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Pnc1p Supports Increases in Cellular NAD(H) Levels in Response to Internal or External Oxidative Stress†

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ABSTRACT: Following transfer from medium with fermentable glucose to medium with nonfermentable acetate as the carbon source, cellular levels of NAD(H) were found to increase ~2-fold in a parental yeast strain. Similar transfer of a mutant strain subject to endogenous oxidative stress under these conditions produced more dramatic increases in cellular levels of NAD(H), and elevations above parental levels were shown to be due to the nicotinimidase Pnc1p. Similar transient increases in NAD(H) levels observed in the parental strain following addition of exogenous hydrogen peroxide were also attributable to Pnc1p.

Reducing equivalents in the form of NADPH are essential for antioxidant systems involving glutathione and thioredoxin. The major cellular mechanism for reduction of $NADP^+$ to NADPH is purported to be the hexose monophosphate (or pentose phosphate) pathway. However, a yeast mutant with a deletion of the gene (*ZWF1*) encoding glucose-6-phosphate dehydrogenase, the first and rate limiting enzyme in that pathway, was found to be viable unless challenged with exogenous hydrogen peroxide (*1*). In earlier studies of yeast mutants containing various combinations of disruptions in genes encoding potential enzymatic sources of NADPH (*2*, *3*), we identified unique and dramatic growth phenotypes associated with codisruption of *ZWF1* and of *IDP2*, the gene encoding cytosolic $NADP^+$-specific isocitrate dehydrogenase. In comparison with the parental strain or with single *zwf1*Δ or *idp2*Δ mutants, a *zwf1*Δ*idp2*Δ mutant not only failed to grow but exhibited a dramatic loss in viability when shifted from glucose medium to medium with either oleate or acetate as the carbon source. This loss in viability correlated with a substantial increase in levels of endogenous cellular oxidants (*3*). Utilization of acetate or oleate as a carbon source requires rapid respiration which is associated with an increase in the generation of reactive oxygen species. In addition, since β-oxidation is strictly peroxisomal in yeast (*4*, *5*), utilization of a fatty acid carbon source results in a stoichiometric production of hydrogen peroxide by acyl CoA oxidase during each round of β-oxidation. We showed that peroxidases including catalase were ineffective in removal of hydrogen peroxide. Instead, the decrease in viability of the *zwf1*Δ*idp2*Δ mutant and associated increase in levels of intracellular oxidants correlated with damage to cellular macromolecules (*3*, *6*), suggesting that Zwf1p and Idp2p have redundant functions in reducing $NADP^+$ to NADPH for thiol-based antioxidant systems needed to eliminate detrimental byproducts of endogenous oxidative metabolism.

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Previous measurements of cellular levels of $NADP^+$ and NADPH in the *zwf1*Δ*idp2*Δ strain following a shift from permissive glucose to nonpermissive acetate medium indicated, relative to the parental strain, a substantial increase in the ratio of oxidized to reduced cofactor ([$NADP^+$]/[NADP]) (*7*), as might be expected for a strain lacking the two cytosolic dehydrogenases. Interestingly, as the mutant strain lost viability, the total cellular levels of cofactor ([$NADP^+$] + [NADPH]) increased 2−3-fold above parental strain levels. This was interpreted to represent a possible compensatory mechanism to attempt to restore redox ratios for survival. NADP is produced from NAD by NAD kinases (*8*, *9*), and cellular NAD levels are maintained by de novo and salvage pathways. Therefore, in the current study, we examined changes in levels of both NAD(H) in the *zwf1*Δ*idp2*Δ mutant as well as the potential involvement of de novo or a salvage pathway enzyme in the synthesis of NAD(H). In yeast, the de novo pathway from tryptophan is catalyzed by enzymes designated Bna1p, Bna2p, and Bna4p-7p (*10*−*12*), whereas the salvage pathway is catalyzed by enzymes partially localized in the nucleus (*13*, *14*). The latter includes Pnc1p, a nicotinamidase that converts nicotinamide to nicotinic acid, but that is not required for utilization of some other salvage pathway substrates. While disruption studies suggested that both types of pathways are redundant sources of NAD(H) (*10*), only a few recent reports (*15*, *16*) have addressed differential contributions of the pathways to various cellular processes.

The parental yeast strain used in current studies can grow with acetate as a carbon source. As shown in Figure 1A (●), following a transfer of parental cells from YP glucose to YP acetate medium, viable cell numbers increased 3−4-fold over a 24 h period prior to entry into stationary phase. In contrast, for the *zwf1*Δ*idp2*Δ mutant lacking the two major cytosolic sources of NADPH, viable cell numbers doubled once over a 24 h period and then decreased ~4-fold by 72 h after a similar shift to acetate medium (○, Figure 1A). As shown in Figure 1B, total cellular levels of NAD(H) in the parental strain initially decreased and then increased ~2-fold from starting glucose levels within 48 h following the shift to acetate medium. This increase is similar to that recently reported for another parental yeast strain following a similar shift in carbon source (*15*) and may reflect a need for increased cofactor levels to support rapid respiratory metabolism and/or changes in chromatin and gene expression during entry into stationary phase (*17*). For the *zwf1*Δ*idp2*Δ mutant, cellular levels of NAD(H) increased ~4-fold relative to glucose levels 72 h following the shift to acetate medium. The magnitude of this increase above parental strain levels is quite similar to that previously observed for NADP(H) in the mutant strain (*7*). The high levels of both NAD(H) and NADP(H) cofactors in the mutant strain are remarkable considering that they occur when the cells are losing viability in acetate medium.

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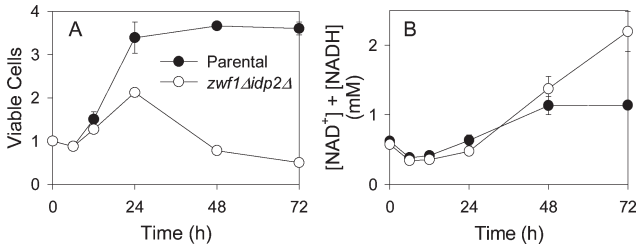

FIGURE 1: Growth and total NAD(H) levels in parental and *zwf1*Δ*idp2*Δ strains following transfer to medium with acetate as the carbon source. Yeast strains growing logarithmically in medium with glucose as the carbon source were harvested and resuspended in YP medium with acetate as the carbon source. Samples of cultures taken before the shift (time 0) and at indicated times following the shift were diluted and plated to determine viable cell numbers (A), which are expressed relative to initial values set at 1.0. Cells were also harvested to prepare extracts for measurements of NAD^+ and NADH (B) as described under Experimental Procedures in Supporting Information. Concentrations are expressed as total oxidized and reduced cofactors/viable cell/cell volume.

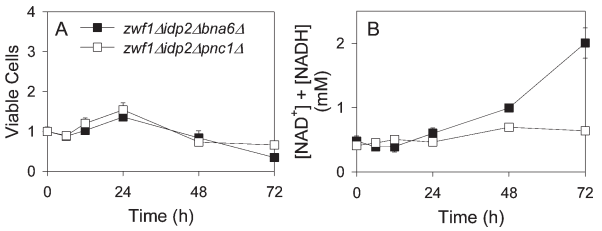

FIGURE 2: Growth and total NAD(H) levels in *zwf1*Δ*idp2*Δ*bna6*Δ and *zwf1*Δ*idp2*Δ*pnc1*Δ strains following transfer to medium with acetate as the carbon source. Determination of viable cell numbers (A) and measurements of NAD^+ and NADH (B) are described in the caption for Figure 1.

To examine the source for increased levels of nicotinamide cofactors in the *zwf1*Δ*idp2*Δ mutant shifted to acetate medium, we constructed *zwf1*Δ*idp2*Δ*pnc1*Δ and *zwf1*Δ*idp2*Δ*bna6*Δ mutant strains. The former disruption of *PNC1* reduces flux through the salvage pathways, while the latter disruption of *BNA6* prevents function of the de novo pathway for NAD(H) biosynthesis. The mutant strains were cultivated in YP glucose medium prior to shifts to YP acetate medium as described above. As shown in Figure 2A, both the *zwf1*Δ*idp2*Δ*pnc1*Δ and *zwf1*Δ*idp2*Δ*bna6*Δ mutant strains exhibited changes in viable cell numbers very similar to those observed for the *zwf1*Δ*idp2*Δ strain (Figure 1A), that is, 2−3-fold decreases at 72 h from peaks at 24 h following the medium shift. For the *zwf1*Δ*idp2*Δ*pnc1*Δ mutant (Figure 2B, □), changes in the cellular pool of NAD(H) were similar to those obtained following the medium shift for the parental strain (Figure 1B). These results suggest that the residual de novo pathway in the *zwf1*Δ*idp2*Δ*pnc1*Δ mutant is sufficient to support normal increases in pyridine nucleotide levels as observed in the parental strain; in fact, we have found that either the de novo or salvage pathway can support the increase in the parental strain (Figure S1, Supporting Information). In contrast, for the *zwf1*Δ*idp2*Δ*bna6*Δ mutant (Figure 2B, ■), changes in the cellular pool of NAD(H) were similar to those obtained for the *zwf1*Δ*idp2*Δ mutant (Figure 1B). This suggests that the substantial increase in pyridine nucleotide levels above parental levels observed for the *zwf1*Δ*idp2*Δ strain is due to the presence of Pnc1p in the *zwf1*Δ*idp2*Δ*bna6*Δ mutant.

We have previously used the *zwf1*Δ*idp2*Δ yeast mutant as a novel model to compare macromolecular targets of endogenous

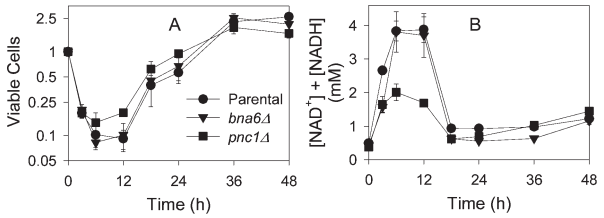

FIGURE 3: Growth and total NAD(H) levels in parental and mutant strains following challenge with hydrogen peroxide. The indicated yeast strains were grown in YP acetate medium for 6 h prior to addition of hydrogen peroxide to 1.5 mM. Samples of cultures taken before (time 0) and at indicated times following the addition of hydrogen peroxide were plated to determine viable cell numbers (A) and to prepare extracts for measurements of NAD^+ and NADH (B) as described in the caption for Figure 1.

oxidative stress with those of exogenous oxidants like hydrogen peroxide in the parental strain. Patterns of DNA damage and protein oxidation were similar in the *zwf1*Δ*idp2*Δ yeast mutant shifted to acetate medium and in the parental strain following a similar shift in the presence of exogenous hydrogen peroxide (*3*, *6*). In the current study, we therefore examined changes in cellular NAD(H) levels following an acute exogenous challenge of the parental strain with hydrogen peroxide during growth in acetate medium. We empirically determined a concentration that would produce a transient decrease in cellular viability similar to that observed following transfer of the *zwf1*Δ*idp2*Δ mutant to acetate medium. As illustrated in Figure 3A, treatment of the parental strain (●) with 1.5 mM hydrogen peroxide at time 0 produced a ∼10-fold decrease in viable cell numbers after 6−12 h. During this loss in viability, levels of NAD(H) increased ∼8-fold (Figure 3B) in viable cells, suggesting substantial rates of cofactor synthesis to counteract the exogenous oxidative stress. After 12 h, the parental strain resumed exponential growth in acetate medium (Figure 3A) and exhibited a reduction in NAD(H) after 18 h to a level approximately equivalent to that measured prior to the oxidative challenge (Figure 3B).

To determine relative contributions to the transient increase in cellular NAD(H) levels observed with hydrogen peroxide challenge of the parental strains, we constructed *bna6*Δ and *pnc1*Δ mutants. Both mutant strains exhibited transient decreases in viability similar to that observed for the parental strain following an acute challenge of acetate-grown cells with hydrogen peroxide (Figure 3A). Following this challenge, similar concomitant transient elevations in cellular NAD(H) levels were observed for the *bna6*Δ mutant (▼) and for the parental strain (Figure 3B). In contrast, the magnitude of the transient elevation in NAD(H) levels observed for the *pnc1*Δ mutant (■, Figure 3B) was ∼50% of those in parental and *bna6*Δ strains. These results suggest that function of Pnc1p in the *bna6*Δ strain is sufficient for the full transient elevation in NAD(H) levels, but that the residual de novo pathway in the *pnc1*Δ mutant can contribute sufficiently to NAD(H) synthesis to allow cells to recover from the hydrogen peroxide challenge. Consistent with our results, Castegna et al. (*18*) recently reported an ∼3-fold increase in cytosolic levels of NAD(H) in yeast strains following a 1 h exposure to hydrogen peroxide. An interesting question for future research is whether the return to prechallenge levels of NAD(H) after the transient increase observed here for all strains is due to active degradation of cofactor or to cessation of synthesis coupled with dilution as the cells grow and divide.

The magnitudes of increases in cellular NAD(H) levels we observed were similar in response to endogenous or exogenous

oxidative stress. An interesting difference was observed in redox ratios (Figure S2, Supporting Information). Shifts of parental or mutant strains to medium with acetate as a carbon source produced substantial transient increases in the cellular ratio of $[NAD^+]/[NADH]$ that returned to preshift levels within six to eight hours of cultivation. In contrast, less dramatic changes in redox ratios observed following challenge of the parental or mutant strains to hydrogen peroxide. This suggests that endogenous metabolic changes in response to acetate as a carbon source involve a rapid transient consumption of reducing equivalents that is much less pronounced with challenge by an exogenous oxidant. While not a direct measure, sampling with time during a transition from one growth condition to another as reported here is reflective of changes in flux in cofactor levels and in redox ratios.

Overall, our current and previous results (*15*) suggest that the de novo pathway for NAD(H) synthesis is sufficient for normal increases in cellular cofactor levels when a parental yeast strain is shifted from glucose to acetate medium. However, flux through the salvage pathways apparently contributes to a higher elevation of cellular cofactor levels when endogenous metabolic oxidants accumulate, as in the *zwf1*Δ*idp2*Δ mutant shifted to nonpermissive medium. In addition, function of Pnc1p is also sufficient for the dramatic transient increase in cofactor levels in parental cells acutely challenged with an exogenous oxidant (i.e., hydrogen peroxide). Thus, in general, flux through the salvage pathways appears to be for the primary mechanism for the increase in NAD(H) levels when cellular needs for the cofactor exceed normal levels. In related studies, we observed a similar contribution of Pnc1p to a substantial increase in NAD(H) levels in a TCA cycle mutant shifted to nonpermissive cultivation conditions (*15*). Also, a study by Sporty et al. (*16*) demonstrated that the de novo pathway contributed little to NAD(H) synthesis if a functional salvage pathway was present during replicative lifespan analyses in yeast, and that the salvage pathway was required for lifespan extension under conditions of caloric restriction.

Mammalian cells have both nuclear/cytosolic and mitochondrial NAD salvage pathways. Flux through the mitochondrial pathway is increased following fasting suggesting nutrient-sensitive regulation (*19*), and many recent studies indicate important links between the nuclear salvage pathway and transcriptional regulation through the activity of SIRT1, a deacetylase that regulates expression of target genes in connection with cell metabolism (*20*). Thus, it will be of considerable interest to examine the extent of alterations in cellular and compartmental NAD(H) levels and redox ratios in mammalian cells as a function of time with changes in metabolic and physiological conditions.

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SUPPORTING INFORMATION AVAILABLE

Details of experimental procedures, Figures S1, S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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