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PURIFICATION AND SOME PROPERTIES OF RAT LIVER CYSTEINE OXIDASE (CYSTEINE DIOXYGENASE)

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Summary

Cysteine oxidase (cysteine dioxygenase, EC 1 13.11.20) was purified approximately 1000-fold from rat liver. The purified enzyme (protein-B) was obtained as an inactive form, which was activated by anaerobic preincubation with L-cysteine. The active form of protein-B was inactivated during aerobic incubation to produce cysteine sulfinate. This inactivation of protein-B was protected by a distinct protein in rat liver cytoplasm, namely stabilizing protein (protein-A) The K_a and K_m values for L-cysteine were $0\,8 \cdot 10^{-3}$ M and $1.3 \cdot 10^{-3}$ M respectively. The enzyme was strongly inhibited by Cu^+ and/or Fe^{2+} chelating agents but not by Cu^{2+} chelating agent The optimum pH of enzyme reaction was 8.5—9 5 while that of enzyme activation was 6 8—9 5, with a broad peak

Introduction

Cysteine oxidase (cysteine dioxygenase, EC 1 13.11.20) is a soluble enzyme catalyzing the oxygenation of cysteine to cysteine sulfinate, which has been considered as a key intermediate of cysteine metabolism to cysteic acid, hypotaurine, taurine, isethionic acid, sulfate and pyruvate in mammalian tissues [1] However, the purification of this enzyme has not been achieved due to its extreme instability with the standard purification procedures

In a previous communication from this laboratory [2], the soluble enzyme cysteine oxidase of rat liver was reported to be composed of two distinct proteins, namely catalytic and activating proteins and the catalytic protein existed as an inactive form which was converted to an active form by a preincubation with L-cysteine under anaerobic conditions. The present paper describes the purification procedures and some properties of the purified catalytic and stabilizing proteins of cysteine oxidase

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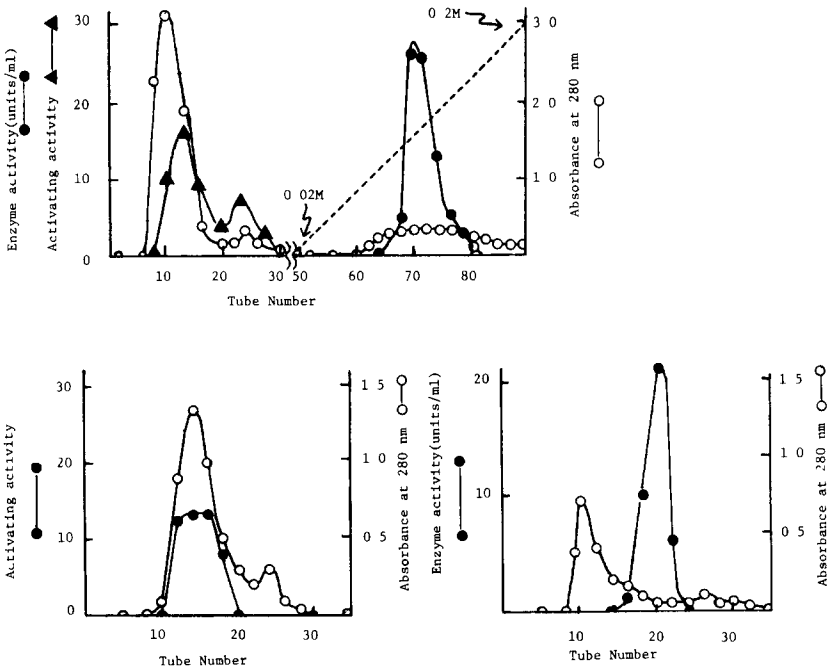
Materials and Methods

The preactivation and the assay of cysteine oxidase activity using Thunberg tubes were carried out as previously reported [2] except for the assay mixtures The assay mixtures consisted of 10 μmol of L-[^{35}S]cysteine (0 4—0 8 μCi), 100 μmol of glycine buffer, pH 9.0, and the enzyme in a final volume of 1 ml The air in the Thunberg tube containing the reaction mixture was replaced 3 times by nitrogen after evacuation with a vacuum pump The mixtures were preincubated at 37° C for 30 min, then the tops of the tubes were removed for aerobic incubation for 10 min, with shaking at 120 strokes per min The determination and identification of the cysteine sulfinate produced were carried out as described previously [2,3] A unit of enzyme was defined as the amount of enzyme producing 1 μmol of cysteine sulfinate in 1 h The amounts of cysteine remaining in reduced form in the assay mixtures after preincubation or incubation were estimated by the method reported by Grassetti et al [4] The reaction products from D-cysteine, L-cystine, DL-homocyst(e)ine and cysteamine were determined by ninhydrin reaction after isolation with a Dowex-50 column as cysteine sulfinate except for the possible reaction product from cysteamine, hypotaurine, which was partially adsorbed by the resin under the above conditions and entirely eluted with 7 ml of water followed by 8 ml of HCl 0 5 M Other materials and chemicals were used as reported previously [2,3]

Results and Discussion

Purification

All procedures for the enzyme purification were carried out at 4° C The animals were killed by cervical dislocation and their livers were excised immediately after perfusion with an ice-cold sterilized 0 9% NaCl solution through the portal vein The livers were homogenized in 2 vols. of 0 25 M sucrose containing 0 05 M sodium phosphate buffer, pH 6 8 The soluble fraction from approx 100 g of rat liver was prepared by a standard procedure [5] To the soluble fraction, the L-cysteine solution neutralized to pH 6 0—7 0 was added to a final concentration of 20 mM, and the mixture was heated to 55—60° C in a water bath under nitrogen and maintained at this temperature for 10 min After cooling in an ice-bath to 4° C, the denatured protein was removed by centrifugation The clear dark-brown supernatant was fractionated with solid ammonium sulfate and the precipitate at 40—55% saturation was dissolved in 0 02 M sodium phosphate buffer, pH 6 8 and dialyzed overnight against 0 002 M sodium phosphate buffer The denatured protein was removed by centrifugation The clear supernatant (55 ml) was placed on a DEAE-cellulose column (2.8 cm × 17 cm) which was previously equilibrated with 0 02 M sodium phosphate buffer, pH 6 8 The column was washed with 400 ml of the same buffer The stabilizing protein (protein-A) was eluted in this fraction as shown in Fig 1 The enyme (protein-B) was subsequently eluted from the column by linear gradient elution, with 0 02 M and 0 2 M of sodium phosphate buffer (200 ml each) as shown in Fig 1 The protein-B fractions were applied on a Sephadex G-200 column (1 5 cm × 70 cm) equilibrated with 0 02 M



Figs 1—3 The DEAE-cellulose chromatography of the catalytic (protein-B) and activating (protein-A) proteins of rat liver cysteine oxidase (step 2) (Fig 1), and the Sephadex G-200 chromatography of protein-A (Fig 2) and of protein-B (Fig 3) isolated by the DEAE-cellulose chromatography The enzymic activities of protein-B fractions were assayed in the presence of 0 1 ml of protein-A (tube No 14 of the Sephadex G-200 chromatography) and the activating activities of protein-A fractions were assayed in the presence of 0 1 ml of protein-B (tube No 18—21 of the Sephadex G-200 chromatography, 12 units/ml, enzyme solution) The activating activity was represented as μmol of cysteine sulfinate produced for 1 h

sodium phosphate buffer, pH 6 8 The chromatography was developed with the same buffer and 5-ml fractions were collected A major protein peak appeared after the void volume of the column and enzyme activity was detected in the fractions (tubes No 18—22, see Fig 3) In a similar chromatography of protein-A, two main protein peaks were usually observed One major peak appeared immediately after the void volume and the other appeared a little later The stabilizing protein was eluted in the first peak as shown in Fig 2 The fractions of protein-B pooled were treated with ammonium sulfate and the precipitate at 60% saturation was dissolved in a minimum volume of 0 02 M sodium phosphate buffer, the solution was then placed on a Sephadex G-100 column (1.5 cm × 70 cm) equilibrated with 0.02 M sodium phosphate buffer The chromatography was developed as described above One major protein peak exhibiting enzyme activity was observed in the fractions collected (tubes No 18—20). No significant loss of activity of the purified enzyme (protein-B) stored at −20° C was observed for at least three months, while the stabilizing activity of protein-A (step 4) decreased remarkably under the same conditions.

A summary of the purification procedure is given in Table I The over-all purification achieved was about 1000-fold with a yield of 3%

TABLE I

PURIFICATION OF CYSTEINE OXIDASE

The enzymic activities were assayed in the presence of excess protein-A (step 4) with preactivating procedure

Step	Treatment	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg protein)
1	Cytoplasmic supernatant	166	9392	4512	2 1
2	Heat treatment and amm sulfate fractionation (40—55%)	55	3828	461	8 3
3	DEAE-cellulose	80	1352	25	54 3
4	Sephadex G-200	15	300	0 53	566 0
5	Sephadex G-100	15	250	0 12	2083

TABLE II

THE EFFECTS OF CHELATING AGENTS ON CYSTEINE OXIDASE ACTIVITY

Addition	Relative activity (%)	
	1 10^{-5} M	1 10^{-4} M
None	100	100
EDTA	41	0
8-hydroxyquinoline	122	41
o-Phenanthroline	87	12
α,α'-Dipyridyl	135	8
Diethyldithiocarbamate	85	89
Bathocuproine sulfonate	20	17
Bathophenanthroline sulfonate	32	0
$CuSO_4$	10	0
$FeSO_4(NH_4)_2SO_4$	50	0

TABLE III

STOICHIOMETRY OF THE FORMATION OF CYSTEINE FULSINATE AND CYSTINE AND THE DEGRADATION OF CYSTEINE

Fe^{2+} Ferrous ammonium sulfate, Cu^{2+} $CuSO_4$

Assay system	(1) Remaining R-SH during over-all reaction (μmol)	(2) 1/2 cystine formed after anaerobic preincubation (μmol)	Aerobic incubation	
			(3) Cysteine sulfinate formed (μmol)	(4) 1/2 cystine formed (μmol)
No enzyme	10 0	n s	0	0
Protein-A alone	9 07	0 50	0	0 43
Protein-B alone	9 71	n s	0	0 29
Complete	8 63	0 50	0 97	0
Complete				
+ 5 10^{-5} *M* Fe^{2+}	1 02	5 40	0 39	3 19
+ 5 10^{-5} *M* Cu^{2+}	0 80	6 21	0	2 99

Properties of the purified enzyme

It has been considered that the prosthetic group of a rat liver cysteine oxidase was Fe^{2+}, on the basis of the observation that enzyme activity was stimulated by exogenous Fe^{2+} and inhibited by Fe^{2+}-chelating agents such as α,α'-dipyridyl, *o*-phenanthrolin [6—8] As shown in Table II, however, the enzymic activity was strongly inhibited by 1 10^{-5} M of bathocuproine sulfonate which is more specific for Cu^{+} than Fe^{2+}, while no inhibition by the same concentration of α,α'-dipyridyl and 8-hydroxyquinoline was observed The Cu^{2+}-chelating agent diethyldithiocarbamate caused no significant inhibition. From these findings, it seems possible that the prosthetic group of cysteine oxidase is Cu^{2+} but the possibility that Cu^{+} and/or Fe^{2+} are the prosthetic group is not to be excluded at present. On the other hand, exogenous Fe^{2+} and Cu^{2+} strongly inhibited the enzymic activity, as shown in Table III. The paradoxical inhibition by these metals may be explained by the results shown in table III, i e. that 85% and 92% of the cysteine was respectively converted to cystine, being inert as a substrate in the presence of 0.5 10^{-4} M of Fe^{2+} and Cu^{2+} through the assay procedures

The optimum pH of the purified enzyme was 8.5—9.0 The addition of ferrous iron caused a strong inhibition of enzymic activity with a shift of the optimum pH to 6.8 The enzymic reaction proceeded under aerobic conditions, but not under anaerobic conditions even in the presence of NAD(P), NAD(P)H, FAD, FMN, tetrahydropteridine, cytochrome C, ubiquinone or methylene blue

As shown in Table IV, the superoxide-generating systems such as hematin plus ascorbate and xanthine oxidase plus hypoxanthine strongly inhibited enzymic activity Furthermore, neither superoxide-generating systems nor catalase could be substituted for protein-A

The purified enzyme was highly specific for L-cysteine The substrate analogues such as L-cystine, D-cysteine, DL-homocyst(e)ine and cysteamine were inert as substrates under standard assay conditions 1 10^{-2} M D-cysteine, 1

TABLE IV

EFFECT OF ADDITIONS ON CYSTEINE OXIDASE ACTIVITY

Additions were added into the reaction mixtures before the preincubation Specific activity of catalase and xanthine oxidase was 39000 units/mg and 0 4 unit/mg protein respectively

Addition	Relative activity
None	100%
Hemoglobin (20 μg)	78
Methemoglobin (20 μg)	78
Ascorbate (1 10^{-3} M)	48
Hematin (1 10^{-6} M)	104
Ascorbate (1 10^{-3} M) + hematin (1 10^{-6} M)	24
H_2O_2 (1 10^{-4} M)	88
Catalase (20 μg)	64
NaCN (1 10^{-4} M)	94
Hypoxanthine (1 10^{-3} M) + xanthine oxidase (100 μg)	0

10^{-2} M DL-homocysteine, 0 5 10^{-2} M L-cystine and 0 5 10^{-2} M DL-homocystine exhibited 20%, 47%, 42% and 47% of inhibition for the enzymic activity respectively

Anaerobic activation of cysteine oxidase (protein-B) by L-cysteine

The purified enzyme was activated by L-cysteine under anaerobic conditions as previously reported [2] The optimum pH for the activation was 6 8—9 5 with a broad peak The maximum activation was observed at 38—40° C for 15 min Protein-A was not an absolute requirement for the preactivation by L-cysteine, but the activated protein-B was rapidly inactivated during the subsequent aerobic incubation in the absence of protein-A, as shown in Fig 4 These results suggest that protein-A may play the role of a modifier and protect the active enzyme from inactivation under aerobic conditions Bovine serum albumin, hemoglobin and methemoglobin had no significant effect on the purified enzyme activity Furthermore, the inactivation of protein-B during the aerobic incubation appears to be dependent on the concentration of protein-B and thus no significant formation of cysteine sulfinate was detected with less than 9 units of protein-B, in the absence of protein-A, as shown in Fig 4 From Fig 5, one can see that the K_a and the apparent K_m value for L-cysteine were 0 8 10^{-3} M and 1.3 10^{-3} M, respectively The enzymic activities of the preparations at step 1 and 2, without preactivation, were approx 25% of the enzymic activities in the preactivated preparations, while no activity could be detected in the preparations at step 3—5 without anaerobic preactivation

Neither enzymic activity nor stimulation effect on the purified cysteine oxidase activity was found in the heart, lung, stomach, brain, spleen, intestine,

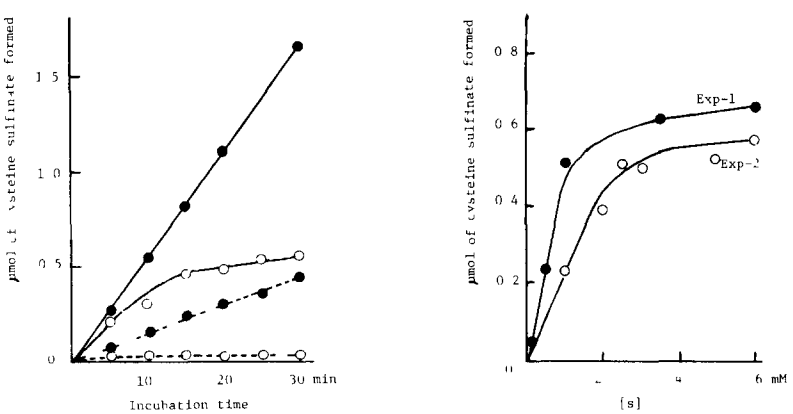


Fig 4 The effect of protein-A on the enzymic activity of protein-B The assay of enzymic activity and the preactivation were carried out as described in text ——— 34 units of enzyme used, - - - - - - 8 units of enzyme used, ● + protein-A, ○ — protein-A

Fig 5 The changes of enzymic activity as a function of cysteine concentration during the activation and the over-all reaction through preactivation and incubation In experiment 1 (●), the reaction mixture containing the enzyme was anaerobically preincubated for 30 min with L-cysteine at the concentration given in the figure and then the aerobic incubation was carried out with L-cysteine at a final concentration of 0 075 M In experiment 2 (○), the assay was carried out with L-cysteine at the concentration given in the figure, throughout assay procedures

pancreas, testis, rat hepatoma cells (AH 2440, AH 109A) and mouse Ehrlich ascites tumor cells

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