Dynamic and Static Light Scattering and Fluorescence Studies of the Interactions between Lactate Dehydrogenase and Poly(ethyleneimine)

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Interactions between poly(ethyleneimine) (PEI) and porcine muscle lactate dehydrogenase (LDH) were studied using static and dynamic light scattering, and intrinsic fluorescence spectroscopy. Time-related aggregation of the enzyme was reduced in the presence of the polymer. PEI of molecular weights 2000 and 25000 were found to complex with the enzyme without significant change in the particle hydrodynamic radius, i.e., the polymer is bound in a flat conformation at the enzyme surface. Interactions with the high molecular weight PEI (2.6×10^6), on the other hand, resulted in a particle of a size significantly larger than the polymer, indicating that several LDH molecules may bind within the large, branched polymer structure. Although it was not found possible to quantitatively determine the degree of binding, estimation of the binding constants by intrinsic fluorescence measurements revealed weak binding between the enzyme and the polymer.

Introduction

The interaction between a polymer and a protein has many practical implications in biotechnology. Complex formation has led to improvement of several important processes. In protein separation, for example, proteins have been recovered in high yield through precipitation with polyelectrolyte[1,2] and by formation of coacervate complexes.[3,4] Addition of polyelectrolytes has been used to achieve high stability and high activity of enzymes in biosensors[5,6] during catalysis in aqueous solution[7,8] as well as in organic solvents.[9]

The primary driving force for the formation of polyelectrolyte−protein complexes are electrostatic interactions[10,11] but hydrophobic effects and hydrogen bonding are also possible.[12,13] In general, the relative weighting of these interactions will depend on the charge density of the protein under the particular conditions used. Complexation of polycations and proteins at pH > pI[14] as well as of polyanions and proteins at pH < pI[15] has been observed. Cases also exist where proteins bearing a negative net charge bind to polyanions[16,17] and proteins bearing a positive net charge may bind to polycations.[18] Such effects have been explained by the polyampholytic characteristics of proteins. For example, clusters of negatively charged residues can exist on the protein surface at a pH value lower than the isoelectric point, providing sites for interaction with the positive charges on a polymer. It is also suggested that the hydrophobic effect may overcome weak electrostatic repulsive forces between a protein and a polyelectrolyte having the same charge sign.[17,19]

Several investigations have dealt with the mechanism of complex formation. Different modes of light scattering (dynamic, static, and electrophoretic) have been employed for this purpose.[18,20−22] These techniques have been supported by others such as turbidimetric titration and capillary electrophoresis.[23] The intrinsic tryptophan (Trp) fluorescence of proteins has been used in some instances as a probe for the changes in conformation or microenvironment of a protein which can occur upon interaction with polymers.[24−26]

The cationic polymer poly(ethyleneimine) (PEI) is a branched polymer in which 25% of the nitrogen atoms are primary amines, 50% secondary amines, and 25% tertiary amines—the polycationic character deriving from the positive charges located on the nitrogen atoms of the polymer backbone. The ionization is ∼10% at pH 7 and 2% at pH 10.5.[27] The polymer has found a number of interesting applications in biotechnology, e.g., in facilitating protein recovery[28] and biocatalyst immobilization.[29] It has also been shown to have a stabilizing effect on several enzymes in solution[30−33] as well as in biosensors.[34]

In our previous study,[30] we demonstrated the improvement in stability of the porcine muscle lactate dehydrogenase (LDH), a four-subunit enzyme, in the presence of PEI both during long-time storage in solution as well as during freeze-drying. This was achieved even at pH 7.2 where the net charge of the protein should be positive (pI of LDH ∼ 8.5). In a later study, we have clearly demonstrated the protective effect of the polymer against oxidative damage of proteins.[35]

The present paper describes further investigations of the interactions between LDH and PEI of different molecular weights using dynamic light scattering (DLS) and intrinsic fluorescence measurements. Static light scattering was used for molecular weight determinations of the protein and polymers used.

Experimental Section

Materials. Poly(ethyleneimine) samples of average molecular weights ca. 2000 and 25000 (50% aqueous solution) were

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purchased from Aldrich Chemical Co. (Milwaukee, WI). Poly(ethyleneimine) of high molecular weight (50% aqueous solution, $M_w \sim (0.6-1) \times 10^6$ according to the manufacturer, but $\sim 2.6 \times 10^6$ according to our measurements using static light scattering) and porcine muscle lactate dehydrogenase (EC 1.1.1.27; 11.3 mg/mL suspension in 2.1 M $(NH_4)_2SO_4$) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from standard sources.

Stock solutions of 5% (w/v) poly(ethyleneimine) were prepared in water and adjusted with HCl to pH 7.2. The LDH suspension was extensively dialyzed against Millipore quality water and subsequently centrifuged at 13000 g for 6 min in order to remove any aggregated material. Total protein concentration of the enzyme solution was determined by the bicinchoninic acid method[36] using bovine serum albumin as the standard protein.

Aliquots of the respective polymer and enzyme stock solutions were mixed in 50 mM Tris-HCl buffer pH 7.2 to the desired concentrations, and the final solutions were filtered through 0.2 μm filters. Light scattering and fluorescence measurements were made at enzyme concentrations of 0.5 and 0.02 g/L, respectively, with different concentrations of PEI.

Dynamic Light Scattering. The light scattering setup consists, as described previously,[37] of a 488 nm Ar-ion laser at a power of 0.2 W. The detector optics were coupled via a 4 μm monomodal fiber to an ITT FW 130 photomultiplier. The ALV-PM-PD amplifier-discriminator was connected in turn to an ALV-5000 autocorrelator-computer. The cylindrical scattering cells were sealed after filtration of the sample through 0.2 μm Millipore filters and immersed in a large-diameter thermostated bath containing Decalin placed on the axis of a goniometer.

Dynamic light scattering measurements were made at an angle of 90° and different sample concentrations at 25 °C. Analysis of the data was performed by fitting the experimentally measured g_2t, the normalized intensity autocorrelation function, which is related to the electrical field correlation function g_1t by the Siegert relation:[38]

$$g_2(t) - 1 = \beta|g_1(t)|^2 \tag{1}$$

where β is a factor accounting for deviation from ideal correlation. For polydisperse samples, $g_1(t)$ can be written as the inverse Laplace transform (ILT) of the relaxation time distribution, $\tau A(\tau)$:

$$g_1(t) = \int \tau A(\tau) \exp(-t/\tau) \mathrm{d} \ln \tau \tag{2}$$

where t is the lag time. The relaxation time distribution, $\tau A(\tau)$, is obtained by performing the inverse Laplace transform (ILT) with the aid of a constrained regularization algorithm (REPES),[39] which minimizes the sum of the squared differences between the experimental and calculated $g_2(t)$. The algorithm allows selection of a "smoothing" parameter, P, the probability to reject. Greater the P, the greater is the smoothing. $P = 0.5$ was chosen in all analyses. Discussions of the analysis of autocorrelation functions are given in ref 40.

The mean diffusion coefficient (D) is calculated from the second moments of the peaks as $D = \Gamma/q^2$, where $q = (4\pi n_D/\lambda)\sin\theta/2$ is the magnitude of the scattering vector and $\Gamma = 1/\tau$ is the relaxation rate with τ being the relaxation time. Here θ is the scattering angle, n_D the refractive index of pure solvent, and λ the wavelength of the incident light.

Within the dilute regime, D varies linearly with sample concentration (C), that is,

$$D = D_0(1 + k_D C + ...) \tag{3}$$

where D_0 is the diffusion coefficient at infinite dilution and k_D is the hydrodynamic "virial" coefficient related to the solute−solute and solute−solvent interactions. The Stokes−Einstein equation relates the infinite dilution diffusion coefficient to the hydrodynamic radius (R_H):

$$D_0 = \frac{kT}{6\pi\eta R_H} \tag{4}$$

where kT is the thermal energy factor and η is the temperature-dependent viscosity of the solvent. The hydrodynamic radius was evaluated assuming an equivalent sphere in all cases.

Static Light Scattering. Static light scattering measurements were made using a Hamamatsu photon-counting device with a 3 mW He−Ne laser. Toluene was used as the reference (Rayleigh ratio, $R_{toluene} = 13.59 \times 10^{-4}$ m^{-1} at 633 nm). The optical constant is

$$K = 4\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2 / N_A \lambda^4 \tag{5}$$

with ($\mathrm{d}n/\mathrm{d}c$) being the refractive index increment measured in a differential refractometer with Rayleigh optics at 25 °C. In the present system (LDH dissolved in 50 mM Tris-HCl buffer at pH 7.2), $\mathrm{d}n/\mathrm{d}c$ was determined to be 0.241 mL g^{-1}. N_A is Avogadro's constant, and λ is the wavelength. R_{90} is the Rayleigh ratio at angle 90° determined using $[(I - I_o)/I_{tol}]R_{tol}\ (n_s/n_{tol})^2$. Here, n_s is the refractive index of the solvent and n_{tol} that of toluene. I is the measured intensity of the solution, I_o that of the solvent, and I_{tol} that of toluene. For molecular weight (M_w) determinations, the reduced scattered intensity, (Kc/R_{90}) was plotted versus concentration and M_w was calculated from the intercept, $1/M_w$. This procedure is valid for small particles (e.g., free LDH) with the product of scattering vector and radius of gyration, $qR_g \ll 1$, for which there is no angle dependence of the reduced scattered intensity. For large particles, e.g., the high M_w polymer−LDH complex, with $qR_g < 1$ and where the reduced scattered intensity is angle-dependent, the radius of gyration, R_g, was determined from the ratio of slope to intercept of plots of $1/I \sin\theta$ vs $\sin^2(\theta/2)$ in the range where there is a negligible concentration dependence of (Kc/R_θ), where R_θ is the Rayleigh ratio at different angles θ.

Fluorescence Measurements. The fluorescence spectra of tryptophan (Trp) in LDH was recorded at 25 °C on a SPEX Fluorolog spectrometer using a 1 × 1 cm quartz cuvette in a thermostated cuvette holder. The excitation wavelength was 295 nm and the bandwidths for excitation and emission were 3 and 3.5 nm, respectively. Emission spectra between 310 and 360 nm were recorded in steps of 0.5 nm. An average of two scans was calculated.

The mixture of LDH and PEI can be treated as a solution containing one fraction of LDH molecules bound to the polymer and another fraction of LDH molecules free in solution. The total concentration of LDH ($[L]_{tot}$) is a sum of free LDH, $[L]_F$ and bound LDH, $[L]_B$:

$$[L]_{tot} = [L]_F + [L]_B \tag{6}$$

The total emitted intensity (I_{tot}) consists of intensity contributions from both free LDH molecules (I_F) and LDH molecules bound to PEI (I_B), and can be represented by the following equation:

$$I_{tot} = I_F \frac{[L]_F}{[L]_{tot}} + I_B \frac{[L]_B}{[L]_{tot}} \tag{7}$$

TABLE 1: Diffusion Coefficients of Three Different Molecular Weights of PEI at Different Concentrations[a]

$M_w = 2000$		$M_w = 25000$		$M_w \sim 2.6 \times 10^6$	
concn (g/L)	$D \times 10^{10}/m^2$ s^{-1} [b]	concn (g/L)	$D \times 10^{10}/m^2$ s^{-1} [b]	concn (g/L)	$D \times 10^{12}/m^2$ s^{-1} [b]
10	5.0	5	3.8	0.02	6.8
20	5.4	10	3.6	0.05	8.6
30	5.1	20	4.7	0.1	7.3
36.6	5.2	30	3.9	0.2	7.3
R_H (nm)	0.5		6.6		33

[a] Their respective hydrodynamic radii are given in the bottom row. [b] Error ± 0.5%.

The equilibrium dissociation constant, K_D, for LDH interacting with PEI is given by the relation

$$K_D = \frac{[L]_F[P]}{[L]_B} \tag{8}$$

where [P] is the concentration of PEI monomers. By combining eqs 7 and 8, the intensity equation can be developed:

$$I_{tot} = I_F \frac{K_D}{[P] + K_D} + I_B \frac{[P]}{[P] + K_D} \tag{9}$$

By chosing the initial values of K_D, I_F, and I_B, the obtained values of maximum intensity at different polymer concentrations were fitted to the best solution with the Levenberg−Marquardt nonlinear fitting algorithm using Origin, Microcal Software. Different initial choices of K_D, I_F, and I_B led to the same final result.

Results and Discussion

Characterization of Free Polymer and Enzyme. Prior to characterizing the interactions between LDH and PEI, light scattering measurements were performed individually on the enzyme and polymer solutions.

Study of samples of PEI with three different molecular weights revealed a small dependence of the diffusion coefficient on concentration in all cases, and values for the hydrodynamic radii calculated from the diffusion coefficients at infinite dilution were 0.5, 6.6, and 33 nm for PEI 2000, 25000, and 2.6×10^6, respectively (Table 1). For comparison with the dimensions of a flexible linear chain, R_H for poly(ethyleneoxide) is 1.1 nm ($M_w = 2000$) and 67 nm ($M_w = 2.5 \times 10^6$) using the relationship between R_H and M_w for this polymer given earlier.[41] A significantly smaller value of R_H is anticipated for the branched analogue. An approximately linear relationship between the logarithmic values of hydrodynamic radius and molecular weight of the PEI fractions was found (Figure 1). We note that, although PEI is a polyelectrolyte, in a buffer of strength 50 mM typical Coulombic forces will be screened out and the polymer would approximate the conformation of an uncharged chain.

A solution of 0.5 g/L LDH was used for dynamic light scattering measurements at 25 °C. The relaxation time distribution from Laplace inversion revealed the presence of two modes, (Figure 2). To confirm that the faster mode corresponds to free LDH molecules in solution, determination of the corresponding molecular weight was made using static light scattering. Preliminary measurements established that the scattering intensity was independent of angle, which will be true for particles having a radius of gyration, R_g, below ~200 Å. Values of (Kc/R_{90}), from the intensities of the fast mode, were estimated by multiplying the total intensity at angle 90° with the relative amplitude of the mode at different concentrations of the enzyme. The plot of (Kc/R_{90}) vs enzyme concentration shown in Figure 3 gives a molecular weight of 125 kDa for the fast mode. This value is lower than anticipated (140 kDa), but serves to confirm that the fast mode corresponds to the free protein.

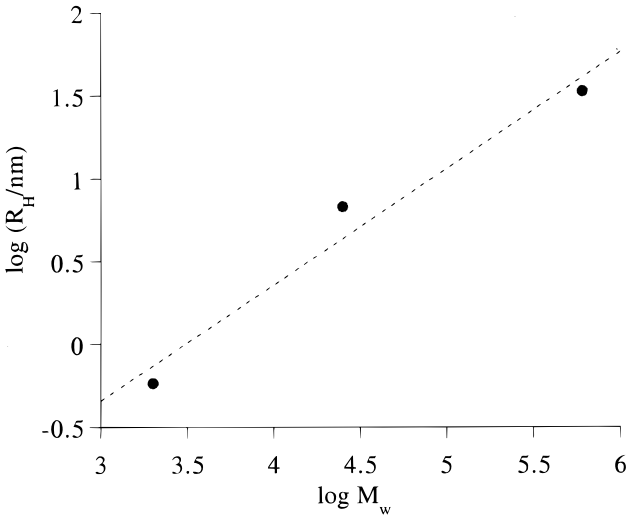

Figure 1. Relation of log hydrodynamic radius (R_H) to log molecular weight of PEI.

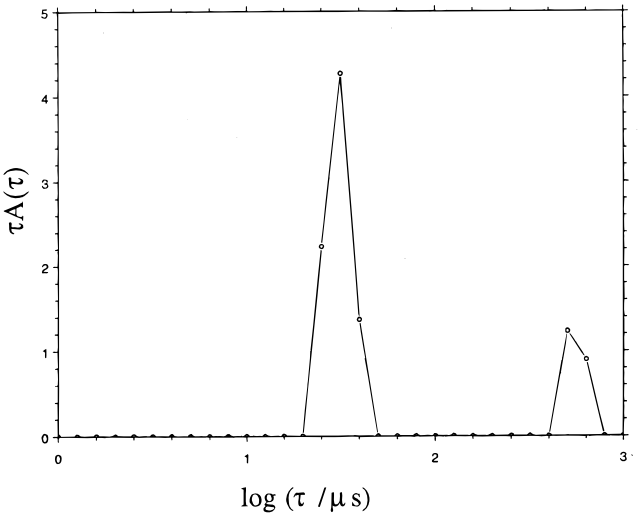

Figure 2. Relaxation time distribution (inverse Laplace transform) from dynamic light scattering of LDH solution (0.5 g/L). Measurements were made at 25 °C and at angle 90°.

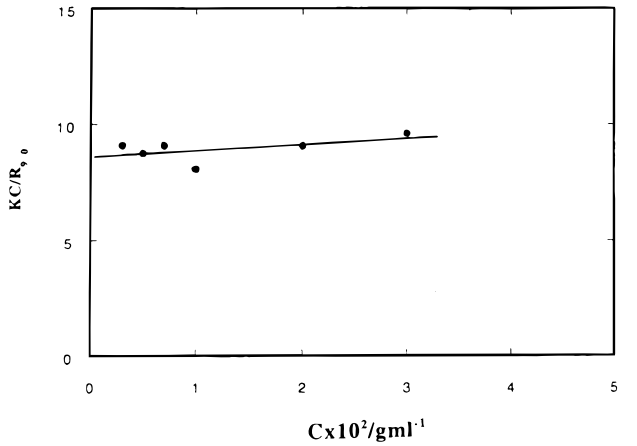

Figure 3. Reduced scattered intensity (Kc/R_{90}) for LDH as a function of concentration obtained by static light scattering.

TABLE 2: Diffusion Coefficients of the LDH Molecule (fast mode) and Aggregates (slow mode) in Solutions of Different Enzyme Concentrations

concn (g/L)	fast mode $D \times 10^{11}/m^2\ s^{-1}$ [a]	slow mode $D \times 10^{13}/m^2\ s^{-1}$ [a]
0.3	5.7	12
0.5	5.6	31
0.7	5.8	10
1	6.0	44
2	5.6	22
3	5.8	14

[a] Error ± 0.5%.

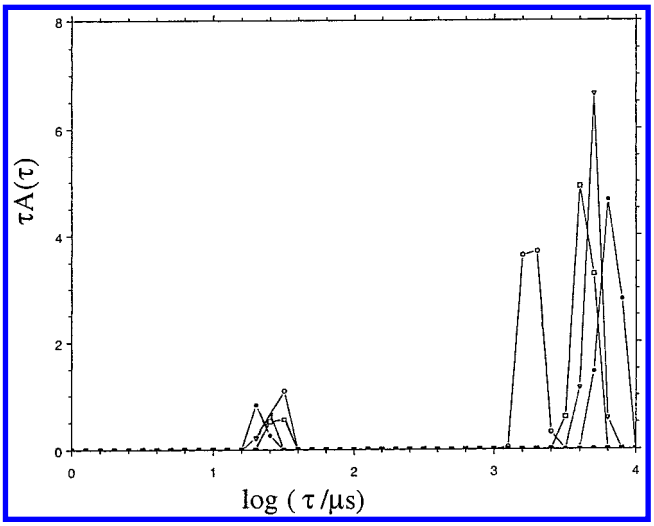

Figure 4. Relaxation time distributions of LDH solution (0.5 g/L) at different time periods of storage at 25 °C. $t = 0$ (○); $t = 2.5$ h (□); $t = 5$ h (▽); and $t = 24$ h (●).

For the fast mode, the diffusion coefficient showed no significant dependence on concentration in the range of 0.3−3 g/L, (Table 2), which can be expected for a compact, globular protein. Using the Stokes−Einstein equation, the hydrodynamic radius of the LDH molecule was estimated to be 4.3 nm (from the value of the fast mode at infinite dilution, D_0), which is in good agreement with the values reported earlier for the enzyme from rabbit muscle and pig heart LDH (∼4.3 nm),[42] and *Thermotoga maritima* (4.2 ± 0.1 nm).[43]

The diffusion coefficients of the slow mode displayed erratic values with increasing concentration (Table 2). An estimate of the hydrodynamic radius of ∼100 nm can be made from the extrapolation of the diffusion coefficient to infinite dilution. However, as the scattered intensity (proportional to the area of the peak) is related to (R_H),[6] slowly relaxing components will have a very low mass concentration weighting.

Time-Related Aggregation of LDH and Influence of PEI. The observed aggregates in the LDH solution were monitored over a period of time at room temperature. This was done by measuring relaxation time distributions by dynamic light scattering at different time intervals after preparation of the LDH solution (Figure 4). These show a clear shift of the slow mode to a longer relaxation time, i.e., decreased diffusion coefficient (Table 3), and a strong enhancement in the corresponding relative intensity with time. It should be noted that the LDH solution used in this particular experiment contained a significantly larger portion of aggregates of larger size already from the start than can be seen in Figure 2, due to a relatively longer time maintained at room temperature before conducting the measurements.

Our earlier studies[30] have shown that addition of PEI to a solution of LDH substantially suppresses the aggregation phenomenon visually observed and concomitantly leads to an increased stability of the enzyme activity. This observation was confirmed also by light scattering. In the presence of 3% (w/v) PEI 2000, the aggregate peak (slow mode) of LDH was seen to decrease in amplitude and was shifted to a shorter relaxation time (not shown). It can be seen in Table 3 that, in the presence of PEI, a significant decrease in the diffusion coefficient of the slow mode does not occur until 24 h after the first measurement, whereas the size of the aggregates starts to increase already after 2.5 h in the absence of the polymer. Figure 5 shows that the addition of PEI clearly reduces the intensity of the slow mode, i.e., it inhibits aggregate formation, serving to maintain the time-stability (and activity) of LDH.

TABLE 3: Time Dependence of Diffusion Coefficients of the Aggregate (slow mode) in LDH Sample (0.5 g/L), in the Absence and Presence of PEI 2000 (3%)

time (h)	LDH $D \times 10^{13}/m^2\ s^{-1}$ [a]	LDH−PEI $D \times 10^{13}/m^2\ s^{-1}$ [a]
0	9.5	14
2.5	4.0	12
5	3.5	11
24	2.6	3.2

[a] Error ± 0.5%.

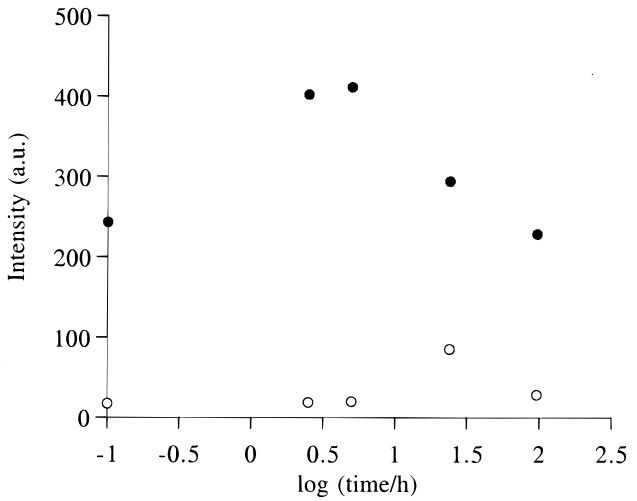

Figure 5. Intensity of the slow mode (aggregate) in solutions of pure LDH (●) and LDH in the presence of 3% PEI 2000 (○), respectively, at 25 °C.

The addition of PEI with higher molecular weights, 25000 and 2.6×10^6, respectively, resulted in either a similar shift of the aggregate peak to a shorter relaxation time and a strongly reduced amplitude or a complete disappearance of the peak (not shown). It has been suggested that such absence of aggregation could be the result of electrostatic repulsion between the protein molecules due to coating of the protein surface with charged polymer molecules.[44] The molecular basis for the observed anti-aggregation effect of PEI was further investigated with light scattering.

Interactions of LDH with PEI of Different Molecular Weights. Dynamic light scattering was used to obtain support for binding between the protein and the polycationic polymer of different molecular weights. Relaxation time distributions for solutions of LDH (0.5 g/L), PEI 2000 (30 g/L) and a mixture of the two components, respectively, are shown in Figure 6. We note that the peaks are well-separated, allowing evaluation of the hydrodynamic radii without ambiguity. The LDH−PEI mixture has a main central peak with a hydrodynamic radius similar to that of pure LDH. A low intensity, fast peak of approximately the same relaxation time as that for pure PEI is

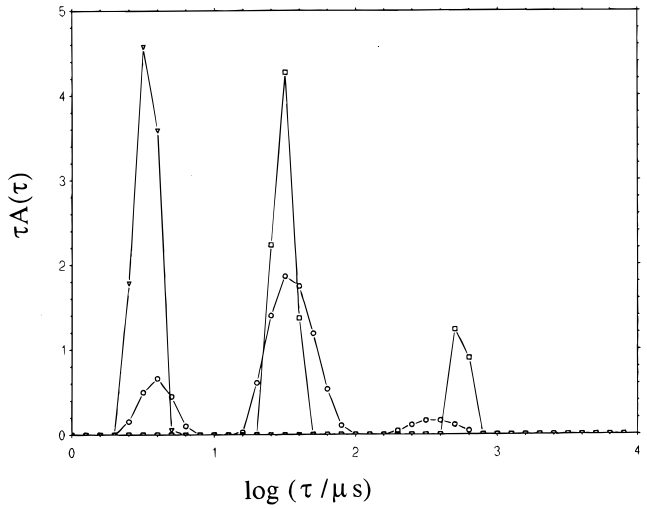

Figure 6. Relaxation time distributions for the complex formed between LDH (0.5 g/L) and 3% PEI 2000 (○) compared with pure LDH (□) and pure PEI 2000 (▽) of similar concentrations.

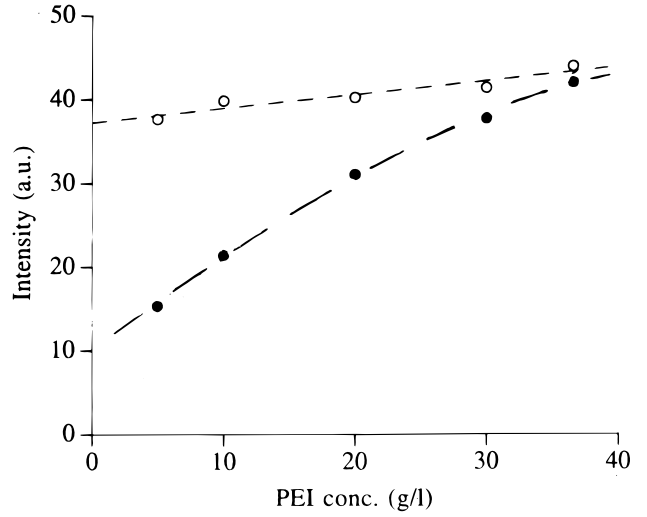

Figure 7. Total intensity of pure PEI 2000 (●) and of its complex with LDH (○) at different polymer concentrations at 25 °C.

also present. A reasonable inference to be drawn from these results is that a significant proportion of the polymer associates with the enzyme to form a complex. Further evidence is provided by the observation that the total scattered intensity of a mixture of LDH and PEI 2000 is similar in magnitude to that of the sample containing only LDH (38 au), (Figure 7); i.e., the intensity increases only weakly on increasing the concentration of PEI. This was in contrast to the intensity profile of PEI, which displays the expected, approximately linear increase with increasing polymer concentration. The interpretation here is that the intensity contribution from the LDH molecule masks that of the polymer on forming the complex. In the absence of complex formation, the contributions to the total intensity of the LDH/PEI mixture would have been expected to be roughly additive and have the same trend as that for free PEI. On the basis of the observations above, we propose that there is a close association of the polymer chain segments with enzyme surface, i.e., the polymer lies in close contact with the surface of the enzyme molecule leading to a complex with a radius similar to that of the pure enzyme.

Figure 6 also shows a low intensity peak of the LDH−PEI mixture close to the position of LDH aggregates, but which has a slightly shorter relaxation time, indicating a significant quantitative reduction in size in comparison with the aggregates in the pure LDH sample, as reported above.

TABLE 4: Diffusion Coefficient of the Fast and Slow Modes in the Mixture of LDH (0.5 g/L) and PEI (M_w = 2000 and 25000)[a]

	LDH−PEI 2000		LDH−PEI 25000
concn (g/L)	fast mode (free PEI) $D \times 10^{10}$/m^2 s^{-1} [c]	slow mode (complex) $D \times 10^{11}$/m^2 s^{-1} [c]	slow mode[b] (complex) D $\times 10^{11}$/m^2 s^{-1} [c]
10	4.6	5.6	5.5
20	4.4	5.4	4.8
30	4.5	5.1	4.2
R_H (nm)	0.5	4.2	4.0

[a] Measurements conducted at time $t = 0$. [b] Only one mode can be seen in the mixture of LDH with PEI 25000. [c] Error ± 0.5%.

Table 4 shows diffusion coefficients calculated from the peaks obtained in a mixture of LDH−PEI 2000 and LDH−PEI 25000, respectively. In the mixture of LDH−PEI 2000, a peak corresponding to free PEI 2000 is observed together with the peak representing the complex. With PEI 25000, a separate peak for free PEI is not observed since its diffusion coefficient lies close to those of free LDH and the formed complex. The calculated hydrodynamic radii of the complexes are very similar to each other and to the radius of the free LDH molecule. Considering the monomer unit mass of 43 g/mol and a monomer unit length of 0.3 nm for the PEI chain, one obtains the maximal lengths of 14 nm for PEI 2000 and 175 nm for PEI 25000 with linear polymer chains. However, since the used polymers are highly branched with 50% of the nitrogens appearing as secondary amines and 25% being tertiary amines, the true contour lengths of the polymers are at least 25% shorter. Since the circumference of the LDH equivalent sphere (radius 4.3 nm) is ∼27 nm, it appears plausible that binding of either PEI molecule at the surface of the LDH particle in a flat, loosely coiled conformation results in a complex with a radius similar to that of the pure enzyme as presented schematically in Figure 8.

With the high molecular weight polymer ($M_w = 2.6 \times 10^6$), a somewhat different picture is obtained. Figure 9 shows the relaxation time distributions for LDH, high M_w PEI, and the mixture of the two components. Although the distribution is broad, the profile of the mixture of LDH and PEI contains a mode with a significantly longer average relaxation time than that of pure PEI, i.e., there is an increase in the size of the high M_w PEI matrix possibly because of binding of LDH molecules. The figure also shows a fast mode peak of low intensity with a relaxation time close to that of pure LDH. This may either represent free LDH or a low M_w fraction of PEI combined with LDH. The picture here is less clear than that for the mixture of LDH with PEI of lower molecular weights, due to the breadth of the distributions. Nevertheless, the same trend was reproducibly found for a series of high M_w PEI concentrations.

Comparison of the diffusion coefficients for pure high M_w PEI and the slow mode in the mixture with LDH at varying polymer concentrations reveals a clear difference as shown in Figure 10 a. From the infinite dilution values of the diffusion coefficients, $R_H = 66$ nm is found for the slow mode in the mixture compared with $R_H = 34$ nm for pure high M_w PEI. A strong intensity enhancement upon addition of LDH to the high M_w PEI solution (Figure 10b) was also observed.

From the relation $\rho = R_g/R_H$, the particle conformation can be estimated[45] (hard sphere $\rho = 0.78$; compact theta solution random coil, $\rho = 1.5$). The radius of gyration (R_g) of large particles ($> \lambda/20$) can be calculated from the ratio of slope to

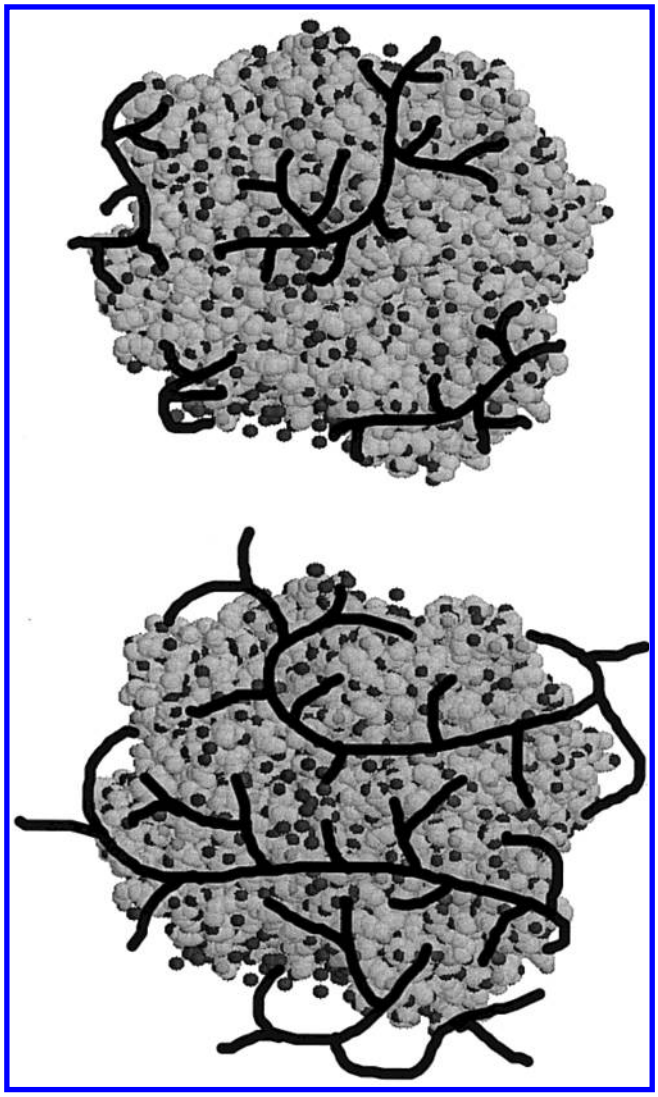

Figure 8. Schematic representation of the complex of LDH with (a) PEI 2000 and (b) PEI 25000.

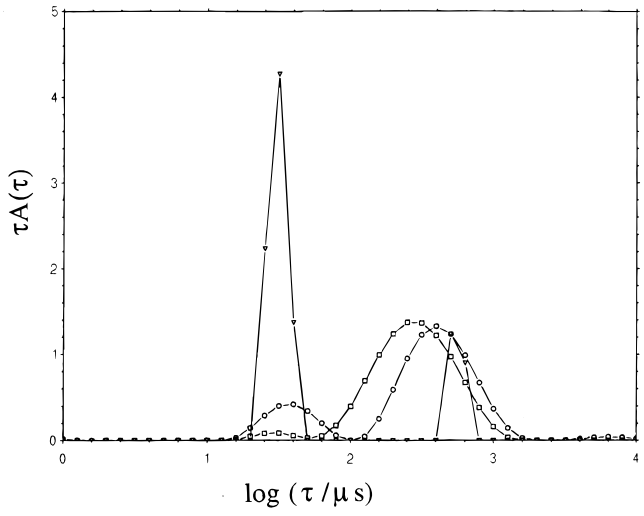

Figure 9. Relaxation time distributions of the complex formed between LDH (0.5 g/L) and 0.002% high molecular weight (2.6×10^6) PEI (○) compared with pure LDH (▽) and pure high molecular weight PEI (□) of similar concentrations.

intercept of the angle-dependent inverse intensity plot without requiring knowledge of the particle mass. Since there is no significant concentration dependence of the reduced scattered

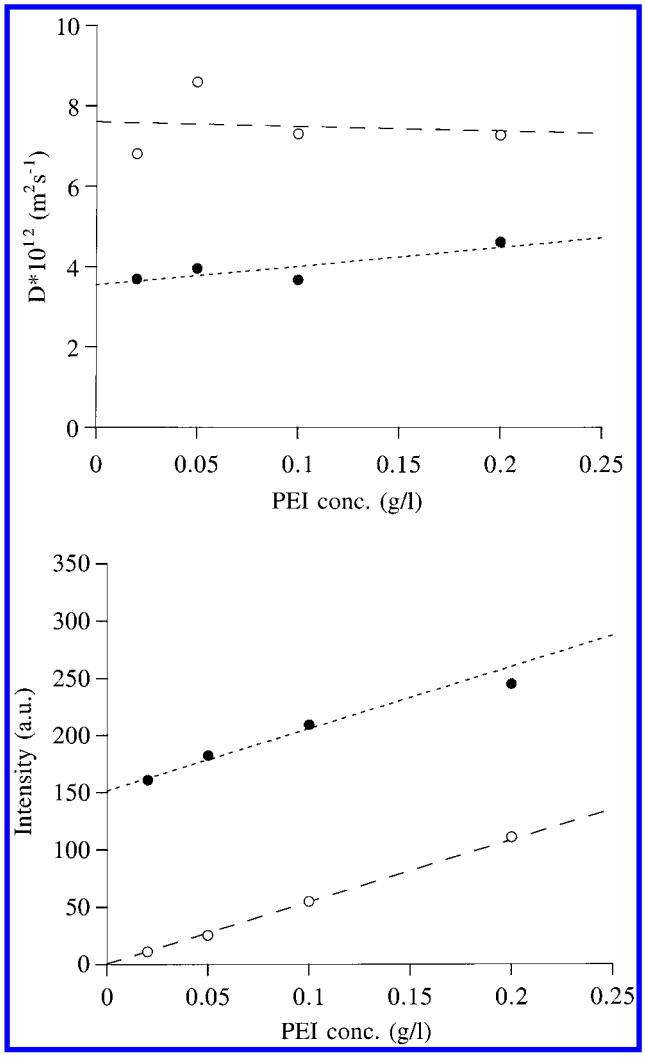

Figure 10. (a) Diffusion coefficients of pure high molecular weight PEI (2.6×10^6) (○) and its complex with LDH (●) as a function of polymer concentration. (b) Total intensities of pure high molecular weight (2.6×10^6) PEI (○) and of its complex with LDH (●) as a function of polymer concentration.

intensity, R_g will be close to the true value. For high M_w PEI, which has a single-peaked relaxation time distribution, the radius of gyration was estimated to be 45 nm. Taken together with an $R_H = 34$ nm, an index $\rho = 1.5$ for pure high M_w PEI can be calculated, indicating a random coil conformation. For the mixture of LDH and high M_w PEI, an R_g of 88 nm was calculated (Figure 11), which taken together with an R_H of 66 nm for the LDH−PEI matrix gives an index of $\rho = 1.3$, suggesting a more compact particle than for PEI alone. The above observations taken together suggest a different model for the enzyme−polymer interaction than that proposed for LDH with PEI of lower molecular weights. With high molecular weight PEI we speculate that several LDH molecules bind within the large, branched PEI molecular structure resulting in a complex having approximately double the size of the polymer but with a lower flexibility (Figure 12). The decrease in flexibility is possibly due to interaction of several branches of PEI which presumably "collapse" around the surface of each LDH molecule to give maximum interaction, yielding on the whole a more dense particle than in the case of pure PEI.

Strength of the Interactions between LDH and PEI. After establishing the formation of complexes between LDH and PEI of different molecular weights, further investigations of the

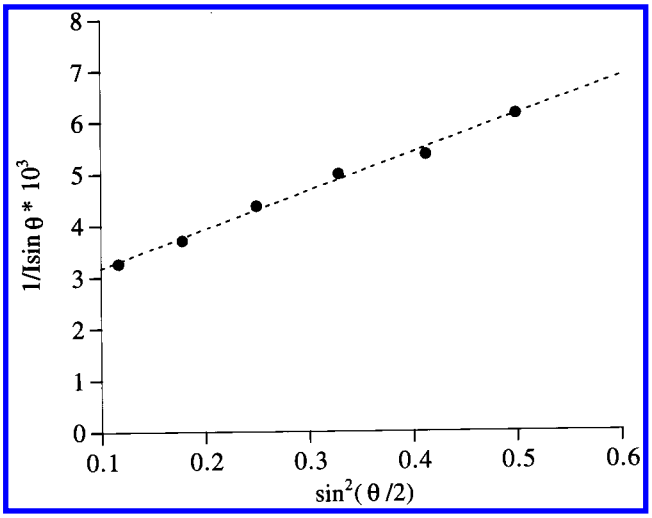

Figure 11. A plot of $(1/I\sin\theta)$ versus $\sin^2(\theta/2)$ of the complex of LDH (0.5 g/L) with 0.002% high molecular weight PEI. See text for more details.

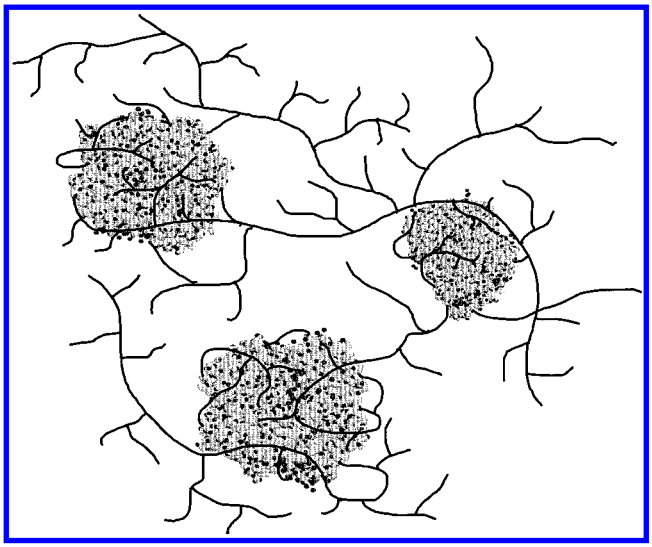

Figure 12. Schematic representation of the complex of LDH with high M_w PEI.

presence of close molecular interactions and estimation of the strength of binding between the components seemed relevant. Fluorescence spectroscopy was chosen as the technique for determining the binding of the components. It is well-known that the intrinsic tryptophan fluorescence of proteins varies with their conformation and the microenvironment around the Trp residues, e.g., shift of the emission maximum (λ_{max}) and/or reduction of the intensity (I_{max})(quenching).[46]

The emission maximum of LDH at 343 nm was seen to shift to shorter wavelengths (blue shift) and become reduced in intensity with increasing polymer concentration [P] (expressed as mol monomer/L), (Figure 13 a). These results are indicative of changes in the local conformation around the Trp residues resulting from interaction of PEI with the enzyme. The blue shift indicates that the environment of the tryptophan becomes more hydrophobic and shielded from the aqueous solvent,[47] which can be understood from the partially hydrophobic nature of the polymer backbone. Earlier studies with papain and potassium poly(vinyl alcohol sulfate) (KVPS) have attributed the blue shift to increased protein/protein interactions within an intrapolymer papain/KVPS complex.[25] In our case, this could only be true for binding to high M_w PEI where we assume several LDH molecules to associate within a polymer network.

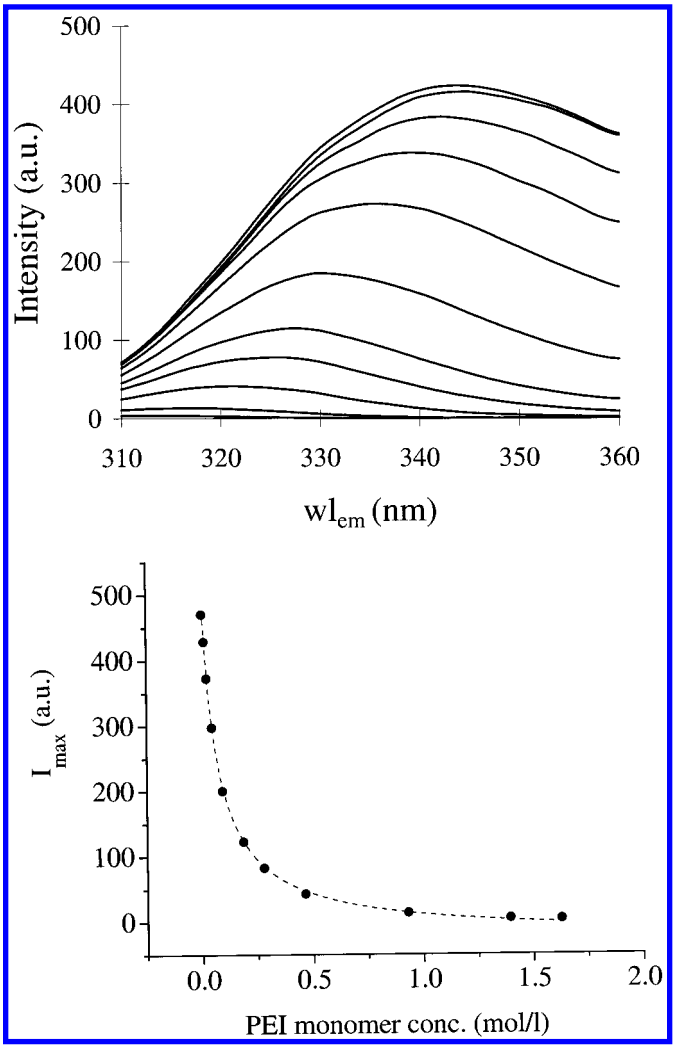

Figure 13. (a) Fluorescence emission spectra of 0.02 g/L LDH at different concentrations of PEI 2000. (b) The fitted binding curve of LDH and PEI 2000 from intrinsic fluorescence measurements.

It is important to point out that the observed blue shift is not likely to originate from aggregation of LDH molecules since the presence of PEI was seen to decrease or eliminate LDH aggregation.

The dependence of total emission intensity on concentration of PEI 2000 is shown in Figure 13 b. It may be noted here that interaction of the polymer with only native (i.e., unaggregated) form of the enzyme was assumed since aggregation of LDH was negligible in the presence of PEI (particularly during the initial hours). This assumption is strengthened by the extremely good fit of the curve to the equation assuming only one component (Figure 13b). By fitting this relation for all PEI molecular weights to the intensity equation (eq 8), the equilibrium dissociation constants for the LDH−PEI interactions were determined to be 7.4×10^{-2} M for PEI 2000, 3.45 M for PEI 25000, and 1.24 M for PEI 2.6×10^6, based on monomer concentration. For all molecular weights, these values suggest rather weak binding. The weaker binding in case of PEI 25000 could be due to greater rigidity of the polymer, which may lead to fewer optimal contact points with the enzyme in comparison with PEI 2000. Similar binding constants were obtained if, instead of reduction in intensity, the blue shift was plotted against PEI concentration. Therefore, even if the amine-containing PEI polymer could possess intrinsic quenching properties, the observed reduction in intensity is more likely

due to close interaction between the enzyme and polymer and subsequent masking of Trp residues.

The binding with PEI 2000 was seen to be somewhat weakened in the presence of 30% ethyleneglycol, 20% ethanol, and 3 M NaCl (not shown). These results may indicate involvement of weak charge−charge interactions and weak hydrophobic contributions at a pH lower than the isoelectric point of the enzyme. It is very likely that hydrogen bonding of protein −COOH groups with the nitrogen of PEI also plays an important role in the complexation.

Conclusions

Light scattering and Trp fluorescence spectroscopy have in this study proven to be valuable complementary techniques for study of the interactions between LDH and PEI. These techniques provide information at the molecular level useful in various aspects of biotechnology. The present study demonstrates that LDH and PEI in solution exist together as a loose complex although endeavors to estimate the composition of the complex were so far unsuccessful. The results obtained so far are, nevertheless, considered important in furthering our understanding of the underlying mechanism of protein stabilization with PEI and possibly other polyelectrolytes or polymers. The observed effect on aggregation probably results from coating of the enzyme surface by PEI molecules to yield a polycationic species; this inhibits protein−protein contacts by electrostatic repulsion which in turn hinders aggregation. The presence of PEI in the close proximity of the enzyme surface could also hinder or slow down deleterious inactivation processes by decreasing the mass transfer rates and also hinder oxygen transfer and oxidation which we report in our latest study.[35]

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