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Short communication

Capillary electrophoresis of the electrochemical oxidation products of Ng-hydroxy-L-arginine at physiological pH

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Abstract

Ng-hydroxy-L-arginine has been described as an intermediate in the nitric oxide pathway. Its electrochemical oxidation was studied by capillary electrophoresis using either electrospray ionisation mass spectrometry or diode array detection. Three stable end products and unstable intermediates were found using these techniques. These results permitted to propose an electrochemical oxidation scheme of this molecule.

Keywords: Ng-hydroxy-arginine; Oxidation; Capillary electrophoresis

1. Introduction

Ng-hydroxy-L-arginine (NHA) has been described as an intermediate in the nitric oxide pathway since its discovery by high performance liquid chromatography fourteen years ago [1]. Its chemical and enzymatic oxidation has been extensively studied, but its oxidation products remained to be studied. Oxidation products of Ng-hydroxy-guanidine were a model for NHA oxidation and were well documented [2]. In an attempt to detect molecules generated through this oxidation and to propose a chemical scheme, capillary electrophoresis was used with two different detection modes (electrospray ionization mass spectrometry and diode array) after electrochemical oxidation of NHA. This type of oxidation was selective of a chemical function depending of the oxidation potential of the electrode surface. Here the NHOH group of NHA was selectively oxidized [3].

2. Materials and methods

2.1. Electrochemical oxidation

Electrochemical oxidation was performed in a commercial microcell of carbon rod (0.141 cm^2, Tacussel, France). Reference and auxiliary electrodes were Ag/AgCl and platinum, respectively. The volume of the microcell was 50 μL. The solution was in a closed box avoiding light during electrolysis at room temperature (20 °C) [3]. The potentiostat (PRG5, Tacussel, France) was used at +1.2 V versus Ag/AgCl during 1 h. Fifty microlitres of NHA (10 g L^{-1}) in PBS (pH 7.4, 0.3 M) was used for electrolysis. Oxygen was absent after argon bubbling of the parent solution. In another experiment 1–3-cyclohexadiene (10 μL in formamide 50/50) was introduced in NHA solution before electrolysis to test nitroso-molecules formation by Diels Alder reaction [4].

2.2. Capillary electrophoresis

2.2.1. Detection by electrospray mass spectrometry

The technique of capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESIMS) was precedently

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used for amino acids [5]. The procedure used for NHA oxidation was identical. Briefly, experiments were performed on a P/ACE system 2100 (Beckman Coulter, France) coupled to a Quattro II (Micromass UK, Altrincham, UK) tandem quadrupole mass spectrometer. Capillaries were fused-silica (50 μM i.d., 105 cm long). Buffer was 50 mM formic acid (pH 2.5). Voltage was set at 30 kV and capillary electrophoretic migration was assisted by application of an overpressure (0.5 psi). Samples were introduced hydrodynamically (10 s). The sheath solvent was pumped at a flow-rate of 10 μL/min (50:50 water–MeOH mixture with 0.1% formic acid). Nitrogen gas flowed to the probe tip through a nebulizer capillary at 35 L/h. The ESI source was operated in the positive mode with 3.2 kV to the probe tip with a 20 V cone voltage. The source was at 80 °C. For full-scan mass spectra the acquisition time was 60 s over the range *m/z* 70–350 with a 1 s scan time at unit mass resolution. For MS–MS spectra, the collision gas was argon at $3.2.10^{-3}$mbar with 20 eV collision energy.

2.2.2. Detection by photodiode array

Capillary electrophoresis was performed with a P/ACE 5510 (Beckman-Coulter, France) equipped with a photodiode array detector in a fused-silica capillary (75 μM i.d., 27 cm). The electrolytic buffer was formic acid/formate (25mM, pH 2.5) with lauryl sulfate (5%). The potential was set at 30 kV and the detector was set at 200 and 230 nm with 3D acquisition (190–600 nm). Electrokinetic injections were realized (10 kV, 10 s).

3. Results and discussion

Theoretical electrochemical oxidation of NHA has been studied by cyclic voltammetry many times. It indicated an oxidation in two steps giving an iminoxyl radical species with an half-life of μs which was followed by generation of NA, an unstable nitroso compound with an half-life of a few seconds (see Fig. 1) [3]. CE-ESIMS permitted to follow the nature of the stable oxidation products of NHA. Three major products were observed (Fig. 1). The major product at 10.87 min with a mass of 176 was identified as L-citrulline. Using MS–MS the principal fragments were 159, 113, 70 which were found using L-citrulline standard. Unmodified NHA was at 7.23 min with 191 for its mass and fragments were 175, 158, 116, 76, 70. Two oxidation

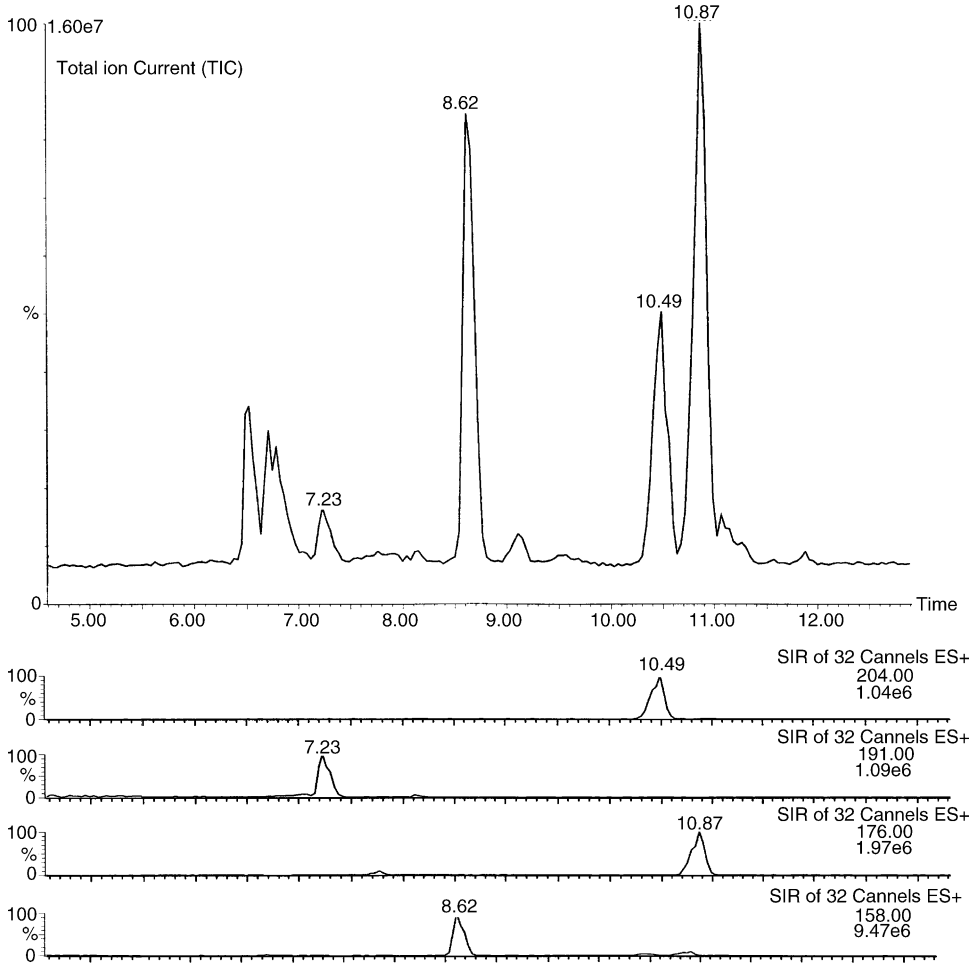

Fig. 1. Total ion current (TIC) and selected mass electropherograms (SIR) after 1 h of electrochemical oxidation of Ng-hydroxy-L-arginine (NHA).

products were identified as cyano-ornithine at 8.62 min (158, fragments 141, 112) and Ng-nitroso-L-arginine at 10.49 min (204, fragments: 116, 87, 70). These compounds had the same migration time as their standards which can be bought or synthesized [6,7]. Cyano-ornithine has been proposed as a product of NHA oxidation by authors studying Ng-hydroxyguanidine oxidation [8]. Its formation was observed when hydrogen peroxide was added to NHA and neuronal nitric oxide synthase [6]. Ng-nitroso-L-arginine was observed in neuronal nitric oxide synthase [7] and heme peroxidase [9]. Their formations were due to the reaction of NHA with acidified nitrite which gave an unstable NO-NOate before its degradation into cyano-ornithine and Ng-nitroso-L-arginine. This degradation depended of the pH of the solution.[2,10]. In neutral solution and basic pH, Ng-nitroso-L-arginine and cyano-ornithine were formed as observed for Ng-hydroxy-guanidine. Here the electrochemical oxidation produced nitrite and H^+. This was sufficient to nitrosate NHA. In the way to study this scheme photodiode array detection was used. All described compounds were commercially available or synthesized [2,3]. Separation needed to be improve for complete resolution of all components. Briefly lauryl sulfate was used. Five percent was retained according to obtain a sufficient separation and an electropherogram migration time not too long. In these conditions cyano-ornithine was at 1.43 min (maximum absorbance peak 230 nm). Ng-nitroso-L-arginine was at 4.30 min (maximum absorbance peak 260 nm). NHA and L-citrulline were at 1.13 and 2.07 min, respectively (Fig. 2). The formation of cyano-ornithine and Ng-nitroso-L-arginine were less than 5% of L-citrulline formation in the chosen oxidation conditions. To test the origin of L-citrulline formation, 1–3-cyclohexadiene was introduced during electrophoresis. L-Citrulline peak disappeared and two new peaks appeared. The 1–3-cyclohexadiene peak at 1.66 min (maximum absorbance peaks 230 and 309 nm) corresponded to unreacted initial product. The other peak at 2.22 min (maximum absorbance peak 230 nm) corresponded to the NHA cyclo-adduct formed by Diels Alder reaction which was precedently described [4]. The nitrosation product were unchanged indicating that they were not produced by the electrochemical oxidation. This also indicated that the unstable NA compound described by the electrochemical oxidation of NHA was the source of L-citrulline. The scheme presented in Fig. 3 for the oxidation of NHA was similar to the oxidation scheme of *N*-hydroxy-guanidine [10]. This showed that L-citrulline was the sole molecule of the NHA oxidation as observed by biochemists in vivo.

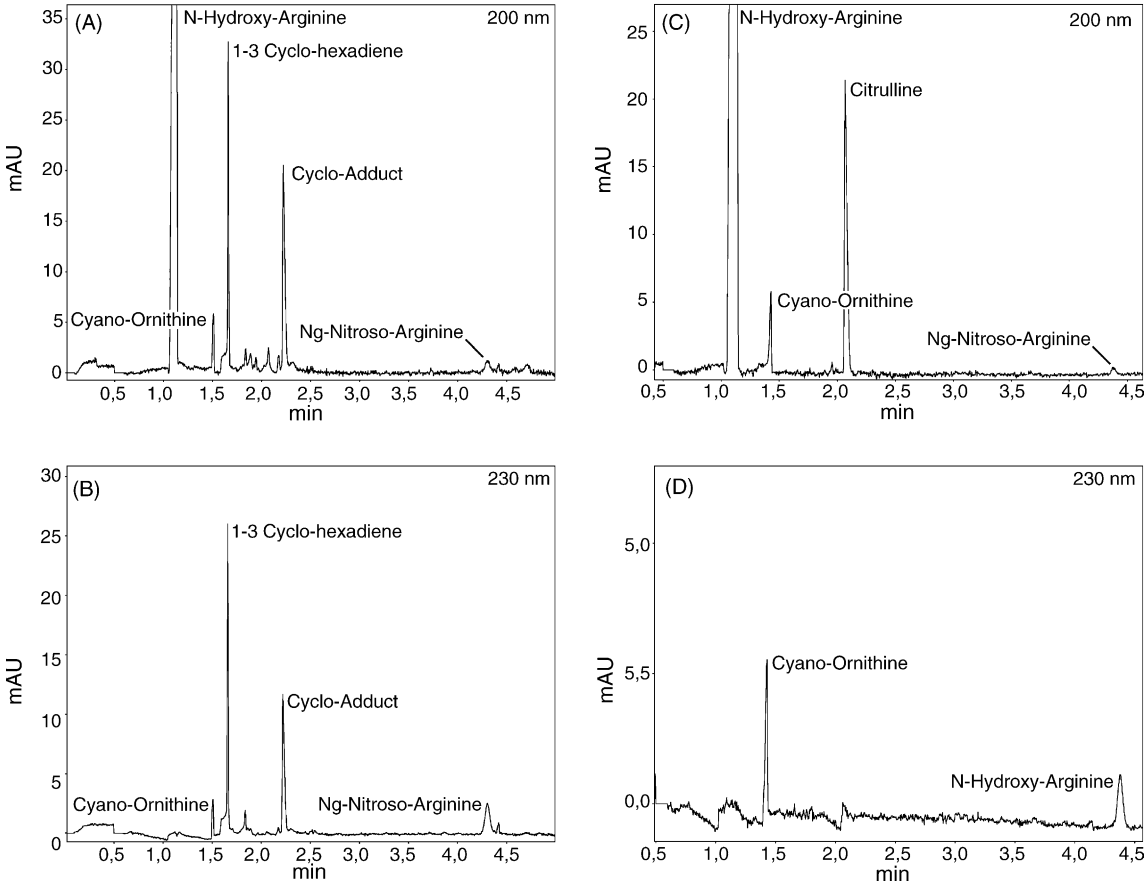

Fig. 2. Electropherograms after electrochemical oxidation of Ng-hydroxy-L-arginine (NHA) in presence of 1–3-cyclohexadiene (A and B) and in its absence (C and D) at 200 and 230 nm using photodiode array detection.

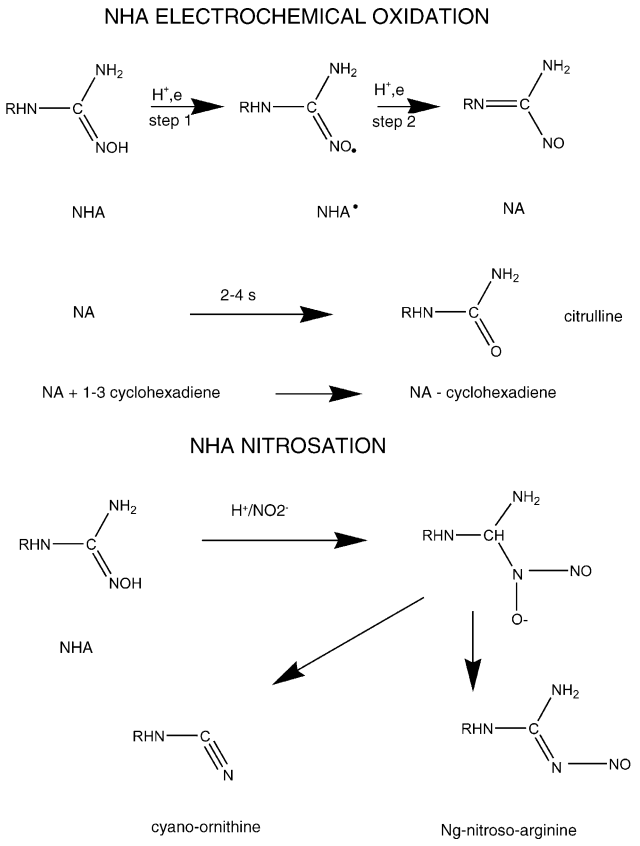

Fig. 3. Proposed scheme for the electrochemical oxidation and nitrosation of Ng-hydroxy-L-arginine (NHA).

4. Conclusion

CE-ESIMS and classical CE with diode array detection permitted to follow easily the oxidation of NHA at physiological pH. These procedures could be improved for analysis in biological prelevements where concentrations could be very low and were in progress in my laboratory.

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