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IR and NMR Studies on the Action of Hypochlorous Acid on Chondroitin Sulfate and Taurine

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Although it has been recently shown by the use of ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy that enzymatically formed hypochlorous acid ($HOCl/Cl^{\ominus}$) reacts with carbohydrates containing *N*-acetyl groups under the formation of acetate and presumably chloramines, mechanistical aspects of this reaction remain unknown. Since NMR spectroscopy suffers a lot from low sensitivity especially toward polymeric components giving less marked resonances, we used here infrared (IR) spectroscopy to characterize the products of the reaction between hypochlorite and chondroitin sulfate. NMR is used for means of comparison. The reaction of $HOCl/Cl^{\ominus}$ with chondroitin sulfate does not change most regions of the IR spectrum of the polysaccharide markedly. Whereas most vibrational bands remain nearly unchanged toward their position and their relative intensities, an additional band at 975 cm^{-1} is clearly detectable. This band is in good accordance with the proposed formation of a N-Cl-bond in chondroitin sulfate upon HOCl treatment. Additionally, NMR experiments clearly indicate that chloramine formation is accompanied by the formation of acetate. For comparison, taurine (2-amino-1-ethanesulfonic acid) was also investigated toward its reactivity with $HOCl/Cl^{\ominus}$. An analogous band at 975 cm^{-1} was found which unequivocally indicates the formation of a chloramine. NMR also detects changes upon this reaction, but these changes cannot be exactly assigned to chloramine formation. Thus, we conclude that IR spectroscopy is most suitable for the detection of chloramines due to its relatively high sensitivity and the possibility to detect directly N-Cl-groups.

Key Words: hypochlorous acid; chondroitin sulfate; taurine; NMR spectroscopy; IR spectroscopy.

INTRODUCTION

An accumulation of large numbers of polymorphonuclear leukocytes (PMNs) occurs in synovial fluids of patients suffering from rheumatoid arthritis (*1*, *2*). It is a widely accepted fact that PMNs are involved in the damage of articular cartilage in chronic inflammatory diseases.

Neutrophils generate potent reactive oxygen species including $O_2^{\cdot -}$, H_2O_2, $HO^{\cdot}$, HOCl, and $^1O_2$ and release different enzymes (e.g., collagenase, elastase, and mye-

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loperoxidase) upon stimulation (*3*, *4*). Although the contribution of neutrophils to these pathologies is well established, detailed mechanisms of damage, regulations, and defense reactions remain widely unknown.

We have focused our recent interest mainly on neutrophil-derived hypochlorous acid due to its strongly oxidizing properties. HOCl is generated in a myeloperoxidase (MPO)-catalyzed reaction between hydrogen peroxide and chloride anions (*5*, *6*):

$$H_2O_2 + Cl^{\ominus} \rightarrow HOCl + HO^{\ominus}.$$

Myeloperoxidase is present in high concentration in azurophilic granules of neutrophils comprising about 5% of the total protein content in these cells (*7*). Thus, high amounts of HOCl are formed under *in vivo* conditions and concentrations up to 340 μM are discussed to be of physiological relevance (*8*).

The predominant role of myeloperoxidase and its product, hypochlorous acid, in cartilage degradation is clearly reflected by two different experimental results: On the one hand, neutrophils isolated from synovial fluids of patients with rheumatoid arthritis are characterized by a markedly increased native chemiluminescence (*9*), which correlates well with MPO activity, i.e., with HOCl concentration (*9*). On the other hand, cell-free synovial fluids from patients with rheumatoid arthritis show intense ^{1}H NMR signals arising from degradation products of cartilage (oligosaccharides with *N*-acetyl groups and acetate). The integral intensities of these resonances correlate extremely well with the myeloperoxidase activity (*10*).

Unfortunately, detailed studies on the reaction of cartilage polysaccharides with hypochlorous acid and on the detailed chemical mechanisms were only scarcely performed. For example, it has been shown by viscosimetry and HPLC (*11*) that a loss of viscosity of hyaluronic acid solutions is observed at relatively small amounts of hypochlorous acid, whereas higher concentrations lead to a cleavage of the polysaccharide chain under the formation of low-molecular-mass breakdown products. Both effects indicate that complex reaction patterns are involved in these oxidation processes (*11*).

Additionally, the molecular mechanism of the involved reactions has been studied using NMR spectroscopy (*12*, *13*). HOCl clearly damages *N*-acetylglucosamine as well as chondroitin sulfate and hyaluronic acid *in vitro*. Two different effects have been observed: a breakdown of large polymers, such as hyaluronic acid, into smaller oligomeric units, and a simultaneous reaction of HOCl with *N*-acetyl side groups of these carbohydrates to yield acetate as final product via a transient chlorinated product (*12*). Only in the presence of extremely high concentrations of hypochlorous acid are further oxidation products like formate detectable (*13*). On the basis of these investigations, the following model has been proposed (Fig. 1). Carbohydrates with *N*-acetyl side chains are chlorinated in a first step at the N-H-amide group. This transient product is hydrolyzed in a second step to the corresponding chloramine and acetate (*12*, *13*).

Although this mechanism is consistent with experimental results, two important drawbacks of NMR spectroscopy must be considered: First, NMR is not suitable for detection directly of the presence of chlorinated products, and NMR spectroscopy suffers a lot from its relatively low sensitivity. Unfortunately, other spectro-

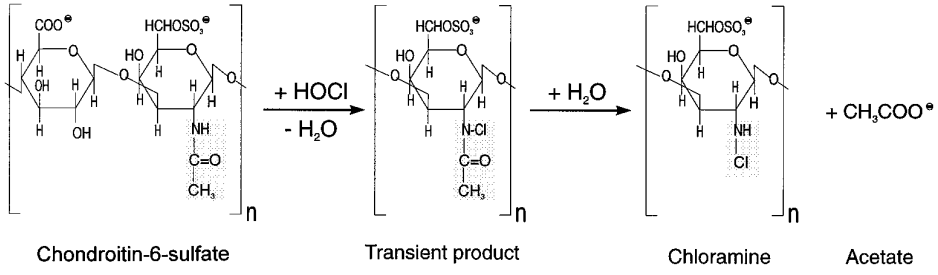

FIG. 1. Proposed mechanism of the reaction between chondroitin sulfate and hypochlorous acid under the formation of a transient product, which is gradually depleted to a chloramine and acetate. Groups of interest are emphasized.

scopic methods such as the detection of chloramines by their strong UV absorption at about 254 nm (*14*) are influenced by the absorption of the carbohydrates (*14*).

On the other hand, infrared spectroscopy should provide a considerably higher sensitivity compared to NMR and should clearly monitor the formation of chloramines due to its high sensitivity toward strongly polar functional groups (*15*).

The present paper deals with the examination of pathways of the reaction of hypochlorite with N-acetylated polysaccharides. These experiments are compared with the reactions of taurine (2-amino-1-ethanesulfonic acid). Taurine, as a simple amino-group-containing component, is well characterized toward its ability to form chloramines (*16*). It is shown that the performed experiments are in good agreement with the previously postulated pathway and that IR spectroscopy is most suitable for the detection of chloramines.

MATERIALS AND METHODS

Chemicals

All chemicals (NaCl, Na_2HPO_4, KH_2PO_4, chondroitin sulfate from bovine trachea, and taurine) were purchased from Fluka Feinchemikalien GmbH (Neu Ulm, Germany) of analytical grade and used without any further purification.

Sodium hypochlorite (as source for HOCl) was from Sigma Chemie (Deisenhofen, Germany). A stock solution of NaOCl was prepared in water. Its concentration was determined spectrophotometrically at pH 12 (ε_{290} = 350 liters mol^{-1} cm^{-1}) each day prior to use (*17*).

Incubation Conditions

All solutions were freshly prepared in 50 mmol/liter phosphate buffer, pH 7.4. Under these conditions, HOCl solutions consist of a nearly 1 : 1 mixture of the acid (HOCl) and the corresponding salt (p*K* 7.53) (*17*). Taurine was used just like the chondroitin sulfate as a 50 mmol/liter solution. In the case of chondroitin sulfate

the molecular mass of one repeating unit (sodium glucuronate and monosulfated *N*-acetylgalactosamine) was used to calculate the molar concentrations (*13*) of the polysaccharide.

Solutions were subsequently incubated in the presence of a 1:1 molar ratio of hypochlorous acid for 2 h at 37°C in a water bath. It has been previously shown by time-dependent NMR spectroscopic investigations that an incubation of 2 h gives most expressed changes (*12*). After this period 400 μl of incubation solutions was used for NMR spectroscopy and the remaining solution was concentrated in a vacuum centrifuge (Jouan, Germany) for infrared spectroscopy.

UV Measurements

UV measurements were performed to obtain information on the extent of chloramine formation ($\lambda \sim 250$–255 nm) (*14*) and the residual content of $HOCl/ClO^{\ominus}$ ($\lambda = 290$ nm) (*17*). These measurements were performed on an Hitachi U-2000 photometer, using quartz cuvettes.

NMR Measurements

NMR measurements were conducted on a Bruker AMX-300 spectrometer operating at 300.13 MHz for ^1H. All spectra were recorded at ambient temperature (25°C). These conditions were the best compromise between enhanced resolution at higher temperature and diminished denaturation of carbohydrates at lower temperature.

Typically 0.40 ml solution was placed in a 5-mm-diameter NMR tube and 50 μl of D_2O was added to provide a field-frequency lock. The intense water signal was suppressed by the application of a 2-s presaturation pulse at the water resonance frequency (*10*).

Thirty-two free induction decays were usually accumulated with a pulse delay of 8 s between two pulses to allow full T_1 relaxation. No line broadening or Gauss broadening was used. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propane-1-sulfonate (TSP) added in a final concentration of 5 mmol/liter.

Infrared Spectra

Concentrated solutions from incubation experiments with hypochlorous acid were spread on one side of a ZnSe attenuated total reflection (ATR) crystal which was mounted on a commercially available horizontal Benchmark unit (Specac, UK). Infrared spectra were measured using a Bio-Rad FTS-60a Fourier-transform infrared spectrometer equipped with a deuterated triglycine detector. Typically, 128 scans were accumulated. Absorbance spectra of the sample were calculated using the respective single-channel spectra of the empty ATR crystal (without sample) as background. Selected absorption bands are characterized in terms of the wavenumber at maximum absorbance (*18*).

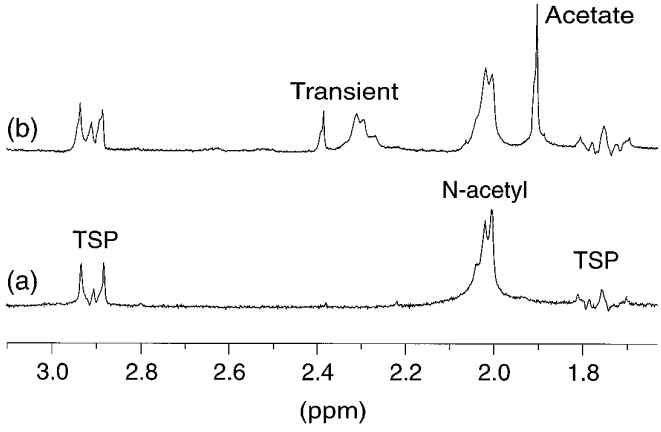

FIG. 2. Proton–NMR spectrum of an aqueous solution of chondroitin sulfate prior (a) and after (b) treatment with an equimolar quantity of sodium hypochlorite (b). The weak resonances at 1.76 and 2.91 ppm result from the standard sodium 3-(trimethylsilyl)-propane-1-sulfonate. Abbreviations used in peak assignment: TSP, standard; *N*-acetyl, mobile *N*-acetyl groups.

RESULTS

Chondroitin Sulfate and Hypochlorous Acid

Since NMR spectroscopy is a powerful tool for the detection of molecular changes in aqueous solutions, chondroitin sulfate samples were analyzed by NMR spectroscopy in the presence (1 : 1 molar ratio) or absence of HOCl (Fig. 2).

The ^{1}H NMR spectrum of an aqueous solution of chondroitin sulfate prior to the addition of hypochlorous acid is given in Fig. 2a. With the exception of the resonances of the TSP standard (1.76 and 2.91 ppm), only a single, broad resonance with two maxima at 2.005 and 2.020 ppm can be detected (*12*). The two maxima are most likely attributable to the isomeric forms of the polysaccharide, chondroitin 4- and chondroitin 6-sulfate. Both isomers are present in nearly equal amounts in commercially available chondroitin sulfate (*19*) resulting in two resonances of comparable intensity for the protons of the *N*-acetyl side group. These resonances are much broader than observed for the monomers, e.g., *N*-acetylglucosamine, because of the high molecular weight of the polymer conferring restricted mobility of the protons in the sample (*12*). Since the *N*-acetyl side chain is by far the most mobile functional group of chondroitin sulfate, C-H protons of carbohydrate rings are only scarcely detectable between 3.3 and 4.0 ppm (data not shown).

The remaining O-H protons from the sugar ring and the N-H proton from the *N*-acetyl side chain are quickly exchanged with the solvent (a H_2O/D_2O mixture) and are not detectable under our experimental conditions using presaturation of the water resonance. Although the use of deuterated dimethyl sulfoxide (*20*) is useful, because it hinders the protons from exchanging, it is not the solvent of choice due to its unphysiological properties.