, protein concentrations 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 averaged automatically except where indicated.

Molecular modelling

The PLP–serine complex present in the crystal structure of serine hydroxymethyltransferase from *B. stearothermophilus* [21] was used to generate both the D-alanine–PLP and L-alanine–PLP conjugates, using the BUILDER package of InsightII (V.2000; MSI, Los Angeles, CA, U.S.A.). The choice of this template as a starting point for the modelling of the external aldimine is based on the following points: (a) both serine hydroxymethyltransferase and cystalysin, belonging to fold type I, are optimized for an α,β-elimination and are also capable of transaminating and racemizing L- and D-alanine at appreciable rates [22], and (b)

Table 1 Steady-state kinetic parameters for L- and D-alanine

	Racemization				Transamination		
	K_D (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_m (mM)	$10^{-4} \times k_{cat}$ (s^{-1})	$10^{-5} \times k_{cat}/K_m$ ($mM^{-1} \cdot s^{-1}$)
L-Alanine	6.8 ± 0.8	10 ± 1	1.05 ± 0.03	0.10 ± 0.01	8.5 ± 0.5	4.50 ± 0.05	5.3 ± 0.3
D-Alanine	n.d.*	10 ± 1	1.4 ± 0.1	0.14 ± 0.02	9.9 ± 0.5†	1.0 ± 0.1†	1.0 ± 0.1

* n.d., not determined.

† These values have been numerically corrected taking into account the conversion of D- into L-alanine.

serine and alanine are structurally similar. The complexes were positioned into the active site of the crystal structure of cystalysin (PDB code 1C7O) in its dimeric assembly, following the binding mode of aminoethoxyvinylglycine reported recently [5]. Particular care was taken to remove the steric clashes in the manually docked structure. Water molecules of the active site were included into the system. Atomic potentials and partial and formal charges were defined using the Cff91 forcefield, and inspected to verify that the proper values had been assigned to the external aldimine and the active-site residues. The following formal charges were assigned to key residues: a net positive charge on $K238N^{\zeta}$ (only with D-alanine), $K72N^{\zeta}$, $R369N^{\epsilon}$, $H206N^{\delta 1}$, PLP atom N1, PLP atom P and alanine substrate, atom N; a net negative charge on $D355O^{\delta 2}$, $D203O^{\delta 1}$, PLP atom O1P, Y123O (only for D-alanine), PLP atom O2P, PLP atom O3P and alanine substrate, atom O^1.

An initial minimization was performed on the whole system to allow added hydrogen atoms to adjust to the crystallographically defined environment. For this purpose, the position of the heavy atoms of the binary complex was fixed, and 481 steepest descent steps were performed, until the maximum energy derivative was less than 41.8 $kJ \cdot mol^{-1} \cdot Å^{-1}$. Next, main chain atoms were fixed, and side chains of every residue comprised in a sphere of 20 Å from the external aldimine were subjected to a gradually decreasing tethering force (from 4180 to 418 $kJ \cdot Å^{-2}$) using again steepest descents, until the maximum derivative was less than 4.18 $kJ \cdot mol^{-1} \cdot Å^{-1}$. Finally, a decreasing tethering force (until the system was totally relaxed) was applied on the external aldimine and on every atom comprised in a sphere of 10 Å from PLP, using conjugated gradients until the maximum derivative was less than 0.0004 $kJ \cdot mol^{-1} \cdot Å^{-1}$. Charges were included in the minimization only during the last step to avoid possible artifacts, due to the overwhelming effect of salt bridges on the other non-bonding interactions [23].

Ten slightly different minimization methods were adopted, in which the tethering force applied on the external aldimine was changed each time. The Cff91 forcefield, a distance-dependent dielectric constant and a cut-off distance of 40 Å were used during each simulation. The potential energy of each conformation of the binary complex after minimization was then compared with the others and the model displaying the lowest energy was chosen for the subsequent analysis. Discover 2.9 and Analysis package of InsightII were used for the minimization.

RESULTS

Racemase activity of cystalysin

Considering the fact that the active-site organization of cystalysin is similar to that of alanine racemase (Figure 1), we decided to check whether a racemase activity towards alanine was displayed by cystalysin. L- and D-alanine are converted by cystalysin into the corresponding enantiomers, as revealed by coupled assay systems using D-amino acid oxidase and L-alanine dehydrogenase respectively. Racemization of both enantiomers is catalysed by cystalysin at pH 7.4 with a robust k_{cat} value measured in seconds, only approx. 10-fold lower than that of the main reaction of cystalysin at the same pH value, i.e. the α,β-elimination of L-cysteine [2]. The kinetic parameters for racemization have been determined and their values are listed in Table 1. The equilibrium constant $K = (k_{cat}/K_m)_{L \to D}/(k_{cat}/K_m)_{D \to L}$ is nearly equal to 1, within the error limits, as predicted by the Haldane relationship [24]. Cystalysin is also capable of catalysing the conversion of L-alanine methyl ester into the D-enantiomer. Initial velocity data of this reaction were fitted using eqn (1) and yielded the following values for the kinetic parameters: $K_m = 31 \pm 3$ mM and $k_{cat} = 0.237 \pm 0.007$ s^{-1}. Racemization of D-alanine methyl ester has not been measured, since L-alanine methyl ester does not behave as a substrate for L-alanine dehydrogenase.

The pH dependence of the kinetic parameters for racemase activity towards alanine in both directions was determined, and the results are shown in Figures 2(A) and 2(B). Neither any effect of buffer as acid–base catalyst on racemase activity nor spontaneous racemization could be observed over the pH range examined. Although the k_{cat} and k_{cat}/K_m values differ only by a 6- to 7-fold change, data for k_{cat} and k_{cat}/K_m fit well to eqn (2). The value of $\log k_{cat}$ decreases below pK_a of 6.63 ± 0.04 and 6.49 ± 0.08 in the D → L and L → D directions respectively. The pH profile for k_{cat}/K_m measured in the D → L direction is identical with that measured in the L → D direction because the equilibrium constant is close to 1. The $\log k_{cat}/K_m$ profile exhibits a single pK_a value of 6.47 ± 0.03.

Molecular modelling

To rationalize the experimental data obtained with both L- and D-alanine, we decided to model their putative binding modes (Figure 3). As a starting point, we retrieved the co-ordinates of the binary complex PLP–serine and PLP-glycine present in the crystal structure of a homologue of cystalysin, serine hydroxymethyltransferase [21]. Both substrate molecules were preorientated into the active site as the external aldimine, with their α-carboxylate group pointing towards Arg^{369}, as observed in the enzyme–intermediate complex between cystalysin and aminoethoxyvinylglycine, and the most severe steric clashes were manually removed. Overall, after minimization of both complexes, the PLP cofactor remains fixed in position, with no major structural changes in its interaction with the active-site residues. The α-carboxylate groups of both alanine and aminoethoxyvinylglycine superpose perfectly, forming two hydrogen bonds at a distance of 2.6 and 2.7 Å with the guanidinium group of Arg^{369}, which are further strengthened by charge–charge interactions. Additional hydrogen bonds are formed with Asn^{175} and with the main-chain amide of Ala^{39}. For both substrates, according to the Dunathan hypothesis, the leaving group is antiperiplanar to the aromatic

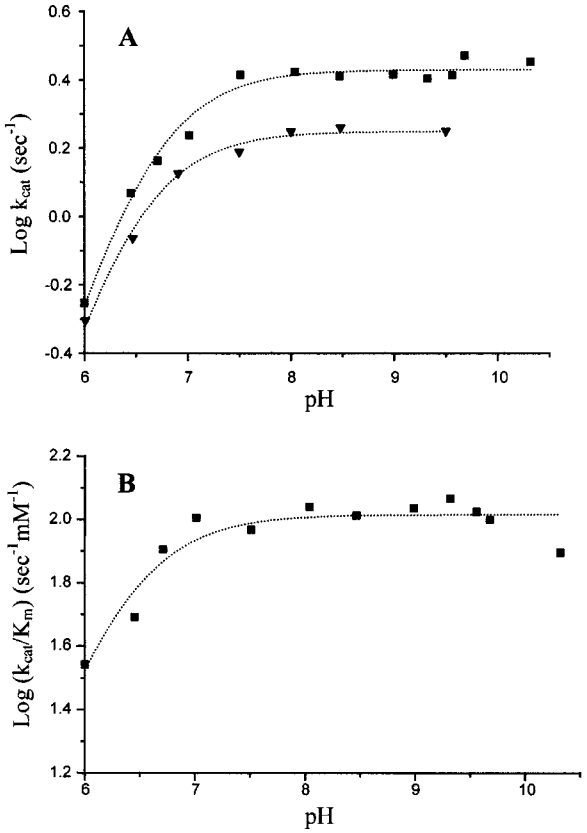


Figure 2 pH dependence of the kinetic parameters for racemase activity of cystalysin at 25 °C

(**A**) k_{cat}, (**B**) k_{cat}/K_m. ■, D → L direction; ▼, L → D direction. For k_{cat} and k_{cat}/K_m, curves are the best fits to eqn (2).

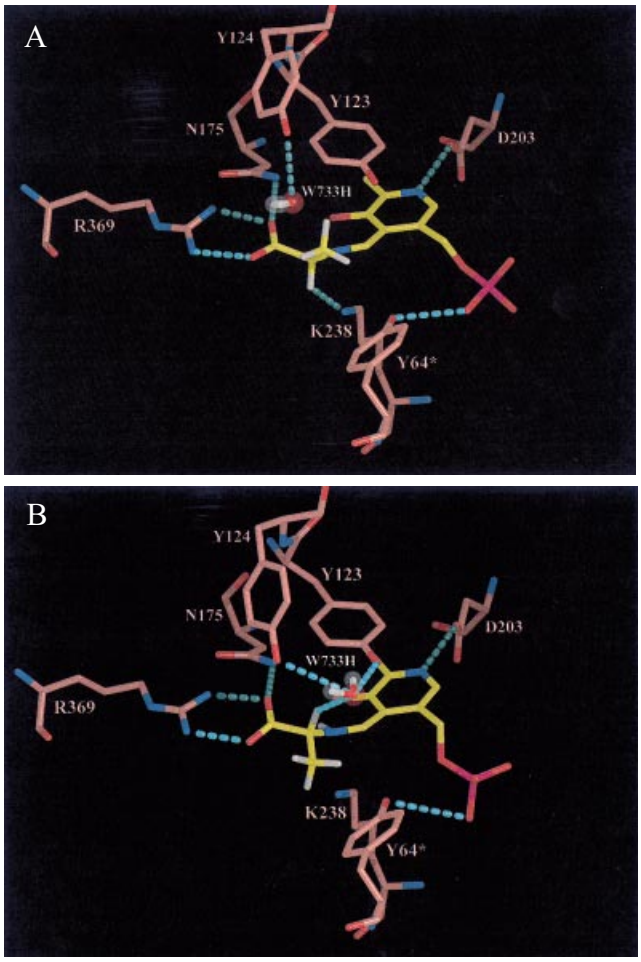


Figure 3 Modelling of the binding modes of L- and D-alanine

Active-site view of the energy-minimized model for cystalysin with (**A**) L-alanine or (**B**) D-alanine bound. The alanine–PLP conjugates are represented as yellow sticks. Oxygen atoms are coloured red, nitrogen atoms blue and phosphorus atoms purple. Hydrogen bonds are shown in cyan. The * denotes a residue that belongs to the neighbouring subunit. This Figure was obtained using pyMOL [39].

moiety of PLP. For L-alanine, the Cα-hydrogen and the C4′ of PLP are located close to the ε-amino group of Lys^{238} (2.5 and 2.9 Å respectively), which also seems to have the proper orientation to act as a catalytic base (Figure 3A). For D-alanine, two tyrosines (Tyr^{123} and Tyr^{124}) and a water molecule (W733H) lie on the *re* side of the PLP cofactor, and are possible candidates for catalysis (Figure 3B). However, according to the minimized model, Tyr^{124} is too far from the α-hydrogen of the substrate (4.1 Å) for the α-proton abstraction. Tyr^{64}*, which is part of the active site, is located 4.6 Å from the Cα-proton and is involved in the formation of a hydrogen bond with the phosphate group of PLP. Tyr^{123}, co-planar with the PLP ring, is placed in a good position to act as a catalyst (3 Å from the Cα-proton and 3.1 Å from C4′), but it should rotate out of its hydrophobic environment for the proton abstraction, losing its stacking interaction with the cofactor. Nevertheless, a water molecule (W733H) located at hydrogen-bond distance with Tyr^{123} (2.8 Å), with the Cα-hydrogen (2.8 Å) and, to a lesser extent, with Tyr^{124} (3.5 Å), could bridge this gap for proton abstraction, acting as a general acid–base catalyst.

Absorption changes of cystalysin with L- and D-alanine and L-alanine methyl ester

The addition of L-alanine to cystalysin immediately leads to the appearance of an external aldimine absorbing at 429 nm and a band with an absorbance maximum at 498 nm, indicative of a quinonoid species (Figure 4). The 429 and 498 nm absorbance bands decrease with time with the concomitant increase in the absorption at 325 nm. The reaction shows a clear isosbestic point at 354 nm (Figure 4). Absorbance values at 429, 498 and 325 nm, measured as function of time, were fitted to the appropriate form of the first-order equation. The apparent rate constant k_{429} shows dependence on the L-alanine concentration in a hyperbolic manner (Figure 4, inset). The value of the apparent dissociation constant and the maximum value of the rate constant are 6.8 ± 0.8 mM and $3.66(\pm 0.08) \times 10^{-4}$ s^{-1} respectively. Identical rate-constant values are obtained by monitoring either the decrease in 429 nm absorbance or the increase in 325 nm absorbance as well as the decrease in 498 nm absorbance.

When the reaction of cystalysin with D-alanine was examined, spectral changes, which were qualitatively identical with those for L-alanine, were observed, but they occurred more slowly. A single isosbestic point is observed at 351 nm (results not shown). The apparent rate constant k_{429}, measured following the 429 nm absorbance change at 200 mM D-alanine, was $9(\pm 1) \times 10^{-5}$ s^{-1}. It should be noted that cystalysin forms a quinonoid intermediate, absorbing at 498 nm by catalysing the loss of the α-proton from both L- and D-alanine.

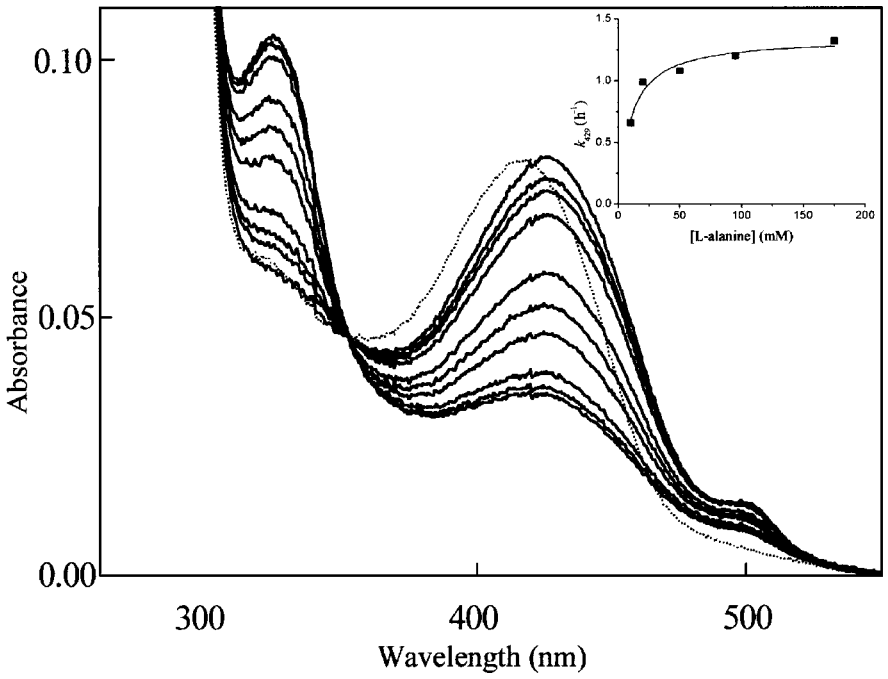


Figure 4 Time-dependent spectral changes that occur on the addition of L-alanine to cystalysin

The enzyme (· · · · ·) (6.5 μM) in 20 mM potassium phosphate buffer (pH 7.4) was incubated with 95 mM L-alanine at 25 °C, and absorbance spectra were recorded at 1, 3, 6, 9, 14, 31, 43, 58, 95 and 123 min. Inset: plot of k_{429} as a function of the L-alanine concentration.

The addition of L-alanine methyl ester to cystalysin causes the immediate appearance of absorbance bands at 425 and 501 nm which undergo time-dependent changes, consisting of a decrease in the absorption at 425 and 501 nm and a concomitant increase at 325 nm. The apparent rate constant, measured following the 425, 501 or 325 nm changes at 200 mM L-alanine methyl ester, was $4.7(\pm 0.3) \times 10^{-4}$ s^{-1} (results not shown).

Taken together, these results strongly suggest an active-site event and the occurrence of a reaction between cystalysin and both enantiomers of alanine or L-alanine methyl ester. However, the reaction cannot be related to the racemization occurring with turnover times measured in seconds.

CD and fluorescence spectral changes of cystalysin with alanine enantiomers

To characterize further the enzyme–intermediate complexes with L- and D-alanine, CD and fluorescence spectra were recorded in the presence of saturating concentrations of both alanine enantiomers. As shown in Figure 5(A), after the addition of either L- or D-alanine, the CD spectrum displays two negative dichroic bands at 441 and 340 nm, and the disappearance of the original negative near-UV CD signal with the concomitant appearance of a positive signal at 275 nm. Excitation of cystalysin at 281 nm in the presence of L- or D-alanine (at a concentration 20-fold higher than K_m) shows a quenching of the tryptophan emission (14 % for L-alanine and 20 % for D-alanine) shifted to 335 nm (Figure 5B), and shows a long-wavelength band which is of low intensity and centred at approx. 510 nm for both L- and D-alanine. When the enzyme was excited at 429 nm in the presence of L- or D-alanine, the emission spectrum exhibits a red shift from 504 to 516 nm and 512 nm respectively. The enhancement was approx. 2-fold for both enantiomers (results not shown). Together, these results suggest that binding of both alanine isomers results in a similar spatial readjustment in the active site.

Transaminase activity catalysed by cystalysin

When the enzyme was preincubated with 200 mM L-alanine, the desulphydrase activity towards L-cysteine decreases as a function of time following a pseudo-first-order kinetics with a $k_{inactivation}$ of $3.6(\pm 0.3) \times 10^{-4}$ s^{-1} (Figure 6). As shown in Figure 6, this time-dependent inactivation is strictly correlated with a decrease in the PLP content in the active site. At the same time, the decrease in PLP can be related to its conversion into PMP, which occurs with a rate constant of $4.2(\pm 0.5) \times 10^{-4}$ s^{-1} (Figure 6), a value in good agreement with the k_{429} value measured by changes in absorbance. Similarly, the decrease in intensity of the 429 nm peak of the D-alanine–cystalysin complex was paralleled by the conversion of PLP into PMP (results not shown).

Addition of 1 mM pyruvate to 2.75 μM apoenzyme in the presence of 5.5 μM PMP in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C results in a time-dependent appearance of a peak at 418 nm (Figure 7). The increase at 418 nm follows a pseudo-first-order behaviour. The apparent rate constant k_{418} shows a hyperbolic dependence on the pyruvate concentration (Figure 7, inset), with resulting k_{max} of $1.80(\pm 0.08) \times 10^{-3}$ s^{-1} and K_D of 0.19 ± 0.03 mM. As shown in Figure 8, the time-dependent increase in the 418 nm absorbance is strictly correlated with the conversion of PMP into PLP and the recovery of desulphydrase activity. The curves for PMP and PLP content as well as the curves for 418 nm absorbance and recovery of desulphydrase activity were theoretical from a fit to the appropriate form of the first-order equation. The k_{PMP}, k_{PLP}, k_{418} and $k_{reactivation}$ values were found to be $1.2(\pm 0.2) \times 10^{-3}$, $1.2(\pm 0.1) \times 10^{-3}$, $1.3(\pm 0.1) \times 10^{-3}$ and $1.2(\pm 0.1) \times 10^{-3}$ s^{-1} re-

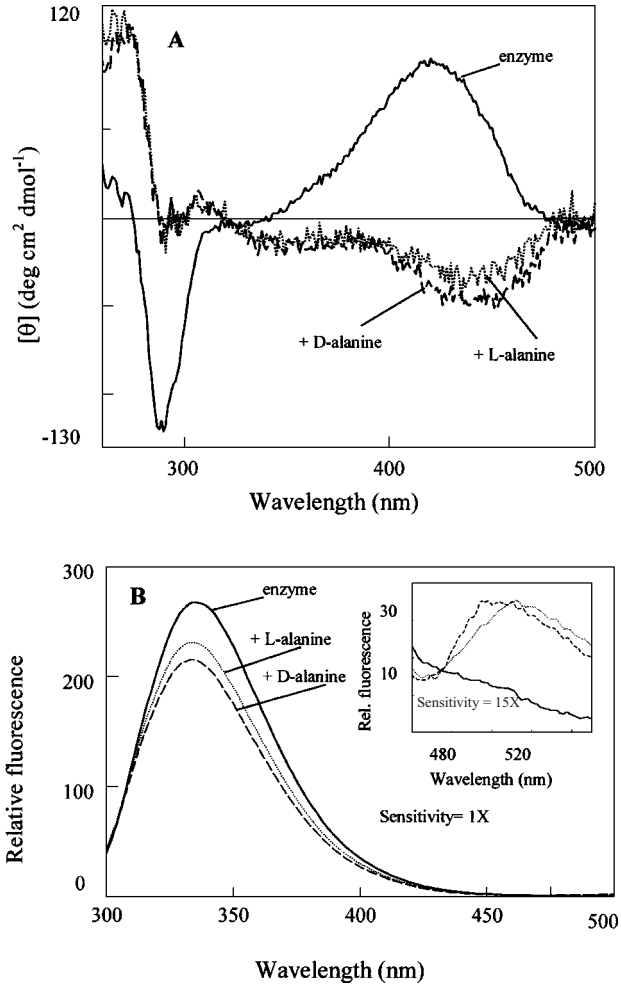


Figure 5 CD and fluorescence spectral changes of cystalysin in the presence of L- or D-alanine at 25 °C

(**A**) CD spectra of 8.2 μM enzyme (——) and immediately after the addition of 200 mM L-alanine (·····) or D-alanine (– – –). (**B**) Emission spectra of the enzyme (0.75 μM) and immediately after the addition of 200 mM L- or D-alanine. Excitation was at 281 nm. Symbols used are as in (**A**). Instrument sensitivity is indicated in the Figure.

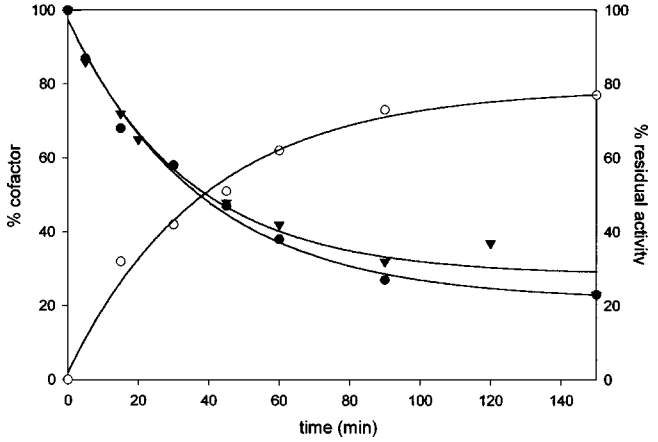


Figure 6 Coenzyme content and activity during the reaction of cystalysin with L-alanine

Cystalysin (4.7 μM) was incubated with 200 mM L-alanine in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C. At the indicated times, aliquots were removed and treated as described in the Experimental section. ●, PLP; ○, PMP; ▼, residual desulphydrase activity measured as described in the Experimental section. Data represent means for three independent experiments; S.E.M. in each case was less than 5%. The curves for PLP and PMP content and for residual activity are theoretical from a fit to a first-order kinetic equation.

spectively. During this reaction, L- and D-alanine are produced in nearly equivalent amounts. Thus the PMP form of cystalysin can be converted back into the PLP form with a catalytic efficiency of $9(\pm 1) \times 10^{-3}$ mM$^{-1} \cdot$ s^{-1}. Taken together, these results clearly indicate that cystalysin catalyses both directions of transamination: from PLP to PMP and from PMP to PLP.

Initial-velocity studies and pH profile for the steady-state kinetic parameters for transamination

Initial velocities of transaminase activity, measured as PMP formation, as a function of different concentrations of L- or D-alanine were determined. The k_{cat} and K_m for L-alanine are $4.50(\pm 0.05) \times 10^{-4}$ s^{-1} and 8.5 ± 0.5 mM, whereas those for D-alanine are $1.0(\pm 0.1) \times 10^{-4}$ s^{-1} and 9.9 ± 0.5 mM (Table 1). Thus cystalysin is capable of transaminating both alanine isomers, the catalytic efficiency towards L-alanine being 5-fold higher than that towards D-alanine. The level of aminotransferase activity is very low. The k_{cat}/K_m value of 0.05 M$^{-1} \cdot$ s^{-1} for L-alanine is similar to the k_{cat}/K_m values of alanine racemase (0.02 M$^{-1} \cdot$ s^{-1}) [13] and 1-aminocyclopropane-1-carboxylate synthase (0.08 M$^{-1} \cdot$ s^{-1}) [25] for the same substrate. Tyrosine phenol-lyase exhibits a somewhat higher catalytic efficiency of transamination (2.5 M$^{-1} \cdot$ s^{-1}) [26] for alanine. It should be noted that the catalytic efficiency of these side reactions is several orders of magnitude lower than that of *E. coli* aspartate aminotransferase for L-aspartate (9×10^4 M$^{-1} \cdot$ s^{-1}) [27]. The major difference (ranging from 5×10^4 to 3×10^6-fold) is reflected in the k_{cat} values of these enzymes catalysing transamination as a side reaction. This may be due to the fact that the orientation of the cofactor and the position of the active-site bases in these enzymes are not fine-tuned for transamination.

The rate constant of PMP formation during the reaction of cystalysin with saturating concentration of L-alanine was measured over the pH range 6–9.5. As shown in Figure 9, the pH profile for $\log k_{PMP}$ increases above a single pK_a value of 6.69 ± 0.05.

Stereospecificity for the hydrogen abstraction from the C4′ carbon of PMP catalysed by cystalysin

When the PMP form of an enzyme is converted into the PLP form by transamination with an amino acid acceptor (oxo acid), one of the two hydrogens at C4′ of PMP is usually transferred stereospecifically to Cα of the oxo acid [28]. However, alanine racemases and amino acid racemases of broad substrate specificity constitute the first class of PLP enzymes catalysing the hydrogen removal, on both sides of the plane, of a substrate–cofactor complex during transamination [10,29]. We studied the stereospecificity of cystalysin for hydrogen abstraction from C4′ of PMP using the method described previously [20]. Stereospecificity is determined by measurements of the radioactivity of ^{3}H released from the PMPs, which are stereospecifically tritiated at C4′. As shown in Table 2, tritium was released equally from both (4′*S*)- or (4′*R*)-[4′-^{3}H]PMP enantiomers. The amount of tritium was 40–50% of the amount that initially existed. No tritium was released from each PMP in the absence of the enzyme. Thus cystalysin catalyses the non-stereospecific abstraction of hydrogen from C4′ of PMP.

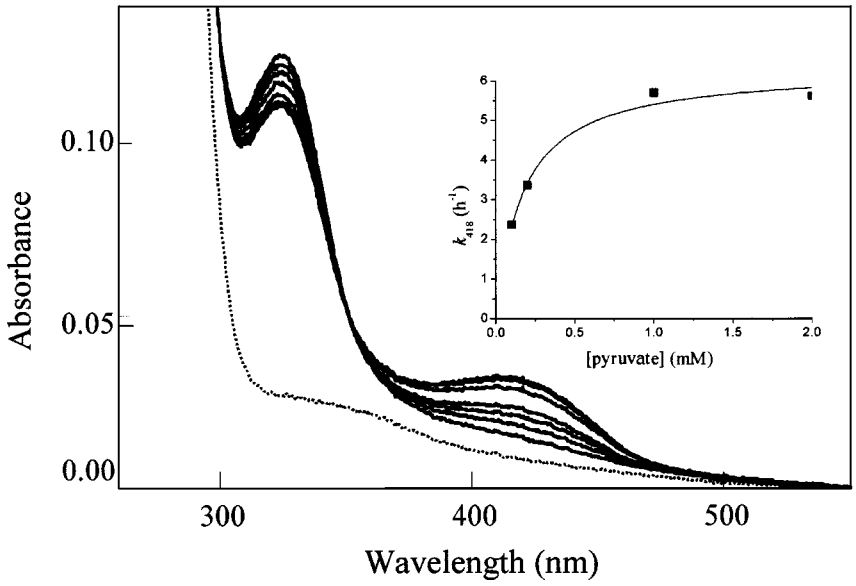


Figure 7 Time-dependent spectral changes that occur on the addition of pyruvate to cystalysin in PMP form

The apoenzyme (· · · · · · · · ·) (2.72 μM) in 20 mM potassium phosphate buffer (pH 7.4) was incubated with 5.5 μM PMP and 1 mM pyruvate at 25 °C, and spectra were recorded at 1, 3, 5, 8, 17, 27 and 36 min. Contribution of pyruvate to the absorbance at 330 nm was not subtracted. Inset shows plot of k_{418} as a function of pyruvate concentration.

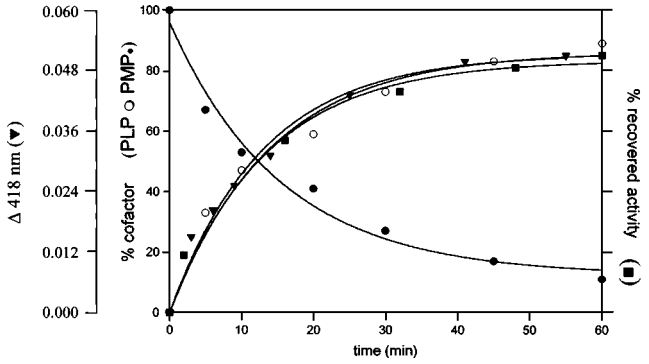


Figure 8 Coenzyme content, absorbance at 418 nm and desulphydrase activity during the reaction of cystalysin in the PMP form with pyruvate

Apocystalysin (2.75 μM) was incubated in the presence of 5.5 μM PMP with 1 mM pyruvate in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C. At the indicated times, aliquots were removed and treated as reported in the Experimental section. ○, PLP; ●, PMP. ■, recovered desulphydrase activity measured as described in the Experimental section; ▼, absorbance at 418 nm at the indicated times. Data represent the means for three independent experiments; S.E. of the mean in each case was less than 5%. The curves for PMP and PLP content, for desulphydrase activity and for 418 nm absorbance were theoretical from a fit to a first-order kinetic equation.

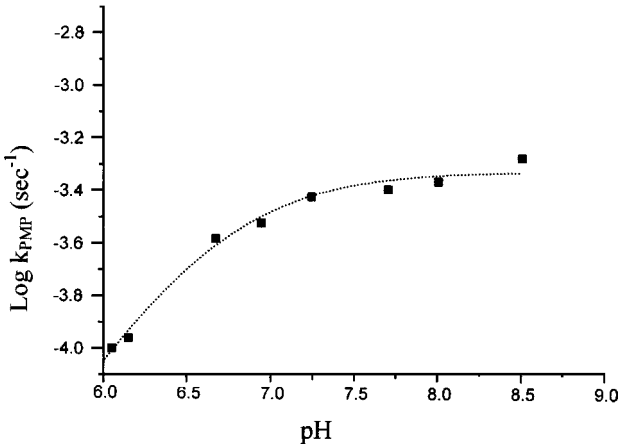


Figure 9 pH dependence of the rate constant of PMP formation during the reaction of cystalysin with L-alanine

Plots of k_{PMP} of the reaction of 4 μM cystalysin with 500 mM L-alanine. The curve is from a fit of the data to a first-order kinetic equation.

DISCUSSION

Along with the main reaction, almost all PLP-dependent enzymes catalyse many side reactions. In this regard, cystalysin is not an exception in that, in addition to the α,β-elimination of sulphur and non-sulphur amino acids [2], the results reported here clearly demonstrate that the enzyme is capable of catalysing racemization and transamination reactions with both alanine isomers as substrates. The occurrence of these side reactions suggests that the active site, which is optimized for catalysing α,β-elimination, also encodes the basic elements required for other PLP-catalysed reactions.

The active enzyme binds both L- and D-alanine with similar K_m values of approx. 10 mM. Binding of both enantiomers of alanine results in a shift in the λ_{max} value from 418 to 429 nm and the appearance of a peak at 498 nm. This spectrum probably represents an equilibrium mixture of an external aldimine and a quinonoid species respectively. The effects of both the isomers on the CD and fluorescence spectra of cystalysin are nearly identical. They consist of quenching of intrinsic fluorescence, enhancement and red shift of the long wavelength PLP emission, change in the dissymmetry of bound cofactor and of aromatic amino acids

Table 2 Release of ^{3}H from stereospecifically labelled [4′-^{3}H]PMPs catalysed by cystalysin

The initial radioactivity in the reaction mixture was 3096 and 10127 c.p.m. for 4′*S*-[4′-^{3}H]PMP and 4′*R*-[4′-^{3}H]PMP respectively.

	% of ^{3}H released*	
	4′*S*-[4′-^{3}H]PMP	4′*R*-[4′-^{3}H]PMP
Apocystalysin	49	39
Without enzyme	0	0

* Volatile radioactivity expressed as percentage of the released radioactivity to that initially added in the reaction mixture.

possibly located at or near the active site. This suggests that binding of both enantiomers of alanine causes almost similar changes in the orientation of bound coenzyme and in the PLP microenvironment with respect to the neighbouring residues. We have calculated the partition ratio between racemization and transamination reactions on the basis of their k_{cat} values: this was found to be 2.3×10^3 s^{-1} for L-alanine and 1.4×10^4 s^{-1} for D-alanine. The racemization reaction is surprisingly robust (k_{cat} of approx. 1 s^{-1}) considering the fact that it is a side reaction of cystalysin. Nevertheless, it has been reported recently [30] that serine racemase catalyses the elimination of L-serine *O*-sulphate with a rate approx. 500 times higher than the rate of its physiological racemization reaction. It should be noted that other PLP-dependent enzymes have been found to catalyse racemization as a side reaction. For example, tryptophan synthase $\alpha_2\beta_2$ complex from *E. coli* racemizes tryptophan slowly [31]. Serine hydroxymethyltransferase [32], aspartate aminotransferase [33] and tyrosine phenol-lyase [34] exhibit an alanine racemase side activity with k_{cat} values ranging from 10^{-5} to 10^{-2} s^{-1}. The quantitatively significant k_{cat} value of racemase activity exhibited by cystalysin might raise the question about its physiological importance. One could speculate its possible importance for the synthesis of bacterial cell walls. On the other hand, considering the high K_m for alanine, it cannot be excluded that this racemizing activity could be a mere corollary of the chemical properties of the enzyme.

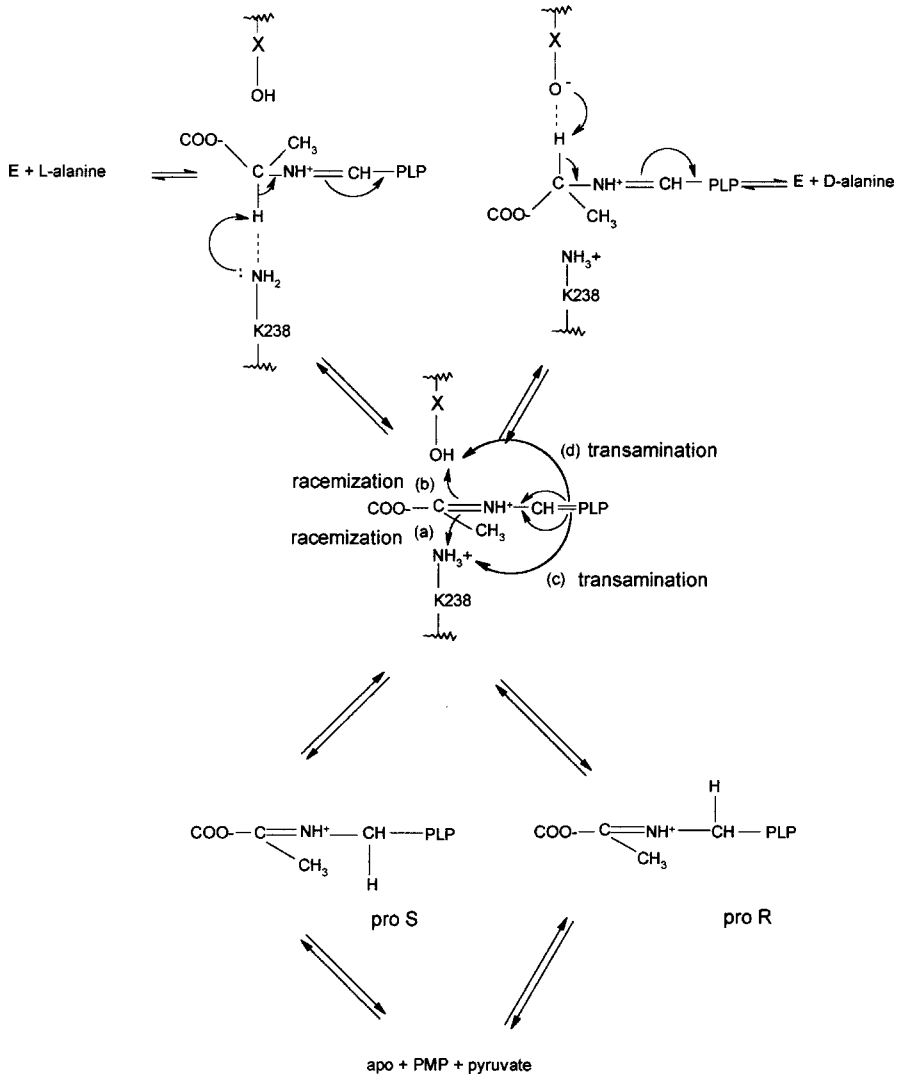
Quinonoid formation is required for both racemization and transamination reactions, but the two following steps, Cα or C4′ protonation, are unique for racemization and transamination respectively. The reaction specificity is, therefore, determined by the reaction pathways following the quinonoid intermediate, and the course of the reaction of the enzyme in the presence of both enantiomers of alanine diverges after quinonoid formation (Scheme 1).

Evidence has been provided for a two-base racemization mechanism for alanine racemase from *B. stearothermophilus*, in which Tyr^{265}* and Lys^{39}, the PLP-binding lysine, participate in the catalysis: one abstracts the hydrogen from a substrate, and the other returns a hydrogen to the deprotonated intermediate [8–12]. Inspection of the model of cystalysin complexed with either L- or D-alanine shows that potential acid–base catalysts are located on the *si* (Lys^{238}) and *re* face (Tyr^{123} and the water molecule W733H) respectively depending on the aldimine linkage (Figure 3). In the coenzyme–L-alanine adduct, the ϵ-amino group of Lys^{238} is situated close enough to Cα of the substrate and in a proper orientation to act as a catalytic base. Although in the coenzyme–D-alanine complex both Tyr^{123} and a water molecule are in a good position to act as catalytic bases, the finding that Tyr^{123} does not seem to orientate correctly in the minimization process allows us to consider a water molecule also as a possible catalyst. In this case, the PLP-binding lysine and a water molecule would be the two acid–base catalysts. A similar situation has been suggested for mutants of Tyr^{265} of alanine racemase [10] and for the racemase activity displayed by aspartate aminotransferase [33]. It is noteworthy that racemization catalysed by cystalysin, Tyr^{265} mutants of alanine racemase and aspartate aminotransferase occur with k_{cat} values that are lower than the k_{cat} value of alanine racemase by approx. 5×10^2-, 3×10^3- and 5×10^7-fold respectively. These data seem to suggest that when the hydroxide ion in solvent water is functionally substituted for an appropriate proton transfer catalyst of the enzyme, racemization takes place with a lower efficiency.

At present, it can be postulated that the racemization pathway of cystalysin proceeds through a proton transfer from Lys^{238} to the α-position of the deprotonated intermediate to form L-alanine [Scheme 1, (a)], and from Tyr^{123} or a water molecule to the α-position of the deprotonated intermediate to form D-alanine [Scheme 1, (b)]. Thus a two-base mechanism can be proposed for the racemization reaction catalysed by cystalysin, based on the fact that proton donors and proton acceptors are situated on both sides of the planar quinonoid intermediate to accomplish removal and return of the α-hydrogen of the substrate amino acid.

Alanine racemase catalyses transamination of both enantiomers of alanine as a side reaction [13,35]. By means of site-directed mutagenesis experiments of *B. stearothermophilus* alanine racemase, it has been demonstrated that the α-hydrogen of L-alanine is transferred suprafacially by Tyr^{265}* to the *pro-S* position at C4′ of PLP on the planar π system of the quinonoid intermediate [10]. Lys^{39} plays the role of a counterpart forTyr^{265}*, being specific to D-alanine and the *pro-R* hydrogen of PMP [8]. Furthermore, non-stereospecific transamination has been shown to be catalysed by PLP-dependent amino acid racemases of broad substrate specificity [29]. All the above enzymes represent the first class of PLP enzymes catalysing the hydrogen removal on both sides of the plane of a substrate–cofactor complex during transamination [29].

Racemization and transamination reactions of L- and D-alanine catalysed by cystalysin could share the step leading to the quinonoid intermediate (Scheme 1), and the same catalytic residues (Lys^{238} and Tyr^{123} or a water molecule) are probably used for α-hydrogen abstraction in both reactions. When half-transamination (i.e. formation of PMP and pyruvate) occurs, the C4′ position of the cofactor moiety of the quinonoid intermediate must be protonated. Taking into account the putative binding modes of both enantiomers of alanine to cystalysin, it can be reasonably postulated that protonation at C4′ could be accomplished by Lys^{238} for L-alanine [Scheme 1, (c)] and by Tyr^{123} or a water molecule for D-alanine [Scheme 1, (d)]. If the formation of the C–H bonds at C4′ of the coenzyme occurs on both sides of the plane of the quinonoid intermediate, the cleavage will take place in a similar non-stereospecific manner. In the present study, we provide evidence that non-stereospecific hydrogen abstraction from C4′ of PMP occurs in the reverse transamination catalysed by cystalysin in the presence of pyruvate. To our knowledge, this is the first example of a non-stereospecific hydrogen abstraction catalysed by an enzyme belonging to the α-family of PLP enzymes. A racemic mixture of alanine is formed by the reverse transamination reaction. This could imply that the hydrogen is introduced to C-2 of the oxo acid moiety of the anionic intermediate on both sides of the planar intermediate during the half reaction of transamination. However, since alanine undergoes racemization, it cannot be excluded that both enantiomers of alanine were formed by racemization of



Scheme 1 Proposed racemization and transamination mechanisms of cystalysin. X represents the base (Tyr[123] or a water molecule) on the *re* face

one enantiomer produced through transamination, rather than directly by transamination. The examination of this aspect will be facilitated by using an appropriate amino acid which is a substrate for transamination but inert for racemization.

There is a general scarcity of information in the literature on the pH dependence of the kinetic parameters for racemases that operate with a two-base mechanism in either a PLP-independent or PLP-dependent fashion. Kinetic studies on *Pseudomonas putida* mandelate racemase led to the identification of two pK_a values of approx. 6.4 and 10 in both log k_{cat} and log k_{cat}/K_m pH profiles [36]. Similarly, glutamate [37] and proline [38] racemases employ two general acid–base residues, identified as cysteine residues, of similar pK_a values during catalysis, one of which must be in a protonated form and the other must be in a deprotonated form for a given reaction direction. It is peculiar that, unlike racemases not requiring PLP, alanine racemase and cystalysin do exhibit only a single pK_a associated with a catalytic group over the pH range examined. A pK_a value of approx. 7.2 is observed in both k_{cat} and k_{cat}/K_m profiles for alanine racemase and an additional pK_a value of 9.7 is found in k_{cat}/K_m. By means of site-directed mutagenesis experiments, the group responsible for the lower pK_a value was assigned to Tyr[265]* [10], whereas the group with the higher pK_a value was assumed to be either some active-site residue or the α-amino group of free alanine by Sun and Toney [9] and by Watanabe et al. [11] respectively. The results on the variation of log k_{cat} with pH for racemase reaction catalysed by cystalysin indicate that a single ionizing group with a pK_a value of approx. 6.6 must be unprotonated to achieve maximum velocity. The same pK_a value is observed in the k_{cat}/K_m profile and, thus, it can be concluded that this pK_a value represents the ionization of a group involved in catalysis but not in binding. This implies that cystalysin is more competent at alkaline pH and reinforces a previous view that, at high pH, binding of substrates to the enzyme converts the inactive aldamine form of the coenzyme into the active ketoenamine form [2]. The pK_a value of approx. 6.6 seems to coincide with the pK_a value observed in the pH profile curves for transamination and α,β-elimination [2]. On the basis of the present information, this pK_a value could be tentatively associated with ionization of Lys[238], even if it cannot be ruled out that it could reflect the ionization of an enzymic group other than Lys[238] involved in catalysis.

According to Watanabe et al. [11], an additional basic residue must be assumed to participate in the reaction catalysed by alanine racemase to return the original states of Lys[39] and

Tyr^{265}* to the original ones in each catalytic turnover. On the basis of X-ray structures of enzyme bound with reaction intermediate analogues, mimicking external aldimine with L- and D-alanine, they suggest a mechanism in which the carboxy group of the substrate directly participates in the catalysis by mediating the proton transfer between the two catalytic bases, Lys^{39} and Tyr^{265}*. However, the finding that cystalysin is capable of catalysing conversion of L-alanine methyl ester into its D-enantiomer, albeit with a k_{cat} value approx. 4-fold lower than that of L-alanine, ruled out the possible involvement of the carboxy group of the substrate in the racemization reaction catalysed by cystalysin.

In conclusion, the kinetic analysis of the side reactions catalysed by cystalysin together with molecular modelling studies have provided mechanistic and stereochemical insight into the topography of its active site. Site-directed mutagenesis experiments are now in progress to check the roles of Lys^{238} and Tyr^{123} residues as general acid–base catalysts.

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