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Interaction of H^+-ions with α-crystallin: solvent accessibility of ionizable side chains and surface charge

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Abstract

Interaction of H^+-ions with α-crystallin from goat lens has been studied at three different ionic strengths using the potentiometric titration method. Titrations have also been carried out in the presence of 1.5 M and 6 M GuHCl (guanidine hydrochloride). The isoionic pH of the protein in water and the effect of KCl on it have been determined. Titration curves have been found to be reversible between pH 3 to 9.25 at all ionic strengths. To aid in the data analyses, the reactivities of α-crystallin lysine residues to trinitrobenzenesulfonic acid have been determined in this work. For α-crystallin aggregate, 130 ± 2 histidine side chains out of a total of 300 and about 134 ± 4 lysine side chains out of 310 have been found to be inaccessible to the solvent in the native condition. The remaining titratable side chains determine the surface charge of the native protein. In 1.5 M GuHCl, however, the nontitratable histidine side chains are found to be available for titration as are the nontitratable lysine and tyrosine side chains in 6 M GuHCl. The theoretical titration curve computed on the basis of Linderstrøm–Lang model is found to fit quite comfortably with the experimental one between pH 4.6 and 9.25. The pK_{int} value for β,γ-carboxyl side chains has been found to be 5.18 which is somewhat higher than usual indicating that the carboxyl groups in the protein are probably in some constrained condition which is released in the presence of a denaturant. Below pH 4.6, there begins a conformational change in the α-crystallin aggregate as is corroborated from the circular dichroism studies. The value of electrostatic interaction factor w which remains more or less constant between pH 4.6 and 9.25 is also found to gradually fall off below pH 4.6. © 1998 Elsevier Science B.V.

Keywords: Interaction of hydrogen ions; α-Crystallin; Solvent accessibility; Ionizable side chains; Surface charge

1. Introduction

α-Crystallin is the most important member among crystallins, the structural proteins of mammalian eye lens. It accounts for about 40% (w/v) of the total protein of the lens fiber [1]. Being the largest in size among the lenticular proteins, α-crystallin is believed to play a prominent role in scattering of light and hence in maintaining the transparency of lens and its impairment during cataractogenesis [2]. This multimeric protein is composed of two 20 kDa subunits, αA and αB, which are highly homologous and probably have similar secondary structures. The A and B subunits undergo a non-covalent self-association to form the oligomeric aggregate composed of approximately 40 subunits in the ratio of about

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3αA:1αB leading to a molecular mass of around 800 kDa [3–7].

Neither α-crystallin nor its individual subunits could be crystallized. So, it has not been possible to know their three-dimensional structure directly by X-ray crystallography. However, some models have been proposed for the quaternary structure of α-crystallin. These include a three-layer model [8], a micelle-like model [9], a combination of miceller and the three-layer model [10], a rhombic dodecahedron [11] and a pore like structure [12]. However, these models are in controversy and it has not been possible yet to attain a general consensus on this issue. Recently, an additional important functional property has been discovered for α-crystallin. α-Crystallin has been found [13] to exhibit chaperone-like activity by preventing heat-induced aggregation and precipitation of other lenticular and non-lenticular target proteins like small heat shock proteins (hsp) with which it shows a good deal of structural homology [14].

The novel interactions of α-crystallin with other crystallins (β, γ-crystallins) of lens through short-range orderly packing is considered to be of paramount importance in maintaining lens transparency. For exhibiting chaperone activity also, α-crystallin has to interact with target proteins. A property of a protein that appears to be important in its interactions with other proteins is electrostatic interaction through its surface charge contributed by the ionizing side chains. In the absence of a 3D structure of α-crystallin, it is still not known how many ionizing side chains are on the surface of the protein. While information regarding the microenvironments of some amino acid residues in α-crystallin are available through modification reactions with specific chemical probes [15–19], little is known until now [20] about the microenvironments of the major titratable groups in this protein. One of the most useful method for assessing the number and nature of ionizable residues on a protein surface is by determining their reactivity towards H^+ ions [21–23]. Anomaly in H^+ ion reactivity can also be an indicator of probable conformational changes that may accompany with the alteration of protein surface charge [21,22,24].

Accordingly, we have studied H^+ ion titration properties of highly purified α-crystallin from goat lens with special emphasis to the titratability and solvent accessibility of different ionizable side chains in the protein. In order to aid in the data analyses the results of some side chain modification studies have also been used. The subsequent analyses of the data deal with the determination of (i) the number of various types of dissociable groups which are accessible and also inaccessible to the solvent per mole of the protein yielding directly an idea regarding the surface charge of the protein, (ii) the intrinsic dissociation constants (pK_{int}) these titratable groups would have in the absence of electrostatic effect and (iii) comparison of the experimental titration curve with the theoretical one computed on the basis of the Linderstrøm–Lang model. Further, it is generally known that when the molecular mass of a globular protein exceeds 100 kDa, the surface area per unit mass begins to become too small to accommodate all the titratable groups which might cause titration anomaly. Reports of complete titration curve of proteins above this size are also rare [22]. Our additional interest to study H^+-ion equilibria of α-crystallin is to know whether this large aggregate can accommodate all the ionizable groups on its surface.

2. Materials and methods

2.1. Materials

TNBS (trinitrobenzenesulfonic acid), GuHCl (guanidine hydrochloride), urea were purchased from Pierce Chemical (USA) and SDS (sodium dodecyl sulfate) from Sigma Chemical (USA). Recrystallization of TNBS was performed prior to use following the method as described by Fields [25]. All other chemicals were of reagent grade.

2.2. Protein purification and protein determination

Purification of α-crystallin was done following essentially the method as described earlier in Ref. [20]. Buffer (Tris–Cl, pH 7.3) soluble protein of the cortical parts of goat lenses was fractionated on BioGel A-1.5 m column. α-Crystallin fraction was then concentrated by pellet formation using ultracentrifugation and the solution of the pelleted material was subjected to Ultrogel AcA-22 chromatography

twice. The final gel filtration chromatography was performed on the same column equilibrated with KCl solutions of appropriate concentrations needed in the subsequent titration studies performed at different ionic strengths. When protein sample was collected in different batches of preparation care was taken to check that the volume at which α-crystallin was finally eluted out from the same column was exactly identical for all the batches. Protein solution belonging only to the topmost fraction of the final chromatographic peak from each batch of preparation was used in this work. This was done in order to ensure that the constant molecular status of α-crystallin was maintained all through the work as far as practicable. Protein concentrations were routinely determined from the absorbance at 280 nm. The value of $E_{1\ \mathrm{cm}}^{1\%}$ determined for the protein at 280 nm is 8 [20].

2.3. Reactivity of lysine residues in α-crystallin towards TNBS

The reactivity of lysine residues in α-crystallin towards TNBS was estimated following essentially the method of Habeeb [26]. A total of 0.3 ml of the protein solution (1 mg/ml) and 0.3 ml of 4% $NaHCO_3$ (pH 8.5) were pipetted into 15 separate test tubes. Varying amounts of TNBS solution prepared from the freshly crystallized reagent were added in different tubes to make the molar excess (reagent: lysine residue) gradually increase to 30 folds. The total volume of each reaction mixture was maintained to 0.9 ml. The reaction was allowed to continue at room temperature ($25 \pm 1°C$) for an hour and stopped by adding 0.15 ml of 1.0 (N) HCl and 0.3 ml of 10% SDS solution. The absorbance of these solutions were then read at 344 nm, the wavelength of maximum absorption, against a proper blank prepared as above but containing 0.3 ml water in place of the protein solution. The number of lysine residues modified at any stage of reaction was calculated from the observed molar absorbance at 344 nm using a value of 1.1×10^4 for the molar extinction of trinitrophynyl amino group in a protein at the same wavelength [27]. Reactivity of lysine residues in the denatured protein was also estimated in the similar way in the presence of 6 M urea.

2.4. Isoionic point determination

Isoionic salt free stock solutions of the protein were prepared by passing protein solutions having different concentrations (between 1.5–2 mg/ml) down a mixed bed ion exchange column according to Dintzis [28]. The pH values of the effluent protein solutions were immediately determined. For determining the isoionic pH value of the denatured protein, GuHCl was added to the deionized native protein solutions to attain the desired concentration of the denaturant, pH values of these solutions having protein concentrations between 1.0–1.5 mg/ml were finally measured.

The effect of KCl on the isoionic pH of the native protein was determined by adding varying amounts of KCl solution to the deionized protein solution of fixed concentration and measuring the corresponding pH values.

2.5. Titration of native α-crystallin at different ionic strengths

Acid-base titration of native α-crystallin was carried out in the pH range 2.5 to 11.0 at three different ionic strengths: 0.3, 0.2 and 0.1. Titration was performed in a discontinuous manner which meant that each experimental point on the titration curve at a particular ionic strength represented the result obtained with a separate reaction mixture. Titrants (HCl or KOH solutions) of desired concentrations were prepared in KCl solution having the same ionic strength at which the titration had to be carried out. Direct titrations were always started at pH 6.3 to 6.7 which was the pH range of the protein solutions prepared in KCl solutions of desired ionic strengths. Generally, in several titration vessels, 0.7 ml of protein solution (2–2.5 mg/ml) was added to each. Different amounts of acid/alkali were then added separately in order to bring the reaction mixtures to different desired pH values. The final volume of the reaction mixtures was made to 1 ml in each case with KCl solution of appropriate ionic strength. Additions of titrants were made in small fractions with constant stirring to avoid any local increase of acid/alkali concentration that might lead to conformational change of the protein. Sufficient time was allowed (up to 2 min) after each addition of acid/al-

kali for the pH to become constant before it was recorded.

Reversibility of some points on the titration curves at different ionic strengths was checked by bringing the pH of the protein solutions initially to pH 2.5, 11.5 or to any other pH values within this range by adding sufficient amounts of acid or base. After standing for about 5 min, the solutions were titrated back by adding base/acid following the procedures described before.

2.6. Titration of α-crystallin in the presence of different concentrations of GuHCl

Direct titrations were also carried out in two different GuHCl concentrations, i.e., in 1.5 M and in 6 M. Reaction mixtures were prepared in 0.3 M KCl as before and appropriate amounts of GuHCl were added from a concentrated stock solution in order to attain the desired concentrations of the denaturant in each reaction mixture.

2.7. Determination of apparent activity coefficients of H^+ ions and OH^- ions

Several protein-free solutions whose pH would lie between 2 to 3 in the acid range and between 10 to 11 in the alkaline range were prepared separately for each of the ionic strengths at which the titration work was carried out using KCl and HCl as well as KCl and KOH solutions respectively both in the absence and in the presence of 1.5 M GuHCl. The exact pH values of these solutions were measured at 25 ± 1°C. The apparent activity coefficients of H^+ ions (γ'_{H^+}) and of OH^- ions (γ'_{OH^-}) were calculated. The mean value obtained for γ'_{H^+} at each ionic strength was assumed to remain constant between pH 2.5 and the isoionic pH of the protein and that for γ'_{OH^-} was assumed to remain constant between the isoionic pH and pH 11 [29].

2.8. Measurements of pH

A Radiometer Model PHM 82 pH meter (Copenhagen, Denmark) equipped with either Radiometer semi-micro electrode system: G2040 C 945-041 and K4040 945-110 or with Radiometer (America) combined electrode: GK 533501 was used. It was routinely standardized at pH 7 ± 0.01 (Radiometer buffer) and checked with two other standard buffers at pH 4.01 ± 0.01 and at pH 9.18 ± 0.01 to ensure accurate response over the entire pH range covered. At the completion of a few measurements, the response of the meter to standard buffers was rechecked. Only those results were accepted where the standardization drifts during titration did not exceed ± 0.02 pH unit. All measurements were carried out at 25 ± 0.1°C. Above pH 9 measurements were made under moist and purified nitrogen atmosphere.

2.9. Construction of titration curves

The number of moles of H^+ ions bound/dissociated per mole of protein ($\bar{Z}_{H^+}$) was estimated from the difference between the total amount of acid or alkali added to the protein solution and the amount of H^+ or OH^- ions left free in the solution at equilibrium. The latter quantity was computed by using the observed pH values with the aid of γ'_{H^+} or γ'_{OH^-} values determined at the relevant ionic strength. For calculating $\bar{Z}_{H^+}$ values from the titration data obtained between pH 3 to 11 in the presence of 6 M GuHCl and those obtained between pH 2.5 to 3.5 in the presence of 1.5 M GuHCl, γ'_{H^+} and γ'_{OH^-} values were not used. Blank titrations were carried out in these cases on similar solutions containing identical amounts of acid/alkali and GuHCl as in the sample solutions but no protein. The amount of H^+ bound by the protein at any pH was obtained by difference [30].

The plot of $\bar{Z}_{H^+}$ vs. pH at each ionic strength constituted the titration curve of the protein; the reference point being the isoionic pH ($\bar{Z}_{H^+} = 0$), i.e., the pH of zero net proton charge. Since the subunits in the α-crystallin aggregate exist in an unequal ratio, i.e., 3:1 and also differ in amino acid compositions [3,4], we expressed the results in terms of 800 kDa aggregate unit rather than in 20 kDa monomer unit.

2.10. CD measurements

Circular dichroism experiments were carried out at room temperature (25 ± 1°C) in both far and near UV regions using a JASCO J720 spectropolarimeter calibrated with a freshly prepared aqueous solution of D-10-camphor-sulphonic acid [31]. Mean residues

elipticity values $[\theta]_{MRW}$ (expressed in deg cm^2 $dmol^{-1}$) were calculated using a value of 115 as the mean residue weight [32–34]

3. Results

3.1. Reactivity of lysine residues of α-crystallin with TNBS

Fig. 1 shows the reactivity profiles of lysine residues in the native and denatured α-crystallin using TNBS as the specific chemical probe. It has been found that at around 20 fold molar excess of the reagent over the lysine residues, the surface exposed lysine residues in the standard proteins, i.e., γ_{II}-crystallin and papain used in this work (two lysine residues in the former and three in the latter are known to be surface exposed) [35,36] are all modified under the conditions of the experiment. However, Fig. 1 shows that with native α-crystallin, 176 ± 4 residues of lysine among a total of 310 are found to be maximally modified by TNBS under this condition. There was no further modification even when the molar excess of the reagent was raised to 32 folds. The simple way to interpret this data is to state that 176 ± 4 lysine residues are exposed on the surface of α-crystallin, the rest are not available for modification in the native protein. Fig. 1 also shows that when the protein was denatured with 6 M urea all the lysine residues in the protein became available for modification. Modification of lysine residues in α-crystallin had been made earlier by other workers using various modifying reagents including TNBS [19]. We have selected TNBS for this work since these workers reported that except TNBS other modifying reagents affected the molecular status of α-crystallin by either dissociation or aggregation. Our result, of course, varied to some extent from that obtained by this group with TNBS. This is not unlikely since the conditions of modification chosen by these workers were somewhat different from those employed in our work. While we tried to modify only the surface exposed lysine residues in the native protein under milder conditions, they carried out the reaction at an elevated temperature where more lysine residues are expected to be modified.

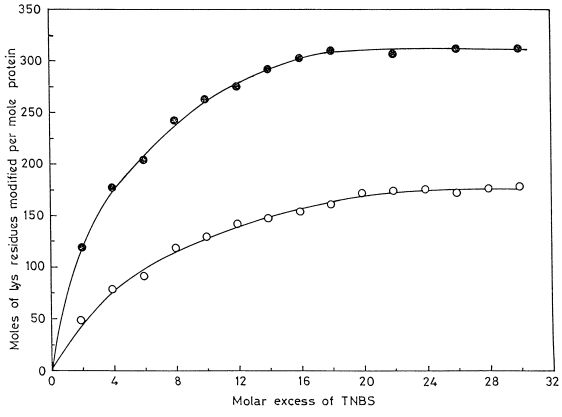

Fig. 1. Reactivities of lysine residues in goat α-crystallin with increasing molar excess of TNBS at room temperature in the presence and absence of urea. Data in the absence of urea (○), data in the presence of 6 M urea (●).

3.2. Isoionic pH of α-crystallin and the effect of KCl on it

In water, the isoionic pH value of α-crystallin preparations with comparable concentrations (from 1.4 mg/ml to 1.8 mg/ml) used in the titration work was found to be 5.88. The protein solutions of higher concentration appeared turbid as these came out of the deionizing column but became clear at a very low salt concentration. The isoionic pH of α-crystallin in the presence of 0.3 M KCl has been found to be 5.84. The isoionic pH values of denatured proteins were found to be 6.12 and 6.29 in the presence of 1.5 M and 6 M GuHCl respectively.

Since H^+-ion titrations were carried out in the presence of KCl, the effect of KCl on the isoionic point of α-crystallin was determined by measuring the change of pH produced by increasing KCl concentrations in the solution of isoionic protein in water. It has been found (Fig. 2) that low concentrations of KCl produces an initial decrease in the isoionic pH of α-crystallin to the extent of about 0.04 pH unit with no further decrease with the gradual increase of KCl concentration. On the basis of usual arguments [37] this can be attributed to the effect of salt on the activity coefficients in the pH region where carboxylic acids are the dominating buffers. It has been found by actual experiment in this work that the pH of an acetic acid/acetate buffer decreases from pH 5.87 in the absence of added salt to pH 5.79 when sufficient KCl has been

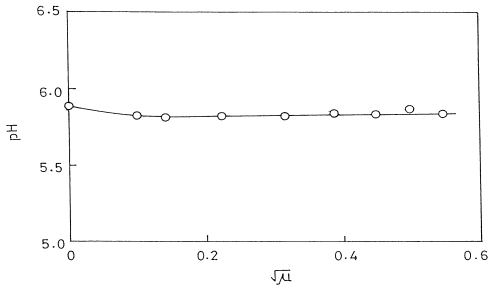

Fig. 2. The effect of KCl on the isoionic pH of goat α-crystallin. In the absence of KCl, 5.88 is the isoionic pH of goat α-crystallin; μ is the ionic strength.

added to make the solution 0.3 M in KCl. This change can be explained theoretically to be resulted from salt effects on activity coefficients [21]. Accordingly, the observed decrease in the isoionic pH of α-crystallin with the addition of KCl is considered to be much smaller than what is generally expected in the case of cation binding to proteins.

3.3. Titrations of α-crystallin in different ionic strengths

Results of titration of α-crystallin at three different ionic strengths: 0.3, 0.2 and 0.1 are shown in Fig. 3. Each curve was constituted with enough experimental points with protein preparations from different batches in order to ascertain unequivocally its natural course. Data designed to test the reversibility of titration indicate that titration to and from any pH value between 3 and 9.25 was rapidly reversible for all the ionic strengths studied with no significant pH drifts noted. Back titrations from pH higher than 9.25, however, showed irreversibility at all the ionic strengths studied. Titration curves between pH 3 to 9.25 were, therefore, considered reversible in the sense that thermodynamic analyses would be permitted only within this region. Between the pH region 4 to 5 during both direct and back titrations, the protein showed some insolubility at all ionic strengths suggesting some aggregate formation. But, this had no effect either on the reversibility of the titration curve or on the steadiness of pH observed within this range. We assumed therefore, that this phenomenon had no influence on the H^+-ion equilibria. However, at 0.3 M KCl precipitation did not appear during the course of titration but did so after a few hours. The titration curve at 0.3 M KCl was, therefore, selected for the subsequent thermodynamic analyses.

3.4. Titration curve of native α-crystallin at 0.3 M KCl and at 25°C

Fig. 4 shows the titration curve at 0.3 M KCl. The data are shown both in terms of $\bar{Z}_{H^+}$ vs. pH and also

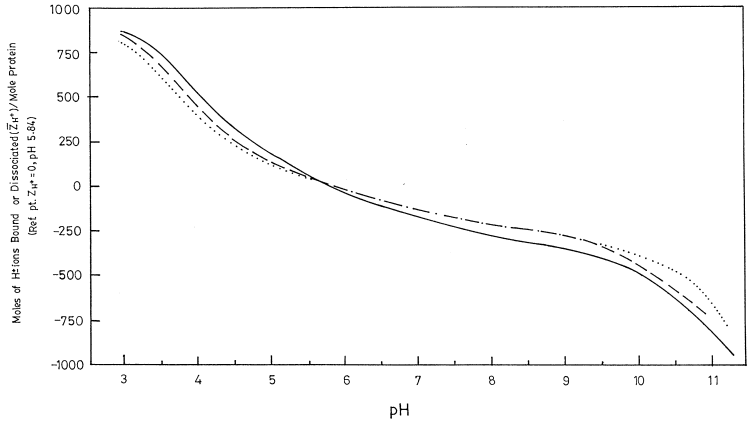

Fig. 3. Titration curves of native goat α-crystallin at 25°C and at different ionic strengths. (· · ·), Ionic strength 0.1; (— — —), ionic strength 0.2; (————), ionic strength 0.3. Since the isoionic pH of goat α-crystallin in the presence of 0.1 M KCl was 5.84 and this value remained unaltered in the other two ionic strengths, the reference point was taken as 5.84 for all the ionic strengths where $\bar{Z}_{H^+}$ is considered to be zero.

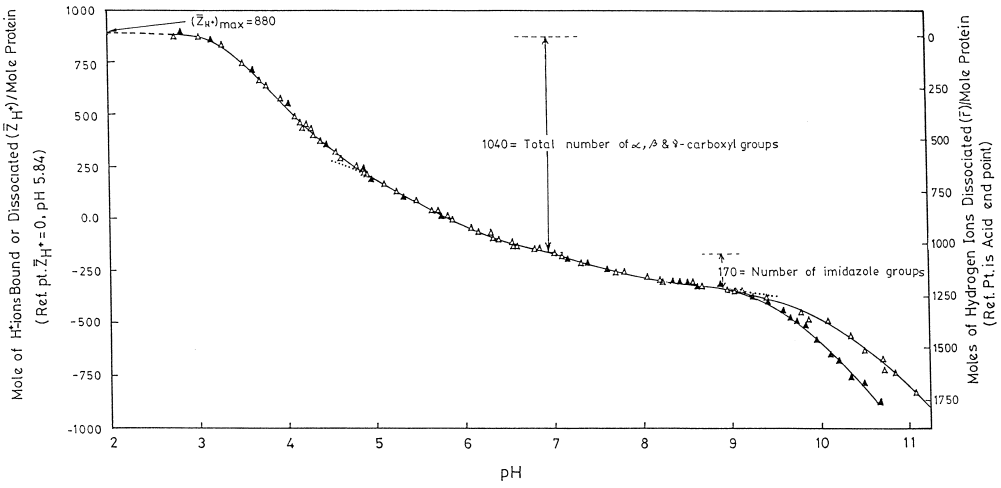

Fig. 4. Titration curve of native goat α-crystallin at ionic strength 0.3. ($\triangle$), Direct titration data; ($\blacktriangle$), reverse titration data. Reference point ($\bar{Z}_{H^+}=0$) is 5.84, the isoionic pH of the protein at 0.3 ionic strength. On the right hand side, the ordinate ($\bar{r}$) represents the number of moles of H^+-ions dissociated per mole protein counted from the acid end point [$(\bar{Z}_{H^+})_{max}$] as the reference point where, $\bar{r}=(\bar{Z}_{H^+})_{max}-\bar{Z}_{H^+}$. The titration curve has been found to be reversible up to pH 9.25 beyond which the reverse titration data are seen to deviate from the direct titration ones. Two downward arrow heads indicate the two inflection points (at pH 6.95 and at 8.9) in the titration curve determined by the derivative plot ($(d\bar{Z}_{H^+})/(dpH)$ vs. pH). These divide the curve into acidic, neutral and alkaline segments. The value of $(\bar{Z}_{H^+})_{max}$ is 880 which represents the total number of cationic groups in the native protein. Up to the first inflection point, a total number of 1040 protons are dissociated which is equal to the total number of α, β, γ-carboxyl groups in the protein. Between the first and the second inflection point, the total number of protons dissociated is 170 representing the titratable histidine residues in the native protein. The theoretical titration curve has been computed by using the parameters of Table 1. The calculation also includes 78 titratable tyrosine with $pK_{int}=10.26$ [38], 13 cysteine residues [39] with $pK_{int}=10.0$ (assumed) and 176 lysine residues with $pK_{int}=10.26$ (assumed). It is found that the theoretical titration curve deviates from the experimental titration curve below pH 4.6 in the acid region and above pH 9.25 in the alkaline region (indicated by dotted line).

in terms of $\bar{r}$ vs. pH where $\bar{r}$ denotes the number of hydrogen ions dissociated from the acid end point. These two parameters are related through the maximum value of $\bar{Z}_{H^+}$ at the acid end point [$(\bar{Z}_{H^+})_{max}$] by the relation: $\bar{Z}_{H^+}=(\bar{Z}_{H^+})_{max}-\bar{r}$; negative values of $\bar{Z}_{H^+}$ signifying hydrogen ions dissociated from the isoionic protein. The titration curve (Fig. 4) can be sub-divided into three distinct segments as is usual, i.e., the acidic, the neutral and the alkaline segments. The end of the acid segment and the beginning of the neutral segment as well as the end the neutral segment and the beginning of the alkaline segment have been obtained from the inflexion points (shown by arrow heads) determined (not shown) from the derivative plots [$(d\bar{Z}_{H^+})/(dpH)$ vs. pH].

The value of $\bar{r}$ at the first inflection point in Fig. 4 at pH 6.95 which is conventionally assigned to the end point of α, β and γ-carboxyl titration has been found to be 1040 ± 2. This number coincides well with the analytical figure for the total of C-terminal amino acid (40), aspartic acid (530) and glutamic acid (470) residues in the protein. In a similar way, the number of H^+-ion dissociated ($\bar{r}$) between the first inflexion point at pH 6.95 and the second inflexion point at pH 8.90 in the titration curve is a measure of total histidine and the terminal amino acid residues in the protein. Since the terminal amino groups in the constituent αA and αB subunits are known to be acetylated, the number of proton dissociated within this pH range can, therefore, be assigned entirely to the imidazole groups of histidine residues titrated in the native protein. It has already been shown in our previous work [20] that out of the total 300 histidine residues in the protein, 130 ± 2 residues are neither titratable nor available for chemical modification. These become titratable when the protein is titrated in the presence of 1.5 M GuHCl (Fig. 5, inset). The maximum acid binding capacity

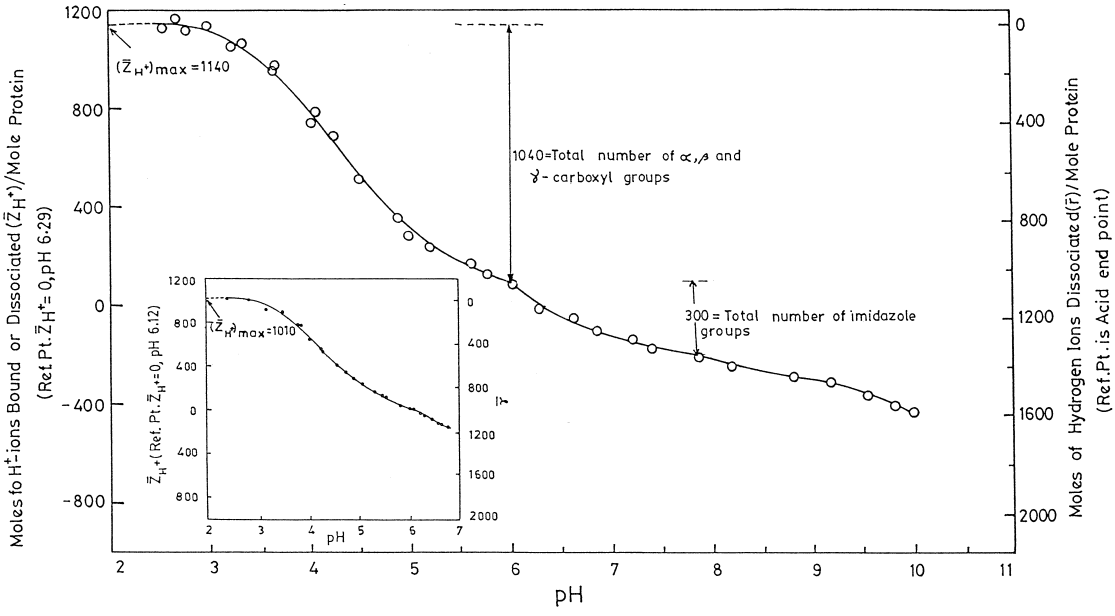

Fig. 5. Titration curve of goat α-crystallin in the presence of 0.3 M KCl and 6 M GuHCl at 25°C. Reference point ($\bar{Z}_{H^+}=0$) is 6.29, the isoionic pH of the protein under above conditions. Arrow heads indicate the positions of the inflection points (at pH 6.0 and pH 7.9) in the titration curve. The value of $(\bar{Z}_{H^+})_{max}$ is 1140 which represents the total number of cationic groups in the protein. Up to the first inflection point, 1040 protons are dissociated which equals the total number of α, β, γ-carboxyl groups. Between the first and second inflection points, total number of protons dissociated is 300 representing the total histidine residues in the protein. Inset shows the titration curve of goat α-crystallin in the presence of 0.3 M KCl and 1.5 M GuHCl at 25°C. Reference point ($\bar{Z}_{H^+}=0$) is 6.12, the isoionic pH of the protein under this condition. The value of $(\bar{Z}_{H^+})_{max}$ is 1010 which represents the total number of cationic groups in the protein.

of the protein, which gives the total number of histidine, lysine and arginine residues (all α-amino groups being acetylated) has been found to be 880 in 0.3 M KCl. But, this value has been found to be increased by 130 ± 2, i.e., 1010 ± 2, when the titration was carried out in the presence of 1.5 M GuHCl (Fig. 5, inset). This is because 130 ± 2 histidine residues which were unavailable in the native protein became available in the presence of 1.5 M GuHCl. The analytical figure for the total number of histidine, lysine and arginine residues in the protein appears to be 1140. Therefore, there is still a discrepancy of 130 ± 2 residues. The chemical modification studies of lysine residues in the protein with TNBS as described earlier indicate that these are the unavailable lysine residues which do not make themselves available during 1.5 M GuHCl treatment. For their availability, the complete denaturation of the protein with 6 M GuHCl is necessary. Fig. 5 which represents the titration curve in the presence of 6 M GuHCl shows that the $(\bar{Z}_{H^+})_{max}$ value under this condition is indeed 1140 ± 2 (Table 1).

Our results thus suggest that all α, β and γ-carboxyl groups of C-terminal amino acid, aspartic acid and glutamic acid residues respectively; about 170 ± 2 of histidine and 176 ± 4 of lysine residues as well as all arginine residues are available for titration in the native α-crystallin aggregate. These titratable residues are expected to be exposed on the protein surface.

3.5. Analyses of the titration curve

For thermodynamic analyses, the reversible part of the titration curve from pH 3 to 9.25 of Fig. 4 has been used. Analyses are based on the tentative assumption that all the dissociable groups of any one kind (i) are intrinsically identical having the same intrinsic dissociation constant $(K_{int})_i$. The number of

Table 1
Titration parameters of α-crystallin from goat lens

Ionizable residues	Number (± 2) per 800 kDa	Class	pK_{int}	Electrostatic interaction factor, w					
				$\mu = 0.3$		$\mu = 0.2$		$\mu = 0.1$	
				w_{cal}	w_{expt}	w_{cal}	w_{expt}	w_{cal}	w_{expt}
α-COOH	40	titratable	3.6						
β,γ-COOH	1000	titratable	5.18	0.004	0.003	0.005	0.005	0.006	0.008
α-NH_2	40	acetylated							
Histidine	$300[^{170}_{130}$	titratable	7.00		0.003				
		nontitratable	–		–				
Lysine	$310[^{176\,a}_{134}$	titratable	nd						
		nontitratable	–						
Arginine	530	titratable	> 12						

Counts of basic amino acid residues from the acid end points

Native condition	In 1.5 M GuHCl	In 6 M GuHCl
$(\bar{Z}_{H^+})_{max} = 880$	$(\bar{Z}_{H^+})_{max} = 1010$	$(\bar{Z}_{H^+})_{max} = 1140$
His = 170	His = 300	His = 300
Lys = 176[a]	Lys = 176[a]	Lys = 310[a]
Arg = 530	Arg = 530	Arg = 530
Total = 876	Total = 1006	Total = 1140

[a]The number of the solvent accessible lysine residues in the native α-crystallin (176 ± 4) and that in the denatured protein (310 ± 4) have been obtained from chemical modification studies (see Section 3.1).

groups of any kind (r_i) dissociated at any pH is given by the Linderstrøm–Lang equation [40,41]:

$$\mathrm{pH} - \log\frac{\alpha_i}{1-\alpha_i} = \mathrm{pH} - \log\frac{r_i}{n_i - r_i} = (pK_{int})_i - 0.868\, w\bar{Z}\ldots \quad (1)$$

where n_i is the total number of groups of kind i and α_i is the fraction $[(r_i)/(n_i)]$ dissociated. The total number, $\bar{r}$, of hydrogen ions dissociated per mole of the protein as given by the ordinate (right hand) of Fig. 4 is therefore the sum of the r_i for all kinds of groups dissociated at that pH, i.e., $\bar{r} = \sum_i r_i$. The electrostatic interaction between the net charge $\bar{Z}$ of the protein at any pH and the dissociating hydrogen ion is taken care of by the factor $0.868\, w\bar{Z}$, where w is considered to be an empirical parameter not necessarily to remain constant over the entire pH range. Since the three dimensional structure of α-crystallin is not yet determined, the mode of distribution of various charged groups on the surface of the protein molecule can not be known at present. The value of the above factor is therefore considered to be independent of the type of group from which the proton is dissociated. Since there is no evidence of any specific ion-binding to the isoionic protein, the charge Z of the protein is primarily owed to the bound or dissociated protons and is equal to $\bar{Z}_{H^+}$ which can be directly obtained from the titration curves (Figs. 4 and 5).

In the region of pH 3 to 6.95, however, virtually all types of carboxyl groups (α, β and γ) are mainly titrated with, of course, a fraction of imidazole groups specially at the higher pH side of this region. If the dissociation of α-carboxyl groups and that of imidazole groups are taken into account assuming their reasonable pK_{int} values, the entire region can be represented by the dissociation of only β, γ-carboxyl groups taken together as a single class. Eq. (1) may, therefore, be applied directly and a value of the intercept at $\bar{Z}_{H^+} = 0$ can be obtained from the plot of $(\mathrm{pH} - \log\alpha)/(1-\alpha)$ vs. $\bar{Z}_{H^+}$. The entire procedure was repeated several times with different sets of assumed pK_{int} values of α-COOH and imidazole groups of histidine residues each time until a constant value of 5.18 was obtained for the intercept at $\bar{Z}_{H^+} = 0$ which was the value for pK_{int}

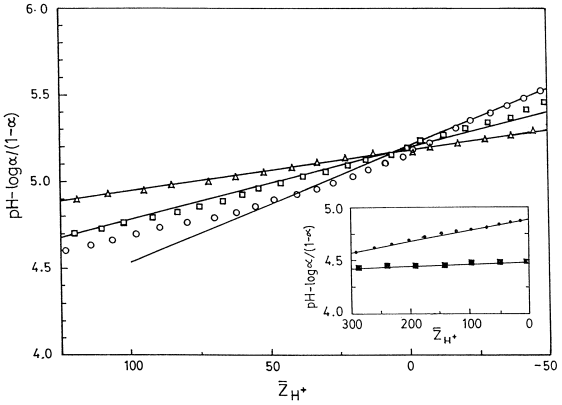

Fig. 6. Plots of titration data for β, γ-carboxyl groups of goat α-crystallin according to Eq. (1). (△), 0.3; (□), 0.2 and (○), 0.1 ionic strengths. Inset shows similar plots of titration data obtained in the presence of 1.5 M GuHCl (●) and 6 M GuHCl (■). The lines were computed by the method of least squares. At 0.1 ionic strength, however, data below $\bar{Z}_{H^+} = +75$ are used in the least square fitting because of considerable scattering of the data higher than this $\bar{Z}_{H^+}$ value.

of β, γ-carboxyl groups (Fig. 6). From the slopes of the plots of Fig. 6, the values of w, the electrostatic interaction parameter, were evaluated at different ionic strengths. The two important criteria of Linderstrøm–Lang equation were fulfilled by our data: (i) the data obtained at 0.3 M and 0.2 M KCl fall comfortably on the respective straight lines from $\bar{Z}_{H^+} \leq +150$ and those obtained at 0.1 M KCl also showed linearity, of course, from $\bar{Z}_{H^+} \leq +70$; (ii) the three straight lines really intersected at $\bar{Z}_{H^+} = 0$, which is expected theoretically in the absence of ion binding. However, the value of pK_{int} determined in this way for the β, γ-carboxyl groups of α-crystallin was considered to be somewhat higher than usual. This value has been found to lie generally within the range 4.3–4.8 for other proteins [22]. The value was found to be normalized as α-crystallin gradually dissociated. Thus, in the presence of 1.5 M GuHCl the pK_{int} value dropped down to 4.88 and in 6 M GuHCl, however, the value was found to normalize to 4.48 (Fig. 6, inset). In the presence of 1.5 M and 6 M GuHCl, however, the values of w were found to decrease to 0.0011 and 0.0002 respectively as expected for denatured proteins.

A value of pK_{int} for imidazole groups of histidine residues at 0.3 ionic strength has been computed in the similar manner extending the linear logarithmic plot to $\bar{Z}_{H^+} = 0$ to the neutral region of the titration curve. The plot (not shown) has the same slope as obtained for carboxyl groups at the same ionic strength as is required by the theory. The pK_{int} value for imidazole groups has been found to be 7.0.

Theoretical values of w at different ionic strengths were calculated from the equation based on the Debye–Hückel theory:

$$w_{cal} = \frac{\varepsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right)\ldots \qquad (2)$$

where b is the radius of the protein sphere (hydrated), a is the distance of the closest approach of salt ion (exceeds b by about 2.5 Å for KCl), κ is the Debye–Hückel constant defined as: $\kappa = (8\pi\varepsilon^2\mu/1000\ DkT)^{1/2}$, μ is the ionic strength of the solution, D, the dielectric constant of water, k, the Boltzmann's constant, ε, the protonic charge and T the temperature in the absolute scale. The assumption of sphericity of α-crystallin molecule required to apply Eq. (2) is not far from reality since our previous work [39] on electron microscopy has established that α-crystallin molecule is a sphere with an average diameter of 160 Å; the ratio of the major and minor axes has been found to be statistically close to unity. Values of w calculated from the above equation were 0.004, 0.005 and 0.006 at ionic strengths 0.3, 0.2 and 0.1 respectively. The respective experimental values are: 0.003, 0.005 and 0.008 as obtained from the corresponding slopes of Fig. 6. The experimental value of w at 0.1 ionic strength appears to be much higher. However, the experimental points at this ionic strength are also found to be more scattered (Fig. 6). The reversible portion of the titration curve at 0.3 M KCl has been theoretically computed using the titration parameters of Table 1. The calculation does not take into account of 130 residues of histidine and 134 residues of lysine since they are not titratable and hence it is assumed that they do not enter into H^+-equilibria. Fig. 4 shows that the experimental data follow closely the calculated ones from pH 4.6 to 9.25. From pH 4.6 to the acid end point, however, there is a considerable deviation between the two sets of data. The reason for this deviation can not be unequivocally settled at this stage but some reasonable possibilities can be discussed (see Section 4).

3.6. Effect of pH on CD spectra

In the far UV CD spectra (Fig. 7A), it is seen that there is practically no significant change in the secondary structure of α-crystallin between pH 4 to 9. To evaluate quantitatively the maximum deviation in mean residue ellipticity at different pH values has been observed to be ± 200 deg when compared with that of the native protein at pH 7. The position of the minima being more or less unaltered at around 217 nm up to pH 4.6, which is found for the native protein at pH 7. Below pH 4.6, there is a slight blue-shift in the minima. However, at about pH 3, there is a drastic change in the ellipticity towards a more negative value with a minima of about 215 nm.

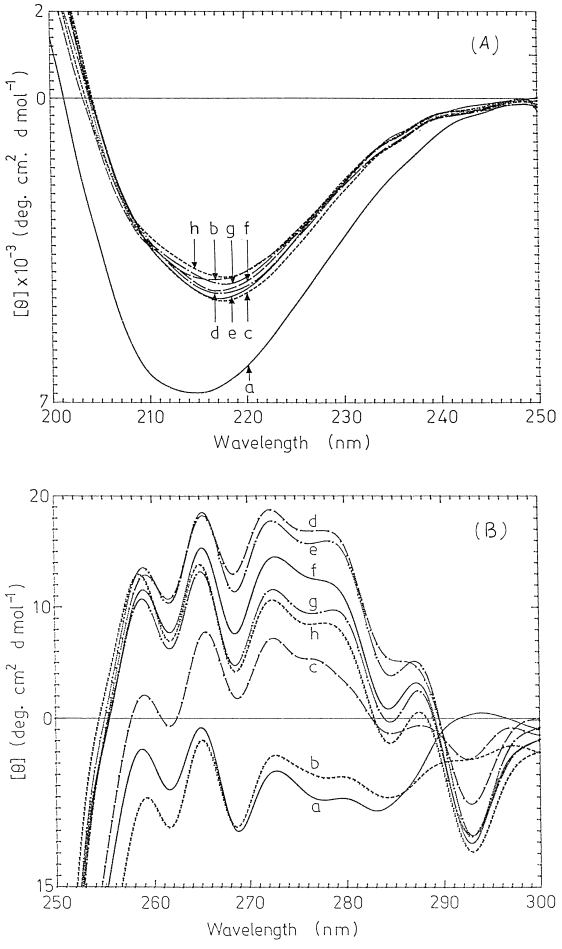

Fig. 7. CD spectra of goat α-crystallin as a function of pH at room temperature in 0.3 M KCl solution. Cylindrical quartz cuvettes of 1 mm (for measurements in the far UV range: 200–250 nm, (A)) or of 10 mm (for measurements in the near UV range: 250–320 nm, (B)) path lengths were used. Protein solutions ($\sim$0.1 mg/ml for far UV and $\sim$1.0 mg/ml for near UV measurements) were prepared in 0.3 M KCl solutions of pH ranging from 3 to 9 adjusted by adding appropriate amount of HCl or KOH solutions prepared also in 0.3 M KCl solution. Five scans were recorded for each sample at a scan speed of 20 nm/min for far UV and 50 nm/min for near UV measurements with a bandwidth of 2 nm. The scans were averaged, corrected for buffer and smoothed for eliminating background noises. Spectral profiles observed at different pH values: (a), 3.0; (b), 4.0: (c), 4.6, (d), 5.0; (e), 6.0; (f), 7.0; (g), 8.0; (h), 9.0.

The typical near UV CD spectra of native goat α-crystallin Fig. 7B (profile: f) is composed of five characteristics maxima at 259, 265, 273, 279 and 287.5 nm and five distinct minima at 262, 268.5, 275, 284.5 and at 292.5 nm. Above 256 nm the elipticity values are always found to be positive except for the minima at 292.5 nm. The negative vibronic transition at 292.5 and the 284.5 nm minima are owing to the tryptophan residues [42]. The maxima at 259 and 265 nm and the minima at 268.5 and 262 nm are characteristics of phenylalanine fine structure. The remaining transitions between 270–280 nm arise from tyrosine and/or tryptophan residues, the 287.5 nm maxima being owing to tyrosine. It is apparent that the effect of pH is manifested (Fig. 7B) more in the alteration of intensities of the spectra rather than in the fine structure or in the positions of maxima or minima except of course, at pH values below 4.6. At both the pH values, i.e., pH 4.05 and 3.06 studied below pH 4.6, the mean residue elipticity values, however, assumed a negative sign throughout the wavelengths scanned. Moreover, the peaks at 292.5 and at 287.5 nm which are known to be characteristics of Tyr and Trp residues in the protein were found to be absent at these pH values and the peak around 278–279 nm has been found to be slightly red-shifted to 280.5 nm. Some conformational change of the α-crystallin aggregate is therefore envisaged below pH 4.6.

4. Discussion

From the results described above, it appears that all C-terminal carboxyl groups, all β, γ-carboxyl groups of aspartic acid and glutamic acid side chains, all arginine residues, and part of lysine and histidine

residues reside on the surface of α-crystallin aggregate. The rest of the lysine and histidine residues are either away from the surface or present on the surface in a manner incapable of being titrated or chemically modified. This offers directly an estimate of the surface charge of α-crystallin aggregate. Our earlier studies indicate that 28 ± 2 tyrosine [38] and 13 ± 1 cysteine [39] residues are also unavailable for titration or chemical modification in the native protein. At pH 5.84 which is the isoionic point of the protein in 0.3 M KCl, the total negative charge of the α,β,γ-carboxyl groups appears to be counterbalanced with the total positive charge of the solvent accessible histidine, lysine and arginine residues within the experimental error (Table 1). This makes the $\bar{Z}_{H^+}$ value to be virtually zero at the isoionic point. It seems that α-crystallin molecule can be represented adequately by the Linderstrøm–Lang model between pH 4.6 to 9.25. The more sophisticated model, Tanford and Kirkwood model [43], could not be used in analyzing our data since this treatment requires precise knowledge about locations of titratable groups in the molecule. Since the crystal structure of γ_{II}-crystallin has already been solved [35], this model has been successfully applied recently to the titration studies of γ_{II}-crystallin [44]. As crystal structure of α-crystallin aggregate is still lacking we are compelled to use the Linderstrøm–Lang treatment as the only available model.

The somewhat high pK value of β, γ-carboxyl groups indicates that carboxyl groups in this protein are in constrained conditions. The pK_{int} value for the imidazole groups in the protein appears, however, to be more or less normal. We did not determine the pK value for the titratable lysine residues in this work as their ionization takes place in the pH region where reversibility of titration has not been found. However, the pK values for the ionizable tyrosine residues of the protein have been obtained in our previous work by independent spectrophotometric titration which showed reversibility up to pH 10.5. Some of the tyrosyl phenolic groups have been found to have a somewhat higher pK value [38]. The unavailability of these side chains appears to be interlinked with the maintenance of the stability as well as of the appropriate surface hydrophobicity of the aggregate. Both the surface charge and surface hydrophobicity of α-crystallin are important for its interaction with other crystallins on one hand for the maintenance of eye lens transparency and with the target proteins on the other for exhibiting its chaperone activity [45–47]. Partial dissociation of the aggregate in the presence of 1.5 M GuHCl exposes the unavailable histidine residues [20] but not the nontitratable lysine residues. Exposure of latter residues needs complete denaturation of the molecule. The pK_{int} value of the carboxyl groups was also found to be normalized partially in 1.5 M GuHCl and totally in 6 M GuHCl.

The question naturally arises: how can a molecule remains stable over a wide pH range with its 260 basic groups removed from participation in H^+-ion equilibria? We believe that this 260 Lys and His side chains are present in their uncharged form and hence they behave as hydrophobic side chains [22]. When the isoionic protein is dissolved in 1.5 M GuHCl the 130 His residues inaccessible in the isoionic protein become accessible and take up protons from the medium raising the pH of the solution to 6.12 compared to the isoionic pH: 5.88. Similarly, when the isoionic protein is dissolved in 6 M GuHCl the 130 more basic groups come out to take up protons from the medium thus raising the pH still further to 6.29. It may be that these uncharged masked residues exist in the hydrophobic cavity that has been suggested in one of the models proposed recently for the quaternary structure of α-crystallin [12]. The observed diameter of the native α-crystallin molecule appears to be larger than what is computed (133 Å) for a compact globular molecule based on 800 kDa molecular mass. The extra volume of space has been considered to be used up in forming a cavity inside the molecule. The smaller surface area per unit mass of α-crystallin aggregate probably cannot accommodate all the titratable groups on its surface and may demand some of these groups to remain away from the surface in order to stabilize the aggregate.

The observed values of electrostatic interaction factor w, at various ionic strengths as determined from slopes of Fig. 6 have been found to deviate from the calculated values based on Debye–Hückel theory Eq. (2). Even though there is a fortuitous agreement between the theoretical value of w at 0.2 ionic strength with that obtained experimentally, the corresponding theoretical and experimental values of this parameter at 0.3 and at 0.1 ionic strengths,

however, differ approximately by $\pm 25\%$. This cannot owe to any deviation of molecular status of α-crystallin since experimental data in the presence of 0.3 M KCl indicate that there has been no change in the molecular status of the native protein unless the pH is lowered below 3.8. This discrepancy can probably be explained in view of the deviation from the basic assumptions which underlie the Linderstrøm–Lang treatment. As for example, the 800 kDa aggregate of α-crystallin has been considered in the present treatment as a single unit rather than an aggregate composed of 40 subunits resulting in a multi-surface entity. However, the high pK value of β,γ-carboxyl groups obtained from the same plot (Fig. 6) is not a reflection of the deviation from the basic assumptions of Linderstrøm–Lang equation as mentioned above since the same high value of pK_{int} has been obtained even when the figure is replotted with the data averaged on the basis of monomer unit.

The observed deviation of the theoretical titration curve based on Linderstrøm–Lang model from the experimental one below pH 4.6 is noteworthy. As we have seen that α-crystallin remains intact up to pH 3.8 below which there is a gradual dissociation of the aggregate. Stevens and Augusteyn [48] reported that the complete dissociation of α-crystallin into an αA aggregate and αB subunits occurred in glycine buffer, pH 2.5. However, both far and near UV CD data indicate that before dissociation some conformational change of α-crystallin starts below pH 4.6, which becomes more and more prominent with further decrease of pH as both far and near UV CD signals alter drastically at lower pH values. This conformational change is expected to affect the value of w. This is probably the reason behind the deviation of the theoretical titration curve from the experimental one below this pH. Since titration data in this pH region show both reversibility and reproducibility, it appears that the conformational change in this pH region, which is probably owed to the repulsive interactions between basic residues in the protein, is also a reversible phenomenon.

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