Study of Poly-L-lysine Conformations in Aqueous Methanol Solution by Using Polarized Raman Techniques

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Raman polarization measurements of the amide I band are reported in ionized poly-L-lysine dissolved in aqueous methanol. The observed changes with methanol concentration, attributed to changes in coil conformation and to the helix-coil transition, represent a novel method of measuring polymer conformation. Polarization measurements as a function of temperature yield values of the energy differences between rotational isomeric states in the coil, ΔH , of 8.8 ± 0.7 , 10.4 ± 1.1 and 10.8 ± 1.5 kJ/mol at methanol concentrations (v/v) of 85, 80 and 70% respectively. The stabilization energy of the helix is estimated at 9.3 kJ/mol.

Polypeptide conformations in solution have been extensively studied by many different techniques, including c.d. (circular dichroism), o.r.d. (optical rotatory dispersion), Raman scattering and calorimetric measurements (e.g. Chen & Lord, 1974; Chou & Scheraga, 1971; Ingwall *et al.*, 1968; Koenig & Sutton, 1970, 1971; Tiffany & Krimm, 1969, 1972; Wallach *et al.*, 1970; Yu *et al.*, 1973). In some cases (Ingwall *et al.*, 1968; Chou & Scheraga, 1971; Hesselink *et al.*, 1973) the values of thermodynamic parameters governing transitions from random to regular structures have been estimated. The importance of such parameters lies in the close connexion between these model systems and proteins where the analogous quantities are determining factors in biological function. The transition from random coil to α -helical structure has been of particular interest and, in addition to the experimental measurements, there has been considerable theoretical work (Gibbs & di Marzio, 1959; Lifson & Roig, 1961; Nagai, 1960; Zimm & Bragg, 1959; Zimm & Rice, 1960). Despite the success in monitoring phase transitions the various experimental techniques have not so far proved effective in studying the fine details of the coil state. This is a severe limitation, particularly as there has been doubt expressed recently about the conformation of ionized polypeptides, e.g. poly-L-lysine and poly-L-glutamic acid. These were considered to be ideal models of random coils, but the c.d. work of Tiffany & Krimm (1969, 1972) and the calculations of Krimm & Mark (1968) have suggested the possibility of an 'extended-helical' arrangement with approx. 2.7 residues per turn. The purpose of the present paper is to show that changes in the backbone formation of poly-L-lysine cause small, though measurable, changes in the polarization of Raman bands, which may help in understanding the structure

of the ionized polymer as well as providing a method of observing coil-helix transitions.

Depolarization ratios for Raman scattering have previously been used with considerable success to study the conformations of synthetic polymers both in the bulk form and in solution (Hartley & Shepherd, 1976; Maxfield & Shepherd, 1973*a,b*, 1974; Shepherd, 1975; Speak & Shepherd, 1974, 1975). Briefly the basis of the technique is as follows. The environment of the vibrating molecules depends on the rotational isomeric state of the monomer unit and this, in turn, implies slight differences in the differential polarizability tensors that govern the Raman scattering. These differences, though small, can be detected by measuring changes in the polarization characteristics of certain Raman bands. In the case of samples of high optical clarity, where there is no crystal structure, the depolarization ratio, ρ , is the most convenient parameter to measure. This is defined as the ratio of Raman intensities polarized perpendicular and parallel to the incident polarization. [See, e.g., Porto (1965), Wilson *et al.* (1955) and Gilson & Hendra (1970) for a discussion of the theoretical relationship between ρ and the tensor elements.] The connexion between the measured ρ values and the rotational isomeric state populations is not simple, but in particular cases detailed information on the conformations can be obtained under a variety of conditions. [See: Hartley & Shepherd (1976); Maxfield & Shepherd (1973*a,b*, 1974); Speak & Shepherd (1974, 1975); Shepherd (1975).] The present measurements, on poly-L-lysine in aqueous methanol, represent the first application of the technique to a model biological system. This system was chosen for two reasons. First, it was suitable for accurate Raman work, because the sharp and well-characterized spectrum of the methanol provided an internal calibration. Secondly, the

system showed a well-defined transition to α -helix as measured by o.r.d. (Epand & Scheraga, 1968a). Depolarization measurements are presented as a function of methanol concentration and of temperature and are shown to be sensitive to the transition to α -helix and also to conformation in the coil. Under certain simplifying assumptions it has proved possible to measure the energy differences between rotational isomeric states, and, when combined with known enthalpy changes on α -helix formation, to estimate energy parameters of the helix.

Experimental

Relatively polydisperse samples of poly-(L-lysine hydrobromide) (i.e. each residue incorporates an HBr molecule) of nominal mol.wts. 6.5×10^4 and 2.3×10^4 were from Sigma, Kingston-on-Thames KT2 7BH, U.K. The samples were dissolved in $^2\text{H}_2\text{O}$ /methanol with the methanol concentration ranging from 0 to 96% (v/v). $^2\text{H}_2\text{O}$ was used, because the Raman bands of normal water obscure the spectral region of the sharp amide I carbonyl stretching band around 1660cm^{-1} . Some $^1\text{H}_2\text{O}$ is present, owing to exchange of the methanol proton, but the concentration is low. The concentration of poly-(L-lysine hydrobromide) was approx. 3% (w/w). The Raman measurements were carried out on a Cary 82

triple-monochromator system with both argon (514.5 nm; power 0.8 W) and helium/neon (632.8 nm; power 0.05 W) laser excitation. When argon excitation was used count rates of $3 \times 10^4\text{s}^{-1}$ were obtained in the amide I band with resolution of 5cm^{-1} ; the corresponding values for helium/neon excitation were 10^3s^{-1} and 7cm^{-1} . A rotatable polarizer placed before the collecting lens permitted the separate measurement of the scattered signals polarized parallel and perpendicular to the incident polarization and the values of ρ were then obtained from the integrated intensities. Detailed discussions of the experimental techniques of measuring Raman spectra can be found in the literature, e.g. Gilson & Hendra (1970). Temperature control was effected up to 65°C by using a square optical-quality cell, round which was wound a wire heater with necessary insulation. Temperatures below ambient down to -5°C were attained by using cold N_2 gas. The temperature was measured to an accuracy of 0.25°C with a Comark thermometer. Initially the samples showed strong impurity fluorescence. This fluorescence was adequately removed from the poly-lysine solutions by successive batch gel filtration through Sephadex G-25. The poly-lysine solutions were equilibrated with an amount of dry Sephadex so that at equilibrium the volume of the swollen gel was approximately equal to half of the volume of the

Fig. 1. Raman spectrum in the region up to 1700cm^{-1} of a 3% solution of poly-(L-lysine hydrobromide) in $^2\text{H}_2\text{O}$

The intensity units are arbitrary, the temperature was 18°C and the poly-(L-lysine hydrobromide) had mol.wt. 6.5×10^4 .

solution plus gel. The supernatant was removed and equilibrated with a further batch of Sephadex, this procedure being repeated 2–3 times. (The purification was carried out by Dr. M. N. Jones, Department of Biochemistry, University of Manchester, Manchester, U.K.)

Results

The Raman spectrum of 3% (w/v) poly-L-lysine (mol.wt. 6.5×10^4) dissolved in $^2\text{H}_2\text{O}$ in the region up to 1700cm^{-1} is shown in Fig. 1, and is in general agreement with previous work at acidic pH (Yu *et al.*, 1973; Chen & Lord, 1974). The strong, broad band in the region of 1200cm^{-1} is due to $^2\text{H}_2\text{O}$. The broad background signal is believed to be due to fluorescence from impurities that remain even after the gel-

filtration procedure. The amide I band at 1659cm^{-1} , which shifts to 1632cm^{-1} in the helical form, is largely comprised of the carbonyl stretching vibration. Because of its sharp resolution this band was chosen for detailed polarization study. Expanded traces of this band in pure $^2\text{H}_2\text{O}$ solution at room temperature (20°C) are shown in Fig. 2 for scattered polarizations parallel and perpendicular to the incident polarization. The depolarization ratio for this band can be measured to an accuracy of better than 1%. At substantial methanol concentrations the accuracy was nearer 2%. There are two reasons for this worsening, both attributed to exchange of the methanol proton. First, as mentioned above, there will be a small concentration of $^1\text{H}_2\text{O}$, which gives rise to an increased background. Secondly, the amide I band corresponding to a protonated residue appears as a shoulder on the side of the deuterated band and, although the band shapes are the same in either polarization, the signal-to-noise ratio is poorer. The depolarization ratios, measured as a function of percentage of methanol in the solvent, are shown for samples of mol.wt. 6.5×10^4 and 2.5×10^4 in Fig. 3; all measurements were at room temperature. Fig. 4 shows the dependence of ρ on temperature at several methanol concentrations. The value ρ_e is the depolarization ratio in pure $^2\text{H}_2\text{O}$ and was independent of temperature within the experimental errors. There will always be some scattering of both the incident laser light and the Raman scattered light caused by the refractive-index

Fig. 2. Raman amide I band of poly-(L-lysine hydrobromide)

(a) This shows the Raman amide I band of poly-(L-lysine hydrobromide) dissolved in $^2\text{H}_2\text{O}$. The gain for the perpendicular relative to the parallel polarization is $\times 2$. (b) The same band was measured with 80% (v/v) methanol as the solvent in parallel polarization. This shows the distortion of the band shape caused by protonation of the residue, with consequent decrease in signal/noise ratio. The gain is the same as in the parallel band of (a). In both cases the mol.wt. was 6.5×10^4 , the temperature 18°C and the concentration 3%. The noise level (gain $\times 5$) is shown in (c).

Fig. 3. Depolarization of the amide I band of poly-(L-lysine hydrobromide) measured as a function of methanol concentration in methanol/ $^2\text{H}_2\text{O}$ solvent

The sharp increase at 87% methanol for the higher-molecular-weight sample indicates the coil-helix transition. The temperature was 18°C . Mol.wt. of sample: $\blacktriangle$, 6.5×10^4 ; $\circ$, 0.2×10^4 .

Fig. 4. Depolarization of the amide I band of poly(L-lysine hydrobromide) in methanol/ H_2O solvent measured as a function of temperature at various solvent compositions

Proportion of methanol (v/v): ■, 95%; Δ , 85%; □, 80%; ○, 70%. The mol. wt. of the sample was 6.5×10^4 . ρ_e is the depolarization measured in pure H_2O .

difference between solvent components and the polymer. The effect of this is to cause random scrambling of the polarization and is frequently referred to as random scattering. This will cause an error in the measured ρ values of the 1659cm^{-1} band and this error is expected to be a function of solvent composition and temperature. The extent of this effect was determined by measuring the depolarization of the highly polarized methanol band near 2830cm^{-1} . Over the whole series of experiments this value was within the range 0.037 ± 0.005 . Thus any variations in depolarization owing to random scattering are small compared with the polymer depolarization changes observed here.

Discussion

The ρ values plotted as a function of methanol concentration (Fig. 3) show two features of interest: first a relatively slow increase with concentration up to approx. 87% (v/v) methanol and then a sharp increase to a constant value. The sharp increase is quite comparable with the o.r.d. measurements in the same system (Epand & Scheraga, 1968a), where a sharp increase in optical rotation was observed at this concentration and was assumed to arise at the random coil-helix transition. The abrupt nature of the increase is consistent with the co-operative nature of such a transition. It seems probable therefore that the sharp change in ρ at this concentration is a mani-

festation of the coil-helix transition where the higher ρ value of 0.29 is that appropriate to the carbonyl stretching mode when the polymer is in the α -helical form. If this is correct, and indeed there is no other reasonable explanation, the measurement of ρ provides a new method of observing transitions, and further, it is a method that is sensitive to the conformation at the level of individual residues.

On the other hand the relatively slow increase in ρ at lower concentrations has no parallel in the o.r.d. work (Epand & Scheraga, 1968a). This can be understood in qualitative terms by making the usual assumption that the polypeptide conformation is changing through rotations of the bond on either side of the C_α atom. In pure H_2O the polymer backbone is in a relatively extended configuration, owing in part to the electrostatic charges on the side group. As the methanol concentration is increased and the effect of the charges becomes less, perhaps by ion-pair formation (Hesslink *et al.*, 1973), some, though relatively few, of the bonds can rotate to allow a less extended conformation. These rotations then alter the environment of the $\text{C}=\text{O}$ vibrating unit and thus give a change in measured Raman polarization. No helical structure is implied as a result of these rotations, and o.r.d. measurements would not be expected to be sensitive to the changes.

The polarization data thus provide a qualitative measure of the transition to α -helix and also of the rotational isomeric configuration in the denatured state. It is tempting to try to gain quantitative information from the data, though this is not a trivial task. Difficulties arise from two basic causes: (1) the relatively large number of possible rotational isomeric states; (2) the complicated nature of the relation between ρ and isomeric populations. However, it turns out that by making a simplifying assumption about the rotamers in the denatured state it is possible to measure the energy differences between rotamer states that disfavour helix formation. We can then separate these contributions from the total enthalpy of the transition and obtain a value of the interactions that favour helix formation, which we refer to as the stabilization energy. This stabilization energy will in general include contributions from solute-solvent interaction. These latter interactions are expected to be temperature dependent, though we shall see below that in the present case the dominant dependence is on solvent composition. The entirely reasonable value deduced for the stabilization energy justifies the initial assumption, at least as a first approximation, and supports the ideas presented here.

The conformation of a unit in the chain is conventionally characterized by the dihedral angles (ϕ , ψ) representing the rotations about the bonds on either side of the C_α atom (Flory, 1969). The accessible region of (ϕ , ψ) space is limited by the various

interactions and varies from system to system (Ramachandran & Sasiskharan, 1968). In rough terms, for polypeptides with at least one carbon atom in the side chain, there is a relatively extensive region where both ϕ and ψ lie approximately within 60° of zero and a much more constrained region near (ϕ, ψ) values (120,120). The latter region represents the α -helix conformation, and is roughly gauche-like. The former region represents extended states of the chain, roughly *trans*-like, and although the allowed (ϕ, ψ) area is quite large, we wish to make the assumption that the appropriate ρ value for the amide I band is the same throughout. The present data do not lead to a more definite conclusion about whether this extended state is more in the nature of a random coil or an extended helix. However, it should be noted that no β structure has been observed in poly-L-lysine dissolved in aqueous methanol over the range of experimental parameters studied (Epand & Scheraga, 1968a). This assumption for ρ should be a reasonable approximation, because the angles are only varying from the energy minimum by approx. $\pm 60^\circ$, which is small compared with the change to the (120,120) position. Under this speculative assumption we then have a two-state system, each state having a well-defined depolarization. The slow change of measured ρ with solvent composition can then be interpreted as an adjustment of the relative populations of these states under the changing conditions. The calculations below suggest that at a methanol concentration just less than that required to cause the transition to α -helix, only about 5% of the residues are in the gauche configuration. In the α -helical state, of course, they are all gauche, though it should be noted that the depolarization value appropriate to the amide I band in the α -helix is not necessarily equal to that in a pre-helical gauche state.

The depolarization can now be written generally as (Maxfield & Shepherd, 1973b):

$$\rho = \frac{n_e I_{1e} + n_g I_{1g}}{n_e I_{1e} + n_g I_{1g}} \quad (1)$$

where n_e and n_g are the numbers of extended and gauche-like isomers respectively. The intensity (I) terms relate to the polarized scattering from particular isomers; thus I_{1e} is the angular averaged intensity of perpendicular scattering from extended isomers, and the other terms are similarly defined. In the approximation that $n_g \ll n_e$, which we assume to be valid at low methanol concentrations, we can write

$$\rho \approx \rho_e + Kx \quad (2)$$

where ρ_e is the depolarization of extended isomers and x is the ratio n_g/n_e . K is a constant, less than unity, that depends on the intensity terms and is assumed to be temperature independent. If we further assume that in pure $^2\text{H}_2\text{O}$ solution the chain is effectively

Fig. 5. Plot of $\ln(\Delta\rho)$ against $1/T$ for various compositions of methanol/ $^2\text{H}_2\text{O}$ solvent

$\Delta\rho$ is the difference between the depolarization, ρ , of the poly-(L-lysine hydrobromide) amide I band measured at the given methanol concentration and ρ_e , the depolarization of the same band measured in pure $^2\text{H}_2\text{O}$. The mol. wt. of the sample was 6.5×10^4 . Proportion of methanol (v/v): Δ , 85%; $\square$, 80%; $\circ$, 70%.

comprised entirely of extended isomers, then ρ_e is given by 0.226 (Fig. 3) and x is related to $\Delta\rho = \rho - \rho_e$ by eqn. (2). The ratio x is connected with the energy difference ΔH between the two rotational states by the Boltzmann relation $x = \exp(-\Delta H/kT)$ and values of ΔH at different methanol concentrations can be deduced from the temperature variation of ρ shown in Fig. 4 under the assumption that ΔH is not temperature dependent. This assumption, although clearly not strictly valid, implying as it does that solute-solvent interactions are constant, appears to be justified within the experimental error limits by the linearity of the plots in Fig. 5, where $\ln(\Delta\rho)$ is plotted against $1/T$ at several methanol concentrations. Further, measurements of diffusion constant over a similar temperature range by using Rayleigh linewidth techniques show no evidence of significant conformation changes that would result from large changes in ΔH . [Dr. M. F. Treadaway, Department of Physics, University of Manchester, Manchester, U.K., carried out the Rayleigh linewidth measurements (Shepherd, 1975).] The values deduced from Fig. 5 are $8.8 \pm 0.7 \text{ kJ/mol}$ at 85%, $10.4 \pm 1.1 \text{ kJ/mol}$ at 80% and $10.8 \pm 1.5 \text{ kJ/mol}$ at 70%. At lower methanol concentrations the change in ρ was too small to be measured with reasonable accuracy. The decrease in ΔH with increasing concentration reflects the decreasing importance of the electrostatic forces. If the gauche rotamer is the configuration appropriate to the α -helix, then ΔH is the energy connected with the parameters ν of Lifson & Roig (1961) or σ^* of Zimm & Bragg (1959) and Zimm & Rice (1960), representing the difficulty

in starting a section of helix relative to extending it. When combined with values of the enthalpy change observed in the helix-coil transition, ΔH_{h-c} , then the values of ΔH deduced here allow the stabilization energy of the helix to be calculated as ($\Delta H_{h-c} + \Delta H$). To the best of our knowledge, enthalpy measurements of the helix-coil transition of poly-L-lysine have not been measured in methanol solvents. However, ΔH_{h-c} has been measured to be 500 J/mol in aqueous 10% (v/v) ethanol (Pederson *et al.*, 1971). Thus by using ΔH at 85% methanol we get a stabilization energy of approx. 9.3 kJ/mol. The fact that this value is typical of hydrogen-bond energies, and is therefore entirely reasonable as a stabilization energy, gives confidence that the method may be generally valid in spite of the gross assumptions.

When the polymer is in the α -helical conformation, that is for methanol concentrations greater than 87%, ρ decreases as the temperature increases (Fig. 4, upper curve), which is a change of opposite sign to that observed at lower concentrations. This effect, which is in qualitative agreement with o.r.d. data (Epand & Scheraga, 1968b), may well represent displacement of equilibrium towards the random coil. I am at present unable to make a quantitative assessment of this effect.

The measurements presented here show that details of poly-L-lysine conformation in the denatured state and in the transition to α -helix can be monitored by careful Raman polarization measurements. Further, under certain simplifying assumptions, quantitative estimates of energy parameters may be obtained. This technique can be extended to a variety of other polypeptides, e.g. poly-glutamic acid and poly-benzylglutamate, under various conditions of solvent and temperature to build up a comprehensive picture of the conformation of model systems for comparison with data obtained by other methods. It is possible, for instance, that the technique will prove sensitive to transitions to ordered structures other than α -helix, for instance β structures, which should be investigated fully.

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