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Subcellular Compartmentation in Control of Converging Pathways for Proline and Arginine Metabolism in *Saccharomyces cerevisiae*

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Enzymes of proline biosynthesis and proline degradation which act on the same compound, Δ^1-pyrroline-5-carboxylate, are physically separated in yeast cells. The enzyme responsible for the final step in proline biosynthesis, pyrroline-5-carboxylate reductase, converts pyrroline-5-carboxylate to proline and is located in the cytoplasm. The last enzyme in the proline degradative pathway, pyrroline-5-carboxylate dehydrogenase, converts pyrroline-5-carboxylate to glutamate and is found in the particulate fraction of the cell, presumably in the mitochondrion. By subcellular compartmentation, yeast cells avoid futile cycling between proline and pyrroline-5-carboxylate.

When a compound is an intermediate in both biosynthetic and degradative pathways, regulatory mechanisms must exist within a cell such that metabolic balance is maintained and the desired end product is generated. In our studies on proline biosynthesis (1) and proline and arginine degradation (2, 3) in the yeast *Saccharomyces cerevisiae*, we have found Δ^1-pyrroline-5-carboxylate (P5C) to be such an intermediate. The following sequences of reactions occur:

$$\text{arginine} \rightarrow \text{ornithine} \rightarrow \text{P5C} \rightarrow \text{proline} \quad (1)$$

$$\text{proline} \rightarrow \text{P5C} \rightarrow \text{glutamate} \quad (2)$$

$$\text{glutamate} \rightarrow \text{glutamyl phosphate} \rightarrow \text{P5C} \rightarrow \text{proline} \quad (3)$$

Line 1 is the pathway of arginine degradation. The sequential action of arginase and ornithine transaminase produces P5C (8, 14). We showed recently (3) that proline is then formed from P5C via P5C reductase, the third enzyme in proline biosynthesis (1). Proline can be degraded to P5C by using proline oxidase; P5C dehydrogenase converts the P5C to glutamate (line 2, also reference 2). The proline formed from arginine breakdown, as well as proline provided exogenously, is degraded by this route (3). In proline biosynthesis, glutamate is converted to P5C in what we believe to be a two-step process; P5C reductase then converts P5C to proline (line 3, also reference 1).

Obviously, P5C is a key intermediate in these processes. Control mechanisms must exist which assure the cell sufficient proline for protein synthesis as well as the ability to utilize the nitrogen contained in arginine and proline when other sources of nitrogen are not present. Therefore, a mechanism to avoid futile cycling between proline and P5C must exist.

In this paper, we show that the proline degradative enzyme P5C dehydrogenase and the proline biosynthetic enzyme P5C reductase are physically separated within the cell. P5C reductase is a cytoplasmic enzyme which converts P5C made from ornithine or glutamate in the cytoplasm to proline. P5C dehydrogenase is a mitochondrial enzyme which acts on P5C derived from proline. Therefore, two P5C pools exist inside yeast cells, and the enzymes which use these pools as substrates do not compete. A futile cycle between P5C and proline is avoided since the final step in proline biosynthesis occurs cytoplasmically and proline degradation is mitochondrial.

MATERIALS AND METHODS

Yeast strains. MB1000 is the wild-type strain of Jean-Marie Wiame, Σ1278b. MB380-13D is *α put2-57*.

Growth of cells for fractionation. A stationary-phase culture of MB1000 (2 ml) was inoculated into a 2-liter flask containing 500 ml of yeast nitrogen base without ammonium sulfate or amino acids (Difco Laboratories) supplemented with galactose (0.5%) and either proline (0.1%), ornithine hydrochloride (0.1%), or arginine hydrochloride (0.1%). The cells were incubated with shaking at 30°C until the culture reached a final density of 70 to 100 Klett units (blue filter, Klett-Summerson colorimeter).

Spheroplast formation. The procedures described here are based on those reported by Jauniaux et al. (6). Exponentially growing cells were harvested by centrifugation at 2,500 × *g* for 5 min. The cells were

then washed twice with water and suspended in 5 volumes (volume per weight of wet cells) of 0.1 M Tris-hydrochloride (pH 9.3)-15 mM mercaptoethanol. The cells were shaken for 10 min at 30°C on a rotary shaker. Cells were washed once with water and once with a solution containing 1.35 M sorbitol-1 mM EDTA (potassium salt)-0.05 M citrate phosphate buffer (pH 5.8). The cells were suspended in 3 volumes (vol/wt) of the latter solution, and 0.4 ml of glusulase (Endo Laboratories) was added per g of wet cells. Conversion to spheroplasts was performed at 30°C with mild shaking for 2 h. The spheroplasts were then washed twice in a solution containing 1.5 M sorbitol-1 mM EDTA-0.1% bovine serum albumin (pH 7.0) and were either used immediately or refrigerated overnight without substantial loss of enzyme activities.

Spheroplast disruption and fractionation (Fig. 1). The spheroplasts were lysed osmotically by suspension in lysis solution containing 0.6 M sorbitol-2 mM EDTA-0.1% bovine serum albumin (pH 7.0) and disrupted by vigorous shaking with a Vortex mixer. This fraction constituted the spheroplast lysate. Unbroken cells and debris were pelleted by one centrifugation at 750 × *g* for 10 min. The supernatant ("decanted lysate") was removed, and the pellet ("low-speed pellet") was suspended in 3.5 to 6 ml of lysis solution and held on ice. The decanted lysate was centrifuged at 30,000 × *g* for 10 min to separate fractions referred to as "cytoplasm" and "particulates." The particulates were resuspended in 3.5 to 6.0 ml of lysis solution. All five fractions were disrupted by sonication (Branson Sonic Power Co.) for 2 min and then centrifuged at 20,200 × *g* for 20 min. The supernatants were removed, and 2.5 ml was applied to a G25 Sephadex column (PD10; Pharmacia Fine Chemicals) to remove small molecules. The samples were eluted with 3.5 ml of 0.05 M tris(hydroxymethyl)methylaminopropane sulfonic acid (pH 7.7) buffer and used in enzyme assays.

Isopycnic gradient analysis. A particulate pellet was obtained as described above and was gently suspended in 2.5 ml of a solution containing 20% sorbitol (wt/vol), 0.1% bovine serum albumin, 1 mM EDTA (potassium salt), and 50 mM Tris-hydrochloride (pH 8.0). A 1.65-ml amount of the suspension was subjected to equilibrium centrifugation in a 12-ml 20 to 75% (wt/vol) linear sorbitol gradient containing 1 mM EDTA (potassium salt) and 50 mM Tris-hydrochloride (pH 8.0). Centrifugation was carried out with a Beckman L5-50 centrifuge in an SW40 rotor at 35,000 rpm (217, 348 × *g* at the bottom) for 2 h at 4°C. The gradient was divided into 0.7-ml fractions. A 0.2-ml amount was removed for subsequent determination of refractive index. The remaining 0.5 ml in each fraction was diluted sevenfold in a solution containing 50 mM tris(hydroxymethyl)methylaminopropane sulfonic acid buffer (pH 7.7). Each fraction was sonically disrupted for a total of 2 min, and the debris was pelleted by centrifugation. The supernatant was used in the enzyme assays.

Enzyme assays. P5C reductase was assayed as described previously (1). P5C dehydrogenase was assayed as described previously (2). Citrate synthase was measured as described by Parvin (9), and glucose 6-phosphate dehydrogenase was measured as described by Clifton et al. (4). Total activity is nanomoles of product formed per minute.

RESULTS

Fractionation of cell extracts. Yeast cells of wild-type strain MB1000 were grown on a medium containing galactose and proline as the sole carbon and nitrogen sources, respectively, and fractionated into cytoplasmic and particulate fractions, as described above. The enzymes glucose 6-phosphate dehydrogenase and citrate synthase were used as markers of the cytoplasm and mitochondrial matrix, respectively. P5C reductase and P5C dehydrogenase were assayed, and the distribution of the enzyme activities was compared with that of the marker enzymes.

Table 1 lists the activities and distribution of the four enzymes found in the fractions from a typical experiment. More than 90% of the activity of each enzyme found in the spheroplast lysate was recovered in the low-speed pellet and decanted lysate fractions. The low-speed pellet trapped substantial mitochondria (Table 1). Of the total spheroplast lysate enzyme activities, 24% of the P5C dehydrogenase and 36% of the citrate synthase activities were found in the low-speed pellet fraction. Only 5 to 10% of the cytoplasmic activities were found there. Similar results were reported by Jauniaux et al. (6). For the purposes of comparison, the enzyme activities found in the decanted lysate were used as 100% activity.

Glucose 6-phosphate dehydrogenase was found in the cytoplasmic fraction as expected, with 4% of the activity contaminating the particulate pellet. P5C reductase behaved in a similar manner, in all attempts, with 90 to 96% of the activity in the cytoplasmic fraction.

Citrate synthase activity was distributed 80 to 85% in the particulate fraction, with 15 to 20% found in the cytoplasmic fraction presumably owing to the breakage of mitochondria in the course of preparation. P5C dehydrogenase be-

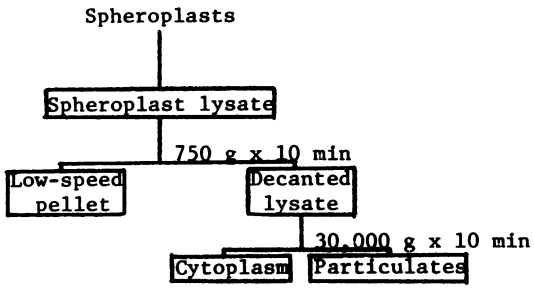

FIG. 1. *Scheme of the fractionation procedure.*

TABLE 1. *Subcellular distribution of enzyme activities in proline-grown cells*

Enzyme	Total activity[a] (% of decanted lysate)				
	Spheroplast lysate	Low-speed pellet	Decanted lysate	Cytoplasm	Particulates
G6P dehydrogenase	3,913	422	3,386 (100)	3,497 (103)	152 (4)
P5C reductase	7,203	422	6,270 (100)	5,778 (92)	985 (16)
Citrate synthase	3,165	1,132	2,322 (100)	551 (24)	1,879 (81)
P5C dehydrogenase	2,032	490	1,423 (100)	109 (8)	636 (45)

[a] Measured as nanomoles of product formed per minute.

haved like a particulate enzyme, in that recovered enzyme was 85 to 90% particulate. However, in every fractionation experiment, almost 50% of the enzyme activity was never recovered. This phenomenon was investigated further and is discussed below.

A similar fractionation experiment was performed with the wild-type strain grown on ornithine as the sole nitrogen source. The results are given in Table 2 and are identical to those of Table 1. The existence of a second P5C dehydrogenase activity specific to the arginine degradative pathway and therefore inducible by ornithine was not found.

Isopycnic density gradient analysis. A 20 to 75% sorbitol gradient was loaded with the particulate fraction derived from the wild-type strain grown on proline as the sole nitrogen source. The mitochondrial marker enzyme citrate synthase and P5C dehydrogenase had identical distributions in the gradient (Fig. 2). The density of the peak fraction was 1.198 g/ml, a density which has been associated with yeast mitochondria (6, 11). Here, too, the recovery of the P5C dehydrogenase was only 46%, whereas that of the citrate synthase was 73%.

Effect of additional centrifugations on P5C dehydrogenase. An enzyme loosely associated with the mitochondrion might be sensitive to additional manipulation of the particulate pellet, resulting in loss of enzyme activity into the cytoplasmic fraction. To test this, the particulate pellet was subjected to an additional 30,000 × *g* centrifugation, and the total activity of P5C dehydrogenase in the pellet as well as that released into the supernatant was compared with the activity found in the pellet after only one high-speed centrifugation (Table 3). When compared with the release of 5 to 7% of the citrate synthase activity, 18 to 20% of the P5C dehydrogense activity was released with the additional manipulation. This result suggests that P5C dehydrogenase is a mitochondrial enzyme but even more loosely associated than citrate synthase, which, as a matrix enzyme, is known to be less tightly bound than inner membrane enzymes (6).

TABLE 2. *Subcellular distribution of enzyme activities in ornithine-grown cells*

Enzyme	Total activity[a] (% of decanted lysate)		
	Decanted lysate	Cytoplasm	Particulates
G6P dehydrogenase	3,545 (100)	3,537 (100)	84 (2)
P5C reductase	6,684 (100)	6,592 (99)	309 (5)
Citrate synthase	2,702 (100)	441 (16)	2,110 (78)
P5C dehydrogenase	1,199 (100)	145 (12)	490 (41)

[a] Nanomoles of product formed per minute.

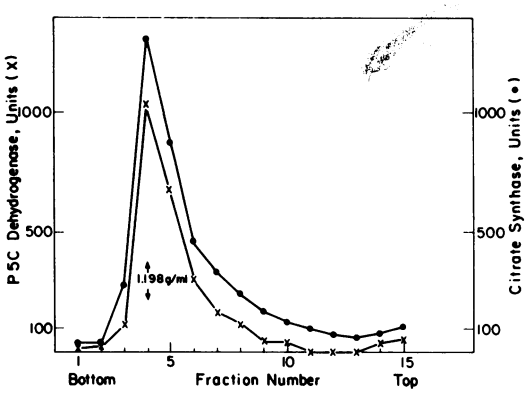

FIG. 2. *Isopycnic sorbitol density gradient centrifugation of a particulate fraction. Experimental details are given in the text. Symbols: ○, citrate synthase; ×, P5C dehydrogenase. One enzyme unit corresponds to 1 nmol of product formed per min. Total activities in the particulate fraction loaded on the gradient were: citrate synthase, 5,861; P5C dehydrogenase, 5,663. Recovery of total activity was (activities layered on the gradient = 100%): citrate synthase, 73%; P5C dehydrogenase, 46%. The density was determined with a Bausch and Lomb Abbe-3L refractometer.*

Loss of P5C dehydrogenase activity. In the typical fractionation experiment, the particulate fraction was resuspended in lysis solution containing sorbitol, EDTA, and bovine serum albumin. The P5C dehydrogenase activity measured in this fraction was repeatedly about 50% of the activity found in the decanted lysate (also suspended in lysis solution). Lines 1 and 3 of Table 4 show this effect. When the particulates

were resuspended in the same cytoplasm from which they had just been fractionated, no loss of enzyme activity was seen (Table 4, line 4). Furthermore, the cytoplasmic fraction which had been "desalted" by passage over a G25 Sephadex column was also capable of preventing the loss of enzyme activity (Table 4, line 5). This suggests, but does not prove, that a macromolecular component in the cytoplasm may stabilize the mitochondrial P5C dehydrogenase activity. It is still possible that, with this technique, there may be up to 4% contamination of small molecules still present, which could be sufficient to prevent the loss of enzyme activity. This finding appears to be specific to the P5C dehydrogenase since citrate synthase activity was unaffected (Table 4, lines 1 to 5).

Mixing experiments were performed to determine the location of the stabilized P5C dehydrogenase activity. In the first experiment, the particulate fraction derived from proline-grown cells was mixed with the cytoplasmic fraction derived from ammonia-grown cells. Conversely, the particulate fraction of ammonia-grown cells was mixed with the cytoplasmic fraction of proline-grown cells. Enzyme activity found in these reconstituted mixtures was compared with that in the decanted lysates of both ammonia-grown and proline-grown cells (Table 5). The cytoplasmic fraction derived from ammonia-grown cells was capable of restoring the full P5C dehydrogenase activity found in the decanted lysate from proline-grown cells. However, the cytoplasmic fraction taken from proline-grown cells did not contribute significantly more enzyme activity to that which was present in the particulate pellet of ammonia-grown cells.

In the second mixing experiment, both wild-type and P5C dehydrogenase-deficient (*put2*) strains were grown in a medium containing arginine as the sole nitrogen source. (Arginine will induce P5C dehydrogenase since it is degraded to form proline; a *put2* strain cannot grow on proline as the sole nitrogen source.) Particulate and cytoplasmic fractions were prepared from each culture. The *put2* cytoplasm was mixed with the wild-type particulate fraction, and the wild-type cytoplasm was mixed with the *put2* particulate fraction. The *put2* cytoplasm stabilized the wild-type particulate enzyme activity (Table 5) in a manner similar to that found in the ammonia-proline mixing experiment. Similarly, the wild-type cytoplasm did not contribute any additional P5C dehydrogenase activity to the *put2* particulate fraction. The four- to five-fold decrease in enzyme activity displayed by this *put2* mutant is characteristic and is low enough to prevent proline utilization (2). The P5C dehydrogenase activities were lower in experiment 2 than in experiment 1, since arginine induces P5C dehydrogenase activity about one-

TABLE 3. *Effects of additional high-speed centrifugation on P5C dehydrogenase activity*

Expt	Enzyme	Enzyme activity[a] after:		Activity released	% released
		One spin	Two spins		
1	Citrate synthase	5,200	4,600	280	5.4
	P5C dehydrogenase	1,900	1,400	390	21
2	Citrate synthase	3,500	3,400	260	7.4
	P5C dehydrogenase	1,400	960	250	18

[a] Nanomoles of product formed per minute.

TABLE 4. *Loss and reconstitution of P5C dehydrogenase activity*

Fraction	P5C dehydrogenase		Citrate synthase	
	Total activity[a]	%	Total activity[a]	%
Decanted lysate	1,266	100	1,724	100
Cytoplasm	67	5	212	12
Particulates in lysis solution	625	49	1,634	95
Particulates in original cytoplasm	1,277	101	1,763	102
Particulates in "desalted" cytoplasm	1,390	110	1,724	100

[a] Nanomoles of product formed per minute.

TABLE 5. *Localization of reconstituted P5C dehydrogenase activity*

Expt	Condition	Total activity[a] of P5C dehydrogenase
1[b]	Decanted lysate, proline-grown cells	942
	Decanted lysate, ammonia-grown cells	55
	Particulates (proline) + cytoplasm (ammonia)	871
	Particulates (ammonia) + cytoplasm (proline)	183
2[c]	Decanted lysate, *PUT*$^+$ cells	350
	Decanted lysate, *put2* cells	93
	Particulates (*PUT*$^+$) + cytoplasm (*put2*)	330
	Particulates (*put2*) + cytoplasm (*PUT*$^+$)	71

[a] Nanomoles of product formed per minute.

[b] The strain used was the wild type, MB1000 α *PUT*$^+$.

[c] The strains used were MB1000 α *PUT*$^+$ and MB380-13B α *put2-57*. Both were grown in a minimal medium containing 0.1% arginine hydrochloride as the sole nitrogen source.

half as well as does proline (3).

On the basis of these experiments, we conclude that P5C dehydrogenase is a mitochondrial enzyme which requires for its full expression an element (possibly a macromolecule) in the cytoplasm. This cytoplasmic element is present in cells grown on ammonia and is, therefore, not proline inducible. It is also present in the cytoplasm of a *put2* mutant and, therefore, is likely to be unrelated to P5C dehydrogenase.

DISCUSSION

On the basis of experiments reported here and previously (3), we postulate that proline degradation in yeast cells occurs in the mitochondrion. Proline oxidase activity depends on a functional electron transport system; respiratory-deficient yeast cells cannot utilize proline as a nitrogen source and have undetectable levels of proline oxidase under conditions in which the wild-type level is high (3). Unfortunately, the activity of this enzyme is highly unstable and at present can only be measured in permeabilized whole cells. This fact precludes more extensive examination of its subcellular location. However, in mammals, proline oxidation is a mitochondrial function (7), and in *E. coli* proline is oxidized by a membrane-bound flavoprotein (12).

We have shown by fractionation experiments and an equilibrium density gradient that P5C dehydrogenase behaves in a manner identical to that of citrate synthase, an enzyme of the mitochondrial matrix. Although 50% of the P5C dehydrogenase activity was lost in the fractionation procedure, the loss could be prevented by the presence of a cytoplasmic fraction (either the original cytoplasm, one derived from wild-type cells grown on ammonia as the sole nitrogen source, or one derived from P5C dehydrogenase-deficient cells grown on arginine as the sole nitrogen source). The nature of this stabilization is not understood at the present time.

Proline oxidase converts proline to P5C inside the yeast mitochondria, which forms a substrate pool of P5C for the P5C dehydrogenase to convert to glutamate.

Proline oxidase and P5C dehydrogenase are encoded by nuclear genes (2). Since the majority of nuclear-encoded mitochondrial proteins are cytoplasmically translated (13), both proline-degrading enzymes are probably translated in the yeast cytoplasm and secreted into the mitochondrion.

Proline can be formed from the degradation of arginine (3) or by de novo synthesis from glutamate (1). Arginase and ornithine transaminase were shown to be cytoplasmic enzymes (6), generating a pool of P5C in the cytoplasm. We have shown here that P5C reductase, the last enzyme of proline biosynthesis, is also cytoplasmic, capable of converting P5C derived from ornithine or from glutamate into proline.

Therefore, the cell contains two pools of P5C, which, on the basis of the behavior of proline-nonutilizing yeast mutants (3), do not mix. P5C dehydrogenase does not have access to ornithine-derived P5C, even in cells lacking P5C reductase activity, and can act only on mitochondrial P5C.

Yeast cells have solved the regulatory problem of the futile cycling between proline and P5C by physically separating into different compartments the enzymes of proline biosynthesis and of proline degradation which share the same substrate. Proline needed for protein synthesis is generated cytoplasmically. To utilize the nitrogen in proline, it must be transported into the mitochondrion for degradation.

Regulation by subcellular compartmentation has been described in several other pathways of lower eucaryotic organisms. In both *S. cerevisiae* and *Neurospora crassa*, the enzymes which function early in arginine biosynthesis are mitochondrial (5, 6), whereas the degradative enzymes are cytoplasmic. In the biosynthesis of the branched-chain amino acids leucine, isoleucine, and valine, the four isoleucine-valine biosynthetic enzymes, as well as the first enzyme unique to leucine biosynthesis, are mitochondrial (10, 11). In these systems, it has been hypothesized that such organization is useful to avoid futile cycling between biosynthetic and degradative pathways as well as a means to accumulate a pool of a necessary intermediate.

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