Two Forms of Acetoacetyl Coenzyme A Thiolase in Yeast

II. INTRACELLULAR LOCATION AND RELATIONSHIP TO GROWTH*

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SUMMARY

Two forms of acetoacetyl-CoA thiolase with different properties have been found in Saccharomyces cerevisiae. The enzyme with isoelectric point pH 5.3 (thiolase 5.3) is found in the cytosol while that with isoelectric point pH 7.8 (thiolase 7.8) occurs in the mitochondria. When yeast are grown on glucose under aerobic conditions, thiolase activity shows two increases in activity during the growth period. Early in log phase, there is an increase in thiolase activity which subsequently reaches a plateau level. A second increase in activity at mid-log phase attains a peak value before decaying in late log to stationary phase.

The biphasic curve is also observed during anaerobic growth on glucose or aerobic growth on galactose, indicating that removal of catabolite repression by glucose is unrelated to the rise in activity during midlog phase growth. A method for distinguishing the activities of both forms was developed. The early log phase activity contains predominantly thiolase 7.8, while the midlog phase activity is due to thiolase 5.3. Growth on galactose, which provides higher activity at mid-log phase, indicates increased mitochondrial development. Thiolase 5.3 was not affected. The possibility that thiolase controls the level of acetoacetyl-CoA and acetyl-CoA, negative and positive effectors of pyruvic carboxylase, is discussed.
spheroplasts were diluted with 30 ml of 0.6 M mannitol, 0.1 M EDTA, pH 7.2, and centrifuged. The pellet was washed with 150 ml of the same medium and suspended in 15 ml of 0.25 M mannitol, 5 mM EDTA, 50 mM Tris, pH 7.2. The spheroplasts were disrupted by homogenization (24 strokes) in a Potter Elvehjem homogenizer. Mannitol (1 M) was added to bring the concentration to 0.6 M.

The homogenate was centrifuged for 5 min at 800 x g and the pellet discarded. The supernatant solution was then centrifuged at 9,000 x g for 10 min. The pellet was resuspended in 0.6 M mannitol, 1 mM EDTA, 20 mM Tris, pH 7.2, centrifuged again at 1,000 x g for 5 min followed by centrifugation at 9,000 x g for 10 min. The pellet sedimenting between 1,000 x g and 9,000 x g is referred to as the mitochondrial fraction. The supernatant solution from the original 9,000 x g centrifugation was centrifuged at 48,000 x g for 30 min. The 48,000 x g supernatant solution is referred to as the cytosol.

Small quantities of yeast were available for studies involving the time course of thiolase appearance during the early portion of the growth curve. Samples of yeast harvested from the microfermentor weighed less than 2.5 g. These samples were diluted to 5 ml with water, 0.5 ml of toluene was added, and the samples were then incubated at 37° with shaking. Autolysis was allowed to take place for 3.5 hours and was stopped by centrifugation of the remaining whole cells and cell debris.

All other methods used were described in the first paper in this series (1).

**RESULTS**

**Intracellular Location**—Middleton and Apps (2) surveyed subcellular fractions of yeast for acetoacetyl-CoA thiolase and found activity in both the cytosol and mitochondrial fractions. They did not determine whether the enzymatic activities were caused by the same enzyme.

Cytoplasmic and mitochondrial protein were subjected to isoelectric focusing as shown in Fig. 1. Thiolase was released from the mitochondrial fraction only after sonication. The mitochondrial fraction (about 25 mg of protein), suspended in 6 ml of 50 mM Tris, pH 8.2, was sonicated for 30 sec with a Branson Sonifier at full power. After that time thiolase activity was no longer pelleted by centrifugation for 10 min at 9,000 x g; in addition there was a 50% increase in the total thiolase activity.

Thiolase 5.3 is the dominant species in yeast cytosol (Fig. 1A). The ratio of thiolase 5.3 to thiolase 7.8 in the cytosol preparation is 5.4, whereas the same yeast, when lysed with toluene followed by electrofocusing, resulted in a thiolase 5.3 to thiolase 7.8 ratio of 1.7 (1). Fig. 1B shows quite clearly that the only thiolase activity associated with the yeast mitochondrial fraction is thiolase 7.8. Thiolase 5.3, except for a small amount of contamination, is absent from the mitochondrial fraction.

Cross-contamination of the subcellular fractions was investigated using cytochrome oxidase as a mitochondrial marker and glucose 6-phosphate dehydrogenase as a cytosol marker. We were not able to detect any contamination of washed mitochondria with cytosol in our best preparation. Similarly, the mitochondrial fraction (not shown) contained no detectable thiolase 5.3. The cytosol, after centrifugation at 48,000 x g for 30 min, contained no cytochrome oxidase activity. As can be seen in Fig. 1A this fraction contains only a trace amount of thiolase 7.8.

**Occurrence of Acetoacetyl-CoA Thiolase during Yeast Growth**—Preliminary experiments on the occurrence of thiolase showed that the total thiolase specific activity varied throughout the growth of the strain. The time course of thiolase activity is shown in Fig. 2. Thiolase activity was measured by the method of Kornblatt and Rudney (3). Inoculum was centrifuged, and lysed under standard conditions. Thiolase specific activity (units per mg of protein) refers to the specific activity of the lysates.

![Fig. 1. Thiolase activity of subcellular fractions of Saccharomyces cerevisiae ATCC 9896. Fractions were prepared by the method of Middleton and Apps (2). Curve A, a K15/30 column containing pH 3 to pH 10 ampholytes was loaded with 20 mg of protein containing 1500 units of thiolase. Isoelectric focusing proceeded for 72 hours at 130 volts, 0.2 watts and 4°. The recovery of units was 70%.](http://www.jbc.org/)
growth period. Growth conditions were standardized in a New Brunswick microfermentor. Fig. 2 shows the growth curve for S. cerevisiae ATCC 8896 grown aerobically on glucose. Plotted in the same figure is the thiolase activity of yeast lysates determined during the growth experiment. The biphasic growth curve is characteristic of catabolite repression (6). The total specific activity of the thiolase is not constant throughout the growth experiment. After an initial period of 3 hours during which the enzyme is barely detectable, the specific activity rises to a level of 40 to 80 units per mg of protein. This value remains constant until about midlog phase of the fermentative portion of growth and will be referred to as early enzyme. At about midlog phase there is a second increase in specific activity which reaches a peak value of about 150 units per mg of protein. This peak occurs at the end of the log phase (13 hours) and will be referred to as peak enzyme. The specific activity then decreases with further growth.

**Relationship of Growth Status to Appearance of Early and Peak Enzymes**—Yeast, when grown aerobically on glucose, do not show much respiratory activity as long as the glucose is present (7). In addition they show few complete mitochondrial structures and respiratory activity. Prior to the exhaustion of glucose from the growth medium the yeast grow fermentatively even though oxygen is present. The first exponential portion of the growth curve in Fig. 2 represents fermentative growth while the second exponential portion represents oxidative growth. Yeast, when grown anaerobically, do not synthesize complete mitochondria but do elaborate promitochondria (8). The latter structures contain some mitochondrial enzymes but not others (10). Promitochondria appear to be more fragile than mitochondria when visualized with the electron microscope (9).

By growing yeast anaerobically, it should be possible to determine whether or not the appearance of peak enzyme is due to the conversion of promitochondria into mitochondria or de novo mitochondrial synthesis. This was done and the results shown in Fig. 3. The growth curve is typical of fermentatively grown yeast. Interestingly, the thiolase activity curve of Fig. 3 is substantially the same as the comparable curve in the aerobic experiment of Fig. 2. It must be concluded, therefore, that the appearance of the peak enzyme is not due to mitochondrial synthesis.

The inhibition of the expression of mitochondrial enzymes by glucose is referred to as catabolite repression. It is responsible for the lack of respiratory activity of yeast grown on glucose. If the expression of the peak enzyme is due to a release from catabolite repression, growth of the organism on galactose (which is much less effective than glucose in causing catabolite repression (11)) should result in substantially different thiolase activity curve during growth. Yeast were grown on galactose and thiolase activity was determined. The results are shown in Fig. 4. It is clear that the over-all shape of the thiolase activity curve is similar to the glucose curves shown in Figs. 2 and 3. It is important to note that the peak enzyme activity still occurs and therefore is not a function of catabolite repression.

An interesting feature of the thiolase activity curve of the galactose-grown yeast is the fact that the early enzyme activity is higher than in the glucose grown yeast as shown in Figs. 2 and 3. Since yeast harvested in early log phase on galactose contained well developed mitochondria, the increased early enzyme activity seen in Fig. 4 was probably due to the thiolase present in these structures. Similarly the early enzyme activity of glucose grown cells seen in Figs. 2 and 3 was probably due to thiolase present in promitochondria.

It is possible to inhibit both promitochondrial and mitochondrial development by growing yeast in the presence of high concentrations of α-threochloramphenicol (12), an inhibitor of mitochondrial protein synthesis. When the yeast were grown in the presence of the antibiotic, there was a total inhibition of the expression of early enzyme (Fig. 5). The expression of peak enzyme was not affected. It must be concluded that it is early enzyme that is associated with the promitochondria and
mitochondria and not peak enzyme. The cause for the late appearance of the peak enzyme is not yet known.

Type of Thiolase in Early and Peak Enzyme Activity—Isoelectric focusing was performed on both early and peak enzyme activities. No activity was detected after isoelectric focusing of the early enzyme; this point will be dealt with later. Isoelectric focusing of the peak enzyme showed that at least 90% of the activity was thiolase 5.3.

In the preceding paper, thiolase 7.8 was shown to use thiol donors other than CoA much more efficiently than thiolase 5.3 (1). Thiolase 7.8 uses dithiothreitol 69% as effectively as CoA. Thiolase 5.3, on the other hand, uses dithiothreitol only 11% as effectively as CoA. This fact was used to probe the nature of the thiolase occurring at different points in the growth cycle. The ratio of thiolase activity using dithiothreitol as substrate to thiolase activity using CoA as substrate was determined for a number of synthetic mixtures of thiolase 5.3 and thiolase 7.8. The dithiothreitol to CoA ratio was shown to vary linearly with the percentage of each thiolase. The dithiothreitol to CoA ratios were then determined in the anaerobic growth experiment, as shown in Table I. From these ratios we were able to determine the percentage of each thiolase by comparison with the known standards. It can be seen in Table I that the majority of the early enzyme is thiolase 7.8. The peak enzyme tends to be almost exclusively thiolase 5.3.

To further substantiate the view that thiolase 7.8 is the early enzyme, samples of thiolase taken from the peak enzyme in the growth curves of glucose-aerobic and glucose-chloramphenicol experiments were subjected to isoelectric focusing. The results are shown in Fig. 6. The chloramphenicol-treated yeast contain no thiolase 7.8, whereas the yeast grown without chloramphenicol do contain it. Although the percentage of thiolase 7.8 in the untreated yeast is small, the value, being 4-fold greater than the limit of detection, is significant. It should be emphasized that the chloramphenicol-treated yeast contain absolutely no detectable thiolase 7.8 in this experiment. Except for the chloramphenicol-treated yeast, we have not yet observed a peak enzyme preparation that did not contain thiolase 7.8. Chloramphenicol had no effect on activity when added directly to either thiolase 5.3 or 7.8.

As mentioned earlier, attempts had been made to isoelectrically focus the early enzyme but no activity was recovered on eluting the column. If thiolase 7.8 is the early enzyme and it

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**Fig. 5.** Thiolase activity during growth on glucose medium containing 4 mg per ml of D(-)-threochloramphenicol. Yeast were grown aerobically in a New Brunswick microfermentor. They were harvested and lysed under standard conditions. **Thiolase Specific Activity** (units per mg of protein) refers to the specific activity of the lysates.

**Fig. 6.** Isoelectric focusing of thiolase from yeast grown with and without chloramphenicol in the growth medium. D(-)-Threochloramphenicol concentration was 4 mg per ml. Recovery of units in the presence of chloramphenicol was 68%, and without chloramphenicol 40%.

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**Table I**

| Hours after onset of growth | 3 | 4 | 5.5 | 6.5 | 7.5 | 9.25 | 10.5 | 12.75 |
|----------------------------|---|---|-----|-----|-----|------|------|-------|
| Total thiolase specific activity | 10 | 24 | 40  | 46  | 33  | 150  | 190  | 79    |
| Ratio of dithiothreitol to CoA | 0.53 | 0.40 | 0.50 | 0.12 | 0.17 | 0.17 |
| Calculated specific activity for thiolase 5.3 | 11 | 22.5 | 11 | 150 | 190 | 71 |
| Calculated specific activity for thiolase 7.8 | 29 | 22.5 | 22 | 19 | 8 |
TABLE II

| pH  | units | pH  | units |
|-----|-------|-----|-------|
| 4.75| 0     | 4.55| 0     |
| 5.0 | 58    | 4.80| 0     |
| 5.22| 0     | 5.10| 40    |
| 5.45| 0     | 5.25| 0     |
| 5.80| 0     | 5.50| 0     |
| 6.15| 0     | 5.85| 0     |
| 6.50| 0     | 6.21| 0     |
| 6.85| 0     | 6.48| 0     |
| 7.20| 0     | 6.85| 0     |
| 7.55| 0     | 7.21| 0     |
| 7.88| 0     | 7.68| 0     |
| 8.12| 0     | 8.12| 0     |

was inactivated or degraded on the column by proteolytic enzymes, then addition of authentic thiolase 7.8 to early enzyme followed by focusing of the mixture should result in total degradation or inactivation of the added thiolase. This experiment was carried out and the results are presented in Table 11.

Of the 400 units of early thiolase added to the column, all but 15% of the enzyme was inactivated. This 15% eluted at the position of thiolase 5.3. In the experiment in which authentic thiolase 7.8 was added to early enzyme extract, all of the added thiolase 7.8 was inactivated plus 90% of the early enzyme. The results of this experiment explain why no thiolase 7.8 is seen when the early enzyme is subjected to isoelectric focusing.

**Discussion**

In *Saccharomyces cerevisiae* acetocetyl-CoA thiolase is found in both the cytosol and mitochondria (2). The mitochondrial form and the cytosol form are not the same (Fig. 1). Cytosol thiolase has an isoelectric point of 5.3, mitochondrial thiolase an isoelectric point of 7.8.

When yeast are grown on glucose, thiolase activity is not constant throughout the growth period (Fig. 2). Prior to midlog phase, the specific activity of the thiolase is 40 to 80 units per mg (early enzyme). After midlog phase the specific activity increases to about 150 units per mg (peak enzyme). We attempted to determine what causes this rapid increase in thiolase activity.

We reasoned that the increase in thiolase activity might be associated with either mitochondrial development or release from catabolite repression. Therefore, yeast were grown under conditions that would prevent the conversion of promitochondria into mitochondria (Fig. 3) or prevent catabolite repression from occurring (Fig. 4). The results of these two experiments demonstrated unequivocally that the appearance of peak enzyme was not related to mitochondrial development or catabolite repression. When the organisms are grown on galactose, the specific activity of the early enzyme was higher than when growth took place on glucose. This indicated that the early enzyme might be associated with both promitochondria and mitochondria.

Since it appeared that the early enzyme was associated with promitochondria, the yeast were grown in the presence of n-(-)-thiocholoramphenicol. This antibiotic prevents mitochondrial and promitochondrial protein synthesis both in vivo (12) and in *vivo* (13). If the early enzyme were really present in the promitochondria, growth of the yeast on media containing chloramphenicol might inhibit the appearance of the early enzyme. Fig. 5 shows this to be the case. When all early enzyme activity was abolished (Fig. 5), chloramphenicol had no effect on peak enzyme. We conclude that it is highly likely that the early enzyme is associated with both promitochondria and mitochondria, and that the peak enzyme activity is probably present in the cytosol.

It is not necessary to postulate that early enzyme is synthesized by the mitochondrial genetic apparatus, although this is a possibility. It is just as likely that a component of the mitochondrial protein is necessary for inclusion of early enzyme in the mitochondrion. In this case, thiolase which was synthesized on the cytoplasmic ribosomes could not enter or attach to the mitochondrion and might be particularly susceptible to degradation. A third possibility is that chloramphenicol inhibits the mitochondrial synthesis of a protein which is required to initiate some cytoplasmic protein synthesis. Since thiolase 7.8 is found in the mitochondria (Fig. 1), and since it is not found in chloramphenicol-treated yeast (Fig. 5), it is quite likely that it corresponds to early enzyme.

It was not possible to show by isoelectric focusing that early enzyme and thiolase 7.8 were the same. All of the evidence directly indicating that the early enzyme is thiolase 7.8 is provided by enzymatic assays based on the differential response of the true enzymes to thiol donors such as CoA and dithiothreitol. It is clearly shown in Table I that the activity in the early enzyme is primarily due to thiolase 7.8 while that in the peak is thiolase 5.3. Attempts to isolate the early enzyme activity by electrofocusing were unsuccessful. This is probably due to proteolytic enzymes expressed during early growth since addition of purified thiolase 7.8 to early growth extracts also resulted in destruction of activity (Table II).

It is likely that yeast contain several proteolytic enzymes, and that the particular proteases present are determined by the growth phase, among other things. Manney (14) has shown yeast tryptophan synthetase is susceptible to proteolysis and that the sensitivity of this enzyme to degradation is a function of growth. He has also shown that different proteases are present during each growth phase. A similar system may be present in the yeast strain used in this study. Here, as mentioned earlier, there would be an additional complication in that mitochondrial protein may protect thiolase 7.8 from degradation late in growth, but not early when there are fewer mitochondria. All of the preceding evidence supports the conclusion that the early enzyme is predominantly thiolase 7.8.

On the basis of studies reported here and the properties of the two forms of acetocetyl-CoA thiolase reported in the preceding paper (1) it seems reasonable to propose physiological roles for the thiolase as outlined in Fig. 7. In most instances definitive
information is unavailable concerning the direction and mode of transport of metabolites through the mitochondrial membrane and these pathways are designated with a question mark. The positive and negative effector roles of acetyl-CoA and acetoacetyl-CoA are indicated by broken lines; a question mark indicating that the effect remains to be studied.

Klein (15) has shown that the major part of yeast nonesterifiable lipid biosynthesis comprised of primarily sterols and isoprenoid compounds occurs on the endoplasmic reticulum in the cytosol fraction of the cell. Fatty acid synthesis by the fatty acid synthetase complex studied by Lynen (16) also occurs in the cytosol. The exclusive location of thiolase 5.3 in the cytosol indicates that it is the enzyme involved in the biosynthesis of sterols and isoprenoids by supplying acetoacetyl-CoA as a substrate for the HMG-CoA synthase. The kinetic properties of thiolase 5.3 are such as to facilitate this requirement. The high Michaelis constants for both substrates and substrate inhibition by both acetoacetyl-CoA and free CoA suggest that this enzyme is "designed" to operate in the direction of acetoacetyl-CoA synthesis (17). It also exhibits extreme specificity for both substrates (1), which may indicate the anabolic function of this enzyme.

The role of thiolase 7.8 in mitochondrial metabolism is more difficult to discern, but it is reasonable to assume that it functions in regulating the levels of acetyl-CoA and acetoacetyl-CoA originating from fatty acid catabolism and that it functions in both acetyl-CoA and acetoacetyl-CoA synthesis. The exceptionally low K_m for CoA, coupled with the absence of substrate inhibition at the concentrations tested (1) points to the likelihood that the enzyme operates primarily in the direction of acetyl-CoA formation. Kinetic constants of mitochondrial enzymes should be considered cautiously since Munkres and Woodward (18) have shown that the association of mitochondrial protein with mitochondrial enzymes may alter the kinetic constants. The mitochondrial location of the thiolase 7.8 would tend to establish a link between energy production via the tricarboxylic acid cycle and the glyoxylate bypass sequence of reactions. Thiolase 7.8 may also function in isoprenoid biosynthesis. Middleton and Apps (2) have found that HMG-CoA synthase, although primarily located in the cytosol, also occurs in mitochondria.

It has long been known that acetyl-CoA is a positive effector for the mitochondrial enzyme pyruvic carboxylase (19). This enzyme plays a key role in gluconeogenesis by being the first enzyme in the series which generates phosphoenolpyruvate. More recently it was shown by Fung and Utter (4) that acetoacetyl-CoA is a negative effector for pyruvic carboxylase of chicken liver; indeed, acetoacetyl-CoA abolished the cooperative effect due to acetyl-CoA. HMG-CoA is also a negative effector, but does not appear to influence the cooperativity. In yeast, acetyl-CoA is also a positive effector for pyruvic carboxylase (19); the effects of acetoacetyl-CoA and HMG-CoA, however, have not yet been studied. Approximately 95% of pyruvate carboxylase in yeast is located in the cytosol. It would appear then that the cytosol enzyme thiolase 5.3 catalyzes the reversible conversion of both positive and possibly negative pyruvate carboxylase effectors. The role of thiolase in the regulation of gluconeogenesis, as well as the significance of the sharp rise in activity in late log phase growth, deserves further study. The same comment applies to the role of acetoacetyl-CoA thiolase in controlling the intracellular concentration of acetyl-CoA and acetoacetyl-CoA in animal systems.

1 The abbreviation used is: HMG, β-hydroxy-β-methylglutaryl.

2 M. F. Utter, personal communication.
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