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Effects of Salts on the Conformation and Catalytic Properties of D-Amino Acid Aminotransferase

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The effects of salts on the biochemical properties of D-amino acid aminotransferase from *Bacillus* sp. YM-1 have been studied to elucidate both the inhibitory effects of salts on the activity and the protective effects of salts on the substrate-induced inactivation. The results from UV-visible spectroscopy studies on the reaction of the enzyme with D-serine revealed that salt significantly reduced the rate of the formation of the quinonoid intermediate and its accumulation. The kinetic and spectroscopy studies of the reaction with α-[^{2}H]-DL-serine in different concentrations of NaCl provided evidence that the rate-limiting step was changed from the deprotonation of the external aldimine to another step(s), presumably to the hydrolysis of the ketimine. Gel filtration chromatography data in the presence of NaCl showed that the enzyme volume was reduced sharply with the increasing NaCl concentration, up to 100 mM. An additional increase of the NaCl concentration did not affect the elution volume, which suggests that the enzyme has a limited number of salt-binding groups. These results provide detailed mechanistic evidence for the way salts inhibit the catalytic activity of D-amino acid aminotransferase.

Keywords: Aminotransferase, Quinonoid, Conformation, Gel filtration

Introduction

The transamination reaction that is catalyzed by D-amino acid aminotransferase (D-AAT) provides bacterial cells with D-amino acids, which are important constituents for the cell wall and secondary metabolites (Martinez-Carrion and Jenkins, 1965a, b; Yonaha *et al.*, 1975; Fotheringham *et al.*, 1998). Some bacterial cells use other enzyme systems, like alanine racemase (Kim and Yu, 2000), glutamate racemase, etc. D-AAT requires the cofactor pyridoxal 5'-phosphate (PLP), which is bound to the enzyme through a Schiff base that is formed between PLP and the lysine residue of the active site (Yonaha *et al.*, 1975; Tanizawa *et al.*, 1989) (E-PLP in Fig. 1). In the absence of a substrate, E-PLP is in equilibrium with E-aldamine, which has a saturated C4' with an unknown functional group (Ro *et al.*, 1996). In the presence of a D-amino acid, the α-amino group of the substrate replaces the active site lysine residue in order to form the Schiff base between the substrate and PLP (E-PLP-D-AA) (Martinez-Carrion and Jenkins, 1965a). In the first half reaction, E-PLP-D-AA is converted to a quinonoid intermediate (E-Q) by deprotonation at the α-carbon of D-amino acid, then E-Q is subsequently converted to the enzyme complex with pyridoxamine phosphate and α-keto acid (E-PMP + α-KA) via E-ketimine and E-carbinolamine (Martinez-Carrion and Jenkins, 1965a; Martinez del Pozo *et al.*, 1989). The second-half reaction is the reverse of the first-half reaction, except that a new α-keto acid reacts with E-PMP to form a new D-amino acid.

DAAT is reported to be inactivated by D-alanine, which is a normal substrate for the enzyme, in the absence of a α-keto acid (Martinez del Pozo *et al.*, 1992; Bhatia *et al.*, 1993; van Opem *et al.*, 1998). This inactivation is due to the formation of the PLP-related adduct (PXP) through the α-decarboxylation of E-Q during the normal catalysis with D-alanine in the absence of a α-keto acid (van Opem *et al.*, 1998). Formation of PXP is proportional to the formation of E-Q, which has a characteristic absorption peak at 493 nm (Martinez del Pozo *et al.*, 1989). A recent report demonstrated that the inactivation could be protected by various salts (van Opem *et al.*, 1998). The report suggested that salts might impede the release of pyruvate, which was suggested as a main cause of inactivation from the active site to enter the second-half reaction, thereby decreasing the overall turnover. Therefore, the protection effect of salts from the D-alanine-induced inactivation was interpreted as being due to the reduced release of free pyruvate. The reaction of the enzyme with its quasisubstrate D-serine mainly accumulates E-Q

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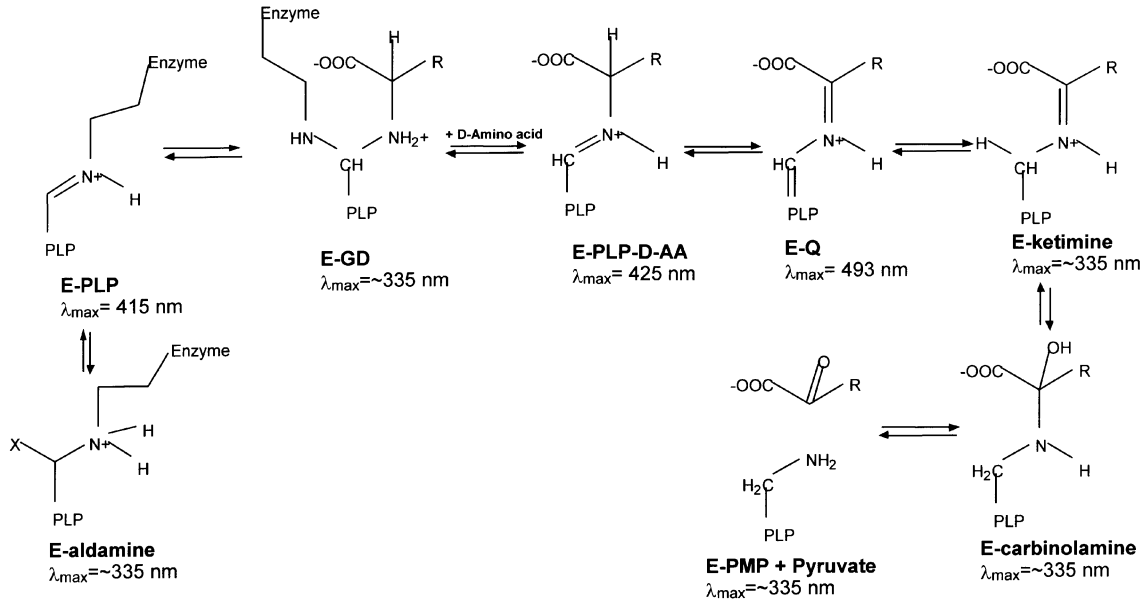

Fig. 1. Reaction mechanism of D-amino acid aminotransferase (E) with D-amino acid (D-AA).

(Martinez del Pozo *et al.*, 1989). The activity of the enzyme toward D-serine is no more than 2% of the activity with D-alanine (Martinez del Pozo *et al.*, 1989; Tanizawa *et al.*, 1989). Therefore, D-serine is a useful substrate for the investigation of the inactivation phenomena, because it provides much more E-Q where the inactivation pathway starts and produces less pyruvate to slow down the inactivation process.

Salts are known to affect the enzyme conformation. Low and Somero (1975a, b) showed that salts affect the protein volume and decrease the activity of some enzymes. They also suggested that salts disrupt the dense hydration spheres around the exposed protein groups, which affects the enzyme volume. In this regard, the salt-induced inhibition and inactivation of D-AAT may be related to the salt-induced enzyme volume change. This report demonstrates that NaCl significantly reduces the enzyme volume. This leads to a change in the rate-limiting step of the catalytic reaction from the deprotonation of substrate to other steps.

Materials and Methods

Bacterial strain, enzyme purification, and assay The D-AAT from *Bacillus* sp. YM-1 was expressed in *Escherichia coli* JM105, as described previously (Ro *et al.*, 1996). The cells were suspended in 30 mM Tris-HCl (pH 7.3) that contained 0.01% β-mercaptoethanol and 20 μM PLP, then broken by sonication. The crude cell extract was incubated at 65°C for 10 min. The pellet was then removed by centrifugation at 15000 rpm for 30 min. The supernatant was applied to a Pharmacia Resource Q anion exchange column (Uppsala, Sweden), then eluted with a linear gradient of NaCl from 0 to 1 M. The enzyme activity was measured in the presence of 100 mM D-alanine, 20 mM α-ketoglutarate, and 50 μM pyridoxal 5'-phosphate at 50°C by a spectrophotometric assay that was coupled with lactic dehydrogenase (Miles and Mcphie, 1974). A triethanolamine buffer (50 mM, pH 8.5) was used to maintain pH of the reaction mixture since Tris or other salt-based buffers were found to inhibit the enzyme activity (see the Results Section). One unit of activity is defined as the amount of the enzyme (mg) that forms 1 mmole of pyruvate in 1 min at 50°C.

Spectroscopic methods Absorption spectra were measured using a Hewlett Packard 8452 diode array spectrophotometer. The reaction mixture contained the enzyme (1 mg/ml), triethanolamine buffer (50 mM, pH 8.5), D-alanine or D-serine (100 mM), and various concentrations of NaCl. Fluorescence spectra were made using a PTI spectrofluorometer (Photon Technology International). The measurement conditions were the same as for the absorption spectra, except the enzyme concentration was 0.5 mg/ml. All spectra were obtained at 25°C.

Gel filtration chromatography Gel filtration chromatography was carried out at 4°C on a FPLC system using a Superose 12HR 10/30 column (1 × 30 cm) at a flow rate of 0.4 ml/min. The enzyme (1 mg in 100 μl of elution buffer) was injected and eluted with a 50 mM triethanolamine buffer (pH 8.3) in the absence and presence of D-alanine (0.1 M) with various concentrations of NaCl (0-5 M).

Preparation of α-[^{2}H]-DL-serine The α-[^{2}H]-DL-Serine was prepared as reported (Miles and Mcphie, 1974). The pyridoxal free base was prepared by loading 1 g pyridoxal hydrochloride onto a column of Dowex 50-H+ and elution with 2 M ammonium hydroxide. The fractions that contained pyridoxal free base were freeze-dried. DL-serine (5 g) and pyridoxal were dissolved in 100 ml of D_2O, and the pD was adjusted to 9.6 with 5 M KOD. The solution was freeze-dried, dissolved in 100 ml of D_2O, and combined with $Al_2(SO_4)_3 \cdot 18H_2O$ (1 g) that had been previously freeze-dried from 5 ml of D_2O. The solution was incubated for 2 days at room temperature in the dark. The reaction mixture was

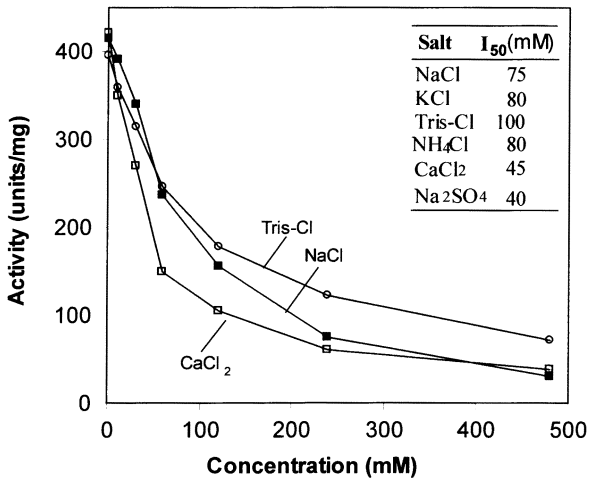

Fig. 2. Inhibition of the enzyme activity in the transamination reaction by various salts. I_{50} is a concentration of salt (mM) that inhibits 50% of the enzyme activity.

precipitated with acetone, washed with 50% ethanol twice, dissolved in water, treated with activated charcoal, and recrystallized. The final yield was 70% (3.5 g α-[^{2}H]-DL-serine).

Results

Effect of salts on the activity In order to determine the effect of salts on the activity of D-AAT, the transamination activity of the enzyme in the presence of various salts (NaCl, KCl, NH_4Cl, $CaCl_2$, $(NH_4)_2SO_4$, Na_2SO_4, Tris-Cl) was examined. The activity was measured in the presence of 100 mM D-alanine, 20 mM α-ketoglutarate with different concentrations of salts at 50°C (Fig. 2). A triethanolamine buffer (50 mM) was used in order to avoid the inhibitory effect of Tris-Cl or other salt-based buffers. The specific activity was 450 units/mg in the absence of salt, but decreased with increasing salt concentration. Only 40% of the activity was expressed at 0.1 M NaCl or KCl, which is a conventional concentration that is used for enzyme studies. With $CaCl_2$, the inhibition was much more significant than NaCl. Forty-five mM of $CaCl_2$ was enough to inhibit 50% of the enzyme activity. Interestingly, the Tris-Cl buffer also showed a similar inhibition curve to the other salts. All of the salts that were tested were inhibitory (see the Table in the *inset* of Fig. 2).

Effects of salts on the absorption spectra The absorption spectrum of D-AAT in the presence of D-alanine is shown in Fig. 3A. The reaction of D-alanine with D-AAT showed a strong absorption band at 335 nm, which is attributed to E-PMP, E-ketimine, and E-carbinolamine (Martinez-Carrion *et al.*, 1965; Martinez del Pozo *et al.*, 1989). The weak absorption band at 420 nm is attributed to the external aldimine form of the enzyme (E-PLP-D-ala). The addition of 500 mM NaCl did not change the distribution of the enzyme-substrate intermediates. The reaction of D-AAT with its

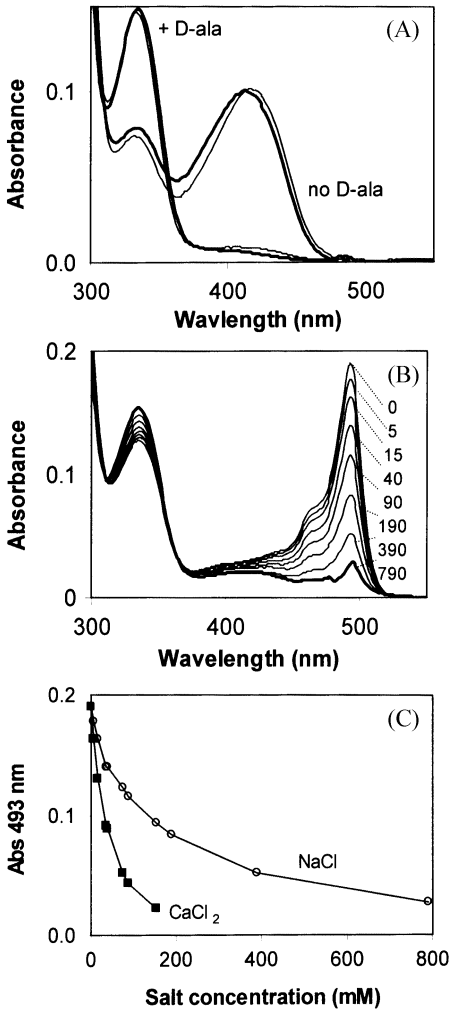

Fig. 3. Effects of salts on the UV/Vis spectral properties. (A) UV/Vis spectrum with or without the substrate (0.1 M D-alanine). The spectra were unaffected by NaCl (thin line: without NaCl, thick line: 0.5 M NaCl). (B) Effect of NaCl concentration on UV/Vis spectrum from the reaction of the enzyme with 100 mM D-serine. The spectrum was taken 2 min after mixing. The numbers shown on the right side of the figure indicate the concentration of NaCl (mM). (C) Effect of salts on the accumulation of E-Q.

quasisubstrate D-serine produced an intense absorption peak at 493 nm, which is attributed to a quinonoid intermediate (E-Q) (Martinez-Carrion *et al.*, 1965; Martinez del Pozo *et al.*, 1989). The intensity of the 493 nm band decreased with the increasing NaCl concentration with a concomitant increase of the 335 nm band (Fig. 3B). Therefore, NaCl may shift the equilibrium between E-Q, and the E-PMP forms towards the latter forms. Half of the absorbance at 493 nm diminished with 100 mM NaCl or 50 mM of $CaCl_2$.

Effects of NaCl on the rate of formation of E-Q The initial rate of the formation of the 493 nm absorption peak was examined in the presence of 100 mM D-serine at various

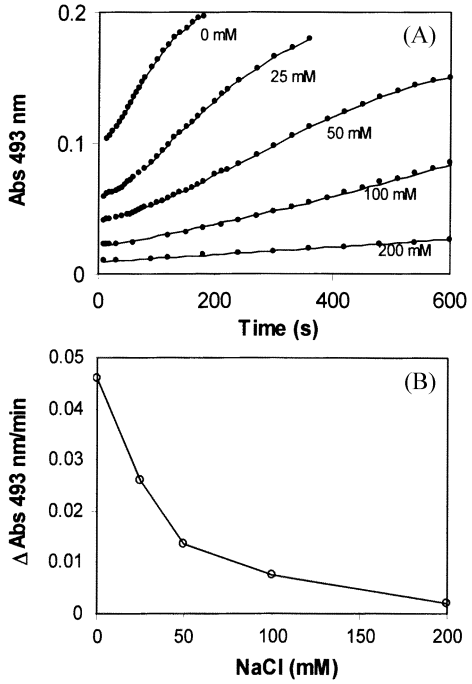

Fig. 4. Effects of NaCl concentration on the rate of the formation of E-Q (493 nm peak) from the reaction of the enzyme with 100 mM D-serine. (A) Time-course of the 493 nm peak at different concentrations of NaCl. (B) Effect of NaCl on the rate of the formation of E-Q. The first-order rate constant was calculated from the linearly increasing region of the data in (A).

concentrations of NaCl (Fig. 4). The rate decreased sharply up to 100 mM NaCl, then showed a slow decrease at higher NaCl concentrations. Since the reaction of D-AAT with D-serine establishes the equilibrium between E-Q and one of the enzyme-substrate intermediates, which absorb at 335 nm, the time-dependent increase of the 493 nm peak (Fig. 4) may not reflect the deprotonation rate at the α-carbon of external aldimine (E-PLP-D-ser) to form E-Q. Rather, it reflects a slow conversion of the 335 nm species to E-Q, which eventually goes into the inactivation pathway through the decarboxylation from the a-carbon of E-Q.

Effect of NaCl on the reaction of D-AAT with α-[^{2}H]-DL-serine The value of the kinetic isotope effect 2-3 was reported from the reaction of D-AAT from *Bacillus sphaericus* with DL-alanine in a 0.3 M potassium phosphate buffer (Soper and Manning, 1981). This indicates that the α-deprotonation step is partially rate-limiting. Assuming that this is true for D-AAT from the *Bacillus* YM-1, the addition of α-[^{2}H]-DL-serine would limit the rate at this step. Fig. 5A shows the spectra of D-AAT with α-[^{2}H]-DL-serine in the absence and presence of 100 mM NaCl. As expected, the E-PLP-D-ser (425 nm) became one of the major peaks and E-Q peak increased in a time-dependent manner, which indicates that α-deprotonation occurs at a slow rate (Fig. 5B). The effect of NaCl on the rate of the formation of E-Q (calculated

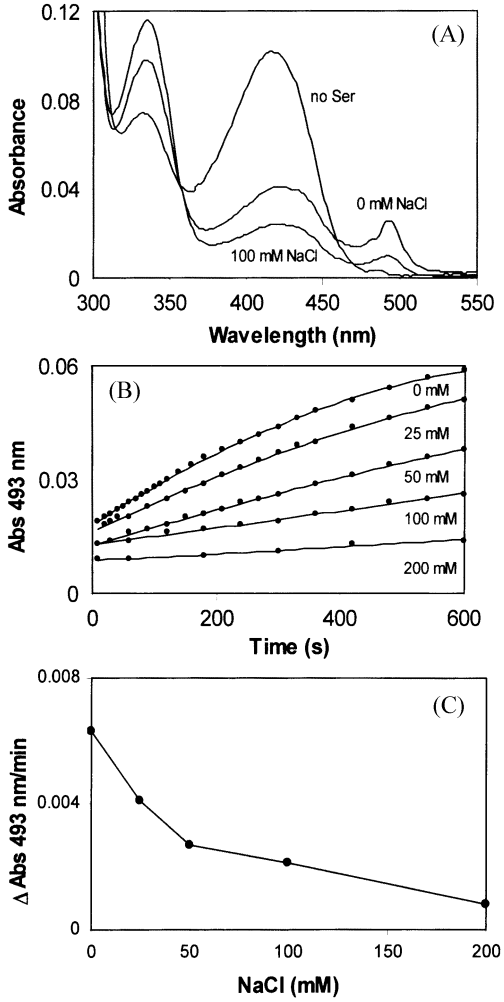

Fig. 5. The reaction of D-AAT with α-[^{2}H]-DL-serine. (A) UV/Vis spectrum with or without substrate (200 mM α-[^{2}H]-DL-serine) at different NaCl concentrations. (B) Time-course of the 493 nm peak at different concentrations of NaCl. (C) Effect of NaCl on the rate of the formation of E-Q.

from Fig. 5B) is shown in Fig. 5C. The data indicate that salt does inhibit the a-deprotonation step.

Gel filtration chromatography Gel filtration chromatography was carried out to elucidate the effect of NaCl on the enzyme conformation. As shown in Fig. 6, the elution volume of the enzyme was highly dependent on the salt concentration. The elution volume increased sharply, up to 100 mM NaCl (indicating a salt-dependent reduction of the enzyme volume), and showed a slow increase at a higher concentration. Further increase of the NaCl concentration from 0.5 M to 5 M showed little effect. The addition of a substrate did not affect the elution volume.

Discussion

Salts inhibit the enzyme activity Many studies have

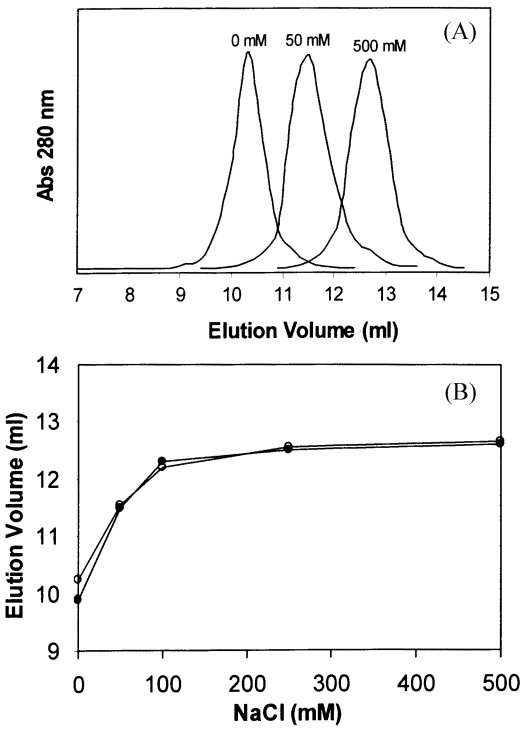

Fig. 6. Gel filtration chromatography. (A) The elution profile of the enzyme in the presence of 0 mM, 50 mM, and 500 mM of NaCl. (B) The dependence of the elution volume on the NaCl concentration in the presence of 50 mM D-alanine (●) and in the absence of the substrate (○).

reported the effects of salts on PLP-dependent enzymes. For example, the tryptophan synthase $\alpha_2\beta_2$ complex is activated by both NaCl and CsCl in the β-replacement reaction (Peracchi *et al.*, 1995), but the β subunit alone is inhibited by NaCl and stimulated by CsCl (H. S. Ro and E. W. Miles, unpublished results). Tyrosine phenol-lyase and tryptophan indole-lyase are activated by K^+ and NH_4^+ ions (Demidkina and Miagkikh, 1989). Dialkylglycine decarboxylase is inhibited by Na^+ and activated by K^+ (Toney *et al.*, 1993; Hohenester *et al.*, 1994). CsCl activates Ornithine decarboxylase (Lee *et al.*, 2001). For these enzymes, monovalent cations bind to the specific sites and affect both conformation and catalysis (Hyde *et al.*, 1988; Toney *et al.*, 1993; Rhee *et al.*, 1996; Isupov *et al.*, 1998). Therefore, the effects that are seen with these enzymes are clearly different from the effects of salt on D-AAT. An activity vs. salt concentration profile demonstrates that D-AAT activity is significantly inhibited by the high salt concentration. This is consistent with previous finding (van Opem *et al.*, 1998). The salts of higher ionic strength, $CaCl_2$, Na_2SO_4 (this work), and $MgCl_2$ (van Opem *et al.*, 1998), inhibited at lower concentrations than those of the lower ionic strength.

Salts decrease the accumulation of E-Q The accumulation of E-Q in the reaction of D-AAT with D-serine decreased with the increase of NaCl or $CaCl_2$ concentrations (Fig. 3BC). The crystal structure of aspartate aminotransferase with *erythro*-β-hydroxy-L-aspartate revealed that a hydrogen bond network between the β-hydroxyl group of substrate and the enzyme groups (Tyr70, Lys258) stabilizes E-Q (absorbs at 493 nm) and E-carbinolamine (absorbs at 330 nm). It may be responsible for the accumulation of E-Q and E-carbinolamine (Hayashi and Kagamiyama, 1995; von Stosch, 1996). A similar study was done with aromatic amino acid aminotransferase (Hayashi *et al.*, 1996). It is, therefore, crucial for the enzyme to have a correct arrangement of the active site groups in order to accumulate E-Q. Although the stereospecificity of D-AAT is opposite to that of aspartate aminotransferase, crystallographic data revealed that the arrangement of the active site groups is quite similar (Sugio *et al.*, 1995). The fact that the reaction of D-AAT with D-serine and *erythro*-β-hydroxy-DL-aspartate results in the accumulation of E-Q as a major peak, but the reaction of D-threonine that has a *threo* β-hydroxy group could not, suggests the requirement of a certain structural arrangement of the active site groups for the accumulation of E-Q (Martinez del Pozo *et al.*, 1989). Therefore, the inhibitory salt effect on the accumulation may be caused by a salt-induced enzyme conformational change that disturbs the hydrogen bond network in the active site that stabilizes E-Q. The destabilizing effects of salt on E-Q influences not only the accumulation, but also the rate of formation (Fig. 4). The protective effect of salt from the substrate-induced inactivation may simply reflect the reduced rate of the E-Q formation since the inactivation is proportional to the formation of E-Q.

NaCl decreases the deprotonation rate at a-carbon The results of the NaCl effect on the reaction of D-AAT with α-[^{2}H]-DL-serine indicate that NaCl decreases the rate of formation of E-Q (Fig. 5). Since the formation of E-Q requires the deprotonation at the α-carbon of the substrate, the rate decrease in the E-Q formation is related to the decrease of the ability of the enzyme groups that are responsible for the deprotonation.

NaCl significantly reduces the enzyme volume From the gel filtration study, it is obvious that the presence of NaCl significantly increases the elution volume, which indicates the salt-dependent reduction of the enzyme volume (Fig. 6). The lack of change in the elution volume above 100 mM of NaCl indicates that the enzyme has a limited number of surface groups. Replacement of these surface groups from water to salt may decrease the total enzyme volume. Low and Somero (1975a, b) reported that the presence of salt disrupts the dense hydration spheres around the exposed protein groups, which leads to either an increase or decrease of the enzyme volume. The paper suggested that the enzyme requires a certain amount of activation volume, which is the volume difference in the enzyme substrate complex between the transition and ground states. Therefore, the constriction of the enzyme that is

induced by salt may not provide the required activation volume, resulting in the inhibition of the enzyme activity.

The studies demonstrate that the binding of salt to the groups in D-AAT reduces the enzyme volume significantly. This may disturb the arrangement of the active site groups that are responsible for the stabilization of E-Q and deprotonation at the α-carbon of the substrate. The effects of salt on protection from the inactivation (van Opem *et al.*, 1998) and inhibition of the activity (this study and van Opem *et al.*, 1998) are a consequence of the salt-induced enzyme volume reduction.

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