

BUOYANT TITRATION OF BOVINE MERCAPTALBUMIN CHEMICALLY MODIFIED AT THE CARBOXYL GROUPS

by

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Bovine mercaptalbumin has been modified at the carboxyl groups by means of 1-ethyl-3 (3-dimethylisopropyl) carbodiimide and glycine amide. A stable derivative was obtained after modification of 54 groups. The modified protein differed only slightly from the unmodified one with respect to the optical rotatory dispersion parameters a_0 , b_0 and λ_c . This preparation was studied in the analytical ultracentrifuge in a CsCl gradient in the pH-interval from 2 to 11. The buoyant densities were determined and compared with those of the unmodified protein in the same pH-interval. The contribution from the carboxyl groups to the buoyant density is discussed and compared with results obtained with other proteins and synthetic polypeptides.

1. INTRODUCTION

The study of nucleic acids by the density gradient sedimentation equilibrium technique has provided useful information about the physico-chemical properties of these macromolecules (5). However, the technique is applicable also to proteins and has been described in detail for these macromolecules by IFFT (11, 12). The »buoyant density«, ρ_0 , which is the parameter determined in this type of ex-

periment is in the case of proteins strongly dependent on pH. This was clearly demonstrated for ovalbumin for which data could be obtained in the pH region 2 to 12.5 (13). The change in buoyant density as a function of pH closely simulated the potentiometric titration curve (14) and the term »buoyant titration« has been introduced for this type of investigations (12).

From the determined ρ_0 's it is possible to es-

timate the extend of hydration or binding of anions and cations to the proteins. In order to get a better understanding of the contribution from the individual ionizable groups to hydration and ion binding, a series of experiments was initiated in which the buoyant titration curves for synthetic polypeptides (1, 21) and proteins (13, 20, 25) were determined.

The present article is a contribution to these studies. Bovine mercaptalbumin (BMA)² has already been studied in the pH region 5 to 13, but due to poor solubility at pH's lower than 5, buoyant density determinations in this region have been avoided (26). However, since carboxyl groups titrate in the pH interval 2 to 6 and since data for these groups in proteins are available for two proteins only, an alternative way of obtaining buoyant titration data for the carboxyl groups of BMA was attempted. The rationale behind the present study was that chemical modification of the carboxyl groups with a neutral group (e.g. glycine amide) would make them unavailable for titration. The difference in buoyant densities at neutral pH between chemically modified BMA and unmodified protein would then be a measure of the contribution of the carboxyl groups to the change in ρ_0 on titration. A similar approach has been taken by IFFT (13) and ELLIS *et al.* (4) in the study of the lysines in ovalbumin and BMA, respectively.

2. MATERIALS AND METHODS

2.1 Materials.

Bovine serum mercaptalbumin (BMA), Nutritional Biochemical Company, twice crystallized, lot nr 8915, was purified according to the method of DINTZIS (7) and stored in the freezer at -20° C as a 5% isoionic solution. Cesium chloride (CsCl) was obtained from Harshaw Chemical Company. The purity was stated to be 99.9%. Glycineamide hydrochloride was bought from Calbiochem as was 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC). Sephadex G25 was purchased from Pharmacia Fine Chemicals, Sweden. All

other reagents used were analytical grade products, and deionized water was used throughout.

2.2 Modification of carboxyl groups

The carboxyl groups of BMA were modified according to the method of KOSHLAND (6). In a typical experiment 500 mg of glycineamide hydrochloride were dissolved in 4 ml of deionized water in the pH-stat vessel and the pH was adjusted to about 6. Two ml of a 5% BMA solution were added followed by 40 mg of EDC. The pH was adjusted to 4.75 and kept at this value for 3 hours at 25°. The reaction was terminated by passing the solution over a Sephadex G25 column (1.6 × 2.8 cm) equilibrated with 10⁻³ M-Na-phosphate buffer, pH 7.0, in the cold room. The same buffer was used for elution and the flow rate was 70 ml/hr. Fractions of 4 ml were collected and those containing protein, as determined by measurements of optical density at 280 nm, were pooled and frozen. In preliminary experiments the protein which had passed over a Sephadex column was subjected to extensive dialysis in order to assure that all glycine, which was not incorporated in the protein, was removed. No difference in the glycine content of modified BMA was detected between gelfiltered and gelfiltered plus dialyzed protein.

2.3 Amino acid analysis

The degree of modification was determined by amino acid analysis according to the method of SPACKMAN, MOORE and STEIN (22). Acid hydrolyses were made in 6N- HCl in sealed, evacuated tubes and the analysis performed on a Beckman amino acid analyzer Model 118. The degree of modification of carboxyl groups was established as the difference in glycine content between native and modified BMA. Fortunately, BMA contains only 16 moles of glycine per mole of BMA, which makes the determination of incorporated glycine amide residues quite reliable, since about 100 carboxyl groups potentially are reactive.

2) Abbreviations used: BMA = bovine mercaptalbumin, EDC = 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide, hydrochloride.

2.4 Absorption measurements

were performed with a Cary 14 recording spectrophotometer. Concentrations of native BMA solutions were calculated using $O. D. \frac{0.1\%}{280 \text{ nm}} = 0.667$ (27). Concentrations of solutions of modified BMA were determined by amino acid analysis.

2.5 Spectropolarimetric measurements

were done on a Perkin-Elmer spectropolarimeter model 241 equipped with a 10 cm water-jacketed microcell kept at 25°. The optical rotations at 578, 546, 436 and 345 nm were recorded and the parameters $[\alpha]$, λ_c , a_o and b_o calculated the usual way (15). In the calculations of a_o and b_o from the Moffitt equation, a value of 218 nm was adopted for λ_o (19).

2.6 Refractive indices

were measured on a Bausch and Lomb, Abbé Model 3L refractometer at 25°.

2.7 pH-measurements

were made with a Beckman Research pH meter, Model 1019 equipped with a combination electrode (39183). Standardization of the instrument was made with Beckman Standard Buffers.

2.8 Ultracentrifuge studies

were performed in a Spinco Model E ultracentrifuge using the schlieren optics. An An-F aluminum rotor was used in conjunction with standard 12 mm Kel-F centerpieces equipped with 0°, -1° and -2° wedge windows. The temperature was kept at 25° and the angular velocity was 50740 rev./min.

Solutions containing protein, CsCl and buffer were prepared following standard procedures (11). At equilibrium, which was established within 24 hrs, pictures were taken using Kodak metallographic plates and after development enlarged for evaluation with a Beseler Model 23c enlarger. Refractive index and pH were measured both before and after each run. Only small differences were noted, but in all instances the latter values were used in calculating the buoyant densities. The calculations were performed by a computer for which a program was written in BASIC.

3. RESULTS AND DISCUSSION

3.1 Extent of modification

Preliminary experiments, in which the reaction mixture was left for an extended period of time, showed that it was possible to modify about 90 carboxyl groups in BMA as judged from the incorporation of glycine residues. However, this high degree of modification rendered the protein quite unstable and a continuous precipitation of material took place when a gelfiltered preparation was left in the cold-room. A reaction time of 3 hrs was finally chosen since in this case a stable but still reasonably extensively modified derivative was obtained. The amino acid composition of such a preparation is given in Table 1 together with that of the unmodified protein. It is seen that 54 glycy residues have been incorporated.

Although modification of tyrosyl residues *a priori* cannot be excluded (2) it appears unlikely in the present study. EDC modified tyrosyl residues are partially stable to acid hydrolyses (3). Amino acid analysis of BMA modified with EDC, but with omission of glycineamide, showed no decrease in tyrosine content (Table 1). Although small changes were noted in the UV-spectrum of modified BMA this was not changed upon treatment with hydroxylamine which was to be expected if modification of tyrosyl residues had taken place (2). It is therefore assumed that the number of glycines incorporated reflects modification of carboxyl groups, only.

3.2 Conformational changes

In order to assure that no major changes in the three dimensional structure had resulted from modification of 54 carboxyl groups the optical rotation of the modified BMA was measured at 4 wavelengths. In Table 2 are shown $[\alpha]_\lambda$, λ_c (calculated from the Drude-equation), and b_o and a_o (calculated from the Moffitt equation). All the ORD data were obtained at pH between 7.0 and 7.3. The concentration of CsCl was identical to the one used in the ultracentrifuge studies at the same pH. Included in the table are data obtained with unmodified BMA. The identical values of λ_c in all four cases indicate that no major change in the tertiary structure has taken place by either modification of

Table I

Amino acid composition of native bovine mercaptalbumin and two chemically modified derivatives.

Amino acid	Native BMA	Modif. BMA ¹⁾	Modif. BMA ²⁾	SPAHR-EDSALL ³⁾	KING-SPENCER ⁴⁾
Lysine	56.8	57.3	57.1	62	58
Histidine	18.2	16.0	16.9	17	17
Arginine	22.9	23.2	22.6	22-23	23
Aspartic acid	56.5	56.1	55.5	54	54
Threonine	32.5	33.1	33.2	34	32
Serine	31.0	30.0	31.0	26	26
Glumatic acid	79.7	82.9	81.1	77-78	80
Proline	28.9	31.8	29.7	30	28
Glycine	17.1	71.1	16.9	16	15
Alanine	47.9	46.7	47.5	46	44
½ Cystine	26.6	28.7	30.8	36	36
Valine	32.5	35.1	32.9	36-37	35
Methionine	4.0	4.0	4.1	4	4
Isoleucine	21.2	13.3	13.5	14	14
Leucine	58.4	61.7	62.5	62	62
Tyrosine	20.2	20.3	20.0	20	19
Phenylalanine	24.9	27.0	27.0	27	26

¹⁾ Modified with EDC and glycineamide. ²⁾ Modified with EDC alone. ³⁾ From ref. (23). ⁴⁾ From ref. (17). The values in column 2-4 are average or extrapolated values from 24, 48 and 72 hrs hydrolysates. Tryptophan was not determined.

carboxyl groups or addition of CsCl. However, the b_0 values indicate a decrease in helical content upon modification, but not by addition of CsCl. On the other hand chemical modification does not seem to influence the a_0 value while a change is observed when CsCl is added to either unmodified or modified BMA. The same observation is made for $[\alpha]_{578}$. It should be pointed out that ORD-studies have only been performed at pH's near neutrality, no systematic studies have been conducted over

the whole pH-range where the ultracentrifuge studies were performed. Anyhow, the changes in tertiary structure as a result of the chemical modification were considered so small that further studies in the ultracentrifuge would be meaningful.

3.3 Density gradient experiments

The results of the density gradient experiments with BMA in which 54 carboxyl groups have been modified are presented in Fig. 1 together

Table II

ORD parameters for native bovine mercaptalbumin and mercaptalbumin modified at carboxyl groups.

	$-\alpha]_{578}$	λ_c	$-a_0$	$-b_0$
Native BMA, literature ¹⁾	-	-	336	253
Native BMA, this study	65.4	265	328	248
Native BMA in 3M CsCl	59.5	265	285	254
COOH-modified BMA	63.0	265	326	190
COOH-modified BMA in 3M CsCl	56.4	265	270	200

¹⁾ From ref. (19).

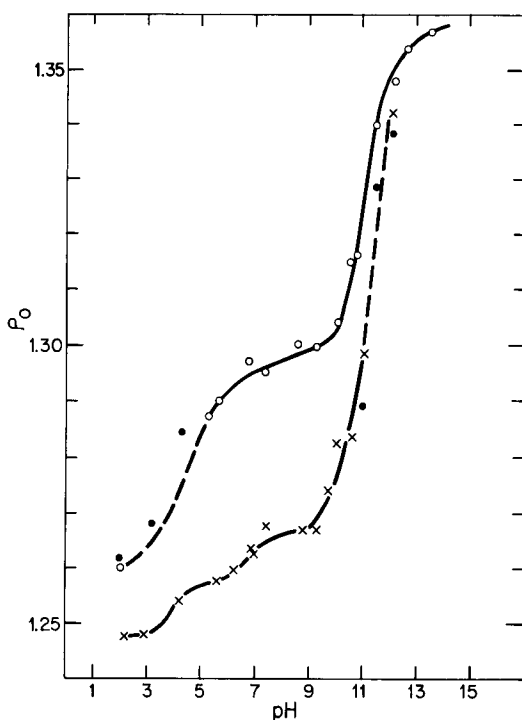


Figure 1. Buoyant titration curve for: -x- bovine mercaptalbumin chemically modified at 54 carboxyl groups, and —○— native bovine mercaptalbumin. All runs were conducted at 25°. —●— indicates buoyant densities of precipitated protein. The curve for native bovine mercaptalbumin was adopted from reference (4).

with those from experiments with native BMA. The curve for the unmodified protein above pH 5 is taken from ELLIS et al. (4). The same purified preparation of BMA has been used as the starting material in the two studies.

It is seen that throughout the pH-range covered by both sets of experiments (pH 2- 11) ρ_0 's calculated for the modified BMA are consistently lower than those calculated for native BMA. Considering experimental errors the difference in ρ_0 from pH 5 to 11 appears to be nearly constant, $\Delta\rho_0 \sim 0.030$ g/ml. While native BMA precipitated at pH lower than 5, the modified protein remained in solution at pH as low as 2. Although the native protein precipitated below pH 5 it was still possible to obtain values for ρ_0 . Three such determinations are included in the curve (Fig. 1). At pH 2 the protein begins to redissolve and both ρ_0 (prec.) and ρ_0

(sol.) were determined. At this pH ρ_0 (sol.) of the native BMA was 0.012 g/ml higher than ρ_0 of the modified protein. On the other hand precipitation of modified BMA was observed at pH 10 and above, a phenomenon not seen with native BMA. In the experiments conducted at pH 10.0 and 10.6 both precipitated and soluble protein were present simultaneously while at pH 11.5 only precipitate was observed. Since 54 negative charges have been removed by the modification of carboxyl groups the isoelectric point of the derivative is expected to be at pH 11. The occurrence of precipitate at high pH is probably due to decreased solubility as well as decreased stability of the modified molecules. It was noted that the precipitate observed in the ultracentrifuge at pH 10.0 and 10.6 redissolved when the experiment was terminated and the cell shaken. At pH 11.5 the precipitate remained at the end of the run.

The change in ρ_0 at high pH shall not be dealt with in any detail in this paper. The contribution of lysyl residues in native BMA to $\Delta\rho_0$ has already been studied by ELLIS et al (4). From Fig. 1, it appears that the sharp rise in ρ_0 due to titration of lysyl, tyrosyl and (perhaps) arginyl residues starts at the same pH in the modified BMA which indicates that modified and native BMA have the same structure at high pH.

poly-L-glutamic acid has been used as a model compound for the study of the contribution from carboxyl groups to the change in buoyant density, $\Delta\rho_0$, when these groups are titrated (1). It was shown that ρ_0 increased by 0.195 g/ml upon ionization.

$\Delta\rho_0$ for the titration of poly-L-aspartic acid has never been measured. It is assumed to be the same as for poly-L-glutamic acid. How close does this value compare to the $\Delta\rho_0$ in proteins in the acid pH region? Until now results are available for only two proteins: ovalbumin and immunoglobulin IgG. Using the value given above, a theoretical change in buoyant density, $\Delta\rho_0'$, of 0.026 g/ml was calculated for the titration of the carboxyl groups in ovalbumin (13). This figure compared fairly well with the observed 0.033 g/ml. With IgG an even closer agreement was found. In this case the theoretical $\Delta\rho_0'$ was 0.023 g/ml and the observed 0.021 g/ml (20).

In native BMA $\Delta\rho_0$ upon titration of the carboxyl groups was found to be 0.031 g/ml (Fig. 1). From the number of amino acid residues determined in the present study, 585, and a total number of carboxyl groups of 100 (24, 25) the change in density, $\Delta\rho_0'$, can be calculated on the basis of the value obtained for poly-L-glutamic acid as

$$\Delta\rho_0' = \frac{0.195 \times 100}{585} = 0.033 \text{ g/ml}$$

A good correspondance between the model compound behavior and experiments with native BMA is demonstrated.

In the modified BMA only 46 carboxyl groups are available for titration. Therefore, one would expect a

$$\Delta\rho_0' \text{ of } \frac{0.195 \times 46}{585} = 0.015 \text{ g/ml.}$$

The actually observed increase was 0.011 g/ml. The deviation from the theoretical value has in this case the opposite trend than observed for ovalbumin.

At pH 2 a difference in ρ_0 between native and modified BMA of 0.012 g/ml is observed. At this pH all the free carboxyl groups are protonated and those that are modified are uncharged as well. One might therefore expect the buoyant density of native and modified BMA to be identical. That this is not the case is most likely due to the introduction of 54 glycine-amide residues, which tend to decrease ρ_0 relative to that of native BMA. A similar, although opposite, effect was observed by IFFT after carbamylation of ovalbumin (13). In this case the addition of $-\text{CONH}_2$ groups to 18 lysyl residues (transferring them into uncharged homocitrullines) led to a positive change in ρ_0 of 0.007 g/ml relative to the unmodified protein.

If the difference in ρ_0 between the native and modified BMA at pH 7 is used to calculate the contribution of carboxyl groups to $\Delta\rho_0$ a correction for the ρ_0 due to glycineamide is necessary. The difference in ρ_0 at pH 7.0 is 0.032 g/ml which therefore should be corrected to $0.032 - 0.012 = 0.020$ g/ml, assuming that the contribution to ρ_0 from the glycineamide group is the same at pH 2 and 7. This value is somewhat higher than the theoretically calculated and

almost twice that calculated from the change in the acid pH region.

The reason for the differences is not immediate apparent. BMA molecules undergo a transition and an expansion at pH values lower than 4 which might influence the buoyant densities measured in the acid pH-region. However, a good agreement between the results for native BMA and poly-L-Glu was found. The conformational changes in modified BMA in the acid pH-region might be more drastic giving rise to a relatively small change in ρ_0 . At pH 7 both native and modified BMA exist in a more compact form (although ORD-studies showed that small conformational changes had taken place in the modified BMA). The $\Delta\rho_0$ for ionization of carboxyl groups determined from the difference between native and modified BMA at pH 7 therefore is believed to be a more representative value than the one determined from $\Delta\rho_0$ in the acid region.

A comparison between the $\Delta\rho_0$'s obtained to date for the ionization of proteins and polypeptides is made in Table 3. All data are normalized to the value that would have been obtained if the protein consisted of glutamic acid residues only. The following equation was used:

$$\Delta\rho_0^* = \Delta\rho_0 \times \frac{\text{total number of amino acid residues}}{\text{number of carboxyl residues}} \text{ g/ml}$$

In view of the empirical equation used, the agreement between the results obtained from the three proteins and the model peptides seems quite good. A comment is appropriate with regard to poly-Glu ionization. During the ionization a phase shift from insoluble to soluble form takes place (1). The agreement between $\Delta\rho_0$ from this polymer and the $\Delta\rho_0$'s of the proteins is found for the *total* change in ρ_0 of poly-Glu. When the insoluble and soluble form of poly-Glu are investigated separately the corresponding $\Delta\rho_0$'s for the two phases can be calculated as 0.730 and 0.073 g/ml, respectively (1). If the intermediate value of 0.195 g/ml found for the overall ionization of poly-Glu was connected to the phase-shift only, one might expect the determined values for proteins and

Table III

Change in buoyant density on the ionization of carboxyl groups.

Source	ρ_0 *(g/ml)
COOH in poly-Glu ⁶⁰ Lys ⁴⁰	0.246
COOH in poly-Glu ⁵⁵ Tyr ⁴⁵	0.249
COOH in poly-Glu ¹	0.195
COOH in poly-Glu (insoluble)	0.730
COOH in poly-Glu (soluble)	0.073
COOH in ovalbumin	0.214
COOH in BMA ²⁾	0.219
COOH in BMA ³⁾	0.146
COOH in BMA ⁴⁾	0.181
COOH in immunoglobulin IgG	0.178

1) Taken as the difference between the flat portions of the ρ_0 vs pH curve at high and low pH.

2) Calculated from the corrected difference at pH 7 between the buoyant density titration curves for native and COOH-modified BMA.

3) Calculated from the difference in ρ_0 of COOH-modified BMA between pH 2 and 6.

4) Calculated from the difference in ρ_0 of native BMA between pH 2 and 6.

polypeptides which do not undergo phase-shifts from pH 2 to 6, to lie close to one of the extreme values determined for poly-Glu. This is not the case. Thus poly-Glu⁶⁰Lys⁴⁰ is soluble throughout the titration range, but has $\Delta\rho_0$ of the same magnitude as poly-Glu⁵⁵Tyr⁴⁵ which is insoluble throughout the titration range of the carboxyl groups³. The normalized $\Delta\rho_0$ for the carboxyl groups of all the soluble proteins also are far from the 0.073 g/ml obtained with soluble poly-Glu.

In the experiments with ovalbumin at low pH IFFT (13) observed small amounts of precipitated material both with native and carbamylated protein. In both cases, ρ_0 of the insoluble material was 0.010 g/ml higher than that of the soluble protein. For native BMA at pH 2 both

soluble and precipitated protein is present. Also in this case the precipitate is more dense, but the difference in ρ_0 is only about 0.002 g/ml. However, the precipitate observed with modified BMA at high pH's had ρ_0 's which were lower than that observed with soluble modified BMA when both phases were present simultaneously. Thus at pH 11 ρ_0 (prec.) was 1.290 and ρ_0 (sol.) was 1.299, while at pH 12.2 ρ_0 (prec.) was 1.338 v.s. 1.342 for the soluble derivative. At the present there is no way to comment on the relative importance of changes in hydration or ion binding for the observed differences.

The number of Cs⁺ and Cl⁻ ions bound to a protein can be calculated from the following equations:

$$\rho_0 = \frac{1 + (v_{Cl^-} + NNC) (M_{Cs^+}/M_3) + v_{Cl^-} (M_{Cl^-}/M_3) + \Gamma'_*}{\bar{v}_3 + (v_{Cl^-} + NNC) (M_{Cs^+}/M_3) \bar{v}_{Cs^+} + v_{Cl^-} (M_{Cl^-}/M_3) \bar{v}_{Cl^-} + \Gamma'_* v_1} \quad (1)$$

$$\rho_0 = \frac{1 + v_{Cs^+} (M_{Cs^+}/M_3) + (v_{Cs^+} + NPC) (M_{Cl^-}/M_3) + \Gamma'_*}{\bar{v}_3 + v_{Cs^+} (M_{Cs^+}/M_3) \bar{v}_{Cs^+} + (v_{Cs^+} + NPC) (M_{Cl^-}/M_3) \bar{v}_{Cl^-} + \Gamma'_* v_1} \quad (2)$$

3) The value of 0.249 g/ml was calculated by SHARP et al. (21) from the slope of a ρ_0 vs degree of ionization plot. This plot was found unsatisfactory by the same authors due to scatter of the points. If the above calculations are made on the basis of ρ_0 between pH 2 and 6 a value of 0.354 g/ml is obtained which is somewhat closer to, but still far from the 0.730 g/ml obtained for insoluble poly-Glu.

where eq. (1) applies above the isoelectric point of the protein and eq. (2) applies below. ρ_0 = observed buoyant density, v_{Cl^-} and v_{Cs^+} are numbers of chloride and cesium ions bound, M_{Cs^+} , M_{Cl^-} and M_3 are the molecular weights of Cs^+ , Cl^- and the protein, \bar{v}_3 = partial specific volume of protein, \bar{v}_{Cs^+} and \bar{v}_{Cl^-} = the partial specific volumes of cesium and chloride at 25° (10), NNC and NPC = net negative and net positive charge. Γ_* in g H_2O per g protein is the net hydration of the protein-salt complex. In this equation (which has been derived thermodynamically by SHARP et al. (21), ρ_0 is known, and \bar{v}_{Cl^-} and \bar{v}_{Cs^+} can be obtained from ref. (10). When equations (1) and (2) are used for the calculation of Γ_* it is assumed that \bar{v}_3 is constant throughout the pH-region studied. This assumption is probably an oversimplification, but no separate studies of the variation of \bar{v}_3 with pH in high salt concentrations have been performed. The apparent specific volume for BMA has been determined at the isoelectric point in 2.47M-CsCl by pycnometry (8). The value 0.736 g/ml obtained does not differ from the one determined in the absence of CsCl. This value was used in the following calculation of Γ_* of the protein-salt complex.

Potentiometric titration of a protein under the same conditions as in the ultracentrifuge experiments provides the NPC or NNC of eq. (1) and (2), respectively (ref. 14). Such a titration has not been performed in the present study. However, at pH 2 all the carboxyl groups are assumed to be in their protonated form and the protein will carry a positive net charge of 96. In order to obtain electroneutrality 96 chloride ions must be bound. Using eq. (1) Γ_* of 0.36 g of H_2O /g of protein-salt complex was calculated for the modified BMA and 0.31 g of H_2O /g of protein-salt complex for the native BMA. These figures are equivalent to about 1350 H_2O molecules/mole of modified BMA and about 1100 H_2O molecules/mole of native BMA.

The preferential hydration, Γ' , which is the minimum amount of water bound by the

protein can be calculated from the following equation:

$$\rho_0 = \frac{1 + \Gamma'}{\bar{v}_3 + \Gamma' \bar{v}_1}$$

\bar{v}_3 being the partial specific volume of the protein and \bar{v}_1 the partial specific volume of the solvent (assumed to be 1.00 ml/g for H_2O). Calculations showed Γ' for modified BMA at pH 2 to be 0.33 g of H_2O /g of protein and for the native BMA to be 0.28 g of H_2O /g of protein or about 1220 molecules of H_2O /mole of modified and 1000 moles of H_2O /mole of native BMA, respectively.

It is interesting to note that at pH 2 only a small difference in Γ_* and Γ' for both modified and unmodified BMA is observed. $\Gamma_* - \Gamma'$, which represents the further solvation due to Cl^- ion binding is in both cases about 0.03 g of H_2O /g of protein: In contrast $\Gamma_* - \Gamma'$ for unmodified BMA at its isoelectric point was shown to be 0.31 g of H_2O /g of protein (9). The reason for the small $\Gamma_* - \Gamma'$ at pH 2 might be that the 98 Cl^- ions are much more loosely bound than the 53 Cl^- ions at the isoelectric point.

Since the same number of ions is bound to both modified and native BMA at pH 2 one would expect Γ_* to be identical for the two proteins. However, a difference corresponding to 235 moles of H_2O /mole of protein was observed. A similar calculation using the Γ' s gave 220 moles of H_2O /mole of protein. These differences probably reflect changes in hydration number due to the introduction of 54 glycineamide residues. Thus each glycineamide residue binds approximately 4 moles of H_2O .

The increase in ρ_0 on titration of the carboxyl groups can be caused by 1) a gain in Cs^+ ions, 2) a loss in Cl^- ions or 3) a change in volume. Each of these possibilities will now be considered separately.

1) In modified BMA 46 carboxyl residues are available for titration. If we assume that the creation of 46 negative charges between pH 2 and 6 leads to binding of 46 Cs^+ ions a »theoretical« ρ_0 can be calculated:

$$\rho_0 = \frac{67000 \text{ g protein} + (46 \times 132.9) \text{ g } Cs^+}{(67000 \text{ g}/1.248 \text{ g/ml}) \text{ ml} + (46 \times 19.6) \text{ ml } Cs^+} = 1.339 \text{ g/ml}$$

However, a ρ_0 of 1.259 g/ml was observed at pH 6. Therefore, as expected, the Cs^+ must be hydrated. If the terms $\times \text{g H}_2\text{O}$ and $\times \text{ml H}_2\text{O}$ are added to the numerator and denominator, respectively, and if ρ_0 is set to 1.259 g/ml, it can be calculated that 940 moles of H_2O must be added concomitantly with the 46 Cs^+ , or 20 water molecules per Cs^+ . This is an unreasonably high number for hydration of Cs^+ .

2) If a similar calculation is made assuming the loss of 46 Cl^- a resulting loss of 4 molecules of water per Cl^- is obtained. This number is a reasonable figure for hydration of chloride ions.

3) As already pointed out by KAUZMANN (13, 18) at least part of the change in ρ_0 might be due to a change in volume. From the equation

$$\Delta\rho_0 = \frac{-\Delta V \times \rho_0}{V}$$

and a $\Delta V = 11 \text{ ml/mole of COOH}$, IFFT (13) calculated that about $\frac{2}{3}$ of the change in ovalbumin on ionization was accounted for in this way. The value $-11 \text{ ml/mole of COOH}$ was obtained at low salt concentration and is assumed also to be valid in 3 M-CsCl. A similar calculation for modified BMA shows a $\Delta\rho_0$ of 0.013 g/ml. The observed $\Delta\rho_0$ was 0.011 g/ml. Thus the change in buoyant density of modified BMA can be explained almost solely in terms of change in volume when the carboxyl groups titrate.

Similar calculations performed with native BMA showed that 18 moles of H_2O must be bound per Cs^+ bound or 4 moles of H_2O lost per Cl^- lost when 100 carboxyl groups titrate. Of these possibilities loss of Cl^- as the carboxyl groups dissociate is the most likely explanation. When the change in ρ_0 for native BMA is calculated in terms of change in volume a close agreement with the observed value is observed: 0.029 g/ml vs 0.031 g/ml.

The results of these calculations therefore would indicate that the change in buoyant density between pH 2 and 6 can be accounted for almost exclusively in terms of changes in the volume of both native and modified BMA. It should be pointed out that the calculations involving volume changes are somewhat uncertain in light of the anomalous volume changes which take place in the acid pH region (see f. ex. KATZ et al. (16)). However, the data

obtained in this study together with those obtained by IFFT (13) with ovalbumin stresses the importance of obtaining independent data on \bar{v}_3 as a function of pH in high concentrations of salt. Such data would be of great importance for a more conclusive interpretation of data obtained by buoyant titration of proteins.

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