Mechanism of Protein−Poly(ethylene glycol) Interaction from a Diffusive Point of View

Alessandro Vergara, Luigi Paduano, and Roberto Sartorio*

Department of Chemistry, University of Naples, "Federico II" Via Cinthia, Complesso Monte S. Angelo, 80126 Naples, Italy

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ABSTRACT: The first multicomponent diffusion data ever determined in protein−polymer systems are presented for the system lysozyme(1)−PEG 400(2)−water. Although there are no specific interactions between protein and polymer, the cross-term diffusion coefficient D_{21}, that links the PEG flow to the protein concentration gradient, is up to 35 times the main-term diffusion coefficient of the protein. This observation can only be due to a "crowding effect" and not to specific interactions, such as electrostatic ones. The exclusion effect is also qualitatively confirmed by the measured counter-flow associated with the protein motion. On the base of a hard core potential, our recent predictive equations are used to predict diffusion coefficients in this ternary system, and a good agreement with the experimental D_{21} is obtained. The PEG concentration dependence of the main-term diffusion coefficient of the protein cannot be interpreted exclusively by the excluded volume effect. Some dielectric effect or aggregation phenomena must be invoked to completely describe diffusive behavior in protein−PEG systems. A strong dielectric constant decrease and an anomalous pH dependence on PEG concentration in this system have been observed. We have extended to this nonelectrolyte system a recent procedure for extracting thermodynamic data from ternary diffusion coefficients that uses the Onsager reciprocal relations and the coupling of D_{ij} and second virial coefficient data. Thus we obtained the change of the lysozyme chemical potential with increasing PEG concentration. We emphasize that it is incorrect to neglect the nonideality of PEG−water systems, as was done in some previous preferential solvation analyses.

I. Introduction

The major types of molecular biology applications in which PEG is used are macromolecular crowding,[1] purification methods,[1] and macromolecular crystallization.[2−5] PEG has also many cell-fusion applications (e.g., fusion of mammalian cells and hybridoma production). It is often used even in PEG-modified protein for therapeutic use and to change the enzyme solubility in organic solvent.[6]

Macromolecular crowding of PEG is based on its large volume occupying space in solutions (crowding). When added to a multicomponent system, PEG acts on the other components increasing their effective concentrations. The effects of such crowding are self-association (as in tubuline, chymotrypsinogen, TMV, and cytoplasm), protein precipitation[7] or crystallization,[8] and increase of chemical rates (the nucleic acid hybridization, T4 DNA ligase-mediated ligation of DNA, nucleation time in protein crystallization, and protein refolding).[9−14] Because the osmotic stress, induced by the PEG, decreases the water activity,[15] it was used even for conformational and hydration studies in solution.

Earlier studies of protein−polymer systems were performed using different experimental and theoretical approaches. The main results are summarized below.

Initially investigators had the erroneous opinion that the interaction in aqueous solutions between a protein and a polymer involves the formation of a complex. Laurent[16] first offered another explanation, based on Ogston's idea;[17] namely, that the polymer sterically excludes the protein from part of the solvent. Hermans gave a clear description of the excluded volume theory of protein−polymer interaction.[18] The preferential solvation studies showed that the proteins are preferentially hydrated; i.e., PEG is excluded from the protein domain.[1] The preferential solvation concept was able to rationalize the solubility of proteins in water−PEG media, but it was not successful in universally rationalizing the protein stability.[19] In Timasheff's terminology the PEG is a cosolvent classified as salting-out destabilizer. Studies of static light scattering[20] showed that the cross derivatives of the protein chemical potential with respect to the PEG concentration are in qualitative agreement with values predicted by excluded volume theories. The solubility data of protein for different PEG molecular weight were reported by the pioneering study of Atha−Ingham.[7] These data were analyzed with different and more or less accurate excluded volume descriptions.[7,21−22] Second virial coefficients (B_2) in lysozyme−PEG−water systems, recently presented by Zukoski,[23] showed that the excluded volume, described through an Asakura−Osawa protein−protein potential, cannot justify the nonmonotone concentration dependence of B_2. On the other hand the PRISM approach,[22] dealing with the nonspherical but segmental structure of the PEG, provided a better explanation. This behavior and the incomplete understanding of the solubility can also be qualitatively justified by the hypothesis of an aggregation PEG−PEG up to a network formation, proposed by some of us in intradiffusion studies on binary systems water−PEG (from 200 to 10 000 Da)[24] and hypothesized elsewhere.[25] In fact at very high PEG concentration the intradiffusion data can be interpreted by a network formation based on the PEG−PEG interaction mediated by water bridges. We note that this network should make the PEG exclusion efficiency lower. The network formation condition is dependent

* Corresponding author. Fax +39081674090. E-mail: sartorio@chemistry.unina.it.

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on the polymer size and aggregation status, as are the solubility of proteins[7] and the range of protein−protein attractions.[23]

Only few papers are devoted to a noncovalent complex protein−PEG.[1,26] For chymotrypsin, the complex was observed under pressure and at elevated temperatures. For human serum albumin and peptine, an intrapolymer complex was observed at high concentration of protein, but only for high PEG molecular weight (higher than 100 000 Da). In the case of lysozyme, no complex was observed with PEG, even if nonpolar polymer binding on the surface of the lysozyme was identified by ^{1}H NMR. Lee and Lee discussed some specific PEG interaction with the denatured form of proteins.[19]

The dielectric effect in PEG−water systems is less well investigated in the literature. The dielectric constants of some aqueous solutions of PEG were determined at 20 °C.[27] The pK_a variation for some amino acids with PEG concentration[28] and a hypothesis of dielectric effect on the membrane fusion[27] were discussed. Hall presented a theory including the dielectric effect to interpret the pH dependence of the protein solubility in the presence of PEG.

The major purpose of this study is to measure the multicomponent diffusion coefficients of the protein−PEG−water system. We use these coefficients to study the crowding effect, test predictive equations for diffusion coefficients, and extract thermodynamic data using the Onsager reciprocal relations.

Another purpose of this work is to collect information on some physical-chemistry properties on PEG−water systems, such as dielectric constant and pH of mixtures, and to discuss the consequences for protein solutions when PEG is present. Thus dielectric constant and pH measurements have been performed on the PEG−water systems. The results are briefly discussed in this report.

Multicomponent Diffusion in Systems Containing Macromolecules. In this paper we are interested in the effect of the macromolecular crowding on matter transport in systems containing a protein. While an intradiffusion analysis of the crowding effect was presented by Herzfeld,[29] we present here an analysis of the mutual diffusion phenomenon.

Only one mutual diffusion study on the protein−PEG systems is present in the literature. It was performed by Phillies,[30] author of many basical theoretical and experimental works on diffusion and viscosity in colloidal solutions.[31] In the Phillies work,[30] as well as in all other papers dealing with the dynamic light scattering technique, the pseudobinary approximation is used. This means that only $n - 1$ diffusion coefficients are used to describe the matter transport in an n component system. A recent study has shown that dynamic light scattering diffusion coefficients are numerically close to the corresponding eigenvalue of the diffusion coefficient matrix[32] when the system is correctly treated as a multicomponent system, i.e., including the cross fluxes.

Fick's equations includes such a multicomponent effect, and give the phenomenological description of the mutual diffusion by

$$J_i = -\sum_{j=1}^{n-1} D_{ij} \nabla C_j \quad i = 1, ..., n-1 \qquad (1)$$

Equation 1 contains the main terms D_{ii}, which account for the flux of each component on its own concentration gradient. It also contains the off-diagonal terms D_{ij}, which account for the flux of each component on the gradient of the other component.

The pseudobinary approximation, in which the cross-term diffusion coefficients are neglected and the main-term ones are approximated by the corresponding binary ones, is widely used. Although this is a very simple approach, it cannot provide a full description of diffusion process in multicomponent systems. In this paper, the authors emphasize that it is seriously incorrect, especially when macromolecules "crowd" the solution.

During the past decade, the pseudobinary approximation was widely used to describe the mass transport in citoplasmatic systems[33] and to model crystal growth of macromolecules as protein,[34] virus, DNA,[35] etc. without any attention to the possible erroneous consequences. Space missions attracted much attention on models of crystallization in microgravity environment[36] where, because the buoyancy-driven convection flow is absent, just diffusive transport is present. At present, the authors are involved in a research program, financed by the Italian Space Agency (ASI), to investigate this aspect. On the ground, diffusion also has an important role in protein crystal growth, as one of us discussed in ref 30, as well as in the cases of highly viscous systems and of crystallization in gel matrix.[37]

In the last 50 years, many systems have been found to show cross-terms that are significant or even larger than main terms. The case of a large gradient concentration, as in phase transition phenomena, is a self-evident situation when cross fluxes could become significant. Large cross-terms are expected for three categories: (1) strong interactions (e.g., electrolyte mixtures[38]); (2) binding equilibrium (e.g., enzyme−substrate and cyclodextrin−guest[39−41]); (3) crowded solutions (presence of macromolecules as polymers[42,43] and proteins[32,44−46] or in mixed solvents[47]).

Although the case of electrolyte mixtures[48] and inclusion phenomena[39,49] are well interpreted from a theoretical and experimental point of view, the multicomponent diffusion in systems containing macromolecules is the subject of current research.

In this paper, we describe the effect of macromolecular crowding by the multicomponent approach (eq 1). Few studies have been devoted to the correct multicomponent diffusion approach in systems containing macromolecules. Cussler studied a ternary system containing two polystyrenes at different molecular weights.[43] We have also analyzed many ternary systems containing PEGs with different molecular weights.[42,50−53] In these systems, the cross-term diffusion coefficients were found quite large and in some case larger than the main terms. The same features were found in system containing polyelectrolyte[54,55] or protein. However in these latter cases, because of the presence of a third component often a salt,[32,44−46] the magnitude of the cross-term reflects the presence of strong electrostatic interaction between the components, as well as an excluded volume effect. In other words, a strong electrostatic effect overlaps any other potential of interaction. Because of the nature of the components, the system lysozyme−PEG 400−water presented here is the first one that can clearly show the excluded volume effect in macromolecular systems without long-range electrostatic interactions between solutes.

Table 1. Volumetric Parameters (d, H_i, $\bar{V}_i$) and Gravitational Instability Range ($\Delta C_2/\Delta C_1$) for Overstability, Fingers, and Isodensity

	A	B	C	D	E
$\bar{C}_1$ (mol dm^{-3})	0.6000	0.6000	0.6000	0.6000	0.6000
$\bar{C}_2$ (mol dm^{-3})	0.0625	0.1250	0.2499	0.4999	0.7501
H_1 (kg mol^{-1})	3.853 ± 0.070	3.832 ± 0.085	3.833 ± 0.065	3.886 ± 0.095	3.786 ± 0.047
H_2 (kg mol^{-1})	0.0623 ± 0.0015	0.0625 ± 0.0011	0.0629 ± 0.0017	0.0644 ± 0.0029	0.0647 ± 0.0009
d (kg dm^{-3})	1.003106 ± 0.000012	1.007159 ± 0.000013	1.015007 ± 0.000009	1.030944 ± 0.000028	1.047000 ± 0.000011
$10^3\bar{V}_1$ (dm^3 mol^{-1})	10486 ± 69	10506 ± 84	10506 ± 63	10458 ± 89	10561 ± 44
$10^3\bar{V}_2$ (dm^3 mol^{-1})	338.7 ± 1.5	338.5 ± 1.1	338.1 ± 1.6	336.8 ± 2.4	336.58 ± 0.68
$10^3\bar{V}_0$ (dm^3 mol^{-1})	18.074 ± 0.002	18.072 ± 0.003	18.069 ± 0.008	18.081 ± 0.023	18.085 ± 0.010
overstability	−298	−51	−16	−12	−15
isodensity	−0.014	−0.020	−0.015	−0.019	−0.014
fingers	1.4	3.1	7.0	25	77

Recently one of us was involved in diffusion studies of supersaturated solutions of lysozyme and NaCl, a system relevant in crystal growth studies.[32,45,46] In one of these papers,[45] a procedure was presented to extract the variation of the chemical potential of lysozyme as a function of the salt concentration from ternary diffusion coefficients. It uses two thermodynamic conditions, the Euler relations and the Onsager reciprocal relations (ORR).[57] This procedure also requires that the concentration of one solute be very small compared to that of the other. This condition is satisfied in our system, and so we tried such an extraction in the present work.

The comparison between the diffusion behavior in protein−salt−water systems and this protein−PEG−water system is also discussed.

Since the PEG as precipitant makes the system more and more unstable thermodynamically as its concentration is raised, a check of the determinant of the diffusion matrix was performed. A few studies verified experimentally, as theory predicts,[58] that the determinant of the diffusion matrix approaches zero as the spinodal curve is approached.[59] Furthermore, some theoretical and experimental studies of the concentration dependence of the diffusion coefficients in supersaturated solutions were performed by Izmailov−Myerson[60] and showed that the strong concentration dependence, predicted by cluster theory, is sometimes experimentally observed.[61]

The prediction of mutual diffusion coefficients in crowded solutions is one of our goals. Recently the authors developed predictive equations, based on hard sphere theory,[50] to evaluate the main and cross-term diffusion coefficients in systems containing nonionic macromolecules. These predictive equations for D_{ij} were applied successful to several ternary systems containing two PEGs at different molecular weight.[50−53] They succeeded also in predicting the lysozyme−NaCl data[32] after adding the electrostatic interaction[49] through the Nernst−Hartley equations extended to ternary systems.[62] Elsewhere,[47] authors compared the predictive efficiency of our equations with other literature theories.[64,43] Our predictive equations for D_{ij}s were also used by the authors in a numerical simulation of the free interface diffusion hydrodynamics in prenucleation conditions of interest in crystallization processes.[63] These predictive equations have been applied to our present data to evaluate how much the excluded volume effect can be considered part of the overall diffusive mechanism.

II. Experimental Section

Materials. Poly(ethylene glycol) with numerical average molecular weight 400 (PEG 400) was purchased from Aldrich and used without further purification. For Aldrich PEG samples the ratio between the weighted and numerical molecular weight is typically about $M_w/M_n \simeq 1.04$[42] indicating a mild polydispersity. Hen egg-white lysozyme (14,023 Da), recrystallized six times and lyophilized (lot E96302), was purchased from Seikagaku. Rosenberger and co-workers suggested this choice because of its high purity.[65] The counterion is the chloride (2.27%) with moisture content of 3.93 wt %. No impurity effect on the interferometric data was detected in previous works,[32,45,46] where the same supplier was used.

Solution Preparation. The solutions have been prepared by weight, with double distilled water, in the same day of the diffusion experiments, withdrawing solutes under dry conditions, as described earlier.[32] In contrast to systems of lysozyme−salt−water,[32,45,46] the protein−PEG−water systems have a low ionic strength, and a combined electrode with a good performance under these conditions has been used (Radiometer pHM220). The pH-meter is a DeMori pM520. The pH-meter was calibrated with the IUPAC standard buffer solutions each day that the diffusion measurements were performed. No standardization of the pH[66] has been presented in the literature for the "mixed solvent" water−PEG, and so the usual standards for pH in aqueous solutions have been used. The pH must be equal for both top and bottom solutions in the mutual diffusion runs in order to keep the number of components in solution to three. Therefore, the pH was adjusted for both solutions by adding hydrochloric acid. A buffer was not used because the low buffer capacity in water−PEG medium is well-known.[5]

Density Measurements. Density measurements were performed for all solutions prepared for diffusion experiments to convert from the mass concentration to the molarity. The density, d, has been measured at 25.00 ± 0.01 °C using an Anton PAAR densimeter, model 602. The instrument was calibrated with double distilled water and with air whose density was based on the ambient humidity and pressure, where the humidity was corrected to 25.00 °C. The following equation was fitted to the experimental data

$$d = \bar{d} + \sum_{i=1}^{n-1} H_i(C_i - \bar{C}_i) \tag{2}$$

where

$$H_i = \left(\frac{\partial d}{\partial C_i}\right)_{C_j,\bar{C}_i} \tag{3}$$

and $\bar{d}$ is the density at the average concentration. The volumetric results are reported in Table 1. Also reported in Table 1 are the partial molar volume evaluated as recommended in ref 67.

Dielectric Measurements. Measurements of the static dielectric constant of aqueous PEG 400 solutions were made at 25 °C using a commercial apparatus (DIEM) working at 1 MHz and 0.6 V. The experimental cell is made of nickel plated brass and has a volume of 20 mL. The instrument was calibrated with two pure solvents of known dielectric constant

Table 2. D_{ij}^{γ} with Different Solvent (γ) Choices and Determinant of the Diffusion Matrix, $|D_{ij}|$, for the Five Compositions Investigated (A−E)

	A	B	C	D	E
10^3C_1 (mol dm^{-3})	0.6000	0.6000	0.6000	0.6000	0.6000
C_2 (mol dm^{-3})	0.0625	0.1250	0.2500	0.4999	0.7501
$10^9D_{11}^0$ (m^2 s^{-1})	0.496 ± 0.002	0.428 ± 0.008	0.326 ± 0.003	0.1970 ± 0.0027	0.105 ± 0.002
$10^9D_{12}^0$ (m^2 s^{-1})	0.00020 ± 0.00008	0.0002 ± 0.0001	0.0002 ± 0.0001	0.0002 ± 0.0001	0.0002 ± 0.0001
$10^9D_{21}^0$ (m^2 s^{-1})	0.47 ± 0.04	0.89 ± 0.36	1.43 ± 0.45	2.68 ± 0.19	3.77 ± 0.18
$10^9D_{22}^0$ (m^2 s^{-1})	0.438 ± 0.002	0.435 ± 0.005	0.412 ± 0.007	0.409 ± 0.007	0.357 ± 0.007
$10^9D_{00}^1$ (m^2 s^{-1})	0.511 ± 0.003	0.457 ± 0.017	0.372 ± 0.022	0.283 ± 0.014	0.225 ± 0.013
$10^9D_{02}^1$ (m^2 s^{-1})	1.26 ± 0.015	0.29 ± 0.53	−0.86 ± 0.52	−2.46 ± 0.037	−2.57 ± 0.034
$10^9D_{20}^1$ (m^2 s^{-1})	−0.0008 ± 0.0001	−0.0015 ± 0.0006	−0.0025 ± 0.0008	−0.0046 ± 0.0003	−0.0065 ± 0.0003
$10^9D_{22}^1$ (m^2 s^{-1})	0.4228 ± 0.0035	0.406 ± 0.017	0.366 ± 0.022	0.323 ± 0.014	0.237 ± 0.0014
$10^9D_{11}^2$ (m^2 s^{-1})	0.490 ± 0.004	0.422 ± 0.011	0.320 ± 0.006	0.191 ± 0.006	0.0987 ± 0.0052
$10^{12}D_{10}^2$ (m^2 s^{-1})	−0.011 ± 0. 004	−0. 011 ± 0. 005	−0.011 ± 0.005	−0.011 ± 0.005	−0. 011 ± 0. 005
$10^9D_{01}^2$ (m^2 s^{-1})	−40 ± 5	−9 ± 16	27 ± 16	76 ± 12	81 ± 11
$10^9D_{00}^2$ (m^2 s^{-1})	0.444 ± 0.004	0.441 ± 0.011	0.418 ± 0.006	0.415 ± 0.006	0.363 ± 0.005
$10^{18}\|D_{ij}\|$ (m^4 s^{-2})	0.217 ± 0.002	0.186 ± 0.006	0.134 ± 0.004	0.080 ± 0.003	0.037 ± 0.002

with values outside the range investigated. We used cyclohexanol (15.00), DMSO (46.68), and water (78.39) as reference solvents.

Mutual Diffusion Measurements. The four D_{ij}s have been obtained by Gouy interferometry[68] at five compositions, keeping the protein concentration constant at 0.6 mM but the PEG 400 concentration ranging from 0.125 up to 0.75 M at 25.00 ± 0.02 °C and at pH = 4.5.

The concentration range of the PEG 400 in our experiments has been limited by the pH value. Above 0.75 M, any further addition of PEG to the system produces a lowering of the pH value below 4.5. Adjustment of the pH in this case implies the use of some base that would have the disadvantage of adding another component to the system, so that the system could no longer be treated as a ternary system. For each pair of solutions used in the diffusion experiments, the difference of pH is very small and lower than about 0.05 of a pH unit. Because of the small pH dependence of the diffusion coefficients found in a previous work,[45] it is reasonable to presume that even a difference of a tenth of a pH unit allows reasonable comparison of data at the five compositions in this system too.

In each free diffusion experiment, two solutions, top and bottom, at different compositions of at least one solute, are layered using the siphoning technique. The large viscosity increase[67] due to the solute 2 (PEG in our system) concentration makes the pulling of the boundary a more and more delicate effort. We temporarily stop pulling the boundary during the sharpening period in order to get good step function-like initial conditions, as suggested by Gosting.[62]

The Gouy interferometer at the University of Naples uses a He−Ne laser source (λ = 632.8 nm), a single channel cell (a = 2.5000 cm), and the Gouy minima were recorded with a photodiode on line. When the light goes through the cell the presence of the concentration (refractive index) gradient produces an interference pattern that evolves with time. A set of four measurements at the same mean concentration but at different values of ratio $\Delta C_i/\Delta C_j$, where the ΔC_i is the difference in concentration of component i between top and bottom solution, were performed.

Twenty scans of this changing fringe pattern are usually recorded at different times for each experiment. The experimental data are the positions of the fringes (50 on average) at each of the 20 scans. From these data, some literature programs are used to obtain the three Gouy parameters:[70] J_m, the total number of Gouy interference fringes; D_A, apparent diffusion coefficient; and Q_0; the "area under the deviation function".[71]

From the set of four experiments collected at different ratio $\Delta C_i/\Delta C_j$ the four D_{ij} can be determined by the Fujita−Gosting procedure.[71] The presence of a polymer with a mild polydispersity (Poisson distributions[72]) makes the errors from the analysis of the interferometric data slightly higher than usual.

Table 3. Dielectric Constants, ϵ, of the PEG 400(2)−Water System with the Corresponding Weight Percent, $(\%)_{W,2}$, and Molar Concentration, C_2

$(\%)_{W,2}$	C_2 (mol dm^{-3})	ϵ
0.00	0.0000	78.39
18.83	0.4840	68.38
29.53	0.7734	62.10
39.92	1.0630	54.91
50.01	1.3507	49.97
59.95	1.6389	43.47
80.42	2.2435	28.50
100.00		12.17

The effects of the polydispersity on the diffusion of several polymers, including PEG, was elsewhere discussed by the authors.[68] The high errors associated with the D_{ij} data at C_2 = 0.125 M are due to the very similar values of the eigenvalues of the diffusion matrix.[73]

The Gouy interferometry analysis is based on the hypothesis of no volume change on mixing associated with the diffusive flow, i.e.,

$$\sum_i^{n-1} J_i \bar{V}_i = 0 \qquad (4)$$

and so provides the D_{ij} expressed in the volume fixed reference frame,[74] reported in Table 2. We have also evaluated the D_{ij} expressed in the solvent fixed reference frame, according to ref 74, for applying the ORR.

III. Results

Partial Molar Volume. The partial molar volumes, reported in Table 1 with the corresponding errors obtained using the error propagation formula, have been evaluated from the parameters $\bar{d}$, H_1, and H_2 obtained from the density data, using the procedure reported by Dunlop and Gosting.[67] The partial molar volume of the protein is almost constant within the experimental error in the range of PEG concentration explored. In contrast, at increasing PEG 400 concentration, the partial molar volume of the PEG decreases slightly, with values similar to those of the corresponding binary system. The water partial molar volume shows a small increase. The general small change of the $\bar{V}_i$ seems to confirm the absence of significant solute−solute specific interactions.

Dielectric Constant. The dielectric constant, ϵ, of the system PEG 400(2)−water(0), reported in Table 3, shows a sharp decrease as the concentration of PEG 400

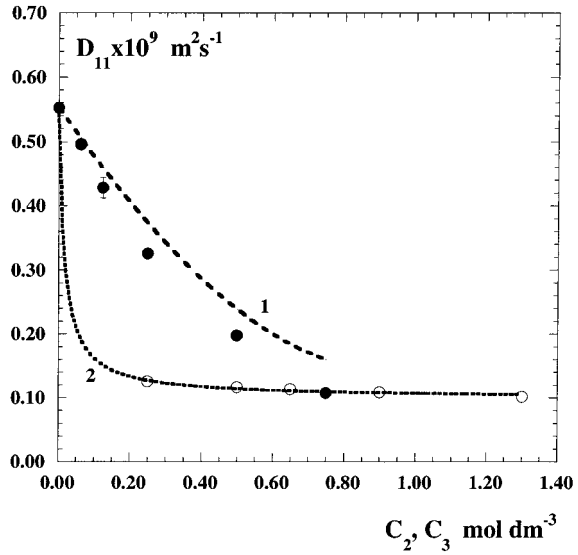

Figure 1. D_{11}, experimental data and calculated values from eq 5 (curve 1). Comparison between the two ternary systems Lys(1)−PEG 400(2)−water (●) and lysozyme(1)−NaCl(3)−water (○ and curve 2).

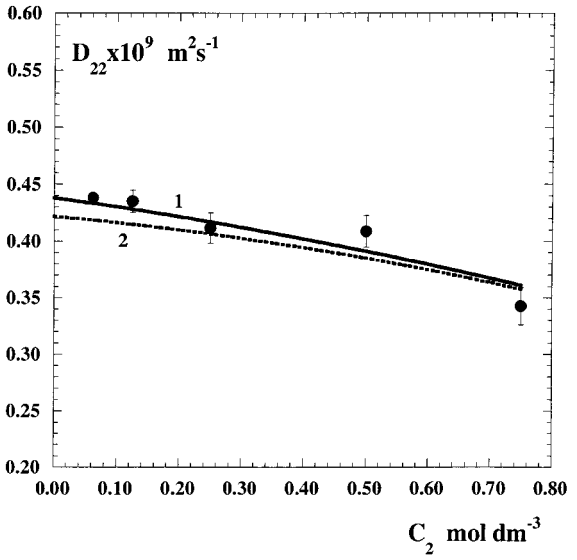

Figure 2. D_{22}, experimental data and calculated values (curve 2) according to eq 5. In curve 1 the trend of the D_2 is reported.

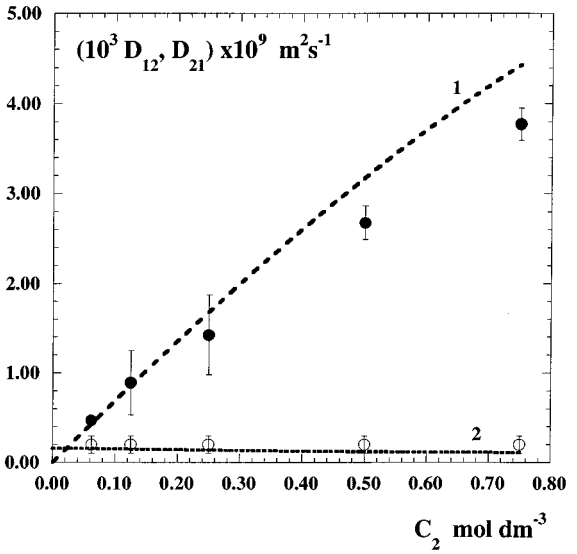

Figure 3. D_{ij}, experimental data and calculated values according to eq 6 (curves 1 and 2): D_{21} (●) and D_{12} (○).

increases. (This trend at 25 °C is different from that observed at 20 °C with a frequency of 7 MHz,[27] where there is a slight increase of the dielectric constant between 0 and 20 wt. % of PEG 400). Thus our diffusion experiments were performed in the medium PEG 400(2)−water(0), with a static dielectric constant ranging from 78.36 up to 65. It is therefore reasonable to consider that the strength of the ionic interactions will rise at increasing PEG 400 concentration. An issue strictly related to dielectric properties is the pH of PEG−water solution. Preliminary pH measurements in PEG−water systems[75] showed an anomalous trend, being nonmonotone both as a function of PEG weight percent (for a fixed PEG molecular weight) and as a function of the PEG molecular weight (for a fixed PEG percent). Changing the pH values in PEG−water requires a pH standardization in this medium. A pH standardization according to the Bates procedure[66] is in progress in the authors' laboratory on some PEG−water systems.

Diffusion Data. The experimental diffusion coefficients of lysozyme(1)−PEG 400(2)−water(0) systems are reported in Table 2 and shown in Figures 1−3.

In Figure 1, the values of D_{11} are reported as a function of the PEG 400 concentration. A large decrease of D_{11} can be observed with increasing C_2. The D_{11} value in the absence of PEG 400 corresponds to binary diffusion coefficient D_1 of the system lysozyme(1)−water(0) at the same lysozyme concentration ($D_1 = 0.5443 \times 10^{-9}$ m^2 s^{-1}).[45] For comparison, the values of D_{11} of the ternary system lysozyme(1)−NaCl(2)−water-(0) are also reported. This latter system was investigated at the same lysozyme concentration, with the simple salt concentration ranging between $C_2 = 0.250$ and 1.300 M. For further comparison, the trend of the main lysozyme diffusion coefficient predicted by Sartorio equations[50] is also reported; see below.

In Figure 2, the values of the diffusion coefficient D_{22} are reported as a function of the PEG 400 concentration. They decrease smoothly with increasing PEG 400 concentration as observed for the corresponding binary diffusion coefficient D_2,[76] also reported in figure for comparison. The D_{22} values obtained by the Sartorio predictive equation are also reported.

In Figure 3, the values of the cross-term diffusion coefficients, D_{12} and D_{21}, are reported in function of the PEG 400 concentration. The D_{21} values increase very sharply with PEG concentration, having values larger than all other diffusion coefficients in the whole range of concentration explored. In contrast, the D_{12} values are small and quite constant within the experimental error.

Finally the determinant values of the diffusion matrix, $|D_{ij}|$, are reported in Figure 4.

The coupling of D_{ij} and densimetric data allows a change of solvent analysis.[74] Our crowded solutions have two very abundant components (water and PEG), so we can choose either of these two constituents as the solvent. Hence a different choice of solvent can be a useful tool to make clearer the dragging effects associated with large macromolecules. This is another way to see how the counter-flow associated with a macromolecule motion is due only to the water, because of its abundance and speed.[47] Table 2 also contains the D_{ij}^{γ} referred to the solvent γ:[74] water(0), lysozyme(1), and PEG(2). In contrast to the recently investigated PEG

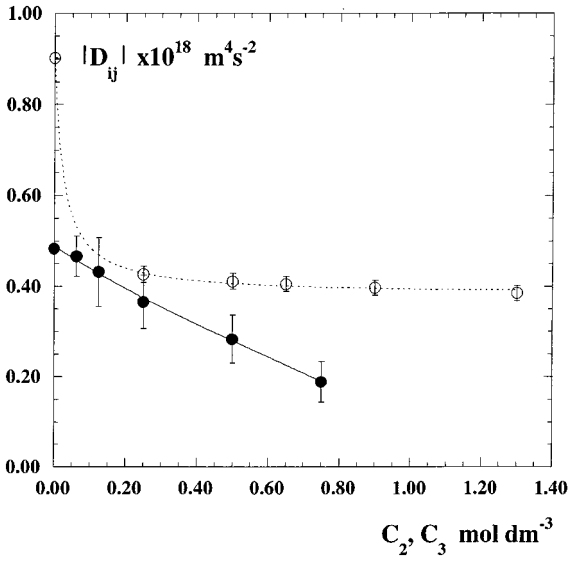

Figure 4. Determinant of the diffusion coefficient matrix. Comparison between lysozyme(1)−PEG 400(2)−water (●) and lysozyme(1)−NaCl(3)−water (○).

400−NaCl−water system,[47] the main-term diffusion coefficients are all positive. This can be due to the diffusivity of the protein that is much lower than that of NaCl. Analogously with PEG 400−NaCl−water system, the PEG and water molecules always move in opposite directions, i.e., are anticorrelated. The coupled flow of water(0) and of protein(1) indicates a different behavior. In the presence of a concentration gradient of protein, the water moves in the opposite direction. Therefore, the flows of both protein and PEG 400 produce a counter-flow of water molecules.

IV. Discussion

Diffusion Coefficients and Test of Sartorio's Predictive Equations. The change of solvent analysis, the literature studies about protein−PEG interaction, and previous studies on systems containing different PEGs suggest testing the excluded volume contribution to these systems. The authors recently presented some predictive equations to evaluate this effect semiempirically.[50−53] The intuitive physical origin and the algebra development of these equations are given elsewhere[50]

The predictive equations for main and cross-terms are

$$D_{ii} = D_i^{\infty}(1 - 0.898\phi_i)\frac{1 + 2.5\phi_i}{1 + 2.5(\phi_i + \phi_j)} \qquad (5)$$

$$D_{ij} = D_{ii}\frac{C_i\phi_j}{C_j(1 - \phi_j)^2} \quad \text{with } i \neq j \qquad (6)$$

where ϕ_i is the solute volume fraction, $\phi_i = C_i V_i$ with V_i an effective volume.[50] Then using a good estimate of D_{ii} from binary data, we can get a good estimate of D_{ij} too.

***D*$_{11}$.** This coefficient links the diffusion of lysozyme to its own concentration gradient. Due to the polyelectrolyte nature of lysozyme (Lys^{z+}), which is a polycation with chloride counterions (Cl^-), D_{11} depends on the diffusivity of both Cl^- and Lys^{z+}. The presence of an electrostatic potential between the polyion and the counterions implies that the faster Cl^- ions drag the slower polyion Lys^{z+}. The experimental diffusion coefficient accounts for both the diffusivities. Because of the nature of lysozyme and PEG 400, the dependence of the main lysozyme diffusion coefficient on PEG 400 concentration seems mainly due to an obstruction effect. The presence of PEG 400 molecules in solution, which obstruct the motion of both Cl^- and Lys^{z+}, should decrease the value of D_{11}. To test the contribution of this obstruction effect, we used the Sartorio predictive equation for the main diffusion coefficients of a ternary system containing hard sphere noninteracting solutes (eq 5).

The "effective" volumes of lysozyme[51] and PEG 400[47] were evaluated in previous papers, and they correspond to $V_1 = 16.20$ dm^3 mol^{-1} and $V_2 = 0.472$ dm^3 mol^{-1}. The comparison between the experimental and predicted D_{11} shows that the predicted values are systematically larger than the experimental ones, indicating the presence of other effects on the main diffusion coefficient of lysozyme. The observed variation of the dielectric constant or a protein self-association as the PEG 400 concentration increases can justify this difference. Leaist and Lyons discussed the effect of dielectric constant on the binary and pseudobinary diffusion of electrolyte in term of electrophoretic effect and ion−pairing.[77] The extension of these theories to ternary systems is in progress. Therefore the hard core potential itself can not fully describe the self-velocity correlation of the protein.

***D*$_{22}$.** This coefficient links the diffusion of PEG 400 to its own concentration gradient. Because of the nonelectrolytic nature of PEG, this coefficient is simply related to the motion of PEG molecules themselves. The lysozyme obstruction effect on the PEG 400 motion is small because of the small protein concentration. In fact the value of the protein volume fraction is only $\phi_i = C_i V_i = 9.72 \times 10^{-3}$. The small difference between of D_2 and D_{22} confirms the predicted small effect of the protein obstruction. The predicted values are in very good agreement with the experimental ones, showing that the lysozyme effect on the main-term diffusion coefficient of PEG 400 is mostly a volumetric effect.

***D*$_{12}$.** This coefficient links the protein flux to the PEG 400 concentration gradient. The experimental values are always positive, are very small, and can be considered constant within the associated errors. In absence of solute−solute interactions, positive cross-diffusion coefficients can arise from an excluded volume effect, as explained by eq 6. According to this equation, the value of the cross-term diffusion coefficient relative to the motion of component *i* under the concentration gradient of component *j* is directly proportional to the main-term diffusion coefficient of component *i*, to its molar concentration, and to the "effective" volume of component *j*, and it is inversely proportional to the free volume in solution. Because the product C_1V_2 is constant for the ternary compositions examined, the almost constant value of D_{12} is due to a comparable decrease of both D_{11} and $(1 - C_2V_2)$ with C_2.

***D*$_{21}$.** This coefficient links the PEG 400 flux to the protein concentration gradient. This coefficient is zero by definition at zero PEG concentration. Its values are positive and increase very sharply at increasing C_2, reaching values larger than both main-term diffusion coefficients. According to eq 6, this trend can be qualitatively explained. Because the quantity $(1 - C_1V_1)$ is almost unity, and the main diffusion coefficient D_{11} does not change a lot with C_2, the increase of D_{21} at increasing PEG 400 concentration is mainly driven by the increase of C_2 itself. On the other hand, the very

large increase observed experimentally is due to the large value of the "effective" volume of the protein, V_1. From a quantitative point of view, the agreement between the experimental and predicted values is very good at small PEG 400 concentration, while it is fairly good at high values of C_2. It is interesting to note that the ratio D_{21}/D_{11}, which represents the number of PEG 400 molecules driven by one lysozyme molecule in the same direction, reaches values of about $D_{21}/D_{11} \cong 35$. Finally the hard core potential itself can fully describe the cross-velocity correlation between the protein and PEG.

Thermodynamic Stability. The values of the determinant of the diffusion matrix, reported in Figure 4, decrease sharply as the PEG concentration increases. To understand the behavior of the $|D_{ij}|$, we need to consider that for each diffusion coefficient the following relation holds

$$D_{ij} = \sum_{k=1}^{n-1} L_{ij}\left(\frac{\partial \mu_k}{\partial C_j}\right) \tag{7}$$

This equation links the mutual diffusion coefficients to thermodynamic factors and to dynamic factors L_{ik}. Thus the values of $|D_{ij}|$ depend on thermodynamic and dynamic factors. The theory of thermodynamic stability claims that the determinant of the diffusion matrix must be zero along the spinodal curve. Actually at 25 °C, no phase separation occurs and so no a spinodal composition is expected for this system.[12] Therefore the decrease of the determinant value seems to be essentially due to a mobility effect. The analysis of the determinant of the diffusion coefficient matrix is a nice example of how nonequilibrium properties can be useful in determining equilibrium properties. Another example is discussed below.

Extraction of Chemical Potential Derivatives. In a previous paper, one of us was involved in the development of a procedure for obtaining the cross derivatives of the chemical potential

$$\mu_{12} = \left(\frac{\partial \mu_1}{\partial C_2}\right)_{C_1,T,p} \mu_{21} = \left(\frac{\partial \mu_2}{\partial C_1}\right)_{C_2,T,p} \tag{8}$$

in a system containing a macromolecule (1) and a simple solute (2) at low and high concentrations, respectively. The main hypothesis of this procedure holds here too (the molar concentrations of lysozyme and PEG are small and large respectively). The only difference from the previous lysozyme−NaCl−water system is that in the lysozyme−PEG−water system no common ion is present and so the chemical potentials of the solutes are "uncoupled". Using Euler's relations on the Gibbs free energy, the following expression can be obtained

$$\mu_{12}(1 - C_2\bar{V}_2) - \mu_{11}C_1\bar{V}_2 = \mu_{21}(1 - C_1\,\bar{V}_1) - \mu_{22}C_2\bar{V}_1 \tag{9}$$

and using the ORR for a ternary system, we obtain

$$\mu_{11}(D_{12})_0 - \mu_{12}(D_{11})_0 = \mu_{22}(D_{21})_0 - \mu_{21}(D_{22})_0 \tag{10}$$

where $(D_{ij})_0$ represent the diffusion coefficients expressed in the solvent fixed reference frame.[74]

By coupling of these two expressions, it was shown[44] how we can obtain the cross derivatives μ_{12} and μ_{21} as a function of the main derivatives μ_{11} and μ_{22}, the experimental $(D_{ij})_0$, and $\bar{V}_i$:

$$\mu_{12} = \{\mu_{11}[C_1\bar{V}_2(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{12})_0] - \mu_{22}[C_2\bar{V}_1(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{21})_0]\}/\{(1 - C_2\bar{V}_2)(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{11})_0\} \tag{11}$$

$$\mu_{21} = \{\mu_{11}[C_1\bar{V}_2(D_{11})_0 - (1 - C_2\bar{V}_2)(D_{12})_0] - \mu_{22}[C_2\bar{V}_1(D_{11})_0 - (1 - C_2\bar{V}_2)(D_{21})_0]\}/\{(1 - C_2\bar{V}_2)(D_{22})_0 - (1 - C_1\bar{V}_1)(D_{11})_0\} \tag{12}$$

Table 4. Chemical Potential Derivatives of Species *i* with Respect to the Concentration of Species *j*, μ_{ij}, Corresponding to Three Compositions (C−E)[a]

	C	D	E
$10^3\ C_1$ (mol dm^{-3})	0.6000	0.6000	0.6000
C_2 (mol dm^{-3})	0.2500	0.4999	0.7501
μ_{11}/RT (dm^3 mol^{-1})	1821 ± 80	1845 ± 90	1850 ± 80
μ_{22}/RT (dm^3 mol^{-1})	6.5 ± 0.6	4.6 ± 0.4	4.15 ± 0.4
μ_{12}/RT (dm^3 mol^{-1})	93 ± 9	35 ± 5	39 ± 4
μ_{21}/RT (dm^3 mol^{-1})	103 ± 10	56 ± 6	65 ± 6
μ_{21}^{id}/RT (dm^3 mol^{-1})	12 ± 1	11 ± 1	14 ± 2
μ_{21}^{PS}/RT (dm^3 mol^{-1})	17 ± 6	12 ± 6	10 ± 6

[a] The comparison with the preferential solvation results from ref 79, μ_{ij}^{PS}, and the derivatives evaluated assuming PEG as an ideal solute, μ_{ij}^{id}, are reported too.

The μ_{11} and μ_{22} values are not directly accessible, and therefore, we need to estimate them. We first assume that the μ_{22} for the ternary and binary (PEG−water) systems are essentially the same. This assumption is a comparable, if not better, approximation for PEG than for NaCl[45] or NH_4Cl[46] because of the absence of specific lysozyme−PEG interactions. Therefore μ_{22} was evaluated using recent activity data for the PEG 400−water system at 25 °C.[15] These values are reported in Table 4 and are in good agreement with our previous analysis on PEG−water systems,[76] based on data interpolated from activity coefficients at different temperatures (20, 30, 40 °C)[78] using the van Laar equation. The errors in Table 4 have been evaluated by propagating the maximum errors associated with the experimental quantities $\bar{V}$, D_{ij}, and μ_{ii}. Out of the five investigated compositions, two compositions (the most dilute ones) provide μ_{ij} affected by too large errors, therefore they are not reported in Table 4.

For μ_{11} we need a new route, because the expression used in the previous paper holds only for the special case of electrolytes with a common ion, for which an expression for μ_{11} as a function of C_1, C_2, and the protein charge can be written. The use of the experimental second virial coefficients, B_2, for ternary lysozyme−PEG−water systems, seems to be the best route to evaluate μ_{11}. The third virial coefficient term can be neglected because of the low lysozyme concentration. Unfortunately the experimental B_2 values are not present in the literature for ternary systems lysozyme−PEG−water, and for quaternary systems lysozyme−PEG−NaCl−water, they are reported only at low but not zero salt concentration.[79] We have extrapolated B_2 at zero salt concentration from some of these published data.[23] We note that fortunately the cross derivatives obtained are very insensitive to the B_2 value. In fact, even using a hard sphere model ($B_2 = 4V_1$), the cross derivatives have the same value within the standard

errors. A similar insensitivity was also shown in previous Lys−NaCl−water[45] or Lys−NH_4Cl−water[46] studies.

This procedure, using the coupling of D_{ij} and B_2 data, can be considered very general and thus not limited to the important case of electrolyte with a common ion. Nevertheless it requires data from two different sources, and usually B_2 data are less accurate than our D_{ij}. It is worth pointing out that the numerical evaluation of the μ_{21} and μ_{12}, as in the case of lysozyme−NaCl−water system, depends mostly on the μ_{22} and $(D_{21})_0$, so larger errors on μ_{11} should not affect the μ_{21} and μ_{12} evaluation much.

The μ_{ij} values evaluated by eqs 11 and 12 are collected in Table 4. The results at the five compositions explored can be compared with the preferential interaction parameters, μ_{21}^{PS}, of Lee−Lee.[19] The Lee−Lee approach involves a very crude approximation. In fact the μ_{22} values were approximated by RT/C_2, since they wrote "there is no data on the variation of the activity coefficient of PEG on concentration". Actually some activity data were already present in the literature in 1981. A comprehensive study of PEG activity coefficients was published by Hasse.[80] All these studies showed how incorrect it is to assume an ideal system value of μ_{22}. In Table 4, μ_{12} evaluated for the ideal case (PEG as an ideal solute) is also reported as μ_{21}^{id} for a comparison. The ideal case here evaluated, μ_{21}^{id}, and the preferential solvation results from ref 19, μ_{21}^{PS}, are in good agreement, but the real chemical potential derivatives, μ_{21}, are very different, even in 1 order of magnitude.

The positive μ_{12} values show that, according to the Timasheff definition of PEG as a salting-out destabilizer, PEG increases the chemical potential of the protein. We have not integrated the chemical potential of the protein from the PEG dependence of μ_{12} on concentration for two reasons. First, the solubility of lysozyme in water−PEG 400 is very high.[7] At $C_1 = 0.6$ mM, the solubility condition is at a C_2 higher than 70% w/w, very far from the compositions investigated. Second, From the Knoll−Hermans paper,[20] it is hard to assume that the composition of the crystal is independent of the PEG concentration. In fact for PEG with a low molecular weight, like PEG 400, the concentration of radiolabeled PEG was higher in the precipitate than in the supernatant. Hence partition equilibrium, related to the segregation phenomenon, must be included in the thermodynamic model to derive the chemical potential from integration. Unfortunately these data are unavailable.

Gravitational Stability. We checked the effect of the PEG concentration on the gravitational instability behavior of the system.[1] The opinion of the authors is that the effect of the PEG on nucleation time in protein crystallization can also be due to a variation of the time evolution of the large fluctuation[82] associated with the concentration gradients associated to the metastability of the nucleation phenomena.

Usually the limiting conditions for overstability and fingering are reported on a clockwise diagram (a plane ΔC_1 and ΔC_2) reported in Figure 5. The arrows indicate the effect of increasing D_{21} or decreasing D_{12} (in our case an increase of PEG concentration). The results of the analysis on the limits of gravitational stability for the system lysozyme−PEG 400−water are collected in Table 1. The conditions for static gravitational instability are satisfied in the region of the concentration gradient plane between the fingering and overstability, discussed

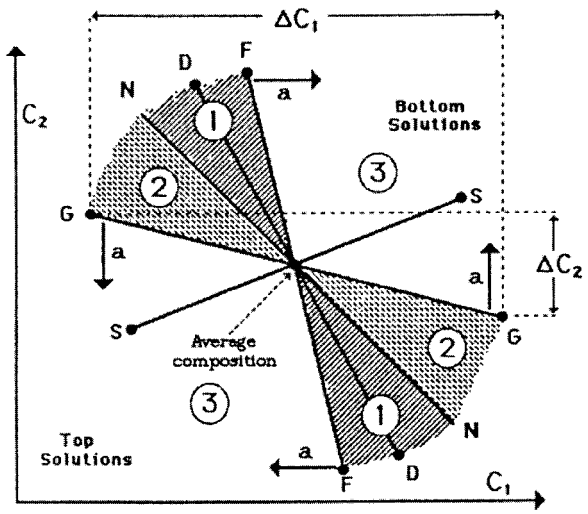

Figure 5. Clockwise diagram to define the limiting condition of gravitational stability through the boundary. The straight lines correspond to the following conditions: overstability (G), isodensity (D), fingers (F), isorefraction (N), and a generic pair of two solutions useful for diffusion measurement (S). The arrows *a* indicate the effect of an increase of PEG concentration.

elsewhere by some of us. PEG appears to have the typical behavior of an increase of the gravitational stability, analogously to the similar ternary system (PEG 400−NaCl−water).[47] These findings imply that the presence of PEG makes longer the lifetime for fluctuations associated with free diffusion in the presence of macroscopic concentration gradients.[82] This can be relevant in nucleation phenomena where protein is crystallized by salt and PEG,[13] as discussed in Section I.

Comparison with Other Ternary Systems Containing Lysozyme. All diffusion studies on protein systems have been performed in multicomponent systems. All except the early works of Leaist[44] and the recent publications involving one of us[32,45,46] have used a pseudobinary approach to describe diffusion in protein systems. As mentioned above this approach is incorrect. Therefore, a realistic comparison between the system presented here and others containing lysozyme is possible only with the lysozyme(1)−NaCl−water(0) system under identical conditions (25 °C, pH = 4.5, and $C_1 = 0.6\ 10^{-3}$ mol dm^{-3}).

The major feature for both systems is the large excluded volume effect due to the protein, even if it is present at very low concentration. However, there are significant differences between the two ternary systems protein−PEG−water and protein−salt−water: In the first one we have strong viscosity and dielectric effects and in the second one essentially electrostatic shielding effects.

Let us compare in some detail the four D_{ij} in these two ternary systems. The concentration dependence of the D_{11} on the other solute concentration is quite different in the two systems. In the lysozyme(1)−NaCl−water system, the sharp dependence of D_{11} on C_{NaCl} at small salt molar concentration is essentially due to a sharp decrease of the dragging effect of Cl^- counterions on the positive macroion. In our system, the D_{11} variation on C_{NaCl} is mainly due to an obstruction effect, as noted above. D_{11} decreases slowly with PEG 400 concentration. However, it is interesting to point out that, for $C_{PEG} > 0.780$ M, it reaches values smaller than the

corresponding main diffusion coefficient of lysozyme in the lysozyme−NaCl−water system. This is due to the large increase of viscosity in the present system. It is also worth pointing out that the dielectric effect, which we consider a second-order effect in our system, is in some way equivalent to the effect of adding NaCl to the protein solution. In fact in both cases, there is a decrease of the dragging effect of the faster Cl^- counterions on the slow lysozyme polycation, even if for different reasons, i.e., counterion condensation or a decrease of the electrostatic potential between the polyions and the solution.

Below we indicate both the two precipitants (NaCl and PEG 400) as component 2. There is not much to say about the D_{22} for the two systems because the solutes (PEG and NaCl) have very different physical properties. The main terms, however, can be compared with their corresponding binaries. In the NaCl system the D_{22} lies within 1−2% below that of the binary, whereas for the PEG system this difference is much smaller. Due to the protein obstruction for both solutes a slightly smaller values of the D_{22} in the ternary systems with the respect to the corresponding binary ones are expected. However in PEG-containing systems the protein obstruction effect is less evident because of the lower PEG diffusivity with respect to the NaCl one.

Although their values are quite small, the concentration dependence of the D_{12} is quite different in the two systems. This reflects, more than for the other diffusion coefficients, the different nature of the two solutes, ionic for the NaCl and non ionic for the PEG. Probably the values of D_{12} in the system containing NaCl, at least at low concentrations, is due to the electric field that is generated by the different diffusivity of the salt ions. This effect, of course, is absent in the system containing PEG, and the D_{12} is quite constant. Its values are due only to the excluded volume effect produced by the PEG in solution. As discussed above, this effect does not appear very large because of the very low concentration of protein; see eq 6. Finally, we note that as for the NaCl system, this diffusion coefficient should remain positive on increasing the PEG concentration if this polymer leads to the crystallization of the protein. In fact, the positive values of D_{12} do correspond to the salting out effect that either NaCl or the PEG has on the lysozyme.

In both systems, D_{21} is a linear function of the concentration of the component 2 in the concentration range investigated. On the other hand, in the system containing NaCl, D_{21} is also linear with C_2, but its magnitude is always larger than that observed in the system containing PEG. This can be attributed to the quite different diffusivity of PEG with respect to that of NaCl.

The comparison of the chemical potential derivatives of PEG and salt (with the respect to the weight concentration) shows that PEG is less a destabilizer than salts, in the case of lysozyme. To compare the effect of polymer size, protein size, and charge, other protein−polymer systems are under study. These studies involve changing the PEG molecular weight and the protein (human serum albumin). Furthermore, the literature shows that many properties of protein−polymers solutions are strongly affected by ionic strength.[83] Therefore, some quaternary protein−polymer−salt systems are now in progress in collaboration with Prof. J. G. Albright at the Texas Christian University.[84]

Finally we note that, in supersaturated solutions of lysozyme−NaCl systems, the determinant of the matrix is very far from zero[32] (see Figure 4). This highlights once again the different mechanism of salts and polymers in protein crystallization. According to the μ_{ij} values determined here, the above difference between the two systems is due essentially to mobility factors, associated with the large viscosity of the PEG−water systems.[69,47]

V. Conclusions

In this paper, we discussed the experimental mutual diffusion coefficients D_{ij} in protein−PEG−water systems. We used these intrinsically nonequilibrium physical quantities for obtaining some equilibrium properties, such as chemical potential cross derivatives with respect to the solute concentration.

The cross derivatives are in disagreement with a previous preferential solvation analysis, due to the rather drastic approximation, not used here, of considering the PEG as an ideal solute. Therefore, these can be considered the first thermodynamic data in such crowded solutions.

A strong dielectric constant decrease and an anomalous pH dependence on PEG concentration in this ternary system are observed. Thus a systematic dielectric constant study and pH standardization are in progress for several PEG−water systems in our laboratory.

Recent predictive equations for D_{ij} were tested on this system and give good agreement with three of the four diffusion coefficients. For the main-term diffusion coefficient of the protein the prediction is poor, probably due to a dielectric effect or an association that cannot be excluded. This findings claim that the hard core potential describes well the cross-velocity correlation between protein and PEG, but it does not work well to describe the self-velocity correlation of the protein.

The large decrease of the diffusion matrix determinant, $|D_{ij}|$, can be ascribed to a mobility factor rather than a thermodynamic one. Therefore the $|D_{ij}|$ is not expected to approach zero at all, according to the absence of a spinodal composition at the temperature investigated.

The gravitational instability analysis confirms, as recently observed in aqueous PEG 400−NaCl solution, that PEG addition increases the gravitational stability of the systems, thus damping the convective flow occurrence. This issue can be relevant in understanding the kinetics in protein nucleation and crystal growth where PEG is present.

The effect of the molecular size of the polymer and protein is being studied by changing the PEG molecular weight and the protein size and charge.

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