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Explanation of Ionic Sequences in Various Phenomena. VI. The Formation and Destruction of Helices in Bovine Plasma Albumin in Neutral and Acidic Solutions

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SUMMARY

The structure of bovine plasma albumin (BPA) was examined by optical rotatory dispersion studies at both low (pH 1.5 and 2.0) and high (pH 9.0) pH values in various aqueous salt solutions. The resulting cationic sequences were compared to those observed by Pedersen for values of the sedimentation constant. At pH 9.0 the destruction of the "helix" produces an acidic sequence. The relative pH position of the "helix" transition, the fact that addition of salt increases the apparent helical content of BPA, and the observed acidic-type of sequence rule out the possibility of (1) ionic bonds between carboxylate and ϵ -amino groups, (2) hydrophobic bonds, or (3) hydrogen bonds between peptide linkages as major contributing forces in the formation of the helix. The stability of the "helix" in BPA between pH 3.0 and 9.0 must therefore be due to hydrogen bonds between carboxylate ions and hydroxyl groups such as those of serine, threonine, and tyrosine. Repulsive forces between the positively charged groups on BPA strengthen these bonds by preventing the expanded form of BPA from collapsing. At pH 2.0 two types of sequences were observed: The $s_{20,w}^0$, $[\alpha]_D$, and a_0 values

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gave an acidic-type cationic sequence. The b_0 (helix content), λ_c , and $[\alpha]_{233}$ values gave essentially a nonpolar sequence. The nonpolar or hydrophobic salting-out sequences show that the formation of hydrophobic bonds at pH 2.0 hinders the formation of the helix or folded structure. The acidic sequences show that hydrogen bonds between carboxylic acid groups stabilize both the apparent helix or helices and the intermolecular aggregation of the BPA molecules. From a comparison of the $s_{20,w}^0$ values and the helical content of BPA at pH 9.0 it is also concluded that the formation of these apparent helices or folded structures expands or stiffens the BPA molecule.

INTRODUCTION

By using a common anion or cation, cationic and anionic sequences can be obtained for polyelectrolytes by measuring the relative ability of the added ion to change the physical properties of the polyelectrolyte. It was previously pointed out [1] that such sequences can be used to detect the type of bonds being formed or destroyed in aqueous salt solutions. Such sequences are determined by the structure of the hydrated ion. Ions such as Li^+ and Na^+ have tightly bound water (A regions according to Frank and Wen [2] or positively hydrated water according to Samoilov [3]). The position of these ions varies in the cationic sequence because their A regions react differently to various hydrogen and hydrophobic bonds. If no tightly bound water exists on the cations, the sequence for such cations will always remain the same for hydrogen and hydrophobic bonds. The ability of such cations to destroy these bonds will be according to their effective dielectric constant (D_+). The greater the value of D_+ , the greater will be the cation's ability to destroy the hydrogen or hydrophobic bond [1, 4].

The A regions on the cations Li^+ and Na^+ , in addition to those on the divalent cations, interact favorably (salt in) with neutral nitrogen bases such as in peptide bonds but unfavorably (salt out) with polar hydroxyl groups such as those on neutral carboxylic acids ($\text{R}-\text{CO}_2\text{H}$), tyrosine ($\text{R}-\text{OH}$), or starch ($\text{R}-\text{OH}$) [1, 5]. The A regions have no effect on hydrophobic bonds, and hence in this case the ability of a cation to destroy this type of bond is related to the value of D_+ of its B region. Consequently, various cationic sequences can be obtained for these types of bonds. In addition, the formation of insoluble salt bonds (destruction of ionic bonds) or soluble ionic interactions (destruction of repulsive forces) with ionic groups on the polymers must also be taken into account [1].

In a previous paper [5] it was shown that an acidic sequence for the decrease in the sedimentation coefficient of bovine plasma albu-

min (BPA) at pH 2.0 is obtained from the data of Pedersen [6]. Hence the addition of salt at this low pH salts out the neutral carboxylic acids on BPA. A more detailed correlation of this sedimentation sequence will now be made by correlating the cationic sequence obtained from $s_{20,w}^0$ data with those sequences obtained from optical rotatory dispersion data.

EXPERIMENTAL

Equations

Optical rotatory dispersion data can be expressed either by the simple one-term Drude equation or by the Moffitt-Yang equation [7-10]. The one-term Drude equation is [9]

$$[\alpha]_{\lambda} = A/(\lambda^2 - \lambda_c^2) \quad (1)$$

where $[\alpha]_{\lambda}$ equals the specific rotation at the wavelength λ and λ_c is the critical wavelength. A plot of $[\alpha]_{\lambda} \lambda^2$ versus $[\alpha]_{\lambda}$ is made and the resulting slope yields λ_c .

The Moffitt-Yang equation is [9]

$$[m']_{\lambda} = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (2)$$

Here $[m']_{\lambda}(\lambda^2 - \lambda_0^2)$ is plotted versus $(\lambda^2 - \lambda_0^2)^{-1}$. The value of λ_0 is assumed in order to get the best straight line through the experimental data. The value of λ_0 is approximately $212 \pm 5 \text{ m}\mu$ [9] and this value ($\lambda_0 = 212$) was used in our calculations. The term $[m']_{\lambda}$ is defined as

$$[m']_{\lambda} = \frac{3}{n^2 + 2} \frac{\text{MRW}}{100} \quad (3)$$

where n is the refractive index of the solvent at wavelength λ and MRW is the mean residue weight of the polymer. The value of MRW used in this study was 118 g/mole. The fraction (f_H) of helix content in polymer can be calculated from the observed value of b_0 if it is assumed that $b_0 = b_0^H = -636$ for the complete helix using the equation $f_H = b_0^{\text{obs}}/b_0^H$. (See the review by Urnes and Doty [9].)

The two-term Drude equation has also been used. However, according to Yang [10], the Moffitt-Yang equation provides simple and at least semiquantitative estimate of the fraction of helix in proteins, but the two-term Drude equation brings in new unanswered problems, despite claims to the contrary. Consequently, the percentage of helix in BPA will be determined using the Moffitt-Yang equation.

Materials

The bovine serum albumin was obtained from Armour and Co., Lot No. T68204. The salts were obtained from K & K Laboratories, Inc. The molar concentration (M) of the guanidinium and lithium thiocyanate was obtained from the refractive index (n) of the aqueous solution according to the equation

$$M = (n - 1.3325)/A \quad (4)$$

The constant $A = 0.02521$ for guanidinium thocyanate (GSCN) and $A = 0.01606$ for lithium thiocyanate [11]. The percentage moisture in the BPA sample was obtained by dissolving the BPA in water and measuring the concentration with a Beckman Model DU spectrophotometer using $E_{1\text{cm}}^{1\%} = 6.67$ at $279 \text{ m}\mu$ [12].

Methods

The optical rotatory dispersion of the BPA in the various salt solutions was obtained on undialyzed samples with a Cary-60 recording spectropolarimeter using 1-cm cells at wavelengths of 600 to $200 \text{ m}\mu$ at 23°C . A solvent blank was subtracted from the observed rotation of the protein solution. The BPA solutions in acid and base mediums were centrifuged in a Spinco Model L preparatory centrifuge at 25,000 rpm for 30 min using a swinging bucket rotor. The bottom 80% of each tube was retained by using a syringe.

RESULTS AND DISCUSSION

Correlation of a_0 with Degree of Aggregation of Polymer

A comparison is given in Table 1 of the sedimentation constants of BPA (as obtained by Pedersen [6]) with the various constants and the apparent percentage of helix content (as obtained from optical rotatory dispersion studies at pH 2.0). The results show that the acidic sequence is obtained both for values of $s_{20,w}^0$ ($\text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{Cs}^+$) and for values of $[\alpha]_D$ and a_0 ($\text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{Cs}^+$). As noted previously [1], this acidic sequence can only be correlated with the salting out of the $\text{R}-\text{CO}_2\text{H}$ groups on the BPA. Moreover, the larger values of $s_{20,w}^0$ are due to either a contraction or to an aggregation of the swollen BPA molecules or both. It will now be shown that they must in part be due to an aggregation phenomenon involving hydrogen bonding between the $\text{R}-\text{CO}_2\text{H}$ groups of BPA molecules.

Jennings et al. [13] have recently studied the aggregation of poly(L-glutamic acid) (PGA) and observed that the formation of the aggregate containing four helical chains can be correlated with the

Table 1. Sedimentation and Optical Rotatory Dispersion Results of BPA at pH 2.0 in 0.1 M H_3PO_4 + 0.1 M NaH_2PO_4 plus 0.2 M of Added Salt^a

Added salt (0.2 M)	$s_{20,w}^0$	$[\alpha]_D$	a_0	b_0	% Helix	$[\alpha]_{233m\mu}^{\max}$	λ_c , m μ
LiBr	4.58	-48	-247	-247	39	-6100	253
NaBr	4.42	-49	-298	-152	24	-3900	241
KBr	4.29	-59	-367	-196	31	-5700	244
CsBr	4.07	-62	-372	-253	40	-6200	249

^aThe $s_{20,w}^0$ values were obtained from Fig. 7 of Pedersen [6]. The % helix was obtained assuming $b_H = 630$ in this table as well as in the others.

values of a_0 and $[\alpha]$. Both a_0 and $[\alpha]$ become more negative with increasing aggregation. Consequently, even though the value of a_0 may be effected by solvent interaction effects in addition to aggregation effects, their data show that the aggregation effect may in some cases be the major contributor to changes in a_0 . If their results on a_0 and $[\alpha]$ can be applied to BPA, the more negative values of a_0 and $[\alpha]$ should correspond with the larger values of $s_{20,w}^0$. However, examination of Table 1 shows the exact opposite: The CsBr solution gives the most negative values of a_0 and $[\alpha]_D$ and the smallest value of $s_{20,w}^0$. In all cases the same concentration of salt (0.2 M salt plus the phosphate buffer) was used. Moreover, the solvent was subtracted from the readings. Consequently, the changes in a_0 most likely are associated with the aggregation phenomenon. Yet the results show that the correlation observed by Jennings et al. [13] between a_0 and aggregation of PGA is exactly the reverse for BPA.

Further proof of this conclusion is seen in Table 2. At pH 1.5 the sedimentation-equilibrium molecular weights were found to be $\bar{M}_w = 125,000$ for 0.005 M GSCN and $\bar{M}_w = 121,000$ for 2.5 M GSCN, showing that at pH 1.5 the BPA is aggregated if the salt concentration is between 0.005 M GSCN and 2.5 M GSCN. In contrast with this, the low value of $\bar{M}_w = 75,500$ was obtained in 5.0 M GSCN. This latter value corresponds to unaggregated BPA as shown by our [14] previous studies ($\bar{M}_w = 73,000$ at pH 3.2 in 0.0005 M NaCl and $\bar{M}_w = 76,100$ at pH 1.8 in 0.1 M NaCl plus HCl). Yet the value of a_0 increases to more negative values in going from 0.5 M GSCN to

Table 2. Optical Rotatory Dispersion Results for BPA at pH 1.5 in Various Aqueous Salt Solutions

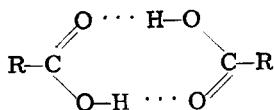
Solvent	$[\alpha]_D$	a_0	b_0	% Helix	λ_c , m μ
0.5 M LiCl	-59.2	-354	-373	59.2	259
0.5 M GSCN	-62.3	-370	-368	58.4	260
5 M LiSCN	-68.3	-428	-149	23.7	235
5 M GSCN	-75.0	-570	-120	19.1	238

5 M GSCN, as shown in Table 2. Again it is seen that the greater the value of a_0 , the lower will be its degree of aggregation. Consequently, it appears that a_0 values can be correlated with the degree of aggregation of a polymer. This correlation is not quantitative because other, more minor, factors must be involved. However, as seen in the examples of PGA and BPA, the degree of aggregation may correspond to an increase or a decrease in a_0 .

Formation of Aggregates at pH 2.0 by Production of Hydrogen Bonds between Carboxylic Acid Groups

Comparison of Table 2 with Table 1 indicates that the change in the value of $s_{20,w}^0$ with a change in cation is due in part to a change in the state of aggregation of the BPA. Only the addition of an anion should cause an electrostatic contraction in the volume of BPA, because at pH 2.0 or lower, only positive electrostatic charges exist on BPA. In Table 1 it is seen that the same anion (Br^-) is used and that its concentration is kept constant. Therefore, the positive charges on BPA must maintain the same repulsive force in all these salt solutions. Consequently, because only positive electrostatic charges exist on BPA, a variation in the cation at constant ionic strength cannot vary the ability of the salt to reduce the electrostatic repulsive forces on BPA. Hence the only manner in which the variation in salting out of $\text{R-CO}_2\text{H}$ groups can occur on BPA is by means of aggregation of BPA molecules or, as will be shown below, a change in the apparent helical content of the BPA. Thus it is concluded from the above molecular weight studies that the acidic sequence for $s_{20,w}^0$ values at pH 2.0 as obtained by Pedersen [6] is caused in part by an aggregation of BPA molecules. Intermolecular rather than intramolecular bonds can thus be exemplified by the a_0 and $[\alpha]$ cationic sequence. The type of intermolecular bonds formed

between BPA molecules to give such an acidic sequence would most likely be those between neutral carboxylic acid groups:



The formation of these bonds is enhanced by the addition of anions which reduce the electrostatic repulsive forces of the positively charged groups. The effect can be seen by the anionic sequence as discussed previously [5].

Formation by Hydrophobic Bonds Decreases the Apparent Helical Content of BPA at pH 2.0

If the b_0 and λ_C constants are now considered, it is seen that the sequence is not acidic but rather is essentially a nonpolar sequence. These changes in b_0 may be correlated to changes in the helical content or folded structure of the BPA molecule. That is, a change in b_0 does not necessarily mean a change in helical content but may be due to conformational changes caused by destruction of folded structures. Consider, for example, the apparent percent helix content of BPA in these solvent systems. The apparent percent helix as obtained from the observed b_0 value varies according to the cationic sequence $\text{Na}^+ < \text{K}^+ < \text{Li}^+ \leq \text{Cs}^+$. This sequence, which is essentially nonpolar, occurs according to the effective dielectric constant of the ion. The Cs^+ ion has the greatest ability to destroy hydrophobic bonds, whereas the Na^+ ion has the least [1, 5]. Thus the data of Table 1 indicate that the apparent percentage of helix in BPA is increased with an increase in the destruction of hydrophobic bonds. The helices or folded structures of BPA are therefore not stabilized by such bonds. Consequently, if the above interpretation of b_0 is correct, the formation of hydrophobic bonds thus prevents the BPA chains from forming the folded or helical structure.

The position of the Li^+ ion in the sequence is too close to that of the Cs^+ ion to make the sequence entirely a hydrophobic sequence [1, 5]. Consequently, the A region on the Li^+ ion must be effective in forming helical or folded structures. The position of the Li^+ ion therefore indicates that the salting out of the carboxylic acid groups further enhances the formation of these apparent helices. However, if hydrogen bonds between peptide linkages were being destroyed, the sequence would be basic and not hydrophobic. Consequently, the apparent helices exist at these low pH values and their stability is not due to hydrophobic bonds. Rather their stability must be due to hydrogen bonds between peptide linkages and/or hydrogen bonds between carboxylic acid groups. If hydrogen bonds between peptide linkages are involved, these bonds must be protected from the Li^+

ion by closely associated positive charges on the BPA molecule. In other words, the positive charges on BPA must gather around each peptide bond. Therefore, it is more reasonable to conclude that the apparent helices as well as the aggregate are stabilized by means of hydrogen bonds between carboxylic acid groups. However, the important point is that in the formation of these helices or folded structures, hydrophobic bonds must first be destroyed.

Further Evidence for the Formation of Carboxylic Acid Hydrogen Bonds at pH 2.0

Comparison of the results in Table 2 with those in Table 1 shows that by decreasing the pH, the apparent helical content of BPA is increased. This result further substantiates the above conclusion that the apparent helices at low pH values are stabilized by hydrogen bonds between carboxylic acid groups because more RCO_2H groups exist at this lower pH. Moreover, because of the strong A regions on the Li^+ ion, the LiCl can destroy hydrogen bonds between peptide linkages but instigates the formation of hydrogen bonds between carboxylic acid groups [1]. If hydrogen bonds between peptide linkages held the helix together, then the addition of LiCl would destroy such bonds. Hence hydrogen bonds between peptide linkages do not stabilize the helices or folded structures in these acidic solutions. This result again shows that neutralization of the positive charges on BPA as well as destruction of its hydrophobic bonds are important steps in the formation of such structures.

In the 5 M GSCN solution, both the G^+ and the SCN^- ions can destroy hydrogen bonds because their effective dielectric constants are greater than that of water. Consequently, at this molarity, where the hydrogen bonds between RCO_2H groups are virtually engulfed with these B regions, the apparent helices and aggregate are destroyed. However, for the 5 M LiSCN solution the salting out effect of the A regions for $\text{R-CO}_2\text{H}$ groups and the low value of D_+ for the B regions of the Li^+ ions is seen. That is, there is more apparent helix content in BPA in the 5 M LiSCN solution than in the 5 M GSCN solution. A summary of the ionic sequences observed for BPA in these acidic solutions is given in Table 3.

Existence of Hydroxyl-Carboxylate Bonds between pH 3 and 9

Leonard et al. [12] examined the optical rotatory dispersion effects of BPA between pH 6 and 11. They observed that at about pH 7.5 to 9.0 the BPA undergoes structural changes. These structural changes produce variations in the constant a_0 and $[\alpha]_{313}$ as well as in b_0 or the apparent helical content. As the pH is increased from 7.5 to 9.0, there is an increase in the values of a_0 (-339 to -328), $[\alpha]_{313}$ (-580 to -560), and b_0 (-251 to -240) [12]. As noted above, both the data of Jennings et al. [13] for the aggregation of

Table 3. Ionic Sequences for BPA at pH 1.5, 2.0, and 9.0

Molarity of salt	pH	Measurement	Ionic sequence	Type of sequence
0.2	2.0	$s_{20,w}^0, [\alpha]_D, a_0$	$Li^+ > Na^+ > K^+ > Cs^+$	Acidic
		% helix	$Na^+ < K^+ < Li^+ \leq Cs^+$	Nonpolar
		$[\alpha]_{233m\mu}^{max}$	$Na^+ < K^+ < Cs^+ < Li^+$	Nonpolar
		λ_c		
0.5	1.5	$[\alpha]_D, a_0$	$LiCl < GSCN$	—
		% helix	$LiCl > GSCN$	—
5.0	1.5	$[\alpha]_D, a_0$	$Li^+ < G^+$	
		% helix	$Li^+ > G^+$	
1.0	9.0	a_0	$Li^+ < Na^+ < K^+ < Cs^+$	Acidic
		% helix	$Li^+ > Na^+ > K^+ > Cs^+$	Acidic
		$[\alpha]_{233m\mu}^{max}$	$Li^+ > Na^+ = K > Cs^+$	Acidic
		$s_{20,w}^0$	$Li^+ > NH_4^+ > Cs^+$	Acidic

PGA and our acidic cationic sequence for the aggregation of BPA (Table 1) show that the change in the value of a_0 can be associated with a change in the degree of aggregation of polymer chains. Consequently, the a_0 and $[\alpha]$ data of Leonard et al. [12] indicate that a change in the association of the peptide chains of BPA is occurring in this pH range. This change may be associated with the unmasking of hydrophobic groups as postulated by them [12]. And as seen below, hydroxyl-carboxylate bonds are involved in stabilizing the "helix" at neutral pH. Consequently, the "unmasking" would be the release of tyrosyl, serine, or threonine residues by destruction of its hydrogen bond with carboxylate groups. The decrease in the degree of helical content may therefore be associated with a destruction of a specific type of conformation which is stabilized by the tyrosine-carboxylate, serine-carboxylate, plus threonine-carboxylate bonds. These points will not be investigated more thoroughly.

As seen in Figs. 1, 2, and 4 of Leonard et al. [12], an increase in the amount of salt at pH 9.0 (from 0.1 to 1.0 M KCl or from 0.02 to 0.10 M KCl) decreases the change in $[\alpha]_{313}$. In other words, not only does the increase in salt concentration shift the transition to a lower pH value, but also the increase in salt concentration decrea-

Table 4. Optical Rotatory Dispersion Results on BPA at pH 9.0 in Various Salt Solutions

Solvent	$[\alpha]_D$	a_0	b_0	% Helix	$[\alpha]_{233m\mu}^{\max}$	λ_c , m μ
1 M LiCl	-59.4	-339	-379	60.2	-9620	258
1 M NaCl	-60.0	-341	-365	58.0	-9540	261
1 M KCl	-59.2	-343	-363	57.6	-9550	259
1 M CsCl	-59.4	-350	-356	56.5	-9360	257
4 M CsCl	-60.0	-350	-376	59.7	—	259
7 M CsCl	-57.8	-312	-382	60.6	—	261

ses the magnitude of the change at pH 9.0. The dependence of both the magnitude of the change and its pH position indicate that ionic groups are involved. Therefore, the optical rotatory dispersion of BPA was examined in alkali solutions to clarify both the role of ionic groups and the role of a_0 (aggregation) in this phenomenon.

In Table 4 the changes occurring at pH 9.0 for various salt solutions are recorded. The variations in $[\alpha]_D$ and λ_c are not large enough to give a discrete sequence. However, if one examines the variations of a_0 , $[\alpha]_{233}$, and b_0 (or per cent helix), the same type of cationic sequence (acidic) is obtained in all three cases (see Tables 3 and 4). The results show that the Li^+ ion promotes the formation of the helical or folded structures more than any other cation examined. Now, the acidic cationic sequence at this pH value can only be correlated with three types of forces: hydroxyl-carboxylate bonds, ion-ion bonds, or electrostatic repulsive forces between carboxylate groups. (Repulsive forces between $\epsilon\text{-NH}_3^+$ groups exist, as will be shown below.) The existence of the ion-ion bond between carboxylate and lysine groups has been suggested recently by Vijai and Foster [15]. If the transition caused by addition of a cation is due to the reduction of electrostatic repulsive forces, it should occur at a lower pH, where the carboxylic acid group becomes ionized. The position of the transition on the pH scale therefore associates it with either hydroxyl groups of the ϵ -amino groups of lysine residues.

In the case of tyrosine, serine, or threonine groups, the Li^+ ion would salt such a bond out of solution more than the other cations. In other words, each of these three amino acids possesses a hydroxyl group which can become associated with the negatively charged carboxylate ion. Such bonds would be enhanced by the A regions of the Li^+ ions. Hence the Li^+ ion would tend to stabilize these hydroxyl-carboxylate bonds.

In the case of the ϵ -amino group, the Li^+ would not salt out but rather would compete with the $\epsilon\text{-NH}_3^+$ group for the R-CO_2^- group. In other words, the Li^+ ion would destroy the salt bond. Furthermore, the Li^+ ion would destroy such an ion-ion bond more than the other cations listed in Table 4 because it forms the most insoluble carboxylate salt [1]. Consequently, if the group that is bonded to these carboxylic acid groups is the $\epsilon\text{-NH}_3^+$ group of the lysine residue as suggested by Vijai and Foster [15], then the destruction of this ion-ion bond increases the helix content of the BPA.

But the data of Leonard et al. [12] and the data in Table 4 for the 1 M, 4 M, and 7 M CsCl solutions show that the addition of salt tends to increase the helix content of the BPA back to what it originally was before the addition of alkali. In other words, the destruction of the ion-ion bond by alkali titration decreases the helix content, and the destruction of the ion-ion bond by addition of salt increases the helix content of BPA. These two conflicting statements show that the observed acidic sequence cannot be due to the existence of a bond between the carboxylate and $\epsilon\text{-NH}_3^+$ groups on BPA. Furthermore, the studies reported in Table 4 were done at pH 9.0. Yet this is where the transition due to titration of one of the groups is almost complete. The addition of salt therefore forces the reformation of the bond, and consequently the bond cannot be an ionic bond. It is concluded, therefore, that the acidic sequence observed at pH 9.0 and the transition observed by Leonard et al. [12] is due to the formation of hydroxyl-carboxylate bonds. The titration of the $\epsilon\text{-NH}_3^+$ groups of BPA must therefore weaken the hydroxyl-carboxylate bond by changing the BPA conformation. The addition of salt strengthens these bonds by salting out the polar hydroxyl group of the hydroxyl-carboxylate bond. Moreover, it is concluded from the observed acidic sequence for the helical content in BPA that this hydrogen bond is necessary for the formation and stabilization of the BPA "helix" at neutral pH values. The change in a_0 values must also be related to this association of the peptide chains in BPA, as discussed above.

Further Evidence for the Hydroxyl-Carboxylate Bond at pH 3 to 9 and Its Stabilization by Either the Addition of Salt or the Retention of the Expanded Structure (Retention of $\epsilon\text{-NH}_3^+$ Groups)

Further evidence also substantiates the above conclusions concerning the formation of a hydroxyl-carboxylate bond in BPA. The data of Foster and Clark [16] and Vijai and Foster [15] show that approximately 40 carboxylate groups are associated with either hydroxyls or cationic amino groups. In conjunction with this, Williams and Foster [17, 18] have shown that BPA undergoes a shift in absorption spectrum of the tyrosine group when the carboxylate ion is titrated. The above observed acidic cationic sequence indicates that the tyrosine-carboxylate bond may exist in BPA and that

the spectral perturbation is a manifestation of the destruction or formation of this bond. Furthermore, the carboxylate group could also interact with the hydroxyl groups of serine and threonine. There are approximately 19 tyrosine, 28 serine, and 29 threonine groups per molecule of BPA [19]. This gives a total of 76 hydroxyl groups, which is more than sufficient to account for the 40 abnormal carboxylate groups observed by Vijai and Foster [15]. The particular groups involved in those hydroxyl-carboxylate interactions cannot, however, be specified by these experiments. And it may very well be that tyrosine does not interact with the carboxylate group as suggested by Williams and Foster [18]. In other words, serine and threonine may be the groups which interact with the 40 abnormal carboxylate groups.

Pedersen [6] also examined the effect of salt on the sedimentation coefficient of BPA at pH 9.0. He observed that lithium salt gives a lower value for the sedimentation constant, $s_{20,w}^0$, than NH_4^+ or Cs^+ salt ($\text{Li}^+ < \text{NH}_4^+ < \text{Cs}^+$). Because the position of the NH_4^+ should be approximately equal to that of the K^+ ion [1, 4], this sequence for the effect of cations of BPA $s_{20,w}^0$ values is acidic, just as in the above optical dispersion studies. The results indicate that the formation of the apparent "helix" involves an expansion or stiffening of the BPA molecule assuming that its molecular weight remains constant. Because the $s_{20,w}$ values are extrapolated to zero polymer concentration, any electrostatic effects will be canceled because of reduction (reversal) of charge effects, and the effects studied by Nagasawa and Eguchi [20] and Pedersen [6] at zero polymer concentration can be correlated with different conformations of the polymer. That is, the ratio of salt to BPA becomes large enough to cancel ionic but not conformational effects. Consequently, the above acidic sequence can be correlated with an expansion of the BPA molecule.

Formation of "Helix" Produces Expanded Form of BPA at Both pH 9 and 2

A comparison can be made with the results at pH 2.0 and those at pH 9.0 with regard to $s_{20,w}^0$ values and "helix" content. The sequences show the following:

$$\begin{array}{l} \text{K}^+ \text{ has less "helix" } \\ \text{and greater } s_{20,w}^0 \text{ values} \end{array} \left\{ \begin{array}{l} s_{20,w}^0 \text{ values: } \text{K}^+ > \text{Cs}^+ \\ \text{"helix" content } \text{K}^+ > \text{Cs}^+ \end{array} \right\} \text{ pH 2.0}$$

$$\begin{array}{l} \text{K}^+ \text{ has more "helix" and } \\ \text{smaller } s_{20,w}^0 \text{ values} \end{array} \left\{ \begin{array}{l} s_{20,w}^0 \text{ values: } \text{K}^+ < \text{Cs}^+ \\ \text{"helix" content: } \text{K}^+ > \text{Cs}^+ \end{array} \right\} \text{ pH 9.0}$$

where the sequence K^+ to Cs^+ always represents an increase in the value of D_+ [4]. The results are similar in one respect. At pH 2.0

an increase in the apparent "helix" content is associated with a decrease in $s_{20,w}^0$, and at pH 9.0 an increase in the apparent "helix" content is also associated with a decrease in $s_{20,w}^0$. However, at pH 9.0 the percentage of helix content in BPA decreases as the value of D_+ increases ($K^+ > Cs^+$ in helix content), whereas at pH 2.0 the per cent helix increases as the value of D_+ decreases ($K^+ < Cs^+$ in helix content). Moreover, at pH 2.0 the value of $s_{20,w}^0$ decreases as the value of D_+ increases because the greater the value of D_+ , the greater will be the cations ability to destroy the aggregates. On the other hand, at pH 9.0 the opposite effect occurs ($s_{20,w}^0$ increases as D_+ increases) because now the greater the value of D_+ , the greater will be the cations ability to destroy the "helix". In other words, at pH 2.0 the major effect of the salt is the destruction of bonds (hydrophobic) so that the "helix" can form. At pH 9.0 the major effect is the destruction of hydrogen bonds so that the "helix" cannot form. The results at pH 9.0 therefore show that the destruction of the apparent helix or folded structure results in a contraction of the BPA molecule, or, vice versa, the formation of the apparent "helix" results in an expansion or stiffening of the BPA.

If this conclusion is now applied to the pH 2.0 results, one would expect that the formation of the apparent helix or helices at pH 2.0 will stiffen or expand the BPA and hence lower the value of $s_{20,w}^0$. As seen in the relation given above, this is what actually occurs. The Cs^+ ion increases the apparent helix content (stiffens or expands the BPA) and hence decreases the value of $s_{20,w}^0$. The results at pH 2.0 therefore indicate that the increase in $s_{20,w}^0$ is not due entirely to an aggregation of the BPA but must also be due to the contraction of the BPA molecules because of a destruction of the apparent helices. It should also be emphasized that the relationship between the ionic sequence and $s_{20,w}^0$ values at pH 2.0 is such that intermolecular aggregates could form (an increase in D_+ decreases $s_{20,w}^0$) but that at pH 9.0 the relationship is not conducive to the formation of intermolecular aggregates (an increase in D_+ increases $s_{20,w}^0$).

CONCLUSIONS

This study shows that the apparent helices in BPA are stabilized by hydroxyl-carboxylate hydrogen bonds between pH 3 and 9. The titration of the $\epsilon-NH_3^+$ on BPA weakens these bonds because it allows the BPA to contract more readily. Repulsive forces between the positively charged groups of BPA therefore strengthen these hydroxyl-carboxylate bonds. Addition of salt (cations) increases the strength of these bonds by salting out the hydroxyl group.

The formation of these hydrogen bonds increases the apparent helical content of the BPA according to the value of b_0 . However,

since this increase in apparent helical content is actually quite small [12], the b_0 term may be a measurement of the twisting of the BPA chains rather than the formation of a tight α helix. There still remains a relatively large amount of apparent helical content in BPA after the hydroxyl-carboxylate bonds are destroyed. This latter helix would not be stabilized by ionic bonds or by hydrogen bonds involving ionic groups. Rather the stability may be due to hydrogen bonds between peptide units. Further studies are needed to clarify this.

As the pH is lowered to 2.0 or 1.5, an apparent helix structure is again formed in BPA. The formation of this helix is hindered by the formation of hydrophobic bonds and is stabilized by the formation of hydrogen bonds between titrated carboxylic acid groups. Moreover, the addition of salt will also reduce the electrostatic charge of the BPA because the anion of the salt will become associated with the BPA. The more soluble the anion (as a counterion to the BPA), the greater will be the reduction in charge [5]. Therefore, the anion effect may also influence the apparent helical content. In other words, the greater the reduction of the positive electrostatic charge (reversal of charge), the greater will be the apparent helical content of the BPA at pH 2.0 or lower. The effect of the anion was not considered in this paper and therefore this postulated effect on the helical content of BPA has not been confirmed.

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