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Tuning Hemoglobin–Poly(acrylic acid) Interactions by Controlled Chemical Modification with Triethylenetetramine

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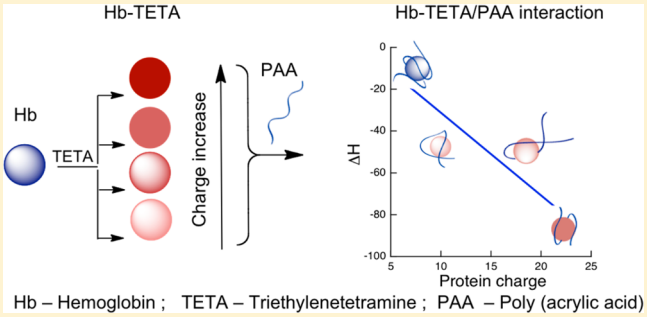
Supporting Information

ABSTRACT: Protein–polymer interactions play a very important role in a number of applications, but details of these interactions are not fully understood. Chemical modification was introduced here to tune protein–polymer interactions in a systematic manner, where methemoglobin (Hb) and poly(acrylic acid) (PAA) served as a model system. Under similar conditions of pH and ionic strength, the influence of protein charge on Hb/PAA interaction was studied using chemically modified Hb by isothermal titration calorimetry (ITC). A small fraction of COOH groups of Hb were amidated with triethylenetetramine (TETA) or ammonium chloride to produce the corresponding charge ladders of Hb-TETA and Hb-ammonia derivatives, respectively. All the Hb/PAA complexes produced here are bioactive, entirely soluble in water, and indicated the retention of Hb structure to a significant extent. Binding of Hb to PAA was exothermic ($\Delta H < 0$). The binding of Hb-TETA charge ladder to PAA indicated decrease of ΔH from -8 ± 0.2 to -89 ± 4 kcal/mol, at a rate of -3.8 kcal/mol per unit charge introduced via modification. The Hb-ammonia charge ladder, in contrast, showed a decrease of ΔH from -8 ± 0.2 to -17 ± 1.5 kcal/mol, at much slower rate of -1.0 kcal/mol per unit charge. Thus, the amine used for the modification played a strong role in tuning Hb/PAA interactions, even after correcting for the charge, synergistically. Charge clustering may be responsible for this synergy, and this interesting observation may be exploited to construct protein/polymer platforms for advanced biomacromolecular applications.



INTRODUCTION

Controlling protein–polymer interactions is vital for the construction of protein–polymer hybrid scaffolding platforms for biocatalytic, biosensing, and biomedical applications. Many of the protein/polymer platforms use protein immobilization with polymers via the layer-by-layer method,[1−4] covalent bonding, precipitation,[5−8] or encapsulation.[9−11]

Protein–polymer interactions, for example, are important in the development of biocompatible, stable, inexpensive, and sterile drug delivery systems.[12] Biotherapeutics such as peptides, proteins, and nucleic acids are susceptible to degradation or denaturation in the biological milieu,[13] and polymeric multilayer capsules have been used to protect these sensitive biologics.[14−16] Vast numbers of multilayer assemblies were fabricated by alternative deposition of oppositely charged polymers, where the polymer–protein interactions play crucial roles in the assembly formation and stability.[17] These polyelectrolyte-based microgels, hydrogels, and interpenetrating polymer networks allowed uptake and delivery of biotherapeutics.[18,19]

Molecular interactions play a significant role in the design and construction of these platforms, and understanding the contributions of specific molecular segments to these interactions can facilitate better design of materials for the above applications.[20] Our understanding of molecular signatures and thermodynamics of the protein–polymer interactions are rudimentary, despite several important studies.[21−25] The goal of the present work is to perturb the protein–polymer interactions precisely and gain insight into the details of these interactions by ITC studies. Very few thermodynamic studies are available which provide a detailed analysis of contributions of each interactive force toward protein/polymer complex formation.[26] This gap in knowledge is because of the complexity of separating these interactions and limited availability of rational approaches to control them in a predictable manner.

Use of different proteins to factor out specific contributions to these interactions, for example, would make the study complicated since each protein has its own structure with different kinds of amino acid residues decorating its surface. These amino acid residues play a major role in defining the hydrophobic and charged patches on the protein surfaces.[27] In this paper, we introduce protein chemical modification as a rational route to introduce specific interactions at the protein–

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polymer interface and study their influence quantitatively. Chemical modification is a powerful tool to introduce specific and precise molecular segments on protein surfaces,[28] but it has not been used to control protein−polymer interactions in a systematic manner. This latter outcome could be quite useful in the design of novel functional biomacromolecules with defined, predictable properties.

Earlier studies from our group showed that protein charge plays a significant role in defining the protein−solid interactions, and we hypothesized that protein charge controls the binding enthalpies in a predictable manner.[29,30] Along these lines, we now test the hypothesis that binding of proteins to charged polymer molecules (polyions) would also depend on protein charge and that chemical modification of the protein can serve as a predictable tool to probe these interactions in a systematic manner by calorimetric methods.

Poly(acrylic acid) (PAA), a water-soluble, negatively charged polymer, and met-hemoglobin (Hb) have been chosen as a model system. Previously, cross-linking of Hb with PAA was shown not to perturb Hb structure or its peroxidase-like activity.[31] Surprisingly, PAA conjugation improved the Hb shelf life. Therefore, understanding the nature of Hb/PAA interactions will be interesting, and it may provide rational approaches to produce functional biomacromolecules with predictable properties.

Hb is negatively charged at pH 7, and its charge can be tuned by converting the carboxyl functions of aspartic and glutamic acid side chains to their corresponding amides. Replacing the negatively charged amino acid side chains, for example, with neutral or positively charged ones alters protein charge in a predictable manner. Control of Hb charge by chemical modification may provide a simple method to gain further insight into Hb/PAA interactions.

The carboxyl groups of Hb, in the current study, are amidated with ammonia or triethylenetetramine (TETA) to produce the corresponding charge ladders. The extent of amidation, protein/polymer concentrations, ionic strength, and pH are adjusted such that only soluble Hb/PAA complexes are produced.[32,33] This aspect is important because precipitation or insoluble complex formation introduces complications. Data show that the complex formation is exothermic, and exothermicity increases with increasing charge contrast. More interestingly, ITC studies indicated that Hb-TETA/PAA interaction is much more enthalpically favorable than Hb-ammonia/PAA interaction, even after correcting for the charge. This increase is over and above that due to the binding of TETA itself to PAA, and thus, a strong synergistic effect has been established where the TETA segment enhanced the binding enthalpies by a new mechanism.

EXPERIMENTAL METHODS

Materials. Bovine methemoglobin (Hb), poly(acrylic acid) (PAA, 450 000 MW), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), triethylenetetramine (TETA), NH_4Cl, *N*-hydroxysuccinimide (NHS), ethanolamine, and 2-methoxyphenol were purchased from Sigma-Aldrich (St. Louis, MO). β-Mercaptoethylamine hydrochloride was obtained from Fisher and used as received. Phosphate buffered saline (PBS, 10 mM phosphate 0.137 M NaCl) at pH 7.4 and 6.4 was used to prepare the solutions.

Isothermal Titration Calorimetry (ITC). Energetics associated with Hb binding to PAA were measured using a nanocalorimeter (VP-ITC from Microcal Inc., Piscataway, NJ). PAA (450 000 MW) solution was prepared by dissolving 0.3 mg/mL or 0.03 wt % of PAA (0.7 μM) in distilled water and pH adjusted to 7.4. Hb (486 μM in PBS), and PAA samples were dialyzed against PBS pH 7.4 or PBS pH 6.4. Calorimeter was thermally equilibrated for 1 h, and dialyzed PAA was loaded into the calorimetric cell (1.4167 mL) while Hb solution has been loaded into the automated syringe. During the experiment, 6 μL aliquots of Hb solution were added in successive injections to PAA solution (0.7 μM) with 300 s intervals, and heat absorbed or released with each injection has been recorded.

The heats of dilution of the protein and the polymer were measured separately and subtracted from the titration data, analyzed by Origin software (v. 5.0, Microcal Inc., Piscataway, NJ). The heat released or absorbed (Q) during the titration is related to the molar heat of protein binding (ΔH), the volume of the sample cell (V_0), the initial concentration of the ligand (X_t), PAA concentration (M_t), the binding constant (K_b), and the number of binding sites (n) by eq 1.[34,35]

$$Q = \frac{nM_t\Delta HV_0}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nK_bM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nK_bM_t}\right)^2 - \frac{4X_t}{nM_t}}\right] \quad (1)$$

The calorimetric data were fitted to a single set of noninteracting, identical binding sites model, and K_b, n, ΔH, and ΔS were extracted from best fits to the data to eq 1. The limitations of the above model to fully describe protein binding to various surfaces is well-known, and hence, we proceeded to test the validity of the above binding enthalpies by using a direct method, described below. Each titration was performed three times, and errors were estimated from multiple measurements. Fits to each curve were performed multiple times with different initial values of K_b, n, and ΔH to find the best fit, and the values of ΔS and ΔG were calculated, using standard equations of thermodynamics.

Model-Independent Method for the Determination of Binding Enthalpies. Hb and PAA concentrations were adjusted such that when an aliquot of Hb solution was added to PAA solution, most of the protein binds to PAA to produce soluble protein/polymer complex. Four successive additions of 60 μL of Hb solution (4 mg/mL 60 μM, in PBS pH 6.4) into the cell (PAA, 4 mg/mL in PBS pH 6.4) were done at intervals of 300 s each. The injections were separated by 48 s of mixing time, at a stirrer speed of 300 rpm. Heat released or absorbed during the reaction time was recorded, and the data have been corrected for the corresponding dilutions of Hb and PAA solutions. To factor out the enthalpy changes due to chemical modification, the binding enthalpies of TETA and NH_4Cl ligands (no protein) with PAA were also measured. Each measurement was repeated multiple times, and the errors were estimated from these.

Dynamic Light Scattering (DLS). Hydrodynamic radii of Hb/PAA and Hb-TETA/PAA soluble complexes were measured using CoolBatch+ dynamic light scattering apparatus, where a Precision detector (Varian Inc.) using a 0.5 × 0.5 cm^2 square cuvette and 658 nm excitation laser source at 90° geometry. Hb and Hb-TETA samples (60 μM) were diluted once and filtered with a 0.2 μm filter (PVDF, 13 mm, Fisher

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Scientific) prior to the measurements. Hb/PAA and Hb-TETA/PAA soluble complexes from the ITC chamber (8.7 μM protein and 7.7 μM PAA) were diluted 10× and filtered. All samples were equilibrated for 300 s at 26 °C, and five repetitions with 60 accumulations were done at the same temperature. Precision Elucidate Version 1.1.0.9 was used to run the experiment, and Deconvolve Version 5.5 was used to process the data.

Surface Plasmon Resonance Studies for Hb Binding to PAA. Surface plasmon resonance (SPR) was performed using an SR7000DC dual channel flow SPR spectrometer from Reichert Analytical Instruments (Depew, NY) with a semi-automatic injection setup with a 500 μL PEEK injection loop and a Harvard Apparatus flow pump. The flow cell was setup with a Y-connector to facilitate parallel flow through the sample and reference channels, at 25 °C. The running buffer (PBS, 0.1 M phosphate, 0.1 M NaCl, 2.7 mM KCl) was thoroughly degassed before use. The data were analyzed using the Scrubber 2.0 software (BioLogic Software, Australia) and Kaleidagraph (Synergy Software, Reading, PA).

The bare gold chips were obtained from Reichert (catlog # 13206060) and functionalized with 100 mM β-mercaptoethylamine hydrochloride in ethanol/water (80/20 v/v) mixture (Supporting Information Scheme S1). The chip was immersed in the solution, purged with nitrogen gas, sealed, and equilibrated overnight (12 h) followed by rinsing with ethanol and drying in nitrogen flow. The aminothiol self-assembled monolayer (SAM)-covered Au chips were used immediately to avoid oxidation.

The functionalized Au chip was mounted in the Reichert SPR flow cell, and PAA was attached to the amine SAM by carbodiimide coupling. The carboxylic acid groups of PAA (13.9 mM COOH groups, 1 mg PAA/mL) were activated by flowing a mixture of EDC (100 mM) and NHS (40 mM) over the SAM for 2000 s at a flow rate of 20 μL/min. The functionalized PAA surface was rinsed by washing with PBS buffer (50 μL/min flow rate) until the baseline remained constant. Any remaining activated PAA COOH groups were blocked by reaction with ethanolamine (1 M).

Hb solutions (1−25 μM, as needed) were flowed over the PAA-coated Au chip for 300 s at a flow rate of 20 μL/min, and the association/dissociation curves have been obtained in real time. These kinetic curves were fit to obtain the corresponding k_a and k_d values by Scrubber. The reference channel showed a significant amount of direct binding of Hb to the amine SAM, and for this reason the reference curves were not subtracted from the corresponding sample channel curves. To recycle the chip, Hb bound to PAA was removed by injection of NaOH solution (pH 12.5), and the regenerated polymer surface indicated consistent binding of Hb to PAA during the second and subsequent cycles. Multiple regeneration cycles were used to examine the binding at increasing concentrations of Hb, at a constant loading of PAA on the Au chip. Each measurement was repeated multiple times to check for reproducibility and obtain errors in the measurements.

Chemical Modification of Hemoglobin. Aspartic and glutamic acid residues of Hb were modified by activating them with carbodiimide and by reaction with either NH_4Cl or TETA, by adopting reported methods.[36] Hb (4 mg/mL) dissolved in deionized water (DI) was stirred with the appropriate amine (pH adjusted to 5 or 7) for half an hour followed by the addition of EDC (10 mM). The reaction mixture was stirred for an additional 4 h at room temperature, and unreacted EDC, amine, and byproducts were removed by dialysis against PBS buffer at pH 6.4.

The degree of modification, and therefore the net charge on the modified Hb, was controlled by adjusting the pH (5 or 7) or amine concentration (40 mM to 2.5 M), at a constant reaction time (4 h, Supporting Information Table 1). Chemically modified Hb samples are labeled as Hb-TETA40-7, where the derivative has been obtained by reacting TETA (40 mM) with Hb at pH 7.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed using horizontal gel electrophoresis apparatus (Gibco model 200, Life Technologies Inc., Gaithersburg, MD) and agarose (0.5% w/w) in Tris acetate (40 mM) buffer. Modified Hb samples were loaded with 50% loading buffer (50% v/v glycerol and 0.01% w/w bromophenol blue). The running buffer was Tris acetate (40 mM) adjusted to specific pHs to determine the isoelectric points of the Hb derivatives. Samples were spotted into wells placed at the middle of the gel, so that the protein could migrate toward the negative or the positive electrode, based on net charge. A potential of 100 V was applied for appropriate duration; gels were stained overnight with 10% v/v acetic acid and 0.02% w/w Coomassie blue, followed by destaining in 10% v/v acetic acid, overnight.

Circular Dichroism Measurements. Structural changes of Hb, if any, upon chemical modification, as well as after complexation with PAA, were monitored using circular dichroism studies. Far-UV and Soret CD spectra of unmodified Hb, TETA-modified Hb, and PAA/Hb mixtures were recorded using a Jasco 710 spectropolarimeter. Same concentrations of Hb and PAA used for ITC experiments were also used to for CD studies. All samples were in PBS pH 6.4, and the buffer scan was subtracted during processing. Step resolution was kept at 0.2 nm/data point, and bandwidth and sensitivity were 1 nm and 20 mdeg, respectively. The far-UV CD spectra were scanned from 200 to 260 nm, and the scan speed was maintained at 50 nm/min. An average of four accumulations was recorded using 0.05 cm path length cuvette. For the Soret CD spectra, samples were scanned from 350 to 450 nm at a scan speed of 50 nm/min and 0.2 cm path length, and eight accumulations were averaged.

Hemoglobin Peroxidase-like Activity Studies. Peroxidase-like activity of Hb was used to compare the activities of Hb in the presence and absence of PAA. The substrate, *O*-methoxyphenol (5 mM), and the oxidant, H_2O_2 (0.5 mM), were added to the solution containing 1 μM Hb in PBS, pH 6.4. Absorbance at 470 nm, due to the product formation, was monitored as a function of time, using an HP 8453 diode array spectrophotometer (Agilent Inc., Totowa, NJ).[37] From the plots of absorbance vs time, initial velocities and specific activities were calculated, and the activities were compared under the same conditions of pH, ionic strength, substrate concentration, and temperature.

RESULTS

The hypothesis that chemical modification of proteins serves as a convenient method to systematically modulate hemoglobin−PAA interactions has been tested here. Chemical modification of Hb with TETA, for example, served to probe the contributions of charge and TETA segments to these interactions, which revealed strong synergy.

Energetics of Hb Binding to PAA. Using Hb as a model protein and PAA as a model polymer, we examined the binding of Hb to PAA to form a soluble complex, under a specific set of

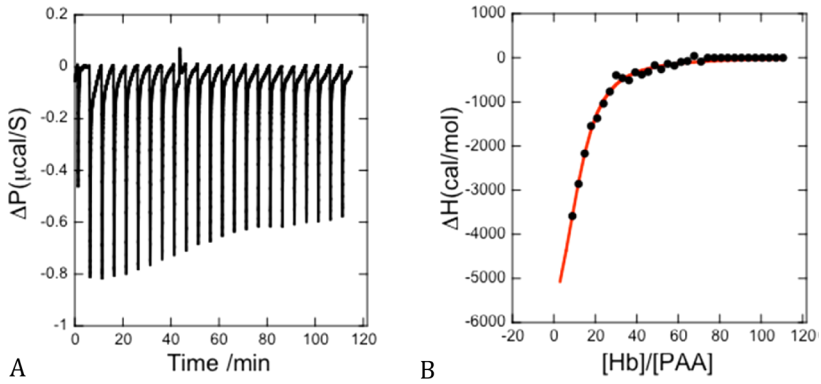
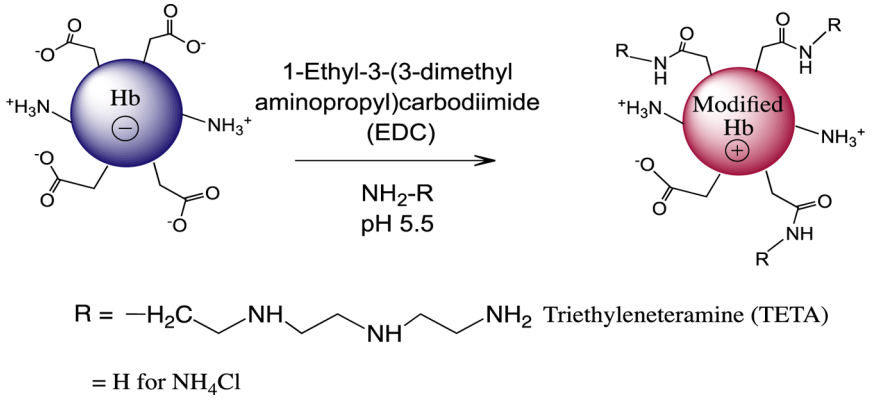


Figure 1. Titration of Hb (486 μM) with PAA (0.7 μM) in PBS, pH 7.4 at 25 °C. (A) Change in power vs time plot when PAA solution was titrated with Hb solution. (B) Enthalpy change vs [Hb]/[PAA] for titration. Red line is the best fit to the data, according to the single, identical, noninteracting binding site model. Best fit indicated a binding stoichiometry of 14 Hb per mole of PAA, binding constant of 3.2×10^5 M^{-1}, ΔH and ΔS values of −7.4 kcal/mol of Hb bound and 0.1715 cal/mol, respectively.

Scheme 1. Chemical Modification of COOH Groups of Hb via EDC Chemistry



conditions by ITC. Exothermic binding of Hb to PAA, at pH 7.4 in PBS buffer, was demonstrated (Figure 1), and binding saturated at a Hb to PAA mole ratio of ∼14.

Enthalpy change associated with each addition of Hb solution to PAA solution was plotted as a function of the ratio of molar concentrations of Hb to PAA (Figure 1B, black dots), and the data were fitted to a single set of indistinguishable, noninteracting binding sites model (Figure 1B, red curve, using eq 1). The best fit to the data indicated K_b, ΔG, ΔH, ΔS, and the binding stoichiometry to be $(3.2 \pm 0.75) \times 10^5$ M^{-1}, −7.3 ± 0.95 kcal/mol, −7.4 ± 0.2 kcal/mol, 0.17 cal/mol, and 14 Hb molecules bound per each PAA molecule, respectively. Hb binding to PAA is exothermic, with significant affinity, and large loading on the polymer of 14:1 protein to polymer (monomer) mole ratio, and the binding is primarily enthalpy driven (−7.4 ± 0.2 kcal/mol) but it is also entropy favored. The above single-site binding model assumes that there are no interactions between the bound protein molecules and that the binding sites on PAA are nonoverlapping. Introduction of these assumptions simplifies data analysis but could introduce potential errors in the estimated parameters.[38,39] Hence, we have used a model-independent method to measure binding enthalpies, directly, and compared them with these values, as described later. The binding enthalpy determined above is in good agreement with the values obtained from the direct measurement, described below.

SPR Studies. Hb binding to PAA is also verified by SPR studies. PAA was immobilized on Au chip using the aminothiol SAM and EDC coupling (Scheme S1) and from the SPR signal of 500 μRIU, and the conversion factor of 1 μRIU corresponding to 0.73 ng/mm^2 of mass,[40] we estimated that 365 ng of PAA was attached to 1 mm^2 of the chip. Using the known density of PAA,[41] we estimate an average polymer layer thickness of 300 nm, and this is a lower limit on the PAA film thickness, as the polymer can swell substantially due to hydration as well as the pH of the medium. PAA film was used to monitor Hb binding by SPR. Flowing Hb solutions over the PAA surface in the sample channel resulted in huge SPR signal increase, while the reference channel indicated only weak signals. We monitored the on and off rates of Hb binding to the PAA SAM (Figure S1), and best fits to the data indicated average on and off rate constants of 1.2×10^3 M^{-1} s^{-1} and 2.8×10^{-3} s^{-1}, respectively (5 μM, PBS, pH 7.2), to PAA film (Table S2). The ratio of the on to off rate constants, the binding constant, is 7.1×10^5 M^{-1}, and this value is comparable to that obtained from the ITC data, $(3.2 \pm 0.7) \times 10^5$ M^{-1}. Therefore, the calorimetric and SPR measurements agree reasonably and indicate substantial interaction of Hb with PAA but note that SPR cannot provide direct enthalpy measurements.

Chemical Modification. Several factors could contribute to the observed Hb and PAA interactions, and chemical modification is a convenient tool to evaluate specific contributions to these interactions. For example, the conversion of the COOH groups of the aspartate and glutamate residues in Hb to the corresponding amides would neutralize at least one

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negative charge per COOH modified (Scheme 1). For example, amidation with TETA would lower the protein negative charge by the conversion of COOH groups to the corresponding amides, and additional charge neutralization can occur by the protonation of the basic nitrogens of TETA. Thus, chemical modification provided an appealing method to continuously tune net charge of Hb at constant pH and ionic strength, in a systematic and predictable manner.

Hb was reacted with increasing concentrations of TETA or NH_4Cl under specific conditions of pH, temperature, amine concentration, and reaction time using EDC chemistry (Scheme 1). Nine different Hb-TETA and Hb-ammonia derivatives were prepared and purified by extensive dialysis to remove unreacted reagents and byproducts. The progress of Hb modification has been monitored by agarose gel electrophoresis, described below.

Agarose Gel Electrophoresis. Chemical modification altered the net charge on Hb, as monitored in agarose gels (Figure 2), and modified samples began to migrate toward the cathode. The samples were loaded into wells at the center of the gel, and Hb did not migrate out of the wells (lane 1, Figure 2). The isoelectric point (pI) of Hb is ~6.77,[42] at pH 7 Hb is nearly neutral, and hence it did not move out of the well. As the extent of amidation increased with increasing TETA concentration, the net negative charge on the Hb-derivative decreased, or positive charge increased, and samples began to migrate toward the cathode (lanes 2–4, Figure 2). All the Hb-TETA samples migrated toward the cathode, directly proving that they bear net positive charge. Thus, Hb-TETA charge ladder consisting of Hb-TETA40-7, Hb-TETA40-5, and Hb-TETA80-5 are produced successfully.

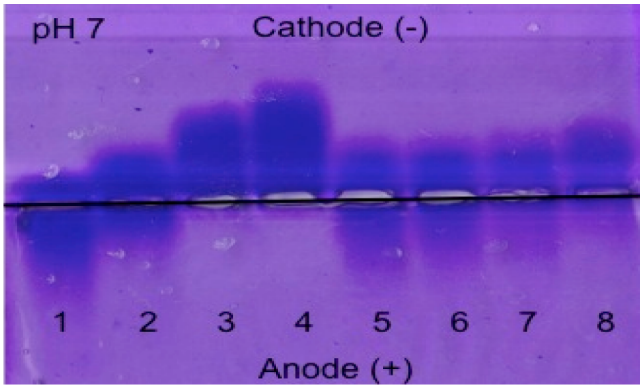

Figure 2. Agarose gel electrophoresis of Hb-TETA and Hb-ammonia derivatives at pH 7 (samples spotted at the center of the gel). Lane 1 is Hb, and lanes 2–4 are Hb-TETA40-7, Hb-TETA40-5, and Hb-TETA80-5, respectively, while lanes 5–8 are the Hb-ammonia derivatives, Hb-ammonia400-5, Hb-ammonia800-5, Hb-ammonia1K-5, and Hb-ammonia1.5K-5, respectively, produced under specific reaction conditions.

The reaction of ammonia with Hb facilitated by EDC chemistry, under specific reaction conditions (Supporting Information Table 1), also resulted in a charge ladder of Hb-ammonia derivatives (lanes 5–8). Note that these samples, migrated farther toward cathode than native Hb, bore less positive charge when compared to those of the Hb-TETA derivatives. Additionally, Hb-ammonia derivatives also indicated better mobilities than Hb.

The electrophoresis data are consistent with progressive conversion of Hb to Hb-TETA or Hb-ammonia derivatives with decreasing negative charge or increasing positive charge, as the case may be. Thus, chemical modification has successfully produced two different charge ladders of Hb-derivatives where the protein charge was controlled in a gradual and systematic manner.

Charge and Isoelectric Points of Hb Charge Ladders. Since the mass difference between Hb and Hb-derivatives is negligible, migration distance of each band in the agarose gel is proportional to its charge.[43] Therefore, agarose gels were run at specific pH values (Figures S2 and S3), and at each pH, certain samples migrated toward the positive or negative electrode. Using the known charge of Hb,[42] charges of all Hb-derivatives were determined (Table S3).

When pH of the running buffer is equal to the pI of the protein, net charge on the protein will be zero, and the sample will not move out of the loading well, even under the influence of the applied electric field. Therefore, charge obtained from the band mobility of each sample was plotted as a function of pH, and from these plots we obtained the pH at which the charge on the protein is zero (pI value) (Figure S4 and Table 1). The pI values of the samples, for example, increased from ~7 to ~9, with increased chemical modification, and chemical modification successfully resulted in the construction of Hb charge ladders.

Table 1. Key Properties of Hb-Derivatives[a]

sample	pI	charge at pH 6.4	ΔH (kcal/mol)
Hb	7	+6	−8 ± 0.2
Hb-TETA40-7	7.4	+10 ± 0.5	−45 ± 2
Hb-TETA40-5	8.4	+19 ± 1	−55 ± 3
Hb-TETA80-5	8.8	+23 ± 1	−89 ± 4
Hb-TETA60-7	7.5	+14 ± 0.5	−28.9 ± 4.1
Hb-TETA60-5	8.7	22 ± 1	−68.04 ± 8.8
Hb-NH_4Cl400-5	7.3	+10 ± 0.5	−10 ± 0.9
Hb- NH_4Cl800-5	7.4	+10.8 ± 0.5	−14 ± 0.4
Hb- NH_4Cl1K-5	7.6	+11.6 ± 0.5	−16 ± 0.9
Hb-NH_4Cl1.5K-5	7.9	+15 ± 0.5	−17 ± 1.5

[a]The isoelectric points (pI), average charge at pH 6.4, and binding enthalpies (kcal/mol), in PBS, pH 6.4, at 25 °C.

Circular Dichroism Studies. To assess any distortions in secondary and tertiary structure of HB in Hb-TETA derivatives due to chemical modification, circular dichroism spectra of the samples were compared with that of the unmodified Hb. The UV-CD spectra are highly sensitive to the changes in protein secondary structure, and they serve as excellent probes to examine protein structure.[44] The UV CD spectra of Hb has a strong peak maximum at 190 nm and double minima at 208 and 222 nm.[45] The CD spectra of Hb-TETA derivatives overlapped with that of the unmodified Hb, under similar conditions (Figure S5). Thus, the secondary structure of Hb has largely been retained after chemical modification with TETA. Similarly, the CD spectra of H-derivatives reordered in the presence of PAA showed that the spectra overlapped well with that of Hb, except for a minor distortion in the case of Hb-TETA80-5/PAA (Figure S6). Hence, the majority of the samples retained their structure after chemical modification and binding to PAA.

We also examined the structure by recording the Soret CD, which is a sensitive measure of the asymmetry of the heme

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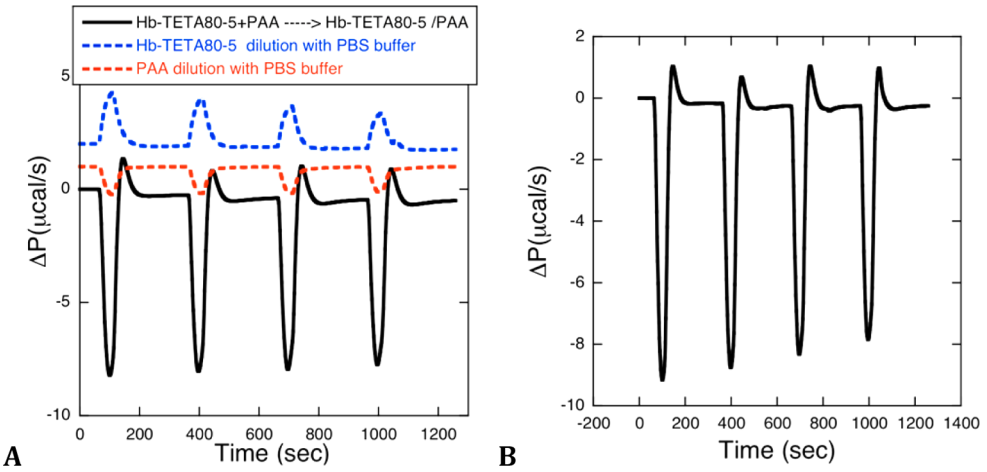

Figure 3. (A) Change in power due to the addition of Hb-TETA80-5 (60 μM) to PAA (9 μM, polymer) (black curve), the dilution of Hb-TETA80-5 (60 μM) (blue curve), and dilution of PAA (9 μM) (red curve). (B) Change in power due to the binding of Hb-TETA80-5 to PAA, after correcting for the dilutions of Hb-TETA80-5 and PAA (PBS, pH 6.4, 25 °C). These peaks were integrated to extract the corresponding ΔH values.

environment. The Soret CD spectra of the Hb-derivatives and their complexes with PAA are shown (Figure S7). Since peak positions and intensities are the same, the heme coordination environment is well preserved when the protein was modified or when the modified protein was bound to PAA. Encouraged by the fact that PAA did not distort the structures of Hb or Hb-derivatives to a significant extent, only minor changes noted, we proceeded to examine the binding enthalpies.

Model-Independent Binding Enthalpies. As mentioned earlier, the estimation of the binding enthalpies from ITC curves using the model-dependent method could introduce potential errors due to the tacit assumptions made in the model. Hence, we chose to measure the binding enthalpies of the Hb-derivatives with PAA, directly by the model-independent method. The protein and polymer concentrations have been chosen such that each addition of the protein solution resulted in complete binding to PAA and soluble complexes. The heat released or absorbed, under these conditions, was corrected for dilution, and the corresponding binding enthalpies were evaluated.

The calorimetric curves representing the addition of Hb-TETA80-5 to PAA (black), Hb-TETA80-5 dilution (blue), and PAA dilution (red), in PBS at pH 6.4, are shown in Figure 3A. The area under the peak for each injection (q) was used to calculate the binding enthalpy ($\Delta H_{\mathrm{Hb/PAA}}$), and appropriate corrections have been made for the dilution enthalpies of the polymer ($\Delta H_{\mathrm{PAAdil}}$) and the protein ($\Delta H_{\mathrm{Hbdil}}$), using eq 2. The resulting, corrected binding enthalpies per mole of Hb-TETA80-5 bound to PAA (Figure 3B) was extracted to be −89 ± 4 kcal/mol (Table 1).

$$\Delta H_{\mathrm{interaction}} = \Delta H_{\mathrm{Hb/PAA}} - (\Delta H_{\mathrm{PAAdil}} + \Delta H_{\mathrm{Hbdil}}) \qquad (2)$$

The binding enthalpies of Hb and all Hb-TETA derivatives were measured by this method, at pH 6.4, and their binding to PAA has been strongly exothermic. The binding enthalpies, measured under the same conditions of ionic strength, pH, and temperature, varied from −45 to −89 kcal/mol (Table 1). The binding enthalpy of Hb measured by the titration method was −7.4 ± 0.2 kcal/mol, which is in good agreement with the value obtained from direct method (−8.0 ± 0.2 kcal/mol, Table 1). None of these protein/polymer complexes precipitated under these conditions, and proper corrections were done for dilution to obtain heat release due to protein interactions with PAA.

The Hb-TETA/PAA binding enthalpies are analyzed with respect to the protein charge, and plot of the binding enthalpies vs their charge is shown in Figure 4 (blue dots). These showed

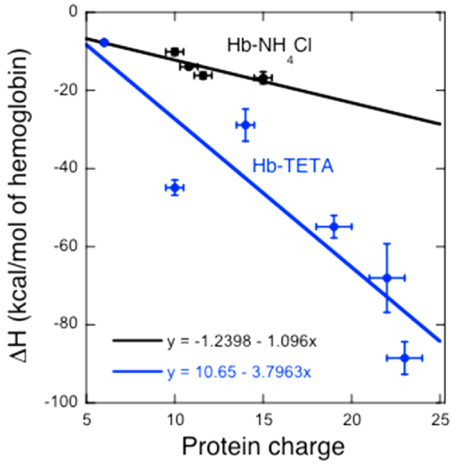

Figure 4. Binding enthalpies of Hb-TETA (blue dots) and Hb-ammonia derivatives (black dots) (60 μM) to PAA (9 μM) as a function of their charge. Slopes of the linear fits to the TETA and ammonia derivatives are −3.8 and −1.0 kcal/mol per unit charge, respectively (PBS, pH 6.4, 25 °C).

a strong trend, and linear fit to the data had a slope of −3.8 kcal/mol per unit charge. The steep, favorable, dependence of ΔH on protein charge was observed with the TETA derivatives, and TETA modification substantially increased the binding exothermicity.

Binding Enthalpies of the Hb-Ammonia Derivatives. Several factors contribute to the binding enthalpies measured above, and to separate the enthalpy contributions from protein charge vs the specific interactions of TETA side chains, we measured the binding enthalpies of similarly charged Hb-derivatives prepared from the chemical modification with ammonium chloride, rather than TETA. The TETA side chains may specific contributions to the binding enthalpies, and these have been evaluated using the Hb-ammonia charge ladder.

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The binding enthalpies of the members of the Hb-ammonia charge ladder were obtained, under identical conditions of pH, ionic strength, and temperature as those of the TETA derivatives. Concentrations and the extent of modification have been adjusted such that all protein/PAA complexes were completely soluble, and there has been no precipitation. The binding of this charge ladder was also exothermic, but the exothermicity increased much more slowly from −10 to −17 kcal/mol, while their charge varied over a range of +10 to +15 (Table 1), at the same pH, buffer, and ionic strength.

A plot of the binding enthalpies of the ammonia derivatives vs charge (black dots, Figure 4) was also linear but had a slope of −1 kcal/mol per unit charge. The magnitude of this slope is ∼4 times smaller than that noted for the TETA derivatives. Therefore, it is clear that appending TETA to Hb has a strong synergistic effect to the binding enthalpies.

Activities of Polymer−Protein Complexes. The anomalous binding enthalpies of Hb-TETA derivatives could be due to extensive unwinding of the protein, before or after interaction with the oppositely charged PAA molecules. Therefore, we set out to assess the biological activities of the Hb-derivatives and their complexes with PAA. Hb does not function as an enzyme in nature, but its peroxidase-like activity is well documented.[46] Therefore, the peroxidase-like activities have been determined, and compared, under the same conditions of pH, ionic strength, buffer, and temperature.

The catalytic activities of Hb-TETA samples and Hb-TETA/PAA physical mixtures were determined using *o*-methoxyphenol as the substrate and H_2O_2 as the oxidant. The absorbance change at 470 nm due to product formation was monitored as a function of time (Figure S8). All samples indicated a high degree of activity, and chemical modification did not inhibit their activities to a significant extent. Specific activities were determined from the initial rates of the activity curves, and these ranged from 0.74 to 0.94 per μM per s (Table S5).

The Hb-TETA derivatives also retained their activities after complexation with PAA, and these have been compared with those obtained in the absence of the polymer (Figure 5). The specific activities of the Hb-TETA/PAA complexes varied from 118 to 90% of that of Hb, and some of them improved slightly when compared to that of Hb. Therefore, neither chemical modification nor complexation with PAA had a significant effect on the peroxidase-like activities of the Hb-TETA charge ladder.

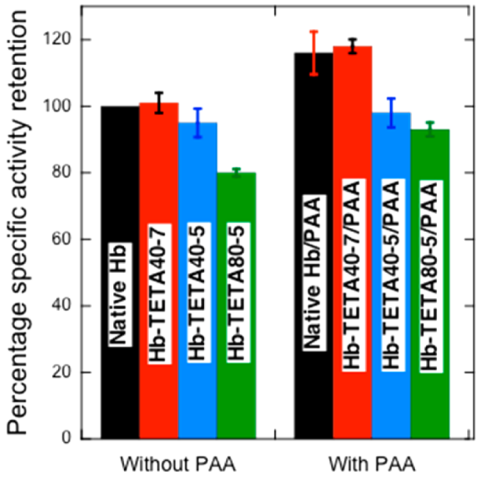

Figure 5. Relative activities of Hb-derivatives (1 μM) in the presence or absence of PAA (0.15 μM), with respect to that of Hb, in PBS, pH 6.4, at 25 °C.

The excess enthalpy change observed for Hb-TETA derivatives is not due to the distortion or denaturation or precipitation of the protein−polymer complexes. It must be originating from intrinsic interactions between the modified protein and the polymer.

DISCUSSION

Unlike the interaction of a small, charged ligand with polymers, the interaction of proteins with polymers is more complicated. The presence of 20 different amino acid building blocks of the protein introduce various chemical and physical properties to the protein, and these make it a complex moiety to study. For example, the use of different proteins with specific charges to study the charge dependency on protein−polymer complex formation, at a particular pH, is complicated, since each protein has a unique way of folding which results in variability of the hydrophobic and charged patches on the protein surface.

Many factors could contribute to the formation of soluble protein/polymer complexes,[25,26,47] and electrostatic interactions have been implicated to play a major role.[33] Contributions of interactions other than electrostatic interactions are much less understood.[48] Hydrophobic interactions were shown to play a major role in the binding of bovine serum albumin (BSA) to hydrophobically modified PAA.[49] Gelation and precipitation of proteins by polymers are suggested to be due to hydrophobic interactions between proteins and polymers.[50,26,51] H-bonding and electrostatic interactions, hydration, or dehydration or ion release or ion uptake were implicated in protein binding to inorganic polymeric materials.[29,30]

As a model system, here, we first examined the binding of Hb with a water-soluble weak polyelectrolyte, PAA (MW 450 000). Binding constant from data for Hb/PAA soluble complex formation is $(3.2 \pm 0.7) \times 10^5$ M^{-1}, which is in fair agreement with the binding constant obtained from SPR (7.1×10^5 M^{-1}). On one hand, this value is similar to the binding constant of Hb observed with calf thymus DNA, an anionic biopolymer, of 4.9×10^5 M^{-1}, in Tris buffer at pH 7.0.[52] On the other hand, the binding constant of Hb with α-Zr(IV) phosphate, anionic rigid, inorganic polymer, was 5.4×10^6 M^{-1}, an order of magnitude higher than observed with PAA.[53] Note that this solid is strongly negatively charged, one negative charge per 25 Å^2, and the higher binding affinity is consistent with the idea that electrostatic interactions contribute substantially for Hb binding to its partners. In support of this notion, positively charged lysozyme was reported to bind to PAA with comparable affinities.[21]

From the observed stoichiometric ratio of 14:1, we propose that each Hb molecule occupies nearly 120 nm along the PAA chain, spanning 400 monomeric units, and that PAA wraps around the protein (beads-on-a-string model) due to favorable interactions with surface groups of the protein. The positively charged patches on Hb could interact favorably with the negatively charged carboxyl groups of PAA.

According to the ITC data, the binding of Hb to PAA is exothermic (−7.4 kcal/mol, obtained at pH 7.4 in PBS), and the enthalpy change could be due to patches of positive charges on its surface despite its weak negative charge at pH 7.4 (pI ∼7). When the pH was dropped to 6.4, the ΔH decreased from -7.4 ± 0.2 to -8 ± 0.4 kcal/mol, and this is consistent with the fact that Hb is slightly positively charged at pH 6.4 and greater

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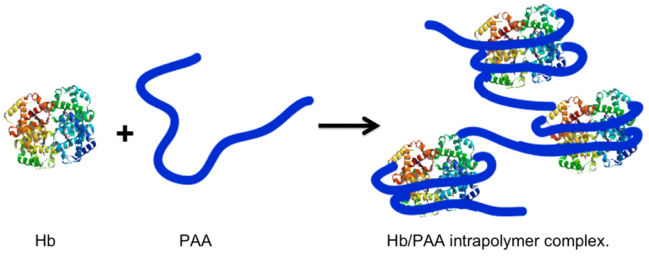
Scheme 2. Schematic Representation of Soluble Intrapolymer Complex Formation between Hb and PAA



electrostatic interactions contribute at lower pH. The binding of Hb to calf thymus DNA was weakly endothermic (ΔH = 3.1 kcal/mol) at pH 7 in Tris buffer.[52] Along these lines, the binding of Hb to α-Zr(IV) phosphate was found to be strongly exothermic −24 kcal/mol, at pH 7.2, in phosphate buffer[30] due to the high charge density of this solid, as discussed earlier. The binding enthalpy for the interaction of strongly positively charged lysozyme to PAA (MW, 240 000) was reported to be −10 kcal/mol, at pH 5.5, and also supports the role of electrostatic interactions in its binding to PAA.[21] Thus, binding enthalpies or entropies differed in terms of the type of host, pH, molecular weight of PAA, and the buffer used for the studies. Therefore, we decided to chemically modify the net charge on Hb and examine the trends in the binding parameters with PAA as a model polymer, under a consistent set of conditions to gain insight into the binding.

To evaluate the role of electrostatic interactions in these, at constant pH and ionic strength, without using different proteins or different host materials, we have prepared a number of Hb-derivatives of two separate charge ladders, TETA and ammonia. Note that modification of Hb with ammonium chloride would convert the glutamic or aspartic COOH groups to the corresponding CO-NH_2 groups, while reaction with TETA would produce CO-TETA groups on the protein. The basic nitrogens of TETA can be potentially protonated to produce additional positive charges. Therefore, these differences are to be kept in mind in the interpretation of the observed data. At a given pH, the net charge on the protein is systematically varied to study its contribution to the binding interactions.

Hb modification with TETA resulted in the charge ladder (Figure 1), which indicated progressive decrease of binding enthalpies from −8 ± 0.2 kcal/mol for Hb to −89 ± 4 kcal/mol for Hb-TETA80-5 (Figure 4). The slope of the linear fit to these data indicated enthalpy decreases of −3.8 kcal/mol per unit charge. In contrast, the enthalpy decrease noted with the Hb-ammonia ladder has been from −8 ± 0.2 for Hb to −17 + 1.5 kcal/mol for Hb-Ammonia1.5K-5 with a slope of −1.0 kcal/mol per unit charge. The latter value is in good agreement with reported values for Coulombic interactions, ΔH_{Ele},[21] but it is much smaller than noted for Hb-TETA charge ladder.

This discrepancy between the two sets of data could be attributed to additional contributions from the TETA ligand, such as hydrogen bonding with the amino groups and hydrophobic interactions from the ethyl segments of TETA (Scheme S2). To test this hypothesis, we have examined TETA binding to PAA by ITC, under identical conditions of pH, ionic strength, and temperature. The binding of TETA to PAA was also exothermic with a ΔH of −2 kcal/mol per TETA. The estimated charge on TETA at pH 6.4 is +2, and hence, the ΔH per unit charge is −1.0 kcal/mol of charge on TETA, which is in good agreement with the ΔH value noted for the Hb-ammonia derivatives but much lower than that of Hb-TETA derivatives (−3.8 kcal/mol charge).

The dependence of binding enthalpies on protein charge observed here is in good agreement with previous related work. Several key studies examined the interactions between oppositely charged peptides and polyelectrolyte microgels.[54−56] The strength of peptide−microgel interaction depended on the charge contrast between the peptide and the microgel. The binding and release kinetics of the peptide with the microgel depended directly on the net charge on the peptide as well as the microgel, and these are consistent with the current observations, despite the fact that two distinct approaches are being used to establish the dependence. These previous data are in support of our current findings, in that the binding enthalpies of the Hb-derivatives gradually increased with increasing charge contrast, just as the on−off rates of the peptides increased with charge contrast. Unfortunately, these earlier studies did not quantify the relationship between the charge and binding enthalpies but related the peptide charge to the kinetics of association and release. Strong roles of electrostatic contributions are clearly supported by these studies, and current data are consistent with earlier reports.

The difference between the binding enthalpies of the Hb-TETA and Hb-ammonia derivatives (−3.8 vs −1 kcal/mol of charge) still needs to be explained. Increased substitution with TETA chains have greater chance to produce clusters of multiple charges by the close proximity of two or more TETA ligands on the protein surface. When these charges are separated by distances shorter than the Debye length (8 Å) at the ionic strength (150 mM ionic strength), they could function as a multivalent point charge, which can enhance the electrostatic interactions. This explanation is along the lines of previously reported peptide−microgel interactions where enhanced interactions due to charge localization was invoked.[56] Clustering of charges along the TETA chain could function as a multivalent point charge, as opposed to uniform distribution on the protein surface, and charges farther than Debye length would not substantially contribute to binding.

To make sure that observed excess enthalpy change (synergy) is not due to the unwinding of protein structure by PAA and subsequent enhanced interaction of the denatured protein with PAA, we proceeded to examine the secondary structures of the protein/PAA complexes. Unwinding of Hb by PAA will substantially decrease Hb secondary structure. However, we find that the CD spectra of the Hb/PAA complexes and their enzymatic activities are similar to Hb without PAA (Supporting Information Figures 4 and 5). Therefore, these structural changes in Hb-TETA are only minor, if any, and cannot account for the enhanced binding enthalpies of Hb-TETA derivatives.

If there are protein structure distortions that are not readily visible in the CD studies, they might influence its peroxidase-like activity. The peroxidase-like activities of the Hb-derivatives and their complexes with PAA are comparable to that of Hb (Figure 5). Interestingly, there have been small improvements in activities in specific cases on binding to PAA. Therefore, it is unlikely that the excess binding enthalpies can be accounted for by the unwinding of the protein upon interaction with PAA. Neither chemical modification nor binding to PAA resulted in substantial loss of activity. Therefore, PAA-induced conformational changes in Hb are minimal and cannot account for enhanced binding enthalpies. PAA conformational changes

accompanying Hb binding, if any, are expected to be very small as the polymer adopts the conformations of a random coil.[57]

One other issue is that the conversion of the COOH groups on the protein surface to CO-NH_2 neutralizes the local negative charge and could alter the Hb/PAA contact points but may not necessarily involve the newly introduced CO-NH_2 groups in the interaction, since these are polar but not charged. In contrast, TETA chains attached to Hb would favorably interact directly with COOH groups of PAA (Scheme S2), and this interaction could promote additional contacts with functional groups on the protein surface. These new interactions could potentially alter the points of contact between Hb and PAA. In simple terms, chemical modification alters the overall charge, but it might also alter the points of contact between the protein and the polymer, and charge localization or clustering of TETA charges could enhance the interactions further. These aspects depend on the type and nature of chemical modification that has been carried out. Thus, controlled chemical modification provided a powerful new tool to alter binding enthalpies in a quantitative manner, without adversely affecting the biological activity, in a predictable, linear manner, in the charge range examined here. This powerful approach could be expanded to other systems, and it might be useful to fine-tune the protein−polymer interactions in a systematic manner via benign, controlled chemical modification of protein side chains.

CONCLUSIONS

The Hb and PAA interaction is exothermic at pH 7, and the binding is mostly enthalpy driven but entropically favorable. Approximately 14 Hb molecules associate with one PAA polymer chain, on an average, or occupy about 120 nm along the polymer backbone. One reason to form such discrete structures could be due to the extremely low concentrations of the polymer used here (0.7 μM). Regardless of the binding motif, discrete Hb-PAA complexes are produced, which are completely water-soluble and did not form macrogels or insoluble precipitates.

Carefully controlled chemical functionalization and consequent introduction of charge groups on Hb is a novel strategy to manipulate Hb interaction with PAA. Binding exothermicity has been substantially improved by chemical modification of the COOH groups of Hb with TETA, at a rate of −3.8 kcal/mol per unit charge. This provided a powerful approach to fine-tune the binding enthalpies, at least in this charge and concentration regions. In comparison, the binding enthalpies of the Hb-ammonia charge ladder increased at a low rate of −1.0 kcal/mol per unit charge. We speculate that the excess exothermicity associated with the TETA derivatives as compared to that of the Hb-ammonia derivatives can be due to changes in the contact points between the protein and the polymer and possible localization of charge on protein surface with increased modification. The binding enthalpies of the ammonia or the TETA derivatives can be predicted within the region examined here.

While chemical modification appeared to be a simple approach to factor out the electrostatic contributions to the protein−polymer interactions, it could potentially alter the protein−polymer contact points and hence alter the strength/nature of these interactions further. Nevertheless, this synergy could be useful in tuning the binding enthalpy in a systematic manner, and the enthalpy change can be controlled by controlling the type and extent of modification. This type of ability to manipulate the molecular nature of protein/polymer interactions could be useful in designing novel protein/polymer hybrid materials for biomaterial and biotechnological applications.

ASSOCIATED CONTENT

Supporting Information

Figures of SPR studies for Hb binding to PAA, agarose gel electrophoresis showing chemically modified Hb at different pHs, CD spectra, and activity traces of hemoglobin derivatives in the presence and absence of PAA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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