Interaction of Calf Skin Collagen with Glycerol: Linked Function Analysis

ABSTRACT: Glycerol stabilizes the triple-helical structure of solubilized calf skin collagen. The equilibrium melting temperature of the protein increased linearly from 38.0 °C in AS buffer (0.01 M NaOAc and 0.02 M NaCl, pH 4.0) to 43.0 °C in AS and 6 M glycerol buffer. To understand the thermodynamic basis of this effect on the equilibrium melting temperature and the glycerol inhibition of collagen self-association, the preferential interactions of native and denatured calf skin collagens in AS buffer containing 1.5, 3, and 4.5 M glycerol were measured with a precision densimeter. The results indicated that native collagen binds glycerol preferentially whereas denatured collagen neither binds nor repels glycerol. The preferential binding of glycerol by native collagen, when interpreted in terms of the three-component solution thermodynamics, suggests that the surface interaction of native collagen with glycerol is energetically more favorable than its interaction with water. By use of the Wyman linked function, the negative chemical potential change of collagen derived from its preferential binding of glycerol can account for both the glycerol stabilization of the triple-helical structure of collagen and the inhibition of in vitro self-association of monomers into fibrils.

In the preceding paper, we have shown that glycerol inhibits the in vitro self-association of monomeric collagen into fibrils (Na et al., 1986). This effect appears to be contrary to the enhancing effect of glycerol toward the self-assemblies of globular proteins such as L-asparaginase, actin, and tubulin (Shifrin & Parrott, 1975; Kasai et al., 1965; Shelanski et al., 1973; Lee & Timasheff, 1977). However, glycerol has also been shown to stabilize the triple-helical structure of collagen (Hart et al., 1971; Russell, 1973; Gekko & Koga, 1983) just as it stabilizes the native structure of many globular proteins (Gekko & Timasheff, 1981a; Na & Timasheff, 1981).

In an attempt to understand the thermodynamic basis of the above effects of glycerol toward globular proteins, Timasheff and co-workers measured the preferential interactions of eight globular proteins in aqueous glycerol solutions (Na & Timasheff, 1981; Gekko & Timasheff, 1981a,b). Their results showed that all of these globular proteins are preferentially hydrated in aqueous glycerol solutions; i.e., glycerol is repelled from the surface domain of the protein. Furthermore, at pH 2.0, the degree of exclusion of glycerol by these proteins appeared to be inversely proportional to their polarity. Although the exact molecular mechanism of the preferential interaction remains unclear, in terms of thermodynamics the unfavorable free energy change resulting from the mutual exclusion can satisfactorily account for both the glycerol stabilization of the native structure and the enhancement of the self-association of the globular proteins (Na & Timasheff, 1981; Gekko & Timasheff, 1981a,b).

The inhibiting effect of glycerol toward in vitro collagen self-association, however, cannot be understood on the basis of the same arguments used for the globular proteins. In order to reconcile these apparently contradictory effects and to further test the previously proposed thermodynamic rationale of the effects of glycerol on protein structures, the preferential interactions of both native and denatured calf skin collagen in glycerol solutions were measured, and the results are reported here.

Materials and Methods

Materials. The chemicals and the type I calf skin collagen used were the same as described in the preceding paper (Na et al., 1986).

Thermal Denaturation of Collagen. The thermal denaturation of collagen was monitored by measuring UV hyperchromicity at 224 nm (Na & Butz, 1983). A Perkin-Elmer Lambda-7 UV–visible spectrophotometer was used.[1] The temperature of the collagen solutions was regulated through use of both jacketed cuvettes and a cell holder connected in series to a Neslab Model RTE-8 heating/cooling water bath. Temperature was increased linearly with a Neslab Model ETP-3 electronic temperature programmer. The actual temperature of the solution in the cuvette differed from that of the water bath by about 0.1–0.2 °C. Consequently, the true temperature of the solution was always monitored with a digital thermometer using a microthermistor immersed in the solution above the light path. At each glycerol concentration, the apparent melting temperature, defined as the temperature at 50% transition, was measured at four different heating rates, ranging from 1 to 6 °C/h, and these were extrapolated to zero heating rate to obtain the equilibrium melting temperature.

Circular Dichroism Spectroscopy. The circular dichroism (CD)[2] spectra of collagen were measured with a JASCO J41-C circular dichroism spectropolarimeter. Collagen was dialyzed exhaustively against 1 mM HOAc and adjusted to 0.25 mg/mL. The final collagen solution, either in AS (0.01 M NaOAc and 0.02 M NaCl, pH 7.0) or in PS (0.03 M NaP_i and 0.1 M NaCl, pH 7.0) buffer and with or without 1 M glycerol, was prepared by mixing with a proper ratio of the above collagen stock solution and the corresponding single and double-strength buffers. For measurement of the CD spectra, a cell with a 1-cm light path was used to obtain the positive peak at 215–250 nm whereas a cell with a 0.05-cm light path was used for the negative peak at 190–215 nm. The concentration of the collagen solution used was approximately 0.05 mg/mL in the former measurement and 0.1 mg/mL in the latter one. A mean residue weight of 91.5 g/mol derived from the amino acid composition of collagen was used in calculating the molar ellipticity.

[1] Reference to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

[2] Abbreviations: AS, 0.01 M NaOAc and 0.02 M NaCl, pH 4.0; PS, 0.03 M NaP_i and 0.1 M NaCl, pH 7.0; CD, circular dichroism; GdnHCl, guanidine hydrochloride.

Precision Density Measurement. The preferential interactions of calf skin collagen in aqueous glycerol solutions were determined through precision solution density measurements using an Anton-Parr DMA-2 precision densimeter. The experimental protocol was the same as described by Lee et al. (1979). Aliquots of calf skin collagen were adjusted to the concentration range of 3–12 mg/mL and dialyzed against AS buffer containing 0, 1.5, 3, or 4.5 M glycerol. The dialysis was carried out at 4 °C for 3–5 days with one change of buffer. The denatured collagen samples were prepared by heating the collagen samples for 1 h at 50 °C and were dialyzed at 40 °C for 24 h. All dialyzed samples were equilibrated at room temperature for 0.5 h before the density measurement. Solution densities were measured at 20 °C by connecting a Neslab Model RTE-8 heating/cooling circulating water bath to the jacketed cell of the densimeter. Measurement of the molar ellipticity and far-UV absorbance of the protein indicated that the renaturation of the denatured collagen was negligible within the time required for the density measurement. After the density measurement, the solution was withdrawn from the densimeter, and its protein concentration was determined by gravimetric dilution with 6 M guanidine hydrochloride and measurement of the absorbance at 218 nm as described in the preceding paper (Na et al., 1986). The volume of each aliquot was subsequently calculated from its weight and density in order to obtain the collagen concentration on a volume basis.

Data Treatment. For a three-component solution, using the notation of Scatchard (1949) and Stockmeyer (1950), the principal solvent, water, is designated as component 1, the protein as component 2, and the cosolvent, in this case glycerol, as component 3. The apparent specific volume of the protein, ϕ_2, can be calculated from (Schachman, 1957; Casassa & Eisenberg, 1961, 1964; Kupki, 1973)

$$\phi_2 = \frac{1}{\rho_0}\left(1 - \frac{\rho - \rho_0}{C_2}\right) \tag{1}$$

where ρ and ρ_0 are the densities of the solution and the solvent, respectively, in units of grams per milliliter. C_2 is the weight concentration of the protein, also in units of grams per milliliter.

To determine the preferential interaction between collagen and the glycerol solvent with a densimeter, the apparent isopotential specific volumes of the protein, ϕ_2', were determined under constant chemical potential of the solvent components achieved through equilibrium dialysis. Because collagen, once dried, is difficult to redissolve into a homogeneous solution of high concentration, the isomolal volume of the protein was approximated by the isopotential volume determined in the absence of glycerol. Data have shown that the isomolal volume of protein is not affected by the presence of cosolvents such as salt, sugar, or alcohol (Gekko & Timasheff, 1981a,b; Lee & Timasheff, 1981; Arakawa & Timasheff, 1982, 1984a,b). The preferential interaction of the macromolecule with component 3 of the solution is defined as

$$\xi_3 \equiv (\partial g_3/\partial g_2)_{T,P,\mu_3} \tag{2}$$

where g_i is the weight concentration of component i in units of grams per gram of H_2O. ξ_3 is related to the apparent specific volumes by (Casassa & Eisenberg, 1964)

$$\xi_3 = \left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3} = \frac{\phi_2^0 - \phi_2'^0}{1/\rho_0 - \bar{v}_3} \tag{3}$$

where $\bar{v}_3$ is the partial specific volume of component 3. The superscript 0 indicates that the apparent specific volumes were extrapolated to zero protein concentration to eliminate the effect of interprotein interactions.

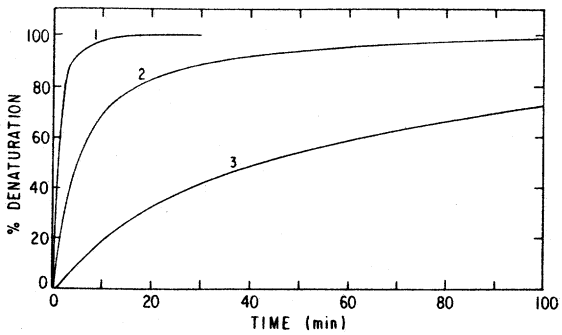

FIGURE 1: Effect of glycerol on the kinetic stability of calf skin collagen. Collagen (0.32 mg/mL) was dissolved in AS buffer containing (1) 0, (2) 1.5, and (3) 3.0 M glycerol. The solution was heated to 40.5 °C by opening the valve of the water bath connected to the jacketed cuvettes. The solution took approximately 2 min to rise from 20 to 40.5 °C. The unfolding of the triple-helical structure was followed by measuring the hyperchromicity as described under Materials and Methods.

According to the three-component solution thermodynamics, the chemical potential of the protein is related to its preferential interaction with the solvent components by

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_2} = \frac{M_{r_2}}{M_{r_3}}\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_2} = -\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_3} \Big/ \left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2} \tag{4}$$

where M_{r_i} and m_i are the molecular weight and molal concentration of component i, respectively, and μ_i is its chemical potential. On the right-hand side of eq 4, the denominator, $(\partial\mu_3/\partial m_3)_{T,P,m_2}$, is related to the nonideality of the glycerol solvent and can be calculated from the osmotic pressure of glycerol solution reported by Scatchard et al. (1938) with the equation:

$$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2} = \frac{RT}{m_3} + RT\left(\frac{\partial \ln \gamma_3}{\partial m_3}\right)_{T,P,m_2} \tag{5}$$

as described by Lewis and Randall (1961).

RESULTS

Effect of Glycerol on the Thermal Stability of Calf Skin Collagen. As shown in Figures 1 and 2a, introducing glycerol to the solvent resulted in a decreased rate of denaturation of calf skin collagen at a constant temperature of 40.5 °C and an increase in the apparent melting temperature of the protein. The apparent melting temperature (T_m) of calf skin collagen in AS buffer was measured at four different heating rates. As shown in Figure 2a, within this range of heating rates, the apparent melting temperature decreased linearly with decreasing heating rate. However, the presence of glycerol did not affect the dependence of T_m on the heating rate. Extrapolation of the apparent melting temperature to zero heating rate gave the equilibrium melting temperature (T_m^0) of the protein. As shown in Figure 2b, the latter increased linearly with glycerol concentration, from 38.0 °C in AS buffer to 43.0 °C in AS and 6 M glycerol buffer.

Effect of Changing the pH and Addition of Glycerol on the Structure of Calf Skin Collagen. The effect of changing the solution pH and adding glycerol to the solution on the conformational structure of collagen was probed with circular dichroism spectroscopy. As shown in Figure 3, native calf skin

Table I: Preferential Interaction of Native and Denatured Collagen in Glycerol–AS Buffers

[glycerol] (M)	$\phi_2'^0$ (mL/g)	ϕ_2^0 (mL/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (m/m)	$(\partial \mu_2/\partial m_3)_{T,P,m_2}$ (kcal/mol² in 1000 g of H_2O)	$(\partial m_3/\partial m_2)_T^c$ (m/m)
		Native Collagen			
0	0.685 ± 0.004	0.685			
1.5	0.680 ± 0.004	0.685	76.4	−29.5	237
3.0	0.674 ± 0.004	0.685	198.7	−34.7	1145
4.5	0.662 ± 0.004	0.685	493.0	−50.8	2229
		Denatured Collagen			
0	0.702 ± 0.002	0.702			
1.5	0.704 ± 0.002	0.702	−29.4	11.3	
3.0	0.694 ± 0.002	0.702	143.3	−25.1	
4.5	0.697 ± 0.002	0.702	108.3	−11.1	

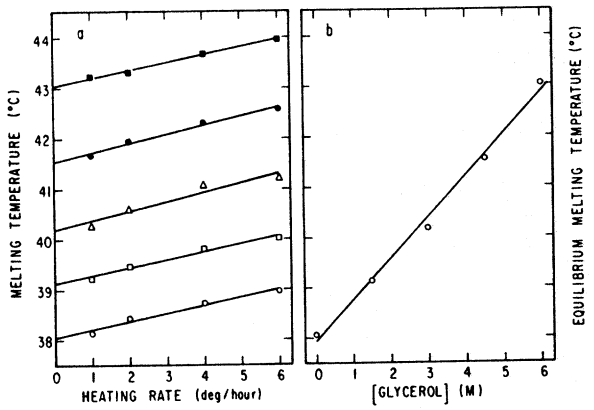

FIGURE 2: Effect of glycerol on the equilibrium stability of calf skin collagen. AS buffer (0.01 M NaOAc and 0.02 M NaCl, pH 4.0) was used. (a) Apparent melting temperature as a function of heating rate. The glycerol concentrations were 0 (○), 1.5 (□), 3 (△), 4.5 (●), and 6 M (■). (b) Dependence of the equilibrium melting temperature of collagen on glycerol concentration.

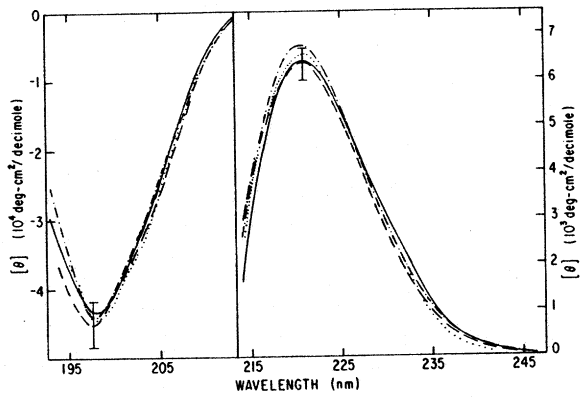

FIGURE 3: Circular dichroism spectra of calf skin collagen. The protein was dissolved in AS buffer (—), PS buffer (-·-), 1 M glycerol–AS buffer (--), and 1 M glycerol–PS buffer (···). All spectra were taken at ambient temperature (20–25 °C) except those in PS buffer which were taken at 10° C by using a jacketed cuvette. Each spectrum represents an average of at least three measurements, and the bars indicate the standard deviations.

collagen exhibits a positive circular dichroism peak with its apex located at 221 nm and a negative peak with its apex found at 198 nm. Within experimental error, neither the change from the pH 4.0 AS buffer to the pH 7.0 PS buffer nor the introduction of 1 M glycerol to the solution affected the CD spectrum of collagen.

Preferential Interaction of Native and Denatured Collagen in Glycerol Solutions. The preferential interactions of both native and denatured collagen in glycerol solutions were determined by precision solution density measurements. The isopotential specific volumes ($\phi_2'^0$) of calf skin collagen in AS buffer containing 0, 1.5, 3, and 4.5 M glycerol are listed in Table I. Native calf skin collagen in AS buffer has an isopotential specific volume of 0.685 ± 0.004 mL/g. It decreased with increasing concentration of glycerol, to 0.662 ± 0.004 mL/g in 4.5 M glycerol solutions. Denatured collagen has an isopotential specific volume of 0.702 ± 0.002 mL/g in AS buffer which is slightly higher than that of the native collagen. This volume remained essentially constant within experimental error in the presence of 1.5, 3.0, and 4.5 M glycerol.

The preferential interactions of the native and denatured collagen in the glycerol solutions were calculated from the isopotential volumes of the protein by using eq 4. The results shown in column 4 of Table I indicated that the values of ξ_3 are positive for native collagen; i.e., the protein binds glycerol preferentially. On the other hand, the denatured collagen showed a slight exclusion of glycerol in the 1.5 M glycerol solution but a slight binding of glycerol in the 3 and 4.5 M glycerol solutions. None of these were of significant magnitude relative to the standard deviation of the measurement.

The changes in chemical potential of the protein resulting from the presence of glycerol in the solvent were calculated from eq 5 and are listed in column 5 of Table I. Addition of glycerol to the solution decreased the chemical potential of the native collagen without significantly affecting that of the denatured protein.

Discussion

A number of polyalcohols including glycerol, 1,3-propanediol, ethylene glycol, and poly(ethylene glycol) have been shown to increase the apparent melting temperature of solubilized type I collagen (Harrap, 1969; Hart et al., 1971; Russell, 1973; Gekko & Koga, 1983). The results shown in Figure 1 indicate that glycerol decreases the rate of collagen denaturation and, therefore, increases the kinetic stability of the triple-helical structure. To determine the effect of glycerol on the equilibrium stability of collagen, the apparent melting temperature of collagen was measured at several different heating rates and extrapolated to zero heating rate to obtain the equilibrium melting temperature. This cautionary measure is necessary because the apparent melting temperature of collagen is dependent on the heating rate. Within the range of heating rates commonly used (1–5 °C/h), the apparent melting temperature increases with the heating rate, suggesting that the unfolding of the triple helix is too slow to keep up with the temperature change. Consequently, the higher apparent melting temperature of collagen in glycerol solution could have reflected a slower rate of denaturation and not an increased equilibrium stability of the protein. By obtaining the equilibrium melting temperature, the results shown in Figure 2b demonstrated that glycerol indeed brings equilibrium stabilization as well as kinetic stabilization to the triple-helical structure of collagen.

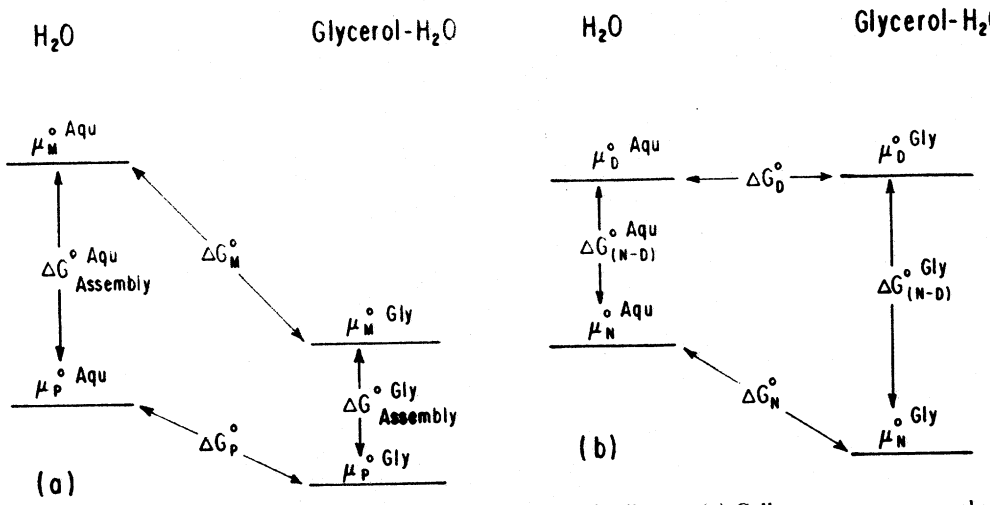

FIGURE 4: Schematic diagram of the effect of glycerol on the chemical potential of collagen. (a) Collagen monomer vs. polymer and (b) native collagen vs. denatured collagen. The notations used were the same as those in eq 7 and 8. In panel a, $\Delta G^\circ_{assembly}$ is the standard free energy change of the assembly reaction. ΔG°_M and ΔG°_P are the standard free energy changes of transferring the monomer and polymer, respectively, from an aqueous buffer to a glycerol solution. In panel b, $\Delta G^\circ_{N\text{-}D}$ is the standard free energy change of the denaturation reaction. ΔG°_N and ΔG°_D are the standard free energy changes of transferring the native and the denatured collagen, respectively, from an aqueous buffer to a glycerol solution.

The stabilizing effects of glycerol on the native structures of globular proteins and the enhancement of the self-association of L-asparaginase, actin, and tubulin by glycerol have been well documented. Since high concentrations of glycerol, in the range of several molar, are needed and since these effects are indiscriminate toward different globular proteins, they must be resulting from certain weak nonspecific interactions between the protein and the solvent components. Studies carried out by Timasheff and co-workers have shown that all eight globular proteins examined are preferentially hydrated in glycerol solution, i.e., glycerol is preferentially excluded from the surface domain of these proteins (Na & Timasheff, 1981; Gekko & Timasheff, 1981a,b). Furthermore, at pH 2.0, the degree of exclusion of glycerol was found to be inversely related to the polarity of the protein (Gekko & Timasheff, 1981a).[3] In terms of multicomponent solution thermodynamics, the preferential exclusion of glycerol by the protein means that the introduction of glycerol to the buffer should raise the chemical potential of both the protein and glycerol which would result in destabilization of the system. Therefore, the interaction of the protein with the glycerol solution must be energetically less favorable than that with water. To account for the above-described structural stabilization and self-association enhancement effects of glycerol in terms of the unfavorable interaction, the effect of glycerol on the chemical potential of the protein, in its native vs. denatured state and in the monomeric vs. the polymeric state, was examined (Na & Timasheff, 1981). It was reasoned that since most of the hydrophobic groups of the globular proteins are buried in the interior and become exposed to the solvent as the protein denatures, the denatured state of the protein is expected to show even stronger repulsion of glycerol than the native state does. Consequently, the presence of glycerol should more strongly destabilize the denatured state of the protein than the native one. Thus, the introduction of glycerol to the solvent should inhibit the denaturation reaction. It was further argued that the protein in its polymeric state should have a smaller surface of contact with the solvent than in the monomeric state, due to the formation of intersubunit contact. Assuming that the repulsion of glycerol by the protein is distributed evenly on its surface, then the polymers, with a smaller surface area per unit weight than the monomers, should be less destabilized by the presence of glycerol than the monomers. Consequently, the introduction of glycerol to the solvent should favor the polymeric state and enhance the polymerization reaction.

While the above arguments are valid toward all of the globular proteins examined, they are evidently not applicable in a straightforward manner to the fibrous protein collagen; the self-association of the latter was found to be inhibited rather than enhanced by glycerol. The effects of glycerol on the structure of collagen also appear to result from weak nonspecific interactions, since high concentrations of glycerol are required and several other polyalcohols are known to exhibit similar effects. Furthermore, according to the circular dichroism results of Figure 3, the presence of 1 M glycerol in the solution did not affect the triple-helical structure of collagen. The results shown in column 4 of Table I indicate that glycerol interacts with collagen in a completely different manner than it interacts with the globular proteins. Glycerol is preferentially attracted to collagen rather than being repelled as is the case in globular proteins (Na & Timasheff, 1981; Gekko & Timasheff, 1981a). Consequently, the surface contact of the protein with the glycerol solution should be more favorable than with water alone. Indeed, as shown in column 5 of Table I, because of the preferential binding of glycerol to collagen, the presence of glycerol in the solution should lower the chemical potential of the protein. Figure 4a presents a schematic diagram of the effect of glycerol on the chemical potentials of collagen in its monomeric and polymeric states. The polymers, because of their smaller surface area per unit weight, should be less stabilized by glycerol than the monomer, and the presence of glycerol in the solution should shift the chemical equilibrium from the polymer to the monomer. Consequently, one can still use the preferential interaction parameters, interpreting them in terms of the three-component solution thermodynamics, to account for the glycerol inhibition of collagen self-association. In other words, although opposite effects are imposed by glycerol on the self-associations of globular proteins and collagen, they can be attributed to the different manner of interaction of these two different classes of proteins with glycerol; the interpretation of the effects in terms of three-component thermodynamics remains valid in both cases.

[3] The polarity of a protein has been defined as the ratio of its total number of polar amino acids over the apolar ones (Bigelow, 1967).

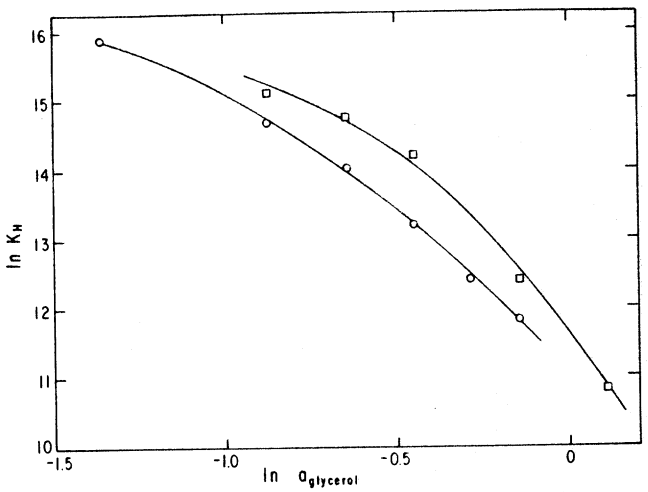

FIGURE 5: Wyman plot of collagen self-association as a function of glycerol concentration. The collagen self-association constants (K_H) were taken from the preceding paper (Na et al., 1986). The circles are the $NaBH_4$-reduced collagen whereas the squares are the native collagen.

According to the linked function, the effect of a ligand on the chemical equilibrium of a macromolecular reaction can be expressed as (Wyman, 1964)

$$(\partial \ln K/\partial \ln a_3)_{T,P,m_2} = (\partial m_3/\partial m_2)^{\text{product}}_{T,P,\mu_3} - (\partial m_3/\partial m_2)^{\text{reactant}}_{T,P,\mu_3} \quad (6)$$

where K is the macromolecular equilibrium constant and a_3 is the activity of the ligand (component 3). $(\partial m_3/\partial m_2)^{\text{product}}_{T,P,\mu_3}$ and $(\partial m_3/\partial m_2)^{\text{reactant}}_{T,P,\mu_3}$ are the sum of the preferential interactions of the product(s) and reactant(s) with the ligand, respectively. Figure 5 shows a Wyman plot of the apparent self-association constants of in vitro collagen fibril assembly (K_H) derived from the critical concentration measurements described in the preceding paper (Na et al., 1986) against the activity of glycerol in the solution. The data were curvilinear and showed a negative slope which ranged from −2.5 to −4.8 mol of glycerol/mol of collagen for the $NaBH_4$-reduced collagen and from −1.5 to −6.5 mol of glycerol/mol of collagen for the native collagen. According to eq 6, the slope of the Wyman plot should be equal to the change in the preferential binding of glycerol by the protein as it polymerizes. If one linearly extrapolates the data of column 4, Table I to zero glycerol concentration to estimate the glycerol binding by the protein at the lower glycerol concentrations used in measuring the fibril assembly, the results show a reduction of approximately 11–15% of the total preferential binding of glycerol as collagen polymerizes. If one further assumes that the preferential binding of glycerol by collagen is evenly distributed on its surface area, then the above results lead to the conclusion that approximately 11–15% of the surface area is removed from the solvent contact as collagen self-associates into microfibrils. Microfibrils appear to be the dominating species at equilibrium with the monomers as shown in the preceding paper (Na et al., 1986).

It is interesting to note that in the polymerization of tubulin, glycerol served as a "thermodynamic booster" in the sense that it enhanced the polymerization reaction through weak nonspecific mutual exclusion with the protein. The effect of glycerol is manifested in a shifted chemical equilibrium but not an altered association mechanism (Timasheff, 1978). This "boosting" effect permitted in vitro studies of the microtubule assembly at convenient protein concentrations on the order of 1 mg/mL instead of the 10–20 mg/mL concentrations required in the absence of glycerol (Lee & Timasheff, 1977; Na & Timasheff, 1981). By the same token, glycerol can be considered to be a "thermodynamic inhibitor" toward the self-association of collagen since it appears that glycerol weakens the fibril assembly reaction through a weak nonspecific mutual attraction with the protein. As shown in the preceding paper (Na et al., 1986), this inhibiting effect of glycerol does not seem to have altered the overall association mechanism. This effect can be used beneficially in in vitro studies of the fibril assembly reaction to increase the critical concentration of microfibril and fibril formation from less than 10 μg/mL to a range that is easily measurable. This critical concentration, when interpreted in terms of the helical cooperative assembly mechanism, permits one to determine the equilibrium constant of the polymer elongation reaction. It should be stressed that the sensitivities of the present-day physicochemical techniques of measuring protein self-association are limited to the free energy range of approximately −3 to −10 kcal/mol. Consequently, a self-association reaction will look as if it were nonassociating if it is weaker than −3 kcal/mol but as if it were nondissociating if it is stronger than −10 kcal/mol. This limited sensitivity essentially imposes a window through which all biomacromolecular interactions must be examined. One virtue of glycerol lies in its ability to bring a self-association reaction that is outside this window into an observable range.

Since collagen binds glycerol preferentially, how can one explain the stabilization of its triple-helical structure by glycerol? To answer this question, the preferential interaction of denatured collagen was measured. The results shown in Table I indicate that within experimental error, the denatured collagen neither binds nor excludes glycerol. In terms of eq 4, this means that the chemical potential of the denatured collagen will remain relatively constant with or without glycerol in the solution. A schematic description of the effect of glycerol on the chemical potential of native and denatured collagen is shown in Figure 4b. $\Delta G°_{stab}$, the free energy of stabilization of the native collagen in the glycerol solution as compared to aqueous solvent, can be expressed in terms of the chemical potential of each state:

$$\Delta G°_{stab} = (\mu_N^{gly} - \mu_D^{gly}) - (\mu_N^{aq} - \mu_D^{aq}) \quad (7)$$

where μ is the chemical potential of the protein. The subscripts N and D denote the native and denatured states of the protein, respectively, and the superscripts gly and aq refer to the glycerol solution and aqueous buffer, respectively. Since the presence of glycerol does not have much effect on the chemical potential of the denatured collagen, eq 7 can be rearranged to

$$\Delta G°_{stab} = (\mu_N^{gly} - \mu_N^{aq}) - (\mu_D^{gly} - \mu_D^{aq}) \simeq \mu_N^{gly} - \mu_N^{aq} \quad (8)$$

Therefore, it is evident that the driving force for the stabilization of the triple-helical structure of collagen stems mainly from the favorable interaction of the native collagen with glycerol, rather than the unfavorable interaction of the denatured state with glycerol, as shown in the globular proteins (Na & Timasheff, 1981; Gekko & Timasheff, 1981a,b). In this regard, it is interesting to note that a similar difference in the driving force of the guanidine hydrochloride (Gdn·HCl) induced denaturation of lysozyme and ribonuclease has been proposed by Schellman (1978). It was suggested that the Gdn·HCl-induced denaturation of lysozyme is driven by the favorable binding of the denaturant to the denatured state of the protein, whereas in the case of ribonuclease it is driven by the unfavorable exclusion of Gdn·HCl by the native state of the protein.

Why does collagen bind glycerol instead of repel it as the globular proteins do? This question may be answered by examining the structural differences between these two classes of protein. Collagen has a unique amino acid composition that is approximately one-third glycine and one-fourth proline and hydroxyproline. The secondary structure of the protein is also unique in that three peptide chains, each a left-handed helix, intertwine into a triple-helical structure. Water molecules appear to play a major and direct role in stabilizing the triple-helical structure by serving as bridges of interchain hydrogen bonds and forming chained structures on the surface of the helix (Privalov, 1982). The primary sites of such water bridges are the carboxyl groups of the second amino acid of each Gly-X-Y triplet, the amino groups, and the hydroxy groups of hydroxyproline side chains which occur almost exclusively at the Y position of the triplet (Ramachandran & Ramakrishnan, 1976). It seems possible that glycerol, with three hydroxy groups on each molecule, can become bound to the collagen molecule through formation of multiple hydrogen bonds, replacing the tightly bound water molecules. This mechanism becomes particularly attractive in light of the fact that several other polyalcohols such as ethylene glycol and 1,3-propanediol can stabilize the triple-helical structure whereas monoalcohols and 1,2-propanediol, each with an apolar group at one end of the molecule, destabilize the triple helix (Schnell, 1968; Bianchi et al., 1970). If this mechanism is correct, one would expect the collagen denaturation be accompanied by an increasing enthalpy change with increasing concentration of glycerol. This remains to be determined through microcalorimetric studies.

Another possible mechanism that could explain the attraction of glycerol by collagen is the "solvophobic effect" (Sinanoglu & Abdulnur, 1965). It is known that glycerol reduces the surface tension of aqueous solvent. In other words, the surface contact of air with glycerol solvent is more favorable than with water. If one creates a hypothetical miniature cavity in a glycerol solvent and fills it with air, one would expect glycerol molecules to distribute themselves preferentially at the air–solvent interface. In measurements of preferential interaction, this preferential distribution will appear essentially as if glycerol is "attracted" to the cavity. What if this small hypothetical cavity is filled with a protein molecule? As Gekko and Timasheff (1981a) indicated, in the case of globular proteins, glycerol is preferentially excluded from their surface, and at pH 2.0, the degree of this exclusion is inversely proportional to the polarity of the protein. Since glycerol is a very polar chemical and it mixes readily with polar solvents, the above results suggest that glycerol is actually repelled by the apolar amino acids on the surface of the protein. In other words, for globular proteins to exhibit preferential exclusion of glycerol, the repulsion effect of the apolar amino acids on the surface area must first overcome the preferential distribution of glycerol at the protein–solvent interface due to the decreased surface tension of the solvent. In the case of interstitial collagen, the protein has a calculated polarity of 0.89 (Bigelow, 1967) which, by the scale established for globular proteins, is not high enough to make it attract instead of repel glycerol (Gekko & Timasheff, 1981a). However, because the structures of these two classes of protein are totally different, it is clearly inappropriate to apply to collagen the scale established for globular proteins at a single pH. one-third of the amino acids of collagen are glycine. Although the glycine side chain is considered apolar, collectively they occupy a small volume, and, according to the well-accepted supercoiled structure of the protein, they are well concealed from the solvent (Ramachandran & Ramakrishnan, 1976). Consequently, the surface of the collagen molecule could be a lot more polar than indicated by the polarity of the protein calculated from its amino acid composition.

One can estimate the percentage of the total preferential binding of glycerol by collagen that is contributed by the decreased surface tension of the solvent. According to the Gibbs adsorption isotherm (Gibbs, 1878):

$$\left(\frac{\partial m_3}{\partial m_2}\right)_T^{\text{surface tension}} = \frac{-sa_3}{RT}\left(\frac{\partial \sigma}{\partial a_3}\right)_T \qquad (9)$$

where the term on the left-hand side of the equation is the preferential attraction of glycerol to the protein–solvent interface due to the reduced surface tension of the solvent. s is the surface area of the molecule which can be estimated from the molecular dimensions. Collagen monomer can be taken as a cylindrical rod with a length of 300 nm and a diameter of 1.5 nm. σ is the surface tension of the solvent. a_3 is the activity of component 3. The results of such a calculation are shown in column 6 of Table I. It is evident that the preferential distribution of glycerol at the protein–solvent interface due to the decreased surface tension of the solvent actually surpasses the total preferential binding displayed by the protein. Therefore, the surface of collagen is probably more apolar than an air surface and actually repels glycerol. However, it appears that this repelling effect is not strong enough to overcome the surface tension effect and the net result is preferential binding of glycerol to the protein–solvent interface. It should be cautioned, however, that the macroscopic surface tension of glycerol solvent that was used in the above calculation was for a flat surface. For a microscopic surface with a strong curvature such as the cavity created by inserting a collagen molecule into the solvent, its surface tension could be lower than the macroscopic value by as much as a factor of 3 (Choi et al., 1970; Tanford, 1979). Consequently, the calculated surface tension effect should be viewed only as a semiquantitative rather than as a quantitative estimation.

It has been reported that the presence of glycerol in the solution can reduce the shrinkage temperature of fibrous collagen (Russell, 1973). The shrinkage temperature of fibrous collagen is related to the melting temperature of solubilized collagen in that it reflects the unfolding of the triple-helical structure of the protein within the fibers. Shrinkage temperature is higher than melting temperature due to the contribution of the intermolecular interaction within the fibers to the stability of the helical structure. Although glycerol stabilizes the triple-helical structure, it weakens intercollagen interactions. The reduced shrinkage temperature of collagen fibers in glycerol solution seems to reflect that the latter effect is stronger than the former one.

Finally, it should be mentioned that the preferential interaction parameters shown in Table I were measured in the pH 4.0 AS buffer used in examining thermal denaturation and not in the pH 7.0 PS buffer used in studying fibril assembly. This is because collagen polymerizes into fibrils in PS buffer which interfere with the solution density measurement. Since glycerol is uncharged and, according to the results of Figure 3, changing the solution pH from 4.0 to 7.0 does not affect the triple-helical structure of collagen, it seems reasonable to expect that glycerol interacts with collagen in PS buffer in the same manner as in AS buffer. The partial specific volumes of native collagen shown in Table I have standard deviations of approximately 0.004 mL/g. This is higher than the usually observed values of 0.001–0.002 mL/g for globular proteins. The lower precision of the data can be attributed to the high

viscosity of the sample which traps air bubbles in the solution and interferes with the density measurement. More precise densities and partial specific volumes were obtained from solutions of denatured collagen which have lower viscosities. Also, the results shown in Table I indicate that in AS buffer, the partial specific volume of the denatured collagen is higher than that of the native protein. This is consistent with the observations that the denaturation of collagen is accompanied by a solution volume increase (Christensen & Cassel, 1967) and that the melting temperature of the protein increases with increased pressure (Gekko & Koga, 1983).

In summary, in aqueous glycerol solutions, native calf skin collagen binds glycerol preferentially whereas denatured collagen neither binds nor repels glycerol. Such interactions are different from those displayed by the globular proteins and can explain the increase in thermal stability and inhibition of self-assembly of calf skin collagen in the glycerol solution.

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