Rapid Exchange of Subunits of Mammalian Ornithine Decarboxylase*

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The subunit structure of mouse L-ornithine decarboxylase (ODC) was investigated using mutants involving single amino acid changes that greatly reduced the catalytic activity. Studies were carried out both by expressing the enzyme protein in a coupled transcription/translation system and mixing the various purified mutant ODCs and wild type enzyme together. The results confirm that ODC activity requires the formation of a dimer and that this dimer contains two active sites, each made up from part of one subunit that contains amino acids lysine 69, lysine 169, and histidine 197 and a part of the other subunit that contains cysteine 360. Mixing of the purified ODC mutant enzymes with each other and with the wild type enzyme indicated that there was a very rapid exchange of subunits between the enzyme dimers even under physiological conditions without addition of chaotropic agents. This rapid exchange may facilitate the binding of antizyme and the rapid turnover of ODC in vivo.

L-Ornithine decarboxylase (ODC) is an essential enzyme for polyamine synthesis and growth in mammalian cells (1, 2). ODC is a PLP-dependent amino acid decarboxylase that has a very rapid rate of turnover in the cell (3, 4). Many details of the mechanism by which this rapid degradation is brought about remain unclear, but it is known that ODC destruction is facilitated by the binding of an inhibitory protein termed antizyme (5–7), and occurs via the action of the 26 S proteasome (6, 8).

Although no crystal structure for this protein is yet available, some information on the key residues in the protein has been obtained by studying the formation of adducts with the cofactor PLP or inhibitors such as DFMO (9, 10), by site-specific mutagenesis (11–14), and by the production of chimeras between the mouse and the trypanosome enzyme that differ in regulatory properties (15, 16).

Mouse ODC is a protein with 461 amino acid residues giving a subunit Mr of ~51,000. The lysine responsible for the formation of a Schiff base is located at position 69 (9, 12). Both this residue and the cysteine located at position 360 form adducts with the enzyme-activated irreversible inhibitor DFMO, showing that they are located near the active site (9, 12, 13). In agreement with this, we have found that mutation of these residues to alanines leads to a major, but not complete, loss of ODC activity in the recombinant enzyme expressed in Escherichia coli (13). This finding is supported by experiments in which plasmids containing ODC mutated at these residues have been used for in vitro transcription/translation studies in reticulocyte lysates followed by measurements of ODC activity. These experiments have indicated that mutations K69A, K69R, K115R, K169A, K169R, H197A, C360A, and C360S (10–14) lead to a substantial loss of activity.

Recently, Tobias and Kahana (14) have provided evidence that the active form of ODC is a dimer containing two active sites located at the interface between the subunits and that each active site contains amino acid residues from each subunit. They reached this conclusion by translating mRNAs derived from plasmids containing cDNAs containing the mutations K69A and C360A. Translation of the individual mutant mRNAs gave rise to no ODC activity, but activity was obtained when both mRNAs were translated together. This implies that each active site is made up of a region containing Lys-69 from one subunit and a region containing Cys-360 from the other.

These conclusions are consistent with previous studies using gel filtration chromatography, which suggest that the normal form of ODC is a dimer and that it can be dissociated with loss of activity into the monomer form by the addition of high concentrations of salt (17–19).

In order to investigate the structure of the active site of ODC and the association between dimers in more detail, we have combined ODC molecules containing inactivating mutations and examined the restoration of activity by the formation of heterodimers. These experiments confirm that the active site of the enzyme is made up of regions at the interface between the subunits involving amino acids from each subunit and show that the subunits exchange very rapidly under physiological conditions. This rapid exchange may be of importance in the physiological turnover of ODC.

EXPERIMENTAL PROCEDURES

We have described previously the preparation of plasmids containing mutations in ODC in the pGEM vector for transcription/translation studies for mutations K69A, K169A, H197A, and C360A and for P426Stop (11, 13). The double mutant K69A/C360A was constructed by digesting each of the single mutant plasmids, pGEMK69A, and pGEMC360A, with XcmI and BamHI. XcmI cuts within the cDNA coding region between the nucleotides coding for amino acid residues 69 and 360, while BamHI cuts 3' to nucleotides corresponding to amino acid 380. The larger 3.8-kilobase fragment containing the K69A mutation and the smaller 1034-base pair fragment that contained the C360A mutation resulting from these digests were purified after agarose gel electrophoresis as described previously (13) and the fragments ligated to form a plasmid encoding the double mutant pGEMK69A/C360A. The presence of the double mutation was confirmed by sequencing (13). For expression of the ODC proteins in E. coli, the pGEM plasmids were digested with EcoRI and BamHI and the resulting fragment, which contains the ODC coding sequence, was inserted into plN-III-lopp*-A3 protein expression vector (20) as previously described (9).
Table I

Activity of ODC after transcription/translation from plasmids containing mutant cDNAs

| Plasmids added | ODC activity (cpm) |
|----------------|--------------------|
| None           | 26                 |
| Wild type ODC  | 41,678             |
| K69A ODC       | 476                |
| K169A ODC      | 196                |
| H197A ODC      | 1,835              |
| K69A/C360A ODC | 33                 |
| C360A ODC + K69A ODC | 4,160 |
| C360A ODC + K169A ODC | 4,395 |
| C360A ODC + H197A ODC | 5,234 |
| K69A ODC + K169A ODC | 158               |
| K69A ODC + H197A ODC | 689               |
| K169A ODC + H197A ODC | 1,053             |
| P426Stop       | 62,975             |

Matched to the manufacturer's instructions but using a total volume of 12.5 μl containing 0.5 μg of plasmid DNA (unless stated otherwise). After a 30-min incubation to synthesize the ODC protein, aliquots of 5 μl were removed and used for the assay of ODC activity.

The ODC proteins were purified to homogeneity using affinity chromatography on a pyridoxamine 5'-phosphate-agarose column eluted with PLP (9). ODC activity was assayed by measuring the production of 14CO2 from [1-14C]ornithine in an assay mix that contained 70 mm Tris-HCl, pH 7.5, 4.5 mm diethanolamine, 0.08 mm EDTA, 0.016% Brij 35, 40 μM PLP, and [1-14C]ornithine at low (17 μM for TNT assays) and saturating (407 μM for assay of purified ODC preparations) concentrations. The reactions were incubated for 30 min at 37°C (9). Protein was measured by the method of Bradford (21).

Theoretical values for ODC activity were calculated as follows. For this calculation, it was assumed that the enzyme is a dimer with two active sites formed at the interface between the subunits, that each active site is composed of amino acid residues from each subunit (14), that there is no interaction between the two sites, and that the subunits associate at random. If a quantity of ODC equal to 4y molecules of monomers is considered, this will form 2y homodimers which have 4y active sites. If random association of the ODC monomers to form dimers occurs, addition of an equivalent amount of an essentially inactive single mutant such as K69A will lead to the formation of y homodimers of wild type with 2y active sites having full activity, y homodimers of the mutant with no activity, and 2y heterodimers each containing one competent and one incompetent active site. There will, therefore, still be 4y competent active sites, and the total activity remains constant although the specific activity will be reduced by 50% since there is twice as much enzyme protein. In contrast, an equal amount of an inactive double mutant such as K69A/C360A, where both regions making up the active sites are mutated, the heterodimers will be inactive and the total number of active sites will be 2y. The total activity will therefore be reduced by 50% and the specific activity by 75%. Similar calculations can be made for situations where increased amounts of the mutant ODC are added, which increases the proportion of the wild type ODC in the heterodimers. All calculations are affected very slightly by the fact that the inactive mutants do have a small activity, but this only becomes significant when very large amounts of the mutants are added.

RESULTS AND DISCUSSION

It has been shown previously that sufficient ODC protein is synthesized in a reticulocyte lysate supplemented with RNA transcribed from the T7 promoter of pGEM-ODC for the measurement of ODC activity and comparison of potentially inactivating point mutations (11, 13, 14). These studies indicated that ODC mutants K69A, K169A, H197A, and C360A all led to >90% loss of activity. These results were confirmed using the TNT system of coupled transcription and translation (Table I). Translation of 0.5 μg of the pGEM-ODC plasmid in this system, in a 12.5-μl assay volume, gave rise to sufficient ODC to release more than 40,000 cpm in a standard assay with a background of only 26 cpm. ODC mutant K69A had an activity of <0.1% of wild type, whereas the activities of mutants K169A, H197A, and C360A were 0.4, 4.0, and 1.1% of the wild type, respectively. The double mutant ODC K69A/C360A was also inactive. The results with plasmids encoding mutants K69A and C360A are in agreement with the recent report from Tobias and Kahana (14), with the exception of the slight activity for C360A, which they found to be inactive.

Co-translation of the C360A mutant pGEM-ODC with any one of the K69A, K169A, or H197A mutant pGEM-ODCs led to a significant increase in activity over that expected from the sum of the two activities. No complementation was observed when any two of the K69A, K169A, or H197A mutant pGEM-ODCs were used (Table I). These results confirm previous studies showing that mutants K69A and C360A complement one another and that the active form of ODC is a homodimer (14). They also indicate that the active sites involve Cys-360 from one subunit and the Lys-69, Lys-169, and His-197 from the other. If this is the case, the co-expression of either of the single mutants K69A or C360A with the wild type ODC plasmid should not affect the activity since the heterodimers formed would have one complete active site and would contain only one subunit derived from the wild type enzyme (see "Experimental Procedures"). In contrast, the co-expression of the double mutant K69A/C360A with the wild type ODC would inactivate the enzyme since both active sites in the heterodimer would be defective. This result was obtained when the products from the transcription/translation reactions were mixed and assayed (Fig. 1). This result suggests that there may be a rapid exchange of the subunits but is not unequivocal proof of this, since the extent to which complete folding of the nascent ODC occurs during the assay rather than during the translation is unknown.

Although the extent of synthesis of each of the ODC proteins from these plasmids in the TNT system should be the same, it is possible that differences arise in amounts of protein present for the ODC assays due to differences in synthesis related to the quality of the plasmid preparations, or that folding of the nascent proteins may occur to differing extents due to the mutations or to altered stability of the mutant ODC protein. This is emphasized by the fact that expression of ODC mutant P426Stop, which is known to be more stable (11, 15), gave significantly more activity that the wild type ODC (Table I). Furthermore, there is no certainty that random association of
the nascent protein subunits occurs in this system. Therefore, in order to confirm these results and to study the interaction of the ODC dimers more quantitatively, the wild type ODC and ODC mutants K69A, C360A, K69A/C360A, and P426Stop were expressed in E. coli and purified to homogeneity. Mixing experiments were then carried out with the purified proteins. All of the mutant ODC proteins were readily purified by affinity chromatography on pyridoxamine phosphate linked to agarose, suggesting that no major distortion of the active site had occurred. The activities of the enzyme preparations used are shown in Table II. Although the mutations produced dramatic decreases in ODC activity, all of the mutant enzymes had sufficient activity to release more than 1,000 cpm of $^{14}$CO$_2$ by adding an appropriate amount of enzyme, and all of the activities could therefore be measured accurately (Table II).

Initial experiments with the purified proteins were carried out by unfolding the proteins with 8 M urea or using a high ionic strength of up to 1 M NaCl to dissociate the subunits in order to ensure that subunit exchange might occur upon renaturation by dialysis. However, quite remarkably, it was found that such treatment was unnecessary. Simply adding the C360A and K69A mutant ODC proteins together in the standard ODC assay buffer and measurement of the activity over a 30-min assay period led to the reconstitution of the activity in an amount equivalent to 27% of the activity of the control enzyme. This is indistinguishable from the amount expected, assuming that there was a random association of the ODC subunits (Table II). Addition to the wild type enzyme of an equal amount of the C360A/K69A double mutant reduced the specific activity to the expected values of 50.2 or 51.2%, respectively. (The expected values were calculated as described under “Experimental Procedures” and represent the contribution of both the wild type and the mutant ODCs.)

A more detailed study adding increasing amounts of these mutant ODC proteins to the wild type ODC was carried out (Fig. 2). These results are plotted as the total ODC activity (not specific activity), and it can be seen that the single mutants do not reduce the total activity, whereas the double mutant leads to a substantial loss. (The increase in total activity at higher values of the addition of the mutant is consistent with the fact that these mutant enzymes do have some ODC activity. When the mutant protein is added in great excess of the wild type enzyme, this becomes a significant fraction of the total.) Fig. 2 also shows the theoretical curves for the expected results if these mixtures were completely randomized mixtures of the subunits to form dimers and if there is no interaction between the two active sites present in an ODC dimer. The results found are close to these theoretical values.

Attempts were made to estimate how quickly the randomization of ODC subunits occurred by measuring both the appearance of ODC activity when the C360A and K69A mutants were mixed and by measuring the inhibition of wild type and P426Stop mutant ODCs by addition of the K69A/C360A double mutant. However, this rearrangement occurred in less than 5 min at 37 °C in the presence of ODC assay mix containing saturating ornithine concentration irrespective of the presence or absence of 0.2 M NaCl. The assay technique for ODC is not suitable for assays over a very short time period and so much exchange occurs during the assay period itself that accurate measurements could not be made.

There are many examples of enzymes containing an active site made up of components from both subunits at their interface where active heterodimers have been prepared using inactive mutants (e.g. mercuric reductase (22), thymidylate synthase (23), aspartate transcarbamylase (24), and phosphofructokinase (25)). However, in these examples either co-expression of the mutant proteins or the exposure of the proteins to denaturing conditions or chaotropic agents was needed in order to promote interchange of the subunits to produce the needed heterodimers. In contrast, the ODC subunits exchange so rapidly that randomization is complete within 5 min.

Our findings in both the transcription/translation assays and the purified enzyme mixing experiments are not in agreement.

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**Table II**

**Complementation of ODC mutants by mixing of purified proteins**

Results are given as a mean of at least four determinations. The ODC activity of each protein and of an equimolar mixture of the combinations shown was determined as described under “Experimental Procedures.” One unit of ODC activity is defined as the amount of enzyme producing 1 nmol of $^{14}$CO$_2$/min.

| Protein                | Specific activity | Wild type |
|------------------------|-------------------|-----------|
|                        | units/mg ODC protein | %        |
| Wild type ODC          | 12.8 ± 0.6        | 100       |
| C360A ODC              | 0.27 ± 0.03       | 2.1       |
| K69A ODC               | 0.66 ± 0.003      | 4.4       |
| C360A/K69A ODC         | 0.004 ± 0.0007    | 0.03      |
| C360A ODC + K69A ODC (1:1) | 3.5 ± 0.5    | 27        |
| Wild type ODC + C360A ODC (1:1) | 6.5 ± 0.7 | 51        |
| Wild type ODC + K69A ODC (1:1) | 6.3 ± 0.6 | 49        |
| Wild type ODC + C360A/K69A ODC (1:1) | 2.5 ± 0.4 | 27        |

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**Fig. 2. Effect of addition of mutant ODC proteins on control ODC activity.** Increasing proportions (up to a total of 1.98 μg) of purified K69A, C360A, or the double mutant K69A/C360A were added to a fixed amount of wild type protein (0.02 μg), and the resulting effect of the combination on total ODC activity (not specific activity) after a 30-min assay at 37 °C was determined as described under “Experimental Procedures.” The results were converted to a percentage of the activity when 0.02 μg of wild type protein alone was used (100% corresponds to 0.256 nmol of CO$_2$ released). Results are shown for the actual activity determined (solid lines) together with the theoretical lines for random association of the subunits (broken lines) based on assumptions given in the text and the calculations described under “Experimental Procedures.”
physiological conditions. The very rapid turnover of ODC may be related to this unusual property. The degradation of ODC is brought about by the action of the 26S proteasome acting on an ODC-antizyme complex (6). Antizyme is a protein that binds to and inactivates ODC as well as enhancing its degradation (3, 5–7). Antizyme has a very high affinity for ODC, and its binding to ODC is facilitated by conditions that promote ODC dimer dissociation (26). Its binding to ODC occurs at a region involving residues 117–140 (15). The facile dissociation of the ODC subunits may facilitate antizyme binding, fixing the protein in the monomer form and thus inactivating it and rendering it susceptible to degradation (Fig. 3). A critical signal for ODC degradation resides in a domain at the carboxyl terminus of the molecule (7, 10, 14, 15, 27). Truncation of the molecule removing portions of the residues from positions 425–461 renders ODC stable, and a point mutation of C441W or C441A has the same effect (28). The recognition signal present in this sequence may become exposed to the proteasome in the monomer form of the ODC and thus lead to the rapid destruction of the protein.

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**Fig. 3. Scheme for the structure and degradation of ODC.**