Yeast Epiarginase Regulation, an Enzyme-Enzyme Activity Control

IDENTIFICATION OF RESIDUES OF ORNITHINE CARBAMOYLTRANSFERASE AND ARGINASE RESPONSIBLE FOR ENZYME CATALYTIC AND REGULATORY ACTIVITIES*

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In the presence of ornithine and arginine, ornithine carbamoyltransferase (OTCase) and arginase form a one-to-one enzyme complex in which the activity of OTCase is inhibited whereas arginase remains catalytically active (5). The epiarginase regulation prevents the recycling by OTCase of ornithine produced by arginase and, because both enzymes are cytosolic in S. cerevisiae, is expected to avoid the operation of a futile urea cycle when yeast is growing on arginine as a nitrogen source (4). This control was found in yeast species showing a strong Pasteur effect and taxonomically related to Saccharomyces (6) and was also reported in Bacillus subtilis (7). The absence of the epiarginase regulation in some species can be linked to a mitochondrial localization of OTCase, whereas arginase is cytosolic, as for example in Debaryomyces hansenii, Hansenula anomala, and Schizosaccharomyces pombe (8).

Direct interaction between OTCase and arginase, which are trimeric, was demonstrated by molecular sieving, a complex containing both enzymes being only observed in the presence of ornithine and arginine (4). Purification in the presence of these effectors (4) and equilibrium sedimentation and electron microscopy (9) identified an OTCase-arginase hexamer. The association of the two enzymes was rapidly reversible in vivo as well as in vitro under specific conditions, such as protein dilution, suggesting that no enzymatic modification was involved in this inhibitory mechanism (4, 10, 11). It was proposed that the ornithine regulatory receptor site on OTCase is distinct from the catalytic site, because by mutation, heating, or chemical alteration inhibition by arginase can be lost without impairing the catalytic activity of OTCase (5). However, the occurrence of a second ornithine binding site was not supported by the data of Hensley and co-workers (9) who proposed that the binding of ornithine to the active site promotes a conformational change needed for the complex formation with arginase.

Vissers et al. (6) have investigated the occurrence of the epiarginase control in 32 yeast species belonging to 12 different genera and obtained some correlation between the sensitivity of OTCases to excess ornithine and their sensitivity to arginase. Indeed, all OTCases inhibited by arginase present a marked inhibition by excess ornithine. However, the reverse is not true, and some arginase insensitive OTCases are inhibited by excess ornithine. To get deeper insight at the molecular level into the mechanism involved in the inhibition of OTCase by arginase, we created and analyzed a series of mutations leading to the loss of inhibition of its activity by arginase. We also identified residues in the arginase that are required for its regulatory function.

EXPERIMENTAL PROCEDURES

Strains and Media

S. cerevisiae HY is a diploid strain obtained by crossing strains HP7c (MATa, ura3–52, his3–200, lys2–801, ade2–101, trpl–901, leu2–3,112,
gal4Δ452, gal80Δ–583, lys2Δ::GAL1-HIS3, ura3Δ::(GAL1Δ7,Δ15::CYC1-loxZ) and YEp [MATa, ura3·52, his3·200, ade2·101, trp1·601, leu2·3,112, met ·, gal4A, gal80A, URA3::GAL1-loxZ (12)] and was the recipient strain for experiments using the two-hybrid system. The yeast strain MaV99 (MATa, SPAL10::URA3), which contains an integrated GAL1-URA3 reporter gene activated by the GAL upstream activation sequence and derives from the strain MaV2 (MATa, ura3·52, leu2·3,112, met ·,his3·200, gal80Δ, gal80Δ), was used as primers the appropriate oligonucleotides flanked by a BamHI site for the 5' end and a XhoI site for the 3' end. This DNA fragment was digested by BamHI and XhoI and inserted into the pACTII vector, leading to plasmid pGAD-ARG3. In these experiments, we determined the nucleotide sequence to ensure that the fusions were in-frame and that no mutation had been introduced during the PCR procedure.

**Selection of Mutations in the ARG3 Gene by the "Reverse Two-hybrid" Assay**

Plasmid pGAD-ARG3 was used to transform E. coli strain XL1-Red (Stratagene) to obtain mutations in the ARG3 gene, because this strain is highly mutagenic. Plasmids containing the mutated GAD-arg3 gene were extracted from a pool of bacterial transformants and used to transform yeast strain MaV99 containing plasmid GAD-CAR1. This strain is suitable for the use of the reverse two-hybrid assay, because it contains the URA3 reporter gene under the control of the UASau sequences. The URA3 gene allowed the selection of mutated GAD-OTCases having lost their interaction with GAD-arginase, because only the strains that did not express the URA3 gene could grow on a medium containing 5-fluoroorotic acid plus 25 μg of uracil. 15 candidates were obtained, and their DNA was extracted and used to transform E. coli strain HB101 (λ-, pro+, ampicillin). This allowed the selection of plasmids containing a functional LEU2 gene and the GAD-ARG3 gene, respectively.

**Construction of Plasmids Overexpressing OTCase and Arginase under the GAL10 Promoter**

To overexpress OTCase and arginase, we amplified by PCR the full-length ARG3 and CAR1 genes, using as template the genomic DNA from the wild type strain Σ1278b as well as primers the appropriate oligonucleotides extended with BamHI restriction sites. These fragments were amplified in the BamHI and XhoI sites and used as primers for the construction of fusions containing the CAR1 gene inserted into the pACTII vector (pGAD-CAR1, 2μ, URA3) and pE24 (pGAL1-CAR1, 2μ, URA3). The two genes were sequenced to ensure that no mutation was introduced during the PCR procedure.

**Construction of DNA-binding Domain and Activation Domain Fusions**

The DNA binding domain of the Gal4 activator, Gal4 (1–147), is referred to as GBD, and its activation domain, Gal4 (768–881), is referred to as GAD. The underlined GBD and GAD refer to the DNA sequences encoding these domains. The GBD-containing fusion genes were constructed in vector pAS2 (19). Transformants harboring the vector or a derivative thereof, were selected by omitting tryptophan from the yeast nitrogen base medium (Difco) in the presence of Leu2. The GAL2-containing fusion genes were constructed in vector pACTII (19), transformants harboring the vector, or a derivative thereof, were selected by omitting leucine from the yeast nitrogen base medium.

**GBD-ARG3 and GAD-ARG3 Fusions**

To construct the GBD-ARG3 and GAD-ARG3 gene fusions, we PCR-amplified the ARG3 gene, using as template the genomic DNA from the wild type Σ1278b strain, and as primers the appropriate oligonucleotides flanked by a BamHI site for the 5' end and a XhoI site for the 3' end. This DNA fragment was digested by BamHI and XhoI and inserted into the pAS2 vector, then digested by BamHI and SalI, leading to plasmid pGBD-ARG3, and inserted into the pACTII vector, finally digested again by BamHI and XhoI, leading to plasmid pGAD-ARG3. In these experiments, we determined whether the fusions were in-frame and that no mutation had been introduced during the PCR procedure.

**GBD-CAR1 and GAD-CAR1 Fusions**

To construct the GBD-CAR1 and GAD-CAR1 gene fusions, we amplified by PCR the CAR1 gene, using as template the genomic DNA from the wild type Σ1278b strain and as primers the appropriate oligonucleotides flanked by a BamHI site for the 5' end and a XhoI site for the 3' end. This DNA fragment was digested by BamHI and XhoI and inserted into the pAS2 vector, then digested by BamHI and SalI, leading to plasmid pGBD-CAR1, and inserted into the pACTII vector, then finally digested again by BamHI and XhoI, leading to plasmid pGAD-CAR1. To these plasmids, we ligated CAR1 fusions, confirming that the nucleotide sequence to ensure that the fusions were in-frame and that no mutation had been introduced during the PCR procedure.

**Construction of Deletion and Point Mutations in the ARG3 Gene**

The deletion of the region between amino acids 166 and 169 (YENVK) was created by in vitro mutagenesis on double-stranded DNA from plasmid pKVO2 (pBB322, 2μ, URA3, ARG3), which contains the ARG3 gene, leading to plasmid parg3-M1. The substitutions T86G, E123S, E123A, G181R, D182N, N184Q, N185Q, C191M/C194N/F197V, E256Q, E256A, K260R, K260A, K263R, K263A, K265R, K265A, K268R, K268A, C289S, L290S, L290Q, and Q294P were introduced in OTCase by site-directed in vitro mutagenesis on plasmid pGAD-ARG3, 2μ, URA3), leading to plasmid pME223 (TS2G), pME204 (E123S), pME206 (E123A), pME216 (G181R), pfV48 (D182N), pME234 (N184Q), pME235 (N185Q), pME211 (C191M/C194N/F197V), pME205 (E256Q), pME207 (E256A), pME217 (K260R), pME218 (K260A), pME213 (K263R), pME208 (K263A), pME240 (K265R), pME219 (K265A), pME241 (K268R), pME220 (K268A), pME257 (C289S), pME221 (C289G), pME222 (C289S) were sequenced to ensure that no additional mutation had been introduced during the PCR procedure.

**Construction of Deletion and Point Mutations in the CAR1 Gene**

The deletion of the region between amino acids 78 and 86 (ΔGQSS-VMDG) was generated by in vitro mutagenesis on double-stranded DNA from plasmid pKVO2, 2μ, URA3), leading to plasmid pGAD-CAR1. The gene was amplified by PCR and cloned into the pGAD-CAR1 vector pUTC24, generating the plasmid pUTC25. The substitutions H309K/H312E and C321R/C326T were introduced during the PCR procedure.

**Enzyme Assays**

β-Galactosidase activity was assayed as described by Miller (20). Protein contents were determined by the Folin method. Arginase activity was assayed as described previously (5). OTCase activity was determined by citrate detection (21). Routinely the OTCase activities were measured in the presence of 50 mM Tris-HCl buffer, pH 8.0, 5 mM carbamoyl phosphate and at ornithine saturation. The reaction was carried out at 30 °C for 30 min and was stopped by addition of HCl at a
final concentration of 0.5 M. In the experiments of OTCase inhibition by arginase, 1 mM agmatine, an arginine analog, and increasing amounts of arginase (up to 1000 arginase units) were added in the incubation mixture. This enzyme concentration corresponded to an amount of wild type arginase 4-fold higher than the amount required for maximal inhibition of OTCase. Agmatine was used in the OTCase-arginase binding assays instead of arginine to avoid arginine degradation at high arginase concentration. The experiments shown in Fig. 5 (see below) were performed using the more sensitive assay of Prescott and Jones (22), for which standard curves for citrulline were generated in each experiment. Specific activities were expressed in micromoles of citrulline formed per hour and per milligram of protein.

Modeling of Yeast OTCase

Alignment of OTCase amino acid sequences of S. cerevisiae, S. pombe, human, P. furiosus, E. coli, and Pseudomonas aeruginosa has been performed with the ClustalW program (23, 24). Based on this multiple sequence alignment, the structure of E. coli was selected as template to construct a three-dimensional model of S. cerevisiae OTCase. Assignment of the coordinates, construction of additional loops (residues 45–50 and residues 160–169 in the yeast sequence), and minimization of the structure were carried out using the Modeller4 program (25). The quality of the model has been checked using the Procheck program (26). The geometry of the final model complies with statistical criteria. About 98% of the residues fall in allowed regions (86.2% and 11.5% of phi/psi angles are, respectively, in the most favored or additional allowed regions) of the Ramachandran plot, and all bond lengths and valence angles corresponded to expected values. Graphical outputs were generated with Molscript (27). All calculations were carried out on an O2 SGI workstation operated with Irix 6.2. The coordinates of the model are available upon request to the authors.

Gel Filtration on the Superdex 200 Column

The gel filtration procedure was carried out at 4°C. A Superdex 200 column (1.6 x 60 cm, Amersham Biosciences) was equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing either 15 mM ornithine or a concentration stated in the legends of Figs. 2 and 3. The gel filtration machine was operated with Irix 6.2. The coordinates of the model are available upon request to the authors.
Residues for OTCase-Arginase Interaction and Activity Control

OTCase activity was assayed at 30 °C in extracts from strain 02859D[arg3-car1 (aro3, leu2, trp1, arg3::kanMX4, car1Δ) transformed with pYeF2 plasmid containing wild type ARG3 gene or the mutated arg3 genes after growth at 30 °C on M. am plus 2% galactose plus 25 μg of tryptophan plus 25 μg of arginine. The assays were performed in 50 mM Tris-HCl buffer, pH 8.0, and in the presence of 5 mM CP. The ornithine concentration used in the inhibition tests varied from 15 mM up to 2-fold the concentration of the apparent Km of mutated OTCases for ornithine. At least three independent transformants were tested, and the standard error was typically 10-15% of the mean. The OTCase specific activity is expressed in micromoles of citrulline formed/h/mg of protein.

FIG. 2. In vitro inhibition of wild type OTCase and mutated OTCase-D182N by wild type arginase. The inhibition test was performed at 30 °C in the presence of 50 mM Tris-HCl, pH 8.0, 15 mM ornithine for the wild type OTCase and 200 mM for the D182N variant, 1 mM agmatine, and increasing amounts of arginase (4). Maximum OTCase activity measured in the absence of arginase was taken as 100%. Open circles, WT OTCase; filled squares, OTCase-D182N.

### TABLE I

| Plasmids | Nature of the mutation in OTCase | OTCase specific activity measured at saturation of ornithine and CP | Apparent Km for ornithine | Remaining activity of OTCase after inhibition by 500 units of WT arginase |
|----------|----------------------------------|---------------------------------------------------------------|--------------------------|-------------------------------------------------|
| pDC2     | WT                               | 1570                                                         | 1.6                      | 14                                             |
| pME223   | T86G                             | 17                                                           | 3.4                      | 18                                             |
| pME204   | E123S                            | 1390                                                         | 2.1                      | 16                                             |
| pME206   | E125A                            | 1520                                                         | 2.6                      | 15                                             |
| pME216   | G181R                            | 0                                                            | ND                       | ND*                                            |
| pFV48    | C191M-C194N-P197V                 | 250                                                          | 2.8                      | 18                                             |
| pME205   | E256Q                            | 760                                                          | 1.2                      | 15                                             |
| pME207   | E256A                            | 900                                                          | 10                       | 94*                                            |
| pME217   | K260R                            | 1510                                                         | 1.2                      | 16                                             |
| pME218   | K260A                            | 1550                                                         | 1.1                      | 16*                                            |
| pME213   | K263R                            | 890                                                          | 20                       | 100                                            |
| pME208   | K263A                            | 450                                                          | 30                       | 98                                             |
| pME240   | K265R                            | 1418                                                         | 1.1                      | 18                                             |
| pME219   | K265A                            | 1010                                                         | 1.2                      | 47*                                            |
| pME241   | K268R                            | 1444                                                         | 1.1                      | 18                                             |
| pME220   | K268A                            | 1500                                                         | ≤0.05                    | 5%                                            |
| pME237   | C289S                            | 170                                                          | 10                       | ND*                                            |
| pME238   | L290S                            | 280                                                          | 2                        | ND*                                            |
| pME239   | L290Q                            | 800                                                          | 2                        | 22                                             |
| pME221   | Q294P                            | 1460                                                         | 1.3                      | 17                                             |

* ND, not detectable. Interaction with arginase in molecular sieving experiment (data not shown).

a Interaction with arginase is absent or reduced in the molecular sieving experiment.

b See Fig. 5B.
c See Fig. 5D.

d See Fig. 5D.

RESULTS

Selection of a Mutated OTCase Resistant to Inhibition by Arginase Using the Reverse Two-hybrid Assay—As a first attempt to select mutations in OTCase that impair its association with arginase, we took advantage of assays that had been developed in yeast (13, 29). We first tested whether OTCase and arginase do interact in vivo using the two-hybrid assay (29). Therefore, we fused OTCase and arginase coding sequences to the DNA binding domain (GBD) and the activation domain (GAD) of the Gal4 protein, leading to the chimeric proteins GBD-OTCase, GAD-OTCase, GBD-arginase, and GAD-arginase (see “Experimental Procedures”), which were tested in vivo by the two-hybrid assay. The results indicated a significant interaction between GBD-OTCase and GAD-arginase and a stronger interaction between GBD-arginase and GAD-OTCase (data not shown). We used the reverse two-hybrid assay to select mutants impaired in the interaction between the two proteins (see “Experimental Procedures”). The plasmids of strains growing on 5-fluoroorotic acid were extracted, purified, and used to transform yeast strain 12516c for OTCase activity determination, and strain HY (containing plasmid GBD-CAR1) was used to confirm the loss of interaction of these mutated OTCases with arginase. Only one candidate, named M3, retained sufficient OTCase activity to sustain the growth of a strain deleted of the ARG3 gene. This residual OTCase activity was resistant to the inhibition by arginase, even at ornithine concentrations higher than 100 mM (data not shown). The GAD-arg3-M3 gene was sequenced and found to harbor a single replacement Asn for Asp at position 182, a highly conserved residue present in all OTCases (Fig. 1). The mutation was introduced in the ARG3 gene yielding plasmid pFV48 (pGAL10-arg3D182N, 2µ, URA3). The substitution re-
sulted in a slight reduction of OTCase specific activity, a strong decrease of the apparent affinity of the mutant enzyme for ornithine and a total loss of inhibition of its activity by arginase, even at high ornithine concentration (200 mM instead of 15 mM) (Table I and Fig. 2). Moreover, OTCase-D182N had lost the capacity to interact with arginase, as shown by molecular sieving (Fig. 3). In the presence of ornithine and arginine, wild type OTCase and arginase formed a complex of about 200 kDa, whereas, in contrast, OTCase-D182N and wild type arginase were unable to form such a complex, even at a concentration of ornithine equivalent to 2-fold the apparent $K_m$ value (Fig. 3 and Table I).

**Construction of Additional Mutations in OTCase by in Vitro Mutagenesis**—At present more than 60 OTCase amino acid sequences are available (SwissProt search, 2002), and the crystal structure of OTCases from *P. aeruginosa*, *E. coli*, *P. furiosus*, and human are known (30–33). Each polypeptide chain from these trimeric enzymes folds into two domains, a carbamoyl phosphate binding domain and an L-ornithine binding domain (Fig. 4A). As shown in Fig. 1, alignment of the amino acid sequences of these OTCases and those of *S. cerevisiae* and *S. pombe* shows large regions of identity but also reveals several regions where the sequences diverge significantly. The region from residue 161 to 168 in particular is unique to the *S. cerevisiae* OTCase. However, deletion of amino acids E166/V167/N168/K169 (Fig. 1) had no effect on the OTCase-arginase interaction (data not shown). Surprisingly, the mutation D182N rendering OTCase insensitive to arginase occurs in a conserved stretch (Gly-181, Asp-182, Asn-184, and Asn-185) in which some amino acids might interact with ornithine, ac-
Residues for OTCase-Arginase Interaction and Activity Control

The velocity of the reaction catalyzed by wild type and mutated OTCases was measured at 30 °C in 50 mM Tris-HCl buffer, pH 8.0, in the presence of 5 mM CP, 1 mM agmatine, different concentrations of ornithine, and in the presence or absence of wild type arginase. The maximum OTCase activity measured in the absence of arginase was taken as 100%. A, wild type OTCase in the absence of arginase (open circles), in the presence of 200 units of arginase (filled circles), and in the presence of 500 units of arginase (filled circles), and in the presence of 200 units of arginase (filled circles), and in the presence of 500 units of arginase (filled circles). B, OTCase-K260A in the absence of arginase (open circles), in the presence of 200 units of arginase (filled circles), and in the presence of 500 units of arginase (filled circles). C, OTCase-K265A in the absence of arginase (open circles), in the presence of 200 units of arginase (filled circles), and in the presence of 500 units of arginase (filled circles). D, OTCase-K268A in the absence of arginase (open circles) and in the presence of 200 units of arginase (filled circles). Note the change of scale in this figure compared with Fig. 5 (A–C).

The data presented in Table I show the specific activities of the mutated OTCases determined at saturation for both substrates, their apparent $K_m$ for ornithine, and their sensitivity to arginase, determined as in Fig. 2. In none of the mutated OTCases was the apparent $K_m$ for CP significantly modified, except for the protein bearing the T68G substitution, which increased the apparent $K_m$ for CP from 0.1 to 2.4 mM. According to Western blot analysis, the amounts of the mutated OTCases were comparable to those of the wild type OTCase, indicating that the decrease in enzyme specific activities did not result from a lack of protein stability or reduced enzyme production (data not shown). Moreover, molecular sieving experiments showed that the mutations did not affect the trimeric structure of the different OTCases (data not shown).

The mutants fell into several categories. Some mutations did not affect any of the enzyme properties: E123S or E123A, K260R, and Q294P. The simultaneous replacements of Cys-191, Cys-194, and Phe-197 by the corresponding residues present in the S. pombe OTCase (MNV) reduced the enzyme activity, as did the T68G substitution. However, these two mutations did not modify the sensitivity of the enzyme to arginase (Table I). The replacement G181R completely abolished the enzyme activity but did not compromise its interaction with arginase, a 200-kDa complex being formed with wild type OTCase (data not shown).

In contrast, the substitutions N184Q, N185Q, E256A, K263R, K263A, and C289S led to an important modification of the affinity of the mutant OTCases for ornithine: the specific activities in crude cell extracts were reduced, and the enzymes were insensitive to arginase inhibition, even at high ornithine
Residues for OTCase-Arginase Interaction and Activity Control

| S. cerevisiae | S. pombe | Rattus norvegicus | B. caldovelox |
|--------------|----------|------------------|--------------|
| 26 | 28 | 26 | 28 |
| K260A | E256A | K265A | D182N |

Fig. 6. Amino acid alignment of arginase sequences from different organisms. Conserved amino acid residues are indicated by black letters, while the positions of the first and last residues are indicated. The histidine and cysteine residues are indicated by white letters in black boxes. “Δ” represents the deletion of the residues covered by the bracket.

transformatns bearing either the wild type or the mutated CAR1 genes, after growth on M-am-galactose supplemented with 25 μg of arginine, 25 μg of leucine, and 25 μg of tryptophan, per milliliter. Deletion of the 9-amino acid stretch (78–86) unique to the S. cerevisiae arginase had no effect on arginase activity and did not modify its inhibitory capacity on OTCase activity (data not shown). Replacing the two cysteines completely abolished the regulatory function of arginase, which had lost the capacity to bind and inhibit OTCase. In contrast, the substitution of the two histidine residues had only a minor effect (Table II and Fig. 7A). These mutations did not alter significantly the enzymatic properties of the enzymes; their catalytic activity and their affinity for arginine were unaffected. Thus, the regulatory properties of yeast arginase multienzyme complex. As a first attempt to localize in the arginase residues involved in its interaction with OTCase, we generated four mutants. The deletion of amino acids 78–86 (pAM4) and the replacements of H309K/H312E (pME209), C321R/C326T (pME210), and H309K/H312E/C321R/C326T (pME222) in the C-terminal end of arginase were constructed by in vitro mutagenesis in plasmid pEJ24 expressing the CAR1 gene under the GAL10 promoter, as described under “Experimental Procedures.” It must be emphasized that the amino acid replacements mentioned above introduce the cognate residues selected arginase was comparable to that of the wild type enzyme shown). As shown in Fig. 7C, the molecular mass of the mutated arginase was comparable to that of the wild type enzyme (about 114 kDa, shown in Fig. 3), indicating that the mutated enzyme was still a trimer. The two cysteine residues are thus important for the epiarginase function, whereas the binding of concentration (up to 10-fold the apparent $K_m$ value). The effects were comparable to those observed for the D182N mutation selected in vivo. The E256A substitution caused a reduction of the apparent affinity of the enzyme for ornithine and impaired its interaction with arginase, whereas the E256Q substitution only reduced the specific activity of the enzyme. The K260A, but not K260R, replacement had no effect on the interaction of OTCase with arginase when the test was performed with 15 μM ornithine, but the interaction was reduced at lower ornithine concentrations, as shown in Fig. 5B (compared with Fig. 5A with wild type OTCase). In contrast to the mutations described above, the substitution K268A, but not K268R, resulted in an enhanced affinity of the enzyme for ornithine. The OTCase-K268A was extremely sensitive to inhibition by excess of ornithine as compared with the wild type enzyme and exhibited a higher sensitivity to arginase as shown in Fig. 5D. Interestingly, the two mutations K265A (Fig. 5C) and L290S rendered the OTCase less sensitive to arginase without affecting the apparent $K_m$ of the mutated enzymes for ornithine. The substitution L290S was not expected to affect the catalytic properties of yeast OTCase, because its main chain only is involved in the direct stabilization of CP and ornithine (33), whereas the side chain of leucine is probably involved in inhibition by arginase. As indicated in Table I, most of the mutated OTCases had lost the capacity to interact with wild type arginase in molecular sieving experiments. Moreover, for some mutated enzymes, D182N, E256A, and K265A, the reduction of inhibition by arginase was correlated with the loss of interaction between the two proteins using the two-hybrid assay (data not shown). Fig. 4B shows those amino acids that appear to be important for the association of OTCase with arginase, whereas Fig. 4C shows those residues that were found not to be involved.

Construction of Mutations in Arginase by in Vitro Mutagenesis—The comparison of the available arginase amino acid sequences (S. cerevisiae, S. pombe, Rattus norvegicus, and Bacillus caldovelox) revealed the presence in the S. cerevisiae enzyme of several short sequences that are absent or different in the arginases devoid of regulatory properties (Fig. 6). Among the known arginase sequences, R. norvegicus and B. caldovelox arginases were chosen for this comparison because their crystal structures had been determined (35, 36). Regions of interest in the S. cerevisiae arginase were those between amino acids 78 and 86 (indicated by the arrow in Fig. 6), and more interestingly, the C-terminal end of the enzyme, which contains several cysteine and histidine residues that could constitute a zinc finger-like element (white residues in black boxes). The group of P. Hensley (37) has demonstrated that the binding of a weakly bound Mn$^{2+}$ ion confers catalytic activity, whereas the binding of a more tightly associated Zn$^{2+}$ ion confers substantial stability to the tertiary and quaternary structure of the enzyme, thus possibly playing a role in the formation of the OTCase-arginase multienzyme complex. As a first attempt to localize in the arginase residues involved in its interaction with OTCase, we generated four mutants. The deletion of amino acids 78–86 (pAM4) and the replacements of H309K/H312E (pME209), C321R/C326T (pME210), and H309K/H312E/C321R/C326T (pME222) in the C-terminal end of arginase were constructed by in vitro mutagenesis in plasmid pEJ24 expressing the CAR1 gene under the GAL10 promoter, as described under “Experimental Procedures.” It must be emphasized that the amino acid replacements mentioned above introduce the cognate residues present in S. pombe arginase. The plasmids obtained were used to transform yeast strain 02859darg3-car1 (arg3-kamMX4, car1Δ, leu2, trp1, ura3) on M-am-glucose supplemented with 25 μg of arginine, 25 μg of leucine, and 25 μg of tryptophan, per milliliter. Arginase specific activities were measured in the
zinc to the enzyme, although necessary to maintain its quaternary structure (37), does not require the two cysteines. The arginase from Pichia sorbitophila (sequence provided by the Genolevures project) (38), which contains the two cysteines, is unable to inhibit S. cerevisiae OTCase (data not shown). Cys-321 and Cys-326 are thus necessary but not sufficient to confer a regulatory function to arginase.

**DISCUSSION**

For the yeast OTCase, as for most of OTCases, the catalysis of carbamoylation was predicted to follow a preferred ordered Bi-Bi binding mechanism with carbamoyl phosphate (CP) being the first substrate bound and ornithine the second one (enzyme-CP-ornithine) (39, 40). In such a kinetic mechanism, a high ornithine concentration could favor a slower pathway of central ternary complex formation (enzyme-ornithine-CP) leading to inhibition of OTCase activity. In agreement with such a mechanism, it was shown that increasing the concentration of the preferred substrate (CP) overcame the inhibition of OTCase by ornithine and reduced its inhibition by arginase (4). Ornithine binding to the free enzyme could promote a conformational change in the enzyme, which could potentiate its association with arginase. The crucial role of ornithine in this regulatory process is emphasized by the fact that carbamoyl phosphate cannot replace ornithine in the complex with arginase,\(^2\) and mutating Thr-68 to Gly in the STRTR carbamoyl phosphate binding domain, which had a dramatic effect on the catalytic activity of the enzyme, had no effect on its interaction with arginase (Table I).

By mutation analysis we identified in S. cerevisiae OTCase two regions required for the OTCase-arginase interaction. Residues Asp-182, Asn-184, Asn-185, Cys-289, and Leu-290 are crucial for the regulation by arginase and are probably involved in the binding of ornithine, based on the structure of the E. coli OTCase bound to the bisubstrate analog PALO (33, 34). In the E. coli enzyme, Asp-231, Ser-235, and Met-236 are part of the flexible SMG loop, the main binding site for ornithine. In yeast OTCase, Glu-256 located in the SMG loop could also participate in ornithine binding, because its replacement strongly reduced the apparent affinity of OTCase for ornithine to the same extent as the replacement of residues Asp-182, Asn-184, Asn-185, and Cys-289. In contrast, the four lysine residues, Lys-260, Lys-263, Lys-265, and Lys-268 in the SMG loop were not reported to bind PALO in E. coli and human OTCases but appear to play an important role in the interaction between OTCase and arginase. Changing Lys-260, Lys-265, or Lys-268 to arginine had no effect, but their replacement by alanine modified the interaction with arginase, attesting the importance of a basic residue at those positions. OTCase-K260A was only slightly less sensitive to arginase at low ornithine concentration, whereas OTCase-K265A had lost 50% of the inhibition capacity, but exhibited a normal Km for arginine,\(^2\) and mutating Thr-68 to Gly in the STRTR carbamoyl phosphate binding domain, which had a dramatic effect on the catalytic activity of the enzyme, had no effect on its interaction with arginase (Table I).

**TABLE II**

**Characteristics of the mutated arginases**

Arginase activity was assayed in 30 °C in extracts from strains 02859darg3-car1 (ura3, leu2, trp1, arg3:kanMX4, car1Δ) transformed with pYeF2 plasmid containing wild type car1 gene or the mutated car1 gene. After growth at 30 °C on MMa plus 2% galactose plus 25 µg of leucine plus 25 µg of tryptophan plus 25 µg of arginine. At least three independent transformants were tested and the standard error was typically 10-15% of the mean. The arginase specific activity is expressed in Micromoles of urea formed/h/mg of protein.

| Plasmids | Nature of the mutation in arginase | Arginase specific activity | Km for arginine | Remaining activity of WT OTCase after inhibition by 500 units of arginase |
|----------|-----------------------------------|---------------------------|-----------------|---------------------------------------------------------------------|
| pME209   | H309K-H312E                        | 5800                      | 6.9             | 16                                                                   |
| pME210   | C321R-C326T                        | 4700                      | 7.1             | 91                                                                   |
| pME222   | H309K-H312E-C321R-C326T             | 3000                      | 6.7             | 90                                                                   |

\(^a\) M. El Alami, E. Dubois, Y. Oudjama, C. Tricot, J. Wouters, V. Stalon, and F. Messenguy, unpublished results.
the amino acid present at that position in many other OTCases, the apparent $K_m$ of the yeast enzyme for ornithine dropped about 15-fold, rendering OTCase-K268R insensitive to arginase. Thus Lys-268 seems to retain the ability to inhibitory distance on these lysine residues remains questionable, but it is still possible that the yeast enzyme undergoes the transition from the open to the closed states with more ease than other OTCases and that the binding of arginase enhances this effect. Lysine 268 seems to restrain the transition, because K268A substitution results in an improved affinity for ornithine and an enhanced sensitivity to excess ornithine and to inhibition by arginase. Whether ornithine acts directly on these lysine residues remains questionable, but they could be involved in transducing and enhancing the signal given by ornithine for catalytic domain closure.

The regulatory function of arginase requires at least the residues Cys-321 and Cys-326. The group of Hensley (37) had given by ornithine for catalytic domain closure. Directly on these lysine residues remains questionable, but it is still possible that the yeast enzyme undergoes the transition from the open to the closed states with more ease than other OTCases and that the binding of arginase enhances this effect. Lysine 268 seems to restrain the transition, because K268A substitution results in an improved affinity for ornithine and an enhanced sensitivity to excess ornithine and to inhibition by arginase. Whether ornithine acts directly on these lysine residues remains questionable, but they could be involved in transducing and enhancing the signal given by ornithine for catalytic domain closure.

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