Matthews, B. F.; Cress, D. E. *Planta* **1981**, *153*, 90-94.
Ohki, S.; Leonards, K. *Chem. Phys. Lipids* **1982**, *31*, 307-318.
Ohtsuru, M.; Kito, M. *Agric. Biol. Chem.* **1983**, *47*, 1907-1908.
Ohtsuru, M.; Yamashita, Y.; Kanamoto, R.; Kito, M. *Agric. Biol. Chem.* **1979**, *43*, 765-770.
Osborn, T. W. *J. Agric. Food Chem.* **1977**, *26*, 229-232.
Patel, K. M.; Morrisett, J. a.; Sparrow, J. T. *Lipids* **1979**, *14*, 596-597.
Pick, U. *Arch. Biochem. Biophys.* **1981**, *212*, 186-194.
Schenkman, S.; Soares de Araujo, P.; Sesso, A.; Quinz, F. H.; Chaimovich, H. *Chem. Phys. Lipids* **1981**, *28*, 165-180.
Schlieper, P.; Michaelis, L. *Biophys. Struct. Mech.* **1983**, *10*, 1-9.
Sessa, D. J. *J. Argic. Food Chem.* **1979**, *27*, 234-239.
Szoka, F., Jr.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4194-4198.
Thanh, V. H.; Okubo, K.; Shibasaki, K. *Plant Physiol.*]8(4m *56*, 19-22.
Thanh, V. H.; Shibasaki, K. *J. Agric. Food Chem.* **1976**, *24*, 1117-1121.
Wang, H. H.; Earnest, J.; Limbacher, H. P. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5297-5301.
Weiss, U.; Funes, J.; Karel, M. *J. Agric. Food Chem.* **1983**, *31*, 517-522.
Wolf, W. J. In "Soybeans: Chemistry and Technology"; Smith, A. K.; Circle, S. J., Eds.; Avi Publishing Co.: Westport, CT, 1972; Vol. I, Chapter 4, pp 93-143.
Wu, G.; Stein, R. A.; Mead, J. F. *Lipids* **1982**, *17*, 403-413.

Received for review May 2, 1984. Revised manuscript received August 6, 1984. Accepted August 22, 1984. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agricultue over other firms or similar products not mentioned.

Effect of Temperature on the Conformation of Soybean Glycinin in 8 M Urea or 6 M Guanidine Hydrochloride Solution

B. R. Suresh Chandra, A. G. Appu Rao, and M. S. Narasinga Rao*

The effect of temperature on soybean glycinin in 8 M urea or 6 M Gdn·HCl solution has been studied by CD and ORD measurements. At room temperature the protein assumes an unordered conformation in the presence of the denaturant. With an increase in temperature, $[\Theta]_{MRW}$ in the range 250-210 nm assumes more negative values, suggesting formation of ordered conformations at higher temperatures.

The study of denaturation of proteins by various agents is aimed at understanding the molecular forces responsible for maintaining the native state of the protein (Kauzmann, 1959; Tanford, 1968). Urea, guanidine hydrochloride (Gdn·HCl), and heat have been extensively used as denaturants (Joly, 1965; Lapanje, 1978). Urea is less effective than Gdn·HCl in denaturing proteins (Tanford, 1968; Pace, 1975). Thermodynamics of denaturation of proteins as also the mechanism have been the subject of extensive studies (Tanford, 1968, 1970; Pace, 1975; Privalov, 1979, 1982).

There are only a few reports on the effect of temperature on proteins in concentrated urea or guanidine hydrochloride solution (Pace and Tanford, 1968; Ahmed and Salahuddin 1974; Cortijo et al., 1973). In 6 M Gdn·HCl solution, the proportion of α-helix in charged polypeptide chains appears to increase with temperature (Cortijo et al., 1973). Proteins of unordered conformation show conformational anomalies in denaturing solvents at higher temperatures that are independent of amino acid composition, chain length, and the nature of the denaturing solvent (Ahmed and Salahuddin, 1974). In the present investigation, the effect of temperature on soybean glycinin (also called the 11S protein of soybean) in 8 M urea and 6 M Gdn·HCl solution has been studied by CD and ORD measurements. The effect of temperature on the structure of glycinin has been reported (Fukushima, 1968; Koshiyama et al., 1980-81; German et al., 1982).

MATERIALS AND METHODS

Soybean (Bragg variety), grown in the State of Karnataka, India, was purchased from the local market. Bovine serum albumin (recrystallized), Gdn·HCl, and *N*-ethylmaleimide were from Sigma Chemical Co., and mercaptoethanol was from Fluka, Switzerland. Urea from British Drug House Co., India, was recrystallized from ethanol. Gdn·HCl was recrystallized by the method of Nozaki (1972). The buffer salts and NaCl were of analytical reagent quality.

Soybean glycinin was prepared by the method of Appu Rao and Narasinga Rao (1977). It was found to be homogeneous (>95%) by gel filtration on Sepharose 6-B, sedimentation velocity, and polyacrylamide gel electrophoresis on 7.5% gels at pH 7.8.

CD and ORD measurements were made with Jasco J20-C automatic recording spectropolarimeter. The instrument was calibrated with (+)-10-camphorsulfonic acid for CD and sucrose solution for ORD measurements. Quartz cells of different path lengths (1, 0.1, or 0.05 cm) were used for measurements in the region 330-200 nm. Slits were programmed to yield a 10-Å bandwidth at each wavelength. Protein concentration in the range 0.3-2.0 mg/mL was used. Mean residue rotation, $[m]_{MRW}$, and mean residue elipticity $[\Theta]_{MRW}$, were calculated by the standard procedure (Adler et al., 1973). On the basis of the amino acid composition of glycinin, determined with an LKB α-amino acid analyzer, a value of 115 was used for the mean residue weight (MRW). For measurements at different temperatures, water from a preset water thermostat was circulated through the double-walled cell and the temperature maintained for 15-20 min for thermal equilibrium before making measurements. The temperature was controlled to ±0.05 °C.

All the measurements were made in 0.05 M phosphate buffer of pH 7.8 containing 0.35 M NaCl and 0.01 M β-mercaptoethanol and in triplicate.

Protein concentration was estimated by absorbance measurements at 280 nm, using a value of $E_{1cm}^{1\%}$ = 7.9

Central Food Technological Research Institute, Mysore 570013, India.

0021-8561/84/1432-1402$01.50/0 © 1984 American Chemical Society

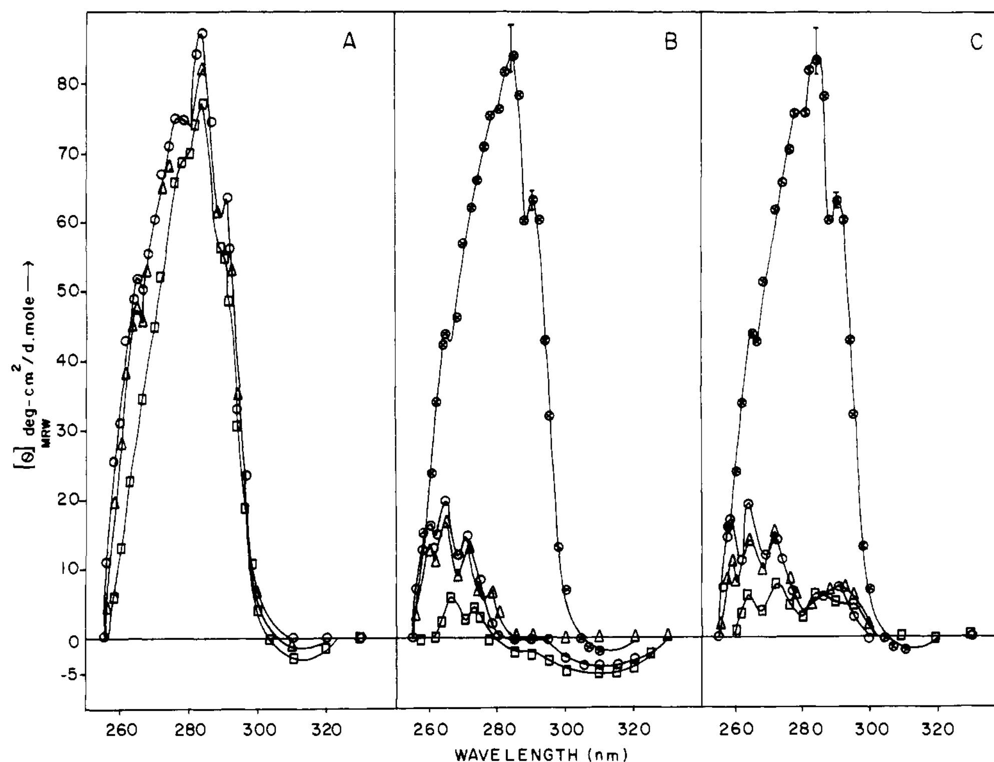

Figure 1. Effect of temperature on the near-UV CD spectrum of glycinin. (A) Glycinin: (○) 15 °C; (△) 30 °C; (□) 60 °C. (B) Glycinin in 8 M urea: (⊗) glycinin (control) at 30 °C in the absence of denaturant; (○) 15 °C; (△) 30 °C; (□) 60 °C. (C) Glycinin in 6 M Gdn·HCl: (⊗) glycinin (control) at 30 °C in the absence of denaturant; (○) 15 °C; (△) 30 °C; (□) 60 °C.

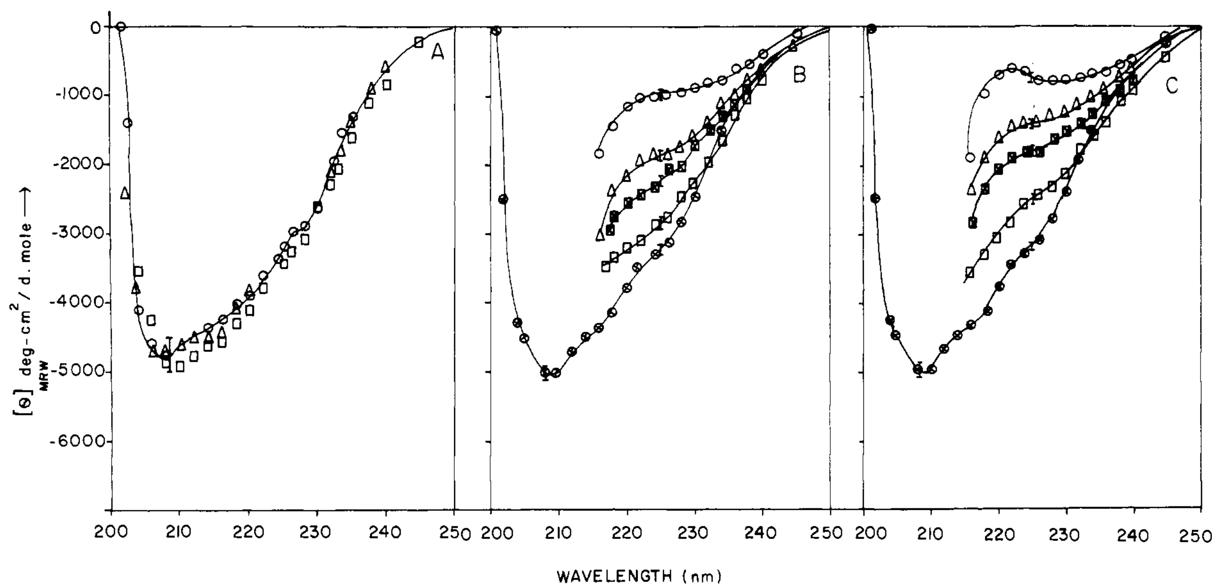

Figure 2. Effect of temperature on the far-UV CD spectrum of glycinin. (A) Glycinin: (○) 15 °C; (△) 30 °C; (□) 60 °C. (B) Glycinin in 8 M urea: (⊗) glycinin (control) at 30 °C in the absence of denaturant; (○) 15 °C; (△) 30 °C; (⊠) 40 °C; (□) 60 °C. (C) Glycinin in 6 M Gdn·HCl: (⊗) glycinin (control) at 30 °C in the absence of denaturant; (○) 15 °C; (△) 30 °C; (□) 40 °C; (□) 60 °C.

(Appu Rao and Narasinga Rao, 1977).

RESULTS

Near-UV CD Spectra. The near-UV CD spectrum of glycinin exhibited a small trough at 315 nm and peaks at 290, 285, 279, and 265 nm (Figure 1A). Increasing the temperature of the protein solution up to 60 °C did not cause significant changes in the spectrum; there was a slight decrease in the band intensities. The addition of 8 M urea markedly altered the spectrum; the 290- and 285-nm bands were abolished, and the intensity of the 279- and 265-nm bands was considerably reduced (Figure 1B). Increasing the temperature of the solution in 8 M urea caused further decreases in band intensity. The same effect was observed in 6 M Gdn·HCl solution also (Figure 1C). In this solvent, however, the 290-nm band was not totally abolished.

Far-UV CD Spectra. The far-UV CD spectrum in the region 250–200 nm exhibited shoulders at 225 and 215 nm and a trough at 208 nm (Figure 2A). The spectrum at 60 °C was practically the same as at 15, 30, and 40 °C; all the fine features of the spectrum were retained at 60 °C also. These results are in agreement with those reported by Koshiyama et al. (1980–81) and German et al. (1982). The addition of 8 M urea reduced $[\Theta]_{MRW}$ values in the 250–215-nm region. Thus, 8 M urea disrupted the native conformation of the protein (Cortijo et al., 1973; Fasman et al., 1970). Increasing the temperature of the solution

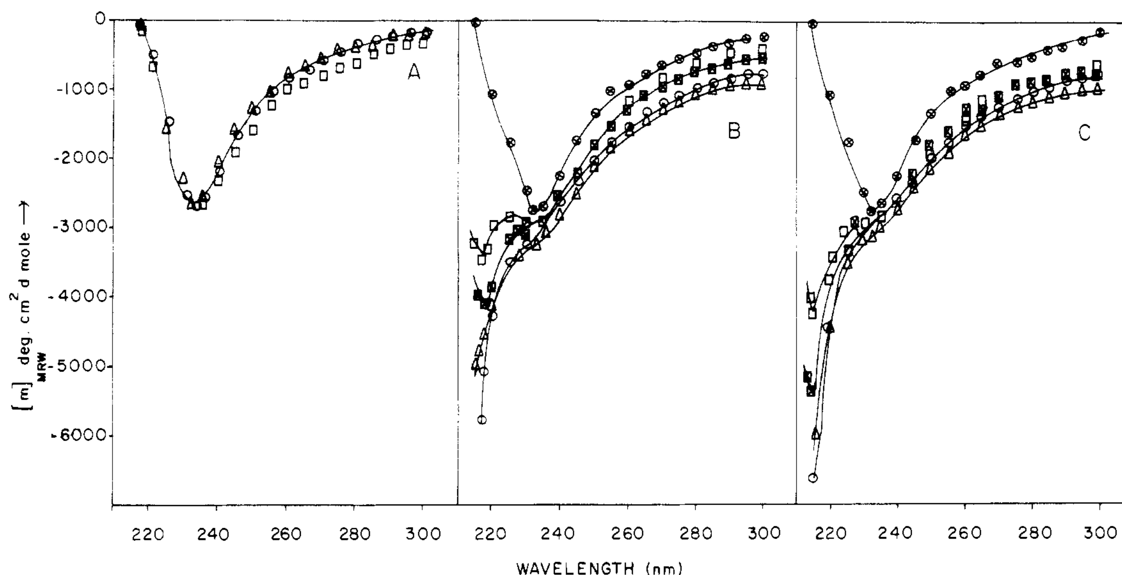

Figure 3. Effect of temperature on the ORD spectrum of glycinin. (A) Glycinin: (○) 15 °C; (△) 30 °C; (□) 60 °C. (B) Glycinin in 8 M urea: (⊗) glycinin (control) at 30 °C in the absence of denaturant; (○) 15 °C; (△) 30 °C; (■) 40 °C; (□) 60 °C. (C) Glycinin in 6 M Gdn·HCl: (⊗) glycinin (control) at 30 °C in the absence of denaturant; (○) 15 °C; (△) 30 °C; (⊠ 40 °C; (□) 60 °C.

in 8 M urea from 15 to 60 °C resulted in more negative values of $[\Theta]_{MRW}$ in the 240–215-nm region (Figure 2B). Although the values became more negative, the values at 60 °C were not identical with those of the native protein. The same effect was observed in 6 M Gdn·HCl solution also (Figure 2C). Gdn·HCl was more effective than urea in disrupting the native structure of soybean glycinin. At any temperature, the $[\Theta]_{MRW}$ values in 6 M Gdn·HCl were less negative than those in 8 M urea.

ORD Spectra. The ORD spectrum of the protein in the 300–210-nm region consisted of a trough at 232 nm and a crossover point at 215 nm (Figure 3A). Increasing the temperature of the protein solution to 60 °C did not affect the spectrum.

The addition of 8 M urea caused the following changes: (i) $[m]_{300nm}$ values assumed slightly more negative values; (ii) $[m]_{232nm}$ did not change significantly; (iii) the crossover point shifted to shorter wavelengths. Increasing the temperature of the solution in 8 M urea did not have much effect on the spectrum in the 300–230-nm region. However below 230 nm, $[m]_{MRW}$ values became less negative. Also, a clear minimum at 215 nm was seen in the case of solutions at 40 and 60 °C. Interestingly enough, the $[m]_{MRW}$ value at 232 nm was nearly the same for the native protein as for the protein in 8 M urea solution at different temperatures. The value of −2700 deg·cm²/dmol falls within the range of values reported by Tanford et al. (1967) for proteins in 6 M Gdn·HCl, with their S–S bonds intact. The behavior of the protein in 6 M Gdn·HCl was the same as in 8 M urea solution (Figure 3C).

DISCUSSION

Soybean glycinin has 1.36% tryptophan, 5.10% tyrosine, 5.03% phenylalanine, and 1.36% cystine (Suresh Chandra, 1984). In the near-UV CD spectrum of glycinin at 30 °C, the bands at 290 and 279 nm may be assigned to tryptophan and tyrosine, respectively. The band at 265 nm could be due to phenylalanine. The negative band at 315 nm could be due to the contribution of disulfide residues. The shape of the band suggests that the aromatic bands are positive and superimposed on a broad negative background due to cystine (Strickland, 1974).

As the temperature of the protein in buffer solution was increased from 15 to 60 °C, the intensity of the bands did not change significantly. However, in 8 M urea or 6 M Gdn·HCl solution the band intensities decreased considerably, indicating gross conformational changes. The denaturants changed the environment around the aromatic chromophores. From UV difference spectral measurements, Catsimpoolas et al. (1970) have reported that in 6 M urea and 6 M Gdn·HCl solutions, all the tryptophan residues of glycinin become accessible to the solvent. Further, in these solvents the protein would be completely dissociated (Catsimpoolos et al., 1967). Increasing the temperature of the denatured protein solution in 8 M urea or 6 M Gdn·HCl caused further decrease in the intensities of the near-UV CD bands. The fact that the CD contributions of nonpeptide chromophores in urea or Gdn·HCl solution decreased with an increase in temperature indicated that at least the local conformations around these chromophores became less rigid at higher temperatures.

However, the far-UV CD and ORD spectra showed some anomalies on heating the protein in 8 M urea or 6 M Gdn·HCl solution. The far-UV CD spectrum of the native glycinin consisted of a trough at 208 nm and shoulders at 225 and 215 nm. The shoulders could possibly be due to antiparallel and parallel β-structure (Raghavendra and Ananthanarayana, 1981). The calculation of the secondary structures by the method of Greenfield and Fasman (1969) showed that the protein contained about 4% α-helix, 33% β-structure, and the rest unordered conformation. These values agree well with the reported values for glycinin (Koshiyama, 1972; Koshiyama and Fukushima, 1973; Sakakibara and Noguchi, 1977). Thus, glycinin has predominantly β-structure and unordered conformation. Raising the temperature of the protein solution from 15 to 60 °C did not affect significantly the far-UV CD spectrum; temperature as such did not unfold the protein molecule.

Due to the addition of urea or Gdn·HCl to the glycinin solution, the ellipticity values became less negative. A decrease in ellipticity values was a function of added denaturant concentration. The values of $[\Theta]_{MRW}$ of the protein in the presence of 8 M urea or 6 M Gdn·HCl were markedly less negative than those of the native protein. The CD spectrum of the protein in 6 M Gdn·HCl was typical of a protein fully denatured and in unordered conformation (Cortijo et al., 1973; Fasman et al., 1970).

However, when the temperature was increased from 15 to 60 °C, the $[\Theta]_{MRW}$ values became more negative and tended toward the values of the native protein. Although the value of $[\Theta]_{MRW}$ at 225 nm at 60 °C in 6 M Gdn·HCl or 8 M urea was close to that of the native protein, it was not identical, suggesting that the protein did not fold back to the native conformation. Increasing the temperature of the denatured protein restored some ordered structure of glycinin; such ordered structures were likely to be different from that of native protein as indicated by near-UV CD spectra.

The ORD spectrum of the protein in 8 M urea or 6 M Gdn·HCl at different temperatures showed that the value of $[m]_{232nm}$ did not change with temperature. The $[m]_{MRW}$ values below 225 nm were less negative with an increase in temperature. Unordered conformation has a minimum at 205 nm (Greenfield et al., 1967). The less negative $[m]_{MRW}$ values below 220 nm could possibly mean a decrease in the proportion of unordered conformation.

In the case of proteins such as lysozyme (Edelhoch and Steiner, 1962) and soybean trypsin inhibitor (Edelhoch and Steiner, 1963), the effect of temperature on the protein denatured in 9 M urea solution is to denature them further. In the case of glycinin denatured in 8 M urea or 6 M Gdn·HCl solution, temperature seems to have a contrary effect.

Soybean glycinin is an oligomeric protein. The anomalous effect observed may not be due to subunit interactions. In 8 M urea or 6 M Gdn·HCl the protein is completely dissociated (Catsimpoolas et al., 1967). Further, in the case of bovine serum albumin, a protein consisting of a single polypeptide chain, Ahmed and Salahuddin (1974) observed that the intrinsic viscosity of the protein in 6 M Gdn·HCl solution decreased with an increase in temperature. This has been attributed to a decrease in the end-to-end distance between residues, with an increase in temperature.

Both urea and Gdn·HCl denature proteins by their preferential interaction with the proteins (Prakash et al., 1981). The interaction parameter may be temperature dependent, and binding of denaturant molecules may be less at higher temperatures. Consequently, the extent of denaturation may be less. Optical activity of any substance is affected by temperature in two ways: (i) by changing the extent of interaction between solute and solvent and (ii) by changing the conformation of the optically active solute. Generally, as the temperature increases, the interaction between solute and solvent decreases and at higher temperatures the optical activity of the substance tends to become independent of solvent (Kauzman and Eyring, 1941).

In case of charged polypeptides in 6 M Gdn·HCl solution temperature increases the amplitude of the CD bands, suggesting that the proportion of α-helix has increased. This was attributed to a solvation-exchange phenomenon (Cortijo et al, 1973).

Micro calorimetric experiments of Pfeil and Privalov (1978) suggest that there is no difference in the extent of unfolding of proteins due to either temperature or other denaturing agents. Our experiments show that temperature in the range 15–60 °C has no effect on glycinin but urea or Gdn·HCl denatures the protein.

Our interpretation that higher temperatures favor the formation of ordered structures from denatured glycinin should be viewed with caution since Fasman et al. (1970) have reported a variety of CD curves for polypeptide chains in unordered conformation.

Abbreviations Used: CD, circular dichroism; ORD, optical rotatory dispersion; UV, ultraviolet; Gdn·HCl, guanidine hydrochloride.

LITERATURE CITED

Adler, A. J.; Greenfield, N. J.; Fasman, G. D. *Methods Enzymol.* **1973**, *27*, 675.
Ahmed, F.; Salahuddin, A. *Biochemistry* **1974**, *13*, 245.
Appu Rao, A. G.; Narasinga Rao, M. S. *Prep. Biochem.* **1977**, *7*, 89.
Catsimpoolas, N.; Rogers, D. A.; Circle, S. J.; Meyer, E. W. *Cereal Chem.* **1967**, *44*, 631.
Catsimpoolas, N.; Wang, J.; Berg, T. *Int. J. Pept. Protein Res.* **1970**, *2*, 277.
Cortijo, M.; Panijpan, B.; Gratzer, W. B. *Int. J. Pept. Protein Res.* **1973**, *5*, 179.
Edelhoch, H.; Steiner, R. F. *Biochim. Biophys. Acta* **1962**, *60*, 365.
Edelhoch, H.; Steiner, R. F. *J. Biol. Chem.* **1963**, *238*, 931.
Fasman, G. D.; Hoving, H.; Timasheff, S. N. *Biochemistry* **1970**, *9*, 3316.
Fukushima, D. *Cereal Chem.* **1968**, *45*, 203.
German, B.; Damodaran, S.; Kinsella, J. E. *J. Agric. Food Chem.* **1982**, *30*, 807.
Greenfield, N.; Davidson, B.; Fasman, G. D. *Biochemistry* **1967**, *6*, 1630.
Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108.
Joly, M. "A Physico-Chemical approach to the denaturation of proteins"; Academic Press: London and New York, 1965.
Kauzman, W. *Adv. Protein Chem.* **1959**, *14*, 1.
Kauzman, W.; Eyring, H. *J. Chem. Phys.* **1941**, *9*, 41.
Koshiyama, I. *Int. J. Pept. Protein Res.* **1972**, *4*, 167.
Koshiyama, I.; Fukushima D. *Cereal Chem.* **1973**, *50*, 114.
Koshiyama, I.; Hamano, M.; Fukushima, D. *Food Chem.* **1980–81**, *6*, 309.
Lapanje, S. "Physico-chemical aspects of protein denaturation"; Wiley: New York, 1978.
Nozaki, Y. *Methods Enzymol.* **1972**, *26*, 43.
Pace, C. N. *CRC Crit. Rev. Biochem.* **1975**, *3*, 1.
Pace, C. N.; Tanford, C. *Biochemistry* **1968**, *7*, 198.
Pfeil, W.; Privalov, P. L. In "Biochemical Thermodynamics"; Skinner, H., Ed.; Elsevier: Amsterdam, 1978.
Prakash, V.; Loucheux, C.; Scheufele, S.; Gorbunoff, M. J., Timasheff, S. N. *Arch, Biochem. Biophys.* **1981**, *210*, 455.
Privalov, P. *Adv. Protein Chem.* **1979**, *33*, 167.
Privalov, P. *Adv. Protein Chem.* **1982**, *35*, 1.
Raghavendra, K.; Ananthanarayana, V. S. *Int. J. Pept. Protein Res.* **1981**, *17*, 412.
Sakakibara, M.; Noguchi, H. *Agric. Biol. Chem.* **1977**, *41*, 1575.
Strickland, H. E. *CRC Crit. Rev. Biochem.* **1974**, *2*, 113.
Suresh Chandra, B. R. Ph.D. Dissertation, Mysore University, 1984.
Tanford, C. *Adv. Protein Chem.* **1968**, *23*, 121.
Tanford, C. *Adv. Protein Chem.* **1970**, *24*, 1.
Tanford, C.; Kawahara, K.; Lapanje, S.; Hooker, T. M., Jr.; Zarlengo, M. H.; Salahuddin, A.; Aune, K. C.; Takagi, T. *J. Am. Chem. Soc.* **1967**, *89*, 5023.

Received for review December 19, 1983. Accepted August 2, 1984.