with the subunit structures. Besides, since α' subunit has three times the histidine content of α subunit, we may relate the relatively high content of histidine in B_1, B_3 and B_5 conglycinins to the presence of α' subunit in their structures.

The proposed structures are in agreement with N-terminal amino acid composition and carbohydrate content of the conglycinins. Calculation of the carbohydrate content (mannose and glucosamine) from that of the subunits (Thanh and Shibasaki, 1977) gave values that are consistent with the experimental values (Table II). The immunological relationship between the conglycinins, attributable to their structural relationship (Thanh and Shibasaki, 1976b, 1977), is also consistent with the structure proposal. A further support for the structures is furnished by investigations on self-association of the subunits and reconstitution of the conglycinins from their subcomponents, which will be discussed in a subsequent report.

These structures, presenting three subunits per molecule at first, seem to be uncommon. Most of protein oligomers have an even number of subunits. Of the 300 proteins of which the subunit compositions have been established (Klotz et al., 1975), only ten have three subunits per oligomers and only four of them (troponin, $\alpha_2\beta$; bovine procarboxypeptidase A, $\alpha\beta\gamma$; molybdoferredoxin, $\alpha_2\beta$; and cysteine synthetase, $\alpha_2\beta$) are composed of nonidentical subunits. The present proposal becomes common as we look at the "dimer" form (9S form) of β-conglycinin which would possess a hexameric structure, i.e., an even number of subunits.

Taking into consideration that only one type of geometry, namely a cyclic structure, is possible for the arrangement of monomers in a homotrimer (Klotz et al., 1975), from the structure α_3 of B_6 conglycinin, we can speculate that the 7S molecules might have a cyclic structure. The 9S molecules, "dimer" of the 7S, therefore, might be composed of two cyclic ensembles packed one on top of another. This may be the only possible type of geometric arrangement in a dimer (Klotz et al., 1975).

In the light of a recent report which showed clearly that the acidic and basic polypeptides in glycinin (the 11S soybean globulin) are linked by disulfide bond(s) (Kitamura et al., 1976), the glycinin molecules would be considered to consist of six subunits (12 polypeptide chains) packed in two cyclic ensembles. On the basis of the consideration, the molecular model of β-conglycinin appears to be similar to that of glycinin. Whether the subunit structure presented here is an universal structure of vicilin-type proteins deserves further investigations.

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Major Proteins of Soybean Seeds. Reconstitution of β-Conglycinin from Its Subunits

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Isolated α, α', and β subunits of β-conglycinin were unfolded in phosphate buffer containing 6 M urea. Upon removal of urea, self-association of the subunits occurred. The self-association and the reconstitution of β-conglycinin from the three subunits were investigated by disc electrophoresis and ultracentrifugal analysis. The α subunit reassociated to form a 7S protein identical with B_6 conglycinin. Most of α' subunit recombined to build a 7S aggregate which had no ability to dimerize at 0.1 ionic strength. The β subunit associated to form a 16S aggregate at 0.05 ionic strength, pH 8.4. The six molecular species of β-conglycinin (B_1 to B_6) could be reconstituted by mixing the three subunits in urea solution, and subsequently dialysis of the solution against phosphate buffer. A combination of α and α' subunits produced B_5 conglycinin. Mixtures of α and β subunits gave B_2, B_4, and B_6 conglycinins; α, α', and β gave B_3; and α' and β gave B_1 conglycinin. The results are discussed with regard to the ten possible molecular species ($\alpha_x\alpha'_y\beta_z$) of β-conglycinin.

Recent studies have revealed that a number of proteins have the ability of renaturation (Anfinsen and Scheraga, 1975; Baldwin, 1975). Proteins were unfolded by urea or guanidine hydrochloride and by reduction of disulfide bonds. Refolding could be achieved under appropriate conditions by reoxidation of the sulfhydryl groups and removal of the denaturing reagents. On the other hand, some proteins could be reconstituted from their subunits

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which were reactivated separately (Bernini and Borri-Voltattorni, 1970; Bjork and Tanford, 1971) or simultaneously (Crombrugghe and Edelhoch, 1966; Greaser and Gergely, 1971; Ingham et al., 1974, 1976; Bewley et al., 1974; Kitamura et al., 1977).

Most of the studies have been on the renaturation or reconstitution of biologically active proteins. Their activities provided a useful means of following the renaturation. Storage proteins, which are generally considered to possess no biological activities, therefore, were much less studied. However, the storage proteins, like other proteins, can elicit antibodies production; and the antigen–antibody reaction can serve as a very effective, simple tool for investigating the renaturation or reconstruction.

In a previous report, we have observed the immunoreactivity of β-conglycinin, a major 7S globulin of soybean proteins, which was previously denatured in urea solution (Thanh and Shibasaki, 1977). The recovery of immunoreactivity and the regaining of the 7S sedimenting form, first noted by Roberts and Briggs (1965) and Koshiyama (1970), suggested renaturation of the protein. It seems interesting that each of the constituent polypeptides (α, α', and β subunits) isolated from the protein could restore the immunological properties of the native molecules (Thanh and Shibasaki, 1977). These observations lead to the assumption that the polypeptides of β-conglycinin could spontaneously refold and associate with the same or different species to build the molecular forms identical with those of the native protein.

This paper presents some evidence supporting the above assumption. Besides immunodiffusion method, disc electrophoresis and ultracentrifugal analysis were used to investigate the self-association of the α, α', and β polypeptide chains upon removal of urea, the renaturation of β-conglycinin, and the reconstitution of the multiple molecular species (B_1 to B_6 conglycinins) from their constituent polypeptides. The present study may offer some information about the quaternary structure of β-conglycinin and furnishes a further support for the subunit structures proposed in a recent paper (Thanh and Shibasaki, 1978).

MATERIALS AND METHODS

Protein Samples. β-Conglycinin, α, α', and β subunits were isolated from soybean seeds (*Glycine max*. var. *Raiden*) and purified by the methods described previously (Thanh and Shibasaki, 1976a,b, 1977).

Self-Association of Subunits. The α and α' subunits dissolved in 0.5 ionic strength (I = 0.5) buffer (standard phosphate buffer without 2-mercaptoethanol) containing 6 M urea at 2% protein concentration were treated at 30 °C for 1 h and subsequently dialyzed against the standard buffer (I = 0.5) overnight at 25 °C. The β subunit in the urea/phosphate buffer (0.7% protein concentration) was dialyzed against 50 mM Tris–HCl buffer, pH 8.4.

Reconstitution of β-Conglycinin. The isolated subunits (α, α', and β) and β-conglycinin were dissolved in phosphate buffer (I = 0.1) containing 6 M urea (pH 7.8) at 0.5% protein concentration and treated at 30 °C for 1 h. Portions of the protein solutions were mixed in various molar ratios (the molecular weight of the subunits was taken as 57 000 (α and α') and 42 000 (β) (Thanh and Shibasaki, 1977)) and dialyzed against the phosphate buffer (I = 0.1) overnight at 25 °C to remove urea.

Analytical Methods. Standard disc electrophoresis was performed on 5% polyacrylamide gel, 1:30 cross-linkage, using 5 × 130 mm columns. The gels were stained with Coomassie Blue G-250, destained by leaching method, and scanned on a Gilford linear transport attachment to the Beckman DU monochromator.

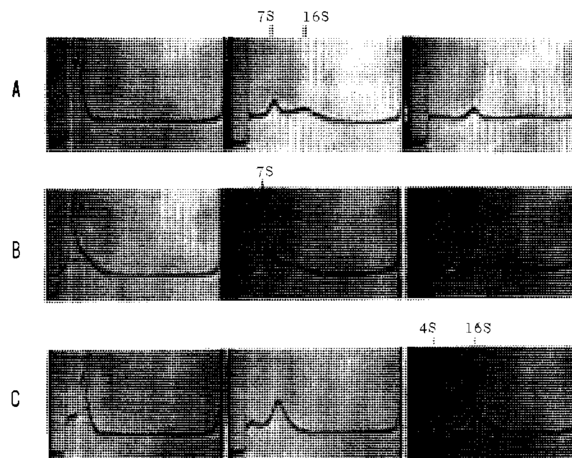

Figure 1. Self-association of the subunits upon removal of urea: (A), α subunit; (B), α' subunit; and (C), β subunit. The isolated subunits dissolved in phosphate buffer containing 6 M urea were dialyzed against 0.5 ionic strength standard buffer (A and B), and 50 mM Tris–HCl buffer (C). Photographs were taken at intervals of 18 min (A), 12 min (B), and 6 min (C) after reaching 55 430 rpm. The protein concentration was 0.7%.

Ultracentrifugal analysis was carried out at 20 °C with a Hitachi UCA-1 ultracentrifuge at 55 430 rpm and 0.7% protein concentration.

RESULTS

Self-Association of the Subunits. Upon removal of urea by dialysis against standard buffer (I = 0.5, pH 7.6), α and α' subunits associated itself to form a 7S and 16S aggregates (Figures 1A and 1B). Lower sedimenting forms (subunits) were not observed.

About 60% of α subunit formed a 7S aggregate which may be, on the basis of its sedimentation coefficient, constructed by three α subunits. Disc gel electrophoresis (Figure 2, patterns a and c) indicated that the 7S aggregate was identical with B_6 conglycinin. Under low ionic strength condition (I = 0.06) of the gel electrophoresis, the observed bands of B_1 to B_6 conglycinins are considered to correspond to the 9S (dimer of 7S) of β-conglycinin. The identification of the 7S aggregate with B_6 conglycinin on disc gels suggests that the aggregate could dimerize to form 9S at low ionic strength.

Most of α' subunit (80%) recombined to build a 7S aggregate (Figure 1B) that was most likely composed of three α' subunits. The 7S aggregate showed a distinct band on disc gel that migrated in front of B_1 to B_6 conglycinins (Figure 2d). Ultracentrifugal analysis indicated that the 7S aggregate kept a 7S sedimenting form even at low ionic strength (I = 0.1). The aggregate of α' subunit observed on disc gel, therefore, corresponded to a 7S form of the protein.

The association of β subunit was investigated at 0.05 ionic strength (Tris–HCl buffer, pH 8.4) since at higher ionic strength (I = 0.5) gelation and precipitation of the protein occurred upon removal of urea. Neither 7S nor 9S aggregates were observed. The subunit associated to form a 16S aggregate (Figure 1C). The aggregate, because of its large molecular size, remained at the top of disc gels and was not seen in Figure 2e. The low sedimenting form ($s_{20,w}$ = 3.8 S) observed on the ultracentrifugal pattern (Figure 1C) may be correlated to the multiple bands (Figure 2e) which represented unassociated β subunits. The multiple bands of β subunit was also detected by gel isoelectrofocusing (Thanh and Shibasaki, 1977).

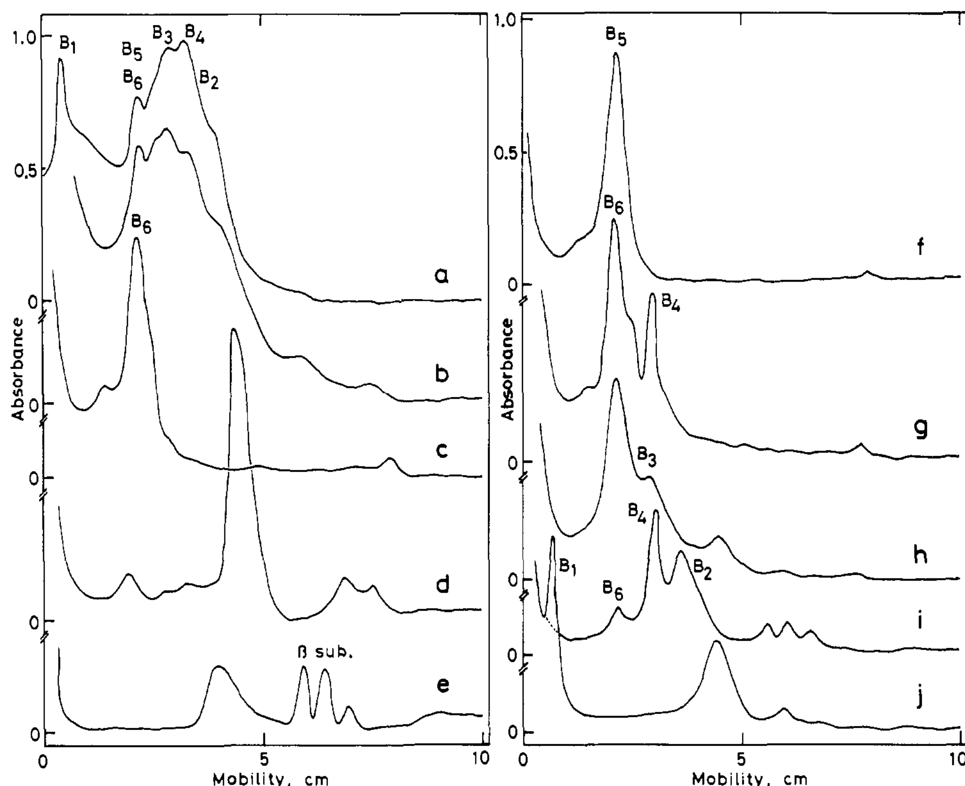

Figure 2. Disc electrophoresis patterns of the reconstituted conglycinins and the aggregates from the subunits. (a) Native β-conglycinin; (b) mixture of subunits dissociated from β-conglycinin; (c) α subunit; (d) α′ subunit; (e) β subunit; (f) mixture of α and α′ subunits (2:1); (g) mixture of α and β (2:1); (h) mixture of α, α′, and β (1:1:1); (i) mixture of α and β (1:2); and (j) mixture of α′ and β (1:2).

Reconstitution of β-Conglycinin. In previous papers (Thanh and Shibasaki, 1976b, 1978) we reported that β-conglycinin consisted of six different conglycinins (B_1 to B_6) and assigned the structures $\alpha'\beta_2$, $\alpha\beta_2$, $\alpha\alpha'\beta$, $\alpha_2\beta$, $\alpha_2\alpha'$, and α_3 to B_1 to B_6 conglycinins, respectively. These structures present a homooligomer (α_3) and five heterooligomers (B_1 to B_5 conglycinins). The self-association result indicated that the homooligomer (B_6 conglycinin) could be easily reconstituted from α subunit. The reconstitution of the other conglycinins from their subunits was attempted by mixing the subunits in urea-containing buffer and then dialysis against phosphate buffer ($I = 0.1$) to remove the denaturing reagent. The reconstituted products were characterized by disc electrophoresis.

Upon removal of urea, a mixture of the subunits that were dissociated from β-conglycinin showed multiple bands identical with B_1 to B_6 conglycinins (Figure 2, patterns a and b). Ultracentrifugal analysis confirmed that the reassociated products had a 7S sedimenting form at $I = 0.5$ and a 9S form ($s_{20,w} = 10.2$ S) at $I = 0.1$. The interconversion between the 7S and 9S (dimer of 7S) forms with the change of ionic strength has been considered as a characteristic feature of the native β-conglycinin. Immunodiffusion indicated that the reassociated products were immunologically identical with the native protein (Thanh and Shibasaki, 1977). The recovery of electrophoretic mobility, ultracentrifugal characteristic, and immunoproperties was unambiguous evidence supporting the renaturation of β-conglycinin upon removal of urea.

By varying the composition and ratio of different subunits in the reconstituted systems we would expect the reconstitution of one or several conglycinins. As shown in Figure 2f, a combination of α and α′ subunits (α/α', 2:1) produced a component identical with B_5 (B_6) conglycinins. The absence of the α′ aggregate, which was formed by the self-association of α′ subunit (Figure 2d), suggests that α′ subunit combined with α subunit to form the reconstituted component. Therefore, the component may be chiefly heteromer (B_5 conglycinin).

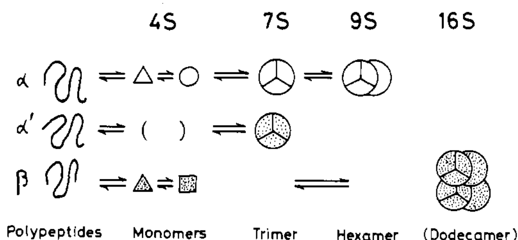

Figure 3. Schematic representation of the refolding and self-association of the subunits. The α, α′, and β polypeptide chains refold into 4S monomers. Conformation changes may occur before they are packed into a 7S (trimer) or a 16S aggregate. The 7S aggregate of α subunit undergoes dimerization at 0.1 ionic strength to form 9S (hexamer).

Several conglycinins were reconstituted from mixtures of α (or α and α′) and β subunits. Mixtures of α and β subunits (α/β, 2:1 and 1:2) gave B_2, B_4, and B_6 conglycinins (Figure 2, patterns g and i). A mixture of α, α′, and β subunits (ca. ratio 1:1:1) gave B_3 and B_5 (B_6) conglycinins (Figure 2h). Conglycinin B_1 was reconstituted from a mixture of α′ and β subunits (α'/β, 1:2) (Figure 2j).

DISCUSSION

Refolding and subsequent self-association of the subunits of β-conglycinin could be represented schematically as in Figure 3. The α, α′, and β polypeptide chains refold into structured monomers (4S form) upon removal of urea by dialysis. Conformation changes may occur before they are packed into a 7S (trimer) or a 16S (probably, dodecamer) aggregates. The 7S aggregate of α subunit (α_3) is capable of dimerizing at low ionic strength and, therefore, is identical with B_6 conglycinin, whereas the 7S aggregate of α′ subunit (α'_3) undergoes no conformation change with the change of ionic strength. In contrast with α and α′

subunits, β subunit builds only a 16S aggregate. The higher content of hydrophobic amino acids in β subunit (Thanh and Shibasaki, 1977) might be responsible for its characteristic aggregation.

The existence of a structured α' monomer is questionable. However, there are some observations supporting the assumption that α and β subunits could assume an organized structure prior to self-association: (1) The structured monomers could be identified on disc gels without denaturing reagents. (2) The α subunit could dissociate reversibly from β-conglycinin at low ionic strength without the use of an unfolding agent. These observations will be discussed with regard to the association–dissociation phenomena of β-conglycinin in a future report.

The association of different subunits to build heteromers is rather complicated. Theoretically, if we assume a random association of the subunits to form trimeric aggregates we could predict the structures of the reconstituted products and could calculate their relative amounts from the composition of subunits in reconstituted systems. Some of the recombination experiments in this investigation yielded results that seem to be in accord with the random-association assumption. Combination systems of α and β subunits gave three conglycinins: B_6, B_4, and B_2. Assuming the random association of α and β subunits we could expect four aggregates: α_3, $\alpha_2\beta$, $\alpha\beta_2$, and β_3 in a ratio 1:6:12:8 (combination $\alpha\beta$, 1:2). Since self-association of β subunit produced no β_3 (7S form) but a larger aggregate (16S form) which could not be seen on disc gels, the reconstituted B_6, B_4, and B_2 could be assigned to the expected aggregates α_3, $\alpha_2\beta$, and $\alpha\beta_2$, respectively. The three conglycinins were reconstituted in relative amounts that are likely in consistent with the theoretical calculation 1:6:12 (Figure 2i). However, in other combination systems that contained α subunit, the relative amount of the reconstituted B_6 (B_5) conglycinins was far higher than the calculation (Figure 2, patterns g and h). This suggests that α subunit had a greater tendency of self-association (or association with α' subunit). Besides, α' subunit, in the presence of α subunit, showed no tendency of self-association (Figure 2f), and β subunit, as mentioned above, formed a 16S aggregate. Therefore, the association of α, α', and β subunits to build various aggregates may not follow exactly the random assumption.

In a recent paper (Thanh and Shibasaki, 1978), the general structure of β-conglycinin has been proposed as $\alpha_x\alpha'_y\beta_z$ where x, y, and z represent the number of α, α', and β subunits, respectively, in the molecules. If α, α', and β subunits were able to associate at random to form trimers, they could constitute ten molecular species. In fact, six molecular species (B_1 to B_6 conglycinins) have been isolated and characterized (Thanh and Shibasaki, 1976b). The other possible molecular species corresponding to the values $y = 2$ and 3, and $z = 3$ would be $\alpha'_2\beta$, $\alpha'_2\alpha$, α'_3, and β_3. Of the four possible species, the homotrimer β_3 could not be constituted. The aggregate of β subunit, if it exists in soybean seeds, might be a 16S component (probably, $(\beta_3)_4$). The homotrimer α'_3 could be constituted in vitro in the absence of α and β subunits. However, its existence in vivo seems unlikely because α' subunit tend to form $\alpha\alpha'$ complex(es). Furthermore, the reconstituted α'_3 having no ability to dimerize at low ionic strength is considered not to belong to the β-conglycinin that possesses the 7S $\rightleftharpoons$ 9S dimerization characteristic. There remain to be considered the two possible molecular species containing α' dimer ($\alpha'_2\beta$ and $\alpha'_2\alpha$). Although it cannot be ruled out, the possibility that the two molecular species may exist in soybeans, they might, like the homotrimer α'_3, lack the dimerization ability and, therefore, be excluded from our β-conglycinin preparation.

From the above consideration, the present results are in accord with the proposed subunit structures of the six conglycinins (Thanh and Shibasaki, 1978). The characteristic of self-association of the α, α', and β subunits and the energetics involved may deserve further investigation.

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