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Guanidine Hydrochloride-Induced Denaturation of *Pseudomonas cepacia* Lipase

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The guanidine hydrochloride-induced denaturation of *Pseudomonas cepacia* lipase (PCL) was studied at pH 7 by monitoring the changes in the fluorescence and circular dichroism of the enzyme. The denaturation was irreversible as a whole, and the addition of Ca^{2+} ions decreased the velocity of the denaturation. The denaturation process was well explained consistently by a two-step mechanism, as follows:

$$N \cdot Ca^{2+} \underset{Ca^{2+}}{\overset{Ca^{2+}}{\rightleftharpoons}} D_I \rightarrow D_F$$

where N is the native state of PCL, D_I an intermediate denatured-state which can be refolded into the native state, and D_F the final denatured-state that can not be renatured. Ethanol (10%) increased the denaturation velocity by decreasing the refolding step, $D_I + Ca^{2+} \rightarrow N \cdot Ca^{2+}$, which would be caused by the stabilization of D_I by ethanol.

Key words: denaturation, guanidine hydrochloride, lipase from *Pseudomonas cepacia*, unfolding.

Lipase [EC 3.1.1.3] catalyzes the hydrolysis of triglycerides to produce free fatty acids and glycerols. This enzyme is widely used in the food and detergent industries, and is also useful for the synthesis of fine chemicals of high added value in the pharmaceutical industry (*1-5*). Lipases are even active in organic solvents, and catalyze a wide range of esterification and transesterification reactions under low-water conditions.

Pseudomonas cepacia lipase (PCL) is a representative microbial lipase, which consists of 320 amino acids, including three tryptophan residues (Trp30, Trp209, and Trp284), with a molecular weight of 33,210 (*6*). The detailed three-dimensional structure of PCL has been elucidated at 2 Å resolution (*7-9*). PCL contains a tightly bound Ca^{2+} ion, which is six-coordinated by Asp242, Asp288, Gln292, Val296, and two water molecules (*9*).

In the preceding study involving adiabatic differential scanning calorimetry (DSC) (*10*), it was shown that the thermal denaturation of PCL is well explained by the following two-state scheme with dissociation of the bound Ca^{2+} ion upon denaturation;

$$N \cdot Ca^{2+} \rightleftharpoons D + Ca^{2+} \quad (1)$$

where N and D are the native and denatured states of PCL, respectively. The denaturation step itself is considered to be essentially reversible, but when the PCL solution was reheated, after the initial scan, no endothermic peak was observed, which suggests that the denaturation might be an irreversible process. A DSC study also showed that alcohols, such as methanol and ethanol, decreased the denaturation temperature of PCL with both increasing alcohol concentration and length of the alkyl chain.

In this study, we aimed to elucidate the guanidine hydrochloride-induced denaturation process of PCL by monitoring the fluorescence and circular dichroism (CD) of PCL. The effects of alcohols on the denaturation were also examined to characterize the denaturation process. It is expected that the detailed characterization of the denaturation process of PCL will enable us to improve the stability of the enzyme and thus increase its application.

MATERIALS AND METHODS

The lyophilized preparation of purified *P. cepacia* lipase was a generous gift from Amano Pharmaceutical (Nagoya). Protein concentrations were determined spectrophotometrically using a molar absorbance of $\varepsilon = 37{,}000$ at 280 nm (*11*). Calcium chloride, guanidine hydrochloride (GuHCl), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), and ethanol were obtained from Wako Pure Chemical Industries (Osaka).

Fluorescence spectra were obtained with a fluorescence spectrophotometer, Hitachi 650-60, with an excitation wavelength, λ_{ex}, of 280 nm, unless otherwise stated. The protein concentration was fixed at 0.27 μM. The time course of the denaturation of PCL by GuHCl (2.8 M) was observed in the absence and presence of Ca^{2+} by monitoring the decrease in the protein fluorescence intensity at 332 nm

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Abbreviations: CD, circular dichroism; GuHCl, guanidine hydrochloride; PCL, *Pseudomonas cepacia* lipase.

in 20 mM PIPES buffer, pH 7, 34°C. Possible renaturation of the denatured PLC was examined by diluting the PCL solution containing 2.8 M GuHCl ten times, the fluorescence intensity being compared before and after the dilution with correction of the dilution factor.

CD spectra were obtained with a spectropolarimeter, Jasco J-720M, using a quartz cell with a 1 mm-light path and 13.5 μM PCL.

The buffers used were PIPES (pH 7), phosphate (pH 6, 7), acetate (pH 4-4.5), citrate (pH 2.6-3.6), and glycine (pH 2-3), the concentration being 20 mM. HCl (0.1 N) was used as the pH 1 solution. Except in acid-induced denaturation experiments, the fluorescence and CD spectra of PCL were observed at pH 7.

To remove the bound Ca^{2+} ion from the enzyme molecule, the PCL solution (approximately 50 μM) was kept with 10 mM EDTA for an hour at pH 7, and then dialyzed several times against 20 mM PIPES buffer without EDTA.

RESULTS AND DISCUSSION

Acid Denaturation of PCL—The fluorescence spectrum of the native PCL (Fig. 1A) has a peak at 332-333 nm with both $\lambda_{ex} = 280$ and 295 nm, which suggests that tryptophan residues are shielded from a solvent in the native state, as is expected from the three-dimensional structure of PCL (*8, 9*). The fluorescence intensity decreased with decreasing pH (solid lines in Fig. 1A). Around pH 1-2.6, the fluorescence intensity was approximately 40% of that observed at pH 7. In the acidic region, the fluorescence peak slightly shifted to the longer wavelength side by 2-3 nm. The dependence of the fluorescence intensity at 332 nm on pH is shown in Fig. 2A.

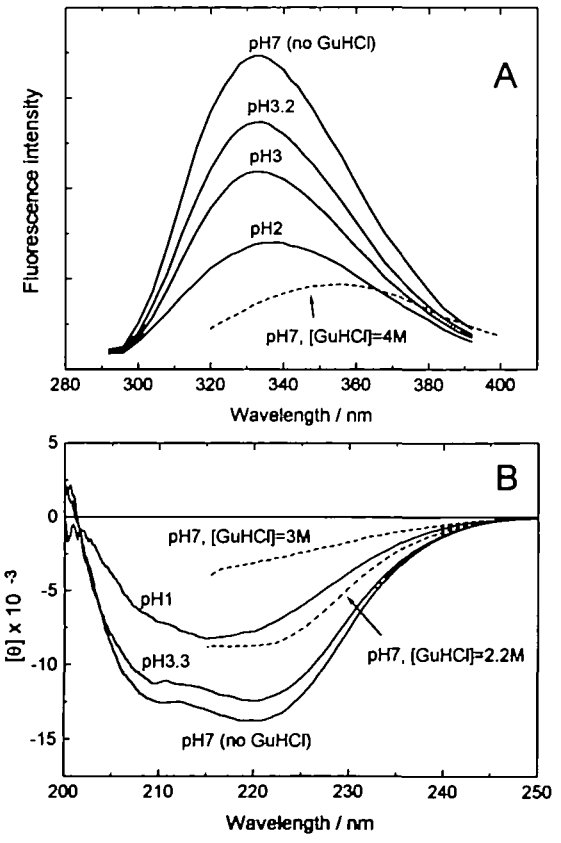

Fig. 1. **Acid and guanidine hydrochloride-induced denaturation of PCL observed as to fluorescence (A) and CD (B).** The concentrations used were 0.17 (A) and 13.5 μM (B). Observed at 25°C. Solid lines are fluorescence/CD spectra observed at various pHs in the absence of guanidine hydrochloride, and dashed lines those observed at pH 7 in the presence of the denaturant.

The magnitude of the ellipticity $[\theta]$ at 222 nm of the CD spectrum of PCL decreased with decreasing pH, as shown in Fig. 1B (solid lines), and percent values of $[\theta]$ relative to that observed at pH 7 were plotted against pH (Fig. 2B). The pH-profiles of the fluorescence intensity and ellipticity were almost the same, and it has been shown that PCL is stable at >pH 4 but is denatured at <pH 2.5.

GuHCl-Induced Denaturation of PCL—Fluorescence and CD spectra of PCL were obtained in the presence of various concentrations of GuHCl at pH 7 (*cf.* dashed lines in Fig. 1, A and B). Figure 3 shows the degree of GuHCl-induced denaturation observed as the fluorescence intensity at 332 nm and as the CD signal at 222 nm at 25°C. These data were obtained after keeping the PCL solution with GuHCl at pH 7 for approximately 1 h and for 1 day, respectively, at 25°C. One hour after the addition of GuHCl, PCL was stable up to 1-2 M GuHCl, but was denatured above 3 M GuHCl. It can be seen that there is a slow denaturation process in the presence of 1-3 M GuHCl. When PCL solutions were diluted ten times with the buffer after being kept with 2.8 M GuHCl for 0.5-5 h in the absence and presence of Ca^{2+} (50 μM), no prominent recovery of the fluorescence intensity was observed, suggesting that the denaturation is irreversible as a whole.[2]

The maximum degrees of the changes in the fluorescence intensity at 332 nm and the ellipticity at 222 nm caused by GuHCl, approximately 80% for both (Figs. 1 and 3), were larger than those on acid denaturation, 50-60% (Figs. 1 and 2). The fluorescence spectrum with the higher GuHCl concentration exhibits a peak at around 355 nm (dashed line in Fig. 1A), which is almost the same as that of free tryptophan. This suggests that the acid-induced denatured state of PCL retains the partially folded structures compared with the GuHCl-induced one.

It should be noted that, between 1-3 M GuHCl, the degree of denaturation observed as to the fluorescence was greater than that observed as to CD (Fig. 3). For example, 1 day after the addition of 2 M GuHCl, approximately 80% of the PCL was denatured judging from the fluorescence intensity, but only 35% judging from the CD signal. This difference might be due to that these two probes reflect different states of denaturation of PCL or that the denaturation velocity might be different due to the difference in the protein concentrations used for the observations.

Effect of the Ca^{2+} Ion—To examine the effect of bound Ca^{2+}, the molar ellipticity of PLC after removal of the bound Ca^{2+} by EDTA-treatment was observed in the presence of various concentrations of GuHCl (Fig. 3B). It

[2] The fluorescence intensity of the diluted PCL solution was not stable. When PCL solutions kept with 2.8 M GuHCl for 0.5-5 h were diluted, 1-20% of the fluorescence intensity was apparently recovered right after the dilution, and then it decreased again. The final fluorescence intensity observed a few hours after the dilution was almost the same as that right before the dilution. This might suggest that the denaturation mechanism expressed as Eq. 2 is not sufficient to describe the whole event.

can be seen that PCL was destabilized on the removal of Ca^{2+}.

In the presence of 1 mM $CaCl_2$, on the other hand, PCL was stable up to 2.5 M GuHCl even at 37°C, judging from the fluorescence intensity observed 1 h after the addition of GuHCl. No fluorescence intensity change was seen on the addition of 1 mM $CaCl_2$.

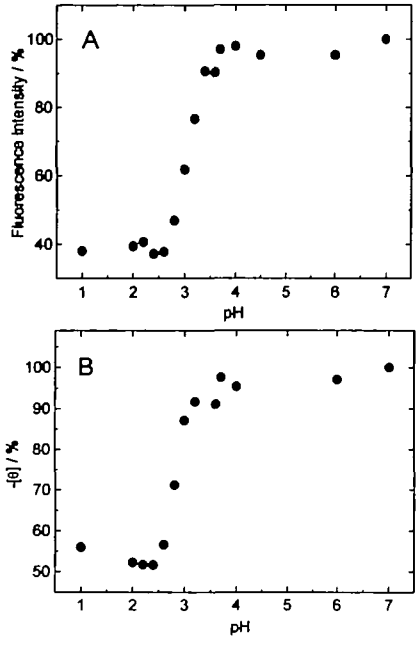

Fig. 2. **Degree of acid-induced denaturation of PCL.** (A) Relative fluorescence intensity values at 332 nm. (B) Relative ellipticity values at 222 nm. Observed at 25°C.

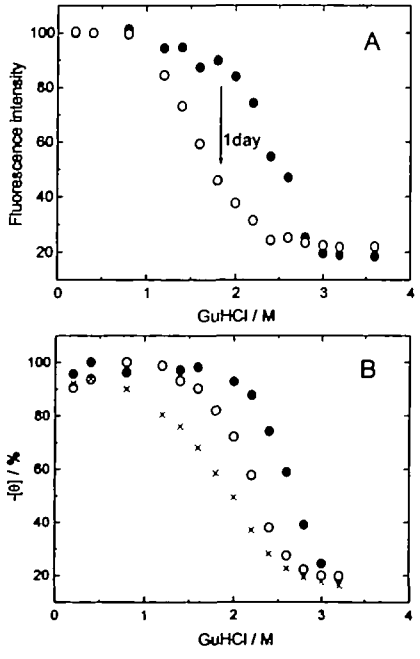

Fig. 3. **Degree of GuHCl-induced denaturation of PCL.** (A) Relative fluorescence intensity values at 332 nm observed approximately 1 h (●) and 1 day (○) after the addition of GuHCl. Observed at 25°C, pH 7. (B) Relative ellipticity values at 222 nm observed 1 h (●) and 1 day (○) after the addition of GuHCl. Crosses (×) indicate those observed after EDTA-treatment (see the text). Observed at 25°C, pH 7.

Denaturation Kinetics—The time-course of the denaturation of PCL by 2.8 M GuHCl was monitored as the decrease in the protein fluorescence in the presence of various concentrations of $CaCl_2$ (Fig. 4A) at 34°C. At each Ca^{2+} concentration, the semilogarithmic plot obtained was not linear, which suggests that the denaturation reaction might be at least biphasic. An increased concentration of Ca^{2+} decreased the apparent rate of denaturation. Although the denaturation of PCL is irreversible, this effect of Ca^{2+} implies that the denaturation is not a simple one-step irreversible process such as $N \cdot Ca^{2+} \rightarrow D + Ca^{2+}$, for which the addition of Ca^{2+} cannot slow the reaction velocity, but includes an equilibrium step, as was suggested by a DSC study (*10*).

One of the minimum mechanisms that can explain the whole denaturation of PCL is as follows:

$$N \cdot Ca^{2+} \underset{v_{-1},\, k_{-1}}{\overset{v_{+1},\, k_{+1}}{\rightleftharpoons}} D_I + Ca^{2+} \qquad (2)$$

$$\downarrow v_2,\, k_2$$

$$D_F$$

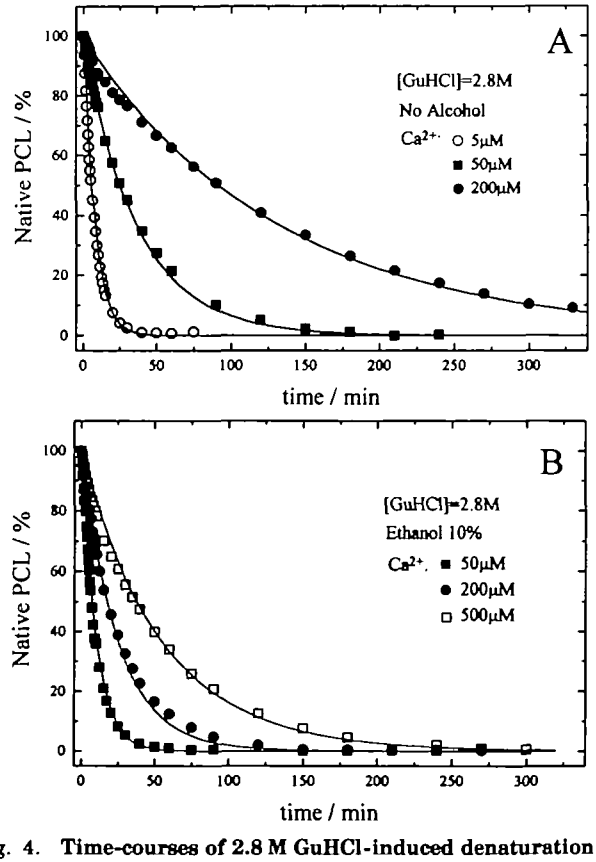

Fig. 4. **Time-courses of 2.8 M GuHCl-induced denaturation of PCL in the presence of various concentrations of Ca^{2+}.** Observed using the fluorescence intensity at 332 nm as a probe in the presence of no alcohol (A) and 10% ethanol (B). Symbols, ■ and ●, are the same in the two figures. Observed at pH 7, 34°C. The solid lines were drawn according to Eqs. 3-8 using k_{+1}, k_{-1}, and k_2 values of 0.226 min^{-1}, 0.368 $\mu M^{-1} \cdot min^{-1}$, and 2.57 min^{-1} (A), and 0.193 min^{-1}, 0.0439 $\mu M^{-1} \cdot min^{-1}$, and 2.35 min^{-1} (B), respectively, with the $[Ca^{2+}]$ values indicated in the figures.

where D_I is a denatured state of PCL that can be refolded to the native state, and D_F is the final and irreversibly denatured state of PCL; v_{+1}, v_{-1}, and v_2 are the reaction velocities, and k_{+1}, k_2, and k_{-1} are the rate constants for the steps indicated. Hereafter, we will refer to the first forward step ($N \cdot Ca^{2+} \rightarrow D_I + Ca^{2+}$), the reverse step, and the second step ($D_I \rightarrow D_F$) as k_{+1}-step, k_{-1}-step, and k_2-step, respectively, for simplicity.

In the presence of a excess amount of Ca^{2+} ions, the k_{-1}-step ($D_I + Ca^{2+} \rightarrow N \cdot Ca^{2+}$) can be regarded as a pseudo-first order reaction, and the concentration of $N \cdot Ca^{2+}$ at time t is given as follows:

$$[N \cdot Ca^{2+}] = (\lambda_1^* - \lambda_2^*)^{-1}[(1-\lambda_2^*)\exp(-\lambda_1^* t^*) + (\lambda_1^* - 1)\exp(-\lambda_2^* t^*)] \times [PCL]_0 \quad (3)$$

where

$$\lambda_1^* = [1 + (k_{-1})_{app}^* + k_2^* + \sqrt{(1+(k_{-1})_{app}^* + k_2^*)^2 - 4k_2^*}\,]/2 \quad (4)$$

$$\lambda_2^* = [1 + (k_{-1})_{app}^* + k_2^* - \sqrt{(1+(k_{-1})_{app}^* + k_2^*)^2 - 4k_2^*}\,]/2 \quad (5)$$

$$t^* = k_{+1} t \quad (6)$$

$$k_2^* = k_2/k_{+1} \quad (7)$$

and

$$(k_{-1})_{app}^* = k_{-1}[Ca^{2+}]_0/k_{+1}. \quad (8)$$

It is assumed that $[N \cdot Ca^{2+}]$ at $t=0$ is the same as the initial (total) concentration of PCL, $[PCL]_0$. The k_{+1}, k_{-1}, and k_2 values were calculated by means of a least squares procedure to minimize the sum of the deviation of the observed fluorescence data from the calculated ones. The k_{+1}, k_{-1}, and k_2 values thus obtained were 0.226 min^{-1}, 0.368 $\mu M^{-1} \cdot min^{-1}$, and 2.57 min^{-1}, respectively. The solid lines in Fig. 4A are the theoretical curves drawn according to Eqs. 3-8 using the values described above and the corresponding concentrations of Ca^{2+}. Agreement between the experimental and theoretical values is satisfactory for the first approximation. Apparent stabilization of the native PCL on the addition of Ca^{2+} is thus explained by the increased velocity of the renaturation (k_{-1})-step, $v_{-1} = k_{-1}[D_I][Ca^{2+}]$. In other words, this stabilization effect of excess Ca^{2+} is a result of Le Chatelier's Law, not intrinsic stabilization of the PCL molecule itself.

The difference in the degree of denaturation between that observed as to the fluorescence and that as to CD (Fig. 3) may be explained similarly as follows: The PCL concentration used for the fluorescence observation was 0.27 μM and that for CD was 13.5 μM. When the concentration of PCL increases, the equilibrium, $N \cdot Ca^{2+} \rightleftharpoons D_I + Ca^{2+}$, shifts remarkably to the native state side because v_{+1} increases proportionally with increasing concentration of PCL ($v_{+1} = k_{+1}[N \cdot Ca^{2+}]$), but v_{-1} increases by the second power ($v_{-1} = k_{-1}[D_I][Ca^{2+}]$), resulting in a slower rate of denaturation.

No detectable renaturation was seen on dilution with 2.8 M GuHCl ten times, as described above, which suggests that the concentration of the intermediate molecule, D_I, is negligibly low during the denaturation reaction. This is consistent with the concentration of D_I calculated with the following equation:

$$[D_I] = (\lambda_1^* - \lambda_2^*)^{-1}[\exp(-\lambda_2^* t^*) - \exp(-\lambda_1^* t^*)] \times [PCL]_0 \quad (9)$$

and the values of the rate parameters. The $[D_I]/[PCL]_0$ value thus calculated is small; the maximum $[D_I]/[PCL]_0$ value is seen at around $t=1$ min, and is 5% at $[Ca^{2+}]=5$ μM and 1% at 200 μM.

The activation Gibbs energy, ΔG^*, of each step in Eq. 2 can be obtained with the following equation:

$$\Delta G^* = RT\ln(k_B T/kh) \quad (10)$$

where R, T, k_B, k, and h are the gas constant, absolute temperature, Boltzmann's constant, rate constant, and Planck's constant, respectively. The ΔG^* values of the k_{+1}-step and k_2-step were calculated to be 90 and 83 $kJ \cdot mol^{-1}$, and those for the k_{-1}-step in the presence of 5 and 200 μM Ca^{2+} to be 84 and 75 $kJ \cdot mol^{-1}$, respectively. Thus, the D_I-state was destabilized by 9 $kJ \cdot mol^{-1}$ by an increase in the concentration of Ca^{2+} from 5 to 200 μM (Fig. 5).

Hobson *et al.* reported (*12, 13*) that a molecular chaperone, Lim, is required to obtain active PCL when it is produced in *Escherichia coli*, although Lim is not required for transcription or translation of the PCL gene. When PCL is denatured with 8 M urea, it can be renatured to yield active PCL only when Lim is present during the renaturation, that is, dialysis against decreasing concentrations of urea. These facts suggest that Lim is essential for the renaturation of PCL from the denatured state, even though guanidine hydrochloride or urea is removed. If we assume that the process of renaturation is the reverse of that of denaturation, $D_F \rightarrow D_I \rightarrow N \cdot Ca^{2+}$ or similar, Lim may act on the $D_F \rightarrow D_I$ process, not on the second reversible process, to yield the intermediate-state molecule, D_I, that can bind Ca^{2+} and is ready to fold into the active state, $N \cdot Ca^{2+}$.

Effects of Alcohols on the Rate of Denaturation—DSC studies have shown that alcohols decrease the denaturation temperature for the thermal denaturation of PCL. We observed the effect of ethanol on GuHCl-induced denaturation. Figure 4B shows the time courses of denaturation of PCL at 34°C observed as the fluorescence intensity change in the presence of 2.8 M GuHCl, 10% (v/v) ethanol, and various concentrations of Ca^{2+}. The k_{+1}, k_{-1}, and k_2 values were determined by the same procedure as described above by assuming a two-step denaturation mechanism (Eq. 2),

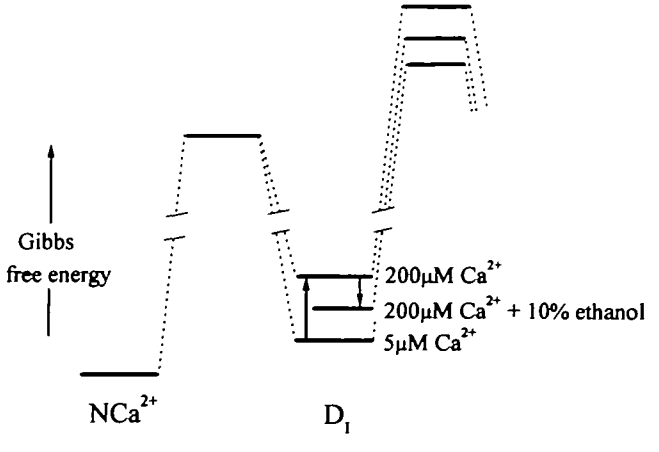

Fig. 5. **Schematic energy diagram of the process of GuHCl-induced denaturation of PCL.** The intermediate, D_I, is destabilized by 9 $kJ \cdot mol^{-1}$ with the increase in the concentration of Ca^{2+} from 5 to 200 μM, as indicated by the upward arrow. Ethanol (10%) in the presence of 200 μM Ca^{2+} stabilizes D_I by 5 $kJ \cdot mol^{-1}$ (downward arrow).

and were found to be 0.193 min^{-1}, 0.0439 $\mu M^{-1} \cdot min^{-1}$, and 2.35 min^{-1}, respectively. The solid lines in Fig. 4B are the theoretical curves drawn using these values. There is no essential difference in the k_{+1} and k_2 values between those obtained in the absence and presence of ethanol, but k_{-1} decreased remarkably on the addition of ethanol, which implies that the D_I-state is stabilized by ethanol. The ΔG^* values for the k_{+1}-step and k_2-step are 90 and 84 $kJ \cdot mol^{-1}$, and that for the k_{-1}-step, in the presence of 200 μM Ca^{2+} for example, is 80 $kJ \cdot mol^{-1}$. Ethanol (10%) thus stabilizes the D_I-state by about 5 $kJ \cdot mol^{-1}$ (Fig. 5). This effect can be explained as follows: The inner hydrophobic residues of the native PCL are exposed to the solvent in the initial step of denaturation into the D_I-state, which is stabilized by the increased hydrophobicity of the solvent on the addition of ethanol.

In conclusion, the native PCL is denatured into the final state, D_F, *via* the intermediate state, D_I. The apparent stabilization effect of the addition of Ca^{2+} is caused by the destabilization of D_I. On the other hand, 10% ethanol is considered to destabilize PCL by stabilizing D_I.

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REFERENCES

1. Antonian, E. (1988) Recent advances in the purification, characterization and structure determination of lipase. *Lipids* **23**, 1101-1106
2. Arbige, M.V. and Pitcher, W.H. (1989) Industrial enzymology: a look towards the future. *Trends Biotech.* **7**, 330-335
3. Gilbert, E.J. (1993) *Pseudomonas* lipase: Biochemical properties and molecular cloning. *Enzyme Microb. Technol.* **15**, 634-645
4. Jaeger, K.-E., Ransac, S., Dijkstra, B.W., Colson, C., Heuvel, M.v., and Misset, O. (1994) Bacterial lipases. *FEMS Microbiol. Rev.* **15**, 29-63
5. Soberon-Chavez, G. and Palmeros, B. (1994) *Pseudomonas* lipases: Molecular genetics and potential industrial applications. *Crit. Rev. Microbiol.* **20**, 95-105
6. Jørgensen, S., Skov, K.W., and Diderichsen, B. (1991) Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: Lipase production in Heterologous hosts requires two *Pseudomonas* genes. *J. Bacteriol.* **173**, 559-567
7. Kim, K.K., Hwang, K.Y., Jeon, H.S., Kim, S., Sweet, R.M., Yang, C.H., and Suh, S.W. (1992) Crystallization and preliminary X-ray crystallographic analysis of lipase from *Pseudomonas cepacia*. *J. Mol. Biol.* **227**, 1258-1262
8. Kim, K.K., Song, H.K., Shin, D.H., Hwang, K.Y., and Suh, S.W. (1997) The crystal structure of a triacylglycerol lipase from *Pseudomonas cepacia* reveals a highly open conformation in the absence of a bound inhibitor. *Structure* **5**, 173-185
9. Schrag, J.D., Li, Y., Cygler, M., Lang, D., Burgdorf, T., Hecht, H.-J., Schmid, R., Schomburg, D., Rydel, T.J., Oliver, J.D., Strickland, L.C., Dunaway, C.M., Larson, S.B., Day, J., and McPherson, A. (1997) The open conformation of a *Pseudomonas* lipase. *Structure* **5**, 187-202
10. Tanaka, A. (1998) Differential scanning calorimetric studies on the thermal unfolding of *Pseudomonas cepacia* lipase in the absence and presence of alcohols. *J. Biochem.* **123**, 289-293
11. Sugiura, M. and Oikawa, T. (1977) Physicochemical properties of a lipase from *Pseudomonas fluorescence*. *Biochim. Biophys. Acta* **489**, 262-268
12. Hobson, A.H., Buckley, C.M., Aamand, J.L., Jørgensen, S.T., Diderichsen, B., and McConnell, D.J. (1993) Activation of a bacterial lipase by its chaperone. *Proc. Natl. Acad. Sci. USA* **90**, 5682-5686
13. Aamand, J.L., Hobson, A.H., Buckley, C.M., Jørgensen, S.T., Diderichsen, B., and McConnell, D.J. (1994) Chaperone-mediated activation in vivo of a *Pseudomonas cepacia* lipase. *Mol. Gen. Genet.* **245**, 556-564