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Two applications of pressure tuning spectroscopy to processes in liquids

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Abstract

In this short review, we describe two problems involving pressure tuning spectroscopy. The first of these concerns reversible pressure induced changes in conformation of the protein lysozyme with consequent changes in its affinity for *N*-acetyl-D glucosamine. The second problem involves the effect of solvent viscosity on the rate of isomerization of 4, *N*, *N*′-dimethylaminobenzonitrile (DMABN) in a series of linear alcohols, isobutanol and glycerol. An important result of this study is that there can be apparent differences in resistance to shear near a solute molecule for solvents with the same bulk viscosity but different geometries. This concept may be extended to other bulk properties like density, compressibility and dielectric constant.

Keywords: Pressure tuning spectroscopy; Reversible pressure; Solvent viscosity; Lysozime; *N*-acetyl-D glucosamine; 4, *N*, *N*′-dimethylaminobenzonitrile

1. Introduction

It is my great pleasure to contribute to this issue honoring Dr. John H. Sinfelt, my former student. The research in my group has, in general, no direct connection with catalysis, but in this paper I discuss two kinds of high pressure experiments which may be of interest to people involved in reactivity and kinetics.

2. Modification of protein conformation by pressure

This experiment involves the use of pressure to bring about reversible changes in protein conformation which, incidentally, effect its enzymatic activity [1,2]. The work was done in collaboration with Professor Gregorio Weber who pioneered in the use of fluorescence as a probe of protein conformation. In earlier studies, Weber demonstrated the nature of changes in protein conformation induced by temperature or pH which are invariably irreversible. We have shown that, under a variety of circumstances pressure can introduce reversible changes [2].

Two kinds of probes are involved: (1) a fluorescent amino acid of which tryptophan is the most useful; (2) a ligand which has drastically different luminescent efficiency when attached to a protein substrate than when free in solution. One such ligand is 1-anilino-8-naphthalene sulphonate (ANS), that has a very low fluorescent yield when free in solution but which emits strongly when attached to a substrate. The structures of these two probes are presented in Fig. 1.

The protein discussed here is lysozyme, a single stranded protein of molecular weight ∼15,000. It is a very efficient catalyst for digesting glucose. It consists of two attached globules (or domains) with a cleft between them. Each domain contains a tryptophan in an organic environment, but the environments differ significantly. In the native protein there is no site at which the ANS can attach.

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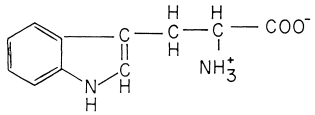

ANS (1-ANILINO-8-NAPHTHALENE SULPHONATE)

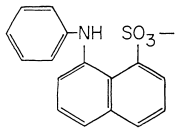

Fig. 1. Structure of Tryptophan and 1-anilino-8-naphthalene sulphonate (ANS).

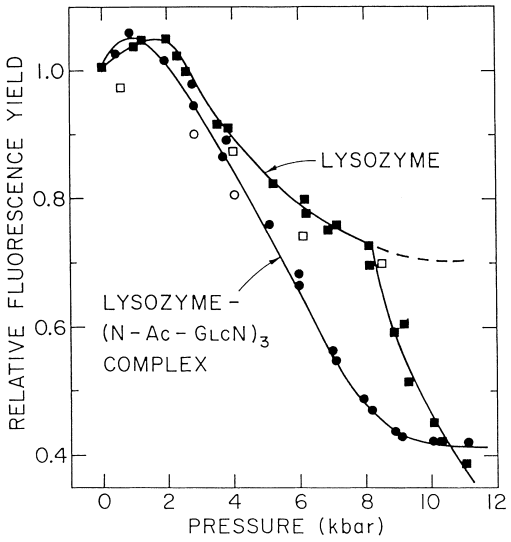

Fig. 2. Pressure dependence of the relative fluorescence yield of lysozyme (■) and lysozyme-(N-Ac-GlcN)$_3$ complex (●). Open symbols were data obtained upon lowering the pressure of the samples. Conditions as in Fig. 1. At 1 atm., the lysozyme-substrate complex has a relative fluorescence equal to 1.4 of the value for lysozyme.

It is well established that free tryptophan emits 4–5 times more efficiently in an organic environment than in an aqueous one [3,4]. In either environment the emission efficiency increases 30–40% in 110 kbar [1].

In Fig. 2 the curve on the right represents the emission efficiency of tryptophan in lysozyme as a function of pressure in a buffered aqueous solution. In the first kilobar the efficiency increases as for free tryptophan, then it decreases in an S shaped curve to about 8 kbar, beyond which pressure the efficiency drops sharply and then again levels. It is reasonable to assume that the behavior between 1 and 8 kbar represents the change in fraction of the tryptophan in one globule or domain exposed to an aqueous environment, while the behavior in the range 8–12 kbar represents the corresponding equilibrium in domain or globule two. With this assumption one can extract equilibrium constants between 'native' and 'denatured' protein in each region as a function of pressure. From the pressure derivatives of these equilibrium constants one can extract the volume change associated with denaturation in each region. In region one $\Delta V \cong -20\,\mathrm{cc\,mol^{-1}}$, while in region two $\Delta V \cong -40\,\mathrm{cc\,mol^{-1}}$. In either case, the fraction of the total volume occupied by the protein is small.

The unfolding is reversible. Immediately after release of pressure the efficiency immediately returns to ~90% of its original value. After about 1–2 h the reversal is complete. It is of interest to note that it takes 12–24 h for the biological activity to be 100% restored, indicating that there are more subtle changes in geometry not detected by the tryptophan probes.

As indicated above, the function of lysozyme as an enzyme is to digest glucose, which it does in a matter of seconds. If *N*-acetyl-D glucosamine (abbreviated as glucosamine) is substituted for glucose the lysozyme will enclose it in the cleft and gradually destroy it, but the process is very inefficient and takes several days. The curve on the left in Fig. 2 represents the change in emission efficiency of lysozyme as a function of pressure in a solution containing glucosamine.

One can represent the process in the presence of glucosamine by the following equations for the equilibrium in regions (1) and (2):

$$K_1 = \left[\exp\left(p_{1/2} - p\right)_1 \frac{\Delta V_1^0}{RT}\right]\left[\exp\left(-\frac{\delta G_1^0}{RT}\right)\right] \quad (1)$$

$$K_2 = \left[\exp\left(p_{1/2} - p\right)_2 \frac{\Delta V_2^0}{RT}\right]\left[\exp\left(-\frac{\delta G_2^0}{RT}\right)\right] \quad (2)$$

Here the first factor in square brackets in each equation represents the equilibrium of lysozyme unattached to the glucosamine. $p_{1/2}$ in each case represents the pressure at which 50% of the tryptophan in region (1) or

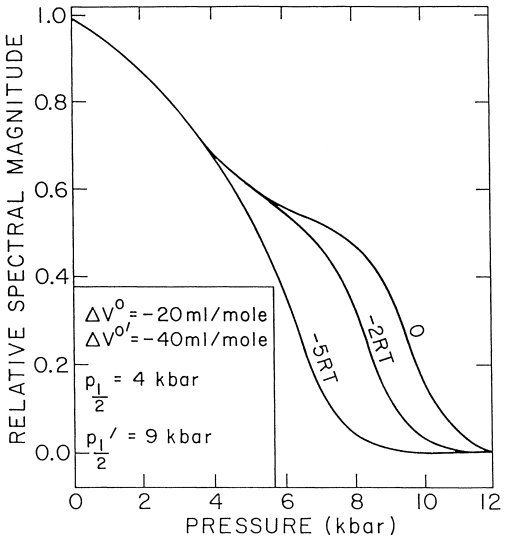

Fig. 3. Relative spectral magnitude S vs. pressure-lysozyme. (Calculated as described in text).

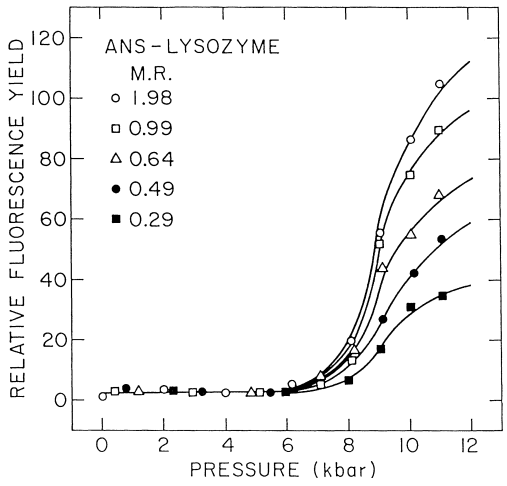

Fig. 4. Pressure dependence of the relative fluorescence yield of 20.3 μM ANS in the presence of (○) 40.3 μM lysozyme; (□) 20.2 μM lysozyme; (△1) 12.9 μM lysozyme; (●) 10 μM lysozyme; (■) 6 μM lysozyme. Conditions as in Fig. 4. M. R.: Molar ratio of lysozyme to ANS.

(2) are exposed to an aqueous environment and $\Delta V_1{}^0$ and $\Delta V_2{}^0$ are the volume changes mentioned above. The second bracket refers to the interaction of domains (1) and (2) with the glucosamine. δG_1^0, and δG_2^0 are the differences in free energy of the 'native' and 'denatured' protein to glucosamine in the two domains.

In Fig. 3, we present calculations for several assumed values of δG_1^0, and δG_2^0. The relative spectral magnitude S is given by:

$$S = \frac{1 + 2\alpha K_1}{1 + 2K_1 + K_1 K_2} \tag{3}$$

where K_1 and K_2 are defined above and α is the fraction converted in the first domain. If one assumes $\delta G_1^0 = \delta G_2^0 = 0$ then, as expected, one reproduces the behavior of free lysozyme in solution. If we assume $\delta G_1^0 = 0$ while $\delta G_2^0 = -5RT$ $(-3\,\mathrm{cal\,mol^{-1}})$, we reproduce the behavior of the protein attached to glucosamine. This implies that in domain (2) the 'denatured' protein binds better to the glucosamine substrate than does the 'native' protein. In a sense, we have created with pressure an enzyme more specific for glucosamine than native lysozyme.

In Fig. 4, we present the emission intensity of ANS in the presence of lysozyme as a function of pressure for various molar ratios of lysozyme to ANS [1,2]. Up to 7 kbar there is negligible emission indicating that no sites are available for ANS to attach to the protein. Above this pressure there is a rapid increase in emission intensity the amount of which depends on the molar ratio mentioned above. In Fig. 5, we plot the relative fluorescence intensity versus lysozyme concentration on the bottom axis and versus molar ratio in the top axis. Clearly just one site opens up for attachment of ANS at high pressure, initially becoming available at ~7 kbar (See Fig. 4). A similar study on the protein chymotrypsinogen indicates that for this protein two sites open up beginning at ~6 kbar [1,2].

The above discussion on abbreviated version of the presentation in [1,2]. A wide variety of high pressure investigations of biological molecules have been undertaken in laboratories around the world. A number of these are reviewed e.g. in [2,5].

3. Macroscopic versus local properties

It is frequently useful to apply theories involving bulk properties (e.g. density, compressibility, viscosity, dielectric constant) to predict the behavior of solute or dopant molecules or ions in liquid or solid solution. The following study [6] shows that this approach may not always be without possible hazard, especially if quantitative relationships are needed.

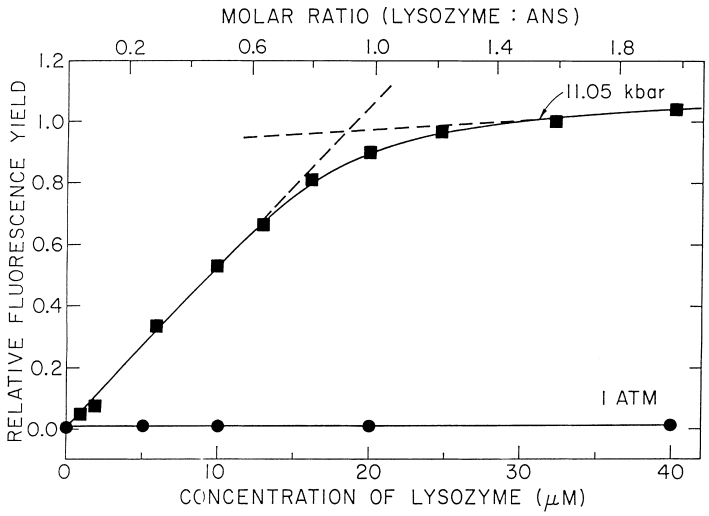

Fig. 5. Relative fluorescence yield vs. concentration of lysozyme at (●) 1 atm.; (■) 11.05 kbars.

The study in question relates the bulk viscosity of a series of solvents to the rate of isomerization of 4, *N*, *N*′ dimethylaminobenzonitrile (DMABN). It is well established [7] that DMABN can exist in the excited state, either as a planar molecule (LE) as in the ground state, or in a charge transfer state in which the dimethylamino group is rotated (TICT). The TICT state fluoresces at ∼20–21 000 cm^{-1} while the LE state emits at ∼26–28 000 cm^{-1}.

In this set of experiments, the relative intensity of emission from the two states is measured as a function of pressure to 10 kbar in three linear alcohols (ethanol, *n*-butanol and pentanol) as well as in isobutanol and glycerol. The glycerol data are not directly germane to our conclusions, but are included for completeness. The relevant physical properties of the solvents are given in Table 1.

The major effect of pressure is to increase the viscosities of the solvents, with a modest increase in polarity as indicated by the change of dielectric constant (ε) and refractive index calculated from the Clausius-Mosotti and Lorenz-Lorentz relationships and the density. The changes of density and viscosity with pressure are taken from the work of Bridgman [8,9].

In Fig. 6 typical spectra in *n*-butanol are given for several pressures. At one atmosphere the emission from the TICT state predominates. With increasing pressure the major change is an increase in the relative intensity of the emission from the LE state. Qualitatively similar behavior is observe in all solvents.

Table 1
Physical properties of solvents[a]

Solvents	ε	n	d	η	$\Delta L'$	$\nu_{(TICT)}$	$\nu_{(LE)}$
Ethanol	25.3	1.360	0.785	0.011	0.78	20.78	27.54
n-Butanol	17.8	1.399	0.810	0.027	0.73	21.04	27.90
iso-Butanol	17.9	1.396	0.803	0.036	0.73	21.05	27.92
Pentanol	13.9	1.409	0.811	0.039	0.69	21.14	27.97
Glycerol	46.5	1.474	1.261	6.25	0.80	20.70	27.43

[a] ε: Dielectric constant; n: Refractive index; d: Density; η: Viscosity (Poise); $\Delta L'$: Solvent polarity parameter; $\Delta L' = [(2\varepsilon+1)/(\varepsilon-1)-1]^{-1} - (1/2)[(2n^2+1)/(n^2-1)-1]^{-1}$; $\nu_{(TICT)}$: Maximum of the TICT fluorescence band (10^3 cm^{-1}); $\nu_{(LE)}$: Maximum of the LE fluorescence band (10^3 cm^{-1}).

The dual fluorescence observed can be described in terms of the model presented in Fig. 7 which is due to Grabowski et al. [10]. The ratio of intensities can be expressed as:

$$\frac{I_{TICT}}{I_{LE}} = \frac{k'_f}{k_f} \frac{k_a}{k'_f + k_{nf} + k_d} \quad (4)$$

where k_a and k_d are respectively, the rates of the forward reaction for forming the TICT state and the rate of backward reaction (TICT → LE). k_f and k_{nf} are the radiative fluorescence and non-radiative decay rates for the LE state and the primed terms are the corresponding rates for the TICT state. It is conve-

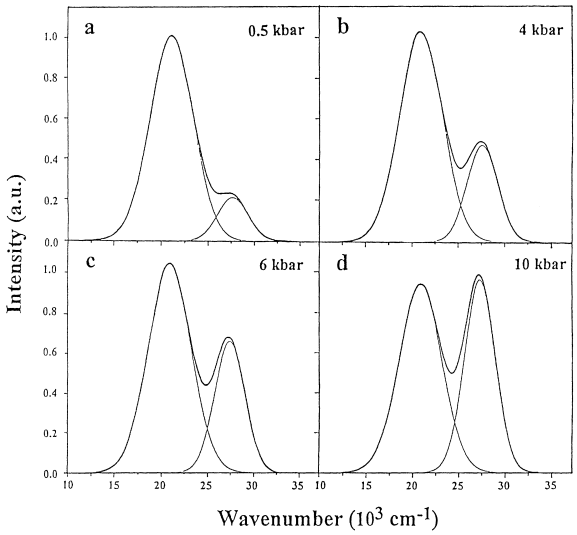

Fig. 6. Fluorescence emission spectra of DMABN in *n*-butanol at different pressures (kbar): (a) 0.5, (b) 4, (c) 6, and (d) 10.

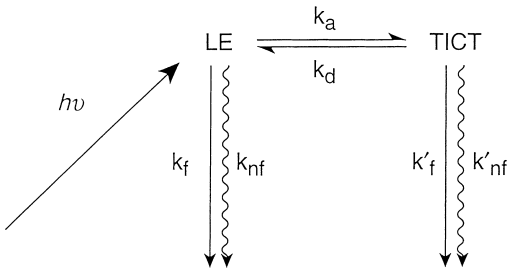

Fig. 7. Scheme of physical and chemical rate processes according to Grabowski et al. [10].

nient to consider two limiting cases: $k_d \gg k'_f + k'_{nf}$ and $k_d \ll \hat{k}'_f + k'_{nf}$. In the former case, the data describe a chemical equilibrium between the two excited states. In this case, since all ethanol has the highest polarity and pentanol the lowest (see Table 1) ethanol should have the highest TICT yield and pentanol the lowest. As seen in Fig. 5 discussed below the order is reversed, so the equilibrium case does not describe the results.

Since the viscosity is quite large it is reasonable to assume that $k_d \ll k'_f + k'_{nf}$. Radiative rates do not vary significantly with pressure especially in this modest pressure range. The peak shifts are small so that, according to the energy gap law [11], one can also assume that k'_{nf} is not significantly pressure dependent. Thus, we can rewrite Eq. (1) as:

$$\frac{I_{TICT}}{I_{LE}} \cong \frac{k'_f}{k_f} \frac{k_a}{k'_f + k'_{nf}} \cong C k_a \tag{5}$$

A recent review of isomerization dynamics by Bagchi [11] indicates that excited state isomerization rate constants depend on the nature of the solvent, its viscosity and the sharpness of the activation barrier. The well established expression for k_a from Bagchi can be written in the form

$$k_a = A\eta^{-\alpha} \exp\left(-\frac{E_0}{k_B T}\right) \tag{6}$$

where A is a viscosity independent constant and E_0 is the intrinsic activation energy. The exponent α is a parameter in the range $0.1 < \alpha < 1$. In barrierless cases $\alpha \to 1$ which would indicate that the rate is viscosity controlled. If $\alpha \to 0.1$ the reaction is essentially barrier controlled.

In Fig. 8, we present plots of $\log(I_{TICT}/I_{LE})$ versus $\log \eta$ for the three linear alcohols. The values of α for all three alcohols are identical and equal to $0.5 + 0.02$. Velsko et al. [12], found $\alpha = 0.43$ for the excited state isomerization of the cyanine dye molecule 3, 3′dicthyloxadicarbocyanine iodide, while Velsko and Fleming [13,14] obtained $\alpha = 0.59$ for the photoisomerization of diphenylbutadiene, so that the values obtained for the linear alcohols are reasonable.

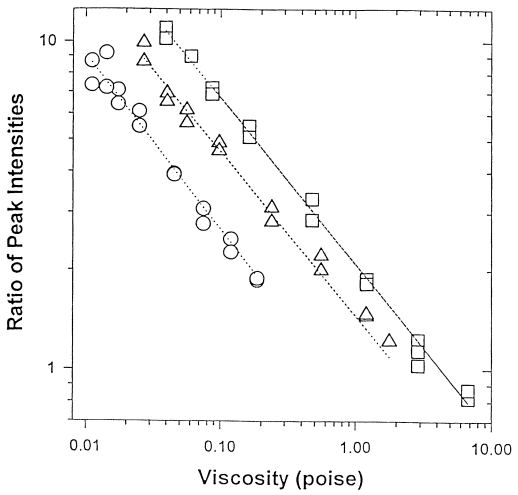

Fig. 8. Viscosity dependence of the ratio of the TICT to the LE intensities of DMABN in (○) ethanol, (△) *n*-butanol, and (□) pentanol. The dashed lines are the fitting results using Eqs. (2) and (3).

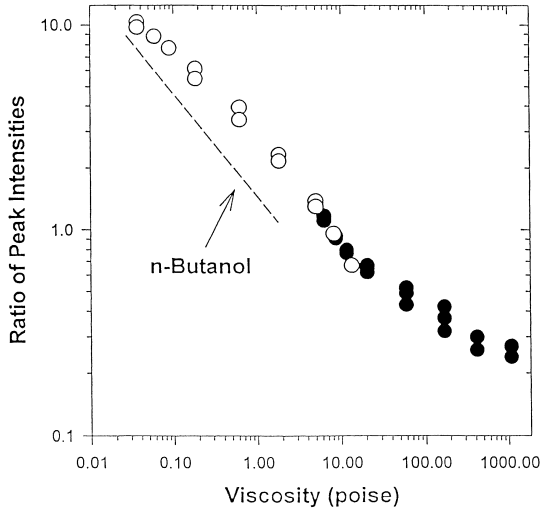

Fig. 9. Viscosity dependence of the ratio of the TICT to the LE intensities of DMABN in (○) iso-butanol and (●) glycerol. The dashed line represents the results for *n*-butanol from Fig. 4.

In Fig. 9, we present results for isobutanol and glycerol together with a dashed line representing the data for *n*-butanol from Fig. 8. In view of the large differences in molecular structure, geometry, bulk detection constant and compressibility for glycerol and isobutanol it is probably safer to attribute the continuity in results between these two solvents to coincidence or cancelling effects of various properties than to attempt a more explicit explanation.

The difference in behavior of *n*-butanol and isobutanol are significant. In the first place, the ratio of intensities is a factor of 1.5–2 greater in isobutanol than in *n*-butanol at the same viscosity. In the second place, the isobutanol results exhibit a marked curvature in contrast to the results for the linear alcohols. From Table 1, we see that the relative parameters ε, n, d and $\Delta L'$ for the two alcohols are essentially identical. The compressibilities of most simple alcohols are nearly identical [9], so the variation with pressure of ε and n should be the same. The viscosity of isobutanol covers a greater range, but the comparison is made at constant viscosity.

It is quite possible that the resistance to shear adjacent to a solvent molecule may differ from the bulk viscosity and that the magnitude of the difference may vary from solvent to solvent. In the same way, the reaction to an elective field for solvent molecules adjacent to a solute molecule may differ from the bulk dielectric constant. Similar arguments apply to density, compressibility and thermal expansion coefficient. The points concerning, ε, ρ, $(-1/\nu\,\delta V/P)_t$ and $(1/\nu\,\delta V/\delta T)_P$ apply also to the properties adjacent to a dopant in a solid.

It is known that theories like Onsager's reaction field model [14] and Marcus's theory of electron transfer [15,16] give semiquantitative agreement with experiment, and that, in particular, Marcus's theory predicts correctly a variety of behaviors not previously anticipated, so that the discrepancies mentioned in the previous paragraph may frequently be second order. Nevertheless, they should be considered whenever one is trying to make quantitative comparisons.

4. Summary

We review here two studies, one involving the reversible change of conformation of a protein molecule with pressure and some consequences for its enzymatic activity, the second demonstrating the potential problems in relating quantitatively bulk properties of solvents to the corresponding properties adjacent to a salute molecule. These are just two out of a very large number of available examples where pressure tuning spectroscopy has revealed new information about molecular behavior and improved our understanding of processes at one atmosphere.

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