Coevolution of transcriptional and allosteric regulation at the chorismate metabolic branch point of *Saccharomyces cerevisiae*

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Control of transcription and enzyme activities are two interwoven regulatory systems essential for the function of a metabolic node. *Saccharomyces cerevisiae* strains differing in enzyme activities at the chorismate branch point of aromatic amino acid biosynthesis were constructed by recombinant DNA technology. Expression of an allosterically unregulated, constitutively activated chorismate mutase encoded by the *ARO7*^{T226I} (*ARO7*^{c}) allele depleted the chorismate pool. The resulting tryptophan limitation caused growth defects, which could be counteracted only by transcriptional induction of *TRP2* encoding the competing enzyme anthranilate synthase. *ARO7* expression is not transcriptionally regulated by amino acids. Transcriptional activation of the *ARO7*^{c} allele led to stronger growth retardation upon tryptophan limitation. The same effect was achieved by removing the competing enzyme anthranilate synthase, which is encoded by the *TRP2* gene, from the transcriptional control. The allelic situation of *ARO7*^{c} being under general control instead of *TRP2* resulted in severe growth defects when cells were starved for tryptophan. In conclusion, the specific regulatory pattern acting on enzymatic activities at the first metabolic node of aromatic amino acid biosynthesis is necessary to maintain proper flux distribution. Therefore, the evolution of the sophisticated allosteric regulation of yeast chorismate mutase requires as prerequisite (*i*) that the encoding *ARO7* gene is not transcriptionally regulated, whereas (*ii*) the transcription of the competing feedback-regulated anthranilate synthase-encoding gene is controlled by availability of amino acids.

Supply of precursor metabolites and energy for anabolic pathways to synthesize cellular components is necessary for growth and maintenance of a living cell. Metabolic pathways are numerous and extremely plastic, and different modes of regulation are possible to channel intermediates from the input reactions to the formation of end products. Branched reaction cascades are of special interest, as most metabolic networks are constituted by such pathways. To ensure proper distribution of intermediates, specific regulatory systems have evolved to trigger the enzymatic activities at a metabolic node. Two main mechanisms are possible to regulate catalytic turnover at a given enzyme. Either the amount of protein is altered by means of gene expression, protein synthesis, or protein degradation, or enzymatic activity itself is varied by the action of effectors, modifications, or conformational changes.

In the baker's yeast, *Saccharomyces cerevisiae*, specific mechanisms contribute to regulation of catalytic turnover, with transcriptional regulation being the most important feature to control protein levels. Amino acid biosynthesis is the target of a key regulatory network acting upon amino acid starvation conditions and imbalances (reviewed in ref. 1). Starvation for almost any of the 20 amino acids found in proteins can initiate the network response. The final effector of this "general control of amino acid biosynthesis" system is the transcription factor Gcn4p, which binds as a homodimer to conserved sequence elements within promoters of specified target genes (2). Biosynthesis of aromatic amino acids in *S. cerevisiae* is a model pathway for a strictly regulated, branched reaction cascade (reviewed in ref. 3). From the last common intermediate, chorismic acid, two main branches emerge to initiate the tyrosine/phenylalanine- and tryptophan-specific routes, respectively. The enzymatic activities constituting this metabolic node, chorismate mutase (CM; EC 5.4.99.5) and anthranilate synthase (AAS; EC 4.1.3.27), are regulated in their activities by different means (Fig. 1). While the amount of CM molecules is not varied by the general control transcriptional system, the expression of both genes encoding the competing AAS complex is transcriptionally regulated by the general control activator Gcn4p. In addition, both enzymatic activities are targets of allosteric effectors, namely the pathway end products tyrosine and tryptophan, that modulate catalytic turnover rates.

CMs are unique enzymatic activities that are found only in microorganisms and plants but never in animals (4). They accelerate the one-step conversion of chorismate to prephenate, a reaction formally resembling a Claisen rearrangement, to initiate the tyrosine/phenylalanine-specific branch at the first metabolic branch point of aromatic amino acid biosynthesis (5, 6). Whereas bacterial CM activities are often found to reside on a distinct domain within a bifunctional enzyme, all eukaryotic enzymes characterized to date are monofunctional. One prototype of bacterial CMs is represented by the CM domain (EcCM) of the *Escherichia coli* P-protein, which contains additional prephenate dehydratase activity (7). Both activities are subject to feedback inhibition by the end product phenylalanine, and catalytic turnover rates of the CM activity are characterized by Michaelis–Menten-like kinetics. As an exemplar of eukaryotic CMs, the enzyme of *S. cerevisiae* (ScCM), the *ARO7* gene product, has been studied extensively (8–10). This CM activity is allosterically regulated in its activity. Substrate saturation kinetics of the unliganded enzyme display cooperativity, with the substrate chorismate acting as homotropic, positive effector. In addition, two end products of the pathway act as heterotropic effectors on ScCM. Tyrosine inhibits activity by decreasing the affinity of the enzyme toward its substrate, whereas tryptophan, the end product of the opposite branch, acts as a strong activator of catalytic turnover, resulting in Michaelis–Menten kinetics without cooperativity. Expression of the *ARO7*^{T226I} mutant allele, here referred to as *ARO7*^{c}, conserves the latter situation and leads to an unregulated, noncooperative yeast CM that is locked in its allosteric R state (11). In contrast to most amino acid biosynthetic genes, the CM-encoding *ARO7* gene of *S. cerevisiae* is not transcriptionally regulated by the general control activator Gcn4p (12). The *TRP2* gene of *S. cerevisiae* encodes the AAS activity, which is competing with the CM for the common substrate chorismate and channels it toward the tryptophan-specific

Abbreviations: CM, chorismate mutase; AAS, anthranilate synthase; 5-MT, 5-methyltryptophan; U, unit(s).

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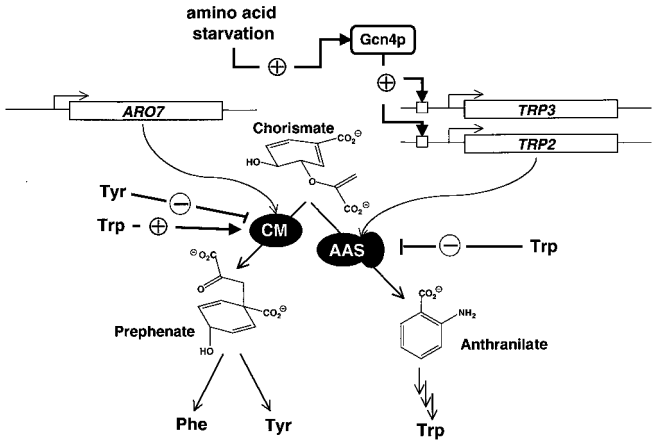

Fig. 1. Interplay between transcriptional and enzyme regulation at the first branch point of aromatic amino acid biosynthesis in yeast. The metabolic node emerging from chorismate is schematically shown with the enzymatic activities chorismate mutase (CM) and anthranilate synthase (AAS) as black ovals. The encoding genomic loci are drawn as bars with transcriptional start sites indicated by broken arrows. Positive feedbacks and inductions are indicated by ⊕, feedback inhibition by ⊖.

branch. *In vivo*, an additional glutamine amidotransferase activity, encoded by part of the *TRP3* gene, is necessary to fulfil catalytic activity, and both genes coding for the AAS heterodimer are regulated by Gcn4p (3, 13). Furthermore, the AAS activity is regulated in this yeast via feedback inhibition by the end product, tryptophan. A *TRP2*^{S76L} (*TRP2*^{fbr}) mutant allele is impaired in feedback inhibition by tryptophan and confers resistance to the structural analogue 5-methyltryptophan (5-MT) (14).

In summary, the two enzymatic activities constituting the first metabolic branch point of aromatic amino acid biosynthesis in *S. cerevisiae* are regulated by different means. Given the complex interwoven regulatory pattern modulating both enzymatic activities, we were interested in the impact of an unregulated, noncooperative CM enzyme upon starvation conditions. Therefore, the *ARO7* gene was replaced by the *ARO7*^{c} allele and growth was monitored under conditions of tryptophan starvation. Additionally, the unregulated CM was expressed in a Gcn4p-dependent manner to investigate the influence of the general control network on flux partitioning at the metabolic node. To investigate the necessity of transcriptional regulation of AAS expression, the *TRP2* gene was removed from the general control system. Additionally, the impact of the feedback-unresponsive *TRP2*^{fbr} allele was monitored. We found that the conserved regulatory pattern controlling CM activity is strictly necessary when AAS activity is limited and that the interplay of allosteric and transcriptional regulation is a prerequisite for proper chorismate distribution to both branches at the first metabolic node of aromatic amino acid biosynthesis in baker's yeast.

Materials and Methods

Materials. Chorismic acid as barium salt and DL-5-MT were purchased from Sigma. 5-Fluoroorotic acid (5-FOA) was obtained from Toronto Research Chemicals (Toronto). *Platinum Pfx* DNA polymerase from Life Technologies (Karlsruhe, Germany) was used for PCRs. All other chemicals were supplied by Fluka or Sigma–Aldrich.

Yeast Strains and Growth Conditions. All yeast strains in this study are isogenic to the S288C genetic background and are listed in Table 1. The *aro7*::*hisG* and *trp2*::*hisG* deletion mutations were introduced into the progenitor strain RH1408 [*gcn4-103*; *ura3-52*] (15) by using deletion plasmids pME1901 and pME1902, respectively, followed by counterselection on 5-FOA-supplemented medium (16). All mutant alleles of *ARO7* and *TRP2* were reintroduced at the homologous loci in single copy as verified by Southern hybridization analyses.

Complex medium for growth of *S. cerevisiae* was YEPD (1% yeast extract/2% peptone/2% glucose). Minimal MV medium contained 0.14% yeast nitrogen base (without amino acids and without ammonium sulfate), 0.5% ammonium sulfate, and 2% glucose, and was buffered to acidic pH of 4.0 with succinic acid and KOH as described previously (17). Because cells harboring no functional Gcn4p starve for arginine, this amino acid was supplemented in all minimal growth media. Supplements were added according to Guthrie and Fink (18). Growth rates were determined turbidimetrically at 595 nm, and the specific growth rate is given as μ defined by $(\ln x_2 - \ln x_1)/(t_2 - t_1)$, where x stands for the optical density at the corresponding time t.

Plasmids. Plasmid DNAs were generally propagated in *E. coli* strain DH5α (19). Plasmids used in this study are listed in Table 2. Deletion cassettes for *ARO7* and *TRP2* were created by replacement of coding sequences by the *hisG*::*URA3*::*hisG* marker (20). Plasmid pME1905, carrying the *ARO7* gene under the control of the *TRP2* promoter, was constructed by separate

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
RH1408	*MAT***a**, *ura3-52, gcn4-103*	Ref. 15
RH2457	*MAT***a**, *ura3-52, gcn4-103, aro7*::*hisG–URA3–hisG*	This study
RH2458	*MAT***a**, *ura3-52, gcn4-103, aro7*::*hisG*	This study
RH2459	*MAT***a**, *ura3-52, gcn4-103, trp2*::*hisG–URA3–hisG*	This study
RH2460	*MAT***a**, *ura3-52, gcn4-103, trp2*::*hisG*	This study
RH2461	*MAT***a**, *ura3-52, gcn4-103, aro7*::*hisG, trp2*::*hisG–URA3–hisG*	This study
RH2462	*MAT***a**, *ura3-52, gcn4-103, aro7*::*hisG, trp2*::*hisG*	This study
RH2463	*MAT***a**, *ura3-52, gcn4-103, ARO7*^{c}	This study
RH2465	*MAT***a**, *ura3-52, gcn4-103, ARO7*^{c}, *TRP2*^{fbr}	This study
RH2466	*MAT***a**, *ura3-52, gcn4-103,* ^{p}*TRP2*::*ARO7*	This study
RH2467	*MAT***a**, *ura3-52, gcn4-103,* ^{p}*ARO7*::*TRP2*	This study
RH2468	*MAT***a**, *ura3-52, gcn4-103,* ^{p}*TRP2*::*ARO7,* ^{p}*ARO7*::*TRP2*	This study
RH2469	*MAT***a**, *ura3-52, gcn4-103,* ^{p}*TRP2*::*ARO7*^{c}	This study
RH2470	*MAT***a**, *ura3-52, gcn4-103, ARO7*^{c}, ^{p}*ARO7*::*TRP2*	This study
RH2471	*MAT***a**, *ura3-52, gcn4-103,* ^{p}*TRP2*::*ARO7*^{c}, *TRP2*^{fbr}	This study
RH2472	*MAT***a**, *ura3-52, gcn4-103, ARO7*^{c}, ^{p}*ARO7*::*TRP2*^{fbr}	This study
RH2473	*MAT***a**, *ura3-52, gcn4-103,* ^{p}*TRP2*::*ARO7*^{c}, ^{p}*ARO7*::*TRP2*	This study
RH2474	*MAT***a**, *ura3-52, gcn4-103,* ^{p}*TRP2*::*ARO7*^{c}, ^{p}*ARO7*::*TRP2*^{fbr}	This study

Table 2. Plasmids used in this study

Plasmid	Description	Source
pME1901	*aro7*::*hisG–URA3–hisG* cassette for deletion of *ARO7* ORF	This study
pME1902	*trp2*::*hisG–URA3–hisG* cassette for deletion of *TRP2* ORF	This study
pME1187	2-kb *Eco*RI fragment containing *ARO7* in pGEM7(+)	This study
pME606	2-kb *Eco*RI fragment containing *ARO7*^{c} in pJDB207	Ref. 8
pME1903	2.3-kb *Xba*I/*Bam*HI fragment containing *TRP2* in pGEM7(+)	This study
pME1904	2.3-kb *Xba*I/*Bam*HI fragment containing *TRP2*^{fbr} in pGEM7(+)	This study
pME1905	3-kb *Aat*II/*Eco*RI fragment containing 5′-*ARO7*::^{p}*TRP2*::*ARO7* replacement cassette in pGEM7(+)	This study
pME1906	3-kb *Aat*II/*Eco*RI fragment containing 5′-*ARO7*::^{p}*TRP2*::*ARO7*^{c} replacement cassette in pUC19	This study
pME1907	3-kb *Aat*II/*Bam*HI fragment containing 5′-*TRP2*::^{p}*ARO7*::*TRP2* replacement cassette in pGEM7(+)	This study
pME1908	3-kb *Aat*II/*Bam*HI fragment containing 5′-*TRP2*::^{p}*ARO7*::*TRP2*^{fbr} replacement cassette in pUC19	This study
p164	2.8-kb *Sal*I/*Eco*RI fragment containing *GCN4* in YCp50	Ref. 22
p238	2.8-kb *Sal*I/*Eco*RI fragment containing *GCN4* with all four upstream ORFs mutated in YCp50	Ref. 22
pME1909	470-bp *Ssp*I/*Eco*RV fragment of *ARO7* ORF and 465-bp *Eco*72I/*Eco*RV fragment of *TRP2* ORF in pBluescript II KS	This study

amplification of the *TRP2* promoter region from pME1903 and the *ARO7* coding sequence from pME1187 by means of PCR using oligonucleotide combinations OLSK24 (5′-GGCAAAAAATGGATTTCACAAAACCAGAAAC-3′)/OLSK15 (5′-TCCTATAGAATTTATGAGCCATCG-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′)/OLSK25 (5′-GTGAAATCCATTTTTTGCCTTTTTTCCAATC-3′), respectively, and a second PCR using both products as template in combination with T7/OLSK15. The amplified DNA was ligated as *Xba*I fragment to an *Aat*II/*Xba*I DNA fragment comprising part of the 5′ region of *ARO7* (position −1482 to −652 relative to the translational start codon) and cloned in the plasmid pGEM7(+) (Promega). Plasmid pME1907 was constructed as described for pME1905 by using oligonucleotide combinations T7/OLSK27 (5′-GCGGTCATATCTTATACCAATTTTATGCAG-3′) and OLSK26 (5′-GGTATAAGATATGACCGCTTCCATCAAAATTC-3′)/OLSK17 (5′-ACAGAGAATGCCCTTTTTAAGC-3′). The resulting ^{p}*ARO7*::*trp2 Eco*RI/*Eco*72I fragment carrying a chimeric construct with the *ARO7* promoter, and part of the *TRP* ORF was ligated in pME1903 together with an *Aat*II/*Eco*RI fragment comprising part of the *TRP2* 5′ region (position −1505 to −735). Plasmid pME1906 with the *ARO7*^{c} allele driven by the *TRP2* promoter (^{p}*TRP2*::*ARO7*^{c}) was constructed by combination of an *Aat*II/*Hin*dIII fragment from pME1905 and a *Hin*dIII/*Eco*RI fragment from pME606. For construction of pME1908 with the *TRP2*^{fbr} allele under the control of the *ARO7* (^{p}*ARO7*::*TRP2*^{fbr}) promoter, an *Aat*II/*Eco*72I fragment from pME1907 was fused to an *Eco*72I/*Bam*HI fragment of pME1904. Gcn4p was expressed either at low levels from plasmid p164, which carries the wild-type *GCN4* gene on the low-copy vector YCp50 (21), or at high levels from p238, which carries a mutant allele of *GCN4* in YCp50 with mutated upstream ORFs (22). YCp50 was used as empty vector control for strains expressing no functional Gcn4p. Plasmid pME1909 containing a 470-bp *Ssp*I/*Eco*RV fragment of *ARO7* as well as a 465-bp *Eco*72I/*Eco*RV fragment of *TRP2* in pBluescript II KS was used for probe preparation in Southern analyses and Northern experiments.

Transformation Procedures. Transformation of *E. coli* was performed as described by Inoue *et al.* (23), and *S. cerevisiae* strains were transformed by following a modified protocol of Elble (24).

Nucleic Acid Preparation and Analyses. For isolation of plasmid DNA from bacterial strains the plasmid purification system from Qiagen (Hilden, Germany) was used. Genomic DNA from yeast was isolated according to ref. 25 and analyzed by Southern hybridization (26) or diagnostic PCR (27). Total RNAs from *S. cerevisiae* cultures were prepared according to Cross and Tinkelenberg (28), and transcript levels were quantified by Northern hybridization (29) using a Bio-Imaging Analyzer from Fuji Photo Film (Tokyo). Sequencing reactions were carried out by using a BigDye sequencing kit (30) and analyzed on an ABI Prism 310 Genetic Analyzer (PE Biosystems, Foster City, CA).

Enzyme Assays. Enzymatic assays were performed at 37°C with Triton X-100-treated cell suspensions prepared by the method of Miozzari *et al.* (17). Glutamine-dependent AAS activities were determined according to Egan and Gibson (31) at 0.5 mM substrate concentration. CM activity was measured spectrophotometrically as described (8) with the modification that permeabilized cells were spun down and resuspended in cold buffer containing 125 mM potassium phosphate (pH 7.6), 25 mM DL-dithiothreitol, 2.5 mM EDTA, and 0.125 mM phenylmethylsulfonyl fluoride before chorismate was added to 1 mM final concentration. The concentration of phenylpyruvate was determined after cells had been removed from the assay mixture by brief centrifugation. Specific activities are quantified in units (U), 1 U equaling 1 nmol of product formed in 1 min of turnover by 1 mg of total protein. Protein content of the detergent-treated cell suspensions was measured by the method of Herbert *et al.* (32), using the Bradford assay (33).

Results

An Unregulated CM Allele and a *gcn4* Deletion Are Synthetically Lethal in Yeast After Tryptophan Starvation. The yeast *ARO7*^{c} allele encoding an unregulated CM displays high catalytic activity that preferentially channels chorismate toward the tyrosine/phenylalanine branch when expressed in high amounts (11). To modulate distribution of chorismate, we replaced the wild-type *ARO7* gene in strain RH1408 [*gcn4-103*, *ura3-52*] at its original locus with the *ARO7*^{c} allele, resulting in strain RH2463. Because of the *gcn4* mutation these strains are unable to adapt their transcription to the availability of amino acids. Enzymatic activities of the branch point enzymes, CM and AAS, altered the flux at the metabolic node (Fig. 2). In the progenitor strain RH1408 used as wild-type control, a specific CM activity of 1.0 U was determined, and specific AAS activity was at a basal level of 1.3 U. RH2463, which expresses *ARO7*^{c}, displayed a specific CM activity of 12.2 U, whereas AAS activity remained at 1.4 U. Determination of growth rate constants μ revealed no nutritional requirements of the two strains when cultured in minimal medium (Table 3). However, when both strains were starved for tryptophan by the action of the structural analogue 5-MT, significant differences in viability were present. 5-MT acts as a false feedback inhibitor on AAS and therefore decreases the input into the tryptophan-specific branch of the pathway. In

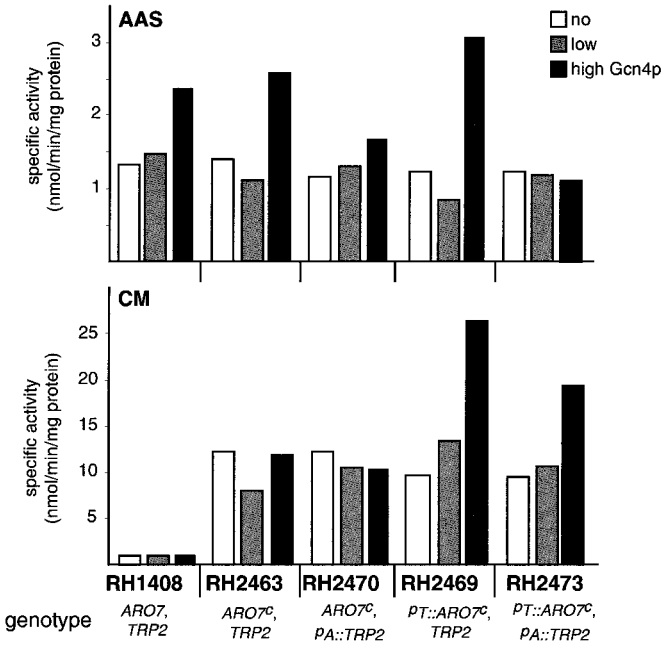

Fig. 2. Specific AAS (*Upper*) and CM (*Lower*) activities of *S. cerevisiae* strains with altered regulatory properties of the branch point enzymes. Activities were determined from cells cultivated in minimal medium expressing either no functional Gcn4p (−), low levels (+), or high levels (+++) of Gcn4p. The genotypes of all strains are indicated with ^{p}A:: and ^{p}T:: representing *ARO7* and *TRP2* promoter fusions, respectively. All values are the means of two independent measurements with a standard deviation not exceeding 20%.

minimal medium supplemented with 0.1 mM 5-MT, wild-type strain RH1408 grew at a reduced rate of 0.18 h^{-1}. Strain RH2463 expressing the unregulated, constitutively active, CM enzyme was not viable under these conditions.

To investigate the impact of transcriptional regulation by amino acids that acts in the wild-type situation on *TRP2* expression but not on *ARO7*, both strains were transformed with plasmids expressing the activator protein Gcn4p at wild-type levels and in high amounts, respectively. Specific CM activities of both strains were unaffected by Gcn4p, whereas AAS activities were induced to a similar degree by high levels of the transcriptional activator to 2.4 U (RH1408) and 2.6 U (RH2463), respectively (Fig. 2). Growth rates determined in minimal medium displayed no differences between the wild-type control RH1408 and RH2463 when Gcn4p was expressed (Table 3). When cultured in the presence of 5-MT, RH1408 grew at rates comparable to rates determined in unsupplemented medium. Strain RH2463, however, was viable in the presence of 5-MT when Gcn4p was expressed at low levels but displayed a growth reduction of 53% (μ of 0.094 vs. 0.20 h^{-1}). This reduction was diminished by high levels of Gcn4p, which resulted in a growth rate of 0.13 h^{-1}, corresponding to a growth reduction of 22% in comparison to the wild-type *ARO7* strain RH1408. Expression of the allosterically unregulated, feedback resistant, $TRP2^{fbr}$ allele counteracted the growth defects caused by the unregulated CM activity and resulted in a strain (RH2465) that was fully viable in the presence of 5-MT without the transcriptional activator Gcn4p (not shown).

These results suggest that depletion of the intracellular chorismate pool caused by an unregulated constitutively active CM is lethal when the competing enzyme AAS is reduced in its enzymatic activity. The general control "backup" system is able to counteract this starvation situation by increasing the amount of AAS molecules by transcriptional derepression, suggesting that the enzyme and transcriptional regulation represent strongly interwoven systems.

Removing *TRP2* from the General Control Transcription System in the Presence of $ARO7^{c}$ Results in Strong Growth Reduction Under Tryptophan Starvation Conditions. To monitor the impact of the transcriptional regulation-mediated changes in AAS activities, strains were constructed that carry the encoding *TRP2* gene under the control of a Gcn4p-independent promoter. For that purpose, the *ARO7* promoter seemed most appropriate. Strain RH2470 expresses the unregulated CM activity encoded by the $ARO7^{c}$ allele in combination with an *ARO7* promoter::*TRP2* fusion integrated at the *trp2*::*hisG* locus of strain RH2462 (Table 1). Again, this strain was transformed with plasmids to express functional Gcn4p at low and high levels. Steady-state transcript levels in RH2470 showed a constant ratio of $ARO7^{c}$ and *TRP2* mRNAs independent of the general control network (Fig. 3). Specific activities of both branch point enzymes were not significantly changed in RH2470 at different levels of Gcn4p, with AAS activity ranging from 1.2 U to 1.7 U and CM activity from 12.2 U to 10.4 U (Fig. 2). Whereas no difference in growth of RH2470 compared with the wild-type strain RH1408 was observed under minimal conditions, starvation for tryptophan had a strong influence on viability of that strain (Table 3). Without any functional Gcn4p present, RH2470 showed no growth upon tryptophan starvation induced by 5-MT. Expression of wild-type levels of Gcn4p restored growth to a rate of 0.094 h^{-1}, corresponding to 53% growth reduction in comparison with RH1408. High amounts of the transcriptional activator increased the growth rate slightly to 0.096 h^{-1}, which corresponds to a decreased reduction of 41% compared with the wild-type level. Growth reductions of RH2470 were suppressed either by the $TRP2^{fbr}$ allele (RH2472) or by restoring allosteric regulation of

Table 3. Growth behavior of *S. cerevisiae* strains with altered regulatory properties at the branch point of aromatic amino acid biosynthesis emerging from chorismate

		Growth rate, h^{-1} or % reduction					
		No limitation			Trp starvation		
Strain	Genotype	−	+	+++	−	+	+++
RH1408	*ARO7, TRP2*	0.21 h^{-1}	0.21 h^{-1}	0.17 h^{-1}	0.18 h^{-1}	0.20 h^{-1}	0.16 h^{-1}
RH2463	$ARO7^{c}$, *TRP2*	0%	0%	0%	100%	53%	22%
RH2470	$ARO7^{c}$, ^{p}*ARO7*::*TRP2*	0%	0%	0%	100%	53%	41%
RH2469	^{p}*TRP2*::$ARO7^{c}$, *TRP2*	0%	0%	0%	100%	60%	43%
RH2473	^{p}*TRP2*::$ARO7^{c}$, ^{p}*ARO7*::*TRP2*	0%	0%	0%	100%	78%	76%

Growth rates were determined in minimal medium (No limitation) and in minimal medium supplemented with 10^{-4} M 5-MT (Trp starvation). Strains expressed no functional Gcn4p (−), low wild-type levels (+), or high levels (+++) of the transcriptional activator as indicated. For the wild-type strain RH1408, growth rate constants μ are indicated, whereas for all other strains the growth reduction as determined from growth rates with respect to RH1408 is given. Values are the mean of three independent measurements with a standard deviation not exceeding 20%.

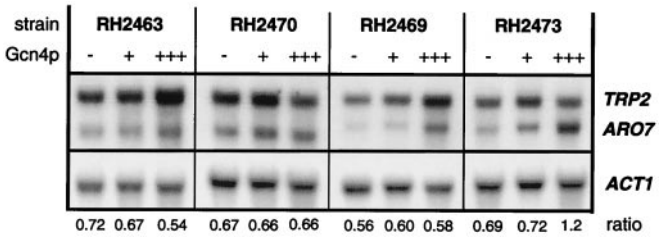

Fig. 3. Northern hybridization analyses of *S. cerevisiae* strains expressing *ARO7*^{c} and *TRP2* with altered dependence on the general control activator Gcn4p. Cells were cultivated in minimal medium and expressed no functional Gcn4p (−) or low levels (+) or high levels (+++) of Gcn4p, as indicated. In each lane, 25 μg of total RNAs was hybridized successively with probes specific for *ARO7*/*TRP2* and *ACT1* (encoding actin). For quantification, steady-state transcript levels were standardized with respect to *ACT1* levels and the ratio between *ARO7*^{c} and *TRP2* transcript levels is indicated at the bottom. All values are the means of two independent measurements with a standard deviation not exceeding 20%.

Aro7p (RH2467), as both strains grew well at wild-type levels in the presence of 5-MT (not shown).

In conclusion, these data demonstrate that transcriptional derepression of AAS activity is crucial under tryptophan starvation conditions when allosteric regulation of CM activity is not present.

Placing *ARO7*^{c} Under General Control Impairs Proper Flux Partitioning at the Branch Point. To investigate the effects when an unregulated CM activity of *S. cerevisiae* was expressed in a Gcn4p-dependent manner, strains were constructed in which the encoding *ARO7*^{c} allele was fused to the *TRP2* promoter. Strain RH2469 carries a *TRP2* promoter::*ARO7*^{c} cassette integrated at the *aro7*::*hisG* locus of RH2460 (Table 1). As a result, the synthesis of the two competing enzymatic activities at the metabolic node is increased in a general control-dependent manner driven by the *TRP2* promoter after starvation for almost any amino acid. To modulate the general control in RH2469, Gcn4p was expressed from plasmids in low and high amounts. The changes in transcript levels resulted in a constant ratio between *ARO7*^{c} and *TRP2* mRNAs that was not altered by the level of Gcn4p (Fig. 3). Specific enzymatic activities of CM and AAS reflected the impact of the transcriptional activator (Fig. 2). In the absence of Gcn4p, AAS activity was at a basal level of 1.2 U that was elevated to 3.1 U when Gcn4p was present in high amounts. Accordingly, CM activity was induced from 9.5 U to 26.4 U by high levels of the transcriptional activator. Growth rates were determined from RH2469 expressing different levels of Gcn4p. Whereas no effect on growth fitness was observed in minimal medium, severe growth reductions were present upon starvation for the aromatic amino acid tryptophan as induced by 5-MT (Table 3). No growth in the presence of the drug was observed for RH2469 when no functional Gcn4p was expressed. In the presence of Gcn4p, growth rates were reduced in comparison with strain RH1408. Whereas a low level of Gcn4p restored growth with 60% growth reduction, high levels of the transcriptional activator counteracted the starvation situation, resulting in 43% growth reduction with respect to the wild-type situation of RH1408. Again, this reduction in viability of RH2469 was suppressed by the *TRP2*^{fbr} allele (RH2471) or alternatively by an allosterically regulated CM encoded by the *ARO7* wild-type gene (RH2466). Both control strains RH2471 and RH2466 displayed no growth reduction upon starvation for tryptophan (not shown).

Furthermore, inversion of chorismate flux was achieved in strain RH2473, which expresses the *ARO7*^{c} allele driven by the *TRP2* promoter as well as the *TRP2* gene from the *ARO7* promoter. Northern analyses after transformation of this strain with Gcn4p-expressing plasmids demonstrated an inversion in the ratio between *ARO7*^{c} and *TRP2* mRNAs when the transcriptional activator was present in high amounts (Fig. 3). This altered expression pattern was reflected by specific enzymatic activities of CM and AAS (Fig. 2). AAS activity of RH2473 was unaffected by the level of Gcn4p at a basal level of 1.2 U, in contrast to CM activities, which ranged from 9.4 U to 19.6 U in the absence and presence of the transcriptional activator, respectively. The *in vivo* effect of this allelic situation was monitored by determination of growth rate constants and compared with the wild-type situation of strain RH1408 (Table 3). No significant reduction in growth rates was detected when cells were cultured in minimal medium. In contrast, tryptophan limitation induced severe reductions in growth. In the absence of Gcn4p, RH2473 displayed no growth when starved for tryptophan. Reintroduction of the transcriptional activator resulted in strongly retarded growth that was not elevated by expression of Gcn4p in high amounts. These two growth reductions in comparison with RH1408 were 78% and 76%, respectively. In contrast, strain RH2474, which expresses the feedback-resistant *TRP2*^{fbr} allele from the *ARO7* promoter, showed no significant growth retardation under tryptophan starvation conditions induced by 5-MT. Accordingly, strain RH2468, in which an allosterically regulated CM is present, grew well in minimal medium supplemented with the false feedback inhibitor (not shown).

In summary, we demonstrate here that transcriptional regulation of an allosterically unregulated CM enzyme in *S. cerevisiae* leads to severe growth defects when the competing enzyme is down-modulated in its activity, even when the general control system is present. Removing AAS expression from the transcriptional control amplifies this effect, as no other mechanism is present to increase enzyme activities for catalytic turnover feeding the tryptophan trunk of the branch point.

Discussion

Biosynthesis of aromatic amino acids in the yeast *S. cerevisiae* is a model for a branched, strictly regulated, metabolic reaction cascade. Different modes of regulation acting on enzyme synthesis as well as catalytic turnover are present in yeast to modulate the enzymatic activities constituting the pathway. For the amount of a given enzyme, different mechanisms of modulation are possible, involving synthesis and degradation of the encoding transcript, translational regulation, or stability of the gene product. Additionally, enzymatic activity itself can be modulated by a variety of mechanisms such as specific localization, (covalent) modification, or the action of effector molecules.

Here, we present data illustrating the necessity of the interplay of allosteric and transcriptional regulation acting on the enzymatic activities at the first metabolic node of aromatic amino acid biosynthesis in *S. cerevisiae*. Two enzymatic activities constitute the branch point emerging from chorismate. The amount of CM enzyme is not regulated, but catalytic activity is carefully modulated and fine-tuned by a positive as well as a negative effector. In contrast to this, expression of AAS activity is transcriptionally induced upon amino acid starvation, and catalytic turnover can only be reduced by negative feedback.

We have expressed an allosterically unregulated, constitutively active, CM to favor the tyrosine/phenylalanine-specific branch of this pathway. When no starvation situation was present, no reduction in growth was determined for all strains expressing the *ARO7*^{c} allele. The *ARO7*^{c} allele used in this study reflects a CM activity that is not regulated allosterically. This is reminiscent of bacterial CMs, which are found to display Michaelis–Menten-like kinetics in substrate saturation assays. Accordingly, catalytic efficiency (k_{cat}/K_m) of this unregulated yeast CM activity of approximately 13 $min^{-1}\cdot\mu M^{-1}$ is in the same range as the value reported for the *E. coli* P-protein CM activity: 10 $min^{-1}\cdot\mu M^{-1}$ (7, 11). The *ARO7*^{c} gene product is locked in its active R state because of an amino acid exchange in a flexible loop (loop 220s). Several mutant enzymes with substitutions in this loop have been

characterized, but the precise role of this structural element in the allosteric transitions remains unclear (34).

By making the *ARO7*^{c} allele a target of the general control, a regulation of the amount of CM molecules was achieved that is not present in yeast wild-type cells. Because growth rates were not reduced even when CM activity was elevated, the cellular chorismate pool has to be sufficiently high to maintain the flux into the tryptophan-specific branch, even when the competing sink is strongly favored. This accounts for a large reserve capacity of the tryptophan-specific branch as initiated by the AAS activity.

Starvation for tryptophan induced by the structural analogue 5-MT, which reduces AAS activity, resulted in severe growth defects when the unregulated CM activity was expressed. In the absence of the general control effector Gcn4p, starvation for tryptophan because of depletion of the chorismate pool was so severe that the cells were no longer viable. The general control system counteracts this starvation situation but shows dependence on the transcriptional pattern at the branch point up to different levels. When both encoding genes, *ARO7*^{c} and *TRP2*, were not under general control, growth was restored to 60% of wild-type levels. This reflects a replenished chorismate pool, as all enzymatic activities leading to chorismate are elevated by the general control system under starvation conditions. Making both branch point enzymes a target of the general control resulted in growth rates half as high as wild type. Here, the elevated chorismate pool is channeled symmetrically into both main branches as expression of both enzymatic activities is induced by high levels of Gcn4p. Inverting the regulatory pattern at the node with concern to general control dependency leads to severe growth retardation even in the presence of Gcn4p. In conclusion, the asymmetrical regulation of the two branch point enzymes by the general control as observed in the wild-type situation is necessary to maintain proper chorismate distribution under tryptophan starvation conditions. When allosteric regulation of CM activity was restored by the wild-type *ARO7* allele, growth was maintained at wild-type levels even under starvation conditions.

In a first view it seemed surprising that the CM-encoding gene of *S. cerevisiae* is not regulated by the general control system. However, our detailed analysis in the study presented here showed that the *ARO7* gene must not be regulated by the general control because of the careful interplay and fine tuning between activation and feedback regulation of a constant number of CM molecules vs. the feedback regulation of an increasable number of AAS molecules. This situation is likely to be conserved for other eukaryotic CMs. Two additional fungal CM enzymes have been characterized in detail, the *aroC* gene product of the filamentous fungus *Aspergillus nidulans* and the *HARO7*-encoded enzyme of the methylotrophic yeast *Hansenula polymorpha* (35, 36). For both fungal enzymes no induction of gene expression was monitored under amino acid starvation conditions, but strict allosteric regulation by homotropic and heterotropic effectors was present.

The allelic situation with an allosterically unregulated CM expressed in a Gcn4p-dependent manner might reflect an early evolutionary situation; the existence of a reversed Gcn4p recognition element in the *ARO7* promoter region that is able to bind the transcriptional activator *in vitro* implies that this gene formerly was subjected to the general control system (12). From structural studies it has been deduced that the dimeric allosterically regulated CM might have evolved from a monomeric unregulated ancestor by a gene duplication/gene fusion event (37). Given the drastic effects observed for the *ARO7*^{c} allele being subject to the general control, we speculate that either CM expression was removed from the general control system before this gene duplication and fusion or, alternatively, it was never subjected to it and *TRP2* acquired the transcriptional regulation after this evolutionary event. By dimerization and remodeling of distinct domains, allosteric behavior was achieved for the yeast CM. Because the balance between transcriptional and enzymatic regulation is so crucial, subtle changes on one regulatory level immediately required the adaptation on the other level to guarantee that the subtle fine tuning permanently worked. This is an interesting result of coevolution of transcriptional and enzymatic regulation. In *S. cerevisiae*, the different regulation of the two branch point enzymes with respect to the general control provides that flux imbalances can be counteracted in an asymmetric manner. Furthermore, our results demonstrate that allosteric and transcriptional regulation at the first branch point of aromatic amino acid biosynthesis in *S. cerevisiae* are interconnected, implying a continuous coevolution of both regulatory mechanisms controlling CM activity as well as AAS activity.

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