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Biochemical characterization of Alanine racemase- a spore protein produced by *Bacillus anthracis*

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Alanine racemase catalyzes the interconversion of L-alanine and D-alanine and plays a crucial role in spore germination and cell wall biosynthesis. In this study, alanine racemase produced by *Bacillus anthracis* was expressed and purified as a monomer in *Escherichia coli* and the importance of lysine 41 in the cofactor binding octapeptide and tyrosine 270 in catalysis was evaluated. The native enzyme exhibited an apparent K_m of 3 mM for L-alanine, and a V_{max} of 295 μmoles/min/mg, with the optimum activity occurring at 37°C and a pH of 8-9. The activity observed in the absence of exogenous pyridoxal 5′-phosphate suggested that the cofactor is bound to the enzyme. Additionally, the UV-visible absorption spectra indicated that the activity was pH independece, of VV-visible absorption spectra suggests that the bound PLP exists as a protonated Schiff's base. Furthermore, the loss of activity observed in the apoenzyme suggested that bound PLP is required for catalysis. Finally, the enzyme followed non-competitive and mixed inhibition kinetics for hydroxylamine and propionate with a K_i of 160 μM and 30 mM, respectively. [BMB reports 2009; 42(1): 47-52]

INTRODUCTION

Alanine racemase (EC 5.1.1.1) (Alr) is a pyridoxal 5'-phosphate (PLP) dependent enzyme that catalyzes the racemization between L- and D- alanine. This enzyme is indispensable to prokaryotes because D- alanine is essential for peptidoglycan biosynthesis in the cell wall of gram-positive and gram-negative bacteria (1, 2). *Escherichia coli* and other gram-negative bacteria possess two independent alanine racemases with distinct functions (3). Alanine racemase is ubiquitous in ubiquitous, which makes it an attractive target for the development of antimicrobial drugs. As a result, L-fluoroalanine, a structural analog of L-alanine, is currently being evaluated to determine its potential as an antibacterial drug (4). Alanine racemization proceeds through a two-base mechanism that involves lysine (pyridoxal 5'-phosphate binding residue) and tyrosine (plays a role in binding/catalysis). Crystallographic analysis of *B. stearothermophilus* alanine racemase has shown that the residues at the active site play a role in its racemization (5). Based on its known structure, it is plausible that *B. anthracis* produces alanine racemase via an analogous catalytic site, wherein Lys41 and Tyr270 remove α-hydrogen from the alanyl-PLP aldimine and protonate the carbanion to induce racemization.

Immunoproteome analysis of the spore antigens produced by *Bacillus anthracis* has led to the identification of several immunogenic proteins including exosporium associated alanine racemase (6, 7). However, several studies have demonstrated that few antigens produced by anthrax spores can augment the protective efficacy of currently used protective antigen based vaccines (7). *B. anthracis spores* being a potential biological warfare agent, development of vaccines or inhibitors against proteins/enzymes crucial for *B. anthracis* germination and outgrowth-associated factors or combining PA with one of the immunodominant antigens in vaccine formulation may lead to anefficacious safer vaccine.

The development of mechanism-based inhibitors of alanine racemase requires understanding the mechanism by which alanine racemase enforces substrate specificity and maintains enantioselectivity. Understanding these mechanisms requires enzyme-substrate complex analysis to determine if it requires a PLP cofactor. Therefore, the present study was conducted to characterize alanine racemase (YP_026523), a spore-specific enzyme produced by *B. anthracis* (8).

RESULTS AND DISCUSSION

Biochemical characterization of Alanine racemase

PCR amplification of *alr* resulted in amplification of an 1170 bp fragment, which was subsequently cloned into an expression vector. Blast P analysis of the deduced amino acid sequence of *B. anthracis* Alr revealed that it was 96-99% homologous with the Alr produced by closely related bacillus species. The PLP binding octapeptide of *B. anthracis* Alr, KGNAYGHD, was also found to be conserved among species.

SDS-PAGE of *E. coli* BL21 (λDE3) cells harboring pPSAlr following IPTG induction revealed that a high level of an ≈45

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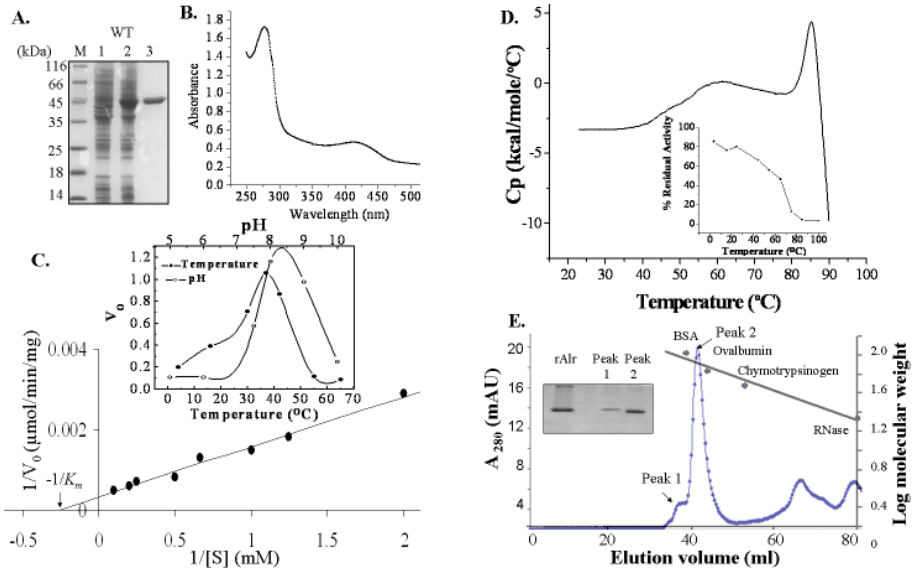

Fig. 1. (A) Expression and purification of wild type rAlr. Lanes 1 and 2: uninduced and induced cell lysates; 3: purified rAlr; M: molecular mass standards. (B) UV-Visible spectrum of rAlr. (C) Kinetics of wild type rAlr. Lineweaver-Burk plot of 1/(S) vs. $1/V_o$. Inset: temperature and pH optimum for rAlr. (D) Determination of T_m for rAlr. Denaturation transition of rAlr obtained using DSC. Inset: percentage of residual activity for rAlr at each temperature indicated. (E) Determination of the molecular weight of rAlr.

kDa rAlr was expressed (Fig. 1A, Lane 2), and that this protein was purified to≈98% homogeneity (Fig. 1A, Lane 3). A shake flask culture of the transformed organisms yielded approximately 180 mg of the purified protein per liter of culture. The protein produced using the technique described here had a specific activity of 295 U/mg. The specific activity of the rAlr produced *B. anthracis* was found to be lower than that reported for dimeric alanine racemases (2000-7700 U/mg) (9-13); however, it was comparable to that of monomeric alanine racemases (36-220 U/mg) (2, 14-16).

The UV-Visible spectrum of rAlr revealed a small shoulder at 420 nm, which is characteristic of a Schiff's base complex between PLP and the ε-amino group of the lysine residue (Fig. 1B). The absorption maximum for free PLP was observed at 291 nm.

The rAlr produced here was active within a temperature range of 25°C to 45°C, with the optimum activity being observed at 37°C. In addition, rAlr was active over a wide pH range, with the optimum activity being observed between pH 8-9. These findings are in accordance with those of all other alanine racemases that have been evaluated to date (14-18) (Fig. 1C, Inset).

The wild type rAlr followed classical Michaelis-Menten's kinetics; therefore, the kinetic coefficients were calculated using a Lineweaver-Burk plot (Fig. 1C). The K_m was calculated to be 3 mM for L-alanine with a V_{max} of 295 U/mg. The k_{cat} for *B. anthracis* rAlr was determined to be 22 s^{-1}, which differs greatly from the values reported for *H. pylori* (632 s^{-1}) (1), *P. monodon* (2568 s^{-1}) (19) and *crayfish* (7504 s^{-1}) (9). These findings indicate that rAlr has a high catalytic efficiency.

The thermal stability and T_m determination for rAlr were assessed by Differential Scanning Calorimetry (DSC). The results of this analysis revealed that rAlr is an extremely stable enzyme with an apparent T_m of 85°C (Fig. 1D). However, rAlr was prone to aggregation at temperatures higher than 90°C, which resulted in irreversible unfolding. As a result, the post transitional baseline could not be evaluated. Additionally, the residual activity of rAlr (functional stability) resulted in a 50% reduction in the enzyme activity between 60 and 70°C (Fig. 1D, inset). Additionally, the T_m (structural stability) suggests that 50% of the protein molecules are completely denatured at 85°C. This finding indicates that the remainder of the protein molecules in the solution may have already undergone conformational perturbations at this temperature, thereby reducing its catalytic efficiency (4% residual activity). The high degree of thermostability associated with rAlr could be attributed to its presence on the spore surface. The optimum activity for the dimeric enzyme from *Acidiphilium organovorum* and *B. stearothermophilus* also falls within the range of 50-60°C (10, 16). However, even though rAlr produced by *B. anthracis* is monomeric; it is thermostable, which indicates that it is a potential candidate for the development of a recombinant vaccine.

Determination of the molecular mass of rAlr

Bacterial alanine racemases are classified as monomeric (13, 15, 18) or homodimeric (3, 11, 12) based on the structure of their subunits. SDS-PAGE revealed that the molecular mass of the purified rAlr of *B. anthracis* was≈45 kDa. In addition, gel filtration analysis of purified rAlr (1 and 10 mg/ml) in its native form revealed that it was a monomer (Fig. 1E). No multimerization was observed at this concentration. The eluted fraction of rAlr (peak 2) exhibited a specific activity of 260 U/mg, which demonstrates that the rAlr monomer is catalytically active. Peak 1 shown in the inset, which was eluted just before rAlr, possessed no activity.

Substrate specificity of rAlr

rAlr acted exclusively on L- alanine, and the formation of D-ala-

nine was demonstrated by a decrease in the CD signal following racemization (Data not shown). Other amino acids were inactive as substrates, which suggests that *B. anthracis* rAlr is highly specific for L-alanine. However, the 20% racemization that was observed for L-leucine could be due to its structural similarity with L-alanine, which is the true substrate of rAlr.

Effect of inhibitors on enzyme activity

Hydroxylamine is a general inhibitor of PLP-dependent enzymes (20). Small chain carboxylic acids (acetate, propionate and butyrate) that resemble alanine also bind to the active site of alanine racemase (21). Therefore, we evaluated the role that these inhibitors played in modulating the enzyme activity. To accomplish this, K_i (the equilibrium constant for binding of the inhibitor to the enzyme, EI) and K_i' (the equilibrium constant for binding the inhibitor to the enzyme-substrate complex, ESI) were calculated using a Dixon plot (Fig. 2B and D) and a Cornish Bowden plot, respectively (Fig. 2A and C). A Dixon plot cannot discern between competitive and mixed inhibition and a Cornish Bowden plot cannot differentiate uncompetitive inhibition from mixed inhibition; therefore, both plots were used to determine the type of inhibition that occurred (22).

Steady state kinetic analysis of rAlr at various concentrations of propionate yielded a Dixon plot with an intersection in the lower left quadrant. Slope and intercept replots of the data were linear and consistent with a mixed-type inhibition pattern (Fig. 2A and B), with a K_i of 30 $\pm$ 5 mM being observed for propionate (Fig. 2B). This value is 1.5-fold higher than those reported for *B. stearothermophilus* (23). The mixed inhibition obtained for propionate suggests that the inhibitor binds both to the enzyme and enzyme-substrate complex, with lower affinity for the enzyme being indicated by a higher K_i (30 mM) when compared to K_i' (7 mM). This finding indicates that propionate influences both K_m (affinity for substrate) and V_{max} (enzyme catalysis), which has also been proposed for the alanine racemase of *B. Stearothermophilus*. Propionate, which resembles alanine more closely than acetate, is a stronger inhibitor of rAlr (Data not shown). Taken together, these findings suggest that size of the binding pocket for alanine allows efficient binding of substrate analogues such as propionate, while allowing smaller compounds such as acetate to be recognized, albeit with much lower affinity.

Classical non-competitive inhibition kinetics (Fig. 2C and D) with a K_i of 160 $\pm$ 20 μM were observed for hydroxylamine (Fig. 2D). Specifically, the mechanism of action of hydroxylamine occurs via disruption of the schiff's linkage between the lysine residue and the PLP moiety, which results in a loss of activity. These findings further reemphasize the dependency of rAlr on PLP.

EDTA did not exert any discernible effect on the enzyme activity, which suggest that divalent metal ions/cations do not play a plausible role in catalysis. This finding is similar to the findings of all other alanine racemases that have been studied to date.

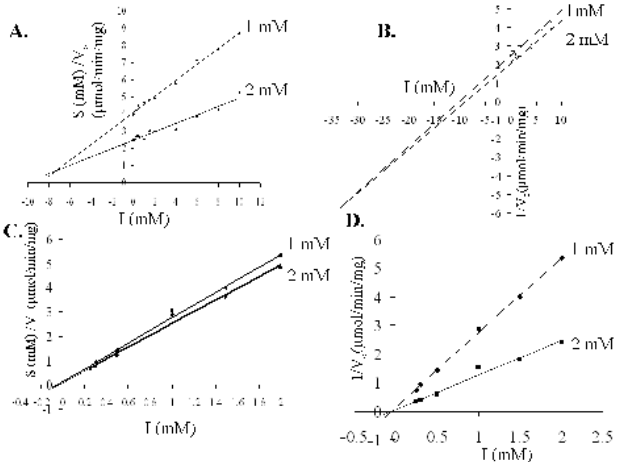

Fig. 2. Inhibition kinetics of rAlr. Cornish Bowden plot of S/V_o (Y-axis) versus I (mM) (X-axis) for propionate (A) and hydroxylamine (C) Dixon plot of the reciprocal of the velocity plotted against the inhibitor concentration (mM) for propionate (B) and hydroxylamine (D).

Kinetic properties of catalytic variants of alanine racemase

The *Bacillus anthracis* octapeptide differs from the conserved octapeptide only at glycine at the 42nd position and the aspartate at the 48th position (supplementary Fig.1). In almost all other species, including *B. stearothermophilus*, alanine and glycine are located at these potions (23). The glycine to alanine substitution does not represent a major difference in the protein (non polar, uncharged); however, replacement of glycine with aspartate (non polar, uncharged to a polar charged) in *B. anthracis* alanine racemase is believed to have a significant effect on the activity of the enzyme. Because this marked alteration in amino acid substitution occurs in only a few *Bacillus* species, we evaluated the role that the aspartate at the 48th position of the otherwise conserved octapeptide played. However, the tyrosine at the 270th position was conserved invariably among all bacterial species. Therefore, to specifically delineate the active site residues of alanine racemase and to define its exact catalytic mechanism, site specific mutants were generated. The mutant proteins, K41A rAlr, D48A rAlr and Y270A rAlr were expressed in *E. coli* and were purified to near homogeneity (Fig. 3A).

The racemization potential of the purified mutants, K41A rAlr, D48A rAlr and Y270A rAlr was assessed and K_m and V_{max} were then derived using a Lineweaver-Burk plot (supplementary Fig. 2 and 3). K41A substitution in rAlr resulted in the complete loss of activity, even at higher substrate and enzyme concentrations. Table 1b summarizes the relative catalytic parameters obtained for the native and the mutant proteins. The enzyme inactivation that occurred after substitution of the highly conserved residues, Lys41 and Tyr270, indicated that these residues play an essential role in catalysis. However, substitution of the aspartate at the 48th position with alanine did not have a deleterious effect on the enzyme activity. This indicates that replacement of aspartic acid with glycine in the conventional oc-

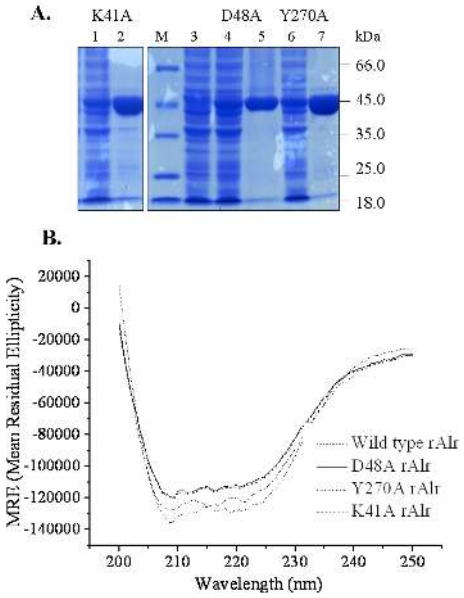

Fig. 3. (A) Expression and Purification of mutants of rAlr. Lanes 1, 4 and 6: induced cell lysates expressing K41A, D48A and Y270A rAlr; respectively; 2, 5 and 7: purified K41A, D48A and Y270A rAlr, respectively; 3: uninduced cell lysate; M: molecular mass standards. (B) Circular dichroism spectra of wild type and mutants of rAlr.

tapeptide sequence of *Bacillus* species is only an evolutionary event with no significant functional consequences.

Far UV circular dichroism spectroscopic analysis (Fig. 3B) of wild type rAlr and the mutants suggested that site-directed replacement did not cause substantial unfolding or other gross significant changes in the content of the secondary structure.

Requirement of cofactor: PLP dependency

Amino acid racemases are categorized into two groups based on their requirement for cofactors or metals. All alanine racemases studied to date require PLP as a cofactor (17). However, *B. anthracis* rAlr exhibited considerable activity without the addition of exogenous PLP. Nevertheless, the activity was slightly enhanced upon addition of 10 μM PLP, which suggests that PLP is inherently bound to the enzyme (Fig. 1B). This is similar to the alanine racemase produced by *Bacillus stearothermophilus*, in which PLP is covalently linked to lysine 39 via an aldimine linkage. It has been reported that deprotonated internal aldimines have substantial blue shifts when compared to the protonated species (21). In the present study, the UV-visible absorption spectra of the internal aldimine form of the enzyme revealed that it was pH independent (Data not shown). This finding indicates that the internal aldimine exists as a protonated Schiff's base, which is the reactive form of the enzyme.

To evaluate the requirement for PLP, apoenzyme (rAlr without PLP) was generated by extensive dialysis of native rAlr against hydroxylamine. The complete removal of PLP from the enzyme was then confirmed by the disappearance of a characteristic peak in aporAlr at 420 nm. Although this apoenzyme was inactive, exogenous addition of PLP restored its activity. However, K41A rAlr could not be activated by the addition of PLP due to the irreversible loss of lysine. Conversely, a slight activation was observed when PLP was added to D48A and Y270A rAlr due to the presence of an intact Lys41 in the octapeptide. The PLP requirement clearly indicated the extent of the bound PLP (Table 1b). The lower affinity of PLP for Y270A rAlr may be due to the destabilization of the hydrogen bond formed between Tyr-OH and the phosphate moiety of PLP (23). In addition, 20% PLP binding was observed in K41A rAlr, which is attributed to the existence of a functional Y270 residue. However, substitution of the D48 residue may have destabilized the PLP binding octapeptide, resulting in a slight reduction in PLP binding.

The present study focused on biochemical characterization of alanine racemase, an immunodominant antigen constituting 75% of the exosporium (8). Further studies are currently in progress to evaluate the protective efficacy of antibodies generated against this protein alone and in conjunction with PA in response to *B. anthracis* spore challenge.

MATERIALS AND METHODS

Materials

All of the restriction enzymes used in this study were obtained from *Fermentas GmbH* (Germany). Ni^{2+}-NTA agarose resin was obtained from *Qiagen* (Hilden, Germany). Sephacryl S-200, a HiPrepTM 16/60 FPLC column, IPTG and bovine serum albumin were purchased from *Amersham Biosciences* (Uppsala, Sweden). Bovine liver catalase, L- and D- alanine and D- amino acid oxidase (porcine kidney) were obtained from *Sigma* (St. Louis, MO, U.S.A.). A QuickChange site-directed mutagenesis kit was obtained from *Stratagene* (La Jolla, CA, USA). Oligonucleotides were custom synthesized by *Microsynth* (Switzerland).

Bacterial strains and vectors

E. coli strains DH5α and BL21(λDE3) (*Novagen*) were used as the hosts for cloning and expression, respectively. Genomic DNA was isolated from an avirulent strain of *Bacillus anthracis* (Sterne 34F2, $pXO1^{+}$, $pXO2^{-}$). Plasmid pET-28a (+) obtained from *Novagen* (Madison, Wisconsin, USA) was used for heterologous gene expression.

Expression and purification of Alanine racemase from *B. anthracis*

The open reading frame corresponding to *alr* of *B. anthracis* was PCR amplified employing forward and reverse primers (Table 1a), using genomic DNA as a template. The amplified DNA fragment was then ligated into the *Nhe* I/Sal I sites of pET-28a expression vector to obtain pPSAlr. The integrity of the clone was verified by automated dideoxy DNA sequencing.

The protein expression was analyzed in *E. coli* BL21(λDE3) cells harboring pPSAlr following induction with 1 mM IPTG at 30°C ($O.D_{600}$ 0.6-0.8). Alanine racemase with hexahistidine at the N-terminus (rAlr) was purified from the soluble fraction using Ni^{2+}-NTA affinity chromatography. The purified protein was then dialyzed against 10 mM Tris- HCl, pH 8.0. Finally, the protein concentration was determined using Bradford reagent with bovine serum albumin as the standard.

Determination of the molecular mass of rAlr

Purified rAlr (1 and 10 mg/ml) was loaded onto a Sephacryl S-200 gel filtration column that had been pre-equilibrated with 10 mM Tris-HCl, pH 8.0. A calibration curve was then constructed using bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa) and lysozyme (14.4 kDa). The molecular weight of rAlr was calculated by plotting the elution volumes against molecular weight standards on a logarithmic scale.

Determination of enzyme activity enzyme coupled colorimetric hydrazone method

The enzymatic activity of rAlr was determined spectrophotometerically using a colorimetric assay (9). The assay mixture contained 100 mM 2-(N-cyclohexylamino) ethanesulfonic acid (CHES) buffer, (pH 9.0), 2 mM L-alanine, 0.2 U of D amino-acid oxidase, 100 U of catalase, 10 μM PLP and rAlr in a final volume of 0.5 ml. One unit of enzyme activity is defined as 1 μm of the racemized product formed per minute. To determine the substrate specificity of rAlr, a CD based enzyme assay was conducted (24). The reaction mixture, which contained 2 mM L-amino acids, 10 μM PLP and 200 μg of rAlr in 100 mM CHES buffer (pH 9.0), was incubated at 37°C for different time intervals. The reaction was terminated by boiling the samples for 10 min.

Determination of the optimal temperature and pH for rAlr

The optimum temperature for rAlr was determined by assaying its activity at different temperatures (4-65°C) in 100 mM CHES buffer, pH 9.0. Similarly, the optimum pH for rAlr was determined by assaying its activity in 100 mM buffer at pHs that ranged 4 to 10. The coupled colorimeteric assay was performed as described above.

Thermal stability of rAlr

A. T_m measurement by differential scanning calorimetry

Calorimeteric measurements were performed on a VP-DSC scanning calorimeter (Microcal Inc., Northampton, MA, USA). Data were accumulated at a scan rate of 1°C per minute for 100 min. T_m was calculated by plotting C_p (kcal/mole) values on the Y-axis versus temperature (°C) on the X-axis.

B. Residual activity of rAlr

The thermal stability of rAlr was assessed by incubating the native rAlr at a temperature range of 20°C to 90°C in 20 mM Tris-HCl buffer (pH 8.0) for 30 min prior to the enzyme assay. The activity measurements were then performed as described above. The activity at 37°C was used as control.

Site directed mutagenesis of Alanine racemase

Mutations were generated using a QuickChange site-directed mutagenesis kit (25). Mutagenized plasmids were subjected to dideoxy sequencing to confirm that the specific mutations were only incorporated at the desired positions and that no random mutation(s) occurred. All mutant proteins were purified using a Ni^{2+}-NTA affinity chromatography as described for the wild type rAlr.

Far UV CD spectra of the wild type rAlr and mutants (50 μg/ml) were obtained using a J-710 JASCO spectropolarimeter (Jasco Corp.) at 25°C using a 10 mm cell, wavelengths between 200 and 250 nm, and a scanning speed of 50 nm/min. Five spectra were accumulated for each sample, averaged and then subjected to a buffer baseline correction. The CD signals were then converted to Mean Residual Ellipticity (MRE) using the following relationship: $\theta_m = \theta_o \times 100/lc$, where θ_m is the molar ellipticity (degrees per square centimeter per decimole), θ_o is the observed ellipticity (degrees), l is the path length (centimeters) and c is the molar concentration.

Kinetic properties of rAlr

The Michaelis-Menten constant was determined using 0.5 mM -10 mM of the substrate, L-alanine, at 37°C in 100 mM CHES buffer (pH 9.0). The K_m, V_{max} and k_{cat} (V_{max}/molar concentration of the enzyme) were determined using a Lineweaver-Burk plot. The molecular weight of rAlr was taken as 45,000 Da for the k_{cat} determination.

Effect of activators and inhibitors

The requirement of pyridoxal 5´-phosphate for the activity of wild type and mutant enzymes was assessed by conducting enzyme catalysis in the presence (10 μM) and absence of PLP. The percentage of bound PLP in the native and mutant enzymes was determined based on the A_{420}/A_{280} (protein concentration normalization) ratio. The role that divalent ions play in the enzyme catalysis was demonstrated by including EDTA (1 mM-20 mM) in the reaction mixture. The effect of specific alanine racemase inhibitors on enzyme activity was determined using hydroxylamine and propionate. The inhibitor constants were calculated using a Dixon and a Cornish Bowden plot (22).

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