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Effect of Pressure on Catalytic Properties of Glutamate Racemase from *Aquifex pyrophilus*, an Extremophilic Bacteria

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Abstract The effect of pressure on the catalytic properties of glutamate racemase from *Aquifex pyrophilus*, an extremophilic bacterium, was investigated. The activation volume for the overall reaction ($\Delta V^{\neq}$) and catalysis ($\Delta V^{\neq}_{cat}$) was -96.97 ml/mol and 4.97 ml/mol, respectively, while the reaction volume for the substrate binding ($\Delta V_{K_m^{-1}}$) was -101.94 ml/mol. The large negative $\Delta V^{\neq}$ for the overall reaction indicated that the pressurization of glutamate racemase resulted in enhanced catalytic efficiencies. In addition, this value was also due to the large negative $\Delta V_{K_m^{-1}}$ for the substrate binding. The negative value of $\Delta V_{K_m^{-1}}$ implied that the conformational changes in the enzyme molecule occurred during the substrate binding process, thereby increasing the degree of hydration. The small value of $\Delta V^{\neq}_{cat}$ suggested that the pressure did not affect the glutamate racemase catalysis after the substrate binding.

Key words: Activation volume, *Aquifex pyrophilus*, extremophiles, glutamate racemase, high pressure

Of all the environmental factors that influence biological systems, pressure is the least understood and appreciated. However, pressure is the most important factor that has to be considered in the study of deep-sea organisms or of proteins from extremophilic microorganisms [15]. Extremophilic microorganisms are adapted to survive in ecological niches, such as high temperatures, extreme pHs, high salt concentrations, and high pressure [2]. Therefore, the effect of pressure on extremophiles has attracted much attention in the last decade, because of their evolutionary significance and ability to maintain their cellular integrity at high pressure [1, 5, 12]. Furthermore, since extremozymes offer new opportunities for biocatalysis and biotransformation due to their extreme stability, study on the effect of pressure and temperature on extremophile enzymes has attracted increased interest from a biotechnological point of view [3, 13].

High-pressure kinetics provide a powerful tool for investigating the properties of the reaction transition state, especially the charge distribution [4, 6, 17]. The effect of pressure on an enzyme reaction, as on a chemical reaction, is quantified by the activation volume ($\Delta V^{\neq}$). For example, the activation volume for the catalytic step of an enzyme reaction equals the volume of the system containing the activated complex ($V^{\neq}_{ES}$) minus the volume of the system containing the ground-state complex (V_{ES}) [10]. Changes of the activation volume in enzyme reactions result from two sources [11, 16]. The first is structural contributions due to changes in the volume of protein itself, while the other is due to rearrangement of the solvent molecules due to a change in the solvent exposure of the protein groups, which modifies the water density. However, according to the results of Low and Somero, the latter effect dominates the former in enzyme reactions [10]. For example, the creation of a charge in the transition state during the reaction increases intermolecular electrostatic forces between charged molecules and solvating molecules, and this electrostricted complex then leads to a reduction in volume, whereas the reverse process has the opposite effect. Moreover, Low and Somero, indicated that the energy barrier ($\Delta G^{\neq}$) and activation volume ($\Delta V^{\neq}$) of an enzyme reaction are lowered by increasing the water density during the formation of the activated enzyme-substrate complex, thereby accelerating the velocity of the reaction [9].

Glutamate racemase catalyzes the interconversion of L-glutamate to D-glutamate and vice versa. Since D-glutamate is one of the essential amino acids present in peptidoglycan, glutamate racemase has been considered as an attractive target for designing new antibacterial drugs [18]. One of the current authors (Y. G. Yu) previously cloned a gene encoding the glutamate racemase from *Aquifex pyrophilus*, an extremophilic bacterium, and expressed it in *Escherichia coli* [7]. Accordingly, in order to examine how pressure affects the substrate binding and catalysis of

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the glutamate racemase, and evaluate which of these two processes causes the conformational changes during the catalytic process, the current project on high-pressure kinetic studies was conducted.

The transformants of *E. coli* strain BL21 were grown in Luria-Bertanis (LB) media supplemented with ampicillin (100 μg/ml). The glutamate racemase was purified using the method of Kim *et al.* [7]. The protein concentration was determined by Bradford's method using bovine serum albumin as the standard. The pressurization was performed hyperbarically using N_2 from a high-pressure gas booster (Haskel. Inc, U.S.A.), while the reaction pressure was regulated using a back pressure regulator (Tescom. Co, U.S.A.). The high-pressure reactor for the enzymatic reaction was made of stainless steel, and the reaction temperatures were controlled by circulating preheated water. The reaction mixture was added to the high-pressure reactor and full pressure was achieved in less than 1.5 min; a short time compared to the linear time course of about 20 min. The reaction mixture inside the high-pressure reactor was magnetically stirred and aliquots were removed periodically. In order to conserve the amount of high pressure required to maintain the reaction, sampling was done through a needle valve and metering valve in series (Fig. 1).

The glutamate racemase activity was measured at pH 8.5 and 30°C by determining the conversion of D-glutamate to L-glutamate using a coupled enzyme assay method that employs *Aquifex pyrophilus* glutamate racemase and L-glutamate dehydrogenase (Fluka, U.S.A.)/NAD^+ (Sigma, U.S.A.) [7]. The first reaction for racemization of the D-glutamate to L-glutamate direction was performed in a high-pressure reactor containing 30 ml of 50 mM Tris-HCl buffer and 2 mM dithiothreitol (Sigma, U.S.A.) at pH 8.5 and 30°C. The concentration of D-glutamate was varied from 0.025 to 0.50 mM. After the first reaction was terminated, L-glutamate produced in sampled aliquots was measured using L-glutamate dehydrogenase/NAD^+. The kinetic parameters of k_{cat} and K_m within the pressure range of 1–600 atm were determined from Lineweaver-Burk and Hanes-Woolf plots based on the initial rate according to the substrate concentration. All the reaction kinetics were obtained in triplicate. The active enzyme was assayed by active-site titration using 5,5'-dithiobis(2-nitrobenzoate) (Sigma, U.S.A.) [17]. The amount of 2-nitro-5-mercaptobenzoic acids released was measured at 412 nm using an HP 8453 UV-VIS spectrophotometer (Hewlett-Packard, U.S.A.), and these results were then used to determine molarity of the active enzyme.

According to the transition state theory, the pressure dependence of a reaction rate is given by the relationship [11]:

$$\left(\frac{\partial \ln v}{\partial P}\right)_T = -\frac{\Delta V^{\neq}}{RT} \tag{1}$$

where v is the reaction rate at pressure P, $\Delta V^{\neq}$ is the activation volume, and R is the gas constant (82.0578 ml · atm/K · mol). Negative activation volumes correspond to an activity enhancement induced by pressure. The Michaelis-Menten equation for the reaction rate v may also be written:

$$\ln v = \ln V_{max} - \ln\left(1 + \frac{K_m}{[S]}\right) \tag{2}$$

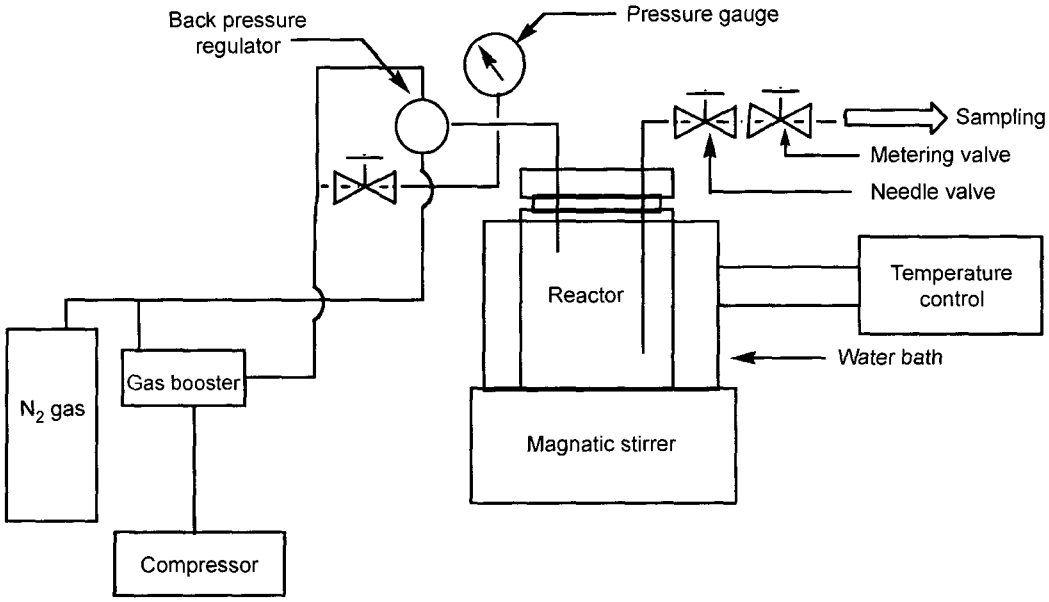

Fig. 1. Schematic diagram of the high-pressure reactor for enzymatic catalysis.

so differentiation in respect to pressure gives:

$$\left(\frac{\partial \ln v}{\partial P}\right)_T = \left(\frac{\partial \ln V_{max}}{\partial P}\right)_T - \frac{K_m/[S]}{1+K_m/[S]} \cdot \left(\frac{\partial \ln K_m}{\partial P}\right)_T \quad (3)$$

$$-\frac{\Delta V^{\neq}}{RT} = -\frac{\Delta V^{\neq}_{cat}}{RT} - \frac{K_m}{K_m+[S]} \cdot \frac{\Delta V_{K_m^{-1}}}{RT} \quad (4)$$

$$\Delta V^{\neq} = \Delta V^{\neq}_{cat} + \frac{K_m}{K_m+[S]} \cdot \Delta V_{K_m^{-1}} \quad (5)$$

At low substrate concentrations, Eq. (5) becomes:

$$\Delta V^{\neq} = \Delta V^{\neq}_{cat} + \Delta V_{K_m^{-1}} \quad (6)$$

According to Morild [11], the volume change in an enzyme reaction during a catalytic process can be defined by Eq. (7), where k_{cat}/K_m is the catalytic efficiency of the enzyme reaction.

$$\Delta V^{\neq} = -RT\left(\frac{\partial \ln(k_{cat}/K_m)}{\partial P}\right)_T \quad (7)$$

The catalytic efficiency term can be split into the activation volume ($\Delta V^{\neq}_{cat}$) for the catalysis and the reaction volume ($\Delta V_{K_m^{-1}}$) for the substrate binding, as depicted in Eqs. (8) and (9), respectively.

$$\Delta V^{\neq}_{cat} = -RT\left(\frac{\partial \ln k_{cat}}{\partial P}\right)_T \quad (8)$$

$$\Delta V_{K_m^{-1}} = -RT\left(\frac{\partial \ln K_m^{-1}}{\partial P}\right)_T \quad (9)$$

The volume changes can result from substrate binding to the active site, conformational changes of the enzyme during the catalytic process, or interaction of the enzyme with the bulk reaction medium. Therefore, studying the influence of pressure on enzyme function can provide information on the nature of enzyme hydration.

Figure 2 shows the variation in the k_{cat}, K_m, and k_{cat}/K_m values of the glutamate racemase-catalyzed interconversion of D-glutamate to L-glutamate as a function of pressure in 50 mM Tris-HCl buffer at pH 8.5 and 30°C. As the pressure increased, the K_m values (Fig. 2B) decreased, indicating an enhanced affinity between the enzyme and the substrate, while the k_{cat} values (Fig. 2A) remained apparently unaffected within the pressure range examined. The activation volume for the overall reaction ($\Delta V^{\neq}$) and catalysis ($\Delta V^{\neq}_{cat}$) and the reaction volume for the substrate binding ($\Delta V_{K_m^{-1}}$) were obtained from the slope of the plots, and the linear character of the curves in Fig. 2 suggests that the catalytic mechanism and active conformation of glutamate racemase remained unchanged within the pressure range investigated. Table 1 shows the activation volumes and reaction volume of the glutamate racemase reaction. The activation volume for the overall reaction ($\Delta V^{\neq}$) and catalysis ($\Delta V^{\neq}_{cat}$) was -96.97 ml/mol and 4.97 ml/mol, respectively, while the

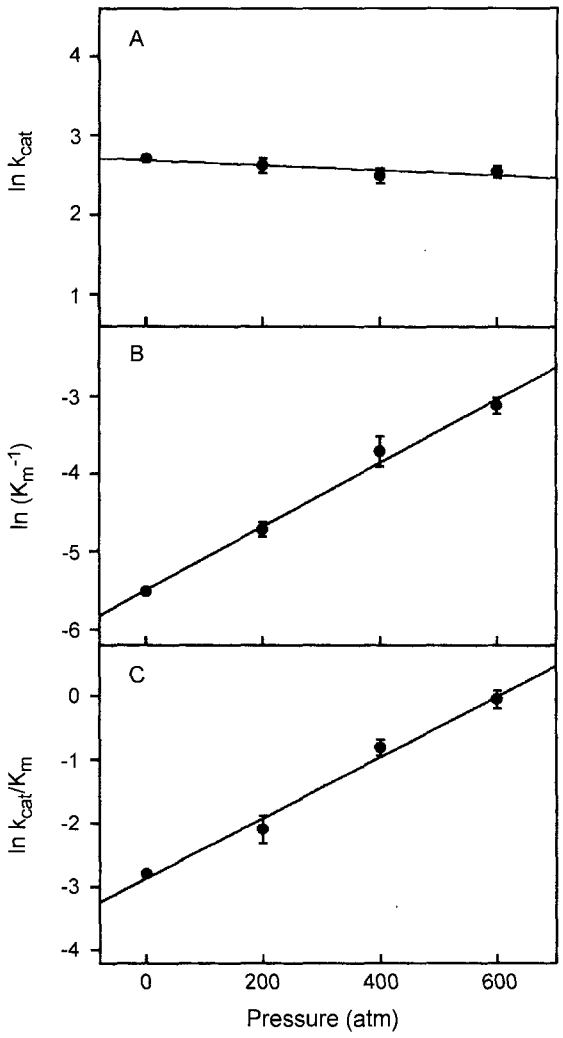

Fig. 2. Effect of pressure on catalytic parameters for the glutamate racemase-catalyzed conversion reaction of D-glutamate to L-glutamate.
The reaction was carried out at pH 8.5 and 30°C. $[E]_o$= 1.077 μM, [S]=0.025 to 0.50 mM, The kinetic parameters were determined within a pressure range of 1–600 atm. Panel A, ln k_{cat}; Panel B, ln (K_m^{-1}); Panel C, ln k_{cat}/K_m.

reaction volume for the substrate binding ($\Delta V_{K_m^{-1}}$) was -101.94 ml/mol. Morild has compiled a list of over 50 enzymes that are affected by pressure, either catalytically or structurally. Activation volumes typically range from -90 to +60 ml/mol for an enzymatic reaction [8, 11, 14]. The large negative $\Delta V^{\neq}$ for the overall reaction in the present study indicated that the pressurization of glutamate racemase resulted in enhanced catalytic efficiencies. In addition, this value was also due to the large negative value of $\Delta V_{K_m^{-1}}$ for the substrate binding. Water is generally involved in all noncovalent interactions that help to maintain the proper catalytic state for enzymatic catalysis to occur, and the greater the number of electrostricted water molecules around the enzyme molecules, the more the

Table 1. Activation volumes and reaction volume of glutamate racemase reaction in 50 mM Tris-HCl buffer at pH 8.5 and 30 °C.

Pressure (atm)	Activation volumes and reaction volume (ml/mol)		
	$\Delta V_{K_m^{-1}}^{a}$ (for K_m^{-1})	$\Delta V_{cat}^{\neq b}$ (for k_{cat})	$\Delta V^{\neq c}$ (for k_{cat}/K_m)
1 to 600	-101.94	4.97	-96.97

[a]The reaction volume for the substrate binding was obtained from the slope of the plots in Fig. 2B.
[b]The activation volume for the catalysis was obtained from the slope of the plots in Fig. 2A.
[c]The activation volume for the overall reaction was obtained from the slope of the plots in Fig. 2C.

activation energy is lowered [20]. Furthermore, as mentioned earlier, negative volume changes can result from interaction between the charged molecules of the enzyme and the solvating molecules. Therefore, the large negative $\Delta V_{K_m^{-1}}$ indicates that the conformational change in the glutamate racemase took place during the substrate binding process, thereby enhancing the degree of hydration. Accordingly, it can be concluded that this enhanced hydration accelerated the reaction rate when the reaction system was pressurized. Conversely, the $\Delta V_{cat}^{\neq}$ value was small, suggesting that the catalysis step after the substrate binding was unaffected by the pressure.

The present study showed that the glutamate racemase obtained from *Aquifex pyrophilus*, an extremophilic bacterium, was more functional under high pressure than at atmospheric pressure. In particular, the affinity between the enzyme and substrate was significantly enhanced, further confirming that pressure is an another important factor that modulates the enzyme activity.

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