Dependence of Peroxisomal $\beta$-Oxidation on Cytosolic Sources of NADPH*

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Growth of Saccharomyces cerevisiae with a fatty acid as carbon source was shown previously to require function of either glucose-6-phosphate dehydrogenase (ZWF1) or cytosolic NADP$^+$-specific isocitrate dehydrogenase (IDP2), suggesting dependence of $\beta$-oxidation on a cytosolic source of NADPH. In this study, we find that IDP2ΔZWF1 strains containing disruptions in genes encoding both enzymes exhibit a rapid loss of viability when transferred to medium containing oleate as the carbon source. This loss of viability is not observed following transfer of a IDP3 strain lacking peroxisomal isocitrate dehydrogenase to medium with docosahexaenoate, a nonpermissive carbon source that requires function of IDP3 for $\beta$-oxidation. This suggests that the fatty acid$^+$ phenotype of IDP2ΔZWF1 strains is not a simple defect in utilization. Instead, we propose that the common function shared by IDP2 and ZWF1 is maintenance of significant levels of NADPH for enzymatic removal of the hydrogen peroxide generated in the first step of peroxisomal $\beta$-oxidation in yeast and that inadequate levels of the reduced form of the cofactor can produce lethality. This proposal is supported by the finding that the sensitivity to exogenous hydrogen peroxide previously reported for ZWF1 mutant strains is less pronounced when analyses are conducted with a nonfermentable carbon source, a condition associated with elevated expression of IDP2. Under those conditions, similar slow growth phenotypes are observed for ZWF1 and IDP2 strains, and co-disruption of both genes dramatically exacerbates the $H_2O_2^+$ phenotype. Collectively, these results suggest that IDP2, when expressed, and ZWF1 have critical overlapping functions in provision of reducing equivalents for defense against endogenous or exogenous sources of $H_2O_2$.

Reducing equivalents in the form of NADPH are essential for many enzymatic steps involved in biosynthesis of cellular macromolecules. NADPH is also the essential cofactor for glutathione- and thioredoxin-dependent enzymes that constitute major cellular defenses against oxidative damage (reviewed in Refs. 1 and 2). In eucaryotic cells, the majority of these NADPH-dependent reactions occur in the cytosol. Availability of the reduced form of the cofactor requires maintenance of high steady-state cytosolic redox ratios for NADPH:NADP$^+$ and values as high as ~70:1 have been reported (3).

Enzymatic sources of NADPH are relatively few in number.

In a recent study (4), we utilized gene disruption and phenotype analyses to examine the relative contributions by the oxidative branch of the hexose monophosphate pathway and by differentially compartmentalized isozymes of NADP$^+$-specific isocitrate dehydrogenases in Saccharomyces cerevisiae. Results indicate that yeast cells are remarkably flexible in terms of enzymatic sources of NADPH for growth, since mutants containing multiple gene disruptions grow well under various cultivation conditions. In fact, the only dramatic growth phenotypes observed were the inability of strains containing co-disruptions of genes encoding glucose-6-phosphate dehydrogenase (ZWF1) and cytosolic NADP$^+$-specific isocitrate dehydrogenase (IDP2) to grow with fatty acids as a carbon source and the inability of homozygous IDP2ΔZWF1 diploid strains to sporulate.

In this report, we further examine the fatty acid$^+$ growth phenotype demonstrated by IDP2ΔZWF1 yeast disruption mutants. This phenotype is not observed for mutant strains containing a single gene disruption of ZWF1 or of IDP2, and the mutant strain containing both gene disruptions is capable of growth on acetate as a carbon source (4). These observations suggest that ZWF1 and IDP1 have some overlapping function in support of $\beta$-oxidation of fatty acids, a process that is strictly peroxisomal in S. cerevisiae (reviewed in Refs. 5 and 6).

Disruption of the yeast ZWF1 gene has been reported to result in methionine (and cysteine) auxotrophy and an increased sensitivity to oxidizing agents including hydrogen peroxide and diamide (7, 8). The Met$^+$ phenotype has been interpreted as a deficiency in NADPH required for a thioredoxin-dependent reaction in methionine/cysteine biosynthesis (9). The $H_2O_2^+$ phenotype may reflect insufficient levels of NADPH to combat oxidative stress. Results of several recent studies support the importance of the hexose monophosphate pathway in this stress response; Juhneke et al. (10) report that $H_2O_2^+$ is a common phenotype for yeast mutants with defects in enzymes of this pathway. Based on results of complementation studies, Slekar et al. (9) suggest that the methionine auxotrophy displayed by yeast strains lacking the cytosolic Cu/Zn superoxide dismutase (SOD1) is due to a drain of NADPH from biosynthetic pools and that increased flux through the hexose monophosphate pathway can alleviate this auxotrophy in SOD1 disruption mutants. In addition, Godon et al. (11) reported recently that overall changes in expression of various cellular proteins in yeast cells following exposure to hydrogen peroxide suggest a major redirection of carbon flux from glycolysis into the hexose monophosphate pathway.

These previous studies of ZWF1 function were conducted using yeast cells grown with glucose as a carbon source, a condition that represses expression of IDP2 (12, 13). We report here that, under conditions when IDP2 is normally expressed, disruption of IDP2 or of ZWF1 produces similar phenotypes and growth defects are compounded by co-disruption of both genes. Also, we provide evidence that, in addition to a requirement for either ZWF1 or IDP2 to support growth with fatty
acids as a carbon source, the absence of both enzymes results in rapid loss of viability. We suggest that the cause of lethality is probably oxidative damage due to accumulation of hydrogen peroxide, a major byproduct of peroxisomal β-oxidation, and that significant levels of cytosolic NADPH are normally required for efficient degradation of that metabolite in vivo.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Yeast strains used in this study were the parental haploid strain S173–6B (MATa leu2-3, 112 his3-1 araU3-52 trpl-289, Ref. 14) and mutant strains containing coding region deletions and URA3 insertions in IDP1, IDP2, or ZWF1 loci constructed as described previously (4, 13, 15). The ΔIDP2ΔZWF1 haploid strain was obtained by mating and sporulation, and the ΔIDP3 mutant was constructed by deletion/insertion of the selectable kanMX4 gene (4, 16). Strains were cultivated in liquid medium or on 2% agar plates with rich YP medium (1% yeast extract, 2% Bacto-peptone) or with minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.0) with supplements of 20 μg/ml to satisfy auxotrophic requirements for growth. Carbon sources were glucose, ethanol, glycerol, or lactate added to 2%. Fatty acids (oleate or docosahexaenoate) used as carbon sources were added to 0.1% with 0.2% Tween 40 or glycerol, or lactate added to 2%. Fatty acids (oleate or docosahexaenoate) used as carbon sources were added to 0.1% with 0.2% Tween 40 (17).

For tabulation of viable cell numbers, yeast strains were cultivated in YP glycerol/lactate medium for 24 h then diluted into YP medium with a fatty acid carbon source, with no carbon source, or with oleate plus 200 μg/ml Geneticin. Aliquots of cultures were taken at 12-h intervals and several dilutions plated on YP glucose plates. Viable cells were scored as colonies after 2–3 days incubation at 30 °C. For measurements of intracellular peroxides as described by Lee and Park (18), 10-nl cultures grown for 24 h with fatty acid carbon sources were divided and incubated for 30 min with or without 10 μM 2"-dichlorofluorescein acetate (Molecular Probes, Eugene, OR). The cells were pelleted, washed twice with water, then lysed with glass beads in 300 μl of water. The lysates were cleared by centrifugation and various dilutions used for fluorescence measurements at peak excitation and emission wavelengths of 504 and 524 nm for dichlorofluorescein.

Sensitivity to exogenous hydrogen peroxide was tested by plating strains on YP glucose or ethanol plates containing 0, 0.5, 1.0, 2.0, or 4.0 mM H2O2. Methionine auxotrophs were analyzed on YNB glucose or ethanol/glycerol plates containing all supplements needed for growth in the presence or absence of 20 μg/ml methionine.

**Protein Analyses**—Whole cell protein samples were obtained by glass bead lysis of cells harvested from cultures in logarithmic growth. Assays for NADPH-specific isocitrate dehydrogenase activity were conducted as described previously (15). Protein concentrations were determined using the Bradford assay (20) with bovine serum albumin as the standard. Specific activities are expressed as μmoles of NADPH produced per minute per milligram protein. Immunoblot analysis was conducted with an antiserum prepared against yeast IDP1 (12) which cross-reacts with yeast IDP2. Immunoreactivity was detected by the enhanced chemiluminescent method (ECL, Amersham Pharmacia Biotech) and autoradiography.

**Saccharomyces Genome Data base designations for various proteins include:** ZWF1 (YNL241C), IDP1 (YDL066W), IDP2 (YLR174W), IDP3 (YNL099W), CTA1 (YDR256C), CTT1 (YGR088W), POX1 (YGL205W), SOD1 (YJR104C), and TSA1 (YML208W).

**RESULTS AND DISCUSSION**

*Saccharomyces* cells are able to utilize fatty acids as carbon sources because the acetyl CoA produced by peroxisomal β-oxidation serves as a substrate both for energy metabolism and for net carbon biosynthesis via the glyoxylate pathway (reviewed in Ref. 21). Growth of yeast on oleate (9) oleic acid (C18:1) results in proliferation of peroxisomes and in dramatic increase in peroxisomal NADPH for β-oxidation of fatty acids like petroselinate (6) petroselenic acid (C18:1) and docosahexaenoate ((4, 7, 10, 13, 16, 19) docosahexaenoic acid (C22:6)) that require NADPH for reduction of even-numbered double bonds (e.g., docosahexaenoic) requires two ancillary enzymes, 2,4-dienoyl-CoA reductase (1) and 3,32-enzyme-CoA isomerase (2). β-Oxidation of fatty acids with odd-numbered double bonds (e.g. oleate) requires the same ancillary enzymes, but the second, as shown, requires reductase but not reductase function. The NADPH required by the reductase is provided by peroxisomal NADPH-specific isocitrate dehydrogenase (IDP3, Refs. 17 and 23). Removal of the H2O2 produced during each round of β-oxidation is attributed to peroxisomal catalase (CTA1). Ancillary roles in this process are proposed for cytosolic catalase (CTT1) and thiol-dependent peroxidases (thioredoxin peroxidase, TPX, and glutathione peroxidase, GPX). Other abbreviations are: TRR, thioredoxin reductase; GLR, glutathione reductase; IDP2, cytosolic NADP—specific isocitrate dehydrogenase, and ZWF1, glucose-6-phosphate dehydrogenase.

We previously compared rates of growth with various fatty acids as carbon sources for yeast strains containing disruptions of IDP and/or ZWF1 loci (4). Other than replication of the growth phenotypes (e.g. petroselenate”) described by others for strains with IDP3 gene disruptions (17, 23), the only dramatic growth phenotype observed with various combinations of these gene disruptions was an inability of strains containing disruptions of both IDP2 and ZWF1 loci to grow with any fatty acid (stearate, oleate, or petroselenate) as the carbon source.

We further investigated this growth phenotype of ΔIDP2ΔZWF1 strains by analyzing viable cell numbers as described under “Experimental Procedures” following a shift of cultures from medium with glycerol/lactate to medium with oleate as the carbon source. As illustrated in Fig. 3A, logarithmic-phase doubling times of 3 h were observed for the parental strain, whereas the mutant strain containing both gene disruptions exhibits a rapid decline in viability when shifted to oleate as carbon source. The decrease in viable cell numbers for the ΔIDP2ΔZWF1 strain contrasts sharply with results obtained following a shift of the parental culture to medium with no added carbon source (Fig. 3A); the parental...
culture ceases to grow after an initial doubling but exhibits no loss of viability over this 60-h period. Instead, the decrease in viability for the ΔIDP2ΔZWF1 mutant strain is similar to but slower than the lethality produced by shifting a parental culture to oleate medium containing Geneticin (G418) at levels used for selective protocols. The calculated t₁⁄₂ values for loss of viability are ~16 h for the ΔIDP2ΔZWF1 strain in medium with oleate and ~4 h for the parental strain in medium with oleate plus Geneticin.

These results suggest that transfer of the ΔIDP2ΔZWF1 strain to medium with a fatty acid carbon source activates an endogenous cellular mechanism that results in lethality. The time lag (~12 h) before loss of viability is measurable (Fig. 3A), relative to that observed with the parental strain with Geneticin, may be due induction of this mechanism as the cells adapt to oleate as a carbon source. Thus, a potential cause of lethality in the ΔIDP2ΔZWF1 strain is accumulation of toxic levels of H₂O₂ during the process of β-oxidation (Fig. 1), which is induced by this shift. To examine this possibility, similar shifts were conducted using a ΔIDP3 mutant strain. Counts of viable cell numbers (Fig. 3B) give a doubling time of ~7 h with oleate, a permissive carbon source for this strain, since β-oxidation does not require a peroxisomal source of NADPH (17, 23). In contrast, when shifted to medium with docosahexaenoate, a nonpermissive fatty acid carbon source, growth of the ΔIDP3 strain (Fig. 3B) resembles that of the parental strain shifted to no carbon source (Fig. 3A). The parental strain exhibits a doubling time of ~11 h with docosahexanoate (Fig. 3B). Significantly, there is little loss of viability for the ΔIDP3 mutant strain in docosahexaenoate medium over the time frame of this experiment. In this mutant strain, as illustrated in Fig. 1, docosahexanoate is expected to be a substrate for acyl-CoA synthetase and oxidase but to undergo no further metabolism in the absence of NADPH normally generated by IDP3.

Van Roermund et al. (17) have demonstrated an accumulation of the enoyl CoA product of acyl oxidation in a ΔIDP3 strain incubated with docosahexanoate, suggesting that some H₂O₂ is also produced. The absence of lethality for the ΔIDP3 strain in medium with docosahexanoate as carbon source may thus be due to the presence of normal cellular mechanisms (e.g. ZWF1 and IDP2) for removal of this toxic intermediate and/or to reduced production of this metabolite by acyl-CoA oxidase.

To try to distinguish between these possibilities, we examined growth responses of a strain containing disruptions of IDP2, ZWF1, and IDP3 loci. As illustrated in Fig. 3B, a shift of the ΔIDP2ΔZWF1ΔIDP3 strain to docosahexaenoate medium produces a decrease of approximately 20% in viable cell numbers after 12 h, but these numbers do not change significantly over the course of the experiment. In contrast, the ΔIDP2ΔZWF1ΔIDP3 mutant strain exhibits a significant loss of viability (t₁⁄₂ ~ 12 h) following a shift to oleate medium. Our interpretation of these results is that this loss of viability is due to production of H₂O₂ in multiple rounds of β-oxidation when oleate is the substrate. The absence of this dramatic loss of viability for the mutant strain when docosahexanoate is the substrate suggests that production of H₂O₂ may be limited, perhaps due to product inhibition of oxidase activity.

To try to compare intracellular levels of H₂O₂, we used 2',7'-dichlorofluorescein diacetate, a fluorescent probe previously used to measure cellular oxidant production in mammalian (24) and yeast cells (18, 25). This compound is believed to cross cell membranes by passive diffusion, to be trapped after deacetylation by intracellular esterases, and to undergo oxidation by reactive oxygen species to form a fluorescent dichlorofluorescein derivative. Measurements were made with cultures grown with a fatty acid for 24 h, a time chosen to ensure adaptation to this carbon source but prior to any significant decrease in cell number (Fig. 3). As shown in Table 1, two different series of experiments produced different absolute but similar relative fluorescence values. These values are con-

**Table 1**

| Strain         | Increase in fluorescence/mg of cellular protein% |
|---------------|-----------------------------------------------|
|               | Oleate | Docosahexaenoate |
| Parental      |        |                  |
| ΔIDP3         | 1, ND  | ND, ND           |
| ΔIDP2ΔZWF1    | 90, 139| ND, ND           |
| ΔIDP2ΔZWF1ΔIDP3 | 23, 32 | ND, ND           |

* Yeast strains were cultivated for 24 h as described in the legend for Fig. 3 in YP medium with the indicated fatty acid as carbon source. The cultures were divided, and half of the cells were exposed to 2',7'-dichlorofluorescein diacetate for 30 min. Extracts were prepared for fluorescence measurements as described under “Experimental Procedures.” Values shown for two independent experimental determinations represent the percentage increase in cell associated fluorescence relative to nontreated controls.

ND = no detectable difference in fluorescence relative to nontreated controls.

**Fig. 3. Growth and viability of yeast strains shifted to medium with a fatty acid carbon source.** Parental and mutant yeast strains were cultivated in YP medium with a fatty acid or no carbon source as indicated. Viable cell numbers (log scale) were determined by plating viable cells/ml, and these initial numbers are normalized to one for comparison. Each point represents an average of two or three values obtained from independent experiments.

**Table 2**

| Strain         | Increase in fluorescence/mg of cellular protein% |
|---------------|-----------------------------------------------|
|               | Oleate | Docosahexaenoate |
| Parental      |        |                  |
| ΔIDP3         | 1, ND  | ND, ND           |
| ΔIDP2ΔZWF1    | 90, 139| ND, ND           |
| ΔIDP2ΔZWF1ΔIDP3 | 23, 32 | ND, ND           |

* Yeast strains were cultivated for 24 h as described in the legend for Fig. 3 in YP medium with the indicated fatty acid as carbon source. The cultures were divided, and half of the cells were exposed to 2',7'-dichlorofluorescein diacetate for 30 min. Extracts were prepared for fluorescence measurements as described under “Experimental Procedures.” Values shown for two independent experimental determinations represent the percentage increase in cell associated fluorescence relative to nontreated controls.

ND = no detectable difference in fluorescence relative to nontreated controls.
Figure 4. Growth phenotypes of yeast strains with disruptions in IDP2 and/or ZWF1 loci. A, parental and mutant yeast strains were streaked as indicated onto YP agar plates (1.0 mM H$_2$O$_2$) containing either glucose or ethanol as the carbon source, and the plates were incubated at 30 °C for 2 (glucose) or 4 days (ethanol). B, the same strains were streaked onto YNB agar plates with all necessary auxotrophic supplements except methionine and with either glucose or glycerol/ethanol as the carbon source and the plates incubated at 30 °C for 3 days (glucose) or 5 days (ethanol).

Consistent with significant increases in intracellular oxidant levels in ∆IDP2ΔZWF1 and ∆IDP2ΔZWF1ΔIDP3 strains cultivated with oleate and a slight increase in these levels in the ∆IDP3 strain cultivated with oleate. Fluorescence differences were not measurable with other strains and growth conditions. Thus, increased fluorescence appears to correlate with conditions that lead to loss of culture viability.

Collectively, these results suggest that either ZWF1 or IDP2 is essential to prevent the loss of viability that otherwise occurs during β-oxidation in yeast cells. We propose that the shared function is maintenance of high cytosolic redox ratios to ensure efficient function of NADPH-dependent enzymes involved in degradation of H$_2$O$_2$ or in prevention of oxidative damage from that metabolite and that these thiol-dependent reactions are an essential adjunct to catalase function (Fig. 1).

One correlate of this proposal is that IDP2, when expressed, may alleviate some of the detrimental effects (e.g. H$_2$O$_2$) reported previously for disruption of ZWF1 (8) and, conversely, that co-disruption of IDP2 may exacerbate these effects. This was tested by comparing growth of parental and mutant strains on plates with YP medium containing as the carbon source either glucose, which represses expression of IDP2, or ethanol, which is conducive to IDP2 expression (12). Effects on growth with both carbon sources of addition of H$_2$O$_2$ was tested by titration on a series of plates; results with concentrations (1 mM) producing definitive effects are illustrated in Fig. 4A. As expected, on plates with glucose as the carbon source, the H$_2$O$_2$-phenotypes are comparable for ∆ZWF1 and ∆IDP2ΔZWF1 strains, whereas the ∆IDP2 strain exhibits resistance equivalent to that of the parental strain. With ethanol as a carbon source, however, both the ∆ZWF1 strain and the ∆IDP2 strain exhibit reduced growth (colony size) relative to the parental strain with concentrations of H$_2$O$_2$ that clearly eliminate growth of the ∆IDP2ΔZWF1 strain. These results suggest a “synthetic lethal” relationship between these cytosolic sources of NADPH for protection from exogenous H$_2$O$_2$ when both are expressed. Interestingly, despite this apparently essential function, levels of ZWF1 are elevated at most 2-fold (11) and levels of IDP2 are not significantly elevated (data not shown) in cells exposed to H$_2$O$_2$ under conditions that result in over 10-fold increases in levels of other proteins with antioxidant functions including cytosolic catalase and thioredoxin reductase (11).

Since ∆ZWF1 mutant strains are also reported to be methionine auxotrophs on glucose medium, we planned similar experiments to examine potential compensation by IDP2 on nonfermentable carbon sources. However, as illustrated in Fig. 4B, while the methionine auxotrophies of ∆ZWF1 and ∆IDP2ΔZWF1 mutant strains are clearly apparent on plates with glucose as the carbon source, no obvious growth phenotypes are exhibited by any of the mutant strains on plates with glycerol/ethanol as the carbon source. Thus, methionine biosynthesis on nonfermentable carbon sources apparently does not require ZWF1 and/or IDP2. Since a specific reaction in this biosynthetic pathway, that catalyzed by NADPH/thioredoxin-dependent phosphoethenyl sulfite reductase, is believed to be the site affected by disruption of ZWF1 in glucose-grown cells (9), it would appear that there is an alternative source(s) of NADPH for this reaction under other growth conditions.

In this report, we provide evidence that the process of β-oxidation in peroxisomes can be lethal in the absence of major sources of cytosolic NADPH. The most logical cause of lethality is damage resulting from accumulation of H$_2$O$_2$, a stoichiometric product of each round of oxidation. Accumulation of H$_2$O$_2$ is believed to be particularly hazardous because of the increased potential for generation of hydroxyl radicals (‘OH) by the iron-mediated Fenton reaction (26). We propose that maintenance of a significant cytosolic pool of the reduced form of the NADP(H) cofactor is an essential adjunct to catalase function during this challenge and that the most likely fate of the reducing equivalents may be delivery to thioredoxin/glutathione-dependent peroxidases via respective reductases for enzymatic removal of H$_2$O$_2$ (Fig. 1). For S. cerevisiae, a thioredoxin peroxidase (also called thiol-specific antioxidant protein) has been described and kinetically characterized (27, 28). Based on the Saccharomyces Genome Database base, there are several other open reading frames potentially encoding thioredoxin and glutathione peroxidases that to date have not been otherwise characterized. Because of the current implication of a role for NADPH in protection of the cell from adverse consequences of endogenous metabolism, further study of expression and compartmental localization of these thiol-dependent enzymes is of significant interest.

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