Pressure Inactivation of α-Chymotrypsin[1]

Yoshihiro Taniguchi* and Keizo Suzuki

Department of Chemistry, Faculty of Science and Engineering, Ritsumeikan University, Kita-ku, Kyoto, 603, Japan
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Pressure effects on the inactivation of α-chymotrypsin (α-CHT) have been examined by measuring the rate of hydroysis of *p*-nitrophenyl acetate (PNPA) catalyzed by α-chymotrypsin at pressures up to 5 kbar and in a temperature range from 20 to 55 °C. At each pressure, the apparent rate increases with increasing temperature and then suddenly decreases above a certain temperature to zero. The increasing rate reflects the temperature dependence of the deacylation and the decreasing rate corresponds to the heat inactivation of α-chymotrypsin, which agrees with other optical methods. In the range from 20 to 50 °C, the activation volumes of deacylation are in the range from −4.4 to −35 cm^3/mol and the volume changes accompanying the pressure inactivation are from −72 to −99 cm^3/mol. These volume changes are considered carefully from the following points of view for volumetric properties of hydrophobic interactions: (1) transfer of a simple solute from water to organic solvents, (2) dimerization of two simple solutes in an aqueous solution, (3) aggregation of a large number of solute molecules in aqueous solution, (4) binary mixture of simple solutes and water, (5) polymers in aqueous solution. These results support the proposal that pressure inactivation is caused by the rupture of hydrophobic interactions among nonpolar groups of α-chymotrypsin (Kauzmann model).

Introduction

Thermodynamic aspects of denaturation of proteins are important to the understanding of the mechanism of denaturation. Volume changes accompanying the denaturation of ribonuclease A,[2] chymotrypsinogen,[3,4] metmyoglobin,[5] and lysozyme[4] in terms of a two-state model have been determined by measuring absorption and fluorescence spectra under high pressure. At 1 atm they are of the order of −50 to −100 cm^3/mol for metmyoglobin, and for other proteins they are of the order of −5 to −70 cm^3/mol. These absolute values are too small to show that pressure denaturation is caused by the rupture of hydrophobic interactions among nonpolar groups if the exposure of each nonpolar side chain of the protein to water brings a contraction of 10–20 cm^3 as shown by Kauzmann.[6] In order to explain this problem, it is necessary to change two viewpoints. One of them is to consider whether the volume change for hydrophobic interactions estimated by Kauzmann[6] is reasonable or not. The other is to apply other physicochemical approaches to the pressure denaturation of proteins.

In the preceding study,[7] the rate of hydrolysis of *p*-nitrophenyl acetate (PNPA) catalyzed by α-chymotrypsin (α-CHT) was measured up to 2 kbar. The rates at 25 and 30 °C by the compression up to 2 kbar. The rates at 25 and 30 °C by the compression up to 2 kbar are accelerated, while the rates at 35 °C are depressed above 1.5 kbar. These results coincide with the change of the fluorescence spectra of α-CHT up to 5 kbar.[8] Therefore, it is possible to approach the pressure denaturation of α-CHT from the measurement of the hydrolysis rate of PNPA under high pressure.

The detailed mechanism of the charge-relay system of esters catalyzed by α-CHT is well-known from kinetic and X-ray studies.[9] The rate-determining step of hydrolysis of PNPA catalyzed by α-CHT at higher concentration of PNPA than that of α-CHT is deacylation.[10] This is the first report of the investigation of the pressure inactivation of an enzyme using a kinetic method. It should be emphasized that the denaturation process can be checked in very dilute enzyme solution (2×10^{-6} M). The rate of hydrolysis of PNPA catalyzed by α-CHT at pH 7.8 in 0.05 M Tris buffer was measured up to 5 kbar at various temperatures.

Experimental Section

Chemicals. The PNPA from Aldrich Chemical Co. was recrystallized twice from hexane, mp 79–80 °C (lit.[10] mp 79.5–80 °C). The α-CHT was obtained from Sigma Chemical Co. (crystallized 3 times and used without additional purification). The acetonitrile was distilled from P_2O_5 before use. The protein concentration at the fluorescence experiments was determined from the absorption coefficient of 5×10^4 M^{-1} cm^{-1} at 282 nm. The enzyme concentration in the kinetic experiments was obtained by titration of PNPA to indicate 83% initial activity. The PNPA concentration was also determined from the absorption coefficient of 1.8×10^4 M^{-1} cm^{-1} at 400 nm of *p*-nitrophenolate ion formed stoichiometrically after basic hydrolysis in 0.1 M NaOH. Both stock solutions of 2×10^{-4} M at pH 4.0 in 0.05 M Tris buffer solution for α-CHT and of 10^{-2} M in pure acetonitrile for PNPA were kept in the refrigerator.

Apparatus and Procedure. A high-pressure vessel of the Drickamer type with two sapphire windows was used for the optical measurements up to 5 kbar. The vessel to be aligned with the light beam was placed in a copper jacket which was maintained at a constant temperature of ±0.1 °C in the range of 20–55 °C by circulating water from a thermostated bath. The hydrolysis was monitored by observing the change of the optical density at 400 nm due to the *p*-nitrophenolate ion by means of a Hitachi-Perkin-Elmer EP 139 type spectrophotometer directly under pressure. The sample cell for the measurement was made of quartz and the pressure-transmitting fluid was

(1) (a) Studies of Polymer Effects under Pressure. Part 7. (b) For part 6, see: Makimoto, S.; Suzuki, K.; Taniguchi, Y. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 1223.
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silicone oil (Shinetsu Chemicals Co.). It was found to be free from absorbance near 400 nm. The 50 μL of acetonitrile solution of PNPA was added to 5 mL of 0.05 M Tris buffer solution (pH 7.8) containing 2×10^{-6} M α-CHT. The enzyme stock solutions were assayed by using PNPA before each experiment. Each solution and the quartz cell were kept at a constant temperature in the thermostated bath prior to mixing. It took about 3–5 min to compress the reaction mixture to a desired pressure. The data were taken during the first 15 min of each run. Fluorescence emission spectra at 280 nm and at 1 atm were measured by a Hitachi 204 A spectrofluorometer with the thermojacket maintained at ±0.1 °C in the range of 20–90 °C.

Results

PNPA is hydrolyzed spontaneously in the absence of α-CHT. The rate of the spontaneous hydrolysis was proportional to the ester concentration. Therefore, at given temperature and pressure, the rate of hydrolysis in the presence of α-CHT $(\text{rate}_{app})_{T,P}$ is separated into two terms, the uncatalyzed $(\text{rate}_w)_{T,P}$ and the α-CHT-catalyzed $(\text{rate}_{cat})_{T,P}$

$$(\text{rate}_{app})_{T,P} = (\text{rate}_{cat})_{T,P} + (\text{rate}_w)_{T,P} \quad (1)$$

The hydrolysis rates $(\text{rate}_{cat})_{T,P}$ for the enzyme reaction were obtained at given temperatures and pressures by means of eq 1. At PNPA concentrations higher than 1.6×10^{-5} M[9], the reaction rate can be considered to be substrate independent and to be proportional to the enzyme concentration [E]. The rate constant $(k_{cat})_{T,P}$ was obtained by means of eq 2. α-CHT-catalyzed hydrolysis of PNPA

$$(k_{cat})_{T,P} = (\text{rate}_{cat})_{T,P}/[\text{E}] \quad (2)$$

follows the pathway of eq 3, where P_1 is *p*-nitrophenol and

$$\alpha\text{-CHT} + \text{PNPA} \underset{K_m}{\rightleftharpoons} \alpha\text{-CHT·PNPA} \xrightarrow[k_2]{} \text{acyl-CHT} + P_1 \xrightarrow[k_3]{} \alpha\text{-CHT} + P_2 \quad (3)$$

P_2 is acetic acid, and the rate-determining step is the deacylation process (k_3).

The temperature dependence of the pH value of Tris-HCl buffer is given to be −0.031 (pK_a) per degree,[11] but the pressure dependence of the pH value is negligible as the volume change of the dissociation process is −1 cm^3/mol.[12] Therefore, the pH effect on k_{cat} mainly comes from the effect of temperature on the pH value of the Tris buffer. The pH dependence of the deacylation follows sigmoid pH–rate profiles for which the pK_a value is pH 6.85 and the rate above pH 7.8 is pH independent. As a pH of 7.8 for the Tris buffer is prepared at 30 °C in this experiment, the effect of pH 7.3 estimated at 50 °C on the rate is only a 10% drop of the rate at pH 7.8.

Figure 1 shows the temperature effect on k_{cat} at pH 7.8 (30 °C) and 1 atm. The values of k_{cat} increase from 0.125×10^{-2} s^{-1} at 10 °C to 1.36×10^{-2} s^{-1} at 40 °C with increasing temperature and then decrease to zero at 57 °C. The reaction temperature is decreased rapidly until 40 °C after the first 15 min of the run at 57 °C, and the activity was checked to be completely recovered; i.e., this process is reversible. The activation parameters of the deacylation process in the temperature range 10–35 °C were $\Delta F^* = 20.68 \pm 2$ kcal/mol (30 °C), $\Delta H^* = 11.4 \pm 2$ kcal/mol, and $-T\Delta S^* = 9.27 \pm 2$ kcal/mol (30 °C), respectively. These values correlate well with $\Delta F^* = 20.4 \pm 1$ kcal/mol, ΔH^* = 9.7 ± 1 kcal/mol, and $-T\Delta S^* = 10.7$ kcal/mol at pH 8.6 (Bender et al.[13]).

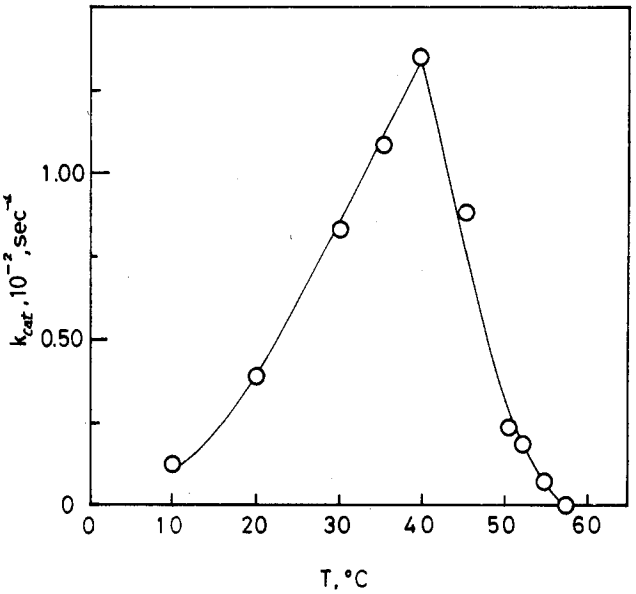

Figure 1. k_{cat} of α-CHT hydrolysis of PNPA vs. temperature at 1 atm. Measurements were obtained with 2×10^{-6} M α-CHT and 10^{-4} M PNPA in 0.05 M Tris-HCl buffer pH 7.8 (30 °C) in 1% acetonitrile solution.

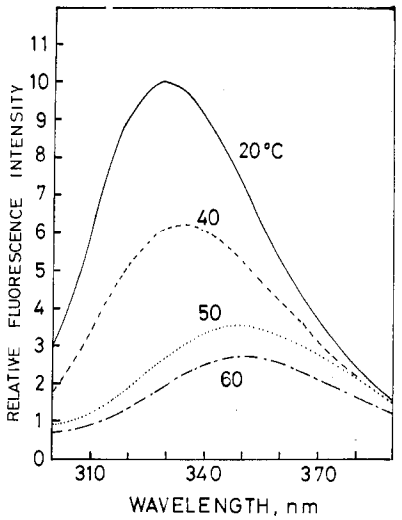

Figure 2. Fluorescence spectra of α-CHT at temperatures in the range of 20–60 °C. Measurements were obtained with 6.4×10^{-5} M α-CHT at 1 atm in 0.2 M Tris-HCl buffer, pH 8.0 (20 °C). The excitation light was at 280 nm.

The fluorescence spectra of α-CHT at pH 8.0 (20 °C) for 0.2 M Tris buffer at various temperatures and at 1 atm are shown in Figure 2. Figure 3 shows the temperature dependence of the emission maximum and the relative fluorescence yield. The emission maximum of the native form of α-CHT due to tryptophan residues appeared at about 333 nm. Between 40 and 57 °C, the spectra shift from 333 to 350 nm. The relative fluorescence yield decreases more rapidly with increasing temperature in this temperature range. Such a change in the fluorescence spectrum corresponds to the unfolding process of the globular structure of α-CHT and is consistent with the phenomenon of the temperature dependence of k_{cat} between 40 and 57 °C as shown in Figure 1. Therefore, it is concluded that the profile of the k_{cat} vs. temperature

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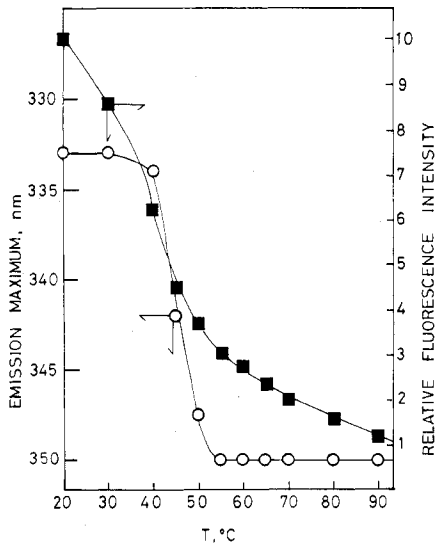

Figure 3. Emission maxima and relative fluorescence yield vs. temperatures.

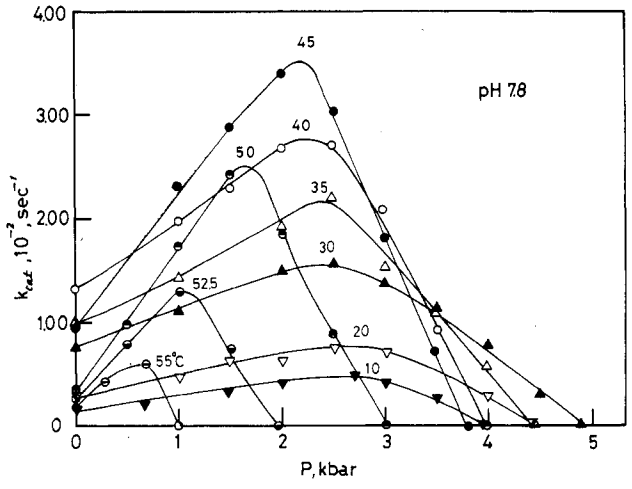

Figure 4. k_{cat} of α-CHT hydrolysis of PNPA vs. pressure at temperatures in the range of 10–65 °C.

plots between 40 and 57 °C of Figure 1 is the reversible heat denaturation of α-CHT. If one applies the process between 40 and 57 °C to the transition of a two-state model, native proteins (N) ⇌ denatured protein (D), the equilibrium constant K_{eq} can be determined from the relation

$$(K_{eq})_{T,P} = [(k_{cat}^{max} - k_{cat})/(k_{cat} - k_{cat}^{min})]_{T,P} \quad (4)$$

where the k_{cat}^{min} value for inactivity of α-CHT was zero and k_{cat}^{max} was the value at the optimum temperature (Figure 1) or pressure (Figure 4). As a result, the equilibrium constant at each temperature and pressure is given by

$$(K_{eq})_{T,P} = [(k_{cat}^{max} - k_{cat})/k_{cat}]_{T,P} \quad (5)$$

According to the temperature dependence of k_{cat} at the transition region (40–57 °C) at pH 7.8, the enthalpy change ΔH^{eff} for the denatured process from van't Hoff plots was 83 ± 10 kcal/mol, which is consistent with the value of 87 ± 10 kcal/mol obtained by the temperature effect on the spectral shift (Figure 3). These data are also in good agreement with those of the solubility (82 kcal/mol), optical rotatory dispersion (84 kcal/mol), and difference absorption spectra (83–90 kcal/mol) at pH 2.0 and around 30 °C.[14] From calorimetric studies, the ΔH^{cal} values of

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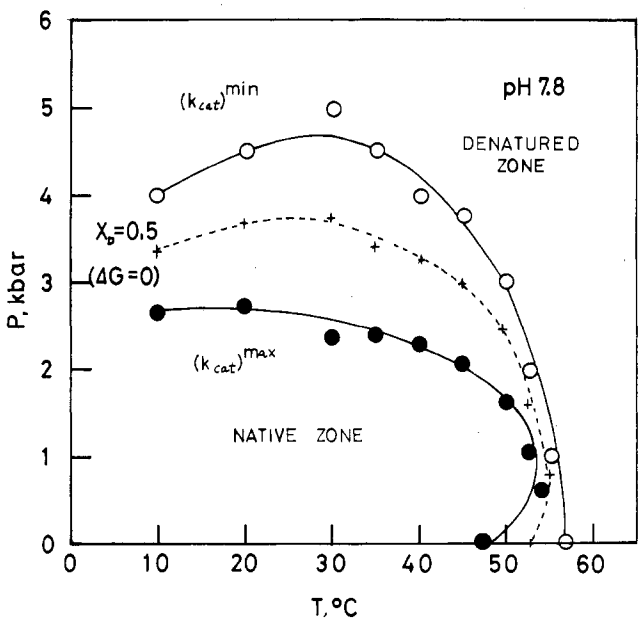

Figure 5. Contours of constant fraction denatured X_D, on the pressure–temperature plane.

TABLE I: Volume Changes for Pressure Inactivation of α-Chymotrypsin and Activation Volumes for the Deacylation Process at pH 7.8 in 0.05 M Tris Buffer and Various Temperatures

temp, °C	20	30	35	40	45	50
$-\Delta V$, cm³/mol	72	44	51	76	85	99
$-\Delta V^*$, cm³/mol	4.4 (3)[a] (6.0)[b]	6.3 (4)[a]	6.4 (4)[a]	6.5	13	35

[a] Reference 7. [b] Reference 17.

110[15] and 110–140 kcal/mol[16] are larger than those from the van't Hoff plots. As the denaturation of α-CHT can be regarded as a two-state model, we apply the transition region of the k_{cat} vs. pressure plots in Figure 4 to a two-state model using eq 5.

The contours of the constant fraction X_D denatured on the pressure–temperature plane for α-CHT are given in Figure 5. The free energy difference between two states, $(\Delta G)_{T,P}$, is calculated from $(\Delta G)_{T,P} = -RT \ln (K_{eq})_{T,P}$. Along the constant-pressure axis up to 2 kbar, the free energy difference decreases with increasing temperatures. The entropy change associated with the transition must be positive. As the slope of $(dP/dT)_{\Delta G=0}$ at the $\Delta G = 0$ ($X_D = 0.5$) contour at 1 atm is positive, the volume change for the denaturation at 1 atm is also positive following the Calusius–Clapeyron equation. The $(dT/dP)_{\Delta G=0}$ curve at 1 kbar shows zero and then changes negative above 1 kbar. At higher pressures up to 3 kbar and at lower temperatures below 30 °C the transition point at $\Delta G = 0$ increases with increasing temperatures. The maximum point exists at a temperature between 20 and 30 °C and at a pressure between 3.5 and 4.0 kbar, respectively. This region shows both ΔV and ΔS as negative.

The volume change ΔV for the pressure denaturation of α-CHT and the activation volume for the deacylation process are calculated from the relations

$$(\partial \ln K_{eq}/\partial P)_T = -\Delta V/RT \quad (6)$$

$$(\partial \ln k_{deacyl}/\partial P)_T = -\Delta V^*/RT \quad (7)$$

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TABLE II: Volume Changes for Pressure Denaturation

protein	$-\Delta V$, cm^3/mol	ref
ribonuclease A	4.5 (55 °C)-46 (24 °C)	2
α-chymotrypsinogen	14.3 (0 °C), 31.2 (23 °C)	3, 4
lysozyme	19.7 (23 °C)	4
metmyoglobin	51-114	5
FMN-binding protein	74 (23 °C)	18
flavodoxins	63-74 (23 °C)	19

where T is the absolute temperature and R is the gas constant. The volume parameters in Table I were given from the straight line of the plots of ln K_{eq} or ln k_{deacyl} vs. pressure within experimental error in the range from 20 to 50 °C.

Discussion

Pressure Effects on Deacylation. The activation volumes (ΔV^*_{deacyl}) accompanying the deacylation of acyl-α-CHT come mainly from the rupture of the hydrophobic interaction between the alkyl chain of acyl-α-CHT and the hydrophobic cavity of α-CHT.[6] The activation volumes decrease with increasing temperatures. Such a small negative value of $\Delta V^*_{deacyl} = -35$ cm^3/mol at 50 °C can never be explained simply by the hydrophobic interaction between acyl groups and the active center of the enzyme. The expected values accompanying the rupture of the hydrophobic interaction were from −4 to −6 cm^3/mol.[6,17] Therefore, above 40 °C the decrease in the activation volume may be expected to come from the conformational change of α-CHT.

Pressure Inactivation. It is generally accepted that the volume change for the pressure-induced denaturation of globular proteins does not take a value below −100 cm^3/mol as shown in Table II. This fact indicates an exposure of only 2–10 groups if the exposure of each nonpolar aliphatic side chain of the protein brought about a contraction of 10–20 cm^3/mol. According to Kauzmann's estimation,[6] this number seems too small to explain protein denaturation. We know that the thermodynamic properties of thermal denaturation of proteins are quite similar to those of the solution process of a nonpolar molecule in water.[20] So it is believed that the native protein in aqueous solution is stabilized chiefly by hydrophobic interactions. This discrepancy in thermodynamic parameters from the effects of both pressure and temperature confuses protein chemists. In order to explain this problem, it is necessary to accumulate volumetric data for pressure denaturation observed by other physicochemical methods. We also doubt whether the dissolution process of a hydrocarbon like methane in water is truly the model of protein denaturation. Recently, considering a conformational change of a biopolymer, Ben-Naim[21] pointed out that there are two kinds of processes. One of the simplest processes is the transfer of a simple solute like methane from a nonpolar liquid such as n-hexane to pure water. The other is the dimerization or aggregation processes of solute molecules from monodispersion in water. At this time, it is important to check carefully the volume change for each process from the data of density measurements and the effect of pressure on these processes.

(1) It has been believed that the volumetric model system for hydrophobic interactions is the transfer process of methane or ethane in hexane into pure water, and that the volume changes are −23 cm^3/mol for methane and −18 cm^3/mol for ethane on the basis of density measurements of aliphatic hydrocarbons (gas) in water.[22] In 1965, Friedman and Scheraga[23] measured the density of several aqueous aliphatic alcohol solutions and showed that the volume changes were −1.1 to −1.3 cm^3/mol for CH_2 and −1.9 to −2.3 cm^3/mol for C_2H_4 in the range of 1–50 °C, accompanying the transfer of nonpolar groups from a nonpolar medium into an infinitely dilute aqueous solution. These data are based on the important assumption that the difference between the partial molal volume $\bar{V}_2°$ of aqueous alcohol solutions and the molar volume v of pure alcohol, Δv ($=\bar{V}_2° - v$), contains additive contributions from the polar and nonpolar parts of the alcohol, so a subtraction of two values of Δv between two alcohols with different alkyl chains should give the volume change for the above transfer processes. From the data of the partial molal volume of aqueous sulfopropyldextran solution,[24] the volume change for hydrophobic hydration around the propyl group ($CH_3CH_2CH_2$) was −7.5 cm^3/mol, which is satisfactory enough to be a good correlation to Friedman and Scheraga's data rather than Masterton's. This small value of ca. 1 cm^3/mol per methylene group (CH_2) for the transfer process of the hydrophobic model system is supported by recent measurements of the apparent molal volume of 1-alkanols,[25] α,ω-alkanediols,[25] and ethylene glycol derivatives.[26] The excess partial molal volume $\bar{V}_2^E$ is defined by eq 8,

$$\bar{V}_2° = V + \bar{V}_2^E \tag{8}$$

where V is the molal volume of a pure solute. The $\bar{V}_2^E$ values are interpreted by the above idea about the volume contraction accompanying the structure change of water caused by the hydrophobic hydration. The values of 1-alkanols are −2.5 cm^3/mol for CH_2, −3.5 cm^3/mol for CH_2CH_2, and −4.5 cm^3/mol for $CH_2CH_2CH_2$ at 25 °C, respectively. In the case of n-alkoxyethanol in water,[27] they are −4.1 cm^3/mol for CH_2, −6.2 cm^3/mol for CH_2CH_2, and −7.5 cm^3/mol for $CH_2CH_2CH_2$ at 25 °C, respectively. The last one is in good agreement with the value found by Gekko and Noguchi.[24]

The most interesting and successful quantitative approach to the transfer of a molecule from the gas phase to solution is the scaled particle theory (SPT).[28] The transfer consists of two steps. First, a cavity is created in the solvent of the proper size to accommodate the solute molecule. Secondly, the solute is introduced into this cavity and interacts with the solvent molecules. The $\bar{V}_2°$ is defined by the SPT expression

$$\bar{V}_2° = \bar{V}_{cav} + \Delta\bar{V} + \beta_T RT \tag{9}$$

where $\bar{V}_{cav}$ is the partial molal volume associated with cavity formation, $\Delta\bar{V}$ is the partial molal volume contribution from solute–solvent interactions, and $\beta_T RT$ (β_T is the isothermal compressibility of the pure liquid, R is the gas constant, and T is the absolute temperature) arises

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from the change in the standard state between the gas and the solution. As $\Delta\bar{V}$ values in water were 0.8 cm^3/mol for CH_4 and −0.5 cm^3/mol for C_2H_6, the $\Delta\bar{V}$ values for the formation of the hydrophobic hydration for methane or ethane may be less than 1 cm^3/mol. Other interesting results also show that the $\Delta\bar{V}$ values accompanying the transfer of argon into various solvents are in the range of 1.7 cm^3/mol for H_2O to −34.3 cm^3/mol for n-C_7F_{16} (−15 cm^3/mol for n-hexane). As $\bar{V}_{cav}$ strongly depends on the solvent mediums, it is meaningless to select n-hexane for the transfer process of hydrophobic solute from nonpolar medium to pure water.

To study the effect of pressure on the solubility of hydrophobic solute in water is one way to obtain volumetric information on transfer processes. The relationship between the solubility and the volume change is defined by

$$(\partial \ln m_s/\partial P)_T = -\Delta V/RT = -1/RT((\bar{v}_2)_p - v_p) \quad (10)$$

where m_s is the molar concentration in the saturated solution, v_p is the molar volume of pure organic liquids, and $(\bar{v}_2)_p$ is the partial molar volume of organic molecules in water at given pressure and at a constant temperature. Also, each molar volume is approximately given by the following equations related to the corresponding isothermal compressibility $(\beta_T)_1$ and isothermal partial molar compressibility $(\bar{\beta}_T)_1$ at 1 atm:

$$v_p = v_1[1 - (\beta_T)_1 P] \quad (11)$$

$$(\bar{v}_2) = (\bar{v}_2)_1[1 - (\bar{\beta}_T)_1 P] \quad (12)$$

The volume changes for the transfer processes of benzene and toluene into water at 1 atm were −4.5 cm^3/mol[29] at 35 °C and −9.6 cm^3/mol[29] at 25 °C, respectively. Kliman[30] carefully studied 4-octanone ($CH_3CH_2CH_2CH_2COCH_2CH_2CH_3$) and got −30 cm^3/mol at 1 atm and 25 °C. The solubility datum of benzene is similar to the −6.4 cm^3/mol given by the density measurement.[22] When we adapt the solubility data to the additive rule, $\Delta V(C_6H_5CH_3) = \Delta V(C_6H_6) + \Delta V(CH_3)$, the volume contraction ΔV_{CH_3} per methyl group is −5.1 (= −9.6 − (−4.5)) cm^3/mol, which is reasonable for the density data of n-alkoxyethanol derivatives.[27] Compared with toluene and 4-octanone containing the same hydrocarbon number, we notice that ΔV values for hydrophobic probes with benzene rings are smaller than those for alkyl hydrocarbons. This fact tells us the clear differences of the hydrophobic hydration between the alkyl chain and the aromatic ring. As the volume changes at each pressure accompanying the pressure dependences of the solubility are also defined by the differences between $(\bar{v}_2)_p$ and v_p in eq 11 and 12, the small negative volume changes at 1 atm mean that $(\bar{v}_2)_1$ of the solutions is smaller than v_1 of pure liquids. In general, $(\beta_T)_1$ values of pure nonpolar liquids (1.56×10^{-4} bar^{-1} for isooctane, 1.69×10^{-4} bar^{-1} for n-hexane, 0.98×10^{-4} bar^{-1} for benzene)[28] are more positive than for pure water (0.46×10^{-4} bar^{-1}), so that v_p values of pure organic liquids decrease with increasing pressure. The adiabatic partial molar compressibility $\bar{\beta}_s°$ for 2-butoxyethanol, which has a structure similar to that of 4-octanone, is -0.45×10^{-4} bar^{-1} at infinite dilution at 25 °C,[26] and $\bar{\beta}_s$ at a certain concentration is determined by the linear empirical equation of $\bar{\beta}_s° + bm$, in which b is positive at 12.3×10^{-4} bar^{-1} (ref 26) at 25 °C and m is the experimental concentration. So, $\bar{\beta}_s$ at the concentration of 10^{-2} mol/kg at 25 °C corresponding to the process of the solubility of 4-octanone may exist near -0.33×10^{-4} bar^{-1}, which is corrected to be $\bar{\beta}_T = -0.29 \times 10^{-4}$ bar^{-1} by using the corresponding data of the thermal expansion $\alpha = 0.878 \times 10^{-3}$ deg^{-1} (ref 26) and the heat capacity $C_p = 555.6$ J/(deg mol) of the 2-butoxyethanol aqueous solution at 25 °C.[27] When one uses the $\bar{\beta}_T$ value of 2-butoxyethanol instead of 4-octanone, the pressure dependence of $(\bar{v}_2)_p$ is positive, but that of v_p is negative. This is the reason that the solubility of 4-octanone in water passes through maxima along the pressure axis. The smaller $(\bar{v}_2)_1$ compared with v_1 is due to the partial disappearance of the volume of the solute molecule like methane itself, when the solute molecule is accommodated in the cage of the water structure accompanying the transfer processes. The formation of the cage is supported by the thermodynamic parameters for the dissolution process calculated by SPT and was comparable to experimental values.[31] From the measurement of adiabatic partial molar compressibilities in terms of CH_2 for an alkoxyethanol series in water, Harada et al.[26] observed that the $\bar{\beta}_s°$ increment per CH_2 group is -1.8×10^{-4} bar^{-1} at 25 °C. The small increment of $(v_2°)_p$ per CH_2 under pressure is due to the exposure of part of the solute molecules that disappear in the cagelike open structure as the cage is destroyed by compression.

The effect of pressure on the substrate-binding process of enzyme model reactions following Michaelis–Menten kinetics, such as with micelles,[32-34] is one approach for getting volumetric data on hydrophobic model processes. The including processes of nonpolar parts of substrates from the aqueous medium into the hydrophobic pocket of catalysts correspond to the reverse processes of the transfer of nonpolar molecules from the nonpolar medium into pure water. The volume changes for the incorporated processes of n-alkyl p-nitrophenyl esters are 0 cm^3/mol for methyl, 4 cm^3/mol for ethyl, and 9 cm^3/mol for propyl groups,[32] respectively. These values correlated well with all the data discussed above except for Masterton's.

(2) One of the typical studies of the volume change for the dimerization processes of organic compounds containing nonpolar parts involves the effect of pressure on the dimerization of carboxylic acids in water. The dissociation constants (K_D) of the dimers for the different kinds of n-alkyl groups of carboxylic acids were measured by the conductive method up to about 6 kbar at 30 °C.[35] The volume change accompanying the dimerization in water is given by

$$(\partial \ln (1/K_D)/\partial P)_T = -\Delta V_D/RT \quad (13)$$

The ΔV_D for the dimerization at 1 atm was calculated to be −14 cm^3/mol for formic acid, −13 cm^3/mol for acetic acid, −8.8 cm^3/mol for propionic acid, and −6.2 cm^3/mol for n-butyric acid. If the ΔV_D except that for formic acid is assumed to contain additive contributions from the polar (hydrogen bonding) and nonpolar parts (hydrophobic interactions) of carboxylic acids, the decrease in the absolute value of ΔV_D with the increase in the alkyl chain length must be attributed to hydrophobic interactions. That is, the ΔV_D value accompanying the formation of the pairwise hydrophobic interactions is calculated by $\Delta V_{H\phi} = \Delta V_D - \Delta V_D$ (formic acid). The $\Delta V_{H\phi}$ at 1 atm was 1 cm^3/mol for methyl, 5 cm^3/mol for ethyl, and 8 cm^3/mol for propyl

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TABLE III: Volume Changes, ΔV_m, for Micellar Formation of Surfactants and Differences between $\Delta V_m(C_{n+2})$ and $\Delta V_m(C_n)$ at 1 bar

surfactant	temp, °C	ΔV_m, cm³/mol	$\Delta\Delta V_m$, cm³/mol	ref
anionic				
C_8SO_4Na	35 (25)	4.9 (6.3)		
$C_{10}SO_4Na$	35 (25)	7.9 (9.1)	3.0 (2.8)	39[a] (42[b])
$C_{12}SO_4Na$	35 (25)	10.0 (10.8)	2.1 (1.7)	
$C_{14}SO_4Na$	35	11.3	1.3	
C_7COONa	25	9.5		
C_9COOK	25	10.6	1.1	40[b]
$C_{11}COONa$	25	11.0	0.4	
C_9COORb	25	9.7		
$C_{11}COORb$	25	11.2	1.5	40
$C_9COON(CH_3)_4$	25	7.4		
$C_{11}COON(CH_3)_4$	25	9.1	1.7	40
$C_4C_6H_4SO_3Na$	25	11		
$C_8C_6H_4SO_3Na$	25	16	$5/(C_{n+4}\text{-}C_n)$	41[b]
cationic				
$C_9N(CH_3)_3Br$	25	2.5		
$C_{10}N(CH_3)_3Br$	25	3.7	$1.2/(C_{n+1}\text{-}C_n)$	42
$C_8N(CH_3)_3Br$	25	3.8		
$C_{10}N(CH_3)_3Br$	25	6.9	3.1	43[b]
$C_{12}N(CH_3)_3Br$	25	8.5	1.6	
nonionic				
$C_6(OC_2H_4)_6OH$	30	4		44[b]
$C_8(OC_2H_4)_6OH$	30	6	2	
$C_6SO(CH_2)_2OH$	25	3.2		
$C_8SO(CH_2)_2OH$	25	6.3	3.1	43
$C_6SO(CH_2)_3OH$	25	3.0		
$C_8SO(CH_2)_3OH$	25	6.1	3.1	43
$C_6SO(CH_2)_4OH$	25	2.7		
$C_8SO(CH_2)_4OH$	25	5.5	2.8	43

[a] Pressure dependence of cmc. [b] Density measurement.

groups, which increases with increasing alkyl chain length. These data correspond to those of the density measurement of alkanols. The values of ΔV_D for the dyes in water determined by the effect of pressure on the absorption spectra of aqueous dye solution[36] are quite surprising at −10.4 cm³/mol for rhodamine B (RB) and −10.6 cm³/mol for methylene blue (MB) at 20 °C. These values do not agree with the positive sign for the formation of the pariwise hydrophobic interaction. This may be the reason that other factors of electrostatic interactions apart from the hydrophobic interactions predominantly contribute to the ΔV_D values.

(3) There are two kinds of approaches for getting volumetric information accompanying aggregation processes such as micelle formation. One is density measurements of surfactant solutions as a function of concentration. The other is the effect of pressure on the critical micelle concentration (cmc) of surfactant solutions. There are two nice reviews of the properties and micelle formation of surfactant solutions under pressure.[37,38] From these reviews, it is made clear that the volume change ΔV_m for the transfer from a monodispersed state to a micellar state in aqueous solution is positive (i.e., the partial molar volume ($\bar{v}_m$) of the micelle is larger than the partial molar volume ($\bar{v}_s$) of the monodispersed state) and that ΔV_m values from the density measurements nearly coincide with those of the pressure effects. The ΔV_m data presented in Table III carefully compare results obtained by the same experimental methods and the same scientists for a series of surfactants and the average value for 18 data is 2.0 cm³/mol per CH_2CH_2 for the micellar formation. Tanaka et al.[45] studied the pressure effect on the $\bar{v}_m$ and $\bar{v}_s$ of the series of sodium *n*-alkyl sulfates C_nSO_4Na (n = 2–10) as anionic surfactants and *n*-alkyltrimethylammonium bromide C_nMe_3NBr (n = 2–10, only odd) as cationic surfactants at 25 °C. Plots of $\bar{v}_s$ against the length of alkyl chain for both surfactants at each pressure produce a straight line represented by the following expression:

$$\bar{v}_s = \bar{v}_{ion} + \bar{v}_{me}n \quad (14)$$

where $\bar{v}_{me}$ is the partial molar volume per CH_2 group and $\bar{v}_{ion}$ may be assumed to be the contribution of the ionic part which is obtained by extrapolating n to zero. The values of $\bar{v}_{me}$ at 1 atm are 15.4 cm³/mol for C_nSO_4Na and 15.5 cm³/mol for C_nMe_3NBr, respectively. The $\bar{v}_{me}$ values of 15.5–15.4 cm³/mol correspond to the volume contraction of 1 cm³/mol per CH_2 when the molar volume of CH_2 is calculated to be 16.1–16.5 cm³/mol.[46] It is also supported by the molar volume of *n*-alkane (C_5–C_{18}) at 20 °C.[47] This value nearly coincides with the positive volume change of 1.5 cm³/mol per methylene group accompanying micelle formation. The values of $\bar{v}_m$ for both kinds of surfactants decrease with increasing pressure and the partial molar isothermal compressibilities $(\bar{\beta}_T)_m$ are 2.8×10^{-5} bar^{-1} for $C_{10}SO_4Na$ and 3.7×10^{-5} bar^{-1} for $C_{10}Me_3NBr$.[45] The $(\bar{\beta}_T)_s$ of the monodispersion is -0.05×10^{-5} bar^{-1} for $C_{10}SO_4Na$ and 1.2×10^{-5} bar^{-1} for $C_{10}Me_3NBr$[45] due to the small positive or negative pressure dependence of $\bar{v}_s$. The same results were supported by studies of the partial molal

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volume ($\bar{V}$) and adiabatic partial molal compressibilities ($\bar{\beta}_s$) of dodecylhexaoxyethylene glycol monoether ($C_{12}E_6$).[48] That is, the $(\bar{\beta}_s)_s$ value of the monodispersion was -0.52×10^{-5} bar^{-1} and $(\bar{\beta}_s)_m$ of the micelle was 4.2×10^{-5} bar^{-1}. They also reported that the calculated compressibilities of the dodecyl group in the micellar state are in agreement with the model compressibility of *n*-dodecane at 25 °C.[49] This fact supports the idea that the interior of the micelle resembles liquid hydrocarbon. This is why the pressure dependence of $\bar{v}_m$ ($d\bar{v}_m/dP < 0$) is larger than that of $\bar{v}_s$ ($d\bar{v}_s/dP > 0$ or $\simeq 0$). Accordingly, the above results are similar to the inversion phenomena of plots of log cmc vs. pressure, which show maxima around 1 kbar.

(4) There is an alternative approach to the hydrophobic hydration from the volume properties of aqueous mixtures of nonelectrolytes studied by Hvidt et al.[50-54] They argued against the above discussion related to model systems of the unfolding process of the native conformations of proteins. In macromolecular solutions, the local concentrations of the constituent groups of macromolecules never approach zero, so that the volume change for a given group of a folded protein molecule is not comparable with a corresponding volume change accompanying small molecules at infinite dilution. This positive volume change accompanying the rupture of hydrophobic interactions is not in line with general concepts (negative volume change) of aliphatic groups in water, even if it is acceptable that the local concentration of aliphatic groups of unfolded proteins does not approach zero. This can be explained as follows. The decrease of the apparent molar volume of the solute with increasing concentration of alcohols, ketones, or alkyl amides has such a strong concentration dependence that it can hardly be explained by overlapping effects due to cospheres of bulky water surrounding the alkyl groups of the solute molecules. Therefore, they proposed two types of equilibria in dilute aqueous solution. One is the solvation equilibria of alkyl groups (R) between solvated and nonsolvated states: $R + nH_2O \rightleftharpoons R(H_2O)_n$ ($\Delta V > 0$). The other is solute–solute association: $R + R \rightleftharpoons R\cdots R$ ($\Delta V > 0$). With increasing solute concentration both processes are displaced in opposite directions, to the left for the solvation process and to the right for the association. In dilute solutions, the negative volume changes following the displacement of the desolvation process of alkyl groups with increasing solute concentration appear to be numerically larger than the positive volume changes following the displacement of the association.

The above explanation of the solvation and association equilibria of alkyl groups, however, is not supported by structure studies of clathrate hydrate formation in the binary mixture of *tert*-butyl alcohol (TBA)–water. From ultrasonic absorption studies,[55] there are two relaxation processes involving the formation of clathrate and association reactions among alcohol molecules. At the maximum excess absorptions near $x_{TBA} = 0.067$ corresponding to the minimum partial molal volume, where x_{TBA} is the mole fraction of TBA, the adiabatic volume change for the formation of the clathrate $2[(H_2O)_{25}\cdot TBA]$ is -9 cm^3/mol and that for the association process yielding $(TBA)_3$ is 0.7 cm^3/mol. At the low concentrations below which the concentration shows a maximum excess absorption near $x_{TBA} = 0.067$, each alcohol molecule is enclathrated in a water cage. Therefore, the positive volume change for the formation of the icelike water structure around nonpolar groups in the solvation equilibria proposed by Hvidt et al. must be corrected to small negative values, so that, in the dilute solution near the minimum $\bar{v}_2$, the decrease of $\bar{v}_2$ of the solute with increasing concentrations should be due to the formation of the clathrate and the increase of $\bar{v}_2$ mainly due to the association process. This explanation is also supported in detail by light-scattering studies of binary mixtures of TBA–water.[56] In the low concentration range below $x_{TBA} = 0.045$, the solution only consists of the mixtures composed of $(H_2O)_r$ ($1 \le r \le 20$) and $(H_2O)_{21}\cdot$TBA. Near the minimum $\bar{v}_2$, the mixtures are composed of $5[(H_2O)_{21}\cdot TBA]$ and $(TBA)_3$. In the concentration range above the minimum $\bar{v}_2$, the associated complex $5[(H_2O)_{21}\cdot TBA]$ breaks down gradually with an increase of x_{TBA}. Considering the experimental data of the apparent molar volume (ϕ_v) as a function of the TBA concentration in dilute solution just below $x_{TBA} = 0.05$, the negative strong concentration dependence of ϕ_v is due to the formation of clathrate hydration ($\Delta V < 0$). This minimum point is also quite sensitive to change in temperature or pressure.[57] When one increases the temperature and pressure, the absolute values of ϕ_v increase at the minimum point and with increasing alkyl chain length of the alcohols.

TABLE IV: Volume Changes for Hydrophobic Hydration of Macroions at 25 °C[a]

macroion	$-\Delta V_{H\phi}$, (cm^3/mol)/CH$_2$CH
NaPAA ($\alpha = 1.0$)[b]	17.5
KPMA[c] ($\alpha = 0.95$)	9.1
PEI	21.5
NaPS	6.0
NaPES	20.0

[a] Reference 58. [b] α is the degree of dissociation. [c] Potassium polymethacrylate.

(5) The hydrophobic effects in polymer solutions have been studied by Ise and Okubo.[58] They measured the densities of aqueous solutions of polyelectrolytes of polyacrylates (NaPAA)

$$(-CH_2-\underset{\displaystyle COO^-Na^+}{\underset{|}{CH}}-)_n$$

poly(styrenesulfonates) (NaPS)

$$(-CH_2-\underset{\displaystyle C_6H_4SO_3^-Na^+}{\underset{|}{CH}}-)_n$$

poly(ethylenesulfonates) (NaPES)

$$(-CH_2-\underset{\displaystyle SO_3^-Na^+}{\underset{|}{CH}}-)_n$$

and salts of poly(ethylenimines) (PEI)

$$(-CH_2-\underset{\displaystyle NH_2HCl}{\underset{|}{CH}}-)_n$$

by a pycnometric method. It was interesting that ϕ_v values of the polyelectrolytes were found to be practically concentration independent, in contrast to the strong dependence of the partial molal volume of the binary mixtures

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TABLE V: Specific Volumes and Isothermal Partial Specific Compressibilities of Random Coil Polymers[a] and Proteins[b] in Water

polymer	$\bar{v}_{sp}^{\circ}$, cm³/g	$10^5\bar{\beta}_T$, bar⁻¹
hydrophobic		
(hydroxyethyl)cellulose	0.678	1.05
poly(ethylene glycol)	0.846	1.07
poly(vinylpyrrolidone)	0.781	1.04
hydrophilic		
dextran	0.600	0.23 (−0.73)[c]
poly(vinyl alcohol)	0.750	0.65
ionic		
sodium (carboxymethyl)cellulose (0.01 M NaOH, pH 12.0)	0.505	−0.86
sodium polymethacrylate (0.01 M NaOH, pH 12.0)	0.385	−6.48
native		
ribonuclease A	0.704	0.548
α-chymotrypsinogen A	0.733	0.695
lysozyme	0.712	0.467
β-lactoglobulin	0.751	1.18
ovalbumin	0.746	1.21
bovine serum albumin	0.735 (0.738)[a]	1.46 (1.23)[a]
denatured		
ribonuclease A		0.70 (1.5)[d]
α-chymotrypsinogen A		0.85 (1.57)[e]

[a] Reference 60. [b] Reference 59. [c] Adiabatic compressibility, reference 63. [d] Reference 2. [e] Reference 3.

of alcohol-, ketone-, or amide–water systems.[50,52,54,57] Thus, the observed insensitivity of ϕ_v for the concentration indicates a delicate balance between hydrophobic and electrostrictional effects; the hydrophobic effect tends to lower ϕ_v with concentration and the charge effect operates to cause a positive concentration dependence of ϕ_v. From the partial molal volumes of macroions, $\bar{V}_{H\phi}$ values calculated for the hydrophobic hydration were negative for all polyelectrolytes in the range of −6.0 to −22 cm³/mol as shown in Table IV,[58] where the average value is −17 cm³ per CH_2CH group. This value is much smaller than those of surfactant molecules.

(6) It is also necessary to look at the behavior of the macromolecular configurations in solution in order to understand the mechanism of pressure denaturation. When enzymes are inactivated, the compact globular forms with partial helixes are released to take the random coil form. At the same time, nonpolar side chains in the interior of the molecules are exposed to the solvent water. From the aspect of polymer configuration, such a native structure is unique with a high-density structure in water.[59] On the other hand, the denatured form has a random coil form with a low of nonpolar groups exposed in water, the so-called hydrophobic random polymers. The isothermal partial specific compressibilities $(\bar{\beta}_T)^D$ of two kinds of denatured proteins[2,3,59] are larger than those of native ones (Table V). From the superior studies of the pressure dependence of the partial specific volume $(\bar{v}_{sp})$ of polymers in solution by Andersson,[60] we can get the following rule of behavior of $\bar{v}_{sp}$ and $\bar{\beta}_T$ for three kinds of random coil polymers in water: $\bar{v}_{sp}$, $\bar{\beta}_T$ for hydrophobic > for hydrophilic > for ionic, as shown in Table V. The $\bar{\beta}_T$ values of the denatured form correspond to those of the hydrophobic random coil polymers. From these results we can estimate that both $(\bar{v}_{sp})^D$ and $(\bar{\beta}_T)^D$ values are larger than those of native ones. From the properties of solvent water itself, water molecules behave as a poor solvent for the hydrophobic polymers, and so are a good solvent for the hydrophilic polymers. The relationship of $\bar{\beta}_T$ between good and poor solvents for random coil polymers is supported by the fact that the $\bar{\beta}_T$ of polymers in poor solvents are larger than those in good solvents.[60-62] As a result, we can draw a scheme of the pressure inactivation of enzymes as follows in Figure 6 from the data of α-chymotrypsinogen instead of α-CHT. (i) The $(\bar{v}_{sp})_1^D$ value of the denatured form extrapolated to 1 atm is larger than the $(\bar{v}_{sp})_1^N$ value of the native form. (ii) The pressure where the inactivation begins corresponds to the pressure at the cross point of both linear lines of $(\bar{v}_{sp})_p^N$ vs. pressure and $(\bar{v}_{sp})_p^D$ vs. pressure, where $(\bar{v}_{sp})_p^N$ and $(\bar{v}_{sp})_p^D$ are expressed by eq 15.

$$(\bar{v}_{sp})_p = (\bar{v}_{sp})_1[1 - (\bar{\beta}_T)_1 P] \quad (15)$$

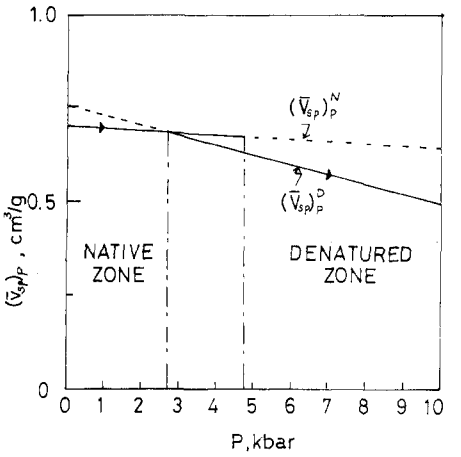

Figure 6. Schematic drawing of the pressure inactivation of α-CHT at room temperature. The pressure inactivation goes along the solid line.

Conclusion

It was established as in the optical methods that the kinetic method is a useful tool in studying reversible enzyme denaturation by heat or pressure. The volume change of −72 to −99 cm³/mol accompanying the reversible pressure inactivation of α-CHT obtained by the kinetic method coincides at −5 to −100 cm³/mol for six proteins already obtained by optical methods. The extent of these volume changes corresponds to an exposure of 20–100 nonpolar aliphatic side chains of proteins at least on the basis of the volume contraction of 1–2 cm³/mol per CH_3 group accompanying the formation of the hydrophobic hydration. These values were supported by hydrophobic model processes containing the transfer of a simple solute like methane in nonpolar solvent to pure water, dimerization of carboxylic acids, and aggregations like micelles. The inversion phenomena of both the transfer and aggregation processes against the pressure were explained by the fact that the molar volumes and the isothermal compressibilities of pure hydrophobic liquids or micelles are larger than the corresponding partial molar volumes of hydrophobic solutes in aqueous solutions. The pressure inactivation of α-CHT was also explained by the differences of $(\bar{v}_{sp})_p$ and $(\bar{\beta}_T)_p$ between native and denatured enzymes if we assume that denatured enzymes are like hydrophobic random coiled polymers. The $(\bar{v}_{sp})_1^N$ value

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for the native enzyme at 1 atm is smaller than that for the denatured one extrapolated from $(\bar{v}_{sp})_p^D$ values for the denatured one at high pressure.

The mechanism of the inactivation of α-CHT is understood as follows. As $(\bar{v}_{sp})_p^N$ values are smaller than $(\bar{v}_{sp})_p^D$ values up to about 3 kbar, the compact globular structure of the active enzyme is not drastically influenced by the compression. Above 3 kbar, where native enzyme is denatured, the order of the values of $(\bar{v}_{sp})_p$ is reversed, i.e., $(\bar{v}_{sp})_p^N > (\bar{v}_{sp})_p^D$, as hydrophobic random coiled polymers (denatured enzymes) are much more compressed than globular structures. The above-described mechanism of pressure inactivation of enzymes agrees with Kauzmann's model in which protein is stabilized by the hydrophobic interaction among nonpolar side chains of protein molecules in water even if we can never accept the volume change of -20 cm^3/mol accompanying the hydrophobic hydration per CH_3 group.

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Dynamics of Nitrate and Carbonate Anions in Aqueous Solutions by Raman Fluctuation Spectroscopy

Michel Perrot,* François Guillaume, and Walter G. Rothschild*†

Laboratoire de Spectroscopie Infrarouge, associé au C.N.R.S., Université de Bordeaux I, 33405 Talence, France
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We have undertaken a Raman study between −10 and +80 °C of the rotational and vibrational relaxation of the C–O and N–O stretching fundamental of the anions of sodium carbonate and nitrate in 1 M aqueous solutions. These planar anions of point group symmetry D_{3h} differ essentially only by their net electronic charge. We find that end-over-end rotation (of the threefold symmetry axis) of the anions does not carry along tightly bound water molecules and does not cause rupture of hydrogen bonds in the surrounding water layers. The correlation time of this orientational motion is four times longer for the carbonate than for the nitrate anion, but has the same activation energy for both. We propose that CO_3^{2-} and Na^+ associate to form a dynamic 1:1 complex of one unit net electronic charge and of a lifetime intermediate to the anionic rotational correlation time (10^{-12} to 10^{-11} s) and the average lifetime of a water cage configuration (>10^{-11} s). From the vibrational relaxation data, we compute a typical rate of fluctuation of molecular positions in such water–anion cage of $\sim 3 \times 10^{12}$ s^{-1}.

Introduction

Improvement in Raman techniques and in the numerical evaluation of spectral data as well as the introduction of useful theoretical models make it now possible to study the dynamics of polyatomic ions in water solution by analyzing their Raman profiles. The method involves numerical Fourier transformation of the spectral frequency data of a convenient vibrational fundamental of the solvated anion into the time domain ("fluctuation spectroscopy"). Thereby, direct information on the mechanisms of anion–water interactions and on the temporal structures of ionic solutions is obtained. No longer is it necessary to extract this information, indirectly, from the profile of the OH-valence motions of the solvent water.[1,2]

We report here our results on the orientational and vibrational dynamics of the anions of sodium nitrate and sodium carbonate in aqueous solutions. Whereas most previous work[3-11] dealt with the concentration dependence of these phenomena, we have examined their temperature dependence, intentionally restricting this study to a fixed, low salt concentration (1 M). In this way band profile changes from increasing anion–cation interactions, such as asymmetries or even splittings, are avoided or, at least, do not introduce additional variables.[11]

We have analyzed the totally symmetric C–O or N–O stretching fundamental (ν_1), near 1050 cm^{-1}, of the respective anion, separating the Raman profile into its anisotropic (rotational–vibrational) and isotropic (vibrational relaxation) contributions by a standard VV–VH polarization experiment.[12]

The two planar anions NO_3^- and CO_3^{2-} essentially differ solely by their electronic charge; our results, which we can only explain by postulating a type of 1:1 dynamic anion–cation association complex between carbonate and sodium ions, are therefore of general interest.

Experimental Section

Solutions. Na_2CO_3 and $NaNO_3$ solutions (1 M) were prepared by weighing. We estimate negligible equilibrium concentrations of CO_3H^-. The solutions were filtered (millipore filter) and placed, within 0.3-cm^3 fused silica cells, into a Coderg cryostat at regulated temperatures between −10 and +80 °C.

†Permanent address: Research & Engineering Staff, Ford Motor Company, P.O. Box 2053, Department of Chemistry, Dearborn, MI 48121.

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