J. Biosci., Vol. 9, Numbers 3 & 4, December 1985, pp. 177–184. © Printed in India.

Association-dissociation of glycinin in urea, guanidine hydrochloride and sodium dodecyl sulphate solutions

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MS received 25 March 1985

Abstract. The effect of urea, guanidine hydrochloride and sodium dodecyl sulphate on glycinin, the high molecular weight protein fraction from soybean has been investigated by analytical ultracentrifugation. Urea and guanidine hydrochloride dissociate the protein to a '2S' protein through the intermediary 7S and 4S proteins. However, in sodium dodecyl sulphate the protein directly dissociates to a 2S protein. Analysis of the data by calculation of per cent fraction and $S_{20,w}$ value indicates that dissociation and denaturation of glycinin occur simultaneously in the presence of the above reagents but to different extents.

Keywords. Association; dissociation; guanidine hydrochloride; sodium dodecyl sulphate; urea; glycinin; denaturation.

Introduction

Urea and guanidine hydrochloride (GuHCl) and sodium dodecyl sulphate (SDS) are well known protein-dissociating and denaturing reagents and are used extensively in protein chemistry to understand the forces responsible for maintaining the native structure of a protein molecule (Joly, 1965; Tanford, 1968; Jancks, 1969). Both urea and GuHCl are effective at high concentrations (> 6 M) whereas SDS is effective at low concentrations (Tanford, 1968,1970; Pace, 1975; Reynolds, 1982). Many proteins loose their ordered structure upon addition of these reagents and in multimeric proteins both dissociation and denaturation take place (Tanford, 1968,1970; Lapanje, 1968; Prakash and Nandi, 1976,1977; Gururaj a Rao and Narasinga Rao, 1979; Rahma and Narasinga Rao, 1981; Prakash and Narasinga Rao, 1984).

The high molecular weight protein component of soybean (*Glycine max*) proteins, termed often as 11 S component or glycinin, is a multimeric protein containing 12 non-identical subunits with a molecular weight of 350,000 (Catsimpoolas, 1967). The dissociation of glycinin has been studied in detail mainly with changes in pH, ionic strength, in sodium octyl benzene sulphonate and urea solutions of low concentration (Naismith, 1955; Wolf and Briggs, 1958; Wolf *et al.,* 1958). Wolf and his co-workers have used urea mostly to dissociate the subunits of glycinin. However, urea, GuHCl and

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Abbreviations used: GuHCl, Guanidine hydrochloride; SDS, sodium dodecyl sulphate; LMW, low molecular weight; $S_{20,w}$, sedimentation coefficient corrected for temperature and viscosity.

SDS have been mainly used to dissociate glycinin into subunits principally for the quantitation of the number of subunits (Catsimpoolas, 1969; Catsimpoolas *et al.,* 1969; Koshiyama and Fukushima, 1976). The present work deals mainly with the dissociation and denaturation of glycinin in urea, GuHCl and SDS solution. The object of this investigation was to determine if there were any differences in the effect of these reagents on glycinin. In addition it was also of interest to determine if dissociation and denaturation of the protein occur simultaneously or sequentially.

Materials and methods

Materials

Bragg variety soybean (*Glycine max*) seeds cultivated in a farm near Mysore, were purchased locally. Sephacryl S-200, ultrapure urea and GuHCl were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Stock solutions of concentrated urea and GuHCl were prepared, treated with activated charcoal and then appropriate buffer salts were added to obtain the required pH. The solutions were used within 7 days of preparation (Prakash *et al.,* 1981) and β-mercaptoethanol was obtained from Fluka; SDS obtained from Loba Chemie was used after recrystallizing twice with ethanol. Bufer salts, sodium chloride and all other reagents were of analytical reagent grade.

Methods

Isolation of glycinin: Glycinin was isolated by a minor modification of the method of Appu Rao and Narasinga Rao (1977). The protein solution in buffer obtained after final precipitation with ammonium sulphate was purified on Sephacryl S-200 column, the major peak portion pooled and dialysed against large quantities of water for 24 h with repeated changes of water. The solution was then centrifuged at 16000 *g* for 1 h and lyophilized.

Determination of protein concentration: The protein in native state and in different concentrations of denaturant were all taken in 0·05 M sodium phosphate buffer of pH 7·8 containing 0·35 M NaCl and 0·1 % β-mercaptoethanol. For experiments with SDS, NaCl was not added. The protein concentration was determined by absorbance measurements using a value of 7·9 for at 280 nm (Appu Rao and Narasinga Rao, 1977).

Sedimentation velocity: Sedimentation velocity runs were made with Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics and rotor temperature indicator and control unit. All runs were made using 1 % protein solution prepared with lyophilised protein. The protein solutions were dialysed against the solvent in which they were prepared for 24 h before the runs were made. A standard 12 mm duraluminium or KelF cell was used for the runs at a speed of 59780 rpm and ~ 27°C. The plates were read on a Gaertner microcomparator or an Abbe Comparator, modified to read ultracentrifuge plates, and $S_{20,w}$ values were calculated by the standard procedure (Schachman, 1959). For density corrections, the density of

solutions was calculated by the procedure of Kawahara and Tanford (1966). Viscosities of solutions were measured with an Ostwald viscometer. The percentage composition of different fractions was determined by measuring the area under each peak of enlarged tracings of ultracentrifugal pattern.

Results

Figure 1 A, shows the sedimentation velocity pattern of glycinin. It indicates a single major peak with an $S_{20,w}$ value of 11·5 with a small quantity (< 5 %) of 7S component

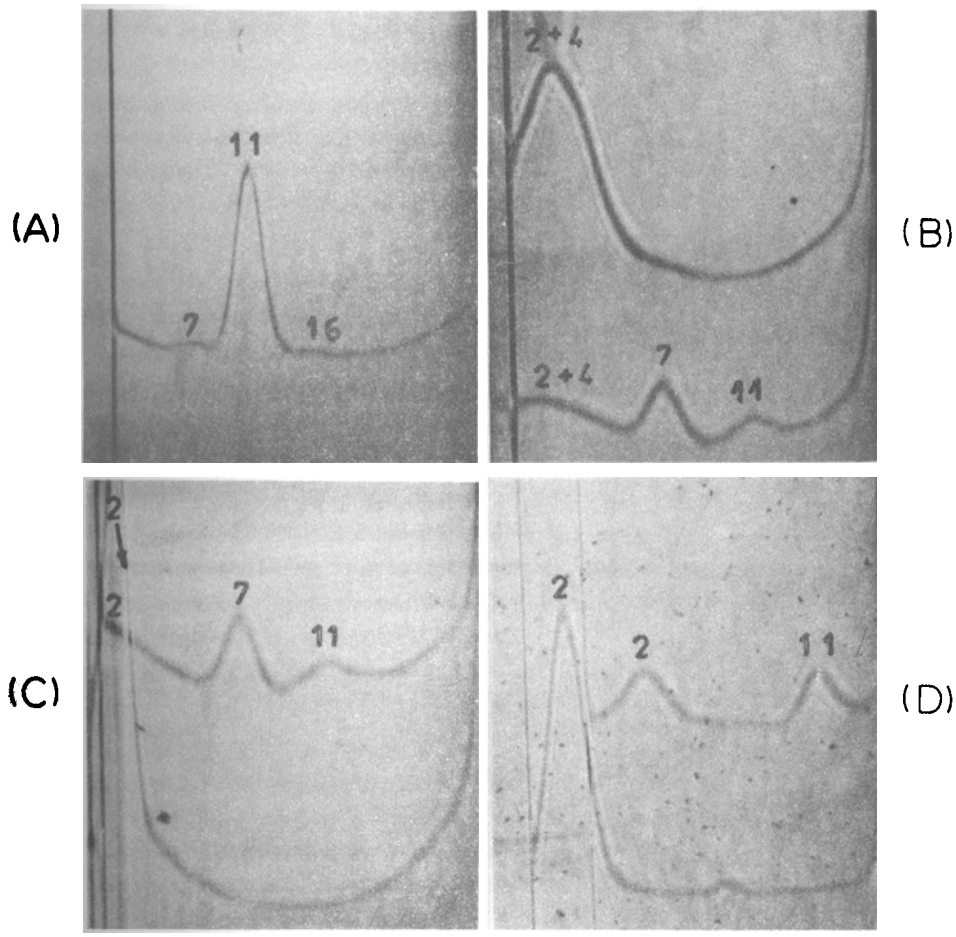

Figure 1. Representative velocity sedimentation patterns of glycinin under various conditions of experimentations. All runs were carried out at 59,780 rpm, 27°C and 1 % protein concentration unless otherwise stated. The photographs are taken 32 min after attaining the maximum speed. Glycinin in (A) phosphate buffer, pH 7·8, 0-05 M containing 0·35 M sodium chloride and 0·1 % 2-mercaptoethanol; (B) top, 5 M urea; bottom, 2 M urea; (C) top, 1 M GuHCl; bottom, 4M GuHCl; (D) top, 0·7mM SDS and bottom, 3·5mM SDS.

and 16S component. This is due to the fact that although the isolated 11S is homogeneous, during freeze-drying and storage it can rapidly associate and dissociate (Wolf *et al.,* 1962; Catsimpoolas *et al.,* 1969). Hence proper corrections are incorporated in analysing the data for the presence of both 7S and 16S components.

Effect of urea

In figure 1B, the velocity sedimentation patterns of glycinin at urea concentrations of 1 M and 3 M are given. The protein dissociates progressively to 7S, 4S and 2S components. However, due to the viscosity of solution, it was difficult to achieve total separation of 2S and 4S components. The following sequence appears to be taking place in the dissociation reaction.

$$11S \rightarrow 7S \rightarrow (4S + 2S) \text{ or LMW component.}$$

The (4S + 2S) component is termed as low molecular weight (LMW) component for the purposes of discussion. From figure 2a it is apparent that even at 1 M concentration, there is considerable LMW component. Also there is a decrease in 11S protein and the 7S protein is also seen. With further increase in urea concentration to 2 M, the 7S protein reaches a maximum concentration, and the LMW component increases rapidly to about 65 %; the 16S protein is absent. At 5 M urea concentration the pattern consists essentially of LMW component. Also the $S_{20,w}$ value ofthe LMW fraction is 2S and the peak is gaussian in shape indicating the homogeneity of the fraction. Prakash and Nandi (1977) have reported similar observations with the multimeric protein α-globulin of sesame seed where the dissociation is complete at 5 M urea.

Effect of GuHCl

In figure 1C, the velocity sedimentation patterns of glycinin in GuHCl solution of 1 M and 4 M are given.

The protein dissociates in these solutions also (figure 1C). The dissociation follows the sequence

$$11S \rightarrow 7S \rightarrow \text{LMW component.}$$

The 7S protein reaches a maximum at 1·5 M GuHCl and is absent at 3 M GuHCl concentration (figure 2b). Also at 3 M GuHCl, most of the LMW component (nearly 90%) is already formed and has an $S_{20,w}$ value of 2S. At-4M GuHCl the pattern consists of 2S protein only. However, with α-globulin from sesame seed, Prakash and Nandi (1977) have reported that above 1·5 M GuHCl most of the protein was present as 2S component. These results indicate that GuHCl is more effective than urea in dissociating the protein.

Effect of SDS

In figure 1D, are shown the velocity sedimentation patterns of glycinin in SDS solutions of 0·7 mM and 3·5 mM concentrations. Here the protein appears to dissociate directly to the 2S protein (figure 1D and figure 2c).

$$11S \rightarrow 2S.$$

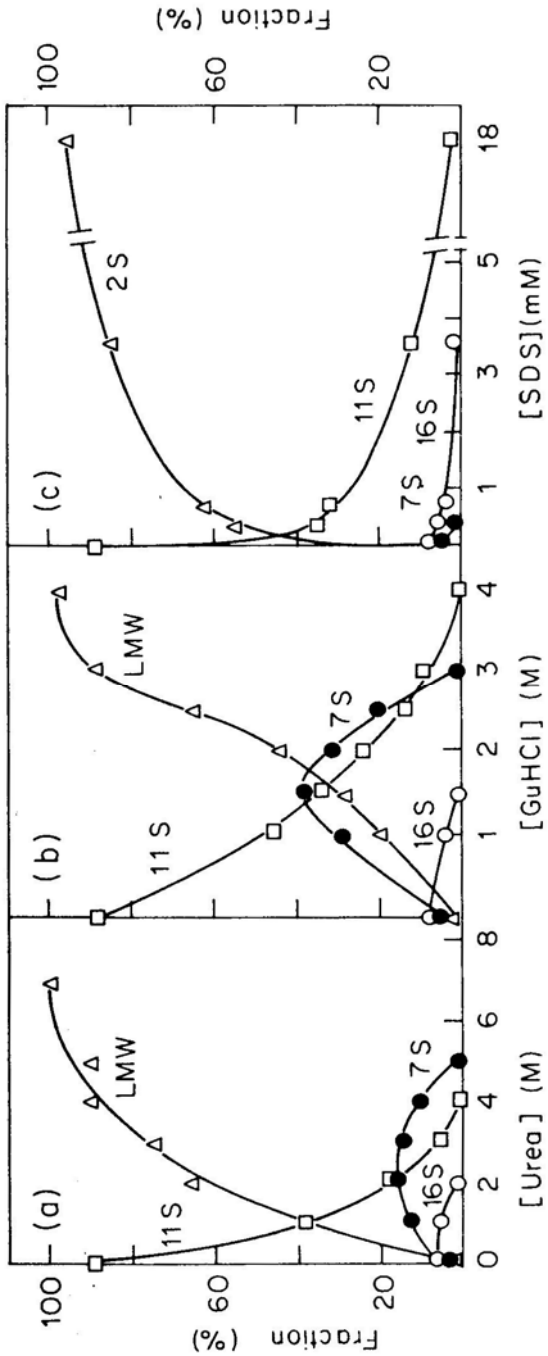

Figure 2. The proportion of various protein fractions as a function of (a) urea (b) GuHCl and (c) SDS concentrations. The various fractions are indicated in each figure.

Around 3·5 mM SDS most of the protein is dissociated to the 2S protein. This is not unusual SDS is known to be a more powerful dissociating and denaturing reagent than urea or GuHCl. Gururaj Rao and Narasinga Rao (1979) have in their study of the effect of SDS on the high molecular weight protein of mustard and rapeseed have reported that the protein directly dissociates to the 2S component. However, α-globulin, does not dissociate directly to the 2S protein in SDS solutions. 7S and 4S intermediates in both urea and GuHCl solutions were observed (Prakash and Nandi, 1977).

Discussion

A comparison of the effect of urea, GuHCl and SDS on glycinin, shows the following.

SDS is more effective than GuHCl and in turn GuHCl is more effective than urea in dissociating the protein. The order is

$$\text{SDS} \gg \text{GuHCl} > \text{Urea.}$$

Meyer and Kauzmann (1962) have reported that unlike urea and GuHCl, detergents do not compete for peptide hydrogen bonds, but instead weaken the hydrophobic bonds. These results suggest the possibility of the predominance of hydrophobic bonds and ionic linkages over hydrogen bonds in the stabilization of the native structure of glycinin. This strong dissociative action of SDS on glycinin may be due to its greater ability to break hydrophobic bonds and ionic linkages (Jencks, 1969).

The 7S protein completely dissociated at 4 M urea and 3 M GuHCl at which concentration the 11S protein is still present. These results suggest that (i) the subunits of 7S component are not held predominantly by hydrophobic bonds and (ii) the conformational stability of 7S protein is low compared to the 11S protein. These results are in conformity with the results of Robert and Briggs (1963) and Wolf *et al.* (1964) who have studied the effect of alcohols on glycinin. Prakash *et al.,* (1980) have reported similar results in their study of the effect of SDS, urea and GuHCl on the conformation of α-globulin.

A comparison of the per cent fraction of 7S component both in urea and GuHCl solutions shows (figured 2a, b) that the maximum proportion of 7S component is ~ 16% in 2 M urea and ~ 39 % in 1·5 M GuHCl. This suggests that the sequence of dissociation in urea and GuHCl could be different due to differences in the mechanism of their action. Alternatively urea may have a greater penetration in to the interior of the protein molecule as compared to GuHCl (Hibband and Tulinsky, 1978).

The $S_{20,w}$ value of different protein fractions decreases with the increase in denaturant concentrations. This decrease possibly reflects the extent of denaturation (Holcomb and Vanholde, 1962). Figure 3 shows a plot of $S_{20,w}$ value of glycinin against denaturant concentration till it is completely dissociated. The $S_{20,w}$ value of 11·5 in native state decreases only very slightly in SDS solution. In 18 mM SDS solution, the $S_{20,w}$ is 10·9 and the maximum change in $S_{20,w}$ value is observed between zero and 3·5mM SDS concentration. With the increase in urea and GuHCl concentration the $S_{20,w}$ value of the protein decreases. Greater change is observed in GuHCl solution. The value of 7·8S in 2·5 M GuHCl is almwost equal to that in 5 M urea. This may reflect the known fact that urea is a less effective denaturing agent than GuHCl at the same

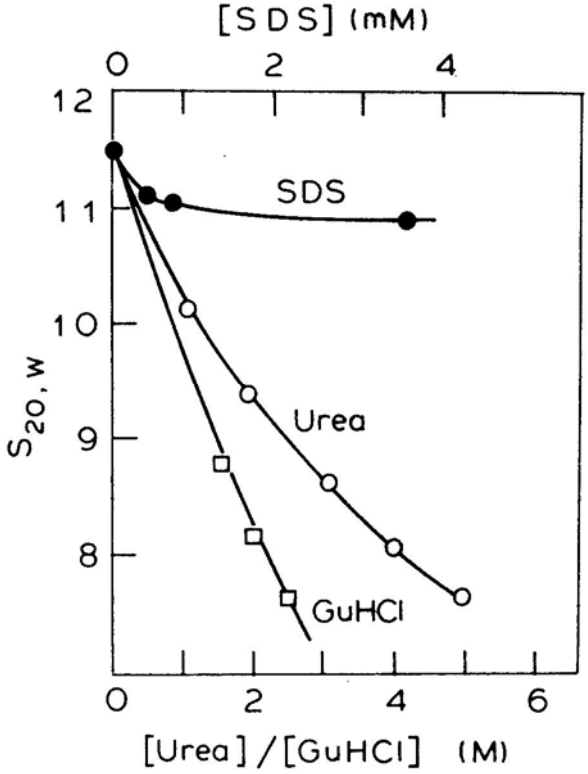

Figure 3. $S_{20,w}$ value of glycinin as a function of denaturant concentration.

molar concentration (Tanford, 1968,1970; Green and Pace, 1974). The $S_{20,w}$ value of 7S protein also decreases with increase in denaturant concentration. Analysis of 2S protein has not been possible because of the very diffuse peaks obtained with the protein.

When studying the effect of reagents such as urea, GuHCl and SDS on multimeric proteins one faces the question whether dissociation and denaturation reactions occur sequentially or simultaneously? Very often it is difficulty to get a clear answer. In this investigation we have studied the effect of urea, GuHCl and SDS on glycinin by sedimentation velocity techniques. The dissociation of glycinin by these reagents is clearly seen by the appearance of components of low $S_{20,w}$ value.

In the case of homogeneous proteins, $S_{20,w}$ value of the protein decreases when it is denatured (Wales and Coll, 1969). Determination of the $S_{20,w}$ value of the peak of undissociated glycinin at various concentrations of urea, GuHCl or SDS shows that it decreases considerably. This is also supported by secondary structural changes in the protein at these concentrations of reagents as measured by circular dichroism (Suresh Chandra, B. R., Appu Rao, A. G. and Narasinga Rao, M. S., unpublished results). This would suggest that the dissociation and denaturation of glycinin occur simultaneously, in the presence of these reagents.

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