THE INTERACTION OF ALPHA-LACTALBUMIN WITH TRIS CATION AND OTHER CATIONIC HYDROXAMINES

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SUMMARY: In the course of physical studies on alpha-lactalbumin, the "B protein" of the lactose synthetase system, anomalous behavior was observed in starch gel electrophoresis experiments when they were carried out in Tris buffer at pH 7.0. Further investigation has confirmed this anomalous behavior both in starch gel and in cellulose acetate electrophoresis experiments. The sharp pH dependence of this effect near the pK_a of the Tris amino group suggests a requirement for the cationic amine. A simple series of dilution experiments and electrophoretic runs in various buffer salts were carried out. The effect was found to be reversible upon dilution or addition of a second salt or buffer system. Anomalous behavior was also found in D-glucosamine buffers at pH 6.5. This alteration in the apparent net charge of alpha-lactalbumin is attributed to the binding of the protonated hydroxyamine species.

In the past several years, interest in the milk protein alpha-lactalbumin has intensified, particularly since the description of its role in the lactose synthetase system (1) and the striking homology between this milk protein and hen's egg white lysozyme (2). While preparing some alpha-lactalbumin for fluorescence polarization studies, a starch gel electrophoresis experiment was carried out in order to judge the state of purity of the protein. The results of the experiment which was carried out in a Tris buffer at pH 7.0, indicated a significantly altered charge on the alpha-lactalbumin molecule. The anomalous behavior was readily apparent

since the alpha-lactalbumin migrated as a cation, in spite of the fact that it was in a solution of over two pH units above its isoelectric point (3). The alpha-lactalbumin was found to behave completely normally (anionic behavior) when the same experiment was carried out in sodium phosphate buffer. The effects of pH, ionic strength and a second salt were explored.

In looking for compounds analogous to Tris which might show a similar affinity for alpha-lactalbumin, a series of experiments were carried out in D-glucosamine buffers. While anomalous electrophoretic behavior was not observed at pH 7 in Glucosamine buffer, when the pH was lowered to 6.5 once again cationic behavior was evident. In addition, the possible binding of two zwitterionic buffers containing cationic hydroxyamines was studied.

Starch gel electrophoresis - Initial experiments were carried out in 15% starch according to the technique of Carsten and Pierce (4). One mg samples were dissolved in 20 µl of buffer and the solution was soaked up into a small square of filter paper. The filter papers were carefully inserted into the horizontal gel and electrophoresis carried out for 3 1/2 hours at 15-20 volts per centimeter at 4°. The gels were sliced horizontally with a sharp Stadie blade and stained for 3 minutes in methanol, acetic acid, water (5:1:5), saturated with Amidoschwartz 10B. Destaining was accomplished by washing with the same solvent mixture.

Cellulose acetate electrophoresis - 2% solutions of alpha-lactalbumin or lysozyme in the appropriate buffer were spotted in the center of strips of cellulose acetate (Sephrapore III, Gelman Instrument Company, Ann Arbor, Michigan) which had been presoaked in buffer for 8-12 hours at 4°. Electrophoresis was carried out for 25 minutes at 25 volts per cm. The electrophoretograms were fixed and stained in a Ponceau S-5% trichloroacetic acid mixture

and then destained by washing with 5% acetic acid. The finished electrophoretograms were then dried and preserved under transparent mending tape. Glucosamine buffers were made from D-glucosamine·HCl and sodium hydroxide. Relative electrophoretic mobility was calculated in all cases as the ratio of the distance migrated by alpha-lactalbumin to that migrated by hens egg white lysozyme.

A typical example of the anomalous electrophoretic behavior in the presence of Tris cation is shown in Figure 1a along with "normal migration" in an experiment conducted under identical

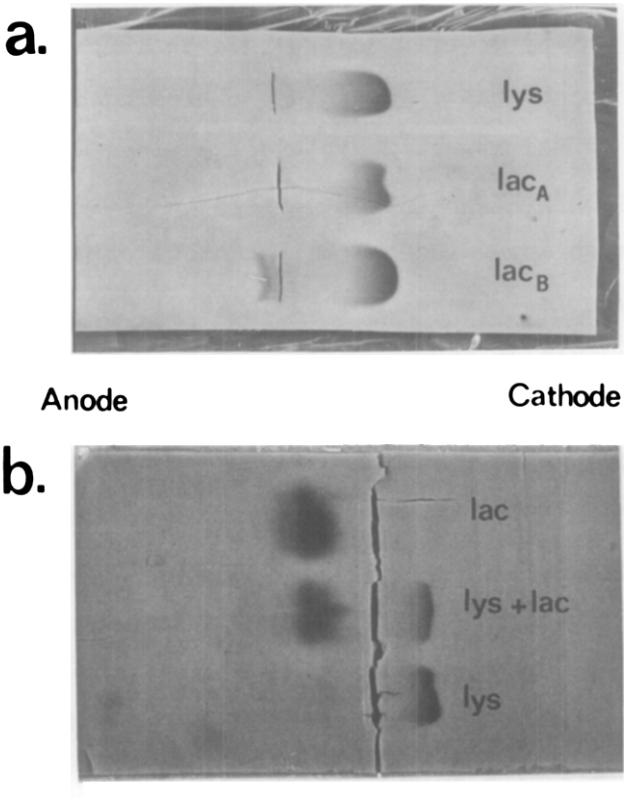

Figure 1a Starch gel electrophoresis in 0.025 M Tris buffer pH 7.0. 1 mg samples of each protein were electrophoresed for 3 1/2 hours at 15 volts per cm in the cold room (4°). Lac$_A$ was prepared in our laboratory, lac$_B$ was provided by Professor R.L. Hill, Duke University and contained a small amount of a second protein.

Figure 1b Starch gel electrophoresis in 0.025 M sodium phosphate buffer, pH 7.0. Conditions were identical to Figure 1a. Lac$_A$, a mixture of lac$_A$ and lysozyme and lysozyme alone are shown from top to bottom respectively.

conditions but in phosphate buffer, Figure 1b. An investigation of the effect of pH on this apparent interaction with Tris showed apparent binding of Tris cation between pH 6.5 and 7. At pH values below 6.5 and above 7 the effect rapidly disappeared. These observations suggest that both the charge of the Tris and that of the protein are important factors in this interaction. Additional evidence that the interaction is primarily an electrostatic one is provided by the fact that upon the inclusion in the electrophoretic medium of a second buffer salt, such as sodium phosphate or neutral salt, such as NaCl the anomalous migration was virtually eliminated. While it is difficult to eliminate the possibility that the uncharged Tris species also binds, since it would have a negligable effect on the electrophoretic migration of the protein, preliminary studies using the intrinsic fluorescence of alpha-lactal bumin suggest no such interaction occurs (5).

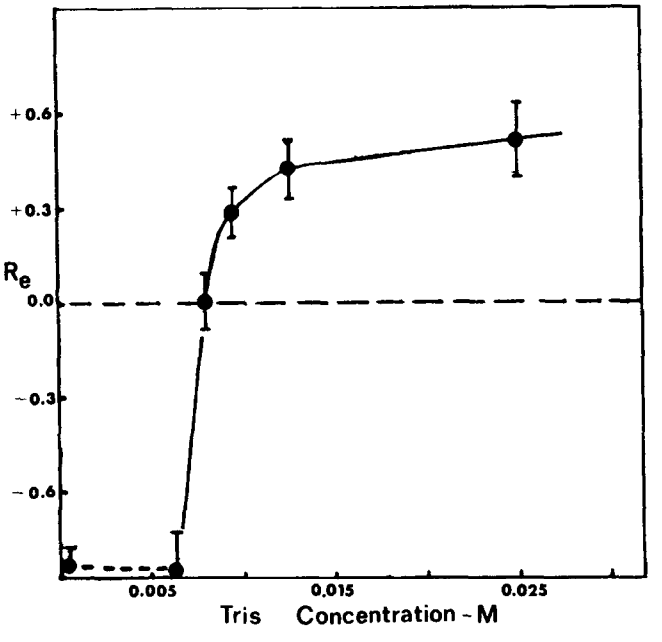

Figure 2 A plot of relative electrophoretic mobility of alpha-lactalbumin with respect to lysozyme (R_e) as a function of Tris buffer concentration at pH 7.0.

In order to establish that the association of Tris with alpha-lactalbumin was reversible, a series of experiments carried out in buffers of decreasing concentration and the relative mobility of alpha-lactalbumin with respect to lysozyme was measured. Figure 2 shows a plot of relative electrophoretic mobility versus Tris concentration. As may be seen in Figure 2, dilution results in a dissociation of the complex and the protein approaches normal mobility at concentrations near 0.005 M. Figure 3 shows cellulose acetate electrophoretograms of a mixture of alpha-lactalbumin and lysozyme in 0.05 M D-glucosamine, pH 6.5

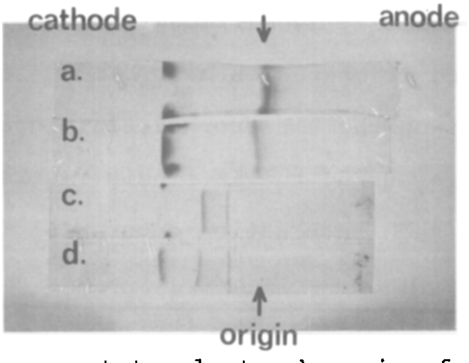

Figure 3 Cellulose acetate electrophoresis of lysozyme and alpha lactalbumin: a. alpha-lactalbumin spotted across the strip, lysozyme at edges only. Electrophoresis was in 0.025 M sodium phosphate at pH 6.5 for 25 minutes at 25 volts/cm. b. Alpha-lactalbumin and lysozyme cospotted across the strip. Electrophoresis buffer and conditions were the same as in a. c. Alpha-lactalbumin spotted across the strip, lysozyme at edges only. Electrophoresis was in 0.025 M glucosamine buffer at pH 6.5 for 25 minutes at 25 volts/cm. d. Alpha-lactalbumin and lysozyme co-spotted across the strip. Electrophoresis buffer and conditions were the same as in c.

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and at the same pH in a phosphate buffer. D-glucosamine cation also appears to bind to alpha-lactalbumin under appropriate conditions. Figure 4 shows a dilution study on the alpha-lactalbumin-glucosamine complex. In this case once again normal behavior is approached at concentrations below 0.001 M.

In order to evaluate the effect of a negative charge on the ion binding, two zwitterionic buffer species were investigated.

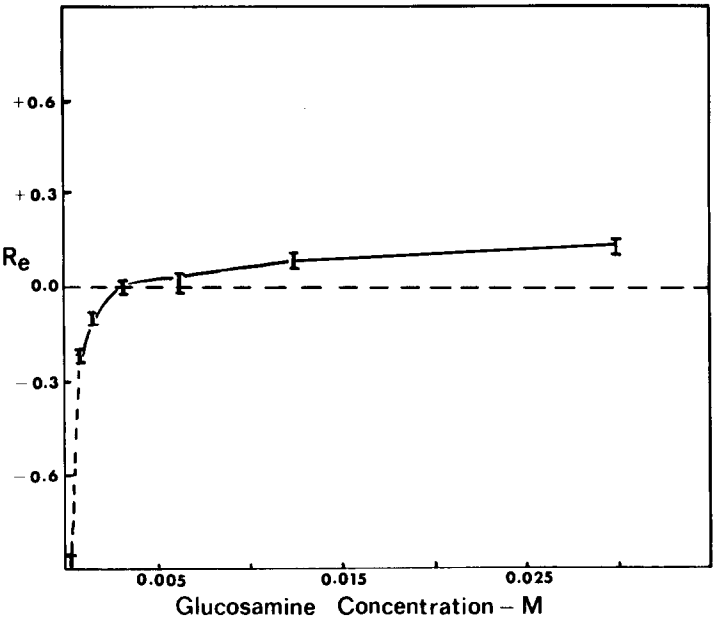

Figure 4 A plot of relative electrophoretic mobility of alpha-lactalbumin (R_e) as a function of glucosamine buffer concentration at pH 6.5.

The two compounds were Bicine (N,N, bis(2-hydroxyethylglycine) and Tricine (N-Tris(hydroxymethyl)methyl glycine) as expected no reversal of mobility was observed in the zwitterionic buffers and little buffer concentration effect was evident. The latter suggesting little or no interaction between Bicine and Tricine zwitterions and alpha-lactalbumin. Figure 5 illustrates a control experiment in phosphate buffer. The slowly decreasing electrophoretic mobility of alpha-lactalbumin in very dilute phosphate buffers reflects the general electrostatic influence of the environment on the rate of migration of the protein and possibly some interaction with the cellulose acetate support medium under conditions where we believe no significant ion binding is occurring.

While the cation binding described here is not likely to be of physiological significance in the case of Tris, the observation

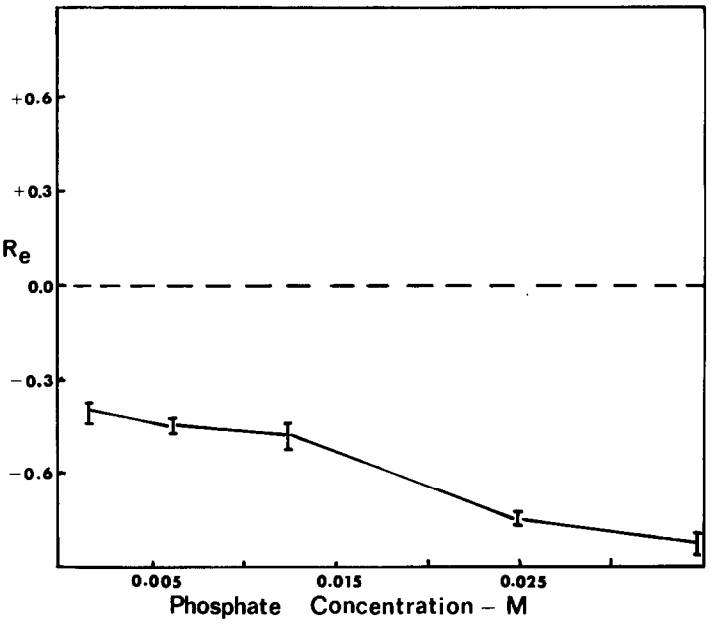

Figure 5 A plot of relative electrophoretic mobility of alpha-lactalbumin (R_e) as a function of phosphate buffer concentration at pH 6.5.

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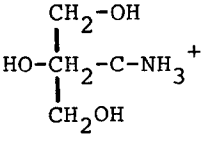
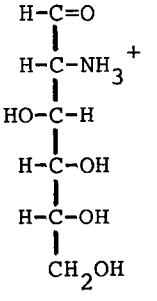
that D-glucosamine interacts with alpha-lactalbumin is of interest in view of both the role of this protein in disacharide synthesis and its homology with lysozyme, a protein which is known to bind glucosamine derivatives. One may readily see the homology between the two cations Tris (a) and D-glucosamine (b).

(a)



(b)



It is necessary, based on the observations reported here, to avoid Tris buffers in most studies of the milk protein, alpha-lactalbumin. This is an interesting case, never-the-less, of a

class of buffer ions which reversibly bind to a protein species and may merit further investigation as a potential tool in probing the specificity of alpha-lactalbumin. It is interesting to contrast this binding of a cationic species with the report in 1956 by Zittle (6) of anion binding to alpha-lactalbumin near its isoelectric point. In the latter case, ion binding significantly influenced both electrophoretic mobility and solubility.

In the case of Tris binding, alpha-lactalbumin has been shown to exhibit anomalous properties in electrophoresis, in isoelectric focusing (7) and in rotational diffusion experiments (8). Further study with other hydroxyamine cations may yield information concerning the ion binding properties of alpha-lactalbumin.

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