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Collagen type II modification by hypochlorite✪

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Oxidation of proteins is a common phenomenon in the inflammatory process mediated by highly reactive agents such as hypochlorite ($HOCl/OCl^-$) produced by activated neutrophils. For instance, in rheumatoid arthritis hypochlorite plays an important role in joint destruction. One of the major targets for $HOCl/OCl^-$ is collagen type II (CII) – the primary cartilage protein. In our study, $HOCl/OCl^-$ mediated collagen II modifications were tested using various methods: circular dichroism (CD), HPLC, ELISA, dynamic light scattering (DLS), fluorimetry and spectrophotometry. It was shown that hypochlorite action causes deamination with consecutive carbonyl group formation and transformation of tyrosine residues to dichlorotyrosine. Moreover, it was shown that ammonium chloramine (NH_2Cl) formed in the reaction mixture reacts with CII. However, in this case the yield of carbonyl groups and dichlorotyrosine is lower than that observed for $HOCl/OCl^-$ by 50%. CD data revealed that collagen II exists as a random coil in the samples and that chlorination is followed by CII fragmentation. In the range of low $HOCl/OCl^-$ concentrations (up to 1 mM) 10–90 kDa peptides are predominant whereas massive production of shorter peptides was observed for high (5 mM) hypochlorite concentration. DLS measurements showed that chlorination with $HOCl/OCl^-$ decreases the radius of collagen II aggregates from 30 to 6.8 nm. Taking into account the fact that chlorinated collagen is partially degraded, the DLS results suggest that smaller micelles are formed of the 10–90 kDa peptide fraction. Moreover, collagen chlorination results in epitope modification which affects CII recognition by anti-CII antibodies. Finally, since in the synovial fluid the plausible hypochlorite concentration is smaller than that used in the model the change of size of molecular aggregates seems to be the best marker of hypochlorite-mediated collagen oxidation.

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Abbreviations: BSA, bovine serum albumin; CII, collagen type II; HRP, horseradish peroxidase; OPD, *o*-phenylenediamine dihydrochloride; PMNs, neutrophils; RA, rheumatoid arthritis; ROS, reactive oxygen species; TFA, trifluoroacetic acid.

Protein oxidation by reactive oxygen species (ROS) plays an important role in the pathomechanism of inflammatory diseases. ROS are produced by neutrophils, the main cells of inflammed synovial fluid in rheumatoid arthritis (RA). They are responsible for cartilage damage and joint erosion observed in this disease. The most toxic ROS generated by activated neutrophils is hypochlorite ($HOCl/OCl^-$) – the product of the myeloperoxidase/Cl^-/hydrogen peroxide system (Weiss *et al.*, 1982). Collagen type II (CII) is a primary protein component of cartilage and destruction of this protein caused by hypochlorite and by collagenase is one of the principal pathological reactions observed in RA (Edwards & Hallett, 1997).

It has been demonstrated (Weiss *et al.*, 1983) that $HOCl/OCl^-$ can react rapidly with amino acids and proteins to form low molecular and protein *N*-mono(di)chloramines. Davies *et al.* (1993) have shown that hypochlorite action leads to intensive fragmentation of tritiated acid soluble bovine collagen type II and that some of the amino acid-derived chloramines (*N*-chloroleucine or *N*-chloroalanine) increase the proteolytic susceptibility of *N*-chloramine modified collagen. At present, however, the knowledge of the early stages of $HOCl/OCl^-$ mediated collagen oxidation is poor. In our study we used various methods to monitor hypochlorite-mediated changes in CII amino-acid residues as well as changes in the structure of this protein.

MATERIALS AND METHODS

Chemicals. Collagen type II (CII) from chicken sternal cartilage, pronase E, biotinylated goat anti-mouse IgG antibody, potassium hydroxide, acetonitrile, sulfuric acid, hydrochloric acid, citric acid, sodium mono- and dihydrophosphate, ammonium dihydrophosphate, guanidinium hydrochloride, sodium thiosulfate, hydrogen peroxide, *o*-phenylenediamine dihydrochloride, 2,4,6-trinitrobenzenesulfonic acid, ethanol and ethyl acetate were obtained from Sigma (St. Louis, U.S.A.). 2,4-Dinitrophenylhydrazine was from Merck (Darmstadt, Germany). Sodium hypochlorite (NaOCl) and sodium chloride were from Aldrich Chemie (Steinheim, Germany). Taurine, Nessler reagent and trifluoroacetic acid (TFA) were from Fluka Chemica AG (Buchs, Switzerland). Anti-human collagen type II antibody monoclonal purified IgG (clone II 4011) was from ICN Biomedicals, Inc. (U.S.A.). Horseradish peroxidase streptavidin was from Vector Lab (Burlingame, CA, U.S.A.). NaOCl solutions were prepared prior to use from 0.57 M stock solution and standardized iodometrically (Stelmaszyńska & Zgliczyński, 1978).

Ammonium monochloramine synthesis. Two millimolar NH_2Cl was prepared prior to use in a cuvette. Forty microliters of 4 mM $HOCl/OCl^-$ in 0.2 M phosphate buffer, pH 7.4, was added to 0.96 ml of 4 mM $NH_4H_2PO_4$ in the same buffer.

Taurine monochloramine synthesis. Taurine monochloramine solutions (1–5 mM) were prepared as follows. One millilitre of 2–10 mM hypochlorite solution in 0.2 M phosphate buffer, pH 7.4, was added dropwise to 1 ml of 2–10 mM taurine in the same buffer. The final taurine monochloramine concentration was calculated using $\varepsilon_{253nm} = 0.4\ mM^{-1} \times cm^{-1}$ (Stelmaszyńska & Zgliczyński, 1978).

Collagen chlorination with $HOCl/OCl^-$ (1), ammonium monochloramine (2) or taurine monochloramine (3). (**1**) Samples of collagen (2 mg/ml) in 0.2 M phosphate buffer, pH 7.4, with 0.2 M NaCl were incubated for 2 h with 1, 3 or 5 mM $HOCl/OCl^-$ or for 30 min with 0.2, 1, 2 or 4 mM $HOCl/OCl^-$. To stop the reaction the samples were treated with a steichiometric amount of thiosulfate (1/1). The dichlorotyrosine, tyrosine and aldehyde groups were assayed in the samples and additionally in the fractions of molecular mass < 10 kDa.

(**2** and **3**). Collagen samples (1 mg/ml) in 0.2 M phosphate buffer, pH 7.4, with 0.2 M NaCl

were incubated with 2 mM ammonium chloramine or with 1, 3 or 5 mM taurine chloramine for 2 h at 37°C. The progress of reaction (2) was monitored by the decrease of absorption at 253 nm and the resulting NH_2Cl concentrations were calculated as above.

Estimation of dichlorotyrosine. Dichlorotyrosine concentration was calculated from difference spectra of chlorinated collagen (2 mg/ml) using ε_{306nm}= 4.7 $mM^{-1} \times cm^{-1}$ (Rudie *et al.,* 1980). The reference cuvette contained native collagen solution (2 mg/ml). To reduce turbidity the samples were incubated with a 10 μg/ml solution of pronase E for 30 min prior to measurements.

Estimation of tyrosine and bityrosine. The fraction (%) of intact tyrosine moieties in collagen samples (2 mg/ml) was calculated from the relative fluorescence intensity (RFI) for emission at 307–309 nm (excitation at 275 nm). Bityrosine fluorescence was excited at 310 nm and monitored for emission at 410 nm.

Assay of carbonyl groups. Carbonyl groups were determined as dinitrophenylhydrazones according to Oliver (1987). Assays were carried out in 0.1 ml samples of native and chlorinated collagen (0.2 mg) and the concentration of protein dinitrophenylhydrazones was calculated using absorption coefficient ε_{368nm} = 21 $mM^{-1} \times cm^{-1}$.

Assay of ammonium. Ammonium, a HOCl-mediated deamination product, was assayed using Nessler reagent. Fifty microliters of collagen (0.1 mg) sample was added to the assay buffer containing Nessler stock solution diluted with 0.12 M KOH (1:4, v/v). Absorption at 430 nm was measured after 30 min incubation at 37°C and ammonium concentration was evaluated from a standard curve obtained for 5–100 μM $NH_4H_2PO_4$ solutions.

HPLC assays. (1) Assays were performed using an apparatus (Waters Millipore Comp., U.S.A.) equipped with a μBondpack C-18 column (3.9 × 300 nm) and the buffer set as follows: (A) 0.1% TFA in water, (B) 0.07% TFA in 80% acetonitrile in water. Gradient was formed from 0 to 100% of B within 15 min. Twenty micrograms of unchlorinated or HOCl-modified collagen was loaded onto the column. (2) Assays were performed using a Shimadzu SCL 10A apparatus equipped with a Supelcosil ^{TM} LC-318 C-18 column (4 × 250 mm) and the buffer set as follows: (A) 0.1% TFA in water, (B) 0.1% TFA in acetonitrile. Gradient was formed from 0 to 100% of B within 30 min. One hundred microliters of sample was filtered through a Milipore YM 10 kDa membrane and loaded on to the column.

Electrophoresis. SDS/PAGE was performed in a MiniProtean II apparatus (BioRad, U.S.A.) using Tris/Tricine protein gels, 3.25 μ g of total protein was loaded into each well. The gels were stained with Coomassie Blue R-250 or silver stained.

Competitive ELISA. To determine collagen epitope modification by chlorination competitive ELISA was used. Briefly, microtiter plates (Corning, U.S.A.) were coated with 5 μg/ml of CII. The antigens tested (CII modified by HOCl/OCl^- or taurine monochloramine) at 0.5–50 μg/ml were mixed with an anti-CII monoclonal antibody (12.5 μg) and then added and incubated with a fixed competitive antigen for 1 h at room temperature and washed 3× to remove nonadsorbed immune complexes. The plates were then incubated with biotinylated goat anti-mouse antibody for 45 min at room temperature. Horseradish peroxidase (HRP) conjugated streptavidin diluted 1:1000 in 1% BSA/PBS was added and the plates were incubated for 45 min at room temperature. Then *o*-phenylenediamine dihydrochloride (OPD) was used as a substrate (5 mg of OPD in 10 ml of phosphate/citrate buffer pH 5.0) and incubated with 40 μl of 30% H_2O_2 for 30 min at room temperature. The reaction was stopped with 3 M H_2SO_4. Absorbance was measured at 492 nm. As a control we used AgAb complex formation without preincubation with free test antigen (A_{490} = 1.25).

Spectrophotometric measurements were carried out with a U-2000 spectrophotometer

(Hitachi, Japan) and fluorescence spectra were recorded with an F-2000 fluorescence spectrophotometer (Hitachi, Japan). Circular dichroism spectra (CD) of 2.2 μM collagen solutions were measured with a J710 spectropolarimeter (Jasco, Japan).

Dynamic light scattering measurements were performed with an MS200 (831.6 nm) laser unit (DynaPro, England) for collagen solutions of 1.5 mg/ml in 0.2 M phosphate buffer with 0.2 M NaCl. Samples of collagen unchlorinated and chlorinated with 1, 3 or 5 mM HOCl were filtered (100 nm) prior to the measurements. Radii (nm) were evaluated on the assumption that collagen exists in solution as a randomly coiled structure. Calculations were performed using an updated version of Dynamics Software (DynaPro, England).

Table 1. HOCl/OCl$^-$ mediated amino-acid side chain modification and its influence on CII aggregate formation

Content	Unchlorinated CII	CII chlorinated with HOCl/OCl$^-$		
		1 mM	3 mM	5 mM
Dichlorotyrosine (μM)	0	26	34	47
Tyrosine (%)	100	46.7	23.2	12.0
Carbonyl (μM)	5.7	11.9	24.13	44.7
Ammonium (μM)	0.0	614.0	45.0	0.0
Radius (nm)*	30	9.2	8.5	6.8

Data represent averages of three measurements and refer to 22 μM CII concentration (calculated for 90 kDa CII species). *The evaluated radii represent the most abundant fraction (70–90%) of total content. Artificial radii over 100 nm were out of measurement range. All samples showed an additional artificial radius of 0.2 nm in the assay buffer control.

RESULTS

It is widely known that protein chlorination results in modification of reactive amino-acid side chains containing such functional groups as: –SH, –SS–, RSR, –NH_2, OH (in Tyr) or indole (in Trp) (Hampton *et al.,* 1998). Chlorination of collagen II with HOCl/OCl$^-$ leads to the conversion of tyrosine to dichlorotyrosine as it was observed for other proteins (Kettle, 1996; Olszowski *et al.,* 1996). As shown in Table 1 dichlorotyrosine formation could be easily traced as absorbance increase at 305–308 nm in the difference spectra of chlorinated CII. Since CII does not contain tryptophan moieties it was possible to record the weak tyrosine fluorescence at 305 nm. This emission diminished with increasing HOCl/OCl$^-$ concentration and could be treated as a measure of the content of intact tyrosine moieties in CII (Table 1). The dichlorotyrosine formation in CII was dose dependent and correlated with the loss of tyrosine moieties in the samples. Moreover, a shorter chlorination time (30 min) at the same range of HOCl/OCl$^-$ concentrations (0.1–4 mM) produced a similar content (up to 40 μM) of dichlorotyrosine in 22 μM collagen (not shown). On the other hand, the 5 mM taurine monochloramine-mediated collagen chlorination did not lead to dichlorotyrosine formation although the more reactive 5 mM NH_2Cl did produce dichlorotyrosine. The yield of the latter process was, however, lower (50%) than that of corresponding 5 mM HOCl/OCl$^-$ solution (not shown).

Additionally bityrosine formation was detected in CII samples treated with 5 mM HOCl/OCl$^-$. However, the bityrosine fluorescence at 410 nm was too weak to permit evaluation of its concentration. Since CII does not contain other (e.g. tryptophan) moieties which could serve as markers of oxidation, the dichlorotyrosine content seems to correlate with the degree of CII modification.

The $HOCl/OCl^-$ mediated chlorination of lysine ε-amino groups in collagen converted them to aldehydes with concomitant deamination. Ammonium evolved in the reaction mixture was quickly converted with an excess of HOCl to mono- and dichloramines which in turn could chlorinate the protein. Ammonium chloramine-mediated CII chlorination was confirmed spectrophotometrically. Our results suggest that up to 15% of the initial amount of ammonium monochloramine reacted with CII during a 2 h incubation (27 ammonium monochloramines/collagen, mol/mol). Ammonium and carbonyl group contents in $HOCl/OCl^-$ treated CII are shown in Table 1. Whereas the carbonyl group content gradually increased with $HOCl/OCl^-$ concentration to reach 45 μM (approx. 2 carbonyl groups for a 90 kDa fragment), the ammonium concentration was the highest for relatively low (1 mM) $HOCl/OCl^-$ concentration (Table 1). The latter result suggests that intensive deamination prevails up to 1 mM hypochlorite concentration, whereas at higher $HOCl/OCl^-$ concentration the deamination is followed by the reaction that transforms the ammonium produced to chloramines (NH_2Cl and $NHCl_2$).

Circular dichroism spectra (CD) revealed changes in the content of secondary structures in CII samples. As shown in Fig. 1, an increase of the $HOCl/OCl^-$ concentration decreased the ellipticity in the range of 190–240 nm (n-π*). All spectra correspond to random coil structure and suggest an absence of triple helix in all samples. Thus, the lower θ corresponds to a higher content of degraded uncoiled α(II) collagen chains. Since the spectra showed the same position (220 nm) of θ maximum it could be concluded that no other secondary collagen structures formed during chlorination. These results correlate with the concomitant decrease of the content of high molecular CII fraction – calculated from the peak height for 10.9 min retention time (Table 2). In addition, the presence of low molecular mass peptides in collagen samples filtered through a 10 kDa membrane was confirmed. The HPLC profiles shown in Fig. 2 suggest that only the highest $HOCl/OCl^-$ concentration let to a massive formation of low molecular mass (< 10 kDa) peptides.

The antigen–antibody reaction between chlorinated collagen and a monoclonal antibody specific to native collagen was used to determine epitope deterioration caused by $HOCl/OCl^-$ and taurine monochloramine. $HOCl/OCl^-$ in a dose dependent manner affected the formation of immune complexes as shown in Fig. 3. This indicates that $HOCl/OCl^-$, used at concentrations higher than 1 mM, seriously changed the epitope structure of CII, which did not compete with native CII for the specific antibody. In contrast to $HOCl/OCl^-$, taurine monochloramine did not affect the ability of collagen to react with the antibody specific for the native protein (Fig. 4).

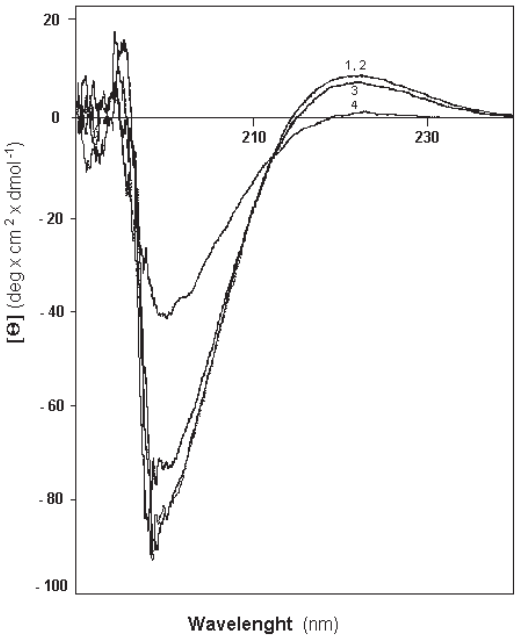

Figure 1. CD spectra of unchlorinated CII (1) and CII chlorinated with 0.2 mM (2), 1.0 mM (3) and 4 mM (4) $HOCl/OCl^-$.

Samples contained 2.2 μM CII in 0.2 M phosphate buffer with 0.2 M NaCl, pH 7.4.

SDS profiles (data not shown) indicated that the molar mass of unchlorinated CII was

Table 2. Influence of $HOCl/OCl^-$ on CII structure

Content	Unchlorinated CII	CII chlorinated with $HOCl/OCl^-$		
		0.2 mM	1 mM	4 mM
Secondary structure (%)*	100	96.1	82.2	16.4
Peptide bonds (%)**	100	78.6	32.5	17.5

*CD data for 2.2 μM CII; **HPLC data derived from CII peak height (10.9 min retention time). For other details see Methods – HPLC procedure (1).

90–95 kDa and that the $HOCl/OCl^-$-mediated fragmentation of CII could be monitored even within low $HOCl/OCl^-$ concentration range (up to 1 mM). Samples of chlorinated CII gave one main band whose thickness diminished proportionally to the chlorination level both on Commassie and silver stained gels. Although no additional low molecular bands were found, dichlorotyrosine and carbonyls were detected in fractions (molecular mass <10 kDa) derived from CII samples chlorinated with 3 and 5 mM $HOCl/OCl^-$ (not shown). The latter result suggests the presence of low molecular mass oxidatively modified peptides in the samples.

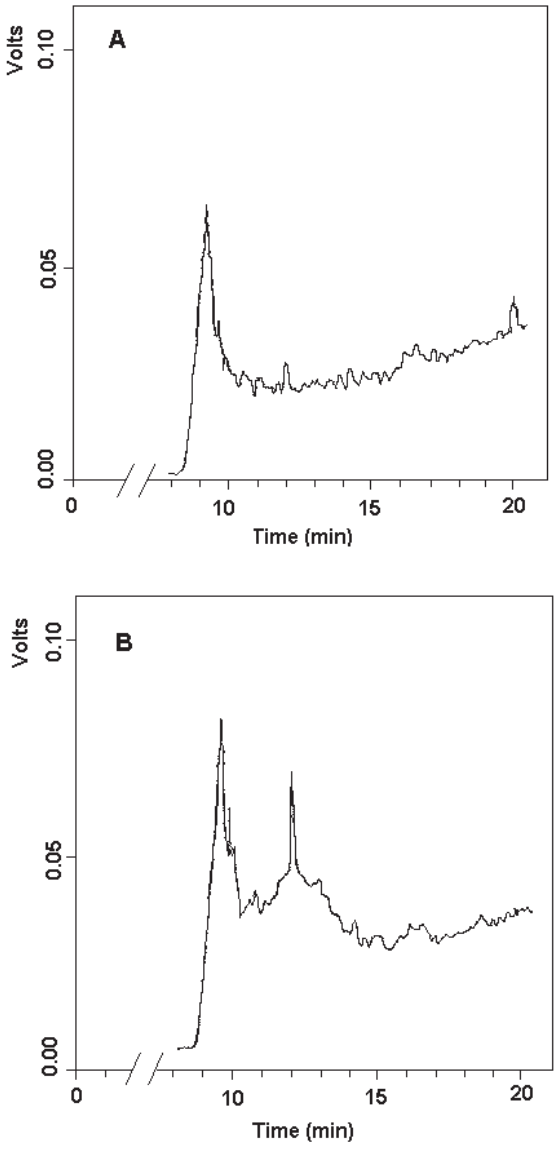

Figure 2. HPLC profiles of CII derived peptide (< 10 kDa) fraction of unchlorinated CII (A) and CII chlorinated with 5 mM $HOCl/OCl^-$ (B).

Fractions of CII chlorinated with 1 and 3 mM $HOCl/OCl^-$ showed the same profile as profile A.

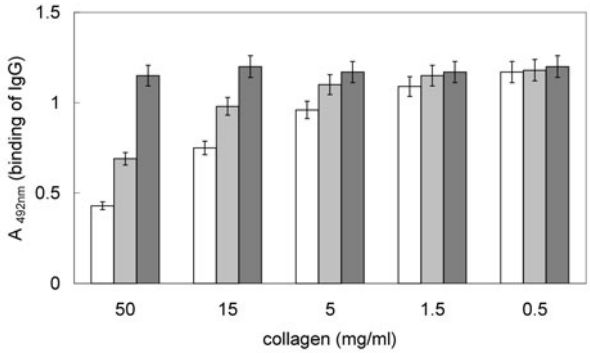

Figure 3. Effect of $HOCl/OCl^-$ modification on immune complex formation.

Competitive ELISA was used. Bars represent: unchlorinated CII (❑) and CII chlorinated with 1 (▒) or 3 (■) mM $HOCl/OCl^-$. The results represent three independent experiments and are expressed as mean ±S.E.M.

The dynamic light scattering method (DLS) was used to monitor changes in CII aggregates in water. The DLS data shown in Table 1 suggest that $HOCl/OCl^-$ chlorination decreased the radius of existing CII structures. Unchlorinated CII micelles had the radius of

30 nm which corresponds to a structure of molecular mass 900–9000 kDa, much higher (10–100 times) than the mass of a single collagen molecule (90 kDa). Although the calcu-

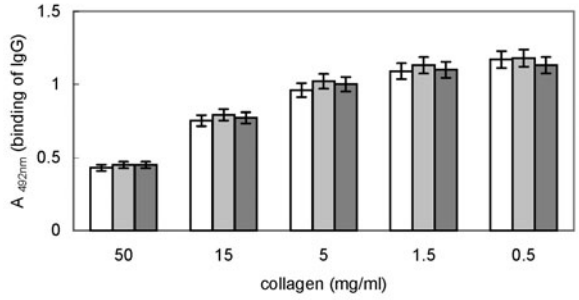

Figure 4. Effect of taurine monochloramine modification on immune complex formation.

Bars represent unchlorinated CII (❑) and CII chlorinated with 1 (▒) or 3 (■) mM taurine monochloramine. Other details as for Fig. 3.

lated mass depends strongly on the assumptions made (globular proteins or pollulans) the hypochlorite mediated relative decrease of the radius is unquestionable. Samples containing chlorinated collagen showed a radius corresponding to the molecular mass 150–200 kDa.

DISCUSSION

In this study we tested the ability of HOCl/OCl^-, a product of activated neutrophils, to modify the structure of collagen type II – the major protein present in joint cartilage. The present data suggest that hypochlorite modifies amino-acid side chains, secondary structure and type of aggregate. Assuming the molecular mass of CII to be approx. 90 kDa one could estimate the dichlorotyrosine and aldehyde content per one collagen molecule. The numbers of dichlorotyrosine moieties as well as of aldehyde groups vary from 0.25 to 2 per collagen molecule in collagen II samples modified with 1–5 mM HOCl/OCl^-. Unlike the stable dichlorotyrosine moieties, aldehydes are easily oxidized with an excess of HOCl/OCl^-. Therefore, due to further oxidation, carbonyl groups were not detected in collagen samples chlorinated with more concentrated HOCl/OCl^- (> 5 mM).

At low hypochlorite concentrations (up to 1 mM) ammonium content seems to be a better marker of lysine group oxidation since carbonyl formation is concomitant with deamination. These results are also supported by the fact that free amino group level in CII samples was not elevated as shown by the 2,4,6-trinitrobenzenesulfonic method (not shown) and are consistent with the suggestion that HOCl/OCl^--modified amino groups are unavailable for reaction with fluorescamine (Davies *et al.,* 1987). At higher HOCl/OCl^- concentrations (3 and 5 mM) ammonium level was decreased which could be interpreted in terms of the ammonium chloramine formation in the presence of HOCl/OCl^- (Table 1). All these facts taken together suggest that collagen is chlorinated with both HOCl/OCl^- and ammonium chloramine and that the higher the initial HOCl/OCl^- concentration, the higher the ammonium monochloramine content in the reaction mixture.

As suggested by the HPLC, SDS/PAGE and CD data, hypochlorite chlorination was followed by CII fragmentation even at low hypochlorite concentrations (less than 1 mM). Unlike in the case of albumin (Olszowski *et al.,* 1996) and fibronectin (Vissers & Winterbourn, 1991), for collagen this tendency seems to prevail over aggregation. The degree of CII fragmentation evaluated from CD measurements was very similar to that calculated from experiments with tritium labelled type II bovine articular collagen (Davies *et al.,* 1993). The data from competitive ELISA showed that HOCl/OCl^- at low concentrations (up to 1 mM) did not affect the formation of immune complexes between chlorinated CII and anti-collagen specific antibodies. This may suggest that the structure of the epitopes was not affected. Nevertheless, lysine and tyrosine modifications in CII molecules were observed. On the other hand, HOCl/OCl^- at higher concentrations (35 mM) significantly diminished

the binding of the antibody due to the fragmentation of CII. In contrast to $HOCl/OCl^-$, taurine monochloramine did not affect CII binding to the antibody. Therefore, these results suggest that taurine may prevent collagen structure deterioration *in vivo*, since taurine monochloramine is formed from hypochlorite at the inflammation site (Weiss *et al.,* 1982).

Our results demonstrating that chlorination was followed by fragmentation, it leads to the conclusion that the structures found in chlorinated CII samples (DLS) are aggregates of chlorinated low molecular CII-derived peptides. However, since physiological hypochlorite or chloramine concentrations are lower (μM) than those used in our model system, only slight (< 10%) collagen degradation could be expected in the synovial fluid. Thus it could be suggested that in the inflammatory process the radius of CII micelles decreases approx. 3–5 times. The components of these modified micelles are probably weakly bound peptides (10–90 kDa) containing carbonyl groups and exhibiting more anionic character than intact collagen (Olszowski *et al.,* 1996). The dramatic change in the size and composition of CII aggregates may influence the properties of the synovial fluid. At present, the biological role of this process is unknown, although, as it has been observed for fibronectin (Olszowski *et al.,* 1994), it is possible that this modification may influence PMN activation in the synovial fluid.

Finally, our data confirm previous observations that chlorination of a protein by $HOCl/OCl^-$ may alter its properties, including structure, susceptibility to proteolysis, immunogenity and pathogenecity (Marcinkiewicz *et al.*, 1991; 1992; Kwaśny-Krochin *et al.*, 2002). However, the effect of $HOCl/OCl^-$ on the protein properties will be different, depending on the exposure of target amino acids in the chlorinated protein (e.g., ovalbumin *vs.* collagen).

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