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The oxidation of sulfur containing cyclic ketimines The sulfoxide is the main product of S-aminoethyl-cysteine ketimine autoxidation

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Summary. The products of autoxidation of S-aminoethyl-L-cysteine ketimine (AECK) have been analysed with the amino acid analyzer, with thin layer chromatography and with high performance liquid chromatography. Under the conditions of the assay (pH 8.5, 38°C, O_2 bubbling) AECK is almost totally oxidized in 1.5 hours. Among the final products a component running fast in HPLC, named Cx1, has been isolated, reduced with $NaBH_4$ and analysed. Reduced Cx1 resulted to show the same properties of synthetic thiomorpholine-3-carboxylic acid-S-oxide, known in the past literature with the name of "chondrine". On the basis of these results and by specific chromatographic tests, Cx1 has been identified as the sulfoxide of AECK. Among the other autoxidation products, thiomorpholine-3-one has been identified. The detection, after HCl hydrolysis, of glyoxylic acid and mesoxalic semialdehyde together with cysteamine indicates that compounds provided with easily cleavable S–C bonds, possibly thiohemiacetals or (and) thioesters, are the likely intermediates for other products. AECK sulfoxide and thiomorpholine-3-one are relatively stable and cannot be taken as the main intermediates for the remaining oxidation products.

Keywords: Amino acids – Aminoethylcysteine – Thialysine – Ketimines – Chondrine

Abbreviations: AAA: amino acid analyzer; TLC: thin layer chromatography; HPLC: high performance liquid chromatography; AECK: S-aminoethyl-L-cysteine ketimine; AECK-SO: aminoethylcysteine ketimine sulfoxide; TMA: thiomorpholine-3-carboxylic acid; TMA-SO: thiomorpholine-3-carboxylic acid-S-oxide; CMCA: S-carboxymethylcysteamine; DNPH: 2,4-dinitrophenylhydrazine.

Introduction

S-aminoethyl-L-cysteine ketimine (AECK, Fig. 5, **I**) is the product of the enzymatic (Cini et al., 1978a; Costa et al., 1986) and non enzymatic (Hermann et al., 1968) α-deamination and cyclization of the parent amino acid (called also thialysine). Occurrence of AECK in brain (Nardini et al., 1990) and of its product of reduction in urine (Matarese et al., 1989) has been reported indicating the metabolic nature of this compound. Like other members of the ketimine group AECK displays reducing properties towards oxygen (Cini et al., 1978a; Pecci et al., 1991), various chemical reagents, cytochrome *c* and other hemoproteins (Solinas et al., 1992). As the result of the reducing activity ketimines are converted into oxidation products of not yet fully understood nature. In the present study we have taken AECK as representative member of the ketimine group and have shown the formation of some oxidation products detected by three different chromatographic procedures. The identification of AECK sulfoxide (AECK-SO, Fig. 5, **IV**) as the main autoxidation product is reported in this first note.

Materials and methods

Products

AECK and its dimer (Hermann, 1961; Pecci et al., 1991), S-carboxy-methylcysteamine (CMCA, De Marco et al., 1964), CMCA sulfone and sulfoxide (Hermann et al., 1969), thiomorpholine-3-one (Matarese et al., 1984), thiomorpholine-3-carboxylic acid (TMA, Carson et al., 1964), TMA sulfoxide (TMA-SO, Däbritz et al., 1965), were prepared as indicated.

Analyses

Spectral curves were recorded with a Varian DMS 90 spectrophotometer. The amino acid analyzer (AAA) was a Carlo Erba 3A30. Thin layer chromatography (TLC) was performed on cellulose aluminium sheets (0.1 mm) with methanol:butanol:acetic acid:water (4:4:1:2) as solvent. The UV lamp was from Spectroline. High performance liquid chromatography (HPLC) analyses were carried out with a Waters chromatograph equipped with two Model 501 pumps, a Model 680 gradient controller, a U6K sample injector, a Model 490 variable wavelength detector and a Maxima 820 chromatography Workstation D.O. The column was a 250 mm × 4 mm Hypersil ODS, 5 micron. Mobile phases and gradients were as reported in the legends of figures. Cysteamine plus cystamine after reduction by dithiothreitol were determined by HPLC after reaction with monobromobimane (Newton et al., 1981). For these analyses eluates from the column were detected by a Perkin- Elmer Model LS-1 LC fluorescence detector using a 340 nm filter for excitation with an emission wavelength of 480 nm. Glyoxylic acid and mesoxalic semialdehyde were determined by HPLC after reaction with 2,4-dinitrophenylhydrazine (Antonucci et al., 1990).

Standard oxidation system

The procedure was as reported earlier (Solinas et al., 1992). In brief: 4.4 mg AECK (20 μmoles) per ml H_2O were rised to pH 8.5 by the addition of aliquots 2N NaOH. The solution was kept at 38°C under O_2 bubbling.

Results

Spectral analyses

Spectral changes in the course of AECK autoxidation, reported in the previous paper (Solinas et al., 1992), indicate the decrease of the 296 nm absorbance to a lower steady value of about 1/3 of the original one and the appearance of a new absorbance at 250 nm. Further absorbance changes are minimal after 1.5 hours and we took this time as the end of autoxidation of AECK. Figure 1 reports the spectral curves of samples of the standard solution, diluted (30 μl to 3 ml) with 0.1 M, pH 8, K phosphate buffer, at zero time (curve a) and after 1.5 hrs autoxidation (curve b). The absorbance spectra of the main oxidation product (Cx1) and of the reduced Cx1 (see later) are also shown (curves c and d respectively).

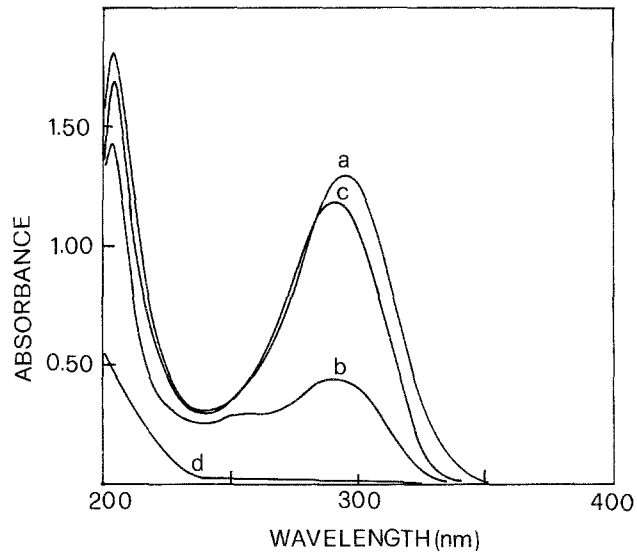

Fig. 1. Absorption spectra of AECK in water brought to pH 8.5 with 2 N NaOH as soon as dissolved (**a**) and after 90 min incubation with O_2 at 38°C (**b**). Spectra c and d are of Cx1, isolated by HPLC, before and after reduction with $NaBH_4$ respectively

AAA assay

AECK shows in the AAA an asimmetric peak (retention time 39 min) close to the area occupied by methionine. At the end of autoxidation this peak is no more visible and two ninhydrin weakly reacting products appear, one running fast, located in the area before taurine, and the other slower close to the area occupied by phenylalanine (retention time 49 min). After hydrolysis with 6N HCl (30 min at 100°C, in stoppered vials) followed by drying in the rotovap, CMCA is detected as the main product. Oxidation with H_2O_2 and molibdate (Toennies et al., 1939) either before or after HCl hydrolysis produces taurine and CMCA sulfone together with small amount of other unidentified compounds.

HPLC assay

More informative data are obtained by HPLC analysis at the end of autoxidation (Fig. 2). Apart traces of AECK still present, a large peak running faster than AECK, previously named Cx (Pecci et al., 1991) and now renamed Cx1, appears together with other compounds absorbing at 250 nm. Figure 2 shows the elution profile detected at 250 nm. In order to differentiate the peaks to be identified, these have been labeled from Cx1 to Cx5. Some preparations of AECK show the presence of traces of its dimer indicated in the chromatogram with *D*. Insert in Fig. 2 illustrates the progress of the AECK decrease with time and the increase of Cx1 on the basis of the integration of peaks registered by the HPLC apparatus.

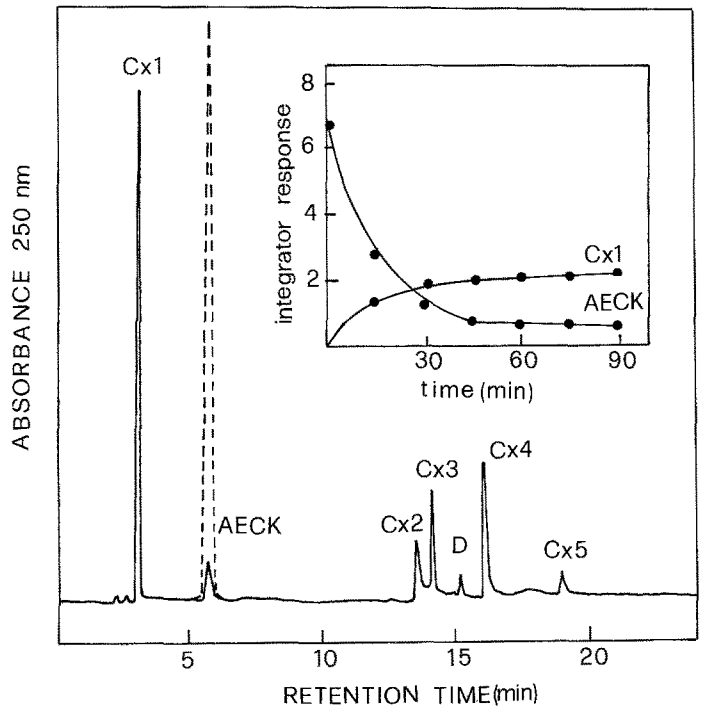

Fig. 2. HPLC elution profile of the oxidized AECK solution. The broken line indicates the peak of AECK before the oxidation. Conditions: solvent A was 50 mM ammonium acetate, solvent B was acetonitrile-water (70:30, v/v); linear program from A to 100% B over 30 min. Flow rate 1 ml/min at room temperature. Insert: AECK decrease and Cx1 increase with time on the basis of peaks integration

Cx1 extraction and identification

Cx1 shown in HPLC appears to be the main product of the AECK autoxidation. By using HPLC in a semipreparative scale the Cx1 area has been collected from 20 HPLC runs. The pooled solutions dried in the rotovap yielded a solid product that when submitted again to the HPLC analysis showed the presence of the single peak of Cx1. The spectral curve was similar to that of AECK, with the maximum slightly shifted to 292 nm, and free of the 250 nm absorbance (Fig. 1, c). TLC shows a spot dark under the UV lamp, positive to the iodoplatinate test

(Toennies et al., 1951) and reacting yellow with ninhydrin. The extracted Cx1 resulted to contain same inorganic material and attempts to deionize it by the usual ion-exchange procedures were unsatisfactory. In order to obtain a compound suitable for further analyses a sample of 50 mg of Cx1, prepared as described, was dissolved in 1 ml of H_2O and reduced by adding solid $NaBH_4$ in portions until the 292 nm absorbance stopped to decrease (about 50 mg). The solution slightly acidified by adding 2N HCl, was introduced in a 10x1 cm Dowex 50 column (200–400 mesh, H^+ form), washed with H_2O to neutrality and eluted with 2N ammonia. The effluent, monitored with the iodoplatinate reagent (Awwad et al., 1966), gave a solution of reduced Cx1 which was dried in the rotovap, redissolved in 1 ml H_2O and kept frozen for further analyses. Spectral analysis of reduced Cx1 shows a slight absorbance at 210–220 nm (Fig. 1, d) and in TLC produces a spot running slower than Cx1 ($Rf = 0.38$ compared with $Rf = 0.56$ for Cx1), no more dark under the UV lamp and reacting brownish with ninhydrin. Submitted to the tests for the oxidation level of sulfur (Cavallini et al., 1959) reduced Cx1 resulted positive to the iodoplatinate and the KI + HCl test (the latter should be done on filter paper or using cellulose on glass plates to avoid interaction of excess HCl with the aluminium support). The latter test is typical for partially oxidized sulfur derivatives and the assumption was made that reduced Cx1 could be the sulfoxide of reduced AECK, i.e., thiomorpholine-3-carboxylate-S-oxide (TMA-SO, Fig. 5, **V**), a compound known in the past literature with the name of "chondrine" (Kuriyama et al., 1960; Tominaga et al., 1963). The identification of reduced Cx1 (TMA-SO) with

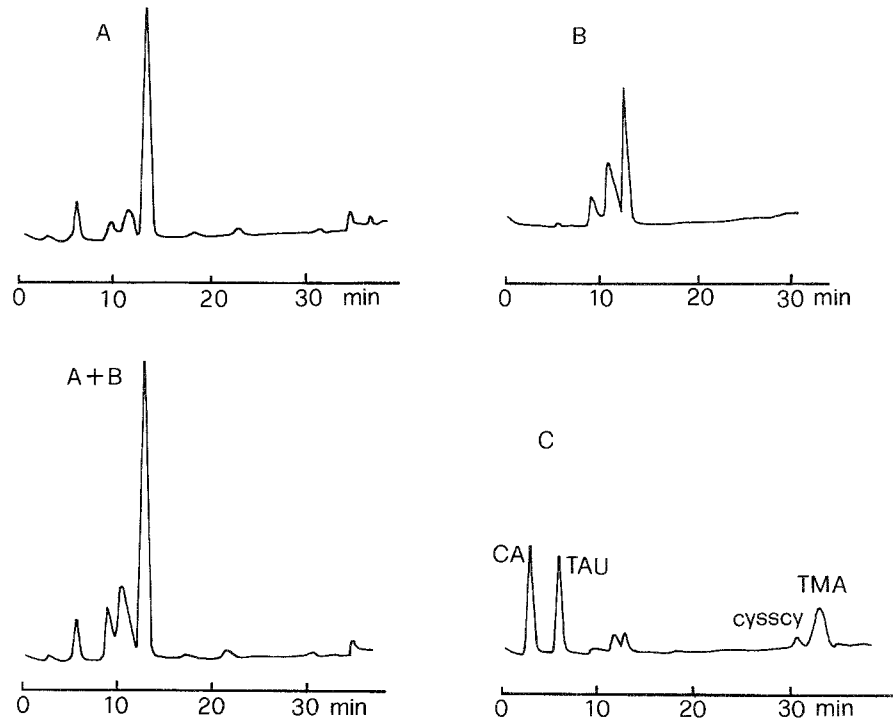

Fig. 3. AAA of Cx1 reduced with $NaBH_4$ (**A**), of synthetic TMA-SO (**B**), of reduced Cx1 plus synthetic TMA-SO (**A** + **B**) and of reduced Cx1 boiled in 6N HCl for 2 hrs (**C**)

chondrine has been confirmed by cochromatography on TLC with the synthetic TMA-SO prepared according to Däbritz et al. (1965). AAA analysis of either reduced Cx1 and synthetic TMA-SO produces a three peaks picture superimposing perfectly when the two samples are added together (Fig. 3). The nature of the three peaks is likely to be due to the different conformation and stereochemical forms known for the oxidized thiomorpholines (Däbritz et al., 1965; Carson et al., 1968). This point not being the object of our present investigation has not been explored further and the identical behaviour on the AAA of the two products has been taken as a valid preliminary indication of the identity of reduced Cx1 with the synthetic TMA-SO. Sulfoxides are known to be reduced back to the respective thioeters by boiling in 6N HCl; thus methionine sulfoxide is reduced to methionine by such treatment (Savige et al., 1977). In the case of TMA-SO heating in 6N HCl leads to reduction to TMA (Tominaga et al., 1963) but also to disproportionation to cysteic acid, taurine and cystine (Däbritz et al., 1965). This reaction applied to reduced Cx1 yielded the expected products (Fig. 3, C) adding further support to the identification of reduced Cx1 with

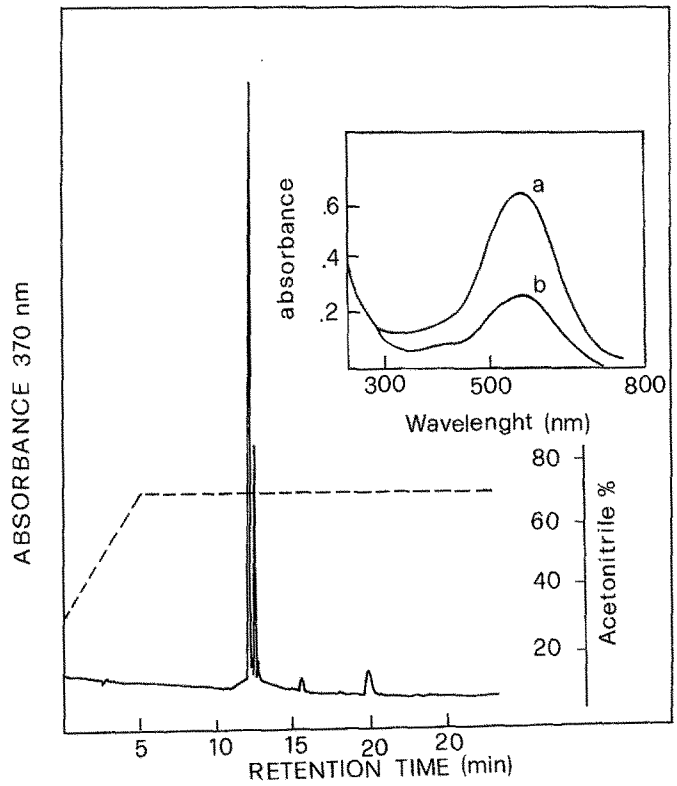

Fig. 4. HPLC elution pattern of dinitrophenylhydrazone precipitated by reacting oxidized AECK with DNPH. Conditions: solvent A was 50 mM ammonium acetate-acetonitrile (70:30, v/v), solvent B was acetonitrile; linear program from 30 to 70% acetonitrile over 5 min followed by a 20 min isocratic hold at 70% acetonitrile. Flow rate 1 ml/min at room temperature. Insert: Absorbance spectra of synthetic bis-dinitrophenylhydrazone of mesoxalic semialdehyde in 0.3% NaOH in ethanol (**a**) and of the higher peak of the chromatogram eluted from the column and diluted in 0.3% NaOH in ethanol (**b**). The smaller peak gave the same spectrum (not shown)

TMA-SO and consequently to the identification of Cx1 as the sulfoxide of AECK (Fig. 5, **IV**).

Other products

The chromatographic analyses of the final oxidation solutions of AECK show also the presence of other products. On TLC a spot, seen only by spraying the iodoplatinate reagent, has the same *Rf* (0.83) of synthetic thiomorpholine-3-one (Fig. 5, **III**). This product eluted from TLC and hydrolyzed (6N HCl, 100°C, 30 min) yields, as expected, carboxymethyl-cysteamine (CMCA). Work is in progress for the isolation and identification of other compounds. Preliminary studies have been performed on the final oxidation solution of AECK after 6N HCl hydrolysis. TLC of hydrolysates demonstrate, together with CMCA, the presence of cysteamine and cystamine which are not visible in the AAA because retained in the column. Using the monobromobimane assay (Newton et al., 1981) the sum of cysteamine plus cystamine (half-cystamine) is calculated to be 25% of the initial AECK. Other informations have been obtained by reacting oxidized AECK with 2,4-dinitrophenylhydrazine (DNPH) (1 ml of oxidized standard solution of AECK + 15 ml 0.2% DNPH in 2N HCl, heated at 45°C for 30 min). HPLC analysis of the reaction mixture shows the presence of a main peak eluting before the two peaks obtained by reacting AECK with DNPH (Antonucci et al., 1992). After boiling the reaction mixture for 15 min, a new HPLC profile is obtained with two peaks having the same elution time of synthetic phenylhydrazone of glyoxylic acid. Moreover when the oxidized standard solution of AECK added with dinitrophenylhydrazine is left at room temperature for 24 hrs a precipitate is produced which was collected by centri-

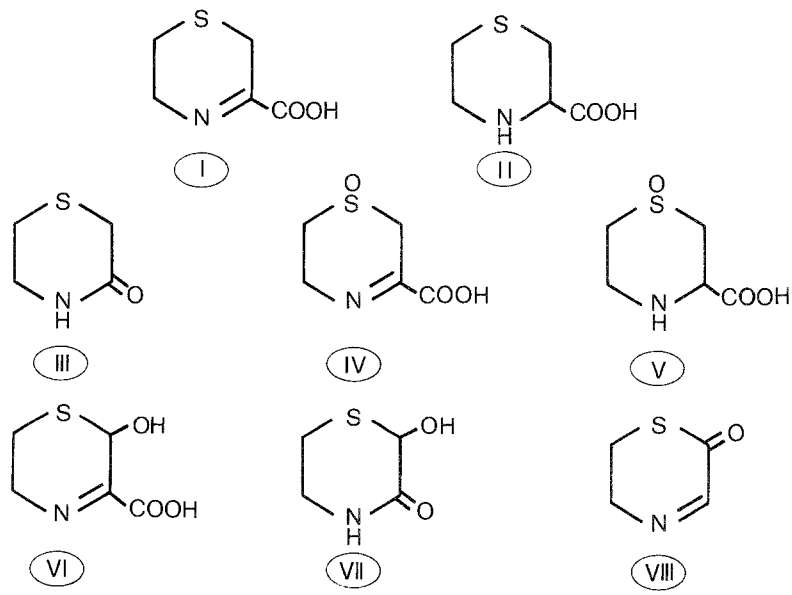

Fig. 5. Structures of: AECK (**I**), TMA (**II**), thiomorpholine-3-one (**III**), AECK-SO (Cx1) (**IV**), TMA-SO (reduced Cx1, chondrine) (**V**), cysteamine-mesoxalic semialdehyde adduct (**VI**), cysteamine-glyoxylic acid adducts (**VII**, **VIII**)

fugation and washed with 2N HCl. Dissolved in acetonitrile it gave the HPLC picture reported in Fig. 4. By addition of the bis-dinitrophenylhydrazone of mesoxalic semialdehyde prepared according to Sprinson et al. (1946) the two peaks have been identified as the isomeric derivatives of mesoxalic semialdehyde. The identification is confirmed by the identical spectral curves of the compounds isolated by HPLC with the synthetic mesoxalic semialdehydephenylhydrazone (insert Fig. 4). Spectral curves appear to be specific for these compounds because they show a maximum at 560 nm which has not been reported for any phenylhydrazone of keto acids so far analyzed (Cavallini, 1950), whereas it is similar to that of glyoxal prepared according to Jones et al. (1960).

Discussion

The data reported point to AECK-SO (Fig. 5, **IV**) as the main product of the spontaneous oxidation of AECK. A number of other compounds are also seen in the chromatograms and other could have escaped observation because not detected by the analytical methods employed. The relative stability of AECK-SO (Cx1) formed after 1 hr incubation of AECK with O_2, when most of the ketimine has been consumed (insert Fig. 2), rules out the intermediate role of AECK-SO for the production of other compounds. Thus two different oxidation routes appear to be operative in the course of the autoxidation, one leading to the sulfoxide, probably as a terminal product, and other(s) to compounds of different nature. Among these products thiomorpholine-3-one has been identified (Fig. 5, **III**). Moreover the detection of cyst(e)amine after HCl hydrolysis of the final oxidation solution of AECK indicates the formation of easily cleavable S–C bonds in some of the oxidation products. The identification of glyoxylic acid and of mesoxalic semialdehyde is consistent with this assumption indicating the formation of a thiohemiacetal or thioester bond as illustrated in Fig. 5, **VI–VIII**. The spontaneous production of AECK sulfoxide, under the mild conditions of this study rises some important conclusions. The first is that it is possible that AECK-SO could be produced *in vivo* together with AECK already reported to be present in mammalian tissues (Nardini et al., 1990). The second is that TMA-SO (chondrine, Fig. 5, **V**) could be easily produced from AECK-SO using the same unspecific reductase able to reduce all the ketimines so far investigated (Nardini et al., 1988). This possibility, worthy of a future confirmation, could promote chondrine from the present state of a biochemical curiosity to the level of a mammalian metabolite. Chondrine has been detected in marine algae (Kuriymama et al., 1960; Tominaga et al., 1963) and its presence in urine of Japanese people has been assumed so far to be due to the habit of this population to eat algae as a food additive instead of normal metabolic product.

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