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Correlation between catalytic activity and monomer–dimer equilibrium of bacterial alanine racemases

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From the reaction mechanism and crystal structure analysis, a bacterial alanine racemase is believed to work as a homodimer with a substrate, L-alanine or D-alanine. We analysed oligomerization states of seven alanine racemases, biosynthetic and catabolic, from *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *P. putida* and *P. fluorescens*, with three different methods, gel filtration chromatography, native PAGE and analytical ultracentrifugation. All alanine racemases were proved to be in a dynamic equilibrium between monomeric and dimeric form with every methods used in this study. In both biosynthetic and catabolic alanine racemases, association constants for dimerization were high for the enzymes with high V_{max} values. The enzymes with low V_{max} values gave the low association constants. We proposed that alanine racemases are classified into two types; the enzymes with low and high-equilibrium association constants for dimerization.

Keywords: alanine racemase/analytical ultracentrifugation/association constant/dynamic equilibrium/gel filtration chromatography/native PAGE.

Abbreviations: $dadX_{Pa}$, *Pseudomonas aeruginosa dadX*; alr_{Pa}, *Pseudomonas aeruginosa alr*.

Gram-positive and Gram-negative bacteria require the D-isomer of alanine as an essential building block in the synthesis of the peptidoglycan layer of cell walls and spore cortex peptidoglycan (*1*, *2*). D-Alanine is formed from L-alanine by a specific enzyme, alanine racemase (EC 5.1.1.1). Alanine racemase belongs to the fold-type III group of pyridoxal 5′-phosphate (PLP)-dependent enzyme, catalysing the interconversion of L-alanine and D-alanine. In some bacteria, such as *Escherichia coli* and *Salmonella*, there are two independent alanine racemase genes; biosynthetic (*alr*) and catabolic (*dadX*). The *alr* gene is constitutively expressed in the cells for peptidoglycan biosynthesis, while *dadX* gene expression is induced when cells are grown in high concentrations of L- or D-alanine. The enzyme encoded by the *dadX* gene catalyses the conversion of L-alanine to the directly oxidizable D-alanine, which is used as carbon and energy sources (*3*). In some other bacteria, such as *Bacillus pseudofirmus* OF4, whose genome sequence has been deposited recently, there is only one gene encoding catabolic alanine racemase (*4*). Little is known about advantages and disadvantages of having two enzymes or about the interaction between the enzymes (*5*).

Crystal structure analysis has demonstrated that almost all of the alanine racemases are dimeric forms in *Geobacillus stearothermophilus* (*6*), *Pseudomonas aeruginosa* (*7*), *Streptomyces lavendulae* (*8*), *Mycobacterium tuberculosis* (*9*), *S. pneumoniae* (*10*) and *B. anthracis* (*11*). Moreover, intermolecular-complementation analysis between two defective mutants of *alr* in *P. aeruginosa* and *E. coli* clearly indicates that these enzymes function exclusively as homodimers (*5*).

Mutagenic, structural and modeling analyses indicate that Lys39 and Tyr265′ of *G. stearothermophilus* alanine racemase are residues involving in proton transfer in the racemase reaction (*12*). The residues Lys39 and Tyr265′ are from two identical polypeptides. These two residues are conserved well in other alanine racemases.

Although gel-filtration chromatography analyses of several purified alanine racemases show monomeric forms, such as those from *Salmonella* (*13*), crayfish (*14*), *Thermus thermophilus* (*15*) and marine gastropod *Cellana grata* (*16*), Yokoigawa *et al.* (*17*) reported that *Shigella* alanine racemases are in monomer–dimer equilibrium. We demonstrated that *P. fluorescens* alanine racemases exist in monomer–dimer equilibrium in the cells (*18*). In this study, seven alanine racemases, both biosynthetic and catabolic, were subjected to analyse dimerization states using three different methods, gel filtration analysis, native PAGE and analytical ultracentrifugation, and we elucidated the relationship of oligomerization efficacies and catalytic activities of these alanine racemases.

Materials and Methods

Bacterial strains, plasmids and materials

Escherichia coli DH12S (Invitrogen, USA) and BL21(DE3) (Novagen, USA) were used for cloning and gene expression, respectively. MB2795 (*alr::frt dad::frt*) is a D-alanine auxotroph (*19*). Bacteria were routinely grown at 30°C or 37°C in LB medium (1% bacto tryptone, 0.5% yeast extract and 0.5% NaCl).

D-Alanine was added to LB medium at 0.5 mM when necessary. Ampicillin was added at 100 μg ml^{-1}. Two expression vectors, pTrc99A (*20*) and pET-22b (+) (Novagen), and two cloning vectors, pUC18 and p3T (Mo Bi Tec, USA), were used (Table I). D-Amino acid oxidase (EC 1.4.3.3) from porcine kidney (3.4 U/mg) was obtained from Sigma (St Louis, USA). All other chemicals were of analytical grade.

Cloning of alanine racemase genes

Chromosomal DNAs of *P. aeruginosa* PAO1, and *P. putida* KT2440 were isolated by AquaPure Genomic DNA Isolation Kit (BIO-RAD, USA) according to the manufacturer's instruction. Isolated DNAs were used for amplifying fragments of *P. aeruginosa alr* (alr_{Pa}), *P. aeruginosa dadX* ($dadX_{Pa}$) and *P. putida dadX* ($dadX_{Pp}$). PCR was carried out with *Pfu* Turbo (Stratagene, USA) at 30 cycles of 95°C for 45 s, 55°C for 1 min and 72°C for 2 min using respective primers (Table II). Amplified fragments, alr_{Pa}, $dadX_{Pa}$ and $dadX_{Pp}$, were separated on 0.8% agarose gel electrophoresis, purified by E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA) and TA cloned into *Xcm*I-digested p3T, to construct p3TPaalr, p3TdadPa and p3TdadKT, respectively. Nucleotide sequences of cloned *alr* and *dadX* gene fragments confirmed no mutations. Plasmids, p3TPaalr, p3TdadPa and p3TdadKT, were digested with *Nde*I and *Hin*dIII, and cloned into pET-22b(+), to generate pETPaalr1, pETPadadX1 and pETKTdadX1, respectively.

The *alr* and *dadX* gene fragments on pETPaalr1, pETPadadX1 and pETKTdadX1, were isolated by *Xba*I and *Hin*dIII cleavage, and recloned into the same restriction sites of pTrc99A. The resultant plasmids were named pPaalr2, pPadadX2 and pKTdadX2, respectively. Each gene contains a ribosomal binding site of pET-22b(+) vector.

Phylogenetic analysis

The translated amino acid sequences of seven alanine racemases were aligned by using CLUSTALW (*21*). A phylogenetic tree was constructed with Mega 4.1 program package (*22*).

Gel filtration chromatography

Pre-cultured MB2795 cells with alanine racemase gene cloned on pTrc99A were incubated at 30°C in fresh LB medium. The expression of gene was induced by adding 1 mM isopropyl thio-β-D-galactoside (IPTG). After incubation for 4 h, cells were harvested, resuspended in 50 mM potassium phosphate buffer pH 7.2 containing 0.5 mM EDTA, 20 μM PLP and 0.01% (v/v) 2-mercaptoethanol, and sonicated for 30 s several times until the solution became clear. After removal of cell debris, cell lysate was loaded on a HiLoad Superdex 200 prep grade (1.6 × 60 cm, Amersham Biosciences, USA) equilibrated with 20 mM potassium phosphate buffer pH 7.2 containing 150 mM NaCl, 0.5 mM EDTA, 20 μM PLP and 0.01% (v/v) 2-mercaptoethanol at a flow rate of 1 ml min^{-1}, and fractionated in 1-ml fractions. Cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66 kDa) and alcohol dehydrogenase (150 kDa) were used as molecular mass standards (Sigma).

Purification of racemases

Pre-cultured BL21(DE3) cells with alanine racemase gene cloned on pET-22b(+) were inoculated into 40 ml fresh LB culture, and incubated at 28°C. When cell density at OD_{600} reached 0.6–1.0, IPTG was added at the final concentration of 1 mM, and incubation was continued for 5 or 8 h. Cells were harvested, re-suspended in 4 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl and 10 mM imidazole), and sonicated for 30 s several times until the solution became clear. Sonicated cells were centrifuged to remove cell debris, and the supernatant was mixed with 1 ml 50% Ni-NTA slurry (QIAGEN, USA) and incubated gently at 4°C for 1 h. Unbound proteins to the Ni-NTA were washed three times by 1 ml of wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl and 20 mM imidazole). Bound proteins were eluted four times by 0.5 ml of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl and 250 mM imidazole). The purity of the enzyme was checked by SDS–PAGE.

Table I. Plasmids used in this study.

Plasmid	Genotype	References
pET-22b(+)	T7 expression vector, Ap^r	Novagen
pTrc99A	*tac* expression vector, AP^r	*(20)*
P3T	TA cloning vector, Ap^r	MO Bi Tec
pETEcalr	*Escherichia coli alr* on pET-22b(+)	*(31)*
pETStalr	*Salmonella alr* on pET-22b(+)	*(31)*
pETPaalr1	*Pseudomonas aeruginosa alr* on pET-22b(+)	This study
pETPadadX1	*Pseudomonas aeruginosa dadX* on pET-22b(+)	This study
pETKTdadX1	*Pseudomonas putida dadX* on pET-22b(+)	This study
pTMdadX1	*Pseudomonas fluorescence* TM5-2 *dadX* on pET-22b(+)	*(18)*
pLRBdadX1	*Pseudomonas fluorescence* LRB3W1 *dadX* on pET-22b(+)	*(18)*
pEcalr	*E. coli alr* on pTrc99A	*(31)*
pStalr	*Salmonella alr* on pTrc99A	*(31)*
pPaalr2	*Pseudomonas aeruginosa alr* on pTrc99A	This study
pPadadX2	*Pseudomonas aeruginosa dadX* on pTrc99A	This study
pKTdadX2	*Pseudomonas putida dadX* on pTrc99A	This study
pTMdadX2	*Pseudomonas fluorescence* TM5-2 *dadX* on pTrc99A	*(18)*
pLRBdadX2	*Pseudomonas fluorescence* LRB3W1 *dadX* on pTrc99A	*(18)*

Table II. Primers used in this study.

Primer	Sequence [5′–3′]	Description
PaalrA2	CATATGCGTCCCCTCGTTGCC	PAO1 *alr*
PaalrB2	**AAGCTT**TCAGTGGTGGTGGTGGTGGTGGCTGTAGCGTCGCGG	PAO1 *alr*
PadadA1	CATATGCGCCCCGCCCGTGCC	PAO1 *dadX*
PadadB1	**AAGCTT**TCAGTGGTGGTGGTGGTGAGCCCCGGAATAGACGCG	PAO1 *dadX*
KTdadA1	CATATGCGTCCCGCCCGCGCC	KT2440 *dadX*
KTdadB1	**AAGCTT**TCAGTGGTGGTGGTGGTGTTCGCCGATGTAGTCCCG	KT2440 *dadX*
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Universal primer
RV-M	GAGCGGATAACAATTTCACACAGG	Universal primer

The underlined and bold sequences are *Nde*I and *Hin*dIII restriction sites, respectively.

Enzyme assay

Micro-plate assay for the alanine racemase activity was carried out. The standard racemization mixture contained 50 mM potassium phosphate buffer pH 7.4, 10 μM PLP, 50 mM L-alanine and 10 μl of fractionated cell lysate in a final volume of 200 μl. After 10-min incubation at 30°C, the reaction was terminated by adding 25 μl of 2 N HCl. Following kept on ice for 2 min, the reaction mixture was spun down at 15,000 rpm for 10 min at 4°C. An aliquot (180 μl) of supernatant was mixed with 20 μl of 2 N NaOH for neutralization. The amount of D-alanine was measured with D-amino acid oxidase at 37°C for 20 min (*18*). D-Amino acid oxidase reaction mixture contained 200 mM Tris–HCl pH 8.0, 0.1 mg/ml 4-aminoantipyrine, 0.1 mg/ml TOOS, 2 U peroxidase and 0.1 U D-amino acid oxidase (Sigma) in a final volume of 200 μl. The absorbance at 550 nm was measured by Model 550 Microplate Reader (Bio-Rad). The relative enzyme activity in each fraction was calculated based on the absorbance at 550 nm.

Kinetic parameters of alanine racemase activity at 30°C were determined by measuring the amounts of D- and L-alanines by HPLC (*18*).

Protein concentration of the purified enzyme was determined by BCA Protein Assay Reagent Kit (Pierce, USA). Bovine serum albumin was used as a protein standard.

Native PAGE analysis

Standard proteins and purified alanine racemases were run on different concentrations of acrylamide without SDS: 5, 7.5, 10 and 12.5% at constant current 15 mA. To determine the relative mobility (R_f) of a protein, the migration distance of each protein (M_p) was divided by the distance of tracking dye migration (M_d). $100 \times \log(R_f \times 100)$ values was plotted against the percent gel concentration (T%), and the slope of each protein was determined by linear regression. Log of negative slope was plotted against log of molecular mass to draw a standard curve. α-Lactalbumin, carbonic anhydrase, albumin chicken egg and BSA were used as standards. In the case of BSA, two forms, monomer and dimer, exist. Molecular masses are 14.2, 29, 45, 66 kDa for monomer and 132 kDa for dimer, respectively.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were done in a Beckman XL-A analytical ultracentrifuge with a 60Ti rotor using Epon charcoal filled six-channel centrepieces (*23*). Rotor speed was between 12,000 and 22,000 rpm for biosynthetic alanine racemases, 14,000 and 24,000 rpm for catabolic alanine racemases at 20°C. Purified proteins were prepared in diluted buffer (50 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, 0.1 M NaCl, 10 μM PLP, 0.01% 2--mercaptoethanol). Three different concentrations of proteins, 0.2, 0.3 and 0.4 at 280 nm, were prepared. Sample of 100 μl and 110-μl diluted buffer as blank were loaded in well.

The absorbance versus radius distribution was recorded at 280 nm. The association constant in terms of absorbance (K_{abs}) was measured. The association constant was converted to units of M^{-1} using an extinction coefficient $E^{1\%}{}_{280}$ of 20.4, and assuming a value of twice that for the dimer (*24*),

$$K_{conc} = K_{abs} \times \varepsilon l/2$$

where K_{conc} = the association constant in M^{-1}; K_{abs} = the association constant in terms of absorbance (estimated directly from a best-fit curve of a monomer–dimer self-association system); ε = the extinction coefficient in l/mol-cm (= a monomer molecular weight × OD_{280} at 1 mg/ml); l = the pathlength in cm (1.2 for a 12-mm centerpiece); OD_{280} at 1 mg/ml = OD_{280}/protein concentration.

Results

Phylogenetic analysis of seven alanine racemases

Seven alanine racemases were aligned based on translated amino acid sequences using ClustalW. The PLP binding site residue Lys39 and active residue Tyr265 in *G. stearothermophilus* alanine racemase (*12*) are conserved in all alanine racemases. Similarities of these seven alanine racemases are between 42 and 91%. There are two distinct groups: one is composed of biosynthetic alanine racemases and the other is catabolic alanine racemases including alanine racemases from two *P. fluorescens* strains (Fig. 1).

Gel filtration chromatography

Although the purified alanine racemases are normally subjected to the gel-filtration chromatography for oligomerization analysis, we loaded cell lysate of MB2795 strain expressing one of the alanine racemases on the gel-filtration column, and alanine racemase activity in each fraction was measured in this study. When cell lysate of MB2795/pTrc99A grown in the presence of D-alanine was fractionated, no alanine racemase activity was detected in any fractions. Alanine racemase activities of $DadX_{Pa}$ and $DadX_{Pp}$ in cells of MB2795/pPadadX2 and MB2795/pKTdadX2 were observed in two peak fractions. One peak fraction corresponded to the molecular mass of dimeric form (~80 kDa, D in Fig. 2D and E), and the other peak fraction matched to that of monomeric form (~40 kDa, M in Fig 2D and E). The same distributions of racemase activities are observed in *P. fluorescens* LRB3W1 ($DadX_{LRB}$) and *P. fluorescens* TM5-2 ($DadX_{TM}$) (*18*). In the case of $DadX_{Pp}$, the molecular mass of the peak fraction was slightly larger than that of dimeric form (Fig. 2E). Although we do not have a clear explanation for this phenomenon, the equilibrium of $DadX_{Pp}$ was shifted most to dimeric form among alanine racemases, even in the other two methods, native PAGE analysis and analytical ultracentrifugation analysis, as shown in the later sections.

On the contrary, alanine racemase activities of Alr_{Ec} and Alr_{Pa} in cells of MB2795/pEcalr and MB2795/pPaalr2 were detected only in one peak fraction, which did not correspond to either monomeric or dimeric form (Fig. 2B and C). The peak fraction matched to the intermediate molecular mass between monomer and dimer. Alanine racemase activities of Alr_{St} in cells of MB2795/pStalr were detected in two peak fractions. One peak fraction corresponded to the molecular mass of monomer form (~40 kDa, M in Fig. 2A), and the other peak fraction corresponded

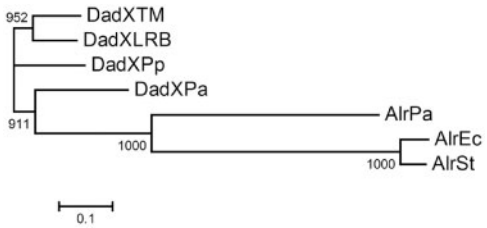

Fig. 1 Phylogenetic tree of seven alanine racemases. Amino acid sequences of three biosynthetic alanine racemases from *E. coli* K-12 (AlrEc), *S. typhimurium* (AlrSt) and *P. aeruginosa* PAO1 (AlrPa), and four catabolic alanine racemases from *P. aeruginosa* PAO1 (DadXPa), *P. putida* KT2440 (DadXPp), *P. fluorescens* LRB3W1 (DadXLRB) and *P. fluorescens* TM5-2 (DadXTM) were aligned with ClustalW. The tree was constructed by using the NJ method and drawn by Mega 4.1. The bootstrap values are shown at branching points. The scale bar indicates 0.1 amino acid substitutions per site.

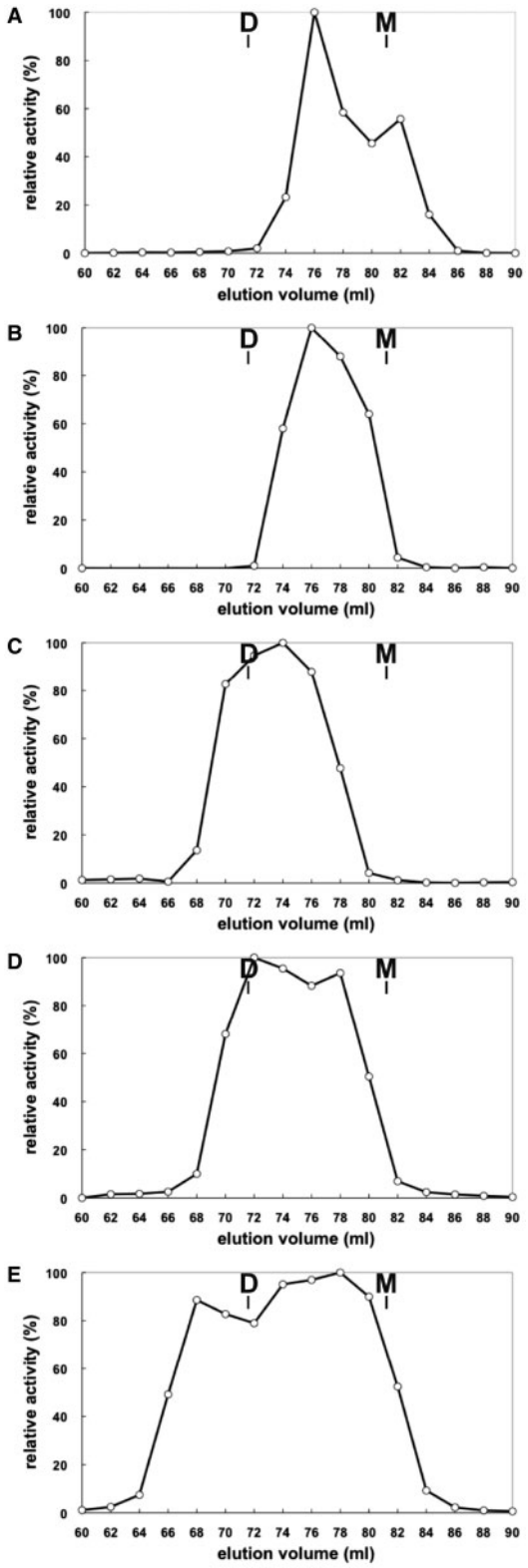

Fig. 2 Gel-filtration chromatograms of cell lysate. Cell lysate of *E. coli* MB2795 containing pETStalr (A), pETEcalr (B), pETPaalr1 (C), pETPadadX1 (D) and pETKTdadX1 (E), were loaded on the HiLoad Superdex 200 column (1.6 × 60 cm) and fractionated. Alanine racemase activity in each fraction was assayed with L-alanine by D-amino acid oxidase method, and the relative enzyme activity in each fraction was calculated based on the absorbance at 550 nm measured by Model 550 Microplate Reader (Bio-Rad). The relative activity was plotted as percentage to the maximum activity. D and M indicate the fractions corresponding to dimers and monomers of the enzymes.

to the intermediate molecular mass between monomer and dimer.

All these data indicated that alanine racemases are in a dynamic equilibrium of monomeric and dimeric forms in the cells, and monomer–dimer transition in biosynthetic alanine racemase is faster than in catabolic alanine racemases. Especially the equilibrium of Alr_{St} is shifted to monomeric form.

Purification of racemases

Seven alanine racemases with C-terminal histidines were overproduced in BL21(DE3) cells and purified to electrophoretic homogeneity (Fig. 3). Molecular masses of purified alanine racemases were all ~40 kDa. Kinetic parameters of purified alanine racemases were determined (Table III). The values of His-tagged alanine racemases were in the same ranges of enzymes without His-tags (*13*, *25–27*). While the K_m values of all the alanine racemases used in this study were not different in ~10 mM, the V_{max} values of catabolic alanine racemases were much higher than those of biosynthetic alanine racemases, especially in the case of L-alanine to D-alanine (Table III).

Native PAGE analysis

In native PAGE gel, proteins migrate based on both shape and charge. Four standard proteins were run on different concentration of acrylamide without SDS (Supplementary Fig. S1A). Values of $100 \times \log(R_f \times 100)$ were plotted against the percent gel concentration T%, and the slope of each protein was determined by linear regression (Supplementary Fig. S1B). Log of negative slope was plotted against log of molecular mass to draw a standard curve (Supplementary Fig. S1C).

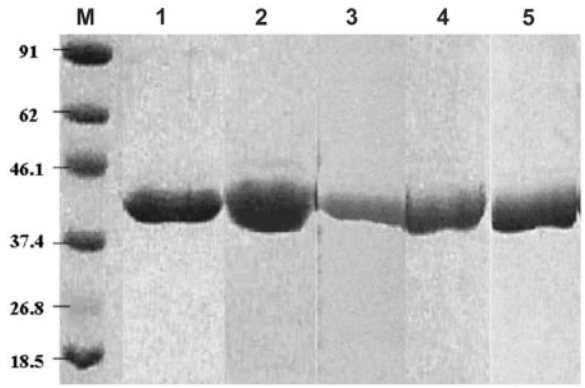

Fig. 3 SDS–PAGE profile of purified enzymes. Alanine racemases with six histidines on C-termini were purified from BL21(DE3) cells expressing alr_{St} (lane 1), alr_{Ec} (lane 2), alr_{Pa} (lane 3), $dadX_{Pa}$ (lane 4) and $dadX_{Pp}$ (lane 5). Cells of BL21(DE3) containing either of plasmid, pETEcalr, pETStalr, pETPaalr1, pETPadadX1 or pETKTdadX1, were incubated at 37°C for 5 or 8 h after 1 mM IPTG addition, harvested and re-suspended in 50 mM sodium phosphate buffer, pH 8.0 containing 300 mM NaCl and 10 mM imidazole. Cell lysate were prepared by sonication, and His-tagged enzymes were purified with Ni-NTA agarose (QIAGEN) according to the manufacturer's instruction. Purified enzymes were analysed on 12.5% SDS–PAGE. Molecular weight standards (lane M) were shown on the left in kilo Dalton.

Table III. Molecular weights, kinetic parameters and association constants of alanine racemases.

			L- → D-Ala		D- → L-Ala		
Enzyme	**Predicted molecular weight of monomer[a]**	**Molecular weight from native-PAGE**	**K_m (mM)**	**V_{max} (U/mg)**	**K_m (mM)**	**V_{max} (U/mg)**	**K_{conc} (M^{-1})**
Alr_{St}	39,075 (40,127)	43.7 ± 3.9	9.3[b]	11[b]	6.1[b]	6.5[b]	1.3×10^5
Alr_{Ec}	39,152 (40,204)	66.3 ± 5.7	17.4[b]	13[b]	6.9[b]	6.7[b]	2.1×10^5
Alr_{Pa}	38,337 (39,160)	63.8 ± 6.4	4.1	2.1×10^2	5.6	1.4×10^2	1.2×10^6
$DadX_{Pa}$	39,068 (39,891)	65.6 ± 1.4	9.8	1.1×10^3	4.2	3.7×10^2	7.7×10^5
$DadX_{Pp}$	38,765 (39,588)	78.8 ± 1.8	7.4	1.5×10^3	7.3	7.7×10^2	1.6×10^6
$DadX_{LRB}$	38,978 (39,800)	62.4 ± 1.3	18.4[c]	1.4×10^{3c}	8.7[c]	5.0×10^{2c}	5.9×10^5
$DadX_{TM}$	38,883 (39,706)	66.2 ± 1.4	8.1[c]	1.3×10^{3c}	4.7[c]	4.7×10^{2c}	1.1×10^6

[a]Values in parentheses are molecular masses of His-tag fusion enzymes at C-terminal ends. [b]Values are cited from (*31*). [c]Values are cited from (*18*).

Seven purified alanine racemases were separated on native PAGE with 7.5, 10 and 12.5% acrylamide without SDS (Supplementary Fig. S2). The R_f of each enzyme was calculated. The slope of each protein was determined (Supplementary Fig. S3), and molecular mass of each alanine racemase was calculated (Table III). The calculated molecular mass of Alr_{St} was 43.7 kDa, which is close to the predicted molecular mass of monomeric form. On the contrary, the calculated molecular mass of $DadX_{Pp}$ was 78.8 kDa, which corresponds to the predicted molecular mass of dimeric form. The calculated molecular masses of other alanine racemases, Alr_{Ec}, Alr_{Pa}, $DadX_{Pa}$, $DadX_{LRB}$ and $DadX_{TM}$, were the intermediate between monomeric and dimeric forms. These data again indicated that alanine racemases are in a dynamic equilibrium of monomeric and dimeric forms. Monomer–dimer transition is faster in Alr_{St} and slower in $DadX_{Pp}$.

Analytical ultracentrifugation

In order to understand the dynamic equilibrium of monomeric and dimeric forms of alanine racemases, sedimentation equilibrium experiments were done to estimate the equilibrium association constant (K_{conc}) (Supplementary Figs S4–S10) (*23*). Equilibrium association constants (K_{conc}) for dimerization of alanine racemases were calculated from the association constant in terms of absorbance (K_{abs}) (Supplementary Table SI). Association constant of $DadX_{Pp}$ was largest and that of Alr_{St} was smallest (Table III), which was good agreement with data of gel-filtration chromatography and native PAGE analysis. The maximum velocity V_{max} of alanine racemase was plotted against the association constant (Fig. 4). Biosynthetic alanine racemases with higher V_{max} values, in both L-alanine to D-alanine (Fig. 4A) and D-alanine to L-alanine (Fig. 4C), gave higher association constants for dimerization. Catabolic alanine racemases had basically the similar tendency. Enzymes with higher maximum velocities exhibited higher association constants for dimerization (Fig. 4B and D).

Discussion

The bacterial alanine racemase is shown to work as a homodimer in the enzyme reaction with substrates, L-alanine and D-alanine, based on the proposed reaction mechanism and crystal structure analysis. However, many of alanine racemases have been shown to be monomeric mainly by the analysis of gel-filtration chromatography (*13–16*). In this study, we used seven alanine racemases, biosynthetic and catobolic and elucidated dimerization states of these enzymes using different methods, gel-filtration chromatography, native PAGE and analytical ultracentrifugation. We applied the whole cell lysate on gel-filtration chromatography, so we could detect even small portions of monomeric or dimeric forms of alanine racemases. We could calculate the association constant for dimerization using analytical ultracentrifugation. All three methods indicated that alanine racemases used in this study are in the dynamic equilibrium between monomeric and dimeric forms.

Alanine racemases with higher specific activities showed higher association constants for dimerization. Since the affinities of alanine racemases to the substrate, L-alanine or D-alanine, were not different, we hypothesized that the slow rates of dimmer formation of Alr_{St} and Alr_{Ec} in the presence of substrate results in the low-specific activities. We obtained an *alr*$_{St}$ mutant, which increased the specific activity >10-fold compred to the wild-type. The association constant of the mutant Alr_{St} for dimerization increased by a factor of 40 (Ju, unpublised data). This supports the above hypothesis. When we added L-alanine in the buffer of gel-filtration chromatography of Alr_{St} and Alr_{Ec} for expecting that the substrate binds to the enzyme, and the enzyme is kept as the dimeric form, we could not detect the dimeric forms (data not shown). Yokoigawa *et al*. (*17*) analysed *Shigella* alanine racemases on gel filtration in the presence of D-cycloserine. In the absence of D-cycloserine, the enzymes were eluted as a monomeric form and in the presence of D-cycloserine, the apparent molecular masses of the enzymes corresponded to a dimeric form. D-Cycloserine acts as a suicide inhibitor of alanine racemase, and the mechanism of alanine racemase inactivation involves an initial transamination step followed by tautomerization to form a stable aromatic adduct (*28*). This could explain that alanine racemases with D-cycloserine are in the dimeric forms. In conclusion, alanine racemase classification into monomeric and dimeric enzymes should be reconsidered. We propose that alanine racemases should be classified as enzymes with low- or

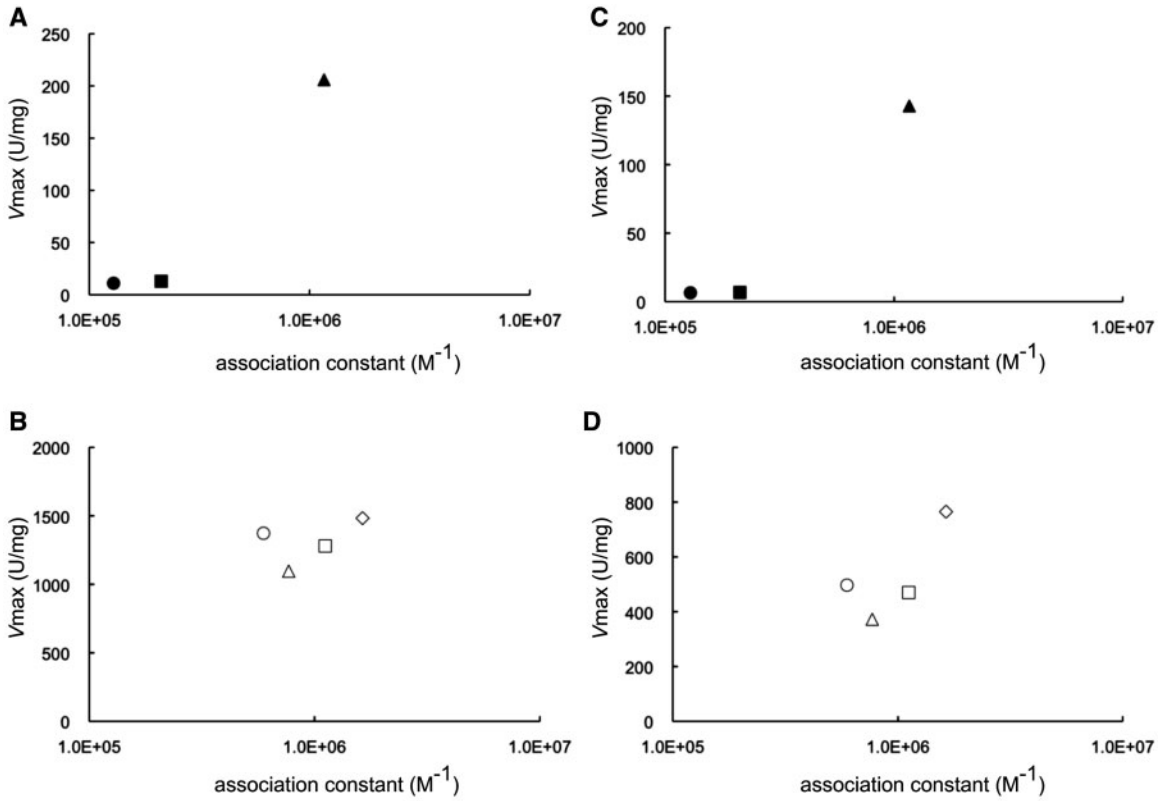

Fig. 4 Relationship between specific activity and the association constant. The association constants of dimers were plotted against the specific activities, V_{max}, of L-alanine to D-alanine (A and B) and of D-alanine to L-alanine (C and D). Purified biosynthetic alanine racemases (A and C) and catabolic alanine racemases (B and D) were used. Closed circles, Alr_{St}; closed squares, Alr_{Ec}; closed triangles, Alr_{Pa}; open triangles, $DadX_{Pa}$; open diamonds, $DadX_{Pp}$; open circles, $DadX_{LRB}$; open squares, $DadX_{KT}$.

high-equilibrium association constants for dimerization.

In this study, we used seven alanine racemases from gamma proteobacteria, and demonstrated that the enzymes with high-specific activities have high association constants for dimerization. The V_{max} values of gram-positive bacillus strains, such as *G. stearothermophilus* (*29*), *B. psychrosaccharolyticus* (*30*) and *B. pseudofirmus* (*4*), are high, >1,000 U/mg. Since the reaction mechanism of alanine racemases should be common in all bacteria, the equilibrium association constants for dimerization could be high in the alanine racemases of these gram-positive bacteria. We need to evaluate the association constants of these racemases at the next step in the future experiment.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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