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Intramolecular β-Pleated-Sheet Formation by Poly-L-lysine in Solution*

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ABSTRACT: In order to learn whether the β form of poly-L-lysine is intramolecular or intermolecular, the rate of the heat-induced conformational transition of this polymer from the α-helical to the β-pleated-sheet conformation was studied polarimetrically over the concentration range 1.5×10^{-4} to 7.0×10^{-2} g per 100 ml at pH 10.8. Interpretation of the rates for polymer with degree of polymerization 240 or 1000 shows that, at concentrations of 10^{-2} or less, poly-L-lysine folds into the intramolecular β form. In contrast, at higher concentrations both inter- and intramolecular β forms are generated simultaneously. For degree of polymerization 70, however, the β form is intermolecular at all concentrations.

These observations support the assignment of the peptide circular dichroism extremum at 216 nm to the anti-parallel-chain β-pleated-sheet structure.

Poly-L-lysine, a synthetic polypeptide, is shown by unambiguous kinetic measurements, presented in this study, to be capable of folding into *intramolecular* as well as intermolecular β-pleated-sheet structures in aqueous solution. Poly-L-lysine thus exhibits in solution a versatility of conformational transformations rivaling that of proteins. The solution properties of poly-L-Lys have been extensively studied previously (Applequist and Doty, 1962; Rosenheck and Doty, 1961; Davidson and Fasman, 1967). These previous studies have shown that at neutral or acid pH, poly-L-Lys is disordered, somewhat like a protein in 6 M guanidine hydrochloride. At pH's greater than 10.5, the molecule adopts the α-helical conformation if the temperature is 30° or less. At more elevated temperatures, for example, 50°, poly-L-Lys molecules in alkaline solution adopt the β form.

However, it has been a matter of dispute whether the β form adopted involves parallel or anti-parallel chains, and whether the β form is intermolecular or intramolecular. From molecular weight measurement through sucrose gradient centrifugation on the three forms of poly-L-Lys, Sarkar and Doty (1966) suggested that the β form exists in an intramolecular H-bonded pleated sheet. By contrast, studies by Davidson and Fasman (1967) showed that the initial rate of the thermal transition of the α helix to the β structure is dependent upon concentration under appropriate conditions. These data led Davidson and Fasman to conclude that the β form of poly-L-Lys is an intermolecular pleated sheet. In addition, the effect of molecular weight on the circular dichroism of heated alkaline poly-L-Lys solutions has been reported by Li and Spector (1969), who sought accurate values of the circular dichroism of the β form in order to estimate β content in proteins. Their studies, which were carried out at pH 11.5, supported the view that at concentrations of 0.01 g/100 ml of poly-L-Lys forms large intermolecular β sheets. The detergent sodium dodecyl sulfate, shown by Sarkar and Doty to cause poly-L-Lys in neutral aqueous solutions to adopt the β conformation, was shown by Li and Spector to lead to very small, thus presumably intramolecular, β form.

It occurred to us that such opposed conclusions could have arisen from the difference in the experimental conditions used by the two groups. Sarkar and Doty worked at a relatively low concentration (0.01 g/100 ml) at pH 11; Davidson and Fasman measured kinetics over a concentration range (0.007–0.11 g/100 ml) at pH 11.6–11.7 but most of the data reported are for concentrations greater than 0.01. In the study presented here, the kinetics of the α–β thermal transition are investigated over the concentration range 1.5×10^{-4} to 7×10^{-2} g per 100 ml, *i.e.*, to concentrations 50-fold more dilute than any previously studied. Moreover, by studying the kinetics of the α–β transition for polymers with degree of polymerization 70, 240, and 1000, the effect of chain length upon the β structure is examined. It is to be expected that a competition between inter- and intramolecular processes would be reflected in dependence of the kinetics upon molecular weight.

Materials and Methods

*Poly-*L*-Lys.* Three poly-L-Lys samples having three different average molecular weights were used in these experiments. The two samples of higher molecular weight were purchased as the hydrobromide salt from Pilot Chemicals, Inc. The

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bromide ions, which absorb ultraviolet light, were removed by exhaustively dialyzing the polymer solutions first against 0.1 M NaF and then against deionized water. Poly-L-Lys–HF was recovered from the final solution by lyophilization. Low molecular weight poly-L-Lys–HBr was obtained from Miles Laboratories and used in the experiments without further purification.

The average molecular weight of the three poly-L-Lys samples was determined by measuring viscosities with a Cannon-Ubbelohde viscometer designed for measurements of successively diluted solutions. The flow time for the solvent and the solution ranged from 400 to 500 sec; the kinetic energy corrections are therefore negligible. The viscometer temperature was maintained at 25.00 ± 0.01°. In aqueous pH 7 solution, 0.2 M in NaCl, the intrinsic viscosities of the three samples were 0.199, 0.595, and 2.56 dl per g. The degree of polymerization corresponding to each is estimated as 70, 240, and 1000, respectively.

Preparation of Solutions and Measurement of Kinetics. For optical and kinetic experiments, poly-L-Lys was dissolved in degassed, neutral, deionized H_2O; the solution was filtered through an 8 μ Millipore filter. The polymer concentration was then determined spectrophotometrically, using an ϵ value of 5500 (Rosenheck and Doty, 1961) at 200 nm. The pH was thereupon adjusted at room temperature with NaOH to a pH value between 10.5 and 11.5 using a low leakage reference electrode, to an accuracy of ±0.01 pH unit. The pH values reported elsewhere in this manuscript are all room-temperature values. The pH utilized for most of the experiments was 10.8; this is substantially greater than the pH value, 9.3, we find to be required to convert poly-L-Lys into a 50:50 helix–coil mixture at low ionic strength. The room-temperature average degree of dissociation of the NH_3^+ group to NH_2 in poly-L-Lys at pH 10.8 is then at least 0.95 (Applequist and Doty, 1962).

Solutions containing the β form of poly-L-Lys were prepared by heating the alkaline solutions of α-helical poly-L-Lys at 55.5° for 1–2 hr. For measurement of the kinetics of the α–β transitions, optical rotation was measured in jacketed optical cells of 1- and 10-cm path length which were connected to an external water-circulating bath. The bath temperature was regulated to an accuracy of ±0.2°. All cells were equipped with ground-glass stoppers to minimize evaporation and CO_2 contamination. The polarimeter was set at 233 or, for very dilute solutions, 199 nm, and the rotation at 27° was recorded for several minutes as a function of time. The flow of hot fluid from the external bath (55.5°) to the cell was then initiated abruptly by opening a valve. The optical rotation was continuously recorded for 1–2 hr while the cell remained at 55.5°. Since the rotation of the α and β forms differs greatly at 233 (or 199) nm (Davidson *et al.*, 1966; Sarkar and Doty, 1966), the amount of polymer in the α and β conformation at any time can be easily established from the rotation data. This procedure for measuring kinetics is similar to that of Davidson and Fasman (1967).

Spectra. Ultraviolet absorption spectra were measured with a Cary 15 spectrophotometer. Circular dichroism and optical rotatory dispersion were measured with a Cary 60 spectropolarimeter with 6001 circular dichroism attachment. Both instruments were constantly purged with nitrogen gas in order to remove oxygen from the light path.

Results

Thermally Induced α–β Transition. The thermally induced α–β transition is recorded by following the decrease in optical rotation at 233 nm as a function of time after the cell jacket is heated to 55.5°. A typical transition curve, as Davidson and Fasman (1967) have noted, consists of two steps, a rapid first step superposed onto the slower second step. The first step seems to reflect the rate at which the solution changes temperature after the jacket temperature is altered. The kinetics of this step are not treated in this study; from the work of Applequist and Doty (1962), Hermans (1966), and Davidson and Fasman (1967), however, an explanation for the first step can be proposed. Hermans demonstrated that the free energy for the formation of the uncharged α helix of poly-L-Lys from the charged random coil approaches zero in the vicinity of 55° and becomes positive above this temperature. Therefore, at the transition temperature used in these experiments (approximately 55.5°), the poly-L-Lys undergoes a rapid, reversible transition from 100% α helix to 50% α helix:50% random coil.

The second phase corresponds to the formation of the β form from the mixture of random coil and α helix. In our apparatus, the second step is the rate-limiting step for times longer than 100 sec after the jacket temperature is changed. The concentration dependence of the rate of this second step provides useful information on the nature of the β form.

For the purposes of analysis, we tentatively assume that the reaction can be described by a simple first-order rate expression

$$\frac{d\alpha(t)}{dt} = -k\alpha(t) + k\alpha(\infty) \qquad (1)$$

where $\alpha(t)$ is the optical rotation at time t. (The transition is irreversible during the time period of measurement so only one term is required.) Thus, additional variables required by any deviation from first order are lumped together in the effective first-order rate constant. The rate constant can be obtained by the conventional method, that is, by plotting log $[\alpha(t) - \alpha(\infty)]$ *vs.* t. The initial slope multiplied by 2.303 gives the observed effective first-order rate constant k.

A semilogarithmic plot of k *vs.* concentration for the three samples of DP 70, 240, and 1000, shown in Figure 1, reveals a strong dependence of k on concentration and molecular weight. Let us examine the kinetic behavior of each molecular weight separately.

DP 1000. For DP 1000, at polymer concentrations of 0.06 g/100 ml, the α–β conversion occurs rapidly with initial rates of 0.01 sec^{-1} (see Figure 1). If the initial concentration of the α form decreases 10-fold to 0.006, the rate decreases sharply 25-fold to 0.004, in agreement with the earlier conclusions of Davidson and Fasman (1967). For concentrations less than 0.007 (the lowest concentration studied in earlier work) the rate decreases further but does not go to zero. Indeed, for a concentration of 1.5×10^{-4}, the rate is still 0.002, *i.e.*, a 50-fold concentration change causes only a 2-fold change in rate, for low concentration and high molecular weight. Under these conditions, then, the rate-determining step of the reaction is essentially independent of polymer concentration. This implies that the β structure formed by high molecular weight poly-L-Lys at low concentration is

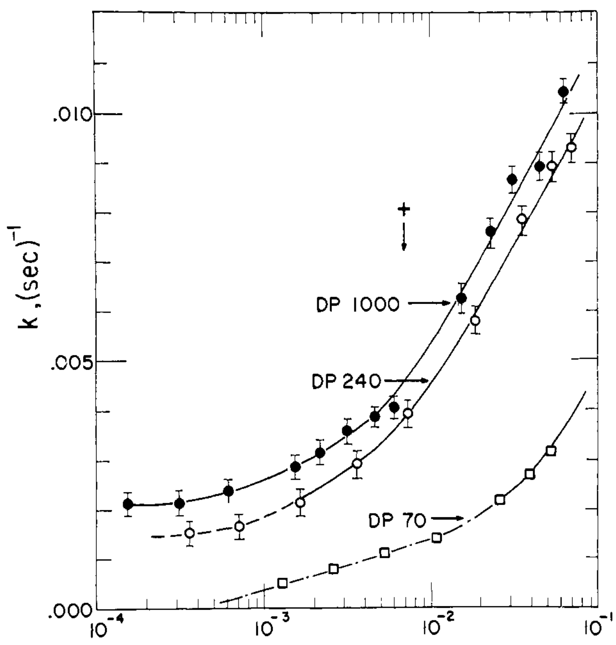

FIGURE 1: The effective first-order rate constant in sec^{-1} for conversion of poly-L-Lys into the β conformation at pH 10.8, 55.5°. Each point corresponds to the total poly-L-Lys concentration in a given experiment. The values of the rate constant are shown for poly-L-Lys of DP 1000, 240, and 70. The plus symbol indicates the lowest concentration used by previous workers.

intramolecular. Filtration evidence supporting this conclusion is presented below. Although it is conceivable that a slow conversion of intramolecular β to intermolecular β could occur, we saw no evidence for such a process.

DP 240. The concentration dependence of the effective first-order initial rate for α–β conversion of poly-L-Lys with DP 240 follows closely that of DP 1000, except that the rates are reduced by 25–50%. The rate of 0.0085 sec^{-1} is obtained for initial concentration 0.07 g/100 ml. The rate decreases rapidly as the concentration decreases, but remains at 0.0015 as the concentration goes to 3.5×10^{-4} g/100 ml. The β form generated at low concentration must therefore be intramolecular.

DP 70. As the degree of polymerization decreases to 70, a more pronounced change in rate occurs (Figure 1). The rate at all concentrations is much reduced, and the rate appears to approach zero as the concentration goes to zero. The results suggest that low molecular weight poly-L-Lys cannot form intramolecular β structure.

We attempted to establish the cause of the slight dependence of rate on concentration for DP 1000 even at the lowest concentrations we were able to study. It seemed possible that the rate changes might reflect a systematic decrease in ionic strength with decreasing polymer concentration. However, experiment shows that the changes in ionic strength required to account for the effects are much larger than those actually occurring in the solutions. For example, at a polymer concentration of 6×10^{-4} g/100 ml, addition of NaF to increase the ionic strength from 8×10^{-4} to 7×10^{-3} caused only a 10% change in the rate.

A second possible source for the low-concentration residual dependence of rate upon concentration in the high molecular weight sample can be sought in the known dependence of rate on pH (Davidson and Fasman, 1967). Since the degree of dissociation of the coil, helix, and presumably also β conformations are slightly different for a given pH value (Applequist and Doty, 1962), one might expect that the pH of the solutions having different poly-L-Lys concentration, although set identical at room temperature, could be systematically concentration dependent at 55°. However, measurements of the change in pH upon heating poly-L-Lys solutions having concentrations 0.01 and 0.001 g per 100 ml reveal that the pH difference is less than 0.01, *i.e.*, this source of systematic error is negligible.

A third possible source of the residual slope in k at low concentration is polydispersity of the sample.

Pseudo Order of Reaction during the Course of α–β Conversion at a Single Initial Concentration. The fractional lifetime method (Frost and Pearson, 1961) can be used to estimate the pseudo order of reaction at various times during conversion of α to β for a given initial concentration. Assuming the reaction is of order n with respect to the α-form component, the overall reaction, as measured by the change of optical rotation, can be represented by an equation of the following form

$$\frac{d\phi}{dt} = k'\phi^n \tag{2}$$

where n is the pseudo order of reaction; ϕ is the difference of the optical rotation between time, t, and infinite time, that is, $\phi(t) = \alpha(t) - \alpha(\infty)$; k' is equivalent to the effective rate constant.

The integrated form of eq 2 is

$$\frac{1}{1-n}(\phi_0{}^{-n+1} - \phi^{-n+1}) = kt \tag{3}$$

where $\phi(t) = \phi_0$ when $t = 0$. The fractional lifetime τ_x is defined as the time required for ϕ to decay to fraction x of its original value ϕ_0. Therefore

$$\tau_x = \frac{(x^{1-n} - 1)}{k'(n-1)}\phi_0{}^{1-n} = f(x,n,k')\phi_0{}^{1-n}$$

This expression implies that a log–log plot of τ_x *vs.* ϕ_0 should be linear with a slope equal to $1 - n$. Although this method was derived for observations on two or more experiments with different initial concentrations, it can be generalized and applied to a single run by selecting two or more points along the curve as a new set of initial points. The quantity ϕ_0 is in the present case easily converted into a value for the instantaneous concentration C_α of α-helical polymer.

A plot of fractional lifetime $\tau_{1/2}$ *vs.* C_α for three different initial concentrations is given in Figure 2 for the sample with DP 1000. Using an initial concentration of 4×10^{-2} g/100 ml (curve C in Figure 2) the pseudo order is found at first to be 1.4–1.5, suggesting that an intermolecular process is occurring. As the concentration of α material becomes reduced, the reaction pseudo-order changes, becoming 1.0 (slope equal to zero) for instantaneous α concentrations less than 6×10^{-4} g/100 ml. For initial concentration 7.5×10^{-3} (curve B in Figure 2), similar results are obtained: the rate initially has pseudo order 1.3, then decreases to 1.0. Finally for initial concentration 1.0×10^{-3} (curve A in Figure 2) the pseudo order is 1.0 (slope equal to zero) throughout the reaction. These data fully support the conclusions derived from the dependence of initial rates on concentration presented in Figure 1.

*Optical Rotatory Dispersion and Circular Dichroism of Poly-*L*-Lys in Intermolecular and Intramolecular β Forms.* Having found conditions of molecular weight and concentration which permit us to generate both intermolecular and intramolecular β poly-L-Lys, we measured the circular dichroism and optical rotatory dispersion of these conformations to 200 nm. For polymer of DP 1000, no major difference in circular dichroism patterns is found for the β form prepared at high and low concentration. The intramolecular form exhibits a negative extremum in $[\theta]$ of -18×10^3 deg cm^2 dmole^{-1} at 216 nm, compared with $[\theta] = -15 \times 10^3$ at 217 nm in the intermolecular form.

Filtration Experiments. Following the procedures first explored by Li and Spector (1969), we tested the hypothesis of inter- and intramolecular β form by measuring the circular dichroism of solutions before and after passage through a 1.2 μ Millipore filter. A solution of β-poly-L-Lys prepared at pH 10.8 with concentrations of 5×10^{-2} g/100 ml or less is observed to be turbid and to contain some large heterogeneous suspended particles. About 75% of the material is recovered after passage through the 1.2 μ filter. If the β form is prepared at pH 11.5, the pH used by Li and Spector and by Davidson and Fasman, one obtains a turbid solution with homogeneous finely suspended particles. In this case essentially no material passes through the filter. In neither case did we observe the residual α-helical material reported by Li and Spector, probably because our solutions were heated for longer times.

Discussion

The major conclusion of this work is that high molecular weight poly-L-Lys forms intramolecular β structures when generated at concentrations less than 0.01 g/100 ml at pH 10.8, whereas at greater concentrations intermolecular and intramolecular β forms are generated simultaneously. These findings are supported by two somewhat independent types of data given in Figures 1 and 2. On the one hand, the data in Figure 1 show that the effective first-order rate constant for initial poly-L-Lys concentrations less than 0.01 g/100 ml is essentially independent of initial concentration for DP 240 and 1000. This strongly supports the idea that the β form is intramolecular. For higher concentrations the rate is dependent upon concentration, suggesting a mixture of inter- and intramolecular β form. On the other hand, examination of the rate of α–β conversion during the conversion of a particular solution (Figure 2) shows that the reaction proceeds differently for different initial concentrations. For very low concentrations, the reaction rigorously obeys a first-order rate law at all times. As the initial concentration increases, however, the reaction is *not* first order at all times. Indeed, the curves suggest that the pseudo order is about 1.5 for instantaneous α-helix concentrations greater than 1×10^{-3} but becomes 1.0 as the concentration of reactants falls below 1×10^{-3}. The highest α-helix concentration at which the reaction is first order is thus quite close to half the total concentration at which the effective rate constant (Figure 1) becomes largely independent of concentration. These findings reconcile the conclusions of Sarkar and Doty (1966) with those of Davidson and Fasman (1967) by drawing attention to the different concentrations at which these groups made their measurements.

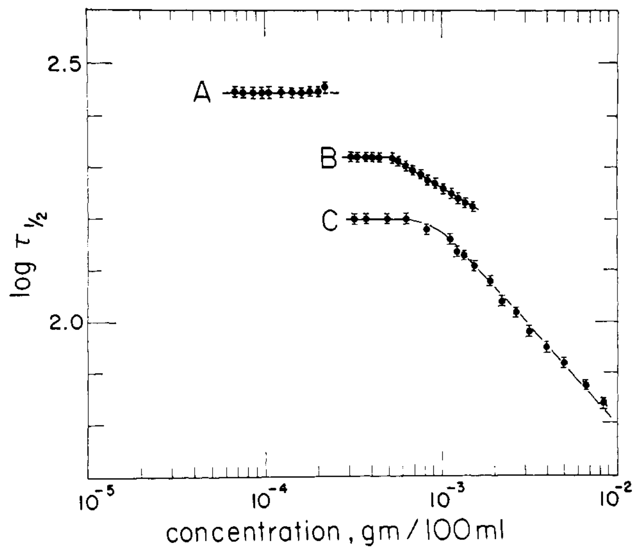

FIGURE 2: Plot of half-lifetime $\tau_{1/2}$ for the generation of β-poly-L-Lys *vs.* instantaneous concentration of α helix at 55.5° for three different total poly-L-Lys concentrations: 10^{-3} g/100 ml in curve A, 7.5×10^{-3} in B, and 4×10^{-2} in curve C. The pseudo-order $\bar{n}$ of reaction is given by $1 - S$, where S is the slope of the curve. During the time course of a given experiment, the initial values of $\tau_{1/2}$ are at the right-hand end of the lines; the value of $\tau_{1/2}$ at the termination of the experiment occurs at the left-hand end of the curve.

Our data on filtration of β-poly-L-Lys through Millipore filters show that a large fraction of both inter- and intramolecular β forms prepared at pH 10.8 can pass through the filters. These observations are contrary to those of Li and Spector (1969), who report that intermolecular β-poly-L-Lys prepared at pH 11.5 cannot pass such a filter. The discrepancy is probably due to a larger average sheet size for β-poly-L-Lys formed at pH 11.5 than for that formed at pH 10.8. Indeed, as noted above, visual inspection of the scattering by β solutions prepared at the two pH values shows that the suspensions are qualitatively different from one another.

The observation that the β form can be intramolecular implies strongly, as Sarkar and Doty have already suggested, that the pleated sheet contains anti-parallel chains. This follows from the fact that the topology for parallel-chain sheet prevents a large fraction of a single molecule from forming intramolecular hydrogen and hydrophobic bonds. Moreover, since the circular dichroism of the β form generated at low concentration is essentially identical with that at high concentration, it would appear that the anti-parallel-chain β form is also dominant when intermolecular β form is generated in poly-L-Lys. The observed circular dichroism patterns (Sarkar and Doty, 1966; Townend *et al.*, 1966; Timasheff *et al.*, 1967) are then in reasonable accord with the theoretical calculations of Pysh (1966, 1970) and of Woody (1969). They support Woody's arguments favoring the anti-parallel chain over the parallel chain arrangement proposed by Rosenheck and Sommer (1967) for poly-L-Lys films. The anti-parallel-chain assignment also yields agreement with circular dichroism observations on silk fibroin (Iizuka and Yang, 1966), which is known to be of anti-parallel-chain β conformation in the solid state. Our circular dichroism data for the intramolecular β form are unfortunately limited to wavelengths of 200 nm or greater because of solvent (OH^-) absorption. Thus we cannot explore the spectral region 185–200 nm for which Woody (1969) predicts the largest differences between parallel and anti-parallel β sheets as well as dependence of circular dichroism band positions on sheet size.

The observations on poly-L-Lys have obvious application to studies of the reversibility of protein denaturation. The reversibility of protein denaturation requires that the renaturing molecule refold into globular form in a short time compared with the time for aggregation. The kinetic results for poly-L-Lys show the sensitivity of spectropolarimetry as a probe of conformation. They suggest that it may be possible to monitor reversible denaturation of protein solutions, *e.g.*, myoglobin at pH 7 (Acampora and Hermans, 1967), under conditions dilute enough to prevent the aggregation (and hence irreversibility) usually found at this pH.

In conclusion, by making kinetic measurements at concentrations 50 times more dilute than those utilized heretofore, we have shown that the heat-induced conformational transition of poly-L-Lys from α helix to β form occurs at a rate which is nonzero but essentially independent of concentration for dilute solutions, provided the polymer is of sufficiently high molecular weight. This implies that intramolecular, and hence anti-parallel, β-pleated sheet is being formed. At higher concentrations, however, the reaction proceeds with pseudo order 1.5, implying a mixture of inter- and intramolecular β sheet. These data support the general assumption that the polymer chains in β-poly-L-Lys solutions are anti-parallel.

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