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Isozymic nature of spore coat-associated alanine racemase of *Bacillus subtilis*

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Summary. Spore coat-associated alanine racemase of *Bacillus subtilis*, which converts L-alanine to D-alanine, that is, the germinant to the competitive inhibitor, to regulate spore germination for survival of the organism under unfavorable growth conditions, was examined. The dormant spores, L-alanine-initiated germination of which is inhibited by diphenylamine, were used to characterize the enzyme in the native form because of its unextractablility from dormant spores. The presence of isozymes, Enz-I and Enz-II with Km for L-alanine of about 20 mM and 50 mM and optimum activity at around 40°C and 65°C, respectively, was proposed. The enzymes were selectively used depending on the L-alanine concentration and the temperature. The pH profiles of the activity (optimun at pH 9.0) and the stability (stable between pH 6–11 at 60°C) were similar, but Enz-II was more heat-stable than Enz-I and the denaturation curve demonstrated a two-domain structure for Enz-II. Sensitivity to D-penicillamine, hydroxylamine and $HgCl_2$ was similar between Enz-I and Enz-II, while that to D-cycloserine, L- and D-aminoethylphosphonic acid, monoiodoacetate and *N*-ethylmaleimide was different; $HgCl_2$ was the most effective inhibitor among these compounds.

Keywords: Amino acid – Alanine racemase – Isozyme – Kinetics – Inhibitors – *Bacillus subtilis* – Spore coat

Introduction

Alanine racemase (EC 5.1.1.1) is a widely distributed enzyme for providing D-alanine as an essential component of cell wall peptidoglycan of most bacteria. D-Alanine is also required for the syntheses of spore cortex peptidoglycan of spore forming bacteria (Tipper and Gauthier, 1972) and lipoteichoic acids of some gram-positive organisms (Fischer, 1990). In eucaryotes, the presence of alanine racemase in a fungus *Tolypocladium niveum* for biosynthesis of cyclosporin A (Hoffmann et al., 1994) and in muscle of crayfish *Procambarus clarkii* for accumulation of D-alanine as an

osmoprotectant (Shirasuna et al., 1999) has been reported. Alanine racemase of *Bacillus stearothermophilus* is one of the most extensively studied alanine racemases for catalytic mechanisms of the active site and other enzymatic properties, DNA and protein sequences, subunit and domain structures, and X-ray crystallographic structure (Inagaki et al., 1986; Tanizawa et al., 1988; Soda and Tanizawa, 1990; Shaw et al., 1997; Watanabe et al., 1999). Vegetative-cell enzymes or overproduced recombinant enzymes from several other bacteria, such as *B. subtilis* (Yonaha et al., 1975; Ferrari et al., 1985), *Salmonella typhimurium* (Wasserman et al., 1983; Galakatos et al., 1986), *Escherichia coli* (Lambert and Neuhaus, 1972; Wild et al., 1985), *Streptococcus faecalis* (Badet and Walsh, 1985), *Listeria monocytogenes* (Thompson et al., 1998) and *Bacillus psychorosaccharolyticus* (Okubo et al., 1999), show essentially similar properties to those of *B. stearothermophilus*.

On the other hand, alanine racemase activity has been also detected in spores of *Bacillus* species and the spores generally show a 3 to 16-fold higher order of racemase activity relative to vegetative cells (Stewart and Halvorson, 1953; Reusch et al., 1982; Preston and Douthit, 1984). The spore enzyme of *B. subtilis* is also active in the dormant state of the spore and is associated with the spore coat (Yasuda et al., 1993). We have investigated the role of spore-alanine racemase on spore germination using diphenylamine as an inhibitor of L-alanine-initiated germination and D-penicillamine as a racemase inhibitor, and proposed that under the unfavorable conditions for vegetative growth such as high population of the spores in limited nutrient, high temperature and high pH of the medium, spore alanine racemase converts the germinant L-alanine to its competitive inhibitor D-alanine and may regulate spore germination for survival of the organism (Yasuda et al., 1993). We have also shown a possibility for the presence of two forms of spore-associated alanine racemase with different optimum temperature for enzymatic activity (Yasuda et al., 1993).

In this report, the presence of an isozyme of the spore-associated alanine racemase of *B. subtilis* is proposed on the basis of the experimental data, such as kinetic parameters, heat and pH stabilities and inhibitors for enzymatic activity.

Materials and methods

Preparation of spores

Bacillus subtilis PCI219 was grown and sporulated on nutrient agar at 37°C. After 5 days spores were harvested and washed repeatedly by centrifugation with distilled water at 4°C.

Spore-associated alanine racemase activity

Alanine racemase activity (L- to D-isomer) was assayed by incubating spores (6×10^8) with various concentrations of L-Ala in a total volume of 1 ml of 35 mM *N*-cyclohexyl-2-aminoethane sulfonic acid (CHES)-NaOH, pH 9.0, containing 1.5 mM diphenylamine, an inhibitor of L-alanine-initiated germination (Yasuda et al., 1978; Yasuda and Tochikubo,

1984) dissolved in a small amount of methanol, at 37°C or 65°C for 10 min, except otherwise stated. A cofactor, pyridoxal 5′-phosphate (PLP), was not used, because the addition of 0.5 mM PLP was not stimulatory in the assay system as reported previously in the case of spore alanine racemase (Stewart and Halvorson, 1953). The racemase reaction was stopped by adding 0.5 ml of 2 N HCl, allowed to stand for 10 min, and neutralized with 0.5 ml of 2 N NaOH. Spores were removed by centrifugation and the supernatant was assayed for D-alanine by the D-amino acid oxidase coupled method according to Nagata et al. (1985). The reaction mixture, containing 200 μl of the supernatant, 200 μl of 200 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic (TAPS)-NaOH, pH 8.3, 3.3 μg FAD and 0.3 unit of D-amino acid oxidase (Boehringer-Mannheim GmbH) was incubated for 15 min at 37°C. After stopping the oxidase reaction by addition of 200 μl of 1 mM 2,4-dinitrophenylhydrazine-1 N HCl, the mixture was allowed to stand for 10 min at 37°C, followed by addition of 1.4 ml of 0.6 N NaOH, and the absorbance at 445 nm was measured. Alanine racemase activity was expressed as initial velocity (v), nmoles D-alanine formed per min per 6×10^8 of spores.

Kinetics

The kinetic parameters, Km and Vmax, were determined at 37°C and 65°C using v – [S] plot and [S]/v – [S] plot (Hanes plot, Hanes, 1932), where [S] was the concentration of L-Ala between 1 to 400 mM. In the presence of various concentrations of inhibitors, such as D-penicillamine, D-cycloserine, L-aminoethyl phosphonic acid (L-Ala-P), D-aminoethyl phosphonic acid (D-Ala-P), hydroxylamine, mercuric chloride ($HgCl_2$), monoiodoacetic acid and *N*-ethylmaleimide, 50% inhibitory concentration of each inhibitor (I_{50}) with 100 mM L-Ala was determined at 37°C and 65°C from the v – [I] plot, where [I] was the concentration of an inhibitor.

Reagents

Good's buffers: CHES-NaOH, TAPS-NaOH, 2-morpholinoethanesulfonic acid (MES)-NaOH, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS)-NaOH were prepared using the reagents from Dojindo Labs. D-Cycloserine was purchased from Meiji Seika Co., *N*-ethylmaleimide from Tokyo Kasei Kogyo Co., L-Ala-P and D-Ala-P from Fluka Chemie AG., and the other chemicals from Katayama Chemical Co.

Results

Unextractability of the alanine racemase from the spores

Since we have demonstrated a significant level of alanine racemase activity of the spores of *B. subtilis* PCI219 in the dormant state and the activity was associated with the spore coat fraction obtained by sonication and lysozyme digestion (Yasuda et al., 1993), solubilization of the enzyme using extraction methods for some spore coat proteins was attempted. After treatment of the dormant spores with 50 mM KOH at 0°C for 3 h, 50 mM KOH at 37°C for 3 h (Kato, 1994) and 1% sodium dodecylsulfate (SDS) -10 mM dithiothreitol–50 mM Na_2CO_3-$NaHCO_3$, pH10, at 65°C for 30 min (Mitani and Kadota, 1976), followed by repeated washes with distilled water, alanine racemase activities of the treated spores were 125%, 109% and 79%, respectively, of that of the untreated ones. There were no solubilized protein bands with

molecular weight of the size expected for the alanine racemase of *B. subtilis* (Ferrari et al., 1985) by SDS-polyacrylamide gel electrophoresis (Kato, 1994). These results showed that the spore alanine racemase was difficult to be solubilized from the spore, and was stable at alkali pH and in the detergent plus reducing agents. Thus, dormant spores were used to characterize the enzyme in the native form.

Kinetic parameters

Previously we have suggested the presence of an isozyme or different forms of the spore-associated alanine racemase because of the characteristic biphasic profile of the temperature-activity curve, in which the maximum activity at 65°C and a shoulder around 40°C have been observed (Yasuda et al., 1993). In this study, alanine racemase activity was determined at 37°C and 65°C to confirm the presence of the different enzyme forms. Relation between [v] and [S] was plotted at 37°C and 65°C. Saturation-curves with a bending at 30mM L-Ala were observed (Fig. 1A, B). Hanes plots at 37°C of less than 30mM and

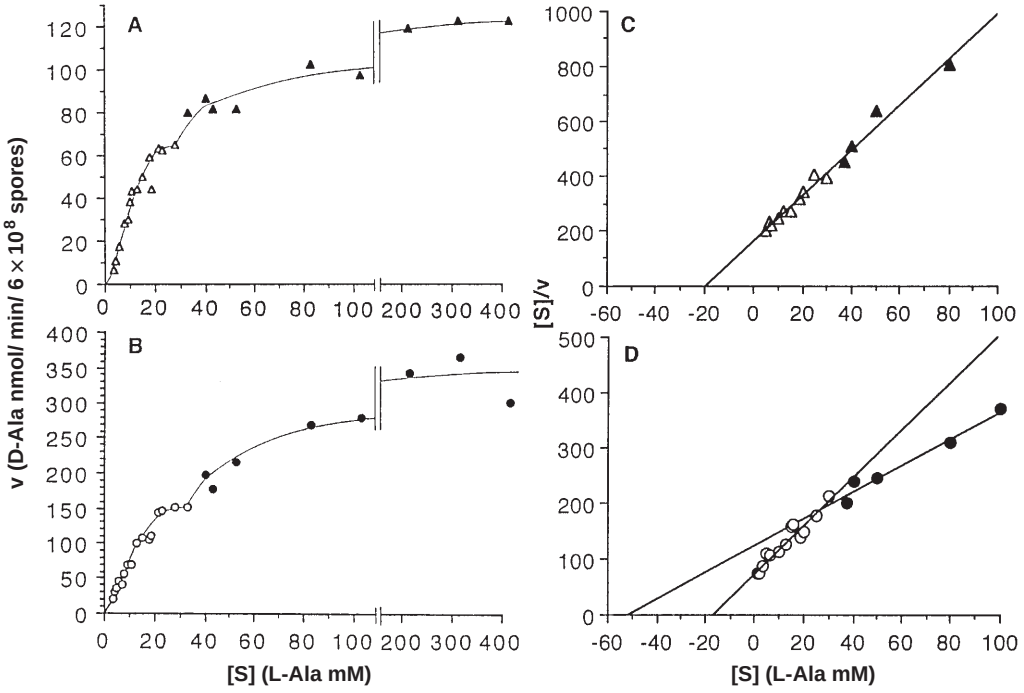

Fig. 1. Effect of L-alanine concentrations and temperatures on the spore-associated alanine racemase activity of *B. subtilis*. Initial velocity, v was measured during the first 10min of incubation with 6×10^8 spores/ml in 35mM CHES-NaOH, pH 9.0, containing 1–30mM (open symbol) and 37.5–400mM (closed symbol) of L-Ala and 1.5mM diphenylamine as an inhibitor for spore germination at 37°C (**A** and **C**) and 65°C (**B** and **D**). **C** and **D** are Hanes plots of **A** and **B**, respectively

Table 1. Km and Vmax values for L-alanine of spore-associated alanine racemase of *B. subtilis*

Temperature	[S] L-Ala (mM)	Km (mM)	Vmax (nmole/ml/min)	Vmax/Km (1/min)
37°C	1–400	19.6	119.9	6.12×10^{-3}
65°C	1–30	18.4	235.4	1.28×10^{-2}
65°C	37.5–400	52.4	418.2	7.98×10^{-3}

Alanine racemase activity was measured using 6×10^8 spores/ml in 35 mM CHES-NaOH, pH 9.0, containing 1.5 mM diphenylamine as an inhibitor for spore germination. Km and Vmax values were calculated from Hanes plot (Fig. 1C and D). Vmax/Km, relative catalytic efficiency.

over 30 mM of L-Ala were indistinguishable (Fig. 1C), but those at 65°C were different in slope (Fig. 1D). Apparent Km and Vmax values are summarized in Table 1. From the Km values, the presence of two kinds of enzymes, one with lower Km (about 20 mM) and the other with higher Km (52 mM), was evident. We designate here tentatively the former as Enz-I and the latter as Enz-II; at 65°C mainly Enz-I in ≦30 mM L-alanine and mainly Enz-II in >30 mM L-alanine are active and at 37°C mainly Enz-I is active irrespective of the substrate concentration. Therefore, we tried to characterize these enzymes further by measuring enzymatic activities in the presence of >30 mM L-alanine at 37°C for Enz-I and at 65°C for Enz-II, although both activities could not be measured completely separately. The highest relative catalytic efficiency was obtained at 65°C in ≦30 mM L-Ala (Table 1).

Effect of pH on the spore alanine racemase activity

The effect of pH on the alanine racemase activities of Enz-I and Enz-II was examined at 37°C and 65°C, respectively, in the presence of 40 mM L-alanine. Both activities could be detected at a wide range of pHs, and the optimal pHs of Enz-I and Enz-II were 8.7–9.0 and 9.0–9.1, respectively (Fig. 2). pH profiles of both enzymes were basically similar.

Temperature stability of the spore alanine racemase

Alanine racemase activities were determined at 37°C (Enz-I) and 65°C (Enz-II) in the presence of 40 mM L-alanine after heating the spore suspensions in distilled water at 50 to 100°C for 30 min. Activities decreased linearly in case of Enz-I and stepwisely in case of Enz-II as the temperature increased, suggesting that structural denaturation proceeded discontinuously in Enz-II. The residual activities at 60°C were about 72% and 100%, and at 75°C were 37% and 63% with Enz-I and Enz-II, respectively, indicating that the latter was more heat-stable than the former (Fig. 3).

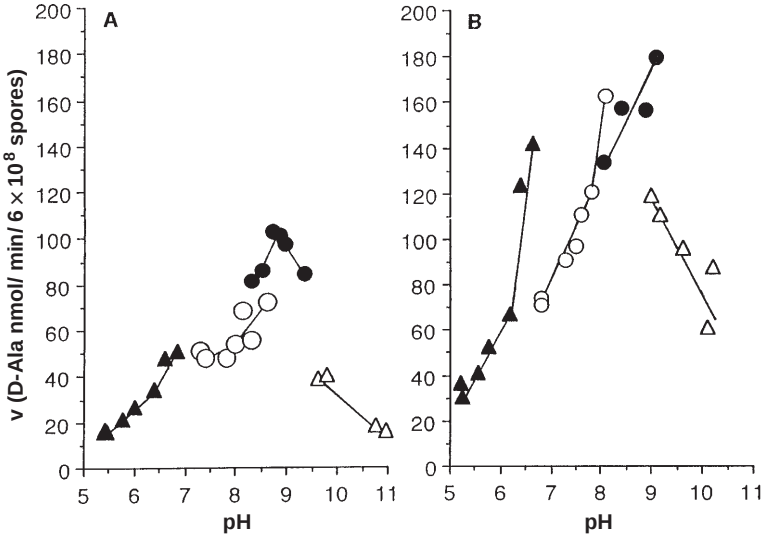

Fig. 2. Effect of pH on the spore-associated alanine racemase activity of *B. subtilis*. Initial velocity, v was measured during the first 10 min of incubation with 6×10^8 spores/ml in 35 mM MES-NaOH (▲), TAPS-NaOH (○), CHES-NaOH (●) and CAPS-NaOH(△) containing 40 mM L-Ala and 1.5 mM diphenylamine at 37°C for Enz-I (**A**) and 65°C for Enz-II (**B**)

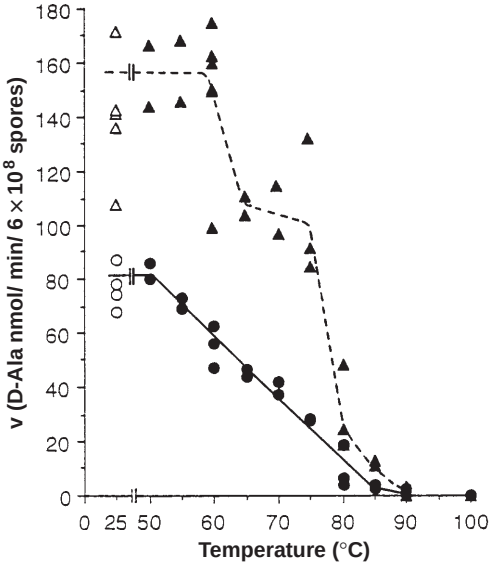

Fig. 3. Temperature stability of the spore-associated alanine racemase activity of *B. subtilis*. Preheated spores at the indicated temperatures for 30 min (closed symbols) and unheated spores (open symbols) were incubated in 35 mM CHES-NaOH, pH 9.0, containing 40 mM L-Ala and 1.5 mM diphenylamine at 37°C for Enz-I (○, ●) and 65°C for Enz-II (△, ▲)

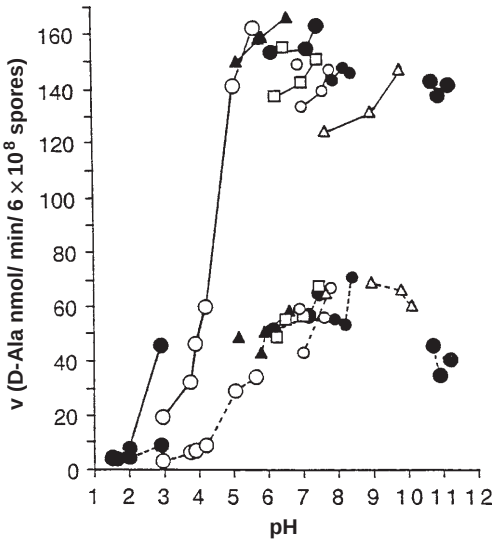

Fig. 4. pH stability of the spore-associated alanine racemase activity of *B. subtilis*. Spores were preincubated in 35 mM of various buffers (●, NaH_2PO_4-HCl and Na_2HPO_4-NaOH; ○, citric acid-NaOH; ▲, MES-NaOH; □, TES-NaOH; ○, TAPS-NaOH; ●,CHES-NaOH; △, CAPS-NaOH) at 60°C for 60 min, and washed with distilled water. Alanine racemase activities of the treated spores were measured in 35 mM CHES-NaOH, pH 9.0, containing 40 mM L-Ala and 1.5 mM diphenylamine at 37°C for Enz-I (dotted line) and 65°C for Enz-II (solid line)

pH stability of the spore alanine racemase

After heating the spore suspensions in various buffers between pH 1.5–11 at 60°C for 60 min, alanine racemase activities were measured at 37°C (Enz-I) and 65°C (Enz-II). Both enzymes were stable between pH 6–11 and inactivated at pH 5 or less (Fig. 4).

Inhibitors of spore alanine racemase

Inhibition of spore alanine racemase activities was examined at 37°C (Enz-I) and 65°C (Enz-II) using various compounds in the presence of 100 mM L-Ala. Similar I_{50} values were obtained from the inhibitory curves by D-penicillamine with both Enz-I and Enz-II (Fig. 5A, B; Table 2). By D-cycloserine, L-Ala-P and D-Ala-P, Enz-II was inhibited more strongly than Enz-I (Fig. 5A–D; Table 2). Hydroxylamine, which is a common inhibitor for PLP-dependent amino acid racemases, acted as an inhibitor with similar affinity for both Enz-I and Enz-II (Table 2). But the profiles of inhibitory curves of hydroxylamine were different from those of the other inhibitors; hydroxylamine showed residual activity even at high concentrations with both Enz-I and Enz-II (Fig. 5E, F). $HgCl_2$ was the most effective inhibitor for Enz-I and Enz-II (Fig. 5E, F; Table 2). Other SH-reagents inhibited at lesser extent; Enz-I was a little more sensitive to monoiodoacetate than Enz-II and Enz-II was more sensitive to *N*-ethylmaleimide than Enz-I (Fig. 5E, F; Table 2).

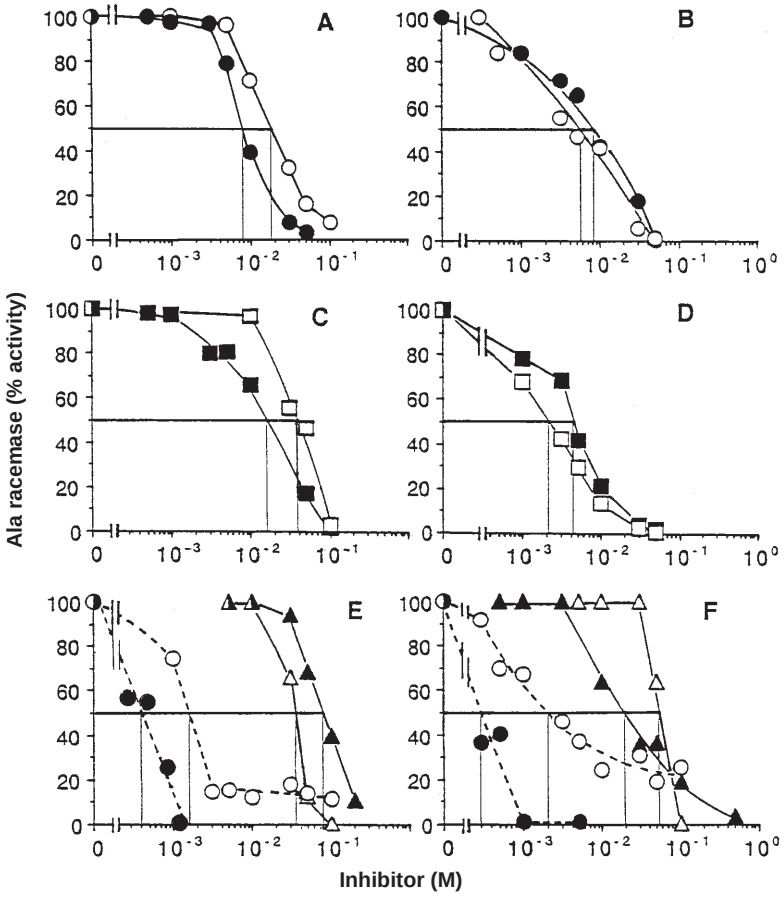

Fig. 5. Effect of inhibitors on the spore-associated alanine racemase activity of *B. subtilis*. In the presence of the various concentrations of inhibitors, enzymatic activity was measured with 6×10^8 spores in 100 mM L-Ala and 1.5 mM diphenylamine at pH 9.0 at 37°C for Enz-I (**A**, **C**, **E**) and 65°C for Enz-II (**B**, **D**, **F**). Inhibitor: D-penicillamine (**A**, **B**, ●); D-cycloserine (**A**, **B**, ○); L-Ala-P (**C**, **D**, □); D-Ala-P (**C**, **D**, ■); hydroxylamine (**E**, **F**, ○); $HgCl_2$ (**E**, **F**, ●); monoiodoacetate (**E**, **F**, △); *N*-ethylmaleimide (**E**, **F**,▲). The 50% inhibitory concentrations were obtained from the curves (see Table 2)

Discussion

Kinetic analysis of spore-associated alanine racemase of *B. subtilis* proved the presence of two kinds of enzyme forms, Enz-I and Enz-II. Enz-I (Km for L-alanine, about 20 mM) and Enz-II (Km, about 50 mM) were selectively used depending on the L-alanine concentration and the temperature. Alanine racemase activities at 37°C and 65°C could be assumed to be those of Enz-I and Enz-II, respectively, if concentrations of L-alanine were >30 mM (Fig. 1; Table 1). Accordingly, the enzymes with optimum temperatures at around 40°C and at 65°C, which have been reported previously (Yasuda et al., 1993) were considered to be Enz-I and Enz-II, respectively. The pH profiles of the activity and pH stability were similar (Figs. 2 and 4), but heat stability seemed different between Enz-I and Enz-II (Fig. 3). The biphasic curve with a plateau

Table 2. Apparent affinity of the inhibitors with spore-associated alanine racemase of *B. subtilis*

Inhibitor	I_{50} (mM)	
	37°C (Enz-I)	65°C (Enz-II)
D-penicillamine	8	8
D-cycloserine	19	6
D-Ala-P	16	4
L-Ala-P	41	2
Hydroxylamine	2	2
$HgCl_2$	0.4	0.3
Monoiodoacetate	36	60
N-Ethylmaleimide	69	19

Alanine racemase activity was measured as shown in Fig. 5 and 50% inhibitory concentration (I_{50}) of the inhibitor was calculated from each curve.

of Enz-II might be related to the two-domain structure of the enzyme which has been clearly demonstrated with *B. stearothermophilus* during denaturation by guanidine hydrochloride (Soda and Tanizawa, 1990). Sensitivities to D-penicillamine, hydroxylamine and $HgCl_2$ were similar between Enz-I and Enz-II, while those to D-cycloserine, D-Ala-P, L-Ala-P, monoiodoacetate and *N*-ethylmaleimide were different (Fig. 5; Table 2).

In case of the psychrophilic alanine racemase of *B. psychrosaccharolyticus*, different optimum temperature and heat-stability are observed in the presence and absence of PLP cofactor in the purified single enzyme (Okubo et al., 1999). In case of the spore enzyme of *B. subtilis*, shifting of optimum temperature depending on PLP had not been examined; the content of PLP in the spore enzyme was not measurable and addition of PLP did not alter enzymatic activity, showing that the spore enzyme contained a sufficient amount of PLP. Moreover, the inhibitory curves of hydroxylamine showed that PLP cofactor was gradually removed from the active site of the enzyme upon increasing the concentration of hydroxylamine, and residual activity was observed even at high concentrations (Fig. 5E, F). If the residual activity represented the activity without cofactor, ratios of the activity in the presence and absence of PLP were similar with both Enz-I and Enz-II. From these facts, the existence of an isozyme of the spore enzyme of *B. subtilis* is more likely rather than a single enzyme with different structural states. The alanine racemase isozymes, which are encoded by two distinct genes and differ in induction mechanisms, were demonstrated in *S. typhimurium* (Wasserman et al., 1983), and *E. coli* (Wild et al., 1985). In *B. subtilis*, besides the gene for alanine racemase, *dal*, coding a 389 amino acid-product (Ferrari et al., 1985), another gene similar to alanine racemase, *yncD*, coding a 394 amino acid-product was predicted by the complete genome sequence project using strain 168 (Kunst et al., 1997). The predicted *yncD* product and several alanine racemases from different sources show a highly conserved amino acid

sequence around the active site lysine (Tanizawa et al., 1988), suggesting a possibility of the presence of isozymes also in *B. subtilis*.

Apparent Km values of L-alanine for the spore enzymes (approximately 20mM for Enz-I and 50mM for Enz-II) were higher than that for the vegetative enzyme (8mM, Yonaha et al., 1975); the difference might depend on the fixed enzymes and soluble enzyme. Potent inhibitors of PLP-enzymes, such as D-penicillamine, D-cycloserine, and hydroxylamine, and sulfhydryl reagents, such as $HgCl_2$, monoiodoacetate and *N*-ethylmaleimide, showed inhibitory effect on the spore enzymes as reported earlier on the vegetative enzyme (Yonaha et al., 1975). The antibiotic derivatives, L-Ala-P and D-Ala-P which are inhibitors useful for examining the details of catalytic mechanisms of the enzyme of *B. stearothermophilus* (Badet et al., 1986) were also potent inhibitors for the spore enzymes of *B. subtilis*.

By the treatment with alkali, *B. subtilis* spores are known to lose several alkali-soluble proteins from the spore coat and become lysozyme sensitive (Zheng et al., 1988). In the present study, the treatment enhanced the alanine racemase activity, indicating that the rigid construction of the spore coat was modified to increase accessibility of the substrate to the enzyme. From this fact, spore alanine racemase seemed to present not only on the surface, but also in the spore coat layer. And tight association of the enzyme with the spore coat was confirmed by retention of the activity after treatment with a combination of reducing and denaturing agents at alkaline pH which removes significant amounts of coat proteins (Aronson and Fitz-James, 1976). The enzymes may be cross-linked to the coat structure after deposition. Some coat proteins of *B. subtilis* spores are thought to be enzyme candidates responsible for cross-linking of coat proteins during spore maturation because of the similarity of DNA sequence of the known enzymes, such as CotA to manganese oxidase (van Waasbergen et al., 1993), CotJC to catalase (Seyler et al., 1997), CotE to peroxidase (Driks, 1999) and transglutaminase encoded by *tgl* (Kobayashi et al., 1996).

Dormant spores are metabolically inactive in the protoplast, but some spore coat-associated enzyme activities without contamination of vegetative cells or debris have been reported, e.g. catalase in *B. cereus* spores (Lawrence and Halvorson, 1954), ribosidase in *B. cereus* spores (Lawrence, 1955), alpha-glucosidase in *B. stearothermophilus* spores (Albert et al., 1998) and 3,3′,5,5′-tetramethylbenzidine oxidizing activity in *B. subtilis* spores (Saito et al., submitted) as well as alanine racemase. These enzymes are generally heat-stable and unextractable, and involvement in spore germination and resistance against various environmental conditions for survival of the spores may be possible as suggested with the ribosidase in *B. cereus* spores (Lawrence, 1955) and the alanine racemase in *B. subtilis* spores (Yasuda et al., 1993). Whether the spore-associated enzymes are derived from the vegetative forms and how the modification proceeds to produce the spore-associated forms, remain unclear. It will be informative to use site-directed mutagenesis to delete region(s) of the enzyme gene and determine whether the coat-association of the enzyme activity remains in the mutant spores. In case of the alanine racemase isozymes of *B. subtilis*, perhaps a deletion of one of the *dal*

and *yncD* genes at a time would lead to the identity of which ones code for Enz-I and Enz-II and more definitely establish their identities and their isozyme forms.

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