TWO COMPONENTS OF CYSTEINE OXIDASE IN RAT LIVER*

Shigeki Sakakibara, Kenji Yamaguchi# and Iwao Ueda

Department of Medical Chemistry, Osaka Medical College
2-7, Daigakucho, Takatsuki, Osaka

Yukiya Sakamoto
Institute of Cancer Research, Osaka University
Medical School, Osaka, Japan

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SUMMARY. Purified cysteine oxidase in rat liver is composed of two distinct proteins. These proteins are able to be fractionated by DEAE-cellulose column chromatography. It appears that one of them is a catalytic protein named protein-B having tightly bound iron as a prosthetic group, while the other is either a modifier or activating protein named protein-A. Protein-B is found to exist in both an active and an inactive form. Inactive protein-B is activated by incubation with substrate cysteine under anaerobic condition. Activated protein-B alone exhibited an extremely low catalytic activity but in the presence of protein-A a remarkable increase in activity was observed.

Cysteine oxidase is an enzyme catalyzing the oxygenation reaction of cysteine to cysteine sulfinate(1-5). It has been reported previously in our laboratory that hepatic cysteine oxidase of rats is an inducible enzyme by cysteine, nicotinamide and hydrocortisone(5) and that this enzymic reaction may play an important role as a rate-limiting enzyme on the regulation of cysteine catabolism in rat.(6). However, the purification of this enzyme has not been achieved due to its extreme instability with standard purification procedures. This instability may be due either to a loss of cofactor(s) or to a conversion to the inactive form during purification procedures. The data reported here demonstrate for the first time that rat liver cysteine oxidase is composed of two distinct proteins, namely catalytic and activating proteins, and that the catalytic protein exists in both an active and an inactive form.

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All correspondence should be addressed to this author.

MATERIALS AND METHODS. The radio-chemical assay of cysteine oxidase activity using L-^{35}S-cysteine was carried out as previously reported(5). The standard assay mixture consisted of 20 μmoles of L-^{35}S-cysteine(0.4-0.8 μC), 0.5 μmoles of $Fe_2(NH_4)_2(SO_4)_3$, 30 μmoles of hydroxylamine-HCl, 100 μmoles of phosphate buffer pH 6.8 and enzyme in a final volume of 2 ml. The assay was carried out under the conditions previously reported(5). Anaerobic preincubation was carried out using Thunberg tubes in which the air was replaced by nitrogen after evacuation with a vacum pump. The amount of cysteine sulfinate produced was monitored by colorimetric analysis with ninhydrin and by enzymic analysis using commercial preparations of asparatate aminotransferase and lactate dehydrogenase as described by Lombardini et al(3). The protein amount was measured by the method of Lowry et al(7). The enzyme cysteine oxidase of rat liver was purified about 200 fold with regard to protein-B from a 20% liver homogenate in 0.25M sucrose by acid treatment, ammonium sulfate fractionation and column chromatography with DEAE-cellulose. The details of the purification procedures will be published elsewhere. Other materials and chemicals were used as previously reported (5,6).

RESULTS AND DISCUSSION.

Fractionation of Two Components of Cysteine oxidase. The dialyzed fraction of ammonium sulfate fractionation was applied on a DEAE-cellulose column (2 X 10 cm) equilibrated with 0.002M sodium phosphate buffer pH 6.8. The column was washed with 70 ml of the same buffer at a flow rate of 30 ml per hour and then eluted with linear gradient elution between 100 ml each of 0.002M and 0.2M phosphate buffer pH 6.8. Ten ml fractions were collected. As shown in Figure 1, protein-A was found in tubes 4 to 6 and protein-B was eluted between tubes 18 and 21. The fractions containing protein-A and protein-B were pooled and used for further purification or experiments. In this experiment, these preparations were used as an enzyme. The fractions of protein-B and protein-A contained protein in a concentration of 0.5-0.7 mg and 3.0-7.0 mg per ml of enzyme solution respectively. Protein-B and

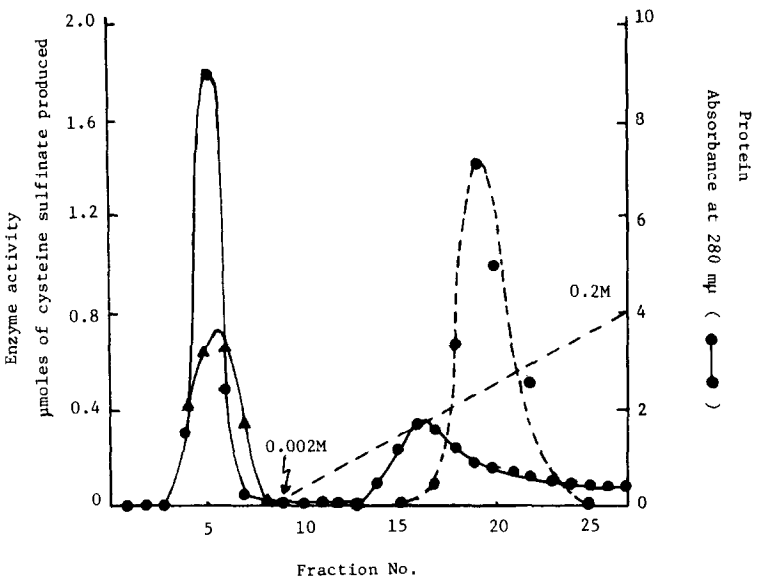

FIGURE 1. Fractionation of the two components of cysteine oxidase of rat liver with DEAE-cellulose column chromatography. Catalytic activity of protein-B fractions was assayed in the presence of 0.5 ml of the fraction in tube 5. Activating activity of protein-A fractions was assayed in the presence of 0.8 ml of protein-B fraction, mixture of tubes 18-21. Enzymic activity was assayed under standard conditions with the addition of bovine serum albumin to inhibit the activity exhibited by protein-B alone. Activating activity of protein-A fractions; ▲—▲ . Catalytic activity of protein-B fractions; ●—● .

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protein-A was added to the assay mixture in amounts of 0.5-0,8 ml and 0.5 ml respectively.

Requirement of Protein-A for the Catalytic Activity of Protein-B and Cysteine Mediated Activation of Protein-B. As shown in Table 1, the enzymic activity of protein-B alone was extremely low. Protein-A alone did not exhibit catalytic activity under any of the conditions so far tested. However, a significant activity was observed in the presence of both fractions. Furthermore, when protein-B was preincubated with 0.01M cysteine for 30 min under anaerobic condition, the enzymic activity of protein-B was remarkably increased in the presence of protein-A. It was found that L-cysteine could not be replaced neither by thiol compounds such as glutathione and dithiothreitol nor by the substrate analogue cystine. The catalytic activity

TABLE 1. Anaerobic activation of protein-B and requirement of protein-A for full enzymic activity of protein-B

Addition in Preincubation Mixture for Activation of Protein-B	Enzymic Activity			
	Enzyme System in Assay Mixture			
	Experiment I		Experiment II	
	Protein-B —	Protein-B + Protein-A	Protein-B* —	Protein-B* + Protein-A
Non Preincubated	0.489	0.775	0.094	0.997
Preincubated				
No Addition	—	—	0.064	1.038
$1X10^{-2}$M Cysteine	0.625	1.511	0.191	2.454
$1X10^{-2}$M Cysteine with Protein-A, 0.5ml	—	—	—	2.436

Bovine serum albumin free protein-B was used in Experiment I and protein-B* used in Experiment II contained bovine serum albumin as described in text. Preincubation of protein-B was carried out under nitrogen as gas phase using Thunberg tube at 37° for 30 min. The preincubation mixture had the same composition as standard assay mixture except for the additions designated above. Isotopic tracer L-^{35}S-cysteine was added after preincubation. Enzymic activity was expressed as μmoles of cysteine sulfinate produced per hour per ml of enzyme. Hydroxylamine and ferrous iron were added in preincubation and assay mixtures.

exhibited by protein-B alone was remarkably inhibited by bovine serum albumin. However, this inhibitory effect was not observed when protein-A was present. As shown in Table 1, it appears likely that serum albumin did not exhibit the activation effect of protein-B by anaerobic preincubation but that protein-B was stabilized by serum albumin during the preactivation process. This anaerobic activation of protein-B mediated by cysteine was not affected by protein-A being added to the preincubation mixture. The possibility of an intermediate formation from cysteine during the preactivation process and the anerobic conversion of this intermediate to cysteine sulfinate by subcequent incubation with protein-A was ruled out due to the following findings: 1. When a preincubated mixture was filtered using an ultrafiltration membrane to remove protein-B or was boiled after preincubation, no

formation of cysteine sulfinate was observed after aerobic incubation of the filtrate or boiled mixture with protein-A. 2. When the isotopic tracer L-^{35}S-cysteine was added either before or after preincubation with non labelled cysteine, a similar formation of ^{35}S-cysteine sulfinate was observed.

Requirement of Cofactor. The effects of hydroxylamine and ferrous iron on the enzymic activity of preincubated and non preincubated protein-B are shown in Table 2. The enzymic activity of protein-B without preincubation

TABLE 2. Effects of hydroxylamine and ferrous iron on the anaerobic activation by cysteine and on the catalytic activity of protein-B

Addition in Preincubation Mixture	Addition in Assay Mixture	Enzymic Activity Preincubated	Enzymic Activity Non Preincubated
None	None	2.111	0.438
	$1X10^{-4}$M Fe^{2+}	0.795	0.330
	$1X10^{-2}$M NH_2OH	2.280	0.803
	$1X10^{-4}$M Fe^{2+} plus $1X10^{-2}$M NH_2OH	1.583	0.997
$1X10^{-4}$M Fe^{2+}		0.566	—
$1X10^{-2}$M NH_2OH		2.943	—
$1X10^{-4}$M Fe^{2+} plus $1X10^{-2}$M NH_2OH		2.454	—

Protein-B was preincubated with $1X10^{-2}$M cysteine under anaerobic condition. Enzymic activity of protein-B was assayed in the presence of protein-A under standard conditions except for the additions of ferrous iron and hydroxylamine. Enzymic activity was expressed as μmoles of cysteine sulfinate produced per hour per ml of enzyme protein-B containing bovine serum albumin as described in text.

was two-fold by hydroxylamine with ferrous iron. However, ferrous iron alone inhibited the enzymic activity of both preactivated and non preactivated protein-B. The anaerobic activation of protein-B was also slightly inhibited by ferrous iron. These inhibitions by ferrous iron were restored by hydroxylamine. Hydroxylamine and ferrous iron were not required for the full enzymic activity of protein-B activated by preincubation.

TABLE 3. Effects of metal chelates on the enzymic activity of preincubated and non preincubated protein-B

Preincubation of Protein-B	Additions in Assay Mixture ($1X10^{-3}M$)	Enzymic Activity	Inhibition (%)
Non Preincubated	None	0.654	0
	α,α'-Dipyridyl	0.160	76
	o-Phenanthroline	0.058	91
	EDTA	0.058	91
Preincubated	None	2.499	0
	α,α'-Dipyridyl	2.704	0
	o-Phenanthroline	0.824	67
	EDTA	1.224	51

Protein-B was preincubated with $1X10^{-2}M$ cysteine under anaerobic condition for 30 min at 37°. The enzymic activity of protein-B was assayed in the presence of protein-A under standard conditions except for the additions designated above. The enzymic activity was expressed as μmoles of cysteine sulfinate produced per hour per ml of enzyme protein-B containing bovine serum albumin. Hydroxylamine and ferrous iron were added to preincubation and assay mixtures.

It has been previously demonstrated that the activity of cysteine oxidase in crude preparations is significantly increased by pyridine nucleotides(2-5). However, no requirement of NAD, NADP, NADH and NADPH was observed for the enzymic activity of protein-B.

Effects of Various Chelates. The enzymic activity of protein-B without preactivation was strongly inhibited by $1 X 10^{-3}M$ α,α'-dipyridyl, o-phenanthroline and ethylenediaminetetracetate. However, when protein-B was preactivated by cysteine, the inhibitory effects of these chelates were remarkably decreased.

From the findings obtained here, it may be concluded that protein-B has as prosthetic group a tightly bound iron atom which oscillates between Fe^{3+} and Fe^{2+} during the cysteine mediated anaerobic activating process of protein-B. This oscillation of the bound iron of protein-B

may be partially caused also by hydroxylamine during the catalytic process. The role of protein-A in this catalytic process still remained to be elucidated. However, two tentative explanations can be considered. One is that protein-A participates in the catalytic process as a generator of the superoxide radical required for oxygenation reaction(8) and the other is that protein-A is an activating modifier of protein-B. We have previously demonstrated that the hepatic cysteine oxidase activity of rats is remarkably increased by L-cysteine injection(5) and that the activity of cysteine oxidase assayed by in vivo measurement was also remarkably increased by cysteine. In this regard, the findings of anaerobic cysteine mediated activation of protein-B and of the requirement of protein-A for the full activity of protein B are of interest. The kinetic properties and the reaction mechanisms of purified cysteine oxidase will be published elsewhere.

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REFERENCES.

1. Wainer, A., Biochim. Biophys. Acta, 141 : 466, 1967.
2. Ewetz, L. and Sörbo, B., Biochim. Biophys. Acta, 128 : 296, 1966.
3. Lombardini, J. B., Turini, P., Biggs, D. R. and Singer, T. P., Physiol. Chem. Phys., 1 : 1, 1969.
4. Lombardini, J. B., Singer, T. P. and Boyer, P. D., J. Biol. Chem., 244 : 1172, 1969.
5. Yamaguchi, K,, Sakakibara, S., Koga, K. and Ueda, I., Biochim. Biophys. Acta, 237 : 502, 1971.
6. Yamaguchi, K., Sakakibara, S., Asamizu, J. and Ueda, I., Biochim. Biophys. Acta, 297 : 48, 1973.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193 : 265, 1951.
8. Hirata, F. and Hayaishi, O., J. Biol. Chem., 246 : 7825, 1971.