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APPLICABILITY OF THE POTENTIOMETRIC TITRATION METHOD TO THE ANALYSIS OF THE EXPANSION PROCESS OF BOVINE PLASMA ALBUMIN IN ACIDIC SOLUTIONS

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To study the expansion process of bovine plasma albumin in acidic solutions, observed potentiometric titration curves at three different ionic strengths were compared with theoretical curves, using the radii of the protein determined by small angle X-ray scattering (SAXS). From the comparison, it was concluded that the expansion is completed via two different transitions and that the conformation of the protein before the first transition is stable and common at all ionic strengths, whereas the form of the protein becomes a more swollen and unstable one after the first transition. Moreover, the charge-independent part of the standard free energy change, ΔG^0, in the first transition was estimated from the potentiometric titration curves. The numerical value of ΔG^0 is 2350 ± 50 cal/mol, which is very small compared with the corresponding one for ordinary biopolymers.

1. Introduction

It was already demonstrated that the theoretical potentiometric titration curves, derived from the solution of the Poisson–Boltzmann equation without the Debye-Hückel approximation, shows satisfactory agreement with the observed potentiometric titration curves of various ionic biopolymers, if there is no change in their conformations [1,2]. Moreover, it was also shown that the potentiometric titration is useful for clarifying the mechanism of conformational change of bipolymers and for determining the non-electrostatic part of free energy change accompanied by the conformational change of ionic biopolymers [1,3].

In 1952, Tanford [4] suggested from potentiometric titration studies that albumin molecule undergoes rapid and reversible expansion as the pH-level is lowered from 4 to 2. Aoki and Foster [5] made the same suggestion from electrophoretic behavior of albumin, and they termed the expansion "N-F isomerism" and "Acid expansion". Afterwards, quite a few studies have been published to investigate the expansion process or the exact structure of the protein at various pH-levels. Yang and Foster [6] followed the expansion process by viscosity and optical rotational studies, and Tanford et al. [7] by viscosity and potentiometric titration studies. Thus, the qualitative feature of the expansion of albumin is now well clarified. Quantitatively, however, the opinions of those researchers do not appear to be always conclusive.

The purpose of this paper is to examine the applicability of the potentiometric titration method to the analysis of the expansion process of bovine plasma albumin. The expansion process of the samples were followed by potentiometric titration and small angle X-ray scattering (SAXS). The radii of gyration of the samples were determined by SAXS. The electrostatic potential at the surface of the protein was computed from the Poisson–Boltzmann equation without the Debye-Hückel approximation [8], and then the expected potentiometric titration curves were compared with observed ones.

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2. Experimental

2.1. Materials and preparations

Crystallized BPA was purchased from the Armour Pharmaceutical Co. (Lot M8A 5417) and Miles Lab. Inc. (Lot 33 and 34). Difference in the quality of the protein between lots and between suppliers was assumed to be insignificant for the present purpose. In order to protect against the denaturation during preparation and storage in a cold room; all samples were dissolved in 0.15 M NaCl solution including 0.02% sodium azide. Just prior to use, sample solutions were deionized on a Dintzis column [9], and a measured amount of concentrated NaCl solution was added, as rapidly as possible, to the solution in order to adjust the salt concentration to a desired value.

The removal of polymeric forms of BPA and fatty acids from commercially available BPA (referred to as "original BPA") was performed by the following procedure: About 2 g of original BPA was dissolved in 60 ml of 0.15 NaCl solution including 0.02% sodium azide, and fractionated by column of Sephadex G-150 (Pharmacia Fine Chemicals) [10]. The protein concentration of each eluent (ca. 7 ml) was determined by UV absorption at 279 nm using the extinction coefficient $E^{1\%}_{1\ cm} = 6.67$ [11]. The elution curve thus obtained has peaks at 50 and 77 in fraction number, as shown in fig. 1. The ratio of monomeric BPA to polymeric one in the fractions was determined by SDS gel electrophoresis [11], as shown by a broken line in fig. 1. From the graph the content of the polymeric components in the original BPA was estimated to be approximately 10%. The solution of pure monomeric BPA was concentrated and desalted by ultrafiltration apparatus Amicon ultrafiltration cell Model 202) in a low temperature room (ca. 4°). The resultant solution was defatted with charcoal by the procedure of Sogami and Foster [12]. BPA thus purified is referred to as "treated BPA" in this paper. SDS gel electrophoresis patterns of the original and treated BPA and also of the polymeric component are shown in fig. 2.

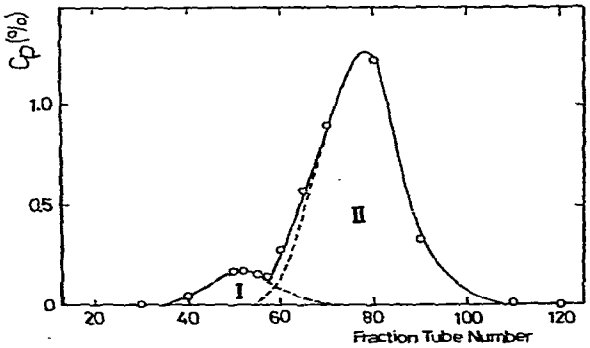

Fig. 1. Fractionation of commercial BPA by Sephadex column. The solid line is an observed elution curve. A portion of this line in the overlapped region was resolved into two broken lines, one belonging to polymeric BPA (I) and the other belonging to monomeric BPA (II), from analysis of SDS gel electrophoresis data.

2.2. Potentiometric titration

Measurements of pH of the protein solutions were carried out at 25 ± 0.1° with a Beckman Research pH meter and NBS pH standards in the atmosphere of argon. The salt molarities used were 0.01, 0.03 and 0.15 M. Salt concentrations were brought to the desired values by addition of measured volumes of concentrated NaCl solutions. The concentration of the sample solutions ranged from 0.15 to 0.77 g/dl.

2.3. Small angle X-ray scattering

The small angle X-ray scattering measurements were performed with a Kratky U-slit camera of Anton Paar Co. The X-ray source was a water-cooled copper

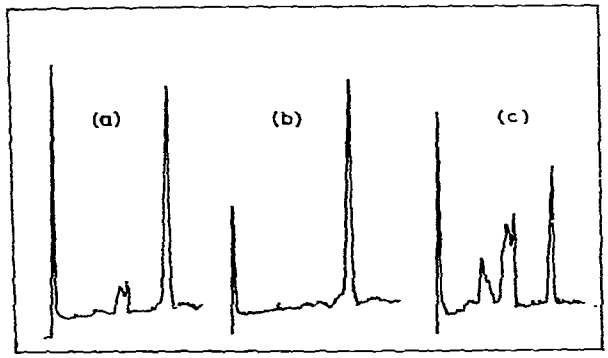

Fig. 2. Patterns of gel electrophoresis of original BPA (a), treated BPA (b) and polymeric BPA (c). A sharp peak at the most left in each pattern shows the absorption by one starting end of a SDS gel rod, i.e., a starting line in this gel electrophoresis experiment.

anode tube operated at 45 kV and 35 mA powered by a JEOL (Japan Electron Optics Laboratory Ltd.) X-ray generator Model DX-GE-2D. The temperature of cooling water was regulated near 30° to the accuracy of 1° and the room temperature was kept at 25 ± 1° in order to gain a stable X-ray intensity. The fluctuation of primary beam intensity was within 0.05% during a measurement.

The widths of entrance and counter slits were 60 and 150 μm, respectively, and the distance between a sample and the plane of registration was 208 mm. The alignment procedure was carried out following the instruction manual from Anton Paar Co. For measurement of the scattered intensity, a scintillation counter was used in connection with a nickel filter and a differential pulse-height analyzer (Philips PW 4280/01) set to receive the 1.54 Å CuKα radiation. The scattered intensity of the sample was measured by the step-scanning method by means of an automatic step controller of Anton Paar Co.

A thin-walled quarz-glass capillary, whose inside diameter was 1.95 mm, was used for a sample cell. Concentrations of the protein ranged from about 0.4 to 3 g/dl, and a concentration of added salt (NaCl) was 0.15 M.

In the perfect collimation system, the scattering intensity $I(\theta)$, at sufficiently small angles, is related to the apparent radius of gyration $\langle s^2\rangle_{app}^{1/2}$ of particles as follows [13]:

$$I(\theta) = I(0)\exp\left(-\frac{4\pi^2\langle s^2\rangle_{app}\theta^2}{3\lambda^2}\right), \qquad (1)$$

where θ is the scattering angle and λ is the wavelength of X-ray (1.54 Å for CuKα line). At a small angle, θ can be regarded as approximately equal to m/L, where m is the distance between the center of gravity of primary beam and a scattered point in the plane of registration and L is the distance between a sample and the plane of registration. Therefore, $\langle s^2\rangle_{app}$ can be obtained from the initial slope of the Guinier plot, $\ln I(m)$ versus m^2. The true radius of gyration, $\langle s^2\rangle^{1/2}$ of a particle at infinite dilution can be determined utilizing the linear relationship between $\langle s^2\rangle_{app}^{1/2}$ and sample concentration C, as pointed out by Anderegg et al. [14].

2.4. Numerical computations

The Schmidt process [15] of converting smeared scattering intensity into desmeared one (perfect collimation intensity) was carried out at Nagoya University Computer Center using a Facom 230-60/75 electronic computer. The numerical solution of the Poisson–Boltzmann equation was obtained by the same computer using a program of Emerson and Höltzer [16].

3. Results

The potentiometric titration curves at different concentrations of NaCl were obtained by the method of Tanford [17]. The ionization degree of carboxyl group α can be estimated from the graph and then the apparent ionization constant of a carboxylic acid group $pK \equiv pH + \log[(1-\alpha)/\alpha]$ is obtained as a function of α or the net charge of the protein Z.

In fig. 3 are plotted the values of pK against α or Z at three different ionic strengths. The titration curve differs with the protein concentration, but the

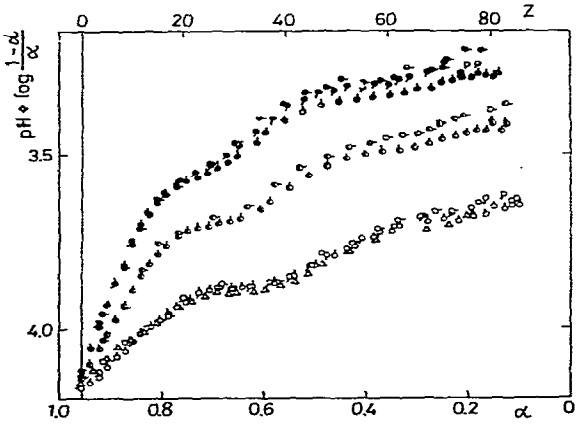

Fig. 3. Potentiometric titration curves for the carboxylic acid groups of original BPA (shown by circles) and treated BPA (shown by triangles) at various protein concentrations C_p (g/dl) and NaCl concentrations C_s (M). (1) Filled circles show the data at $C_s = 0.01$; ●, $C_p = 0.772$. ◕, $C_p = 0.386$. ◔, $C_p = 0.154$. (2) Half-filled circles show the data at $C_s = 0.03$; ◐, $C_p = 0.772$. ◔, $C_p = 0.154$. (3) Open symbols show the data at $C_s = 0.15$; ○, $C_p = 0.772$. ◴, $C_p = 0.386$. ◔, $C_p = 0.154$. △, $C_p = 0.538$.

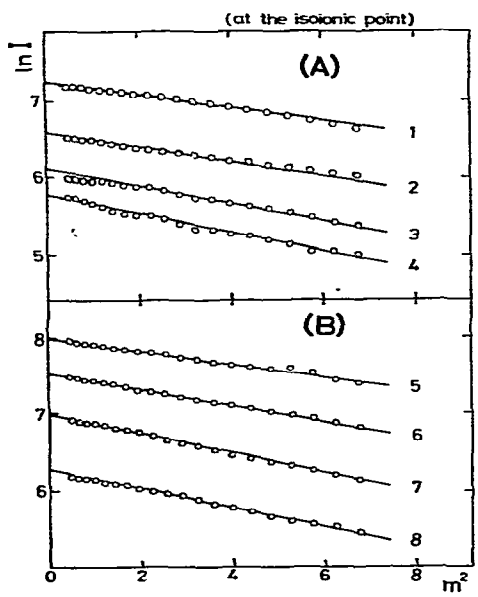

Fig. 4. Guinier plots of SAXS data for treated BPA (A) and original BPA (B) at the isoionic point. Protein concentrations: 1, 2.23; 2, 1.11; 3, 0.05; 4, 0.38; 5, 4.57; 6, 2.29; 7, 1.14; 8, 0.57 w/v %.

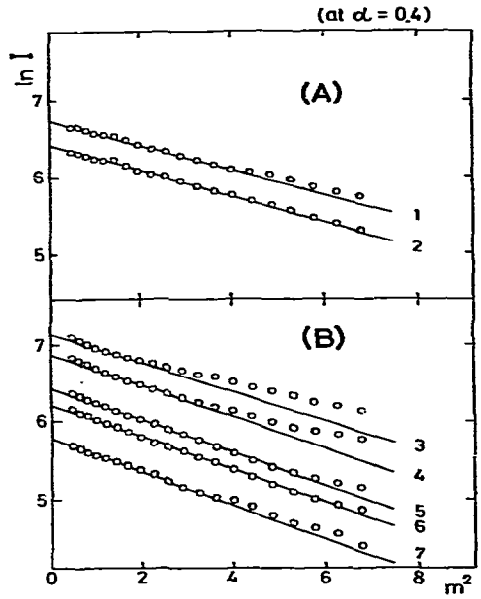

Fig. 5. Guinier plots of SAXS data for treated BPA (A) and original BPA (B) at $\alpha \approx 0.4$. Protein concentrations: 1, 1.08; 2, 0.72; 3, 2.84; 4, 1.89; 5, 1.16; 6, 0.95; 7, 0.47 w/v %.

protein concentration dependence is minor for the present purpose as seen in fig. 3. Comparison between the data of the original sample and treated one at $I = 0.15$ in fig. 3 shows that the difference in their titration curves is also negligibly small. From the ordinate at $Z \approx 0$, the intrinsic ionization constant of carboxylic acid group can be estimated to be 4.18, 4.15 and 4.12 at three ionic strengths, 0.15, 0.03 and 0.01, respectively, which are comparable to the corresponding values reported by Tanford et al. [18] 4.02, 3.95 and 3.92, respectively. However, it should be noted that the titration behavior of BPA is considerably different from that reported by Tanford et al. [18].

X-ray scattering intensities of both original and treated BPA molecules in 0.15 M NaCl solution at the isoionic point are shown in the form of Guinier plot in fig. 4. The observed points from both original and treated samples form straight lines over all employed protein concentrations and up to a higher scattering angle (ca. 0.7°). Similar plots at $\alpha \approx 0.4$ ($Z \approx 56$) and $\alpha = 0.05$ ($Z = 90$) are shown in figs. 5 and 6. In both figures, the observed points deviate from straight lines giving initial slopes as the scattering angle increases if the protein concentration is beyond 1 g/dl. The deviation is more marked in the original BPA than in treated one. However, as the concentration of the sample decreases, this deviation becomes smaller and, at sufficient dilution, the observed points form straight lines. Therefore, the radius of gyration of BPA was estimated from the initial slope in all three cases, i.e., at the isoionic point, $\alpha = 0.4$ and $\alpha = 0.05$.

In fig. 7 is plotted the apparent radius of gyration at finite concentrations, $\langle s^2 \rangle_{app}^{1/2}$, estimated at each concentration of the sample, against its concentration, C. It is seen that the linear relationship between $\langle s^2 \rangle_{app}^{1/2}$ and C holds satisfactorily at lower sample concentrations. The true radius of gyration at the infinite dilution, $\langle s^2 \rangle^{1/2}$, can be obtained as the intercept at $C = 0$. For original samples, the values $\langle s^2 \rangle^{1/2}$ are estimated to be 32, 42 and 44 ± 1 Å at the isoionic point, $\alpha = 0.4$ and $\alpha = 0.05$, respectively, and the corresponding values for treated samples are estimated to be 31, 38 and 42 ± 2 Å, respectively. This

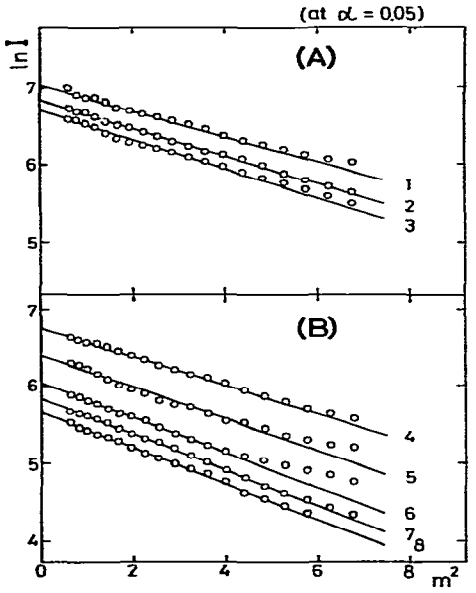

Fig. 6. Guinier plots of SAXS data for treated BPA (A) and original BPA (B) at $\alpha = 0.05$. Protein concentrations: 1, 1.72; 2, 1.29; 3, 1.08; 4, 2.27; 5, 1.51; 6, 0.95; 7, 0.63; 8, 0.47 w/v %.

difference in the values of $\langle s^2 \rangle^{1/2}$ between original and treated BPA may be explained by taking into account the existence of about 10 w/v % of polymeric forms in the original BPA.

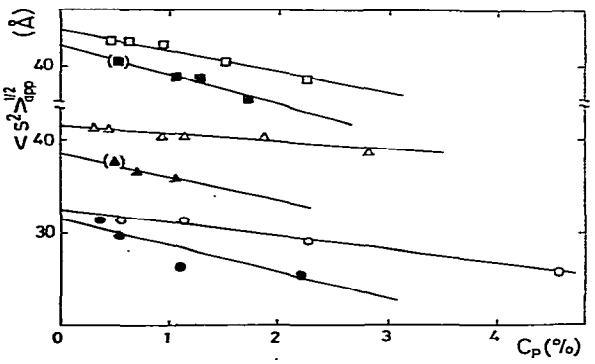

Fig. 7. Apparent radius of gyration as a function of protein concentration for original BPA (open symbols) and treated BPA (filled symbols). Circles, triangles and squares shows the data at the isoionic point, $\alpha = 0.4$ and $\alpha = 0.05$, respectively.

4. Discussion

Theoretical treatment of the problem of potentiometric titration of polyelectrolytes having a uniform distribution of ionizable groups of one kind leads to an expression for the pH of the solution [19–24].

$$pH = pK_0 + \log\left(\frac{1-\alpha}{\alpha}\right) - \frac{0.434}{kT}\epsilon\Phi_b, \qquad (2)$$

where pK_0 is the intrinsic ionization constant of the group, α the degree of dissociation of carboxylic acid group, k the Boltzmann constant, T the absolute temperature, ϵ the magnitude of the electronic charge, and Φ_b the electrostatic potential at the point from which the proton is ionized. Φ_b can be calculated from the Poisson–Boltzmann equation assuming that Φ_b of BPA with the radius of gyration $\langle s^2 \rangle^{1/2}$ should be identical with that of the corresponding equivalent sphere with the radius R, where R is related to $\langle s^2 \rangle^{1/2}$ through the relation $R^2 = \frac{5}{3}\langle s^2 \rangle$. The values of $\langle s^2 \rangle$ were supplied from the experimental results of SAXS.

In fig. 8 are compared the observed titration curve at $I = 0.15$ with three theoretical titration curves. Theoretical curves I, II and III correspond to $\langle s^2 \rangle$ = 32, 40 and 42 Å, respectively. Satisfactory agreement is found between theoretical curve I and experimental points from 0 to 25 in the Z scale (from 5.5 to

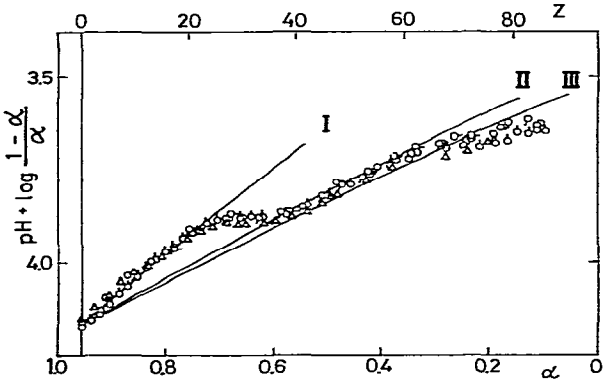

Fig. 8. Comparison between theoretical and experimental potentiometric titration curves for carboxylic acid group of BPA in 0.15 NaCl. Each symbol has the same meaning as in fig. 3. Three theoretical curves, I, II and III correspond to $\langle s^2 \rangle^{1/2}$ = 32, 40 and 42 Å, respectively.

4.5 in pH scale) and between theoretical curve II and experimental points from 35 to 60 (from 4.0 to 3.5). From this fact is derived the following image of the expansion process of BPA induced by increasing its net charge. At the isoionic state, BPA molecule in 0.15 M NaCl solution has the radius of gyration of ca. 32 Å and this size remains unchanged even if the net charge increases until $Z = 25$ where the first expansion process starts to occur. This expansion process ends at $Z = 35$, where the size of BPA amounts to 40 Å. This conformation holds stable to $Z = 60$ where the second expansion process starts to proceed. However, because of ambiguity in the value of α at lower α, it is difficult to decide unequivocally whether curve III represents the final state.

The same value of $\langle s^2 \rangle^{1/2}$ as in fig. 8 were used to calculate the theoretical curves at $I = 0.03$ and 0.01 in fig. 9, for comparison with the observed data. Two pairs of theoretical curves, I and III, II and IV, correspond to $\langle s^2 \rangle^{1/2} = 32$ and 40 Å, respectively.

From the fact that curves I and III are in agreement with the corresponding observed curves, it can be suggested that the conformation of BPA before the first transition is common at all ionic strengths, at least from $I = 0.15$ to 0.01. Moreover, the net

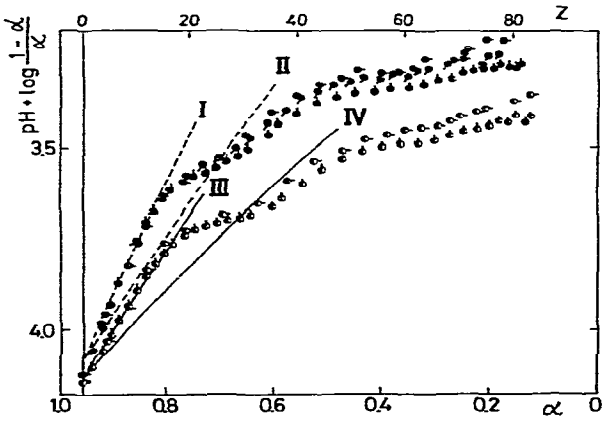

Fig. 9. Comparison between theoretical and experimental potentiometric titration curves for caboxylic acid group of BPA in 0.03 N and 0.01 N NaCl. Each symbol has the same meaning as in fig. 3. Two pairs of theoretical curves, I and III, II and IV, correspond to $\langle s^2 \rangle^{1/2} = 32$ and 40 A, respectively.

charge where the first form starts to collapse appears to become smaller with decrease in ionic strength. After the first transition, no satisfactory agreement is found between the observed curves and the calculated ones if we use the values of $\langle s^2 \rangle^{1/2}$ determined at $I = 0.15$. Therefore, it may be speculated that the form of BPA may have a swollen one and depends on ionic strength after the first transition. Moreover, this swollen form seems to expand gradually with increase of the net charge of the protein.

Foster et al. [25] suggested from the analysis of their titration data that the N-F transition involves the rupture of a number of ion-pair bonds. In their analysis they assumed the presence of two kinds of pK values of carboxyl groups. However, the present data of potentiometric titration could be explained without assuming the presence of two kinds of pK values.

Moreover, a curved Guinier plot observed in insufficiently dilute solution (figs. 5 and 6) was replaced by two straight lines by Luzzati et al. [26] and regarded as a reflection of the unique expanded structure of albumin molecule, which might consist of a highly organized core covered by a less organized coating of polypeptide chain. However, as seen figs. 5 and 6, at sufficiently dilute solutions of treated albumin, the points can be taken to form a straight line up to a higher scattering angle, and, therefore, the curved Guinier plot is not attributable to the unique molecular structure, but to possible intermolecular interaction or interference.

By the procedure reported by Zimm and Rice [3] or Nagasawa and Holtzer [1] can be estimated from figs. 8 and 9 the charge-independent part of the standard free energy change, ΔG^0, in the first expansion process. The numerical value of ΔG^0 amounts to 2350 ± 50 cal/mol (65000 amu) irrespectively of ionic strength. This quantity of ΔG^0 is small compared with the corresponding one in a dissociation reaction of β-lactoglobulin, 10.6 kcal/mol (35500 amu) [2] or with that in the helix–coil transition of poly-L-glutamic acid, 100–300 cal/residue (129 amu) [1,27].

Franglen et al. [28] noted that albumin is remarkable in its ability to combine with a wide range of compounds, and that this may be related to a process involving changes in the relative positions of side-chains of the molecule. Such changes would be created by alterations of conformation. The small value

of ΔG^0 and existence of several expansion processes may contribute to the unique ability of BPA to combine with many different substances.

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