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Thermal stability of calf skin collagen type I in salt solutions

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Abstract

The thermal stability of acid-soluble collagen type I from calf skin in salt solutions is studied by high-sensitivity differential scanning calorimetry. Three concentration ranges have been clearly distinguished in the dependence of collagen thermal stability on ion concentration. At concentrations below 20 mM, all studied salts reduce the temperature of collagen denaturation with a factor of about 0.2°C per 1 mM. This effect is attributed to screening of electrostatic interactions leading to collagen stabilisation. At higher concentrations, roughly in the range 20–500 mM, the different salts either slightly stabilise or further destabilise the collagen molecule in salt-specific way that correlates with their position in the lyotropic series. The effect of anions is dominating and follows the order $H_2PO_4^- \geq SO_4^{2-} > Cl^- > SCN^-$, with sign inversion at about SO_4^{2-}. This effect, generally known as the Hofmeister effect, is associated with indirect protein–salt interactions exerted via competition for water molecules between ions and the protein surface. At still higher salt concentrations (onset concentrations between 200 and 800 mM for the different salts), the temperature of collagen denaturation and solution opacity markedly increase for all studied salts due to protein salting out and aggregation. The ability of salts to salt out collagen also correlates with their position in the lyotropic series and increases for chaotropic ions. The SO_4^{2-} anions interact specifically with collagen — they induce splitting of the protein denaturation peak into two components in the range 100–150 mM Na_2SO_4 and 300–750 mM Li_2SO_4. The variations of the collagen denaturation enthalpy at low and intermediate salt concentrations are consistent with a weak linear increase of the enthalpy with denaturation temperature. Its derivative, $d(\Delta H)/dT$, is approximately equal to the independently measured difference in the heat capacities of the denatured and native states, $\Delta C_p = C_p^D - C_p^N \approx 0.1$ cal · g^{-1}K^{-1}.

Keywords: Collagen type I; Protein–salt interactions; Thermal stability; DSC; Protein electrostatics; Hofmeister effect; (Calf skin)

1. Introduction

Collagen is the major structural component of connective tissues. In vertebrates, it represents about one-third of their total protein content [1]. At least 19 fibrillar and non-fibrillar, genetically different, types of collagen have been distinguished [2]. The fibrillar collagen type I is the major component of tendon, bone, skin, and other tissues. Its primary structure consists of repeating triplets $(Gly\text{-}X\text{-}Y)_n$, where X is often proline (Pro) and Y is often 4-hydroxyproline (Hyp). The collagen molecules consist of three polypeptide chains, each coiled in a left-handed helix. The three chains are thrown into a right-handed triple superhelix stabilised by periodic hydrogen bonds [3,4]. The triple helices, known also as tropocollagen, associate laterally and longitudinally to form microfibrils. These, in turn, form fibrils, aggregates of which constitute various forms of connective tissue. Decrease of pH below 4 results in dissolving of the fibrils and formation of a molecular solution of tropocollagen [5].

Upon heating, collagen undergoes a denaturational transition from the triple helix to a randomly coiled form in which the three chains are separated. A correlation between chemical composition and thermal stability of collagens has been established [6]. Several studies emphasise the stabilising effect of hydroxyproline [7–11]. Altogether, the imino-acid content is considered a dominant determinant of the collagen stability. Since about 40% of the Gly-X-Y triplets of collagen contain at least one charged residue in X or Y position, the electrostatic interactions are also considered important for its molecular organisation [12–14]. The structure of the triple helix allows for formation of both intra- and interchain ion pairs. Examination of collagen primary structure [12,15] shows a considerable

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amount of pairs of closely situated amino-acid residues bearing opposite electric charges (Glu^--Arg^+, Asp^--Arg^+, Asp^--Lys^-, Glu^--Lys^+, etc.). The role of various environmental conditions on the collagen stability is also recognised. However, investigations on the influence of solutes, particularly salts, are scarce. Von Hippel and Wong [16] report the effect of moderate concentrations of different neutral salts on the stability of partially renatured collagens (gelatins) of different origin. They establish a linear dependence between denaturation temperature and salt concentration, with different salts either decreasing or increasing the gelatine stability, according to their position in the lyotropic series. Russel [17] reports for a decreased thermal stability of calf skin collagen in alcohol-buffer media upon addition of up to 1 M KCl. This effect is considered a likely result of modulation of the electrostatic interactions. Burjanadze and Bejitadze [18] observe denaturation at lower temperature upon addition of 0.1 M NaCl to the collagen solution, while sugars and polyols are found to increase the thermal stability of acid-soluble calf skin collagen [19]. The mechanism of stabilisation in the latter case is supposed similar to that for globular proteins — preferential hydration of the protein, originating in the water-structure-making character of these solutes. Various organic solvents such as propanol, 1,2-propanediol, tetrahydrofuran, and acetonitrile are shown to decrease to different extent the collagen stability [20]. A major role of the solvent molecules in stabilising the collagen triple helix has been inferred from these studies.

Our recent UV-spectroscopic measurements showed that low concentrations of NaCl decrease the collagen thermal stability by about 0.2°C/mM [21]. This effect is by two orders of magnitude stronger than that reported by Von Hippel and Wong [16] for moderate NaCl concentrations −1.6°C/M. Such large difference implies that different mechanisms might be responsible for the salt effect on the collagen thermal stability, depending on the range of salt concentration. To clarify this point, in the present work we used differential scanning calorimetry for a systematic study of the thermal stability of acid-soluble collagen type I from calf skin (CSC) in different salt solutions in a concentration interval up to 1 M. Three concentration ranges have been clearly distinguished in the dependence of collagen thermal stability on the salt concentration.

2. Materials and methods

2.1. Isolation, purification and characterization of collagen type I

Calf skin collagen type I (CSC) was isolated according to the procedure described by Fuji and Kuhn [22]. The procedure included acetic acid extraction in the presence of pepsin and salting out with sodium chloride. Further purification by ion-exchange chromatography on DEAE cellulose columns was carried out according to Miller and Rhodes [23]. In control experiments, extraction of CSC in the absence of pepsin was also carried out. The high purity of the collagen preparations was confirmed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE). The absence of type III collagen in the preparations was verified using interrupted electrophoresis according to Sykes et al. [24]. The collagen preparation was freeze-dried from an aqueous solution in vacuum, at −80°C, and the lyophilised material was stored at −18°C. Gravimetric moisture evaluation showed that the residual water in the freeze-dried preparations was about 9 wt%. In control experiments, preparations where the lyophilisation–rehydration step has been omitted, were also measured. The collagen concentration in the solutions was determined from the hydroxyproline content according to the method of Stegemann [25] and by using a modification of the method of Lowry [26].

2.2. Sample preparation

Appropriate salt solutions were prepared in 50 mM acetic acid (pH 3.0). Equal volumes of doubly concentrated solutions of collagen and corresponding salt, both in 50 mM acetic acid, were mixed at 4–5°C 1 hour before the measurements and equilibrated at room temperature. Freshly prepared collagen solutions from lyophilised material were routinely used. The pH of the samples was determined. Quartz-bidistilled water was used. All chemicals were of reagent grade.

2.3. Calorimetric measurements

Calorimetric measurements were performed using high-sensitivity differential scanning microcalorimeters DASM-4 and DASM-1M (Biopribor, Pushchino, Russia) with sensitivity better than $4 \cdot 10^{-6}$ cal $\cdot K^{-1}$ and a noise level less than $5 \cdot 10^{-7}$ W [27]. Sample concentration was 0.5 mg/ml, unless otherwise indicated. Runs were routinely made in the temperature range 20–50°C, with a heating rate of 0.5°C/min. Heating rates of 0.125, 0.25 and 1°C/min were also used. Particular runs were started at 0°C and carried through 90°C, however, no thermal events were registered in the ranges 0–20°C and 50–90°C. The reversibility of the thermally induced transitions was checked by second heating of the sample in the calorimetric cell immediately after cooling to 20°C following the first run. The cooling step together with the equilibration of the instrument for the second run takes about 20–30 min. Samples cooled to 20°C and stored at this temperature for up to 7 days after the first run were also scanned. Particular reheating scans were preceded by taking out the sample immediately after termination of the first run and plunging it into an ice bath for 1 hour. The thermograms were corrected for the instrumental baseline. The temperature at the maximum of the excess heat capacity curve was

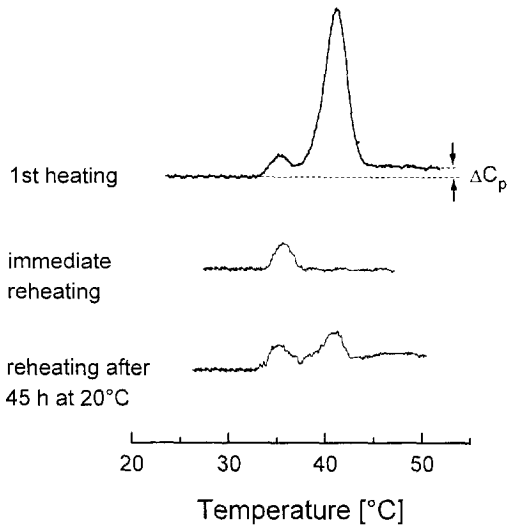

Fig. 1. Heating thermograms of calf skin collagen type I (0.5 mg/ml) in 0.05 M acetic acid recorded during first heating, immediate reheating of the sample, and reheating of the sample after 45 h storage at 20°C. These thermograms were recorded at 0.5°C/min heating rate, but the same picture was obtained also at 0.125, 0.25, and 1°C/min heating rates (data not shown).

taken as the transition temperature and the transition width, $\Delta T_{1/2}$, was determined at the transition half-height. The calorimetric enthalpy ΔH_{cal} of the transition was determined as the area under the excess heat capacity curve. The van't Hoff enthalpy ΔH_{vH} was calculated from the calorimetric data by using the relationship [28]: $\Delta H_{vH} = 4RT^2C_p^{max}/\Delta H_{cal}$, where C_p^{max} is the maximum excess heat capacity. The two peaks on the collagen denaturation thermograms (see below) were separated using peak fit program. The difference in the specific heat capacities of the native and denatured states ΔC_p was determined directly from the thermograms as the difference in the position of the baseline before and after melting (Fig. 1).

2.4. Opacity measurements

The opacity of collagen solutions at different salt concentrations was measured at 430 nm using a Specoll-11 spectrophotometer at 20°C. These measurements served to determine by an independent method the salt concentrations required for initiation of collagen salting out.

3. Results

3.1. Effects of protein concentration, heating rate, and thermal prehistory on the endothermic transitions of calf skin collagen type I

The thermal behavior of calf skin collagen type I in acidic solution is characterized by two endothermic events (Fig. 1). A small pretransition of enthalpy ΔH_{cal} of 1.2 cal/g (345 kcal/mol) takes place at ca. 35°C. It is followed by the main denaturational transition at ca. 40°C with calorimetric enthalpy ΔH_{cal} of 10.9 cal/g (3105 kcal/mol). The temperature of denaturation well agrees with previous data [17,19,29–31] and the total enthalpy −12.1 cal/g (3450 kcal/mol) is also in the range of reported values for acid-soluble CSC type I [6]. The specific heat capacity of the denatured state is higher than that of the native state; the change of the specific heat capacity $\Delta C_p = C_p^D - C_p^N$ upon melting of collagen, as determined from the heating thermograms taken at 0.5°C/min, is about 0.1 cal · g^{-1}K^{-1}, close to a reported earlier value of 0.05 cal · g^{-1}K^{-1} [37]. A pre-denaturational transition at 33–34°C has been recorded in previous studies [30–35], but its nature has not been ultimately identified. Condell et al. [35] ascribe it to shortened or nicked collagen fragments obtained during protein isolation at the step of pepsin digestion. In our thermograms of CSC extracted without pepsin treatment, the pretransition persisted though. In

Table 1
Transition parameters of calf skin collagen type I solutions as a function of collagen concentration and heating rate (subscripts 'p' and 'd' refer to pretransition and denaturational transition, respectively)

Experimental variable	T_p (°C)	T_d (°C)	ΔH_{tot} (cal/g)	ΔH_p (cal/g)	ΔH_d (cal/g)	$\Delta T_d^{1/2}$ (°C)
Collagen concentration (mg/ml) [a]						
0.25	35.25	41.07	12.08	0.94	11.14	2.64
0.50	35.24	40.80	12.11	1.22	10.89	2.80
1.00	34.82	39.96	12.78	1.78	11.00	2.90
1.75	34.58	39.29	13.82	2.50	11.32	2.98
Heating rate (°C/min) [b]						
0.125	34.60	40.06	12.58	1.26	11.33	2.88
0.25	35.00	40.44	12.38	1.67	10.70	2.89
0.5	35.24	40.80	12.11	1.22	10.89	2.80
1.0	35.50	41.31	11.62	1.44	10.19	2.89

[a] Heating rate 0.5°C/min.
[b] Sample concentration 0.5 mg/ml.

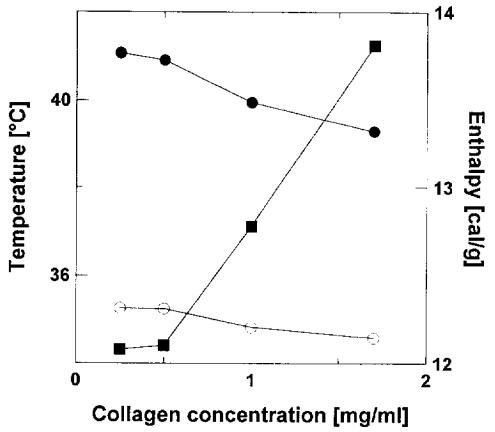

Fig. 2. Effect of the protein concentration on the thermal denaturation of calf skin collagen type I: ○, pretransition temperature; ●, main transition temperature; ■, total enthalpy of denaturation.

addition, no collagen fragments were present in our pepsin-treated samples as verified by electrophoresis tests. The pretransition was considerably suppressed, however, in fresh preparations (both pepsin-treated and untreated) for which the lyophilisation–rehydration step has been omitted (data not shown). Our experiments currently in progress indicate possible involvement of protein oxidation in the formation of this transition.

Decreasing the collagen concentrations from 1.75 to 0.25 mg/ml results in a slight increase of the peak temperatures and a decrease with 10–15% of the total transition enthalpy. At ≈ 0.5 mg/ml these dependencies level out (Fig. 2; Table 1). The denaturation of collagen is known to be slow in comparison with denaturation of other proteins. This is believed to result from the slow *cis–trans* isomerisation of the proline residues abundant in collagen [6,39]. Our measurements at heating rates of 0.125, 0.25, 0.5 and 1°C/min show an increase of the transition temperature accompanied by a decrease of the total transition enthalpy (Fig. 3; Table 1). The main transition is not reversible upon immediate reheating from 20°C, and also after rapid cooling of the denatured sample to 0°C by immersion into ice-water. However, it partially restores after prolonged equilibration at 20°C (Fig. 1). Repeated heating scans demonstrated complete reversibility of the pretransition (Fig. 1). The van't Hoff enthalpies ΔH_{vH} of both transitions are in the range of 200–300 kcal/mol at all studied scan rates and protein concentrations and well agree with the van't Hoff enthalpy obtained from UV-spectroscopy measurement of the hyperchromic effect at 230 nm [36]. The ratio

$\Delta H_{cal}/\Delta H_{vH} \approx 10$ suggests that the denaturation proceeds in about 10 cooperative units along the collagen molecule in the conditions of the present experiment. This estimate should be considered with due caution, however, having in mind that the transition parameters used for calculation of ΔH_{vH} substantially depend on the heating rate, as shown by Fig. 3.

3.2. Influence of salts on the thermal stability of calf skin collagen type I

Salts at different concentrations were added to collagen solutions in 50 mM acetic acid and the thermal behavior of

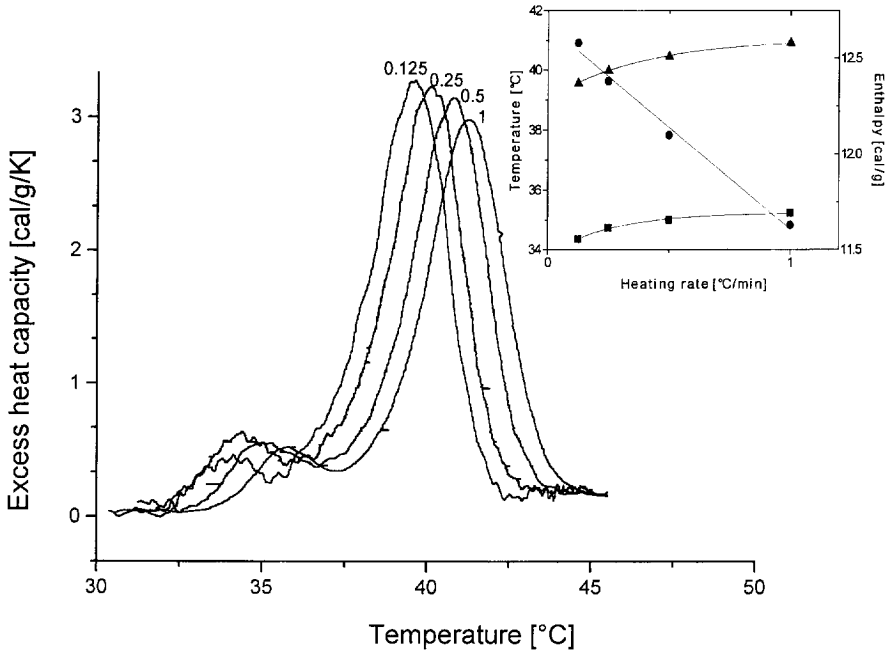

Fig. 3. Heating thermograms of calf skin collagen type I (0.5 mg/ml) in 0.05 M acetic acid recorded at different heating rates (0.125, 0.25, 0.5, and 1°C/min). Inset: effect of heating rate on the thermal denaturation of collagen: ■, pretransition temperature; ▲, main transition temperature; ●, total enthalpy of denaturation.

the samples was registered calorimetrically. Two series of salts were used: sodium salts with Cl^-, SCN^-, $H_2PO_4^-$, HPO_4^{2-}, SO_4^{2-} anions, and chloride salts with Li^+, Na^+, NH_4^+, Ca^{2+} cations. Measurements were also made with Li_2SO_4, KCl and NaN_3 salts. A selection of thermograms of collagen denaturation in the presence of different sodium salts is given in Fig. 4. The thermodynamic characteristics of the collagen transitions are summarised in Table 2. The

Table 2
Dependence of the collagen transition parameters on different salt concentration in the solution (subscripts 'p' and 'd' refer to pretransition and denaturational transition, respectively). Heating rate 0.5°/min

Salt concentration (mM)	T_d (°C)	ΔH_d (cal/g)	ΔH_{tot} (cal/g)	$\Delta T_d^{1/2}$ (°C)	T_p (°C)	ΔH_p (cal/g)
–	40.8	10.89	12.11	2.8	35.2	1.21
LiCl						
5	39.4	10.14	11.97	2.8	34.1	1.83
10	38.6	9.94	11.78	2.7	34.0	1.84
20	38.4	9.39	11.54	2.8	34.0	2.15
50	37.6	9.96	11.52	2.9	33.9	1.56
200	36.1	7.63	11.37	2.5	33.2	3.74
400	35.1	9.60	11.00	2.6	32.3	1.40
600	34.7	6.67	10.91	2.6	32.1	4.24
800	39.7	8.18	8.18	2.8	–	–
NaCl						
5	39.4	10.84	11.75	2.8	34.2	0.91
10	38.7	9.95	11.70	2.7	33.8	1.75
20	38.1	9.77	11.67	3.0	33.2	1.90
50	37.0	9.16	11.30	2.8	33.0	2.13
200	34.7	7.79	11.06	2.7	32.2	3.26
400	33.1	8.83	10.89	2.6	30.8	2.06
450	33.7	7.73	10.44	3.3	31.0	2.71
500	40.3	7.83	9.81	5.9	32.9	1.98
1000	46.0	4.88	6.72	6.3	–	1.85
KCl						
5	39.4	10.11	11.97	2.8	34.1	1.86
50	35.8	8.35	11.13	2.8	32.2	2.78
NH_4Cl						
10	38.5	9.89	11.81	2.8	33.8	1.91
20	36.8	9.05	11.33	2.9	32.1	2.28
50	35.9	7.52	11.12	2.7	32.7	3.60
200	33.8	8.67	11.06	2.7	31.5	2.39
400	32.0	8.14	10.74	2.7	30.1	2.60
600	32.2	7.95	10.53	2.4	30.1	2.58
750	40.3	6.12	8.00	4.2	36.4	1.88
$CaCl_2$						
5	39.2	9.90	11.70	2.8	34.1	1.80
10	38.0	9.69	11.57	2.9	33.5	1.87
20	35.6	9.31	11.24	2.9	31.8	1.92
50	34.9	8.12	10.97	2.6	31.5	2.84
200	32.7	8.13	10.86	2.5	29.8	2.73
300	31.7	7.82	10.68	2.5	28.9	2.86
400	37.3	6.65	8.58	2.7	35.3	1.93
NaSCN						
5	39.4	9.93	11.76	2.8	34.1	1.83
10	38.6	10.02	11.70	2.8	34.0	1.68
20	36.0	8.95	11.34	2.8	32.1	2.39
50	34.2	8.76	11.03	2.6	31.3	2.26
100	31.4	6.63	10.37	2.4	29.3	3.74
150	30.3	8.56	10.29	2.6	28.0	1.73
200	34.8	8.07	8.07	3.1	–	–

Table 2 (continued)

Salt concentration (mM)	T_d (°C)	ΔH_d (cal/g)	ΔH_{tot} (cal/g)	$\Delta T_d^{1/2}$ (°C)	T_p (°C)	ΔH_p (cal/g)
NaH_2PO_4						
10	38.6	10.00	11.79	2.7	33.8	1.79
20	37.8	9.38	11.64	2.8	33.5	2.26
50	37.6	8.60	11.46	2.8	33.4	2.87
200	37.3	8.21	11.42	2.3	34.4	3.21
500	37.8	5.14	8.97	2.1	36.1	3.83
750	45.1	4.88	6.35	2.8	42.9	1.47
Na_2HPO_4						
10	39.6	10.01	11.97	2.6	34.2	1.96
20	40.2	8.03	11.24	2.3	37.0	3.20
50	40.3	8.73	11.66	2.8	36.7	2.93
100	40.7	8.20	11.88	2.6	37.8	3.68
300	42.0	7.49	9.98	2.6	38.3	2.48
500	49.7	9.54	9.99	4.4	38.3	0.45
Na_2SO_4						
5	39.1	9.89	11.73	2.8	34.0	1.84
10	38.0	9.69	11.58	2.6	34.3	1.89
20	36.1	7.87	11.28	2.4	33.3	3.41
50	35.5	8.24	11.21	2.5	32.9	2.96
75	35.5	8.01	11.13	2.5	33.1	3.12
100	35.4 *				32.9	
	42.2 *					
150	32.3 *				29.7	
	45.4 *					
200	46.0	7.26	7.26	4.3	–	
250	48.4	8.025	8.025	5.7	–	
Li_2SO_4						
5	39.0	9.88	11.50	2.7	34.4	1.62
10	37.7	9.66	11.24	2.9	34.1	1.58
20	36.6	9.62	11.19	2.8	33.9	1.57
50	35.9	9.22	11.00	2.9	34.1	1.78
100	35.6	9.16	10.96	2.6	34.1	1.80
200	35.8		11.08		34.6	
300	36.2 *		11.30		35.0	
	43.7 *					
400	37.5 *		11.44		–	
	43.9 *					
500	40.0 *		11.95		–	
	44.4 *					
600	42.5 *		10.92		–	
	44.7 *					
750	43.7 *		12.03		–	
	44.9 *					
900	47.2		11.56	5.5	–	
NaN_3						
1.5	39.5	9.77	11.90	2.6	34.5	2.13

* Double peak.

pH of the solution for all studied salts, except for Na_2HPO_4, was between 3 and 4.5. The collagen thermal stability is only slightly sensitive to pH in this range [7,42]. Our control measurements also showed that increase of pH from 3 up to 4.5 does not alter the collagen denaturation temperature (data not shown). In solutions of Na_2HPO_4, the pH increases from 6.0 at 10 mM Na_2HPO_4 up to 9.6 at 500 mM Na_2HPO_4. Since T_d is known to increase at

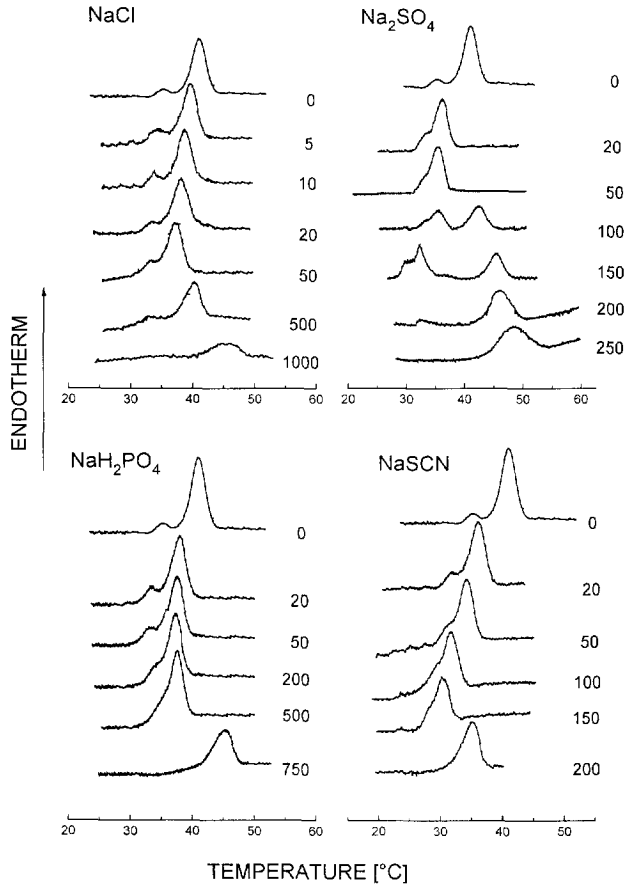

Fig. 4. Heating thermograms of calf skin collagen type I (0.5 mg/ml) in 0.05 M acetic acid in the presence of different sodium salts of concentrations in mM as indicated.

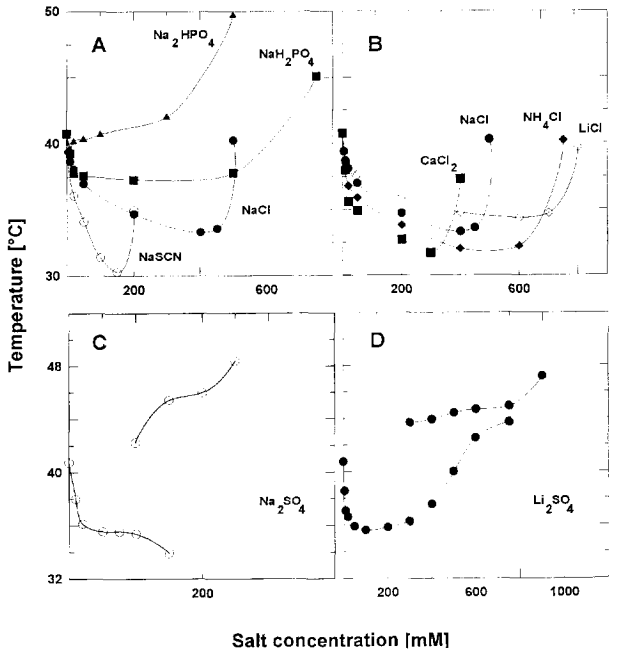

Fig. 5. Dependence of the denaturation temperature of calf skin collagen type I on the salt concentration: (A) selection of sodium salts; (B) selection of chloride salts; (C) Na_2SO_4, (D) Li_2SO_4.

alkaline values of pH [37], the considerable increase of the denaturation temperature in our samples with Na_2HPO_4 might be regarded, at least partially, as a pH effect.

All tested salts at concentrations below 20 mM reduce the thermal stability of collagen in identical way. Both peaks, the pretransition and the main transition, move in parallel (Table 2, Fig. 5). The total enthalpy also decreases with the salt concentration in this range. On further increase of the salt concentration, the effect of different salts becomes salt-dependent. All chloride salts and NaSCN keep on reducing the temperature of collagen denaturation. Intermediate concentrations of NaH_2PO_4 (20–500 mM) and Na_2SO_4 (50–100 mM) practically do not further affect the collagen stability, while Li_2SO_4 (100–300 mM) and Na_2HPO_4 (50–300 mM) increase it. It is clear from Fig. 5 that anions dominate the change in the collagen stability in the intermediate concentration region and follow the order $H_2PO_4^- \geq SO_4^{2-} > Cl^- > SCN^-$. This order represents a typical lyotropic series for anions. The sign inversion point of the anion effect is at about SO_4^{2-}. The HPO_4^{2-} ion is not included in these series as it causes also an increase of pH to alkaline values which, as explained above, may interfere with its lyotropic effect. Cations decrease the temperature of collagen denaturation in the intermediate concentration range, with rather similar slopes (Fig. 5B). According to the magnitude of their effect, they arrange in the order $Li^+ > NH_4^+ > Na^+ > Ca^{2+}$. At still higher salt concentration, a sharp increase of the denaturation temperature takes place. The onset concentration of this increase varies from 100–200 mM in the case of Na_2SO_4 and NaSCN to 800 mM for LiCl. This effect is accompanied by decrease in the transition enthalpy and broadening of the peak (Table 2). Parallel opacity measurements show an increase of the sample opacity at the onset salt concentrations. We assume that the simultaneous increase in denaturation temperature and solution opacity denote the onset of a salting-out and aggregation of collagen. The onset salt

Table 3
Onset concentrations of collagen salting-out as estimated by opacity measurements *

Salt	Collagen salting-out concentration (mol/l) *
NaSCN	0.2
Na_2SO_4	0.3
NaCl	0.5
Na_2HPO_4	0.5
NaF	0.6
NaH_2PO_4	0.7
$CaCl_2$	0.4
NH_4Cl	0.7
KCl	0.7
LiCl	0.8
Li_2SO_4	0.5

* Salt concentration has been increased by steps of 0.1 M and the concentration inducing precipitous opacity increase is indicated as salting out initiating concentration.

concentrations, as estimated from the opacity measurements, are summarised in Table 3. Control measurements show that Gu · HCl, which does not produce salting out up to 2 M concentration [36], does not also cause an increase in the denaturation temperature. A splitting of the main transition into two peaks was observed at Na_2SO_4 concentration 100 and 150 mM (Fig. 4) and Li_2SO_4 concentrations 300–750 mM. Since such splitting was not observed for the other salts in the whole range of concentrations up to 1 M, we consider it a result of specific interactions of the sulfate anions with the collagen molecules.

Table 4
Effect of salts on the denaturation temperature of acid-soluble calf skin collagen per 1 mol/litre at intermediate salt concentrations (slope = $\Delta T_d / \Delta c$)

Salt	Slope (°C · mol^{-1} l^{-1})
NaSCN	−57.5
NaCl	−8.0
Na_2SO_4	0.0
NaH_2PO_4	0.4
Na_2HPO_4	6.8
$CaCl_2$	−12.8
NH_4Cl	−11.1
NaCl	−8.0
LiCl	−3.5
Na_2SO_4	0.0
Li_2SO_4	3.2

4. Discussion

Upward or downward shifts of the denaturation temperature are the most pronounced and easily detectable effect of salts on the collagen stability. Three concentration regions are clearly distinguished from the plots given in Fig. 5 — a salt-independent drop of the transition temperature at low salt concentrations, a salt-dependent change (predominantly decrease) at intermediate concentrations, and an abrupt increase at high concentrations. This behavior indicates more than one mechanism of salt interactions with collagen. At concentrations below 20 mM, all studied salts decrease collagen stability. The denaturation temperature drops by 1.4°C at 5 mM concentration and by 2.2°C at 10 mM concentration, while for 5 mM of divalent ions its reduction is 1.6–1.8°C (Table 2). In general, salts can affect the stability of macromolecules by screening the charged residues. As noted in the Introduction, the CSC triple helix contains a considerable amount of closely situated amino-acid residues bearing opposite electric charges [12,15]. It is thus conceivable that the reduction in collagen stability at low salt concentration results from screening of the electrostatic interactions between these residues. Among them, it seems that the interchain pairs of opposite charges are likely to contribute more to the stabilisation of the collagen triple helix. Their shielding by salts will reduce the stability of the native state with respect to the denatured state since, obviously, no such pairs exist in the latter state where the three chains are separated. This consideration may not be valid for intrachain pairs where the charge separation, and the respective charge interactions, may not substantially change with denaturation. According to measurements on model tripeptides [38], the amino acids expected to form oppositely charged pairs in collagen (Asp^- and Glu^- with Lys^+ and Arg^+) have pK values 4, 4.4, 10.4 and 12.5, respectively. Since the participation of amino acids in ion pairs decreases the pK of acidic residues and increases the pK of basic residues, the Asp^- and Glu^- residues are supposed to be at least partially protonated in the employed pH range. It is thus clear that the contribution of these ion pairs to the collagen stabilisation at low pH would be even enhanced at physiological values of pH. The electrostatic effect of low salt concentrations on collagen stability has the same nature as the effect of opposite sign observed with some globular proteins. For example, screening of the local electrostatic interactions by low amounts of salts stabilises bovine serum albumin against thermal unfolding, independently of the salt species [43]. Closely situated identically charged amino-acid residues are considered responsible for this effect.

At intermediate concentrations, the different salts affect the collagen stability according to their position in the lyotropic series, with a dominating effect of the anions and a sign inversion point located at about SO_4^{2-}. Since the plots of denaturation temperature vs. salt concentration are close to linear in this range, it is appropriate to use the change of denaturation temperature per mol salt to quantify this effect. For the selection of salts studied here, these slopes are as shown in Table 4. The ability of salts and many other low-molecular solutes at moderate concentration to exhibit lyotropic effects on the stability of macromolecules is long known. Solute effects of such kind, generally termed the Hofmeister effect, have also been demonstrated in numerous studies of different interfaces (see, e.g., the review of Collins and Washabaugh [44]). These effects originate from the competition between surface groups and solutes for water molecules. By stabilising the structure of bulk water, kosmotropic solutes tend to reduce interface area and stabilise proteins against thermal unfolding. They are preferentially excluded from the protein interface area, i.e., in their presence proteins are preferentially hydrated. By destabilising the structure of bulk water, chaotropic solutes have the opposite effect. By reviewing the various manifestations of the Hofmeister effect, it has been established that the kosmotropic/chaotropic sign inversion point for the anion series is usually located near the position of Cl^- ion. Thus, HPO_4^{2-}, SO_4^{2-}, F^- ions are considered as kosmotropes (water structure makers), while Br^-, I^-, SCN^- are on the chaotropic (water structure breakers) side of the series [44]. Concerning collagen thermal stability, the sign inversion of the

anion effect is shifted to the kosmotropic side of the series, i.e., only strong kosmotropes can slightly increase collagen stability, while weak kosmotropes decrease it. Similarly, no sign inversion is registered in the cation series even for LiCl, although Li^- has been recognised in other studies as a distinct kosmotropic solute. Such shift of the sign inversion point might be due to at least two different reasons. One possible explanation can be sought in an overlap of the lyotropic effect with the electrostatic screening effect of the salts. It is clear from the Debye–Hückel and Gouy–Chapman theories that the shielding of the electrostatic interactions saturates and levels out at salt concentrations where the Debye screening length assumes values comparable with the charge separation (e.g., at 100 mM univalent salt its value is 9.6 Å). Although saturating with increase of salt concentration, the latter effect may not be fully levelled out at moderate salt concentrations and thus may counterbalance the stabilizing effect of kosmotropes. Another, principally different consideration in this respect is as follows. Generally, the Hofmeister effect is a result of interactions in three-component water–solute–interface systems. While the position of a given ion (solute) in a lyotropic series reflects specifically its effect on water structure in comparison to other ions, the position of the sign inversion point depends also on the properties of the particular interface and its interaction with water. A shift of this point to the kosmotropic end of the series may take place provided that the surface of the collagen molecule bears strong kosmotropic properties and acts as a water structure maker. Such assumption finds some support from the results of a recent structural study of a collagen-like peptide, $(Pro\text{-}Hyp\text{-}Gly)_{10}$, with a single Gly-to-Ala substitution in the centre of the peptide chain [11]. Using X-ray crystallography it was demonstrated that the triple helices formed by the model peptide are surrounded by a highly structured cylinder of hydration, with an extensive hydrogen bonding between water molecules and peptide. The manifestations of the Hofmeister effect are typically dominated by anion effects [44]. The reported here data is in accord with this conclusion. While the denaturation temperature depends on the anion concentration in markedly different and anion-specific way (Fig. 5A), the graphs for the selection of cations (Ca^{2+}, Na^+, NH_4^+, Li^+) show that the ability of cations to regulate protein stability at intermediate concentrations is much less pronounced (Fig. 5B, Table 2).

Except for shifts of the transition temperature, the examined salts induce also changes of the transition enthalpy. It appears that both changes are related through a linear dependence of the denaturational enthalpy of CSC on the transition temperature. It is shown in Fig. 6 where the measured enthalpies of CSC at low and intermediate salt concentrations are plotted versus the corresponding denaturation temperatures. The data points well fit to linear function, with a slope $d(\Delta H)/dT = 0.113$ cal $\cdot g^{-1}K^{-1}$. According to the Kirchhoff's law, the enthalpy derivative

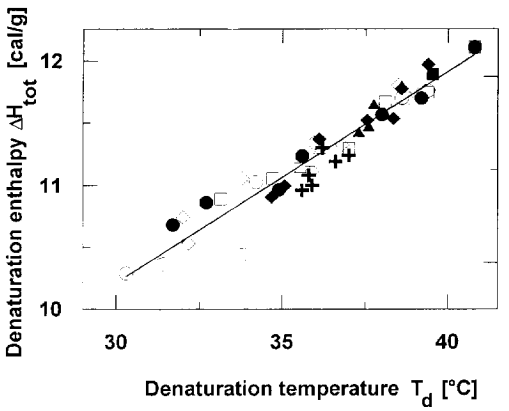

Fig. 6. The denaturation enthalpy versus the denaturation temperature for calf skin collagen type I at salt concentrations lower than the corresponding salting-out concentration: ◆, LiCl; □, NaCl; ◇, NH_4Cl; ●, $CaCl_2$; ○, NaSCN; ▲, NaH_2PO_4; △, Na_2SO_4; +, Li_2SO_4; ▽, KCl; ■, NaN_3.

on temperature is equal to the heat capacity change resulting from denaturation, $d\Delta H/dT = C_p^D - C_p^N = \Delta C_p$. The change of the specific heat capacity of CSC upon denaturation, as determined directly from the heating thermograms, is 0.102 cal $\cdot g^{-1}K^{-1}$. The close coincidence of these two independently determined values indicates that the salt effects on the denaturation enthalpy of CSC indeed originate from the weak linear dependence of the enthalpy on temperature.

On further increase of salt concentration, a precipitous increase of the collagen denaturation temperature takes place. The salt concentration giving rise to this effect, and also to a simultaneous sharp increase of the solution opacity, is salt-specific and ranges from about 200 mM for NaSCN to about 800 mM for LiCl. Obviously, collagen salting out and aggregation take place at these salt concentrations. The linear $\Delta H(T)$ dependence shown in Fig. 6 does not hold in this range, where the increase of T_d is followed by a decrease of the denaturation enthalpy (Table 2). The enhanced stability of aggregated collagen in high salts is consistent with the measured higher stability of collagen fibrils in comparison to dissolved collagen ([8,31], our unpublished results). Similarly, the thermal stability of the collagenous part of the blood plasma protein C1q is higher than that of the monomeric collagen, supposedly as a result of association of the collagen-like helices [40]. One may also speculate that the stability increase of collagen following its aggregation (i.e., removal of the protein from the bulk water phase) has a physical nature similar to the stabilisation observed for some proteins upon freeze-drying, or suspension in anhydrous organic solvents [41].

Noteworthy, the ability of different salts to salt-out collagen also correlates with their position in the lyotropic series and increases for chaotropic ions (Table 2, Fig. 5). Exceptions are the sulfate salts (Na_2SO_4, Li_2SO_4), known from other studies as kosmotropic solutes, which increase the collagen solution opacity at relatively low concentra-

tions of 0.3–0.5 mol/l. According to the calorimetric data, collagen solutions in sulfate salts demonstrate peculiar thermal behavior displayed in splitting of the main denaturation peak (Figs. 4 and 5C,D). The peak splitting takes place at salt concentrations below the onset of the salting-out of collagen, as estimated by the enhanced opacity of the solution (Table 3). The two peaks in the thermograms may represent either a complex two-step denaturation process, or two fractions of dissolved and salted-out protein. Since no splitting of the denaturation peaks was observed for the other salts, the SO_4^{2-} ions appear also to bind to collagen in a specific way. It is worth noting in this connection that sulfated heteropolysaccharides are another major constituent of connective tissues and their specific interactions with collagens might play an important role in the formation of the connective tissue matrix. Unusual binding of sulfates with another connective tissue protein — aortal elastin — has also been reported [45].

5. Conclusions

1. Univalent salts at concentrations below 20 mM reduce the temperature of collagen denaturation with a factor of about 0.2°C per 1 mM. This effect is attributed to screening of electrostatic interactions leading to collagen stabilisation.

2. At higher concentrations, roughly in the range 20–500 mM, the different salts either slightly stabilise or further destabilise the collagen molecule according to their position in the lyotropic series. This is a Hofmeister effect associated with indirect protein–salt interactions exerted via competition for water molecules between ions and the protein surface. The effect of anions is dominating and follows the order

$H_2PO_4^- \geq SO_4^{2-} > Cl^- > SCN^-$ with a sign inversion point at about the SO_4^{2-} anion. Such position of the inversion point may indicate either that the surface of the collagen molecule bears kosmotropic properties, or, alternatively, that the electrostatic shielding by salts partially compensates the lyotropic effect in this concentration range.

3. Above onset concentrations varying between 200 and 800 mM for the different salts, the temperature of collagen denaturation and solution opacity markedly increase for all studied salts due to protein salting out and aggregation. The onset concentrations arrange according to the lyotropic series and decrease for chaotropic ions.

4. The SO_4^{2-} anions interact specifically with collagen and induce splitting of the protein denaturation peak into two components in the range 100–150 mM Na_2SO_4 and 300–750 mM Li_2SO_4.

5. The variations of the collagen denaturation enthalpy at low and intermediate salt concentrations are consistent with a weak linear increase of the enthalpy with the denaturation temperature. Its derivative, $d(\Delta H)/dT$, closely coincides with the independently measured difference in the heat capacities of the denatured and native states, $\Delta C_p = C_p^D - C_p^N \approx 0.1$ cal $\cdot g^{-1} K^{-1}$.

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References

[1] Miller, E.J. (1988) in Collagen (Nimni, M.E., ed.), Chap. 5, pp. 139–156, CRC Press, Boca Raton, FL.
[2] Prockop, D.J. and Kivirikko, K.I. (1995) Annu. Rev. Biochem. 64, 403–434.
[3] Rich, A. and Crick, F.H.C. (1955) Nature (London) 176, 915–916.
[4] Ramachandran, G.N. and Kartha, G. (1955) Nature (London) 176, 593–595.
[5] Ripamonti, A., Roveri, N. and Braga, D. (1980) Biopolymers 19, 965–975.
[6] Privalov, P.L. (1982) Adv. Protein Chem. 55, 1–104.
[7] Burge, E.R. and Hynes, R.D. (1959). J. Mol. Biol. 1, 155–164.
[8] Piez, K.A. and Gross, J. (1960) J. Biol. Chem. 235, 995–998.
[9] Bansal, M., Ramakrishnan, C. and Ramachandran, G.N. (1975) Proc. Ind. Acad. Sci. A82, 152.
[10] Burjanadze, T.V. (1982) Biopolymers 21, 1489–1501.
[11] Bella, J., Eaton, M., Brodsky, B. and Berman, H.M. (1994) Science 266, 75–81.
[12] Traub, W. and Fietzek, P.P. (1976) FEBS Lett. 68, 245–249.
[13] Nemethy, G. and Sheraga, H.A. (1989) Biopolymers 28, 1573–1584.
[14] Venugopal, M.G., Ramshaw, J.A.M., Braswell, E., Zhu, D. and Brodsky, B. (1994) Biochemistry 33, 7948–7956.
[15] Fietzek, P.P. and Kuhn, K. (1976) Int. Rev. Connect. Tissue Res. 7, 1–60.
[16] von Hippel, P.H. and Wong, K.-Y. (1962) Biochemistry 1, 664.
[17] Russel, A.E. (1973) Biochem. J. 131, 335–342.
[18] Burjanadze, T.V. and Bejitadze, M.O. (1988) Biofizika 33, 50–53.
[19] Gekko, K. and Koga, S. (1983) J. Biochem. 94, 199–205.
[20] Bachinger H.P. and Morris, N.P. (1990) Mattrix 10, 331–338.
[21] Komsa-Penkova, R. (1993) Ph.D. Thesis, Medical University of Pleven.
[22] Fuji, T. and Kuhn., K. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 1793–1801.
[23] Miller, E.J. and Rhodes, R.K. (1982) Meth. Enzymol. 82, 33–64.
[24] Sykes, B., Piddle, B., Francis, M. and Smith, R. (1976) Biochem. Biophys. Res. Commun. 72, 1472–1480.
[25] Stegemann, H. (1958) Z. Physiol. Chim. 311, 41–45.
[26] Komsa-Penkova, R., Spirova, R., Bechev, B. (1996) J. Biochem. Biophys. Methods (in press).
[27] Privalov, P.L., Plotnikov, V.V. and Filimonov, V.V. (1975) J. Chem. Thermodyn. 7, 41–47.
[28] Jackson, W.M. and Brands, J.F. (1970) Biochemistry 9, 2294–2301.
[29] McClain, P.E. and Wiley, E.R. (1972) J. Biol. Chem. 247, 692–697.
[30] Danielsen, C.C. (1982) Collagen Rel. Res. 2. 143–150.
[31] Wallace, D.G., Condell, R.A., Donovan, J.W., Paivinen, A., Rhee, W.M. and Wade, S.B. (1986) Biopolymers 25, 1875–1893.
[32] Privalov, P.L. and Tiktopulo, E.I. (1970) Biopolymers 9, 127–139.
[33] Danielsen, C.C. (1984) Biochem. J. 222, 663–668.
[34] Flandin, F., Buffevant, C. and Herbage, D. (1984) Biochim. Biophys. Acta 791, 205–211.
[35] Condell, R.A., Sakai, N., Mercado, R.T. and Larenas, E. (1988) Collagen Rel. Res. 8, 407–418.

[36] Komsa-Penkova, R., Goshev, I. and Alexiev, C. (1994) C.R. Acad. Bulg. Sci. 47, 45–49.
[37] Privalov, P.L., Tiktopulo, E.I. and Tischenko, V.M. (1979) J. Mol. Biol. 127, 203–216.
[38] Nozaki, Y. and Tanford, C. (1967) Methods Enzymol. 11, 715–734.
[39] Bachinger, H.P., Bruckner, P., Timpl, R. and Engel, J. (1978) Eur. J. Biochem. 90, 605–613.
[40] Tischenko, V.M., Ichtchenko, A.M., Andreyev, C.V. and Kajava, A.V. (1993) J. Mol. Biol. 234, 654–660.
[41] Klibanov, A.M. (1989) Trends Mol. Biol. 14, 141–144.
[42] Horgan, D.J., Kurth, L.B. and Kuypers, R. (1991) J. Food Sci. 56, 1203.
[43] Yamasaki, M., Yano, H. and Aoki, K. (1991) Int. J. Biol. Macromol. 13, 322–328.
[44] Collins, K.D. and Washabaugh, M.W. (1985) Q. Rev. Biophys. 18, 323–422.
[45] Winlove, C.P. and Parker, K.H. (1994) Biopolymers 34, 393–401.