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Cysteine-dependent inactivation of hepatic ornithine decarboxylase

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When rat liver homogenate or its postmitochondrial supernatant was incubated with L-cysteine, but not D-cysteine, ornithine decarboxylase (ODC) lost more than half of its catalytic activity within 30 min and, at a slower rate, its immunoreactivity. The inactivation correlated with production of H_2S during the incubation. These changes did not occur in liver homogenates from vitamin B_6-deficient rats. A heat-stable inactivating factor was found in both dialysed cytosol and washed microsomes obtained from the postmitochondrial supernatant incubated with cysteine. The microsomal inactivating factor was solubilized into Tris/HCl buffer, pH 7.4, containing dithiothreitol. Its absorption spectrum in the visible region resembled that of Fe^{2+}·dithiothreitol in Tris/HCl buffer. On the other hand $FeSO_4$ inactivated partially purified ODC in a similar manner to the present inactivating factor. During the incubation of postmitochondrial supernatant with cysteine, there was a marked increase in the contents of Fe^{2+} loosely bound to cytosolic and microsomal macromolecules. Furthermore, the content of such reactive iron in the inactivating factor preparations was enough to account for their inactivating activity. These data suggested that H_2S produced from cysteine by some vitamin B_6-dependent enzyme(s) converted cytosolic and microsomal iron into a reactive loosely bound form that inactivated ODC.

ODC (EC 4.1.1.17), the first and rate-limiting enzyme in polyamine biosynthesis, has the shortest half life *in vivo* among mammalian enzymes studied (Russell & Snyder, 1969; Bachrach, 1980). It is unknown whether this is due to the presence of a specific degradation system for ODC or due to a high susceptibility of ODC protein to a common proteolytic cleavage system. The loss of catalytic activity *in vivo* proceeds significantly faster than does the loss of immunoreactivity (Obenrader & Prouty, 1977), suggesting the presence of an initial inactivation step before proteolytic degradation. Several enzymes appear to undergo oxidative modification before degradation. Thus the inactivation of tyrosine aminotransferase (EC 2.6.1.5) by cytoplasmic membrane in the presence of L-cysteine has been investigated in several laboratories and a modification of thiol groups of the enzyme protein has been proposed (Beneking *et al.*, 1978; Buckley & Milligan, 1978; Federici *et al.*, 1978), although the exact mechanism has not yet been recognized. ODC and tyrosine aminotransferase have some common features; namely, both are PLP-requiring enzymes and rapidly respond to hormonal and dietary stimuli. We therefore examined cysteine-dependent inactivation of ODC, and found that ODC lost both catalytic activity and immunoreactivity to a substantial extent in the presence of cysteine in liver homogenates from normal rats, but not from vitamin B_6-deficient rats, and that microsomal and cytosolic iron was involved in this process.

Abbreviations used: ODC, ornithine decarboxylase; PLP, pyridoxal phosphate; DTT, dithiothreitol; HAVA, DL-δ-amino-α-hydrazinovaleric acid.

Experimental

Materials

DL-[1-^{14}C]Ornithine (sp. radioactivity 59 Ci/mol) was obtained from New England Nuclear Corp. DTT was obtained from Sigma Chemical Co. HAVA was synthesized essentially by the procedure of Sawayama *et al.* (1976). All other compounds were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan).

Animals

Male Sprague–Dawley rats were obtained from CLEA Japan Inc. at 4 or 5 weeks of age and kept in an air-conditioned windowless room lit from 09:00 h to 21:00 h. The rats were fed from 09:30 h

to 17:00h. Water was given *ad libitum*. Vitamin B_6-deficient rats were prepared by feeding for 4 weeks on a vitamin B_6-deficient diet containing 75% vitamin-free casein (CLEA Japan Inc.), 6% dextrin (Ishizu Pure Chemicals, Osaka), 10% sucrose, 4% mineral mixture (Oriental Yeast Co.), 2% oil mixture (Oriental Yeast Co.), 1% vitamin mixture without pyridoxine (Oriental Yeast Co.) and 2% cellulose powder. Normal rats were maintained on laboratory chow for 1 week before use. Rats were killed by decapitation either before feeding (starved; hepatic ODC activity was negligible) or 4h after feeding (fed; hepatic ODC activity was maximally induced).

Inactivation of ODC in homogenate or in postmitochondrial supernatant

Hepatic ODC activity was induced by 4h feeding (Hayashi *et al.*, 1972) or by thioacetamide injection (150mg/kg body wt.) 20h before death. Rats were killed by decapitation and livers were homogenized with 2vol of 0.25M-sucrose containing 1mM-DTT using a Dounce-type all-glass homogenizer. A postmitochondrial supernatant was obtained by centrifugation of the homogenate at 10000*g* for 20min. The homogenate or postmitochondrial supernatant was incubated with or without L-cysteine (5mM) at 37°C with constant shaking. Portions (1.5ml) were taken from the mixture at appropriate intervals, cooled on ice and centrifuged at 100000*g* for 1h. The supernatants were dialysed overnight against 0.1M-Tris/HCl buffer, pH7.4, containing 1mM-DTT and assayed for ODC activity.

Preparation of inactivating factor

A postmitochondrial supernatant not containing endogenous ODC was prepared from the liver of starved rats in a similar manner to that described above. It was incubated with 5mM-L-cysteine at 37°C for 20min and centrifuged for 1h at 100000*g*. The supernatant was dialysed against distilled water (six changes in a 6h period), and used as the cytosolic inactivating factor preparation. Its protein concentration was about 30mg/ml. The microsomal pellet was suspended in a 3-fold original volume of 0.25M-sucrose, and the centrifugation was repeated. The pellet was resuspended in the original volume of 0.25M-sucrose, and used as a microsomal inactivating factor preparation. Its protein concentration was about 8.5mg/ml.

Heat treatment of cytosolic and microsomal inactivating factors

The cytosolic and microsomal inactivating factor preparations were heated for 3min in a boiling-water bath. Denatured proteins of the cytosol were removed by centrifugation at 10000*g* for 20min. The heated microsomal suspension was centrifuged at 100000*g* for 1h, and the pellets were resuspended in the original volume of 0.25M-sucrose.

Extraction of microsomal inactivating factor

Tris/HCl buffer (500mM, 0.1ml) and DTT (62.5mM, 0.1ml) were added to the heated microsomal suspension (1.0ml) in 0.25M-sucrose, pH7.4. After being left at 0°C for 1h, the mixture was centrifuged at 100000*g* for 1h and the supernatant was obtained.

Inactivation of partially purified ODC by cytosolic and microsomal inactivating factors

Hepatic ODC was induced and purified 300-fold from liver extract by pyridoxamine phosphate-succinyldi(aminopropyl)amine–Sepharose affinity chromatography as described previously (Kameji *et al.*, 1982), unless otherwise stated. The partially purified ODC (15–30 units) was pre-incubated with the cytosolic or microsomal inactivating factor preparation in an assay mixture without substrate in a final volume of 1.15ml at 37°C. After 1h, 0.1ml of the substrate solution (radioactively labelled ornithine) was added to initiate ODC assay.

The results are expressed as percentage of ODC activity obtained after pre-incubation without the inactivating factor.

Assays

H_2S was determined by the method of Siegel (1965). This method determines not only free H_2S but also labile sulphur and metal sulphide that liberates H_2S in the acidic assay conditions. Iron was determined by the method of Ramsay (1953), except that boiling was omitted and that hydroxylamine was either replaced with dithionite (50mg in a total volume of 1.05ml) or omitted where indicated. ODC was assayed essentially as described by Hayashi *et al.* (1972). The reaction mixture consisted of 0.125μCi of DL-[1-^{14}C]ornithine, 0.4mM-L-ornithine, 20μM-PLP, 5mM-DTT, 40mM-Tris/HCl buffer, pH7.4, and enzyme solution in a final volume of 1.25ml. Incubation was carried out at 37°C for 1h. One unit of enzyme activity was defined as the amount forming 1nmol of CO_2 per h under the above conditions. Antigenic activity of ODC was determined as previously described by using specific rabbit antiserum raised against the enzyme (Kameji *et al.*, 1981).

Results

When liver homogenates were incubated at 37°C in the presence of 5mM-L-cysteine, their ODC activities decreased with time in a non-linear manner as shown in Table 1. Incubation of post-

Table 1. *Inactivation of ODC in liver homogenate or postmitochondrial supernatant*
Pre-incubation was performed at 37°C for 1h. Pre-incubation mixtures were centrifuged at 100000*g* for 1h. The supernatants were dialysed, and assayed for both catalytic activity and antigenic activity of ODC as described in the Experimental section. The data (percentages of initial value) are expressed as means for two experiments or means ± S.D. for three to seven experiments. The number of experiments is given in parentheses.

	Remaining catalytic activity of ODC (%)					
	−L-Cysteine			+L-Cysteine (5mM)		
Preincubation ...	15min	30min	60min	15min	30min	60min
Homogenate	87 (2)	85 (2)	81 (2)	55 ± 11 (6)	46 ± 11 (7)	45 ± 10 (6)
Postmitochondrial supernatant	100 (2)	92 (2)	78 (2)	48 ± 7 (4)	41 ± 10 (6)	31 ± 6 (3)
Cytosol		100 (2)			93 (2)	
Partially purified ODC + microsomes		95 (2)		100 (2)	88 (2)	

	Remaining antigenic activity (%) +L-Cysteine (5mM)	
	30min	60min
Postmitochondrial supernatant	72 (2)	65 ± 11 (4)

Table 2. *Effect of vitamin B_6-deficiency on ODC inactivation in vitro*
In expt. 1, rats were killed 4h after being fed. Homogenates were pre-incubated with L-cysteine (5mM) for 20min (normal rats) or 30min (vitamin B_6-deficient rats). In expt. 2, three rats were given thioacetamide (150mg/kg body wt.) 20h before death. The postmitochondrial supernatants were pooled and then pre-incubated for 30min with L-cysteine (5mM) in the presence or in the absence of PLP (40μM). In expt. 3, rats were killed 4h after being fed. The postmitochondrial supernatants were pre-incubated with L- or D-cysteine (5mM) for 30min. Results are means ± S.D. Numbers of rats are given in parentheses. Initial ODC activities (units/mg of protein) were 0.48 ± 0.11 ($n = 3$) for 'normal' of expt. 1, 0.83 ± 0.31 ($n = 4$) for 'Vitamin B_6-deficient' of expt. 1, 2.6 for expt. 2, and 0.40 for expt. 3. *$P < 0.005$, compared with 'Normal'.

Expt. no.	Rats	Additions	ODC activity (% of initial activity)
1	Normal	L-Cysteine	46 ± 14 (3)
	Vitamin B_6-deficient	L-Cysteine	83 ± 4 (4)*
2	Vitamin B_6-deficient	L-Cysteine	77
	Vitamin B_6-deficient	L-Cysteine + PLP	58
3	Normal	L-Cysteine	62
	Normal	D-Cysteine	103

mitochondrial supernatants with cysteine also resulted in the inactivation of ODC. However, similar incubation of cytosol fractions (100000*g* supernatant) produced much less inactivation, and only a weak inactivation was observed when partially purified ODC was incubated with washed microsomes in the presence of cysteine. These results indicated that both cytosol and microsomal fractions were necessary for the cysteine-dependent inactivation of ODC. The amount of immunoreactive protein also decreased, at a rate slower than that of enzyme inactivation (Table 1).

As shown in Table 2, the cysteine-dependent inactivation of ODC did not occur in liver homogenates prepared from vitamin B_6-deficient rats and an addition of PLP to the homogenates partially restored the inactivation. Furthermore, D-cysteine was not effective. These results indicated the possibility that cysteine was metabolized by some PLP-dependent enzyme(s) and then some of its metabolite(s) inactivated ODC.

During the incubation with cysteine, either the homogenate or postmitochondrial supernatant became dark to an extent apparently correlating with the rate of ODC inactivation. Since the formation of metal sulphide was suggested by the darkening, we measured the concentration of H_2S, including labile sulphur and metal sulphide, at the end of the incubation. As shown in Table 3 (expt. 1), a marked accumulation of H_2S was observed in both cytosol and microsomal fraction after the incubation of postmitochondrial supernatant with cysteine. The possible effect of various compounds was then tested on ODC inactivation and H_2S formation. As shown in Table 3 (expt. 1), α-oxoglutarate (20mM), homoserine (30mM) and HAVA

Table 3. *ODC activity and the concentration of H_2S before and after incubation with* L-*cysteine*

In expt. 1, postmitochondrial supernatants were incubated with L-cysteine for 20 min, and then supernatants (cytosol) and microsomes were obtained by centrifugation. The microsomes were suspended in the original volume of homogenizing medium. H_2S concentration was determined in the cytosol and microsomal fractions as described in the Experimental section. ODC activity of the cytosol was determined after dialysis. In expt. 2, postmitochondrial supernatant, cytosol or a suspension of washed microsomes was incubated with cysteine. The increase in H_2S concentration was determined after 20 min incubation. Data are the results of a single experiment that is representative of three or four experiments.

			[H_2S] (μM)	
Expt. 1.	Incubation of postmitochondrial supernatant	ODC activity (units/ml)	Cytosol	Microsomes
	No addition	31.1	0	1.2
	Cysteine	20.0	119.5	43.7
	Cysteine + α-oxoglutarate (20 mM)	27.7	38.8	4.4
	Cysteine + HAVA (1 mM)	28.9	8.1	2.6
	Cysteine + EDTA (1 mM)	31.4	136.5	6.5
	Cysteine + homoserine (30 mM)	28.2	40.0	1.2

		[H_2S] (μM)
Expt. 2.	Incubation	
	Postmitochondrial supernatant + cysteine	254
	Cytosol + cysteine	242
	Microsomes + cysteine	1

Table 4. *ODC inactivating activity of various fractions prepared from postmitochondrial supernatant incubated with or without cysteine*

A postmitochondrial supernatant was obtained from liver extracts of starved rats and incubated for 20 min with or without cysteine. Then various fractions were prepared and assayed for inactivating activity against ODC. Further details are given in the Experimental section. Data are the results of a single experiment that is representative of more than four experiments.

			ODC activity remaining (% of control)	
	Fraction added	Volume (ml)	−Cysteine	+Cysteine
(*a*)	Dialysed cytosol	0.1	98	75
(*b*)	Heated supernatant of (*a*)	0.1	100	45
(*c*)	Washed microsomes	0.3	98	59
(*d*)	Heated (*c*)	0.3	87	63
(*e*)	Extract of (*d*) with DTT	0.6	93	91
(*f*)	Extract of (*d*) with DTT and Tris/HCl	0.6	91	47

(1 mM) were found to inhibit not only the enzyme inactivation but also H_2S formation in both cytosol and microsomal fractions, indicating a correlation between the two processes. A discrepancy was noted in the case of EDTA, which protected ODC from the inactivation and reduced the amount of H_2S in the microsomal fraction but did not affect the amount of H_2S in the cytosol. This discrepancy could be explained, however, if we assume that both H_2S and a metal are involved in the inactivation of ODC. A significant accumulation of H_2S was observed when cytosol alone was incubated with cysteine (Table 3, expt. 1). The cytosol, however, became only slightly dark and ODC in the cytosol did not suffer appreciable inactivation under such conditions (Table 1). This also indicated the necessity of both H_2S and microsomal metal for the inactivation of ODC. These results suggested, therefore, that H_2S was formed from cysteine by some PLP-dependent enzyme(s) in the cytosol, and that the H_2S modified some metal in microsomes to a form that could inactivate ODC.

We tried to prepare the possible inactivating factor from a postmitochondrial supernatant derived from livers of starved rats as described in the Experimental section. As shown in Table 4, exogenously added ODC was inactivated by either microsomal or cytosol preparations obtained from the postmitochondrial supernatant pre-incubated with cysteine, whereas the enzyme was not inactivated by control preparations obtained without cysteine. The inactivating factors in the cytosol and the microsomes were stable to heating at 100°C for 3 min; indeed, inactivating activity of

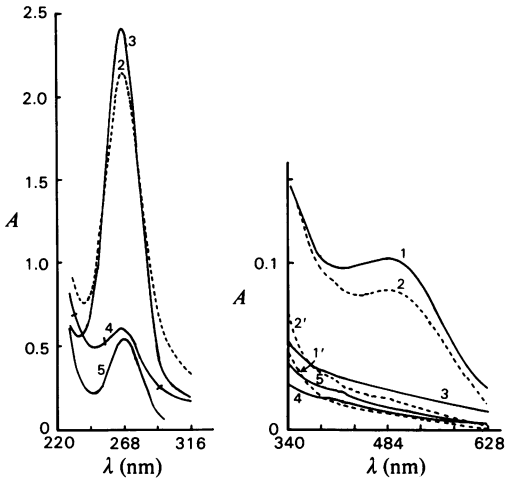


Fig. 1. *Absorption spectra of solubilized microsomal inactivating factor*

Spectra 1 and 1′ were obtained with a solution containing 28.4 μM-$FeSO_4$, 5.2 mM-DTT, 41.7 mM-Tris/HCl and 0.2 M-sucrose. Spectra 2–5 and 2′ were obtained with microsomal extract: DTT (62.5 mM, 0.1 ml) and/or Tris/HCl (500 mM, 0.1 ml) were added to 1.0 ml of microsomal suspension in 0.25 M-sucrose, pH 7.4. After being left at 0°C for 1 h, the mixtures were centrifuged at 100000*g* for 1 h. DTT, Tris/HCl or water was added to the supernatant to adjust their concentrations to be the same in all samples. Spectra 2 and 2′, an extract with Tris/HCl and DTT; 3, an extract with DTT; 4, an extract with Tris/HCl; 5, an extract of control microsomes with Tris/HCl and DTT. Spectra were measured at 0°C (1–4) or at 37°C (1′ and 2′).

the cytosol was rather increased by heating (Table 4). As also shown in Table 4, the factor could be solubilized from microsomes into Tris/HCl buffer containing DTT.

Fig. 1 shows the absorption spectra of the extracts. The extract of microsomes treated with cysteine had a sharp peak at 268 nm and a broad shoulder at 480 nm. The intensity of absorbance in the visible region correlated with the inactivating activity. On the other hand, the peak at 268 nm did not correlate with the inactivating activity: the extract of microsomes with DTT alone showed the peak at 268 nm but did not inactivate ODC. The extract of control microsomes showed an essentially similar spectrum but its intensity was much weaker. The spectrum of the active extract in the visible region resembled that of $FeSO_4$ in the same buffer containing DTT. Furthermore, both spectra changed reversibly depending on the temperature: the shoulder at 480 nm decreased (no colour) at 37°C and it re-appeared (pink) after shaking in an ice bath, indicating possible formation of an iron·thiol complex with $O_2^{-\bullet}$ (Willson, 1977; Lambeth *et al.*, 1982). Since these results suggested a possible involvement of iron, we examined the relationship between the cysteine-dependent inactivation and iron.

First, some metal ions, especially zinc and iron, have been reported to inhibit ODC activity (Haddox & Russell, 1981). We confirmed the inhibitory effect of iron on partially purified ODC. Furthermore, inactivation of ODC either by the present factor or by iron was protected, but not restored, by chelators such as EDTA (4 mM) and αα′-dipyridyl (10 mM) and also by PLP at a high concentration (500 μM) (results not shown).

Although iron is abundant in liver, free iron concentration is assumed to be extremely low (Bezkoravainy, 1980). We examined whether iron was released or exposed as a reactive form during the incubation with cysteine. Iron was determined by its reactivity with αα′-dipyridyl in the presence of either a weak reducing agent (hydroxylamine) or a strong reducing agent (dithionite). We assumed that any reactive iron accessible to ODC would form a chelate with αα′-dipyridyl under weak reducing conditions. As shown in Table 5, such reactive iron increased when postmitochondrial supernatant or cytosol, but not microsomes, were incubated with cysteine. The reactive iron was later shown to be chelatable even without any reducing agent (results not shown). The content of the reactive iron in postmitochondrial supernatant was more than twice as high as that in cytosol that had been pre-incubated without microsomes. This difference in the reactive iron content may have been the cause of the difference in the inactivation of ODC (Table 1). In postmitochondrial supernatant, microsomes may serve, at least partly, as a source of cytosolic reactive iron, since the iron content determined in the presence of dithionite decreased in microsomes and increased in cytosol on the incubation with cysteine. The increase of reactive iron in the cytosol fraction of postmitochondrial supernatant, however, could not be accounted for totally by the supply from the microsomal iron alone. Microsomes may also catalyse the exposure of iron located in cytosol.

As shown in Table 5 (expt. 2), subcellular fractions containing inactivating factor also contained the reactive iron. Thus inactivating activity seemed to correlate with the amount of reactive iron. When compared between different fractions, however, the amount of reactive iron did not precisely correlate with the inactivating activity. For example, it was higher in dialysed cytosol than in its heated supernatant (Table 5, expt. 2), but the inactivating activity was higher in the latter than in the former (Table 4). This suggested the existence in cytosol of some interfering substance(s) that protects the enzyme from inactivation by iron. In fact both cytosol and microsomes prepared from a

Table 5. *Amount of Fe^{2+}·αα'-dipyridyl formed under different reducing conditions after incubation with or without cysteine*
The experimental conditions were as in Table 3 (expt. 1) or Table 4 (expt. 2).

Expt. 1	Fe^{2+}·αα'-dipyridyl formed (nmol·ml^{-1})			
	With hydroxylamine		With dithionite	
Incubation	−Cysteine	+Cysteine	−Cysteine	+Cysteine
Postmitochondrial supernatant				
Cytosol	25	330	331	413
Microsomes	7	93	246	185
Cytosol	19	185	292	271
Washed microsomes	4	10	147	174

Expt. 2	Fe^{2+}·αα'-dipyridyl formed (nmol·ml^{-1}) with hydroxylamine	
Fractions prepared from incubated postmitochondrial supernatant	−Cysteine	+Cysteine
(*a*) Dialysed cytosol	11	323
(*b*) Heated supernatant of (*a*)	4	203
(*c*) Washed microsomes	9	87
(*d*) Heated (*c*)	9	74
(*e*) Extract of (*d*) with Tris/HCl and DTT	3	25

control postmitochondrial supernatant showed a marked protecting effect on the iron-dependent inactivation of ODC (Table 6). Heating of the cytosol abolished the protecting effect, but heating the microsomes did not. The extract of microsomes did not have such a protecting effect (results not shown).

Finally, we determined whether the activity of the inactivating factor can be accounted for by the amount of reactive iron. Since the above results showed that the protecting effect was negligible in the heated supernatant, we prepared the heated supernatant of cytosols containing inactivating factor from several starved rats and examined the relationship between the content of reactive iron and the inactivating activity. As shown in Fig. 2, there was a good correlation between the inactivating activity and the content of reactive iron in the heated supernatant. Inactivating activity per unit concentration of Fe^{2+} was about the same in both the heated supernatant and $FeSO_4$, indicating that the inactivating activity of the heated supernatants was totally accounted for by their content of reactive iron.

In an attempt to understand the mechanism of the iron-dependent inactivation we studied the effect of DTT and oxygen on it. Partially purified ODC was pre-incubated with Fe^{2+} or Fe^{3+} in the absence or in the presence of DTT for 1 h. Then, EDTA was added to stop the action of iron, and ODC activity was assayed in the presence of DTT as usual, since the enzyme used in this experiment was only 3% active if DTT was absent from the assay mixture. As shown in Table 7, ODC inactivation occurred in the presence, but not in the absence, of DTT under aerobic conditions, indicating that Fe^{2+} inactivated ODC by binding to its thiol group(s). Furthermore, the iron-dependent inactivation decreased markedly in the absence of oxygen, suggesting that the bound Fe^{2+} catalysed an autoxidation of ODC. In this experiment, Fe^{3+} exhibited the same effect as Fe^{2+}, probably because Fe^{3+} was easily reduced to Fe^{2+} in the presence of DTT (Lambeth *et al.*, 1982).

Table 6. *Protecting effect of cytosol and microsomes on Fe^{2+}-dependent ODC inactivation*
Partially purified ODC was pre-incubated at 37°C for 1 h with $FeSO_4$ (21.7 μM) and each of various subcellular fractions (0.2 ml) prepared from the liver extract of starved rats. The reaction mixture contained Tris/HCl, pH 7.4, PLP and DTT in a final volume of 1.15 ml. After the pre-incubation, 0.1 ml of the substrate solution was added to initiate the enzyme assay. Control activity was 30 units.

Addition at pre-incubation	ODC activity (%)
None	100
Fe^{2+}	29
Fe^{2+} + cytosol	76
Fe^{2+} + heated supernatant of cytosol	29
Fe^{2+} + microsomes	59
Fe^{2+} + heated microsomes	56

Discussion

Hepatic ODC was found to be inactivated *in vitro* in the presence of L-cysteine, cytosol and

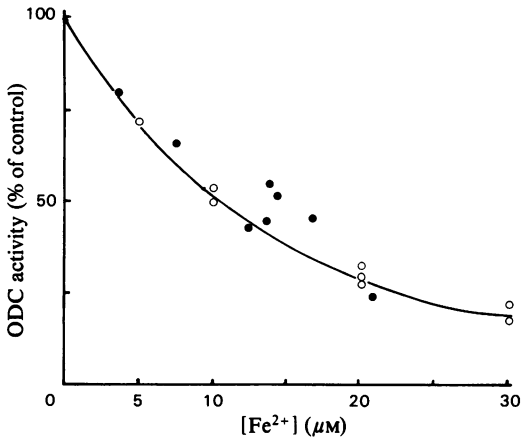


Fig. 2. *Relationship between inactivating activity and the iron content of heated supernatants of cytosols*
Details are given in the text. Inactivation by $FeSO_4$ was also determined for comparison. ○, $FeSO_4$; ●, heated supernatant of cytosol. Control activity was 32 units.

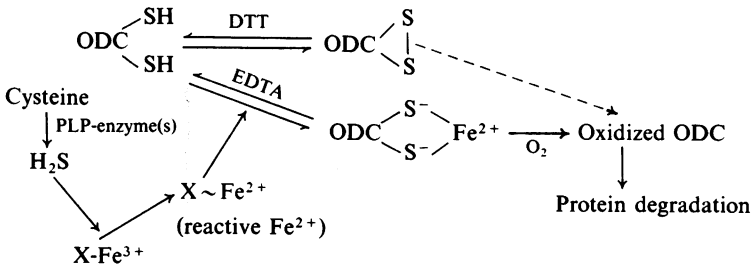
Table 7. *Effect of DTT and oxygen on inactivation of ODC by iron*
The mixture (1 ml), containing ODC (partially purified by DEAE-cellulose column chromatography; Kameji *et al.*, 1982), PLP and Tris/HCl buffer, pH 7.4, was pre-incubated with a final concentration of 0.1 mM-$FeSO_4$ (0.1 ml) or -$FeCl_3$ (0.1 ml) for 1 h at 37°C in the presence or in the absence of DTT (6.25 mM). EDTA (0.05 ml) was added to stop the action of iron, and then DTT (0.05 ml) (*a*) and the substrate (0.1 ml) was added to initiate ODC assay. Each control was similarly pre-incubated and assayed without iron. The concentrations of PLP, EDTA and Tris/HCl buffer in the final assay mixture were 20 μM, 4 mM and 40 mM respectively. Results are means ± S.D. Numbers of rats are given in parentheses. Average values of control activity were 5 units for (*a*), 18 for (*b*), and 16 for (*c*).

		ODC activity (% of control)	
Expt.	Pre-incubation	Fe^{2+}	Fe^{3+}
(*a*)	Aerobic, −DTT	95 ± 29 (4)	90 ± 20 (4)
(*b*)	Aerobic, +DTT	1 ± 1 (3)	5 ± 2 (4)
(*c*)	Anaerobic, +DTT	90 ± 4 (3)	84 (2)

microsomes. The inactivation correlated with a production of H_2S and with a formation of Fe^{2+} loosely bound to cytosolic and microsomal macromolecules. Large amounts of Fe^{2+}·αα′-dipyridyl chelate could be formed in both cytosolic and microsomal fractions in the presence of a strong reducing agent (dithionite), as shown in Table 5. Therefore, the result suggested that some firmly bound endogenous iron was converted by H_2S, a strong reducing agent produced from cysteine, into loosely bound reactive Fe^{2+}, which inactivated ODC. In fact $FeSO_4$ alone inactivated partially purified ODC. Furthermore, reactive Fe^{2+} was contained in the cytosolic and microsomal inactivating factor preparations derived from the post-mitochondrial supernatant incubated with cysteine, and the Fe^{2+} content could totally account for the non-dialysable inactivating activity. The chemical nature of the Fe^{2+}-binding macromolecule is not known. Most of the cytosolic inactivating factor could be precipitated with 1 M-HCl and a pretreatment of the factor with ribonuclease decreased its acid precipitability without affecting the inactivating activity (results not shown). This suggested that at least a part of the Fe^{2+} was bound to RNA.

The physiological significance of the cysteine–H_2S–Fe^{2+}-dependent inactivation of ODC is unknown. Recently, Stipanuk & Beck (1982) reported that cysteine was desulphydrated by cytosolic cystathionine γ-lyase (EC 4.4.1.1) and cystathionine β-synthase (EC 4.2.1.22) in the presence of PLP and suggested that the reaction may be important not only in cysteine catabolism but also as a source of metabolically active reduced sulphur. Although iron is abundant in liver, the free iron concentration is assumed to be extremely low (Bezkoravainy, 1980) and the content of loosely bound reactive Fe^{2+} seemed to be very small under normal conditions (Table 5). Even if firmly bound iron was converted into loosely bound reactive Fe^{2+} by a reductant, such as H_2S, other proteins may compete with ODC for the Fe^{2+}: the result shown in Table 6 indicated that there was abundant heat-labile factor(s) in cytosol that protected ODC from the inactivation by iron. A similar factor modifying iron function has been found with phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (Bentle & Lardy, 1976) and 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) (Ramasarma *et al.*, 1981). On the other hand, it is possible that such protecting factors may have become accessible as a result of tissue homogenization. Therefore, a possible physiological significance of the iron-dependent inactivation could not be ruled out.

As shown in Table 1, the cysteine-dependent inactivation of ODC slowed down with time and almost ceased within 30 min in liver homogenates or postmitochondrial supernatants. This was probably due to the following two facts. First, the formation of H_2S as well as that of the reactive iron also almost ceased within 30 min of incubation (results not shown). Secondly, exogenously added Fe^{2+} inactivated partially purified ODC only in a time-independent manner in the presence of cytosolic protecting factor, although it caused a rapid



Scheme 1. *Proposed mechanism for cysteine-dependent ODC inactivation*

inactivation of the enzyme in both time-independent and time-dependent manner in the absence of cytosolic protecting factor (results not shown). The latter observation also suggested that there are two types of binding of Fe^{2+} to ODC, namely time-independent and time-dependent bindings, the latter being more sensitive to the inhibition by the protecting factor.

The cysteine-dependent inactivation of ODC shares some common characteristics with that of tyrosine aminotransferase: requirement for a particulate fraction (Beneking *et al.*, 1978) and vitamin B_6 (Reynolds, 1978), and protection by PLP at high concentrations (Reynolds & Thompson, 1974). It is uncertain, however, whether or not the two enzymes are inactivated by the same mechanism. Modification of thiol groups has been suggested for the inactivation of tyrosine aminotransferase, and this is also likely to be involved in the inactivation of ODC by Fe^{2+}. The results shown in Table 7 suggested that Fe^{2+} binds to thiol groups at or near the active site of ODC, forming an iron·thiol complex, and catalyses an irreversible oxidation of some functional group essential for the enzyme activity (Scheme 1).

Several studies have pointed out that the thiol group(s) of ODC is essential for catalytic activity and an oxidation of the thiol group(s) results in the inactivation of ODC, as indicated by the broken line in Scheme 1 (Jänne & Williams-Ashman, 1971; Mitchell, 1981; Guarnieri *et al.*, 1982; Zuretti & Garavela, 1983).

Preliminary experiments showed that some decrease in antigenicity took place when ODC inactivated by Fe^{2+}, but not intact ODC, was incubated with liver homogenate. This suggested that such an inactivation of ODC may be a process preceding its degradation *in vitro*.

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