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Sulfur oxidation of free methionine by oxygen free radicals

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The oxidation of free methionine to methionine sulfoxide by chemically or enzymatically generated oxygen free radicals is presented. The physiological significance of this process in living cells is suggested.

Methionine; Methionine sulfoxide; Free radical

1. INTRODUCTION

Among the essential amino acids the metabolism of methionine seems one of the most complicated. In the last two decades several excellent reviews have summarized metabolic routes for methionine [1–5]. Relatively intensive and long-term research has developed our knowledge about the transmethylation and transsulfuration processes [6,7]. Recently evidence has been forthcoming for a pathway involving transamination in the catabolic process of methionine [8,9]. Studies undertaken in our laboratory have shown the ability for conversion of carbons derived from methionine into Krebs cycle metabolites by perfused rat skeletal muscle [10]. During perfusion we have observed the accumulation of large amounts of methionine sulfoxide in the perfusate and tissue as well. To our knowledge, so far the presence of methionine sulfoxide in biological material has been reported mainly in proteins. The proposed mechanism and physiological role of protein-bound methionine sulfoxide is summarized as described in [11,12]. The chemical nature of the oxidation of methionine into methionine sulfoxide is described in [13,14]. Singlet molecular oxygen $^1\Delta gO_2$ is proposed as the major oxidant. The aim of our task is to indicate the possibility of production of methionine sulfoxide in biological systems and briefly to point out physiological implications of its appearance.

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2. MATERIALS AND METHODS

L-Methionine, L-methionine dl-sulfoxide, L-methionine sulfone, diethylenetriaminopentaacetic acid (DETPA), γ-methylthio-α-oxobutyric acid, γ-methylthio-α-hydroxybutyric acid, xanthine oxidase from buttermilk (spec. act. 2 U/mg protein), catalase from bovine liver (spec. act. 12500 U/mg protein), superoxide dismutase from bovine liver (spec. act. 3200 U/mg protein), cytochrome *c* type III and polyester plates coated with silica gel for thin-layer chromatography were purchased from Sigma (St. Louis, MO). Hydrogen hexachloroplatinic acid(IV) hydrate (gold label) and Titanium(III) chloride were from Aldrich (Milwaukee, WI). L-[^{35}S]Methionine (spec. act. 1125 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

2.1. *Production and assay of oxygen free radicals*

(i) Solutions of potassium superoxide were prepared essentially as in [15]. 15 mg KO_2 was added to 5 ml ice-cold 50 mM NaOH + 0.5 mM DETPA. After 3 min 20–100 μl was taken from this solution for the spectrophotometric determination of O_2^-. The concentration of superoxide was

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calculated using an absorbance coefficient of 2000 $M^{-1} \cdot cm^{-1}$ at 250 nm [15]. Methionine was added to the same medium supplemented with 1 μM catalase.

(ii) The generation of superoxide anion by isolated mitochondria was performed according to [19,20]. Briefly, medium contained: 250 mM sucrose, 20 mM Tris-HCl buffer, pH 7.6, 2 μM antimycin A, 0.5 μM catalase, 1 mM epinephrine, and 5 mg rat heart mitochondria. Reactions were started by addition of succinate (5 mM final concentration) or NADH (0.25 mM final concentration). The total volume of the incubation medium was 3 ml and the temperature of incubations 30°C. The rate of adrenochrome production was monitored at 480 minus 575 nm using an Aminco-Chance dual-wavelength spectrophotometer.

Under the same conditions only without epinephrine, mitochondria were incubated in the presence of 1 mM methionine or methionine sulfoxide. Reactions were terminated by ice-cold 10% trichloroacetic acid. The supernatants from these incubations were analyzed by thin-layer chromatography. The qualitative detection of methionine and methionine sulfoxide was carried out with ninhydrin or iodoplatinate reagents.

(iii) Oxidation of NADH. This system of assay of superoxide dismutase activity producing free superoxide was recently recommended by Paoletti et al. [18]. The reaction was monitored at 340 nm.

(iv) The xanthine oxidase reaction. Medium contained 100 mM potassium phosphate buffer (pH 7.0) + 0.1 mM DETPA, 8 mM xanthine, 0.1 U xanthine oxidase, and 0.5 μM catalase. The rate of superoxide production was determined by monitoring the reduction of cytochrome *c* (30 μM) at 550 nm. For quantitative calculation, 21×10^3 $M^{-1} \cdot cm^{-1}$ was the extinction coefficient used [16]. Total incubation volume was 2 ml.

(v) The rate of auto-oxidation of oxymyoglobin (50 μM) was recorded at 581 nm and parallel superoxide anion production was monitored by cytochrome *c* reduction as described above. The total incubation volume was 3 ml. The air-saturated medium was supplemented with 1 μM catalase. To the systems generating superoxide anions (a,d,e) radioactive methionine solution was added. After suitable incubation time the samples were analyzed for the amount of methionine sulfoxide formed.

2.2. *Separation and determination of methionine sulfoxide*

(i) Colorimetric determination of methionine sulfoxide was based on the assay of methionine before and after reduction. Details concerning reagent preparation have been published in [21,22]. This method allowed detection of methionine in the range 5–50 nmol. The presence of methionine sulfoxide and sulfone did not interfere in the assay of methionine. The chemical conversion of methionine sulfoxide into methionine was performed using titanium chloride solution. The samples were saturated with N_2. More details concerning the reduction are reported in [23]. Standard curves for the determination of methionine produced chemically by reduction of methionine sulfoxide were prepared in each experiment due to some variability in the completeness of the reduction reaction.

(ii) Separation of radioactive methionine sulfoxide from methionine was by thin-layer chromatography on silica gel plates. The solvent system was butanol-butanone-2-*t*-butanol-acetic acid-water (4:3:3:2:1) gassed with N_2 to avoid oxidation of methionine. R_f values for methionine and methionine sulfoxide were 0.62 and 0.18, respectively. For detection, plates were sprayed with ninhydrin or iodoplatinate reagent. After localization of standards in the remainder of the plate separate tracks were cut and pieces corresponding to positions of standards were transferred into scintillation vials with 1 ml water. Radioactivity was measured in 10 ml scintillation liquid Scinti-Verse-E (Fisher). Special precautions were taken for preparation of solutions of radioactive methionine. The stock solution of L-[^{35}S]methionine (50 mM) was prepared in nitrogen-saturated water with 10 mM mercaptoethanol. This solution was stored in minivials at −70°C until use. Chromatographic analysis of prepared methionine solutions showed less than 25% presence of radioactive methionine sulfoxide. For each set of experiments with radioactive methionine the results were corrected for the amount of radioactive methionine sulfoxide detected before incubation.

3. RESULTS

In the first series of experiments we used a recently recommended method for the assay of

Fig.1. Effect of superoxide dismutase and metabolites derived from methionine on NADH oxidation. Results are means ± SD (n = 7). The numbers under bars describe: 1, control; 2, superoxide dismutase (0.6 U) added; 3, methionine added; 4, 2 mM γ-methylthio-α-oxobutyric acid; 5, γ-methylthio-α-hydroxybutyric acid; 6, methionine sulfoxide; 7, methionine sulfone. The final concentration of all added compounds was 2 mM.

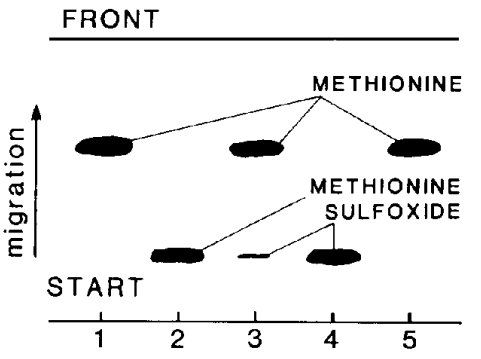

Fig.2. Copy of chromatogram (silica gel plate) of extracts from incubations with mitochondria. Isolated rat heart mitochondria were incubated as described in section 2. 200 μl of deproteinized supernatants were taken for thin-layer chromatography. The presence of methionine and methionine sulfoxide was detected by ninhydrin reagent. No other spots were visualized with iodoplatinate reagent. For the numbers under tracks, additions and incubation times were: (1) 1 mM methionine, 0 time incubation; (2) 1 mM methionine sulfoxide, 0 time incubation; (3) 1 mM methionine, 30 min incubation; (4) 1 mM methionine sulfoxide, 30 min incubation; (5) 1 mM methionine + 2 U superoxide dismutase, 30 min incubation.

Table 1

Superoxide-dependent methionine sulfoxide formation

System producing superoxide anions	Incubation time (min)	Methionine sulfoxide production per sample (nmol)
(A) Solution of KO_2	3	141 ± 17
+ superoxide dismutase (1 U)	3	89 ± 4
(B) Xanthine oxidase reaction	20	24 ± 5
+ superoxide dismutase (0.6 U)	20	7 ± 2
(C) Oxymyoglobin oxidation	60	32 + 6
+ superoxide dismutase (0.6 U)	60	11 ± 2

The values are averages of three separate experiments ± SD. The concentration of methionine was 100 μM. Quantitative analysis of methionine sulfoxide was carried out by thin-layer chromatography. Additionally, the results for systems B and C were confirmed by colorimetric assay of methionine sulfoxide. The assayed initial concentration of superoxide anions in system A was 50 μM. The rate of production of O_2^- by the xanthine oxidase reaction was 2 nmol/min per ml. Autooxidation of oxymyoglobin generated superoxide anions at the rate of 0.16 nmol/min per ml

superoxide dismutase where superoxide anions are generated during NADH oxidation [18]. The data presented in fig.1 show the inhibitory effect of S^{2+}-containing compounds added to the medium with NADH. Methionine sulfoxide and methionine sulfone did not change the rate of NADH oxidation. The maximum effect for methionine and other compounds used was observed at a concentration of 2 mM. The lowest concentration at which NADH oxidation was inhibited with these metabolites was 0.2 mM (not shown).

It is believed that, in the oxidation of adrenaline to adrenochrome, superoxide anion is involved. We used isolated rat heart mitochondria for the generation of superoxide. The rate of superoxide production by isolated heart mitochondria was 0.9 nmol/min per mg protein. Fig.2 is a copy of a developed chromatogram of samples taken from incubations with mitochondria. These results provide qualitative evidence for methionine sulfoxide formation by mitochondria producing O_2^-. No reduction of methionine sulfoxide to methionine was observed under these experimental conditions (track 4). The results collected in table 1 show quantitative conversion of methionine into methionine sulfoxide in three different systems generating superoxide anions (It is difficult to calculate the stoichiometry of conversion of methionine sulfoxide upon reaction of superoxide anions. This is mainly due to the qualitative nature of the methods used for detecting superoxide ions.). In all experiments the presence of catalase excludes the possible action of H_2O_2 on the oxidation of methionine. Diminution in the amount of methionine sulfoxide detected in the samples where superoxide dismutase was present strongly suggests the action of superoxide anions in the oxidation of methionine.

4. DISCUSSION

The general concept of the experiments presented is based on the production of oxygen free radicals in the presence of methionine, and then determination of methionine sulfoxide formation. Our suggestion that free radicals are responsible for methionine sulfoxide production from methionine is not completely new. Recent data published in [24] also suggest an oxidative role of oxygen free radicals generated by human neutrophils in the appearance of protein-bound methionine sulfoxide. Studies on the mechanism of the photo-oxidation of free amino acids showed unambiguously involvement of singlet oxygen $^1O_2$ in this process [14]. The chemical nature of interconversion of oxygen metabolites is still not fully understood. The possibility of interconversion of superoxide anions into singlet oxygen is suggested [25,26]. Estimated steady-state concentrations of oxygen and oxygen metabolites in rat hepatocytes showed low concentrations of singlet oxygen and superoxide radical: 10^{-15} and 10^{-11} M, respectively [27]. However, the oxygen concentration may vary largely in different parts of an organism. Also, heterogeneity of the capillary bed and its dependence on functional state can cause considerable fluctuations. In our in vitro experiments we generated relatively high levels of superoxide anions (solution of KO_2); but also in samples with much lower amounts of free radicals, methionine sulfoxide formation was observed (oxymyoglobin solution). We should also add two other possibilities of methionine oxidation: (i) presence of H_2O_2 in living cells (10^{-9} M) [27]; (ii) enzymatic oxidation of S^{2+}-containing compounds by oxygenases [28]. The present data do not exclude the above-listed possibilities. The formation of methionine sulfoxide at low concentration of methionine (close to physiological [29]) also supports the possibility that methionine can be a target for oxygen radicals in vivo.

Very little is known about the metabolic utilization of methionine sulfoxide by mammalian cells. L-Methionine dl-sulfoxide is not a substrate for methionyl-tRNA synthetase (EC 6.1.1.10) or for ATP:L-methionine *S*-adenosyltransferase (EC 2.4.2.13). Neither does methionine sulfoxide inhibit the above reactions [30,31]. Two enzymatic processes have been previously noted for methionine sulfoxide: (i) reduction to methionine in the presence of NADH or NADPH by liver or kidney homogenates [32]; and (ii) acetylation and excretion in rat urine [33]. Recent studies in our laboratory have shown transamination of methionine sulfoxide by three different transaminases [10]. This transaminative pathway of degradation of methionine sulfoxide can be similar to that proposed for methionine [9]. This suggestion is supported by our unpublished data concerning oxidative decarboxylation of the oxo analog of

methionine sulfoxide by branched-chain oxoacid dehydrogenase isolated from bovine kidney (enzyme provided by Dr Robert A. Harris of this department). The presence of methionine sulfoxide in extracts of rat tissues detected by an amino acid analyzer (unpublished) as well as the recently suggested utilization of methionine sulfoxide by humans [34] indicate the importance of the results presented. Our report opens future directions for studies of methionine metabolism and more possible action of oxygen free radicals as well.

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