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Published in:
Journal of Biological Chemistry

DOI:
10.1074/jbc.M300383200

Publication date:
2003

Document Version
Publisher's PDF, also known as Version of record

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 24, Issue of June 13, pp. 21550–21558, 2003

Printed in U.S.A.

Yeast Epiarginase Regulation, an Enzyme-Enzyme Activity Control

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

Received for publication, January 14, 2003, and in revised form, April 4, 2003
Published, JBC Papers in Press, April 4, 2003, DOI 10.1074/jbc.M300383200

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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. The mechanism by which these nonallosteric enzymes form a stable complex triggered by the binding of their respective substrates raises the question of how such a cooperative association is induced. Analyses of mutations in both enzymes identify residues that are required for their association, some of them being important for catalysis. In arginase, two cysteines at the C terminus of the protein are crucial for its epiarginase function but not for its catalytic activity and trimeric structure. In OTCase, mutations of putative ornithine binding residues, Asp-182, Asn-184, Asn-185, Cys-289, and Glu-256 greatly reduced the affinity for ornithine and impaired the interaction with arginase. The four lysine residues located in the SMG loop, Lys-260, Lys-263, Lys-265, and Lys-268, also play an important role in mediating the sensitivity of OTCase to ornithine and to arginase and appear to be involved in transducing and enhancing the signal given by ornithine for the closure of the catalytic domain.

In *Saccharomyces cerevisiae*, the expression of arginine anabolic and catabolic genes is regulated at multiple levels in response to a wide variety of metabolic signals such as arginine and amino acid concentrations, nitrogen quality, and availability (reviewed in Ref. 1). In addition, two mechanisms are known to operate at the level of enzyme activity. Excess arginine causes feedback inhibition of the activity of the two first enzymes of the anabolic pathway, acetylglutamate synthase and acetylglutamate kinase (2, 3). Another regulation known as the "epiarginase control" leads to the inhibition of the activity of the anabolic ornithine carbamoyltransferase (OTCase)[1] (EC 2.1.3.3) by the catabolic enzyme, arginase (EC 3.5.3.1). In the presence of ornithine and arginine, the respective substrates of the two enzymes (4), 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 epiarginasic 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 in the *S. cerevisiae* OTCase 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 HF7c (*MAT*a, *ura3–52*, *his3–200*, *lys2–801*, *ade2–101*, *trp1–901*, *leu2–3,112*,

* This work was supported by an Action de la Recherche concertée number 98/03-231 between the French Community of Belgium and the Free University of Brussels (U. L. B.) and by the Fonds National de la Recherche Scientifique. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: OTCase, ornithine carbamoyltransferase; CP, carbamoyl phosphate; GBD, Gal4 DNA binding domain; GAD, Gal4 activation domain; M.am, minimal medium; PALO, *N*-(phosphoacetyl)-L-ornithine.

gal4–542, *gal80–538*, *LYS2*::*GAL1-HIS3*, *URA3*::(*GAL4*$_{(17\text{-}mer)3}$-*CYC1-lacZ*) and Y187 (*MAT*α, *ura3–52*, *his3–200*, *ade2–101*, *trp1–901*, *leu2–3,112*, *met*$^-$, *gal4*Δ, *gal80*Δ, *URA3*::*GAL1-lacZ*) (12) and was the recipient strain for experiments using the two-hybrid system. The yeast strain MaV99 (*MAT*a, *SPAL10*::*URA3*), which contains an integrated *GAL1-URA3* reporter gene activated by the *GAL* upstream activation sequence and derives from the strain MaV52 (*MAT*a, *ura3–52*, *leu2–3,112*, *trp1–901*, *his3–200*, *ade2–101*, *gal4*Δ, *gal80*Δ, *can1*^{R}, *cyh2*^{R}, *GAL1*::*HIS3@LYS2*, *GAL1*::*lacZ*) (13), was the recipient strain for experiments using the reverse two-hybrid system. This strain lacks both *GAL4* and *GAL80* genes and contains integrated *GAL1-lacZ* and *GAL1-HIS3* reporter genes. The yeast strain 02859d (*ura3*, *leu2*, *trp1*), isogenic to the wild type strain Σ1278b, was used to delete the *CAR1* and *ARG3* genes. Deletion of the *CAR1* gene was achieved by transformating the strain 02859d with pWX4 plasmid as described by Dubois and Messenguy (14), leading to strain 02859dΔcar1. Deletion of the *ARG3* gene was performed using the PCR strategy developed by Wach (15). The disruption cassette, containing the *kanMX4* cassette with long flanking regions homologous to the *ARG3* gene, was synthesized using PCR. This cassette confers to yeast a resistance to Geneticin (G418). The homologous regions of *ARG3* gene were located upstream from the start codon and downstream from the stop codon. The PCR protocols for preparation of this cassette were identical to those described in Wach (15). Integration of the *kanMX4* cassette at the target locus was confirmed using the PCR strategy as described by Wach (15). Strain 02859dΔarg3-car1 (*ura3*, *leu2*, *trp1*, *arg3*::*kanMX4*, *car1*Δ) was used as recipient strain for transformation with plasmids overexpressing *CAR1* or *ARG3* genes. Strain 12S16c (*ura3*, *leu2*, *arg3*Δ) (a gift from M. Crabeel) was used as recipient strain for transformation with plasmids expressing wild type or mutated *ARG3* genes or with plasmids expressing GBD-OTCase or GAD-OTCase. Strain 12T7cI (*ura3*, *car1*Δ) (14), isogenic to the wild type strain Σ1278b, was used as recipient strain for transformation with plasmids expressing GBD-arginase or GAD-arginase.

The strains used in the two-hybrid analysis were grown on synthetic medium containing 0.7% yeast nitrogen base without amino acids. This medium was supplemented with 2% glucose and all amino acids except those whose omission was required for plasmid selection. All the other strains were grown on minimal medium (M.am) containing 10 mM $(NH_4)_2SO_4$, 3% glucose or 2% galactose, vitamins, and mineral traces as described previously (16). The lithium acetate procedure was used to transform the recipient yeast strains (17) *Escherichia coli* strains XL1-Blue, XL1-Red, and HB101 from Stratagene were used for plasmid amplification, plasmid mutagenesis, and 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 and as primers the appropriate oligonucleotides extended with *Bam*HI restriction sites. These fragments were inserted in the *Bam*HI site of vector pYeF2 (pUC19, 2μ, *URA3*, *GAL10* promoter) (18), yielding plasmids pDC2 (p*GAL10-ARG3*, 2μ, *URA3*) and pEJ24 (p*GAL10-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.

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, Detroit, MI). GAD-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 *Bam*HI site for the 5′-end and a *Xho*I site for the 3′-end. This DNA fragment was digested by *Bam*HI and *Xho*I and inserted into the pAS2 vector, then digested by *Bam*HI and *Sal*I, leading to plasmid pGBD-*ARG3*, and inserted into the pACTII vector, and finally digested again by *Bam*HI and *Xho*I, leading to plasmid pGAD-*ARG3*. In these GBD-*ARG3* and GAD-*ARG3* fusions, we determined the nucleotide sequence to ensure that 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 *Bam*HI site for the 5′-end and a *Xho*I site for the 3′-end. This DNA fragment was digested by *Bam*HI and *Xho*I and inserted into the pAS2 vector, then digested by *Bam*HI and *Sal*I, leading to plasmid pGBD-*CAR1*, and inserted into the pACTII vector, then finally digested again by *Bam*HI and *Xho*I, leading to plasmid pGAD-*CAR1*. In these GBD-*CAR1* and GAD-*CAR1* fusions, 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 GBD-*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 UAS_{GAL} sequences. The *URA3* gene allowed the selection of mutated GAD-OTCases having lost their interaction with GBD-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 (leu$^-$, pro$^-$, amp^s). This allowed the selection of plasmids containing a functional *LEU2* gene and the GAD-*ARG3* gene, and eliminated the pGBD-*CAR1* plasmid.

Construction of Deletion and Point Mutations in the ARG3 Gene

The deletion of the region between amino acids 166 and 169 (ΔEVNK) was created by *in vitro* mutagenesis on double-stranded DNA from plasmid pKV0c2 (pBR322, 2μ, *URA3*, *ARG3*), which contains the *ARG3* gene, leading to plasmid parg3-M1. The substitutions T68G, 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 pDC2 (p*GAL10-ARG3*, 2μ, *URA3*), leading to plasmids pME223 (T68G), 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), pME237 (C289S), pME238 (L290S), pME239 (L290Q), and pME221 (Q294P), respectively. All genes 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 (ΔGGSSVMIDG) was generated by *in vitro* mutagenesis on double-stranded DNA from plasmid pEJ24 (p*GAL10-CAR1*, 2μ, *URA3*), leading to plasmid pAM4. The substitutions H309K/H312E and C321R/C326T were introduced in arginase by site-directed *in vitro* mutagenesis on plasmid pEJ24 (p*GAL10-CAR1*, 2μ, *URA3*), leading to plasmids pME209 (H309K/H312E) and pME210 (C321R/C326T), respectively. The changes of the amino acids H309K/H312E/C321R/C326T were generated in arginase by site-directed *in vitro* mutagenesis on plasmid pME210, leading to plasmid pME222 (H309K/H312E/C321R/C326T). All genes were sequenced to ensure that no additional mutation had been 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 citrulline 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

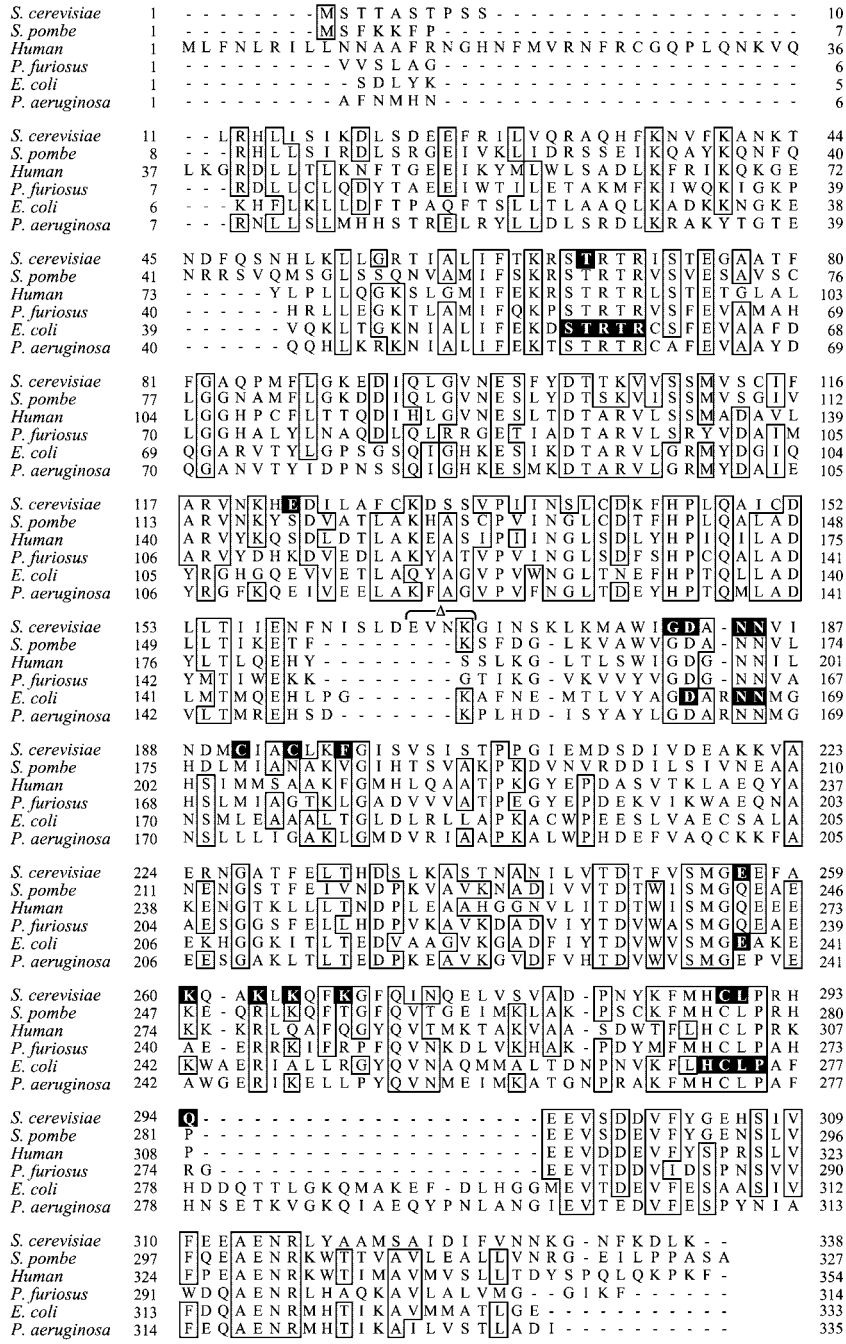

FIG. 1. **Amino acid alignment of OTCase sequences from different organisms.** OTCase sequence from *P. aeruginosa* refers to the sequence of the catabolic enzyme. Conserved amino acid residues are boxed. The position of the first and last residues is indicated. The carbamoyl phosphate and ornithine binding residues in the *E. coli* enzyme (34) are indicated in white and boxed in black. The mutated residues in yeast OTCase are also in white and boxed in black. Δ represents the deletion of the residues covered by the bracket.

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, *Pyrococcus 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 Modeler4 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 × 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*C*, and 1 mM agmatine. This column was pre-calibrated with serum albumin (molecular mass, 66 kDa), *E. coli* OTCase (molecular mass, 120 kDa), *Enterococcus*

TABLE I
Characteristics of the mutated OTCases

OTCase activity was assayed at 30 °C in extracts from strain 02859dΔarg3-car1 (*ura3, leu2, trp1, arg3*::*kanMX4, car1*Δ) 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 leucine plus μg of tryptophan plus 25 μg of arginine. The assays were performed in 50 mM Tris-HCl buffer, pH 8, 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 K_m 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.

Plasmids	Nature of the mutation in OTCase	OTCase specific activity measured at saturation of ornithine and CP	Apparent K_m for ornithine	Remaining activity of OTCase after inhibition by 500 units of WT arginase
			mM	%
pDC2	WT	1570	1.6	14
pME223	T68G	77	3.4	18
pME204	E123S	1390	2.1	16
pME206	E123A	1520	2.6	15
pME216	G181R	0	ND[a]	ND[a]
pFV48	D182N	970	49	100[b]
pME234	N184Q	740	34	95
pME235	N185Q	1500	350	100
pME211	C191M-C194N-F197V	250	2.8	18
pME205	E256Q	760	1.2	15
pME207	E256A	900	10	94[b]
pME217	K260R	1510	1.2	16
pME218	K260A	1550	1	16[c]
pME213	K263R	890	20	100
pME208	K263A	450	30	98
pME240	K265R	1418	1.1	18
pME219	K265A	1010	1.2	47[b]
pME241	K268R	1544	1.1	18
pME220	K268A	1500	≤0.05	<5[d]
pME237	C289S	170	10	100
pME238	L290S	280	2	48[b]
pME239	L290Q	800	2	22
pME221	Q294P	1460	1.3	17

[a] ND, not detectable. Interaction with arginase in molecular sieving experiment (data not shown).
[b] Interaction with arginase is absent or reduced in the molecular sieving experiment.
[c] See Fig. 5*B*.
[d] See Fig. 5*D*.

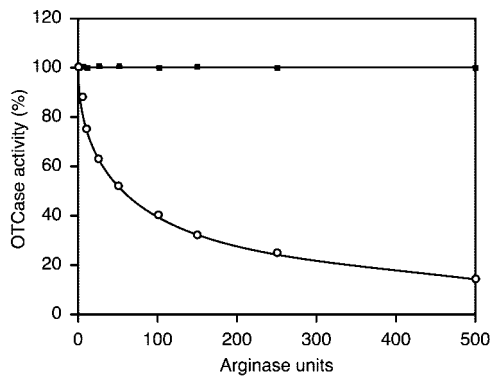

FIG. 2. ***In vitro* inhibition of wild type OTCase and mutated OTCase-D182N by wild type arginase.** The inhibition test was performed at 30 °C in the presence of 50 mM Tris-HCl, pH 8.0, 15 mM ornithine for the wild type OTCase and 200 mM for the D182N variant, 1 mM agmatine, and increasing amounts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

faecalis OTCase (molecular mass, 240 kDa), and *P. furiosus* OTCase (molecular mass, 420 kDa). Elution was carried out with the same buffer, at a flow rate of 0.5 ml/min, and 0.56-ml fractions were collected. Activities of OTCase and arginase were determined in each fraction as described.

DNA Manipulation and DNA Sequencing

Restriction reactions were performed as recommended by the enzyme supplier. Plasmid DNA was prepared using the alkaline lysis method (28). Double-stranded DNA was prepared using Qiagen columns. DNA was sequenced by the Thermosequenase radiolabeled terminator cycle sequencing kit (Amersham Biosciences). Site-directed *in vitro* mutagenesis was performed with the QuikChange site-directed mutagenesis kit from Stratagene.

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 12S16c 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 (p*GAL10-arg3D182N*, *2μ*, *URA3*). The substitution re-

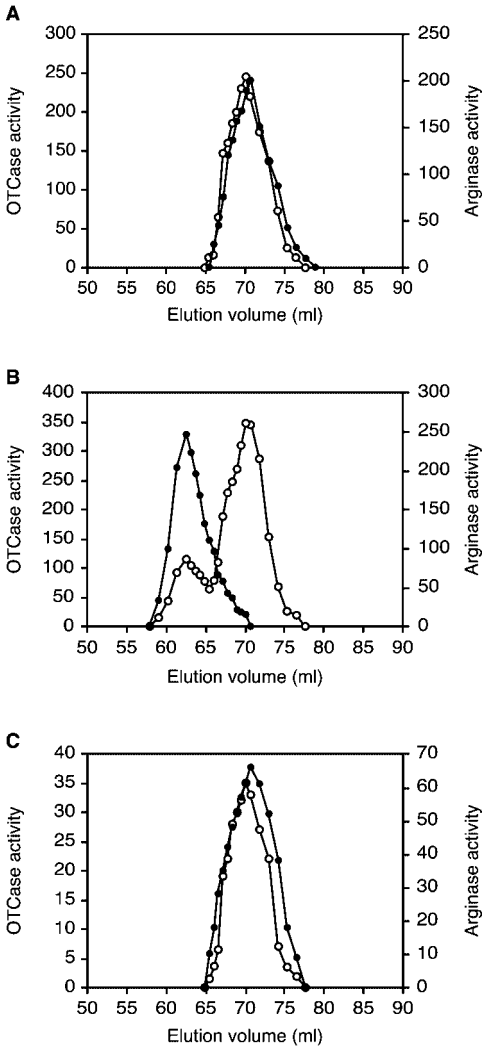

FIG. 3. **Elution profile of a mixture of arginase and OTCase extracted from wild type and mutant strains.** The molecular mass of arginase is about 114 kDa and that of OTCase is about 120 kDa (*A*). When the two enzymes are associated as in *B*, the molecular mass of the associated peak is about 200 kDa. Arginase and OTCase from wild type and mutant strains were eluted on Superdex 200 column with Tris-HCl 50 mM, pH 8.0. *A*, elution profile of 2400 units of WT OTCase (*open circles*) and 1000 units of WT arginase (*filled circles*), in the absence of ornithine and agmatine. *B*, elution profile of a mixture of 3400 units of WT OTCase (*open circles*) and 900 units of WT arginase (*filled circles*), in the presence of 15 mM ornithine and 1 mM agmatine. *C*, elution profile of a mixture of 400 units of mutant OTCase D182N (*open circles*) and 500 units of WT arginase (*filled circles*), in the presence of 200 mM ornithine and 1 mM agmatine.

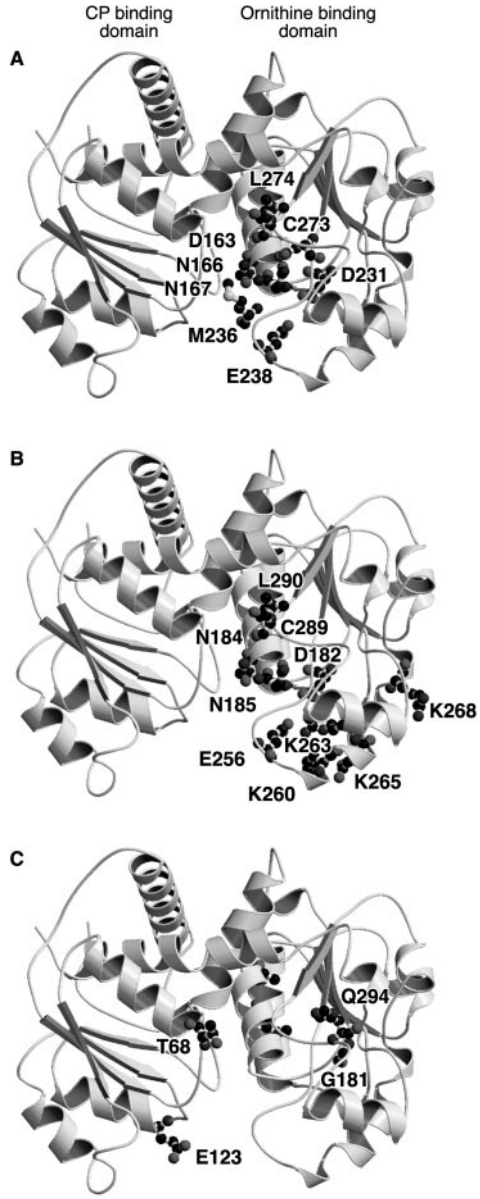

FIG. 4. **View of the α-carbon chain of the predicted structure of the OTCase monomer from *S. cerevisiae* using the structure of the *E. coli* OTCase as template.** *A*, *E. coli* OTCase monomer taken from the crystal structure reported by Ha *et al.* (34). Residues interacting with ornithine are indicated. Secondary structure elements: α helices are represented by *cylinders*, and β strands are represented by *arrows*. *B*, *S. cerevisiae* OTCase monomer modeled on the basis of the structure of *E. coli* OTCase. Residues required for regulation by arginase are indicated. *C*, *S. cerevisiae* OTCase monomer. Residues whose changes did not affect the OTCase-arginase interaction are indicated.

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. 4*A*). 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 (Δ in 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-

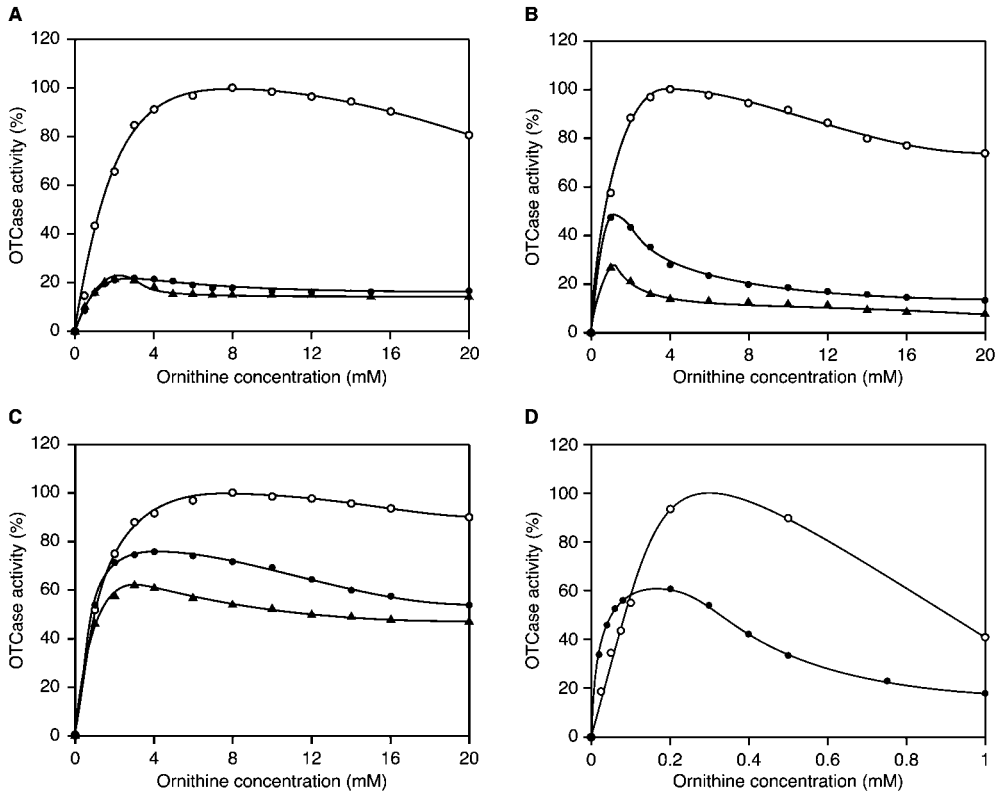

FIG. 5. **Inhibition of wild type and mutated OTCases (K260A, K265A, K268A) by wild type arginase as a function of ornithine concentration.** The velocity of the reaction catalyzed by wild type and mutated OTCases was measured at 30 °C in 50 mM Tris-HCl buffer, pH 8.0, in the presence of 5 mM CP, 1 mM 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 triangles*). *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 triangles*). *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 triangles*). *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*).

cording to studies on *E. coli* OTCase complexed with the bisubstrate analog *N*-(phosphoacetyl)-L-ornithine (PALO) (34). Fig. 4*A* represents a monomer of *E. coli* OTCase, with the CP binding domain on the *left* and the ornithine binding domain on the *right*, and with L-ornithine binding to residues Asp-163, Asn-166, Asn-167, Glu-238, Cys-273, and Leu-274 (indicated in the *E. coli* sequence of Fig. 1, according to the data of Ref. 34). Among the crystal structures of OTCases available in the literature, the structure of *E. coli* (PDB code 1a1s) was retained to construct a model of yeast OTCase by homology modeling (Fig. 4, *B* and *C*, see "Experimental Procedures"). According to this model, Asp-182 in yeast OTCase could interact with residues of the SMG loop (Ser-253, Met-254, and Gly-255) involved in the closure of the ornithine and carbamoyl phosphate domains in the presence of the substrates. We chose to modify a series of residues in the vicinity of Asp-182, within and around the SMG loop, in the CP binding site, and in the ornithine binding site. The following substitutions were introduced: T68G, E123S, E123A, G181R, N184Q, N185Q, C191M/C194N/F197V, E256Q or E256A, K260R or K260A, K263R or K263A, K265R or K265A, K268R or K268A, C289S, L290S or L290Q, and Q294P (see "Experimental Procedures"). These residues are marked in the *S. cerevisiae* OTCase sequence in Fig. 1 (*white* residues *boxed* in *black*). Residue Thr-68 is located in the STRTR sequence known to be involved in the binding of CP in *E. coli* OTCase, whereas Asn-184, Asn-185, Glu-256, Cys-289, and Leu-290 are in yeast OTCase residues that are liable to bind ornithine, in addition to Asp-182. Residue Glu-123 is located in the CP binding domain but opposite to the SMG loop and could therefore make contact with residues in this loop.

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

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 mM ornithine, but the interaction was reduced at lower ornithine concentrations, as shown in Fig. 5*B* (compared with Fig. 5*A* 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. 5*D*. Interestingly, the two mutations K265A (Fig. 5*C*) 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. 4*B* shows those amino acids that appear to be important for the association of OTCase with arginase, whereas Fig. 4*C* 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 02859dΔarg3-car1 (*arg3*::*kanMX4*, *car1*Δ, *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

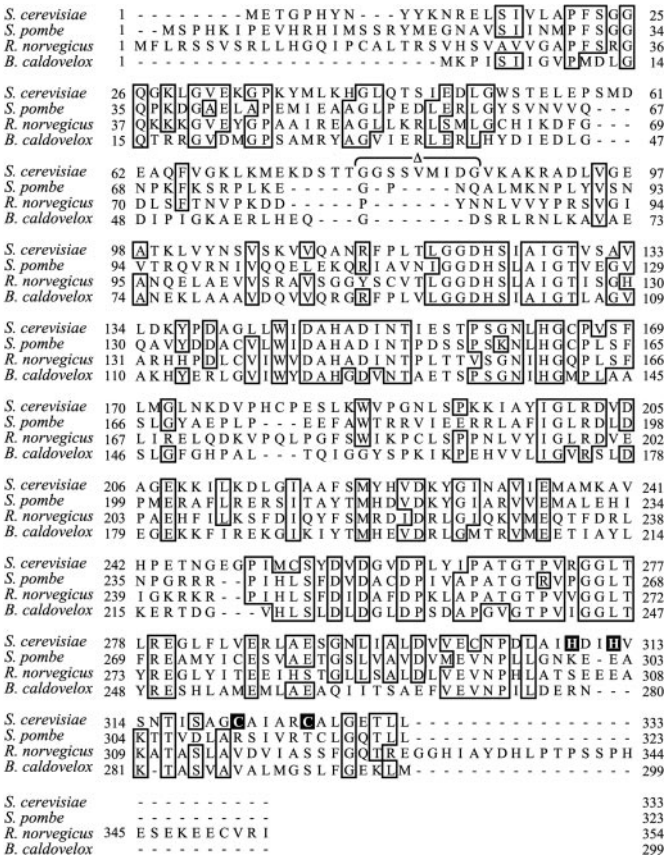

FIG. 6. **Amino acid alignment of arginase sequences from different organisms.** Conserved amino acid residues are *boxed*. The positions of the first and last residues are indicated. The histidine and cysteine residues are indicated by *white letters* in *black boxes*. "Δ" represents the deletion of the residues covered by the *bracket*.

transformants 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. 7*A*). These mutations did not alter significantly the enzymatic properties of the enzymes; their catalytic activity and their affinity for arginine were unaffected (Table II). Moreover, the simultaneous substitution of the two cysteines and the two histidines only led to a 50% reduction of the activity without affecting the K_m of the enzyme for arginine. Such a reduction could result from a slight effect on enzyme stability. The loss of interaction between the arginase-C231R-C326T and the wild type OTCase was confirmed using the two-hybrid assay (data not shown) and by molecular sieving (Fig. 7, *B* and *C*). Addition of mercaptoethanol in the inhibition test (data not shown) did not affect the regulatory capacity of wild type arginase. Moreover, addition of 1 mM zinc acetate in the molecular sieving experiment performed with wild type OTCase and arginase-C321R-C326T did not restore the formation of an OTCase-arginase complex (data not shown). As shown in Fig. 7*C*, 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

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.

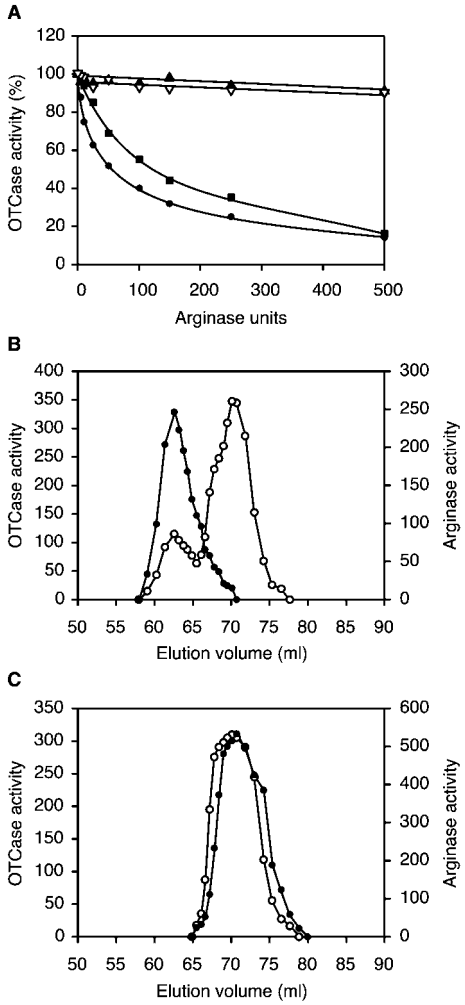

FIG. 7. ***In vitro* inhibition of wild type OTCase by wild type and mutated arginases and elution profiles of a mixture of wild type OTCase and mutated arginases.** *A*, the inhibition test was performed at 30 °C in the presence of 50 mM Tris-HCl, pH 8.0, 15 mM ornithine, and 1 mM agmatine. Wild type arginase (*filled circles*), arginase-H309K, H312E (*filled squares*), arginase-C321R, C326T (*filled triangles*), and arginase-H309K, H312E, C321R, C326T (*open triangles*). *B*, elution profile of a mixture of 3200 units of wild type OTCase (*open circles*) with 900 units of mutated arginase-H309K, H312E (*filled circles*) in the presence of 15 mM ornithine and 1 mM agmatine. *C*, elution profile of a mixture of 3200 units of WT OTCase (*open circles*) and 2500 units of mutated arginase-C321R, C326T (*filled circles*), in the presence of 15 mM ornithine and 1 mM agmatine.

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 K_m for ornithine. In contrast, OTCase-K268A had a better affinity for ornithine than the wild type enzyme and, consequently, was much more sensitive to arginase at an ornithine concentration below 1 mM. Residue Lys-263 is of particular interest. When mutated to arginine,

[2] M. El Alami, E. Dubois, Y. Oudjama, C. Tricot, J. Wouters, V. Stalon, and F. Messenguy, unpublished results.

TABLE II
Characteristics of the mutated arginases

Arginase activity was assayed in 30 °C in extracts from strain 02859dΔ*arg3-car1* (*ura3, leu2, trp1, arg3*::*kanMX4, car1*Δ) transformed with pYeF2 plasmid containing wild type *car1* gene or the mutated *car1* genes after growth at 30 °C on M.am 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	K_m for arginine	Remaining activity of WT OTCase after inhibition by 500 units of arginase
			mM	%
pEJ24	WT	6000	6.5	14
pME209	H309K-H312E	5800	6.9	16
pME210	C321R-C326T	4700	7.1	91
pME222	H309K-H312E-C321R-C326T	3000	6.7	90

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-K263R insensitive to arginase. Thus Lys-263 may constitute one of the key residues that differentiates *S. cerevisiae* OTCase from other OTCases for its inhibition by arginase. The four lysines, Lys-260, Lys-263, Lys-265, and Lys-268, appear to constitute contact points with arginase, when the latter enzyme binds arginine or agmatine, inducing a modification of the catalytic site conformation and reducing the catalytic activity of OTCase. In *E. coli* OTCase, the SMG loop undergoes a large conformational change upon substrate binding. It is 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 proposed that these two cysteines form a putative "Zinc finger-like" element with the residues His-309 and His-312 and that this structure could be involved in stabilizing subunit interactions and, thus, play a role in the formation of the OTCase-arginase complex. However, modifying these four residues had no strong effect on the catalytic activity of arginase and did not modify the trimeric structure of the enzyme, suggesting that these amino acids are not crucial for the zinc-dependent enzyme stability. Moreover, few known arginases contain the two histidine and cysteine residues in their C-terminal end. Our data demonstrate that the two cysteine residues are necessary for the epiarginase function of the yeast arginase but that they are not sufficient, because the arginase from *P. sorbitophila* containing these two amino acids has no regulatory capacity. Based on sequence alignments and on crystal structures, no suitable templates are available to model the C-terminal part of the protein. It is worth noting that the *S. cerevisiae* arginase contains two arginine binding sites (41) that might not be present in arginases of other species. Thus, determining the crystal structure of this unusual arginase and of the OTCase-arginase complex, with and without their effectors, will help to unravel this intricate mechanism of enzyme regulation by protein-protein association.

Acknowledgments—We are very grateful to André Piérard and Daniel Charlier for helpful comments about the manuscript. We thank V. Villeret and B. Clantin for useful suggestions for the creation of some mutations in OTCase, Mark Vidal for the gift of MaV strains and Jean-Luc Souciet for the gift of the *P. sorbitophila* strain, and for the communication of arginase and OTCase sequences determined by the Genolevures project. Bart Scherens was very helpful in exploring the data base for the comparison of these sequences, and J. P. ten Have helped with the figures.

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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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J. Biol. Chem. 2003, 278:21550-21558.
doi: 10.1074/jbc.M300383200 originally published online April 4, 2003
