Thermal Denaturation of β-Conglycinin. Kinetic Resolution of Reaction Mechanism

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Thermal denaturation of soybean β-conglycinin has been studied kinetically at pH 7.5 and ionic strength zero in distilled water by difference spectroscopy measuring the exposure of tyrosyl residues. Progress curves of the difference absorbance changes at 287 nm measured at elevated temperature revealed that the β-conglycinin molecule can assume many different denatured conformations depending on the temperature, so long as the sample solution is maintained at elevated temperature. The thermal denatured state at 87 °C has a degree of unfolding of about 80% compared to a completely unfolded state by GuHCl. Once the heat-denatured β-conglycinin was cooled, the extent of these various $\Delta\epsilon$ values, which represent conformational changes, is lowered to a constant value of around −5600. Thus, cooling induces refolding of the unfolded internal structure of dissociated monomers while the quaternary structure is not regained. From the slope of progress curves, reaction rates and activation energies were calculated.

INTRODUCTION

Heating is one of the most important and frequently used methods for denaturing a protein in the area of food proteins, because functional properties such as gelation, emulsification, and foaming must be a reflection of the protein structure of the denatured state (Kinsella, 1982). Heat treatment of proteins usually results in conformational changes and an intensive aggregation near the isoelectric point and at higher temperature. Therefore, studies on both the denaturation process and conformational changes have been achieved under limited conditions preventing aggregation, i.e., far from the p*I* and at relatively low temperature (Pace, 1975; Tanford, 1968). Thus, the turbidity arising upon heating disturbs the use of spectroscopic methods for studying the conformational state. Thermodynamic parameters of thermal denaturation can be obtained by differential scanning calorimetry (DSC) analysis for many globular proteins as reviewed by Privalov (1979) and Wright (1982). However, information concerning the thermally denatured state of secondary and tertiary structure, which is reported to retain a residual ordered structure (Aune et al., 1967), and the reversible denaturation process is available from spectrophotometric studies by means of UV (Kella and Kinsella, 1988; Kugimiya and Bigelow, 1973), CD (Hiraoka and Sugai, 1984), and optical rotation (Acampora and Hermans, 1967). β-Conglycinin prepared according to the method of Thanh and Shibasaki (1976) consists of six different conglycinins (B_1–B_6) that are made up of three kinds of subunits (α, α′, β) in varying proportions ($\alpha_x\alpha'_y\beta_z$, $x + y + z = 3$) and held together by noncovalent bonds. In the case of β-conglycinin, a complexed protein with a subunit structure of trimers (Thanh and Shibasaki, 1978), thermal denaturation using optical measurements has not been investigated until recently because of the turbidity arising from irreversible aggregation (Hashizume et al., 1975). Previously, we found the appropriate conditions to prepare a transparent solution which is composed of dissociated subunits upon heating to boiling even at neutral pH (Iwabuchi et al., 1991). By using this characteristic, we conducted a kinetic study of the thermal denaturation of β-conglycinin with the aim of elucidating the reaction path of the denaturation process containing dissociation and unfolding. A conformational study of the thermally denatured state of β-conglycinin was also achieved. Thus, optical measurements are expected to gain both information concerning kinetic data and conformational changes. Because β-conglycinin is considered to contain 36–39 tyrosyl residues per protomer (this is calculated by the nucleotide sequence data of α′ and β subunits; Doyle et al., 1986) and because some of the tyrosyls are buried in the interior and the subunit contact regions and some are exposed on the surface, β-conglycinin is particularly convenient for studying the environmental changes of tyrosyl residues by measurements of UV difference spectroscopy at 287 nm. In this paper, we developed progress curves showing the denaturation process between the native and the denatured states keeping an elevated temperature, and we found that their conformational states are different from those at the heat-treated and subsequently cooled stage. Moreover, using these curves, we revealed that the dissociation into subunits is irreversible but the unfolding of the dissociated subunits is reversible.

MATERIALS AND METHODS

Materials. β-Conglycinin was prepared from ground, defatted soybean meals (*Glycine max* var. Raiden) and purified according to the method found in a previous paper (Iwabuchi and Yamauchi, 1987). Protein solutions (0.4%) were prepared from the freeze-dried protein dissolved in distilled water. The protocol for preparation of β-conglycinin without any turbidity upon heating is described elsewhere (Iwabuchi et al., 1991). The final pH was 7.5. β-Conglycinin concentrations were determined spectrophotometrically by using a $E^{1\%}$ at 280 nm of 4.16 (Thanh and Shibasaki, 1978). The GuHCl was obtained from Nakarai Chemicals Co. and purified according to the method of Nozaki (1972). Sepharose GL-6B, DEAE-Sephadex A-50, and ConA–Sepharose 4B were purchased from Pharmacia Co., and all other reagent grade chemicals were obtained from Nakarai.

Heat Treatment. Heating experiments were conducted according to the following two types. In periodic heating experiments, aliquots (1.5 mL) of β-conglycinin solution (0.4%) were transferred to glass ampules (10 cm long and 5.5 mm i.d.) and sealed. The sealed samples were heated to the desired temperatures and held for the desired period of time by immersion in a temperature-controlled water bath (±0.01 °C). The temperature change in the glass tube for the final temperature had a rise time of 1.5 min. The heated samples were removed from

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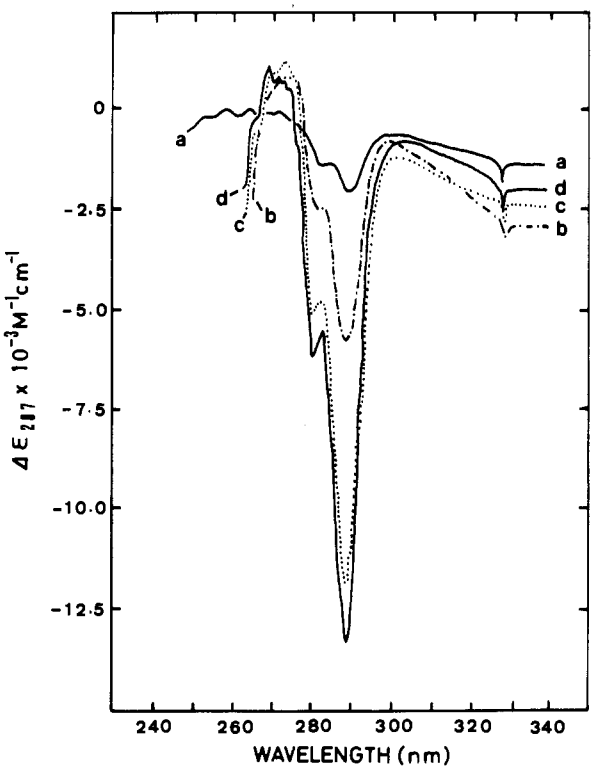

Figure 1. Difference spectra for β-conglycinin under various denaturing conditions: (a) Deletion of salt (β-conglycinin, pH 7.5, was dissolved in distilled water instead of 32.5 mM phosphate buffer, pH 7.6, containing 0.4 M NaCl); (b) temperature (heated at 100 °C for 5 min and cooled to room temperature); (c) 6 M urea; (d) 5.5 M GuHCl. Spectra of β-conglycinin were recorded under various conditions against the same protein dissolved in 32.5 mM phosphate buffer, pH 7.6, containing 0.4 M NaCl as reference by using 4 mg of protein/mL.

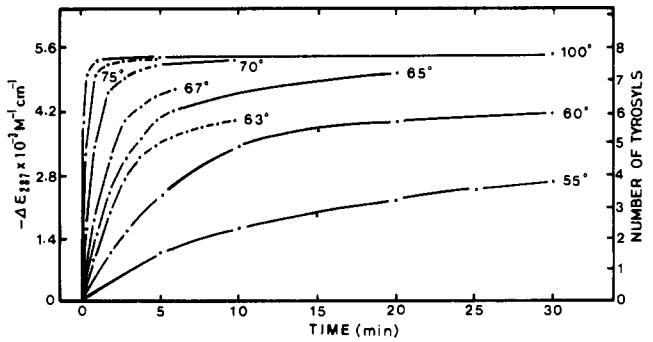

Figure 2. Effect of temperature and duration of heating upon the rate of dissociation of β-conglycinin into subunits measured as difference absorbance changes at 287 nm by using protein solutions after cooling with reference to the unheated protein in distilled water. The reaction was followed by an increase in $\Delta\epsilon_{287}$ and was represented by the number of tyrosyls per mole. The solutions (4 mg of protein/mL) were held in water at various temperatures periodically (see points), cooled immediately, and assayed.

the bath after different intervals and cooled to room temperature by immersion in cold water. The difference spectra were obtained by placing the native (unheated, dissolved in 32.5 mM potassium phosphate buffer, pH 7.6, containing 0.4 M NaCl) and denatured (by heat, 6 M urea or 5.5 M GuHCl treatment) protein solutions in the reference and sample compartments, respectively. It is necessary to use the β-conglycinin at high ionic strength as the native reference sample, because the trimeric structure of β-conglycinin is completely stabilized above 0.5 ionic strength (Thanh and Shibasaki, 1979). The protein concentrations in the two cells were identical. Difference spectra measurements were

Table I. Accessibility to the Solvent of Tyrosine Residues of β-Conglycinin As Measured by Various Treatments

treatment	molar absorptivity diff at 287 nm, $\Delta\epsilon$, M^{-1} cm^{-1}	no. of tyrosine residues[a]
β-conglycinin in 5.5 M GuHCl	−15 100	21.6
β-conglycinin in 6 M urea	−12 600	18.0
heat-denatured β-conglycinin at 100 °C for 5 min	−7 000	10.0
native β-conglycinin dissolved in distilled water (pH 7.5)	−1 600	2.3

[a] The fraction of exposed chromophores in the protein was calculated by use of −700 absorbency units/mol of tyrosine at 287 nm (Donovan, 1973).

carried out on a Hitachi 124 double-beam recording spectrophotometer at room temperature.

In other experiments, progress curves for the thermal dissociation and unfolding of β-conglycinin at elevated temperatures were measured as follows. A difference spectral technique was used, employing two double-compartment cells with a 0.45 cm path length for each of the two compartments. The protein solution was heated by circulating thermostatically controlled water through the outer jacket. In such experiments, the reference solution is preferably maintained at room temperature while the temperature of the sample solution is varied. However, in the equipment employed in this paper, temperature-controlled water was passed through the jacket simultaneously for both references and sample solutions. It has been proven that the completely unfolded β-conglycinin itself can be used as a reference standard, because once β-conglycinin was in 5.5 M GuHCl, there was no further shift with temperature changes. Therefore, it is expected that changes in spectra produced by changes in temperature can be monitored by placing completely unfolded (in 5.5 M GuHCl) and native (unheated, dissolved in water) β-conglycinin solutions in the reference and sample compartments, respectively. In this case, β-conglycinin should be dissolved in distilled water because if the β-conglycinin solution containing salts was heated, aggregates would be produced and disturb the spectrometric measurements. The temperature change in the cuvette for a number of final temperatures in the range 50–90 °C consistently had a rise time of 4–5 min.

The peak of the denaturation difference spectrum was at 287 nm, of tyrosine band, and so the difference absorbance at this wavelength, ΔA_{287}, was measured to monitor the unfolding kinetics for β-conglycinin at elevated temperature. Kinetic runs were performed at a number of temperature between 50 and 90 °C. The results are expressed as changes in molar extinction coefficient ($\Delta\epsilon$). In the calculation of $\Delta\epsilon$, a molecular weight of 186 000 was used for β-conglycinin. The fraction of exposed tyrosyl residues has been calculated by using −700 for the molar absorbance ($\Delta\epsilon_{287}$) of a single tyrosyl residue estimated by Donovan (1973). Measurements were made in matched 0.45-cm cells, but results are calculated for 1-cm cells.

RESULTS AND DISCUSSION

Difference Absorption Spectroscopy. A typical difference spectrum is shown in Figure 1. In all cases, the difference spectra were typical tyrosyl difference spectra having two minima, one at 280 nm and a larger one at 287 nm. When native β-conglycinin was dissolved in distilled water, a slight exposure of tyrosines due to the partial dissociation into subunits occurred even in unheated solution at neutral pH (Figure 1a). This is supported by our other results (Iwabuchi et al., 1991). Upon heating, the exposure of tyrosyls was accelerated up to $\Delta\epsilon$ values of −7000 at 100 °C for 5 min. Another study (Iwabuchi et al., 1991) demonstrated that heating at 100 °C for 5 min induced almost complete dissociation of β-conglycinin into its subunits, although an irreversible aggregation slightly occurred. The large negative difference spectrum indicates

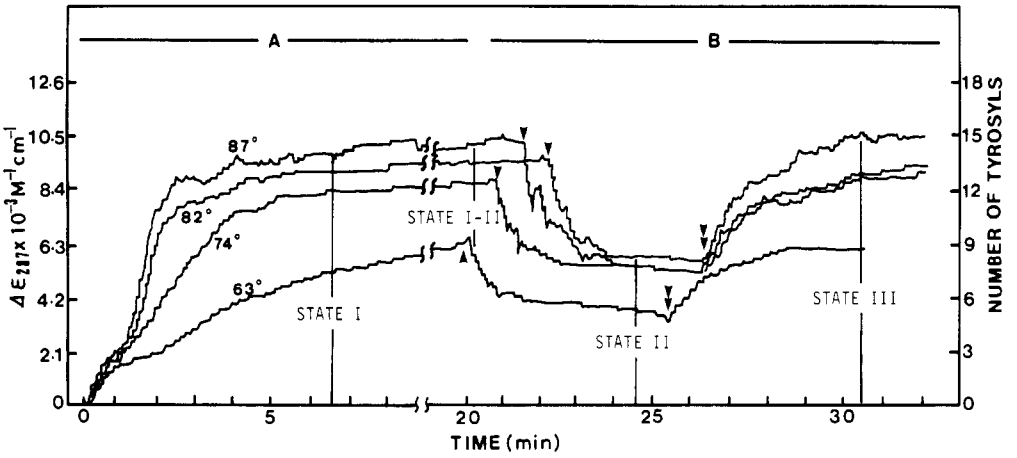

Figure 3. (A) Progress curves showing both dissociation and unfolding of β-conglycinin at elevated temperature. The solution (4 mg of protein/mL) was heated continuously in a quart cuvette with circulating temperature-controlled water. With heating, changes occurring in the protein conformation were monitored by an increase in $\Delta\epsilon_{287}$ values (with reference to the same protein in 5.5 M GuHCl) at elevated temperature. (B) Progress curves showing refolding of unfolded β-conglycinin on cooling. Protein solutions were heated for 10–20 min, then cooled by circulating water (15 °C), and then reheated. The arrow shows the time where the first step (single arrow) "stops heating" and the second step (double arrow) "starts to reheat".

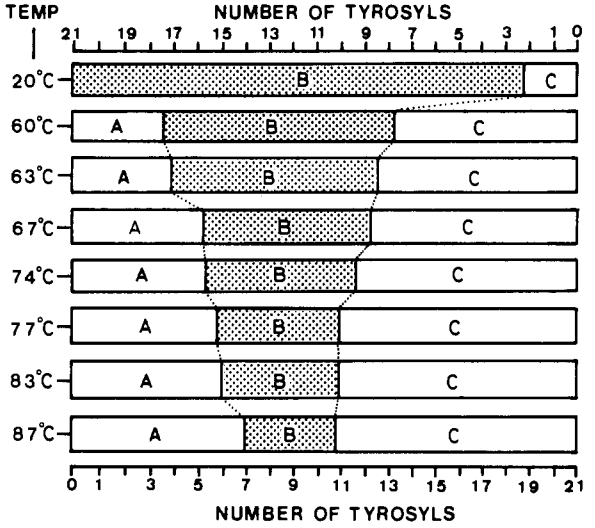

Figure 4. Distribution of 21 tyrosyl residues present in the interior and the subunit contact regions of β-conglycinin. (A) shows the number of tyrosyls that are exposed by heating and able to refold reversibly upon cooling. (B) shows the number of tyrosyls that remain in the interior of the denatured β-conglycinin at the elevated temperature. (C) shows the number of tyrosyls that are present in the subunit contact regions. Deletion of salts in sample solution induced a slight dissociation (2.3 tyrosyls) even under the unheated condition at 20 °C.

that the dissociation of β-conglycinin results in the transfer of a substantial fraction of about 10 tyrosine residues into a more polar environment. The difference spectra of 5.5 M GuHCl and 6 M urea denatured β-conglycinins are also presented (Figure 1c,d). Compared with the completely unfolded value of $\Delta\epsilon$, −15 100, by 5.5 M GuHCl, the $\Delta\epsilon$ value of −7000 for the thermally denatured protein was half. These data indicated that thermal denaturation brings about incomplete unfolding of dissociated subunits, although dissociation is considered to be complete. The fraction of exposed tyrosyl residues is shown in Table I. Thus, it is clarified that the difference absorbance at 287 nm ($\Delta\epsilon_{287}$) is a useful measure of denaturation by heat treatment.

Rates of Dissociation into Subunits by Heat Treatment. At first $\Delta\epsilon_{287}$ values were measured by using the periodically heated solutions which were subsequently cooled. Rates of thermal denaturation at temperatures ranging from 55 to 100 °C and up to 30-min duration are shown in Figure 2. When the β-conglycinin solutions in water were heated above 70 °C, all of the $\Delta\epsilon_{287}$ values reached a constant value of −5600 within 5 min. This $\Delta\epsilon_{287}$ value of −5600 obtained at higher temperatures above 70 °C is different from the value of −7000 at 100 °C shown in Table I and Figure 1b. The difference, −1400, arose from the different ionic strengths of solutions used as the native reference sample solution; that is, the native β-conglycinin exposed the 2.3 tyrosyl residues by the deletion of salts from the sample solution as shown in Table I and Figure 1a.

As evidenced by the results of gel filtration, ultracentrifugation, and electrophoresis (Iwabuchi et al., 1991), it is indicated that at temperatures above 70 °C, dissociation is complete. At 65 °C the thermal denaturation reaction appeared finally to reach the same $\Delta\epsilon_{287}$ value as at 100 °C. However, the extent of dissociation at 55 °C after 30 min is less than 50%, compared with that at 100 °C. Retention of the quaternary structure in the heat-denatured molecule at temperatures below 60 °C was also confirmed (Iwabuchi et al., 1991).

Dissociation into subunits may be accompanied by changes in the tertiary structure of the subunits, resulting in the transfer of tyrosyl residues from interior positions to the solvent. Thermal energy at temperatures below 60 °C is considered to be insufficient to disrupt the hydrophobic bonds (Tanford, 1968). Therefore, the molecular species composed of highly hydrophobic subunits, β, seems to disrupt little the quaternary structure upon heating below 60 °C. Generally, the thermal denaturation process should be reversible as are other denaturant-induced denaturation processes as reported by Tanford (1968). Renaturation will occur on removal of the denaturant, i.e., cooling the heated sample solution.

Upon cooling, the heat-induced dissociates of β-conglycinin will refold their unfolded internal structure but cannot be recombined into the native form of trimer keeping the monomeric form. Previously we found that the heat-dissociated subunits retain secondary structure similar to that of native β-conglycinin by CD spectra and can regain their quaternary structure by the addition of salts to the heated sample solution (Iwabuchi and Shibasaki, 1982). The fact that the addition of salts above 0.1 ionic strength brought about the association of the subunits (mainly α and α′) into 7S or 9S (Iwabuchi and Shibasaki,

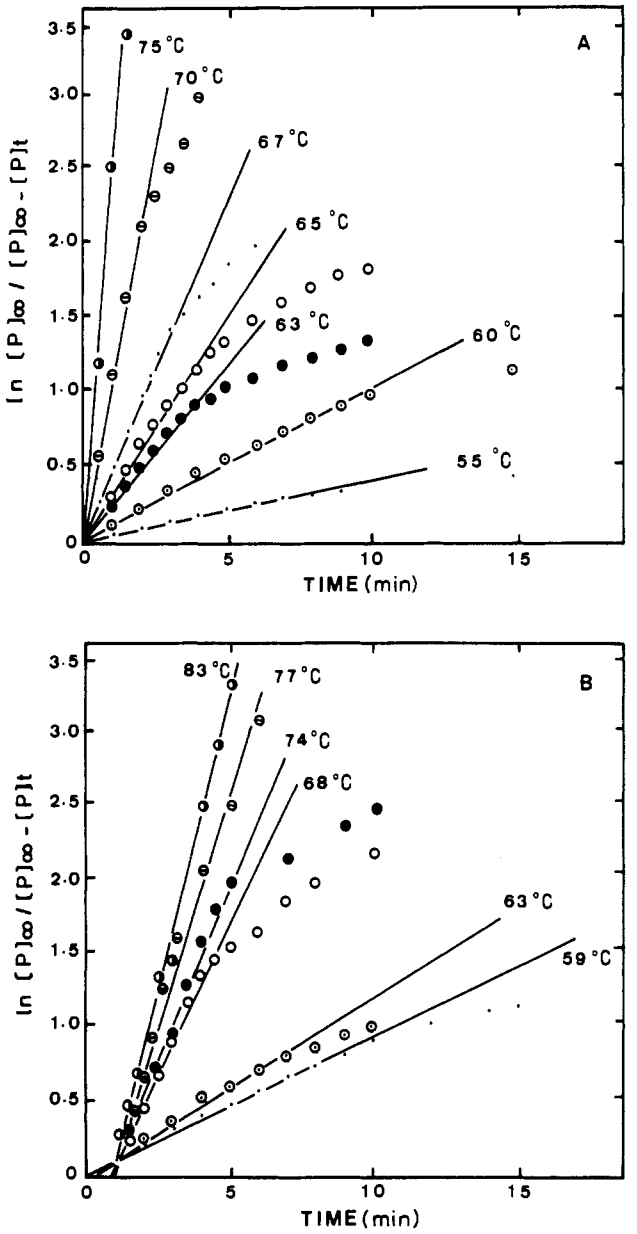

Figure 5. (A) Plots of $\ln [P]_\infty/([P]_\infty - [P]_t)$ versus time showing rate constants k for dissociation of β-conglycinin upon heating and subsequent cooling according to the heating type shown in Figure 2. $[P]_\infty$ shows the maximum number of tyrosyl residues exposed by heating at the highest temperature. $[P]_\infty - [P]_t$ shows the tyrosyl residues remaining in the interior of β-conglycinin at time t. (B) Plots of $\ln [P]_\infty/([P]_\infty - [P]_t)$ versus time showing rate constants k for thermal denaturation of β-conglycinin maintained at elevated temperature according to the heating type shown in Figure 3.

1982) suggests that the individual subunits may appreciably acquire the original tertiary structure of the native β-conglycinin on folding process. Therefore, the progress curves of Figure 2 may yield essentially the kinetic data of dissociation into subunits which represent breakdown of the quaternary structure. The reversibility of the unfolding process of the thermal denaturation of β-conglycinin will be discussed below.

Rates of Thermal Denaturation at Elevated Temperature. In another type of heating experiment, the β-conglycinin solution in the cells was heated by circulating water. Soon after, exposure of tyrosyl began and was monitored in situ by measuring $\Delta\epsilon_{287}$. Figure 3A shows the progress curves at elevated temperature from 63 to 87 °C. Each of the denatured states at different temperatures

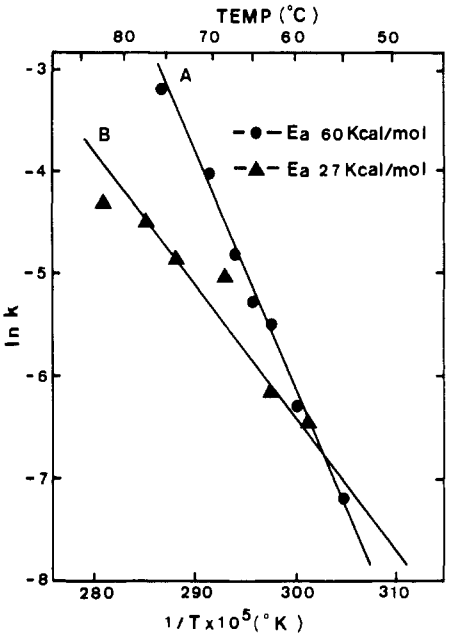

Figure 6. Arrhenius plot relating the rate constants for the denaturation reactions of β-conglycinin to the temperature of heat treatment. The E_a was calculated by $\ln k = -E_a/RT$. (A) ●, k from the $\Delta\epsilon_{287}$ measurements of a protein solution heat-treated and subsequently cooled shown in Figure 2. (B) ▲, k from the $\Delta\epsilon_{287}$ measurements at elevated temperature shown in Figure 3A.

could be distinguished by the number of buried tyrosyls that became exposed. The $\Delta\epsilon_{287}$ values at 10 min did not change up to 20 min. This type of heating experiment shows that heat-treated β-conglycinin gave different denatured states in response to different temperatures (see the scheme of Figure 7).

In contrast, in the sample solutions heat-treated and subsequently cooled, increasing the temperature from 70 to 100 °C produces no increase in all of the final values of the progress curves (Figure 2). The stable $\Delta\epsilon_{287}$ values reached after 10 min in the two types of heating were compared (see Figures 2 and 3A). The extent of total changes in $\Delta\epsilon_{287}$ values measured at elevated temperature was much higher than that achieved with the heated sample after cooling. Thus, this difference in $\Delta\epsilon_{287}$ values observed in the progress curves between Figures 2 and 3A demonstrated that refolding of the unfolded structure actually occurred in the β-conglycinin molecule on cooling. Recently, Damodaran (1988) has reported from the results of CD spectra that soy 11S globulin undergoes partial refolding during the cooling regime of the thermal gelation process.

Reversibility of Thermal Denaturation of β-Conglycinin. We have performed experiments showing the reversible process between the native (N) and the denatured state (U) using the progress curve of Figure 3B. The $\Delta\epsilon_{287}$ value reached after 10–20 min of heating is called state I (Figure 3B). At a point indicated with a single arrow, the temperature was lowered to room temperature with circulating cold water. The $\Delta\epsilon_{287}$ value reached by cooling is called state II. The same sample solution was then again heated at a point indicated with a double arrow. When the sample solution was reheated, the $\Delta\epsilon_{287}$ reached a constant value (state III), which is in fair agreement with state I. Following this procedure, for example, at 87 °C, up to 46% of the $\Delta\epsilon_{287}$ value changes (this part refers to state I minus state II) were found to be reversed. The reproducibility of this refolding process has been confirmed by repeating the heating and cooling experiments (data

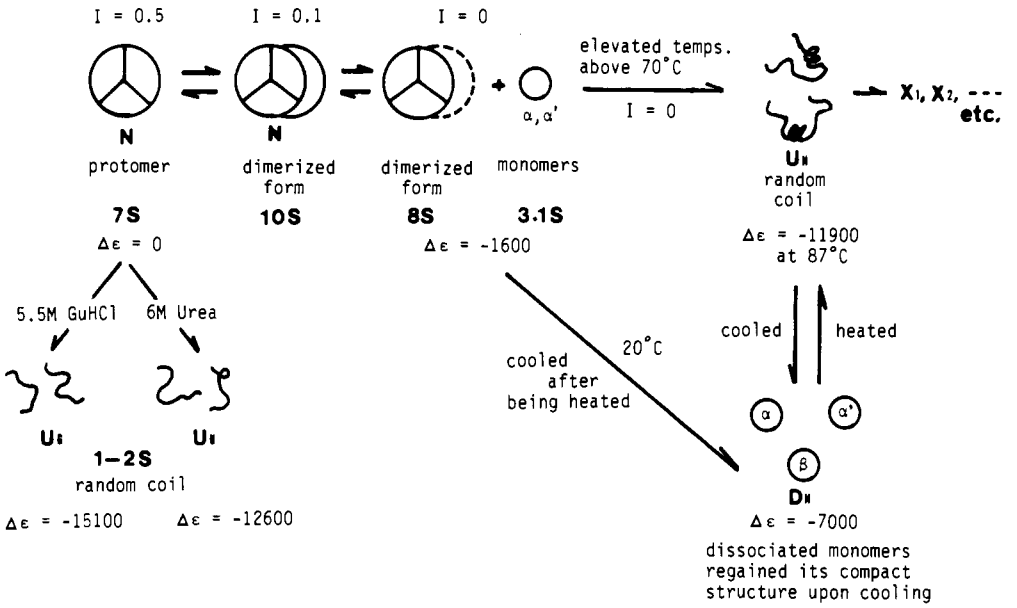

Figure 7. Schematic representation showing the thermal denaturation of β-conglycinin. Thermal denaturation of β-conglycinin involves a disruption of the native structure (protomer) to individual subunits (monomers) which subsequently unfold. Conformational changes induced by different denaturants are expressed as changes of $\Delta\epsilon$. D_H, heat-denatured and subsequently cooled state, refers to the state II. U_H, heat-denatured state standing at elevated temperature, refers to the states I and III. Heat-denatured states of U_H gave different denatured states (X_1, X_2, etc.) in response to different temperatures.

not shown). However, 54% of the $\Delta\epsilon_{287}$ value (this part refers to state II) remained exposed even after cooling (Figure 3B). These results indicate that the reversibility observed upon thermal denaturation of β-conglycinin is limited in state I minus state II.

Assignment of the Exposed Tyrosyl Residues. We tried to assign the tyrosyl residues in state II and state I minus state II as to where they originated. From the results of Figure 1 and Table I that 5.5 M GuHCl produces the most completely denatured state, it has been shown that β-conglycinin contains 21–22 buried tyrosyl residues in the interior of the subunits and in the subunit contact region which became exposed.

The distribution of these 21 tyrosyls at different temperatures was determined through analysis of the progress curves of Figure 3, and the results have been shown in Figure 4. The symbol "C" in Figure 4 shows the number of tyrosines that remained exposed in the heated sample solution after cooling and refers to state II. The deletion of salts from the native solution (20 °C) gave an exposure of 2.3 tyrosyls due to a partial breakdown of quaternary structure, resulting in dissociation even in the unheated solution (Figure 1 and Table I). This treatment makes a great breakdown of the internal structure of the dissociated subunits unlikely. Hence, these tyrosyls are considered to come from the subunit contact region. Upon heating, the number of tyrosyls at "C" gradually increased up to 77 °C but became constant above 77 °C (Figure 4). Moreover, it is noted that the number of tyrosyls distributed at "C" was close to the number observed at the denatured states of the progress curves of Figure 2.

We have speculated that in view of the reversibility of the unfolding, the number of tyrosyls at "C" (state II) may come from essentially the subunit contact region due to dissociation. This speculation is based on the following results: the dissociated subunits formed by heating and subsequent cooling have a characteristic to regain their quaternary structure when salts are added as mentioned above and retain the backbone secondary structure similar to that of the native molecule (Iwabuchi and Shibasaki, 1982). It remains a limitation, however, in an exact quantitative interpretation of the exposed tyrosyls where they have been distributed in the native β-conglycinin. It is difficult to rule out the following possibility: the dissociated subunits have a less ordered structure than the native molecule of protomer; therefore, some tyrosyl residues of the "C" in Figure 4 may belong to irreversibly exposed residues from the interior of the subunits.

The symbol "A" represents the number of tyrosyl residues exposed from the interior of the dissociated subunits by heating when kept at elevated temperatures (Figure 4). These residues of the "A" in Figure 4 correspond to state I minus state II in Figure 3. Hence, it has been shown that upon cooling these exposed tyrosyls are reversibly refolded into the inside and formed the hydrophobic core. The symbol "B" is the number of tyrosyls that still remain unexposed in the interior of the subunits even at the elevated temperature (Figure 4).

At 87 °C, heating induced the exposure of a substantial fraction (17 tyrosyls) of the 21 tyrosyl residues to the more polar aqueous environment. This corresponds "A + C" in Figure 4, that is, about 80% of the total number of tyrosyls present at the inside of the native β-conglycinin. The magnitude of the heat-induced disruption of β-conglycinin at 87 °C demonstrated by the tertiary structure changes was close to that produced in 6 M urea (see Table I and Figure 4). This observation suggests that the dissociated subunits are largely unfolded at the elevated temperature—at least as measured by the exposure of tyrosyl residues. However, the tyrosyl residues of "A", which have been distributed in the interior of the dissociated subunits, have a tendency to refold reversibly on cooling as shown in Figure 3B. Therefore, the conformation of the heat-treated and subsequently cooled molecule is suggested to regain an ordered and rigid structure because all of the tyrosyl residues contained in the dissociated subunits, "A" plus "B" except "C", have been recovered in the interior of the dissociated subunits. Further studies on the difference in the conformations of the thermally unfolded structure and subsequently cooled structure using viscometry and spectrophotometric titration, which measure aspects of overall conformation, are in progress.

Table II. First-Order Rate Constants for Thermal Denaturation of β-Conglycinin at Various Temperatures According to Two Heating Systems

β-conglycinin[a] treatment	temp, °C	rate constant ($k \times 10^4$/s) k
A. periodically heated and subsequently cooled	55	7.3
	60	18.1
	63	40.4
	65	50.0
	67	80.5
	70	176.6
	75	408.3
B. heat-denatured β-conglycinin maintained at elevated temperature	59	15.5
	63	20.9
	68	67.2
	74	77.8
	77	108.3
	83	133.3

[a] Protein was dissolved in distilled water, pH 7.5.

Kinetics of Heat Denaturation. A kinetic study of heat denaturation of β-conglycinin has been performed by using the slopes of the data of Figures 2 and 3A. First- and second-order kinetic analyses provided a poor fit to the slopes of Figures 2 and 3A. Hence, first-order kinetics were used to analyze the data. The following kinetic equation for a first-order reaction should be satisfied

$$\ln [P]_\infty/([P]_\infty - [P]_t) = kt$$

where k is the rate constant of the first-order reaction, $[P]_\infty$ the final value of $\Delta\epsilon_{287}$ reached at the highest temperatures in Figures 2 and 3A, and $[P]_t$ the $\Delta\epsilon_{287}$ at any subsequent time t taken from the progress curves. The $\ln [P]_\infty/([P]_\infty - [P]_t)$ vs time plots for heat denaturation of β-conglycinin were regressed with slope k (Figure 5). The initial part of thermal denaturation follows first-order kinetics in the temperature range up to 83 °C, and a secondary process that takes place after the primary process has occurred. The nonlinear behavior of the ln ($\Delta\epsilon$) plot observed after the primary process reflects complications resulting in deviation from first-order kinetics. This deviation may be attributed to the existence of multiple domains in proteins: β-Conglycinin is an oligomeric protein with three kinds of subunits and may possess regions with different thermal stability. Hence, β-conglycinin may denature via two or more steps. Rate constants (k) at each temperature were calculated from the slopes of the lines in Figure 5. The first-order rate constants are reported in Table II.

To determine the activation energy (E_a), logarithms of the rate constants were plotted as a function of the reciprocal of the temperature (degrees Kelvin) of heat treatment (Figure 6). The slopes of the lines of Figure 6 represent the temperature dependence for the denaturation of β-conglycinins heat-treated to the desired temperature and subsequently cooled to room temperature (slope A in Figure 6) and heat-treated to the desired temperature and kept at the elevated temperature (slope B in Figure 6). These slopes were definitely different, with slope A having a steeper slope than slope B and, therefore, the greater energy of activation (E_a = 60 kcal/mol). With slope B, the slope is less steep than slope A; therefore, the conformational change induced by this type of heat treatment may be less dependent on temperature. This may lead to a lowering in E_a value (27 kcal/mol) of thermal denaturation of β-conglycinin measured at elevated temperature.

With the thermal denaturation of β-conglycinin using the heat-treated and subsequently cooled sample solutions, increasing the temperature from 67 to 70 °C produced an approximately 2-fold increase in rate, whereas with heat-denatured β-conglycinin maintained at high temperature, increasing the temperature from 68 to 74 °C resulted in only a slight increase in rate (Table II). Thus, the higher activation energy (60 kcal/mol) of thermal denaturation observed in the former case may reflect that the heat-induced exposure of tyrosyl residues is highly dependent on temperature. This difference was probably caused by the reversible refolding characteristics of the β-conglycinin molecules upon cooling as mentioned above. Below, 70 °C, a fraction of tyrosyls exposed at the elevated temperature have a tendency to be buried reversibly in the interior upon cooling. As a result, the exposure of tyrosyl residues will be suppressed. Above 70 °C, where the hydrophobic bonds interacting between subunits and the interior of the subunits will be largely disrupted, heat treatment may markedly dissociate the native molecule into subunits.

On the other hand, the conformational changes at the elevated temperature were characterized by holding large amounts of apolar groups accessible to the solvent with no accompanying refolding as after cooling; that is, so long as the temperature of the sample solution is kept high, the exposed tyrosyl residues are maintained. This may explain why thermal denaturation of this type of heating is less dependent on temperature. The activation energy (88 kcal/mol) of thermal denaturation of glycinin (Watanabe, 1988) is higher than E_a of β-conglycinin. These results are consistent with the denaturation temperature (T_d) estimated upon DSC studies (Damodaran, 1988); that is, the T_d of glycinin in water (84.5 °C) is higher than that of β-conglycinin (72 °C).

The molecular events that occur when β-conglycinin is heated above 70 °C at pH 7.5 in distilled water are schematically represented in Figure 7. On the basis of the present study, we tried to show how the various denatured states of β-conglycinin are related to each other. The 5.5 M GuHCl gave a random coil (U_G) of $\Delta\epsilon_{287}$ of −15 100. The 6 M urea gave a random coil (U_U) of $\Delta\epsilon_{287}$ of −12 600, only slightly different from that of GuHCl. The N of 7S form is protomer in the native state with quaternary structure, which is stabilized by the presence of high ionic strength. At 0.1 ionic strength, β-conglycinin dimerizes to 10S (Thanh and Shibasaki, 1979). By the deletion of salt below 0.01 ionic strength, β-conglycinin seems to exist in a dimerized form of 8S (Thanh and Shibasaki, 1979) following dissociation of a fraction of α and α′ subunits (monomer of 3–4S). U_H is a denatured state standing at elevated temperature; that is, heat-induced dissociates were unfolded into a random coil, although a part of the hydrophobic core remains. D_H is a denatured state of heat-induced dissociates formed after cooling which regained the compact internal structure but not quaternary structure. All of the D_H values above 70 °C are very close to −7000. The partial reversibility of thermal denaturation of β-conglycinin has been recognized; that is, the dissociation into monomers ($N \rightarrow D_H$, $N \rightarrow U_H$) is an irreversible process but the unfolding of the dissociated monomer ($D_H \rightleftharpoons U_H$) is a reversible process. The variation in the final $\Delta\epsilon_{287}$ (U_H) indicates that denaturation led to different degrees of unfolding that increased progressively with heating temperature (X_1, X_2, etc. were recognized actually at the elevated temperature).

ABBREVIATIONS USED

UV, ultraviolet; CD, circular dichroism; GuHCl, guanidine hydrochloride.

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