Sources of NADPH in Yeast Vary with Carbon Source*

Received for publication, August 26, 2005 Published, JBC Papers in Press, September 22, 2005, DOI 10.1074/jbc.M509461200

Karyl I. Minard and Lee McAlister-Henn

From the Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229-3900

Production of NADPH in *Saccharomyces cerevisiae* cells grown on glucose has been attributed to glucose-6-phosphate dehydrogenase (Zwf1p) and a cytosolic aldehyde dehydrogenase (Ald6p) (Grabowska, D., and Chelstowska, A. (2003) *J. Biol. Chem.* 278, 13984–13988). This was based on compensation by overexpression of Ald6p for phenotypes associated with ZWF1 gene disruption and on the apparent lethality resulting from co-disruption of ZWF1 and ALD6 genes. However, we have found that a zwf1Δald6Δ mutant can be constructed by mating when tetrads are dissected on plates with a nonfermentable carbon source (lactate), a condition associated with expression of another enzymatic source of NADPH, cytosolic NADP⁺-specific isocitrate dehydrogenase (Idp2p). We demonstrated previously that a zwf1Δidp2Δ mutant loses viability when shifted to medium with oleate or acetate as the carbon source, apparently because of the inadequate supply of NADPH for cellular antioxidant systems. In contrast, the zwf1Δald6Δ mutant grows as well as the parental strain in similar shifts. In addition, the zwf1Δald6Δ mutant grows slowly but does not lose viability when shifted to culture medium with glucose as the carbon source, and the mutant resumes growth when the glucose is exhausted from the medium. Measurements of NADP(H) levels revealed that NADPH may not be rapidly utilized in the zwf1Δald6Δ mutant in glucose medium, perhaps because of a reduction in fatty acid synthesis associated with loss of Ald6p. In contrast, levels of NADP⁺ rise dramatically in the zwf1Δidp2Δ mutant in acetate medium, suggesting a decrease in production of NADPH reducing equivalents needed both for biosynthesis and for antioxidant functions.

Reducing equivalents in the form of NADPH are required for numerous biosynthetic enzymatic reactions and antioxidant mechanisms involving glutathione and/or thioredoxin. The major cellular source of NADPH is thought to be the hexose monophosphate pathway. However, disruption of the *Saccharomyces cerevisiae* ZWF1 gene encoding glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme in that pathway, was found to produce relatively mild growth phenotypes including methionine auxotrophy and an increased sensitivity to exogenous oxidizing agents like hydrogen peroxide (1, 2). In studies designed to identify other crucial sources of NADPH in yeast, co-disruption of ZWF1 and the gene (IDP2) encoding cytosolic NADP⁺-specific isocitrate dehydrogenase was found to produce a rapid loss in cell viability following shifts from medium containing glucose to medium containing either oleate or acetate as the carbon source (3, 4). This loss of viability correlated with an increase in levels of endogenous cellular oxidants. Fatty acid metabolism requires rapid flux through β-oxidation, a peroxiasomal process in yeast that produces hydrogen peroxide in the first reaction of each cycle (5, 6), and acetate is a stringent carbon source that produces rapid flux through mitochondrial respiration, a process associated with the generation of deleterious reactive oxidative species (7, 8). These results suggested that Zwf1p and Idp2p are essential sources of NADPH for thiol-dependent antioxidant functions needed to eliminate detrimental byproducts of oxidative metabolism.

Zwf1p expression in yeast is essentially constitutive (1, 2, 9). In contrast, Idp2p levels are repressed by growth on glucose, elevated with growth on nonfermentable carbon sources, and induced during the diauxic shift as glucose is depleted in the medium (9–11). In a search for alternative sources of NADPH in cells grown on glucose, Grabowska and Chelstowska (12) found the ALD6 gene encoding cytosolic NADP⁺-specific acetaldehyde dehydrogenase to be a multicopy suppressor of the Met⁻ phenotype of a zwf1Δ strain. They also reported that a zwf1Δald6Δ mutant is not viable and concluded that Ald6p is essential for production of NADPH in the absence of Zwf1p.

The conclusion that the zwf1Δald6Δ mutant is not viable (12) was based on the failure of various strategies involving mating and sporulation to obtain this mutant. However, because those experiments were conducted exclusively on glucose medium when Idp2p is not expressed, we postulated that a zwf1Δald6Δ mutant might be viable if a carbon source conducive for Idp2p expression was utilized during construction. Here we describe construction of the zwf1Δald6Δ mutant strain and evaluation of the roles of Zwf1p, Idp2p, and Ald6p as enzymatic sources of NADPH.

**Experimental Procedures**

*Cultivation Conditions*—Yeast strains were cultivated on 2% agar plates or in liquid culture 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 6.5). Supplements (including methionine for zwf1Δ mutants) of 20 μg/ml were added to minimal medium to satisfy auxotrophic requirements or withheld to select for transformants or diploids. Glucose or lactate as the carbon source was added to 2%.

*Yeast Strain Constructions*—Yeast strains used in this study were isogenic parental haploid MATα and MATa strains (lei2−3,112 his3–1 ura3–57 trp1–289; see Ref. 13) and previously constructed mutants of these strains containing deletions and *URA3* disruption insertions in the *IDP2* or *ZWF1* loci (9, 11). For construction of an *ALD6* disruption strain, plasmid pFA6a-kanMX4 (14) was used as a template for polymerase chain reaction (PCR) with oligonucleotides (5’-CTACCTCAGAATACATGACTAAGCTTACACATTGGACACACGTGGACTACCGGTGCAGGTCCGAC and 5’-CTTAAATCCTGACAGCCTTTTACTTCATGATGCA-TGGTAGACTTCTTCATCGATGAATTCCAGCTCG) containing sequences from the 5’- and 3’-regions of the *ALD6* coding sequence (underlined). The resulting ~1.5-kbp DNA fragment was used for targeted gene disruptions in each parental haploid strain and in a MATa strain containing a *URA3* disruption of *IDP2* (9, 11). Disruption of the *ALD6* locus in Kan¹ transformants was confirmed by Southern blot analysis of EcoRI and EcoRV digests of genomic DNA using radiolabeled probes generated by

---

*This research was supported by National Institutes of Health Grant AG017477. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

1 To whom correspondence should be addressed: Biochemistry Dept., University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900, Tel.: 210-567-3782; Fax: 210-567-6595; E-mail: henn@uthscsa.edu.
PCR from ALD6 and kan genes. Disruptions of IDP2 and ZWF1 loci were confirmed by Southern blots, immunochemical analyses, and enzyme assays of cellular protein extracts as described previously (3, 9, 11).

For construction of a zwf1ΔaldΔ strain, a MATa aldΔkan strain was mated with a MATa zwf1::URA3 strain (9). Matings, sporulation, and tetrad dissection were conducted as described by Adams et al. (15) except that tetrads were dissected on YP plates containing 2% lactate as the carbon source. A haploid MATa zwf1ΔaldΔ strain obtained from this mating was mated with a MATa idp2::URA3 strain (9). Resulting tetrads were similarly dissected in attempts to construct a haploid zwf1ΔaldΔidpΔ2 strain.

Analyses of Growth Phenotypes—To analyze effects on viability following carbon source shifts, parental and mutant strains were cultivated overnight in YP medium with 2% lactate as the carbon source. Cells were harvested, washed, and diluted at time 0 to an absorbance at 600 nm (A600) = 0.3 in YP medium with 0.1% oleate plus 0.2% Tween 40, with 2% sodium acetate or 2% glucose as the carbon source. Viable cell numbers were tabulated daily by plating cells on YP lactate plates and counting colonies after 7 days of incubation at 30°C.

Measurements of glucose levels in culture medium were conducted according to instructions for a Sigma diagnostic kit (catalog no. 510-A). Standards were various concentrations (0–2 mg/ml) of glucose in YP medium. The limit of detection was ~0.1 mg glucose/ml medium.

Plate assays for sensitivity to exogenous hydrogen peroxide were conducted by streaking colonies onto YP glucose or ethanol plates containing concentrations of hydrogen peroxide ranging from 0 to 5 mM. Colony size and numbers were evaluated after 7 days of incubation at 30°C.

Enzyme Assays and Immunoblot Analysis—Cellular lysates were prepared for activity assays and denaturing gel electrophoresis by breaking cell pellets with glass beads (11, 16). Protein concentrations were determined using the Bradford dye binding assay (17). Assays for NADPH (NADP(H) Measurements—Enzymatic cycling reactions to measure concentrations of NADP⁺ and NADPH in cellular extracts were conducted essentially as described by Passonneau and Lowery (20) using conditions for spectrophotometric determination. These cycling reactions amplify levels of cofactor in small extract samples and thus minimize effects of inhibitors that might interfere with direct enzymatic assays of larger extract volumes. Parental and mutant strains were subjected to media shifts as described above. At times before and following shifts, cell samples were taken to measure A600 values and viable cell numbers. Two aliquots of cells, each representing A600 = 1.0 (~10⁷ cells), were used to prepare an acid extract to measure NADP⁺ and an alkaline extract to measure NADPH (21, 22). Cycling reactions to amplify levels of NADPH were conducted at 37°C for 4 h. Final concentrations of NADPH were determined spectrophotometrically at A340 nm, by comparison with standard curves conducted with cycling reactions using NADP⁺ and NADPH in concentrations ranging from 0 to 150 μM. Various amounts (2–20 μl) of each cellular extract were used in cycling reactions to produce at least four values within the linear portion of the standard curves. Final experimental values represent averages of such values from at least three independent media shift experiments.

Plate assays for sensitivity to exogenous hydrogen peroxide were conducted as described above. At times before and following shifts, cell samples were taken to measure A600 values and viable cell numbers. Two aliquots of cells, each representing A600 = 1.0 (~10⁷ cells), were used to prepare an acid extract to measure NADP⁺ and an alkaline extract to measure NADPH (21, 22). Cycling reactions to amplify levels of NADPH were conducted at 37°C for 4 h. Final concentrations of NADPH were determined spectrophotometrically at A340 nm, by comparison with standard curves conducted with cycling reactions using NADP⁺ and NADPH in concentrations ranging from 0 to 150 μM. Various amounts (2–20 μl) of each cellular extract were used in cycling reactions to produce at least four values within the linear portion of the standard curves. Final experimental values represent averages of such values from at least three independent media shift experiments.

RESULTS

Construction of a zwf1ΔaldΔ Mutant—Because previous attempts to obtain a zwf1ΔaldΔ mutant strain using methods employing glucose as the carbon source were unsuccessful (12), we attempted to construct this strain by mating and sporulation on plates containing a nonfermentable carbon source. For this, we used kanamycin cassette mutagenesis (14) to replace the ALD6 coding region in a haploid yeast strain and confirmed the disruption by Southern blot analysis. This strain was mated with an isogenic strain of opposite mating type containing a URA3 disruption in the coding region of ZWF1 (9). Tetrads were dissected on YP plates containing 2% lactate as the carbon source. The Ura¹ and Kan¹ markers segregated independently as expected based on the location of ALD6 and ZWF1 genes on different chromosomes. In addition, haploid Ura¹ zwf1ΔaldΔ mutants were obtained at the expected frequency in tetrads dissected on YP lactate plates. Upon replating, the zwf1ΔaldΔ mutants were found to grow well but at slightly reduced rates relative to parental strains on YP plates containing 2% glycerol, lactate, ethanol, or acetate. The mutants did not grow after 7 days on plates containing 2% glucose as expected based on the report by Grabowska and Chelstowska (12). Thus, zwf1ΔaldΔ mutants are viable when constructed and grown on nonfermentable carbon sources.

We also constructed a haploid idp2ΔaldΔ strain by transformation of an idpΔ2 strain with the ALD6 kanamycin disruption cassette. In plate tests, we observed no growth phenotypes for the idp2ΔaldΔ strain other than the slow growth on minimal ethanol medium reported previously for an aldΔ strain (24). Thus, Idp2p and Ald6p do not appear to have essential overlapping functions.

Finally, we attempted to construct a zwf1ΔaldΔidpΔ2 strain by mating haploid zwf1ΔaldΔ and idpΔ2 strains and by sporulation of diploids on YP lactate plates. However, many of the tetrads from this mating contained only two or three spores. Analysis of the viable spores by tests for Ura¹ and Kan¹ markers and by Southern blot analysis indicated that mutants containing one or any two of the gene disruptions could be obtained from this mating. However, haploid mutants containing all three gene disruptions were not identified, suggesting that such strains may not be viable.

Sources of NADPH for Antioxidant Functions—To examine the relative contributions of Zwf1p, Idp2p, and Ald6p as sources of NADPH in protection from endogenous metabolic oxidants, the aldΔ6 and double gene disruption strains (zwf1ΔaldΔΔ; idp2ΔaldΔΔ; zwf1ΔidpΔ2Δ) (Ref. 9) were shifted from permissive YP lactate medium to YP oleate or YP acetate medium. As shown in Fig. 1, the parental strain (●) grows well, whereas numbers of viable zwf1ΔidpΔ2Δ cells (▼) decrease after 24 h on the new carbon sources. We reported previously (3, 4) that the decrease in viability of the zwf1ΔidpΔ2Δ strain following shifts to oleate or acetate medium is coincidental with an increase in levels of cellular oxidants and is presumably due to the loss of critical sources of NADPH needed for cellular antioxidant systems. Both Idp2p and Zwfl1p contribute to this function because no loss in viability was observed in similar shifts of mutants lacking only one of these enzymes (3). In contrast to the zwf1ΔidpΔ2Δ strain, none of the mutant strains lacking Ald6p, importantly including the zwf1ΔaldΔΔ strain (■), show any impairment of growth on oleate or acetate (Fig. 1). This suggests that Ald6p is not a crucial source of NADPH for protection from endogenous oxidants generated by peroxisomal β-oxidation on oleate medium or by rapid mitochondrial respiration on acetate medium.

Saccharomyces genome database (www.yeastgenome.org).
Sources of NADPH in Yeast

To examine the relative roles of enzymatic sources of NADPH in protection from exogenous oxidants, we compared the growth of parental and mutant strains on YP glucose or ethanol plates containing increasing concentrations of hydrogen peroxide (TABLE ONE). With glucose as the carbon source, as reported previously (1, 3), loss of Zwf1p (in the zwf1Δ strain or in the zwf1Δ idp2Δ strain) increases sensitivity to hydrogen peroxide. The inability of the zwf1Δ ald6Δ strain to grow on YP glucose plates precluded analysis of relative sensitivity. With ethanol as the carbon source (TABLE ONE), the parental strains are resistant to hydrogen peroxide. The inability of the parental strains to grow on YP plates containing 2.0 mM hydrogen peroxide, as we reported previously (3, 4), co-disruption of the ZWF1 and IDP2 genes produces an increase in sensitivity to this exogenous oxidant at 2 mM concentrations. Co-disruption of the ZWF1 and ALD6 genes also slightly increases sensitivity relative to disruption of ZWF1 alone.

Collectively, these results suggest a primary role for Zwf1p in resistance to exogenous hydrogen peroxide. However, with endogenous oxidants produced as by-products of metabolic pathways, Idp2p also has a critical antioxidant function.

Viability of a zwf1Δald6Δ Mutant in Cultures with Glucose as the Carbon Source—Because the zwf1Δald6Δ mutant does not grow on glucose plates, we also examined the viability of this strain following a culture shift from permissive YP lactate medium to YP glucose medium. As shown in Fig. 2A, the zwf1Δald6Δ strain (○) shows very little initial growth relative to the parental strain (●), doubling only 1.5 times in 48 h. Importantly, however, there is no indication of a loss of viability during this time, suggesting that the early slow growth of the zwf1Δald6Δ strain does not involve metabolic toxicity. At the 72 h and 96 h time points, growth rates of the zwf1Δald6Δ strain increase substantially. In other experiments (data not shown), we found that using lower initial A_{600} values during the shift to glucose medium similarly delayed the logarithmic growth phase of the parental strain and extended the period of slow growth of the zwf1Δald6Δ strain, again with no apparent effects on cell viability. Similar results were obtained following shifts of the parental and zwf1Δald6Δ strains to minimal medium with glucose.

The patterns of glucose utilization by these strains (Fig. 2B) suggest that glucose is essentially depleted in the parental strain culture within 12 h following the shift; the rate of glucose utilization was ~0.77 mg/h between the 0 and 12 h time points. The zwf1Δald6Δ strain exhibits a much slower rate of glucose metabolism, ~0.06 mg/h between the 12 and 72 h time points, with depletion in the medium observed only after 96 h of cultivation. Because the zwf1Δald6Δ strain does eventually grow in these liquid cultures, we assume that the inability of this strain to grow on YP glucose plates, as described above, is due to inadequate depletion of glucose on the plate surface and/or inadequate oxygenation relative to shaking flask cultures.

We examined immunochemical levels of Idp2p (Fig. 3A, lower band) in these strains using an antiserum that also recognizes mitochondrial NADP⁺-specific isocitrate dehydrogenase (Fig. 3A, upper band). In contrast to Idp2p, expression of the mitochondrial enzyme is essentially constitutive with various carbon sources (18, 19). We also measured total cellular NADP⁺-specific isocitrate dehydrogenase activity (Fig. 3B). In the parental strain, the effect of glucose repression of immunochemical levels of Idp2p (Fig. 3A) is obvious in a comparison of samples taken before (lane 1) and 12 h after (lane 2) the shift to glucose medium, and Idp2p levels are again elevated (lanes 3–6) following the logarithmic stage of growth (see Fig. 2), i.e. as glucose is depleted during the diauxic shift. Changes in levels of Idp2p in the parental strain are also reflected in the transient decrease in total cellular activity 12 h following the shift (Fig. 3B, ●) and in the subsequent recovery of activity to the preshift.

**TABLE ONE**

| Strain             | Glucose | Ethanol |
|--------------------|---------|---------|
|                    | 0 mM H$_2$O$_2$ | 3.0 mM H$_2$O$_2$ | 4.0 mM H$_2$O$_2$ | 5.0 mM H$_2$O$_2$ | 0 mM H$_2$O$_2$ | 1.0 mM H$_2$O$_2$ | 2.0 mM H$_2$O$_2$ | 3.0 mM H$_2$O$_2$ | 4.0 mM H$_2$O$_2$ |
| Parental MATa      | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | -- |
| ald6Δ              | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | -- |
| idp2Δ              | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | -- |
| zwf1Δ              | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | -- |
| zwf1Δidp2Δ         | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | ++ + + + | -- |
| zwf1Δald6Δ         | --      | --      | --      | --      | --      | --      | --      | --      | --   |

**FIGURE 1. Effects of carbon source shifts on viability.** Yeast strains were preincubated in YP lactate medium and diluted into YP medium with oleate (A) or acetate (B) as the carbon source as described under “Experimental Procedures.” Viable cell numbers (averages of two or three independent measurements; log scale) are normalized to the starting value, set as 1.0. Yeast strains were: parental (●), zwf1Δidp2Δ (●), ald6Δ (●), idp2Δald6Δ (○), and zwf1Δald6Δ (●).
level. For the zwf1Δald6Δ strain, immunochemical levels of Idp2p (Fig. 3A) remain repressed during the period of slow growth following the shift to glucose medium (lanes 2–5 relative to lane 1) and are elevated to parental strain levels at the 96 h time point (lane 6) as glucose is depleted (see Fig. 2). Again, there are corresponding changes in total cellular activity (Fig. 3B). Thus, Idp2p levels are elevated in the zwf1Δald6Δ strain during growth on lactate medium and as glucose levels are reduced at late time points following the culture shift to glucose medium.

Changes in Levels of NADP(H) during Carbon Source Shifts—We measured levels of NADP(H) as described under “Experimental Procedures” to directly examine correlations between these levels and the loss of viability of a zwf1Δidp2Δ strain following a shift to acetate medium or with the lag in growth of a zwf1Δald6Δ strain following a shift to glucose medium.

Parental and zwf1Δidp2Δ strains were shifted from glucose to acetate medium as described above. Samples taken at various times were used to calculate viable cell numbers and to prepare extracts for measurement of NADP(H) levels (Fig. 4). Prior to the acetate shift, extracts from the parental strain precultivated in glucose medium exhibited levels of NADP+ ~2-fold higher than those of NADPH (time 0 in Fig. 4). Following the shift to acetate medium, levels of NADP+ (▼) were slightly reduced (by ~40%) over the 72-h period, whereas NADPH levels (■) fell by 4-fold in 24 h and remained low. A ratio of [NADP+]/[NADPH] of ~6 (Fig. 4B, ▶) was attained 24 h following the shift and maintained throughout growth to 72 h. For the zwf1Δidp2Δ strain, levels of NADP+ and NADPH in viable cells were approximately equal prior to the shift (time 0 in Fig. 4), and following the shift to acetate medium, levels of NADPH (○) dropped in a manner similar to those in the parental strain. In contrast to the parental strain, levels of NADP+ in the mutant (▼) rose dramatically after 24 h to ~5-fold the starting value by 72 h, producing a ~25-fold increase in the ratio of [NADP+]:[NADPH] (Fig. 4B, ▶). These results suggest induction of a mechanism in viable zwf1Δidp2Δ cells to produce more NADP+ perhaps to compensate for the decreased rate of conversion of oxidized to reduced cofactor.

Parental and zwf1Δald6Δ strains were similarly shifted from lactate to glucose medium. Levels of NADP(H) were determined and expressed relative to total cell number in this case (Fig. 5) because both strains remain viable during this shift. Levels of NADP+ in the parental strain precultivated in lactate medium exceeded those of NADPH by ~3.4-fold (time 0 in Fig. 5). Following the shift to glucose medium, levels of both NADP+ and NADPH exhibited a transient increase at 12 h (Fig. 5A), a time corresponding to logarithmic growth and rapid utilization of glucose by the parental strain (Fig. 2), and then declined to near preshift levels after 24 h. The overall ratio of [NADP+]:[NADPH] declined steadily to ~1.7 at 48 h (Fig. 5B, ▶). NADPH levels were below detection in parental strain extracts after this time, which corresponds with stationary phase growth (Fig. 2), precluding estimates of the nucleotide ratio at later time points. The general patterns of changes in levels of NADP+ and NADPH in the zwf1Δald6Δ strain shifted from lactate to glucose (Fig. 5A) were quite similar to those for the parental strain, although the peak in the transient increase in levels of both was delayed to 24 h. Also, the ratio of [NADP+]:[NADPH] remained fairly constant (~2.2–2.5) (Fig. 5B, ○) before and after the shift. The lowest ratio value was obtained 96 h following the shift, a time corresponding to rapid growth of the mutant strain and to depletion of glucose in the medium (Fig. 2).

These observations suggest that the dramatic increases in NADP+ levels and in the ratio of [NADP+]:[NADPH] in the zwf1Δidp2Δ mutant are closely correlated with the loss of viability observed upon a shift of this strain to acetate medium. In contrast, there appears to be little correlation between differences in NADP(H) levels or ratios and the
slow growth observed for the zwf1∆ald6Δ strain shifted from lactate to glucose medium.

DISCUSSION

We have demonstrated that construction of a haploid zwf1∆ald6Δ mutant is feasible when haploid yeast strains containing the individual gene disruptions are mated, and tetrads from resulting diploids are sporulated on plates containing lactate as the carbon source. In concurrence with a previous report (12), we were unable to obtain the zwf1∆ald6Δ mutant by sporulation of diploids on glucose plates, and the haploid zwf1∆ald6Δ mutant obtained on lactate plates failed to grow when transferred to glucose plates. Grabowska and Chelstowska (12) also demonstrated that multicopy expression of Ald6p or Zms1p, a putative transcriptional regulator of ALD6 expression, can suppress phenotypes associated with ZWF1 disruption. A recent report (25) suggests additional roles for Ald6p and Zwf1p in protection of yeast from salt stress, a calcineurin-mediated response examined using YP glucose medium containing high concentrations of NaCl or LiCl. Thus, Zwf1p and Ald6p do appear to have an essential overlapping function, presumably production of NADPH, but our results suggest that this function is limited to growth with a fermentable carbon source.

Our results also suggest that production of NADPH may not be the sole reason for the glucose− phenotype of the zwf1∆ald6Δ strain because levels of reduced cofactor are quite similar in parental and mutant strains following a shift to glucose medium (Fig. 5). Another important function of Ald6p during growth on glucose is provision of acetate as a precursor of cytosolic acetyl-CoA for fatty acid biosynthesis (26). Slow rates of fatty acid synthesis in the zwf1∆ald6Δ strain on glucose medium could directly limit growth and substantially reduce utilization of NADPH reducing equivalents that support fatty acid synthesis. This would reduce rates of interconversion of NADPH and NADP+, which, in fact, is what we observe. The ratio of [NADP+]:

\[
\frac{\text{NADP}^+}{\text{NADPH}}
\]
[NADPH] drops in the parental strain following a shift from lactate to glucose medium but remains essentially unchanged in the zwf1Δald6Δ mutant strain (Fig. 5B).

In yeast cells grown on a nonfermentable carbon source or following the diauxic shift on glucose, expression of other enzymatic sources of NADPH including Idp2p is induced (9). We have shown that a culture of the zwf1Δald6Δ mutant exhibits little growth when initially shifted from lactate to glucose medium. However, the mutant retains viability and slowly metabolizes the glucose, permitting the onset of diauxic growth once the glucose is depleted. An interesting correlation is that levels of Idp2p in the zwf1Δald6Δ strain increase at the time growth resumes following the exhaustion of glucose (Figs. 2 and 3). However, we note that numerous other proteins, including mitochondrial Ald4p (27), that might compensate for the loss of Ald6p in producing NADPH are also induced during the diauxic shift. Ald4p and mitochondrial Ald5p, which is induced under anaerobic conditions in the stationary phase (26), can both produce NADPH. However, it is unclear whether this pool of NADPH would be available for cytosolic biosynthetic reactions. Another possibility is suggested by a recent report (26) that Ald5p can compensate for loss of Ald6p in acetate formation. Presumably, induction of Ald4p expression during the diauxic shift and on nonfermentable carbon sources would also increase acetate production for fatty acid biosynthesis. Thus, once glucose is metabolized in the medium, there is an increase in the levels of several proteins that can compensate for different Ald6p functions.

In contrast to the relatively minor effects on levels of NADP(H) in the zwf1Δald6Δ mutant shifted to nonpermissive glucose medium, we observed dramatic effects in the zwf1Δidp2Δ mutant shifted to nonpermissive acetate medium (Fig. 4). Although the levels of NADPH decreased to similar levels in the parental strain, the zwf1Δidp2Δ strain exhibited a dramatic increase in cellular levels of NADP+. This suggests that the reduction of available cofactor may be inefficient in the mutant strain, as would be predicted by loss of the two major enzymatic mechanisms for NADP+ reduction. It also suggests some compensatory mechanism in the stressed mutant cells for dramatically increasing cellular concentrations of NADP+. The most proximal source of NADP+ in yeast cells is NAD+. Three NAD+ kinases have been identified, and all were recently shown to be competent for ATP-dependent phosphorylation of either NAD+ or NADH (29). Interestingly, one of these kinases, Utr1p (30), has been genetically linked with Ald6p, and mutants lacking either protein exhibit similar changes in transcriptional profiles (25). It will be of interest in future studies to examine the role of Utr1p and the other NAD+ kinases not only in the dramatic increase in NADP+ concentrations observed for the zwf1Δidp2Δ mutant following a shift to acetate medium (Fig. 4) but also, in addition, in the transient increase in NADP(H) levels observed in both parental and zwf1Δald6Δ strains following a shift to glucose medium (Fig. 5).

Loss of both Ald6p and Zwflp reduces growth rates on glucose medium but does not dramatically affect growth on nonfermentable carbon sources (Fig. 1). In addition, we find no evidence for metabolic damage causing loss of viability of zwf1Δald6Δ cells in glucose medium. In contrast, Idp2p appears to be the crucial adjunct to Zwflp in providing NADPH both for active growth in the absence of glucose and for prevention of endogenous toxicity during growth on nonfermentable carbon sources like olate and acetate. These differences may be due to the relatively low levels of metabolic oxidants in cells growing on glucose versus in cells growing on a fatty acid or acetate because metabolism of the latter carbon sources requires rapid flux through oxidative pathways. Thus, NADPH may be required primarily for biosynthetic reactions in cells grown on glucose, but in cells grown on nonfermentable carbon sources, these reducing equivalents are apparently needed both for biosynthesis and thiol-based antioxidant systems.

It is interesting that these important sources of NADPH for biosynthesis and antioxidant functions are cytosolic enzymes. For example, we have found no related phenotype associated with loss of the mitochondrial NADP+ -specific isocitrate dehydrogenase (9), and the peroxisomal yeast isozyme functions quite specifically to provide NADPH for a particular enzymatic step in β-oxidation (31, 32). Malic enzyme, which is another critical cytosolic source of NADPH in mammalian cells, is mitochondrial in yeast (33), and loss of this enzyme has not been correlated with phenotypes associated with oxidative stress. It is also of interest that the thiol-related antioxidant systems dependent on NADPH generated by Zwflp and Idp2p appear to be more important than the peroxidative enzymes in the removal of the deleterious byproducts of normal metabolic pathways. This is based on finding no detrimental effects following the shifts of mutants lacking cytosolic catalase and/or mitochondrial cytochrome c peroxidase to medium with olate or acetate as the carbon source (4). Also, addition of dithiothreitol and/or glutathione to the medium was shown to partially compensate for the loss of Zwflp and Idp2p during such shifts, i.e. by providing some protection against rapid lethality.

Some aspects of our results can be correlated with important sources of NADPH in mammalian systems. Defects in glucose-6-phosphate dehydrogenase produce hemolytic anemia in humans, presumably due primarily to an inadequate reducing environment for proper function of hemoglobin (reviewed in Ref. 34). In mammalian cell lines, elevation or reduction of levels of the homologue of IDP2 has been shown to respectively increase or decrease resistance to various oxidative stresses (35), and IDP2 has been shown to be important for fatty acid biosynthesis (36). Unfortunately, however, although numerous pathologies, including the propensity for certain types of cancers and alcoholism, have been associated with various polymorphic forms of the 17 known human aldehyde dehydrogenases (reviewed in Refs. 23 and 37), the exact counterpart in this superfamily of yeast Ald6p is unknown. Also unknown are the impact of cytosolic localization of malic enzyme in mammalian cells and the roles of transhydrogenases, capable of interconverting NADH and NADPH, that are present in mammalian cells but not in yeast cells (28).

Acknowledgments—We thank Sondra Anderson for tetrad analyses and Drs. Jill K. Manchester (Washington University School of Medicine, St. Louis, MO) and Su-Jun Lin (University of California, Davis) for advice on quantitative assays for NADP(H).

REFERENCES

1. Nogae, L., and Johnston, M. (1990) Gene (Amst.) 96, 161–169
2. Thomas, D., Cherest, H., and Surfing-Kerjan, Y. (1991) EMBO J. 10, 547–553
3. Minard, K. I., and McAlister-Henn, L. (1999) J. Biol. Chem. 274, 3402–3406
4. Minard, K. I., and McAlister-Henn, L. (2001) Free Radic. Biol. Med. 31, 832–843
5. Hiltunen, J. K., Mursula, A. M., Rottensteiner, H., Wierenga, R. K., Kastaniotis, A. J., and Gurvits, A. (2003) FEBS Microbiol. Rev. 27, 55–64
6. Kunau, W. H., Dommes, V., and Schulz, H. (1995) Prog. Lipid Res. 34, 267–342
7. Bertlett, R. S., and Stadtmann, E. R. (1997) J. Biol. Chem. 272, 20313–20316
8. Stadtmann, E. R. (2002) Free Radic. Biol. Med. 33, 597–604
9. Minard, K. I., Jennings, G. T., Lofts, T. M., Xuan, D., and McAlister-Henn, L. (1998) J. Biol. Chem. 273, 31486–31493
10. DeRisi, J. L., Iyer, V. R., and Brown, P. O. (1997) Science 278, 680–686
11. Lofts, T. M., Hall, L. V., Anderson, S. L., and McAlister-Henn, L. (1994) Biochemistry 33, 9661–9667

2 K. I. Minard and L. McAlister-Henn, unpublished data.
Sources of NADPH in Yeast

12. Grabowska, D., and Chelstowska, A. (2003) J. Biol. Chem. 278, 13984–13988
13. Botstein, D., Falco, S. C., Stewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K., and Davis, R. W. (1979) Gene (Amst.) 8, 17–24
14. Wach, A., Brachat, A., Pöhlmann, R., and Phillipsen, P. (1994) J. Biol. Chem. 269, 13984–13988
15. Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. (eds) (1997) Methods in Yeast Genetics, 1997 Ed., pp. 19–29, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. McAlister-Henn, L., and Thompson, L. M. (1987) J. Bacteriol. 169, 5157–5166
17. Bradford, M. (1976) Anal. Biochem. 72, 248–254
18. Haselbeck, R. J., and McAlister-Henn, L. (1991) J. Biol. Chem. 266, 2339–2345
19. Haselbeck, R. J., and McAlister-Henn, L. (1993) J. Biol. Chem. 268, 12116–12122
20. Passonneau, J. V., and Lowry, O. H. (eds) (1993) Enzymatic Analysis: A Practical Guide, pp. 1–110, Humana Press, Totowa, NJ
21. Lin, S. S., Manchester, J. K., and Gordon, J. I. (2001) J. Biol. Chem. 276, 36000–36007
22. Lin, S-J., Ford, E., Haigis, M., Liss, G., and Guarente, L. (2004) Genes Dev. 18, 12–16
23. Sophos, N. A., and Vasilou, V. (2003) Chem. Biol. Interact. 143–144, 5–22
24. Meaden, P. G., Dickinson, F. M., Mifud, A., Tessier, W., Westwater, J., Bussey, H., and Midgley, M. (1997) Yeast 13, 1319–1327
25. Butcher, R. A., and Schreiber, S. L. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 7868–7873
26. Saint-Prix, F., Béniquét, L., and Dequin, S. (2004) Microbiology (Read.) 150, 2209–2220
27. Jacobson, M. K., and Bernofsky, C. (1974) Biochim. Biophys. Acta 350, 277–291
28. Rydström, J., Hock, J. B., and Ernst, L. (1976) in The Enzymes (Boyer, P. D., ed) Vol. 13, pp. 51–79, Academic Press, New York
29. Shi, F., Kawai, S., Mori, S., Kono, E., and Murata, K. (2001) FEBS J. 272, 3337–3349
30. Kawai, S., Suzuki, S., Mori, S., and Murata, K. (2001) FEBS Microbiol. Lett. 200, 181–184
31. Henke, B., Girzalsky, W., Berteaux-Lecellier, V., and Erdmann, R. (1998) J. Biol. Chem. 273, 3702–3711
32. van Roermund, C. W., Hetterma, E. H., Kal, A. J., van den Berg, M., Tabak, H. F., and Wanders, R. J. (1998) EMBO J. 17, 677–687
33. Boles, E., de Jong-Gubbels, P., and Pronk, J. T. (1998) J. Bacteriol. 180, 2875–2882
34. Beutler, E. (1996) Blood Rev. 10, 45–52
35. Lee, S. M., Koh, H. J., Park, D. C., Song, B. J., Huh, T. L., and Park, J. W. (2002) Free Radic. Biol. Med. 32, 1185–1196
36. Koh, H. J., Lee, S. M., Son, B. G., Lee, S. H., Ryoo, Z. Y., Chang, K. T., Park, J. W., Park, D. C., Song, B. J., Veech, R. L., Song, H., and Huh, T. L. (2004) J. Biol. Chem. 279, 39968–39974
37. Vasilou, V., Pappa, A., and Petersen, D. R. (2000) Chem. Biol. Interact. 129, 1–19