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Light Scattering, CD, and Ligand Binding Studies of Ferrihemoglobin–Polyelectrolyte Complexes

Abstract: *Quasi-elastic light scattering (QELS), electrophoretic light scattering (ELS), CD spectroscopy, and azide binding titrations were used to study the complexation at pH 6.8 between ferrihemoglobin and three polyelectrolytes that varied in charge density and sign. Both QELS and ELS show that the structure of the soluble complex formed between ferrihemoglobin and poly(diallyldimethylammonium chloride) [PDADMAC] varies with protein concentration. At fixed 1.0 mg/mL polyelectrolyte concentration, protein addition increases complex size and decreases complex mobility in a tightly correlated manner. At 1.0 mg/mL or greater protein concentration, a stable complex is formed between one polyelectrolyte chain and many protein molecules (i.e., an intrapolymer complex) with apparent diameter approximately 2.5 times that of the protein-free polyelectrolyte. Under conditions of excess polyelectrolyte, each of the three ferrihemoglobin–polyelectrolyte solutions exhibits a single diffusion mode in QELS, which indicates that all protein molecules are complexed. CD spectra suggest little or no structural disruption of ferrihemoglobin upon complexation. Azide binding to the ferrihemoglobin–poly(2-acrylamide-2-methylpropanesulfonate)*

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[PAMPS] complex is substantially altered relative to the polyelectrolyte-free protein, but minimal change is induced by complexation with an AMPS-based copolymer of reduced linear charge density. The change in azide binding induced by PDADMAC is intermediate between that of PAMPS and its copolymer. © 1999 John Wiley & Sons, Inc. Biopoly 50: 153–161, 1999

Keywords: *hemoglobin–polyelectrolyte complexes; protein–polyelectrolyte complexes; light scattering; CD; ligand binding*

INTRODUCTION

Proteins interact strongly with both synthetic and natural polyelectrolytes. These interactions are modulated by such variables as pH and ionic strength, and may result in soluble complexes,[1,2] complex coacervation,[3–6] or the formation of amorphous precipitates.[7–9] Protein–polyelectrolyte complexation can change the activity of catalytic proteins (enzymes),[10–12] alter ligand binding to transport proteins,[1,7] and stabilize biological activity against temperature change.[13] Complexation has the potential to alter protein function in several nonmutually exclusive ways, including change in protein conformation, steric hindrance of substrate approach to the active site, and differential partitioning of substrates from bulk solution into the protein–polyelectrolyte complex. A limiting case of protein conformational change is denaturation, which is a large disruption of protein structure causing abolition of function. While the molecular basis(es) for effects on biological function is(are) unclear, complex formation is known mainly, but not exclusively, to be caused by electrostatic interactions between protein molecules and polyelectrolytes. For example, the formation of the trypsin–poly(vinyl alcohol sulphate) complex involves salt bridges between protonated basic groups on the protein and sulphate groups on the polymer.[11] Complicating such interactions are large changes in the radius of gyration of certain polyelectrolytes that are induced by variation of solution pH and ionic strength. Such changes may alter the size, shape, and/or aggregation state of the protein–polyelectrolyte complex.

Hemoglobin is expected to be a sensitive reporter of alteration of protein structure arising from protein–polyelectrolyte interaction, not only because it is structurally designed to change conformation with oxygen binding, but also because this change is modulated by the binding of certain small and moderate size ions at sites separate from those that bind oxygen.[14–16] Hemoglobin is composed of two α- and two β-subunits approximately tetrahedrally disposed ($\alpha_2\beta_2$), with salt bridges, hydrogen bonds, and hydrophobic interactions along the subunit interfaces. Each subunit contains one heme (iron-protoporphyrin). When oxygen or other ligands bind to the heme iron, the latter moves in-plane pulling the F-helix mechanically, which in turn, alters interactions between subunits transmitting a conformational change throughout the protein. This conformational change enhances ligand binding at the other hemes. Indeed, hemoglobin exists in two major conformations in solution, *T* and *R*, with the latter having higher affinity for ligand binding to the heme, and switching between these two produces the cooperative interaction among the four heme binding sites. The equilibrium between these two conformations can also be influenced by the binding of certain anionic effectors [e.g., inositol hexaphosphate (IHP), 2,3-diphosphoglycerate (DPG), etc.], which bind at a specific cationic site separate from the heme, but modulate heme affinity.[16,17] For example, IHP binds tightly to hemoglobin forcing the protein toward the T conformation substantially reducing the affinity of heme for oxygen and other ligands. Ligand binding is also affected by solution pH in that hemoglobin releases protons upon oxygen binding and increasing pH increases oxygen affinity. Because of the potential of polyelectrolyte complexes as blood substitutes, Nguyen[7] and Sacco et al.[1] examined complexes of hemoglobin with sulfated dextran and found that oxygen affinity is greatly decreased upon complexation. A similar effect was also reported for hemoglobin–heparin complexes,[18] as well as lower cooperativity. In these cases decreased oxygen affinity likely involves binding of sulfate groups from the polyelectrolyte to the cationic IHP binding site on the protein; however, other contributions have not been explored.

In this paper, we report formation of ferrihemoglobin complexes with both cationic and anionic polyelectrolytes, with the latter at two different linear charge densities. CD spectroscopy suggests the complexation process causes little or no structural disruption of the protein. The effect of polyelectrolye complexation on ligand binding to the heme iron is probed using the charged ligand azide, and the results are discussed in terms of interactions at the molecular level between the protein and the polyelectrolytes.

EXPERIMENTAL

Materials

Poly(diallyldimethylammonium chloride) (PDADMAC) was a commercial sample "Merquat 100" from Calgon

Corporation (Pittsburgh, PA), with a nominal molecular weight of 2×10^5. Sodium poly(2-acrylamide-2-methylpropane-sulfonate) (PAMPS) and the copolymer of AMPS with N-vinylpyrrolidone (50 mol %; NVP–AMPS), with molecular weights of 2.4×10^6 and 2×10^6, respectively,[19,20] were prepared by free radical polymerization by D. W. McQuigg of Reilly Industries (Indianapolis, IN). Bovine hemoglobin was obtained from Sigma Chemical as 95–99% pure lyophilized protein, with isoelectric point (IEP) of 6.9. Complete oxidation was assured by ferricyanide addition. For oxidation, 3.0 equivalents of potassium ferricyanide was added to hemoglobin in 20 m*M*, pH 6.8 phosphate buffer. Ferrihemoglobin was separated from excess oxidant and further purified by gel filtration with Sephadex G-150 (Pharmacia Biotech AB). All salts used in the present work were AR grade from Sigma Chemical Co.

Sample Preparation

All solutions were prepared with deionized water that was made from glass distilled water subsequently passed through one carbon and two ion-exchange filters. Scattering solutions were made dust-free by filtration through 0.2 μm Acrodisc filters (Gelman Sciences). Buffer was 20 m*M*, pH 6.8 phosphate.

Methods

Quasi-elastic light scattering (QELS) measurements were made at scattering angles from 30° to 150° with a Brookhaven (Holtsville, NY) 72 channel BI-2030 AT digital Correlator and using a Jodon 15 mW He–Ne laser. A 200 μm pinhole aperture was used for the EMI photomultiplier tube, and decahydronaphthalene (Decalin) was used as the refractive index matching fluid to reduce stray light. The homodyne intensity–intensity correlation function $G(q, t)$ was measured with q, the amplitude of the scattering vector, given by $(4\pi n/\lambda)\sin(\theta/2)$, where n is the refractive index of the medium, λ is the wavelength of the excitation light in a vacuum, and θ is the scattering angle. For a Gaussian distribution of the intensity profile of the scattered light, $G(q, t)$ is related to the electric field correlation function $g(q, t)$ by

$$G(q, t) = A[1 + bg(q, t)^2] \tag{1}$$

where A is the experimental baseline and b is a constant, which depends on the number of coherence areas that generates the signal ($0 < b < 1$). The quality of the measurements was verified by determining that the difference between the measured value of A and the calculated one was less than 0.1%. In the present work, we analyzed the autocorrelation functions by using the program CONTIN, which employs the constrained regularization method[21] to calculate the mean diffusion time, $\langle\tau\rangle$, which is related to the diffusion coefficient D by

$$D = \frac{\lambda^2}{16\pi^2 \sin^2(\theta/2)\langle\tau\rangle} \tag{2}$$

More detailed discussions of QELS data analysis may be found elsewhere.[22,23] From each D value, we obtained the Stokes' radius R by the Stokes–Einstein equation:

$$R = \frac{kT}{6\pi\eta D} \tag{3}$$

where k is Boltzmann's constant, T is the absolute temperature, and η is the viscosity of the solvent.

Electrophoretic light scattering (ELS) measurements were made at four scattering angles (8.7°, 17.4°, 26°, and 34.7°), using a Coulter (Hialeah, Florida) DELSA 440 apparatus. The light source was a 5 mW He–Ne laser (λ = 632.8 nm). The total volume of sample chamber was about 1 mL. A rectangular channel ran through a 5 mm thickness of the insert, connecting the hemispherical cavities in each electrode. The electric field was applied at a constant current of 0.4 mA. The temperature of the thermostated chamber was maintained at 25°C. Electroosmotic corrections were determined by measuring the spatial flow profile in the chamber and taking the mobility readings at a distance 16% of the rectangular length from the respective walls of the chamber. This procedure was verified by using a DELSA electrophoretic mobility standard (carboxylated polystyrene latex).

In ELS, the measured Doppler shift frequency $\Delta\omega$ is given by

$$\Delta\omega = \frac{2\pi n}{\lambda} Eu \sin\theta \tag{4}$$

where E (volts/cm) and u [(μm/s)/(V/cm)] are the applied electric field strength and electrophoretic mobility, respectively. Therefore, u can be calculated directly from $\Delta\omega$. The u values obtained in this work were reproducible within an error of less than 10%. Detailed discussion of ELS measurements can been found in several reviews.[24–26]

CD spectra were recorded on an AVIV 61DS (Lakewood, NJ) dichrograph. The instrument was calibrated with (+)-10-camphorsulfonic acid and isoandrosterone. Samples were confined between cylindrical quartz windows with a path length of 0.1 cm. Spectra were recorded with a 2 nm bandwidth, a 0.5 nm step, and an integration time of 0.5 s. Each spectrum was the mean of 5–8 scans and was corrected for solvent contribution. The concentration of ferrihemoglobin was estimated by absorption at 630 nm in 20 m*M*, pH 6.8 phosphate buffer. The ellipticities [Θ] are reported in deg-cm^2-dmol^{-1}. Spectra were obtained with 0.20 mg/mL ferrihemoglobin and excess polyelectrolyte concentration of 2.0 mg/mL to ensure all protein molecules were complexed (see below).

The azide binding titration was quantitated through absorbance measurements with a HP8450 spectrophotometer. Spectra were obtained using a 1 cm path-length quartz cuvette maintained at 25°C. For azide binding titrations,

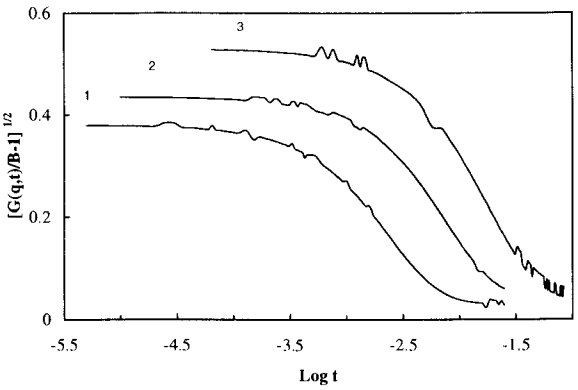

FIGURE 1 Autocorrelation functions obtained for ferrihemoglobin (curve 1), PDADMAC (curve 2), and the ferrihemoglobin–PDADMAC complex (curve 3). Polyelectrolyte and protein concentrations were 1.0 and 3.0 mg/mL, respectively, in 20 m*M*, pH 6.8 phosphate.

equilibrium mixtures containing ferrihemoglobin–polyelectrolytes complex or ferrihemoglobin alone were incubated with varying amounts of NaN_3 in 20 m*M*, pH 6.8 phosphate buffer at 25°C for a sufficient time to achieve constant absorbance. Azide titrations were conducted with 1.0 mg/mL ferrihemoglobin and excess polyelectrolyte concentration of 2.0 mg/mL to ensure all protein molecules were complexed.

RESULTS AND DISCUSSION

Quasi-Elastic and Electrophoretic Light Scattering of the Ferrihemoglobin–PDADMAC Complexes

Coulomb forces drive protein–polyelectrolyte complexation; thus, increasing pH promotes the formation of protein–polycation complexes, and decreasing pH enhances complexation of proteins and polyanions.[27,28] Recently, we have found that the initial complexation between polyelectrolytes and proteins can even occur under conditions where the net protein charge is the same as that of the polyelectrolyte.[29] This result is attributed to protein surface charge heterogeneity. In the present study, soluble complexes were formed between different polyelectrolytes and ferrihemoglobin at a pH near its IEP. QELS provides a powerful technique for detection and characterization of such soluble complexes.

Various data analysis methods have been used in QELS and some yield multiple relaxation time constants. In the present study, we used the program CONTIN to resolve relaxation times for protein, polyelectrolyte, and protein–polyelectrolyte complexes. Concentrations of the protein and the polyelectrolyte were 3.0 and 1.0 mg/mL, respectively. Such an analysis of the autocorrelation functions (including complexes of ferrihemoglobin–NVP–AMPS and ferrihemoglobin–PAMPS) yields single relaxation with diffusion type q^2 dependence (as expected in dilute solution) and good quality of fitting (rms $< 2 \times 10^{-4}$). Figure 1 shows typical autocorrelation functions for ferrihemoglobin, PDADMAC, and the ferrihemoglobin–PDADMAC complex. Diffusion constants obtained with the program CONTIN are $D = 7 \times 10^{-9}$ cm^2/s for the protein; $D = 2.3 \times 10^{-9}$ cm^2/s for PDADMAC; and $D = 9.4 \times 10^{-10}$ cm^2/s for the complex. From these constants and Eq. (3) can be calculated apparent diameters of 7 nm for the protein, 21 nm for the polymer, and 52 nm for the complex. This change in diameter is taken as an indication of complex formation.

Figure 2 shows the apparent diameters and electrophoretic mobilities obtained by QELS and ELS for the ferrihemoglobin–PDADMAC complexes formed at polyelectrolyte concentration (C_p) of 1.0 mg/mL as a function of the protein concentration (C_{pr}) in 20 m*M*, phosphate buffer at a pH of 6.8, chosen because of its proximity to physiological pH. The formation of soluble complexes is evident from the observation by QELS of particles with apparent hydrodynamic diameters larger than those of the individual components (7 nm for the protein and 21 nm for the polyelectrolyte). Clearly, complex size increases with C_{pr} at low C_{pr} and reaches a plateau at higher concentrations. There is essentially no free polyelectrolyte at high C_{pr}, only intrapolymer complex and free protein. This observation is consistent with results found for bovine serium albumin (BSA)–PDADMAC complexes.[29,30] At low protein concentration, the binding capacity of the PDADMAC chain is not saturated, and binding increases with C_{pr}

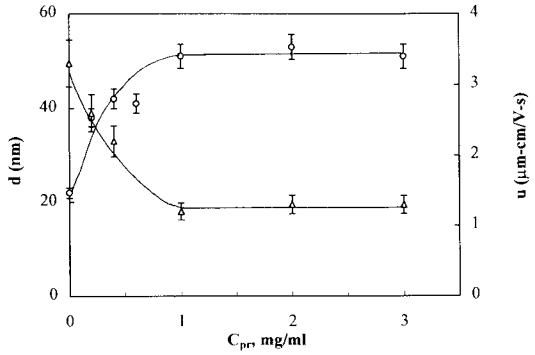

FIGURE 2 Diameter (○) and electrophoretic mobility (△) of ferrihemoglobin–PDADMAC complex as a function of increasing protein concentration. Polyelectrolyte concentration was fixed at 1.0 mg/mL and solution was buffered with 20 m*M*, pH 6.8 phosphate.

along with the apparent size of the complex. While the size measurements alone cannot clearly distinguish between intra- and inter(multi)polymer complexes, the observation of limiting values for diameter and electrophoretic mobility (as described in the next paragraph) with increasing C_{pr}/C_p would be difficult to reconcile with higher-order association. Thus, at higher C_{pr} each PDADMAC chain is apparently saturated by ferrihemoglobin to form an intrapolymer complex, and particle size becomes constant at approximately 52 nm. Neither the polyelectrolyte nor the complex are spherical particles, and the apparent size of the complex is not a matter of solid packing. However, the conformation of protein–polyelectrolyte complexes does strongly depend on solution pH, as discussed in Refs. 27 and 30.

The charge contribution to the ferrihemoglobin–polyelectrolyte complex from ferrihemoglobin is negligible at pH 6.8 because the IEP of the protein is 6.9. Since mobility is the ratio of charge (q) to friction coefficient (f), the complex mobility should decrease upon binding of protein due to an increase of friction coefficient. Thus, the electrophoretic mobility decrease with C_{pr}, as shown in Figure 2, can be attributed to a decrease in complex diffusivity. Further evidence for the formation of a stable intrapolymer complex at high protein concentration arises from the constant electrophoretic mobility observed at $C_{pr} > 1$ mg/mL, which also corresponds to constant hydrodynamic radius. The mobility of the complex (1.3 μm-cm/V-s) is smaller than that of the pure polyelectrolyte (3.3 μm-cm/V-s). Let us assume that the bound protein has zero net charge (because solution pH is very close to its IEP) and that the friction coefficient follows the Stokes relationship:

$$f = 6\pi\eta R \tag{5}$$

where R is the radius. The mobility of the intrapolymer complex can be estimated by

$$\frac{u_{px}}{u_p} = \frac{q_p/f_{px}}{q_p/f_p} = \frac{R_p}{R_{px}},\ q_{px} = q_p \tag{6}$$

where subscripts px and p represent protein–polyelectrolyte complex and protein-free polyelectrolyte, respectively. The QELS data in Figure 2 show that the binding of protein to polyelectrolyte causes the chain to expand, with apparent hydrodynamic radius approximately 2.5 times larger than the value of the protein-free polyelectrolyte. This size change is quantitatively consistent with the mobility change of about $(2.5)^{-1}$.

The existence of intrapolymer complexes, with constant mobility and size, is also consistent with the ultrafiltration results obtained for hemoglobin and dextran sulfate by Nguyen.[7] Nguyen found a linear decrease in the amount of hemoglobin permeating through the membrane with increasing amount of polyelectrolyte. This linear change in the ultrafiltration indicates an intrapolymer structure for the complex.

It is interesting to calculate an apparent concentration of charged species contributed by PDADMAC for a single-chain, protein-free sphere of 21 nm diameter. The result is 470 m*M*, which is large and of unknown effect on the structure of the protein. For the final ferrihemoglobin–PDADMAC complex the concentration is near 30 m*M*.

CD of Ferrihemoglobin–Polyelectrolyte Complexes

The CD spectrum of ferrihemoglobin is in general sensitive to both subtle and large-scale changes in protein structure. Binding of ligands to the heme iron causes small but significant changes near 220 nm,[31] whereas pronounced changes are observed with denaturation by pH extremes or addition of perturbants. Figure 3 shows the essentially identical CD spectra of polyelectrolyte-free ferrihemoglobin and the ferrihemoglobin–PDADMAC complex scanned at protein concentration of 0.20 mg/mL in 20 m*M*, pH 6.8 phosphate buffer. The PDADMAC concentration of 2.0 mg/mL ensured that all protein molecules were bound to the polyelectrolyte. Apparently, there is little or no structural disruption caused by complexation with PDADMAC. Unchanged CD spectra were also observed for complexes formed with ferrihemoglobin and PAMPS or NVP–AMPS (data not shown), and similar results were described by Strelzowa et al.[32] for δ-chymotrypsin with dextran sulfate. However, the absence of structural perturbation upon complexation should not be considered as universal. Since protein–polyelectrolyte complexation is due mainly to electrostatic interactions, the complexed protein could have site-specific interactions as well as sense a different average pH, and thus be subject to destabilizing forces. For example, insulin was totally denatured upon complexation with PDADMAC (unpublished results). However, the complex with ferrihemoglobin was formed at a pH very close to the IEP of the protein lessening the intensity of the overall electrostatic interaction. It is interesting to note that in contrast to cytochrome *c* and tyrpsin, hemoglobin is more sensitive to external conditions in that it exhibits a

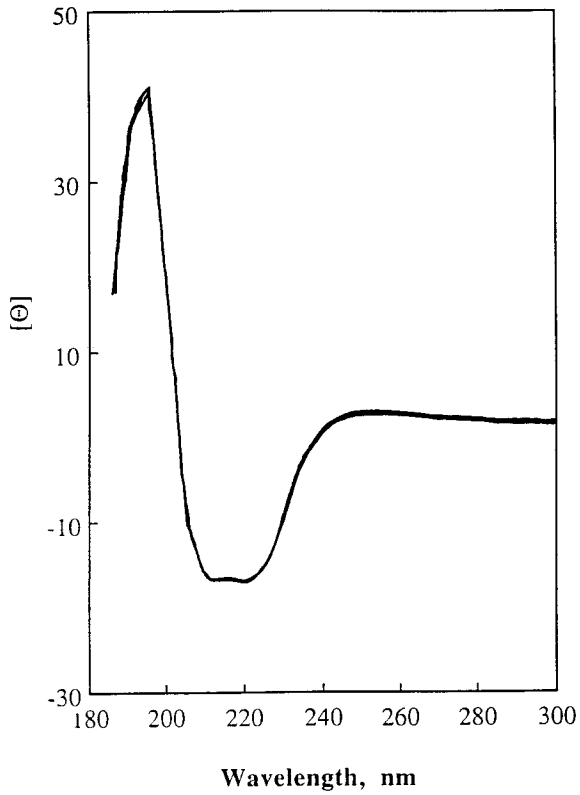

FIGURE 3 Effect of PDADMAC on the CD spectra of ferrihemoglobin. Polyelectrolyte and protein concentrations were 2.0 and 0.20 mg/mL, respectively, in 20 m*M*, pH 6.8 phosphate buffer. Note that CD spectra were coincident in the absence and presence of polyelectrolyte.

conformation change upon adsorption to a solid surface.[33]

Azide Binding Titraton of Ferrihemoglobin–Polyelectrolyte Complexes

A variety of ligands including azide can bind to hemoglobin through its heme iron, and the response of this protein to ligand binding has been characterized extensively.[16] Azide binds to ferrihemoglobin shifting the solution equilibrium toward the *R*-conformation, and IHP binding at a separate site can weaken azide binding by a factor of over 2.5[15] as it attempts to push the conformation toward *T*. Furthermore, the *R*-conformation is favored by high pH, and the *T* by low pH. Thus it is not unexpected that such a complicated set of responses might be significantly influenced by formation of a complex between protein and polyelectrolyte.

The effect of polyelectrolytes on azide binding to ferrihemoglobin was examined by comparing the binding of azide to free and complexed protein, the latter under the condition of excess polyelectrolyte. At concentrations of 2.0 mg/mL polyelectrolyte and 1.0 mg/mL protein, systems based on the three polyelectrolytes exhibit only one diffusion mode in QELS, which indicates that all protein molecules are complexed. The ratio of the apparent diameter of the ferrihemoglobin–polyelectrolyte complex to that of the protein-free polyelectrolyte measured by QELS is given in Table I as β for each of the polyelectrolytes. The sizes of the ferrihemoglobin–PDADMAC and ferrihemoglobin–PAMPS complexes are over two times larger than those of each of the corresponding polyelectrolytes in solution. In contrast to these two cases, the ferrihemoglobin–NVP–AMPS complex has a diameter close to that of the protein-free polyelectrolyte. This may be because the polyelectrolyte has a low charge density, which results in a smaller number of proteins bound per polymer chain.

Figure 4 shows typical optical difference spectra from an azide titration of the ferrihemoglobin–PDADMAC complex. The isosbestics are maintained, implying that the data can be analyzed as a two-state binding process. From these spectra can be calculated the extent of azide binding *Y* according to the equation

$$Y = \frac{\Delta A}{\Delta A_{\max}} \tag{7}$$

where ΔA is the difference in absorbance at 546 nm in the absence and presence of azide, and $\Delta A_{\max}$ is the limiting absorbance change corresponding to maximal heme binding using excess of this ligand. From Table I it is evident that complexation with PAMPS and PDADMAC decreases $\Delta A_{\max}$ 40 and 25%, respectively, vs uncomplexed ferrihemoglobin, but NVP–AMPS causes no change. The decrease in $\Delta A_{\max}$ can be most directly interpreted as the prevention of azide access to a fraction of heme binding sites (see below)

Table I Relative Particle Diameter Measured by QELS (β = Complex/Protein-Free Polyelectrolyte) and Azide Binding Parameters for Ferrihemoglobin and Ferrihemoglobin–Polyelectrolyte Complexes

Sample	Relative Size (β)	$\Delta A_{\max}$	$L_{50\%}$ (mM)	z
Hemoglobin	—	0.35	5.0×10^{-2}	1.4
Hb–NVP–AMPS	1.2	0.34	4.7×10^{-2}	1.6
Hb–PDADMAC	2.6	0.26	4.1×10^{-2}	1.6
Hb–PAMPS	2.3	0.21	2.7×10^{-2}	2.2

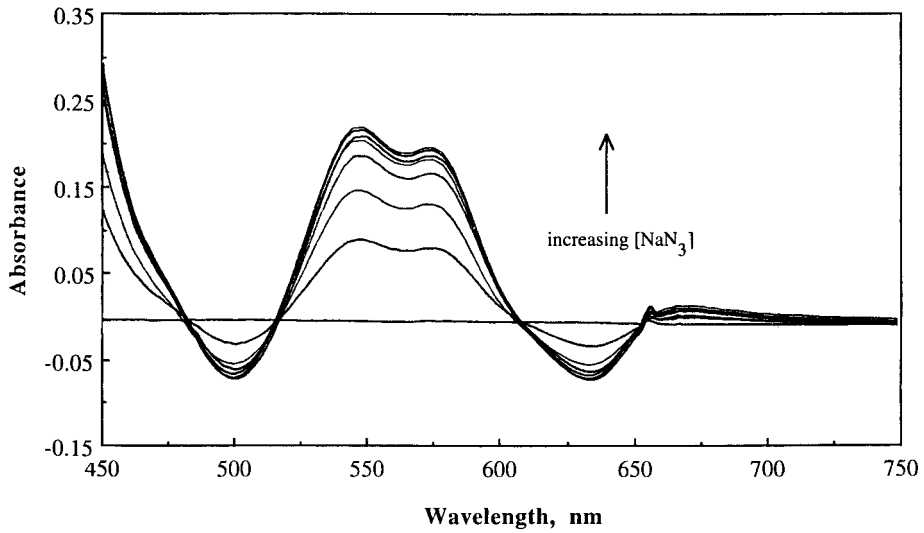

FIGURE 4 A typical example of optical difference spectra from an azide titration of the ferrihemoglobin–PDADMAC complex. Polytelectrolyte and protein concentrations were 2.0 and 1.0 mg/mL, respectively in 20 m*M*, pH 6.8 phosphate buffer.

such that they are excluded from the titration. Figure 5 shows plots of Y vs [NaN_3] for ferrihemoglobin and the three ferrihemoglobin–polyelectrolyte complexes. Clearly, azide binds to the ferrihemoglobin–PAMPS complex in a manner distinctly different from polyelectrolyte-free protein, although differences between free protein and the NVP–AMPS or PDADMAC complexes are less clear without further analysis.

Ligand binding to hemoglobin can be characterized as a two-state process using the Hill equation:

$$Y = \frac{K[L]^z}{1 + K[L]^z} \tag{8}$$

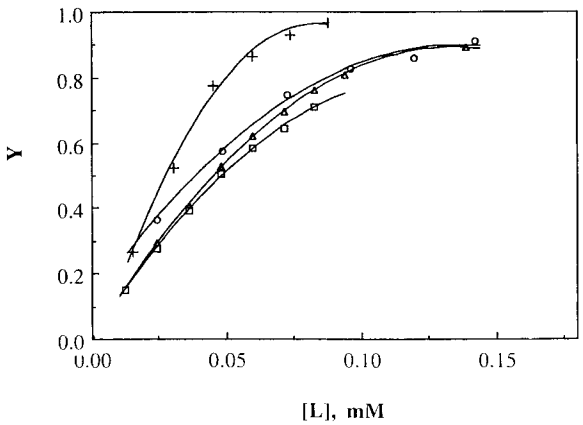

FIGURE 5 Plots of Y vs NaN_3 concentration [L] for azide binding to ferrihemoglobin in the absence and presence of polyelectrolytes. Symbols are pure protein (□) and complexes formed with PDADMAC (○), NVP–AMPS (△) and PAMPS (+).

where K is the "binding" constant and z, the Hill coefficient, is a measure of cooperativity. Manipulation of Eq. (8) yields Eq. (9), which has z as its slope:

$$\log\left(\frac{Y}{1 - Y}\right) = z \log [L] + \log K \tag{9}$$

Figure 6 shows log[Y/(1 − Y)] vs log[NaN_3] plots of the data for the ferrihemoglobin and ferrihemoglobin–polyelectrolyte complexes given in Figure 5. Hill coefficients calculated from these plots are summarized in Table I along with the azide concentration at which Y = 0.5 for each titration ($L_{50\%}$). PAMPS complexation has a substantial effect on $L_{50\%}$ and z, but the effects of PDADMAC and NVP–AMPS are less pronounced. The ferrihemoglobin–PAMPS complex bound azide almost twice as tightly as polyelectrolyte-free protein, as given by $L_{50\%}$. Azide binding appears to be enhanced slightly in the ferrihemoglobin–PDADMAC complex, but it is unlikely that there is a significant change with NVP–AMPS. All the titrations give $z > 1$, which indicates positive cooperativity and the Hill coefficient for the polyelectrolyte-free protein is consistent with the literature value.[34] PAMPS complexation increases cooperativity from 1.4 to 2.2.

Two fundamental alterations in the azide binding titration were observed when polyelectrolyte complexed with ferrihemoglobin, and both decrease in magnitude as follows: PAMPS > PDADMAC > NVP–AMPS ≅ polyelectrolyte-free protein. The first alteration is a decrease in ΔA_{max}, which, with the reasonable assumption of no change in extinction

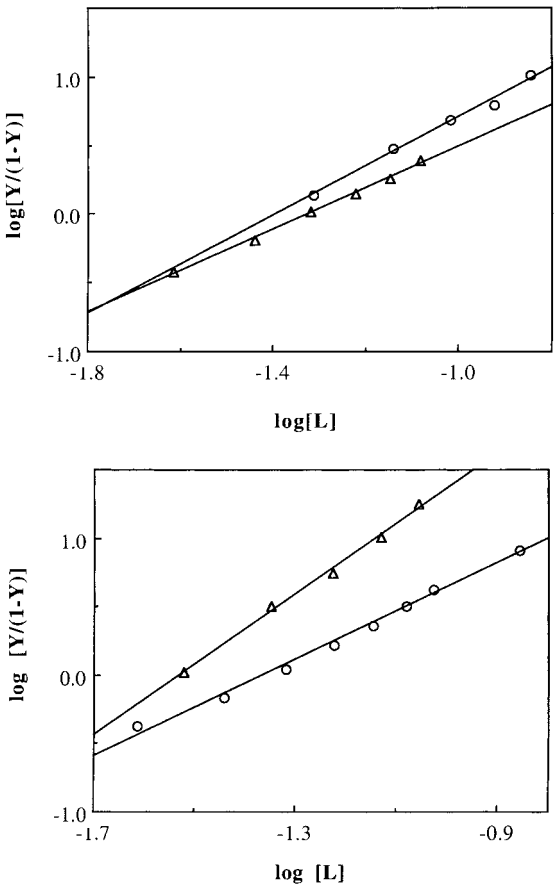

FIGURE 6 Hill plots for ferrihemoglobin–azide binding (top panel) in the absence (△) and presence (○) of PDADMAC and (bottom panel) in the presence of NVP–AMPS (○) and PAMPS (△). Data is taken from Figure 4. Slopes of these plots are Hill coefficients, which are a measure of cooperative ligand binding.

coefficient with progressive azide binding ($\Delta\varepsilon$/azide bound; ε is the extinction coefficient) implies a decrease in the number of heme binding sites available in solution at fixed protein concentration. The unaltered CD spectra of the ferrihemoglobin–polyelectrolyte complex supports the absence of polyelectrolyte-induced denaturation, which could lead to variation in $\Delta\varepsilon$/azide bound. The maintenance of isosbestic points during azide titration of the complex argues against a multistate binding process with different $\Delta\varepsilon$/azide values. Thus the decrease in number of binding sites is consistent with steric blockage of some fraction of the heme. Such blockage is possible in that even in the absence of polyelectrolyte, direct access from solution to the heme iron is limited by amino acid side chains. Indeed, the electrostatic interaction between protein and polyelectrolyte can be substantial, and salt bridges are known to form between charged groups on polyelectrolyte and amino acid side chains.[11] Furthermore, calculation of apparent concentrations of charged groups contributed by PAMPS or PDADMAC yields 32 and 24 m*M*, respectively, for spheres of diameter 101 or 55 nm, the diameters obtained from QELS measurements for ferrihemoglobin–polyelectrolyte complexes under the azide titration conditions. These similar concentrations exceed that of the buffer itself and imply no lack of availability of polyelectrolyte-donated charged groups to interact with the protein. An alternative explanation to steric blockage is that polyelectrolyte-donated sulfonate groups from AMPS interact with the IHP binding site on ferrihemoglobin and shift the equilibrium toward T. This shift is implicated[35] with complexes formed from heparin and sulfated dextran, in which oxygen affinity is decreased.[1,7,18] However, interaction with the IHP site is not possible for PDADMAC because both the polyelectrolyte and the IHP site are cationic, and further, saturating concentration of azide would likely overcome this effect. Thus it is unlikely that the change in ΔA_{max} with polyelectrolyte complexation has its origin in modulation of the IHP site.

The second fundamental alteration is polyelectrolyte-induced enhancement of azide binding to that fraction of ferrihemoglobin with open heme binding sites, as Table I indicates. Enhanced binding is opposite that expected from the interaction of the AMPS sulfonate group with the IHP site. However, a mechanism consistent with enhanced binding is disruption of certain salt bridges on ferrihemoglobin by polyelectrolyte complexation, destabilizing the *T*-conformation. The resulting shift to *R* would enhance the affinity of the heme. Ferrihemoglobin in solution contains protein molecules in both the *T*- and *R*-conformation, and the former is more constrained because of at least eight additional salt bridges.[16,36] Location of many of the associated amino acid side chains is sufficiently superficial to permit their interaction with polyelectrolyte charge groups. The observed smaller enhancement of azide binding by PDADMAC vs PAMPS complexation is as expected in that the positive charge on the nitrogen of each repeat unit is both highly shielded by the polyelectrolyte structure (i.e., the methyl groups) and restricted geometrically in its ability to sample areas of the protein surface. These limitations are in contrast to the sulfonate groups on PAMPS, which extend from the polymer backbone. Indeed, PDADMAC is more rigid and has a lower linear charge density than PAMPS. Geometric restriction and shielding may interfere with formation of multiple, short-range interactions between polyelectrolyte repeat units and protein, which may be necessary for T-state relaxation, but not for gross blockage of heme binding sites effecting ΔA_{max}.

The lack of alteration of azide binding through ferrihemoglobin complexation with NVP–AMPS vs PAMPS can be explained by the lower linear charge density of the former. For example, BSA must be brought to a lower pH to complex with NVP–AMPS compared to PAMPS. This difference arises because BSA binds more strongly to the homopolymer at a given pH and ionic strength.[27] The influence of polyelectrolyte charge density may also be reflected in the number of proteins bound per polyelectrolyte chain at a given pH and ionic strength. If this number were smaller for NVP–AMPS, it would explain why the polyelectrolyte expansion seen upon binding of ferrihemoglobin to PAMPS is not seen for NVP–AMPS (see Table I). The relative weakness of the NVP–AMPS–protein interaction may also result in an increase in the mean distance between polymer repeat units and the protein surface (i.e., the configuration of the bound polyelectrolyte chain is characterized by a larger ratio of "loops" to "trains"). Such a configuration would tend to leave the initial (intraprotein) salt bridges intact and thus have less effect on azide binding to the heme.

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