Rat liver cytosol contains NADPH- and GSH-dependent factors able to restore ornithine decarboxylase inactivated by removal of thiol reducing agents

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Removal of dithiothreitol (DTT) from partially purified ornithine decarboxylase (ODC) led to an almost complete inhibition of enzymic activity. The inactivation was reversed by addition of millimolar concentrations of DTT, whereas natural reductants such as NADPH or NADH were ineffective, and GSH had only a limited effect. Addition of rat liver cytosol to the incubation mixture resulted in a noticeable re-activation of ODC; however, dialysed cytosol had little effect unless NADPH or GSH was present. Fractionation of rat liver cytosol by gel filtration on Sephadex G-75 yielded two fractions involved in the NADPH- and GSH-dependent re-activation of ODC: one designated 'A', eluted near the void volume ($M_r \geq 60000$), and the other designated 'B', eluted later (M_r approx. 12000). The NADPH-dependent mechanism required both fractions A and B for maximal ODC re-activation; the most effective concentration of NADPH was 0.15 mM, although a significant effect was observed at a concentration more than 10-fold lower. The GSH-dependent mechanism involved the mediation of Fraction B only, and operated at millimolar concentrations of GSH. These results suggest the existence of reducing systems in the cytosol, which may play a role in maintaining, and potentially in regulating, ODC activity by modulation of its thiol status.

INTRODUCTION

Ornithine decarboxylase (ODC; EC 4.1.1.17), which catalyses the rate-limiting step in the polyamine-biosynthetic pathway, appears to be a finely regulated enzyme in mammalian cells: transcriptional and translational control of its synthesis [1], changes in its turnover time [2], inhibition by a specific protein, antizyme [3], multiple enzymic forms [4] and phosphorylation [5] have been described, although the exact physiological role and interrelations of these mechanisms have not been clarified. Moreover, a very rapid increase of the enzyme activity has been observed after various membrane-coupled stimuli [6,7].

The reversible oxidation–reduction of protein thiols/disulphides may represent an additional post-translational mechanism that could affect the activity and/or the degradation rate of enzymes [8–10]. Mammalian ODC contains several cysteine residues within its primary structure [11], and it is known to contain reduced thiol groups essential for its activity [12]. ODC activity can be inhibited *in vitro* by compounds able to react with thiol groups, including GSSG [13,14], and by microsomal oxidases [15]. Synthetic dithiols, usually DTT, have to be added to buffers during ODC purification, whereas GSH or other physiological reductants are ineffective or, at best, poor substitutes for DTT [12,16]. Removal of DTT by dialysis results in inactivation of ODC, accompanied by polymerization [12,17], presumably owing to formation of protein disulphides. However, the addition of DTT appears less critical for ODC activity in crude preparations [18,19].

It is not known which physiological thiols or other reducing agents can influence the thiol status of ODC *in vivo*. In the present work we have identified some

cytosolic factors, dependent on GSH or NADPH, that are probably involved in the protection of the thiol groups of ODC.

EXPERIMENTAL

Materials

L-[1- 14 C]Ornithine hydrochloride (sp. radioactivity 54.3 Ci/mol) and Protosol were purchased from New England Nuclear. DTT, NADPH, NADH and bovine serum albumin were obtained from Sigma Chemical Co. GSH was purchased from Calbiochem. Sephadex G-75 (superfine grade) and DEAE-Sephacel were products of Pharmacia.

Preparation of rat liver cytosol

Adult Sprague–Dawley rats, weighing approx. 200 g and fed *ad libitum*, were used. Animals were killed and livers were rinsed in 0.9% NaCl and minced in 3 vol. of 0.125 M-potassium phosphate, pH 7.4, containing 1 mM-EDTA (buffer PBE). The tissue was immediately homogenized in a glass/Teflon Potter–Elvehjem homogenizer and centrifuged at 105000 *g* for 60 min. The resulting supernatant was filtered through glass-wool and was termed 'cytosol'. In some experiments the cytosol was extensively dialysed to remove endogenous reductants of low M_r ; the dialysis was against three changes of PBE (overall 1000 vol.) for 20 h.

Fractionation of cytosol by Sephadex G-75 chromatography and concentration of active fractions

A 1.5 cm $\times$ 4.8 cm Sephadex G-75 (superfine) column was calibrated with bovine serum albumin (M_r 66200),

chymotrypsinogen A (M_r 25000), cytochrome *c* (M_r 12500) and GSH (M_r 307). A 2 ml portion of rat liver cytosol, containing approx. 40 mg of protein, was applied at a flow rate of 4.1 ml/h on to the same column equilibrated with PBE, and 1.37 ml fractions were collected. Fractions showing ODC-re-activating ability were combined in two separated pools (see the text) and concentrated about 5-fold by ultrafiltration with a YM10 membrane (Amicon). The concentrated fractions were stored at -80°C until used (within a few weeks).

Partial purification of ODC

ODC was partially purified in the presence of 1 mM-DTT by $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-Sephacel chromatography from hearts of rats injected with 10 mg of isoprenaline/kg, as previously reported [20]. The enzyme was concentrated by ultrafiltration with a YM30 membrane (Amicon) to a protein concentration of 1.6 mg/ml and stored at -20°C until used.

Preparation of inactivated ODC

Partially purified and concentrated ODC was inactivated by extensive dialysis against an overall 1000-fold volume of buffer (25 mM-Hepes/NaOH, pH 7.4, containing 0.2 mM-EDTA) for about 20 h. The dialysed samples of ODC were stored at -20°C and used for re-activation experiments within a few days.

Reaction system for the re-activation of inactivated ODC, and ODC assay

A portion of the inactivated ODC preparation (corresponding to about 5 μg of protein) was pre-incubated in a mixture containing (final concns.): 50 mM potassium phosphate, pH 7.4, 0.7 mM-EDTA, 20 μM -pyridoxal phosphate and the reducing agents and cytosolic fractions indicated in the text. After 10 min at 37°C , 0.1 μCi of L-[1- ^{14}C]ornithine in 5 μl of 0.01 M-HCl (final concn. of L-ornithine 37 μM) was added to the mixture to give a final volume of 50 μl . The incubation continued at 37°C for 40 min and then was stopped by adding 0.1 ml of 10% (w/v) trichloroacetic acid. The evolved $^{14}\text{CO}_2$ was trapped on a paper disc impregnated with 20 μl of Protosol and counted for radioactivity by liquid-scintillation spectrometry after a further 40 min.

Protein determination

Protein concentration was determined by the method of Bradford [21], with bovine serum albumin as a standard.

RESULTS

Removal of DTT from a partially purified preparation of ODC by prolonged dialysis resulted in a marked, but reversible, inhibition of the enzymic activity. In fact the inhibition could be reversed by re-adding the dithiol, 1 mM being the most effective concentration (Table 1). Natural reducing agents such as NADPH and NADH were completely ineffective as substitutes for DTT, and GSH (2 mM) exerted a moderate effect. Table 1 also shows that addition of rat liver cytosol brought about a more consistent re-activation of ODC, but most of this restoring effect was lost upon extensive dialysis of cytosol. However, dialysed cytosol could acquire the ability to re-activate ODC if NADPH (0.15 mM) was added. The rate of ODC re-activation by dialysed cytosol in the presence

Table 1. Effect of reducing agents and cytosol on inactivated ODC

Partially purified and inactivated ODC was incubated at 37°C with reducing agents at the indicated concentrations or with rat liver cytosol (10 μl , 0.2 mg of protein). After 10 min, [^{14}C]ornithine was added and ODC activity assayed. ODC activity is expressed relative to the maximal ODC activation obtained in the presence of 1 mM-DTT (= 100%). Values are means $\pm$ s.d. for three determinations. Intrinsic ODC activity of rat liver cytosol and dialysed cytosol was negligible when assayed under the same conditions.

Addition	ODC activity (%)
None	4.1 $\pm$ 1.8
NADPH (0.15 mM)	6.2 $\pm$ 1.2
NADH (1 mM)	3.2 $\pm$ 1.1
GSH (2 mM)	19.4 $\pm$ 0.7
DTT (0.15 mM)	82.9 $\pm$ 2.0
DTT (1 mM)	100.0 $\pm$ 3.0
DTT (2 mM)	91.1 $\pm$ 1.6
Cytosol	42.0 $\pm$ 4.9
Dialysed cytosol	9.2 $\pm$ 1.2
Dialysed cytosol + NADPH (0.15 mM)	51.5 $\pm$ 2.6
Dialysed cytosol + NADPH (0.3 mM)	48.8 $\pm$ 1.2
Dialysed cytosol + NADH (0.15 mM)	13.6 $\pm$ 2.0
Dialysed cytosol + NADH (1 mM)	15.9 $\pm$ 1.4
Dialysed cytosol + GSH (2 mM)	28.9 $\pm$ 0.2
Dialysed cytosol + NADPH (0.15 mM) + GSH (2 mM)	65.5 $\pm$ 4.9
Dialysed cytosol + NADPH (0.15 mM) + NaAsO ₂ (0.2 mM)	5.4 $\pm$ 0.2
Dialysed cytosol + GSH (2 mM) + NaAsO ₂ (0.2 mM)	13.3 $\pm$ 0.1

of NADPH was dependent on the amount of cytosol added (Fig. 1a). Finally, it appears from Table 1 that NADH was scarcely effective, even at 1 mM, whereas 2 mM-GSH had a more evident effect in the presence of dialysed cytosol. These results indicate the existence of both diffusible and non-diffusible factors in the cytosol, which must be simultaneously present to re-activate ODC.

Therefore, rat liver cytosol was fractionated by gel filtration on a Sephadex G-75 (superfine) column, and the various fractions were tested for the ability to activate ODC in the presence of NADPH or GSH (Fig. 2a). In both cases, two peaks of ODC-stimulating activity were obtained: one, called 'A', eluted near the void volume at a position approximately equivalent to that of bovine serum albumin (thus with apparent $M_r \geq 60000$), and a later one, called 'B', eluted at a position corresponding to M_r of approx. 12000. When ODC was assayed in the presence of GSH, peak B was clearly predominant compared with peak A, whereas in the presence of NADPH peak B was less evident. It should be noted that the overall NADPH-dependent ODC-stimulating activity recovered after fractionation of the cytosol was much less than expected on the basis of the previous experiments with cytosol. Therefore, samples of fractions of peak A were pooled and mixed with each fraction from the column. Fig. 2(b) shows that this procedure resulted in an increase in peak B. Conversely,

Fig. 1. Effect of increasing amounts of dialysed cytosol and of Fraction A and Fraction B on ODC re-activation in the presence or absence of NADPH

(a) Inactivated ODC was incubated with the indicated amounts of dialysed cytosol (0.2 mg of protein/10 μ l) in the absence (●) or in the presence (▲) of 0.15 mM-NADPH. (b) Inactivated ODC was incubated with the indicated amounts of Fraction A (10 μ l = 0.1 mg of protein) and/or Fraction B (10 μ l = 0.01 mg of protein) in the absence or in the presence of 0.15 mM-NADPH as follows: ○, Fraction A; △, Fraction A with NADPH; ■, Fraction B; ●, Fraction B with NADPH; ▲, Fraction A plus Fraction B; ◆, Fraction A plus Fraction B with NADPH. ODC activity was then assayed and expressed as pmol of CO₂ evolved in 40 min of incubation.

when fractions of peak B were pooled and mixed with each fraction, peak A was increased. No peak other than A or B appeared over the background in these experiments, indicating that the NADPH-dependent ability to re-activate ODC displayed by cytosol was possessed by factors in peaks A and B, which together provoked a synergistic effect.

The pooled and concentrated fractions of peak A ('Fraction A') and peak B ('Fraction B') were assayed for the ability to re-activate ODC in the presence of various reducing agents (Table 2). As expected, NADPH (0.15 mM) produced only a moderate effect when Fraction A or Fraction B was added separately, and an ODC re-activation of about 60 % of that produced by DTT when both fractions were present. Under these conditions the ODC reaction was essentially linear for at least 40 min

Fig. 2. Sephadex G-75 column chromatography of cytosol and ability to re-activate ODC

(a) Fresh rat liver cytosol (2 ml) was fractionated as detailed in the Experimental section, and the collected fractions were assayed for protein concentration (—). In addition, a 20 μ l sample of each fraction was tested for the ability to re-activate ODC in the presence of 0.15 mM-NADPH (····) or in the presence of 2 mM-GSH (----). Two major peaks were noted and designated A and B. The elution positions of bovine serum albumin (1), chymotrypsinogen A (2), cytochrome c (3) and GSH (4) are shown by arrows at the top. (b) A 10 μ l portion of each fraction from the same chromatographic separation as shown above was combined with 10 μ l of pooled fractions of peak B (----) or with 10 μ l of pooled fractions of peak A (····), and tested for the ability to re-activate ODC in the presence of 0.15 mM-NADPH.

Table 2. Effect of cytosolic Fraction A and Fraction B on ODC re-activation in the presence of various reductants

Inactivated ODC was incubated in the presence of various reductants with Fraction A (10 μ l, 0.1 mg of protein) or Fraction B (10 μ l, 0.01 mg). The concentrations of reducing agents were as follows: DTT, 1 mM; NADPH, 0.15 mM; NADH, 1 mM; GSH, 2 mM. The concentration of NaAsO₂ was 0.2 mM. The values represent means of three determinations; S.D. was in each case less than 5%.

Reductants	Fraction	ODC activity (pmol of CO ₂ /40 min)
None	None	8.6
	A	28.1
	B	11.0
	A + B	27.1
DTT	None	140.7
NADPH	None	9.3
	A	30.6
	B	29.0
	A + B	84.8
NADH	A + B + NaAsO ₂	11.7
	None	6.4
	A + B	32.0
GSH	None	30.4
	A	40.4
	B	67.8
	A + B	61.2
NADPH + GSH	B + NaAsO ₂	38.5
	A + B	112.4

(result not shown). NADH could not substitute for NADPH, not even at 1 mM. GSH (2 mM) brought about a consistent re-activation only in the presence of Fraction B, and it should be noted that the simultaneous addition of Fractions A and B did not enhance the effect. These data suggest that the GSH-dependent re-activating effect manifested by the cytosol is essentially mediated by factor(s) in Fraction B. Table 2 also shows that 0.2 mM-

sodium arsenite, which is known to form a complex with dithiols, but not with monothiols [22], strongly inhibited the NADPH-dependent system and, less effectively, the GSH-dependent system (see also Table 1). In the latter case it is possible that this salt interferes only with the enzymic (Fraction-B-mediated) reaction, leaving the spontaneous reaction with GSH unaltered. Sodium arsenite caused only a slight inhibition of ODC activity stored and assayed in the presence of DTT. Heating of Fraction B at 56 °C for 10 min did not inhibit either the NADPH-dependent or the GSH-dependent ODC re-activation (results not shown). Finally, the simultaneous addition of NADPH and GSH, in the presence of both Fractions A and B, resulted in a re-activation of about 80% of that by DTT (Table 2).

Fig. 1(b) shows the effect of various amounts of Fractions A and B in the presence or absence of NADPH. A noticeable re-activation is produced by even very low amounts of Fractions A and B, when both are added together with NADPH. The ODC-reactivating effect mediated by Fraction A plus Fraction B is dependent on the concentration of NADPH (Fig. 3a); although the maximum activity was found at 0.15 mM-NADPH, an appreciable effect was exerted by a NADPH concentration as low as 10 μ M.

The GSH-dependent re-activation of ODC was a function of the amount of Fraction B added (results not shown) and of the concentration of GSH (Fig. 3b). In particular, Fig. 3(b) shows how GSH by itself, i.e. presumably through a spontaneous, non-enzymic, reaction with ODC, produced a significant effect, although the effect was more marked in the presence of Fraction B at any concentration of GSH tested. The most effective GSH concentration was found to be 5 mM; however, the highest ratio of Fraction-B-mediated to spontaneous re-activation was at 1 mM- and 2 mM-GSH.

DISCUSSION

It is well known that ODC is a thiol-dependent enzyme [12–18], and exogenous, non-physiological thiols, usually DTT, are added to purification and assay buffers to avoid

Fig. 3. Effect of the concentration of NADPH and of GSH on ODC re-activation

(a) The reaction mixture contained inactivated ODC, NADPH at the indicated concentrations, 10 μ l of Fraction A (0.1 mg of protein) and 10 μ l of Fraction B (0.01 mg of protein). (b) Inactivated ODC was incubated with increasing concentrations of GSH in the absence (◆) or in the presence (▲) of Fraction B (10 μ l, 0.01 mg of protein). ODC activity was assayed by adding L-[¹⁴C]ornithine, and the reaction was allowed to proceed for 40 min.

a rapid inactivation. Actually, the activity of purified ODC depends on DTT concentration in the assay buffer, although it should be noted that high concentrations of DTT are inhibitory, particularly at low ornithine concentrations, resulting in an apparent increase in K_m [23–25]. On the other hand, the physiological factor(s) and reducing system(s) involved in maintaining ODC in the active status are not known. Zuretti *et al.* [15,26] found that diffusible cytosolic factors, GSH and, more effectively, NADPH exerted a protective/re-activating effect on cytosolic preparations of ODC exposed to a microsomal inactivating system in the absence of DTT. With more purified enzyme, inactivated by dialysis, which presumably provokes thiol/disulphide interchanges, natural reducing agents of low M_r proved ineffective or scarcely effective in restoring ODC activity ([12,16]; the present work).

We have shown here that both diffusible and non-diffusible factors present in the cytosol are needed to re-activate oxidized ODC. Cytosolic NADPH- and GSH-dependent systems may together bring about an ODC re-activation comparable with that by DTT. The characteristics of the NADPH-dependent mechanism appear to be consistent with its identification as that of the thioredoxin system [27]: in fact, the NADPH-dependent ODC re-activation requires two factors of different M_r (Fractions A and B), the effect being very limited when the two fractions are added separately. The M_r of Fraction B coincides with that of rat liver thioredoxin, and the higher- M_r Fraction A may contain thioredoxin reductase [28]; both the ODC-re-activating and the thioredoxin system operate with sub-millimolar concentrations of NADPH (NADH being a very poor substitute), and are strongly inhibited by sodium arsenite [28].

The GSH-dependent mechanism requires Fraction B and physiological concentrations of GSH. Even if the present data cannot exclude that the same component in Fraction B could be activated by GSH or, alternatively, by a NADPH-dependent reductase, present in Fraction A, the possibility also exists that different factors in Fraction B may be involved: GSH-dependent transhydrogenation of protein disulphides may be catalysed by a heat-stable thiol transferase isolated from rat liver cytosol (M_r about 12000) [29]. It is of interest how iodothyronine 5'-deiodinase, another thiol-dependent enzyme, may be activated independently by DTT, by a GSH-dependent thioltransferase-like factor [30] or by a NADPH-dependent thioredoxin-like system [31]. It is thought that both the thioltransferase and the thioredoxin system may function as efficient cytosolic disulphide reductases with a broad specificity for proteins, even if particular substrates may prefer one of the two reducing systems [32]. Glutaredoxin ($M_r \sim 11\,000$), which has been isolated from the cytosol of calf thymus [33], can also catalyse GSH-dependent transhydrogenation. Further purification of Fractions A and B will be required to elucidate more precisely the mechanism of action and the nature of the factors mediating ODC re-activation.

Another point that requires further investigation is the physiological role of these cytosolic factors in the maintenance (and potential regulation) of ODC activity. Thioredoxin-like or thioltransferase-like factors could catalyse reversible thiol/disulphide exchanges in the ODC molecule *in vivo*, depending on the ratio NADPH/NADP⁺ or GSH/GSSG. In this regard, microsomal oxidases may also play a role [15,19,26]. It is not clear if

ODC is always in a fully reduced and thus activated status *in vivo*; however, differences have been found in various conditions in the requirement of DTT for maximal ODC activity [19,25]; furthermore, a decrease in the activity or stability of ODC has been observed in conditions associated with alteration of glutathione status [26,34]. In particular, the perfusion of rat heart with t-butyl hydroperoxide has been found to produce a fall in the GSH/GSSG ratio and an inhibition of ODC activity, which could be removed by adding DTT to the assay mixture or acetylcysteine to the perfusion medium [34].

The possibility that oxidation of thiol groups in ODC may favour its degradation has also been suggested [19], and an altered form of ODC, with a longer half-life *in vivo* and a stronger resistance to inactivation by microsomal fractions, has been described [35]. Characterization of the physiological mechanisms that regulate the thiol status in ODC may help to elucidate the complex regulation of activity and degradation of this enzyme.

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