Modification of Arachin—Effect of Citrate Ions—Structural Implications

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The effect of the anion citrate on the subunit structure of arachin has been monitored by velocity sedimentation, viscosity and circular dichroism techniques. The results indicate that as the concentration of citrate ion increases from 25 mM to 150 mM, arachin dissociates progressively to the low molecular weight 2S component. This dissociation is not accompanied by any conformational changes or denaturation as revealed by the far ultraviolet circular dichroic spectra and viscosity results, respectively. The results are explained by direct anion binding and changes in the solvent structure.

Arachin, the high molecular weight protein component in groundnut, constitutes nearly 65% of the total protein. It is an oligomeric protein of molecular weight around 330,000 daltons and consists of 6 × 2 subunits in the parent molecule (1-3). Various methods have been used to alter the functionality of the protein. We have reported on the acylation of total groundnut proteins and the functional properties of the acylated derivatives (4). In order to understand the implications of these modifications, arachin has been acylated (succinylated and acetylated) and the physicochemical properties of the acylated derivatives have also been reported (5-7). In this study, we have examined the effect of the anion citrate on the subunit structure of arachin by velocity sedimentation and spectroscopic methods. It is hoped that these results will give a deeper insight into the subunit interactions of arachin and the various forces that hold the native arachin molecule together.

EXPERIMENTAL

Groundnut seeds of commercial variety were purchased from the local market. After dehusking, the seeds were decuticled by mild roasting (40-50 C) for 10-15 min and subjected to mechanical abrasion. The decuticled kernels were then flaked and defatted by extraction with n-hexane. The defatted meal was air dried, powdered, passed through 60-mesh sieve and stored. Arachin was isolated from defatted groundnut flour by the procedure of Dawson (8). The final ammonium sulphate precipitate was dissolved in water, dialyzed against the same and finally freeze dried. The freeze dried arachin is designated as native arachin and was used for all the studies. The various chemicals used in the experiments were of the highest purity commercially available. For various experiments, a solution of arachin in 0.06 M phosphate buffer pH 7.5 containing different concentrations of citrate ions was used. These solutions were dialyzed against the same solvent for 24 hr. Protein concentration was measured utilizing a value of $E^{1\%}_{1\,cm}$, 280 nm = 7.9 for arachin in the above solvent.

Analytical ultracentrifugation. These experiments were carried out at room temperature (25 C) at 59,780 rpm in a Spinco Model E analytical ultracentrifuge equipped with phase plate schlieren optics and RTIC unit. A 1% solution of the protein was used for all the experiments. Photographs of the sedimentation velocity patterns were taken at different time intervals of centrifugation, and the sedimentation coefficient was calculated by the standard procedure (9). The proportions of the various components were estimated by area measurements of the enlarged tracings of sedimentation patterns with a Gaertner microcomparator.

Viscosity. Viscosity measurements were made using an Ostwald viscometer in buffer as well as in the presence of 50 and 100 mM citrate at a temperature of 30 ± 0.1 C. The values of reduced viscosity were calculated after determining the concentration of the protein accurately.

Circular dichroism. Circular dichroism (CD) spectra were recorded with a Jasco J20C spectropolarimeter both in the visible and far ultraviolet region in phosphate buffer. Slits were programmed to yield 10Å band width at each wave length. The data were reduced to mean residue ellipticities using a value of 115 for the mean residue weight for arachin. A protein concentration of 0.1% was used in a 1-cm path length cell for the near ultraviolet region and 0.1 cm path length with 0.04% protein solution for the far ultraviolet region. The dichroic absorbance differences were averaged from two recordings, and the mean ellipticity was calculated from the averaged spectrum.

RESULTS AND DISCUSSION

Association and dissociation. The association and dissociation of arachin as a function of different concentrations of citrate was monitored by velocity sedimentation (Fig. 1). Arachin in low ionic strength buffers sediments as a single homogeneous peak with a sedimentation coefficient of 14. This generally is termed the "arachin dimer." A plot of the percent fraction of various sedimenting components in the presence of increasing concentration of citrate is shown in Figure 2. Citrate at concentrations as low as 5, 10 or 20 mM also showed no effect in the shape of the velocity sedimentation profile or formation of any other new components. As the concentration of citrate was increased from 25 to 100 mM, arachin dissociated into a low molecular weight 2S component. At a citrate concentration of 150 mM, dissociation continues and tapers off to a value of nearly 45% of the 2S component. Above these concentrations of citrate, arachin tends to precipitate out of solution.

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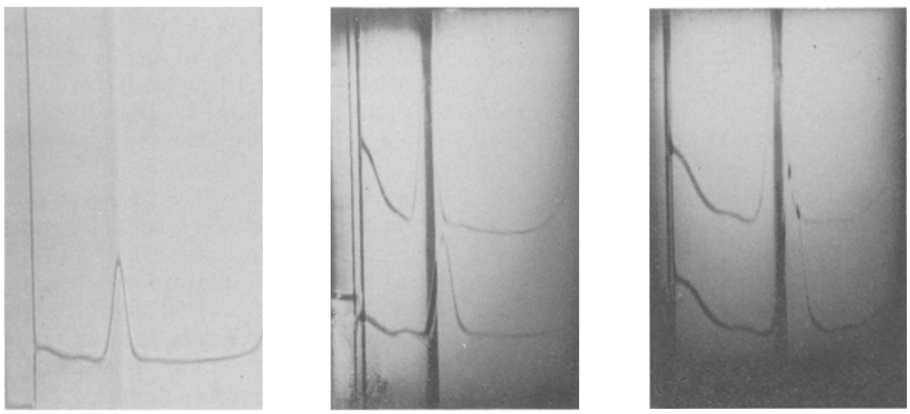

FIG. 1. Sedimentation velocity patterns of arachin. a, in phosphate buffer, pH 7.5 (22 min); b, lower, 25 mM citrate, and upper, 100 mM citrate (22 min); c, lower, 50 mM citrate, and upper, 150 mM citrate (32 min), respectively. The times of photographs are shown in the parentheses above; all runs were performed at 59,780 rpm.

Chemical modification of arachin also dissociates the protein to various low molecular weight components. Succinylation of arachin causes dissociation into low molecular weight protein components (6). At nearly 60% succinylation, the 14, 9 and 4S components are present in nearly equal proportions. At a high degree of succinylation such as 83%, the 4S component was predominant. It is not possible to say clearly whether the 4S component was derived from the 14S or 9S component (6). Polyacrylamide gel electrophoresis of native arachin at pH 8.3 under varying degrees of acylation showed dissociation of arachin (7). Gel filtration results also indicate that, upon increasing the extent of acylation, arachin dissociates to low molecular weight components (7).

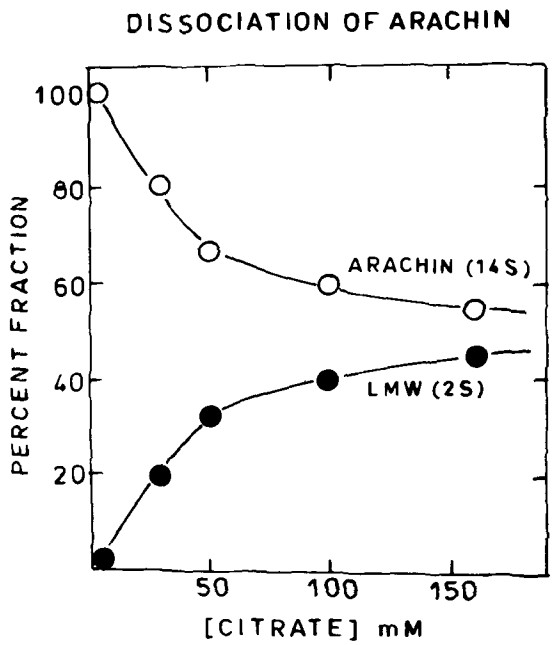

FIG. 2. Percent fraction of arachin (14S) and low molecular weight component (2S) citrate concentration of sedimentation velocity experiment.

The results of dissociation of arachin with an increasing concentration of citrate ion are different from the dissociation phenomenon associated with acylation of arachin. In the acylation of arachin, one could detect a 9S component and the most dissociated peak was the 4S component, while with citrate, the native arachin appears to dissociate directly to form the 2S molecule. It is possible that the 9S and 4S components are present in the citrate system, but the concentrations are too small to be detected by analytical ultracentrifugation. These results indicate that arachin dissociates upon the addition of citrate ion. In order to understand whether this dissociation process is also accompanied by denaturation, the effect of citrate ion on the reduced viscosity of arachin was studied.

Denaturation. The reduced viscosity of arachin is 0.054 dl/g in phosphate buffer. It did not change with an increase in citrate concentrations at either 50 or 100 mM. However, on acylation, there was more than a threefold increase in the reduced viscosity (7). The increase in viscosity was gradual up to 50% acylation, and later there was a marked increase. At comparable levels of acylation, acetylation seemed to have a greater effect on viscosity than did succinylation.

The reduced viscosity of arachin (0.054 dl/g) is slightly higher than that of globular proteins (0.03–0.04 dl/g) (10). Arachin can be considered to have a fairly compact structure similar to that of the major protein fractions, α-globulin from sesame seeds and the 11S component of sunflowerseed, respectively (11,12). Above 100 mM citrate concentration, at 1% protein concentration, the solutions were slightly turbid but the protein did not precipitate out. Hence, viscosity measurements were not performed above these concentrations of citrate.

The reduced viscosity of arachin at 100 mM citrate concentration was 0.06 dl/g.

Conformational stability. Arachin in its native state contains very little α-helix (5%) and is rich in aperiodic (60%) and β-structure (35%) (11,13). In the near UV region, it has characteristic peaks originating from the aromatic amino acids, phenylalanine, tyrosine and tryptophan, and also from cystine residues. In the far UV region, it has a minimum around 208 nm with [Θ]Mrw of -6000 (Fig. 3). Addition of citrate ions up to nearly 150 mM showed no change in the CD spectra of arachin either in the near UV or far UV region. The data indicate that the microenvironment around the various chromophores in arachin is not perturbed, and the secondary structure of arachin is unchanged even at 150 mM citrate concentrations. These results are in excellent agreement with those of Jacks et al. (14), who have shown that the dissociation of arachin did not change the secondary structure of the multimer in acidic hexane.

The above results from sedimentation velocity, circular dichroism and viscosity experiments indicate that citrate ions up to a concentration of 150 mM dissociate arachin molecule and neither denature nor change the conformational status of arachin. Similar observations have been made by Prakash (unpublished data) with the protein α-globulin from sesame seed and from 12S protein fraction of mustard seeds.

These studies indicate that citrate ions have a profound effect on the association-dissociation of arachin and indirectly on the nature of forces that are holding the subunits of arachin together.

Yamada et al. (1-3) have shown that arachin consists of six subunits, three of which are acidic and three basic, as revealed by their isoelectric point in a 'monomer' of arachin (9S) component. The models proposed by Pernollett and Mosse (15) and Prakash and Narasinga Rao (11) for the quaternary structure of seed proteins in general fit in very nicely with the arachin molecule. Their model shows that the trimers are held together by non-covalent interactions leading to their stabilization, and in turn two trimers are held together by a much weaker noncovalent interaction. In addition, the acidic and basic polypeptide chains are juxtapositioned in such a way that one acidic polypeptide chain is always encountered by two basic polypeptide chains and vice versa (11,15). Based on this model of arachin, one can explain the effect of citrate ions on the arachin molecule.

At this stage, it is interesting to speculate on the effect of anions in general on the stability of proteins. Generally these involve larger binding energies (16,17), and the free energy of binding is in the range of -1 to -5 kcal/mol. The large entropy changes presumably reflect changes in the structure of the solvent, the protein, or both, which can take place in such a way as to give compensating enthalpy changes.

However, this effect is quite different from the well-known dissociation of oilseed globulins (11), as in most cases it is either the 11S or 14S dissociating to 7S or 9S, and in the presence of citrate here it is the 2S component.

Now, if we consider the arachin molecule according to the model proposed by Prakash and Narasinga Rao (11), the protein is not undergoing any recognizable conformational changes but rather only dissociation at the concentration of citrate used. Since arachin is a hexamer and these subunits already have been shown to be held by noncovalent interactions and citrate ion being the most potent ion in the series of precipitating anions, it is quite understandable that a destabilizing effect of the compact structure of arachin is brought about by indirect solvent effects resulting from changes in the structure of solvent and by direct anion binding or both.

These specific salt effects reflect rather a direct interaction of a counter-ion with charged groups and may be considered mechanistically in the same way as other direct ionic interactions (16,17), especially considering the equal number of acidic and basic subunits that are reported to be present in the arachin molecule.

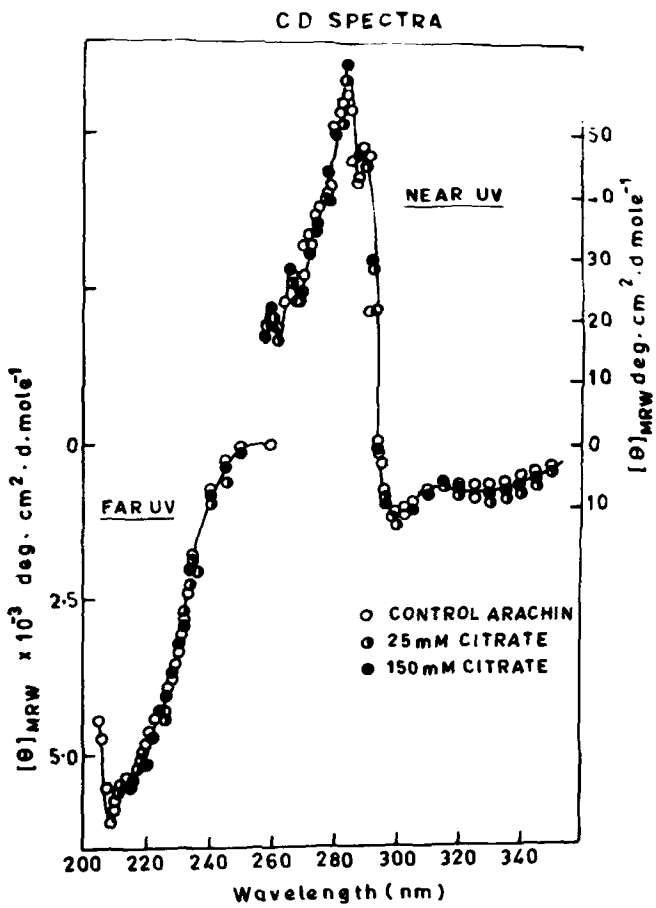

FIG. 3. Near and far ultraviolet CD spectra of arachin in phosphate buffer, pH 7.5, and with different concentrations of citrate.

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