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A Model for the Prediction of Precipitation Curves for Globular Proteins with Nonionic Polymers as the Precipitating Agent

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ABSTRACT

A statistical thermodynamic model for the prediction of precipitation curves of globular proteins using nonionic polymers has been proposed. The model accounts for protein–polymer, polymer–solvent, electrostatic, and hydrophobic interactions as well as the entropy of mixing and employs simplifying assumptions such as spherical globular protein molecule with uniform surface properties and linear, homogeneous polymer uniform with respect to molecular weight. The proposed model can only be employed to predict precipitation curves of charged proteins at sufficiently high ionic strengths since it does not account for electrostatic protein–protein interactions due to overlap of electrical double layers. The model predictions of precipitation curves of human serum albumin (HSA) at the isoelectric point using polyethylene glycol (PEG) for different initial protein concentrations and molecular weights of PEG agreed well with the experimental data. Higher polymer concentrations were found to be required to precipitate proteins for lower molecular weight polymers, lower initial protein concentrations, and more favorable protein–polymer interactions. The HSA–PEG interaction parameter, obtained by fitting the model to experimental data for one molecular weight PEG, was found to be 0.122. Solubility of HSA in PEG solution was found to decrease with increasing salt concentrations, this effect being more pronounced at lower PEG concentrations. The net charge on HSA was found to result in a maximum in its solubility at intermediate salt concentrations as a result of competing salting-in and salting-out effects.

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Key Words. Precipitation; Globular proteins; Protein–polymer interaction; Protein solubility; Thermodynamics of protein precipitation; Nonionic polymers

INTRODUCTION

Protein precipitation represents one of the most important operations for the industrial scale recovery and purification of proteins. These include vegetable and microbial food proteins, human and animal blood plasma proteins, and enzymes for analytical and industrial application. Precipitation is effected by altering the solubility of proteins using various precipitating agents. In addition to more commonly employed techniques such as salting out and isoelectric precipitation, precipitation by nonionic polymers such as polyethylene glycol (PEG) and dextran is attracting increasing attention in the recovery of enzymes because of their ability to preserve the structure of proteins. In other words, unlike most organic solvents which have been used for precipitation, nonionic polymers reportedly have little tendency to denature the proteins even when used at room temperature. Furthermore, the concentration of PEG required to precipitate a given protein is not very dependent on temperature so that precise temperature control is not important. These attractive features account for the considerable interest in developing the use of PEG for large-scale purification of proteins from human plasma and other sources as well as intracellular enzymes. The molecular basis of the protein precipitating action of PEG and other synthetic polymers is not well understood. The major emphasis in the literature has been on excluded volume effects whereby proteins are sterically excluded from regions of aqueous solvent occupied by the synthetic polymers. Excluded volume effect is not able to explain fully the effect of different variables such as types of proteins and polymers, pH, ionic strength, etc. Proper understanding of the mechanism of precipitating action by polymers is necessary in order to effectively separate mixtures through fractional precipitation.

Polson et al. (1) were the first to point out the advantages of polyethylene glycol over other water-soluble polymers for protein precipitation. Iverius and Laurent (2) and Edmond and Ogston (3) suggested that nonionic polymers exclude the proteins from part of the solution and reduce the effective amount of water available for their solvation. This phenomenon is closely related to the formation of a liquid–liquid two-phase system from mixtures of aqueous polymers first studied by Albertsson (4) and more recently by many investigators [see, for example, Kroner et al. (5)]. The effects of initial protein concentration (6, 7), protein size (8, 9), molec-

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ular weight of polymer (1, 8–10), salt type and concentration (8, 11), pH (6–8), protein–protein interaction (12), and temperature (6, 13) on protein precipitation have been investigated. Fractional efficiency of polyethylene glycol (PEG) was found to be impaired by protein–protein interaction at higher protein concentrations (6). Hönig and Kula (10) noted that nominal 300 molecular weight PEG is superior to that of high molecular weight PEG with respect to selectivity of precipitation. It has been demonstrated that the concentration of PEG required to precipitate protein is insensitive to temperature (6, 14). Edmond and Ogston (3) presented a theory based on an osmotic virial equation which was applied by Atha and Ingham (9) to protein precipitation. The precipitation curve was expressed in terms of protein–protein and protein–polymer interaction coefficients. Even though this model was able to predict qualitatively the effect of different variables, the interaction coefficients independently measured employing equilibrium dialysis and light scattering (9, 15) did not agree with the values from solubility experiments. Moreover, their model cannot predict the effect of molecular weight of polymers. Baskir et al. (16) modified the lattice theory developed by Scheutjens and Fleer (17) for adsorption of polymer segments in order to predict the partition coefficient of globular proteins in two aqueous phase systems. Mahadevan and Hall (18) extended the work of Gast et al. (19) and proposed a statistical mechanical model for the prediction of precipitation of proteins in the presence of nonionic polymers. Free energy of interaction of protein molecules, necessary for the prediction of a phase diagram for precipitation, was evaluated by employing perturbation theory and by expressing the protein–protein interaction potential as perturbation around hard sphere potential. Even though they accounted for protein–protein electrostatic interactions and volume exclusion due to polymers, polymer–solvent interactions were not accounted for. Guo and Narsimhan (20) proposed a statistical thermodynamic model for the prediction of solubility of globular proteins in polysaccharide solutions at the isoelectric point wherein they employed the spherical lattice model to describe protein–polysaccharide and polysaccharide–solvent interactions. Even though their model accounted for polymer–solvent interactions, they did not consider electrostatic and hydrophobic interactions.

In the present paper a statistical thermodynamic model for the prediction of precipitation of globular proteins using nonionic polymers is proposed. This model is capable of predicting the effect of nonionic polymer on the solubility of globular protein. In other words, the proposed model can predict protein solubility at different polymer concentrations *given* the solubility of protein in the *absence* of polymer. The model accounts for protein–polymer, polymer–solvent, electrostatic, and hydrophobic in-

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teractions as well as entropy of mixing. The present model, however, neglects protein–protein electrostatic interactions due to overlap of double layer and can therefore be employed to predict precipitation curves of a charged protein only at sufficiently high ionic strengths. Salient features of the model are presented in the next section. Materials and methods and comparison of model predictions with experimental data of precipitation of human serum albumin (HSA) using polyethylene glycol (PEG) are presented in the subsequent two sections, respectively. The last section concludes the paper.

MODEL FOR PRECIPITATION OF GLOBULAR PROTEINS

As pointed out earlier, precipitation of proteins is effected by the addition of nonionic polymers such as polyethylene glycol (PEG) mainly because of the interaction of protein and polymer among other effects. In order to evaluate the precipitation curve of proteins using nonionic polymers, knowledge of the solubility of proteins in such systems is essential. Consider a saturated solution of globular protein in the presence of a nonionic polymer. Since the saturated solution is in equilibrium with the precipitate phase, the chemical potential of protein in both phases should be equal. Experimental investigation of the composition of the precipitate phase (7, 21) seems to suggest that the precipitate phase contains a negligible amount of polymer when PEG is used as the precipitating agent. Consequently, one can make the simplifying assumption that the precipitate solid phase contains only protein. In such a case, the chemical potential of protein in the solid phase can be taken to be that of pure crystalline protein. Since there are three components (protein, polymer, and solvent) and two phases (the solid and the liquid), the number of degrees of freedom is three. Therefore, temperature, pressure, and polymer concentration can be independently varied to vary the solubility of protein. For protein solution in the absence of polymer, however, the solubility is fixed at a fixed temperature and pressure since the number of degrees of freedom is only two. At a fixed temperature and pressure, therefore, protein solubility can be varied by varying the polymer concentration. Since the solid phase is pure crystalline protein, at constant temperature and pressure the chemical potential of protein in the solid phase is constant and is equal to that of protein in the saturated solution (in the presence or in the absence of polymer) at the same temperature and pressure. Consequently, the chemical potential of protein in the saturated solution is the same irrespective of whether polymer is present or not. This property can be used to relate protein solubilities in the presence and in the absence of nonionic polymers. The free energy of protein solution ΔG consisting of n_p mole-

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cules of protein in the presence of polymer is given by

$$\Delta G(n_p) = \Delta G_{pol} + \Delta G_{excess} \quad (1)$$

where ΔG_{pol} refers to the free energy of polymer solution and ΔG_{excess} is the excess free energy because of the protein molecules. The chemical potential of protein molecule μ_2 is therefore given by

$$\mu_2 - \mu_2^0 = \left(\frac{\partial \Delta G_{excess}}{\partial n_p}\right)_{T,P,n_j} = \overline{\Delta G}_{excess} \quad (2)$$

where μ_2^0 refers to the chemical potential of the protein molecule at standard state and $\overline{\Delta G}_{excess}$ is the partial excess free energy. This partial excess free energy can be calculated by evaluating the various interaction energies a protein molecule in a polymer solution experiences. The various interactions energies involved in introducing a protein molecule into a nonionic polymer solution are shown schematically in Fig. 1. At the isoelectric point the system includes interactions of protein–polymer solution, of polymer–solvent, and entropy of mixing. If the pH of the medium is different from the isoelectric point of the protein, the protein molecule will be charged. Therefore, electrostatic energy is required for charging the protein molecule in the presence of electrolyte ions present in the polymer solution. Therefore, the chemical potential of protein equals

$$\mu_2 - \mu_2^0 = \overline{\Delta G}_{excess} = \Delta g + \Delta G_{e\text{-}s} + \Delta G_{mix} \quad (3)$$

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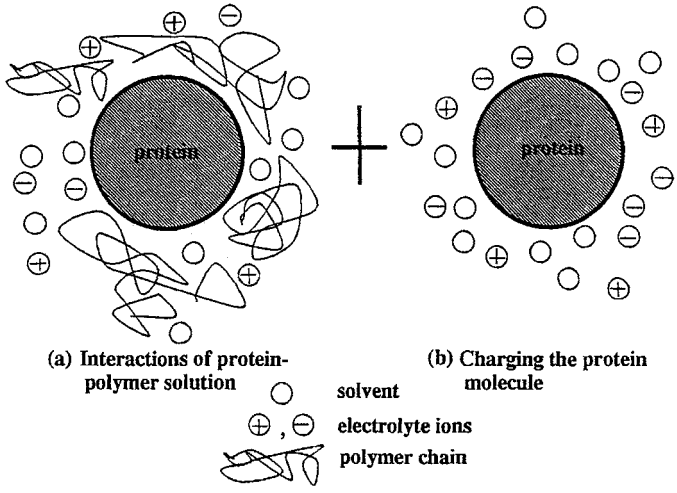

FIG. 1 Interactions involved in the introduction of a globular protein molecule into a polymer solution.

In the above equation, $\Delta G_{e\cdot s}$ is the electrostatic free energy which includes the work required to charge the protein, Δg is the free energy of interaction between a protein molecule and the polymer solution, and ΔG_{mix} is the entropy of mixing of protein molecules in the polymer solution.

The solubility of protein can be evaluated by equating the chemical potential of protein in a saturated solution to that of crystalline protein in the precipitate since the saturated solution is in equilibrium with the precipitate. The condition for equilibrium between the two phases is given by

$$\mu_s - \mu_2^0 = \mu_{2s} - \mu_2^0 = \Delta g + \Delta G_{e\cdot s} + \Delta G_{mix} \tag{4}$$

where μ_s is the chemical potential of crystalline protein in the precipitate and the subscript s refers to saturation. A similar equation can be written for the chemical potential of protein in the saturated solution in the absence of polymer, i.e.,

$$\mu_s - \mu_2^0 = \mu_{2s}' - \mu_2^0 = \Delta g' + \Delta G_{e\cdot s}' + \Delta G_{mix}' \tag{5}$$

where a prime refers to the system in the absence of polymer. From Eqs. (4) and (5), we get

$$\Delta g + \Delta G_{e\cdot s} + \Delta G_{mix} = \Delta g' + \Delta G_{e\cdot s}' + \Delta G_{mix}' \tag{6}$$

The above equation gives the relationship between the solubilities of globular proteins in the presence and in the absence of polymers. Notice that the excess free energy of interaction Δg between the protein and the polymer solution depends not only on protein–solvent, protein–segment, and segment–solvent interactions, but also on the entropy of mixing of polymer segments and solvents. The influence of ionic strength on protein–solvent interactions is accounted for by the variation of surface tension with the ionic strength and by the nonpolar surface area of a protein molecule. Detailed derivation for Δg will be given in the following section. In this model the electrostatic interactions include a charging process of the protein molecule as well as the interaction due to a dipole moment of the protein. Mixing energy ΔG_{mix} of protein molecules in a solution is evaluated based on Tanford's treatment.

In order to simplify the problem of modeling globular proteins in aqueous polymer solution, the following simplifying assumptions are made:

1. The polymer is linear, homogeneous, nonionic, and uniform with respect to molecular weight and constitution.
2. The globular protein molecule is a rigid sphere with homogeneous surface properties.
3. The precipitate phase does not contain any polymer.

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4. The dielectric constant of the aqueous medium is not affected by the addition of polymer.

In the following sections the evaluations of different contributions of the free energy of protein molecule are discussed.

Protein–Polymer Solution Interaction

The interaction energy between protein and polymer solution can be calculated based on a lattice model following the treatments of Scheutjens and Fleer (17) and Baskir et al. (16). The protein molecule is pictured as a sphere in the center of a spherical lattice (Fig. 2). It is assumed that the electrolyte ions influence the protein–solvent hydrophobic interactions but do not affect protein–polymer and polymer–solvent interactions. The central spherical protein molecule is surrounded by random coiled polymer and solvent molecules. Baskir et al. (16) proposed a spherical lattice model to describe the interactions between the polymer segments and the solvent molecules by considering the conformations of polymer segments in the vicinity of a protein molecule. Because of the interaction between protein and polymer segments, the distribution of polymer segments in the vicinity of protein molecule will not be uniform. The equilibrium distribution of polymer segments can be obtained by maximizing the partition function with respect to chain orderings. By the use of statistical mechanics, the excess Gibbs free energy of interaction between a protein molecule and the polymer solution (Δg) (16) is given by

$$\frac{\Delta g}{kT} = L_1\left(\frac{u_{1/s}}{kT} - \chi_s\phi_{3,1}\right) + \sum_{i=1}^{m^*} L_i\left[\phi_{1,i}\ln\left(\frac{\phi_{1,i}}{\phi_{1,*}}\right) + \phi_{3,1}\ln p_i - (\phi_{1,i} - \phi_{1,*}) - \frac{1}{r}(\phi_{3,i} - \phi_{3,*})\right] + \chi\sum_{i=1}^{m^*} L_i\left[\phi_{1,i}(\langle\phi_{3,i}\rangle - \phi_{3,*}) - \phi_{1,*}(\phi_{3,i} - \phi_{3,*})\right] \quad (7)$$

where m^* is the lattice layer where the polymer concentration reaches the bulk; L_i is the number of lattice sites in the ith layer (given by $\frac{4\pi}{3}[3(R + i - 1)(R + i) + 1]$, R being the radius of the protein molecule in lattice units); $\phi_{k,i}$ is the volume fraction of component k (1 = solvent, 2 = protein and 3 = polymer segment) in the ith layer; $\langle\phi_{k,i}\rangle$ is the average volume fraction in the ith layer, $\phi_{3,*}$ and $\phi_{1,*}$ are the bulk volume fractions of polymer segments and solvent, respectively; p_i, the free segment probabil-

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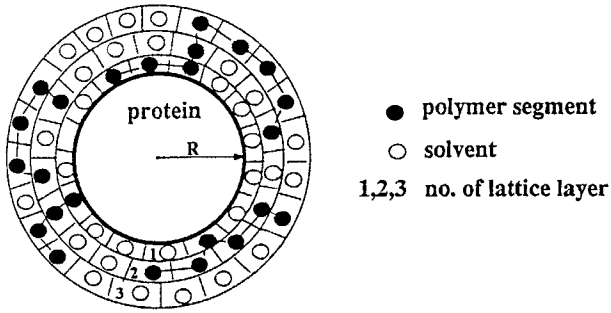

FIG. 2 Schematic diagram of a spherical lattice.

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ity, is a statistical weighting factor that expresses the preference for a free polymer segment to be in layer i instead of bulk; r is the length of the polymer molecule in lattice units; χ is the Flory–Huggins parameter for polymer segments; and the parameter χ_s is the relative adsorption energy of polymer segments on the surface of globular proteins, defined as

$$\chi_s = (u_{1/s} - u_{3/s})/kT \tag{8}$$

where $u_{1/s}$ and $u_{3/s}$ are adsorption energies of the solvent and polymer segments to the protein molecule, respectively.

In Eq. (7) the first term on the right-hand side refers to the adsorption energy whereas the second and the third terms refer to the entropy and the enthalpy of mixing of polymer segments and the solvent molecules, respectively.

The interaction energy $\Delta g'$ between the protein molecule and solvents for the reference solution, for which (a) the polymers are absent, (b) pH = pI, and (c) the salt concentration $m = m'$, is defined as,

$$\frac{\Delta g'}{kT} = L_1 \frac{u'_{1/s}}{kT} \tag{9}$$

The adsorption energy of a solvent molecule onto a protein molecule in a protein solution with polymers and $m \neq m'$ can be written as

$$\frac{u_{1/s}}{kT} = \frac{u'_{1/s}}{kT} + \frac{\Delta u_{1/s}}{kT} \tag{10}$$

where $\Delta u_{1/s}$ is the change of adsorption energy of the solvent onto the surface of a protein molecule due to the change in the salt concentration. Equation (7) can therefore be rewritten as

$$\frac{\Delta g - \Delta g'}{kT} = L_1 \frac{\Delta u_{1/s}}{kT} - L_1 \frac{(u'_{1/s} + \Delta u_{1/s} - u_{3/s})}{kT} \phi_{3,1}$$
$$+ \sum_{i=1}^{m^*} L_i \left[\phi_{1,i} \ln\left(\frac{\phi_{1,i}}{\phi_{1,*}}\right) + \phi_{3,1} \ln p_i - (\phi_{1,i} - \phi_{1,*}) - \frac{(\phi_{3,i} - \phi_{3,*})}{r} \right]$$
$$+ \chi \sum_{i=1}^{m^*} L_i \left[\phi_{1,i}(\langle \phi_{3,i} \rangle - \phi_{3,*}) - \phi_{1,*}(\phi_{3,i} - \phi_{3,*}) \right] \tag{11}$$

It is to be noted that the adsorption energy of solvent molecule onto the surface of protein molecule will be influenced by the salt concentration since the interaction of solvent with the surface hydrophobic residues of protein will change with the salt concentrations. Let f be the fraction of the surface of the globular protein molecule that is covered by hydrophobic residues. Of course, this fraction will depend on the tertiary structure of the protein molecule. Since the rest of the surface hydrophilic residues are compatible with the solvent, it may be reasonable to assume that the average adsorption energy of solvent molecule arises only due to the interaction with the surface hydrophobic residues. Therefore,

$$u_{1/s} = \gamma a f \tag{12}$$

where γ is the interfacial tension of the surface hydrophobic residues and a is the surface area occupied by a solvent molecule. The variation of interfacial tension with salt concentration is given by (24)

$$\gamma = \gamma' + \sigma(m - m') \tag{13}$$

where m is the salt concentration and σ is the surface tension increment. Therefore,

$$u'_{1/s}/kT = \sigma a m' f / kT$$

and

$$\frac{\Delta u_{1/s}}{kT} = \frac{\sigma a \Delta m f}{kT} \tag{14}$$

Furthermore,

$$\frac{\Delta g - \Delta g'}{kT} = L_1 \frac{\sigma a \Delta m f}{kT} (1 - \phi_{3,1}) - \chi'_s \phi_{3,1} L_1$$
$$+ \sum_{i=1}^{m^*} L_i \left[\phi_{1,i} \ln\left(\frac{\phi_{1,i}}{\phi_{1,*}}\right) + \phi_{3,1} \ln p_i - (\phi_{1,i} - \phi_{1,*}) - \frac{(\phi_{3,i} - \phi_{3,*})}{nr} \right]$$
$$+ \chi \sum_{i=1}^{m^*} L_i \left[\phi_{1,i}(\langle \phi_{3,i} \rangle - \phi_{3,*}) - \phi_{1,*}(\phi_{3,i} - \phi_{3,*}) \right] \tag{15}$$

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where

$$\chi_s' = \frac{(u_{1/s}' - u_{3/s})}{kT} \tag{16}$$

or

$$\frac{(\Delta g - \Delta g')}{kT} = \frac{\sigma\Phi\Delta m}{kT}(1 - \phi_{3,1}) - \chi_s'\phi_{3,1}L_1 + \frac{\Delta S_m}{k} + \frac{\Delta H_m}{kT} \tag{17}$$

where χ_s' is the protein–polymer interaction parameter measured at pH = pI and $m = m'$. Φ, the nonpolar surface area of a protein molecule, is equal to L_1af. From the theory of hydrophobic interactions, if γ^0 is the surface tension of pure solvent, the energy of creating a cavity to accommodate a protein molecule can be expressed as (22, 23, 25)

$$\frac{\Delta G_{cav}}{kT} = \frac{\Phi}{kT}(\gamma^0 + \sigma m) \tag{18}$$

Therefore, the first term in eq. (7) represents the contribution of cavitational free energy. The second term refers to the contribution of protein–polymer interactions to the overall interaction energy, $\Delta S_m/k$, the excess entropy of mixing of polymer segments with solvents and $\Delta H_m/kT$, the excess enthalpy of mixing of polymer segments with solvents, are given by the third and the last terms in Eq. (7), respectively. At $m = m'$, the net free energy of interaction between a protein molecule and polymer segments is reduced to

$$\frac{\Delta g - \Delta g'}{kT} = -\chi_s'\phi_{3,1}L_1 + \frac{\Delta S_m}{k} + \frac{\Delta H_m}{kT} \tag{19}$$

At moderate salt concentrations it can be assumed that the adsorption energy of polymer segments $u_{3/s}$ and Flory–Huggins χ parameter are not influenced by salt concentrations.* Consequently, the segment density distribution of polymer segments in the vicinity of a protein molecule will also be insensitive to variations in salt concentration.

Electrostatic Free Energy

The electrostatic interactions between protein and the surrounding ions depend on the ionic strength of the solution. When protein is charged (at pH $\neq$ p*I*), electrolyte ions are distributed in the vicinity of protein and form a layer with a charge density which has the opposite sign to the

* Such an assumption will not be valid at very high salt concentrations since phase separation of polymer solution tends to occur under such conditions.

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protein net charge. Even at their isoelectric point, protein molecules are extremely polar due to the fact that one or more pairs of ionic groups of opposite charge are always attached to protein molecules in which the charges are separated from one another by a considerable distance.

Consider a spherical protein molecule of charge z_p in an electrolytic medium. Because of the net charge on the protein molecule, there will be a profile of electrostatic potential in the vicinity of the protein molecule, the potential becoming zero far away from the molecule. Information with regard to this potential is necessary in order to evaluate the work required to charge the protein molecule.

$$\frac{1}{y^2}\frac{d}{dy}\left(y^2\frac{d\psi}{dy}\right) = -\frac{\rho}{\epsilon_0\epsilon_r} \tag{20}$$

where ψ is the electrostatic potential (V), ρ is the volumetric charge density (Cb/m^3), ϵ_0 is the permittivity of vacuum which has the value 8.85×10^9 C^2/J·m, and ϵ_r is the dielectric constant of the medium, given as 78.54 for water. The solution of the above Poisson–Boltzmann equation with appropriate boundary condition yields the following relationship between the net charge of protein molecule z_p and the surface potential ψ_0 (27, 28):

$$\frac{z_p e}{4\pi R^2} = \frac{\epsilon_0\epsilon_r kT\kappa}{z_i e}\left[2\sinh\left(\frac{z_i\psi_0 e}{2kT}\right) + \frac{4}{kT}\tanh\left(\frac{z_i\psi_0 e}{4kT}\right)\right] \tag{21}$$

where e is the elementary charge, R is the radius of the protein molecule, k is the Boltzmann constant, T is the temperature, κ is the Debye–Hückel parameter, and z_i is the valence number of a z_i:z_i symmetrical electrolyte. The work required to charge the protein molecule ΔG_{ch} is given by

$$\Delta G_{ch} = \int_0^1 \lambda z_p e\psi d\lambda = \frac{1}{2} z_p e\psi_0 \tag{22}$$

where ψ_0 is given by Eq. (21).

When the protein molecule acts as a dipolar ion, the work required to charge this dipolar ion, ΔG_{dip}, can be evaluated (24, 26, 29) by considering the dipolar ion as an ellipsoid of revolution with equal and opposite charges of magnitude $\pm e$ located at the foci to give

$$\Delta G_{dip} = -D\mu I \tag{23}$$

where μ is the dipole moment, I is the ionic strength, and D is given by

$$D = \frac{10002\pi N e^3 g(\lambda_0)}{2.303(4\pi\epsilon_0\epsilon_r kT)^2} \tag{24}$$

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where $g(\lambda_0)$ is a function of the eccentricity of the ellipsoidal cavity. For most ellipsoids, $g(\lambda_0)$ lies between 0.5 and 1.0 (29). Similar expressions for the work required to charge a dipolar ion can be derived when it is considered as either a spherical or an ellipsoidal ion of point dipole (29). They will not be used here since most globular protein molecules can be modeled as ellipsoidal molecules with equal and opposite charges located at their foci.

Therefore, the net work required to charge a protein molecule, $\Delta G_{e\text{-}s}$, is given by

$$\Delta G_{e\text{-}s} = \Delta G_{ch} + \Delta G_{dip} \tag{25}$$

where ΔG_{ch} and ΔG_{dip} are given by Eqs. (22) and (23), respectively.

In addition, as pointed out earlier, there will also be interaction of a protein molecule with the neighboring protein molecules because of the overlap of electrical double layers if the protein molecule is charged. At sufficiently high ionic strengths, however, the average distance of separation between protein molecules is much larger than the thickness of the electrical double layer, so that protein–protein interactions can be neglected. Moreover, the electrostatic interaction due to the overlap of double layers is expected to be small at high ionic strengths. Also, protein–protein interactions will be absent at the isoelectric point since the net charge on the protein molecule is zero. In the present analysis, we do not account for protein–protein interactions. Consequently, this analysis would be valid either at the p*I* of a protein or at sufficiently high ionic strengths.

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Mixing Free Energy

From excluded volume analysis, the entropy of mixing, ΔS_{mix}, of globular protein molecules in a dilute solution in the absence of polymers (protein–solvent system) is given by (30)

$$\Delta S_{mix} = Nkn_2 \ln\left[\frac{n_1\nu_1^0 + n_2\bar{\nu}_2^0}{n_2\bar{\nu}_2^0}\right] - \frac{N^2kn_2^2u}{2(n_1\nu_1^0 + n_2\bar{\nu}_2^0)} + \frac{N^2kn_2u}{2\bar{\nu}_2^0} \tag{26}$$

where N is Avogadro's number, ν_1^0 is the molar volume of the solvent, $\bar{\nu}_2^0$ is the partial molar volume of globular protein, u is the excluded volume of protein molecule, k is Boltzmann's constant, and n_1 and n_2 are the number of moles of solvent and protein molecules, respectively. Substituting c_1'/M_1 for $n_1/(n_1\nu_1^0 + n_2\bar{\nu}_2^0)$ and c_2'/M_2 for $n_2/(n_1\nu_1^0 + n_2\bar{\nu}_2^0)$, the specific entropy of mixing $\bar{S}_2$ is then given by

$$\overline{S}_2 - \overline{S}_2^0 = \left[\frac{\partial \Delta S_{\text{mix}}}{\partial n_2}\right]_{n_1} T$$

$$= Nk \ln\left(\frac{M_2}{c_2'\overline{v}_2^0}\right) - Nk\frac{c_1'v_1^0}{M_1} - \frac{N^2ku}{2}\left(\frac{c_2'}{M_2} + \frac{c_1'c_2'v_1^0}{M_1M_2} - \frac{1}{\overline{v}_2^0}\right)$$

$$= -Nk \ln \phi_2' - Nk\phi_1' - \frac{N^2ku}{2}\left[\frac{c_2'}{M_2} + \frac{c_2'\phi_1'}{M_2} - \frac{1}{\overline{v}_2^0}\right] \quad (27)$$

where c' is the concentration, ϕ' is the volume fraction, M is the molecular weight, and the subscripts 1 and 2 refer to the solvent and the protein molecule, respectively. The excluded volume u of globular proteins is given by (30)

$$u = \frac{8M_2v_2}{N} \quad (28)$$

where v_2 is the specific volume of protein. For one protein molecule,

$$\Delta G'_{\text{mix}} = -T\overline{\Delta S}_{\text{mix}}/N \quad (29)$$

Recognizing that $\overline{v}_2^0 = M_2v_2$, $\phi' = c'v$ and $v_1^0 = M_1v_1$, we have

$$\frac{\Delta G'_{\text{mix}}}{kT} = (\ln \phi_2' + 3\phi_2' + 4\phi_1'\phi_2' - 3) \quad (30)$$

In the presence of polymers,

$$\frac{\Delta G_{\text{mix}}}{kT} = [\ln \phi_2 + 3\phi_2 + 4(\phi_1 + \phi_3)\phi_2 - 3] \quad (31)$$

where ϕ_1 and ϕ_3 refer to the volume fractions of solvent and polymer in the bulk, respectively. Equations (30) and (31) indicate that entropy of mixing of a protein molecule is only a function of volume fractions of the components.

The relationship between the solubilities of globular proteins in the presence and in the absence of polymer is given by Eq. (6). Substituting for different interaction energies, it reduces to

$$\ln \frac{\phi_{2,s}}{\phi_{2,s}'} = 3(\phi_{2,s}' - \phi_{2,s}) + 4[\phi_{1,s}'\phi_{2,s}' - (\phi_{1,s} + \phi_{3,s})\phi_{2,s}]$$
$$-(\Delta g - \Delta g')/kT + (\Delta G'_{e\cdot s} - \Delta G_{e\cdot s})/kT \quad (32)$$

where $(\Delta g - \Delta g')/kT$ is given by Eq. (15) for $m \neq m'$ or by Eq. (19) for $m = m'$. $\Delta G'_{e\cdot s}/kT$ (for pH = pI and $m = m'$) and $\Delta G_{e\cdot s}/kT$ are given by

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Eq. (25). It is to be noted that the reference state (in the absence of polymer) is at a fixed salt concentration m'.

In order to determine the solubility of protein $\phi_{2,S}$ in polymer solution, Eq. (32) is to be solved. The parameters that are to be specified are: protein size (R), molecular weight of polymers (which, in turn, is related to its length r and therefore the number of lattice layers m^*), Flory–Huggins parameter for polymers (χ), protein–polymer interaction parameter (χ_s), solubility of protein in the absence of polymers ($\phi'_{2,S}$), net charge of protein (z_p), and ionic strength (I) [or salt concentration (M)]. The computational procedure for evaluating the segment density distribution of polymer in the vicinity of protein and the subsequent calculation of free energy of interaction of protein and polymer solution is given in the Appendix.

MATERIALS AND METHODS

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Protein precipitation curves were measured for a globular protein, namely, human serum albumin, using polyethylene glycol of molecular weight 4000, 8000, and 10,000. A molecular weight of 8000 was used in experiments to investigate the effects of initial protein concentrations and ionic strength. Both human serum albumin (HSA) and polyethylene glycol (PEG) were purchased from Sigma. Experimental procedure and conditions for different effects are given below.

The buffer solution used for different initial protein concentrations was 0.05 M acetate solution with 0.1 M KCl at pH = 4.5 (pI of HSA) (8). Protein solution (5 mL) was pipetted from a 200-mL flask containing the initial protein concentration of 120 mg/mL and was added to each test tube containing the same volume of PEG solutions of 8, 12, 16, 20, 24, 28, 32, 36, and 40% (g/mL). The molecular weight of PEG was 8000. The test tubes were mixed in a Type M50000 Thermodyne rotary shaker at 200 rpm for 3 to 4 hours. Before transferring these test tubes to a centrifuge, two-phase separation was observed in some of the test tubes which contained higher initial PEG concentrations. The precipitate had the same color as the original HSA. To separate the precipitate, the mixture was centrifuged at 2200g for 40 minutes. The protein concentration in the supernatant was measured by absorbance at 280 nm for a 10-mm pathlength using a Milton-Roy Spectronic 1001 spectrophotometer. The same procedure was repeated for experiments with initial protein concentrations of 100 and 80 mg/mL (before mixed with PEG solutions), resulting in three precipitation curves for initial concentrations of proteins of 41.2, 51.9, and 62.7 mg/mL solvent.

The same type of buffer was used for measuring precipitation curves at different molecular weight of PEG (4000 and 10,000). The initial concen-

trations of all protein solutions were 62.7 mg/mL. Experimental procedure of mixing, centrifugation, and concentration measurement were the same as described previously. It was observed that as the molecular weight of PEG increased, the viscosity of PEG solutions increased, resulting in an increased difficulty of separation.

COMPARISON OF EXPERIMENTAL PRECIPITATION CURVES WITH MODEL PREDICTIONS

Precipitation curves for HSA in PEG solutions for different molecular weights and ionic strengths were calculated for different initial concentrations of HSA. The solubility of HSA in PEG solution was evaluated from Eq. (32). When the calculated solubility of HSA was found to be greater than the initial concentration, no precipitation was deemed to have occurred. When the solubility of HSA was less than the initial concentration, however, precipitation occurred and the concentration of HSA in the supernatant was its solubility. The model parameters employed in the prediction of precipitation curves of HSA in PEG solution are given in Table 1. The Flory–Huggins χ parameter for PEG is taken to be 0.44 (16). The number of segments in a PEG chain was evaluated by assuming PEG to be a monodispersed polymer. The radius of equivalent hydrodynamic spheres for HSA was evaluated from its diffusion coefficient and was found to be 35.2 Å (31). The protein–polymer interaction parameter χ_s was determined by fitting the model predictions to the experimental data

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TABLE 1
Values of Parameters Used in the Lattice Model

Parameter	Value	Reference
Protein radius (R)[a]	8.8	9, 31
Molecular length (r):[a]		9, 16
PEG-4000	76	
PEG-8000	182	
PEG-10000	228	
Interaction parameter χ	0.44	16
Protein solubility in the absence of polymer	125 mg/mL	8

[a] Represented by lattice units (4 Å) (16).

for one molecular weight of PEG. It was found that χ_s of 0.122 gave the best fit for the precipitation of HSA in PEG solution of molecular weight 8000 as shown in Fig. 3. The same value of χ_s was then employed to predict the precipitation of HSA in PEG solution of molecular weights 4000 and 10,000 since the protein–polymer interaction parameter is independent of the molecular weight of PEG. As can be seen from Fig. 3, the model prediction agrees well with the experimental data.

In order to ascertain the model assumption that protein–protein interaction does not influence the distribution of polymer segments in the vicinity of a protein molecule, comparison of the length scale of segment density distribution and the average distance of separation between protein molecules is shown in Table 2. The reported values are for $\chi = 0.44$, $\chi_s = 0.122$, $R = 35.2$ Å, $r = 182$, and an initial protein concentration of 62.7 mg/mL. As can be seen from the table, the length scale of segment density distribution is much smaller than the average distance of separation between protein molecules, thus supporting the model assumption.

In these calculations the expression for the fraction of nearest-neighbor sites in layer j to a site in layer i of curved lattice $\lambda_i(j - i)$ given by Baskir et al. (16) is employed for the calculation of free energy of protein–polymer solution interaction $(\Delta g - \Delta g')/kT$. Van der Shoot and Leemakers (32) criticized these expressions and gave an improved expression for $\lambda_i(j - i)$. Figure 4 compares the values of $\lambda_i(-1)$, $\lambda_i(0)$, and $\lambda_i(1)$ evaluated using the above equations. It can be seen that the difference between the two

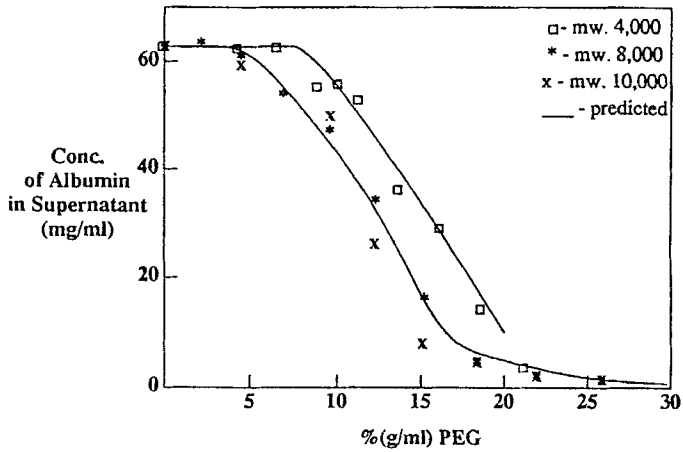

FIG. 3 Comparison of the experimental data with model prediction for the precipitation of HSA using PEG-10,000, PEG-8000, and PEG-4000 at pH 4.5 and 0.1 M KCl.

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TABLE 2
Comparison of Length Scale of Segment Density Distribution with the Average Distance of Separation between Two Protein Molecules

PEG concentration (%)	Length scale of segment density distribution (Å)	Average distance of separation between two protein molecules (Å)
10.34	36	115.84
13.22	24	126.74
19.37	24	238.79
22.67	24	317.24
26.12	24	427.80
29.75	20	544.29
31.63	20	784.38
33.56	16	1008.73
35.55	12	1092.64
37.59	12	1113.87
39.90	12	1134.70

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values is small. Moreover, the values of Δg calculated using these equations did not differ significantly. Consequently, the expression of Baskir et al. (16) was employed in all the other calculations.

Table 3 compares the relative magnitudes of various terms contributing to the free energy for the HSA–PEG system at a net protein charge of

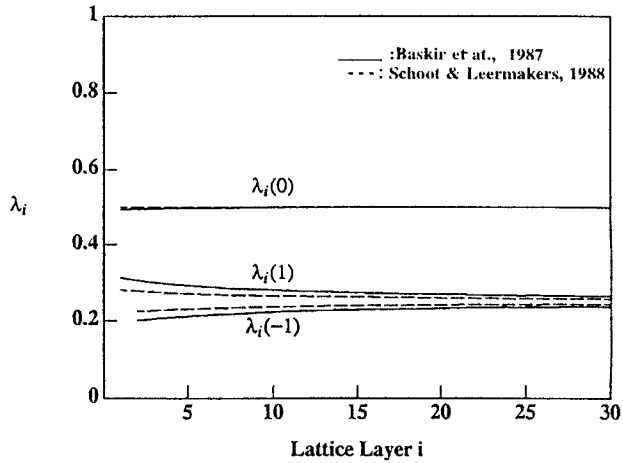

FIG. 4 Comparison of values of fraction of nearest-neighbor sites using Baskir et al. (16) and van der Shoot and Leermaker (32) equations.

TABLE 3
Contributions of Different Terms in Free Energy[a]

% PEG (g/mL)	$(L_1\sigma af(m - m_{ref}) \times (1 - \phi_{3,1}))/kT$	$(\Delta g - \Delta g')/kT$	$(\Delta G_{mix} - \Delta G'_{mix})/kT$	$(\Delta G_{dip} - \Delta G'_{dip})/kT$	$(\Delta G_{ch} - \Delta G'_{ch})/kT$
		0.05 M KCl			
10e-6	−0.3516	10e-7	−0.4725		
2.4	−0.3469	0.3645	−0.5077		
4.9	−0.3423	0.7682	−0.9155		
7.6	−0.3380	1.2659	−1.4160		
10.3	−0.3323	1.3613	−1.5195	0.3302	0.1600
13.2	−0.3274	1.9276	−2.0945		
16.2	−0.3210	2.7022	−2.8636		
19.4	−0.3168	3.2828	−3.4539		
22.67	−0.3108	3.9990	−4.1725		
26.12	−0.3057	4.7884	−4.9665		
		0.2 M KCl			
10e-6	0.7072	10e-7	−0.4725		
2.4	0.6978	0.3645	−0.4974		
4.9	0.6886	0.7682	−0.6628		
7.6	0.6799	1.2659	−1.1510		
10.3	0.6685	1.3612	−1.2351	−0.6604	−0.1350
13.2	0.6586	1.9275	−1.7897		
16.2	0.6456	2.7023	−2.5486		
19.4	0.6371	3.2828	−3.1181		
22.67	0.6251	3.9990	−3.8146		
26.12	0.6149	4.7884	−4.6282		
		0.5 M KCl			
10e-6	2.8613	10e-7	−0.4725		
2.4	2.8231	0.3645	−0.4974		
4.9	2.7859	0.7683	−0.6483		
7.6	2.7508	1.2659	−1.1108		
10.3	2.7045	1.3673	−1.1592	−2.6414	−0.2650
13.2	2.6644	1.9276	−1.6867		
16.2	2.6119	2.7023	−2.4023		
19.4	2.5778	3.2828	−2.9544		
22.67	2.5292	3.9990	−3.6186		
26.12	2.4879	4.7884	−4.3750		
		1.0 M KCl			
10e-6	6.5633	10e-7	−0.4725		
2.4	6.4758	0.3645	−0.5559		
4.9	6.3904	0.7682	−0.8747		
7.6	6.3098	1.2659	−1.2926		
10.3	6.2036	1.3613	−1.2836	−5.9432	−0.3400
13.2	6.1117	1.9276	−1.7566		
16.2	5.9913	2.7023	−2.4138		
19.4	5.9131	3.2828	−2.9172		
22.67	5.8015	3.9990	−3.5167		
26.12	5.7068	4.7884	−4.2356		

[a] $$\frac{(\Delta g - \Delta g')}{kT} = -\chi_s\phi_{3,1}L_1 + \sum_{i=1}^{m^*} L_i\left[\phi_{1,i}\ln\left(\frac{\phi_{1,i}}{\phi_{1,*}}\right) + \phi_{3,i}\ln p_i - (\phi_{1,i} - \phi_{1,*}) - \frac{(\phi_{3,i} - \phi_{3,*})}{r}\right] + \chi\sum_{i=1}^{m^*} L_i[\phi_{1,i}(\langle\phi_{3,i}\rangle - \phi_{3,*}) - \phi_{1,*}(\phi_{3,i} - \phi_{3,*})]$$

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5 for different concentrations of KCl. As expected, the contribution of hydrophobic interactions $\left[\text{i.e., } L_1\sigma af(m - m')\frac{(1 - \phi_{3,1})}{kT}\right]$ increases at high salt concentrations. Also, ΔG_{ch} becomes less important at higher salt concentrations. At very small PEG concentrations, $(\Delta g - \Delta g')/kT$ is negligible and becomes predominant at higher PEG concentrations. ΔG_{mix} is found to decrease (becomes more negative) at higher PEG concentrations.

The effect of χ_s on the segment density distribution of polymer segments in the vicinity of a protein molecule is shown in Fig. 5. Even though protein–polymer interaction is favorable ($\chi_s > 0$), there is depletion of polymer segments in the vicinity of the protein molecule (Fig. 5) because of the predominant steric effect. In other words, χ_s values in the range of 0.122 to 0.15 refer to relatively weak protein–polymer interaction. Higher values of χ_s imply more favorable protein–polymer interactions. Consequently, polymer segments should preferentially orient themselves in the vicinity of a protein molecule for higher χ_s (Fig. 5). As a result, the Gibbs free energy of interaction between protein and polymer solution should decrease for higher χ_s values, thus resulting in higher solubility. The precipitation curves should, therefore, shift to the right for more favorable protein–polymer interactions, as can be seen from Fig. 6. Since the precipitation curve is found to be very sensitive to small variations in χ_s (Fig. 6),

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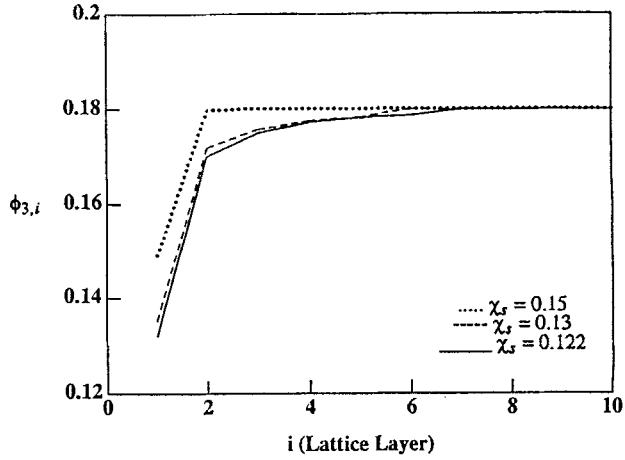

FIG. 5 Effect of χ_s on the segment density distribution of polymer at a concentration of 26%.

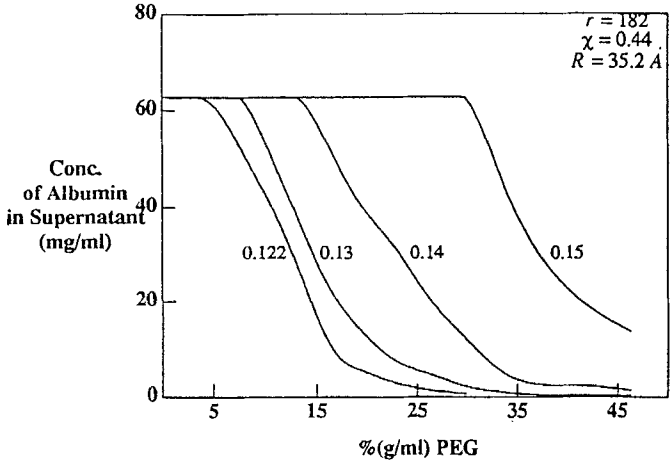

FIG. 6 Effect of χ_s on the precipitation curves of protein at the isoelectric point.

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the proposed model is vindicated by its ability to predict the precipitation curves for different molecular weights of PEG with the same value of χ_s.

The effect of polymer concentration on the solubility of globular protein depends on the nature of protein–polymer interactions. The interaction between HSA and PEG is found to be relatively weak (smaller χ_s value), thus leading to depletion of PEG near the protein molecule as a result of predominant steric exclusion. More depletion of PEG is found to occur at higher polymer concentrations as can be seen from the segment density distributions at different PEG concentrations as shown in Fig. 7. At higher polymer concentrations, favorable protein–polymer interactions ($\chi_s > 0$) tend to decrease the free energy of interaction. On the other hand, loss of entropy of polymer segments in the vicinity of protein molecule tends to increase the free energy of interaction. The latter effect predominates over the former so that the free energy increases, thus resulting in a lower protein solubility, i.e., more precipitation occurs at higher PEG concentrations.

The effect of molecular weight of PEG on precipitation of HSA is shown in Fig. 3. The precipitation curve shifts to the left as the molecular weight increases, i.e., a smaller amount of higher molecular weight PEG is required to precipitate the protein. Steric exclusion effects, which are stronger for larger polymer molecules (higher molecular weight), result in more depletion in the vicinity of a protein molecule. In other words, the depletion region extends over a larger number of layers for higher molecu-

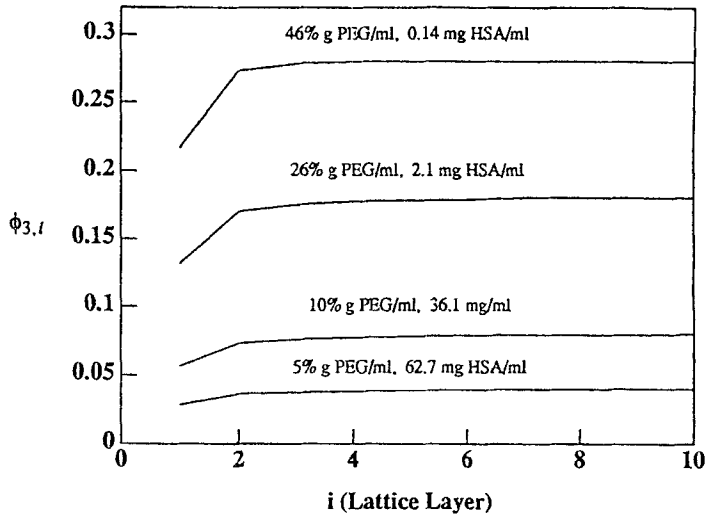

FIG. 7 Effect of PEG concentration on the segment density distribution.

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lar weight PEG. Consequently, the Gibbs free energy of protein–polymer interaction is higher for larger molecular weight of PEG, thus leading to lower protein solubility. Model predictions of precipitation curves of HSA with PEG of molecular weights 4000, 8000, and 10,000 compare well with the experimental data, as shown in Fig. 3. The precipitation curve is found to be insensitive to the molecular weight of PEG at sufficiently high molecular weights. In fact, the predicted precipitation curves for molecular weights of 8000 and 10,000 lie on the same curve, and this is consistent with the experimental data. Such a behavior can be attributed to the fact that steric exclusion of PEG near HSA is insensitive to the variations in polymer length for sufficiently long molecules.

The concentration of PEG required to initiate the precipitation of HSA was found to be higher for lower initial protein concentrations, as can be seen in Fig. 8. When the initial protein concentration is relatively high, protein concentration in the supernatant sharply decreases with increasing PEG concentrations. The precipitation curves for different initial protein concentrations eventually coincide into a single curve at higher PEG concentrations (Fig. 8). Such a result was also found by Haskó and Vaszileva (7). The model predictions of the precipitation curves for different protein concentrations agree well with experiments, as can be seen in Fig. 8.

For favorable polymer–solvent interactions (smaller χ), polymer molecules tend to fully extend themselves in the solvent, increasing the contact

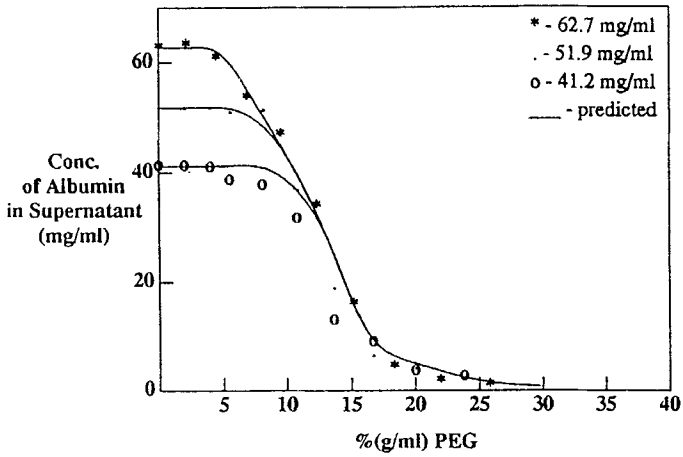

FIG. 8 Comparison of the experimental data with model predictions for the precipitation of HSA using PEG-8000 for different initial protein concentrations at pH 4.5 and 0.1 M KCl.

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area with the solvent and therefore excluding protein molecules. When the interactions of polymer–solvent are less favorable (higher χ), polymer molecules become more compact, minimizing the area of contact with the solvent. Consequently, their ability to exclude protein molecules from the solvent is reduced, resulting in higher protein solubility and shifting the precipitation curves to the right, as can be seen in Fig. 9.

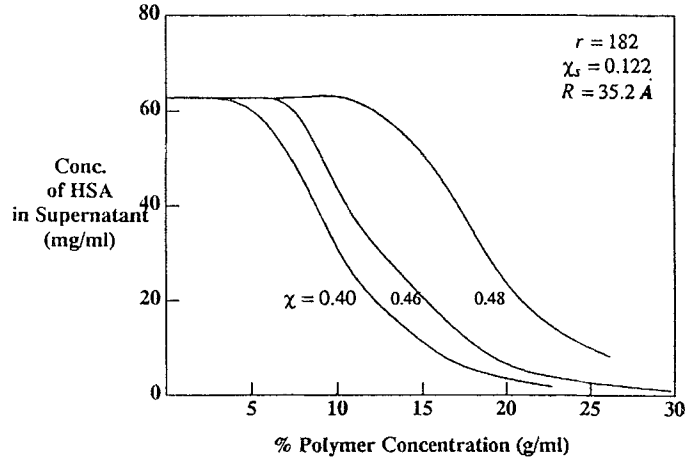

FIG. 9 Effect of Flory–Huggins parameter χ on the precipitation curves.

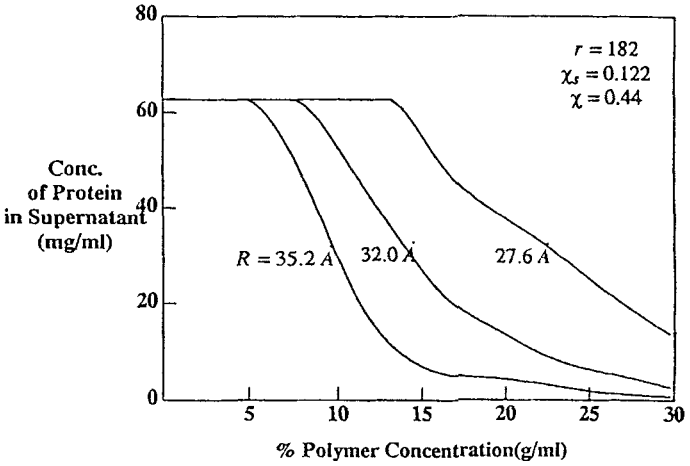

FIG. 10 Effect of protein size (radius) on the precipitation curves.

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Due to steric exclusion, polymer segments are depleted near the protein molecule, and this is more pronounced for larger protein molecules. Therefore, the free energy resulting from a loss of conformational entropy of polymer segments in the vicinity of the protein molecule increases with

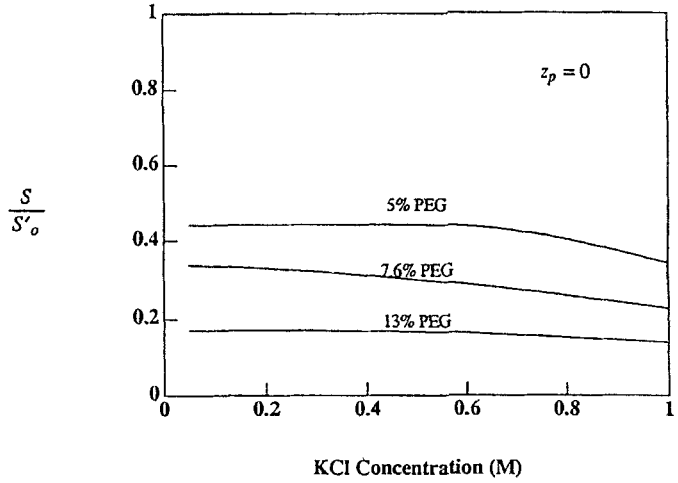

FIG. 11 Plot of predicted dimensionless solubility of HSA versus KCl concentrations for different PEG-8000 concentrations for $Z_p = 0$. S is protein solubility in polymer solutions with various salt concentrations, and S'_0 is that at the reference salt concentration.

TABLE 4
Values of Parameters Used in the Evaluation of Hydrophobic and Electrostatic Interactions

Parameter	Value	Reference
Dipole moment (μ)	380 debyes	24
Nonpolar area (ϕ)	1930 Å^2	24
Average ion radius for KCl	1.5 Å	33
Molal surface tension increment (σ) for KCl	$1.49\left(\times 10^3 \frac{\text{dyne·g}}{\text{cm·mol}}\right)$	24

increasing protein size. Consequently, protein solubility in the polymer solution decreases as the protein molecule becomes larger, shifting precipitation curves to the left as shown in Fig. 10.

Model predictions of the effect of salt concentrations on the solubility of HSA are shown in Fig. 11 for a net protein charge of zero. The parameter values for the model are given in Table 4. The results are expressed as plots of dimensionless protein solubility versus KCl concentration for three different PEG concentrations. As expected, solubility decreases at higher PEG concentrations. Even though solubility decreases slowly with

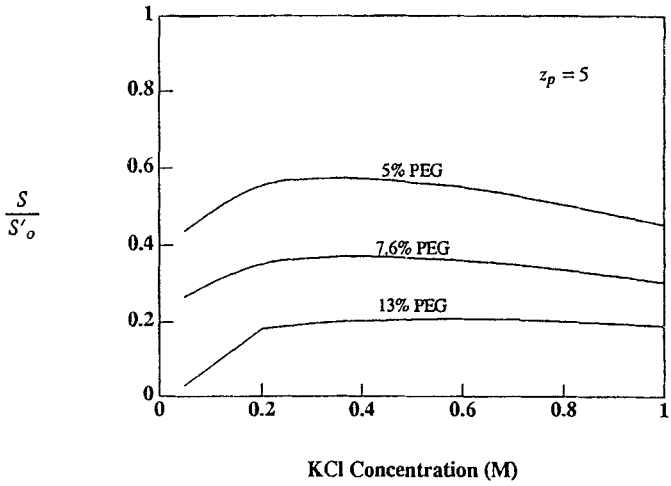

FIG. 12 Plot of predicted dimensionless solubility of HSA versus KCl concentrations for different PEG-8000 concentration for $Z_p = 5$. S is protein solubility in polymer solutions with various salt concentrations, and S'_0 is that at the reference salt concentration.

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salt concentration, this effect is not pronounced because of the predominant precipitating action of PEG. Moreover, the effect of salt is even less pronounced at higher PEG concentrations. Figure 12 presents a similar plot of model predictions for a net protein charge of 5. The solubility is found to exhibit a shallow maximum at intermediate salt concentrations because of competing salting-in and salting-out effects. Such a behavior is consistent with the observed variation of protein solubility in the absence of polymer (24).

CONCLUSIONS

A statistical thermodynamic model has been proposed to predict precipitation curves of globular proteins using nonionic polymers. The proposed model accounts for protein–polymer, polymer–solvent, electrostatic, and hydrophobic interactions as well as the entropy of mixing. The model employed simplifying assumptions such as a spherical globular protein molecule with uniform surface properties and a linear, homogeneous, nonionic polymer uniform with respect to molecular weight and constitution. Since the model does not account for protein–protein interactions due to overlap of electrical double layers, it can only be employed to predict precipitation curves 1) at the isoelectric point of globular proteins and 2) at sufficiently high ionic strengths for charged protein when protein–protein interactions are negligible because of compressed double layers. The model was employed to predict the precipitation curves of human serum albumin (HSA) using polyethylene glycol (PEG) as the precipitating agent. HSA–PEG interaction parameter χ_s was determined by fitting the model predictions to the experimental data for one molecular weight of PEG and was found to be 0.122. The predicted precipitation curves for different molecular weights of PEG and different initial protein concentrations agreed well with the experimental data. Precipitation curves were found to be very sensitive to protein–polymer interaction parameter χ_s. More favorable protein–polymer interactions (higher χ_s values) lead to shifting of the precipitation curves to higher polymer concentrations because of the increase in protein solubility. Segments of PEG were found to be depleted in the vicinity of HSA molecules because of the predominant effect of steric exclusion over relatively weak protein–polymer interactions. Steric exclusion of polymer molecules resulted in lower protein solubility at higher concentrations as well as larger molecular weights of PEG. Higher PEG concentrations were required to initiate precipitation of HSA at lower initial concentrations. At sufficiently high salt concentrations, the solubility of HSA in PEG solution was found to decrease with increasing salt concentrations; this effect is more pronounced at lower

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PEG concentrations. Because of competing salting-in and salting-out effects, the predicted solubility exhibited a maximum at intermediate salt concentration when the protein is charged.

APPENDIX

Computational Procedure for the Evaluation of Segment Density Distribution and Excess Gibbs Free Energy of Protein–Polymer Solution

The procedure of finding segment density distribution ($\phi_{3,i}$) in the lattice model was originally given by Scheutjens and Fleer (17) for a flat lattice model and further applied by Baskir et al. (16) for a spherical model. The lattice unit was chosen as 4 Å (16). We have adapted the same procedure for evaluating Gibbs free energy and it is described as follows. First a set of initial guesses of $\{\phi_{3,i}\}$ was made to calculate free segment probability $\{P_i\}$ (16). Knowing $\phi_{3,i} = n_{3,i}/L_i$, where $n_{3,i}$ is the number of polymer segments in layer i, the segment density distribution is then evaluated based on the following equation.

$$\frac{n_{3,i}}{\sum_{i=1}^{m^*} n_{3,i}} = \frac{L_i \sum_{s=1}^{r} P(s,i,r)}{\sum_{i=1}^{m^*} L_i \sum_{s=1}^{r} P(s,i,r)} \tag{A.1}$$

where L_i is introduced as a weighting factor for spherical lattice model. $P(s,i,r)$ is the statistical weighting factor for the $s$the segment of an r segment chain to be in layer i. $P(s,i,r)$ is expressed in terms of $P(s,i)$ and $P(r - s + 1, i)$, the end segment statistical weighting factors of two smaller polymer chains both having an end segment in layer i.

$$P(s,i,r) = P(s,i)P(r - s + 1, i)/P_i \tag{A.2}$$

The end segment statistical factor for a chain can be expressed in terms of that for chains one unit shorter, given as

$$P(s,i) = P_i \sum_{j=i-1}^{i+1} \lambda_i(j - i)P(s - 1, j) \tag{A.3}$$

$\lambda_i(j - i)$ is the fraction of nearest-neighbor sites in layer j to a site located in layer i. Repeat the same calculating steps until $\{\phi_{3,i}\}$ converges and calculate $\{P_i\}$ from the final $\{\phi_{3,i}\}$. Notice that $\{\phi_{1,i}\} = 1 - \{\phi_{3,i}\}$. Given $\{\phi_{3,i}\}$ and $\{\phi_{1,i}\}$, one can calculate interaction energy of protein–polymer solution from Eqs. (7) and (14) if the adsorption energy of the solvent

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($u_{1/s}$) is known. In our cases, a proper reference system was chosen first, so that the contribution of an exact value of $u_{1/s}$ is not required for evaluating the contribution of the interaction free energy of a protein–polymer solution at the isoelectric point and constant ionic strength in the overall calculation [see Eq. (10)]. For the interaction energy of a protein–polymer solution at various salt concentrations, Eq. (15) is applied. Required model parameters are polymer length (r), polymer bulk concentration ($\phi_{3,*}$), protein radius (R), χ, and χ_s.

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