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Charged Protein Partitioning in Aqueous Polyethylene Glycol–Dextran Two-Phase Systems: Salt Effects

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ABSTRACT

This work continues our examination of the contribution of protein charge to partitioning in polyethylene glycol–dextran aqueous two-phase systems. The partitioning of T4 lysozyme and its charge–change mutants, both point mutations and fusion tails, in aqueous two-phase systems with NaCl, KCl, K_2SO_4, and potassium phosphate salts at concentrations from ca. 0.05–0.5 M are reported. Both salts and proteins have different distributions in systems with the same overall polymer concentration but different salts and salt concentrations. T4 lysozyme point mutants and fusion-tail mutants of the same charge display different partitioning behaviors. The point mutation mutants were best suited for distinguishing nonelectrostatic from electrostatic effects of the different salts on protein partitioning.

INTRODUCTION

Aqueous two-phase systems are typically composed of solutions of two incompatible polymers, such as polyethylene glycol (PEG) and dextran, and provide mild conditions, short processing times, and ease of scale-up that are desirable for purification of biological materials. Abbott et al. (1) and Walter et al. (2) have reviewed theoretical advances while reporting their application of aqueous two-phase systems.

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Previously (3), we summarized the literature which has sought to isolate the role of protein charge (4–7); our own approach was to use a series of T4 lysozymes mutants, differing in net charge and/or charge distribution. We examined the partitioning behavior of charge–change point mutations and charged-tail fusions in PEG–dextran aqueous two-phase systems of differing polymer concentrations. Protein net charge strongly affected protein distribution between the phases, but the difference between point mutations and fusions of the same net charge showed that charge distribution was also a factor. The dependence of the protein partition coefficient, K_p, on phase composition and potential difference was described as

$$\ln K_p = a(w_2^B - w_2^T) + \frac{z_p F}{RT} \Delta\varphi \tag{1}$$

where $(w_2^B - w_2^T)$ is the measure of the polymer concentration difference between the two phases; a is an interaction parameter related to the second virial coefficient between the protein and polymers in a phase solution; and $(z_p F/RT)\Delta\varphi$ is the electrostatic term as a product of protein surface charge z_p and $\Delta\varphi$, the electrostatic potential difference between the two phases. The first term on the right side of the equation is derived from the virial expansion model (6), and it is noted that the same form can also be approximated from the Flory–Huggins model (7). The second term is from the classic model developed by Albertsson (4) for the electrochemical partitioning of proteins in phase systems:

$$\ln K_p = \ln K_0 + \frac{z_p F}{RT} \Delta\varphi \tag{2}$$

where $\ln K_0$ includes all the other factors affecting protein partitioning. However, it has been pointed out (3, 8) that $\Delta\varphi$ in these equations should be the pure electrostatic potential difference, which differs from the potential difference measured by two Ag/AgCl electrodes. The latter has been termed the quasi-electrostatic potential difference (8, 9) and has often been taken as an empirical approximation to the former. The shortcomings of this approximation may have accounted for the earlier observed discrepancy between the potential difference regressed from the protein partitioning results using Eq. (2) and the potential difference from direct measurement (3).

An examination of charge effects on protein partitioning can not be completed without considering salt effects. Different salts and salt concentrations in a phase solution will cause different solvent structures and molecular interactions (10) and, in turn, change phase separation and protein partitioning behavior.

Past studies of salt effects on protein partitioning (1, 4, 11–14, 29) have focused on changes in the potential difference, i.e., $\Delta\varphi$ in Eq. (2), to account for observed changes in partitioning. At low salt concentrations (0.01–0.1 M), the change in protein partitioning with increasing salt concentration was attributed to increasing dominance of the added salt in establishing the interfacial potential difference (4, 11, 13). The alternative, i.e., the presence of salt affects K_0 (Eq. 2), has been neglected. Indeed, independence of K_0 among systems with different salts and salt concentrations was assumed (4, 11, 12) in order to determine the protein isoelectric point from the cross point of curves of protein partition coefficient versus pH in systems with different salts ("cross partitioning").

However, one would expect that different salts and salt concentrations would affect K_0 both through direct changes in protein–polymer interactions and indirectly through alteration of polymer–polymer interactions leading to altered phase compositions. Increasing the concentration of water-structure-making ions, such as SO_4^{2-} and PO_4^{3-}, will shift the binodal to lower polymer concentrations, and increasing the concentration of water-structure-breaking ions, such as Cl^-, will shift the binodal to higher polymer concentrations (15, 16). This may be why it has been very difficult to determine the protein isoelectric point by cross partitioning (10).

An additional complication in past studies focusing on protein charge and salt effects is that charge is usually altered by adjusting the pH of the phase systems through alteration of buffer (typically 0.01 M sodium or potassium phosphate salt) composition. This brings two major limitations: 1) the protein conformation can be changed with the change of pH (17–19), and 2) different buffer salts can have significant effects on solvent properties and phase compositions (10, 17). As a result, the linear relationship between ln K and the protein charge predicted by Eq. (2) is not observed (17–19); therefore, Eq. (2) can not be used to separate the influences of salts on K_0 and $\Delta\varphi$.

In this work we try to separate the effects of protein charge from pH by use of two series of charge–change mutants and to isolate the effects of added salt by using a range of salt concentrations coupled with monitoring of phase composition. Thus, we are able to investigate both electrostatic and nonelectrostatic effects caused by different salts and salt concentrations under the same pH value. The aim is to combine manipulation of protein charge and salt content of the phase system to enhance extraction selectivity.

MATERIALS AND METHODS

Proteins

Two series of genetically engineered T4 lysozymes were produced and purified as described by Luther and Glatz (20). One is the mutant series (strains provided by B. W. Matthews, University of Oregon) that has been modified

TABLE 1
Net Charge of T4 Lysozyme Mutants and Fusions at pH 7.25[a]

T4 Lysozyme	Wild type	Single mutant	Double mutant	Triple mutant	U1 fusion	U2 fusion
Net charge	9.84	7.84	5.84	3.84	5.84	7.84

[a] Charge estimated by using a combination of titration, isoelectric focusing, and calculation from the Henderson–Hasselbach relationship and p*K* values from Lehninger (20, 28).

through site-directed mutagenesis to replace lysine (K) residues with glutamic acid (E) (21). Each mutation results in an expected reduction of two units of charge at neutral pH. Using the numbering notation of Dao-Pin et al. (21) to indicate the mutation site, mutants containing one, two, and three mutations are denoted as K16E, K16/135E, and K16/135/147E, respectively; WT denotes wild type. The “fusion” series consists of fusions of polyarginine tails containing two and four arginines (designated U1 and U2, respectively) to the carboxyl terminus of mutant T4 lysozyme 16/135/147E, effectively restoring the net charges removed by the point mutations. The genes for all the T4 lysozymes are carried on the expression vector pHN1403, which has been transformed into *E. coli* strain RR1.

Lysozyme activity was measured by following the clearing of a *Micrococcus lysodeiktikus* cell suspension versus time (22). Full enzymatic activity is observed for all the mutants and fusions. Lysozyme concentration was determined by measuring the absorbance at 280 nm, using an extinction coefficient of 1.28 (21). The estimation of charge of T4 lysozyme wild type and mutants has been reported previously (20). U1 has the same charge as that of double mutant K16/135E while U2 has the same charge as that of the single mutant K16E. Table 1 lists the estimated charge numbers of all six lysozymes at pH 7.25. The method of charge calculation was to calculate charge contributions as a function of the pH and published p*K*s of amino acids in proteins. The set of p*K*s used was selected on the basis of success in predicting measured isoelectric points and matching a titration curve for one of the proteins.

Aqueous Two-Phase Systems

Stock salt (ACS Certified, Fisher Scientific, Fair Lawn, NJ) solutions, 2 M sodium chloride and potassium chloride, 0.7 M potassium sulfate, and 1 M potassium phosphate at pH 7.25 (with KH_2PO_4:K_2HPO_4 = 0.3:0.7) were prepared. The amount of stock salt solution needed was calculated according to the final bulk salt concentration of the phase systems. Fresh poly(ethylene glycol) (PEG-3350, $M_w = 3350$) and dextran (Dextran-40, $M_w = 39{,}100$) (purchased from Sigma Chemical Co., St. Louis, MO) stock solutions were prepared, and the polymer concentrations were determined from the refractive index (Bausch & Lomb refractometer, Rochester, NY). Bulk phase systems with 8.5% w/w PEG, 8% w/w dextran, 0.01 M potassium phosphate buffer (pH 7.25 with $KH_2PO_4:K_2HPO_4 = 0.3:0.7$), and different salts and salt concentrations were prepared from the stock polymer and salt solutions, potassium phosphate buffer, and deionized water. The phase systems were allowed to equilibrate and separate at room temperature (23°C) for 3 hours, then centrifuged at 10,000*g* for 2 minutes to ensure complete separation of phases.

The dielectric constant, ε, of phase systems with different salts was determined by measuring the refractive index using Snell's law (23):

$$\frac{n_1}{n_2} = \frac{\sqrt{\varepsilon_1}}{\sqrt{\varepsilon_2}} \tag{3}$$

where n is the refractive index of a solution.

Protein Partition Coefficient Measurements

For the same protein, two different concentration levels of stock protein solutions (ca. 0.8 and 0.5 mg/mL) were prepared, and each was dialyzed against 0.01 M potassium phosphate buffer (pH 7.25). Bulk PEG, dextran, and salt solutions were weighed into polystyrene tubes, and 1 g stock protein solution was added. Water was added to a final weight of 8 g, giving an overall composition of 8.5% PEG, 8% dextran, in 0.01 M potassium phosphate buffer at a series of added salt concentrations. Blank phase systems omitted the protein. The systems were mixed by vortexing for 10 seconds, allowed to equilibrate for 3 hours, and then were centrifuged at 10,000*g* for 2 minutes. Top-phase samples were collected using a Pasteur pipette while those from the bottom phase were obtained by piercing the bottom of the tube and allowing the lower phase to drain. The protein concentration of each phase was determined. All partitioning experiments were performed in triplicate and at room temperature, 23°C.

Potential Measurements

Electrodes made of a 1.5-mm glass capillary filled with 2% agar in 3 M KCl were used to measure potential differences as described by Luther and Glatz (20). At least three measurements of each phase system were made. All potential differences reported are expressed as bottom phase minus top phase.

Flame Emission Adsorption Measurements

Sodium and potassium ion concentrations of individual phases were determined by flame emission spectrophotometry using a IL343 Flame Photometer (Instrumentation Laboratory, Inc., Lexington, MA). The phase solutions were diluted with a 15-mM lithium chloride solution to bring the solution concentration level to the flame emission adsorption measuring range. All the measurements were performed in triplicate and at room temperature, 23°C.

RESULTS AND DISCUSSION

Phase compositions and tie-line lengths of PEG–dextran phase systems with the addition of NaCl, KCl, K_2SO_4, and potassium phosphate (pH 7.25) were obtained for the lowest and highest concentrations of added salts used in the partitioning experiments. At the 0.05 M level of added salt, the effect of the 0.01 M phosphate buffer used in each aqueous system may not be negligible. All other cases meet or exceed the criteria that addition of salt with concentration larger than 10 times that of the buffer salt will suppress the influence of buffer salt (4). The addition of salts alters phase compositions and tie-line lengths (Table 2) with the concentrations of K_2SO_4 and potassium phosphate having a larger influence on tie-line lengths than do NaCl and KCl. Zaslavsky (10, 24) also reported that in PEG–dextran systems an increase of K_2SO_4 concentration from 0.05 to 0.2 M reduces by 20% the total polymer concentration required for phase separation while an increase of KCl concentration has little influence (less than 2% reduction for KCl concentration increasing from 0.2 to 1.0 M).

The different salts and salt concentrations also change the measured potential difference between the phases (Table 3). These potential differences, measured by two Ag/AgCl electrodes, are the quasi-electrostatic potential differences and serve as an estimate of the electrostatic potential differences between the two phases (3, 8, 9). Larger magnitude potential differences were observed for phase systems with potassium phosphate and K_2SO_4. While a higher salt concentration results in a smaller potential difference for all four salts, increasing concentration seems to have a larger influence on phase systems with NaCl and KCl. Again, the 0.05 M results may be affected by the buffer salt.

Table 4 lists the partition coefficients of the four salts for these same conditions. The anions determine the partitioning of the three potassium salts as has been reported by others (10, 11, 25) with the order of partition coefficients as chloride salts $>$ sulfate salts $>$ phosphate salts, where the phosphate salts have the most uneven distribution between the two phases. Additionally, for chloride salts, the extent of partitioning decreases with the increase of salt concentration, while for sulfate and phosphate salts the extent of partitioning increases. The same effect of phosphate salt concentration was reported by Bamberger et al. (26). These effects on salt distribution are the result of changes in solvent structure and of polymer–salt and polymer–polymer interactions. Zaslavsky (10) reported that increasing the concentration of water-

TABLE 2

The Phase Compositions of PEG–Dextran Systems with Salts at the Lowest and Highest Salt Concentrations Used

	NaCl		KCl		K_2SO_4		Potassium phosphate	
	0.05 M	0.5 M	0.05 M	0.5 M	0.05 M	0.2 M	0.05 M	0.5 M
Top phase (PEG, dextran), %	(12, 1.6)	(11, 1.4)	(16, 3.3)	(17, 2.9)	(15, 2.4)	(17, 1.1)	(13, 1.9)	(16, 0.8)
Bottom phase (PEG, dextran), %	(2.2, 33)	(2.4, 33)	(4.1, 30)	(4.2, 29)	(3.0, 29)	(1.6, 32)	(3.2, 24)	(1.2, 29)
Tie-line length (%)	32.9	33.0	29.3	29.1	29.2	34.5	24.2	31.9

TABLE 3

Potential Differences (top - bottom) Calculated from the Partition Data of T4 Lysozymes Point Mutants Using the Linear Relationship of Eq. (2) (calculated) (Figs. 2a–d), and Potential Differences (top - bottom) Measured from Ag/AgCl Electrodes (experimental)

Salt concentration	NaCl (mV)		KCl (mV)		K_2SO_4 (mV)		Potassium phosphate (mV)	
	Calcd	Exp	Calcd	Exp	Calcd	Exp	Calcd	Exp
0.05 M	1.5	0.52 ± 0.04	0.20	0.50 ± 0.03	1.7	1.40 ± 0.06	2.8	1.53 ± 0.03
0.1 M	0.01	0.40 ± 0.03	0.34	0.40 ± 0.04	1.0	1.32 ± 0.05	2.6	1.27 ± 0.03
0.2 M	−0.46	0.25 ± 0.04	0.85	0.21 ± 0.02	0.95	1.06 ± 0.04	2.2	1.15 ± 0.05
0.4 M	—	—	—	—	—	—	2.0	1.03 ± 0.03
0.5 M	−0.90	0.12 ± 0.03	0.74	0.10 ± 0.02	—	—	—	—

[a] ± standard deviation of mean.

TABLE 4
The Partition Coefficients of Different Salts in PEG–Dextran Systems with Different Concentration Levels

Salt concentration	NaCl	KCl	K_2SO_4	Potassium phosphate
0.05 M	0.90	0.85	0.74	0.68
0.1 M	0.93	0.91	0.70	0.66
0.2 M	0.97	0.93	0.67	0.59
0.4 M	—	—	—	0.49
0.5 M	0.98	0.96	—	—

structure-making ions such as sulfate and phosphate ions will reduce PEG solubility in the dextran phase and salt solubility in the PEG phase, which leads to a more uneven distribution of polymers and salts between the two phases. This is also the case for PEG–salt systems as an increase of the salt concentration will push more salts from the PEG phase to the salt phase (24). On the other hand, the increase of water-structure-breaking ions like chloride will increase the polymer compatibility (10, 27), which means more PEG transferring from the top phase to the bottom phase and a more even salt distribution between the two phases. The results in Table 2, the salt effects on phase compositions and tie-line lengths, and the results in Table 3, the salt distributions, are related to each other. Zaslavsky et al. (10, 15) reported that for phase systems with the same salt and salt concentration but different polymer concentrations:

$$\ln K_\mathrm{s} \propto \Delta C_{\mathrm{Polymer}_i} \tag{4}$$

where K_s is the salt partition coefficient and $\Delta C_{\mathrm{Polymer}_i}$ is PEG concentration difference between the two phases, which is in turn often proportional to the tie-line length. The same trend predicted from Eq. (4) is evident in our salt partitioning and tie-line length results (Tables 2 and 4); however, in our case the overall polymer concentrations has remained the same and phase compositions were changed indirectly by the addition of salts.

As a result of the salt changes, a protein will encounter differences in both polymer and salt compositions of the phases as well as different interfacial potential differences among the phase systems. Figures 1(a–d) show the resulting partition coefficients of the six T4 lysozymes in all these phase systems. These results not only illustrate the influence of different salts and salt concentrations on the protein partitioning, but also indicate the effects of surface charge and charge distribution of the protein on partitioning. The importance of protein surface charge is evident in the different partitioning behaviors among the six T4 lysozymes. Yet surface charge alone is not a sufficient ex-

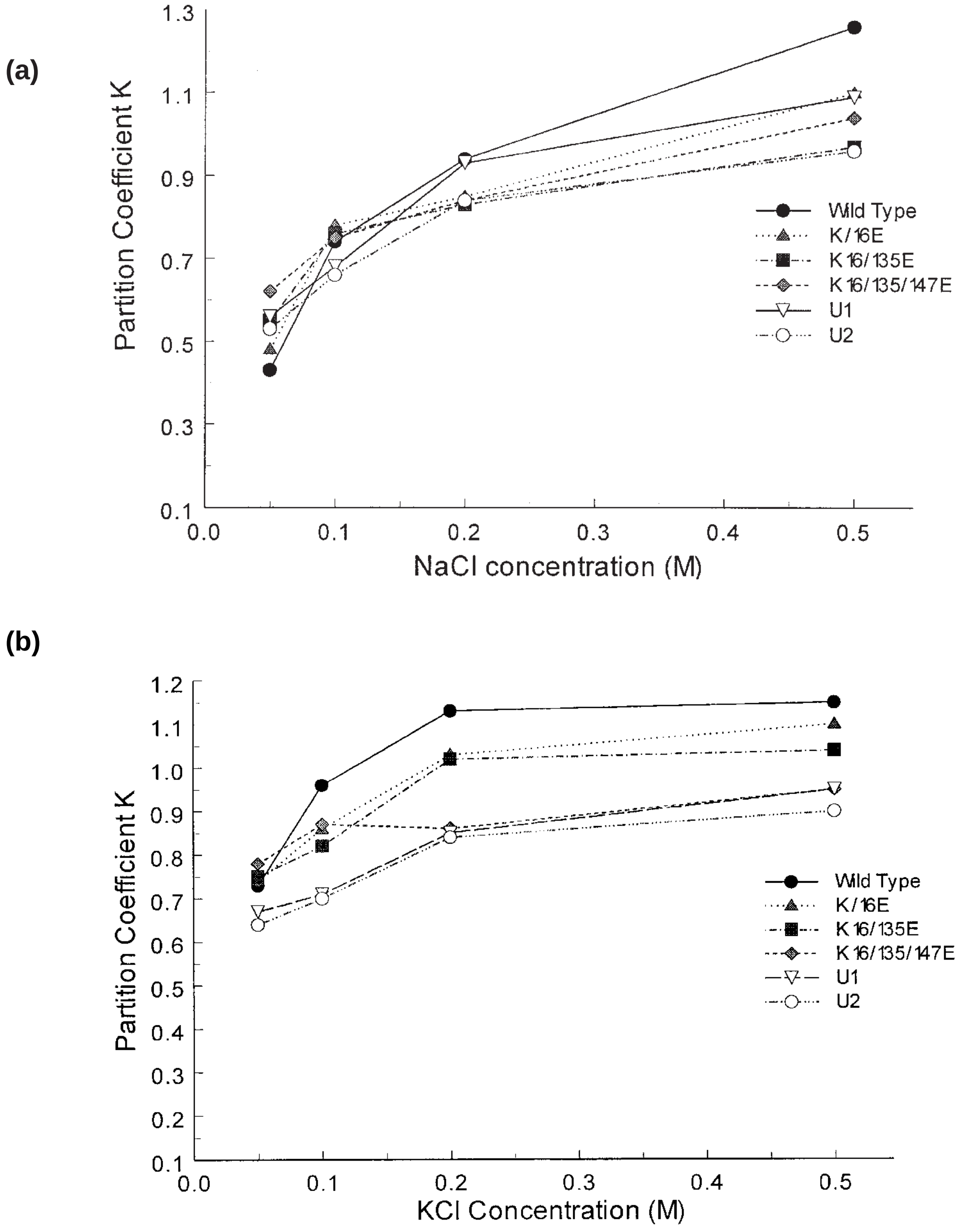
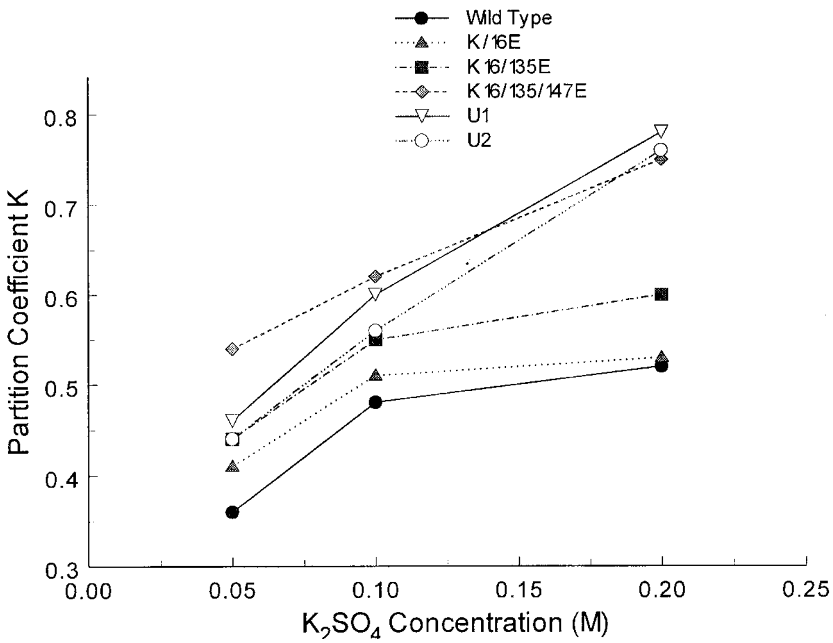


FIG. 1 Partition coefficient of all six T4 lysozymes in the PEG3350/dextran-40 aqueous two-phase systems, at pH 7.25 (0.01 M potassium phosphate buffer), with the addition of different salts: (a) NaCl, (b) KCl, (c) K_2SO_4, and (d) potassium phosphate at pH 7.25 with KH_2PO_4:K_2HPO_4 = 0.3:0.7. The partition results at 0.05 M salt concentration could be influenced by both added salts and 0.01 M phosphate buffer salts.

(*continued*)

(c)



(d)

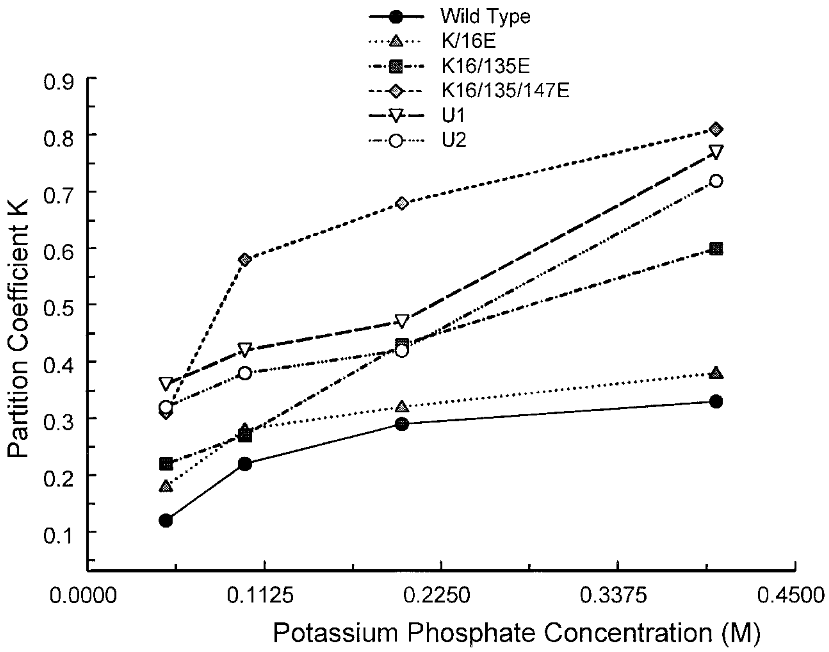


FIG. 1 Continued

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planation as seen in the differing partition coefficients of U1 and K16/136E and U2 and K163 in all phases systems even though these pairs have a matching surface charge. As discussed previously (3), the charge density of the tails may distort the simple charge interaction.

One general trend from Figs. 1(a–d) is that the addition of any of the salts increases the partition coefficient of all the proteins (though the “increase” in these cases means a more equal distribution). The increase of the partition coefficient of positively charged proteins with an increase of salt concentration was also reported by Albertsson (4). However, the magnitude of the change as well as the absolute value of the partition coefficient depends on the salt. First, we can see that the protein net charge has a more significant impact on partitioning in phase systems with K_2SO_4 and potassium phosphate as the differences in partition coefficients among the charge mutants are greater than those observed in phase systems with NaCl and KCl.

The larger influence of protein charge for systems with K_2SO_4 and potassium phosphate is more easily interpreted when replotted according to Eq. (2) in Figs. 2(a–d). The slopes of these plots indicate that the charge effects reflect the magnitudes of the salt-determined potential differences. Table 3 includes the potential differences calculated from the slopes using Eq. (2). While the quantitative agreement with the measured values is not good, qualitatively the larger potentials of phase systems with K_2SO_4 and potassium phosphate are confirmed.

Table 3 also shows that, as a general trend, the potential differences decrease with increased salt concentration. Phase systems with K_2SO_4 and potassium phosphate present a relatively stable electrostatic effect as the potential differences change less significantly with the increase of salt concentrations. For phase systems with NaCl and KCl, we can even observe a change of direction of the potential difference with the increase of salt concentrations; however, this is probably the result of the influence of the phosphate buffer at low added salt concentration.

From the intercepts of the linear relationships in Figs. 2(a–d), we can observe the salt effects on nonelectrostatic interactions, which are included in the K_0 term of Eq. (2). Figures 2(a) and 2(b) show that, for phase systems with NaCl and KCl, different salt concentrations result in small changes of intercept. This means that the nonelectrostatic interactions under different salt concentrations are relatively constant, and the addition of NaCl and KCl affects the protein partitioning mainly through altering the potential difference between the two phases. In contrast, for systems with K_2SO_4 and potassium phosphate, Figs. 2(c) and 2(d) show that changing salt concentration has a smaller effect on the slope but does affect the intercept significantly, which implies that the significant influence of salt concentration on the protein partition coefficients results from nonelectrostatic effects. This is consistent with

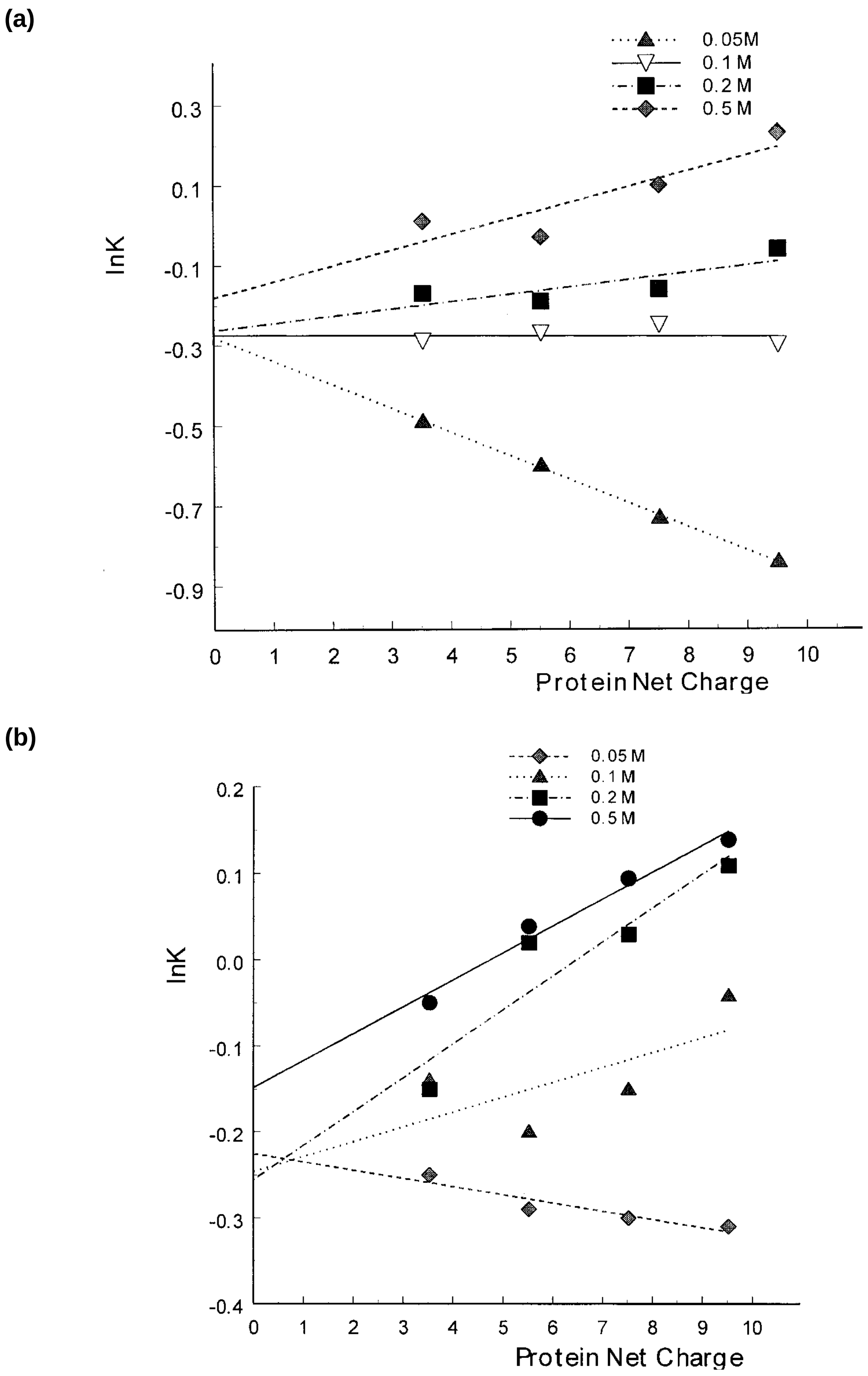

FIG. 2 Partition coefficient of T4 lysozyme Wild Type, K/16E, K/16/135E, and K16/135/147E versus protein net surface charge in phase systems with the addition of (a) NaCl, (b) KCl, (c) K_2SO_4, and (d) potassium phosphate at pH 7.25 with KH_2PO_4:K_2HPO_4 = 0.3:0.7. For systems with 0.05 M NaCl and KCl (a and b), the potential difference (regressed from the slope of the linear relationship) is misleading because the influence of the phosphate buffer salt is not negligible.

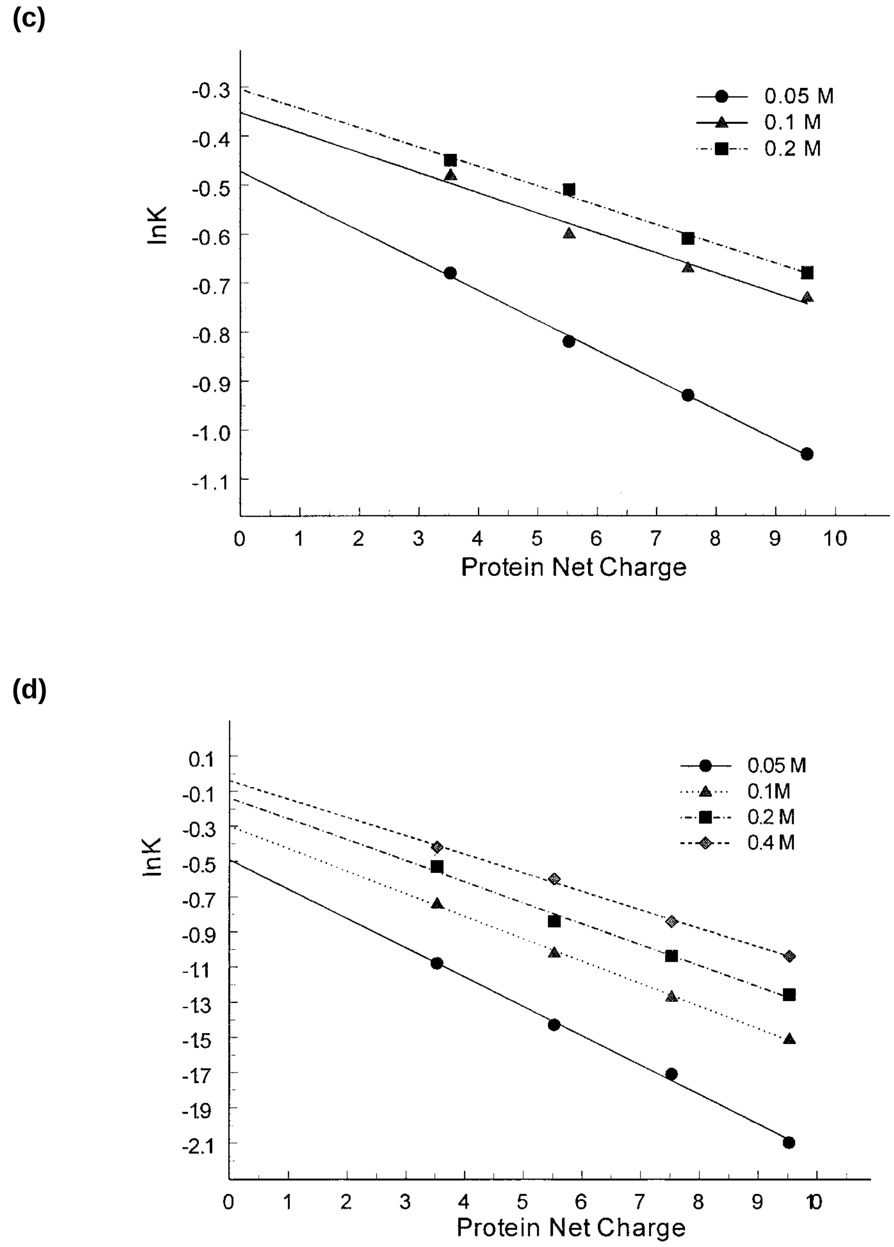

FIG. 2 Continued

our earlier observation that the increase of K_2SO_4 and potassium phosphate concentration has more significant effects on phase compositions, tie-line lengths, and salt partitioning than that of NaCl and KCl as shown in Tables 2 and 4. However, this nonelectrostatic effect cannot be explained by the tie-line effect accounted for by Eq. (1). It predicts that a larger tie-line length will result in a lower (more negative) intercept; however, here higher salt concentration gives a larger tie-line length but a larger (less negative) intercept. This im-

TABLE 5
The Dielectric Constant of the Top and Bottom Phases of PEG–Dextran Systems with Different NaCl Concentrations[a]

	Top	Bottom
0.05 M	81.47 ± 0.02	83.37 ± 0.01
0.1 M	81.51 ± 0.01	83.44 ± 0.02
0.2 M	81.63 ± 0.02	83.55 ± 0.03
0.5 M	82.02 ± 0.02	83.85 ± 0.01

[a] ± standard deviation of mean.

plies that the change of salt concentration has also altered parameter a in Eq. (1), indicating altered protein–polymer molecular interactions.

Therefore, the salt effects on charged protein partitioning can be viewed as a result of the interplay of the electrostatic effects through the salt effects on phase potential differences, and nonelectrostatic effects through the salt effects on phase polymers, salt compositions, and protein–polymer molecular interactions. Equation (2) seems proper in qualitatively explaining the partitioning results of proteins with different surface charge in these systems. Zaslavsky (10) countered with the alternative explanation that salt effects on protein–polymer–salt molecular interactions are caused by different dielectric properties between the two phases resulting from salt effects on solvent structure, and salt and polymer compositions. Table 5, while indicating that there is a small dielectric constant difference between the top and bottom phases of PEG–dextran systems with different concentrations of NaCl, shows that the influence of salt concentration is less than 0.5%, i.e., almost negligible. The same results were found for phase systems with KCl, K_2SO_4, and potassium phosphate. However, this measurement mainly reflects the dielectric properties of bulk phases. Dielectric properties near the surface of the molecules would more directly influence intermolecular interactions.

CONCLUSIONS

We have determined salt effects on the partitioning of different T4 lysozymes in PEG–dextran phase systems. T4 lysozyme fusion-tail mutants display a different partitioning behavior from those of point mutation mutants with a matching surface charge in all the phase systems. Increasing the salt concentration shifts more protein from the bottom phase to the top phase. Phase systems with the addition of sulfate or phosphate salts display a larger potential difference between the two phases, and the change of salt concentration will affect both electrostatic and nonelectrostatic interactions. In phase systems with NaCl and KCl, salt concentration affects the charged protein partitioning largely by influence on the potential difference. The same trend of salt effect on the potential difference between the two phases was also reflected in the results of the potential differences measured by using Ag/AgCl electrodes. The difference between the measured potential difference and the potential difference regressed from protein partitioning results reflects the difference in the definition of the two potential differences (3, 8).

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