Evolution of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase-encoding genes in the yeast *Saccharomyces cerevisiae*

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The shikimate pathway resulting in three aromatic amino acids is initiated in different organisms by two and three 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthases, respectively. Aro3p and Aro4p are the yeast enzymes feedback-inhibited by phenylalanine and tyrosine, respectively. A yeast strain deficient in the general control transcriptional regulatory system of amino acid biosynthesis is unable to live in the presence of high amounts of phenylalanine and tyrosine. Here, we show that this yeast strain can be rescued by the expression of *aroH* from *Escherichia coli* encoding the tryptophan-regulated AroH as third isoenzyme. Yeast carrying Ec AroH as the only enzyme for the initial step of the shikimate pathway can grow in the absence of tryptophan. Without aromatic amino acids, this yeast strain survives only when the yeast *ARO3* promoter instead of the *ARO4* promoter drives *E. coli aroH*. The detailed analysis of Aro3p and Aro4p revealed a triple feedback control by tyrosine/phenylalanine and tryptophan. Dissecting this control allowed engineering of Aro4p S195A as an enzyme, which is inhibited like AroH only by tryptophan. In addition, Aro4p variants were constructed that show an equally strong inhibition by tyrosine and tryptophan (Aro4p P165G Q302R) and in which the regulation by tyrosine and tryptophan was reversed (Aro4p P165G). Our data suggest that yeast possesses only two instead of three isogenes encoding 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthases because both isoenzymes can be fine tuned by tryptophan as additional effector and because transcriptional regulation by the general control system can be induced as backup when aromatic amino acids in the environment are imbalanced.

general control | Aro4p | AroH

The shikimate pathway starts with the stereo specific condensation of erythrose-4-phosphate and phospho*enol*pyruvate to 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) (1) and is catalyzed by the DAHP synthase (DAHPS, EC 4.1.2.15). DAHP is converted to the branch point compound chorismate (2–5), which is a precursor for the aromatic amino acids tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp). The latter two amino acids are synthesized only by microorganisms and plants (6) and are assimilated by animals with the diet. Mammals convert Phe to Tyr and use aromatic amino acids as precursors for numerous important substances including neurotransmitters like serotonin (7). In *Escherichia coli*, the three isogenes *aroF* (8), *aroG* (9) and *aroH* (10) code for three DAHPS isoenzymes, each feedback-regulated by one of the aromatic amino acids Tyr (AroF), Phe (AroG), and Trp (AroH), respectively (11). Total cellular DAHPS activities are unequally distributed to the three isoenzymes. In *E. coli*, the Phe-inhibited AroG (79%) and the Tyr-inhibited AroF (20%) deliver nearly all of the DAHPS activity for the cell. The Trp-inhibited AroH contributes only 1% of the cellular enzyme activity (12). Inhibition of AroG (Phe-inhibited) and AroF (Tyr-inhibited) is highly effective and abolishes >95% of their specific enzyme activity (13, 14),

whereas the Trp inhibition of AroH is only ≈60%, thereby resulting in a 40% constitutive AroH activity (15).

Various fungi such as the yeast *Saccharomyces cerevisiae* and the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* express two differently regulated DAHPSs. The *ARO3*-encoded DAHPS is inhibited by Phe, whereas the *ARO4* gene product is inhibited by Tyr (16–20). In *A. nidulans*, *aroF* and *aroG* code for two DAHPSs that are differentially regulated by Tyr (AROF) and Phe (AROG) (21). In addition to the feedback control of yeast Aro3p and Aro4p, the transcription of the corresponding genes is part of a complex regulatory network that couples transcriptional derepression of >500 genes to the availability of amino acids (22). This regulatory network is conserved between several fungi and is known as "cross-pathway control" in molds or as "general control" of amino acid biosynthesis in yeast (23–25). A key factor is the transcriptional activator Gcn4p of yeast and its interchangeable counterparts including, e.g., CPCA of *A. nidulans* (24). Gcn4p binds to the promoter region of target genes to a well characterized upstream activation site. These upstream sites are present in both *ARO3* and *ARO4* of yeast as well as in the corresponding genes of *A. nidulans*. During amino acid deprivation, *ARO3* and *ARO4* transcription is increased by a factor of two and four, respectively (17, 18).

Alignment of amino acid sequences of DAHPSs from *E. coli* and *S. cerevisiae* showed the sequence identity of 63% between Trp-regulated AroH and Phe-regulated Aro3p and of 64% between AroH and Tyr-regulated Aro4p. To date, the crystal structures of the *aroG* gene product (Phe-inhibited) from *E. coli* (26, 27) and the *ARO4* gene product (Tyr-inhibited) from *S. cerevisiae* (28, 29) have been determined showing that the overall structures of bacterial and yeast DAHPSs are highly conserved.

Here, we started with the analysis of the influence of GCN4p and a heterologously expressed AroH, respectively, on yeast growing on an imbalanced amino acid diet. These experiments resulted in the discovery of an additional residual Trp regulation of Aro3p and Aro4p. Subsequently, the engineering of Aro4p variants with a distinct dual regulation by Tyr and/or Trp verified this subtle regulation of two isoenzymes by three effector amino acids.

Materials and Methods

Yeast Strains, Plasmids, Media, and Growth Conditions. Minimal vitamins (MV) and synthetic complete (SC) medium for the cultivation of yeast have been described (30). Transformation of *S. cerevisiae* was performed by the LiOAc method as described (31). For purification of Aro3p and Aro4p, for overexpression of

yeast *ARO4* alleles and as recipient for the *E. coli aroH* alleles, yeast strain RH2424 (*MAT**a**, can1-100, GAL⁺, aro3Δ::HIS3, aro4Δ::LEU2, ura3-1, his3⁻, leu2⁻*) was used. Strain RH2424 (*aro3Δ::HIS3, aro4Δ::LEU2*) was transformed with a 3.1-kb BamHI/XhoI *gcn4*Δknockout cassette amplified from the *gcn4*Δ Euroscarf Strain ES249 (web.uni-frankfurt.de/fb15/mikro/euroscarf/). Transformants were selected on SC media supplemented with 150 μg/ml geneticin. Correct transformation was verified by Southern hybridization analysis and resulted in strain RH2804 (*gcn4Δ::Kan^R, aro3Δ::HIS3, aro4Δ::LEU2*). Strain RH2424 lacking any DAHPS activity was transformed with the *aroH* gene flanked by the promoter and terminator regions of *ARO3* and *ARO4*, respectively, so that *aroH* was integrated and driven by the *ARO3* or *ARO4* promoter, respectively. The resulting strains RH2487 (*MAT**a**, can1-100, GAL⁺, aro3ΔaroH, aro4Δ::LEU2, ura3-1, his3⁻, leu2⁻*) and RH2803 (*MAT**a**, can1-100, GAL⁺, aro3Δ::HIS3, aro4Δ aroH, ura3-1, his3⁻, leu2⁻*) were verified by Southern hybridization. MV medium was supplemented with arginine, histidine, leucine, isoleucine, and valine to exclude other growth phenotypes produced by the knock-out cassettes used in these strains. For growth tests, strains RH1408 (*MAT**a**, ura3-52, gcn4-103*) (32), RH730 (*MATα* WT) (33), RH1416 (*MAT**a**, ura3-52*) (34), RH1313 (*MATα*, aro4-1) (18), RH1316 (*MAT**a**, aro3-2*) (18) were used as controls.

Mutagenesis of *ARO4*. Site-directed mutations of *ARO4* were introduced by using PCR with specific oligonucleotides carrying single nucleotide exchanges. Mutated alleles were cloned into p426MET25 (pRS426 carrying yeast *MET25* promoter and *CYC1* terminator, *URA3*, 2 μm, *Amp^R*) and pME1513 (p426MET25 (*URA3*, 2 μm, *Amp^R*) with altered MCS: SacI-p*MET25*-XbaI SpeI BamHI SalI SfiI NotI XhoI-t*CYC1*-KpnI), respectively (35, 36). The DNA sequence of all vectors described in this work was determined by sequencing to rule out second-site mutations (37). After transformation of the allelic vectors into RH2424, transformants were selected on SC medium lacking aromatic amino acids to screen for functionally intact DAHPSs.

Enzyme Assays. The DAHPS activity was determined by the stop assay described by Takahashi and Chan (38) with the modifications of Schnappauf *et al.* (20). Measurements of enzyme activities were performed at 550 nm by using an Ultrospec 4000 spectrophotometer (Amersham Pharmacia Biotech). Enzyme assays with crude extracts were performed at least three times at 30°C for 1 min by using 0.5 mM of each substrate and effector. Assays were performed with pretested amounts of protein so that the absorbance of the product was proportional to the amount of enzyme added. Total protein was between 72 μg and 158 μg per reaction, yielding specific activities between 89 and 708 milliunits/mg. In effector titration assays, enzymatic activities were determined at least three times at 30°C after 1 min of reaction time with 150 μg of total protein. Substrate concentrations used were 0.4 mM erythrose-4-phosphate and 0.1 mM phospho*enol*pyruvate. Specific activities measured without effector equaled between 53 and 464 milliunits/mg and were set 100% for each enzyme variant. In the enzyme assays with purified Aro3p or Aro4p, activities were measured with 160 ng and 250 ng of protein, respectively, 0.5 mM of each substrate, and the indicated amount of effector after a reaction time of 10 min. Protein contents of crude extracts were measured by using the Bradford assay (39).

Results

A Trp-Inhibited DAHP Synthase Regenerates Growth of a *gcn4* Mutant Yeast Strain in the Presence of Phe and Tyr. We wanted to know how a *gcn4 S. cerevisiae* mutant strain carrying the WT *ARO3* and *ARO4* genes responds to imbalanced amino acid supply. The
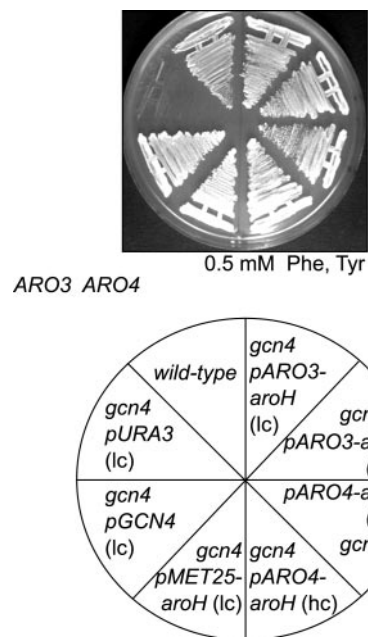

Fig. 1. Suppression of the growth defect of a *gcn4-103* mutation of *S. cerevisiae* on Phe- and Tyr-supplemented medium. Strain RH1408 (*gcn4-103*) was transformed with the low-copy *GCN4*-carrying vector (pME1083), with the low-copy *URA3*-carrying control vector (pRS416), with high-copy (hc) and low-copy (lc) number vectors carrying *E. coli aroH* fused to the *ARO3* promoter [pARO3-aroH (pME1874; pME1873)], by vectors carrying *E. coli aroH* fused to the *ARO4* promoter [pARO4-aroH (pME2391; pME2390)], and streaked out on MV medium. RH1408 was also transformed with *aroH* regulated by the *MET25* promoter (pME1877). The WT strain RH730 was used as positive control.

mutant strain RH1408 (*gcn4-104*) is unable to activate the general control of amino acid biosynthesis due to the lack of the transcriptional activator Gcn4p. As we observed, this yeast is unable to grow on MV medium that is supplemented with 5 mM Phe and 5 mM Tyr to inhibit both DAHPSs (Fig. 1). Addition of Trp regenerates growth of strain RH1408, suggesting that, under these conditions, this strain is starving for Trp. In the presence of only Phe and Tyr, strain RH1408 can be rescued by the expression of a plasmid carrying a copy of the *GCN4* gene. In response to Trp starvation, Gcn4p increases the expression of *ARO3* and *ARO4*, as well as that of the other genes of the general control network, and therefore regenerates growth (Fig. 1).

Similarly, the *E. coli aroH* gene encoding the Trp-regulated DAHPS was heterologously expressed in yeast to prevent Trp starvation. The *aroH*-encoding sequence was driven either by the *ARO3*, by the *ARO4*, or by the *MET25* promoter and expressed on low-copy or high-copy number plasmids in yeast strain RH1408. Expression of all *aroH* constructs rescued growth of strain RH1408 on media supplemented with 5 mM Phe and 5 mM Tyr. Therefore, Ec AroH suppresses this specific yeast *gcn4* phenotype by regenerating the metabolic flux into the shikimate pathway (Fig. 1). Thus, in the presence of a specific unbalanced amino acid diet and in the absence of a functional general control system as backup, the lack of a third DAHPS enzyme is of disadvantage for yeast.

Heterologously Expressed DAHP Synthase^Trp Is Sufficient for Aromatic Amino Acid Biosynthesis in Yeast. The result that a Trp-inhibited third DAHPS isoenzyme improves the physiological potential of yeast *gcn4*Δ mutant strain in the presence of an unbalanced aromatic amino acid diet prompted us to construct a yeast strain with a Trp-regulated enzyme as the only enzyme for the initial step of the shikimate biosynthesis. Strain RH2804 (*gcn4Δ::Kan^R*,
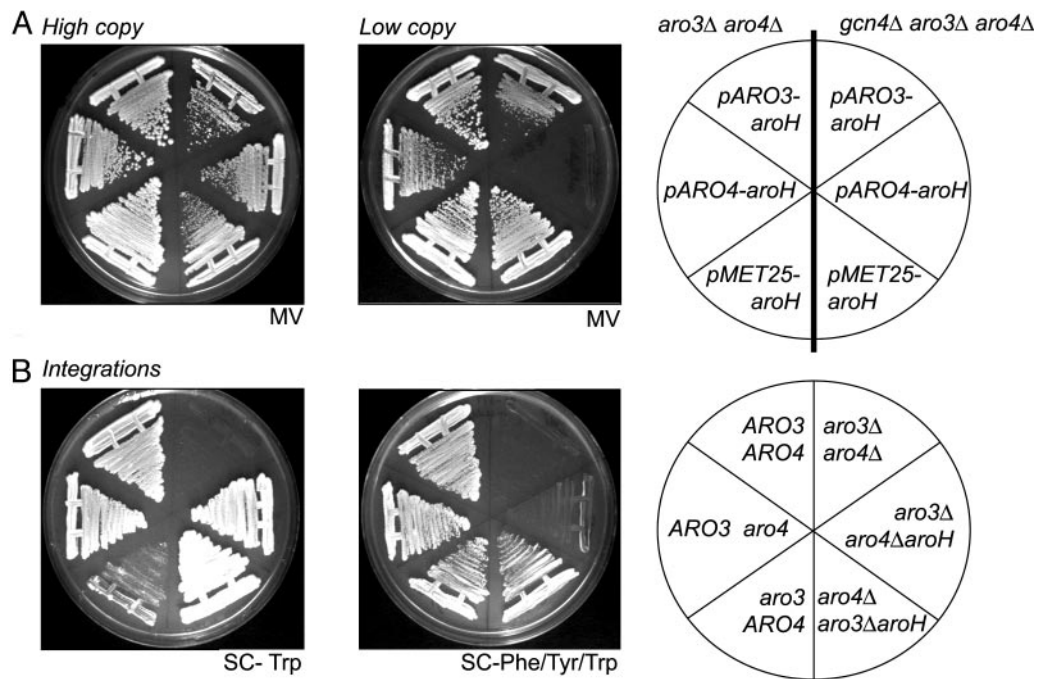
Fig. 2. Growth of different *S. cerevisiae* strains carrying various plasmids expressing the *E. coli aroH* gene or carrying *E. coli aroH* integrated into the genome. (*A*) High-copy and low-copy: Strains RH2424 (*aro3Δ*, *aro4Δ*), and RH2804 (*gcn4Δ*, *aro3Δ*, *aro4Δ*) lacking any DAHPS activity were transformed with high-copy and low-copy number vectors carrying *E. coli aroH* fused to the *ARO3* promoter [p*ARO3-aroH* (pME1874, pME1873)], *ARO4* promoter [p*ARO4-aroH* (pME2391, pME2390)], and *MET25* promoter [p*MET25-aroH* (pME1878, pME1877)], respectively. Vector-carrying yeast strains were streaked out on supplemented MV media lacking all three aromatic amino acids. (*B*) Integrations: *aroH* was integrated at the *ARO3* locus in strain RH2487 (*aro3ΔaroH*), or at the *ARO4* locus in strain RH2803 (*aro4ΔaroH*). As controls, a yeast WT strain (RH1416), an *ARO3*, *aro4-1* mutant strain (RH1313), an *aro3-2*, *ARO4* mutant strain (RH1316), and an *aro3Δ*, *aro4Δ* mutant strain (RH2424) were plated out. All strains were plated on SC medium lacking Trp or on SC medium lacking Phe, Tyr, and Trp, respectively.

aro3Δ::HIS3, *aro4Δ::LEU2*) has no DAHPS activity and no possibility to adapt to imbalances in the amino acid nutrition. Strains RH2804 and as control RH2424 (*aro3Δ::HIS3*, *aro4Δ::LEU2*) were transformed with high-copy and low-copy number vectors, respectively, expressing the *E. coli aroH* gene driven by the *ARO3* promoter (p*ARO3*) and the *ARO4* promoter (p*ARO4*), respectively. As control, the *E. coli aroH* gene was fused to the *MET25* promoter (p*MET25*) that is constitutively expressed on media lacking methionine. A growth test on supplemented MV media demonstrated that a yeast strain expressing high amounts of the Trp-inhibited DAHPS was able to survive on media without aromatic amino acids (Fig. 2*A*). Even without the transcriptional regulator Gcn4p, these artificial yeasts channeled enough flux into the shikimate pathway and were able to grow (Fig. 2*A*).

When expressed from p*ARO3* and p*ARO4* on low-copy vectors, *E. coli* AroH was able to maintain the flux into the shikimate pathway, when the yeast still possessed the master regulator of amino acid biosynthesis, Gcn4p. In a yeast *gcn4Δ* mutant strain, however, only the p*ARO3-aroH* fusion but not the p*ARO4-aroH* fusion resulted in normal growth (Fig. 2*A*). These data suggested that the basal promoter of *ARO3* was stronger than the basal promoter of *ARO4* and is counteracted by the fact that *Aro4* can be induced 2-fold more by the general control than *ARO3* (17, 18).

We constructed yeasts where single copies of p*ARO3-aroH* or p*ARO4-aroH* fusions were integrated into the yeast genome to verify these results. The *E. coli aroH* gene integrated at the *ARO4* locus and driven by the *ARO4* promoter (RH2803) resulted in a specific DAHPS activity that was hardly sufficient to rescue the *aro3Δ/aro4Δ* double deletion on SC media lacking all three aromatic amino acids (Fig. 2*B*). This yeast could grow only if Trp was the only amino acid missing. Integration of *aroH* at the *ARO3*

locus (RH2487), however, resulted in normal growth under all conditions (Fig. 2*B*). Similarly, a yeast strain with intact *ARO4* and deficient in *ARO3* (RH1316, *aro3-2*) was retarded in growth on SC media lacking Trp because the Tyr-inhibited Aro4p did not produce enough DAHP for sufficient Trp, although growth was like the WT control on SC medium without aromatic amino acids when Aro4p was not inhibited. A strain carrying an intact *ARO3*, but a defective *ARO4* (RH1313, *aro4-1*) showed normal growth under all conditions because there were obviously more Aro3p enzymes in the cells to channel sufficient substrate into the shikimate pathway (Fig. 2*B*). Thus, in the presence or absence of Gcn4p, a DAHPS-encoding gene was more efficiently transcribed when expressed from the yeast *ARO3* promoter than from the *ARO4* promoter. The yeast cell thus possesses more Aro3p than Aro4p enzymes as it was described for the DAHPS^Phe and DAHPS^Tyr in *E. coli* (12). All these data suggest that DAHPS^Trp was sufficient for production of aromatic amino acids in yeast, if the enzyme amounts were at least as high as the endogenous basal DAHPS^Phe pool or could be further supported by the general control.

The Yeast DAHPSs Are Regulated by Trp. The high degree of sequence identity and the structural similarities between Aro3p and Aro4p and between the yeast and the *E. coli* enzymes prompted us to ask whether the yeast Phe- and Tyr-regulated DAHPSs might be regulated to a less extent by the other aromatic amino acids. In enzyme activity assays performed with purified DAHPS, the Phe-inhibited DAHPS (Aro3p) exhibited a specific activity of 44 milliunits/mg in the presence of 0.1 mM Trp (97% of unregulated activity) but with 10.5 milliunits/mg only 22% of specific activity at 1.0 mM Trp (Fig. 3). The Trp inhibition was even more obvious for the Tyr-inhibited DAHPS (Aro4p). In the presence of 0.1 mM Trp, the DAPH^Tyr synthase
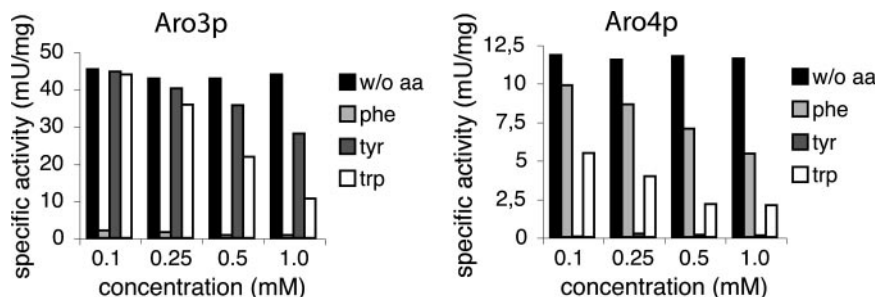
Helmstaedt *et al*.

Fig. 3. Inhibition of Aro3p and Aro4p by aromatic amino acids. The specific Phe-inhibited (Aro3p) and Tyr-inhibited (Aro4p) DAHPS activities of purified enzymes were measured without effector (w/o aa), and with the indicated amount of Phe, Tyr and Trp, respectively.

showed a specific activity of 5.5 milliunits/mg (46%), whereas it displayed a specific activity of 2 milliunits/mg (18%) at 1.0 mM Trp (Fig. 3). At 0.1 mM of Trp the Aro4p enzyme was reduced to half activity, whereas the isoenzyme Aro3p displayed nearly full activity. Similarly, Aro4p was inhibited by the third aromatic amino acid (Phe) more strongly than Aro3p by Tyr at the lowest concentration tested. These results indicate that, in addition to their known major control by Phe (Aro3p) and Tyr (Aro4p), respectively, both enzymes can be down-regulated when high amounts of Trp or Tyr/Phe are present in the diet. Aro4p is more sensible and responds to the effectors in lower concentrations than Aro3p. Thus, the weak triple feedback-regulation of the two yeast isoenzymes might counteract the lack of the third enzyme as long as the Gcn4p-dependent general control is functional as a further backup system.

A Single S195A Substitution Results in an Aro4p Yeast DAHPS Inhibited by Trp. We aimed to dissect the enzyme regulation to verify the triple control of the two yeast DAHPSs. Recent experiments demonstrated that the yeast Tyr-inhibited DAHPS could be turned into a Phe-regulated enzyme by a single amino acid substitution and vice versa (29). Therefore, we performed *in vitro* evolution of Aro4p to change regulation of the Tyr-inhibited DAHPS toward a stronger inhibition by Trp. We applied an amino acid sequence alignment of *E. coli* AroH (Trp-inhibited) and yeast Aro4p (Tyr-inhibited), a comparison of the 3-dimensional structures of *E. coli* AroG (26, 40) and Aro4p (28, 29), as well as results from genetic screens (29). By site-directed mutagenesis, the codons of glycine 193, serine 195, proline 165, alanine 234, and glutamine 302 in the yeast *ARO4*-encoded DAHP^{Tyr} synthase, respectively, were exchanged, and the resulting enzyme variants were examined regarding Tyr and Trp regulation. These residues are mainly located at the putative effector-binding cavity of Aro4p (Fig. 5, which is published as supporting information on the PNAS web site) (29). The structure of this cavity is very similar to the structure published for the Phe-inhibited *aroG* from *E. coli*. The hydrophobic and hydrophilic residues G193 and S195, respectively, are located in the connector region between the half-sites of the α/β barrel and were substituted by a polar lysine (GGT→AAG) and a hydrophobic alanine (TCT→GCT) residue, respectively. Proline 165 in helix $\alpha3$ is located in the bottom of the cavity and was substituted by a glycine (CCT→GGT), which is present in the Trp-regulated AroH at this position. The hydrophobic residue alanine 234 in the internal β strand $\beta6b$, which forms the side of the cavity, was exchanged against a polar threonine residue (GCT→ACT). In addition, glutamine 302 was substituted by arginine (CAA→CGA). This amino acid residue is located in helix $\alpha7$, which is remote from the putative effector binding cavity.

Specific enzyme activities from crude extract measurements with effector concentrations of 0.5 mM revealed that the ex-

change of an A234T did not result in significant alteration of Aro4p regulation. The enzyme was still completely inhibited by Tyr, and activity was reduced to half by Trp (data not shown). Therefore, this amino acid residue seemed not to be involved in the regulation of the yeast DAHP^{Tyr}. Exchange of G193K in Aro4p abolished most of DAHPS activity and thus obviously disturbed the overall structure of Aro4p (data not shown).

The exchange of P165G in the yeast Tyr-inhibited DAHPS resulted in an enzyme for which regulation was shifted more toward Trp inhibition compared with Aro4p wild type, but which maintained a small degree of Tyr inhibition (Fig. 4*A*). In fact, the regulation pattern by Tyr and Trp was reversed in this Aro4p variant. An additional substitution of Q302R in the P165G variant, however, reversed the effect on Tyr regulation, resulting in an enzyme variant that was still Tyr inhibited and equally strongly inhibited by Trp (Fig. 4*A*). Therefore, this enzyme constituted a hybrid of a DAHPS^{Tyr} and DAHPS^{Trp}. In contrast, the exchange of S195A in the yeast Tyr-inhibited DAHPS resulted in an enzyme that no longer was inhibited by its main effector Tyr, but was inhibited by Trp as single effector (Fig. 4*A*). By removing the polar hydroxyl group of serine at the side of the cavity, the yeast DAHPS^{Tyr} became DAHPS^{Trp} although there is also a serine at the respective position in the Trp-regulated AroH. Thus, a single amino acid substitution in the putative effector binding site is sufficient to turn Aro4p into a Trp-inhibited DAHPS.

Effector titration of crude extracts of RH2424 expressing the different Aro4p alleles was performed to measure the sensitivity to Tyr and Trp more precisely. Wild-type Aro4p was inhibited to a maximum of below 9% of original activity above 100 μM Tyr (Fig. 4*B*). A sharp decrease of activity was observed at low effector concentration, so that 50% inhibition was achieved at 10 μM Tyr. The Aro4p P165G and Aro4p P165G/Q203R enzymes were inhibited to a similar maximum of 15% and 7% activity, respectively, but showed only partial inhibition at lower concentrations. At the physiological concentration of 0.5 mM (41), Tyr caused only partial inhibition of the P165G DAHPS (33%) compared with nearly complete inhibition of the Aro4p wild type and P165G/Q302R variant. In comparison, WT AroH and the AroH-like Aro4p S195A variant were not inhibited by Tyr, but were slightly activated instead.

When Trp was used as an effector, maximum inhibition was nearly the same for the AroH wild type, the Aro4p wild type, and the AroH-like S195A variant (21–32% activity), whereas inhibition was nearly complete for Aro4p P165G/Q302R and P165G above 500 μM Trp (Fig. 4*C*). At the physiological Trp concentration in yeast cells of $\approx$20 μM (41), AroH was slightly activated, whereas Aro4p and Aro4p S195A were not affected. For none of the enzymes tested, a rapid and complete inhibition like the Tyr inhibition of Aro4p was observed with Trp, but all enzymes showed a moderate decrease in activity, with half-maximum activity between 120 and 270 μM Trp. These experiments
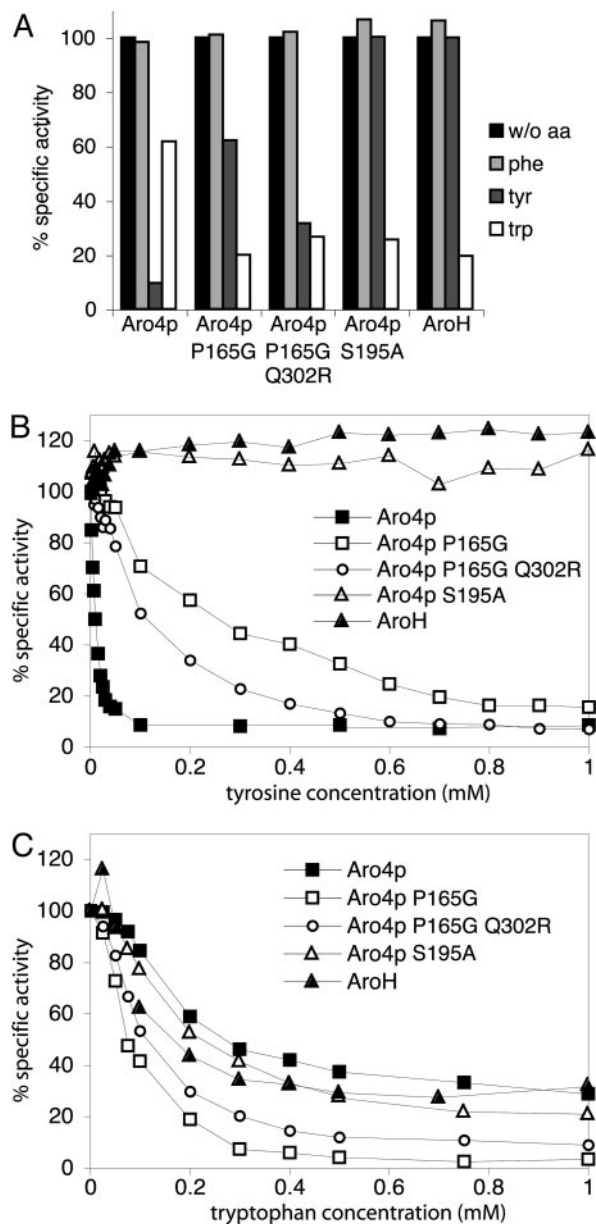
Fig. 4. Inhibition of WT and mutant Aro4p and AroH activity by Tyr and Trp. DAHPS-encoding genes were expressed from the *MET25* promoter on high-copy vectors in strain RH2424 (*aro3Δ*, *aro4Δ*). The vectors carried the WT *ARO4* gene (pME2385), the alleles encoding Aro4p P165G (pME2395), Aro4p P165G Q302R (pME2878), Aro4p S195A (pME2396), and the WT *aroH* gene (pME1878). (*A*) Enzyme assays were performed with 0.5 mM each substrate and effector. (*B* and *C*) Tyr and Trp titration reactions were carried out by using 0.4 mM erythrose-4-phosphate, 0.1 mM phospho*enol*pyruvate, and the indicated effector concentrations.

strongly suggest that Aro4p exhibits a rudimental Trp inhibition that strongly resembles the AroH WT inhibition and can be amplified by specific amino acid substitutions that, however, in part have to be paid for by reduced Tyr inhibition.

Discussion

Evolution of DAHPS enzymes in prokaryotes has been described in detail (42–47). The evolution of prokaryotic DAHPSs presumably started at the level of an allosterically insensitive DAHPS^0 and a tyr-sensitive DAHPS^{Tyr}, and evolutionary processes changed the DAHPS^0 enzyme toward a Trp-inhibited

DAHPS^{Trp} enzyme. In secondary evolutionary processes, some prokaryotes presumably have lost the DAHPS^{Tyr}, carrying only one, highly efficient DAHPS^{Trp} enzyme. Among those microbial representatives with two enzymes, a wide variability of additional chorismate sensitivity of DAHPS^{Trp} was found (42, 45). In the course of evolution, a loss of the chorismate sensitivity of the DAHPS^{Trp} seemed to occur at the same time when microorganisms acquired a third Phe-regulated enzyme activity (DAHPS^{Phe}), presumably by a gene duplication event of a DAHPS^{Trp}-encoding gene (47, 48). Only those prokaryotes carried three DAHPSs, which had a DAHPS^{Trp} enzyme that was inhibited only by Trp but not by chorismate, whereas the allosterical chorismate-dependent regulations of DAHPS^{Phe} or DAHPS^{Tyr} were variable (45).

It is unknown whether *S. cerevisiae* has lost a third DAHPS^{Trp} activity or never acquired one, but the known yeast genome definitely contains only two DAHPS loci (49). We have recently shown that a single amino acid substitution (S219G) was sufficient to switch the *ARO3*-encoded DAHPS^{Phe} into an Aro4p-like DAHPS^{Tyr}, and vice versa the exchange of G226S of the *ARO4*-encoded DAHPS^{Tyr} resulted in an Aro3p-like DAHPS^{Phe} (29). We now extended this study and showed here that the single amino acid substitution S195A turns Aro4p into a DAHPS^{Trp} enzyme very similar to the homologous AroH from *E. coli*. The most important residues controlling binding of Tyr or Trp proved to be those at the bottom and the side of the putative effector binding site. Obviously, the polar S195 is important for contact to Tyr, whereas a more hydrophobic environment or a sterical adjustment due to the loss of proline in helix $\alpha 3$ favors Trp binding. The fact that *E. coli* AroH also possessed a serine at the corresponding position 179 and that substitutions to amino acid residues not present at respective positions in the bacterial enzyme also produced Trp inhibition showed that Trp inhibition in Aro4p S195A was achieved by a different intramolecular signal transduction pathway between allosteric and catalytic site, which depends on further differences in the amino acid sequence. These results suggest that evolution of yeast DAHPS might be different from that described in prokaryotes.

Because we found weak Trp regulation for both fungal isoenzymes, fungal evolution might have started with a Trp-inhibited DAHPS precursor enzyme. Gene duplication and the transcriptional regulation of the general control network of amino acid biosynthesis finally resulted in two Gcn4p-regulated genes encoding for highly effective and closely related DAHPS activities, with additional regulation by Tyr and Phe, DAHPS^{Tyr} (Aro4p) and DAHPS^{Phe} (Aro3p), respectively. Tryptophan regulation in yeast was shifted downstream in the pathway to the first branch point because chorismate mutase evolved as an important key regulator that was activated by Trp and inhibited by Tyr (50). At this strongly regulated branch point, anthranilate synthase encoded by *TRP2* and *TRP3*, which is transcriptionally regulated by the general control (41), competes with chorismate mutase for chorismate. The anthranilate synthase complex has a lower K_m for chorismate than chorismate mutase. When *TRP2* and *TRP3* are transcriptionally induced, the gene products direct the metabolic flux toward Trp production, which ultimately results in their feedback-inhibition by Trp (41, 51). At intracellular Trp levels of 20 μM, at which anthranilate synthase is not yet strongly inhibited by Trp (K_i 56 μM), this amino acid already activates chorismate mutase (K_a, 1.5 μM) leading to increased Phe and Tyr synthesis. When Tyr and Phe are being synthesized, the DAHPSs at the beginning of the pathway (K_i, 10 μM and 1 μM) as well as chorismate mutase (K_i, 50 μM) are inhibited, making an additional Trp control dispensable (19, 20, 41, 52).

In *E. coli*, AroH as third DAHPS together with the residual activity of the other DAHPS, ensures a small but steady flux into the shikimate pathway because it has 40% constitutive activity. Here, we demonstrated that the expression of a Trp-inhibited

DAHPS from *E. coli* had the same effect on Trp production as the general control exerted by GCN4p. Thus, the general control evolved for this pathway in yeast as protection from shutting down the shikimate pathway and preventing Trp starvation through transcriptional GCN4p dependent up-regulation of transcription of *ARO3/ARO4* and *TRP* genes (41) when Aro3p and Aro4p are inhibited. A single Trp-inhibited DAHPS in sufficient amounts or even less DAHPS^{Trp} combined with a functional general control can save yeast from amino acid starvation by unbalanced diets. Thus, the end product Trp can be used as the only feedback regulator of this biosynthetic pathway in an artificial yeast with a DAHPS^{Trp} as first enzyme. The observed mixed inhibition by Tyr and Trp most pronounced in Aro4p reflects a complex evolution and allows yeast cells a subtle fine tuning of the branched pathway even under conditions of very unbalanced amino acid diet, including high Trp concentrations.

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