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International Journal of Pharmaceutics 244 (2002) 1–8

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Shape imposed by secondary structure of a polypeptide affects its free diffusion through liquid-filled pores

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Received 18 January 2002; received in revised form 24 May 2002; accepted 28 May 2002

Abstract

The purpose of the present study was to investigate the effect of secondary structure of three model polypeptides on their apparent permeability (P_{app}) across a synthetic, microporous membrane. Poly-L-lysine (PL), poly-L-glutamate (PGlu), and poly-L-lysine-L-phenylalanine (1:1) (PLP) were selected because a solution environment in which their predominant secondary structure is random coil (RC), α-helix, and β-sheet, respectively, is easily achieved. The conformation of each polypeptide was verified by circular dichroism (CD). Diffusion studies were conducted under sink conditions at 25 °C across a microporous polyester membrane using a donor concentration of 0.02 mM for each model polypeptide. NMR was utilized to obtain a second estimation of the diffusion coefficient for each polypeptide. The equivalent hydrodynamic radii (R_e) of the three model polypeptides were calculated using the values of the diffusion coefficient obtained by both NMR and the classic in vitro diffusion studies. The viscosity of each polypeptide solution was also determined to investigate the effect of viscosity on the aqueous diffusion coefficient. Statistical analysis demonstrated a significant ($P < 0.05$) difference in both P_{app} and the aqueous diffusion coefficient (D_{aq}), as well as the calculated R_e values, between all three model polypeptides and there was no significant ($P > 0.05$) difference in the viscosity of the polypeptide solutions. Values of D_{aq} and R_e calculated from the diffusion studies were in relatively close agreement to those obtained using NMR. The logarithm of P_{app} was highly correlated ($r = -0.961$) with the values of R_e calculated from NMR ($R_{e\ (NMR)}$) rather than the *mw* of the polypeptides ($r = 0.681$). Values of the Perrin or shape factor which deviate substantially from unity are suggestive of a non-spherical or ellipsoid shape and were 1.22 ± 0.20, 1.55 ± 0.11, and 2.38 ± 0.20 for PGlu, PL, and PLP, respectively. In conclusion, the observed difference in the membrane transport/diffusion of the three model polypeptides is suggested to be due to the shape associated with the secondary structure of each macromolecule, rather than the polypeptide's *mw* or the viscosity of the dilute polypeptide solution.

Keywords: Secondary structure; Diffusion; Polypeptide; Molecular shape

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00320-4

A survey of the literature on parameters affecting paracellular transport of polypeptides and proteins (the preferred path of permeation) demonstrates the crucial role of ionic charge, molecular weight, and size on this route of transport. Attempts to enhance the membrane transport of a protein in the unfolded state in vitro (Johnston et al., 1998) and increase the permeability of a variety of small peptides and larger polypeptides through monolayers of various cell types have been conducted to more fully elucidate all of the variables which affect their paracellular transport (Horibe et al., 1997; Matsukawa et al., 1997; Pade and Stavchansky, 1997; Sorensen et al., 1997; Johnston et al., 1998; Dodoo et al., 2000). Other studies have examined the role of increasing molecular weight for a homologous series of compounds on their apparent permeability through various types of cell monolayers or in vivo (Horibe et al., 1997; Matsukawa et al., 1997; Dodoo et al., 2000), but little or no emphasis has been placed on the actual shape of the permeant. There have also been numerous reports, using low-molecular-weight organic molecules, which describe the effect of a molecule's *mw* and ionic charge on its passive diffusion across a Caco-2 cell monolayer by the paracellular route (Burton et al., 1996; Gangwar et al., 1996; Tamura et al., 1996; Knipp et al., 1997a,b; Okumu et al., 1997; Pauletti et al., 1997). Additionally, the effect of overall size and charge on the passive diffusion of peptides across a Caco-2 cell monolayer by the paracellular pathway has been investigated (Pauletti et al., 1997). Unfortunately, peptides no larger than six amino acids were utilized and hence, formation of secondary structures such as α-helices, β-sheets, and extensive random coils (RCs) was not possible (Pauletti et al., 1997). Moreover, most therapeutically useful polypeptide drugs currently being developed by pharmaceutical companies have many more than six amino acids.

The effect of overall ionic charge on the absorption of small (≤ 6 aa) peptides by the paracellular pathways present in a monolayer of Caco-2 cells has also been studied (Pauletti et al., 1997). The rank order for paracellular transport across a Caco-2 cell monolayer with peptides of similar shape and size was charge-dependent (i.e. neutral $\geq$ positive $>$ negative) presumably because the tight-junction complexes associated with the paracellular route are negatively charged (Rojanasakul et al., 1992; Gonzáles-Mariscal et al., 2001). Moreover, charged-molecules are known to be absorbed via the paracellular pathway in a manner consistent with the mechanism of molecular-size restricted diffusion in a fixed electronegative field of force for cylindrical pore channels (Adson et al., 1995). It should be noted that the overall ionic charge associated with a polypeptide becomes less important as the molecular size of the permeant increases and, at the level of a hexapeptide, the contribution of net charge to the overall transport characteristics is virtually negligible (Pauletti et al., 1997).

The aim of the present study was to investigate the aqueous diffusion of three model polypeptides/macromolecules possessing different secondary structures through a porous synthetic membrane. Such a membrane provides a model matrix to determine the intrinsic D_{aq} associated with a molecule's diffusion through aqueous-filled pores without the confounding effects of protein binding, enzymatic degradation/inactivation, endocytotic uptake, membrane charge, and size 'restricted' or 'hindered' diffusion that a macromolecule would experience through the tight junctions of a biological membrane. These studies allowed for an assessment of how shape (induced by predominantly secondary structure with interconvertible and non-permanent tertiary structure), rather than the *mw*, influences the in vitro diffusion of large polypeptides through uncharged, cylindrical, aqueous-filled pores.

To determine whether a difference in the secondary structure of a polypeptide influences its aqueous diffusion through a porous membrane, diffusion experiments were conducted using a synthetic, hydrophilic polyester membrane (Nuclepore®, pore diameter = 1.0 μm, thickness = 12 μm, and porosity = 0.079). The three model polypeptides evaluated were poly-L-lysine (PL), *mw* 46 kDa, in 10 mM phosphate buffer (PB) (pH 7.4) representing primarily a RC (Greenfield and Fasman, 1969), poly-L-glutamate (PGlu), *mw* 61 kDa, in 10 mM PB (pH 4.5) existing mainly as an

α-helix (α) (Johnson and Tinoco, 1972), and poly-L-lysine-L-phenylalanine (1:1) (PLP), *mw* 49 kDa, in 100 mM $NaClO_4$ (pH 5.0) existing predominantly as a β-sheet (β) (Seipke et al., 1974) and were purchased from Sigma (St. Louis, MO). These polymers had a very narrow molecular weight distribution with weight-average to number-average molecular weight ratios (mw/M_n) of approximately 1.1–1.2. The predominant conformation of a given model polypeptide placed in the donor chamber of each of three side-by-side diffusion cells (membrane area = 0.64 cm^2) was verified by circular dichroism (CD) at time $t = 0$ h. All diffusion studies were conducted under sink conditions for 3 h at 25 °C using an initial polypeptide concentration of 0.02 mM. At this donor concentration, PL, PGlu, and PLP retained 92 ± 2.4, 84 ± 0.6, and 74 ± 11% of their predominant conformation at time $t = 0$ h. The percent of each conformation present was calculated using the software called JFit (developed by Dr B. Rupp at the Lawrence Livermore National Laboratory, Livermore, CA; www-structure.llnl.gov/cd/cdtutorial.htm). Polypeptide concentration in the receptor chamber was determined using the micro-BCA protein assay (Pierce, Rockford, IL) with construction of separate calibration curves for each polypeptide.

CD analysis was also conducted with an additional aliquot of the receptor phase samples collected during the diffusion study to ensure that the predominant conformation of each polypeptide was preserved throughout a 3-h experiment. CD spectra were also employed to verify that the ratio of the percent of each conformer present in the donor phase at time $t = 0$ remained relatively constant in the receptor phase following porous diffusion across the polymeric membrane. Using PL as an example, companion CD studies served to verify that the ratio of RC: α-helix conformers present in the donor phase at time $t = 0$ 92%:8% was maintained relatively constant in the receptor phase throughout a diffusion study, since the micro-BCA protein assay does not distinguish between conformer type; rather, it provides only a total protein concentration in solution.

Viscosity measurements were also performed at 25 °C with an Ostwald viscometer to investigate the effect of polypeptide solution viscosity on the value of the D_{aq}. Flow times were determined in triplicate for each polypeptide solution and polypeptide-free buffer using a chronograph accurate to 0.01 min. A 500 MHz model DRX500 Bruker Avance NMR equipped with a PULSED FIELD GRADIENT SPIN ECHO (PFG-SE) software program was used to provide a second estimate of the diffusion coefficient. All NMR measurements were performed at 25 °C using deuterated buffer and polypeptide concentrations identical to those used in the in vitro diffusion studies. The tallest peak in the spectrum was monitored for each polypeptide. For example, this was the epsilon CH_2 at 3.06 ppm for PL and the alpha proton at 4.06 ppm for PGlu. Finally, the Stokes–Einstein equation was employed to obtain an estimate of the equivalent hydrodynamic radii (R_e) of the three model polypeptides from the diffusion coefficient obtained by both NMR (Park et al., 2000) and the classic membrane diffusion studies.

Fig. 1 depicts the cumulative percent of each polypeptide that diffused through the polymeric membrane as a function of time. The cumulative percent of each polypeptide shown in Fig. 1 has been corrected for the predominant conformer associated with a particular model polypeptide as determined by the companion CD studies, since none of the three polypeptides existed exclusively in one specific conformation. P_{app}, D_{aq}, and R_e are listed in Table 1. All mean values were found to be significantly different from one another following a 1-way analysis of variance (ANOVA) (Table 1). The results clearly demonstrate that all three cumulative percent polypeptide transported/diffused-time profiles shown in Fig. 1 are significantly different from one another. The data listed in Table 1 and plotted in Fig. 1 clearly demonstrate that (1) there is only a weak correlation between P_{app} and *mw* [this is easily observed in Table 1, and especially, in Fig. 1, because the polypeptide with the largest *mw* ($PGlu_{\alpha\text{-helix}}$) exhibited a larger membrane flux than either PL_{RC} or $PLP_{\beta\text{-sheet}}$ having *mw*'s of 46 and 49 kDa, respectively], and (2) with two polypeptides having approximately the same *mw* (PL vs. PLP), overall shape plays a more important role in the rate and extent of their membrane permeation

[this is also clearly evident from Table 1 and Fig. 1, since the cumulative percent polypeptide transported/diffused-time profile and the values of P_{app} and D_{aq} for the randomly-coiled PL were all significantly ($P < 0.001$) greater than the corresponding parameters for PLP]. These two key points became even more apparent following plots of log P_{app} versus *mw* (graph not shown) in which the correlation coefficient (r) was only equal to 0.681 and log P_{app} versus $R_{e\,(NMR)}$ in which $r = -0.961$ (Fig. 2).

R_e values estimated using the Stokes–Einstein equation (Table 1) decrease in the order; $PLP_{\beta\text{-sheet}} > PL_{RC} > PGlu_{\alpha\text{-helix}}$. R_e for a molecule possessing an irregular shape is defined as an equivalent hydrodynamic radius and serves as an estimate of its size (Munk, 1989). Even though this parameter has no real physical meaning, it provides some information about the radius of a hypothetical sphere that has the same frictional coefficient, and hence the same diffusion coefficient, in the same liquid as the actual particle. NMR estimated diffusion coefficients for each of the three polypeptides are in relatively close agreement with the values listed in Tables 1 and 2 obtained from the in vitro diffusion experiments. On an average, the diffusion coefficients obtained by NMR were about 20% lower than those determined from the porous-membrane diffusion experiments (Table 2). This discrepancy may be attributed to a slightly higher viscosity of D_2O versus H_2O, since D_2O was used to make the polypeptide solutions used in the NMR experiments.

Based on the results of the in vitro diffusion experiments in the present study, differences in the mean values of the D_{aq} may potentially vary due to the differences in either the viscosity of the polypeptide solutions or the overall equivalent hydrodynamic radius (R_e) associated with each polypeptide's secondary structure. Using a 1-way ANOVA, no significant ($P > 0.05$) difference in the solution viscosity was noted for all three equimolar polypeptide solutions. This observation is significant since viscosity (η) is inversely proportional to D_{aq}. Therefore, differences in the R_e associated with each polypeptide still exists as a potential reason for the observed differences in the experimentally-determined values of D_{aq}.

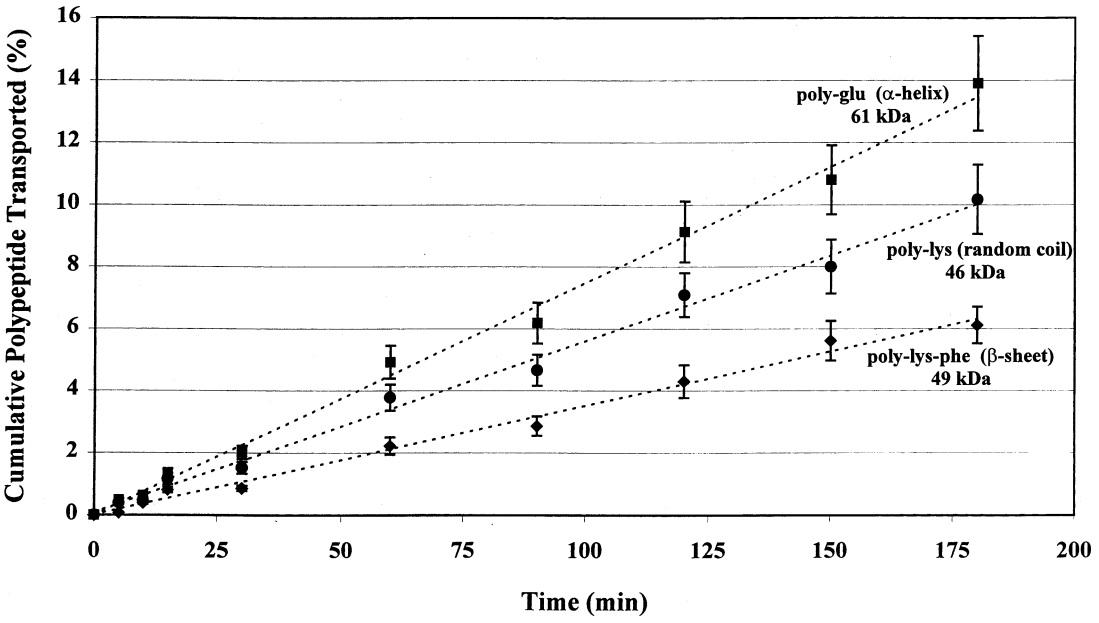

Fig. 1. The cumulative percent of polypeptide which underwent diffusion through liquid-filled pores of the synthetic polyester membrane. PL, ●; PGlu, ■; and PLP, ◆. Lines through the symbols represent a mathematical fit of the data using a linear, least-squares regression analysis.

Table 1
The Effect of secondary structure on the in vitro diffusion of three model polypeptides through aqueous-filled pores of a polyester membrane

Polypeptide	*mw* (kDa)	$P_{app} \times 10^5$ (cm/s)[a]	$D_{aq} \times 10^7$ (cm^2/s)[a]	R_e (Å) [a]
PL	46	4.2 ± 0.3[b]	6.4 ± 0.5	39 ± 3
PGlu	61	5.7 ± 0.8	8.6 ± 1.2	29 ± 4
PLP	49	2.7 ± 0.2	4.1 ± 0.2	60 ± 4

[a] Indicates that all three values for the P_{app}, D_{aq} and R_e were significantly different from one another following a 1-way ANOVA with post hoc testing using the method of Sheffé.
[b] Indicates the mean value $\pm$ the S.D. of three experiments.

Prior to suggesting that the overall shape of each polypeptide influenced its aqueous diffusion through the liquid-filled pores of the membrane, it was first necessary to determine whether the polypeptide's diffusion was 'hindered' or 'restricted' and represented a molecular sieving process. To this end, the Renkin molecular sieving function for a flexible polymer was calculated as follows:

$$F(\lambda) = (1-\lambda)^2(1 - 2.848\lambda + 3.269\lambda^2 - 1.361\lambda^3)$$

where, $\lambda = R_{e(NMR)}/R_{pore}$ and $R_{e\ (NMR)}$ is the equivalent hydrodynamic radius calculated from the diffusion coefficient obtained by NMR for a particular polypeptide as described above and R_{pore} is the radius of the membrane pore (Davidson and Deen, 1988). The following equations were utilized for calculation of the D_{aq}, with and without inclusion of the Renkin molecular sieving function, (i.e. $D_{aq} = P_{app}h/\varepsilon F(\lambda)$ vs. $D_{aq} = P_{app}h/\varepsilon$); where, P_{app} is the apparent permeability, h is the thickness of the membrane, ε is the porosity of the membrane, and $F(\lambda)$ is the Renkin molecular sieving function described above. Table 2 shows the results of these calculations. For each polypeptide, the value of the D_{aq} was extremely similar ($P > 0.05$) regardless of whether the Renkin function was included in the calculations (Table 2). This was true irrespective of whether a particular polypeptide's R_e, as used in the Renkin function [$F(\lambda)$], had been calculated from the diffusion coefficient obtained by NMR [$R_{e(NMR)}$] or determined from the in vitro diffusion experiments. This suggests that the polypeptides' diffusion through the fluid-filled pores of the membrane was not merely a molecular sieving phenomenon. In other words, the diffusion of the three molecules through the membrane pores was 'unrestricted' or 'unhindered'.

Since the observed difference in the values of P_{app} for PL, PGlu, and PLP was not dependent on either the molecular weight of the permeant or the viscosity of the dilute polypeptide solutions and inclusion of the Renkin molecular sieving function in the calculated value of D_{aq} resulted in a negligible change (i.e. the diffusion of the polypeptides through the pores was unhindered), it is reasonable to conclude that the overall shape associated with the secondary structure of the polypeptides is the predominant factor governing the diffusion through liquid-filled pores. Although estimations of R_e can be useful in explaining the

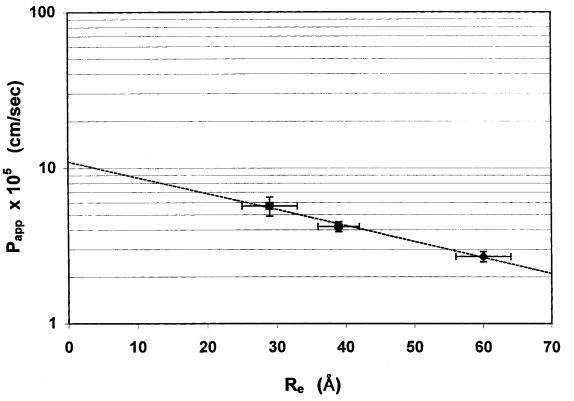

Fig. 2. The relationship between equivalent hydrodynamic radii obtained by NMR [$R_{e\ (NMR)}$] and the logarithm of the apparent membrane permeability (P_{app}) for the three model polypeptides. PL, ●; PGlu, ■; and PLP, ◆. The dashed line through the symbols represents a linear, least-squares regression analysis or fit of the data ($r = -0.961$).

Table 2
Comparison with diffusion coefficients obtained from in vitro diffusion experiments and NMR for three model polypeptides

Polypeptide	$D \times 10^7$ obtained using NMR (cm^2/s)	$D_{aq} \times 10^7$ w/o the Renkin sieving function (cm^2/s)[a]	$F(\lambda)$[b]	$D_{aq} \times 10^7$ with the Renkin sieving function (cm^2/s)[a]
PL	5.5 ± 0.05	6.4 ± 0.5	0.98	6.4 ± 0.5
PGlu	6.1 ± 0.1	8.6 ± 1.2	0.96	9.0 ± 1.2
PLP	3.3 ± 0.4	4.1 ± 0.2	0.93	4.4 ± 0.3

[a] D_{aq} obtained from the in vitro diffusion experiments.
[b] $F(\lambda) = (1-\lambda)^2(1-2.848\lambda+3.269\lambda^2-1.361\lambda^3)$ and $\lambda = R_{e(NMR)}/R_{pore}$ as explained in the text.

results shown in Fig. 1, only limited information on the overall shape of the polypeptide is obtained. If a molecule is highly irregular and/or asymmetric, the value of R_e is only an estimate of the size of an equivalent sphere having the same frictional coefficient as the irregularly-shaped polypeptide, at best.

In the present study, we attempted to use the Perrin or shape factor (F) to better understand the results of our diffusion experiments. The Perrin factor is defined as the ratio of the apparent frictional coefficient for an ellipsoid to that of a sphere having an identical volume and hence, a minimum frictional coefficient (f_0 or f_{min}), and can be informative as an estimate for the deviation from a spherical shape (Tanford, 1961).

The ratio is calculated as follows, $F = f_{exp}/f_0$ where, $f_{exp} = tT/D_{aq}$ and $f_0 = 6\pi\eta R_s$ in which R_s, as defined for a sphere, is equal to $(3mw \cdot \bar{v}/4\pi N)^{1/3}$. In the expressions above, k is the Boltzmann constant, T is the absolute temperature, D_{aq} is the aqueous diffusion coefficient obtained from the porous membrane diffusion experiments, η is the viscosity, $\bar{v}$ is the partial specific volume, and N is Avogadro's number. Calculated F values for PL, PGlu, and PLP were 1.55 ± 0.11, 1.22 ± 0.20, and 2.38 ± 0.20, respectively. A value of F in the range of 1.25–1.40 represents a protein or polypeptide which has minimal deviation from a globular (spherical) shape (as was the case for PGlu). However, molecules with F values which deviate substantially from unity, are suggestive of a non-spherical or ellipsoid shape. The calculated value of F for PL is suggestive of a moderately elongated shape. In contrast, an F value of 2.38 for PLP is suggestive of a highly-elongated prolate ellipsoid as would be expected for an extended β-sheet structure (Schurmann et al., 2001). A larger value of P_{app} for PGlu, despite its higher molecular weight, is suggested to be due to its globular shape. The globular (spherical) shape of PGlu ($F = 1.22$) would have the smallest translational friction in the solution and, therefore, result in a faster rate of diffusion in the liquid-filled channels of the porous membrane. This overall near-spherical shape is quite possible considering the molecular weight of PGlu and the potential for forming a super-secondary globular structure of α-helix bundles as suggested in Fig. 3. It should be mentioned that the suggested helix bundle should be considered only as a highly probable statistical average of many configurations that PGlu could potentially assume by its random

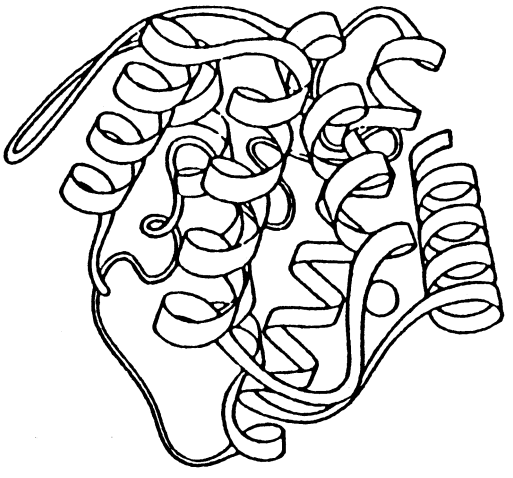

Fig. 3. One potential super-secondary structure of polyglutamate depicted using a ribbon diagram in which numerous α-helices comprise the bundles. With permission from J.S. Richardson (Richardson, 1981).

movement. A difference in the P_{app} between PL ($F = 1.55$) and PLP ($F = 2.38$) may also indicate a more elongated shape for PLP with a higher translational friction and slower diffusion compared with PL with a similar molecular weight.

It should be noted that Perrin or shape factors (F values) are dependent on solvation and asymmetry of a molecule. Since biologically-active polypeptides are highly likely to contain a substantial amount of water and possess asymmetric shapes, the suggestion of an ellipsoidal shape is based primarily on the availability of quantitative models. The postulate should be treated with the understanding that 'asymmetry is described in terms of an ellipsoidal model simply because this is the only model for which quantitative relations are available and not because the hydrodynamic particle is necessarily believed to assume an ellipsoid shape' (Tanford, 1961). Using the Perrin shape factor as a guide, it may be possible that PL behaves as a worm-like chain diffusing through the solvent, with free solvent moving in and out of the polypeptide chain, whereas PGlu may exist as a solvent-impermeable sphere with solvent trapped inside of its globular structure. Similarly, based on the value of F, it may be that the highly-elongated, rod-like shape proposed for PLP is truly descriptive of its overall shape, since this structure has long been observed for extended β-structures for molecules such as fibrinogen (Shulman, 1953).

The question might arise as to whether or not the free diffusion of three model polypeptides across a porous synthetic membrane truly represents diffusion through the tight junctions of a real biological membrane. Diffusion of a molecule through the tight junctions of a biological membrane (paracellular absorption) introduces restriction or hindrance to its diffusion. Restricted or hindered diffusion is a function of two parameters; first, steric hindrance due to the shape and size of the molecule (when a molecule first enters the pore) and second, resistance towards the diffusive movement while the molecule resides in the pore. A molecule with a greater molecular weight does not necessarily possess a larger shape and does not necessarily experience a higher resistance to diffusion while traversing the pore. Myosin with a *mw* of 4.93×10^5 has a f_{exp}/f_0 value of 3.53 and a D_{aq} equal to 1.16×10^{-7} cm^2/s, while collagen, with a *mw* of 3.45×10^5, experiences a greater diffusional resistance as evidenced by a f_{exp}/f_0 value equal to 6.8 and a D_{aq} equal to 0.69×10^{-7} cm^2/s (Tanford, 1961). Possible deformation (change in the overall shape) of a macromolecule may lower its resistance to diffusion in solution and modify any potential steric hindrance normally associated with its diffusion. Our microporous membrane did not impose steric restriction for the model polypeptides and thus allowed us to explore the relationship of shape associated with the secondary structure of a polypeptide to its resistance to movement in solution.

In conclusion, the observed difference in the membrane transport/diffusion of the three model polypeptides is suggested to be due to the shape associated with the secondary structure of each macromolecule, rather than the polypeptide's *mw* or the viscosity of the dilute polypeptide solution. The Perrin or shape factor associated with a polypeptide or protein may serve as a guide to a macromolecule's overall shape and its potential for diffusion through uncharged, cylindrical, liquid-filled pores.

Acknowledgements

The authors would like to thank Dr. D. Vander Velde, Director, NMR Laboratory, University of Kansas, for providing us with estimates of the diffusion coefficients using NMR. We would also like to thank Dr. I. Pezron for insightful scientific discussions. This work was supported by NIH grant GM 64320-01 and a grant from Aventis Pharmaceuticals, Kansas City, MO.

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