Journal of Chromatography, 359 (1986) 131–146
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 796

SOLUTE AND MOBILE PHASE CONTRIBUTIONS TO RETENTION IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS*

J. L. FAUSNAUGH and F. E. REGNIER*
Department of Biochemistry, Purdue University, West Lafayette, In 47907 (U.S.A.)

SUMMARY

Hydrophobic interaction chromatography utilizes high salt concentration mobile phases to induce an interaction between a weakly hydrophobic matrix and exposed hydrophobic amino acids of a native protein. Proteins with a hydrophilic exterior have shorter retention times on a hydrophobic interaction column than do proteins with more hydrophobic exteriors. To examine the effect of amino acid substitutions on protein retention, lysozyme isolated from related bird species was chromatographed on a hydrophobic interaction column at increasing ammonium sulfate concentrations. Chromatographic retention deviated only when amino acid substitutions occurred on the surface of lysozyme opposite the catalytic cleft. This area may constitute a contact surface area and extends from Residue 41 to 102 and from 75 to the α-helical region starting with Residue 89. Retention was analyzed by plotting log k' *versus* the molal concentration of ammonium sulfate. The slope did not deviate significantly for each of the bird lysozymes, indicating a similar contact surface area. However, there was significant deviation in the intercept of each of the lysozyme lines, which probably reflects the strength of the hydrophobic interaction. The intercept increased as the lysozyme became more hydrophobic. Hydrophilic amino acid substitutions affected retention as much as hydrophobic ones. The ionization state of histidine residues within the contact area between lysozyme and the column surface also influenced retention. An uncharged histidine residue increased retention, while a decrease in retention was seen with a charged histidine residue. The amino acid substitutions did not appear to affect the size of the hydrophobic contact surface area, but rather the strength of the hydrophobic interaction. The effect of salt composition on protein retention indicated that factors other than surface tension could influence retention. These factors appear to include protein hydration and specific interactions between the protein and the salt ions. Of these, the latter may or may not result in an alteration in protein structure. The magnitude of the effect of salt composition was found to be dependent upon the protein.

* This is Journal Paper No. 10566 of the Purdue University Agricultural Experiment Station.

0021-9673/86/$03.50

INTRODUCTION

Hydrophobic interaction chromatography (HIC) utilizes high salt concentrations to induce an interaction between the protein and a weakly hydrophobic matrix. Elution is achieved either isocratically or with a descending salt gradient. The descending salt gradient diminishes the hydrophobic interaction and allows the protein to be eluted from the column.

HIC on classical soft gels has been used in the purification of many proteins[1–4]. As with the classical HIC columns, selectivity in high-performance HIC can be easily manipulated through mobile phase and stationary phase variables[5,6]. Stationary phase variables include ligand chain length and ligand density; protein retention increases concomitantly with both ligand density and chain length. Mobile phase variables that influence retention include salt type, salt concentration, and pH. The effect of pH on protein retention depends upon the protein and the nature of the column[6]. Differential variations in retention as a function of pH are a valuable technique for altering selectivity. Protein retention increases logarithmically with isocratic salt concentration. In a gradient chromatogram, an increase in initial salt concentration also results in an increase in protein retention. In general, the equation

$$\log k' = \log k_w - S\Phi_b \tag{1}$$

also applies to HIC. Eqn. 1 relates the logarithm of the capacity factor (k') to the volume fraction (Φ_b) of the low ionic strength buffer, B. The intercept ($\log k_w$) is the value of the capacity factor in pure water, and S is the strength of solvent B as mobile phase. This means that recent mathematical treatments of gradient elution[7] may also be applied to HIC. The effect of salt type on protein retention can be related to the molal surface tension increment of the salt[8]. Salts with higher molal surface tension increments produce increased retention at equal molal salt concentrations. The magnitude of the changes in protein retention that alterations in mobile and stationary phase variables produce is dependent upon the protein.

Melander and Horváth have proposed a thermodynamic model[8,9] based on the cavity theory of Sinanoğlu and Abdulman[10,11] for the hydrophobic interaction between a protein and a hydrophobic matrix. The model (eqn. 2) takes into account the free energy changes in cavity formation (cav), electrostatic effects (es) and Van der Waals interactions (vdw) between free and associated proteins and ligands.

$$\ln k = -\frac{1}{RT}(\Delta G^0_{cav} + \Delta G^0_{es} + \Delta G^0_{vdw} + \Delta G^0_{assoc} + \Delta G^0_{red}) + \ln\frac{RT}{PV} + \varphi \tag{2}$$

ΔG^0_{assoc} is the free energy change for protein–ligand association in the absence of surrounding solvent; ΔG^0_{red} is the reduction in free energy due to solvent–protein and solvent–ligand interactions not considered in the first three terms; V and P are the mean molar volume of solvent and the operating pressure, respectively; the constant φ is related to the density of accessible ligands on the stationary-phase surface.

The free energy change associated with electrostatic effects is given by the equation:

$$\Delta G^0_{es} = A - \frac{B(m^{1/2})}{1 + C(m^{1/2})} - D\mu m \tag{3}$$

where μ is the dipole moment of the protein and m is the molal salt concentration. The coefficients A, B, C, and D are related to the net charge of the protein and stationary phase and the size of the protein.

The free energy change associated with cavity formation is given by the equation:

$$\Delta G^0_{cav} = -\Delta A_s \sigma m + \text{constant} \tag{4}$$

where ΔA_s is the difference in exposed surface area of ligand and protein between the associated and non-associated states and σ is the molal surface tension increment.

Free energy changes related to Van der Waals interactions are given by the equation:

$$\Delta G^0_{vdw} = \Delta G^0_{vdw} + vm + \text{constant} \tag{5}$$

where v is a constant for a given salt–protein pair and stationary phase. These equations are combined to give:

$$\ln (k/k_0) = -Bm^{1/2}/(1 + Cm^{1/2}) - D\mu m + \Delta A_s \sigma m + vm + \text{constant} \tag{6}$$

where k_0 is the retention factor at zero salt concentration. At sufficiently high salt concentrations, the first term on the right side approaches a constant value and the logarithmic retention factor becomes linear with respect to molal salt concentration. Thus,

$$\log (k/k_0) = \lambda m \tag{7}$$

where λ is a parameter related to σ. Plots of log k' *versus* m should yield straight lines with a slope proportional to the contact surface area between the protein and the column.

In most cases, the recovery of enzymatic activity from a HIC column exceeds that from a reversed-phase column. Fausnaugh *et al.*[5,6] found that recoveries of α-amylase and β-glucosidase exceeded 92%, and that of α-chymotrypsin exceeded 86% after chromatography in 1 *M* sodium sulfate on a HIC column containing butyrate ligands, while the recovery of α-chymotrypsin from a C_8 column ranged from 50 to 90%. The organic solvents and the interaction with the reversed-phase column itself were detrimental to the native structure of the protein.

This paper examines the effect of salt type on protein retention. Graphs of log k' *versus* salt molality or surface tension were used to evaluate the Melander–Horváth model for hydrophobic retention. Lysozymes obtained from the egg whites of various bird species were used to determine the contribution of certain amino acid substitutions in the protein to retention.

EXPERIMENTAL

Materials

Sodium sulfate and magnesium sulfate were obtained from Fisher (Fair Lawn, NJ, U.S.A.). Ammonium sulfate and sodium tartrate were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). CM-cellulose was purchased from Sigma (St. Louis, MO, U.S.A.). CM-Sephadex was obtained from Pharmacia (Uppsala, Sweden).

The TSKgel Phenyl-5PW column was a gift from Toyo Soda (Yamaguchi, Japan).

Conalbumin, ovalbumin, and hen egg white lysozyme were purchased from Sigma. All buffers were AR grade.

Lysozyme purification

Lyophilized turkey lysozyme was obtained from Dr. Leo Sercarz of the Microbiology Dept. of UCLA. Peking duck eggs were purchased from Gallo Duck Farms (Patchogue, NY, U.S.A.). The whites were separated from the yolks and frozen until used. Ring-necked pheasant and Japanese quail egg whites were supplied by Dr. Michael Laskowski, Jr., of the Chemistry Dept. of Purdue University. Ring-necked pheasant, Japanese quail, and Peking duck lysozymes were purified from thawed egg white by a procedure based on Prager and Wilson[12]. Egg whites were diluted 1:5 with ammonium acetate buffer (0.05 *M*, pH 9.4), homogenized and filtered through tissue paper (Kimwipes). Approximately 4 g of CM-cellulose, equilibrated in the same buffer, per 100 ml of egg white were added, and the slurry was stirred overnight at 4°C. The remainder of the purification was carried out at room temperature. The resin was allowed to settle and the supernatant was decanted. The resin was poured into a Buchner funnel, lined with eight layers of Kimwipes, and thoroughly washed with the ammonium acetate buffer. The lysozyme was eluted with 0.5 *M* ammonium acetate (pH 9.4). The eluted lysozyme was dialyzed overnight against distilled water. It was next loaded onto a CM-Sephadex column (13 × 1.5 cm I.D.), equilibrated with the ammonium acetate buffer. The lysozyme was eluted in a linear gradient, using 230 ml each of 0.05 *M* ammonium acetate (pH 9.4) and 0.5 *M* ammonium acetate (pH 9.4). After the gradient, duck C lysozyme was eluted with 1.0 *M* ammonium acetate (pH 9.4). The pooled fractions containing lysozyme were dialyzed against distilled water overnight and lyophilized. The purity of each of the bird lysozymes was greater than 95% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Lysozyme assay

Lysozyme activity was assayed by recording the change in percentage transmittance at 540 nm, as the enzyme lysed a suspension of *Micrococcus lysodeikticus*[13].

Instrumentation

Chromatograms were obtained on a Hewlett-Packard 1084B liquid chromatograph with a 79875A scanning UV detector, a 79850B LC terminal and a 79841A variable-volume injector.

Chromatography

Proteins were eluted isocratically from the TSKgel Phenyl-5PW column at

increasing salt concentrations in 10 m*M* morpholinoethanesulphonic acid (pH 6.0), or 10 m*M* potassium phosphate (pH 7.0), or 10 m*M* Tris (pH 8.0) buffer. All separations were conducted at room temperature and a flow-rate of 1.0 ml/min. The effluent was monitored at 254 nm.

RESULTS

The effect of amino acid substitutions on protein retention was analyzed by utilizing lysozymes obtained from the egg whites of the following bird species: chicken (HEW), Peking duck, ring-necked pheasant (RNP). Japanese quail (JPQ), and turkey (TKY). Pooled Peking duck egg whites contain up to three isozymes of lysozyme (LYZ), identified as duck A, duck B, and duck C according to their elution order from a cation-exchange column[14,15]. The lysozymes used in this study differ by between one and 21 amino acid residues (Tables I and II). These amino acid changes are generally on the surface of the molecule and do not alter the three-dimensional structure[20–23].

HEW LYZ consists of 129 amino acids and contains four disulfide bridges. Fig. 1[24] shows a schematic diagram of the peptide backbone of HEW LYZ. The external hydrophobic residues are indicated with an asterisk. Most of these residues are located on the LYZ surface opposite the catalytic cleft. The individual environments of each of the amino acids of lysozyme have been described by Imoto *et al.*[24] and Lee and Richards[25].

Each of the bird lysozymes was chromatographed isocratically on the TSKgel Phenyl-5PW column at increasing ammonium sulfate concentrations in 10 m*M* potassium phosphate buffer (pH 7.0). The high salt concentration probably does not alter the three-dimensional structure of LYZ, since enzymatic activity is retained under these conditions. When log k' was plotted *versus* the molality of the salt solution for each of the lysozymes, a series of parallel lines was produced (Fig. 2). JPQ LYZ appeared to be the most hydrophobic of the lysozymes studied, since it was eluted at longer retention times than the other lysozymes at the same salt concentration. Similarly, duck C LYZ appeared to be the most hydrophilic lysozyme.

Duck A LYZ differs from HEW LYZ by nineteen amino acid residues (Table I). Duck B differs from duck A LYZ by Residues 37 (Ser → Gly) and 71 (Gly → Arg). Duck C LYZ has an additional amino acid substitution at Residue 79 (Pro → Arg), as seen in Table I. All three of these positions are on the surface of the LYZ molecule. Chromatographically (Fig. 2), duck A and duck B lysozymes were indistinguishable, while duck C LYZ was more hydrophilic. All three duck lysozymes were more hydrophilic than HEW LYZ. Von Heijne[26] has assigned to each of the twenty amino acids free energies of transfer, G_t, of residues originally in a helix in water to a helix in a non-polar phase. This value takes into account energy required to break hydrogen bonds and neutralize charged residues and the hydrophobic contribution of the amino acid side chain. A calculation of the change in free energy of transfer, ΔG_t, can be made by subtracting the value of the amino in HEW LYZ from the value of the substituted amino acid[26]. A proline to arginine change results in a ΔG_t of +33.4 kJ/mol. The positive sign indicates the substitution of a more hydrophilic amino acid which would result in duck C LYZ being more hydrophilic than duck B LYZ.

TABLE I

PRIMARY AMINO ACID SEQUENCES OF PEKING DUCK LYSOZYMES*

	1 5 10 15 20 25 30 35 40 45 50 55 60 65
Duck A	KVYSRCELAAAMKRLGLDNYRGYSLGNWVCAANYESSFNTQATNRNTDGSTDYGILQINSRWWCN
Duck B	G
Duck C	G
Chicken	FG H KF N

	66 70 75 80 85 90 95 100 105 110 115 120 125 129
Duck A	DGKTPGSKNACGIPCSVLLRSDITEAVRCAKRIVSDGDGMNAWVAWRNRCRGTDVSKWIRGCRL
Duck B	R
Duck C	R R
Chicken	R R L N A S AS N K K QA

* Sources of primary sequences: Peking duck, ref. 15; chicken, ref. 16.

TABLE II

SPECIES AND PRIMARY AMINO ACID SEQUENCES OF LYSOZYME*

	−1	5	10	15	20	25	30	35	40	45	50	55	60	65
Chicken	K	V	F	G	R	C	E	L	A	A	A	M	K	R
Japanese quail		Y												
Ring-necked pheasant	G	Y		M					G					
Turkey		Y		L					H					
Duck A		YS		L										

	66	70	75	80	85	90	95	100	105	110	115	120	125	129
Chicken	D	G	R	T	P	G	S	R	N	L	C	N	I	P
Japanese quail														
Ring-necked pheasant			K		H									
Turkey			K											
Duck A		K		K	A	G	V	R	E	A	R			

	66	70	75	80	85	90	95	100	105	110	115	120	125	129
Chicken	C	S	A	L	L	S	S	D	I	T	A	S	V	N
Japanese quail														
Ring-necked pheasant														
Turkey														
Duck A														

	66	70	75	80	85	90	95	100	105	110	115	120	125	129
Chicken	C	A	K	K	I	V	S	D	G	N	G	M	N	A
Japanese quail									V	H				
Ring-necked pheasant											K	H		
Turkey								A	G					
Duck A							R				R			

	66	70	75	80	85	90	95	100	105	110	115	120	125	129
Chicken	W	V	A	W	R	N	R	C	K	G	T	D	V	Q
Japanese quail												N		
Ring-necked pheasant												NV		
Turkey												H		
Duck A											R	SK		

	66	70	75	80	85	90	95	100	105	110	115	120	125	129
Chicken	A	W	I	R	G	C	R	L						
Japanese quail														
Ring-necked pheasant														
Turkey														
Duck A														

* Sources of primary sequences: chicken, ref. 16; Japanese quail, ref. 17; ring-necked pheasant, ref. 18; turkey, ref. 19.

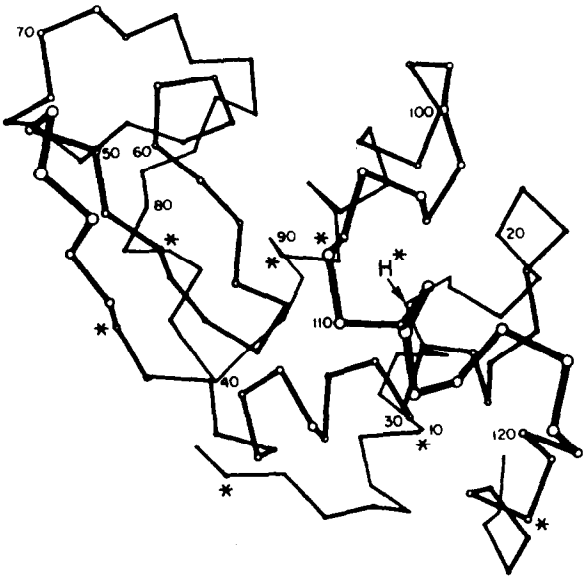

Fig. 1. The three-dimensional structure of the peptide backbone of hen egg white lysozyme. External hydrophobic amino acids are marked with an asterisk.

There are seven amino acid changes between TKY and HEW LYZ (Table II). Five of these substitutions are on the LYZ surface at Residues 15, 41, 73, 101, and 121. Chromatographically, TKY LYZ was more hydrophobic than HEW LYZ (Fig. 2). The amino acid change at position 101 (Asp → Gly) is probably responsible for

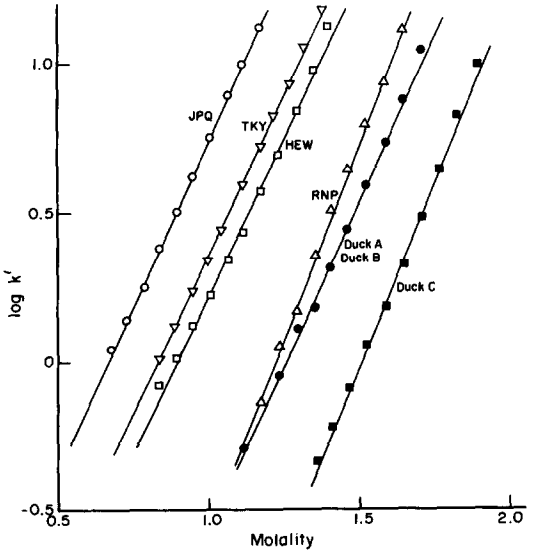

Fig. 2. Graph of log *k' versus* molality for each of the bird lysozymes. Each lysozyme was chromatographed on the TSKgel Phenyl-5PW column in 10 m*M* potassium phosphate buffer (pH 7.0) at increasing ammonium sulfate concentrations.

this increase in hydrophobicity. The ΔG_t for this amino acid substitution is -31.0 kJ/mol.

There are six amino acid substitutions between JPQ and HEW LYZ of which only position 3 is internal (Table II). Chromatographically, JPQ LYZ was more hydrophobic than HEW LYZ (Fig. 2). The amino acid substitutions most likely to be responsible for this increase in hydrophobicity are 21 (Arg → Gln) and 102 (Gly → Val). These substitutions have negative ΔG_t values of -37.2 and -8.4 kJ/mol, respectively. Residues 21 and 102 are spatially fairly close in the three-dimensional structure.

RNP LYZ differs from HEW LYZ by nine amino acid residues and has an additional glycine at position -1 (Table II). This lysozyme was more hydrophilic than HEW LYZ when chromatographed on the TSKgel Phenyl-5PW column at pH 7.0. The amino acid substitution at position 77 (Asn → His) might produce this increase in hydrophobicity, since this exchange has a ΔG_t of $+2.1$ kJ/mol. However, this value assumes that the histidine is charged. At pH 7.0, histidine may be either positively charged or neutral.

To determine the ionization state of histidine on the surface of LYZ, the effect of pH on chromatographic retention of the bird lysozymes was examined. Each lysozyme was chromatographed on the TSKgel Phenyl-5PW column at increasing ammonium sulfate concentrations in buffers of pH 6.0 and 8.0. By raising the pH to 8.0 or lowering it to 6.0, any change in retention can be attributed to a change in the ionization state of histidine, since the column has no charge. Retention increases when the histidine becomes deprotonated and decreases when it becomes charged.

There was no change in retention at pH 6.0 or 8.0 for the three duck lysozymes. This was expected, since these lysozymes do not contain histidine (Table I). HEW LYZ has one histidine at Residue 15 (Table II); however, as with the duck lysozymes, there was no change in retention with pH (Fig. 3).

RNP LYZ contains two histidines at positions 77 and 114. There was a large increase in retention at pH 8.0 and, similarly a large decrease in retention at pH 6.0 (Fig. 3). Residue 114 is spatially quite far from the other substitutions that influenced the chromatographic retention. Therefore, the pH effect on retention is probably due to His 77. This residue appears to be more than half charged at pH 7.0.

JPQ LYZ has two histidines at positions 15 and 103. There was a slight increase in retention at pH 8.0 and a large decrease in retention at pH 6.0 (Fig. 4). Since His 15 is conserved from HEW LYZ and no pH effect was observed for that lysozyme, the pH effect of JPQ LYZ is probably due to His 103. At pH 7.0, this residue appears to be mostly uncharged. The calculated ΔG_t for an uncharged histidine replacing an asparagine residue is -3.8 kJ/mol rather than the $+2.1$ kJ/mol for a charged histidine and, as such, would contribute to the increased hydrophobicity of JPQ LYZ.

TKY LYZ has two histidine residues at positions 41 and 121. There was no increase in retention at pH 8.0 but a significant decrease in retention at pH 6.0 (Fig. 4). The change in retention as a result of pH is probably due to Residue 41. This residue appears to be mostly uncharged at pH 7.0.

Protein retention on the TSKgel Phenyl-5PW column was examined as a function of the chemical composition of the salt in the mobile phase. The effect of salts on chromatographic behavior follows the order of the lyotropic series. For each salt in the lyotropic series a molal surface tension increment value has been calculated.

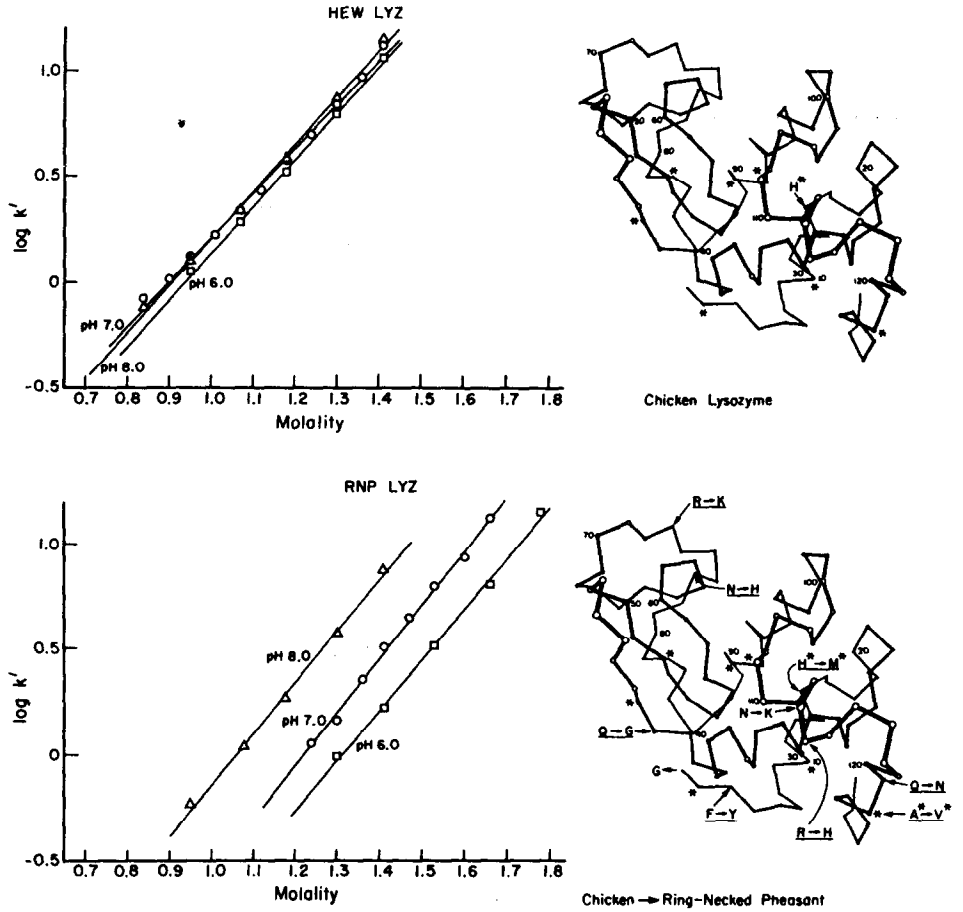

Fig. 3. The effect of pH on the log *k' versus* molality graphs of hen egg white and ring-necked pheasant lysozymes. The lysozymes were chromatographed on the TSKgel Phenyl-5PW column at increasing ammonium sulfate concentrations at pH 6.0, 7.0, and 8.0. The three-dimensional structure of hen egg white lysozyme is also shown with the appropriate amino acid substitutions.

This is a measure of the increase in surface tension upon addition of the salt to a solution. Salts with higher molal surface tension increments produce higher protein retention at equivalent concentrations[8].

Using sodium sulfate, ammonium sulfate, magnesium sulfate and sodium tartrate, graphs of log *k' versus* salt molality were plotted for conalbumin (CON), ovalbumin (OVA) and lysozyme (LYZ) (Fig. 5). The lines were all non-parallel for CON and OVA. The slope of the magnesium sulfate line was the most deviant. The sodium tartrate and sodium sulfate lines of LYZ were parallel, as were the ammonium and magnesium sulfate lines.

When the salts were equalized with respect to surface tension, the salt lines coincided in only two cases, sodium and ammonium sulfate for OVA and ammonium and magnesium sulfate for LYZ (Fig. 6). In all cases, the sodium sulfate, ammonium sulfate and sodium tartrate lines were fairly parallel. The magnesium sulfate line had

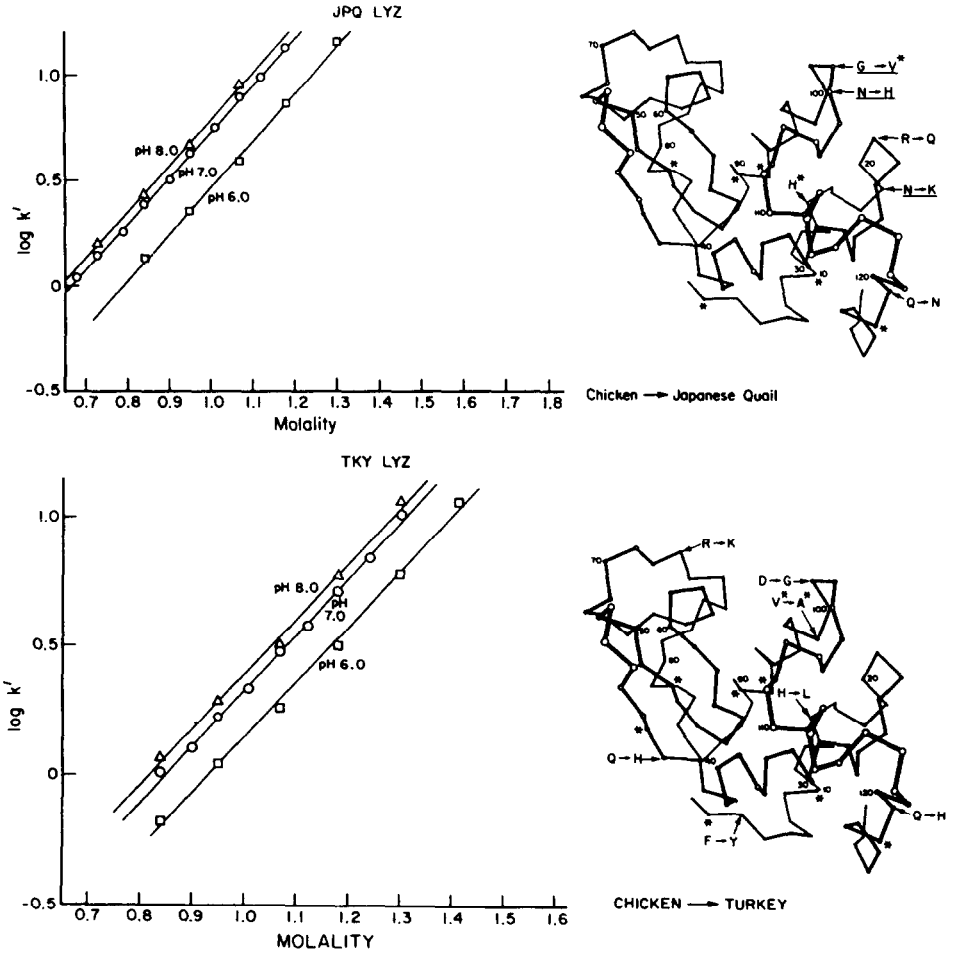

Fig. 4. The effect of pH on the log *k' versus* molality graphs of Japanese quail and turkey lysozymes. The lysozymes were chromatographed on the TSKgel Phenyl-5PW column at increasing ammonium sulfate concentrations at pH 6.0, 7.0, and 8.0. The three-dimensional structure of hen egg white lysozyme is also shown with the appropriate amino acid substitutions.

a much steeper slope for CON and OVA, but was parallel to the other salt lines for LYZ. Furthermore, the protein retention with sodium tartrate did not follow the lyotropic series. The order of molal surface tension increments of the salts is as follows: sodium sulfate, $2.73 \cdot 10^3$ dyn g/cm mol; sodium tartrate, $2.35 \cdot 10^3$ dyn g/cm mol; ammonium sulfate, $2.16 \cdot 10^3$ dyn g/cm mol; and magnesium sulfate, $2.10 \cdot 10^3$ dyn g/cm mol[8]. For all three proteins, retention with sodium tartrate exceeded that with sodium sulfate.

DISCUSSION

Lysozyme obtained from related bird species was used to analyze the effect of amino acid substitutions on protein retention in HIC and, in effect, to map the con-

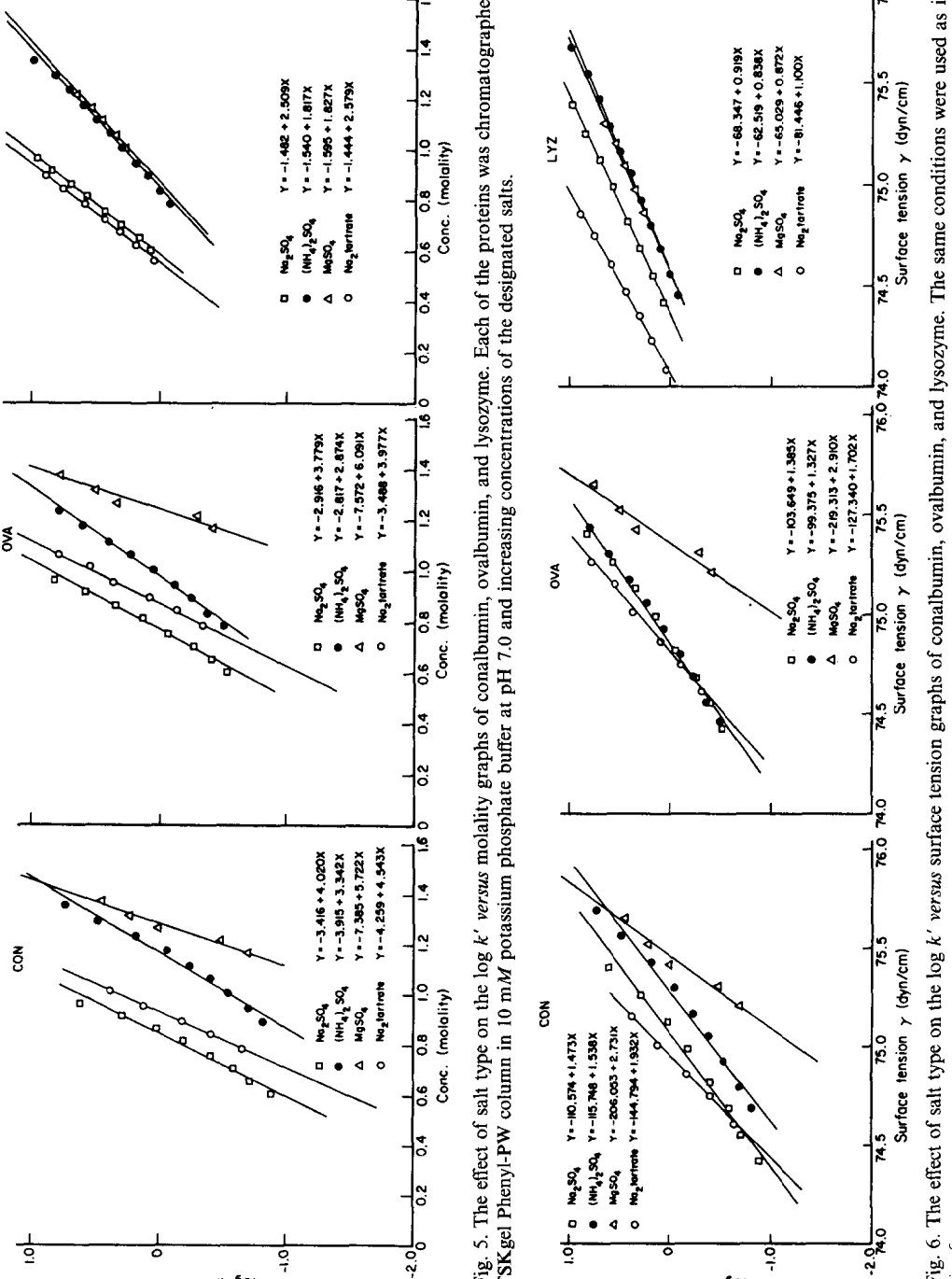

Fig. 5. The effect of salt type on the log k' *versus* molality graphs of conalbumin, ovalbumin, and lysozyme. Each of the proteins was chromatographed on the TSKgel Phenyl-PW column in 10 m*M* potassium phosphate buffer at pH 7.0 and increasing concentrations of the designated salts.

Fig. 6. The effect of salt type on the log k' *versus* surface tension graphs of conalbumin, ovalbumin, and lysozyme. The same conditions were used as in Fig. 5.

tact surface area between the protein and the stationary phase surface. The X-ray crystal structures of HEW, TKY, and human LYZ have been determined and indicate that the amino acid substitutions do not drastically alter the protein structure[20,22].

Not all of the amino acid substitutions had an effect on LYZ retention. Despite the two changes at Residues 37 and 71 between duck A and duck B LYZ, these two proteins could not be distinguished chromatographically. However, duck C LYZ, with an additional substitution at position 79 (Pro → Arg) was more hydrophobic than the other two duck lysozymes. Residue 79 is, therefore, within the contact surface area. Either Residue 37 and 71 are outside the contact surface area or the substitutions in the duck lysozymes at these positions did not alter the protein hydrophobicity chromatographically. The ΔG_t for the (Ser → Gly) substitution at position 37 is −6.3 kJ/mol and +47.3 kJ/mol for the (Gly → Arg) substitution at position 71. The magnitude of these values would appear to indicate a significant change in hydrophobicity.

The possibility of a structural change resulting from the proline to arginine substitution must also be considered. The predicted conformational change by the method of Chou and Fasman is a β-turn to a random coil[15]. However, X-ray crystallography data of HEW, TKY, and human lysozymes indicate that there are no major changes in the backbone of these widely differing lysozymes[20,22]. Duck A and B lysozyme antibodies were also able to cross-react with duck C LYZ, indicating that the structure of duck C LYZ has not been drastically altered[15].

The log k' *versus* molality plots for the lysozymes all had identical slopes. In view of the hydrophobic interaction model of Melander and Horváth[8,9], the contact surface area of the lysozymes was the same. The amino acid substitutions only affected the intercept of the lines or the strength of the hydrophobic interaction.

By examining the effect of pH on the chromatographic behavior of each of the bird lysozymes it was possible to determine the ionization state of several histidine residues within the contact surface area at pH 7.0. His 103 of JPQ LYZ was unprotonated, while His 77 of RNP LYZ was more than half protonated. TKY LYZ has two histidine residues, at positions 41 and 121, which might account for the change in retention with pH. It is doubtful whether position 121 is within the contact surface area, since it is spatially distant from the positions that have influenced retention with the other lysozymes. Position 41 might be within the contact surface area. However, it is close to position 37, which duck B LYZ showed to be outside the contact surface area. Therefore, His 41 is probably just within the contact surface area and is uncharged at pH 7.0.

Alterations in the pH of the mobile phase affected the intercept of the log k' *versus* molality plots, but not the slope. This would indicate that the contact surface area was not altered. The ionization state of amino acids in the contact surface area appears to moderate the strength of the hydrophobic interaction. Therefore, not only hydrophobicity, but hydrophilicity affects HIC retention.

Chromatographic retention was only affected by substitutions on the lysozyme surface opposite the catalytic cleft and can be defined as a possible contact surface area between the protein and the stationary phase surface. This area extends from Residue 41 to 102 and from 75 to the α-helical region starting with Residue 89. Residues outside this area did not affect chromatographic retention. This area in-

cludes most of the external hydrophobic residues in HEW LYZ. It is possible that the protein may have more than one or several overlapping contact areas with the stationary phase surface during the chromatographic process.

The effect of salt composition on protein retention was examined to determine what factors or salt properties influence retention. Protein retention follows the order of the salts in the lyotropic series. Each salt has a calculated molal surface tension increment, and salts with higher values for this term produce longer protein retention.

According to the hydrophobic interaction model of Melander and Horváth[8,9], protein retention is linear with molal salt concentration and dependent upon the surface tension of the mobile phase. Graphs of log k' *versus* surface tension resulted in coincident lines for only two cases, sodium and ammonium sulfate for OVA, and ammonium and magnesium sulfate for LYZ. In all other cases, the sodium tartrate, sodium sulfate, and ammonium sulfate lines were fairly parallel and the magnesium sulfate line exhibited a steeper slope.

The fact that the lines did not coincide when equalized with respect to surface tension indicates that factors other than surface tension influence protein retention. One aspect which the Melander–Horváth model[8,9] does not account for is specific interactions between the salt ions and the protein, which may alter the protein chromatographic behavior.

Arakawa and Timasheff[27,28] have examined the preferential interactions of proteins with solvent components in concentrated aqueous solutions. They found that "salting-out" salts produce a large preferential hydration of proteins and, therefore, an exclusion of the salt ions. For sodium salts, it was found that preferential hydration was primarily a result of surface tension at the protein–solvent interface produced by the addition of the salt to the solution. This is in agreement with the cavity theory of Sinanoğlu and Abdulnur[10,11]. However, for magnesium sulfate and magnesium chloride, there was no correlation between preferential interactions and surface tension increments. For some salts the experimental value of salt exclusion was lower than that calculated from surface tension increment values.

According to Arakawa and Timasheff[28], the observed preferential interactions of salts with proteins should be considered in terms of salt binding and salt exclusion at the protein–solvent interface:

$$\left(\frac{\partial m_{\text{salt}}}{\partial m_{\text{protein}}}\right)_{T,\mu_1,\mu_3}^{\text{obsvd}} = \left(\frac{\partial m_{\text{salt}}}{\partial m_{\text{protein}}}\right)_{T,\mu_1,\mu_3}^{\text{excl}} + \left(\frac{\partial m_{\text{salt}}}{\partial m_{\text{protein}}}\right)_{T,\mu_1,\mu_3}^{\text{bind}} \tag{8}$$

where the two terms on the right side have opposite signs. Among magnesium salts, sulfate was found to contribute more to salt binding and protein stabilization than chloride. The salt binding with sodium salts was much less than with magnesium salts, giving a better correlation with surface tension increments.

The molal surface tension increments can be used to calculate the change in the chemical potential of the protein due to the increase in surface tension[28].

$$\left(\frac{\partial \mu_{\text{protein}}}{\partial m_{\text{salt}}}\right)_{T,P,m_{\text{protein}}} = N_{\text{av}} S_{\text{protein}} \left(\frac{\partial \sigma}{\partial m_{\text{salt}}}\right)_{T,P,m_{\text{protein}}} \tag{9}$$

where N_{av} is Avogadro's number, S is the surface area of the protein, and $\frac{\partial \sigma}{\partial m}$ is the

molal surface tension increment. The ratio of the experimental value to the calculated value is an indication of the contribution of surface tension to preferential interactions of the protein and salt. For the sodium salts, the ratio had a high value, *ca.* 0.70. For magnesium sulfate, however, the value was much lower, indicating that the magnesium ion overcomes the repulsion due to surface tension and penetrates the hydration layer.

With respect to Timasheff's observations, the deviation in protein retention in magnesium sulfate is probably due to salt binding and/or an alteration in protein structure. The deviation was not seen with LYZ, since LYZ has a high net positive charge at pH 7.0 and would exclude the magnesium ion. The elution behavior in sodium tartrate was the most deviant with LYZ, less so with CON, and only slightly deviant with OVA. The proteins may bind the tartrate ion, and the interaction may make the protein appear more hydrophobic than with the other salts. The greater deviation with LYZ could be correlated with the high positive charge of LYZ at pH 7.0. LYZ would, therefore, attract a greater number of tartrate ions. OVA has a net negative charge at pH 7.0 and would therefore, bind fewer tartrate ions and exhibit less deviation in sodium tartrate. In comparing the sodium and ammonium sulfate lines, the sodium ion probably results in a higher preferential hydration of the two proteins than the ammonium ion.

CONCLUSION

The solute and mobile phase effects examined in this study have further characterized the interaction of a protein with a HIC stationary phase surface. The effect of salt composition on protein retention is a very complex phenomenon. It appears to include the surface tension of the solution, any specific interactions between the protein and the salt ions which may or may not alter the protein structure, and the hydration of the protein. Hydrophilic as well as hydrophobic amino acids contribute to the overall hydrophobic interaction with a HIC stationary phase surface. The presence of a hydrophilic amino acid or a change in the charge of an amino acid within the contact surface area can decrease protein retention.

ACKNOWLEDGEMENTS

The authors thank Dr. Dale Deutsch, Dr. William Kopaciewicz, and Mrs. Mary Ann Rounds for their helpful discussions. This work was supported by a subcontract of NIH Grant GM 25431 and performed in the laboratory of Dr. Dale Deutsch at the State University of New York at Stony Brook.

REFERENCES

1 Z. Er-el, Y. Zaidenzaig and S. Shaltiel, *Biochem. Biophys. Res. Commun.*, 49 (1972) 383.
2 B. H. J. Hofstee, *Biochem. Biophys. Res. Commun.*, 91 (1979) 312.
3 S. Shaltiel and Z. Er-el, *Proc. Natl. Acad. Sci. U.S.A.*, 70 (1973) 778.
4 H. A. Arfmann and S. Shaltiel, *Eur. J. Biochem.*, 70 (1976) 269.
5 J. L. Fausnaugh, E. Pfannkoch, S. Gupta and F. E. Regnier, *Anal. Biochem.*, 137 (1984) 464.
6 J. L. Fausnaugh, L. A. Kennedy and F. E. Regnier, *J. Chromatogr.*, 317 (1984) 141.
7 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
8 W. R. Melander and Cs. Horváth, *Arch. Biochem. Biophys.*, 183 (1977) 200.

9 W. R. Melander, D. Corradini and Cs. Horváth, *J. Chromatogr.*, 317 (1984) 67.
10 O. Sinanoğlu, in B. Pullman (Editor), *Molecular Associations in Biology*, Academic Press, New York, 1968, pp. 427–445.
11 O. Sinanoğlu and S. Abdulnur, *Fed. Proc.*, 24(2) (1965) 12.
12 E. M. Prager and A. C. Wilson, *J. Biol. Chem.*, 246 (1971) 5978.
13 R. M. Parry, Jr., R. C. Chandan and K. M. Shahani, *Proc. Soc. Exp. Biol. Med.*, 119 (1965) 384.
14 E. M. Prager and A. C. Wilson, *J. Biol. Chem.*, 246 (1971) 523.
15 K. Kondo, H. Fujio and T. Amano, *J. Biochem.*, 91 (1982) 571.
16 R. E. Canfield and A. K. Liu, *J. Biol. Chem.*, 240 (1965) 1997.
17 M. Kaneda, I. Kato, N. Tomzinaga, K. Titani and K. Narita, *J. Biochem.*, 66 (1969) 747.
18 J. Jolles, I. M. Ibrahimi, E. M. Prager, F. Schoentgen, P. Jolles and A. C. Wilson, *Biochemistry*, 18 (1979) 2744.
19 J. N. LaRue and J. C. Speck, *J. Biol. Chem.*, 245 (1970) 1985.
20 C. C. F. Blake and I. D. A. Swan, *Nature New Biol.*, 232 (1971) 12.
21 S. H. Banyard, C. C. F. Blake and I. D. A. Swan, in E. F. Osserman (Editor), *Lysozyme*, Academic Press, New York, 1974, pp. 71–79.
22 R. Bott and R. Sarma, *J. Mol. Biol.*, 106 (1976) 1037.
23 B. W. Matthews, M. G. Grutter, W. F. Anderson and S. J. Remington, *Nature*, 290 (1981) 334.
24 T. Imoto, L. N. Johnson, A. C. T. North, D. C. Phillips and J. A. Rupley, in P. D. Boyer (Editor), *The Enzymes*, Vol. VII, Academic Press, New York, 1972, pp. 665–868.
25 B. Lee and F. M. Richards, *J. Mol. Biol.*, 55 (1971) 379.
26 G. Von Heijne, *Eur. J. Biochem.*, 116 (1981) 419.
27 T. Araka and S. N. Timasheff, *Biochemistry*, 21 (1982) 6545.
28 T. Arakawa and S. N. Timasheff, *Biochemistry*, 23 (1984) 5912.