Intersubunit Location of the Active Site of Mammalian Ornithine Decarboxylase As Determined by Hybridization of Site-Directed Mutants†

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ABSTRACT: The active form of mammalian ornithine decarboxylase (ODC) is a homodimer consisting of two monomer subunits of 53 kDa each. We have used *in vitro* hybridization of two different catalytically inactive mutants of ODC to determine whether in the wild-type enzyme each monomer contains an independent active site or whether the active sites are shared at the interfaces between the two subunits. Two distinct mutants were obtained using oligonucleotide-directed mutagenesis: In one, cysteine-360, the major α-(difluoromethyl)ornithine (α-DFMO, a suicide inhibitor of ODC) binding site was converted to alanine. In the other, lysine-69, the pyridoxal 5′-phosphate (PLP, the cofactor of ODC) binding residue was converted to alanine. Expression of each mutant, *in vitro*, in reticulocyte lysate translation mix, results in the production of a completely inactive enzyme. In contrast, their coexpression restores enzymatic activity to about 25% of the wild-type enzyme. Moreover, coexpression of wild-type subunits with monomers containing both inactivating mutations reduced their activity to about 25%, while their coexpression with monomers that contain a single inactivating mutation reduced the activity to 50%. Cross-linking analysis has demonstrated that activity restoration and repression are both fully correlated with the formation of heterodimers between mutant subunits and between mutant and wild-type subunits, respectively. We therefore conclude that the active site of ODC is formed at the interface of the two monomers through the interaction of the cysteine-360-containing region of one monomer subunit with the region that contains lysine-69 of the other subunit.

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines (Pegg & Williams-Ashman, 1991). In mammalian cells, ODC provides the only route for the production of putrescine which is then further converted by the action of other enzymes into the polyamines spermidine and spermine (Pegg & Williams-Ashman, 1991). ODC activity and the resulting polyamines have been demonstrated to be essential for the process of cellular proliferation (Metcalf et al., 1978; Pegg, 1988; Pohjanpelto et al., 1985; Steglich & Sceffler, 1982). In accordance with this essential role, ODC activity is greatly and rapidly induced in response to growth-promoting stimuli (Feinstein et al., 1985; Kahana & Nathans, 1984; Katz & Kahana, 1987; Pegg & McCann, 1982; Tabor & Tabor, 1984). In its active form, mammalian ODC is a homodimer, while the 53-kDa monomer retains no enzymatic activity (Solano et al., 1985). The active ODC dimer contains two active sites as evident from the stoichiometry of α-DFMO binding which is very close to 1 mol of inhibitor to 1 mol of subunits (Poulin et al., 1992).

Formation of a functional enzyme from inactive monomer subunits could be explained by assuming either that a conformationally active state within a monomer is achieved only upon its association with other subunits (independent active sites) or that the active sites are formed at the interface between adjoining polypeptide chains in the oligomer (shared active sites).

Wente and Schachman (1987) have presented an elegant experimental approach to distinguish between the possibilities of "shared" versus "independent" active sites. The rationale behind this experimental approach is based on the assumption that in the case of shared active sites, association of two inactive monomers containing defects at distinct positions located on opposite sides of their interface should restore activity, while in the case of independent active sites such association is less likely to restore enzymatic activity. Moreover, in the case of the shared active sites, association between wild-type subunits and subunits that contain both inactivating mutations should result in the formation of a completely inactive heterodimer. If the active sites are independent, wild-type subunits within the heterodimers are expected to retain their enzymatic activity. Several multimeric enzymes have been subjected in the past years to this type of analysis, demonstrating that their active sites are shared between their subunits (Distefano et al., 1990; Larimer et al., 1987; Tat-kwong Lau & Fersht, 1989; Wang et al., 1984; Wente & Schachman, 1987). Performing such experiments, however, requires knowledge of individual residues that are essential for enzyme activity which occupy distinct segments of the active site.

In contrast to the extensive investigations of the mechanisms involved in the regulation of ODC expression (Hayashi, 1989; Pegg & McCann 1982; Tabor & Tabor, 1984), its structural and enzymatic properties received much less attention. Accumulating data concerning the amino acid sequence of an increasing number of ODC proteins from different species, which is deduced from cloned cDNAs, demonstrates the presence of regions that are conserved across a wide evolutionary spectrum. Clearly, such regions must serve fundamental properties of this enzyme. However, only recently lysine-69 and cysteine-360 of mouse ODC were determined as the pyridoxal 5′-phosphate (PLP) and α-(difluoromethyl)-ornithine (α-DFMO) binding residues, respectively, suggesting

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that they constitute an essential part of the active site (Poulin et al., 1992).

We demonstrate here that (1) converting either lysine-69 or cysteine-360 to alanine by site-directed mutagenesis results in complete inactivation of the enzyme, (2) coexpression of the two mutated subunits restores enzymatic activity to a level expected from the shared active-site model, and also in accordance with this model, (3) association of wild-type monomers with monomers containing one or both inactivating mutations within the same polypeptide chain results in respective reduced specific activity. Taken together, our results provide genetic support to the notion that lysine-69 and cysteine-360 are essential constituents of the active site of ODC and strongly suggest that these residues are located in two distinct regions of each subunit that interact to form shared active sites in the active ODC dimer.

EXPERIMENTAL PROCEDURES

Construction of Mutants. Oligonucleotide-directed point mutations were introduced into mouse ODC cDNA cloned in the Bluescript plasmid (Stratagene), using the uracil incorporation method of site-directed mutagenesis (Kunkel, 1985). Mutations were confirmed by sequencing. They were named according to the altered amino acid, its number in the sequence, and the name of the substituting amino acid. The oligonucleotides 5′-C AAG GCC ATC **AGC** TGT TGG TCC C-3′ and 5′-ATC GTT ACA **CGC** GAC TGC GTA-3′ were used to generate C360A and K69A, respectively (both oligonucleotides are presented in their antisense orientation).

Synthesis of ODC in Reticulocyte Lysate. PGEM-1 plasmid harboring wild-type or mutant mouse ODC cDNAs cloned between the *Eco*RI (5′) and *Bam*HI (3′) sites was linearized with *Bam*HI and transcribed *in vitro* using T7 RNA polymerase. The resulting RNA was translated *in vitro* in a reticulocyte lysate based translation mix (Promega) in the presence of [^{35}S]methionine. Synthesized ODC was fractionated in an SDS–polyacrylamide gel and visualized by autoradiography. To quantify the amount of the synthesized ODC, the relevant bands were cut out of the gel, and their radioactivity was determined using a β-scintillation counter.

Cross-Linking and Revelation of Dimers. Equal amounts of *in vitro* synthesized ODCs were incubated for 40 min at 25 °C in ODC buffer (25 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 0.1 mM EDTA, 0.4 mM PLP, and 0.33 mM L-ornithine) containing 1 mM bis(sulfosuccinimidyl suberate) (BS^3, Pierce). Cross-linking was terminated by the addition of 0.1 volume of 1 M glycine/1 M Tris-HCl, pH 8.5. Dimers were visualized by electrophoretic fractionation of the cross-linked material in SDS–polyacrylamide gels followed by autoradiography.

ODC Activity Assay. Equal amounts of *in vitro* synthesized ODCs were incubated for 60 min at 37 °C in ODC buffer (see above section) supplemented with 0.5 μCi of [^{14}C]ornithine (58 mCi/mmol, New England Nuclear). Incubation was performed in a 96-well microtiter plate. Liberated $^{14}CO_2$ was trapped in a covering 3MM paper impregnated with a saturated solution of barium hydroxide. At the end of the incubation period, the paper was washed with acetone, dried, and exposed to X-ray film. To obtain relative activities, the radioactive spots were cut out from the paper, and their radioactivity was determined in a β-scintillation counter. The specific activity of *in vitro* synthesized wild-type ODC was 4.7×10^5 mol of released CO_2/mol of ODC during 1 h of incubation, representing 100% activity.

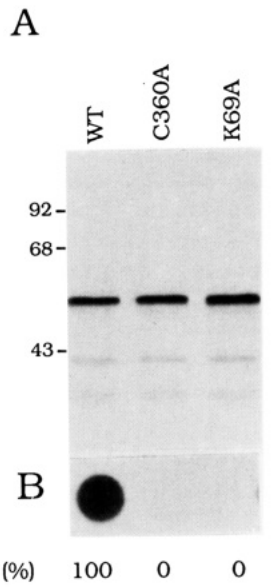

FIGURE 1: K69A and C360A are both inactive mutants. *In vitro* transcribed RNAs encoding wild-type ODC and the K69A and C360A mutants were translated in reticulocyte lysate. One portion of the *in vitro* synthesized material was fractionated by electrophoresis in a 10% SDS–polyacrylamide gel (A) while the other was used to determine ODC activity (B). The position in kilodaltons of marker proteins is indicated on the left. The relative ODC activity is indicated at the bottom.

RESULTS

Conversion of Lysine-69 or Cysteine-360 to Alanine Abolishes Enzymatic Activity. In a recent study, Pegg and co-workers have demonstrated that lysine-69 is the PLP binding site of mouse ODC while cysteine-360 and to a lesser extent also lysine-69 bind α-DMFO (Poulin et al., 1992). On the basis of these results, it was suggested that these two residues are important parts of the active site of this enzyme (Poulin et al., 1992). In order to independently test the importance of these residues for enzymatic activity, we have converted lysine-69 or cysteine-360 of mouse ODC to alanine (denoted K69A and C360A, respectively), using oligonucleotide-directed mutagenesis. Wild-type ODC cDNA and cDNAs encoding each of the two mutants were transcribed *in vitro*, and the resulting RNAs were translated in a reticulocyte lysate based translation mix. ODC activity assay performed with an equal amount of synthesized ODC protein demonstrated that each mutation completely abolished enzymatic activity (Figure 1). Our results therefore directly demonstrate that Lys-69 and Cys-360 are essential for maintaining enzyme activity.

Formation of Active Dimers from Defective Monomers. According to the shared active-site model (Figure 2), association between two types of inactive monomers should result in the formation of active heterodimers (Figure 2A). Demonstration of this possibility relies on the absolute requirement of the mutated residues for enzyme activity and on their location within distinct segments of the active site. On the basis of biochemical analysis (Poulin et al., 1992) and on our present mutational analysis, lysine-69 and cysteine-360 appear to fulfill these demands. *In vitro* transcribed RNAs encoding the K69A and C360A mutants were translated in reticulocyte lysate either separately or together, and ODC activity was determined. As shown above, expression of each mutant alone resulted in the formation of a completely inactive enzyme (Figure 3B). In striking contrast, their coexpression gave rise to 24% of wild-type activity (Figure 3B), a value that is

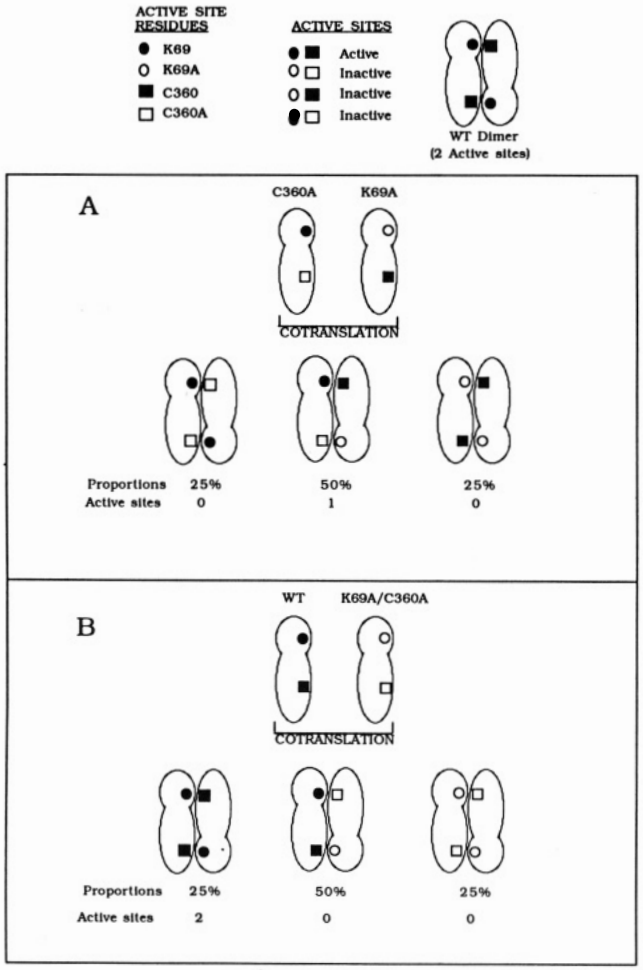

FIGURE 2: Schematic presentation of positive and negative complementation, demonstrating shared active sites. The symbols denoting the wild-type and mutated residues as well as their composition in active and inactive sites are denoted at the top. The structure of an active wild-type dimer is presented as well. (A) Formation of active heterodimers from the two inactive mutant monomers. (B) Inactivation of the wild-type enzyme in heterodimers with double mutant subunits. The expected proportions of each dimeric structure (assuming random interaction) are denoted as are the expected number of active sites.

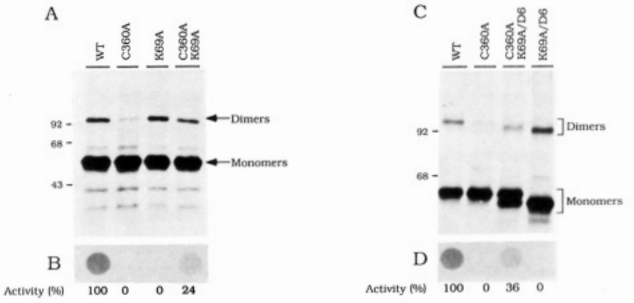

FIGURE 3: Positive complementation between the two inactive mutant monomers. *In vitro* transcribed RNAs encoding the K69A and C360A mutants were translated in reticulocyte lysate either alone or together. One portion of the translation mix was subjected to a cross-linking reaction followed by electrophoretic fractionation in a 10% SDS–polyacrylamide gel (A). The other portion was subjected to an ODC activity assay (B). In order to visualize heterodimers, the K69A mutation was introduced into cDNA encoding the active carboxyl-terminal deletion mutant Del-6 (Rosenberg-Hasson et al., 1991a). *In vitro* transcribed RNA encoding this K69A/Del-6 subunit was cotranslated with mRNA encoding the full-length protein which contains the C360A mutation. As above, one portion was subjected to cross-linking and electrophoretic fractionation (C) while the other was used to determined ODC activity (D). The molecular masses in kilodaltons of proteins used as molecular mass markers are indicated on the left side. The position of the ODC monomers and dimers is indicated on the right side, and the relative ODC activity is indicated at the bottom.

consistent with the shared active-site model, according to which 50% of the mutant subunits are in heterodimers (in respect to the mutated residues) that possess a single functional active site, while the remaining 50% are equally divided between the two types of the inactive mutant homodimers (Figure 2A). We have previously demonstrated that dimers can be clearly visualized by chemical cross-linking of *in vitro* translated ODC followed by electrophoresis in SDS–polyacrylamide gel (Rosenberg-Hasson et al., 1991b). Here we demonstrate that the two inactivating mutations do not prevent dimerization, although the C360A mutant exhibits weaker dimerization compared to the K69A mutant (Figure 3A). However, since the two mutant subunits are identical with respect to their electrophoretic migration, heterodimer formation could only be inferred from the activity observed in the cotranslated material (Figure 3B). In order to be able to directly demonstrate the formation of heterodimers and distinguish them from homodimers, we have converted lysine-69 into alanine in the active ODC deletion mutant Del-6 which lacks 25 amino acids (423–448) from the carboxyl terminus of the enzyme (Rosenberg-Hasson et al., 1991a,b). mRNA representing the K69A/Del-6 mutant was cotranslated with mRNA encoding the full-length enzyme which contains the C360A substitution. One portion of the resulting material was subjected to chemical cross-linking and was then fractionated by electrophoresis in an SDS–polyacrylamide gel, while the other was used to determine ODC activity. ODC activity was restored to 36% of wild-type activity (Figure 3D), a value which is in good agreement with the high proportion of heterodimers which were clearly visualized (Figure 3C).

Inactivation of Wild-Type Subunits Due to Their Association with Monomers Containing Double Mutation. Although the results described above are fully compatible with the shared active-site model presented in Figure 2A, they can also be explained by conformational correction resulting in partial activation of independent active sites residing within each subunit. However, if the shared active-site model is correct, it is expected that association of wild-type subunits with polypeptide chains containing both inactivating mutations should reduce activity to 25%, since only wild-type homodimers are expected to be active. Heterodimers between wild-type and double mutant subunits and homodimers between the two mutant subunits are expected to be completely inactive (Figure 2B). To test for such negative complementation, mRNA encoding Del-6 subunits containing no mutation was cotranslated with mRNA encoding full-length subunits that contain both inactivating mutations (K69A/C360A). In agreement with the shared active-site model (Figure 2B), and the observed formation of heterodimers (Figure 4A), the activity of the cotranslated material was about 25% of Del-6 activity (Figure 4B). Further in agreement with the model, cotranslation of Del-6 subunits with full-length subunits that contain only one inactivating mutation (K69A) exerted only half the inhibitory effect, reducing the activity to about 50% of Del-6 activity (Figure 4B). This result is consistent with Del-6 homodimers that represent 25% of the population exhibiting two active sites and heterodimers that constitute 50% of the population containing a single active site. The higher activity of wild-type homodimers compared with Del-6 homodimers is consistent with our previous demonstration that Del-6 retains 80% of wild-type activity (Rosenberg-Hasson et al., 1991a). Cotranslation of full-length subunits

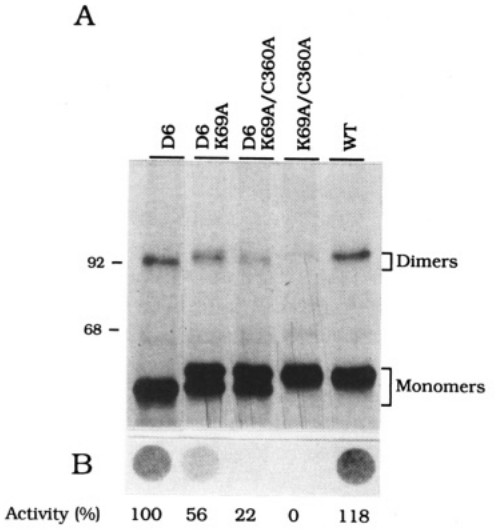

FIGURE 4: Negative complementation between wild-type and single or double mutant subunits. RNA encoding Del-6 subunits (that are wild type with respect to activity) was cotranslated with RNA encoding subunits that contain one inactivating mutation (K69A) or both mutations (K69A/C360A). One portion of the resulting material was cross-linked and fractionated in an SDS–polyacrylamide gel (A), while the other was subjected to an ODC activity assay (B). The molecular masses in kilodaltons of proteins used as molecular mass markers are indicated on the left side. The position of the ODC monomers and dimers is indicated on the right side, and the relative ODC activity is indicated at the bottom.

lacking mutations with full-length subunits that contain one or both mutations yielded practically identical results. Each of the presented experiments was repeated 3 times, resulting in a variation of less then 5%.

DISCUSSION

We demonstrate here the formation of active ODC heterodimers from two mutant ODC subunits that are completely inactive in their homodimeric state. Furthermore, we also demonstrate that association between wild-type and double mutant subunits results in significant reduction of wild-type activity, reflecting the formation of completely inactive heterodimers. Our results provide, therefore, compelling evidence that the active site of mammalian ODC resides at the interface between the two subunits and contains residues originating from separate polypeptide chains.

The cellular regulatory mechanisms that underline the modulation of ODC expression have been at the focus of intensive investigation (Hayashi, 1989; Pegg & McCann, 1982; Tabor & Tabor, 1984). In sharp contrast, the relationships between structural properties and enzymatic activity received very little attention. In a recent study, however, Pegg and co-workers presented a biochemical analysis which identified lysine-69 as the PLP binding site and cysteine-360 as the major α-DFMO binding site (Poulin et al., 1992), suggesting that these residues are essential parts of the active site. We have converted both these residues to alanine using oligonucleotide-directed mutagenesis. Expression of wild-type ODC and each of the two mutants, in reticulocyte lysate, demonstrated that each mutation completely abolished enzyme activity (Figure 1), therefore providing substantial support to the notion that Lys-69 and Cys-360 are essential parts of the active site.

ODC was demonstrated to exist in an equilibrium between active dimers and inactive monomers (Solano et al., 1985). Two models can explain the formation of a functional multimeric enzyme from inactive monomer subunits. In one model, known as the independent active-sites model, a conformationally active state within a monomer is achieved only upon its association with other subunits. Alternatively, according to the shared active-sites model, the active sites are formed at the interface between adjoining polypeptide chains in the oligomer. Crystallographic studies of some oligomeric enzymes such as glucose-6-phosphate isomerase (Shaw & Muirhead, 1976), the PLP-dependent enzyme aspartate aminotransferase (Ford et al., 1980), glutathione reductase (Schultz et al., 1978), and aspartate transcarbamoylase (ATCase) from *Escherichia coli* (Honzatko et al., 1982; Monaco et al., 1978) have indicated that each active site is composed of amino acids originating from adjoining subunits. An elegant experimental approach capable of discriminating between the two models has been used to demonstrate the presence of shared active sites in the catalytic subunits of ATCase (Robey & Schachman, 1985; Wente & Schachman, 1987). This approach is based on measurements of enzyme activity in hybrids formed between different inactive subunits. Several other multimeric enzymes have been subjected in the past several years to this type of analysis. Using such an analysis with *in vitro* mutagenized subunits revealed the presence of shared active sites in ribulosebisphosphate carboxylase/oxygenase (Larimer et al., 1987), phosphofructokinase (Tatkwong Lau & Fersht, 1989), and mercuric reductase (Distefano et al., 1990). Fatty acid synthases have been also shown to contain shared active sites by hybridization of chemically modified variants (Wakil, 1989; Wang et al., 1984). Performing such experiments, however, requires knowledge about individual residues that are part of the enzyme active site, which reside within distinct regions that are locate at the interface between adjoining subunits. Such information is frequently obtained from crystallographic analysis of proteins.

Since the three-dimensional structure of mammalian ODC is still unknown, we have selected mutants in Lys-69 and Cys-360 for hybridization assays based on a biochemical study (Poulin et al., 1992), and on our present genetic analysis which implicated their location within the active site. Coexpression of the two inactive mutants, K69A and C360A, resulted in the formation of partially active heterodimers (Figure 3B) whose activity is quantitatively in agreement with the shared active-site model (Figure 2A). Since partial activity of mutant heterodimers could also reflect a conformational correction resulting in partial activation of each monomer, we have complemented these results with negative hybridization experiments. Coexpression of wild-type subunits with subunits containing one mutation individually or both mutations simultaneously resulted in reduction of activity to about 50% and 25%, respectively (Figure 4B). These results, which are compatible with the shared active-site model (Figure 2B), argue against the possibility of conformational correction.

That heterodimers are actually formed is clearly demonstrated by performing these experiments with two types of subunits exhibiting differential migration in SDS–polyacrylamide gels. Chemical cross-linking, followed by electrophoretic fractionation in SDS–polyacrylamide gels, revealed the formation of heterodimers that caused the observed activation or repression of ODC activity (Figure 3C,D and Figure 4A,B).

Taken together, our results from both the positive and negative complementation experiments provide strong evidence that the active site of mammalian ODC resides at the interface between the two subunits that form the active dimer. Moreover, each subunit has a split active site, with the two

tested residues being located on different segments. Only association with the complementary surface from the other subunit will result in the formation of a functional shared active site. For that reason, association between the wild-type subunit and a subunit containing both mutations yields an inactive heterodimer. It is therefore expected that the double mutant will function as a negative dominant mutant whose overexpression in cells will sequester wild-type subunits into inactive heterodimers resulting in blockage of cellular ODC activity. In supporting this possibility, we have demonstrated that mixing double mutant subunits with wild-type subunits in a ratio of 10:1 completely abolished ODC activity (not shown).

Using a similar approach, formation of shared active sites was previously demonstrated in other enzymes (Distefano et al., 1990; Larimer et al., 1987; Tat-kwong Lau & Fersht, 1989; Wang et al., 1984; Wente & Schachman, 1987). In these studies, heterodimer formation was achieved either by coexpression of the two subunits in cells using appropriate expression vectors (Distefano et al., 1990; Larimer et al., 1987) or by dissociating and reassociating two purified homodimers (Distefano et al., 1990; Tat-kwong Lau & Fersht, 1989; Wang et al., 1984; Wente & Schachman, 1987). As we demonstrate here, cotranslation *in vitro* in reticulocyte lysate provides an alternative rapid and convenient way to perform such analysis.

Lysine-69 and cysteine-360 are both conserved in ODCs from a wide evolutionary spectrum of species that includes mouse (Gupta & Coffino, 1985; Hickok et al., 1986; Kahana & Nathans, 1985), human (Hickok et al., 1987), rat (Van Kranen et al., 1987; Wen et al., 1989), hamster (Grens et al., 1989), *Xenopus laevis* (Bassez et al., 1990), *Trypanosoma brucei* (Phillips et al., 1987), *Saccharomyces cerevisiae* (Fonzi & Sypherd, 1987), *Neurospora crassa* (Williams et al., 1992), and *Drosophila melanogaster* (Rom and Kahana, unpublished results). The two conserved peptides surrounding the amino acids found to bind PLP and α-DFMO (residues 64–70 and 356–364 in mouse ODC) are among the longest contiguous sequences conserved in the various eukaryotic ODCs. Two other relatively conserved sequences are residues 164–171 and 193–201 in mouse ODC. Mutating amino acids within these sequences, namely, Lys-169 to Ala and His-197 to Ala, was demonstrated to abolish enzyme activity (Lu et al., 1991), suggesting that these sequences may be also part of the active site. Comparison between the sequences of various ODC proteins reveals a large number of other conserved residues which, like Lys-69 and Cys-360, must serve fundamental functions required for enzymatic activity. Testing mutants of such conserved residues that abolish ODC activity in combination with K69A or C360A will permit the identification of other residues that are part of the active site of ODC and their location in a specific surface of the split active site.

Intersubunit active sites as determined by association of *in vitro* mutagenized subunits was shown to be in correlation to the three-dimensional structure found by crystallographic studies of the following enzymes: aspartate transcarbamoylase (Honzatko et al., 1982; Monaco et al., 1978; Wente & Schachman, 1987), ribulosebisphosphate carboxylase/oxygenase (Andersson et al., 1989; Larimer et al., 1987; Schneider et al., 1986), and phosphofructokinase (Shirakihara & Evans, 1988; Tat-kwong Lau & Fersht, 1989). Recently, using heterodimer formation, it was concluded that the active site of mercuric reductase resides at the interface between its subunits, containing residues from separate polypeptide chains (Distefano et al., 1990). This predicted structure is in good agreement with the crystal structure of a very similar enzyme, glutathione reductase (Pai & Schulz, 1983; Thieme et al., 1981). In general, it seems that the active-site structures inferred from hybridizations between inactive mutant subunits are in good agreement with parallel crystallographic data.

Structure–function relationships in the ODC protein are at present poorly understood. The experimental approach presented here can distinguish between conserved domains that are part of the active site and domains that serve other fundamental properties of the enzyme. Availability of the three-dimensional structure of ODC will be helpful for validization of our present hybridization experiments predicting the intersubunit location of the ODC active site.

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