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Original Contribution

Reaction of superoxide radicals with glycosaminoglycan chloramides: a kinetic study

B.J. Parsons [a,*], S. Sibanda [a], D.J. Heyes [b], A.W.J. Paterson [a]

[a] Faculty of Health and Social Sciences, Leeds Metropolitan University, Leeds LS1 3HE, UK
[b] Faculty of Life Science, Manchester Institute of Biotechnology, Manchester M1 7DN, UK

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ABSTRACT

Hypochlorous acid and its acid–base counterpart, hypochlorite ions, produced under inflammatory conditions, may produce chloramides of glycosaminoglycans, perhaps through the binding of myeloperoxidase directly to the glycosaminoglycans. The N–Cl group in the chloramides is a potential target for reducing species such as Cu(I) and superoxide radicals. Laser flash photolysis has been used here to obtain, for the first time, the rate constants for the direct reaction of superoxide radicals with the chloramides of hyaluronan and heparin. The rate constants were in the range 2.2–2.7 $\times$ 10^3 M^{-1} s^{-1}. The rate constant for the reaction with the amino acid taurine was found to be much lower, at 3.5–4.0 $\times$ 10^2 M^{-1} s^{-1}. This demonstration that superoxide anion radicals react directly with hyaluronan and heparin chloramides may support the mechanism first proposed by M.D. Rees et al. (*Biochem. J.* **381**, 175–184, 2004) for an efficient fragmentation of these glycosaminoglycans in the extracellular matrix under inflammatory conditions.

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Introduction

The extracellular matrix (ECM) is made up of huge multimolecular complexes with arrays of link proteins and aggrecan molecules along a central hyaluronan backbone. Hyaluronan (HA) is bound by a number of ECM and cell surface proteins. These HA-binding proteins are termed hyaladherins and share a homologous HA-binding domain with the link protein, which is referred to as the link homology domain [1]. Examples include aggrecan, versican, and tumor necrosis factor α-stimulated gene 6 [2].

With this central structural function, HA is a particularly important component of the ECM [3,4], as demonstrated by the fact that a hyaluronan synthase-2 knockout is embryonically lethal in mice [5]. HA also provides a hydrated environment [6] for growing, moving, and renewing cells and tissues [7], activates signaling events in cells, and is involved in moderating many cellular processes, including proliferation, migration, adhesion, and apoptosis [8–11]. HA seems to have a range of significant biological functions dependent upon its molecular mass. Large-molecular-mass fragments are involved in space-filling and immunosuppressive roles, whereas smaller HA fragments have been shown to be proinflammatory and angiogenic; oligosaccharides may be involved in cell signaling (reviewed in [12]).

Oxidative damage of the extracellular components by either enzymatic or nonenzymatic pathways may have implications for the initiation and progression of a range of human diseases. These include arthritis, kidney disease, cardiovascular disease, lung disease, periodontal disease, and chronic inflammation. Oxidative damage to hyaluronan by reactive oxidative species, and in particular free radicals, has received much attention, largely through the ease of monitoring its fragmentation using viscometric techniques, which are reviewed in [13]. The potential mechanism of oxidative damage to the ECM and its role in human pathologies have also been discussed in a recent review [14].

Our previous studies on HA have measured HA fragmentation yields as a proportion of quantifiable fluxes of free radicals produced by ionizing radiation. For this purpose, both viscosity changes and a combination of gel-permeation chromatography with multiangle laser light scattering have been used to measure changes in the molecular weight of the polydispersed hyaluronan. In this way, the efficiencies of fragmentation of HA by a range of free radicals and reactive oxidative species, including hydroxyl radicals, carbonate radicals, dibromide and dichloride radical anions, and peroxynitrite, have been determined [15,16].

The fragmentation of hyaluronan and other glycosaminoglycans has also been investigated intensively by Davies and coworkers using both electron paramagnetic resonance (EPR) spectroscopy and sensitive PAGE techniques. The use of the latter technique showed the novel and potentially biologically significant result that peroxynitrous acid, carbonate, and hydroxyl radicals

* Corresponding author.
E-mail address: b.parsons@leedsmet.ac.uk (B.J. Parsons).

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react largely in a site-specific process to produce an array of HA fragments, in a "ladder-type display," each separated from its neighbor by the molecular mass of the repeating disaccharide unit in HA, thus mimicking to a significant extent the action of the enzyme hyaluronidase [17,18]. Similar site-selective fragmentation was also observed when glycosaminoglycan chloramides (formed through reaction with hypochlorite) were reduced by copper (I) ions and superoxide anion radicals [19,20].

The formation of chloramides and chloramines from the reaction of hypochlorite with amides and amines, respectively, was demonstrated in an early study [21] and is suggested to be a key process in inflammation, in which hypochlorite (from myeloperoxidase) may produce glycosaminoglycan chloramides. Such derivatives may accelerate the fragmentation of glycosaminoglycans within the ECM [22]. Chloramides are weak oxidizing agents and are therefore potential biological targets for reducing radicals and other reducing agents. Indeed, it has been shown that superoxide radicals cause the fragmentation of HA via reaction with its chloramide derivative [19].

Strong reducing agents such as the hydrated electron, e^-_{aq}, may be expected to react rapidly with chloramines and chloramides. Pulse radiolysis studies of the simplest chloramine, NH_2Cl, showed that the hydrated electron reacts with it at diffusion-controlled rates ($k = 2.2 \times 10^{10}\,M^{-1}\,s^{-1}$) [23,24]. In more recent pulse radiolysis studies of chloramines and chloramides of amino acid derivatives [25] and of chloro and bromo derivatives of model compounds, such as *N*-bromoglutarimide (NBG) and *N*-bromosuccinimide (NBS) [26], hydrated electrons were also shown to react at near-diffusion-controlled rates. In the latter study, superoxide radicals were also reacted with NBG and NBS and found to follow complex chain reaction pathways. In the case of *N*-chlorosuccinimide, a direct kinetic measurement could be made, yielding a rate constant of $8 \times 10^5\,M^{-1}\,s^{-1}$. An indirect method based on EPR data has also allowed an estimate of the rate constant for the reaction of superoxide radicals with taurine monochloramine to be made ($k = 5–6 \times 10^2\,M^{-1}\,s^{-1}$) [27].

It seems clear, therefore, that there is no direct, unambiguous kinetic measurement of the rate constants for the reaction of superoxide radicals with the centrally important chloramides of key glycosaminoglycans of the ECM. Consequently, in this work we have used the laser flash photolysis technique to measure rate constants for the chloramide derivatives of both hyaluronan and heparin, the latter being a model for sulfated glycosaminoglycans of the ECM.

Materials and methods

Materials

The following materials were purchased as indicated: hyaluronan (Novozymes); heparin (Alfa Aesar); hypochlorite solution, taurine (Sigma–Aldrich); and superoxide dismutase from bovine erythrocytes, >3 kU/mg protein (Sigma–Aldrich). All other reagents were of analytical grade.

Preparation of chloramides and chloramines

The chloramide derivative of hyaluronan (HACl) was produced by reaction of 4 mg/ml (10 mM in disaccharide units) HA in Chelex-treated 0.1 M phosphate buffer, pH 8.5, with 9 mM hypochlorite for 300 min at 37 °C. The reaction was monitored at 292 nm to ensure that there was less than 0.3 mM unreacted HOCl, which was then quenched by the addition of 2 mM taurine (conditions leading only to taurine monochloramine formation as confirmed by its characteristic absorption maximum at 251 nm), followed by extensive dialysis in 9.5 mM borate buffer.

The HACl concentration was confirmed by 5-thio-2-nitrobenzoic acid (TNB) assay. In this assay, a solution of TNB was prepared by hydrolysis of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 50 mM NaOH through gentle stirring in a dark container until the yellow color development reached a maximum, which took about 5 min. This TNB solution was diluted 40 times in 0.1 M phosphate buffer (pH 7.4) before any assay and added to 10–25 μl of the chloramides to be assayed. These samples were then incubated in the dark at room temperature for 15 min, after which absorbances were read spectrophotometrically at 412 nm and concentrations of chloramides measured using an extinction coefficient for TNB ($\varepsilon_{412} = 14{,}100\,M^{-1}\,cm^{-1}$). In this reaction 1 mol of chloramides oxidizes 2 mol of TNB to 1 mol of DTNB [21].

Typical stock solutions prepared in this way were stored at 4 °C and contained 3.4 mM HACl + 1.6 mM HA.

The chloramide derivative of heparin (HepCl) was synthesized from the reaction of 6.4 mg/ml heparin (10 mM in disaccharide units) hydrated in Chelex-treated 0.1 M phosphate buffer, pH 6.5, with 9 mM hypochlorite for 180 min at 37 °C. The unreacted hypochlorite (less than 0.1 mM) in the reaction was quenched by addition of 1 mM taurine (conditions again ensuring the formation of the monochloramine only). The taurine monochloramine that was formed was removed by extensive dialysis with 10 mM borate buffer (pH 9.5). HepCl was then assayed using TNB to confirm its concentration. Typical stock solutions prepared in this way were stored at 4 °C and contained 6.8 mM HepCl + 2.2 mM Hep.

For solution preparation for the laser flash photolysis experiments, all dilutions were carried out in Chelex-treated borate buffer (pH 9.5) and contained 5 μM EDTA. The sodium formate and sodium persulfate stock solutions were also prepared in Chelex-treated borate buffer (pH 9.5). In all the experiments, the reagents were added just before any saturation with oxygen or air, with the exception of the cytochrome *c* competition experiments in which the cytochrome *c* was added immediately before laser excitation to prevent possible cytochrome *c* oxidation by persulfate.

Laser flash photolysis

For laser photoexcitation experiments, 1-ml samples were excited using the fourth harmonic (266 nm) of a Q-switched Nd-YAG laser (Brilliant B; Quantel) in a 1-ml quartz cuvette of 1-cm path length. The energy output of each laser pulse was approximately 40 mJ and pulses were 6–8 ns in duration. Data were collected using an Applied Photophysics LKS-60 flash photolysis instrument with detection system at right angles to the incident laser beam. The probe light (150-W xenon lamp) was passed through a monochromator before and after passage through the sample. Absorbance changes were measured using a photomultiplier tube and kinetic transients were typically collected over 200 ms. For measurements over faster time scales (typically <1 ms), the output of the xenon arc lamp was pulsed using a xenon arc pulser (Applied Photophysics) and transients were measured using an Infiniium oscilloscope Model 54830B (Agilent Technologies).

Results

Formation of superoxide radical using laser flash photolysis

Laser flash photolysis was used to produce the superoxide radical in yields of up to 4.1×10^{-5} M. This was achieved by excitation, at 266 nm, of solutions containing 30 mM persulfate ($S_2O_8^{2-}$) and 10–50 mM formate ($HCOO^-$) in the presence of either

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