Identification of a Glutaminyl-tRNA Synthetase Mutation in *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae glutaminyl-tRNA synthetase mutants were isolated through systematic screening of tight Gln⁻ derivatives of a leaky glutamine auxotroph. These mutations define a single nuclear gene, *GLN4*. The *gln4-1* mutation is specific for Gln-tRNA synthetase and shows a dosage effect in heterozygous diploids. The wild-type Gln-tRNA synthetase exhibits a K_m for glutamine of 25 μ M; the *gln4-1* mutation increases this value 20-fold. These observations strongly suggest that *GLN4* encodes the Gln-tRNA synthetase.

Aminoacyl-tRNA synthetases are a class of essential enzymes which catalyze formation of an ester linkage between the 3' end of a tRNA molecule and its cognate amino acid. The high specificity of the protein-nucleic acid interaction which occurs during aminoacylation has been of long-standing interest. The enzymes are also the focus of studies on their regulatory roles and on the regulatory functions of aminoacylated tRNA. Isolation of mutant aminoacyl-tRNA synthetases has been of value to these investigations (reviewed in 7 and 9).

Mutations have been studied in *Escherichia coli* that affect such properties of these enzymes as thermal stability, drug resistance, and substrate affinities. Attempts to isolate similar mutants in yeasts have met with limited success. Hartwell and McLaughlin screened a collection of temperature-sensitive mutants for those deficient in protein synthesis (2). By measuring a variety of tRNA aminoacylation activities in these strains, they identified mutants with specific deficiencies in isoleucyl- and methionyl-tRNA synthetases (3, 4). Subsequently, Nass and Poralla (6) selected mutants resistant to borrelidin, a threonyl-tRNA synthetase inhibitor, and found one complementation group in which that enzyme had an altered sensitivity to borrelidin inhibition. More recently, a mutation altering the affinity of the asparaginyl-tRNA synthetase for asparagine was identified as an unusual asparagine auxotroph by Ramos and Wiame (8). This mutation occurred at low frequency because the majority Asn⁻ class is composed of *asn1 asn2* double mutants.

We describe here a strategy to find mutations which cause a reduced affinity of a chosen tRNA synthetase for its cognate amino acid. The approach was used to isolate mutants defective in glutaminyl-tRNA synthetase activity. These mutations are specific for Gln-tRNA synthetase and define a single nuclear gene. One of the mutant alleles is associated with an altered K_m of the enzyme for glutamine.

MATERIALS AND METHODS

Strains. The strains employed were derivatives of *Saccharomyces cerevisiae* Σ 1278b and are listed in Table 1. Strains for which a specific construction is not described were the result of standard genetic manipulations within the Σ 1278b background (5). The mutations *his4-42*, *lys-23*, and *met13-25* were isolated by M. Brandriss (New Jersey Medical Center, Newark, N.J.). *gln1-37* has been described previously (5). Lower-case letters denote a recessive mutant allele.

The *gln1-105* mutation was isolated after ethyl methane-sulfonate mutagenesis of a *GLN1*⁺ strain to 20% survival and was outcrossed twice before its use in this work. The original mutant was a leaky glutamine auxotroph with glutamine synthetase activity reduced 5- to 10-fold due to a single nuclear mutation. This mutation was identified as a *gln1* allele by its failure to recombine with *gln1-37* in 10 tetrads and by its ability to complement *gln1* mutations only weakly. *GLN1* is the structural gene for glutamine synthetase (A. P. Mitchell and B. Magasanik, manuscript in preparation).

To isolate the *gln4-1* mutation, strain 402-5B (containing the *gln1-105* allele) was mutagenized with ethyl methanesulfonate to 10% survival, and survivors were screened by replica plating for glutamine auxotrophs. Mutants were purified and retested. Strains carrying a second *gln1* mutation were eliminated by complementation tests with 444-1C; only mutants capable of weak complementation, as is 402-5B, were chosen for further study. *gln4-2* was isolated from a similar screen, employing 510-1B as the parent strain.

The mutations so isolated were divided into complementation groups by analysis of pairwise crosses, and one representative of each group (either the original mutant or a segregant from the first outcross) was grown to saturation and assayed for Gln-tRNA synthetase activity by the method described below. *gln4-1* was found among 18 mutants in 8 complementation groups; *gln4-2* was isolated as one of 79 mutants in 16 complementation groups.

Media. All media contained (per liter) 20 g of glucose, 3.4 g of yeast nitrogen base (Difco Laboratories) without amino acids or ammonium sulfate, and as nitrogen sources, either 1.0 g of glutamine, 1.28 g of sodium glutamate, or 2.0 g of ammonium sulfate. Concentrations of auxotrophic supplements and other details were as described previously (5).

Cell growth and extraction. Procedures for cell growth, shifting to fresh media, harvesting, and extraction by vortexing with glass beads, have been described previously (5), except that 0.1 M Tris (pH 7.5)-1 mM dithiothreitol was used as extraction buffer in preparation for tRNA synthetase assays. These extracts, at protein concentrations over 2 mg/ml, were dialyzed for at least 4 h at 4°C against 1,000 volumes of 0.1 M Tris (pH 7.5) to remove endogenous free amino acids.

tRNA synthetase assays. Assays were based on the method of Hartwell and McLaughlin (3). Extracts were adjusted to equal protein concentrations (generally, 4 mg/ml) with dialysis buffer, and 16 μ g of extract protein was brought to 50 μ l with 40 mM sodium phosphate (pH 7.5). After preheating these samples to 30°C for 5 min, reactions were initiated by

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TABLE 1. Strain list

Strain	Genotype	Source
Σ1278b	α	M. Brandriss
402-5B	a <i>his4-42 lys-23 gln1-105</i>	A. P. Mitchell
510-1B	α <i>his4-42 met13-25 gln1-105</i>	A. P. Mitchell
318-4D	α <i>met13-25</i>	A. P. Mitchell
444-1C	α <i>met13-25 gln1-37</i>	A. P. Mitchell
105gln-105	a <i>his4-42 lys-23 gln1-105 gln4-1</i>	Present work
2105gln-26	α <i>his4-42 met13-25 gln1-105 gln4-2</i>	Present work
483-5B	a <i>his4-42 lys-23 gln1-105 gln4-1</i>	Present work
646-6C	α	Present work
646-9A	α	Present work
646-8A	α <i>gln4-1</i>	Present work
646-11B	α <i>gln4-1</i>	Present work
483	a <i>his4-42 lys-23</i> + <i>gln4-1 gln1-105</i> α + + <i>met13-25</i> + <i>gln1-37</i>	105gln-105 × 444-1C
512	a <i>his4-42 lys-23</i> + <i>gln4-1 gln1-105</i> α + + <i>met13-25</i> + +	483-5B × 318-4D
633	a <i>his4-42 lys-23</i> + <i>gln4-1 gln1-105</i> α + + <i>met13-25</i> + <i>gln1-105</i>	483-5B × 510-1B
900	a <i>lys-23</i> + α + <i>met13-25</i>	Present work
901	a <i>lys-23</i> + <i>gln4-1</i> α + <i>met13-25 gln4-1</i>	Present work
902	a <i>lys-23</i> + <i>gln4-1</i> α + <i>met13-25</i> +	Present work
903	a <i>lys-23</i> + + α <i>met13-25 gln4-1</i>	Present work

the addition of 50 μl of preheated 40 mM sodium phosphate (pH 7.5) containing 14.4 mg of yeast tRNA per ml, 7.2 mM ATP, 18 mM MgCl₂, 36 mM KCl, 36 mM β-mercaptoethanol, 0.2 mg of bovine serum albumin per ml, and 10 μCi of radioactive amino acid without carrier. At 3, 6, 9, 12 and 15 min after initiation, 18-μl samples were removed, applied to Whatman scintillation pads, and dropped into 1 liter of 5% trichloroacetic acid in an ice bath. When all samples were collected, the filters were washed with three 10-min changes of 5% trichloroacetic acid at 0°C, then with two 10-min changes of 95% ethanol at 0°C, and then with a short rinse of diethyl ether. After drying under a heat lamp, the filters were counted for 2-min intervals in 3 ml of scintillation fluid. Calculations of acylation rates are corrected for background measured in a reaction without extract and for the counting efficiency of the nuclide.

Reaction rates were proportional to extract protein with up to 32 μg per reaction and were constant with respect to time. L-[³H]glutamine was typically at specific activities around 40 Ci/mmol, yielding a final concentration of 2.5 μM in the reaction. The glutamine concentration was low in these reactions to facilitate detection of mutant enzymes with increased *K_m*s for glutamine. Incorporation of radioactivity was reduced by over 90% when freshly dissolved A grade glutamine (Calbiochem-Behring) was added to 0.5 mM. *K_m* determinations were carried out in the presence of 5 mM potassium glutamate to eliminate a possible signal from contaminating [³H]glutamate. Kinetic constants were determined through least squares analysis of the data in double reciprocal form.

There was some variability in the reaction, depending on

the batch of [³H]glutamine used; therefore, a single batch was used for the determinations in any one figure or table.

One unit of activity is that amount converting 10⁻¹² mol of glutamine to trichloroacetic acid-precipitable material in 1 min at 30°C.

Miscellaneous. Methods for glutamine synthetase assays and protein determinations and sources of chemicals have been described previously (5). L-[³H]glutamine was purchased from either New England Nuclear Corp., Boston, Mass., or Amersham Corp., Arlington Heights, Ill. L-[³H]leucine and L-[³⁵S]methionine were obtained from Amersham.

TABLE 2. Segregation of the Gln⁻ phenotype in outcrosses of the *gln4-1* mutation

Diploid no.	Relevant genotype	No. of tetrads ^a with phenotypic ratio among spores (Gln ⁺ :Gln ⁻)				
		0:4	1:3	2:2	3:1	4:0
483	<i>gln4-1 gln1-105</i> + <i>gln1-37</i>	1	6	1	0	0
633	<i>gln4-1 gln1-105</i> + <i>gln1-105</i>	0	0	14	0	0
512	<i>gln4-1 gln1-105</i> + +	0	0	4	10	2

^a Meiotic tetrads from sporulated diploids were dissected by micromanipulation, and the spore phenotypes were analyzed by replica plating.

RESULTS

A collection of ethyl methanesulfonate-induced mutations had been assembled with the following properties: (i) strains carrying one of these mutations in addition to *gln1-105*, which results in 5- to 10-fold-less glutamine synthetase activity, were tight glutamine auxotrophs, and (ii) the mutations were unlinked to *GLN1*, the structural gene for glutamine synthetase, and complemented *gln1* defects. Such mutations define 18 complementation groups, 17 of which have no apparent function relating to glutamine synthetase regulation (Mitchell and Magasanik, in preparation).

We reasoned that mutations resulting in reduced Gln-tRNA synthetase activity might be included in this collection; therefore, we measured its activity in extracts of one member of each complementation group. Only the *gln4* representative had substantially reduced activity; this strain carried *gln4-1* and had 18% of the wild-type level. In subsequent studies, the independently isolated *gln1-105 gln4-2* auxotroph was shown to have 23% of the wild-type activity. Neither allele was associated with heat- or cold-sensitive lethality.

Genetic analysis of the *gln4-1* mutation. The original mutant, of presumed genotype *gln4-1 gln1-105*, was outcrossed to a *GLN4⁺ gln1-37* strain, 444-1C, to test the linkage of *GLN4* and *GLN1*. *gln1-37* is an amber-suppressible mutation in the structural gene for glutamine synthetase, and progeny carrying this mutation are Gln⁻ regardless of the allele at *GLN4*. Segregants of genotype *gln4-1 gln1-105* are Gln⁻ as well, but *GLN4⁺ gln1-105* recombinants are Gln⁺ because the *gln1-105* glutamine synthetase is sufficiently active to support prototrophic growth. The finding that 8 of 24 segregants in tetrads from diploid strain 483 were Gln⁺ (Table 2)

TABLE 3. Segregation of the glutaminyl-tRNA synthetase defect

Strain	Relevant genotype	Phenotype	Gln-tRNA synthetase ^a (U/mg)
483-5B	<i>gln4-1 gln1-105</i>	Gln ⁻	0.8
510-1B	+ <i>gln1-105</i>	Gln ⁺	5.6
633	<i>gln4-1 gln1-105</i>	Gln ⁺	2.4
	+ <i>gln1-105</i>		
633-1A	+ <i>gln1-105</i>	Gln ⁺	4.0
633-1B	<i>gln4-1 gln1-105</i>	Gln ⁻	1.1
633-1C	+ <i>gln1-105</i>	Gln ⁺	4.0
633-1D	<i>gln4-1 gln1-105</i>	Gln ⁻	0.7
633-2A	+ <i>gln1-105</i>	Gln ⁺	4.7
633-2B	<i>gln4-1 gln1-105</i>	Gln ⁻	0.9
633-2C	+ <i>gln1-105</i>	Gln ⁺	4.4
633-2D	<i>gln4-1 gln1-105</i>	Gln ⁻	1.3
483-5B	<i>gln4-1 gln1-105</i>	Gln ⁻	0.9
318-4D	+ +	Gln ⁺	5.3
512	<i>gln4-1 gln1-105</i>	Gln ⁺	2.7
	+ +		
512-15A	<i>gln4-1 gln1-105</i>	Gln ⁻	0.8
512-15B	+ +	Gln ⁺	4.6
512-15C	+ <i>gln1-105</i>	Gln ⁺	4.9
512-15D	<i>gln4-1</i> +	Gln ⁺	0.7

^a Exponential cultures growing on glutamine medium supplemented with histidine, lysine, and methionine were harvested, extracted, and assayed for Gln-tRNA synthetase activity as described in the text.

FIG. 1. Dependence of Gln-tRNA synthetase activity on glutamine concentration. Extracts of 646-9A (●; *GLN4⁺*) and 646-11B (○; *gln4-1*) grown on glutamine medium were assayed for Gln-tRNA synthetase activity with various concentrations of glutamine.

argues that *GLN1* and *GLN4* are not tightly linked. This is confirmed by the results of tetrad analysis of diploid 512 (Table 2; see below).

Segregant 483-5B could complement a *gln1-37*-bearing strain weakly but was phenotypically Gln⁻, indicating that it was of genotype *gln4-1 gln1-105*. This was confirmed by direct assay of Gln-tRNA synthetase activity (Table 3). Tetrads from diploid 633, constructed by crossing 483-5B to 510-1B (*GLN4⁺ gln1-105*), contained two Gln⁺ and two Gln⁻ spores in all 14 cases (Table 2), indicating that *gln4-1* lies at a single nuclear locus. The Gln-tRNA synthetase defect cosegregated with the Gln⁻ phenotype in two tetrads examined (Table 3).

To see whether *gln4-1* conferred a glutamine auxotrophy when coupled with a *GLN1⁺* allele, tetrads were analyzed from a cross of 483-5B to 318-4D (*GLN4⁺ GLN1⁺*). In each of 16 tetrads, two spores complemented *gln1-37* well for glutamine auxotrophy and two complemented weakly, confirming that *gln1-105* segregated as a single mutation. However, the Gln⁻ phenotype segregated as a two-gene trait (Table 2, diploid 512), indicating that the tRNA synthetase defect alone did not cause a glutamine requirement. This interpretation was confirmed by direct assay of Gln-tRNA synthetase activity in the segregants of a 3 Gln⁺:1 Gln⁻ tetrad. As expected, one *GLN1⁺* segregant, 512-15D, had a level characteristic of *gln4-1*-bearing strains (Table 3).

Biochemical nature of the *gln4-1* lesion. *gln4-1* could, in principle, alter either the structure or amount of Gln-tRNA synthetase; alternatively, it could elevate the levels of an inhibitor of the enzyme. However, the activity of mixtures of *gln4-1* and *GLN4⁺* extracts is equal to the sum of the activities of the individual extracts, indicating that *gln4-1* does not produce an excess of a diffusible inhibitor (data not shown).

Because *gln4-1* does not cause an obvious growth defect when glutamine levels are high (e.g., exogenous glutamine provided or coupled with *GLN1⁺*), we anticipated that the mutation would increase the *K_m* of Gln-tRNA synthetase for glutamine. The results of a kinetic study measuring this parameter are presented as a double reciprocal plot in Fig. 1. The wild-type Gln-tRNA synthetase activity exhibits a *K_m* for glutamine of 25 μM. The activity remaining in a *gln4-1* extract displays a *K_m* of ca. 500 μM.

TABLE 4. Dosage effect and specificity of the *gln4-1* mutation

Strain	Relevant genotype	tRNA synthetase activity ^a (U/mg)		
		Glutamine	Leucine	Methionine
900	<i>GLN4</i> ⁺ / <i>GLN4</i> ⁺	8.2	11.8	6.9
901	<i>gln4-1/gln4-1</i>	0.9	11.1	6.8
902	<i>gln4-1/GLN4</i> ⁺	5.0	ND ^b	ND
903	<i>gln4-1/GLN4</i> ⁺	4.4	ND	ND

^a Exponential cultures growing on glutamine medium were harvested, extracted, and assayed for the aminoacyl-tRNA synthetase activities listed as described in the text.

^b ND, Not determined.

To see whether *GLN4* plays a stoichiometric or catalytic role in expression of tRNA synthetase activity, we measured activity in extracts of *gln4-1/gln4-1*, *gln4-1/GLN4*⁺, and *GLN4*⁺/*GLN4*⁺ diploids. Diploids heterozygous for *gln4-1* have levels of Gln-tRNA synthetase activity roughly intermediate between the levels of the two homozygotes (Table 4), arguing in favor of a stoichiometric gene function. The specificity of the mutation is demonstrated by the identical levels of leucyl- and methionyl-tRNA synthetase activities in wild-type and mutant diploid (Table 4).

The possibility remained that *gln4-1* caused a global defect in glutamine metabolism or regulation. Therefore, we examined the regulation of glutamine synthetase in diploids 900 and 901. In both cases, glutamine was completely repressing, ammonia allowed 40-fold derepression, and glutamate brought about 150-fold derepression (data not shown), as previously observed (1, 5).

The above results suggest that *GLN4* is the structural gene for Gln-tRNA synthetase.

Physiological consequences of the *gln4-1* defect. Isogenic *GLN4*⁺ and *gln4-1* strains grow at comparable rates in glutamine-supplemented or glutamine-free media (Table 5). There is no detectable derepression of Gln-tRNA synthetase activity in *gln4-1* strains growing on glutamate. Therefore, the high *K_m* is sufficient to support steady-state growth in this situation.

A different pattern was observed after a shift from glutamine to glutamate. Whereas *GLN4*⁺ strains grew with a 160-min doubling time, the *gln4-1* strains achieved only a 300-min doubling time (Fig. 2). This aberrant response was not due to nonspecific sensitivity of *gln4-1* strains to the manipulations involved in shifting, because all of the strains grew with 140-min doubling times when shifted from glutamine to fresh glutamine. In addition, the induction kinetics of glutamine synthetase were the same in 646-6C and 646-11B (data not shown).

TABLE 5. Response of Gln-tRNA synthetase to growth in glutamine-free medium

Strain	Relevant genotype	Glutamine		Glutamate	
		<i>t_D</i> ^a (min)	Gln-tRNA synthetase ^b (U/mg)	<i>t_D</i> ^a (min)	Gln-tRNA synthetase ^b (U/mg)
646-6C	<i>GLN4</i> ⁺	140	5.2	140	5.2
646-9A	<i>GLN4</i> ⁺	140	5.4	150	4.7
646-8A	<i>gln4-1</i>	140	1.0	150	0.9
646-11B	<i>gln4-1</i>	150	0.9	150	1.2

^a *t_D*, Doubling time of the culture.

^b Exponential cultures growing on the media listed were harvested, extracted, and assayed for Gln-tRNA synthetase activity.

One explanation for the eventual adaptation of *gln4-1* strains to growth on glutamate is that a subpopulation of revertants is selected after long periods of time. This possibility was examined as follows. Samples of 646-6C (*GLN4*⁺) and 646-8A (*gln4-1*) were removed from exponential, adapted glutamate cultures, grown to saturation in glutamine and inoculated into glutamine cultures. In mid-exponential phase, the cultures were shifted to glutamate, and the *gln4-1* strain again displayed impaired growth (Fig. 2). We conclude that adaptation does not involve a heritable change. The fact that Gln-tRNA synthetase activity remains low in adapted *gln4-1* strains (Table 5) also argues against the selective enrichment for revertants during adaptation.

DISCUSSION

The strategy used to isolate *gln4* alleles may be used to find mutations affecting other aminoacyl-tRNA synthetases. After mutagenesis of a leaky amino acid auxotroph, tight auxotrophs are identified by replica plating. Mutants blocked in known biosynthetic steps are eliminated by complementation tests against standard testers, and remaining isolates are assayed for tRNA aminoacylation activity. The efficiency of such screening by assay may be increased enormously if the mutants are divided into complementation groups; *gln4-2* was found in 1 of 79 mutants that complemented a *gln1* tester, but these represented only 16 complementation groups.

A key factor in the success of this approach is the use of a parent strain with reduced pools of the cognate amino acid. Therefore, the sensitivity of the screen is such as to include mutations resulting in subtle structural alterations. This factor should increase the frequency with which mutations in these essential genes are isolated.

tRNA synthetase *K_m* mutants may not be directly useful in examining the regulatory consequences of limitation for an aminoacylated tRNA because the steady-state levels of

FIG. 2. Growth response of *gln4-1* and *GLN4* strains to shifts to a glutamine-free medium. The *gln4-1* strains 646-8A (○, ●) and 646-11B (▽) and *GLN4*⁺ strains 646-6C (△, ▲) and 646-9A (□) were shifted from exponential growth in glutamine medium to fresh, prewarmed glutamate medium. Growth was monitored by turbidity and was normalized to the turbidity immediately after the shift. Solid symbols represent a repetition of the experiment in which the strains had achieved steady-state growth on glutamate before inoculation into the parent glutamine culture, as described in the text.

aminoacylation may not be reduced. *gln4-1* exhibits only a transient growth defect upon an extreme nutritional shift, most likely due to a lowering of the available glutamine concentration. However, a plasmid carrying the *GLN4* gene has been isolated through complementation of a *gln1-105 gln4-1* auxotroph (S. W. Ludmerer, unpublished data). In vitro mutagenesis will provide conditional lethal alleles for a precise evaluation of this function.

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