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Discrete reduction of type I collagen thermal stability upon oxidation

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Abstract

The oxidation of acid-soluble calf skin collagen type I caused by metal-dependent free radical generating systems, Fe(II)/H_2O_2 and Cu(II)/H_2O_2, was found to bring down in a specific, discrete way the collagen thermal stability, as determined by microcalorimetry and scanning densitometry. Initial oxidation results in splitting of the collagen denaturational transition into two components. Along with the endotherm at 41°C typical for non-oxidized collagen, a second, similarly cooperative endotherm appears at 35°C and increases in enthalpy with the oxidant concentration and exposure time, while the first peak correspondingly decreases. The two transitions at 35 and 41°C were registered by densitometry as stepwise increases of the collagen-specific volume. Further oxidation results in massive collagen destruction manifested as abolishment of both denaturational transitions. The two oxidative systems used produce identical effects on the collagen stability but at higher concentrations of Cu(II) in comparison to Fe(II). The discrete reduction of the protein thermal stability is accompanied by a decrease of the free amino groups, suggestive of an oxidation attack of the side chains of lysine residues. Since the denaturation temperature of collagen shifts from above to below body temperature (41°C–35°C) upon oxidation, it appears important to account for this effect in a context of the possible physiological implications of collagen oxidation.

Keywords: Oxidation; Collagen type I; Thermal denaturation; Fe ions; Thermal stability; Specific volume (DSC)

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1. Introduction

Oxidative damage of proteins involves amino acid modification, increased susceptibility to proteolysis, fragmentation, crosslinking and aggregation. It is implicated in various physiological and pathological processes [1–4]. Roles for the oxidative destruction of collagens in inflammatory as well as in age-related processes have also been proposed [1,5–7]. Collagen oxidation has been studied using different oxidative systems including metal ion/H_2O_2, xanthine oxidase system, γ-radiolysis, metal ion/ascorbate system [5–14]. In addition to the above mentioned oxidation effects, specific for collagen effects, such as depolymerization of the triple helix and impaired ability of the triple helices to assemble into fibrils, have also been considered. Many of the studies focus on collagen fragmentation. Such fragmentation may occur either as direct result of an oxidation attack [8], or as a consequence of the enhanced susceptibility of oxidized collagen to enzymatic hydrolysis [8,9]. The degree of fragmentation depends on the collagen type. Collagen type III is more sensitive and type V is much less sensitive to oxidation than collagen type I [10]. Also, the degradation of acid-soluble collagen is higher than that of fibrillar collagen. One possible route for direct collagen fragmentation is given by the oxidation of proline, followed by cleavage of the Gly–Pro peptide bond [11–13]. Except for proline, several other amino acid residues are also sensitive to oxidation attack by metal ion/H_2O_2 systems [12,14]. However, the specific effects of their oxidation on the collagen structure remain at present unclear.

Depending on their type and source, collagens experience cooperative thermal denaturational transitions at temperatures typically in the range 10–40°C [15]. It is worth noting that the thermal transitions of the triple helix into a random coil take place closely above the body temperature of the species from which collagen was isolated. Calf skin collagen type I in acidic solution is characterized by a denaturational transition at approximately 41°C (at commonly used scan rates of 0.5–1°C/min). Also, a small endothermic transition at 33–35°C, referred to as 'pretransition', has been recorded in several studies [16–19], but the nature of structural changes underlying this transition has not been clarified. Condell et al. [19] ascribe it to collagen fragments produced during protein isolation at the step of pepsin digestion. It persists though in collagen samples extracted without enzymatic treatment [20]. Bachinger et al. [21] suggest that the pretransition is caused by unidentified collagen damage during its preparation. In our previous work [20] we noticed that the pretransition was absent in fresh collagen isolated and purified in antioxidant conditions. Oppositely, it was well expressed in collagen solutions stored in aerobic conditions for several months. Since it is conceivable that oxidation may affect the protein thermal stability as well, these observations led us to expect that the pretransition is entirely due to collagen oxidation [20]. Here we demonstrate that the appearance of an endothermic transition at 35°C reflects a discrete reduction of the collagen thermal stability at low degrees of oxidation, while more extensive oxidations result in complete disruption of the protein native structure, manifested as abolishment of both denaturational transitions. From a literature survey, this appears to be the first characterization of oxidation-induced changes in protein thermal stability by thermodynamic methods — differential scanning calorimetry and scanning densitometry.

2. Experimental procedures

2.1. Isolation, purification and characterization of collagen type I

Calf skin collagen type I was isolated by a standard procedure involving acetic acid extraction, salting out with NaCl and using ion exchange chromatography on DEAE cellulose [22]. This preparation, designated as CSC(st), was used in control experiments. In order to prevent spontaneous oxidation of collagen by trace amounts of metal ions, the same procedure of collagen isolation and purification was carried out with solutions containing 10 mmol/l EDTA, 40 mmol/l citrate and 20 mmol/l mannitol (designated fur-

ther as CSC preparation). The high purity (> 95%) of both collagen preparations was verified by using interrupted SDS polyacrylamide electrophoresis, according to Sykes et al. [23]. The collagen concentration was determined from the hydroxyproline content according to the method of Stegemann [24] and by our modification of the Lowry method [25].

2.2. Collagen oxidation procedure

Oxidation was performed by incubation of tropocollagen solution (1 mg/ml in 0.05 M acetic acid, pH ~ 4.3) with different oxidants: (i) Fe(II)/H_2O_2 system: Fe(II) (5–100 μM), H_2O_2 (5–100 mM), 1 μM EDTA; (ii) Cu(II)/H_2O_2 system: Cu(II) (0.1–1.0 mM), H_2O_2 (5–100 mM), 1 μM EDTA; (iii) H_2O_2 alone (0.03–1 M). The incubations were carried out at 22°C for different time periods from 10 min to 18 h. The oxidation was terminated by adding 10 mM EDTA, and the samples were dialyzed against H_2O for 24 h.

2.3. Differential scanning calorimetry

Calorimetric measurements were performed on high-sensitivity differential scanning microcalorimeters DASM-4 and DASM-1M (Biopribor, Pushchino, Russia), with sensitivity greater than 4×10^{-6} cal/K and a noise level less than 5×10^{-7} W [26]. The protein concentration was 0.5 mg/ml in 0.05 M acetic acid. Runs were routinely made in the range 20–50°C, with a heating rate of 0.5°C/min. The reversibility of the thermally-induced transitions was checked by a second heating of the samples immediately after cooling to 20°C following the first run. The thermograms were corrected for the instrumental baseline. The temperature at the maximum of the excess heat capacity curve was taken as the transition temperature. The enthalpy of the transition was determined as the area under the excess heat capacity curve.

2.4. Scanning densitometry

The specific volume of collagen was calculated from the density difference between 0.05 M acetic acid solution and the protein solution. The protein concentrations were 1–2 mg/ml in 0.05 M acetic acid (see Fig. 3). The latter was determined by using two DMA-602H cells (Anton Paar KG, Graz) connected to a home-made unit for data acquisition and temperature control. Linear heating and cooling of the samples was performed at scan rate of 0.5°C/min with a PC-interfaced water bath. The instrument constants were determined according to the specifications of the producer, using distilled water and air as standards. The partial specific volume of protein was calculated according to the equation [27]:

$$\bar{v} = \frac{1}{\rho_{sol}}\left(1 - \frac{\rho_{prot} - \rho_{sol}}{c}\right),$$

where ρ_{prot} and ρ_{sol} are the densities of the protein solution and solvent, respectively, and c is the protein concentration. As evident from the above equation, error in the protein specific volume determination may originate from the uncertainty in the determination of the protein concentration c. A significant error could be introduced by non-identical concentrations of ions (and other non-protein solutes) in the protein solution and the reference sample. Since correct interpretation requires that the exact stoichiometry of protein–non-protein interactions is known, it is argued that in isopotential conditions (attained upon dialysis, as in our case) the measured apparent specific volume may differ significantly from the isomolal one [28,29]. In addition, obtaining strictly isopotential conditions requires sufficiently long dialysis. According to our experience (we did not find related references in the literature), the thin, rigid rod-like collagen molecule is able to penetrate the dialysis membranes. This brings about a formidable difficulty in preparing an isopotential reference sample which does not contain collagen. With these considerations in mind, we only report here the relative, temperature-induced volume changes, and not the absolute specific volume values.

2.5. Other methods

Free amino groups were determined by reaction

with TNBS [30]. A reaction with TNBS was carried out by incubating 100 μl of the sample with 1.5 ml of 0.1 M borate buffer, pH 9.3, and 100 μl of 0.05 M TNBS for 60 min at 40°C. The absorbance was read at 360 nm, after addition of 200 μl of 2 M HCl and 200 μl of 10% SDS, and $\varepsilon = 1.3 \times 10^4$ M^{-1} cm^{-1} was used in the calculations. *Degradation products* were determined by SDS-PAGE using 7.5% gel and stained by 0.5% Coomasie R-250. The samples were resuspended in SDS-PAGE sample buffer and heated to 100°C for 3 min prior to electrophoresis. *Digestibility* by trypsin: after oxidation with Fe(II)/H_2O_2, the amount of amino groups formed as a result of trypsin treatment was estimated by using TNBS (see above). As could be expected, these measurements demonstrated increasing collagen susceptibility to proteolysis with the increase of the Fe(II) concentration (data not shown). *Glycation* (nonenzymatic glycosylation) is the formation of covalent bonds between the carbonyl groups of reducing sugars and protein α- and ε-amino groups. Glycation of collagen was carried out by incubation in 0.5 M glucose solution in PBS (pH 7.2) at 25°C for 5 days, in the presence of 3 mM NaN_3. Glycated collagen was then dialyzed against H_2O for 24 h. Solutions in 0.05 M acetic acid were prepared for the calorimetric measurements.

Table 1
Thermodynamic parameters of the calorimetric peaks recorded with oxidized calf skin collagen samples (0.5 mg/ml, in 0.05 M acetic acid, recorded at 0.5°C/min)[a]

Sample treatment	T_1 (°C) ±0.2	T_2 (°C) ±0.2	ΔH_1 (cal/g) ±5%	ΔH_2 (cal/g) ±5%	$\Delta T_1^{1/2}$ (°C) ±0.2	$\Delta T_2^{1/2}$ (°C) ±0.2
Control CSC	–	40.8	–	11.9	–	2.4
Glycated control	–	42.0	–	7.6	–	2.4
10 μM Fe(II), 10 mM H_2O_2, 18 h	34.6	41.0	0.4	11.2	1.8	2.6
10 μM Fe(II), 100 mM H_2O_2, 18 h	35.5	41.0	0.8	10.4	2.2	2.3
5 μM Fe(II), 5 mM H_2O_2, 18 h	35.6	41.0	0.5	11.2	2.5	2.3
20 μM Fe(II), 5 mM H_2O_2, 18 h	34.9	41.2	2.3	9.3	2.5	2.4
50 μM Fe(II), 5 mM H_2O_2, 18 h	34.5	40.2	5.4	5.3	2.3	2.3
100 μM Fe(II), 5 mM H_2O_2, 1 h	33.1	–	9.1	–	4.2	–
100 μM Fe(II), 5 mM H_2O_2, 18 h	29.3	–	0.8	–	4.4	–
50 μM Fe(II), 5 mM H_2O_2, 10 min	35.3	41.1	1.5	10.5	2.4	2.5
50 μM Fe(II), 5 mM H_2O_2, 1 h	34.9	41.1	1.8	10.2	2.4	2.4
50 μM Fe(II), 5 mM H_2O_2, 18 h	34.5	40.2	5.4	5.3	2.3	2.3
50 μM Fe(II), 5 mM H_2O_2, 18 h, glycated	–	41.8	–	7.0	–	2.2
0.1 mM Cu(II), 5 mM H_2O_2, 18 h	34.9	41.0	1.1	10.2	3.1	2.5
1 mM Cu(II), 5 mM H_2O_2, 18 h	34.7	40.6	1.7	9.3	2.3	2.5
300 mM H_2O_2, 1 h	35.3	41.6	4.7	6.7	2.4	2.6
300 mM H_2O_2, 3 h	35.7	41.9	6.7	5.7	2.5	2.4
300 mM H_2O_2, 6 h	35.8	41.9	7.6	4.0	2.7	2.9
300 mM H_2O_2, 18 h	35.2	–	12.4	–	3.0	–
1 M H_2O_2, 1 h	35.3	41.3	7.8	4.4	2.8	3.3

[a] Indices 1 and 2 refer to the transitions at 35 and 41°C, respectively.

3. Results and discussion

3.1. Thermal stability of native and oxidized calf skin collagen type I

A thermogram of freshly prepared CSC type I is shown in Fig. 1a. It displays a single denaturational transition at 40.8°C, with calorimetric enthalpy ΔH of 11.9 cal/g, in accordance with previously published data [17–21], and with no trace of a pretransition at 35°C (Table 1). This transition is not reversible upon immediate reheating (cf. Fig 1 in [20]). Glycation of collagen results in a slight upward shift, by 1–2°C, of the denaturation temperature and approximately 35–40% decrease of the transition enthalpy (Fig. 1a). In order to examine the effect of collagen oxidation, we applied a widely used radical-generating system, Fe(II)/H_2O_2. Thermograms of CSC samples treated with Fe(II)/H_2O_2 show that oxidation results in splitting of the collagen denaturational transition into two components (Fig. 1). In addition to the endotherm at 41°C typical for non-oxidized collagen, a second, similarly cooperative endotherm appears at 35°C and increases in enthalpy with the oxidant concentration (Fig. 1b,c) and exposure time (Fig. 1d,e). Concomitantly, the first peak correspondingly decreases. The total enthalpy of the two endotherms is conserved upon this redistribution (Table 1). The transition at 35°C is already present after incubation for 10 min at 22°C in media containing 50 μM Fe(II) + 5 mM H_2O_2 (Fig. 1e). Incubation for 1 h in the same media results in transferring of approximately 20% of the denaturation enthalpy to the endotherm at 35°C, and 18-h incubation produces 50% enthalpy redistribution. Upon reducing the Fe(II) concentration, at 5 μM Fe(II) + 5 mM H_2O_2, only initial traces of oxidation are observable after 18-h incubation (Fig. 1c). Increasing

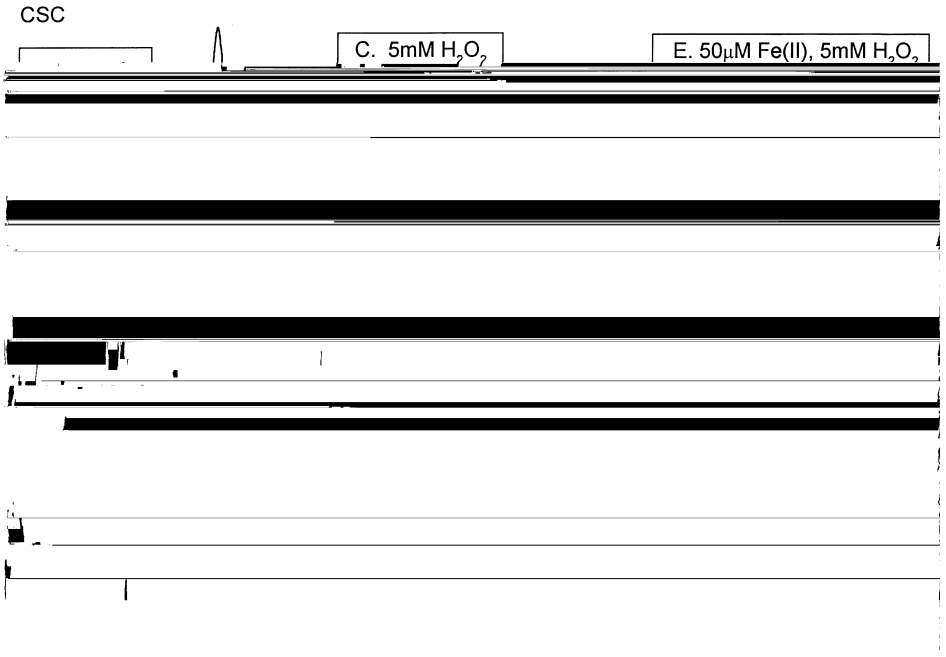

Fig. 1. Heating thermograms of calf skin collagen type I (CSC preparation, 0.5 mg/ml) in 0.05 M acetic acid, recorded at 0.5°C/min. (a) Upper curve: control thermogram of fresh, untreated CSC, lower curve: control thermogram of non-oxidized glycated CSC; (b–e) thermograms recorded after treatment at 22°C with Fe(II)/H_2O_2; (b) for 18 h at 10 μM Fe(II) and different H_2O_2 concentrations as indicated; (c) for 18 h at 5 mM H_2O_2 and different Fe(II) concentrations as indicated; (d) 100 μM Fe(II) and 5 mM H_2O_2, for 1 h and 18 h as indicated; (e) 50 μM Fe(II) and 5 mM H_2O_2, for different exposure times as indicated; the bottom thermogram is for glycated CSC preparation (5 days glycation in 0.5 M glucose solution in PBS, pH 7.2) recorded after 18-h incubation with 50 μM Fe(II) + 5 mM H_2O_2 at 22°C.

the Fe(II) concentration to 100 μM has dramatic effect: 1-h incubation eliminates the original transition at 41°C, while extension of the incubation to 18 h eliminates also the transition at 35°C (Fig. 1d). This is a clear indication of massive destruction of collagen in the latter conditions. However, treatment of glycated collagen with Fe(II)/H_2O_2 does not result in splitting of the denaturational transition and appearance of the 35°C endotherm (Fig. 1e).

Another metal-catalyzed free radical generating system, Cu(II)/H_2O_2, was also examined and was found to affect in a similar way the collagen thermal stability, but with lower efficiency. Incubation of CSC samples for 18 h at 22°C in media containing 0.1 mM Cu(II) + 5 mM H_2O_2 results in the appearance of initial traces of the transition at 35°C (Fig. 2a, Table 1). Increasing the Cu(II) concentration to 1 mM causes approximately 25% oxidation for the same time period. Thus, the system Cu(II)/H_2O_2 appears to be less efficient in the oxidation of CSC type I. This conclusion well agrees with the recently published results that Fe(II)/H_2O_2 exerts significantly higher damage on CSC type I, than the Cu(II)/H_2O_2 system, as judged from the formation of carbonyl compounds upon oxidation [14]. However, it is worth noting that collagen type IV and model peptides such as poly(Pro) and poly(Pro–Gly–Pro) were reported to be more susceptible to hydrolysis by oxidation using Cu(II)/H_2O_2, compared to Fe(II)/H_2O_2 oxidation [11–13].

Oxidation of CSC in H_2O_2 solutions also resulted in the appearance of the transition at 35°C, with the area of the peak being proportional to exposure time and to H_2O_2 concentration (Fig. 2b,c, Table 1). Much higher concentrations of H_2O_2 were needed to produce this effect, however, as compared to those of metal ion/H_2O_2 systems. The H_2O_2 concentration producing 50% enthalpy redistribution for 1 h is between 0.3 and 1 M. Incubation for 18 h in media with 0.3 M H_2O_2 eliminates the transition at 41°C (Fig. 2b).

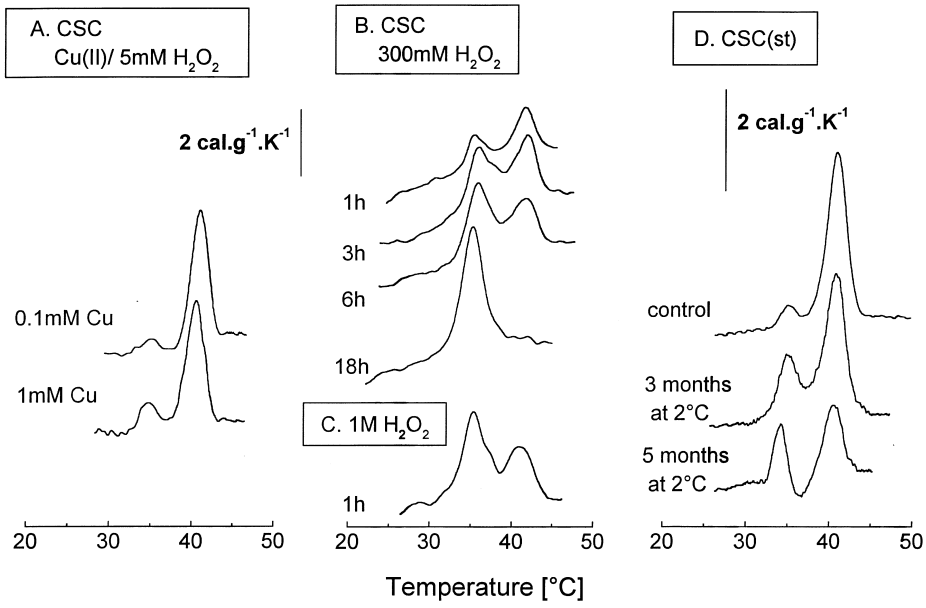

Fig. 2. Heating thermograms of calf skin collagen type I, 0.5 mg/ml in 0.05 M acetic acid, recorded at 0.5°C/min: (a) thermograms of CSC preparations recorded after 18 h treatment at 22°C with 5 mM H_2O_2 and different Cu(II) concentrations as indicated; (b) thermograms of CSC preparation recorded after treatment with 300 mM H_2O_2 at 22°C for different exposure times as indicated; (c) thermograms of CSC preparation recorded after treatment with 1 M H_2O_2 at 22°C for 1 h; (d) thermograms of 'standard' collagen preparation, CSC(st), after storage in solution in aerobic conditions as indicated.

The oxidation of CSC by H_2O_2 was inhibited in the presence of 1 mM EDTA. We therefore assume that, in this case, metal ions were involved in the oxidation process as well. Such ions might be present as trace contamination in some of the reagents used (e.g. 0.0005% Fe in H_2O_2 (Fluka); 0.0001% Fe in NaCl (Fluka) that was used for salting out of collagen).

While fresh CSC samples, extracted and purified in antioxidant conditions, are characterized by a single denaturational transition at 41°C, freshly isolated, 'standard' CSC(st) preparations display also the pretransition at 35°C (Fig. 2d). The enthalpy redistribution to 35°C was strongly expressed in collagen solutions stored in aerobic conditions, at the expense of the main peak at 41°C (Fig. 2d), as well as in lyophilized preparations that have been stored at 0–4°C for several months. We therefore consider the transition at 35°C in all these cases as a result of spontaneous collagen oxidation taking place during the isolation step and also upon prolonged storage of the preparations.

3.2. Specific volume changes of calf skin collagen type I

The splitting of the collagen denaturational transition into two components upon oxidation is also evident from the associated specific volume changes (Fig. 3). These volume changes were determined using differential scanning densitometry. The denaturational transition in freshly prepared, unoxidized CSC takes place with a single-step increase of the protein specific volume by approximately 0.03 ml/g (Fig. 3a). The transition is not reversible upon cooling and immediate reheating, in accordance with the calorimetric data. Upon heating of an oxidized sample (18-h incubation with 50 μM Fe(II) + 5mM H_2O_2 at 22°C), two steps in the specific volume were observed at approximately 35 and 40°C (Fig. 3b), in good agreement with the respective specific heat curves. The total specific volume increase was approximately 0.02–0.03 ml/g. The stepwise specific volume increase accompanying the denaturation of collagen may result, in principle, from protein aggregation [31], from disruption of the ion pairs $-COO^-...-NH_3^+$ between neighbouring acidic and basic amino acids (formation of such pairs in the native state is known to reduce their volume due to electrostriction [32]), or, more likely in the present experiment, from combination of these two effects. As is clear from Fig. 3, the stepwise increases of the specific volume upon denaturation are not reversible in cooling direction. Interestingly, the specific volume of collagen markedly increases upon denaturation, by contrast with the thermal denaturation of globular proteins which is known to take place with very small or no specific volume changes [33,34]. To our knowledge, such distinction between the specific volume behavior of fibrillar and globular proteins has not been earlier reported. It obviously originates from the profound structural difference between fibrillar proteins such as collagen and globular proteins. It has been shown recently that the latter are typified by heterogeneous packing density — a hydrophobic core of lower density is surrounded by a protein/water interfacial layer of higher density [35]. It thus appears reasonable to expect that the lack of a significant volume change upon the thermal unfolding of a globular protein is due to a close compensation of a negative volume change caused by the unfolding of its hydrophobic core and the positive volume changes associated with the above mentioned effects. Furthermore, since tropocollagen type I is a fibrillar protein virtually devoid of a hydrophobic core, its partial specific volume should be less than that of non-conjugated globular proteins (the representative mean value for the latter volume is 0.735 ml/g [29]). According to published data, the partial specific volume of tropocollagen type I is 0.703–0.710 ml/g [36–38]. Comparing the above values, one may expect that denaturation of collagen type I should result in some 0.025–0.030 ml/g volume increase. This well corresponds to the specific volume increases upon denaturation of native and oxidized CSC measured in our experiments (Fig. 3). Thus, the observed significant volume increase upon collagen denaturation is a natural consequence of the protein fibrillar structure for both the native and oxidized states.

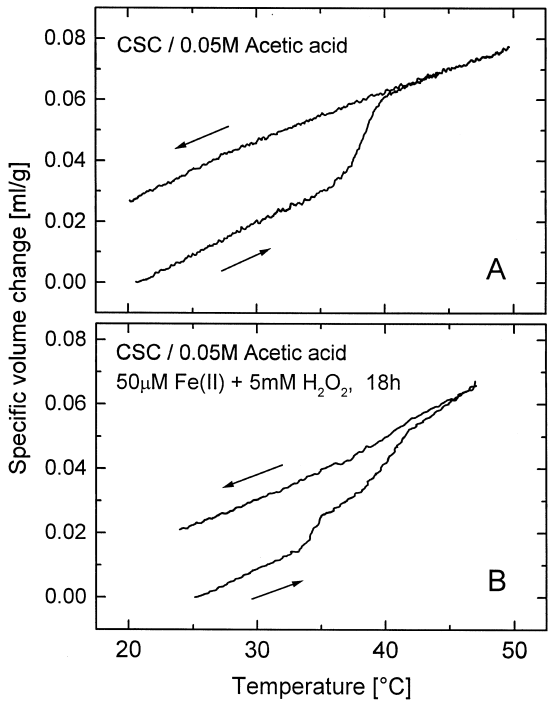

Fig. 3. Densitometric heating and cooling traces of calf skin collagen type I in 0.05 M acetic acid, recorded at 0.5°C/min: (a) fresh, untreated CSC preparation (1.7 mg/ml); (b) CSC preparation (1.9 mg/ml) after 18-h incubation with 50 μM Fe(II) + 5 mM H_2O_2 at 22°C.

3.3. Possible origin of the denaturational transition at 35°C in oxidized calf skin collagen type I

In many of the thermograms given in Figs. 1 and 2 the cooperativity of the transition at 35°C is virtually identical to that of the initial transition at 41°C (the transition halfwidths are given in Table 1). Together with its induction by the lowest oxidant concentrations used, this is a strong indication that the 35°C transition is caused by a single kind of damage to the collagen molecule. Concerted action of multiple oxidation effects, as those enumerated in Section 1, should be expected to reveal itself either in significant broadening and cooperativity drop, or in complete disappearance of the denaturational transition, due to ultimate disruption of the native structure. While both the latter effects are actually observed after oxidation with 100 μM Fe(II) (Fig. 1d), we argue in the following that a likely origin of the cooperative 35°C endotherm is the oxidation of the lysine side chain amino groups.

It is important to know whether the transition peaks at 35 and 41°C originate from different collagen molecules, or they may represent melting of different (oxidized and non-oxidized) parts of the same molecule. A specific insight into this problem was provided by the calorimetric checks on the reversibility of the collagen denaturation. These checks revealed a subtle difference between the denaturational transitions at 35 and 41°C. Upon cooling to 20°C and immediate reheating of denatured samples, the transition at 41°C was completely absent. This is a natural result since, as is long known, the separation of the collagen polypeptide chains upon denaturation precludes rapid reformation of the triple helix upon subsequent cooling. The endotherm at 35°C has different behavior, however. Its reversibility was checked in two ways — by terminating the heating scans above 41°C, where the whole sample is denatured, and at 38°C, where the non-oxidized part of the collagen is still in its native state. In the first case the transition at 35°C was also fully absent in the subsequent heating scans. In the second case, however, it was reversible when its enthalpy was small (approx. 1/10 of the total denaturation enthalpy; a representative thermogram is given in Komsa-Penkova et al. [20]), and it was not reversible when its enthalpy was comparable to that of the 41°C transition. In order to rationalize this peculiar behavior, it is pertinent to recall that, due to its length of approximately 1000 amino acids/chain, the molecule of type I collagen does not melt as a single cooperative unit. Calculations of the ratio between calorimetric and van't Hoff denaturation enthalpies suggest that one protein molecule comprises approximately 10 cooperative units along its length [16,20]. One may hypothesize on this basis that exposure to oxidants initially affects single, isolated cooperative units along the collagen triple helix. These isolated units give rise to a small endotherm at 35°C. When such a sample is heated to a temperature between the endotherms at 35 and 41°C (38°C in our measurements) and then cooled, the endotherm at 35°C is rapidly re-

versible since most of the molecule retains its native conformation and thus keeps the denatured part of the chains in the correct register, needed for reformation of the triple helix. With increase of the oxidation level, the regions melting at 35°C become larger and thus, more difficult to reform into native state. Consequently, the 35°C endotherm becomes irreversible with increase of its enthalpy. If the sample is heated to above 41°C, then naturally, the transition at 35°C does not revert, regardless of its size. This scheme seems speculative, but it is difficult to envisage an alternative, more reasonable explanation for the reversibility of the 35°C transition only observed when its enthalpy is small. We thus believe that, especially at low oxidation levels, the 35 and 41°C transitions may reflect melting of oxidized and non-oxidized units within the same collagen molecule, and that it is very unlikely that the former peak arises from denaturation of randomly-sized [6] collagen fragments. We did not attempt a more precise determination of the boundary between reversible and irreversible 35°C transitions than the order-of-magnitude estimate given above. Actually, such determination would not make much sense, at least for the reason that the result will also depend on the incubation time at 20°C prior to the second heating-storage of denatured CSC type I at room temperature for tens of hours is known to restore partially its triple-helical structure [20,39].

At higher oxidations, resulting in abolishment of either the transition at 41°C, or both denaturational transitions (e.g. 100 μM Fe(II) and 18-h incubation; Fig. 1d), the bands representing the collagen chains in SDS-PAGE were found to decrease in intensity, thus signifying collagen degradation although no accumulation of fragments of a given size was noticeable at the same time. Upon oxidations with Fe(II)/H_2O_2, roughly resulting in up to 50% enthalpy redistribution (cf. Fig. 1), neither collagen degradation, nor noticeable cross-linking could be detected by SDS-PAGE electroforegram analysis. Determinations of the hydroxyproline content before and after dialysis of the sample also did not indicate appearance of collagen hydrolysis products in the latter conditions. On the other hand, treatment with Fe(II)/H_2O_2 at low Fe(II) concentrations results in a substantial decrease of the free amino groups (Fig. 4), suggestive of preferential oxidation attack of the lysine side chains, and in accor-

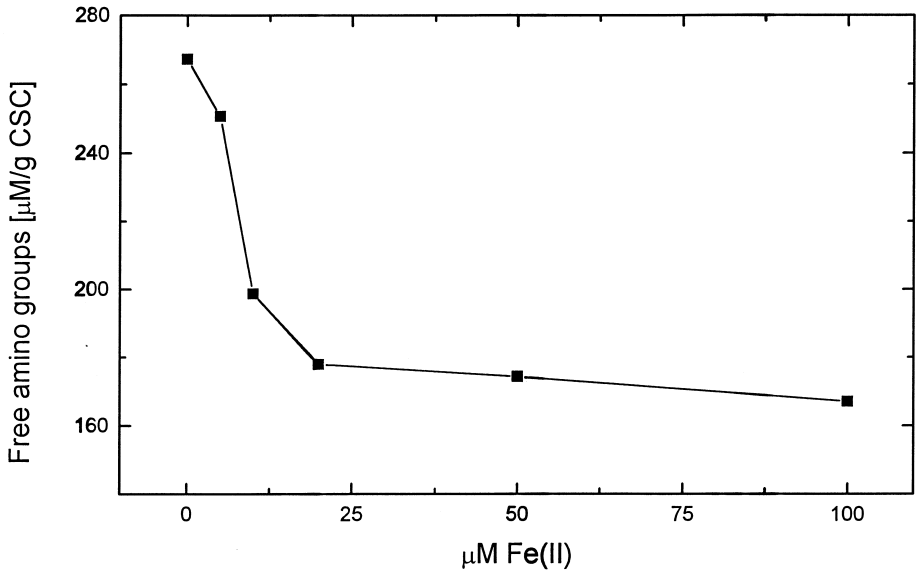

Fig. 4. Decrease of the free amino groups content of acid-soluble calf skin collagen type I following oxidation by Fe(II)/H_2O_2 (5 mM H_2O_2, 18-h incubation at 22°C) at different Fe(II) concentrations.

dance with that obtained by electron paramagnetic resonance evidence for induced by $Fe(II)/H_2O_2$ oxidation of lysine in CSC type I [14]. It is worth noting that in collagen preparations, in which some of the lysine side chain amino groups have been blocked with glucose (glycated collagen preparations), no endotherm at 35°C was detected after 18-h incubation with 50 μM Fe(II) + 5 mM H_2O_2 (Fig. 1e). These data indicate that, at least at low Fe(II) concentrations, the appearance and gradual increase of the endotherm at 35°C does not result from accumulation of collagen fragments, but is more likely due to oxidation of lysine side chains. As explained above, such a conclusion is consistent with the calorimetric data on the reversibility of this transition.

4. Conclusions

The reported here calorimetric and densitometric data unambiguously show that the previously reported pretransition of CSC type I at 35°C is due to oxidation of the protein molecule. We thus distinguish two successive stages in the oxidative damage of collagen by metal ion/H_2O_2 systems. Low oxidations result in discrete reduction of the collagen thermal stability manifested as reduction of the denaturation temperature from 41 to 35°C. More extensive oxidations result in massive collagen destruction manifested as complete abolishment of the denaturational transition. The discrete stability reduction appears to be associated with oxidation of the side chain amino groups, and not with accumulation of collagen fragments. Accordingly, no endothermic transition at 35°C appears upon oxidation of glycated collagen. It is worth noting that the denaturation temperature of oxidized collagen shifts from above (41°C) to below (35°C) body temperature. It appears important to account for this effect in the context of possible physiological and pathophysiological implications of collagen oxidation.

As judged from the low $Fe(II)/H_2O_2$ concentrations required to induce the appearance of the endothermic transition at 35°C, the thermal behavior of type I collagen is rather sensitive to oxidative damage. It is indicative of early oxidation events that may not be readily detectable with other methods. One should be alert in this connection that the frequently used 'standard' collagen preparations, designated here as the CSC(st) preparation, are actually partially oxidized and more susceptible to further damage. We are not aware of other studies on oxidation-induced protein destabilization by means of differential scanning calorimetry and scanning densitometry. From the present results, we expect that these methods would prove advantageous in such kind of studies.

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