Thermal Aggregation of Glycinin Subunits

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ABSTRACT

Glycinin, the major reserve globulin of soybean seeds (*Glycine max*), is dissociated into subunits by exposure to temperatures above 70°C. The thermal aggregation of these subunits was investigated by kinetic scattering absorbance measurements at 320 mμ. The rate and extent of aggregation were enhanced by low ionic strength and the presence of mercaptoethanol. The thermal aggregation of the subunits was depressed at extreme acidic or alkaline pH values, and by high ionic strength. Maximum rate of aggregation was obtained between pH 4.0 and 6.0. Involvement of ionic and hydrophobic bonds in the aggregation process was strongly suggested. Complete dissociation of the protein into subunits, by cleavage of disulfide bonds with mercaptoethanol, appeared to be one of the conditions favoring aggregation. Thermal aggregation which involves dissociation of glycinin into subunits could be distinguished from intermolecular aggregation of the protein at ambient temperatures as the result of pH adjustment in the region of the isoelectric point. These aggregates were dissolved by heat, and hence, disruption of hydrogen bonds was indicated.

The study of association-dissociation phenomena of glycinin[1], the major reserve protein of soybean seeds (*Glycine max*), may contribute to a better understanding of the storage and degradation processes of reserve proteins in seeds, and to improved utilization of these proteins for human nutrition. Glycinin has been isolated in homogeneous form, and some of its properties have been studied in this laboratory (1 to 7) and others (8 to 18). Also, some of the factors affecting heat-denaturation of glycinin as examined ultracentrifugally were reported recently (18). In a previous communication (7), the effects of urea, acid, alkali, and

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[1]Glycinin is equivalent to the 11S component of soybean globulins.

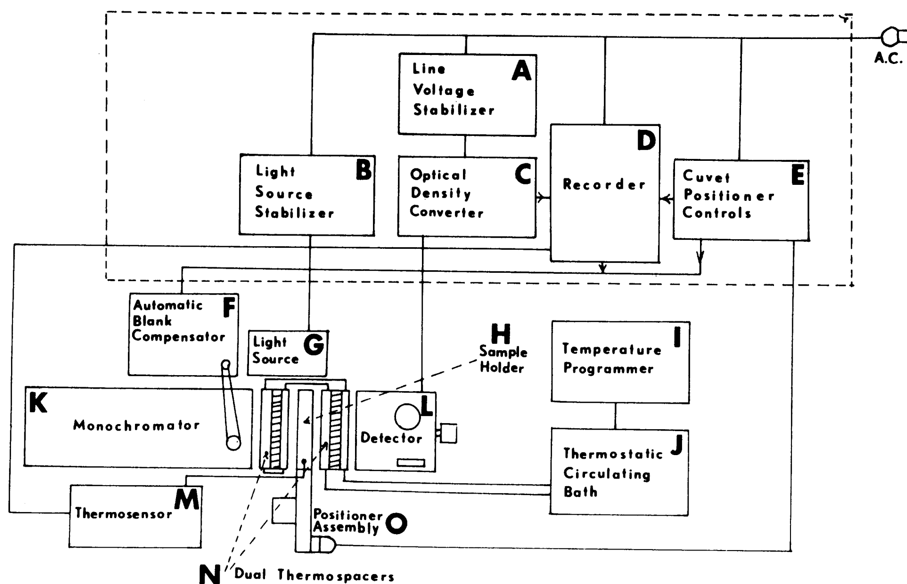

Fig. 1. Block diagram of the optical system used for kinetic scattering absorbance measurements during thermal aggregation of glycinin.

heat-treatment on glycinin were studied by ultraviolet difference spectra, disc electrophoresis, and immunochemical methods. Our interest in the present study stemmed from the previously described observation (7) that glycinin solutions became increasingly turbid when heated at temperatures above 70°C. The observed turbidity was attributed to aggregation of dissociated subunits. The present report describes some of the important factors affecting this type of interaction.

MATERIALS AND METHODS

Glycinin was prepared as described previously (1). Kinetic scattering absorbance measurements at 320 mμ were recorded with a Gilford 2000 spectrophotometer; 1-cm. quartz cuvets were used. The protein samples were heated directly in the cuvets by means of a dual thermospacer set. Sample-compartment temperature control was maintained by circulating water with a Haake thermostatic circulator equipped with a Neslab Model TP-2 temperature programmer. The programmer was set to provide continuous linear temperature variation at the rate of 1°C. per min. Sample temperature was recorded with a Gilford Model 207 linear thermosensor by means of an auxiliary recording channel. The temperature recorded with the thermosensor deviated less than 1°C. from temperatures measured directly in the sample by the use of a YSI Model 42SC telethermometer equipped with a glass thermistor probe. Figure 1 is a block diagram of the system.

When solutions were examined isothermally, the sample compartment was pre-equilibrated to the desired temperature. Usually a sudden temperature drop was recorded upon insertion of the cuvets into the sample holder. The time lapse

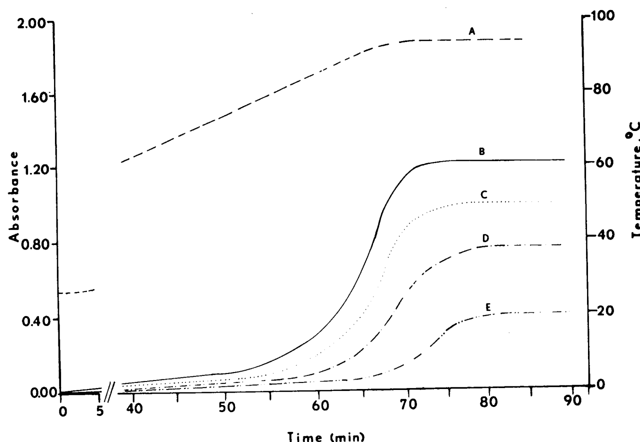

Fig. 2. Kinetic tracings of scattering absorbance at 320 mμ for the thermal aggregation of glycinin at different concentrations. Buffer: phosphate pH 7.6 made 0.4M in NaCl and 0.01M in mercaptoethanol. Protein concentration: B, 0.059%; C, 0.044%; D, 0.029%; and E, 0.014%. A, temperature curve.

between insertion of sample and thermal equilibration could be read directly from the recorder, which was operated at constant speed.

RESULTS

Effect of Protein Concentration

The protein concentration dependence of the kinetic tracings of scattering absorbance at 320 mμ plotted against time and temperature variations is shown in Fig. 2. Samples at 25°C. were heated to a final temperature of approximately 95°C. and absorbance measurements were recorded continuously. The maximum scattering absorbance is increased with increasing protein concentration in the sample. The scattering absorbance changes were insignificant at temperatures below 70°C. for the indicated protein concentrations (0.014 to 0.059%). The glycinin solutions were prepared in pH 7.6 phosphate buffer made 0.4M in sodium chloride and 0.01M in mercaptoethanol. This has been designated as standard buffer (10). When the maximum scattering absorbance was plotted against the protein concentration in the sample, the relation was not linear. This may be due to intraparticle interferences and has been discussed in detail by Oster (19). Thus, meaningful quantitative data with the use of maximum absorbance values could not be considered. Nevertheless, the kinetic tracings, as depicted in Fig. 2, were very useful in exploring the effect of different factors on the thermal aggregation of glycinin on a qualitative basis.

A straight-line relation was obtained under isothermal conditions by plotting the logarithm of the initial relative slopes of aggregation against the concentration of the protein in the samples. The initial relative slopes of aggregation were calculated from the kinetic tracings of the scattering absorbance at 320 mμ vs. time in sec. Such an isothermic (90°C.) diagram is shown in Fig. 3. The straight line fitting the

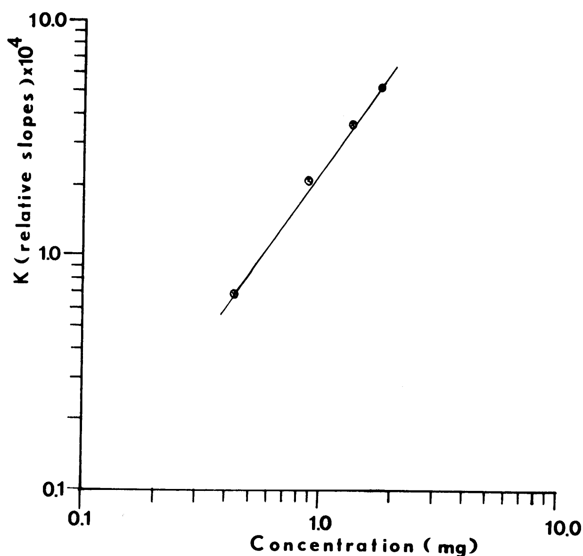

Fig. 3. Protein concentration dependence of the initial slope of scattering absorbance tracings for the isothermal aggregation of glycinin at 90°C. Protein concentration is expressed in mg. per 3 ml. of pH 7.6 phosphate buffer made 0.4M in NaCl and 0.01M in mercaptoethanol.

experimental points has a calculated slope of 1.2. Thus, it appears that the aggregation process can be followed more meaningfully by measuring the initial relative slopes of absorbance measurements at 320 mμ. Lauffer and co-workers (20,21) used a similar approach in their study of the kinetics of polymerization of tobacco mosaic virus A-protein.

Ionic Strength Effect

Glycinin solutions (0.029%) in pH 7.6 phosphate buffer (0.01M mercaptoethanol) in the presence of 0.1, 0.4, 1.0, and 2.0M NaCl were heated at temperatures between 25° and 94°C. for the time indicated in Fig. 4. Aggregation was strongly dependent on the ionic strength of the protein solution. The aggregation below 70°C. was relatively insignificant. The rate and extent of aggregation were greatly facilitated by low ionic strength, and depressed by high ionic strength. The difference in aggregation rate between the 0.1 and 2.0M NaCl-containing solutions (curves B and E, Fig. 4) was dramatic. The ionic strength effect was more pronounced between 0.1 and 1.0M than between 1.0 and 2.0M.

The data presented in Fig. 5 were obtained from a series of isothermic tracings of the thermal aggregation of glycinin (0.029% in pH 7.6 phosphate buffer, 0.01M mercaptoethanol) at different temperatures and ionic strengths. The logarithm of the initial relative slope of aggregation, obtained from the scattering absorbance as a function of time, was plotted against the reciprocal of absolute temperature. Again, the strong dependence of the initial rate of aggregation on the ionic strength of the solution can be readily seen. Calculation of thermodynamic data was not feasible because of the nonlinearity of the family of curves shown in Fig. 5. However, additional information was obtained from these data. It was found that the rate of

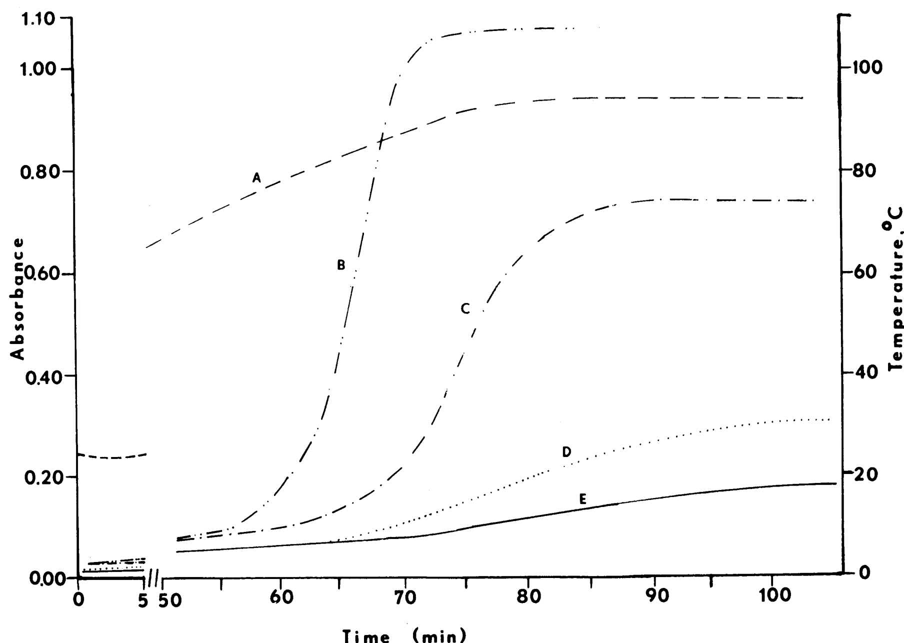

Fig. 4. Kinetic tracings of scattering absorbance at 320 mμ for the thermal aggregation of glycinin at different ionic strengths of solutions. Protein concentration: 0.029% in pH 7.6 phosphate buffer made 0.01M in mercaptoethanol and 0.1M (B), 0.4M (C), 1.0M (D), and 2.0M (E) in NaCl. A, temperature curve.

aggregation was higher at 85° than at 90°C. for the glycinin solutions containing 0.1, 0.4, and 1.0M NaCl. The reverse order was observed for the solutions containing 2.0M NaCl or in the absence of NaCl. At 70°C., glycinin solutions containing 0.4, 1.0, and 2.0M NaCl exhibited similar aggregation rates which were higher than those observed at 75°C. The complexity of the aggregation process does not allow any speculative explanations of this phenomenon at present.

Effect of Mercaptoethanol Concentration

The thermal aggregation of glycinin in the absence and presence of two different concentrations (0.01 and 0.2M) of mercaptoethanol is shown in Fig. 6. Above 90°C., the rate and extent of aggregation were slightly higher in the presence of 0.01M mercaptoethanol than in its absence. However, below 90°C., scattering absorbance readings were higher in the absence of mercaptoethanol than in the presence of 0.01M of the compound. A striking increase in both the rate and extent of aggregation was observed in the presence of 0.2M mercaptoethanol. The initial relative slopes of aggregation (in 0.2M mercaptoethanol) as a function of the reciprocal of absolute temperature (obtained isothermally) are shown in Fig. 7. The experimental points at 70°, 75°, and 80°C. show a straight-line relation, but deviation is observed at higher temperatures. Maximum rate of aggregation was obtained at 90°C., as in Fig. 5, curves A and E.

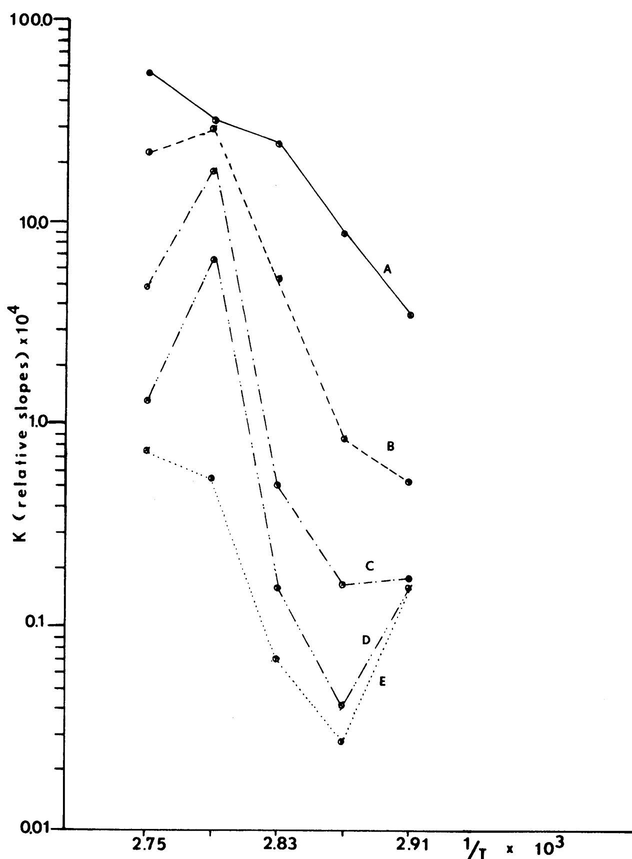

Fig. 5. Ionic strength dependence of the initial slope of scattering absorbance tracings for the thermal aggregation of glycinin at 70°, 75°, 80°, 85°, and 90°C. Protein concentration: 0.029% in pH 7.6 phosphate buffer made 0.01M in mercaptoethanol; in the absence of NaCl (A) and in the presence of 0.1M (B), 0.4M (C), 1.0M (D), and 2.0M (E) of NaCl.

Effect of Trace Amounts of Metal Cations

The effects of 10^{-4}M concentrations of Ca^{++}, Mg^{++}, and Al^{+++} on the thermal aggregation of glycinin were studied isothermally at 90°C. (Fig. 8). Glycinin solutions (0.029%) were prepared in pH 7.6 phosphate buffer containing 0.01M mercaptoethanol and 0.4M NaCl (standard buffer). The metal cations were studied in low concentrations because of the interfering high turbidity obtained at room temperature at higher concentrations. The differences in the rate and extent of aggregation observed in the presence of the metal cations were slight. Calcium and aluminum ions appeared to have a minor depressing effect on the aggregation, whereas magnesium ions caused a relative increase in the rate and extent of aggregation. The addition of Ca^{++} ions to glycinin solutions at room temperature resulted in higher turbidity than the addition of either Mg^{++} or Al^{+++} at equimolar

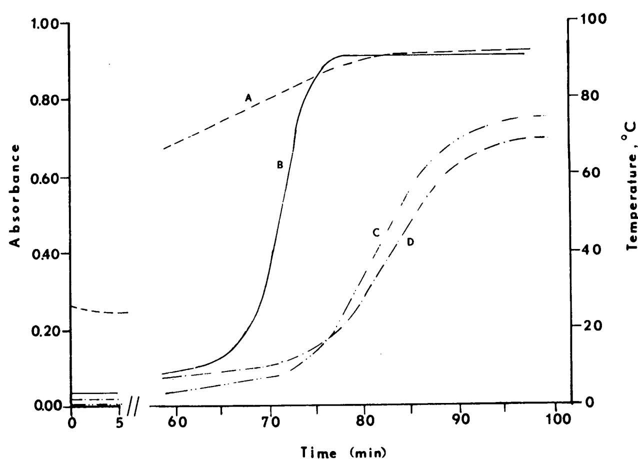

Fig. 6. The effect of mercaptoethanol concentration on the kinetic tracings of scattering absorbance at 320 mμ for the thermal aggregation of glycinin. Protein concentration: 0.029% in pH 7.6 phosphate buffer made 0.4M in NaCl; in the absence of mercaptoethanol (D) and in the presence of 0.01M (C) and 0.2M mercaptoethanol (B). A, temperature curve.

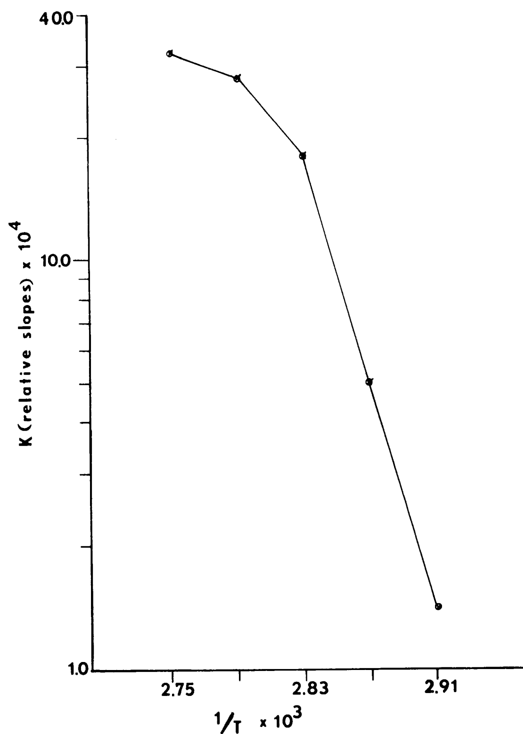

Fig. 7. Temperature dependence of the initial slope of scattering absorbance tracings for the thermal aggregation of 0.029% glycinin in pH 7.6 phosphate buffer made 0.4M in NaCl and 0.2M in mercaptoethanol.

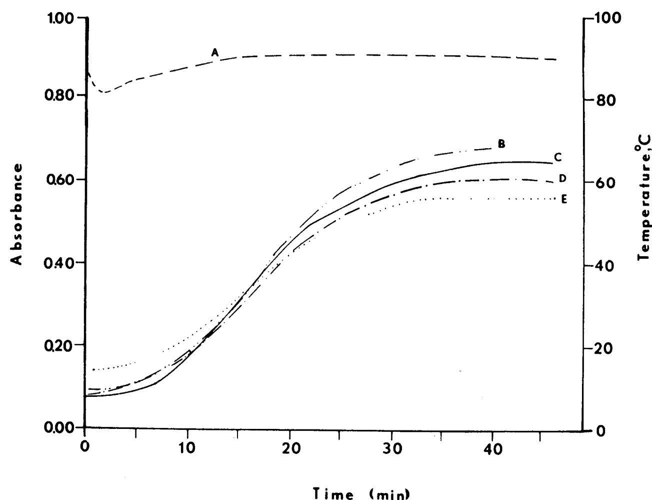

Fig. 8. Effect of metal cations (0.001M) on the kinetic tracings of scattering absorbance at 320 mμ for the thermal aggregation of glycinin. Protein concentration: 0.029% in pH 7.6 phosphate buffer made 0.4M in NaCl and 0.01M in mercaptoethanol. B, Mg^{++}; C, none; D, Al^{+++}; and E, Ca^{++}. A, temperature curve.

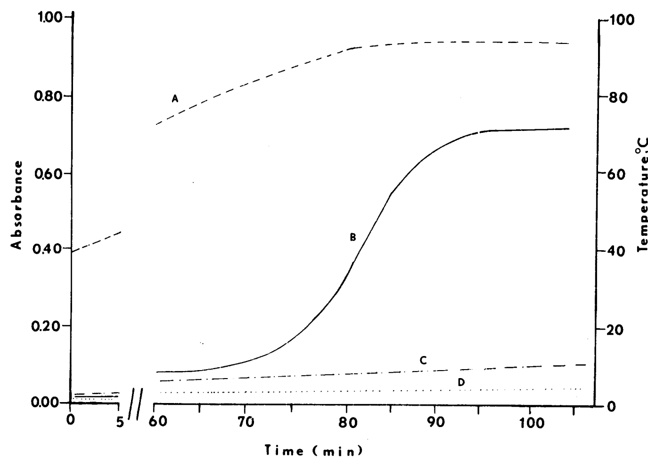

Fig. 9. Effect of pH on the scattering absorbance at 320 mμ for the thermal aggregation of glycinin. Protein concentration: 0.029% at pH 7.6 (B), pH 10.9 (C) adjusted with NaOH, and pH 2.4 (D) adjusted with HCl. A, temperature curve.

concentrations. However, the turbidity due to thermal aggregation in the presence of Ca^{++} was lower than in the presence of the other cations.

Effect of pH on Thermal Aggregation and Disaggregation

Figure 9 shows the kinetic tracings of the thermal aggregation of glycinin at pH

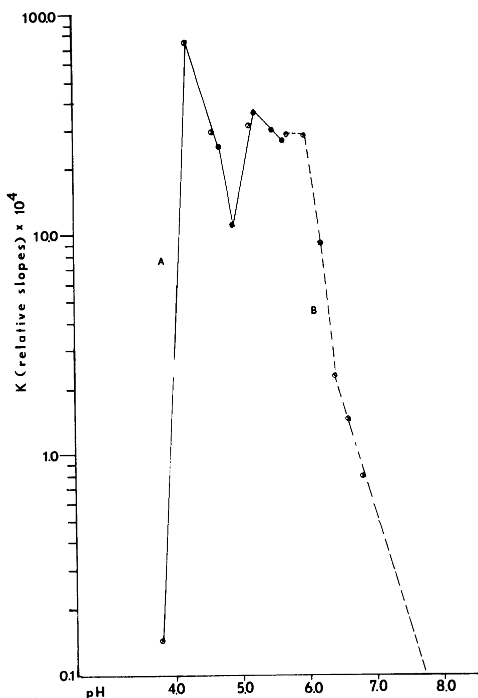

Fig. 10. pH dependence of the initial slope of scattering absorbance tracings for the isothermal (90°C.) aggregation of 0.029% glycinin in 0.05M citrate (solid line) and phosphate (dashed line) buffers.

7.6 (standard buffer) and at two extreme pH values, namely, 2.4 and 10.9. It can be seen that thermal aggregation is markedly depressed at the two extreme pH values. The depression of aggregation is especially pronounced in the acidic solution. The effect of pH on the initial relative slope of the aggregation curves was also investigated in glycinin solutions (0.029%) prepared in two buffer systems. Citrate buffers (0.05M) were chosen to cover the region between pH 3.8 and 5.6, and phosphate buffers (0.05M) for the region between pH 5.7 and 8.0. These regions are of interest because most of the significant changes in aggregation rate occurred here. At pH values lower than 3.8 and higher than 8.0, aggregation rates were depressed to the extent that initial relative slopes could not be meaningfully measured.

A high initial rate of aggregation was observed between pH 4.0 and 6.0 (Fig. 10). Relative minimums in the curve occurred on either side of this pH region and a definite minimum occurred at pH 4.9. The latter coincides with the approximate isoelectric point of the protein. Maximum aggregation rates were obtained at pH 4.0 and 5.2.

It should be noted that glycinin solutions of low concentration become turbid when the pH is adjusted to any value between 3.5 and 6.5. At high concentrations, the protein precipitates. This turbidity is significantly reduced when solutions of

the protein are heated at temperatures below 70°C. Apparently the initially observed precipitate is dissolved by heat. When the protein is heated at temperatures above 70°C., turbidity reappears owing to thermal aggregation. In isothermic studies where the protein is placed in the sample compartment which has been equilibrated at high temperatures (between 70° and 90°C.), the initial scattering absorbance is reduçed to a minimum value within a short period and is followed by the expected increase due to thermal aggregation (Fig. 11). The initial relative slopes of the thermal dissolution of turbidity at 90°C. (isothermal) at various pH values are shown in Fig. 12. The lowest rate of disaggregation was observed at pH 4.9, the approximate isoelectric point of the protein.

DISCUSSION

In an earlier communication (7), we reported that glycinin is dissociated extensively into subunits by heating at temperatures above 70°C. The dissociation into subunits coincided with the appearance of turbidity in the heated solutions. The observed turbidity was attributed to the aggregation of the dissociated subunits. This observation was further substantiated by the present data, since the other possible type of aggregation, namely, intermolecular, can be logically

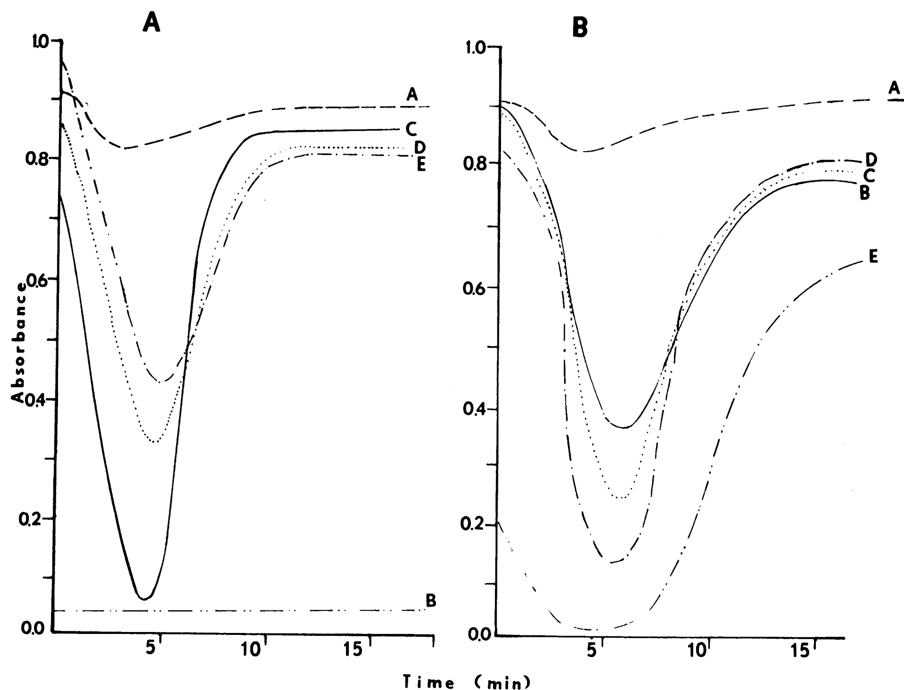

Fig. 11. Kinetic tracings of scattering absorbance at 320 mμ for the thermal aggregation of glycinin (0.029%) at different pH values. Part A (left), B, pH 3.8; C, pH 4.2; D, pH 4.6; E, pH 4.7. Part B (right), B, pH 4.9; C, pH 5.15; D, pH 5.2; E, pH 5.65. A in both figures indicates temperature change (starting at 90°C.) upon insertion of cuvet in sample holder. The temperature after an initial drop reaches 90°C. in approximately 10 min.

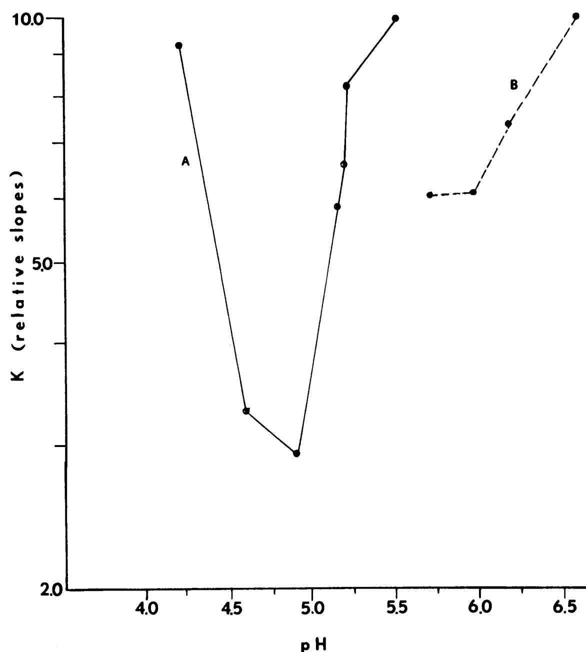

Fig. 12. pH dependence of the initial slope of scattering absorbance tracings for the isothermal (90°C.) disaggregation of 0.029% glycinin in 0.05M citrate (A, solid line) and phosphate (B, dashed line) buffers.

excluded for the following reasons. Ultracentrifugal data (18,22) indicate disruption of the quaternary structure of glycinin (11S component) by heating at high temperatures (80° to 100°C.). The turbidity developed at room temperature, owing to intermolecular interactions of glycinin molecules at pH values near the region of the isoelectric point of the protein, can be dissipated by heat or urea. Therefore, the possibility of hydrogen bonds participating in the intermolecular type of interaction resulting in turbidity is strongly suggested. However, hydrogen bonding can be excluded in the formation of turbidity as a result of thermal aggregation, because these bonds are not favored by a rise in temperature. Thus, one of the major forces of intermolecular interaction in glycinin is not operative during thermal aggregation. The intermolecular hydrogen bonding of glycinin molecules at ambient temperatures may be due to amide group interactions. This assumption is based on the high amide content of glycinin (1). A similar mechanism has been proposed for the aggregation of gluten proteins in aqueous solutions (23,24).

The addition of 0.01M mercaptoethanol to glycinin solutions has been reported to prevent polymerization of the protein through reactions of sulfhydryl groups (8,9,10). The presence of 0.01M mercaptoethanol in glycinin solutions resulted in lower turbidity values. This is consistent with a depolymerization process as suggested previously (8,9,10). However, the rate and extent of thermal aggregation of glycinin in the presence of 0.01M mercaptoethanol were higher than in its absence

(Fig. 6). This is incompatible with the hypothesis of intermolecular thermal polymerization due to sulfhydryl group interactions. Such a process would have been prevented by the addition of mercaptoethanol, and lower values of turbidity would have been obtained. It is possible that during thermal aggregation mercaptoethanol cleaves intramolecular disulfide bonds exposed by the partly dissociated molecule, thus accelerating dissociation into subunits and subsequently increasing aggregation. This is further substantiated by the striking effect of 0.2M mercaptoethanol on the thermal aggregation of glycinin (Fig. 6). We have previously shown that the presence of 0.2M mercaptoethanol is necessary for extensive dissociation of the protein into subunits (5,7). According to reported data (14), glycinin contains approximately 48 half-cystine residues which could form a substantial number of disulfide linkages in the protein. Wolf and Tamura (18) also noted that high concentrations of mercaptoethanol markedly accelerate appearance of the insoluble aggregate state upon heating. Thus, results obtained by two different methods, namely, ultracentrifugation and scattering absorbance, are in agreement on the effect of mercaptoethanol on the heat-aggregation of glycinin and the possible mechanism of aggregation under these conditions.

Dissociation of glycinin into subunits at extreme acidic or alkaline pH values, probably by repulsion of similar charges, has been established (7,11). However, under these conditions thermal aggregation of the subunits is largely depressed. It is apparent that repulsive electrostatic interactions must be overcome for association to occur. This mechanism is supported by the present work, since maximal aggregation rates were observed in the region between pH 4.0 and 6.0 (a region near the isoelectric point of the protein), and were diminished on either side of this region. The maximum rate of aggregation obtained at pH 4.0 (Fig. 10) may be related to the dissociation of the gamma carboxyl groups of aspartic and glutamic acids which are present in large amounts in glycinin (1). The pK values of these groups are 3.87 for aspartic and 4.28 for glutamic acid. Histidine (pK_2 = 6.10) may be involved in the second area of increased aggregation obtained between pH 5.2 and 6.0, although lysine cannot be excluded. The relatively depressed rate of aggregation observed at pH 4.9 which coincides with the isoelectric point of the protein may be due to a more compact configuration and consequent increased stability which the molecule could assume at the pH value where maximum attractive electrostatic forces are operative.

The dependence of thermal aggregation of glycinin on the ionic strength of the solutions also suggests the importance of electrostatic interactions. The depression of thermal aggregation by high-ionic-strength solutions of dissociated glycinin subunits is indicative of decreased electrostatic interaction which is necessary for optimum association. However, the possibility exists that glycinin is more stable to heat at high ionic strengths, and therefore the depression of aggregation can be partly the result of limited dissociation into subunits. Wolf and Tamura (18) also observed that lowering of the ionic strength from 0.5 to 0.1 accelerated the formation of heat-aggregates. Thus, our results are in agreement with theirs.

It has been suggested previously (7,16) that hydrophobic bonds play an important role in the stabilization of the internal structure of glycinin subunits. Earlier experiments utilizing ultraviolet difference spectroscopy (7) failed to detect exposure of chromophoric groups during heat-treatment of glycinin because of

complications arising from the turbidity of the solutions. However, it is conceivable that unfolding of the individual subunits by heat may occur, with consequent exposure of side groups capable of hydrophobic interactions. Since hydrophobic bonding is favored by an increase in temperature, these bonds may be important in the thermal aggregation process. In this case, polar groups may interact more strongly by being buried in hydrophobic regions (25). Thus, the main forces responsible for the thermal aggregation of the dissociated subunits may be a co-operative combination of hydrophobic and ionic bonds, the latter offering the heavier contribution and also being enhanced by the former.

Although the kinetic scattering absorbance method described in this paper does not offer information about the size and shape of the aggregates, it is nevertheless valuable in exploring, in a dynamic rather than in a static fashion, some of the factors involved in glycinin aggregation phenomena. The utilization of the intial rate of aggregation under specified conditions may be applicable to other protein systems where data on the kinetics of aggregation are useful.

Recent data (unpublished) obtained by disc electrophoresis analysis of the aggregates dissociated in phenol-acetic acid-mercaptoethanol-urea (PAMU) solvent (5,7) suggest a relatively selective aggregation of certain subunits under different experimental conditions and especially pH changes. This finding supports recent ultracentrifugal data obtained by Wolf and Tamura (18) on the thermal aggregation of glycinin. These authors suggested that glycinin is composed of two types of subunits which behave differently upon exposure to heat. One fraction consists of soluble subunits of 3-4S which are stable to heating for 30 min. or more. The other fraction of subunits is converted into insoluble aggregates. In recent experimental work (26) we demonstrated that glycinin is composed of acidic and basic subunits. Okubo et al. (27) also showed the presence of alkaline subunits in glycinin. It is possible that, depending on the pH and other experimental conditions, preferential aggregation of acidic or basic subunits may take place.

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