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CYSTEAMINE OXYGENASE: POSSIBLE INVOLVEMENT OF SUPEROXIDE ION IN THE CATALYTIC MECHANISM

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The reaction catalyzed by cysteamine oxygenase on cysteamine in the presence of phenazine methosulphate as cofactor like compound is inhibited by nitroblue tetrazolium, a scavenger of superoxide ions. The reaction is not inhibited by superoxide dismutase and allyl alcohol and it is not activated by superoxide ions produced in solution. Nitroblue tetrazolium is reduced by cysteamine or mercaptoethanol and phenazine methosulphate. This reaction is completely inhibited by superoxide dismutase. In the presence of cysteamine oxygenase the reduction with mercaptoethanol is greatly enhanced and it is only partially inhibited by superoxide dismutase. According to these data a reaction mechanism is proposed in which superoxide ions and thiyl radicals are produced at the active site during catalysis.

KEY WORDS: Cysteamine oxygenase, superoxide, reaction mechanism.

Abbreviations used are: PMS, phenazine methosulfate; NBT, nitro blue tetrazolium; MB, methylene blue, ESR, electron spin resonance.

INTRODUCTION

Cysteamine oxygenase (EC 1.13.11.19.), a non heme iron dioxygenase, oxidizes cysteamine to hypotaurine[1-4]. It also oxidizes a limited number of other thiols to the sulfinate level[5,6]. Although the use of molecular oxygen has been demonstrated[7], the mechanism of the oxygenation has never been understood. In a previous paper the involvement of superoxide ion during the reoxidation of various cofactors of cysteamine oxygenase as methylene blue (MB) or phenazine methosulphate (PMS) in the presence of cysteamine or mercaptoethanol as reducing agents, has been demonstrated[8]. It has been reported that indoleamine 2, 3 dioxygenase uses superoxide as substrate[9,10] and that some oxygenases are inhibited by superoxide dismutase[11-13], thus suggesting a role for superoxide in the function of some oxygenases. In this paper we report data indicating a possible involvement of superoxide ion in the mechanism of cysteamine oxygenase catalysis, although exogeneous superoxide is not substrate of the enzyme and superoxide dismutase does not inhibit the reaction.

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EXPERIMENTAL PROCEDURES

Chemicals

Phenazine methosulfate and nitroblue tetrazolium were obtained from Sigma; potassium superoxide was a K & K Laboratories product; xanthine oxidase and hypoxanthine were from Boehringer, Mannheim; dimethyl-sulfoxide, allyl alcohol, cysteamine hydrochloride, mercaptoethanol, cysteine and methylene blue were from Merck. Dicyclohexyl-18-crown-6- (crown-ether) was obtained from Aldrich Chemical Co. All other chemicals employed were of analytical grade.

Enzyme preparations

Cysteamine oxygenase, purified from horse kidney according to Cavallini et al.[2], has an activity between 2.5 and 3.2 U per mg. Copper/zinc superoxide dismutase, prepared from bovine erythrocytes according to McCord and Fridovich[14], and manganese superoxide dismutase from *Escherichia Coli*[15], both with specific activities of about 3000 U per mg, were a gift of Dr. L. Calabrese. Activity of cysteamine oxygenase has been performed according to Cavallini et al.[1]

Methods

The standard incubation mixture for cysteamine oxygenase activity contained: 0.3 mg (1 unit) of cysteamine oxygenase, 5 μmol cysteamine or mercaptoethanol, 0.2 μmol PMS or MB, in 3 ml (final volume) 0.1 M potassium phosphate buffer pH 7.6. The reaction is stopped by addition of trichloroacetic acid (10% final concentration). The amount of hypotaurine produced was measured by using an Optika amino acid analyzer (Milan, Italy). Samples were applied to an Aminex A6 column (0.9 $\times$ 45 cm) equilibrated with 0.1 M citric acid pH 1.8 containing 0.2 M NaCl, at 56°C. Flow rate was 1.2 ml/min. Variable amount of taurine may also be produced, depending from the experimental conditions[4]. The extent of the enzymatic reaction was thus calculated from the sum of hypotaurine and taurine.

Inhibition by superoxide dismutase has been assayed by adding variable amounts (up to 10 μM) of the enzyme to the standard incubation mixture with or without PMS. Superoxide anions were generated by adding xanthine oxidase (0.05 U) and hypoxanthine (2 mM) in the standard incubation mixture[14]. Experiments in the presence of potassium superoxide were performed by adding (every 2.5 min) aliquots of 10 μl (0.5 μmol), potassium superoxide solution in dimethylsulfoxide containing 0.5% crown ether into the standard enzymatic mixture[16].

Spectrophotometric measurements were done with a Beckman Acta III thermostated at 25°C. Comparative data of the reaction rates with different cofactor-like compounds were obtained with a Warburg apparatus at 37°C using air as gas phase.

RESULTS

Effect of superoxide dismutase, superoxide ion and thiyl radical scavenger on cysteamine oxygenase activity

Table 1 shows that cysteamine oxygenase activity is not inhibited by superoxide dismutase. Both bovine erythrocyte and bacterial (Mn containing) superoxide dis-

TABLE I
Effect of superoxide, superoxide dismutase and thiyl scavenger on cysteamine oxigenase

Additions	PMS	Incubation time	pH	Product (n moles)	% Activity
none	–	5′	7.6	240	100
none	+	5′	7.6	920	100
Mn SOD (3000 U.)	–	5′	7.6	259	107.9
Mn SOD (3000 U.)	+	5′	7.6	900	97.8
Cu SOD (2500 U.)	–	5′	7.6	235	97.9
Cu SOD (2500 U.)	+	5′	7.6	910	98.9
Allyl alcohol	–	5′	7.6	230	95.8
Allyl alcohol	+	5′	7.6	940	102.1
none	–	5′	8.5	42	100
O_2^- Crown	–	5′	8.5	45	107.1
none	–	15′	8.5	121	100
O_2^- Crown	–	15′	8.5	116	95.8
Hypoxant + xant. ox.	–	15′	8.5	118	97.5

Incubation mixture conditions are reported in "Experimental Procedures". The reaction was started by the addition of cysteamine oxygenase. Hypotaurine produced was determined by amino acid analyzer. Reactions performed at pH 8.5 were in 0.1 M Tris-HCl buffer.

mutases have been used in these experiments because of their different sensitivity to sulfhydryl containing compounds[17]. On the other hand, activity is not increased by superoxide ions produced in the enzymatic mixture by hypoxanthine-xanthine oxidase system or by step by step addition of potassium superoxide. Cysteamine oxygenase appears therefore to be unable to use exogeneous added superoxide as substrate unlike other oxygenases[9,10,13]. On the other hand allyl alcohol, an efficient thiyl radical scavenger[18], does not affect the activity.

NBT reduction in enzymatic and non enzymatic systems

Reduced PMS undergoes autoxidation with concomitant production of superoxide ion whose occurrence can be detected by following the reduction of NBT at 560 nm[19]. A parallel has been observed between the ability of PMS and other redox dyes in producing superoxide in the presence of thiols and their property to act as cofactor for cysteamine oxygenase[8]. In order to verify the possibility that cysteamine oxygenase acts as scavenger of superoxide produced by the PMS-thiol system, the rate of NBT reduction was followed in the presence or absence of enzyme. Mercaptoethanol, a good substrate for cysteamine oxygenase[5,6] is used instead of cysteamine which easily reduces NBT also in the absence of PMS. Unespectately the reduction rate of NBT greatly increases in the presence of cysteamine oxygenase. This increase does not occur with heat denatured enzyme (table 2) and no reduction is observed with NBT is added at the end of enzymatic reaction. Moreover PMS mediated NBT reduction by mercaptoethanol is strongly inhibited by Mn containing superoxide dismutase. Addition of cysteamine oxygenase to this system partially abolished this inhibition. This behaviour is strictly related to the catalytic activity of cysteamine oxygenase. In fact cysteine which is not a substrate of the enzyme reduces NBT similarly to mercaptoethanol but the presence of cysteamine oxygenase does not increase the reduction rate of NBT nor protects the system against inhibition by superoxide dismutase (table 2).

Although a number of mechanisms may be hypothesized to explain these results, the involvement of superoxide radicals seems to be probable. Superoxide ion may be

TABLE II
NBT Reduction during cysteamine oxygenase reaction

PMS	NBT	C.O.	SOD	EtSH	Cysteine	NBT reduction Δ A per min
+	+	+	–	+	–	0.410
+	+	–	–	+	–	0.170
+	+	inactivated	–	+	–	0.170
+	+	+	–	–	–	0.015
–	+	+	–	+	–	0.060
–	+	–	–	+	–	0.020
+	+	+	3000 U	+	–	0.190
+	+	–	3000 U	+	–	0.015
+	+	+	3000 U	–	+	0.020
+	+	+	–	–	+	0.150

Standard incubation mixture (25°C) contained: one unit cysteamine oxygenase, 1.66 mM mercaptoethanol, 0.066 mM PMS, 0.33 mM NBT in 3 ml final volume 0.1 M K-phosphate buffer pH 7.6. Bacterial superoxide dismutase (Mn containing) was used.

formed at the active site of cysteamine oxygenase, the site being accessible to NBT but not to superoxide dismutase. On the other hand when the above reported experiments were done in the absence of oxygen, slower reduction rates for NBT were observed (data not shown).

If superoxide ions are involved in the catalytic mechanism of cysteamine oxygenase, the enzymatic reaction should be inhibited by NBT. As expected, a strong inhibition by NBT is observed also in the absence of PMS (table 3). This fact is in accordance with a previous observation that superoxide ions are formed during autoxidation of cysteamine, cysteine and mercaptoethanol in the absence of dyes[8,20,21].

Comparison of cofactor-like activities of PMS and MB

By comparing relative cofactor-like activities of PMS and MB against two substrates it can be seen that the increase of the catalytic activity of cysteamine oxygenase may depend on a substrate-dye interaction. As shown in Figure 1, MB is a better cofactor for cysteamine whereas PMS is more efficient with mercaptoethanol. At 10 mins, 4.03 and 3.13 μmoles hypotaurine are produced with MB and PMS respectively; these values, determined by amino acid analysis, are in agreement with the oxygen consumption date. At the same time only 0.92 and 1.79 μmoles mercaptoethanol (with MB and PMS respectively) are oxidized. If dyes play the role of activators interacting

TABLE III
Inhibition of cysteamine oxygenase activity by NBT

PMS	NBT	Product (nmoles)	Activity (%)
–	–	61	100
–	+	36	59
+	–	301	100
+	+	161	53

Standard incubation mixture (with mM cysteamine) was incubated at 25°C. After 5 min the reaction was stopped and hypotaurine determined on amino acid analyzer.

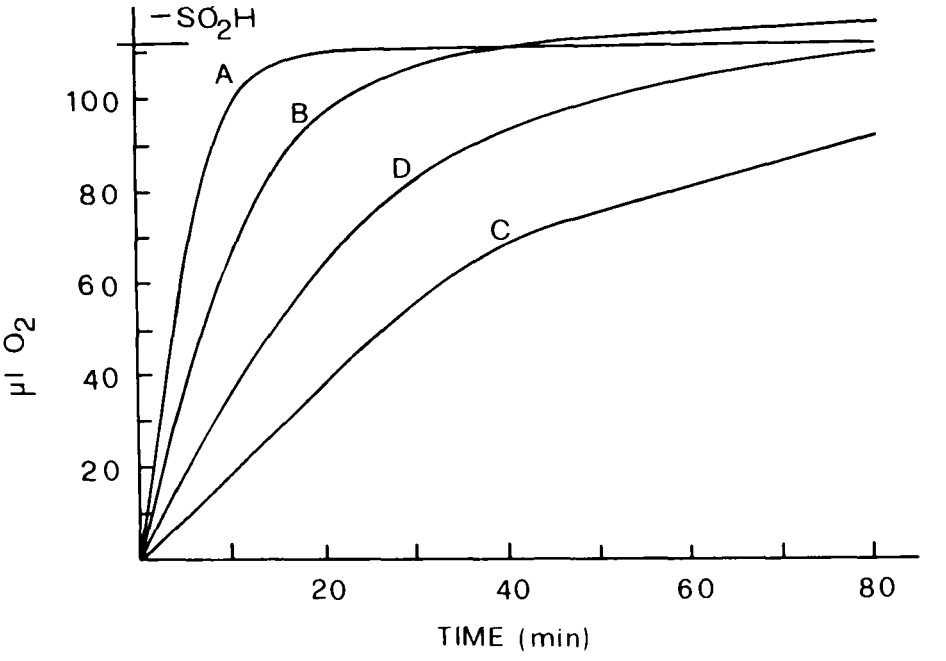


FIGURE 1 *Cysteamine oxygenase activity with cysteamine and mercaptoethanol as substrates and PMS and MB as cofactors.* Enzymatic mixture contained one unit cysteamine oxygenase, 1.66 mM substrate, 0.066 mM cofactor in 3 ml 0.1 M K-phosphate buffer pH 7.6. Oxygen uptake was followed in a Warburg apparatus at 37°C. Curve A: cysteamine and MB; curve B: cysteamine and PMS; curve C: mercaptoethanol and MB; curve D: mercaptoethanol and PMS.

with iron or other groups of the enzyme, the relative activities should be independent upon the kind of substrate.

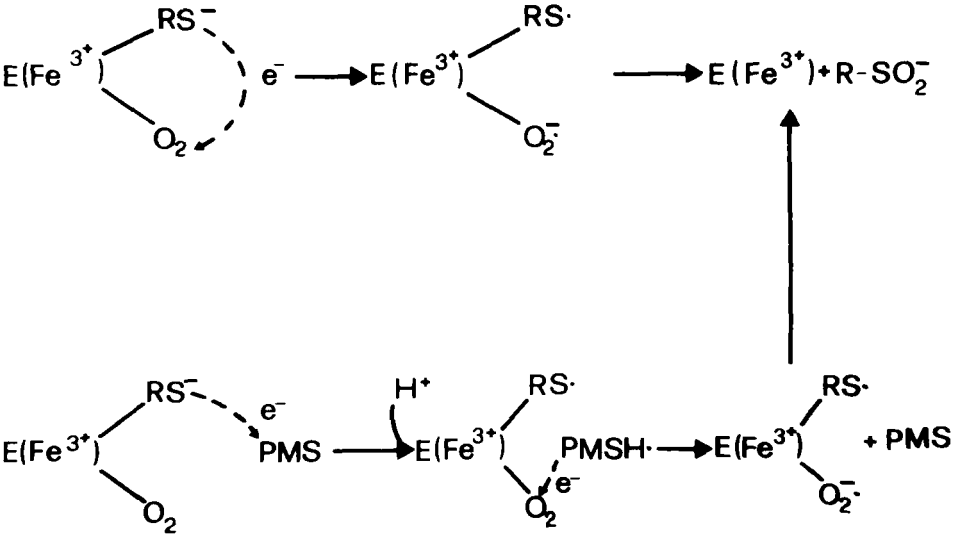
DISCUSSION

Previous studies on the catalytic mechanism of cysteamine oxygenase were concerned mainly with the role of non heme iron in the enzymatic process. One iron atom is present for one mole of enzyme, composed of two subunits (M.W. 50,000)[22,23]. ESR data show that, during oxygenasic activity, iron is present as Fe(III) and does not change its valence state; only the ligand field changes its symmetry, which is rhombic in the resting state and becomes distorted in the presence of the substrate[24]. Furthermore two possible roles for cofactor like compounds have been supposed in the past[25]. The first one claimed for an aspecific activation of the enzyme molecule. This hypothesis seems not tenable in view of the comparative data, for PMS and MB, given in this paper. The second possibility envisaged the production of transient intermediates (as thiyl and oxygen radicals), through the action of the cofactor, which are used by the enzyme to yield the sulfinate.

Some data, emerging from the experiments reported above, should be recalled: (a) autooxidation of PMS or MB in the presence of thiols, or even autooxidation of thiols

produces superoxide ion; (b) cysteamine oxygenase is unable to use superoxide produced in solution as substrate; (c) the enzymatic reaction is not inhibited by superoxide dismutase, and (d) by a thiyl radical scavenger. These facts indicate that the enzyme does not utilize thiyl or superoxide ions arising from the interaction of the cofactor like dye and the thiol in solution. Furthermore the reduction of NBT in the presence of cysteamine oxygenase is faster compared with the reduction rate of NBT in the non enzymatic system; therefore it can be interpreted as a sign that superoxide ions are produced in the active site and are involved in the enzymatic reaction. Superoxide dismutase only partially inhibits the reduction of NBT during enzymatic oxygenation of mercaptoethanol, probably because superoxide ions produced in the active site are protected against superoxide dismutase action. The inhibition of the oxygenase reaction by NBT points again to the involvement of superoxide ion in the active site. Similarly superoxide is involved in the hydroxylation reactions catalyzed by proline hydroxylase and lysine hydroxylase both inhibited by NBT and not inhibited by superoxide dismutase[26-28].

According to these observations a minimal reaction mechanism can be proposed (scheme 1). Pathway A, without cofactor, implies an electron shift between oxygen and substrate. In fact cysteamine oxygenase is able to perform the oxygenation also in the absence of any cofactor although at a low rate[29]. In the pathway B the electron shift is mediated by PMS. Iron has not been considered to be involved in the electron transfer step. This conclusion arises from ESR data[24]. However, the persistance of the ESR signal during catalysis does not exclude that iron undergoes redox cycles, with very low Fe(II) steady state concentration.



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