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The oxidation of aminoethylcysteine ketimine dimer by oxygen reactive species

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Summary. The prominent spontaneous reaction of aminoethylcysteine ketimine in the neutral pH range is the concentration-dependent dimerization (Hermann, 1961). The carboxylated dimer first produced loses the free carboxyl yielding the more stable decarboxylated dimer (named simply the dimer in this note). In the search for a possible biochemical activity of this uncommon tricyclic compound we have assayed whether it could interact with oxygen reactive species (H_2O_2, O_2^-, $^{\cdot}OH$) thus exhibiting a scavenging effect of possible biomedical interest. The dimer interacts with H_2O_2 producing compounds detectable by chromatographic procedures. The presence of Fe^{2+} stimulates the oxidative reaction by yielding the hydroxyl radical (the Fenton reaction). Using the system xanthine oxidase-xanthine as superoxide producer, the dimer oxidation by O_2^- has also been documented. Among the oxidation products the presence of taurine and cysteic acid has been established. Identification of remaining oxidation products and investigation of the possible function of the dimer as a biological scavenger of oxygen reactive species are now oncoming.

Keywords: Amino acids – Aminoethylcysteine ketimine dimer – Hydrogen peroxide – Superoxide anion – Hydroxyl radical

Abbreviations: HPLC: high performance liquid chromatography, AAA: amino acid analyzer, SOD: superoxide dismutase, EDTA: ethylenediaminetetraacetic acid.

Introduction

The carboxylated and decarboxylated dimers of aminoethylcysteine ketimine have been prepared by Hermann (1961) and few details on the conditions of dimerization appeared more recently, Pecci et al. (1991). Dimerization of the parent ketimine is a spontaneous concentration-dependent reaction leading first to the formation of the carboxylated dimer which loses the free carboxyl group

spontaneously and much more rapidly with heating. With the possibility in mind that the decarboxylated dimer (simply named afterward the dimer) could display a biochemical function, the effect of the dimer on some enzymatic activities has been investigated and found that it impairs the mitochondrial respiration by binding at the same rotenone sensitive site used by some neurotoxins (work in progress). It is reported that this type of inhibition induces the formation of oxygen reactive species (H_2O_2, O_2^-, $^{\cdot}OH$) able to damage membrane structures (Hasegawa et al., 1990; Cleeter et al., 1992 and literature therein). We deemed therefore of interest to investigate whether the dimer could react with these oxygen reactants and eventually relieve such damages. As a first approach to this problem we present in this note evidence of interaction of the dimer with oxygen reactive species leading to the production of a number of oxidation compounds envisaged by high performance liquid chromatography (HPLC) and by the amino acid analyzer (AAA).

Materials and methods

Products

Xanthine oxidase Grade III and Superoxide dismutase (SOD) from bovine erythrocyte were obtained from Sigma. Chemicals were from Fluka, from Sigma and from Merck.

Preparation of the dimer

The previous procedure, Hermann (1961), Pecci et al. (1991), has been modified as follows. Cysteamine hydrochloride (2.27 g, 0.02 mol) in 1 ml water is added (without delay) with 10 ml 6N NaOH and bromopyruvic acid (3.34 g, 0.02 mol) previously dissolved in 5 ml water. After 30 min standing 4 ml 6N HCl are added and after 30 min in an ice bath the precipitate is collected in a sintered glass funnel. The product is transferred in a beaker with the aid of 120 ml water and heated in a boiling water bath (20–30 min) where decarboxylation is completed and solution takes place. Kept overnight in the refrigerator the precipitate is filtered, washed with 5 + 5 ml cold water and dried in the dessicator (1.15 g: 50% yield). The product exhibits the spectral and chromatographic properties reported earlier (Herman, 1961; Pecci et al., 1991). Recrystallization may be done by dissolving 0.5 g in 50 ml boiling water and cooling.

The standard oxidation system

This contained in 2 ml: 20 mM K-phosphate buffer pH 7.4, 0.11 mM EDTA, 100 mM H_2O_2, 0.1 mM $FeCl_2$, 0.1 mM dimer. The solution was kept at 38°C and aliquots were taken for analyses at indicated times. Changes from this protocol are reported in the legend of figures.

Oxidation by the xanthine oxidase-xanthine system

This system was used as a superoxide anion (O_2^-) producer according to Beauchamp et al. (1970). The mixture contained in 1 ml: 0.1 M K-phosphate buffer pH 7.4, 0.5 mM KOH neutralized xanthine, 0.5 mM EDTA, 0.14 mU xanthine oxidase measured according to Fridovich (1962), 0.1 mM dimer. The solution was saturated with O_2 and kept at 38°C for an hour. Controls were done either without enzyme or without xanthine.

Analysis of the oxidation products

HPLC and AAA were operated essentially as reported earlier (Pecci et al., 1993).

Results

The dimer dissolved in phosphate buffer pH 7.4 is not appreciably modified when kept for an hour in the presence of air or under O_2 atmosphere (data not shown). Figure 1 illustrates the HPLC profile of the standard solution in the presence of 100 mM H_2O_2 and 0.1 mM $FeCl_2$. The dimer is decreased down to a negligeable amount at the end of an hour incubation while oxidation products are formed. Two products with elution time in the area of 12–14 min have a transient life because present at 30 min incubation but no more visible at the end of an hour. Two other products, eluting very close to each other with elution time 17–18 min, named OX1 and OX2, preceeded by two smaller peaks, appear as the main products detectable by HPLC. Figure 2 shows the decrease of the dimer (A) and appearance of OX1 and OX2 (B) in function of time and other conditions. It is evident the favourable effect of the presence of Fe^{2+} imputable to the formation of hydroxyl radical ($^{\cdot}OH$) by the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$). The favourable effect of iron is much more evident when the concentration of H_2O_2 is lowered to 10 mM. Other experimental data not included in Fig. 2 are: Fe^{2+} can be substituted with Fe^{3+} with similar results; change of pH to 4.5 using 0.1 M K-acetate buffer does not appreciably modify the overall HPLC picture; omission of EDTA lowers the reaction rate and stops the oxidation to half of that in the presence of EDTA; oxidation in the presence of 5 more times of both Fe^{2+} and EDTA produces a slight lower reaction rate.

A form of reduced oxygen involved in a number of oxidative reactions is the superoxide anion (O_2^-). To test for the possible oxidation of the dimer by such radical we have used the system xanthine oxidase-xanthine (Beauchamp

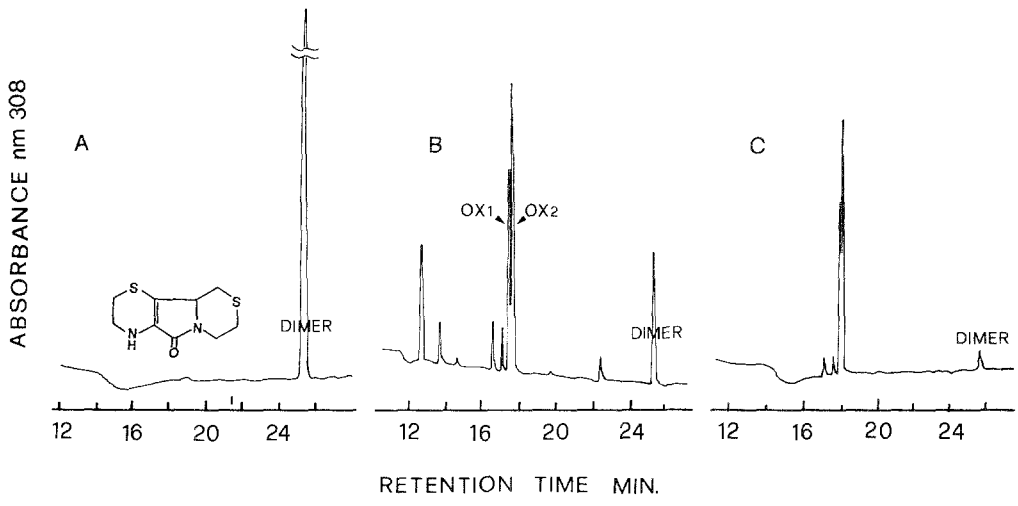

Fig. 1. A Structure and HPLC of aminoethylcysteine ketimine decarboxylated dimer. Injected 5 μl containing 0.5 nmoles dimer. **B, C** The same amount of the dimer as in **A** after 30 and 60 min under the standard oxidation conditions reported in Materials and methods. Effluent monitored at 308 nm

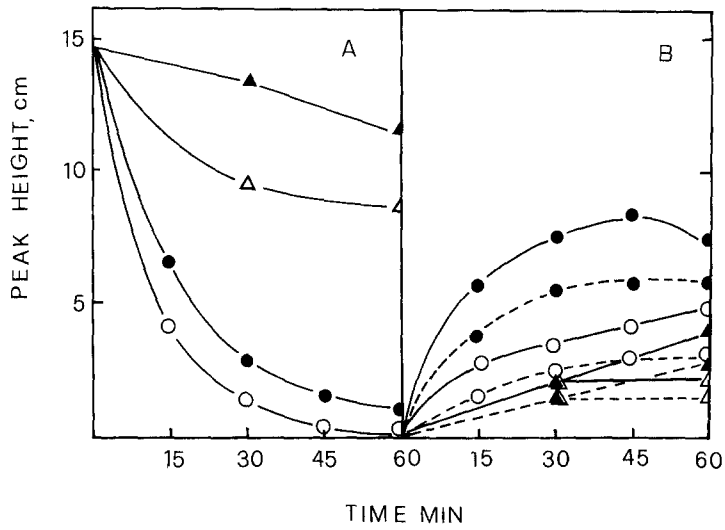

Fig. 2. A Changes of concentration of the dimer in function of time determined by the peak heights of the HPLC profiles. Condition as in Fig. 1. o complete standard system; • with Fe^{2+} omitted; ▵ with 10 mM H_2O_2 instead of 100 mM; ▲ 10 mM H_2O_2 with Fe^{2+} omitted. **B** Increase of the main oxidation products OX1 - - -, OX2—in function of time. Symbols as those in **A**. Note: Quantitation of OX1 and OX2 could be overestimated either by the partial overlapping of the two peaks and by their possible different molar extinction

et al., 1970) under the conditions described in materials and methods. HPLC of the oxidation products at the end of an hour of incubation indicated the decrease of the dimer to 75% of the concentration at zero time and the appearance of OX1 and OX2 in the amount respectively of 5.4% and 9.3% of the dimer peak (calculated by the peaks height; see note on Fig. 2 for a comment). Controls made by the same assay system in the absence of either xanthine oxidase or xanthine did not show any appreciable change of the dimer. Addition of 200 units of SOD to the complete system abolished the oxidation of the dimer. The same xanthine oxidase system run in the presence of 100 μM Fe^{2+} and 300 μM EDTA showed the decrease of the dimer to 29% of that at zero time and formation of OX1 and OX2 respectively of 9% and 18%. This result is consistent with the production of the more reactive hydroxyl radical ($^{\cdot}OH$) through the iron catalyzed Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow O_2 + OH^- + {}^{\cdot}OH$) where H_2O_2 is assumed to be produced by the spontaneous dismutation of part of O_2^- produced by the xanthine oxidase system.

The analysis of the products done by HPLC is based on the UV absorbance of the oxidation products. Products not absorbing in the UV range did escape detection by HPLC. Thus the AAA procedure has been used to detect amino acids and other ninhydrin reactive compounds eventually produced in the course of oxidation. In this assay the oxidation solution (20 ml) contained 0.1 mM dimer and 100 mM H_2O_2, at pH 7.4 by the addition of 1 M KOH. EDTA, Fe^{2+} and phosphate were omitted because oxidation of EDTA by the standard system was found to produce ninhydrin reactive compounds disturbing the chromatographic picture, probably by the partial dealkylation of EDTA. At the end of an hour at 38°C the solution was dried under vacuum in the rotovap and

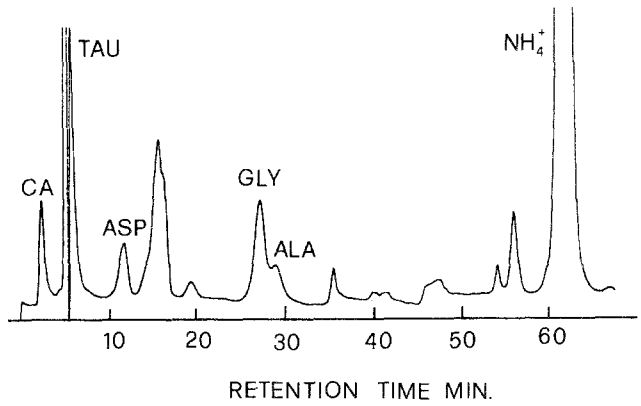

Fig. 3. Amino acid analyzer chromatogram of the standard oxidation system without EDTA, Fe^{2+} and phosphate at the end of an hour of incubation. See text for details

the residue dissolved in the first AAA buffer. The analysis (Fig. 3) indicates taurine as the main oxidation product accompanied by cysteic acid and other compounds. Unexpectedly taurine appeared in the chromatogram split in two peaks owing to the presence of some residual H_2O_2. A more accurate drying permitted the quantitation of taurine accounting for 4% of the initial dimer. Other peaks, whose genesis is of difficult interpretation, eluted on the same position of aspartic acid, glycine and alanine. Another peak at retention time of 17 min has the same position of authentic carboxymethylcysteamine sulfone, but, being asymmetric, it is probably a peak accounting for more than one product. The AAA analysis has shown that oxidation of the dimer by H_2O_2 leads to a much deeper degradation than could be evidenced by the HPLC analysis.

Discussion

The results presented in this note show that the dimer, while it is resistant to the direct oxidation by molecular oxygen, is oxidized by partially reduced oxygen species such as H_2O_2, O_2^-, $^{\cdot}OH$, known as by-products of a number of enzymatic oxidations. The biochemical significance of these radicals and their involvement in many human pathological outcomes has been the object of large discussion and speculation (reviewed by Youngman, 1984; Halliwell et al., 1990; Levine, 1993). At this regard the dimer appears a scavenger of potential biological significance of these reactive oxygen species. Our recent finding of the inhibition of mitochondrial respiration by the dimer adds interest to the results reported here. The dimer appears in fact to have a double activity, one as inhibitor of the mitochondrial electron transport and another as a scavenger of oxygen radicals known to be produced in the course of the mitochondrial activity (Turrens et al., 1980) and enhanced when the electron flux is blocked by certain inhibitors (Hasegawa et al., 1990). Which one of these effects is prevailing in the mitochondrial function appears thus an interesting question to answer. Another interesting result of this study is the formation of a number of new oxidation products of the dimer seen by two analytical procedures. The identifi-

cation of these products and the elucidation of the mechanism of their formation is a problem now under study in our laboratory.

Acknowledgements

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