Metal Catalysis in the Depolymerization of Hyaluronic Acid by Autoxidants[1]

M. J. Harris, A. Herp,* and W. Pigman

Contribution from the Department of Biochemistry, New York Medical College, New York, New York 10029. *Received March 6, 1972*

Abstract: Investigation of the L-ascorbic acid induced depolymerization of hyaluronic acid in phosphate buffer revealed that the oxidative effect of the L-ascorbic acid is largely if not exclusively due to metal catalysis; the metal catalysts in the reaction medium are provided by the metal contamination in both the phosphate buffer and the hyaluronic acid. The function of ascorbic acid in such a system is to maintain the iron ions in the ferrous form by reduction of the inactive ferric ions produced in the course of the oxidative reaction. Cleansing of the various reaction components by means of the chelating resin Chelex 100 drastically reduces the rate of depolymerization of the polysaccharide. The possible implication of the reaction mechanism in biological systems is briefly discussed.

Numerous studies in our laboratory and by other workers have shown that L-ascorbic acid can depolymerize high molecular weight polymers such as hyaluronic acid and alginic acid[2,3] and act as a hydroxylating agent in the presence of iron ions.[4] Ferrous and cuprous ions alone cause similar depolymerizations at millimolar concentrations; however, at micromolar concentrations they accelerate the rate of depolymerization by ascorbic acid, but are relatively inactive alone.[5] We have recently shown that millimolar concentrations of iron are effective if the reaction is carried out in an electrolytic cell in which ferric ions are reduced at the cathode to ferrous ions.[6] These results suggest that ascorbic acid acts to regenerate ferrous ions by the following mechanism.

$$\text{ascorbic acid} + 2\text{Fe(III)} \longrightarrow \text{dehydroascorbic acid} + 2\text{Fe(II)}$$

$$4H^+ + 4\text{Fe(II)} + O_2 \longrightarrow 4\text{Fe(III)} + 2H_2O$$

$$\text{hyaluronic acid} + \text{Fe(II)} + O_2 \longrightarrow \text{Fe(III)} + \text{depolymerized hyaluronic acid}$$

The present work was undertaken in order to determine whether L-ascorbic acid can carry out the previously observed actions alone or whether trace amounts of iron ("impurities") are involved in the above mechanism. For this purpose, a chelating resin (Chelex 100) was used to treat the solutions involved. Poyer and McCay[7] reported that the exposure of a 0.15 *M* solution of phosphate buffer to Chelex 100 reduced its Fe(III) concentration from 2×10^{-6} to 5.5×10^{-8} *M*. Consequently, the buffer and all solutions, including those for rinsing of glassware, were treated with the resin.

Experimental Section

Sodium mono- and dibasic phosphates were mixed for the preparation of phosphate buffer (pH 7.2). Chelex 100 resin mesh size 200–400 (Bio-Rad Laboratories, Richmond, Calif.) regenerated in the hydrogen form was shaken batchwise with the phosphate buffer. The buffer solution was separated, and the operation repeated. The resin was rejected, and the pretreated phosphate buffer applied to a column of fresh Chelex 100 (H^+ form) until the pH of the influent was equal to that of the effluent. At this point, the purified buffer solution was collected for further use. Chelex 100 resin taken from the lower part of such a column was used for treating glass-distilled water and other reagents. After the resin had been stirred with each of the reagents, it was separated by centrifugation, or where appropriate, by filtration. For some experiments, resin-treated water was employed for rinsing all of the glassware used.

Hyaluronic acid was prepared by the procedure described previously.[8] To eliminate contaminating metals from the hyaluronic acid, samples of the polymer solution were stirred with Chelex 100 (Na^+ form) overnight at 4° and freed from the resin by centrifugation. A small though perceptible loss of viscosity was always associated with this treatment.

Solutions of hyaluronic acid were prepared for investigating the oxidative–reductive depolymerization and their viscosities were measured as described elsewhere.[9] The final incubation mixture generally contained 0.4 mg/ml of hyaluronic acid in 0.2 *M* phosphate buffer, pH 7.2, and 0.33 m*M* L-ascorbic acid.

Results

Prior treatment of all constituents of the reaction mixture with Chelex 100, that is L-ascorbic acid, hyaluronic acid, phosphate buffer, and water, produced a significant reduction in the rate of depolymerization of hyaluronic acid as compared with a control consisting of untreated solutions (Figure 1). By preparing reaction mixtures containing one purified reagent at a time, the major sources of catalytic contamination were traced to the phosphate buffer and to the hyaluronic acid.

To the reaction mixture whose constituents (hyaluronic acid, phosphate buffer) had been pretreated with Chelex 100, various quantities of catalyst were introduced: cupric sulfate, ferrous sulfate, or a combination of both. *Copper ions* in concentrations ranging from 0.25 to 2.0 μM were found to augment the rate of depolymerization of the polysaccharide as illustrated in Figure 2. The change in specific fluidity, $\Delta\phi$, over the first 10 min was linear with the amount of metal added (Figure 3). Iron was found to exert a similar effect as copper. Addition of iron (as $FeSO_4 \cdot 7H_2O$) in a concentration of 5 μM to the purified reaction mixture leads to a rate of depolymerization of the polymer that

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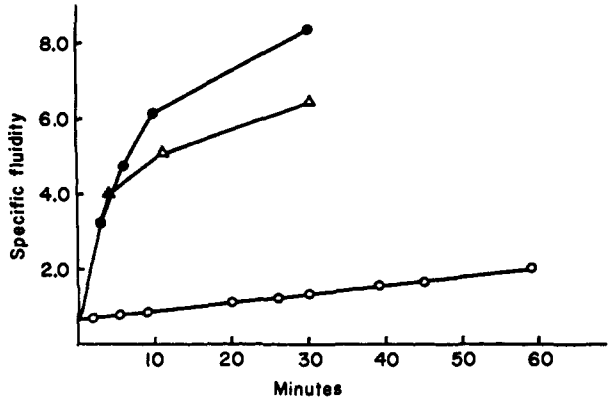

Figure 1. Depolymerization of hyaluronic acid (0.4 mg/ml) by L-ascorbic acid (0.33 mM) in 0.2 M phosphate buffer, pH 7.2: ●, untreated reaction medium; ○, Chelex 100 treated medium; △, Chelex 100 treated solutions, followed by addition of 5 μM $FeSO_4$.

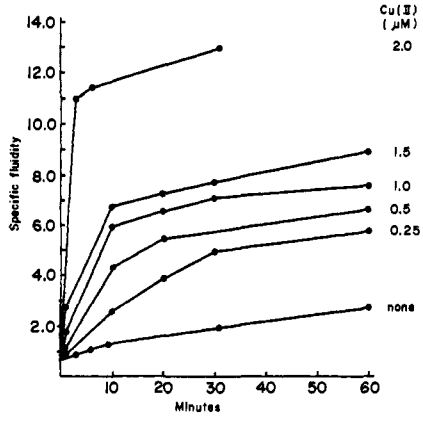

Figure 2. Depolymerization of hyaluronic acid (0.4 mg/ml) in phosphate buffer (0.2 M) at pH 7.2 by a combination of L-ascorbic acid (0.33 mM) and copper (concentration as indicated). Reagents were prepurified by treatment with Chelex 100.

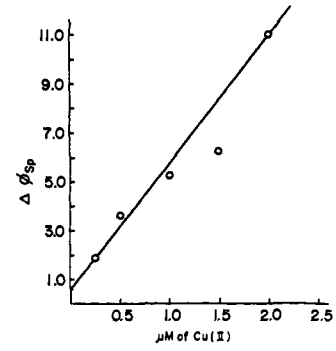

Figure 3. Effect of copper ions on the depolymerization of hyaluronic acid by L-ascorbic acid (0.33 mM) in 0.2 M phosphate buffer, pH 7.2. For details, see text.

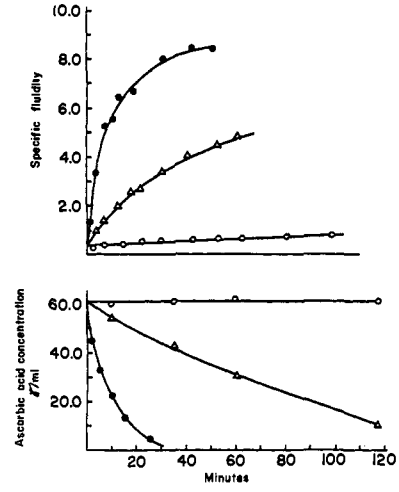

Figure 4. Depolymerization of hyaluronic acid (0.4 mg/ml) by L-ascorbic acid (0.33 mM) in phosphate buffer (0.2 M, pH 7.2) and autoxidation of L-ascorbic acid (0.33 mM) in the same buffer: ●, untreated buffer; △, Chelex 100 pretreated buffer; ○, Chelex 100 in contact with buffered L-ascorbic acid solution.

is close to that obtained for the unpurified system shown in Figure 1. This concentration of iron corresponds to the maximum level of contamination that can be expected for untreated phosphate buffer (0.2 M) according to the analytical specifications quoted for the commercial reagent.

When 0.033 mM L-ascorbic acid was used instead of 0.33 mM, the qualitative effect was the same: increases in the concentration of added copper or iron ions progressively accelerated the depolymerization.

Although "spontaneous" depolymerization of hyaluronic acid in the presence of L-ascorbic acid is still encountered in prepurified solutions (Figure 1), maintaining a suspension of Chelex 100 in contact with the reaction medium further reduced the rates of depolymerization. As this might have been due to a possible scavenging capacity of the suspended resin or to its sequestering activity upon metal ions originating from the atmosphere or the apparatus, the succeeding experiment was devised. A solution of hyaluronic acid in phosphate buffer was pipetted into a dialyzer membrane and equilibrated for 24 hr against a suspension of Chelex 100 in phosphate buffer. The whole mixture was thermostated at 30° and the solution stirred magnetically. L-Ascorbic acid (2 mM) was introduced outside of the dialyzer membrane, and the depolymerization of hyaluronic acid within the membrane was followed as a function of both the time and the concentration of L-ascorbate diffusing into the membrane. The rate of depolymerization was decreased and was similar to that obtained when measurements were conducted with the Chelex 100 *in situ*.

The progressive addition of ethylene(dinitrilo)tetraacetic acid (EDTA) to the system containing Chelex 100 leads to faster rates of reaction until the rate is that observed in the absence of the cleansing resin. This is attributable to competitive chelation of iron ions by EDTA which leads to the formation of a soluble complex that is catalytically active,[10] unlike copper ions which form an inactive complex with EDTA.[5] These observations underline the importance of iron catalysis in the hyaluronic acid–ascorbic acid system.

When EDTA is added to an unpurified reaction mixture containing *N,N*-diethyldithiocarbamate, a strong inhibitor of the ascorbic acid induced depolymerization of hyaluronic acid,[11] the rate of reaction is only partially restored. Possibly then, the mechanism of inhibition by *N,N*-diethyldithiocarbamate results from the com-

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bined effects of free-radical scavenging and metal chelation. Pretreatment with resin results in the stabilization of L-ascorbic acid solutions in phosphate buffer at pH 7.2 and decreases the loss of viscosity of hyaluronic acid, as shown in Figure 4. The amount of L-ascorbic acid present was determined by the 2,6-dichloroindophenol method.[12] In the presence of solid resin, a loss of L-ascorbic acid was barely detectable, and the rate of depolymerization was very slow.

Discussion

Depolymerization of hyaluronic acid and autoxidation of L-ascorbic acid proceed only slowly in solutions that have been rigorously purified by sequestering resin (Chelex 100), and the reaction in untreated solutions is thus dependent on metal catalysis brought about by the impurities that are present in commercial phosphate buffer and in hyaluronic acid solutions. Thus, at 0.2 *M*, commercial phosphate salts contain up to 5 μM iron; in addition, hyaluronic acid binds iron, a property used in the histochemical localization of mucopolysaccharides.[13]

When Chelex 100 is kept in contact with, rather than separated from, the reaction milieu, the rate of autoxidation of L-ascorbic acid and of the concomitant depolymerization of hyaluronic acid is drastically reduced. This effect may possibly arise by prevention of an accumulation of metal impurities that originate either from the atmosphere or that are leached from the glassware by the phosphate buffer.

The rates of depolymerization of hyaluronic acid, as measured by specific fluidity, are directly proportional to the quantity of catalyst for the first 10 min of the reaction period.[5] A similar relationship was observed by Humphreys and Howells[14] for the degradation of alginate by γ-irradiation. The level of copper ions required for measurable increase in the rate of depolymerization of hyaluronic acid (about 0.25 μM) represents 16 ppb of copper ions in water compared to 59 ppm for L-ascorbic acid (0.33 m*M*).

The involvement of metal catalysts in these autoxidative reactions is in agreement with studies on the degradation of citrate by L-ascorbate in Krebs–Ringer phosphate.[15] However, in that study, EDTA was observed to inhibit the ascorbate-induced degradation of citric acid, whereas its presence was shown to aid catalysis in this investigation. The anomaly may perhaps be explained by alternative reliance on iron catalysis, which will be aided by EDTA, as opposed to copper catalysis, which will be inhibited by EDTA.[5] The activity of the iron–EDTA complex in this system for extensively depolymerizing simple and complex carbohydrates has recently been indicated.[16]

It is clear that most if not all previous studies of the oxidation effects of L-ascorbic acid have involved metallic catalysis, presumably by iron or copper ions. The function of the ascorbic acid in such systems is to regenerate the ferrous or cuprous ions which are the actual catalysts. Presumably, this action takes place in biological systems,[17,18] but, since other biological substances and other isomers of L-ascorbic acid are active *in vitro* but not *in vivo*, some sites of action would appear to involve stereoisomeric components such as enzymes.

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Communications to the Editor

A Laser Raman Spectroscopic Study of the Effect of Solvation on the Conformation of Ribonuclease A

Sir:

Lyophilization, freeze drying, is a common method of reducing protein solution to dryness. Most proteins are not denatured by this process and may be stored in the dry state for long periods of time without deterioration of the sample. It is believed tht part of the water is still tightly bonded to protein molecules in the powder and cannot be released even on drastic drying.[1] The major change in this process is the removal of unbonded and loosely bonded water molecules from the frozen sample and the replacement of a certain number of protein-solvent contacts by protein-protein contacts. However, it is not known whether the removal of water from part of the molecular surface may have an effect on the conformation of a protein. In this communication, we wish to present the Raman spectra of a model protein, ribonuclease A, in the powder form and in aqueous solution. These spectra will be compared quantitatively and the spectral differences will be interpreted in terms of main chain and side chain conformational changes.

The Raman spectra of RNase A in solution were first obtained and interpreted by Lord and Yu[2] in 1970. The powder spectra were not reported at that time because of the experimental difficulties associated with high background scattering. Recently, Koenig[3] obtained a preliminary Raman spectrum of the same protein in the solid state and compared it to that in solution. He noted that the Raman lines of the aqueous solution spectra are sharper than the lines of the solid spectra. In Figure 1 we present our higher resolution Raman spectra of RNase A in the powder form and in

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