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Stability of Collagen During Denaturation

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The stability of calf skin collagen (CSC) type I during thermal and chemical denaturation in the presence of glycerol was investigated. Thermal denaturation of type I collagen was performed in the presence of glycerol or in combination with urea and sodium chloride. The denaturation curves obtained in the presence of urea or sodium chloride retained their original shape without glycerol. These curves were shifted upward proportionally to the glycerol concentration in the reaction medium. This means that glycerol and the denaturants act independently. The explanation is based on the difference in the mechanism of their action on the collagen molecule.

KEY WORDS: Collagen; glycerol; urea; sodium chloride; stabilization; denaturation.

1. INTRODUCTION

Certain tissues and cells are often subjected to stresses such as a sharp change in the concentration of salts and metabolic substances. Some of these factors are of great importance for protein structure. In order to minimize such denaturing action, the cells produce specific substances which stabilize protein structure, known as osmolites (Arakawa and Timasheff, 1985; Santoro *et al.,* 1992). Osmolites have different chemical structures: polyols, neutral amino acids, and methylamines. The osmolites limit not only the action of salts, but also effects of urea and heat (Yancey and Burg, 1990). The mechanism of their stabilizing action is unclear. There have been a limited number of investigations concerning their influence on collagen proteins. The stabilizing effect of glycerol upon thermal denaturation of type I collagen was investigated by Na (1986). A similar effect was observed with certain sugars and polyols (Gekko and Koga, 1983). The present paper concerns the stabilizing effect of glycerol on type I calf skin collagen in the presence of urea and sodium chloride, substances which significantly enhance the thermal denaturation of collagen. It was observed that in the presence of glycerol, the collagen molecule was stabilized not only toward heating, but also toward the action of chemical agents. The T_d values increase proportionally to the concentration of glycerol in the reaction medium. Its influence on the thermodynamic characteristics of type I collagen and their dependence on the concentration of urea and sodium chloride was insignificant.

2. MATERIALS AND METHODS

2.1. Sample Preparation

The isolation of type I calf skin collagen (CSC) was carried out by the method of Fuji and Kuhn (1975). Skin samples were washed thoroughly with detergents and subjected to an additional defatting procedure (ethanol or acetone extraction). After air-drying, the starting material was cut into small pieces and the protein was obtained through an acetic acid extraction. Additional purification was achieved by a repeated salting out with 0.7 M NaCl.

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2.2. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE proved the purity of the preparation obtained, according to the method of Furthmayr and Timpl (1971). Extracts were combined, lyophilized, and dissolved in sample buffer, which contained 2 M urea and 0.1% SDS in 0.01 M phosphate buffer, pH 7.2. Then the extracts were boiled for 5 min before being put on the gel. The electrophoresis was run in 6-mm tubes for 4 hr at constant current of 7 mA per tube. Gels were stained for 30 min with 0.25% Coomassie brilliant blue R in methanol–water– acetic acid (5:5:1, v/v) and destained in the same solvent.

2.3. Denaturation

Thermal denaturation experiments were carried out according to the method of Danielsen (1982) on a SPECORD UV VIS M 40 spectrophotometer (Carl Zeiss, Jena, Germany) equipped with a thermogradient device (TSE-1). The heating rate was 0.5°C/min within the interval 22–50°C. A collagen concentration of 0.5 mg/ml in 0.05 M acetic acid was used. The denaturation temperature T_d (T_m) and $\Delta T_{1/2}$ (peak width at the half peak height) were determined from the differential curves. The enthalpy (enthalpy according to van't Hoff) change was calculated from the equation $\Delta H_{vH} = 4RT_d^2/\Delta T$.

3. RESULTS AND DISCUSSION

3.1. Electrophoretic Characterization

The electrophoretic profiles are shown in Fig. 1. Lane 1 shows raw collagen extracted from calf skin. Lane 2 shows the purified preparation. The faint bands with higher electrophoretic mobility than the α chains (noncollagenous proteins or low-molecular-weight fragments) disappeared entirely.

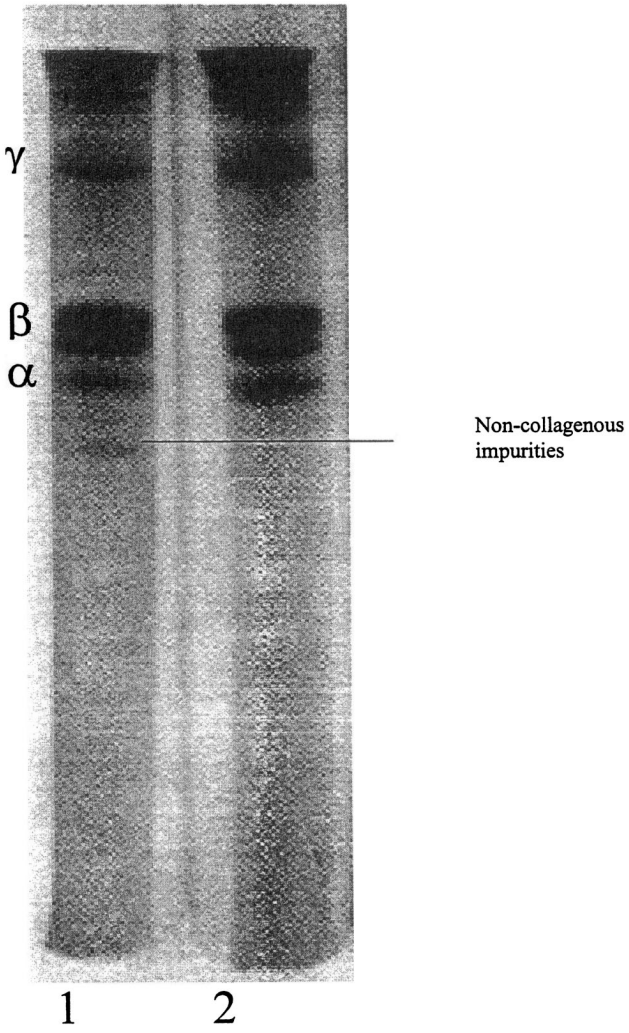

Fig. 1. SDS–PAGE of calf skin collagen. Lane 1, raw collagen extract; lane 2, purified collagen preparation.

3.2. Stabilization Effect of Glycerol on Collagen Proteins

The stabilizing effect of glycerol on type I calf skin collagen (CSC) in acidic medium was investigated by varying the agent's concentration within the range 1–3 M. The denaturation temperature of CSC increased proportionally to the glycerol concentration in the solution (Fig. 2). The $\Delta T_{1/2}$ values remained almost constant (data not shown).

The influence of urea on the stability of type I collagen was described in a previous study (Komsa-Penkova *et al.*, 1995). The denaturation temperatures decreased monotonically with the increasing urea concentration (about 3°C per 1 M urea). Analogous data were obtained with type I collagen species isolated from human placenta, rat tail tendon, and sheep skin (unpublished results).

The combined effect of glycerol and urea was investigated within the concentration range 1–3 M for both agents. The results represented in Fig. 3 and Table I show that glycerol diminishes the denaturing effect of urea. The dependence of T_d upon the urea concentration in the presence of 0–3 M glycerol is given by a set of parallel lines shifted upward by about 1°C per 1 M glycerol.

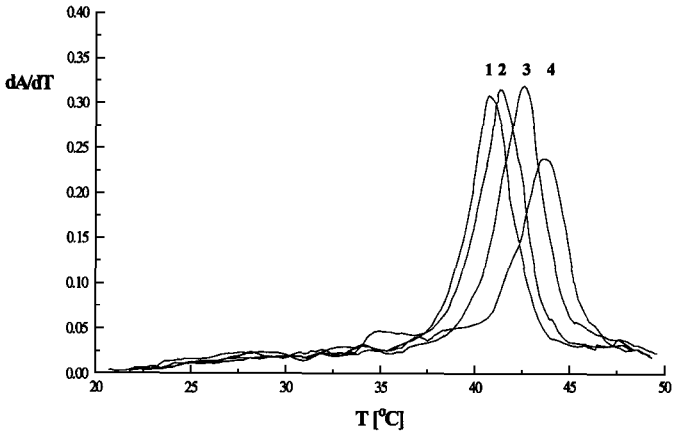

Fig. 2. Thermal denaturation of calf skin collagen type I in 0.05 acetic acid in presence of 0, 1, 2, and 3 M glycerol (curves 1–4).

The results reflecting the effect of sodium chloride on collagen type I in acidic medium alone and in combination with glycerol are summarized in Table II. The concentration ranges studied were 0.05–0.15 M for NaCl and 0–3 M for glycerol, respectively (Fig. 4). The dependence observed is nonlinear, which is better expressed at lower NaCl concentrations. The T_d value changes insignificantly within the range 0.10–0.15 M NaCl.

The addition of glycerol to the reaction medium diminishes the denaturing effect of NaCl. The dependence T_d ([NaCl]) at constant glycerol concentration retains the initial curve shape (in absence of glycerol), but is shifted upward by about 1°C per 1 M glycerol.

It is well known that glycerol stabilizes globular proteins and stimulates their assemblage (Arakawa and Timasheff, 1985). Although its action is not strictly equal for all proteins studied, Gekko and Timasheff (1981) suggested a hypothesis for a preferential hydration (water binding) in the presence of glycerol and a considerable repulsive effect toward glycerol. The degree of its exclusion from the protein surface depends reciprocally on the polarity of the molecule (calculated as percentage content of polar amino acid residues). It is considered that the interaction of glycerol with proteins is energetically less favorable then their interaction with water. The thermal denaturation causes unfolding of the protein molecule and a corresponding exposure of its hydrophobic core. Energetically, this situation is definitively unfavorable, and further, the denatured form of the protein is less stable in the presence of glycerol than the native one. The same relation is valid for monomer–polymer equilibrium, e.g., aggregation phenomena observed in proteins. In the presence of glycerol the aggregate of globular proteins is energetically preferred in comparison with the monomer forms.

This approach cannot be applied to collagen. Glycerol inhibits the fibril formation of collagen type I (Hayashi and

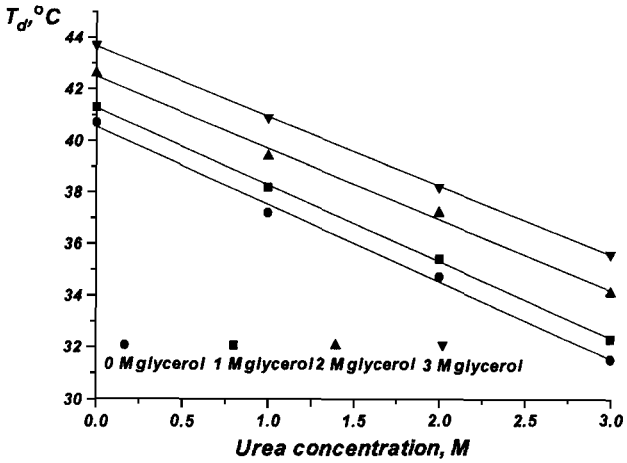

Fig. 3. Stabilizing effect of glycerol in the presence of urea on calf skin collagen type I.

Table I. Denaturation Temperature of Calf Skin Collagen Type I Obtained in Presence of Different Concentrations of Urea and Glycerol

	Denaturation temperature (°C)			
Glycerol (M)	0 M Urea	1 M Urea	2 M Urea	3 M Urea
0	40.7	37.2	34.7	31.5
1	41.3	38.2	35.4	32.3
2	42.6	39.4	37.2	34.1
3	43.7	40.9	38.2	35.6

Table II. Denaturation Temperature of Calf Skin Collagen Type I Obtained in Presence of Different Concentrations of Sodium Chloride and Glycerol

	Denaturation temperature (°C)			
Glycerol (M)	0 M NaCl	0.05 M NaCl	0.10 M NaCl	0.15 M NaCl
0	40.7	36.8	36.0	35.4
1	41.3	37.7	37.2	36.8
2	42.6	38.5	38.1	37.6
3	43.7	39.8	38.4	38.2

Nagai, 1972), acting oppositely in comparison with the globular proteins. Our investigations showed that glycerol stabilizes the collagen molecule and leads to a moderate increase of the denaturation temperature (about 1°C per 1 M glycerol). On the other hand, the $\Delta T_{1/2}$ is almost constant, i.e., does not depend on the agent's concentration. This could mean that glycerol does not affect the transition mechanism. When a combination of agents was applied, either urea–glycerol or NaCl–glycerol, the shape of the dependence remained the same as in the absence of glycerol, but a constant shift upward (see above) was observed in every set of curves. This fact could mean that the denaturing agents and glycerol act independently, i.e., by different mechanisms.

Na (1986) suggested that the stabilizing effect of glycerol is achieved by its binding to the surface of the collagen molecule through incorporation of its hydroxyl groups into the water-chain structure, proposed by Lim (1981). A possible target of its binding might be hydroxyproline or polar amino acid residues. In this way glycerol stabilizes the protein solvation shell and competes with water molecules probably due to its greater ability

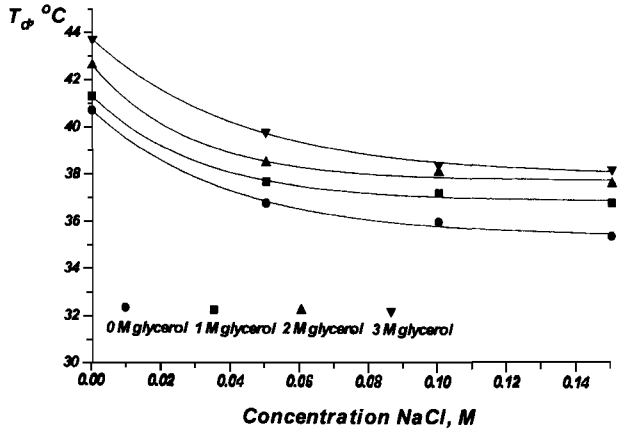

Fig. 4. Stabilizing effect of glycerol on calf skin collagen type I in the presence of NaCl.

for the formation of hydrogen bonds—its molecule contains three hydroxyl groups. As Bachinger and Morris (1990) showed, the presence of hydroxyl groups in the solvent structure is important for stabilization of collagen due to formation of additional, stabilizing hydrogen bonds. On the other hand, the large volume of the glycerol molecule makes impossible its incorporation in the water-chain structure (Lim, 1981) as well as the replacement of water-mediated hydrogen bonds Hyp-OH–Gly-CO (Na, 1986). The glycerol-based salvation shell also hampers collagen fibril formation.

Bachinger and Morris (1990) also showed that glycerol, 1,2-propanediol, and 1,3-propanediol differ in their action on collagen. 1,2-Propanediol does not stabilize collagen, while the other two agents have almost equal positive contribution to the protein molecule stability. One possible explanation might be that the distances between the terminal OH groups in glycerol and in 1,3-propanediol are comparable with that between the OH groups of Hyp residues in position Yyy of two adjacent tripeptides (Gly–Xxx–Yyy). As Fietzek and Kuhn (1976) showed, about 50% of the Hyp residues occupy the Yyy position in the triplets. So we may assume that glycerol could form hydrogen bonds with Hyp–OH groups situated in two neighboring triplets. This leads to stabilization of every individual polypeptide αchain without increasing the number of interchain stabilizing hydrogen bonds.

The enthalpy values did not depend on increasing glycerol concentration, which confirms the suggestion that no additional interchain glycerol-derived hydrogen bonds are formed, as proposed for the water molecules. This fact confirms our suggestion that glycerol binds to the surface of the collagen molecule and competes to a certain extent with the water molecules.

The constant value of $\Delta T_d/\Delta$ glycerol molarity (M), which is not influenced by other agents that destroy different interactions in the collagen molecule, means that it does not affect bonds which stabilize the triple helical collagen structure and serve as a target for the action of these agents. In other words, the action of glycerol and

urea, respectively sodium chloride, when they were applied in combination is independent.

The concentration of free glycerol (there is also glycerol in ester form) in serum is about 120 μmol/L, the reference limits are 40–200 μmol/L. In women the values are somewhat higher than in men. For low-molecular-weight components the serum concentrations and the concentrations in the extracellular milieu are similar (Dyerberg and Hjorne, 1966). So we may conclude further that glycerol stabilizes the collagen molecule toward heating as well as to the action of different chemical agents independently of their nature. The stabilizing effect of glycerol is of great importance in chemical, biochemical, and microbiological practice, where it is widely applied as a cryoprotector of proteins.

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