Mutants of Saccharomyces cerevisiae Defective in
sn-Glycerol-3-phosphate Acyltransferase

SIMULTANEOUS LOSS OF DIHYDROXYACETONE PHOSPHATE ACYLTRANSFERASE INDICATES A
COMMON GENE*

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Fourteen independent mutants of Saccharomyces cerevisiae defective in sn-glycerol-3-phosphate acyltransferase activity were isolated using a colony autoradiographic screening technique. All 14 mutants were similarly defective in dihydroxyacetone phosphate acyltransferase activity. The mutations were recessive and fell into a single complementation group. Tetrad analysis gave results consistent with mutations in a single nuclear gene affecting both activities. sn-Glycerol-3-phosphate acyltransferase activity from different mutant strains exhibited different substrate dependencies and differing responses to temperature, detergent, and pH. In each case, the response of the dihydroxyacetone phosphate acyltransferase activity was similar to that of the sn-glycerol-3-phosphate acyltransferase. These results are consistent with the mutations occurring in the structural gene. The data also establish that the predominant dihydroxyacetone phosphate acyltransferase activity in yeast is a second activity of the sn-glycerol-3-phosphate acyltransferase.

In yeast, as in other eukaryotes, the first committed step of glycerolipid biosynthesis is the formation of lysophosphatidic acid. This occurs either by direct acylation of sn-glycerol-3-phosphate (glycerol-P), or by acylation of dihydroxyacetone phosphate (DHAP) to form acyl-DHAP followed by reduction to lysophosphatic acid. The number and roles of the different acyltransferases catalyzing these reactions, and the relative contributions of these two pathways to overall glycerolipid biosynthesis remain unclear (1, 2). These membrane-bound enzymes have proven difficult to solubilize and purify, and kinetic analysis is complicated by the physical association of the enzyme with the membrane and the physical properties of the acyl-CoA substrate.

In mammalian systems there appear to be at least three triose phosphate acyltransferase activities: a peroxisomal DHAP acyltransferase, a mitochondrial glycerol-P acyltransferase, and a microsomal activity capable of acylating both glycerol-P and DHAP (3). On the basis of kinetic and inhibition studies the microsomal glycerol-P acyltransferase activity from rat liver, brain, kidney, intestinal mucosa, lung, and placenta were inferred to represent activities of a single enzyme (3-7). Each triose phosphate was found to competitively inhibit the acylation of the other. The inhibition profiles for both enzymes were identical with respect to N-ethylmaleimide, heat, trypsin, and detergents. Working with highly purified rat liver microsomes, Declerq et al. (8) also found that the microsomal DHAP acyltransferase was inhibited by glycerol-P and N-ethylmaleimide, and showed a similar pH profile and palmitoyl-CoA dependence to the glycerol-P acyltransferase.

In contrast, Hajra and co-workers (9, 10) reported that while the acylation of glycerol-P is inhibited by DHAP in microsomes from rat liver, brain, and other tissues, DHAP acyltransferase activity was only slightly and non-competitively inhibited by glycerol-P. The two acyltransferase activities were found to have completely different responses to N-ethylmaleimide, heat, trypsin, and detergents (9, 10). The reason for these differing results is not apparent.

In previous work from our laboratory, mutants of Escherichia coli defective in glycerol-P acyltransferase (11) permitted the structural gene for this enzyme to be cloned (12) and the enzyme to be purified to near homogeneity from an overproducing strain (13). The combined approaches of biochemical and genetics permitted molecular analysis of this enzyme.

Saccharomyces cerevisiae offers the possibility of detailed molecular analysis of this enzyme in a eukaryotic system. Recent progress in the study of other yeast lipid biosynthetic enzymes has been reviewed (14). Glycerol-P acyltransferase, DHAP acyltransferase, and acyl-DHAP reductase were all reported present in yeast total particulate fractions (15-17), indicating that both pathways may operate in yeast. Kinetic and inhibition studies on the glycerol-P and DHAP acyltransferase activities in these fractions indicated that these activities may also be due to the same enzyme (15). The behavior of these activities in the yeast system were strikingly similar to that seen in some mammalian systems.

The goal of our present work was to use a combined biochemical and genetic approach to study the glycerol-P acyltransferase activity of S. cerevisiae. This paper reports the isolation of mutants defective in glycerol-P acyltransferase activity. These mutants provide the first genetic evidence that the same enzyme catalyzes both glycerol-P and DHAP acylation in yeast.

EXPERIMENTAL PROCEDURES

Materials—[1,2,3-3H]Glycerol (900 mCi/mmol) was purchased from New England Nuclear and used to prepare [3H]glycerol-P as described previously (18). [14C]DHAP, prepared as described in Ref. 4, was a gift from L. Ballas (North Carolina State University). Glycerol-P, dihydroxyacetone-P, imidazole (grade III), and bovine serum albumin (essentially fatty acid free) were from Sigma. Palmi-
toyl-CoA was from P-L Biochemicals, Triton X-100 was from Research Products International. Yeast nitrogen base, yeast extract, Bacto-peptone, and agar were from Difco. Fluorographic enhancer was ENHANCE spray from New England Nuclear. All other reagents were reagent grade.

**Strains**—All mutant strains were derived from strains DBY746 (a, ade2-1, ura3-52, trpl-289) and DBY747 (as above, except α), obtained from the Yeast Genetic Stock Center. Strains AH22 (α, leu2-3, leu2-112, his7-519, can1) and SHID5C (a, ade6, ura1) used in the genetic analyses were gifts from C. Styles (Massachusetts Institute of Technology) and V. Letts (Albert Einstein College of Medicine), respectively.

**Media and Genetic Analysis**—Cultures were grown on YPD (1% yeast extract, 2% Bacto-peptone, 2% dextrose), YPG (1% yeast extract, 2% Bacto-peptone, 3% glycerol), or SD (0.67% Yeast nitrogen base, 2% dextrose, with the appropriate supplements). Agar was added to 2% for solid media. For complementation analysis, diploids were constructed from the haploid mutants, and from the mutants mated with either AH22 or SHID5C. These were picked into a grid pattern and assayed by filter paper autoradiography as described below. The diploids were compared to the mutant haploids, and to parental haploids and diploids to determine complementation of the mutant phenotype. Stringency was by the method of Roth and Halvorson (19). Normal four-spore ascii were dissected and analyzed by filter paper autoradiography as described below.

**Mutagenesis**—Strains DBY747 and DBY746 were mutagenized with ethylmethanesulfonate (EMS) as described in Ref. 20 to 0.2% survival. This was 3% EMS at room temperature. The mutagenized cells were diluted into 5% sodium thiosulfate and plated onto YPD to give 200–500 colonies/plate after growth at 25 °C.

**Filter Paper Assay**—The filter paper assay used in this study was modified from that of Bulawa et al. (21). The mutagenized colonies were replica printed onto Whatman No. 42 filter paper cut to fit the Petri dish. After regrowth of the colonies at 25 °C, the plates were stored at 4 °C. The replica prints were dried either by incubating at 37 °C for 60 min or by blowing room temperature air on them for 30 min. The replica prints were then placed colony-side up in a 100-mm-diameter Petri dish containing 1.2 ml of a solution containing 1% sodium metabisulfite system (24) and CHCl3:CH30H:10 N HCl, 87:15:0.5, with cystic acid impregnated plates (25), showed that essentially all of the H-labeled material present in the final chloroform phase of glycerol-P acyltransferase assays migrated as lysophosphatidic acid or phosphatidic acid, while all of the P-labeled material in the final chloroform phase of DHAP acyltransferase assay migrated as acyl-DHAP.

**RESULTS AND DISCUSSION**

**Colonies**—The colony autoradiography method of Raetz (26) was modified to assay the glycerol-P acyltransferase activity of *S. cerevisiae*. Cultures were replica printed onto filter paper, permeabilized by desiccation, and assayed for glycerol-P acyltransferase activity with radiolabeled glycerol-P. Fluorography of the replica prints reflected the radioactivity incorporated into the colonies. The assay was useful over a wide range of colony sizes, although colonies less than 0.5 mm in diameter appeared to incorporate a larger amount of radioactivity than expected for their size. Colonies larger than 5 mm in diameter had a tendency to flake off during the desiccation steps. Between 25 and 40 °C, the temperature of the desiccation step had little effect on the wild type level of incorporation. Preincubation in 1 ml of 0.2 mm palmitoyl-CoA, 0.05% Triton X-100 greatly increased the incorporation of label. This step proved necessary to demonstrate that the incorporation of [3H]glycerol-P was dependent on exogenous palmitoyl-CoA levels. This was observed with a variety of permeabilization conditions, including desiccation, freeze-thaw, detergent, toluene/ethanol, and zymolyase treatments; used separately or in combination. The addition of detergent to the preincubation step decreased the amount of palmitoyl-CoA required for the incorporation of a given amount of label. Inclusion of 20% DL-glycerol-3-phosphate in the precipitation step greatly reduced nonspecific binding of the radiolabeled substrate.

**Preparation of Yeast Total Particulate Fractions**—Cultures were grown in 250 ml of YPG at 30 °C for 8 doubling periods with shaking (300 rpm) to an A600 of about 2.0 (or 5 × 10^7 cells/ml). These were harvested by centrifugation at 1,000 × g for 10 min. All subsequent procedures were at 0–4 °C. The pellets were washed once in isolation buffer (0.6 M sorbitol, 10 mM imidazole, pH 7.4, 1 mM EDTA) and resuspended in enough isolation buffer to exclude air from a 1.5-ml mini-bead beater vial containing 0.75 ml of 0.5-mm glass beads. Cells were broken by 3-min bursts on a Biospec Products mini-bead beater with cooling on ice at the intervals between bursts. The homogenate was transferred to 1.5-ml Eppendorf vials and centrifuged 5 s in a microcentrifuge to remove beads, large debris, and unbroken cells. The pellet was resuspended and centrifuged again. The supernatants were then combined and diluted to 10 ml with isolation buffer. The total particulate fraction was the pellet obtained after centrifugation of the diluted supernatant at 200,000 x g for 60 min. These pellets were resuspended in isolation buffer, homogenized by 5 passes in a glass homogenizer, adjusted to a protein concentration of 3.5 mg/ml and stored as 75-μl samples at −70 °C. Protein determinations were made using the method of Lowry et al. (27), using bovine serum albumin as a standard.

**Enzyme Assays**—Glycerol-P and DHAP acyltransferase activities were assayed using the incorporation of [3H]glycerol-P and [3H]DHAP, respectively, into chloroform extractable material essentially as previously described (10). Assays were performed in 13 × 100-mm screw-capped tubes at 25 °C in a final volume of 100 μl.
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FIG. 1. Palmitoyl-CoA dependence of filter paper assay. Colonies were replica printed onto 4-cm papers, dried, and incubated in 0.2 ml of either A, 0.05% Triton X-100 or B, 0.2 mM palmitoyl-CoA, 0.05% Triton X-100, prior to assaying as described under "Experimental Procedures." Exposure time was 4 days. The papers were stained with Coomassie Blue G-250.

FIG. 2. Filter paper assay of mutant strains. The indicated strains were inoculated with a needle onto YPD agar, grown overnight, and assayed by filter paper autoradiography as described under "Experimental Procedures." Exposure time was 5 days. Out of approximately 100,000 colonies, 26 were found to have lower glycerol-P acyltransferase activities by filter paper assay.

Glycerol-P and Dihydroxyacetone-P Acyltransferase Activities in Total Particulate Preparations—The 26 presumptive glycerol-P acyltransferase mutants were further characterized by preparing membranes and measuring their glycerol-P and DHAP acyltransferase activities in vitro. Twenty-one of these had lowered glycerol-P acyltransferase activity. All mutants with lowered glycerol-P acyltransferase activity also showed reduced DHAP acyltransferase activity. The five mutants which contained normal glycerol-P and DHAP acyltransferase activities showed only slightly reduced incorporation in the filter paper assay compared to the dramatically reduced incorporation of the other mutants. Complementation analysis of these mutants showed that these mutations were not allelic with those resulting in defective acyltransferase activities. They were not characterized further.

Fourteen of the remaining mutants were independent. The filter paper assay phenotypes for five of these mutants are shown in Fig. 2. Glycerol-P acyltransferase and DHAP acyltransferase were measured as a function of triose phosphate concentrations for each of these strains. The kinetic parameters were estimated graphically as shown in Fig. 3 for the parent strain, DBY747, and one mutant, TTD6. These data are summarized in Table I. In each case, the defect in glycerol-P acyltransferase activity was accompanied by a defective DHAP acyltransferase activity. Moreover, the change in apparent $K_m$ and $V_{max}$ values for glycerol-P and DHAP acyltransferase activities were similar (Table I). In strains TTB2 and TTD6, which have about 25% and 40% of the parent activities, respectively, the changes in apparent $K_m$ for glycerol-P are closely matched by the changes in apparent $K_m$ for DHAP. In strains TTA1, TTB4, and TTD46 the acyltransferase activities are less than 3% those of the parent, and in these cases the changes in apparent $K_m$ for DHAP are much greater than the changes in apparent $K_m$ for glycerol-P. The extremely low residual activities of these strains limit assessment of $K_m$ values. These remnant activities could be due to...
the mutated enzyme or to contributions from other enzymes. 

Thin layer chromatography of the reaction products showed that both the mutant and the parent strains gave lysophosphatidic acid and phosphatidic acid as reaction products when glycerol-P was the substrate and 1-acyl-DHAP as a reaction product when DHAP was the substrate.

**Genetic Analysis**—Complementation analysis of the mutant strains with defective acyltransferase activities showed all mutations to be recessive. Furthermore, the mutations fell into a single complementation group. Mutant strain TTA1 was mated with strain AH22, the diploids sporulated, and 28 of the resulting asci dissected. The glycerol-P acyltransferase activities of the spore progeny were analyzed by filter paper assay as described under “Experimental Procedures.” Exposure time was 7 days.

**Results**

The tetrads segregated 26 2+:2-, 1 3+:1-, and 1 3+:1-, implying mutation of a single nuclear gene. Homogenates were prepared from the spore progeny of five tetrads, and their DHAP acyltransferase activities were measured in vitro. The results, given in Table II, are consistent with a mutation in a single nuclear gene causing defects in both glycerol-P acyltransferase and DHAP acyltransferase activities.

**Further Enzymatic Analysis**—These experiments suggested that mutation of a single gene was sufficient to alter the activity of both acyltransferases. Each of the five independent mutants examined has an altered $V_{max}$ and an altered apparent $K_m$ for the triose phosphate substrate, consistent with the mutations being in the structural gene. To further investigate this possibility, the effects of temperature, detergent, and pH were examined using strains TTB2 and TTD6. These strains possessed significant residual glycerol-P and DHAP acyltransferase activities.

The effect of temperature on the glycerol-P and DHAP acyltransferase activities from mutant strain TTD6 and its parent strain at 42 °C is shown in Fig. 5. As demonstrated previously, the thermolability curves for the glycerol-P and DHAP acyltransferase activities of the parent strain appeared identical (15). Furthermore, both activities from the mutant strain were clearly thermolabile relative to those from the parent strain. The acyltransferase activities from strain TTB2 were also thermolabile relative to the parental activities, but to a lesser degree than from TTD6 (data not shown).

Surprisingly, the acyltransferase activities of the parent strain stabilized after 5 min under these conditions at about 40% activity remaining. The possibility that these curves reflect the sum of two roughly comparable activities, differing in thermolability and that TTD6 was defective in the thermostable activity was considered. This seemed unlikely in view of the low activities of strains TTA1, TTB4, and TTD46, which have a single mutation allelic with that of strain TTD6. The simplest interpretation of these results is that the mutations reside in the structural gene.

The effect of Triton X-100 on the glycerol-P and DHAP acyltransferase activities from strain TTB2 and its parent is shown in Fig. 6. While the glycerol-P and DHAP acyltransferase activities from each strain showed a similar sensitivity to increasing amounts of detergent, the activities of strain TTB2 were more sensitive to Triton X-100 than those from the parent strain. In contrast, the acyltransferase activities from strain TTD6 were not more sensitive to Triton X-100

**Table II**

**Cosegregation of the acyltransferase defects**

DHAP acyltransferase activity was determined in cell-free homogenates as described under “Experimental Procedures.” Glycerol-P acyltransferase activity was estimated as described in the legend to Fig. 4.

| Strain    | Glycerol-P acyltransferase | DHAP acyltransferase |
|-----------|----------------------------|----------------------|
| TTA1-1A   | +                          | 0.6                  |
| -1B       | +                          | 0.7                  |
| -1C       | -                          | 0.05                 |
| -1D       | -                          | 0.03                 |
| TTA1-2A   | +                          | 0.6                  |
| -2B       | +                          | 0.7                  |
| -2C       | -                          | 0.04                 |
| -2D       | -                          | 0.05                 |
| TTA1-3A   | +                          | 0.6                  |
| -3B       | +                          | 0.6                  |
| -3C       | -                          | 0.03                 |
| -3D       | -                          | 0.02                 |
| TTA1-4A   | -                          | 0.03                 |
| -4B       | +                          | 0.7                  |
| -4C       | -                          | 0.03                 |
| -4D       | +                          | 0.4                  |
| TTA1-5A   | -                          | 0.04                 |
| -5B       | +                          | 0.5                  |
| -5C       | +                          | 0.3                  |
| -5D       | -                          | 0.03                 |

**Fig. 5. Thermolability of strain TTD6.** Total particulate preparations were incubated at 42 °C for the indicated times before being assayed as described under "Experimental Procedures" at 25 °C. At zero time glycerol-P acyltransferase activities were 25.9 and 10.5 nmol/min/mg for strains DBY747 and TTD6, respectively. DHAP acyltransferase activities were 15.7 and 3.7 nmol/min/mg, respectively. O, DBY747 glycerol-P acyltransferase; ●, DBY747 DHAP acyltransferase; □, TTD6 glycerol-P acyltransferase; ■, TTD6 DHAP acyltransferase.

**Fig. 4. Tetrad analysis by filter paper assay.** Ascii were dissected and the spore progeny were inoculated onto YPD, grown overnight, and assayed by filter paper autoradiography as described under "Experimental Procedures." Exposure time was 7 days.
than the parental activities under these conditions (data not shown).

The acyltransferase activities from strain TTB2 also showed a different pH sensitivity from the parental activities. Fig. 7 shows the percent parental activity of the mutant glycerol-P and DHAP acyltransferase activities at each indicated pH. These activities were almost normal at pH 5.0, but declined to less than 25% at pH 7.4. This is in contrast to the behavior of these activities from strain TTD6, which varied between 20 and 40% of parental activities across this range. In both strains, the glycerol-P acyltransferase and the DHAP acyltransferase activities were affected similarly, except for the activities from TTD6 above pH 7. In this range the glycerol-P acyltransferase activity seemed to retain more of the parental activity than the DHAP acyltransferase. However, these activities were not $V_{\text{max}}$ measurements, and when the DHAP concentration was increased from 5 to 25 mM this difference disappeared.

In each mutant strain, the glycerol-P and DHAP acyltransferase activities responded similarly to temperature, detergent, and pH. Furthermore, allelic mutations exhibited activities with different responses to those conditions. Other allelic mutations had less than 2% of the parental acyltransferase activities (Table I). These results imply that the mutations reside within the structural gene, and establish that under these conditions, the predominant dihydroxyacetone phosphate acyltransferase activity in yeast is a second activity of the glycerol-P acyltransferase.

**Absence of Growth Phenotype**— Although in some cases the acyltransferase activities in vitro are reduced over 70-fold, no in vivo phenotype related to the acyltransferase defect has been established for any of the mutant strains. The method of selection required that the mutants grow at 25 °C on YPD in order to be detected. Since the filter paper assay was at 40 °C, temperature-sensitive phenotypes could have been present in the mutant collection. However, none of the mutant strains had temperature-sensitive growth defects linked to their defects in triose phosphate acyltransferase activities. Mutant strains were also tested without success for conditional growth on a variety of carbon sources and in the presence of several inhibitors that may affect the acyltransferase system.

While the triose phosphate acyltransferase activities are thought to be essential for growth, the lack of an obvious in vivo phenotype in these mutant strains is not particularly surprising. Similar phenomena have been reported for other mutants isolated by this method (26-28). This probably reflects the bias of the colony autoradiographic screening technique, which selects those strains with reduced enzyme activity in vitro that also grow well in culture. Mutations in an essential gene must be relatively innocuous in vivo to be detected with these screens. Alternatively, yeast may be able to tolerate wide diversity in their triose phosphate acyltransferase activities. As additional mutations become available, these issues may be resolved.

**Concluding Discussion**—The question of whether the microsomal glycerol-P acyltransferase also catalyzes the acylation of DHAP has important implications in the interpretation of experimental results concerning pathway contributions and regulation. The inherent difficulties of subcellular fractionation and measuring in vitro activities against a background of related activities are reflected in the controversial nature of the literature. Although there may be real differences between tissues, conflicting results have been obtained from different laboratories working with the same tissues. Previous work from this laboratory (4, 5) and others (6-8) presented kinetic and inhibition studies indicating that the acylation of DHAP in microsomes is due to a second activity of the glycerol-P acyltransferase in rat liver, brain, lung, and kidney. Doing similar studies in these tissues, Hajra and co-workers (9, 10) conclude that the microsomal glycerol-P and DHAP acyltransferases are two distinct and separate enzymes. These discrepancies have not been resolved, although they may be explained in part by differing amounts of contamination with microperoxisomal fragments (8).

In yeast, the DHAP acyltransferase activity was found to be comparable to that of glycerol-P acyltransferase, and was shown to behave identically under several different assay conditions (15). Both glycerol-P and DHAP could competi-
tively inhibit the acylation of the other. In this paper we have undertaken a genetic analysis of these activities. Mutation of a single gene was sufficient to alter the activity of both acyltransferases. This conclusion is supported by the fact that 14 independent mutants isolated on the basis of reduced glycerol-P acyltransferase activity showed a similarly reduced DHAP acyltransferase activity, that all these mutations were allelic, and that these mutations segregated as a single nuclear gene.

It is possible that these mutations could be in a locus indirectly affecting the acyltransferase activities. If so, the same locus could affect both acyltransferase activities even if they were separate enzymes. Arguing against this idea is the complex variation of the acyltransferase activities with the different alleles, the in vitro thermostability exhibited by the acyltransferase activities of strains TTB2 and TTD6, and the pH and detergent sensitivities exhibited by the acyltransferase activities of strain TTB2 but not TTD6. The simplest explanation is that these mutations are in the structural gene for glycerol-P acyltransferase, and that in yeast a single enzyme acylates both glycerol-P and DHAP.

These mutations are the first to be isolated in a eukaryotic gene affecting glycerol-P acyltransferase. We are in the process of complementing these mutants with a yeast genomic library, with the intention of pursuing studies on the gene, the gene product, and its regulation.

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