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Differential Scanning Calorimetric Studies on the Thermal Unfolding of *Pseudomonas cepacia* Lipase in the Absence and Presence of Alcohols[1]

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Thermal unfolding of *Pseudomonas cepacia* lipase (PCL) was studied by differential scanning calorimetry (DSC) at pH 7. The peak temperature t_p of the DSC trace increased with increasing concentration of the protein. The DSC traces could be successfully analyzed on the basis of the following mechanism, assuming the dissociation of a calcium ion upon denaturation; N $Ca^{2+} \rightleftharpoons D + Ca^{2+}$, where N and D represent native and denatured states of PCL, respectively. In the presence of 1-5% alcohols (methanol, ethanol, *n*-propanol, and *n*-butanol), t_p decreased with increasing alcohol concentration and longer alkyl chain. In contrast to the case of t_p, the denaturation enthalpy Δh did not depend on the protein concentration or alcohol concentration used. The change in heat capacity on denaturation, $\Delta c_p{}^d$, evaluated directly from the DSC traces, was close to zero both in the absence or presence of alcohol, which could be due to the open conformation of the enzyme exposing a large hydrophobic surface to the solvent.

Key words: alcohol, calorimetry, DSC, *Pseudomonas cepacia* lipase, thermal unfolding.

Lipase [EC 3.1.1.3] hydrolyzes triglycerides to produce free fatty acids and glycerols. This enzyme has industrial versatility, for example, in the detergent and the food industries, and is also useful for the synthesis of fine chemicals of high added value in the pharmaceutical industry (*1-5*). The activity of the enzyme is increased at a lipid-water interface, which is called "interfacial activation." Lipases are active even in organic solvents, and catalyze a wide range of esterification and transesterification under low-water conditions. It is thus interesting to know the effect of the hydrophobic/hydrophilic character of the solvent on the structure and stability of the enzyme.

Various kinds of lipases have been isolated from microorganisms, and the genes of many lipases, including *Pseudomonas cepacia* lipase (*6*), have been cloned. *P. cepacia* lipase (PCL) consists of 320 amino acids with a molecular weight of 33,100, and its three-dimensional structure was elucidated recently by four laboratories (*7-9*). The enzyme molecule has an "open" conformation with a deep cleft. The walls of the cleft surrounding the active site are formed primarily by hydrophobic residues, which are exposed to the solvent. PCL contains a tightly bound Ca^{2+} ion, being six-coordinated by Gln292, Val296, Asp242, Val296, and two water molecules (*9*).

In this study, we observed the thermally induced unfolding of PCL using an adiabatic scanning calorimeter (DSC) at pH 7 in the absence and presence of alcohols with different alkyl chain length to elucidate the mechanism of the unfolding reaction and the effect of alcohols.

MATERIALS AND METHODS

Lipase Sample—A lyophilized preparation of purified *P. cepacia* lipase was a generous gift from Amano Pharmaceutical. The protein concentration was determined spectrophotometrically using a molar absorbance $\varepsilon = 37{,}000$ at 280 nm (*10*). (The microorganism used in Ref. *10* referred to as *Pseudomonas fluorescence*, is now classified as *P. cepacia*.)

Calorimetry—The thermal unfolding of the protein was observed using a DSC instrument, DASM-4 (*11*). A scan rate of 1 K min^{-1} was used throughout. Phosphate buffer, pH 7, at a final concentration of 20 mM was used unless otherwise mentioned. In all experiments the reference cell was filled with the corresponding buffer, plus alcohol if needed, and instrumental base lines were determined with both cells filled with the buffer. The observed DSC data were analyzed after subtraction of the instrumental baseline.

Data Analysis—All the DSC curves were analyzed by a least-squares curve-fitting method based on the procedures outlined by Privalov and Khechinashvili (*12*) and by Sturtevant (*13*) with baselines drawn according to the method of Takahashi and Sturtevant (*14*). The standard deviation of the calculated values from the observed data was expressed as a relative value to the maximal value of the excess specific heat c_{max}.

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Abbreviations: DSC, differential scanning calorimeter (calorimetry); PCL, *Pseudomonas cepacia* lipase; PGL, *Pseudomonas glumae* lipase; CVL, *Chromobacterium viscosum* lipase; BuOH, butanol; EtOH, ethanol; MeOH, methanol; PrOH, propanol.

RESULTS

Thermal Unfolding of PCL and Concentration Dependence—Figure 1 shows DSC traces of PCL observed at pH 7 in the absence of alcohols. A single asymmetric endothermic peak was obtained over the concentration range used (29.3-236 μM; 0.97-7.8 mg/ml). After completion of initial DSC scans, the sample solution was cooled in the calorimeter cell and immediately rescanned to check the reversibility of the unfolding reaction, but no endothermic peak was observed. This irreversibility was expected, since renaturation of PCL activity, after denaturation with guanidine-HCl, requires the presence of a molecular chaperone (*15, 16*).

The peak temperature t_p (in degree Celsius) increased with increasing concentration of the protein (Fig. 2A), indicating either a decrease in the degree of oligomerization of the protein during the unfolding reaction or dissociation of a bound ligand upon denaturation. The latter seems to be the case, since PCL exists as a monomer in solution (*7*) and PCL contains a calcium ion (see "DISCUSSION").

The specific enthalpy of the unfolding reaction, Δh_{cal}, was evaluated from the area of the DSC curves. In contrast to the case of t_p, there was no significant dependence of Δh_{cal} on the protein concentration (Fig. 2B). Δh_{cal} was evaluated to be 25.6±0.8 J·g^{-1} (6.13±0.18 cal·g^{-1}; average and standard deviation of 6 observations), giving a molar calorimetric enthalpy ΔH_{cal} of 849 kJ·mol^{-1} (203 kcal·mol^{-1}). The values of t_p and Δh_{cal} obtained at various concentrations of the protein are summarized in Table I.

The value of Δc_p[d], the heat capacity change upon denaturation obtained directly from DSC traces at t_p, was close to zero; 0.055±0.075 J·g^{-1}·K^{-1}. The temperature dependencies of the apparent heat capacity, *i.e.*, the slope of the DSC scan, of the protein in the pre- and the post-denaturation regions had small positive values.

pH Dependence—The DSC traces were obtained in the acidic pH region. At pHs 4.0 (acetate buffer), 3.1 (citrate buffer), and 2.6 (glycine buffer), t_p was approximately 74, 42, and 32°C at the protein concentration of 90, 100, and 150 μM, respectively. There was no essential difference in t_p between pH 7 and 4, but t_p decreased remarkably between pH 4 and 3. This suggests that protons are taken up by the protein during denaturation in the lower pH region, which could be due to the presence in the native protein of carboxyl groups with low pK values (*17*). No DSC trace could be obtained at around pH 3.5 since the sample precipitated at room temperature.

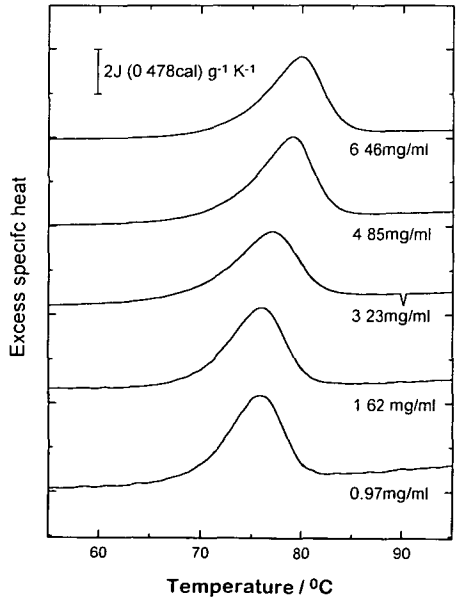

Fig. 1. **DSC traces of the thermal denaturation of *Pseudomonas cepacia* lipase.** Protein concentration is shown in the figure. Observed at a scan rate of 1 K min^{-1} and at pH 7. No alcohol.

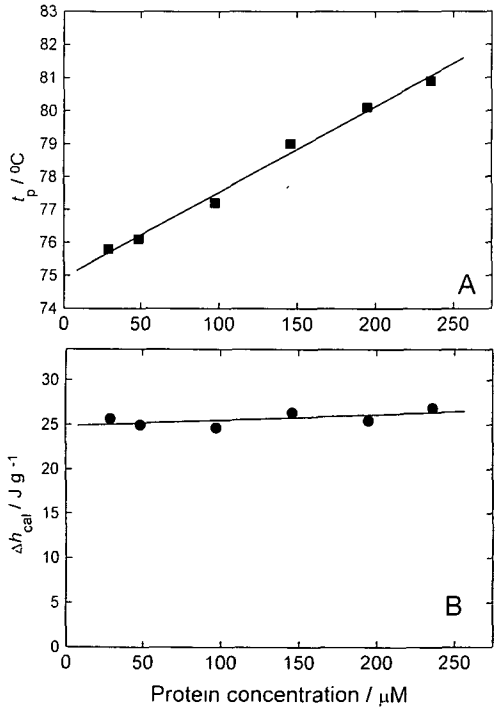

Fig. 2. **Protein concentration dependence of t_p (A) and of Δh_{cal} (B) for the thermal denaturation of PCL.**

TABLE I. **Thermodynamic parameters for the thermal unfolding of *Pseudomonas cepacia* lipase in the absence and presence of alcohols observed at pH 7.0.**

Protein conc. (μM)	No. of expts.	Alcohol conc. (M)	t_p (°C)	Δh_{cal} (J·g^{-1})[a]	ΔH_{cal} (kJ·mol^{-1})[a]	$t_{1/2}$ (°C)	Δh_{cal} (J·g^{-1})[b]	SD (%)
29.3-236	6	–	75.8-80.9	25.6±0.8	849±25	75.2-79.8	24.9±0.9	2.3±0.8
98	5	MeOH 0.24-1.12	76.9-74.6	25.3±0.5	836±15	75.9-73.6	23.7±0.2	3.6±0.7
98	5	EtOH 0.17-0.86	76.7-73.9	25.3±0.9	837±30	75.9-72.6	24.0±0.2	2.7±1.6
98	5	PrOH 0.13-0.67	76.9-70.8	23.7±1.8	784±60	75.7-69.7	22.1±1.2	3.1±1.0
98	2	BuOH 0.11, 0.22	75.6, 74.0	25.0, 22.5	828, 745	73.8, 72.3	22.3, 20.7	6.5, 4.4

[a]Evaluated from the area of DSC curves. [b]Evaluated from the curve fitting.

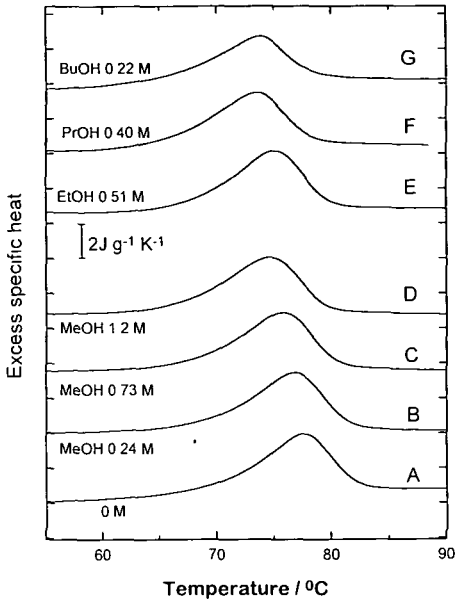

Fig. 3. **DSC traces of PCL in the presence of alcohols.** A-D: Observed at different concentrations of methanol. E-G: DSC traces in the presence of ethanol (E), propanol (F), and butanol (G). Protein concentration was 98.4 μM (3.26 mg/ml) throughout.

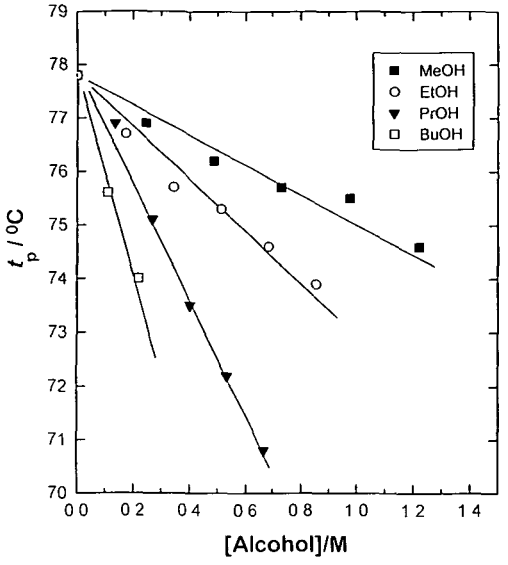

Fig. 4. **Dependence of t_p on alcohol concentration.** ■, methanol; ○, ethanol; ▼, propanol; and □, butanol.

Effect of Alcohols—The thermal unfolding of PCL was observed at constant concentration of the protein in the presence of moderate concentration of alcohols (methanol, ethanol, *n*-propanol, and *n*-butanol) to elucidate the effect of the concentration and the longer hydrophobic alkyl chain of alcohols. Figure 3, A-D, displays the DSC curves at a protein concentration at 98.4 μM (3.26 mg/ml) observed in the presence of 0-1.22 M methanol (MeOH), and Fig. 3, E, F, and G, shows those obtained in the presence of ethanol (EtOH, 0.51 M), propanol (PrOH, 0.40 M), and butanol (BuOH, 0.22 M), respectively. Alcohols decreased the value of t_p, destabilizing PCL, and the degree of destabilization increased with increasing length of the alkyl chain as shown in Fig. 4, where t_p is plotted against the concentration of each alcohol. This phenomenon seems reasonable since, in general, alcohols destabilize proteins by weakening hydrophobic interactions between nonpolar residues and by perturbing the characteristic water structure around the protein molecule (*18, 19*). On the other hand, alcohols in the concentration range used do not change the value of Δh_{cal}. In the case of lysozyme, alcohols decreased the value of $\Delta c_p{}^d$ and significantly increased the temperature dependence of the apparent heat capacity in the pre- and the post-denaturation regions (*19*), but such effects were inconspicuous in the case of PCL at the alcohol concentration used (Fig. 3).

At a high concentration of ethanol (9 M), t_p significantly decreased to 62°C. Protein concentration dependence of t_p in the presence of alcohol was checked at [MeOH] = 1.2 M and at [EtOH] = 0.86 M with [PCL] = 250 μM. Compared with the value obtained at [PCL] = 98 μM, t_p increased by about 4 degrees.

DISCUSSION

Mechanism of the Thermal Unfolding of PCL—The asymmetric feature and concentration dependence of the DSC trace suggest that the mechanism of the thermal unfolding of PCL is not a simple two-state unfolding $N \rightleftharpoons D$, where N and D denote the native and the denatured states of the protein. For the simple two-state mechanism, the van't Hoff enthalpy ΔH_{vH} can be obtained from the following equation (*13*);

$$\Delta H_{vH} = 4RT_m{}^2(c_{max}/\Delta h_{cal}) \quad (1)$$

where R is the gas constant and T_m is the absolute temperature at which the excess specific heat reaches its maximal value c_{max}. The value of ΔH_{vH} thus obtained for the DSC traces in the absence of alcohols was approximately 590 kJ/mol, giving the ratio $\Delta H_{vH}/\Delta H_{cal} = 0.69$, which indicates again that the unfolding is not a simple two-state process.

As described above, the concentration dependence of t_p suggests dissociation of a bound ligand upon denaturation. Since PCL contains a single calcium ion, we can assume the following model,

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

For this type of mechanism, ΔH_{vH} is given by the following equation, similar to Eq. 1 (*13*),

$$\Delta H_{vH} = 5.83RT_m{}^2(c_{max}/\Delta h_{cal}). \quad (3)$$

ΔH_{vH} thus obtained is approximately 850 kJ·mol^{-1}, and hence the ratio $\Delta H_{vH}/\Delta H_{cal} = 1.0$, indicating the validity of Eq. 2.

All the DSC data, including those obtained in the presence of alcohols, were then subjected to numerical analysis based on the mechanism of Eq. 2. The adjustable parameters are $t_{1/2}$, the temperature of half denaturation ([D]/[N] = 1), and Δh_{cal} the specific enthalpy at $t_{1/2}$. The ratio $\Delta H_{vH}/\Delta H_{cal}$ was fixed at unity. An example of the curve resolution is shown in Fig. 5. Values of the adjustable parameters thus obtained are also summarized in Table I. Δh_{cal} did not depend on the concentration or length of alkyl chain of the alcohols, and the average values are listed in the table.

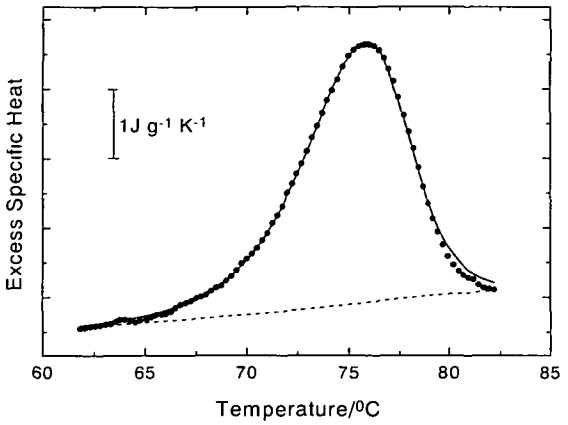

Fig. 5. **An example of curve resolution of a DSC trace of PCL.** Protein concentration; 195 μM. No alcohol. (····) Observed data; (·····) calculated base line; (—) theoretical DSC curve drawn using the following values; $t_{1/2}=75.2$°C, $\Delta h_{cal}=25.9$ J·g^{-1} and $\Delta H_{vH}/\Delta H_{cal}=1$. In this case, the standard deviation of the calculated from the observed data relative to the maximal value of the excess specific heat c_{max} was 1.4%.

The thermal unfolding of PCL was apparently irreversible, but, strictly speaking, the curve-fitting procedure used here is based on the van't Hoff equation and is valid for reversible processes. However, it may be argued that the concentration dependence of t_p would not be observed if the denaturation process were truly irreversible, and the DSC curves were well analyzed by assuming the reversible process shown in Eq. 2. Similar phenomena have been observed with other apparently irreversible unfolding of proteins (*20-22*). Moreover, it was also shown that at least some apparently irreversible denaturations accurately follow predictions expected from equilibrium thermodynamics. It may thus be reasonable to analyze the DSC curves based on the van't Hoff equation as a first approximation, although the validity of the approximation should be confirmed. The values of the thermodynamic parameters may be slightly changed if the reaction rate and the enthalpy change of the irreversible denaturation step that would follow the equilibrium process (Eq. 2) are taken into account.

In the presence of alcohols, when $\Delta H_{vH}/\Delta H_{cal}$ was allowed to vary, there was a tendency for the ratio to be somewhat smaller than unity. This may indicate that, in the presence of alcohols, the unfolding of PCL includes intermediate(s) or there are domains for unfolding in the PCL molecule, but no experimental evidence to distinguish these possibilities is available at present.

Effect of Alcohols—The only conspicuous effect of alcohols on the thermal denaturation of PCL is to lower the denaturation temperature. The value of t_p decreased more or less linearly with increasing alcohol concentration at moderate concentrations of alcohols. This effect increases with the length of the alkyl chain. The values of the slope of the t_p *vs.* alcohol concentration plot (Fig. 4) are approximately −2.7, −4.8, −10, and −19°C/M for methanol, ethanol, propanol, and butanol, respectively. Similar decrease in t_p was observed for the case of lysozyme (*19, 23*), cytochrome *c* (*24*), ribonuclease A (*23, 25-28*), pepsin (*29*), pepsinogen (*30*), orosomucoid (*31*), and luciferase (*32*). The effect is interpreted as follows: increased hydrophobicity of the solvent diminishes hydrophobic interactions between nonpolar groups and stabilizes the unfolded state of a protein. If this is the case, it is expected in general that the value of Δc_p^d is positive and decreases with increasing hydrophobicity of the solvent, since a positive Δc_p^d value is attributed to increased exposure of hydrophobic groups to the solvent resulting from the unfolding (*33*). This is true in the case of lysozyme (*19*). However, the value of Δc_p^d is close to zero in the case of PCL, which could be due to the open conformation of PCL, exposing a large hydrophobic surface to the solvent (*8, 9*). In this connection, it would be interesting to investigate the effect of alcohol and to evaluate the Δc_p^d value of the unfolding of lipases from *Pseudomonas glumae* (PGL) (*34*) and *Chromobacterium viscosum* (CVL) (*35*). Contrary to the case of PCL, PGL and CVL have closed conformations and the molecular surface of these lipases is less hydrophobic than that of PCL, although the amino acid sequence of PCL has 84% identity with those of PGL and CVL, and the structural topology of PCL is similar to that of PGL and CVL (*8, 9*).

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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. Soberón-Chávez, 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. Sugiura, M. and Oikawa, T. (1977) Physicochemical properties of a lipase from *Pseudomonas fluorescence*. *Biochim. Biophys. Acta* **489**, 262-268
11. Privalov, P.L. (1980) Scanning microcalorimeters for studying macromolecules. *Pure Appl. Chem.* **52**, 479-497
12. Privalov, P.L. and Khechinashvili, N.N. (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: A calorimetric study. *J. Mol. Biol.* **86**, 665-684

13. Sturtevant, J.M. (1987) Biochemical applications of differential scanning calorimetry. *Annu. Rev. Phys. Chem.* **38**, 463-488
14. Takahashi, K. and Sturtevant, J.M. (1981) Thermal denaturation of *Streptomyces* subtilisin inhibitor, subtilisin BPN′ and the inhibitor-subtilisin complex. *Biochemistry* **20**, 6185-6190
15. 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
16. 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
17. Anderson, D.E., Becktel, W.J., and Dahlquist, F.W. (1990) pH-Induced denaturation of proteins: A single salt bridge contributes 3-5 kcal/mol to the free energy of folding of T4 lysozyme. *Biochemistry* **29**, 2403-2408
18. Brandts, J.F. and Hunt, L. (1967) The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. *J. Am. Chem. Soc.* **89**, 4826-4838
19. Velicelebi, G. and Sturtevant, J.M. (1979) Thermodynamics of the denaturation of lysozyme in alcohol-water mixtures. *Biochemistry* **18**, 1180-1186
20. Manly, S.P., Matthews, K.S., and Sturtevant, J.M. (1985) Thermal denaturation of the core protein of *lac* repressor. *Biochemistry* **24**, 3842-3846
21. Edge, V., Allewell, N.M., and Sturtevant, J.M. (1985) High-resolution differential scanning calorimetric analysis of the subunits of *Escherichia coli* aspartate transcarbamoylase. *Biochemistry* **24**, 5899-5906
22. Fukada, H., Takahashi, K., and Sturtevant, J.M. (1987) Differential scanning calorimetric study of the thermal unfolding of Taka-amylase A from *Aspergillus oryzae*. *Biochemistry* **26**, 4063-4068
23. Gerlsma, S.Y. and Stuur, E.R. (1972) The effect of polyhydric and monohydric alcohols on the heat-induced reversible denaturation of lysozyme and ribonuclease. *Int. J. Pept. Protein Res.* **4**, 377-383
24. Fu, L. and Freire, E. (1992) On the origin of the enthalpy and entropy convergence temperatures in protein folding. *Proc. Natl. Acad. Sci. USA* **89**, 9335-9338
25. Gerlsma, S.Y. and Stuur, E.R. (1974) The effect of combining two different alcohols on the heat-induced reversible denaturation of ribonuclease. *Int. J. Pept. Protein Res.* **6**, 65-74
26. Gerlsma, S.Y. and Stuur, E.R. (1976) The effects from combining urea and an alcohol on the heat-induced reversible denaturation of ribonuclease. *Int. J. Pept. Protein Res.* **8**, 3-12
27. Brandts, J.F., Hu, C.Q., Lin, L.N., and Mas, M.T. (1989) A simple model for proteins with interacting domains: Application of scanning calorimetry data. *Biochemistry* **28**, 8588-8596
28. Fink, A.L. and Painter, B. (1987) Characterization of the unfolding of ribonuclease A in aqueous methanol solvents. *Biochemistry* **26**, 1665-1671
29. Makarov, A.A., Protasevich, I.I., Frank, E.G., Grishina, I.B., Bolotina, I.A., and Esipova, N.G. (1991) The number of cooperative regions (energetic domains) in a pepsin molecule depends on the pH of the medium. *Biochim. Biophys. Acta* **1078**, 283-288
30. Makarov, A.A., Protasevich, I.I., Bazhulina, N.P., and Esipova, N.G. (1995) Heat denaturation of pepsinogen in a water-ethanol mixture. *FEBS Lett.* **357**, 58-61
31. Kodíček, M., Infanzón, A., and Karpenko, V. (1995) Heat denaturation of human orosomucoid in water/methanol mixtures. *Biochim. Biophys. Acta* **1246**, 10-16
32. Chiou, J.-S. and Ueda, I. (1994) Ethanol unfolds firefly luciferase while competitive inhibitors antagonize unfolding: DSC and FTIR analyses. *J. Pharm. Biomed. Anal.* **12**, 969-975
33. Sturtevant, J.M. (1977) Heat capacity and entropy changes in processes involving proteins. *Proc. Natl. Acad. Sci. USA* **74**, 2236-2240
34. Noble, M.E.M., Cleasby, A., Johnson, L.N., Egmond, M.R., and Frenken, L.G.J. (1993) The crystal structure of triacylglycerol lipase from *Pseudomonas glumae* reveals a partially redundant catalytic aspartate. *FEBS Lett.* **331**, 123-128
35. Lang, D., Hofmann, B., Haalck, L., Hecht, H.-J., Spener, F., Schmid, R.D., and Schomburg, D. (1996) Crystal structure of a bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.6 Å resolution. *J. Mol. Biol.* **259**, 704-717