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Poly(L-lysine) as a model drug macromolecule with which to investigate secondary structure and membrane transport, part I: physicochemical and stability studies

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Abstract

Low oral bioavailability of therapeutic peptides and proteins generally results from their poor permeability through biological membranes and enzymatic degradation in the gastrointestinal tract. Since different secondary structures exhibit different physicochemical properties such as hydrophobicity, size and shape, changing the secondary structure of a therapeutic polypeptide may be another approach to increasing its membrane permeation. Poly(L-lysine) was used as a model polypeptide. The objectives of this study were to induce secondary structural changes in poly(L-lysine) and to determine the time course over which a given conformer was retained. In addition, the hydrophobicity of each secondary structure of poly(L-lysine) was assessed. The circular dichroism (CD) studies demonstrated that the conditions employed could successfully induce the desired secondary structural changes in poly(L-lysine). The α-helix conformer appeared to be more stable at 25°C whereas the β-sheet conformer could be preserved at 37°C. On the other hand, the random coil conformer was retained at both temperatures. Significant losses of the α-helix and the β-sheet conformers were observed when the pH was reduced. The change in ionic strength did not affect any of the conformers. The octanol/buffer partitioning studies indicated that the α-helix and the β-sheet conformers exhibited significantly different ($P < 0.05$) hydrophobicities. In conclusion, variation of pH and temperature conditions can be used to induce secondary structural changes in poly(L-lysine). These changes are reversible when the stimuli are removed. The α-helix and the β-sheet conformers of poly(L-lysine) are more lipophilic than the native random coil conformer. Thus, poly(L-lysine) may represent an ideal model polypeptide with which to further investigate the effects of secondary structure on membrane diffusion or permeation.

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Introduction

Low oral bioavailability of therapeutic peptides and proteins generally results from their poor permeability through biological membranes and enzymatic degradation in the gastrointestinal tract (Harris & Robinson 1990). Poor permeability can be attributed to their physicochemical properties, including charge at physiological pH and hydrophilic characteristics coupled with their globular/spherical three-dimensional shape. These properties also render them good candidates for primarily the paracellular pathway of membrane transport. Unfortunately, due to their rather large molecular dimensions, most therapeutic polypeptides are excluded

from paracellular transport. It has been estimated that tight junctions are only 7–9 Å in diameter (Pauletti et al 1997). Permeation enhancers and chemical modifications have been used to improve the permeability of polypeptides with various degrees of success. Most oral absorption enhancers are non-selective and may cause disruption of cell membranes as well as potentially induce cell death when used in large quantities (Wu & Robinson 1999).

Polypeptides and proteins in solutions exhibit different secondary structures such as random coil, α-helix and β-sheet. These secondary structures are in a dynamic equilibrium and the proportions of each conformer depend on the solution microenvironment. In addition, different secondary structures exhibit different physicochemical properties such as hydrophobicity, size and shape (Gray et al 1994). Generally, hydrophobicity increases in the order: random coil $<$ α-helix $<$ β-sheet, and an α-helix secondary structure appears to be more compact than a random coil (Gray et al 1994). A potential alternative approach to increase the membrane permeation of polypeptides would be to use a secondary structure with optimal physicochemical properties. Therefore, changing the secondary structure of a therapeutic polypeptide may be another approach to increasing its membrane permeation.

Poly(L-lysine) was used as a model polypeptide in this study. It is a polycation that has been used as a carrier for targeted delivery of anticancer (Ryser & Shen 1978; Ryser et al 1978) and antiviral (Di Stefano et al 1995) drugs. Some of the low-molecular-weight polymers of poly(L-lysine) have been shown to have antineoplastic activity in some tumours, and limited toxicity to normal cells (Arnold et al 1979). Poly(L-lysine) is a homopolymer of L-lysine with a vast majority of secondary structure and limited tertiary structure. This is advantageous because the effects of secondary structural changes are not complicated by the occurrence of a variety of tertiary structural interactions that are found in heteropolypeptides such as proteins (Arunkumar et al 1997). A major proportion of poly(L-lysine) exists as random coil at neutral pH, but various stimuli can be used to induce changes in its secondary structure, such as organic solvents (Epand & Scheraga 1968; Arunkumar et al 1997), salts (Tiffany 1975) and phospholipids (Fukushima et al 1994). In addition, pH and temperature, which were used as the stimuli in this study, can also induce changes in the secondary structure of poly(L-lysine) (Greenfield & Fasman 1969). Although the effects of various stimuli on induction of secondary structures of poly(L-lysine) have been well studied and characterized, no studies where either the hydrophobicity of the induced conformer or the time course over which an induced conformer could be retained have been reported. Therefore, our objectives were to investigate the hydrophobicity of each induced secondary structure of poly(L-lysine) and to determine the duration over which a specific conformer was retained at two temperatures. The knowledge obtained from this study will be applied to future mass transport studies which will examine the effects of secondary structural changes on membrane permeability in-vitro.

Materials and Methods

Materials

Poly(L-lysine) HCl of molecular weight range 15000–30000 daltons with an average molecular weight of 26500 daltons (as determined by the supplier using viscosity measurements), potassium phosphate (monobasic, anhydrous), boric acid (anhydrous), potassium chloride (anhydrous), sodium hydroxide, sodium chloride and octanol were purchased from Sigma Chemical Company (St Louis, MO). Micro BCA reagents were purchased from Pierce (Rockford, IL). All materials were used as received.

Methods

All experiments were performed in triplicate, unless otherwise stated, and the results are expressed as the mean value $\pm$ standard deviation.

Induction of secondary structural changes

Sufficient amount of poly(L-lysine) HCl was dissolved to achieve a concentration of 1 mg mL^{-1} in two different buffers. Phosphate buffer (10 mM, pH 7.4) was used for the random coil conformer whereas alkaline borate buffer (10 mM, pH 11.0) was used to induce the α-helix conformation. Before the addition of poly(L-lysine), the pH of both the phosphate and alkaline borate buffers was adjusted to 7.4 and 11.0, respectively. Following complete dissolution of poly(L-lysine), the pH of the solutions was determined and adjusted to their respective values, if required. The β-sheet conformer was then obtained by heating the solution of poly(L-lysine) in alkaline borate buffer (pH 11.0) at 55°C for 30 min. Resulting conformations were verified by circular dichroism (CD) studies. The effect of the heating temperatures on the percentage of the heat-induced β-sheet conformer was also examined by heating the solution of

poly(L-lysine) in alkaline borate buffer (pH 11.0) at 37, 40, 45, 50, 55 or 60°C for 30 min, followed by the CD measurements.

All CD measurements were conducted using a Jasco J-720 spectropolarimeter and a quartz cell with a volume of 150 μL and a 0.2-mm light path length. Each sample was measured with three accumulations and the average ellipticity values were reported in millidegrees. The obtained spectra were analysed in the region of 190–250 nm for the percentages of each conformer present in the solutions using the JFIT program (developed by Bernhard Rupp, Lawrence Livermore National Laboratory, Livermore, CA; www.structure.unl.gov/cd/cdtutorial.htm).

Conformer retention studies

Effect of temperature

Samples (5 mL) of each conformer prepared as described above were maintained at either 25±1°C or 37±1°C for 4 h. The CD measurements were performed on a 150-μL sample withdrawn every 30 min. Samples used for CD spectropolarimetry were discarded following the scan.

Effect of pH

Samples of the α-helix and β-sheet conformers (2 mg mL^{-1}) prepared as described above were mixed with an equal volume of 10 mM phosphate buffer (pH 7.4). Controls were prepared by mixing another set of samples with an equal volume of 10 mM alkaline borate buffer (pH 11.0). For the heat-induced β-sheet conformers, all solutions were maintained at 37±1°C throughout the experiment. The CD measurements were performed immediately after mixing and the percentages of each conformer were estimated. The pH of the final solutions was also measured.

Effect of ionic strength

Samples (2 mg mL^{-1}) of each conformer prepared as described above were mixed with an equal volume of sodium chloride solutions (0.01–0.5 M in the corresponding buffers). Controls were prepared by mixing another set of samples with an equal volume of the relevant buffer. For the heat-induced β-sheet conformer, all solutions were maintained at 37±1°C throughout the experiment. The CD measurements were performed immediately after mixing and the percentages of each conformer were estimated.

Octanol/buffer partitioning studies

The following method was used to determine the octanol/buffer partition coefficient of each conformer. Phosphate buffer (10 mM, pH 7.4) and alkaline borate buffer (10 mM, pH 11.0) were presaturated with octanol overnight before use. Each conformer of poly(L-lysine) was then prepared in the relevant octanol-saturated buffer using the same conditions as described previously. The aqueous phase (5 mL) was vigorously mixed with an equal volume of the presaturated octanol and the mixture was continuously agitated in a shaking water bath at either 25°C or 37°C for 24 h to ensure equilibrium. The two phases were then separated and the aqueous phase was cooled to room temperature and analysed for poly(L-lysine) using the Micro BCA Protein Assay. Since each conformer may react with the Micro BCA reagents differently, separate standard curves were constructed for each conformer. Moreover, standard solutions for the generation of the standard curves were prepared under similar conditions to that of the samples. The apparent partition coefficient (K) was calculated using equation 1 (Narurkar 1988).

$$K = \frac{C_{aq} - C_{eq}}{C_{eq}} \quad (1)$$

where C_{aq} is the original poly(L-lysine) concentration in the aqueous phase and C_{eq} is the poly(L-lysine) concentration in the aqueous phase at equilibrium.

Results and Discussion

Circular dichroism (CD) spectroscopy has traditionally been used to gain information about the secondary structures of proteins and polypeptides in solutions. Proteins and polypeptides assume a biologically meaningful conformation by their interaction with water (Chiou et al 1992). Therefore, in solution, polypeptides exhibit different secondary structures which are in dynamic equilibrium and the proportions of each conformer present at any one time depend on the solution microenvironment. Each of the three basic secondary structures of a polypeptide chain (α-helix, β-sheet and random coil) demonstrates a characteristic CD spectrum. Consequently, a spectrum of a polypeptide consisting of these three elements can be deconvoluted into the three individual contributions (Greenfield & Fasman 1969). Using CD spectroscopy, the effects of changes in solution environment (pH, temperature, denaturants, etc.) on the secondary structures of proteins and polypeptides may be determined. In this study, we used the

JFIT program to analyse the resulting spectra for the percentages of each conformer present in the solutions. This software program fits the data to poly(L-lysine) standard curves proposed by Greenfield & Fasman (1969).

Induction of secondary structural changes

Various stimuli have been used to induce secondary structural changes in poly(L-lysine). Organic solvents have been used as co-solvents with aqueous-based buffers to induce the α-helix conformation of poly(L-lysine) (Epand & Scheraga 1968; Nelson & Kallenbach 1986; Sonnichsen et al 1992; Arunkumar et al 1997). At neutral pH (i.e., poly(L-lysine) in 10 mM Tris-HCl, pH 7.2), high concentrations of acetonitrile (90% v/v) have been shown by Arunkumar et al (1997) to induce the α-helix conformation of poly(L-lysine). Epand & Scheraga (1968) found that alcohol–water solvent systems which contained 87–98% v/v methanol or 76% v/v isopropanol caused poly(L-lysine) to undergo a transition from the random coil to the α-helix conformation. Davidson et al (1966) have reported that 50% v/v methanol in 0.2 M KCl at pH 11.6 stabilized the α-helix conformation of poly(L-lysine), preventing the thermal-induced transition from the α-helix to the β-sheet structure.

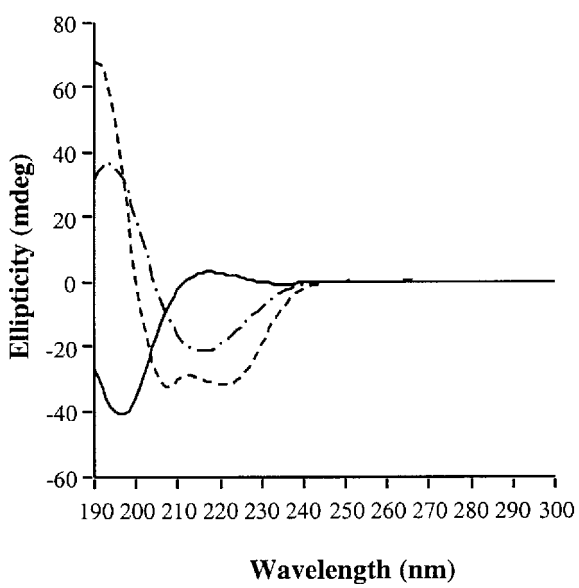

Figure 1 CD spectra of poly(L-lysine) in phosphate buffer (pH 7.4) (——), poly(L-lysine) in alkaline borate buffer (pH 11.0) (- - - -) and poly(L-lysine) in alkaline borate buffer (pH 11.0) heated to 55°C for 30 min (— · — · — · —). Each sample was measured three times and the mean value was reported in millidegrees.

Neutralizing charged side chains by certain anions (e.g. CCl_3COO^-, CF_3COO^-, ClO_4^-) and competition of salts known to produce the salting out phenomenon (e.g. NaH_2PO_4, $(NH_4)_2SO_4$, NH_4F) lead to the induction of α-helicity in poly(L-lysine) (Tiffany 1975). Fukushima et al (1994) have studied the interactions between poly(L-lysine) and phospholipids and found that dilauroylphosphatidic acid could induce the β-sheet from either the random coil or the pH-induced α-helix.

In this study, pH and temperature were used as the stimuli to induce the changes in the secondary structure of poly(L-lysine). As a result, the samples were devoid of any compound that absorbs UV light in the region of interest (190–250 nm) that could potentially contribute to the recorded CD signals. CD spectra of poly(L-lysine) are shown in Figure 1 and the extents of each conformer in the solutions estimated by the JFIT program are listed in Table 1. At neutral pH in phosphate buffer (pH 7.4), the vast majority ($94.77 \pm 0.95\%$) of poly(L-lysine) existed as the random coil conformer. The corresponding spectrum in Figure 1 demonstrates a negative extrema at 198 nm. When the pH of the solution exceeds the pK_a of the terminal ε-amino group of lysine (reported by Hermans (1966) and Pederson et al (1971) as pK_a = 10.0–10.3 in 0.1 M KCl at 25°C), deprotonation of the lysyl chain results in formation of the α-helix conformation of poly(L-lysine) (Tiffany 1975). In this study, alkaline borate buffer (pH 11.0) was used for this purpose and $75.36 \pm 0.43\%$ of the poly(L-lysine) existed as the α-helix while the rest of the poly(L-lysine) molecules remained in the random coil conformation. The spectrum illustrates negative extrema at both 208 and 222 nm, which are the characteristic peaks (Fasman 1989) associated with a molecule existing in the α-helix conformation (Figure 1). When poly(L-lysine) in the α-helix conformation in alkaline aqueous solutions is heated to a temperature of 50–55°C, the molecule's α-helix conformation is transformed into the β-sheet structure (Miyazawa 1967; Fasman 1989). In this study, we used a temperature of 55°C for 30 min to induce the β-sheet conformer. The CD spectrum (Figure 1) clearly shows the characteristic β-sheet negative extrema at 216 nm (Fasman 1989) and $73.57 \pm 1.88\%$ of the poly(L-lysine) appeared to exist as the β-sheet conformer. These results indicated that the conditions used in this study could successfully induce the desired secondary structural changes in poly(L-lysine).

The effect of the heating temperature on the percentage of the β-sheet conformer is presented in Figure 2. The result was in good agreement with the literature that the minimum temperature required for induction of the β-sheet conformer was 50°C. However, a tempera-

Table 1 Percentages of each conformer estimated using the JFIT program.

Poly(L-lysine) solution	Random coil (%)	α-helix (%)	β-sheet (%)
Phosphate buffer (pH 7.4)	**94.77±0.95**	5.24±0.95	0.00±0.00
Alkaline borate buffer (pH 11.0)	24.64±0.43	**75.36±0.43**	0.00±0.00
Alkaline borate buffer (pH 11.0) heated to 55°C for 30 min	9.21±3.45	17.23±2.90	**73.57±1.88**

Data represent the mean±s.d. of six experiments. Bold type represents the percentage of the major conformer in each solution.

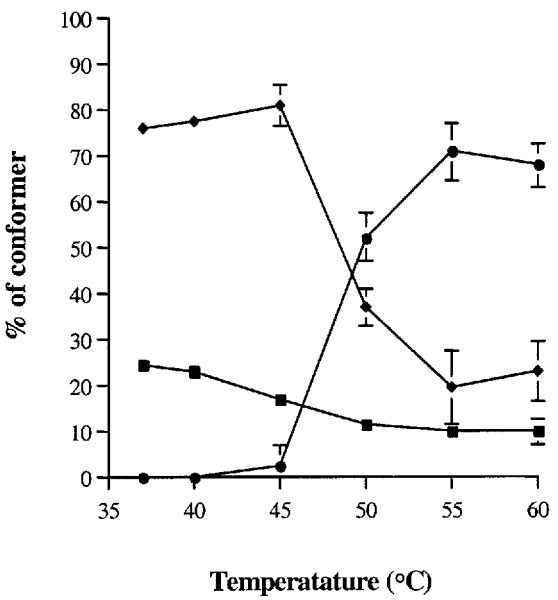

Figure 2 Effect of heating temperatures (for 30 min) on the percentages of each conformer in a heated solution of poly(L-lysine) in alkaline borate buffer (pH 11.0): random coil (■), α-helix (◆) and β-sheet (●). All data points represent the mean values and the error bars represent the s.d. of three experiments.

ture of 55°C yielded a much greater percentage of the β-sheet conformer while 60°C did not further increase the proportion of the β-sheet conformer. Therefore, as mentioned previously, a heating temperature of 55°C for 30 min was used to induce the β-sheet secondary structure in all experiments.

Conformer retention studies

Effect of temperature

Once a particular secondary structure of poly(L-lysine) was induced, it was necessary to determine how long the conformer would retain its specific secondary structure. Therefore, using CD spectroscopy, we conducted studies using solutions of each of the three conformers when stored at either 25°C or 37°C for 4 h. In phosphate buffer (pH 7.4), the random coil conformer of poly(L-lysine) appeared to be very stable at both 25°C and 37°C as the percentage of the random coil conformer was relatively constant throughout the 4-h experiment (data not shown). This may potentially be explained by the fact that the random coil is the native secondary structure for this polypeptide.

Similarly, the α-helix, the major conformer of poly(L-lysine) present in alkaline borate buffer (pH 11.0), was very stable at 25°C (Figure 3A). In contrast, after the solution containing the α-helix was stored at 37°C for 1.5 h, the α-helix conformer was rapidly converted to the β-sheet conformer (Figure 3B). This result suggests that even a temperature as low as 37°C may induce the transition of the α-helix to the β-sheet conformer, albeit much more slowly than at higher temperatures, and that a lag period is required for this transition at 37°C. As illustrated in Figure 4A, a slow transition of the heat-induced β-sheet conformer back to the α-helix conformation was noted at 25°C. At 37°C, the percentage of the β-sheet conformer gradually increased throughout the duration of the 4-h experiment (Figure 4B) with a simultaneous slight decrease in the percentages of the random coil and the α-helix conformers.

The results of these studies aimed at determining the time course over which each induced secondary structure of poly(L-lysine) was stable at both 25°C and 37°C suggest that future experiments employing the heat-induced β-sheet conformer need to be conducted at a temperature greater than, or equal to, 37°C. In addition, the α-helix conformer must be investigated at a temperature lower than, or equal to, 25°C to avoid slow conversion of the α-helix conformer to the β-sheet structure while the native random coil conformation may be studied at either 25°C or 37°C and, hence, may serve as a control to investigate the effect of temperature.

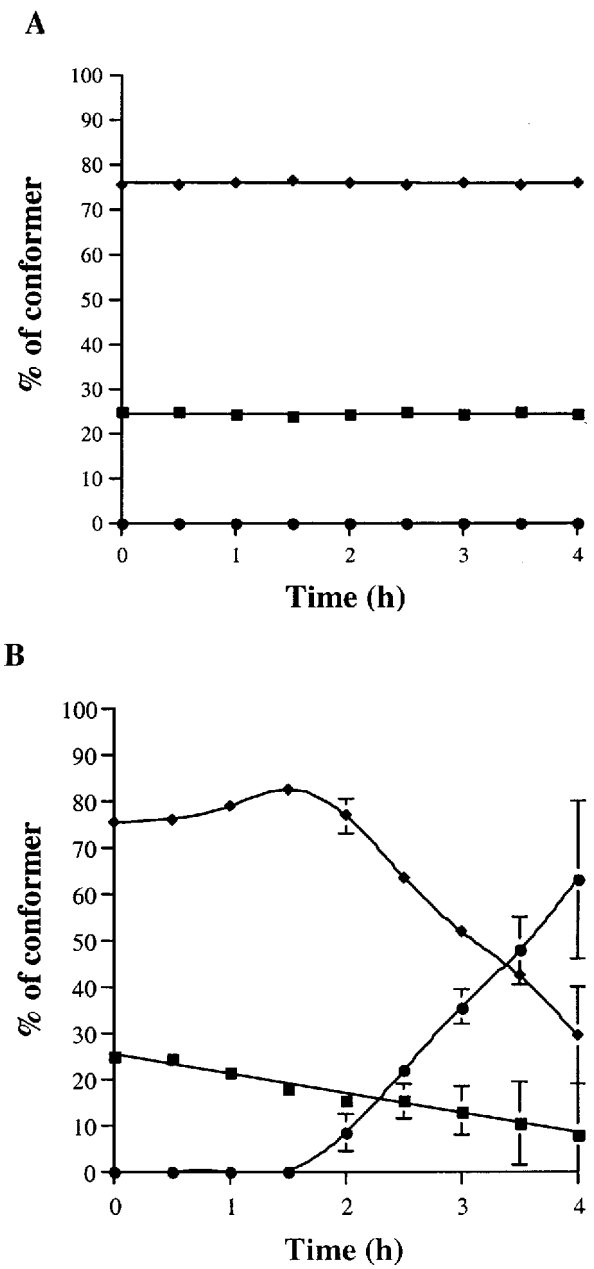

Figure 3 Percentages of each conformer of poly(L-lysine) in alkaline borate buffer (pH 11.0) stored at 25°C (A) and 37°C (B) for 4 h: random coil (■), α-helix (◆) and β-sheet (●). All data points represent the mean values and the error bars represent the s.d. of three experiments. In A, lines through the mean values represent a mathematical fit of the data using a linear least-square regression analysis.

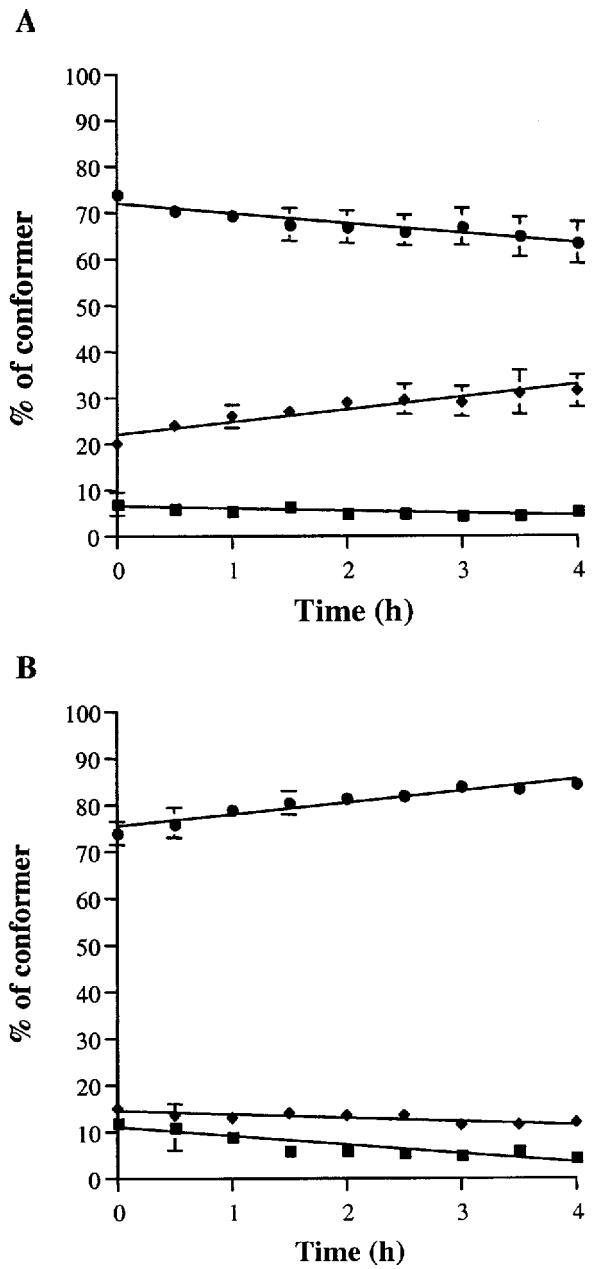

Figure 4 Percentages of each conformer of poly(L-lysine) in alkaline borate buffer (pH 11.0) heated to 55°C for 30 min and stored at 25°C (A) and 37°C (B) for 4 h: random coil (■), α-helix (◆) and β-sheet (●). All data points represent the mean values and the error bars represent the s.d. of three experiments. Lines through the mean values represent a mathematical fit of the data using a linear least square regression analysis.

Effect of pH

Since pH was one of the three stimuli employed in this study, it was interesting to see how a change in the pH affected the percentages of each conformer in the solutions. The results are presented in Table 2. Once the solution of poly(L-lysine) in borate buffer (pH 11.0) was mixed with an equal volume of phosphate buffer (pH 7.4), the pH dropped to 10.1 ± 0.1 and the percentage of

Table 2 Effect of pH on the percentages of each conformer.

Poly(L-lysine) solution	Solution	Random coil (%)	α-helix (%)	β-sheet (%)
Alkaline borate buffer (pH 11.0)	Control	26.15±0.57	**73.85±0.57**	0.00±0.00
	Sample	41.56±1.71*	**58.44±1.71***	0.00±0.00
Alkaline borate buffer (pH 11.0) heated to 55°C for 30 min	Control	10.03±1.63	10.09±0.44	**79.88±1.59**
	Sample	34.70±4.66*	**62.19±2.92***	3.11±3.03*

Data represent the mean±s.d. of three experiments. *$P < 0.05$, compared with the control. Bold type represents the percentage of the major conformer in each solution. The sample was mixed with an equal volume of 10 mM phosphate buffer (pH 7.4) whereas the corresponding solution mixed with an equal volume of 10 mM alkaline borate buffer (pH 11.0) served as the control. In the case of poly(L-lysine) in alkaline borate buffer (pH 11.0) heated to 55°C for 30 min, all solutions were maintained at 37°C.

the α-helix conformer (the predominant secondary structure in this solution environment) significantly ($P < 0.05$) decreased to 58.44±1.71% compared with 73.85±0.57% for the control (Table 2). This suggests that the mechanism associated with the induction of the α-helix was reversible charge neutralization. When the pH was approximately equal to the pK_a of the lysyl side chains, only about 50% of the ionizable groups were neutralized. If the pH were to be further decreased, data from the present study would seem to indicate that the random coil might become the predominant secondary structure.

Interestingly, the heat-induced β-sheet conformer was almost totally lost when mixed with an equal volume of phosphate buffer (pH 7.4) even though the temperature was maintained at 37°C (Table 2). The final pH was 9.6±0.3 and the α-helix became the major secondary structure of poly(L-lysine) in this solution. This result indicates that the β-sheet conformer could not be maintained when the pH requirement of 11.0 was not met. We have also found that the β-sheet conformer could not be thermally induced from the random coil conformer at neutral pH (data not shown). This could be because charge repulsion between the ionized lysyl side chains would not allow either intra- or intermolecular hydrogen bonding.

Effect of ionic strength

Since salts have been used as a stimulus to induce secondary structural changes in poly(L-lysine), one would expect that a change in the ionic strength would affect the relative percentages of a given conformer present in the solutions. Sodium chloride was used for this purpose. We found that all major conformers in the solutions were relatively stable to the change in ionic strength over the concentration range tested, which included an isotonic NaCl concentration of 0.9% w/v or 0.16 M (data not shown).

Octanol/buffer partitioning studies

To examine whether the hydrophobicity was changed following a change in the secondary structure of poly(L-lysine), we conducted octanol/buffer partitioning studies. One of the factors determining membrane permeability is the lipophilicity of the compound (Rosenberg et al 1991). Octanol/water systems have long been used to predict the potential for peptide permeability across the cell membrane (Burton et al 1996).

To parallel the results of our conformer-retention studies, octanol/buffer partitioning studies were conducted at two different temperatures. The random coil and the α-helix conformers were studied at 25°C while the β-sheet conformer was compared with the random coil conformer at 37°C (Table 3). The difference between the values of the apparent partition coefficient (K) of the random coil conformer at both temperatures was

Table 3 Octanol/buffer partition coefficients of poly(L-lysine).

Temperature	Conformer	K	logK
25°C	Random coil	0.47±0.03	−0.32±0.02
	α-helix	0.74±0.02*	−0.13±0.01*
37°C	Random coil	0.39±0.08	−0.41±0.10
	β-sheet	1.69±0.10*	0.23±0.03*

All data represent the mean±s.d. of three experiments. *$P < 0.05$, compared with the random coil conformer at a given temperature.

statistically insignificant ($P > 0.05$). This indicates that temperature does not play a significant role in the partitioning behaviour of the random coil conformer between the two phases. It can be unequivocally noted from Table 3 that the hydrophobicity significantly increased in the order: random coil < α-helix < β-sheet.

At neutral pH, the ε-amino groups of poly(L-lysine) are positively charged. Thus, the random coil conformer is highly hydrated. As the α-helix conformer is formed by charge neutralization, intramolecular hydrogen bonding results in fewer interactions with water and the molecule becomes more hydrophobic. In the β-sheet conformation, the full hydrogen-bonding capacity of the polypeptide backbone is utilized through the interactions between neighbouring chains (Voet & Voet 1995). As a result, the β-sheet conformer is the most hydrophobic of the three conformations. Blout & Lenormant (1957) have shown that the surfaces of the α-helix conformation of poly(L-lysine) are hydrated by four to six molecules of water per lysine residue, whereas the surfaces of the β-sheet conformer have less than two molecules of water per lysine residue.

Although the α-helix and the β-sheet conformers of poly(L-lysine) are more lipophilic than the native random coil conformer, the change in lipophilicity is not large enough to fall within the log K range of 1.5–3.5 for maximal membrane permeation (Lipinski et al 2001). Because most therapeutic proteins and polypeptides are extremely hydrophilic, attempts to increase their intrinsic lipophilicity (e.g. by chemical modifications) to facilitate transmembrane permeation have not been successful.

Conclusions

Variation of pH and temperature conditions can be successfully used to induce the secondary structures (α-helix and β-sheet) of poly(L-lysine). These changes are either partially (α-helix) or wholly (β-sheet) reversible when the stimuli are attenuated. Such reversible changes would be advantageous for therapeutic peptides following absorption for them to resume their native structure, which is generally a requirement for their pharmacological activity. Future experiments employing the heat-induced β-sheet conformer of poly(L-lysine) need to be conducted at a temperature greater than, or equal to, 37°C. On the other hand, the α-helix conformer must be investigated at a temperature lower than, or equal to, 25°C, while the random coil conformation may be studied at either 25°C or 37°C. Hence, the randomly-coiled conformer of poly(L-lysine) may serve as an appropriate control for future experiments which investigate membrane transport, protein binding, etc. Although the α-helix and the β-sheet conformers of poly(L-lysine) are significantly more lipophilic than the native randomly-coiled conformer, neither conformer is lipophilic enough to be a candidate for diffusion through a biological membrane. Thus, future in-vitro work will investigate the effect that modulation of a polypeptide's secondary structure has on membrane permeation via aqueous pore diffusion.

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