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Subcellular Localization of the Leucine Biosynthetic Enzymes in Yeast[1]

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When baker's yeast spheroplasts were lysed by mild osmotic shock, practically all of the isopropylmalate isomerase and the β-isopropylmalate dehydrogenase was released into the 30,000 × *g* supernatant fraction, as was the cytosol marker enzyme, glucose-6-phosphate dehydrogenase. α-Isopropylmalate synthase, however, was not detected in the initial supernatant, but could be progressively solubilized by homogenization, appearing more slowly than citrate synthase but faster than cytochrome oxidase. Of the total glutamate-α-ketoisocaproate transaminase activity, approximately 20% was in the initial soluble fraction, whereas solubilization of the remainder again required homogenization of the spheroplast lysate. Results from sucrose density gradient centrifugation of a cell-free particulate fraction and comparison with marker enzymes suggested that α-isopropylmalate synthase was located in the mitochondria. It thus appears that, in yeast, the first specific enzyme in the leucine biosynthetic pathway (α-isopropylmalate synthase) is particulate, whereas the next two enzymes in the pathway (isopropylmalate isomerase and β-isopropylmalate dehydrogenase) are "soluble," with glutamate-α-ketoisocaproate transaminase activity being located in both the cytosol and particulate cell fractions.

In recent years, the spatial organization of metabolic systems within the (eukaryotic) cell has received increased attention. It has been shown that, in addition to the well-known intracellular organization of catabolic systems, a number of enzymes involved in biosynthetic pathways are localized in organelles. For example, in *Neurospora crassa*, a group of four enzymes involved in the biosynthesis of isoleucine and valine appear to be localized in the mitochondrial matrix (3), and analysis of a mutant with an altered dihydroxy acid dehydratase (1) has suggested that incorporation of this enzyme into mitochondria is essential for in vivo synthesis of valine and isoleucine. In other work, evidence has been presented, again with *N. crassa* (summarized in reference 2), indicating that the maintenance of two pathway-specific pools of carbamyl phosphate can be explained by a spatial separation of carbamyl phosphate synthetase A-ornithine transcarbamylase (arginine pathway) from carbamyl phosphate synthetase P-aspartate transcarbamylase (pyrimidine pathway).

We previously described some properties of α-isopropylmalate (α-IPM) synthase from yeast (12), the first specific enzyme for the biosynthesis of leucine in this and other organisms. In a continuing effort to characterize this enzyme and its relation to the regulation of leucine biosynthesis, we have now studied the intracellular localization of α-IPM synthase as well as of the other enzymes of the leucine pathway. Of the three leucine pathway-specific enzymes, i.e., α-IPM synthase, IPM isomerase, and β-IPM dehydrogenase, only α-IPM synthase appears to be particle-bound.

MATERIALS AND METHODS

Organism. Commercially grown baker's yeast (Budweiser yeast, grown by Anheuser-Busch, Inc., St. Louis, Mo.) was used throughout.

Chemicals. Sodium α-ketoisovalerate, sodium α-ketoisocaproate, sodium oxaloacetate, dipotassium D-glucose-6-phosphate, β-mercaptoethylamine·hydrochloride, nicotinamide adenine dinucleotide, oxidized form, nicotinamide adenine dinucleotide phosphate, oxidized form, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid, 5,5′-dithiobis-(2-nitrobenzoate), sorbitol, and cytochrome *c* (horse heart type III) were obtained from Sigma Chemical Co., St. Louis, Mo. Glusulase (snail digestive juice) was purchased from Endo Laboratories, Garden City,

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N.Y. Bovine serum albumin was a product of Pentex Inc., Kankakee, Ill. Racemic β-IPM was obtained from Reef Laboratories, Santa Paula, Calif. Coenzyme A (CoA) (lithium salt, "chromatopure") was obtained from P-L Biochemicals, Milwaukee, Wis. Acetyl-CoA was synthesized from CoA and acetic anhydride by the method of Simon and Shemin (11). All other chemicals were of the best grade available.

Enzyme assays. All enzymes were assayed optically, using either a Zeiss PMQII or a Gilford model 240 spectrophotometer coupled to a Sargent SRLG recorder. α-IPM synthase was assayed at pH 7.2 by the 5,5′-dithiobis-(2-nitrobenzoate) method as described by Ulm et al. (12). Citrate synthase was assayed by the same method but with oxaloacetate used in place of α-ketoisovalerate. IPM isomerase was assayed by the method of Gross (7); β-IPM dehydrogenase by the method of Parsons and Burns (9); glucose-6-phosphate dehydrogenase by the method of Noltmann et al. (8); glutamate-α-ketoisocaproate transaminase by the method of Collier and Kohlhaw (4); and cytochrome oxidase by the method of Wharton and Tzagoloff (13). Enzyme activity is expressed in units of micromoles of product formed per minute per milliliter.

Preparation of yeast spheroplasts. Spheroplasts were prepared by a modification of the method of Duell, Inoue, and Utter (5). Yeast cells were washed five times with distilled water and incubated in four volumes (wt/vol) of 0.4 M 2-mercaptoethylamine plus 0.04 M ethylenediaminetetraacetate for 16 hr at 30 C on a gyro-rotary table. The cells were then washed twice with distilled water and suspended in four volumes (wt/vol) of a solution containing 1.2 M sorbitol, 0.1 M citrate-potassium phosphate buffer (pH 5.8), 0.03 M 2-mercaptoethylamine, and 0.001 M ethylenediaminetetraacetate. At a noted time, 0.1 ml of undiluted glusulase solution was added per gram of cells, and the suspension was gassed with nitrogen and incubated at 30 C on a gyro-rotary table. Spheroplast formation was estimated by differential counting of samples diluted 1:100 with either distilled water (yields whole cells only) or 1.2 M sorbitol (yields whole cells plus spheroplasts), using a Petroff-Hausser cell counter. After 90 min of incubation, 80 to 90% of the cells were converted to spheroplasts. The spheroplasts (and remaining whole cells) were washed twice with 1.2 M sorbitol, and the pellet obtained was normally disrupted immediately as outlined below. Alternatively, the spheroplast pellet could be stored at 4 C for several days without adverse effect.

Spheroplast disruption. A combination of osmotic shock and mechanical agitation was found to be the most effective means of rupturing the spheroplasts prior to subcellular fractionation. The spheroplast pellet was suspended in 8 volumes (wt/vol) of a solution containing 0.45 M sorbitol, 0.05% bovine serum albumin, and 0.2 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid, and homogenized at half maximum speed with a Virtis homogenizer. The effect of homogenization time is described in Results (see also Fig. 1). All procedures were normally performed at 4 C. However, the cold-labile enzyme β-IPM dehydrogenase was rapidly inactivated at this temperature, and thus replicate experiments were performed at room temperature (23 C) to monitor this enzyme. Spheroplasts were also disrupted by passing the suspension through a French pressure cell twice at 1,500 lb/in², and enzyme levels in the resulting supernatant solution were used as the 100% reference point.

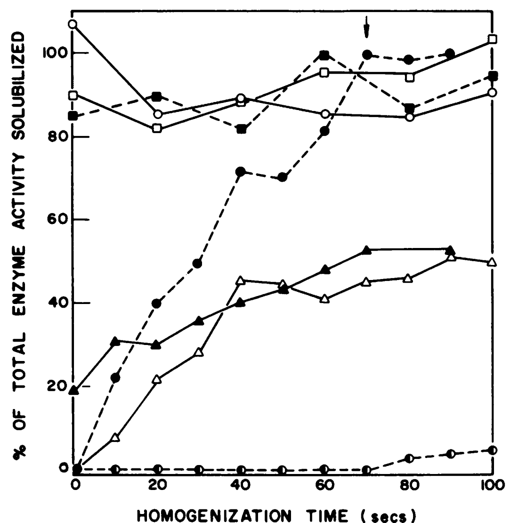

FIG. 1. *Effect of homogenization on release of the enzymes IPM isomerase (□), β-IPM dehydrogenase (○), glucose-6-phosphate dehydrogenase (■), citrate synthase (●), α-IPM synthase (△), glutamate-α-ketoisocaproate transaminase (▲), and cytochrome oxidase (◐) from a spheroplast lysate. Dashed curves represent the marker enzymes. The lysate was obtained by mild osmotic shock, as described in Materials and Methods, and was agitated with a Virtis homogenizer operated at half-maximal speed. Samples of 1 ml were removed at zero time, i.e., before homogenization, and also after each 10-s period of homogenization. Triton X-100 (final concentration, 0.03%) was added after 70 s of homogenization (arrow) to aid solubilization. All samples were then centrifuged at 30,000 × g for 20 min. The resulting supernatant solutions were used as enzyme source. Enzyme activities were measured immediately after processing and are expressed as percentage of the activities obtained when spheroplasts were disrupted using the French pressure cell. The reference (100%) levels, expressed in micromoles of product formed per minute per gram of yeast cells, of the leucine pathway enzymes were as follows: α-IPM synthase, 1.06; IPM isomerase, 0.33; β-IPM dehydrogenase, 0.89; and glutamate-α-ketoisocaproate transaminase, 17.25.*

Differential centrifugation. Spheroplasts were disrupted as outlined above, using a 30-s homogenization period. Whole cells, cell debris, nuclei, etc. were removed by repeated centrifugation at 2,000 × *g*. When no further precipitate was obtained at this *g* value, the supernatant solution was centrifuged at 30,000 × *g* for 20 min, and the resulting pellet was gently suspended in 30% (wt/vol) sucrose, usually 1 ml per 2 g of starting material. Samples (0.5 ml) of this suspension were layered on 4.0 ml of 30 to 70% linear

sucrose gradients. The gradients contained 0.01 M KCl plus 0.01 M tris(hydroxymethyl)aminomethane-phosphate buffer (pH 7.2) throughout. Centrifugation was for 2 h at 20,000 rpm in a Beckman-Spinco SW-65 Rotor ($g_{average}$ = 28,620). The particles had reached their equilibrium position at this time, since essentially identical results were obtained with a 1-h centrifugation period. The centrifuge tubes were punctured, and each gradient was divided into approximately 20 fractions of 15 drops each. Before assay, 200 μliters of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2) and 10 μliters of 10% Triton X-100 was added, and each fraction was sonically treated for 2 s, using a Branson sonifier equipped with a small tip.

RESULTS

Effect of homogenization time on enzyme solubilization. The osmotic shock of transferring from a 1.2 M to a 0.45 M sorbitol solution caused complete spheroplast lysis as determined by light microscopy. However, only three of the enzymes investigated here were solubilized by this treatment, the remainder requiring some degree of mechanical agitation in addition. Thus, as shown in Fig. 1, almost the entire IPM isomerase and β-IPM dehydrogenase content of the spheroplasts, together with the cytosol marker enzyme glucose-6-phosphate dehydrogenase, were present in the supernatant fraction at zero time. On the other hand, α-IPM synthase and the mitochondrial marker enzymes cytochrome oxidase and citrate synthase were absent initially but were progressively released into the supernatant fraction by mechanical homogenization of the spheroplast lysate. It is evident that cytochrome oxidase was much more tightly bound to the particulate fraction than was citrate synthase, whereas α-IPM synthase was solubilized at a rate intermediate between the two marker enzymes. None of these three latter enzymes were solubilized when the spheroplast lysate was allowed to stand for several hours.

It is noteworthy that the activity of α-IPM synthase appeared to level off after about 50% had been released. This was not due to inactivation of the enzyme, since subsequent passage through the pressure cell resulted in total recovery of the activity.

With respect to glutamate-α-ketoisocaproate transaminase, Fig. 1 indicates that 20% of the total activity of this enzyme was present in the initial soluble fraction of the lysate, while the remainder was progressively released by homogenization. In terms of micromoles of product formed per minute per milliliter, the transaminase was much more active than the other enzymes of the leucine pathway (see legend of Fig. 1), although it should be realized that the conditions employed to assay the enzymes may not have been optimal in all cases.

To learn more about the nature of the particle with which α-IPM synthase appeared to be associated, a differential centrifugation was performed, followed by a density gradient centrifugation.

Enzyme profiles after sucrose density gradient centrifugation. Figure 2 illustrates the enzyme profiles obtained after a sucrose density gradient separation of the 30,000 $\times$ g pellet. α-IPM synthase activity appeared as a distinct peak at density 1.19 g/cm^3 and corresponded closely with the mitochondrial marker enzymes cytochrome oxidase and citrate synthase. Care had to be taken to very gently resuspend the 30,000 $\times$ g pellet, since significant amounts of α-IPM synthase and citrate synthase activities were found at the top of the gradient, presumably solubilized from damaged mitochondria, unless shearing forces were avoided. IPM isomerase or β-IPM dehydrogenase were not detected in the particulate pellet applied to the gradient nor in any gradient fraction.

DISCUSSION

The results presented here show that the first specific enzyme in the pathway leading to leucine, α-IPM synthase, is particulate. The two arguments presently available in favor of a mitochondrial organization of α-IPM synthase

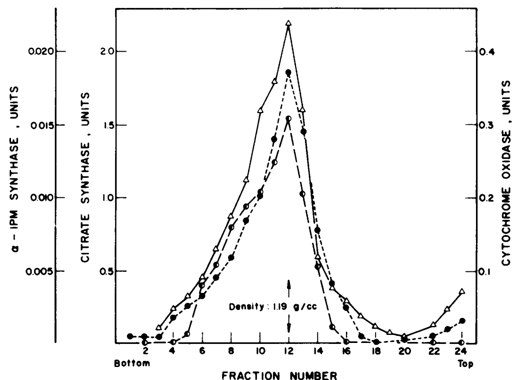

FIG. 2. Isopycnic sucrose density gradient centrifugation of a resuspended 30,000 × g pellet. Experimental details are given in Materials and Methods section. Symbols: Δ, α-IPM synthase; ●, citrate synthase; ◐, cytochrome oxidase. One enzyme unit corresponds to 1 µmol of product formed per min per ml. Recovery of the activities was as follows (activities layered on the gradient = 100%): α-IPM synthase, 82%; citrate synthase, 85%; cytochrome oxidase, 100%. The density was determined with a Bausch and Lomb Abbe-3L refractometer.

are: (i) the cosedimentation of mitochondrial markers like cytochrome oxidase and citrate synthase, and (ii) a density of 1.19 g/cm^3 at the peak position of α-IPM synthase in the sucrose gradient.

The two enzymes following α-IPM synthase in the leucine biosynthetic pathway were found in the supernatant fraction after mild osmotic lysis of the spheroplasts. Whereas this suggests that they belong to the "soluble" fraction of the cell contents, it does not rule out a very loose in vivo attachment to some particle. This, however, is true for every "soluble" enzyme.

Glutamate-α-ketoisocaproate transaminase activity was found in both the particulate and the soluble fractions. A branched-chain amino acid transaminase has been described by Cassady et al. (3) as part of a four enzyme cluster localized in the mitochondrial matrix of *N. crassa*. A similar situation might exist in yeast. Nevertheless, the amount of transaminase present in the cytosol would appear more than sufficient to allow completion of leucine synthesis in this compartment.

The question arises as to what benefit the yeast cell might derive from a particulate organization of the first, but not of the subsequent enzymes of the leucine pathway. If the isomerase and the β-IPM dehydrogenase were induced by α-IPM, a mechanism which is apparently operative in the leucine biosynthesis in *N. crassa* (6) and which has been discussed as a possibility for the regulation of leucine pathway enzymes in yeast (10), a separate production of α-IPM could be useful, since it would provide a means for a more effective accumulation of α-IPM. Such accumulation would be difficult to achieve with all enzymes in the same compartment, particularly if one assumes that, in vivo, the formation of α-IPM is the rate-limiting step of the pathway. Although there is no conclusive evidence yet for an induction of the isomerase and the β-IPM dehydrogenase by α-IPM in yeast, there is some evidence that these two enzymes are regulated in a manner different from the regulation of α-IPM synthase. For example, the intracellular level of both the isomerase and the dehydrogenase is decreased by excess leucine, while under the same conditions the level of the α-IPM synthase activity increases (10).

It should be noted that all experiments described in this report were performed with commercially grown baker's yeast. It is, therefore, of interest that Brown and Umbarger, using *Saccharomyces* sp. strain 60615 of the Lindegren collection, have also observed that at least part of the α-IPM synthase activity is particulate, whereas β-IPM dehydrogenase is not (H. Brown, personal communication). Further pertinent information is expected to come from a determination of the subcellular distribution of the leucine pathway enzymes under various well-defined growth conditions, including anaerobic growth which should provide cells lacking functional mitochondria.

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