Derepression of Citrate Synthase in *Saccharomyces cerevisiae* May Occur at the Level of Transcription

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Pulse-chase labeling in whole cells and cell-free protein synthesis were used to establish that the mitochondrial enzyme citrate synthase is made as a larger precursor in *Saccharomyces cerevisiae*. A 54,000 *M*<sub>r</sub> precursor form appeared to be a primary translation product since it could be labeled with N-[<sup>35</sup>S]formylmethionine in vitro. The induction of citrate synthase was monitored in *S. cerevisiae* cells grown on fermentable (glucose) and nonfermentable (ethanol and glycerol) carbon sources. The amount of citrate synthase activity and immune-reactive protein increased more than 15-fold as *S. cerevisiae* cells entered the stationary growth phase on glucose-containing medium. This increase was paralleled by an increase in translatable RNA for the enzyme. When cells were grown on a nonfermentable carbon source, no increase in either citrate synthase or its mRNA was detected. The results suggest that the release of citrate synthase from catabolite repression may occur at the level of transcription.

The level of mitochondrial enzymes is finely tuned to the energy demands of the cell. In bakers' yeast (*Saccharomyces cerevisiae*), the synthesis of respiratory chain components and enzymes of the citric acid cycle is repressed in the absence of oxygen or in the presence of glucose (9, 18, 24, 25). The reverse of this process, the derepression of mitochondrial enzymes, is most easily monitored spectroscopically by the reappearance of cytochromes, but the synthesis of many achrionic mitochondrial enzymes is also stimulated by the removal of glucose from the culture medium or by the introduction of air to an anaerobic culture. Recent evidence suggests that synthesis of iso-1-cytochrome c in *S. cerevisiae* is regulated at the stage of transcription by the availability of heme (10, 30).

We have studied the induction of citrate synthase (citrate oxaloacetate lyase, EC 4.1.3.7) during the growth of yeast cells in the presence of glucose. In *Neurospora crassa*, this enzyme is made in the cytoplasm as a larger precursor which is processed to a smaller mature form as it is imported into the mitochondrion (12). Preliminary reports suggest that a similar mode of synthesis and transport occurs in *S. cerevisiae* (2). Like many other yeast mitochondrial proteins (21), citrate synthase is made primarily on “free” polyribosomes in the cytoplasm and is transported into the organelle independently of protein synthesis (28). Our interest in this enzyme was stimulated by the report (25) that citrate synthase activity can be derepressed in *S. cerevisiae* even in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis. This result may reflect the presence of a pool of latent enzyme whose activity can be induced by the appropriate stimulus.

We have begun by investigating whether the derepression of citrate synthase occurs at the level of transcription. We report that the amount of translatable RNA which encodes a precursor form of citrate synthase increases as the enzyme level is induced.

**MATERIALS AND METHODS**

* *S. cerevisiae* strains and growth conditions. Two haploid *S. cerevisiae* strains were employed in this study. These were D273-10B (MATa, ATCC 25657) and D273-10B/1a, a rho<sup>-</sup> strain derived from D273-10B by ethidium bromide mutagenesis. For mitochondrial isolation, cells were grown on medium consisting of 1% yeast extract, 1% Bacto-Peptone, and 1% galactose. For growth curves, either 1% glucose or 1% each of glycerol and ethanol were used as carbon sources. For pulse-labeling of *S. cerevisiae* strains with L-[<sup>35</sup>S]methionine, cells were cultured on the sulfate-limiting medium described by Nelson and Schatz (20). Strains were grown to mid-log phase at 28°C for 15 to 17 h with vigorous rotary shaking.

**Purification of citrate synthase.** Citrate synthase was purified from isolated *S. cerevisiae* mitochondria by affinity elution chromatography (5, 19), using a modification of the method of Davies and Scopes (5). We replaced the carboxymethyl cellulose chromatography with a Biorex 70 column in the same buffer system. Nearly all of the loaded citrate synthase activity was bound to the column, and no activity could be measured in either of the wash fractions. Final specific activity of the isolated enzyme was greater than 1,400 U/mg of protein and represented a 150-fold purification over the crude lysate. Enzyme units are defined as micromoles of reduced coenzyme A formed per minute and were assayed by the method of Parvin (22).

Antibodies to citrate synthase were prepared in rabbits and were the generous gift of G. Schatz of the Biocenter of the University of Basel, Switzerland. To demonstrate their specificity, we used a sensitive immune-replica technique (see below), testing the antisera with purified citrate synthase and whole-cell lysates of *S. cerevisiae*. The antibody reacted with only one protein band, corresponding to the purified enzyme (data not shown).

**Pulse-labeling of yeast cells.** Pulse-labeling of *S. cerevisiae* cells was performed as described by Nelson and Schatz (20). Cells were grown overnight in low-sulfate medium with 0.3% galactose as a carbon source until mid-log phase, harvested by centrifugation, and converted to spheroplasts with Zymolyase 5000. Spheroplasts were then regenerates to restore protein synthetic capacity in low-sulfate medium containing 1.2 M sorbitol as osmotic buffer and 0.3% galactose as carbon source. Labeling was initiated by the addition of 100 μCi of L-[<sup>35</sup>S]methionine to 5 ml of *S. cerevisiae* cells suspended at 100 mg (wet weight) per ml. Incorporation proceeded for 15 min at 12°C and was halted by the addition of Casamino Acids (casein hydrolysate) to a final concentra-
tion of 1%. The time course of incorporation of radioactive methionine into proteins was followed by precipitation of samples of the cell suspension with trichloroacetic acid. The yeast cells were incubated for an additional 60 min after the addition of unlabeled amino acids. At 5-min intervals, beginning with the end of pulse labeling (15 min), portions of 1 ml were removed from the cell suspension and were rapidly injected into tubes containing 0.1 ml of 25% (wt/vol) sodium dodecyl sulfate (SDS) and 10 μl of 0.1 M ortho-phenanthroline at 100°C. Samples were boiled for 2 min. After cooling, phenylmethylsulfonyl fluoride (1 mM) and aprotinin (10 U) were added.

Mitochondrial isolation and cell-free import of mitochondrial proteins. Mitochondria were isolated from S. cerevisiae spheroplasts by differential centrifugation as previously reported (16). Precursors to mitochondrial proteins were synthesized in a rabbit reticulocyte lysate protein synthesis system (23) in the presence of L-[35S]methionine. Protein synthesis was directed by LiCl-fractionated S. cerevisiae RNA (17). Reactions were terminated by the addition of phenylmethylsulfonyl fluoride (1 mM) and aprotinin (10 U/ml) and by centrifugation at 140,000 g for 45 min at 4°C. In vitro protein synthesis in the presence of L-[35S]methionyl tRNAf was performed as described previously (16). Purified yeast initiator tRNA was donated by P. Sigler of the University of Chicago. For import reactions, lysates were filtered through Sephadex G-25 columns equilibrated with import buffer (0.6 M mannitol, 150 mM KCl, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.4], 1 mM MgCl2). The incubation mixture for importation of labeled proteins into mitochondria contained 160 μl of reticulocyte lysate (representing ca. 5 × 10^6 cpm in protein-bound ^35S), 200 μg of mitochondrial protein, 1 mM ATP, 5 mM phosphoenolpyruvate, 1 mM dithiothreitol, and 4 U of pyruvate kinase in a volume of 400 μl. To monitor protease resistance of imported proteins, 100 μg of trypsin per ml was added to the import reaction, which was then incubated for an additional 20 min on ice. After the import incubation, protease inhibitors were added as above, and mitochondria were isolated by centrifugation. They were washed once by centrifugation in 0.6 M mannitol–20 mM HEPES-KOH (pH 7.4), suspended in the same buffer, and lysed by the addition of SDS to 2.5% and boiling for 3 min. Supernatants from import incubations were boiled for 3 min in the presence of 2.5% SDS. To ensure comparable efficiency of immunoprecipitation between mitochondrial and supernatant fractions, 200 μg of mitochondrial protein was added to each supernatant fraction as it was dissociated with detergent.

Immunofluorescence techniques. After dissociation of radiolabeled samples in SDS, samples were diluted 15-fold in TNET buffer (1% Triton X-100, 0.15 M NaCl, 2 mM EDTA, 50 mM Tris-hydrochloride [pH 8]). Antibody specific for the citrate synthase was added to each diluted sample, and the mixture was allowed to react overnight at 4°C. Facilitated immunoprecipitation with glutaraldehyde-fixed Staphylococcus aureus cells was performed as described previously (15, 17). Radiolabeled citrate synthase standard was prepared by immunoprecipitation of extracts of continuously labeled S. cerevisiae cells as described previously (17). Proteins were separated on polyacrylamide gels in the presence of SDS by the procedure of Douglas et al. (8). The presence of radiolabeled proteins was detected by impregnating gels with 1 M sodium salicylate before drying and exposure to Kodak XAR-5 medical X-ray film at −80°C (3). Immunoreplicas were prepared by transferring S. cerevisiae proteins separated on SDS-polyacrylamide gels to sheets of nitrocellulose and reacting with antiserum by the procedure of Tobin et al. (29).

Materials. Yeast extract, Bacto-Peptone, and Casamino Acids were obtained from Difco Laboratories, Detroit, Mich. Zymolyase 5000 is marketed in the United States by Miles Laboratories, Inc., Elkhardt, Ind., which also provided the peroxidase-conjugated goat antiserum which recognizes rabbit immunoglobulin G. L-[35S]Methionine was bought from Amersham Corp., Arlington Heights, Ill., and ^35SO4 was obtained from New England Nuclear Corp., Boston, Mass. BDH reagents were used for electrophoresis. Biorex 70 is a product of Bio-Rad Laboratories, Richmond, Calif. Other reagents, fine chemicals, and enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., or from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

RESULTS

Citrate synthase is made as a larger precursor in vivo. When S. cerevisiae cells, grown overnight in the presence of ^35SO4, were lysed and immunoprecipitated with antibody specific for S. cerevisiae citrate synthase, a single radioactive protein was obtained and visualized by autoradiography (Fig. 1). The molecular weight of this protein was 52,000.

![FIG. 1. A 54,000 M protein, which may be a precursor of citrate synthase in vivo. Respiratory-competent (rho+) and respiratory-deficient (rho-) S. cerevisiae strains were pulse-labeled as described in the text. Unlabeled amino acids were added at 15 min (arrow); samples were drawn and processed for immunoprecipitation at the times indicated. The percentage of immunoprecipitated citrate synthase present as the larger form was determined by densimetric tracings of the autoradiogram on a Joyce-Loebell microdensitometer. Tracings were made with several exposures of the film to assure linearity of exposure time with band intensity. Standard (Std) was immunoprecipitated from S. cerevisiae cells labeled overnight with H235SO4.](http://mcb.asm.org/)

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corresponding to the size of purified enzyme monomer. In contrast, when wild-type *S. cerevisiae* cells were labeled for short periods with \(^{35}\)S-methionine, two species of immune-reactive citrate synthase were seen in autoradiograms of SDS-polyacrylamide gels. One band corresponds to the mature enzyme subunit, and the other is ca. 2,000 M\(_r\) larger. Upon a subsequent chase by continued incubation of labeled cells with excess unlabeled amino acids, the larger form of the enzyme disappeared, whereas the relative radioactivity of the mature subunit increased. No additional incorporation of \(^{35}\)S into protein could be detected during this chase period. If a [rho⁻] *S. cerevisiae* strain was labeled by the same protocol, the larger form of immune-reactive citrate synthase was observed long into the chase period, and a trace amount was detected even after 20 min of incubation in unlabeled amino acids.

These results suggest that in *S. cerevisiae*, just as in *N. crassa*, citrate synthase is made as a larger precursor. More precursor form is observed in [rho⁻] cells relative to [rho⁺] at all time points. The rate of conversion of precursor to mature form is approximately the same in both cell types (Fig. 1), indicating that during the initial 10 min of labeling, relatively less of the precursor is competent for processing in [rho⁻] cells. A similar observation for the F\(_1\) ATPase \(\beta\) subunit in *S. cerevisiae* has been made (A. S. Lewin, D. K. Norman, and L. J. Wells, in F. Kaudewitz and R. Schweyen, ed., *Mitochondria*, in press).

**Cell-free synthesis of a precursor to citrate synthase.** A larger form of citrate synthase was observed when specific antiserum was used to precipitate proteins made in a rabbit reticulocyte lysate programmed with *S. cerevisiae* RNA in the presence of L-\(^{35}\)S-methionine. In this case, a labeled protein band ca. 2,000 M\(_r\) larger than the mature enzyme standard and an additional protein slightly smaller than the mature citrate synthase monomer were seen (Fig. 2). We believe the lower band to be a proteolytic fragment of citrate synthase, because a one-dimensional V8 protease “fingerprint” of this band was very similar to the fingerprints of the precursor band and of the mature enzyme precipitated from continuously labeled cells (data not shown) (4). In addition, the precursor of the mature form of citrate synthase can be converted to this smaller form by a partial cleavage reaction with trypsin (25 \(\mu\)g/ml, 20 min, 0°C).

Both forms of immune-reactive citrate synthase made in vitro could be labeled when \(N\)-\(^{35}\)S-formylmethionyl initiator tRNA was used as the sole source of radioactivity in the reticulocyte lysate (Fig. 2). Since excess cold methionine was included in this reaction, no labeling of proteins with free methionine from deacylated tRNA was expected. Since both bands were labeled, it is likely that the smaller protein immunoprecipitated with citrate synthase-specific antisera is derived from the larger by a C-terminal proteolytic cleavage. However, the faster-migrating protein contained less radioactivity when labeled with trypsin or added as charged initiator tRNA, whereas the bands were of equal intensity when carrier-free methionine was employed. This suggests some amino-terminal degradation as well. The extent of cleavage varies between individual protein synthesis reactions.

When precursor to citrate synthase made in vitro was incubated with isolated *S. cerevisiae* mitochondria, some of the 54,000 M\(_r\) form was converted to mature 52,000 M\(_r\) form and was found associated with the mitochondrial pellet after centrifugation and washing of the membranes (Fig. 3, lane 1). Some of the 54,000 M\(_r\) precursor was also associated with the pellet fraction. This band was sensitive to added prote-
FIG. 4. Import of citrate synthase. Import requires an energized inner mitochondrial membrane. Lane 1, Mitochondrial fraction after normal import reaction as in Fig. 5A; lane 2, supernatant fraction after import; lane 3, mitochondrial fraction after import reaction in the presence of 10 μM carbonyl cyanide m-chlorophenylhydrazone; lane 4, supernatant after import reaction in the presence of carbonyl cyanide m-chlorophenylhydrazone.

ase, whereas the mature citrate synthase band was protected from proteolysis (Fig. 3, lane 3). Precursor and partially degraded forms of the enzyme which remained in the supernatant after the import incubation were completely sensitive to added trypsin (Fig. 3, lanes 2 and 4). This experiment demonstrated that the larger form of immune-reactive citrate synthase observed in vitro can be processed to the mature form of the protein. This maturation step accompanies transport of the protein into mitochondria, rendering the mature protein resistant to added trypsin. Much of the precursor form remains in the supernatant, and the conversion of larger form to processed form is poor relative to similar experiments with other mitochondrial proteins (16). This may reflect the protease lability of citrate synthase or, simply, more stringent import requirements of this protein. The partially degraded form remains in the supernatant, indicating that the conformation of the protein may be altered by C-terminal proteolysis in a way that prevents import.

The import of citrate synthase into mitochondria requires an energized mitochondrial membrane (Fig. 4). Lanes 1 and 2 of Fig. 4 show citrate synthase immunoprecipitated from the pellet and supernatant fractions after the importation of labeled proteins into isolation mitochondria. Mature protein is found in the pellet, whereas most precursor remains in the supernatant. In lanes 3 and 4, the uncoupler carbonyl cyanide m-chlorophenylhydrazone was added to an analogous import reaction. In this case, conversion of the precursor to the mature form was completely inhibited, although some precursor form adhered to the mitochondria pellet. The inhibitor had no effect on the proteolytic artifact, and the partially degraded form of citrate synthase remained in the supernatant. Carbonyl cyanide m-chlorophenylhydrazone probably prevents the importation of citrate synthase and other mitochondria proteins into the organelle by eliminating the proton electrochemical gradient across the mitochondrial inner membrane (20, 21, 26).

FIG. 5. Citrate synthase activity. Activity increased dramatically during release from glucose repression but remained at a constant intermediate level when cells were cultured on glycerol and ethanol. (A) Growth curve of [rho⁻] S. cerevisiae cells grown in 1% glucose. Cell density (number of cells per milliliter of culture) was determined with a Neubauer hemacytometer (X). At the times indicated, samples were removed and assayed for citrate synthase activity (O). (B) Same procedures and symbols as in (A), but cells were grown in medium containing 1% glycerol and 1% ethanol.
Citrate synthase activity and protein derepressed during growth on glucose-containing media. The activities of Krebs cycle enzymes are induced as yeast cells are grown to stationary phase on glucose-containing media or when yeast are shifted from high-glucose medium to derepressing conditions (24). We have grown S. cerevisiae on medium containing either 1% glucose or 1% each of glycerol and ethanol as carbon sources. Samples were extracted at various points along the growth curve and assayed for citrate synthase-specific activity and for immunoreactive citrate synthase. Some cells removed from the culture were used to prepare total translatable RNA, using repeated organic extractions and LiCl fractionation. The induction of enzyme levels during growth on glucose is depicted in Fig. 5 and 6.

During growth of S. cerevisiae cells on glucose (Fig. 5A), the level of citrate synthase activity was observed to increase 16-fold, from 13 to 213 U/mg of protein beginning late in exponential growth phase. In contrast, cells grown in ethanol-glycerol-containing medium showed no induction in enzyme level during growth phase. Rather, activity remained at an intermediate level (~100 U/mg) at the time points assayed (Fig. 5B).

Because an increase in specific activity can reflect enzyme activation rather than increase in enzyme amount, we used a sensitive immunochemical method (29) to measure enzyme levels in the whole-cell homogenates prepared from cells drawn at successive stages of growth (Fig. 6). These results correspond to those shown in Fig. 5: the amount of citrate synthase antigen increased during growth of S. cerevisiae on glucose but remained at a constant level during growth on nonfermentable carbon sources. Note, however, that detection of citrate synthase by immune replica is not quantitative and only provides an estimate of whole-cell levels of the protein.

Derepression of citrate synthase is attributable to an increase in translatable RNA. RNA was extracted from cells at the same time points for which immunoassays were made (see above) and translated in the reticulocyte lysate protein synthesis system. Samples equal in terms of protein-bound [35S]methionine were then dissociated with detergent and reacted with citrate synthase antiserum as described above. The resulting precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). As the culture grown on glucose reached mid-exponential phase, the level of RNA which could be translated into immune-reactive citrate synthase increased (Fig. 7A), whereas the level of translatable mRNA for citrate synthase remained fairly constant in the cells grown in ethanol-glycerol-containing medium (Fig. 7B). RNA extracted from both cultures was of greater structural integrity at early growth stages than in late exponential and stationary-phase cultures. This probably accounts for the fact that more citrate synthase was immunoprecipitated from cells grown in glucose for 12 h (lane 3) than from later samples of cells. Similarly, RNA from the last sample of derepressed yeast (Fig. 7B, lanes 4 and 5) directed less enzyme synthesis than early samples. This experiment suggests that the increase in enzyme level during growth on glucose medium is due to new enzyme synthesis. This new synthesis may be attributable to new transcription of the citrate synthase gene or to RNA processing resulting in the increased availability of citrate synthase mRNA.

**DISCUSSION**

The first conclusion we have drawn, that citrate synthase is made in bakers’ yeast as a larger precursor which is cleaved to its active form in a process associated with its transport into mitochondria, was expected based on previously published data (2, 12, 28). However, the means that we used to establish this result directly contradicts claims made in one of these studies. Namely, we successfully labeled citrate synthase with [35S]methionine or H35SO4 in living cells and in a cell-free protein synthesis system. Alam et al. (2) were unable to do this and suggested that citrate synthase contains no sulfur-containing amino acids. That this protein

**FIG. 6.** Induction of citrate synthase due to the synthesis of new enzyme. Samples of cells from the cultures shown in Fig. 5 were homogenized with glass beads, extracted with SDS, and separated on 10% SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose sheets and reacted with citrate synthase antibodies. A 70-μg sample of protein was loaded into each well except the standard, which contained less than 1 μg of purified enzyme. Lane 1 corresponds to a sample drawn at 7 h from the glucose culture; lane 2, 10 h; lane 3, 12 h; lane 4, 15 h; lane 5, 17 h. Glycerol-ethanol aliquots were drawn at 10, 16, 18, 22, and 28 h, corresponding to lanes 1 to 5, respectively. (Decreased reaction in lane 2 of the glycerol-ethanol cells is attributable to uneven transfer of protein to that portion of membrane.)

**FIG. 7.** Increase in citrate synthase levels accompanied by an increase in translatable RNA for citrate synthase. RNA was prepared from S. cerevisiae cells drawn from cultures grown on either glucose or glycerol and ethanol. RNA was then translated in a reticulocyte lysate protein synthesis system in the presence of [35S]methionine, and the products were immunoprecipitated with antibody directed against citrate synthase. (A) Lanes 1 to 5. RNA samples prepared from the glucose-grown cells removed from culture at the times indicated in Fig. 6; lane 6 (arrow), mature citrate synthase standard from labeled S. cerevisiae cells. (B) Lanes 1 to 5. RNA samples prepared from cells drawn from the ethanol-glycerol culture at times listed in Fig. 6; lane 6 (arrow), mature citrate synthase standard.
of 52,000 $M_r$ has no methionine or cysteine would be surprising, especially considering that the same enzyme isolated from other organisms contains these residues (27). We isolated our enzyme by affinity chromatography and determined that our antibody reacts specifically with citrate synthase (data not shown). Since the antibody does precipitate a $^{35}$S-labeled protein of the same size as citrate synthase from labeled cells, we suggest that the enzyme does indeed contain methionine, cysteine, or both. Nevertheless, we draw a conclusion similar to that of Alam et al., i.e., that the enzyme is made in a larger precursor form.

We are the first to demonstrate that the induction of citrate synthase activity during derepression is the result of new enzyme synthesis (Fig. 6). The increase in enzyme level is accompanied by an increase in the amount of translatable RNA for this protein (Fig. 7), which suggests, but does not prove, that the regulation of citrate synthase induction occurs at the level of RNA synthesis. Conceivably, synthesis could also be controlled at a posttranscriptional level. However, this mechanism would have to operate in an analogous manner in both $S$. cerevisiae and reticulocytes to explain our results. We are attempting to isolate a cloned copy of the gene for citrate synthase and to use this as a hybridization probe to monitor RNA levels during release from glucos repression.

The synthesis of several enzymes which are repressed in the presence of glucose has been shown to be regulated at the level of transcription. These include cytochrome $c$ (10, 31), alcohol dehydrogenase (6), and enzymes of the galactose utilization pathway (11, 14). In the CYC1 and GAL10 genes, DNA sequences more than 100 nucleotides upstream (5') from the transcriptional initiation site are deemed important to the control of transcription. Similarly, upstream regulation sites have been recognized for genes encoding amino acid biosynthetic enzymes under the general control system in $S$. cerevisiae (1, 7, 13, 30). We intend to examine the regulation of citrate synthase transcription and determine whether similar DNA sequence elements can be identified.

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LITERATURE CITED

