Involvement of thiol transferase- and thioredoxin-dependent systems in the protection of 'essential' thiol groups of ornithine decarboxylase

Flavio FLAMIGNI,* Sandra MARMIROLI, Claudio Marcello CALDARERA and Carlo GUARNIERI

Dipartimento di Biochimica, Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

Ornithine decarboxylase (ODC), an enzyme with 'essential' thiol group(s), may be inactivated *in vitro* by removal of thiol reducing agents and re-activated by soluble factors from rat liver in the presence of NADPH or GSH. The NADPH- and GSH-dependent reducing systems were separated and resolved into three components, called factors A, B₁ and B₂, by chromatographic techniques. Factor B₁ (*M_r* 12000) could re-activate ODC in the presence of GSH and co-purified with thiol transferase activity. Factor B₂ (*M_r* 12000) and factor A (*M_r* approx. 110000) were both needed to re-activate ODC in the presence of NADPH, and co-purified with thioredoxin and thioredoxin reductase activity respectively. In an attempt to investigate the physiological role of the 'essential' thiol group(s) of ODC, erythroleukaemia cells were incubated with *NN*-bis-(2-chloroethyl)-*N'*-nitrosourea, *t*-butyl hydroperoxide and vinblastine, which are known to increase the cellular GSSG/GSH ratio, azelaic acid, an inhibitor of thioredoxin reductase, and sodium arsenite, a strong inhibitor of the ODC-re-activating factors. All these compounds were able to decrease significantly the ODC activity induced in these cells. These results suggest that the thiol transferase- and thioredoxin-dependent systems may be physiologically relevant in maintaining ODC in the active, reduced, state.

INTRODUCTION

Ornithine decarboxylase (ODC; EC 4.1.1.17), the rate-limiting enzyme in polyamine biosynthesis, is characterized by a very short turnover time and a rapid inducibility in response to growth signals [1,2]. ODC contains thiol groups essential for enzymic activity, and *in vitro* requires added dithiols, such as dithiothreitol (DTT), for maximal activity and stability [3–7]. Furthermore, it has been suggested that oxidation of ODC thiol groups by microsomal fractions may be involved in the degradation of ODC protein [8,9]. However, the physiological role of this post-translational modification is still uncertain, and even the identity of the natural thiol(s) involved in the protection of the 'essential' thiol group(s) of ODC *in vivo* is as yet unknown.

We have recently published that rat liver cytosol contains non-diffusible fractions, dependent on NADPH or GSH, which re-activate oxidized ODC [10]. The present paper concerns studies undertaken to investigate this problem further, and reports data indicating that thioredoxin and thiol transferase may be the naturally occurring dithiols that preserve ODC in the reduced, active, state.

EXPERIMENTAL

Materials

L-[1-¹⁴C]Ornithine hydrochloride (sp. radioactivity 54.3 Ci/mol) was purchased from New England Nuclear. Azelaic acid, vinblastine, DTT and NADPH were obtained from Sigma Chemical Co. GSH and thioredoxin (from *Escherichia coli*) were purchased from Calbiochem.

NN-Bis-(2-chloroethyl)-*N'*-nitrosourea (BCNU) was generously given by Simes, Milan, Italy. Sephadex G-75 (superfine grade) and CM-Sephadex C-50 were products of Pharmacia. 2-Hydroxyethyl disulphide was from Aldrich Chemical Co. Bio-Gel TSK DEAE-5PW and Bio-Sil TSK 250 columns were obtained from Bio-Rad Laboratories and connected to a Waters h.p.l.c. apparatus.

Enzyme assays

Partially purified ODC was inactivated by exhaustive dialysis and utilized in a re-activation system in the presence of reducing agents and various fractions as previously detailed [10]. For some experiments, DTT was rapidly removed from partially purified ODC by diafiltration in a 8MC Amicon apparatus against 0.1 mM-EDTA/50 mM-Tris/HCl buffer, pH 7.2, containing 50 μ M-pyridoxal 5'-phosphate. This procedure preserved ODC activity. ODC activity was assayed essentially as described in [11] by incubation with L-[1-¹⁴C]ornithine (final concn. of L-ornithine 37 μ M) for 20 min, unless stated otherwise. ODC activity is expressed as pmol of CO₂ formed/min. Thiol transferase activity was assayed as described by Axelsson *et al.* [12] with 3 mM-2-hydroxyethyl disulphide as a substrate. Thioredoxin and thioredoxin reductase activities were determined as described by Luthman & Holmgren ([13], method 3 and method 1 respectively). Thiol transferase, thioredoxin and thioredoxin reductase activities are expressed as nmol of NADPH oxidized/min at 30 °C.

Purification of the ODC-re-activating factors

Rat liver cytosol was fractionated on a Sephadex G-75 column (1.5 cm $\times$ 48 cm) as described in [10]. Two

Abbreviations used: ODC, ornithine decarboxylase; DTT, dithiothreitol; BCNU, *NN*-bis-(2-chloroethyl)-*N'*-nitrosourea.

* To whom correspondence should be addressed.

fractions, designated A and B, were shown to be involved in the re-activation of oxidized ODC in the presence of NADPH or GSH. Fractions A and B were concentrated in an Amicon 8MC apparatus by ultrafiltration on a YM30 or a YM5 membrane respectively, and then diafiltered against 1 mM-EDTA/50 mM-Tris/HCl buffer, pH 7.4 (buffer TEB). Portions of diafiltered fraction A (4.5 mg of protein) and Fraction B (0.3 mg of protein) were loaded on a Bio-Gel TSK DEAE-5PW column (0.7 cm $\times$ 7.5 cm) equilibrated in TEB buffer containing 0.005 M-NaCl. Proteins were eluted by a 25 ml linear gradient of 0.005–0.5 M-NaCl in TEB. Anion-exchange chromatography was performed at room temperature at a flow rate of 1 ml/min, and 0.5 ml fractions were collected. A calibrated 0.75 cm $\times$ 60 cm Bio-Sil TSK 250 column was utilized for determination of the M_r

of Fraction A. Elution was carried out with 1 mM-EDTA/50 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl at a flow rate of 0.5 ml/min at room temperature.

Cell culture

Friend erythroleukaemia cells (clone 745) were grown in a RPM-I 1640 medium (Sigma Chemical Co.) supplemented with 10% (v/v) foetal-calf serum. In order to induce ODC activity, stationary-phase cells were seeded at a density of 3×10^5 cells/ml in fresh medium plus 10% serum. At the time indicated, batches of 6×10^6 cells were harvested and washed with phosphate-buffered saline (137.0 mM-NaCl/2.7 mM-KCl/8.0 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 buffer, pH 7.2). The cell pellet was extracted with 0.3 ml of 0.1 mM-EDTA/

Fig. 1. Bio-Gel TSK DEAE-5PW chromatography of GSH- and NADPH-dependent factors re-activating ODC

Fraction A (a) or fraction B (b) obtained from Sephadex G-75 was chromatographed on a Bio-Gel DEAE-5PW column as detailed in the Experimental section and monitored for absorbance at 280 nm (—). Fractions (0.5 ml) were collected and assayed as follows: (a) $\circ$, thiorodoxin reductase activity; $\bullet$, ODC-restoring activity in the presence of 0.15 mM-NADPH and fraction B; (b) $\square$, thiorodoxin activity; $\triangle$, thiol transferase activity; $\blacksquare$, ODC-restoring activity in the presence of 0.15 mM-NADPH and fraction A; $\blacklozenge$, ODC-restoring activity in the presence of 2 mM-GSH. ODC-restoring activity was determined by measuring the ability to re-activate ODC inactivated by exhaustive dialysis and was calculated by subtracting, from the total ODC activity, the basal ODC activity obtained in the absence of the active fractions.

50 mM-Tris/HCl buffer, pH 7.2, containing 50 μ M-pyridoxal 5'-phosphate and 0.5% Triton X-100 for 2 min. The cell lysate was centrifuged at 400 *g* for 5 min and the supernatant was immediately assayed for ODC activity and for protein concentration [14].

RESULTS

Preparation and properties of factors re-activating oxidized ODC

We have previously shown that oxidized ODC can be re-activated by incubation with cytosolic factors in the presence of NADPH or GSH [10]. In order to ascertain if the thiol transferase- and thioredoxin-dependent systems were involved in the protection of the 'essential'

thiol groups of the enzyme, the ODC-re-activating factors were subjected to extensive purification and assayed also for thiol transferase, thioredoxin and thioredoxin reductase activities. The sequential chromatography of rat liver cytosol on Sephadex G-75 and Bio-Gel TSK DEAE-5PW led to the isolation of three factors involved in restoring ODC activity. In particular, Sephadex G-75 gel filtration showed a 12000- M_r fraction, which was designated 'B' [10]. This fraction could re-activate ODC in the presence of GSH or, alternatively, of NADPH plus another fraction of higher M_r , which was called 'A'. Both fractions A and B were then subjected to ion-exchange chromatography on Bio-Gel TSK DEAE-5PW (Fig. 1); fraction A was eluted at about 0.21 M-NaCl as a single peak, which possessed thioredoxin reductase activity (Fig. 1*a*), whereas fraction B was resolved into

Table 1. Purification of GSH- and NADPH-dependent factors re-activating ODC

ODC-restoring activity was measured as described in Fig. 1 legend in the presence of 0.15 mM-NADPH and fraction B (factor A), 2 mM-GSH (factor B₁) or 0.15 mM-NADPH and fraction A (factor B₂). The same factors were assayed for thioredoxin reductase, thiol transferase or thioredoxin respectively and for protein content. Specific ODC-restoring activity is expressed as pmol/min per mg; specific activities of thioredoxin reductase, thiol transferase or thioredoxin are expressed as nmol/min per mg.

Purification step	Factor A		Factor B ₁		Factor B ₂	
	ODC-restoring activity	Thioredoxin reductase	ODC-restoring activity	Thiol transferase	ODC restoring activity	Thioredoxin
1. Cytosol	7	22	8	18	10	2
2. Sephadex G-75	21	54	167	148	536	40
3. Bio-Gel TSK DEAE-5PW	317	665	424	311	10037	847

Table 2. Effect of several agents on ODC activity *in vivo* and *in vitro*

(a) Erythroleukaemia cells were diluted to induce ODC and harvested 7 h later. The indicated compounds were added 30 min before harvesting (or 60 min for vinblastine). ODC activity was assayed in the absence or in the presence of 1 mM-DTT. Specific ODC activity of untreated cells (control) was 124 pmol/min per mg. (b) Erythroleukaemia cells were treated with the agents immediately after the dilution and harvested 5 h later. ODC specific activity of control cells was 102 or 106 pmol/min per mg when assayed respectively in the absence or in the presence of 1 mM-DTT. (c) Partially purified ODC was rapidly diafiltered to remove DTT and incubated for 5 min at 37 °C with the indicated compounds before assay with or without DTT. Control ODC activity was 11.1 or 13.5 pmol/min in the absence or in the presence of 1 mM-DTT respectively. The values are means of three determinations; S.D. was in each case less than 10%.

Agent	Concn. (mM)	ODC activity (% of control)					
		(a)		(b)		(c)	
		-DTT	+DTT	-DTT	+DTT	-DTT	+DTT
Control	—	100	100	100	100	100	100
t-Butyl hydroperoxide	0.08	78	87	1	< 1	95	97
	0.16	59	66	2	3	99	94
Vinblastine	0.10	80	89	< 1	< 1	96	101
BCNU	0.04	—	—	10	11	—	—
	0.10	54	62	< 1	< 1	78	84
Azelaic acid	0.01	—	—	88	93	—	—
	0.10	—	—	75	77	100	97
	0.50	—	—	36	37	99	96
NaAsO ₂	0.02	—	—	< 1	< 1	98	—
	0.20	32	37	< 1	< 1	89	101

two peaks (Fig. 1 b): one, termed B₁, was not retained on the column and showed thiol transferase activity and ODC-restoring activity in the presence of GSH; the other, termed B₂, was eluted at about 0.12 M-NaCl and showed thioredoxin activity and ODC-stimulating activity in the presence of NADPH and fraction A. In addition, Table 1 shows how purification of factors A, B₁ and B₂ involved in the re-activation of ODC is accompanied by a progressive increase in the specific activity of thioredoxin reductase, thiol transferase and thioredoxin respectively. Other features of factors A, B₁ and B₂ are consistent with the above-suggested identifications: gel filtration of factor A, performed in non-denaturing conditions on Bio-Sil TSK-250, indicated an *M_r* of 114000 [13]; factors B₁ and B₂ were heat-stable [13,15]; factor B₁ was retained on a CM-Sephadex C-50 column equilibrated with 10 mM-sodium phosphate buffer, pH 6.7, and released by stepwise elution with 0.15 M-NaCl in the same buffer [16]. In addition, commercially available thioredoxin from *E. coli* was able to substitute for factor B₂ in the ODC re-activation.

Finally, it may be noted that the ODC-re-activating factors, and particularly the GSH-dependent one, were effective in protecting ODC even from inactivation after incubation of the enzyme with ornithine (results not shown). This inactivation is also due to oxidation of the 'essential' thiol group(s) [17].

Effect of altered thiol status on ODC activity *in vivo*

In an attempt to assess the role of essential thiol groups of ODC *in vivo*, erythroleukaemia cells in culture were incubated with compounds known to inhibit the thioredoxin system or however to alter the redox equilibrium of the thiol groups of the cells. Dilution of the cells and change of the medium induced ODC activity within a few hours. In these cells, ODC appeared to be in a fully reduced and therefore activated state since adding DTT to the assay mixture did not result in a significant increase in ODC activity (Table 2, parts a and b). Compounds such as t-butyl hydroperoxide, vinblastine and BCNU, which are known to decrease the GSH/GSSG ratio within cells [18–20], were able to provoke a rapid decrease in ODC activity (Table 2, part a). Sodium arsenite, which is an effective inhibitor of both NADPH- and GSH-dependent ODC re-activation at 0.2 mM [10], could also decrease ODC activity significantly within 30 min. It should be noted that a limited recovery in ODC activity was obtained when assays were performed in the presence of DTT. When these compounds were added at the moment of cell dilution, they were able to prevent the induction of ODC activity almost completely (Table 2, part b). Azelaic acid, which has been found to be an effective inhibitor of thioredoxin reductase *in vivo* [21], was also able to provoke a dose-dependent decrease of ODC activity. The same compounds *in vitro* exerted no effect or only a slight inhibition on partially purified ODC (Table 2, part c). In particular, 0.2 mM-sodium arsenite provoked only about 10% inhibition of ODC *in vitro*, which was relieved by adding DTT.

DISCUSSION

It is not known which physiological thiols may replace DTT or other synthetic dithiols added to ODC *in vitro* in order to maximize the activity and stability of this thiol-

dependent enzyme [3–8]. We report here that two distinct systems, identifiable as the thiol transferase- and the thioredoxin-dependent systems, may operate in rat liver cytosol to fulfil this role. They were found effective by two different criteria: the re-activation of ODC oxidized by exhaustive dialysis, and the prevention of oxidation of ODC provoked by incubation with ornithine. The thioredoxin and thiol transferase systems have been implicated in the control of various physiological processes, and in particular they have been proposed to exert a thiol redox control of proteins by acting as effective thiol-disulphide oxidoreductases [22–24]. However, a precise role for thioredoxin or thiol transferase has been shown only for a few thiol-dependent enzymes [22,24–29]. *In vivo*, thioredoxin, GSH and thiol transferase may protect the thiol groups of ODC and counteract the oxidizing action of oxygen radicals [7], GSSG [6] or microsomal oxidases [8]. The thiol redox status of ODC may depend on the balance between these opposite effectors. Thus an excess of oxidizing agents or an impairment of the reducing systems may lead to oxidation of the enzyme. ODC induced in erythroleukaemia cells appears to be in a fully reduced and active state, suggesting that the enzyme is adequately protected *in vivo* in these conditions. Incubation with agents known to alter the thiol status in cells by different mechanisms caused a marked and rapid decrease in ODC activity. Addition of DTT to ODC resulted in a limited recovery of the activity. This indicates that, in these conditions of altered thiol status, a large fraction of enzymic activity was irreversibly lost. The possibility of an accelerated degradation of the enzyme, possibly favoured by its oxidation, should be considered [8,9]; however, the generally complete inhibition of ODC induction observed when these compounds were added at the moment of cell dilution also hints at inhibition of ODC synthesis. Finally it may be noted that the sensitivity to arsenic in the +3 oxidation state *in vitro* may be used to discriminate between 'monothiol' and 'dithiol' enzymes [30]. Thus the lack of inhibition of ODC by arsenite *in vitro* suggests that the ODC molecule has either a unique essential thiol group or essential thiol groups that cannot form intramolecular disulphides. This would agree with the tendency of oxidized ODC to polymerize, presumably as a consequence of the formation of intermolecular disulphide bridges [3,4].

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