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Proline: an Essential Intermediate in Arginine Degradation in *Saccharomyces cerevisiae*

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Results of studies on proline-nonutilizing (Put^-) mutants of the yeast *Saccharomyces cerevisiae* indicate that proline is an essential intermediate in the degradation of arginine. Put^- mutants excreted proline when grown on arginine or ornithine as the sole nitrogen source. Yeast cells contained a single enzyme, Δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase, which is essential for the complete degradation of both proline and arginine. The sole inducer of this enzyme was found to be proline. P5C dehydrogenase converted P5C to glutamate, but only when the P5C was derived directly from proline. When the P5C was derived from ornithine, it was first converted to proline by the enzyme P5C reductase. Proline was then converted back to P5C and finally to glutamate by the Put enzymes proline oxidase and P5C dehydrogenase.

Earlier studies (9, 17) on arginine degradation indicated the pathway to be arginine → ornithine → Δ^1-pyrroline-5-carboxylate (P5C) → glutamate, with arginase, ornithine transaminase, and P5C dehydrogenase acting sequentially. The first two enzymes were found to be inducible by both arginine and ornithine; the P5C dehydrogenase was reported variously to be inducible (1) or constitutive (17).

However, a recent study (8) on the action of ornithine transaminase in three species of higher plants cast doubt on the existence of this pathway of arginine degradation. It showed that the product of the ornithine transaminase reaction was not P5C, but Δ^1-pyrroline-2-carboxylate (P2C), which in turn was converted to proline.

Our reexamination of the pathway of arginine degradation in the yeast *Saccharomyces cerevisiae* described in this paper shows that P5C is indeed the product of the ornithine transaminase reaction. This compound, therefore, is an intermediate common to three pathways: arginine degradation, proline degradation, and proline biosynthesis.

We recently described the pathway of proline degradation (3, 4). Proline is oxidized to P5C by proline oxidase; P5C is then converted to glutamate by P5C dehydrogenase. Both enzymes are inducible by proline. In addition, P5C can be converted back to proline by P5C reductase, which catalyzes the final step in the synthesis of proline from glutamate (2).

Since both the proline and arginine degradative pathways appear to have the same final step, we undertook to determine whether there were arginine-specific and proline-specific isozymes of P5C dehydrogenase and whether there were any regulatory elements common to the two pathways. Our results have led us to conclude that the two proline-induced enzymes, as well as P5C reductase, are essential for the conversion of arginine to glutamate.

MATERIALS AND METHODS

Yeast strains. The strains of *S. cerevisiae* employed in this study are listed in Table 1. They are all isogenic, apart from the specified genotype.

Media, mutagenesis, and genetic analysis. These have been previously described (3).

Isolation of arginase-deficient mutant. Mutagenized cells of strain MB1000 were spread on agar plates containing 0.5% galactose and 0.2% ammonium sulfate and were incubated at 30°C for 3 to 5 days. The colonies were replica-plated to plates containing galactose and 0.1% arginine-hydrochloride. Colonies which failed to utilize arginine as sole nitrogen source were purified by subcloning on permissive medium and assayed for arginase and ornithine transaminase activities. The mutation studied in this report has been named *car1-107* in accordance with previously isolated arginase-deficient strains (5). It fails to complement an independently isolated arginase-deficient strain supplied by P. Whitney.

Isolation of ethidium bromide-induced respiratory-deficient (ρ^-) yeast strains. Lawns of yeast strains were spread on agar plates containing yeast extract (1%), peptone (2%), and glucose (2%) or a minimal medium with 2% glucose, 0.2% ammonium sulfate, and supplements as appropriate. One drop of an ethidium bromide solution (1 mg/ml) was absorbed onto a sterile concentration disk (Difco) placed in the center of each plate, and the plates were incubated for 24 h at 30°C. Cells from near the area of the disk were subcloned onto similar glucose-containing plates, and single colonies were tested for their inability to grow on plates containing acetate as carbon source. These

TABLE 1. *Strains used*

Strain	Genotype	Enzyme deficiency	Reference
MB1000	α wild type	None	Σ1278b (17)
MB209-8D	α *put1-54*	Proline oxidase	(3)
MB211-3B	α *put2-57*	P5C dehydrogenase	(3)
MB281-8B	α *pro3-66*	P5C reductase	
MB281-10C	a *pro3-66 put2-57*	P5C reductase, P5C dehydrogenase	(3)
MB342-2B	α *car1-107*	Arginase	
MB379-11D	α *put1-54 car1-107*	Proline oxidase, arginase	
MB380-1C	α *put2-57 car1-107*	P5C dehydrogenase, arginase	
MB386-5D	α *put1-54 pro3-66*	Proline oxidase, P5C reductase	
MB1001	α ρ^-	Respiratory deficient	
MB1142	α *pro3-66*	P5C reductase	(2)
MB1142 ρ^-	α *pro3-66* ρ^-	P5C reductase, respiratory deficient	

glucose-utilizing, acetate-nonutilizing colonies were chosen as respiratory-deficient strains.

Cross-feeding experiments. A lawn of the proline auxotrophic strain MB1142 (α *pro3-66*) was spread on plates containing as sole nitrogen source one of the following: ammonium sulfate, urea, arginine-hydrochloride, ornithine-hydrochloride, ammonium sulfate and arginine-hydrochloride, ammonium sulfate and ornithine-hydrochloride. Patches of *put1*, *put2*, ρ^-, and wild-type strains (all α *MAT*) were replica-plated from plates containing permissive medium to the plates with the lawns of MB1142 and incubated for 2 days at 30°C. Halos of growth of MB1142 around the patches were indicative of the excretion of proline by the strains tested.

Chemicals and substrates. Materials required for the proline oxidase and P5C dehydrogenase assays have been described (3). Pyridoxal-5-phosphate was purchased from Sigma Chemical Co., St. Louis, Mo. 1-Phenyl-1,2-propane-dione 2-oxime was purchased from Eastman Kodak Co., Rochester, N. Y.

Growth of cells for enzyme assays. The preparation of cells for the proline oxidase and P5C dehydrogenase assays has been described (3). For ornithine transaminase assays, 15 ml of appropriately supplemented minimal medium in 125-ml side-arm flasks was inoculated to a density of 2 to 10 Klett units (blue filter, Klett-Summerson colorimeter) with a stationary phase inoculum. Cultures were incubated at 30°C with shaking until their density reached approximately 100 Klett units (about 1×10^7 to 2×10^7 cells per ml). For arginase assays, cells were grown in 50 ml of medium in a 500-ml side-arm flask and were treated similarly.

Enzyme assays. (i) Ornithine transaminase. The assay for ornithine transaminase (EC 2.6.1.13) is a modification of the procedure used by Middelhoven (9) and has been adapted for use on whole cells rendered permeable by liquid nitrogen. Cells from a 3-ml sample of the culture were collected by filtration. The filter was immersed in liquid nitrogen as described for the proline oxidase assay (3). Each filter was placed in a small test tube containing sodium phosphate buffer (pH 7.4, 0.1 M final concentration) and kept on ice. After vigorous blending with a Vortex mixer, the assay mixture was added (final concentration per 1.5 ml: 15 mM α-ketoglutarate, pH 7.0; 1 mM pyridoxal-5-phosphate; 10 mM *o*-aminobenzaldehyde; 10 mM $MgCl_2$; and 15 mM ornithine-hydrochloride), and the reaction was allowed to proceed at 30°C for 30 min. The assay was terminated by the addition of 1.0 ml of 15% perchloric acid to each tube. The filters were removed, debris was pelleted by centrifugation, and 1 ml of the supernatant was diluted into 3 ml of water to read the absorbance at 440 nm. For calculations of specific activity, the molar extinction coefficient of the P5C–*o*-aminobenzaldehyde complex was 1,900 (15).

The assay was linear with both time (to 45 min, at least) and cell concentration. Blank values, where water was substituted for the ornithine, were subtracted to yield net absorbance.

To generate larger quantities of the end products of the ornithine transaminase reaction, the procedure outlined above was scaled up. Cells from 250-ml cultures of the wild-type strain grown on a medium containing galactose (0.5%) and arginine (0.1%) were harvested, washed in water, and concentrated approximately 100-fold in sodium phosphate buffer (pH 7.4, 0.1 M final concentration). The cells were frozen in liquid nitrogen for about 5 min, thawed at room temperature, and kept on ice until used. The reaction mixture contained (final concentrations per 6 ml): 30 mM α-ketoglutarate (pH 7.0), 1 mM pyridoxal-5-phosphate, 30 mM ornithine-hydrochloride, 100 mM sodium phosphate buffer (pH 7.4), and 1.5 ml of concentrated, permeabilized cells. Incubation was at 30°C for 60 min, and the reaction was terminated with 4 ml of 0.6 N HCl. The cell debris was removed, and the amount of pyrroline carboxylate in the supernatant was determined by diluting a fraction into buffer and complexing with *o*-aminobenzaldehyde, as described above. A control reaction, in which ornithine was omitted during the incubation but added back after the addition of the acid, was carried out in parallel.

(ii) Arginase. The procedure described by Whitney and Magasanik (16) for the colorimetric assay of arginase (EC 3.5.3.1) in whole cells was followed. The harvested cells were suspended in 2 ml of 0.01 M Tris-hydrochloride buffer (pH 7.0) containing 0.01 M $MnCl_2$ and 0.02 M glycine, frozen in liquid nitrogen, and stored at −20°C overnight.

(iii and iv) Proline oxidase and P5C dehydrogenase. Proline oxidase (EC 1.4.3.2) and P5C dehydrogenase (EC 1.5.1.12) were assayed as described previously (3).

Protein determinations. Protein was determined by the method of Lowry et al. (7) with bovine serum

albumin as standard. A modification of this procedure was used on whole cells (12).

RESULTS

Action of ornithine transaminase in *S. cerevisiae*. To determine whether ornithine transaminase removed the α-amino group of ornithine to form P2C or the δ-amino group to form P5C, we investigated the abilities of extracts prepared from wild-type cells and from those of a mutant lacking P5C dehydrogenase to catalyze the reduction of NAD^+ in the presence of the product of ornithine transaminase, prepared as described in the previous section.

The product of the ornithine transaminase reaction, if P5C, would cause the formation of NADH by P5C dehydrogenase; if the product were P2C, no increase in NADH would be observed. The initial rate of NADH formation with this product was compared with that with genuine P5C generated by the periodate oxidation of hydroxylysine by the procedure of Williams and Frank (18). P5C, and not P2C, is formed in this reaction.

Table 2 lists the specific activities of P5C dehydrogenase with both P5C and ornithine transaminase product as the substrate in the assay. Comparable specific activities in the *put2*$^+$ strains were measured for the ornithine transaminase product and P5C, which dropped to very low values in the P5C dehydrogenase-deficient *put2* strain. The ornithine transaminase assay mixture may be contributing an additional factor which enhances P5C dehydrogenase activity compared with pure P5C. Table 2 also lists P5C dehydrogenase specific activities of the *put2*$^+$ strain grown in a medium (proline as the sole nitrogen source) in which P5C dehydrogenase is fully expressed. The *put2* mutant cannot grow on this medium.

TABLE 2. *P5C dehydrogenase specific activity with the product of the ornithine transaminase reaction*

Strain	Relevant genotype[a]	Substrate[b]	P5C dehydrogenase sp act[c]	
			Ammpro[d]	Pro[d]
MB281-8B	*pro3-66 put2*$^+$	OTA product[e]	23.2	113
		P5C	14.1	93
MB281-10C	*pro3-66 put2-57*	OTA product[e]	0.9	NG[f]
		P5C	2.3	NG

[a] The *pro3-66* mutation eliminates P5C reductase activity to minimize competition for substrate and loss of NADH.

[b] The substrate was supplied at 1.3 to 1.4 mM.

[c] Expressed as nanomoles of NADH formed per minute per milligram of protein.

[d] The strains were cultured on media containing ammonium sulfate supplied at 0.2%, or proline at 0.1% and glucose at 2.0%, or both.

[e] The P5C dehydrogenase specific activity with the product of the ornithine transaminase reaction as substrate is a net value. A background specific activity was subtracted from the total value obtained. The background activity was measured by using the product of an ornithine transaminase reaction in which ornithine was omitted during the reaction (see text). This background specific activity, found in both *put2*$^+$ and *put2-57* strains, was 18 to 22. Additional controls were performed in which glutamate or ammonia was added to the reaction mix. These additions did not alter the results, eliminating the possibility that NADH formation was due to the anabolic or catabolic glutamic dehydrogenases.

[f] NG, No growth.

The ornithine transaminase product was also used as substrate for the P5C reductase (2) reaction, and its behavior was compared with that of pure P5C by using extracts from a strain deficient in P5C dehydrogenase. The reactions were carried to completion, and with ornithine transaminase product as well as pure P5C, the number of nanomoles of P5C which disappeared was equal to the number of nanomoles of NAD^+ formed (data not shown).

Therefore, we conclude that in yeast ornithine transaminase removes the δ-amino group of ornithine and transfers it to α-ketoglutarate to form P5C and glutamate. In addition, proline is formed from P5C, not P2C, by the P5C reductase.

Number of P5C dehydrogenases in *S. cerevisiae*. Since P5C is formed both from arginine and from proline and is subsequently converted to glutamate by P5C dehydrogenase, it was of interest to determine whether *S. cerevisiae* possesses isozymes for P5C dehydrogenase, one under arginine control and one under proline control. We used for this study a strain carrying the *put2-57* mutation, which results in the inability to utilize proline as sole nitrogen source because of a deficiency in P5C dehydrogenase (3).

Attempts were made to measure P5C dehydrogenase activity in a *put2* strain grown under conditions in which an arginine-specific P5C dehydrogenase activity would have been induced if one existed. The same low levels of enzyme activity were found when the mutant cells were grown on media containing arginine as the sole nitrogen source (data not shown) or ammonia and arginine (Table 3) or ammonia and proline (Table 3 of reference 3). These levels were about fivefold lower than those of the wild-type strain.

Furthermore, strains deficient in P5C dehydrogenase activity grow very poorly on a medium containing ornithine as the sole nitrogen source when compared with the wild-type strain. Growth was monitored in liquid cultures containing glucose (2%) as carbon source and ornithine-hydrochloride (0.1%) as nitrogen source. Whereas the wild-type strain doubled in 5 h, the *put2* strain doubled in 15.5 h.

TABLE 3. *Enzyme activities in mutant and wild-type yeast strains*

Strain	Genotype	Sp act[a,b]			
		Arginase	Ornithine transaminase	Proline oxidase	P5C dehydrogenase
MB1000	α wild type	0.75 ± 0.14	15.0 ± 4.9	1.7 ± 1.4	24 ± 5
MB209-8D	α *put1-54*	0.91 ± 0.08	8.9 ± 0.6	0.1 ± 0.1	67 ± 12
MB211-3B	α *put2-57*	1.15 ± 0.03	15.7 ± 5.9	13.2 ± 3.4	3 ± 0.6
MB342-2B	α *car1-107*	0.14 ± 0.01	12.6 ± 0.8	1.4 ± 0.6	18 ± 9

[a] The cells were grown on 0.5% galactose, 0.2% ammonium sulfate, and 0.1% arginine-hydrochloride.
[b] For arginase, units are micromoles of urea formed per minute per milligram of protein; for ornithine transaminase and proline oxidase, units are nanomoles of P5C formed per minute per milligram of protein; and for P5C dehydrogenase, units are nanomoles of NADH formed per minute per milligram of protein.

These two observations together suggest that an arginine- or ornithine-inducible P5C dehydrogenase does not exist and that a single P5C dehydrogenase is responsible for the conversion of P5C to glutamate in both the proline- and arginine-degradative pathways.

Arginine and proline both induce the proline-degradative enzymes. Since the proline- and arginine-degradative pathways intersect at P5C and share P5C dehydrogenase, we examined the regulation of the four enzymes for coordinate and reciprocal control by their inducers. Table 4 lists the specific activities of proline oxidase, P5C dehydrogenase, arginase, and ornithine transaminase when cells of the wild-type strain were grown on various nitrogen sources. When arginine was used as the sole nitrogen source, the levels of arginase and ornithine transaminase were elevated, confirming earlier results reported by Middelhoven (9). Similarly, the activities of the proline-degrading enzymes were elevated in the arginine-grown cells, although to a level lower than that found when the cells were grown on proline as sole nitrogen source. Proline was shown to be the inducer of proline oxidase and P5C dehydrogenase in a previous report (3). As shown in Table 4, proline did not induce the first two arginine-degrading enzymes. This lack of reciprocal induction was examined further.

Arginine itself is not an inducer of the proline-degrading enzymes. Since arginine can serve as a precursor to proline (2, 3; Fig. 1), the induction of proline oxidase and P5C dehydrogenase by arginine shown in Table 4 could be an indirect effect dependent on the cell's ability to convert arginine to proline. To determine whether arginine is an independent inducer of the proline-degrading enzymes, strains were constructed which carried the *car1-107* mutation, leading to a deficiency in arginase activity, and the *put2-57* or *put1-54* mutation, leading to deficiencies in P5C dehydrogenase or proline oxidase, respectively. Activities for all four enzymes in the mutants are listed in Table 3. The *put1* and *put2* mutations had been shown to result in hyperinduction of the remaining proline-degradative enzyme on a medium containing ammonia and proline (3). The Car$^-$ Put$^-$ double mutant strains are therefore highly sensitive to induction by proline and cannot convert arginine to proline.

Table 5 gives the results of proline oxidase

TABLE 4. *Induction of arginine- and proline-degrading enzymes in the wild-type strain MB1000*

Nitrogen source[b]	Sp act[a]			
	Arginase	Ornithine transaminase	Proline oxidase	P5C dehydrogenase
Ammonia	0.10 ± 0.01	2.5 ± 0.6	1.7 ± 1.4	14 ± 4
Proline	0.16 ± 0.03	1.6 ± 0.0	27.2 ± 2.1	124 ± 15
Arginine	3.41 ± 0.30	34.2 ± 5.3	17.4 ± 2.0	47 ± 7

[a] Expressed as in Table 3.
[b] Ammonium sulfate was supplied at 0.2%; arginine-hydrochloride and proline were supplied at 0.1%. The carbon source was 0.5% galactose.

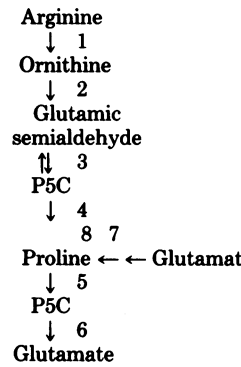

FIG. 1. *Pathways of arginine and proline degradation and proline biosynthesis. Enzymes: 1, arginase; 2, ornithine transaminase; 3, spontaneous; 4, P5C reductase; 5, proline oxidase; 6, P5C dehydrogenase; 7, glutamate kinase; 8, glutamate phosphate reductase.*

TABLE 5. *Induction of the Put enzymes in arginase-deficient mutants*

Strain	Genotype	Proline oxidase sp act[a] on:[b]			P5C dehydrogenase sp act[a] on:[b]		
		Amm	Amm-Arg	Amm-Pro	Amm	Amm-Arg	Amm-Pro
MB211-3B	α *put2-57*	0.9 ± 0.5	12.4 ± 2.8	15.3 ± 2.5			
MB342-2B	α *car1-107*	1.1 ± 0.1	1.4 ± 0.6	5.0 ± 0.3	36 ± 8	34 ± 6	47 ± 7
MB380-1C	α *put2-57 car1-107*	1.6 ± 1.1	0.6 ± 0.2	21.3 ± 1.3			
MB209-8D	α *put1-54*				41 ± 3	67 ± 12	122 ± 17
MB379-11D	α *put1-54 car1-107*				29 ± 6	34 ± 11	114 ± 27

[a] Expressed as in Table 3.

[b] The carbon source was galactose (0.5%). Arginine-hydrochloride (Arg) and proline (Pro) were provided at 0.1% and ammonium sulfate (Amm) was provided at 0.2% as nitrogen sources.

and P5C dehydrogenase assays on the double mutants and the parent strains. The *put2* parent strain showed the hyperinduction of proline oxidase on both ammonia-arginine and ammonia-proline media. The *car1* strain behaved as would a wild-type strain with respect to induction by proline and arginine. The double mutant showed the hyperinduction by proline characteristic of the *put2* mutation, but failed to give a high level of proline oxidase in the presence of arginine. We conclude that it is the presence of a functional arginase which allows arginine to serve as an inducer of proline oxidase. In other words, arginine itself is not the inducer but it is converted to the inducer, proline, by the action of arginase, ornithine transaminase, and P5C reductase.

In an analogous manner, the hyperinduction of P5C dehydrogenase in the *put1* parent strain when grown on ammonia-arginine medium was abolished in the *put1 car1* double mutant. The P5C dehydrogenase assay gives more variability and the effect is smaller, but the conclusion is the same: arginine is not itself the inducer of P5C dehydrogenase but can be converted to the true inducer, proline.

Effect of nitrogen starvation on enzyme induction. Middelhoven (10) reported the induction of arginase and ornithine transaminase upon removal of the nitrogen source from ammonia-grown cells. Subsequently, Whitney and Magasanik (16) showed that the increase in arginase activity was due to internal induction by arginine.

Table 6 gives the results of an analogous series of experiments measuring the effect of nitrogen starvation on proline oxidase activity. When the wild-type strain MB1000 was incubated without a nitrogen source for 2 h, proline oxidase activity increased 12-fold, and arginase activity increased 7-fold. To determine whether proline oxidase activity increased due to internal induction by proline or by arginine which had been converted to proline, the arginase-deficient strain MB342-2B was starved of nitrogen in an identical manner. Proline oxidase activity increased as before, indicating that the starvation-induced proline oxidase activity was not caused by the accumulation of arginine subsequently converted to proline.

TABLE 6. *Induction of proline oxidase and arginase by nitrogen starvation*

Strain	Genotype	Proline oxidase sp act[a]		Arginase sp act[a]	
		Pre-starvation[b]	2-h Starvation[c]	Prestarvation[b]	2-h Starvation[c]
MB1000	α wild-type	0.6	11.9	0.11	0.74
MB342-2B	α *car1-107*	0.5	9.9	ND[d]	ND
MB1142[e]	α *pro3-66*	3.7[f]	3.6	0.21	0.48
		6.3	9.6[g]	ND	ND

[a] Expressed as in Table 3.

[b] Except where noted, cultures of each strain were grown to 100 Klett units on a medium containing 0.5% galactose and 0.02% ammonium sulfate and assayed as described in the text.

[c] At 50 to 70 Klett units, cultures growing on a medium containing 0.02% ammonium sulfate were filtered, washed, and resuspended in an ammonia-free medium containing 0.5% galactose. Incubation continued for 2 h, and the enzymes were assayed as described in the text.

[d] ND, Not determined.

[e] Cultures of MB1142 were grown on 0.5% galactose, 0.02% ammonium sulfate, and 0.1% proline.

[f] Proline was removed for 2 h before the assay. Ammonium sulfate was still present. The proline oxidase specific activity before removal of proline was 6.3; the arginase specific activity was 0.099.

[g] Ammonium sulfate and proline were removed.

When a proline auxotroph (MB1142, blocked in the last step of proline biosynthesis, the P5C reductase) was deprived of proline for 2 h and then starved of ammonia, the proline oxidase specific activity dropped from 6.3 (ammonia-proline level) to 3.7 and remained at this level. That this was not due to an inhibition of protein synthesis was seen in the induction of arginase

activity. In contrast, when ammonia and proline were removed simultaneously for 2 h, the proline oxidase specific activity increased from 6.3 to 9.6. These two results taken together suggest that when the cells cannot synthesize proline and the proline pool is exhausted, proline oxidase is not induced. We conclude that the increase in proline oxidase activity upon nitrogen starvation is a consequence of internal induction by proline.

Analogous experiments in which the levels of P5C dehydrogenase were measured failed to reveal an increase in enzyme activity after nitrogen starvation (data not shown). It appears that P5C dehydrogenase is less sensitive to induction by proline than proline oxidase, which may reflect differences in inducer-DNA interactions between the *put1* and *put2* genes.

Fate of arginine and ornithine in proline-nonutilizing yeast strains. The initial observation that *put2* mutants grew poorly on ornithine as sole nitrogen source led to an examination of other proline-utilizing strains for their ability to utilize ornithine. These included a proline oxidase-deficient (*put1*) strain and a strain made respiratory deficient by treatment with ethidium bromide. Such cytoplasmic petite or ρ^- strains typically lack cytochromes aa_3, b, and c_1 as well as an energy transfer system (11). The ρ^- strains are proline nonutilizers presumably because proline oxidase activity requires the presence of a functional electron transport chain. The specific activity of proline oxidase in the ρ^- strain growing on arginine as nitrogen source was less than 1, compared with a wild-type specific activity of 11.8.

When *put1* and ρ^- strains were compared with *put2* strains for utilization of ornithine, they exhibited similar slow growth, as if these strains were assimilating only one of the two available nitrogen atoms of ornithine. The *put1* strain doubled in 13 h and the ρ^- strain doubled in 16.5 h in liquid cultures containing glucose and ornithine, compared with the wild-type doubling time of 5 h.

The levels of P5C dehydrogenase were measured in the *put1*, ρ^-, and wild-type strains grown on 2% glucose and 0.1% arginine. Table 7 lists the results. The *put1* strain had a level of enzyme about threefold higher than the wild-type strain, consistent with previous findings (3). The ρ^- strain MB1001 had a similar hyperinduced enzyme level. Although both strains have very high P5C dehydrogenase levels as measured in vitro, the enzyme apparently does not function in vivo, as indicated by the poor growth on ornithine.

The fate of the P5C formed from ornithine was determined by a series of cross-feeding experiments in which the excretion of proline was detected by the growth of a lawn of a yeast proline auxotroph (see Materials and Methods). Figure 2 demonstrates that when tested on solid media containing either arginine or ornithine as sole nitrogen source, *put1*, *put2*, and ρ^- (not shown) strains excreted proline, whereas the wild-type strain did not. When grown on ammonia, urea, ammonia plus arginine, or ammonia plus ornithine, none of the strains excreted proline.

The excretion of proline by proline-nonutilizing mutants grown on arginine or ornithine suggests that the P5C formed is converted to proline by P5C reductase, the last enzyme in proline biosynthesis. In the presence of a functional

TABLE 7. *P5C dehydrogenase levels in the wild type and Put⁻ mutants*

Strain	Genotype	Sp act[a] on 2% glucose–0.1% arginine
MB1000	Wild type	73 ± 1
MB209-8D	*put1-54*	168 ± 32
MB1001	ρ^-	245 ± 42

[a] Expressed as in Table 3.

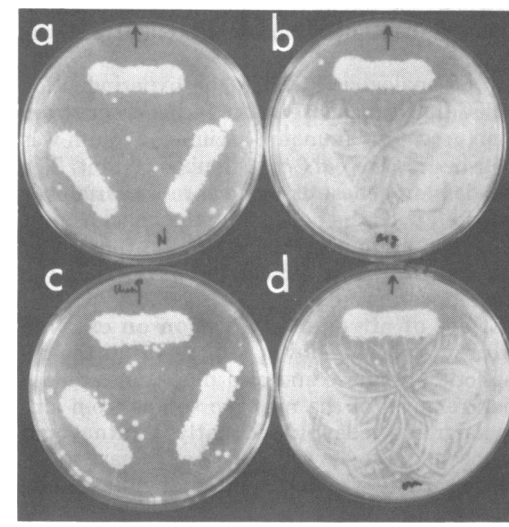

FIG. 2. *Example of cross-feeding of a proline auxotroph through proline excretion by proline-nonutilizing mutants of yeast. Each petri plate contained a different nitrogen source (a, ammonium sulfate; b, arginine-hydrochloride; c, urea; d, ornithine-hydrochloride) and was spread with a lawn of MB1142 (α pro3-66). Patches of strains grown on a rich medium were replica plated onto the above plates and incubated at 30°C for 2 days. Top, MB1000 (wild type); left, MB209-8D (α put1-54); right, MB211-3B (α put2-57).*

proline-degrading system, proline apparently does not accumulate, but is degraded. When P5C production is low, as when ammonia or urea serves as the nitrogen source, proline fails to accumulate in the Put⁻ strains.

Does P5C dehydrogenase compete with P5C reductase for P5C? One possible explanation for the poor growth on ornithine of the Put⁻ mutants is that P5C reductase converts P5C to proline at a more rapid rate than P5C dehydrogenase can convert P5C to glutamate. Proline accumulates and is excreted. The second nitrogen atom contained in ornithine is lost and cannot be used to form a second molecule of glutamate to facilitate growth.

To test this, we reasoned that the removal of the P5C reductase activity by mutation of the *pro3* gene should enhance the ability of the cell to grow on ornithine. Strains were constructed which either were deficient in proline oxidase activity (*put1*) or were respiratory deficient (ρ^-), and also lacked P5C reductase activity (*pro3*). Their growth rates were compared in liquid cultures containing ornithine as a nitrogen source and proline as an auxotrophic supplement. The results given in Table 8 indicated no improvement in growth rate in the double mutant strains. In fact, the *pro3* mutation alone appears to be detrimental, perhaps due to accumulation of a toxic intermediate. (Since a 10-h doubling time is very long, it is not clear whether 26- and 54-h doublings are significantly different.) These results suggest that, although P5C derived from ornithine is available to P5C dehydrogenase in a P5C reductase-deficient strain, P5C may not be accessible to that enzyme.

DISCUSSION

The pathway of arginine degradation in *S. cerevisiae* has been viewed as a three-step sequence involving arginase, ornithine transaminase, and P5C dehydrogenase in the formation of glutamate (1, 9, 16). From our study of mutants unable to utilize proline as sole nitrogen source and the examination of the fate of arginine and ornithine in these mutants, we conclude that the formation of proline is an essential step in the complete degradation of arginine and that glutamate is then formed from proline by using the proline degrading enzymes.

TABLE 8. *Growth rates of strains defective in proline metabolism*

Strain	Enzyme deficiency	Doubling time (h)[a]
MB1000	None	4.5
MB209-8D	Proline oxidase	10
MB1001	Respiratory deficient	14
MB281-8B	P5C reductase	26.5
MB386-5D	Proline oxidase, P5C reductase	54
MB1142 ρ^-	Respiratory deficient, P5C reductase	31

[a] Ornithine-hydrochloride and proline were supplied at 0.1%; glucose was supplied at 2.0%.

It has recently been suggested that P2C, not P5C, may be the product of ornithine transaminase (8). In that case, conversion of P2C to proline would be a necessary step in the conversion of arginine to glutamate via P5C. However, we have shown in this paper that in *S. cerevisiae* the product of ornithine transaminase is as good a substrate of P5C dehydrogenase as chemically prepared P5C. The open chain forms of P5C and P2C are glutamate semialdehyde and 2-keto-5-amino valerate, respectively. The possibility that the same dehydrogenase could with equal ease convert the former to glutamate and the latter, presumably, to 4-aminobutyrate and CO_2 is highly unlikely. Furthermore, we have identified glutamate as the product of proline degradation (unpublished data). We may therefore conclude that P5C is the product of the transamination of ornithine.

Thus, the arginine-degradative pathway appears to be a five-step sequence consisting of arginase, ornithine transaminase, P5C reductase, proline oxidase, and P5C dehydrogenase. The first two enzymes are induced by arginine and ornithine, the third is constitutive, and the fourth and fifth are induced by proline (Fig. 1). The pathway of proline degradation is therefore an integral part of the pathway of arginine degradation.

Because of the behavior of *put1* and ρ^- mutants when grown on a medium containing ornithine as sole nitrogen source, we conclude that P5C derived from ornithine, in contrast to that derived from proline, is not directly available to P5C dehydrogenase for conversion to glutamate. Thus, the yeast cell has devised a control mechanism to prevent futile cycling in these pathways which have P5C as a common intermediate. Such a mechanism could involve physical separation of the pathways by compartmentation of the enzymes or by aggregation of the enzyme proteins, preventing the release of intermediates into the cytoplasm.

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