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Stabilizing effect of glycerol on collagen type I isolated from different species

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Abstract

The stabilizing effect of glycerol on type I collagens (C) from rat tail tendon (RTC), calf skin (CSC), human placenta (HPC), and sheep skin (SSC) at elevated temperature and in urea was investigated. The protein denaturation was followed by means of differential UV-spectroscopy. The denaturation temperatures (T_d) increased proportionally to the concentration of glycerol in the reaction medium. Equations for the dependence of T_d glycerol concentration were derived. The calculated thermodynamic characteristics do not change significantly with increasing glycerol concentration. It was observed that, in the presence of glycerol, the collagen molecule was stabilized, not only by heating, but also by the action of urea. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Collagen; Different species; Glycerol; Stabilization; Urea; Denaturation

1. Introduction

A sharp change of the concentration of salts and metabolic substances influences human tissues. These factors are important for the protein structure. In order to minimize any denaturing action, the cells produce specific substances, which stabilize the protein structure. There are many investigations concerning their influence on the collagen proteins. The stabilizing effect of glycerol on thermal denaturation of type I collagen was investigated by Na (1986). Sheep skin collagen (SSC) is a traditionally prepared collagen. Calf skin collagen (CSC) and rat tail tendon (RTC) are commonly used for different fundamental purposes (Fietzek & Kuhn, 1975; Gay, Walter & Khun, 1976). Human placenta (Mardi, Foellmer & Furthmayr, 1982) has been used for isolation of different collagens (types V, VI, XI). The mechanism of stabilizing action is unknown for the collagens from different species. Therefore, in this paper, the stabilizing effect of glycerol on type I collagens (C) from rat tail tendon (RTC), calf skin (CSC), human placenta (HPC), and sheep skin (SSC), during thermal denaturation in urea is presented.

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2. Materials and methods

2.1. Materials

All reagents were of analytical grade. Deionized and distilled water was used throughout. All chemicals were purchased from Sigma chemical Co.

2.2. Sample preparation

The isolation of type I calf skin collagen (CSC) was carried out by the method of Fujii and Kuhn (1975), using modifications of Gay et al. (1976) and Fietzek and Kuhn, (1975). After a preliminary depilation and delipidation, the sample was cut into small pieces (1×1 cm) and treated with pepsin (1 mg/g wet weight) in 0.5 M acetic acid for 48 h. After centrifugation (2000 rpm/30 min) it was salted out with 0.7 M NaCl and dialyzed against 0.02 M Na_2HPO_4, pH 7.2. Finally, the preparation was purified by chromatography on DEAE-Sepharose in 0.05 M Tris–HCl–0.2 M NaCl–2 M urea, pH 8.6, dialyzed against 0.05 M acetic acid and freeze-dried. Rat tail tendon collagen (RTC) and human placenta collagen (HPC) were isolated by standard procedures, including acetic acid extraction in the presence of pepsin and salting out with sodium chloride.

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The human placenta serves as a source for isolation of different collagens. The method for their isolation and separation has been developed by Miller and Rhodes (1982). The horiotic placental membrane was washed free of blood residues, cut into small pieces and extracted with 0.4 M sodium acetate, pH 4.8 and phosphate-buffered saline, pH 7.4 in 2 h alternative runs at 20°C under constant stirring. After centrifugation and exhaustive washing, the material was subjected to pepsinization. The enzyme:substrate ratio applied depends on the collagen type isolated. For collagen type I, it is 1:100/48–72 h at room temperature. The extraction was repeated several times, the extracts were combined and centrifuged at 9000 rpm/30 min. The clear supernatant was fractionated according to Madri et al. (1982). The solubility of RTC in diluted acetic acid was significantly higher than that of HPC and CSC. Additional purification may be achieved by repeated precipitation with NaCl, followed by a dialysis against 0.02 M Na_2HPO_4, pH 7.2. Sheep skin collagen (SSC) was isolated and purified from untanned sheep skin scraps according to the procedure of Nedkov, Glanville, Goshev, and Kuhn (1983). HPC was available from the Medical University of Pleven as also were the rat tail tendons. Calf skin collagen was easily available from slaughter houses.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was done according to the method of Furthmayr and Timpl (1971).

The thermal denaturation of all collagen samples at different glycerol concentrations was investigated by means of UV-spectroscopy on a SPECORD UV VIS M 40 spectrophotometer (Carl Zeiss, Jena, Germany), equipped with a thermo-gradient device (TSE-1). The change of the absorption at 230 nm vs temperature was followed within the range 22–50°C according to the method of Danielsen (1982).

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 Vant Hoff) change was calculated from the equation: $\Delta H_{vH} = 4RT_d^2/\Delta T$

3. Results and discussion

The electrophoretic profiles proved the purity of the collagen preparations. In all species two lanes, were found showing the raw collagen and the purified preparation. Initially we have investigated the stabilizing effect of glycerol on type I collagens, such as calf skin (CSC). The stability was estimated in acidic medium with varying concentrations from 1 to 4 M of glycerol. The results obtained are shown in Figs. 1, 2, 3 and 4 for type I collagens (C) from calf skin (CSC), rat tail tendon (RTC), sheep skin (SSC) and human placenta (HPC) at

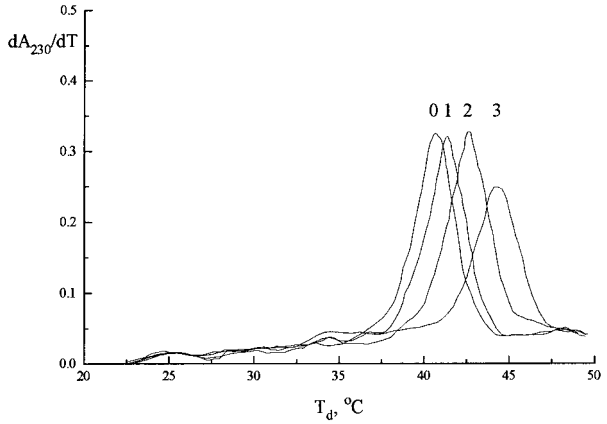

Fig. 1. Thermal denaturation of calf skin collagen type I in 0.05 M acetic acid in the presence of glycerol. Curves 0, 1, 2 and 3: 0, 1, 2 and 3 M glycerol, respectively.

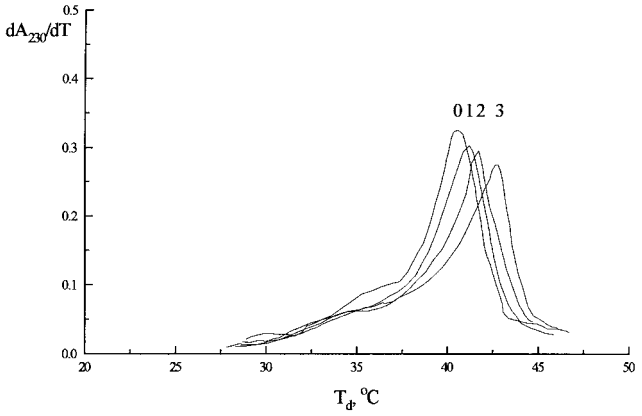

Fig. 2. Thermal denaturation of rat tail tendon collagen type I in 0.05 M acetic acid in the presence of glycerol. Curves 0, 1, 2 and 3: 0, 1, 2 and 3 M glycerol, respectively.

elevated temperature, respectively. The obtained curves were similar for all investigated samples. The denaturation temperatures of CSC, RTC, SSC and HPC increased proportionally to the glycerol concentration in the solution (Fig. 5, Table 1). The $\Delta T_{1/2}$-values remained almost constant. The T_d-values increased proportionally to the concentration of glycerol in the reaction medium. The following equations can be derived for the dependence of T_d upon the glycerol concentration in the reaction medium:

SSC $T_d = 40.68 + 0.84^*$ [Glycerol]
HPC $T_d = 40.74 + 0.95^*$ [Glycerol]
CSC $T_d = 40.45 + 1.15^*$ [Glycerol]
RTC $T_d = 40.46 + 0.71^*$ [Glycerol]

The equations give the dependence on glycerol concentration. It is speculative to draw immediate conclusions, but CSC is evidently the best stabilized. The slope of the dependence is the highest. The dependences

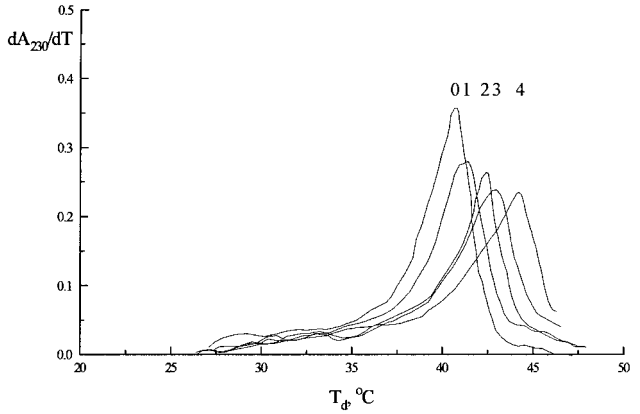

Fig. 3. Thermal denaturation of sheep skin collagen type I in 0.05 M acetic acid in the presence of glycerol. Curves 0, 1, 2, 3 and 4: 0, 1, 2, 3 and 4 M glycerol, respectively.

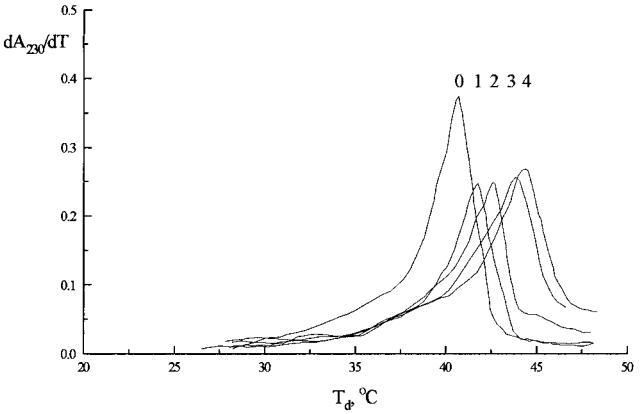

Fig. 4. Thermal denaturation of human placenta collagen type I in 0.05 M acetic acid in presence of glycerol. Curves 0, 1, 2, 3 and 4: 0, 1, 2, 3 and 4 M glycerol, respectively.

obtained are linear. The trend is almost equal in all samples; the T_d value increases with the increasing glycerol concentration (Privalov, 1982). The ΔT value within every set remains constant. This could mean that glycerol does not practically affect the cooperativity of the transition. The influence of urea on the stability of type I CSC was described in previous studies (Komsa-Penkova, Goshev & Alexandrova, 1995; Penkova, Goshhev, Gorinstein & Nedkov, 1999). The denaturation temperatures decreased monotonously with the increasing of urea concentration (about 3°C per 1 M urea).

The combined effect of glycerol and urea was investigated within the concentration range 1–3 M for both agents. The results represented in Fig. 6 show that glycerol diminishes the denaturing effect of urea. The dependence of T_d upon urea concentration in the presence of 0–3 M glycerol is a set of parallel lines shifted upwards by about 1°C per 1 M glycerol. Analogous data were obtained with type I collagen species isolated

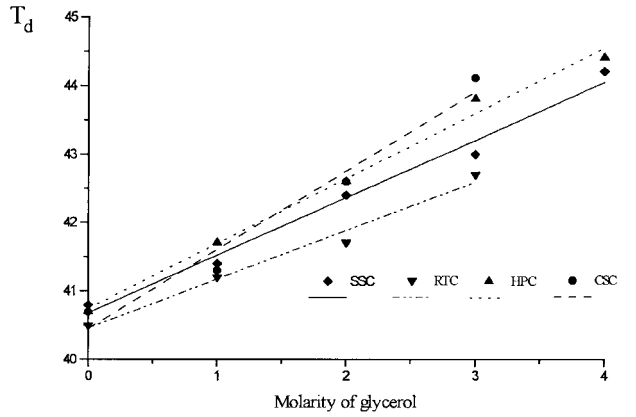

Fig. 5. Dependence of the temperature of denaturation on the molarity of glycerol in different collagen species.

Table 1
Denaturation temperatures [°C] of sheep skin collagen (SSC), rat tail tendon collagen (RTC), human placenta collagen (HPC) and calf skin collagen (CSC) at different concentrations of glycerol

Collagen	Glycerol [M]	T_d [°C]	T_d [K]	ΔT [°C]	ΔH_{vH} [kcal/M]
SSC	0	40.8	313.95	2.0	391.46
	1	41.4	314.55	2.1	374.24
	2	42.4	315.55	2.0	395.45
	3	43.0	316.15	2.0	396.96
	4	44.2	317.35	2.1	380.94
RTC	0	40.5	313.65	1.7	459.66
	1	41.2	314.35	1.8	436.06
	2	41.7	314.85	1.8	437.45
	3	42.7	315.85	1.9	417.06
HPC	0	40.7	313.85	2.2	355.64
	1	41.7	314.85	2.6	302.85
	2	42.6	315.75	2.5	316.76
	3	43.8	316.95	2.4	332.47
	4	44.4	317.55	2.4	333.74
CSC	0	40.7	313.85	2.4	326.00
	1	41.3	314.45	2.6	302.07
	2	42.6	315.79	2.6	304.65
	3	44.1	317.99	2.6	308.91

from human placenta, rat tail tendon and sheep skin (unpublished results).

It was observed that, in the presence of glycerol, the collagen molecule was stabilized, not only by heating, but also by the action of urea. The influence of glycerol concentration on the thermodynamic characteristics of types I collagen and their dependence on the concentration of urea were insignificant.

Although the glycerol action is not strictly equal for all proteins studied, Gekko and Timasheff (1981) have suggested a hypothesis for a preferential hydration (water binding) in the presence of glycerol and a considerable repulsive effect towards glycerol.

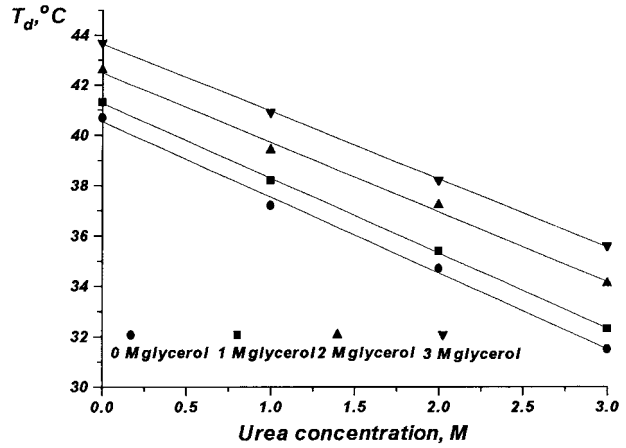

Fig. 6. Stabilizing effect of glycerol in the presence of urea on calf skin collagen type I.

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 favourable than 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 unfavourable, and the denatured form of the protein is less stable in the presence of glycerol than the native one. The same relationship is valid for the monomer–polymer equilibrium, e.g. the aggregation phenomena observed in proteins. In the presence of glycerol, the aggregates of globular proteins are energetically preferred in comparison with the monomer forms.

Glycerol inhibits the fibril formation of collagen type I, 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. it does not depend on the glycerol concentration. This could mean that glycerol does not affect the transition mechanism. When a combination of agents was applied, the shape of the dependence remained the same as in the absence of glycerol, but a constant shifting upwards was observed in every set of curves. This could mean that the denaturing agent and glycerol act independently, i.e. by different mechanisms.

Na (1986) suggests 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. Possible targets of its binding might be hydroxyproline or polar amino acid residues (Fietzek & Kuhn, 1975; Fietzek, Breitkreutz & Kuhn, 1974). In this way glycerol stabilizes the protein solvation shell and competes with water molecules, probably due to its greater ability for formation of hydrogen bonds; each molecule contains 3 hydroxyl groups. 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, as a replacement for water-mediated hydrogen bonds e.g. Hyp-OH–Gly-CO (Na, 1986). 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 neighbouring triplets Fietzek and Kuhn (1975). This leads to stabilization of every individual polypeptide α-chain 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 is 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 actions of these agents. In other words, the action of glycerol and urea, when they were applied in combination, were independent.

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