Multiple active conformers of mouse ornithine decarboxylase

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Purified recombinant mouse ornithine decarboxylase (ODC) was denatured with urea or with guanidinium chloride. Enzymic activity was efficiently recovered upon dilution of the denaturing agent. ODC renatured after urea treatment was further characterized. Kinetics of decarboxylation of the natural substrate ornithine or of the suicide substrate α-difluoromethylornithine (DFMO) were not significantly changed by denaturation/renaturation. Surprisingly, the renatured enzyme was not stably labelled with radioactive DFMO, in contrast with the native enzyme not subjected to denaturation. Native and renatured ODC did not differ in their c.d. spectra, but the former contained four reactive cysteine residues and the latter seven. These data indicate that a conformational change results from denaturation/renaturation that does not alter decarboxylation of substrates, but does change the accessibility or stability of the cysteine-360 residue modified by decarboxylated DFMO.

INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the first and rate-limiting enzyme in polyamine biosynthesis, catalysing the pyridoxal phosphate-dependent decarboxylation of ornithine to putrescine [1]. Mouse ODC is a homodimer of 51 kDa polypeptides, each with 461 amino acids [2]. Diverse mitogens cause a rapid and dramatic increase of ODC upon stimulation of cell growth [1]. Transcriptional, translational and post-translational mechanisms contribute to regulation of its activity [3–6]. The enzyme has a very fast rate of intracellular turnover [7,8].

The folding and assembly of some proteins are mediated *in vivo* by intracellular components, known as molecular chaperones, which do not themselves form part of the final assembled structures. Instead, the chaperones bind non-covalently to unfolded proteins and form a stable complex that suppresses incorrect assembly pathways. Subsequently, the complex is dissolved and the mature functional protein is released. Folding is assumed to take place *in vivo* as a co-translational or post-translational process [9,10].

The work described here was initially directed at determining whether folding and assembly of ODC into a functional form requires the participation of chaperones. We first set out to determine the extent to which spontaneous unassisted folding of ODC can occur *in vitro*. We used urea and guanidinium chloride (GuHCl) to denature the active functional homodimer of recombinant ODC to a more disordered form. Subsequent removal of the denaturing agents resulted in significant recovery of ODC activity. Surprisingly, the new renatured ODC species acquired new physical characteristics: the enzyme retained the same catalytic properties as native recombinant ODC, but showed an immunity to modification by the suicide inhibitor α-difluoromethylornithine (DFMO).

Decarboxylated DFMO is an efficient killer of enzymic activity; about one in three enzymic decarboxylations results in enzyme suicide [11]. Previously, covalent binding of DFMO to ODC was thought to provide an accurate indication of the existence of active protein, since DFMO must be decarboxylated by ODC before it can modify the enzyme and cause irreversible inhibition of activity [12]. Recently, Pegg and co-workers reported the mechanism of irreversible inhibition of ODC by DFMO, and they isolated and identified the adducts of a DFMO-modified amino acid of ODC [13].

We report here the existence of another conformation for ODC, one that is catalytically active, yet does not remain associated with DFMO. We have identified cysteine-360 of the native ODC protein as the residue that predominantly becomes modified by DFMO, in agreement with the data recently published by Poulin et al. [13]. Mutation of cysteine-360 to alanine results in inactive ODC, which indicates that this amino acid plays an important role in the formation of the active site of ODC.

EXPERIMENTAL

Materials

Ultra-pure urea was purchased from ICN Biochemicals. All other reagents were of analytical grade. Recombinant mouse ODC was purified as previously described [14], by using a PMP-Affigel and a Mono Q f.p.l.c. column, and it was stored at 4 °C. The purified recombinant mouse ODC had five additional amino acids, Met-Asp-Pro-Arg-Thr, N-terminal to the initiating methionine. The addition of these amino acids had no significant effect on the specific activity of the recombinant protein (1.1×10^6 nmol/h per mg), compared with that of the ODC purified from mouse kidney [15,16]. Partially purified *Escherichia coli* ODC was purchased from Sigma. The concentration of ODC was determined by the method of Bradford [17], as modified by Bearden [18] with BSA as a standard.

ODC assay

ODC activity was measured as previously described [19], by the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine from an assay medium

Abbreviations used: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; GuHCl, guanidinium chloride; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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that contained 1.5 mM L-[1-^{14}C]ornithine (0.82 Ci/mol) and assay cocktail (100 μM pyridoxal phosphate, 2 mM dithiothreitol, 2 mg/ml BSA and 10 mM potassium phosphate, pH 7.5). One unit of enzyme activity is defined by the release of 1 nmol of CO_2 in a 60 min incubation at 37 °C.

Interaction of ODC with DFMO

The labelled ornithine in the assay cocktail described above was replaced by DL-[1-^{14}C]DFMO (18.3 Ci/mol), in order to measure the decarboxylation of DFMO. The [*carboxy*-^{14}C]DFMO was generously given by Dr. A. E. Pegg. Covalent binding of DFMO to ODC protein was determined by incubating ODC with DL-[3,4-^{3}H]DFMO (28 Ci/mmol) in assay cocktail. Labelled protein was analysed by SDS/PAGE (10% gel) [20], and binding was determined either by fluorography or by excising the Coomassie-Blue-stained protein bands and determining the amount of bound radioactive label in a liquid scintillator.

Unfolding/refolding

For unfolding and refolding studies, ODC was incubated in 6 M urea or 2 M GuHCl in assay cocktail for at least 1 h. After unfolding, the incubation mixture was diluted in assay cocktail without BSA, so that the final concentrations were 0.1 M or 0.055 M for urea and GuHCl respectively. The protein was then allowed to refold for 1–4 h at 30 °C. The recovery of enzymic activity was used to monitor refolding.

Reactivity of thiol groups

The reactivities of thiol groups were measured in 10 mM Hepes, pH 7.5, by the method of Ellman [21]. The differences in A_{412} were measured at 30 °C, after addition of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).

Mutagenesis

The plasmid pSPODC7 [22], containing mouse ODC cDNA under the control of the lambda pR promoter and a mutant cI gene, was mutagenized by the *dUT-UNG* mutagenesis method [23]. The new mutated ODC plasmid, pSPODC7-C360A, contains an alanine in the position of cysteine-360, as confirmed by restriction analysis and sequencing. The bacteria containing the mutagenized plasmid were grown overnight at 32 °C. Induction of ODC synthesis was initiated by a temperature shift to 40 °C for 45 min. The cells were harvested and resuspended in buffer A (10 mM potassium phosphate, pH 7.5, 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM phenylmethanesulphonyl fluoride).

Cleavage of ODC with BNPS-skatole

ODC (2 nmol) was labelled with [^{3}H]DFMO, as described above, and the DFMO that had not reacted was removed by extensive dilutions and centrifugations through Centricon-30. The labelled enzyme was incubated with 0.975 mg/ml BNPS-skatole in acetic acid [24]. A control cleavage reaction without BNPS-skatole was included. The tubes were covered in aluminium foil and incubated for 1 h at 45 °C. An equal volume of water was added, and the mixture was centrifuged for 5 min in a microfuge. The supernatant was freeze-dried to dryness. The pellet was resuspended in water and dried again by vacuum centrifugation. The sample was analysed on a Vydak C_{18} reverse-phase column. Radioactive fractions were subjected to protein-sequence analysis with an ABI model 475A sequencer (Applied Biosystems, Foster City, CA, U.S.A.). A sample from each sequence cycle was counted for radioactivity in liquid-scintillation fluid.

RESULTS

Effect of urea on ODC activity

The activity of ODC was used to monitor the effect of urea on the protein, since activity is the most sensitive indicator of the integrity of the enzyme. When recombinant ODC was incubated with increasing concentrations of urea, up to 6 M, a decrease in enzymic activity was observed (Figure 1). For concentrations higher than 3 M, enzyme activity was completely abolished (Table 1).

The ODC (12 μg) which had been unfolded in 6 M urea was then diluted in assay cocktail to a final urea concentration of 0.1 M and the protein concentration was adjusted with BSA to 0.036 mg/ml. Under these conditions, ODC enzymic activity was assayed and approx. 52% of the activity was regained. When GuHCl was used as an alternative denaturing reagent, complete inhibition of ODC activity was observed with 2 M GuHCl. Dilution of the inactive ODC to a final GuHCl concentration of 0.055 M resulted in approx. 30% recovery of the enzymic activity (Table 1).

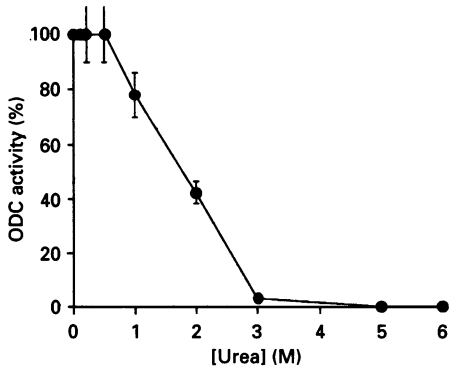

Figure 1 Effect of increasing urea concentrations on the activity of ODC

Recombinant ODC (12 μg/ml; sp. activity 1.1×10^6 units/mg of protein) was incubated with urea in assay cocktail at room temperature for 30 min. ODC activity was then assayed as described, while in the presence of urea; 100% activity in this Figure corresponds to 18 units of ODC activity.

Table 1 Effect of denaturing agents on ODC activity

Recombinant ODC was incubated with different concentrations of urea or GuHCl in assay cocktail, and the enzymic activity was measured before or after renaturation, as described in the Experimental section. The data show the percentage of ODC activity and are means ± S.E.M. ($n = 4$); 100% activity corresponds to approx. 10 units of ODC.

Treatment	ODC activity (%)
None (native enzyme)	100.0
0.1 M urea	100.0 (±2.6)
6 M urea	1.5 (±0.9)
6 M → 0.1 M urea	52.4 (±1.5)
0.055 M GuHCl	98.0 (±0.8)
2 M GuHCl	0.7 (±1.2)
2 M → 0.055 M GuHCl	30.8 (±1.1)

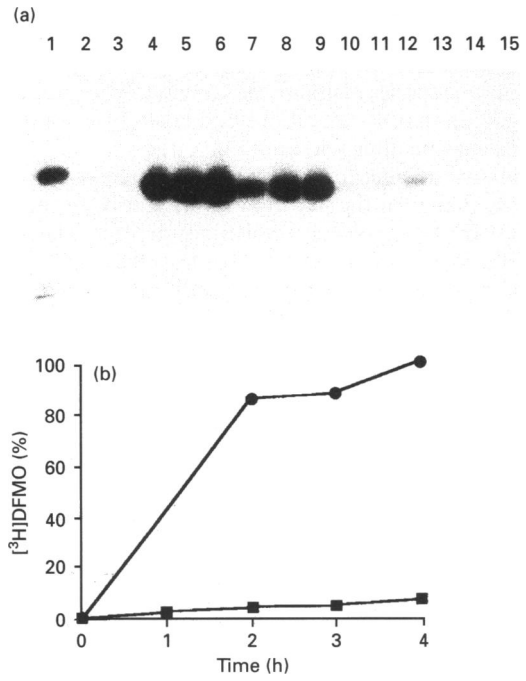

Figure 2 Modification of native and reconstituted ODC by [^{3}H]DFMO

(**a**) ODC (12 μg; sp. activity 1.1×10^6 units/mg of protein) was incubated with 6 M urea or 2 M GuHCl at 30 °C for 60 min, and then the denaturation mixture was diluted to a final urea concentration of 0.1 M, a GuHCl concentration of 0.055 M and a protein concentration (adjusted with BSA) of 36 μg/ml. Immediately after the dilution, DL-[3,4-^{3}H]DFMO was added to the mixture at 10 μM concentration and the samples were incubated at 30 °C for the indicated time periods. The labelled proteins were analysed by SDS/PAGE (10% gels). Control (native) ODC was incubated with 0.1 M urea for the same period of time. Lane 1, native ODC in 0.055 M GuHCl (1 h). Lane 2, reconstituted ODC in 0.1 M urea (1 h). Lane 3, reconstituted ODC in 0.055 M GuHCl (1 h). Lanes 4–6, native ODC in 0.1 M urea (2, 3, 4 h respectively). Lanes 7–9, native ODC in 0.055 M GuHCl (2, 3, 4 h respectively). Lanes 10–12, reconstituted ODC in 0.1 M urea (2, 3, 4 h respectively). Lanes 13–15, reconstituted ODC in 0.055 M GuHCl (2, 3, 4 h respectively). (**b**) The lanes of the above fluorogram for urea-treated ODC were scanned with a PDI gel analyser (Discovery series) and the intensities of the labelled bands plotted, with the extent of modification of native ODC at 4 h taken as 100%. ●, Native ODC; ■, reconstituted ODC.

Temperature-dependence of refolding

The experiments described above were carried out at 30 °C and resulted in ~ 50% recovery of activity. When experiments for the refolding of ODC were performed at 24 °C, the recovery of ODC activity was ~ 40%. Temperatures below 24 °C resulted in even less activity recovered. Incubation at 37 °C resulted in ~ 46% recovery of enzyme activity. Higher temperatures ($\geq$ 45 °C) led to loss of ODC activity in both the native and the renatured samples. Therefore, in all subsequent experiments, renaturation was done at 30 °C.

Kinetics of refolding

To estimate the kinetics of refolding of ODC, we used the substrate analogue DFMO. DFMO can act in two different ways on ODC activity: the structural similarity of ornithine and DFMO confer on DFMO properties of a competitive inhibitor. After DFMO has been decarboxylated by ODC, one out of three molecules binds to the protein covalently, resulting in irreversible inhibition of ODC activity [11,12]. Thus DFMO is used to monitor the presence of active enzyme, most readily by observing the labelling of ODC by DL-[3,4-^{3}H]DFMO.

A control time course was first done, wherein ODC (12 μg/ml, 0.23 μM) was incubated at 30 °C with 10 μM DL-[3,4-^{3}H]DFMO for various periods of time, as described in the Experimental section. The reactions were stopped by the addition of Laemmli sample buffer and boiling. Samples were then analysed by SDS/PAGE (10% gels) and fluorography. Labelling of ODC could be readily detected after incubation of the protein for 2.5 min with DFMO (results not shown). The experiment was repeated under identical conditions for both native ODC and ODC that had been denatured with urea as described and then allowed to refold by dilution to a final urea concentration of 0.1 M (the native ODC was exposed to 0.1 M urea for the same time periods). In a parallel experiment, ODC activity assays were performed to ensure that refolding of the enzyme took place. As shown in Figure 2(a), ODC that was never exposed to a high urea concentration labelled intensely with DFMO at all of the time periods tested, whereas the refolded enzyme showed faint DFMO labelling, accounting at most for about 1% of the signal obtained for the native protein. The fluorogram was scanned and the labelled bands were quantified, as presented in Figure 2(b). The native enzyme had a more than 10-fold greater extent of labelling than the renatured one. Even after very long (up to 24 h) incubations of reconstituted ODC with [^{3}H]DFMO, only a very weak signal could be detected by fluorography. This was unexpected, because in the experiments in which ODC activity was monitored by examining decarboxylation of ornithine rather than labelling with [^{3}H]DFMO, a recovery ranging from 47 to 68% was obtained at the different time points.

The dissociation of ornithine decarboxylation and modification by DFMO in the reconstituted enzyme suggested that a conformational change had taken place in ODC that had either decreased the kinetic efficiency of decarboxylation of DFMO but not of ornithine, or, alternatively, that decarboxylation proceeded as before for both substrates, but the reactive decarboxylated DFMO had less access to the target site of modification.

To test the first possibility, we measured the kinetics of decarboxylation of ornithine and of DFMO by native and renatured ODCs; for both enzymes the reactions were carried out in the presence of 0.1 M urea. As shown in Figure 3(a), with [1-^{14}C]ornithine as substrate, the two enzymes have the same K_m for ornithine (approx. 1.15 mM) and they differ only in their $V_{max.}$ (2.5 nmol/min for the wild-type enzyme versus 1.5 for the renatured one). The decrease in $V_{max.}$ was simply a reflection of less than complete recovery of activity. When native and renatured enzymes were preincubated with unlabelled DFMO at 30 °C for different time periods and then were diluted in assay cocktail with [^{14}C]ornithine as substrate (Figure 3b), DFMO caused a similar extent and rate of loss of ornithine-decarboxylating activity. The K_i for DFMO was found to be 0.3 mM for both enzymic forms. The time of inactivation of the two enzymes differed: the τ_{50} for the native enzyme was 2.1 min and for the renatured ODC 2.8 min. The increased time of half-inactivation associated with the renatured enzyme suggests either that the renatured enzyme decarboxylates DFMO more slowly than the native enzyme, or that decarboxylation of DFMO results in irreversible inactivation of the renatured enzyme less frequently than of the native enzyme.

A second inhibitor was used to determine whether there is a difference in the conformation between native and refolded protein. The protein antizyme, which is a polyamine-inducible inhibitor of ODC [25,26], was tested on both the native and the

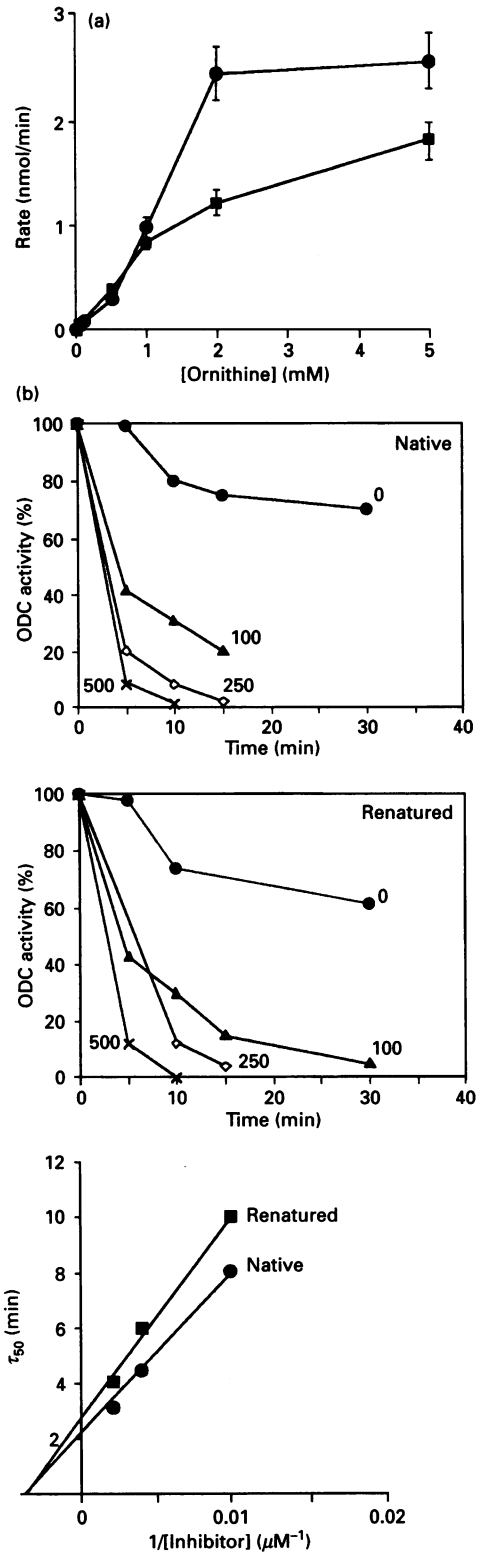

Figure 3 Kinetic properties of native and reconstituted ODC

(**a**) K_m for ornithine. Recombinant ODC (12 μg/ml; 0.235 μg of protein) was incubated with increasing concentrations of L-[1-^{14}C]ornithine for 30 min at 37 °C. Native ODC activity was assayed in the presence of 0.1 M urea. The reconstituted ODC was prepared as in Figure 2. The K_m and the $V_{max.}$ of the decarboxylating reaction were calculated by least-squares fitting of the data to a Lineweaver–Burk plot. ●, Native ODC; ■, reconstituted ODC. (**b**) K_i for DFMO. Native (top) and renatured (middle) ODC, as above, were incubated with increasing concentrations (μM) of DFMO. At each time point, a 20 μl sample was removed from the assay mixture and was kept in ice in assay cocktail. When all the time points were collected, [^{14}C]ornithine was added to each tube and ODC activity was assayed for 60 min at 37 °C. The τ_{50} for inhibition by DFMO for the native and reconstituted enzymes was determined by a least-squares fit to the data plotted as reciprocal inhibitor concentration versus time of half-maximum inhibition (bottom); 100% in this Figure corresponds to 23 units of ODC activity.

reconstituted enzyme. Recombinant rat antizyme [27] inhibited the activity of the two enzymes in a similar dose-dependent manner (results not shown), consistent with the conclusion that the two enzyme species maintain the same catalytic characteristics and suggesting that no alterations had taken place in the active site of the enzyme after renaturation.

The relative inability of DFMO to label reconstituted ODC indicates a change in the partition ratio, that is, in the average number of DFMO molecules which are decarboxylated before irreversible inactivation occurs. To test this possibility, we therefore determined the partition ratio for the native and reconstituted ODC. We measured the rate of DFMO decarboxylation, using 5 μM [1-^{14}C]DFMO as substrate, and in parallel we measured the binding of 5 μM DL-[3,4-^{3}H]DFMO to the protein. To examine if the inactivation of ODC was irreversible and progressive, we also determined the remaining [^{14}C]ornithine-decarboxylating activity by removing a sample from the above incubation mixture of the enzyme with [1-^{14}C]DFMO and diluting 20-fold in ODC assay cocktail. The dilution of DFMO in the assay cocktail significantly decreased the concentration of DFMO in the mixture. The very low specific radioactivity of the labelled DFMO as compared with that of [^{14}C]ornithine made the contribution of $^{14}CO_2$ derived from [1-^{14}C]DFMO negligible. As shown in Figure 4(a), the decarboxylation curves were parallel, with the renatured enzyme being as efficient in decarboxylating DFMO and being inhibited to almost the same degree by DFMO as the native enzyme. Covalent binding of internally labelled DFMO, however, was dramatically different (Figure 4b), thus showing the partition ratio of the renatured enzyme to be approx. 10-fold higher than the 3.3 value found for the native enzyme [11]. The inhibition of ODC activity was progressive and irreversible for both enzymes, arguing that inactivation follows similar mechanisms for the native and renatured ODCs.

When ODC purified from the overproducing mouse lymphoma cell Z12 [28] instead of recombinant mouse ODC produced in bacteria was used as source of native and renatured enzyme, the results obtained were similar: DFMO was decarboxylated at the same rate from both enzymes, ODC activity was inhibited to the same extent, but DFMO did not stably label the renatured Z12-derived enzyme (results not shown). Thus we conclude that refolding and re-acquisition of enzymic activity is not solely a property of the recombinant protein, but it is shared by endogenous mouse ODC.

The refolded enzyme retained its properties for more than 1 month, as determined by activity assays and SDS/PAGE mobility, as well as by the extent of DFMO labelling.

Conformational change?

To investigate further whether there is a change in the secondary structure of ODC after refolding, c.d. spectra of native, denatured and refolded enzymes were obtained (results not shown). The experimental conditions were the same as for the labelling experiment and the reconstitution of the enzyme was allowed to proceed at 30 °C. The c.d. spectrum characteristic of the presence of secondary structure of the wild-type enzyme was completely absent when 6 M urea was present in the sample. Upon dilution of the unfolded ODC to a final urea concentration of 0.1 M, reconstitution of the secondary structure was observed, resulting

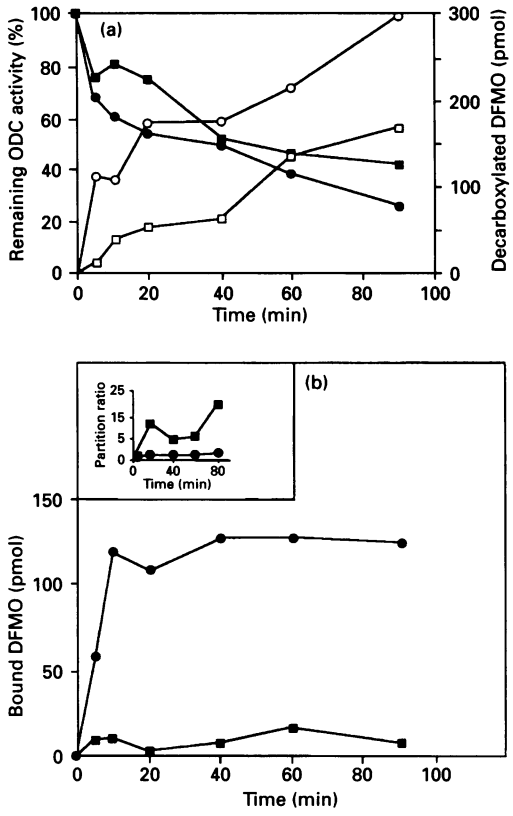

Figure 4 Determination of the partition ratio for native and reconstituted ODC

(**a**) Recombinant native (○) and reconstituted (□) ODC (100 μg for each point), as previously, were incubated with 5 μM [1-^{14}C]DFMO at 37 °C for the time periods indicated. The reactions were stopped by addition of 10% trichloroacetic acid, and the release of $^{14}CO_2$ was counted in liquid-scintillation fluid. Before addition of trichloroacetic acid, a sample was removed at the indicated time points and diluted 20-fold in ODC assay cocktail. [^{14}C]Ornithine was added and the remaining ODC activity was assayed for 30 min at 37 °C (●, native ODC; ■, reconstituted ODC). 100% in this Figure corresponds to 4.5 units of ODC activity. (**b**) In a similar experiment, 10 μg of ODC protein for each point was incubated with 5 μM DL-[3,4-^{3}H]DFMO. At the indicated time points, Laemmli sample buffer was added and the reactions were analysed by SDS/PAGE. The bands were detected by Coomassie Blue staining, excised and counted for radioactivity in liquid-scintillation fluid (●, native ODC; ■, reconstituted ODC). In the inset the partition ratios (pmol of decarboxylated DFMO/pmol of DFMO covalently bound to the protein) for the native and the reconstituted enzyme are shown.

Table 2 Titration of thiol groups in native, denatured and renatured species

The reactivities of thiol groups of recombinant ODC not exposed to urea, or native, denatured or reconstituted enzyme, were measured from the differences in the A_{412} of the samples after addition of DTNB, as described in the Experimental section. The data are averages of two individual experiments.

ODC	Number of accessible thiol groups
Native (not exposed to urea)	4.0
Native in 0.1 M urea	4.0
Denatured in 6 M urea	10.0
Denatured in 6 M urea and reconstituted in 0.1 M urea	6.8

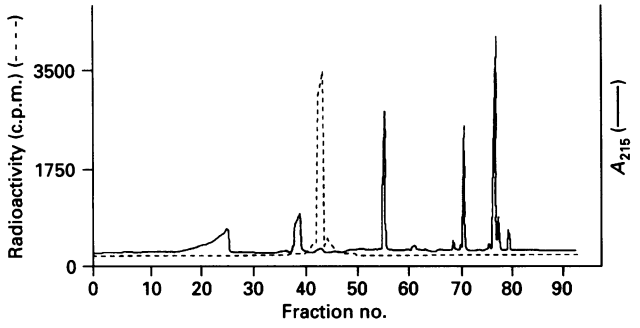

Figure 5 H.p.l.c. analysis of BNPS-skatole fragments of [^{3}H]DFMO-labelled ODC

[^{3}H]DFMO-modified ODC (2 nmol) was cleaved with BNPS-skatole and the fragments were analysed on a Vydac C_{18} reverse-phase column with a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid. Fractions (1 ml) were collected and their A_{215} was plotted. Samples of each fraction were counted for radioactivity in liquid-scintillation fluid.

in a spectrum that did not appear to differ significantly from that of the native enzyme.

Since the c.d. spectra did not provide any information about modifications in the higher-order structures, an alternative method was used to determine differences in the conformations of the two enzymes. The reactivities of the thiol groups of the two protein species were determined by the method of Ellman [21]. After exposure of the native enzyme to 6 M urea, all ten of the cysteine residues of the molecule were capable of being rapidly modified by DTNB (Table 2). When the activity of the enzyme was reconstituted, the number of reactive cysteines decreased slowly (over a time period of approx. 20 min) to 7. The wild-type enzyme was found to contain only four rapidly modifiable cysteine residues. The difference in reactivity of thiol groups between the two enzyme species could account for two alternatives: (1) the refolded enzyme is a homogeneous preparation of the new species with seven exposed cysteines and that its conformation is modified when compared to that of the native enzyme, or (2) the preparation of the renatured enzyme consists of a mixture of fully renatured native-like enzyme (four reactive cysteines) and denatured inactive ODC protein (ten exposed cysteines).

DFMO-binding site

To examine the possibility that the DFMO-modified site was no longer available to be modified in the refolded enzyme, we determined the site on ODC that is modified by DFMO. For this purpose, ODC was labelled with DL-[3,4-^{3}H]DFMO, as described in the Experimental section. The protein was then cleaved with BNPS-skatole and the fragments were separated by h.p.l.c. (Figure 5). When radioactivity in the peptide fragments was measured, the label was found to be associated primarily with a single fragment. The remaining label resided in a large peptide of approx. 33 kDa. The peptide containing 90% of the label was subjected to N-terminal sequencing. Radioactivity was measured in the amino acid products of Edman degradation (Table 3). The sequence of the labelled peptide corresponded to that of mouse ODC beginning at amino acid 357. Sequence data were obtained for 14 cycles. Label was detected predominantly in the position corresponding to cysteine-360 (found as a non-identifiable amino acid, presumably because of its modification). The recovery of radioactivity was approx. 30%, consistent with the percentage of derivatized amino acids that was collected

Table 3 Analysis of N-terminal sequence of the [^{3}H]DFMO-modified peptide

Xaa denotes an unidentified residue.

Residue no.	Amino acid	Radioactivity (c.p.m.)
1	Gly	53
2	Pro	26
3	Thr	26
4	Xaa	938
5	Asp	41
6	Gly	26
7	Leu	30
8	Asp	27
9	Arg	26
10	Ile	18
11	Val	26

(Table 3). The 33 kDa peptide was also subjected to N-terminal sequencing. Its N-terminal amino acid corresponded to leucine-56 of mouse ODC sequence. Pegg and co-workers recently reported also that cysteine-360 is the major site of modification of ODC by DFMO [13], and they identified lysine-69 as a secondary site of modification, and the pyridoxal phosphate cofactor-binding site.

Mutagenesis of cysteine-360 to alanine

Since evidence was accumulating that the difference in DFMO labelling of the refolded enzyme results from an altered conformation assumed upon renaturation, it was important to investigate whether cysteine-360 is essential for activity. DFMO binds to cysteine via the electron pair of the S of the SH group. In order to eliminate the reactive component of cysteine, cysteine-360 was mutagenized to alanine (C360A). Production of recombinant mutant and wild-type ODC protein was confirmed by Western blotting and an ODC activity assay was performed. The C360A mutant was found to have no activity. This result indicates that cysteine-360 is either involved in the formation of the active site or is implicated in helping the protein to maintain the active conformation. Mutation of lysine-69 to arginine (K69R) also renders the protein inactive, retaining only 4% of the wild-type activity [29]. When we attempted to modify the C360A or the K69R mutant proteins with DL-[3,4-^{3}H]DFMO, no labelling was observed.

DISCUSSION

We wanted to test whether unfolded ODC was able to refold unassisted into its active conformation. Ornithine-decarboxylating activity was the highly specific and most sensitive criterion available to us to monitor the unfolding and refolding of ODC. Two different denaturants, urea and GuHCl, caused complete abolition of ODC activity (Table 1). C.d. spectra of the urea-denatured enzyme showed that the secondary structure of ODC was also eliminated. When the concentration of urea was lower than 0.5 M, the effect of denaturant on ODC activity was minimal, whereas at higher concentrations inhibition was observed. The critical concentration, at which the transition from a not strikingly denatured to a thoroughly denatured form occurred, was ~ 3 M urea, as determined both from activity data (Figure 1) and c.d. spectra (results not shown). The inhibition of ODC activity was reversible; when the concentration of the denaturant was decreased by dilution to 0.1 M urea or 0.055 M GuHCl, ODC activity was recovered.

The preparations of recombinant ODC which were used for these experiments, as well as ODC from mouse Z12 cells, were homogeneous as estimated by SDS/PAGE and by N-terminal sequencing. The purity of the preparation and the extensive recovery of ODC activity after dilution of the denaturing agents suggests that *in vitro* no exogenous factors are required to facilitate the refolding of ODC.

Re-acquisition of activity was observed after native enzyme was unfolded with either urea or GuHCl and then allowed to refold in the presence of low concentrations of denaturants (Table 1). Since the recovery of activity was greater after denaturation/renaturation with urea (52.4% after renaturation in urea versus 30.8% in GuHCl), we carried out subsequent experiments using urea.

The reconstituted enzyme, unlike the native one, was not labelled by the suicide inhibitor DFMO (Figure 2). The native enzyme decarboxylates DFMO, which then binds covalently to the protein [12]. The modification of ODC by DFMO is thought to reflect the presence of active enzyme [11]. Unexpectedly, the major decrease in intensity of labelling of the renatured enzyme (less than 10% of that of the native-enzyme control) did not correspond to the activity recovered after the reconstitution step (~ 50%). This discrepancy raised the possibility that either (1) native and renatured enzymes are approximately equally active with respect to both ornithine and DFMO as substrates, but the renatured protein has nevertheless acquired a conformation less susceptible to stable modification by decarboxylated DFMO, or (2) a change in the kinetic parameters relative to native ODC has occurred, leading to altered affinities between the reconstituted enzyme and its substrates.

Examining the latter possibility, we compared the native and renatured ODCs to determine if there were any differences in their affinities for their substrates. Any differences found would presumably reflect an alteration in the active site of the denatured/renatured enzyme. As shown in Figure 3, native ODC has K_m for ornithine that is similar to that of the denatured/renatured enzyme and the two enzyme preparations also have similar K_i values for DFMO. The fact that the native and renatured enzymes have the same kinetic properties suggests that the active site in the refolded enzyme returns to an intact state upon reconstitution. The ability of each of the two enzymes to decarboxylate DFMO was then compared with its ability to become labelled with [^{3}H]DFMO. The number of moles of decarboxylated DFMO divided by the number of moles of DFMO that is bound on ODC is defined as the partition ratio. The renatured enzyme was found to decarboxylate DFMO as efficiently as the native ODC (Figure 4). A dramatic difference was, however, observed in the ability of the two ODCs to become labelled with DFMO: the renatured enzyme was labelled only to a very low extent, whereas the native ODC was strongly labelled. Therefore the partition ratios differed significantly for the renatured and native ODCs. The inactivation of ODC by DFMO, however, was similar for both enzymes. Inactivation by DFMO without labelling implies one or both of the following: (i) the conformations of the native and renatured ODCs are different in the region of the protein that is involved in DFMO binding, thereby modifying the nature or accessibility of the target; (ii) the modification of the renatured protein by DFMO inactivates without producing a covalent modification sufficiently stable to survive fractionation on SDS/PAGE.

Table 4 Activity of recombinant wild-type and mutant ODCs

DH5αF′ *E. coli* cells containing the wild-type ODC cDNA (pSPODC7) or the mutants (pSPODC7-C360A, pSPODC7-K69R) were grown, as described in the Experimental section, and ODC activity was measured in cell extracts. The data show the percentage of ODC activity and are averages of five individual experiments; 100% corresponds to 17 units of ODC activity.

Recombinant ODC	Activity (%)
Wild-type	100
Cys-360 → Ala	0
Lys-69 → Arg	4

We sought to identify the residue that is modified in the native ODC by DFMO, because it would enable us to know more about the active site of the enzyme. After reaction with [^{3}H]DFMO and cleavage, label was predominantly associated with a single peptide, which was then sequenced and found to contain a labelled and modified amino acid, corresponding to the cysteine at position 360 of mouse ODC. This residue is part of a stretch of amino acids conserved among ODCs from different species. The remaining label, approx. 10%, was found associated with a larger peptide of ~ 33 kDa. The major and minor sites where ODC is modified by DFMO, which are cysteine-360 and lysine-69 respectively, have recently been identified by Pegg and co-workers [13], using endoproteinase Lys-C digestion of the DFMO-labelled ODC and subsequent digestion of the DFMO-labelled peptide with endoproteinase Glu-C. Our results are in conformity with theirs. We mutated the modified residues to examine how significant they are for the formation of the active site of ODC. When we mutated cysteine-360 to alanine, the mutant enzyme lacked detectable ODC activity and could not be modified by DFMO. The same was observed for the K69R mutant ODC (Table 4), when the secondary site of modification by DFMO was altered to arginine [29]. This result gives evidence that both cysteine-360 and lysine-69 participate in the formation of the active site. We do not know, however, if the mutation of cysteine-360 to alanine has a major effect on the higher structure of ODC.

The increased number of reactive thiol groups in the renatured protein, compared with that of the native enzyme, suggests either that there is a conformational difference between the renatured and native ODCs that leaves these groups more accessible in the renatured ODC, or that the renatured ODC preparation is composed roughly of 50% native-like protein and 50% fully denatured protein (Table 2). If the latter were true, we would expect that the renatured preparation would be labelled to half the intensity of the native preparation when it was incubated with [^{3}H]DFMO. This was not, however, the case: the renatured enzyme was labelled to less than one-tenth the extent observed for the native ODC (Figure 2). Therefore, since the renatured enzyme has unique characteristics relative to either the native or denatured enzyme, it appears to represent a novel protein species.

The reconstituted enzyme was found to be stable both as a protein and with respect to its altered reactivity, indicating that the conformation studied does not represent a transient intermediate step between fully unfolded, denatured, form of ODC and the native enzyme.

All the above results lead us to conclude that mouse recombinant ODC can exist *in vitro* in more than one active conformation. The new conformer acts catalytically in the same way as the native recombinant ODC, decarboxylating ornithine and DFMO and being inhibited by DFMO. The surprising property of the new enzyme is that it does not become stably modified by DFMO, as shown by the lack of labelling after it is incubated with [^{3}H]DFMO.

Our work also demonstrates that mouse recombinant ODC can attain an active conformation without the participation of exogenous factors. These results do not prove, however, that molecular chaperones are not involved in the assembly and folding of ODC *in vivo*, since it has been shown that (*a*) they do not associate with already assembled proteins [10] and (*b*) they are not always required for the renaturation *in vitro* of chemically denatured proteins [9,30].

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