cis → *trans* isomerization was achieved by irradiation with visible light from a Xenon lamp (UV Spot Light Source: HAMAMATSU PHOTONICS) for 1 min through a L-42 filter (from Asahi Technoglass). For the experiments represented in Figure 1 and Figure 2, the solution was UV-irradiated immediately after incubation at 37 °C started. In the experiment represented by Figure 3, UV and visible-light irradiation was carried out when 40 and 80 min, respectively, had passed after incubation started.

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[6] All the modified DNA sequences were characterized by MALDI-TOF MS: found: Azo-**9**, 8030.7; Azo-**10**, 8032.9; Azo-**11**, 8029.9; Azo-**12**, 8029.8; Azo-**13**, 8029.9; calcd for all $[\text{Azo-}\mathbf{n} - \text{H}]^+$: 8030.4.

[7] About 25% azobenzene in the Azo-**10**/**T** duplex was isomerized to the *cis* form under the conditions employed. Note that *trans* → *cis* isomerization by UV irradiation is rather suppressed when the azobenzene is placed in the duplex.

[8] L-Threoninol was also available as a linker for the azobenzene instead of D-threoninol. Although photoregulated transcription was also possible with modified DNA that included an azobenzene tethered on L-threoninol, an azobenzene on D-threoninol was more effective (see Figure 1 in the Supporting Information).

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[11] Since *trans*-azobenzene has a planar structure, it readily intercalates between adjacent base pairs[2a,c]. The intercalated azobenzene should make the duplex re-wind and widen the distance between the neighboring base pairs.

[12] Note that the linker used in this study is rather flexible compared with the natural deoxyribose framework. Therefore, it is possible for *cis*-azobenzene to be positioned in the groove, where it does not disturb the binding of RNAP.

[13] Introduction of multiple azobenzenes or chemical modification of azobenzene is promising for still more effective photoregulation.

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Site-Directed Mutagenesis of Tyr354 in *Geobacillus stearothermophilus* Alanine Racemase Identifies a Role in Controlling Substrate Specificity and a Possible Role in the Evolution of Antibiotic Resistance

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KEYWORDS:

antibiotics · mutagenesis · protein evolution · racemases · substrate specificity

The pyridoxal 5′-phosphate (PLP) dependent enzyme alanine racemase (EC 5.1.1.1) catalyses the interconversion of the L and D isomers of alanine. In both Gram-negative and Gram-positive bacteria, the D-Ala produced by this enzyme is incorporated into the cell wall as an essential component of the peptidoglycan layer. The structure of the *Geobacillus stearothermophilus*[1] alanine racemase (Alr) has been solved at 1.9 Å resolution,[2] revealing a homodimer in which each subunit consists of two domains: a $(\beta\alpha)_8$ barrel and a C-terminal domain essentially composed of three β sheets (11 strands in total). On dimerisation the $(\beta\alpha)_8$ barrel of one monomer interacts with the C-terminal domain of the other; the active site is a cleft between these two domains. Mutagenic, structural and modelling analyses[3, 4] confirm a catalytic mechanism involving two bases, Lys39 and Tyr265′.[5] The cofactor PLP forms a Schiff base with one of these, Lys39, in the resting enzyme, and in addition to this covalent attachment, a large number of hydrogen bonds fixes PLP rather rigidly in the active site. In particular, the three oxygen atoms of the cofactor phosphate group are involved in an extensive network of hydrogen bonds involving Tyr43, Ser204, Gly221, Ile222 and Tyr354[4] (Figure 1 A). Interestingly however, the structure of an external aldimine form of Alr in which PLP forms a Schiff base with the inhibitor (*R*)-1-aminoethylphosphonic acid (L-Ala-P) suggests that there is ample space in the active site for larger side chains than that of alanine and provides no simple structural explanation for the observed substrate specificity.[4] In the present study we have demonstrated that, rather than merely contributing to the immobilisation of the cofactor phosphate, Tyr354 plays a crucial role in defining the strict specificity of Alr for alanine by preventing turnover of other potential substrates.

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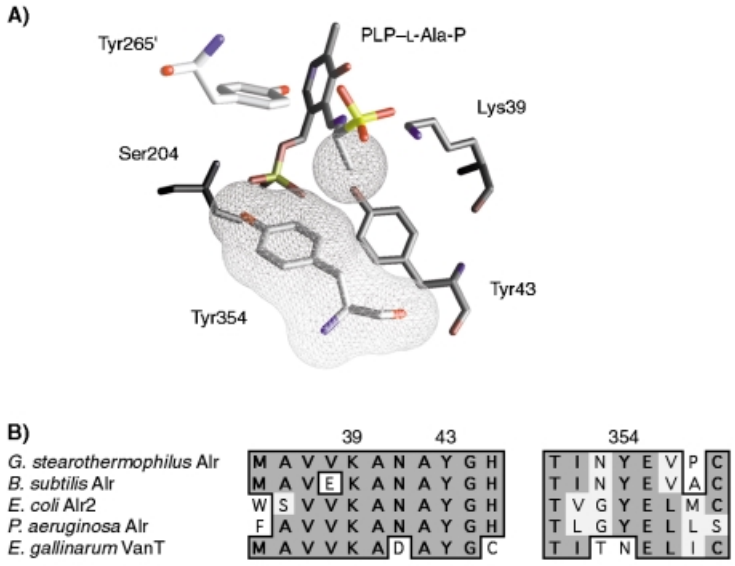

Figure 1. *A) Coordination of the phosphate group of PLP in* G. stearothermophilus *alanine racemase by active site residues Tyr43, Ser204 and Tyr354. The PLP – L-Ala-P external aldimine[4] is shown; note that the methyl side chain of the inhibitor (with Van der Waals radius drawn) will rotate towards Tyr354 in attaining an intermediate planar with the pyridoxal ring of PLP. B) ClustalW sequence alignment of bacterial alanine racemases and the* E. gallinarum *serine racemase VanT in the regions of Lys39 and Tyr43, and Tyr354.*

Alanine racemases have been identified in many bacterial species and, recently, in a number of eukaryotes.[6] Sequence alignments of the prokaryotic enzymes demonstrate very strong conservation of active site residues, including Tyr354 (Figure 1 B), and therefore shed little light on the origin of the observed specificity. Interestingly however, VanT from *Enterococcus gallinarum* shares a strikingly high level of sequence homology with the *G. stearothermophilus* alanine racemase, yet possesses a serine racemase activity that is required for vancomycin resistance.[7] Alignment of the sequences of VanT and Alr revealed 31 % identity and 66 % similarity and, further, suggested that the only active site residue that is not conserved between the two enzymes is Tyr354, which is replaced with an asparagine in VanT (Figure 1 B).[7] Comparative modelling of the two active sites suggests that the shorter side chain of this asparagine would not be able to hydrogen bond to the phosphate oxygen atom of PLP. We were therefore intrigued as to whether Tyr354 of Alr plays the simple cofactor binding role implied by crystallography, or whether it plays some further role in defining the substrate specificity of Alr.

In the present work, we used site-directed mutagenesis to analyse the role of Tyr354 in Alr and to explore the significance of the Asn-for-Tyr (Y354N) substitution found in VanT. We chose to replace Tyr354 with Ala to open an additional pocket in the active site and with Asn and Gln to mimic VanT while allowing for inaccuracies in modelling one active site (VanT) based on another (Alr). Each Alr mutant was over-expressed in *Escherichia coli* and purified to apparent homogeneity. The racemase activity of each protein acting on 40 mM L-Ser was determined by using an assay modified from that previously described for rat brain serine racemase (see the Experimental Section for details). The results are displayed in Table 1. Each of the three mutant enzymes showed a specific activity on L-Ser 51- to 81-fold higher than that of wild-type Alr, which immediately appeared to confirm our hypothesis that Tyr354 plays a role in determining the substrate range of Alr. The highest serine racemase activity was shown by the Y354N-Alr mutant, which is particularly interesting in light of possible mechanisms for the evolution of an ancestral serine racemase activity in response to vancomycin-induced selection pressures, so the kinetic parameters of this mutant were analysed in more detail.

Table 1. *Serine racemase activities of wild-type and Tyr354 mutant Alr enzymes.*

Enzyme	Serine racemase activity [μmol min^{-1} mg^{-1}][a]	Relative activity
Alr	1.1 ± 0.1	1
Y354N-Alr	89 ± 7	81
Y354A-Alr	59 ± 8	54
Y354Q-Alr	56 ± 6	51

[a] Results were measured at a substrate concentration of 40 mM L-Ser. Data represent the means of four independent assays, each done in duplicate, $\pm$ the standard error of mean (S.E.M.).

The Michaelis – Menten (K_M) constants for Alr and Y354N-Alr acting as both alanine and serine racemases were determined by using the assay described, with some modifications to ensure steady state conditions. The results (Table 2) confirm the efficacy of Y354N-Alr as a racemase with dual specificity for L-Ala and L-Ser. Introduction of the Y354N mutation had a negligible effect on the turnover number for L-Ala racemisation, although it

Table 2. *Steady state parameters for racemisation of L-Ala and L-Ser by Alr and Y354N-Alr.*

Enzyme	L-Ala $\rightarrow$D-Ala			L-Ser $\rightarrow$D-Ser		
	K_M [mM]	k_{cat} [min^{-1}]	k_{cat}/K_M [min^{-1} mM^{-1}]	K_M [mM]	k_{cat} [min^{-1}]	k_{cat}/K_M [min^{-1} mM^{-1}]
Alr[a]	4.4 ± 0.2	$1.1 \pm 0.1 \times 10^5$	2.5×10^4	nd[a]	nd[a]	nd[a]
Alr[b]	2.3 ± 0.1	$5.1 \pm 0.2 \times 10^4$	2.2×10^4	110 ± 10	$9.2 \pm 0.9 \times 10^2$	8.4
Y354N-Alr[b]	9.4 ± 0.3	$4.8 \pm 0.2 \times 10^4$	5.1×10^3	75 ± 6	$3.9 \pm 0.1 \times 10^4$	520

[a] Previously reported;[8] nd = not determined. [b] Results from this study. Values are listed as the mean $\pm$ S.E.M. ($n = 3$), and each independent value represents the mean of duplicate assays.

  1439-4227/02/03/08 $ 20.00+.50/0 ChemBioChem **2002**, No. 8

resulted in a four-fold increase in K_M, possibly attributable to cooperative effects on substrate binding in an altered active site environment. A considerably more pronounced effect was observed when L-Ser was the substrate, with Y354N-Alr displaying a racemase activity (as measured by $k_{cat.}/K_M$) 62-fold higher than the wild-type enzyme and a turnover number for L-Ser comparable to that of wild-type Alr acting on L-Ala (Table 2). The ratio of the $k_{cat.}/K_M$ values for wild-type Alr suggests a preference for L-Ala as a substrate over L-Ser by a factor of 2600-fold; this is relaxed to favouring L-Ala only ten-fold in Y354N-Alr. Interestingly, this preference has been relaxed even further in VanT, to the extent that activity against L-Ala is six-fold lower than against L-Ser.[9] Significantly, our data demonstrate that the broadening of specificity observed in Y354N-Alr is due to a newly introduced ability to turn over L-Ser, rather than loss of activity against L-Ala; the K_M of this enzyme for L-Ser is only slightly improved over that of wild-type Alr.

Given that Tyr354 plays no direct role in catalysis, initially it appeared counterintuitive that its mutation to asparagine should lead to a 42-fold increase in the $k_{cat.}$ for L-Ser (Table 2). However, by considering the catalytic mechanism of Alr alongside structural data, the increased serine racemase activity observed for Y354N-Alr can be rationalised. The active site architecture of Alr in the PLP – L-Ala-P external aldimine form suggests that neither Tyr354 nor any other active site residue physically blocks the binding of amino acid substrates other than alanine.[4] This is consistent with our observation that the Y354N mutation had a relatively minor effect on the K_M for L-Ser and with the fact that D,L-cycloserine is a known inhibitor of Alr. Mechanistically, the next step after formation of the external aldimine involves abstraction of the α proton of the substrate by either Lys39 or Tyr265′ (depending on which substrate enantiomer is bound) to generate a planar carbanion intermediate that is stabilised by delocalisation of the negative charge over the pyridoxal ring. Critically, formation of this intermediate requires rotation of the substrate side chain such that it becomes oriented in the plane of the PLP ring, and points directly towards the side chain of Tyr354 (Figure 1 A); simple space-filling models suggest that the presence of any side chain larger than the methyl group of alanine would result in significant steric hindrance of this process.

We therefore propose that the specificity of Alr for alanine is determined largely by the presence of Tyr354 in a subtle blocking role, by preventing intermediate formation and subsequent catalytic turnover of larger substrates. Consistent with this model, the data presented here show that the principal effect of the Y354N mutation is on turnover, rather than K_M.

A number of corollaries can be drawn from this observation. It seems probable that the PLP phosphate is held rigidly in place by the five charged-neutral hydrogen bonds it forms with the side-chain hydroxy group of Tyr43, the side-chain hydroxy group and backbone amide NH groups of Ser204, and the backbone amide NH groups of Gly221 and Ile222. It follows that the primary role of the Tyr354 – PLP hydrogen bond is not immobilisation of the cofactor as previously supposed, but rather anchoring the side chain of Tyr354 in its specificity-determining orientation. That Y354N-Alr was the most active mutant analysed here may imply an additional role for the asparagine side chain in stabilising a transition state or intermediate in serine racemisation through provision of additional hydrogen bonds to the serine side chain hydroxy group, though this is less certain at this stage. Finally, the data presented here provide experimental support for one avenue by which resistance to vancomycin might be acquired in susceptible bacteria. At the genetic level, a single base substitution (*TAY* → *AAY*) is sufficient to confer substantial serine racemase activity on Alr without seriously compromising alanine racemase activity; this in turn suggests that Y354N-Alr represents a plausible intermediate in the evolution of an ancestral VanT-like enzyme. The turnover number of Y354N-Alr for L-Ser closely matches that of wild-type Alr for L-Ala so would presumably be sufficient to allow the host cell to synthesise physiologically relevant levels of D-Ser, albeit inefficiently due to the high K_M. Having achieved catalytic turnover of L-Ser, the next step in the evolution of VanT would logically involve improvement in the recognition of serine, not necessarily at the expense of alanine racemisation; it is perhaps relevant in this regard that VanT shows significant racemase activity against alanine as well as serine.[9]

In summary, we have identified a novel and key role of an active site residue in controlling the substrate specificity of alanine racemase by sterically blocking turnover, rather than binding, of alternate substrates and, in addition, we have provided experimental support for the evolutionary relationship between alanine racemase and VanT.

Experimental Section

Mutagenesis and protein expression

The *alr* gene was amplified by PCR from *G. stearothermothilus* genomic DNA and cloned into pUC19, with addition of a C-terminal $(His)_6$ tag. Mutagenesis of Tyr354 was performed by the QuikChange method (Stratagene, La Jolla, CA). Protein over-expression in mid-log phase *E. coli* XL1-Blue (Stratagene) cultures was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to 100 μM. The $(His)_6$-tagged proteins were purified to apparent homogeneity, as judged by SDS-PAGE, by metal chelate chromatography on a HiTrap column (Amersham Pharmacia Biotech) charged with Ni^{2+}. Use of the $(His)_6$ tag precluded copurification of the *E. coli* alanine racemase.

Serine racemase assays

Racemase assays were carried out essentially as described previously.[10] Each reaction was performed in 100 mM CHES (2-(*N*-cyclohexylamino)ethanesulfonic acid, pH 9.1), 40 mM L-Ser and 60 – 7000 ng enzyme. After 20 min incubation at 37 °C, the reaction was terminated by boiling for 5 min. The amount of D-Ser produced was determined by adding a 10-μL sample aliquot to colour development buffer (90 μL final concentrations: sodium phosphate buffer (pH 7.0), 100 mM; D-amino acid oxidase (DAAO), 3.6 Units ml^{-1}; horseradish peroxidase, 20 Units ml^{-1}; *o*-phenylenediamine (OPD), 2 mg ml^{-1}). This coupled assay allowed quantification of H_2O_2 (produced by DAAO acting specifically on D-Ser) by its peroxidase-catalysed reaction with the chromogenic substrate OPD. Colour development was for 45 – 60 min at 37 °C, after which the reaction was stopped by addition of HCl (3 M, 100 μL). Signals were detected at 492 nm and quantified by interpolation on a D-Ser standard curve, linear over the range 0 – 40 nmol.

Steady-state kinetics

Assays were as described above, except the incubation time for racemisation was shortened to 6 min to ensure that final product concentration remained less than 10% of the initial substrate concentration. Enzyme concentrations used in the analyses were: Alr with L-Ala, 2.0 nM; Alr with L-Ser, 2.5 μM; Y354N-Alr with L-Ala, 2.3 nM; Y354N-Alr with L-Ser, 40 nM. Products were quantified with D-Ala and D-Ser standard curves, which were constructed for each independent assay. Substrate ranges assayed were approximately 10% – 200% the K_M value and analyses routinely included 5 data points.

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