Aldo-Keto Reductases in the Stress Response of the Budding Yeast *Saccharomyces cerevisiae*

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Aldose reductase has been implicated as a factor in the pathogenesis of diabetic complications. However, little is known about the physiological role of this enzyme and of related aldo-keto reductases (AKRs) in humans. We recently turned to the budding yeast *Saccharomyces cerevisiae* as a model system for a functional genomics study of AKRs. Single gene deletions involving each of six putative yeast AKR open reading frames yielded strains with no measurable phenotypic difference from the wild type strain. However, a strain created by ablating open reading frames corresponding to the three most catalytically competent yeast AKRs (*YPR1*, *GRE3*, *GCY1*) demonstrated a marked increase in heat shock sensitivity. Oligonucleotide array experiments showed that the transcriptional response of the triple AKR null strain to heat shock was dramatically altered in comparison to the wild type strain. Transcription profiling of the triple AKR null strain also revealed substantial upregulation of stress response genes under basal (unstressed) growth conditions. Taken together, these results suggesting that yeast aldo-keto reductases may

play an important role in modulating the stress response.

Aldose reductase (AKR1B1) is thought to play an important role in the pathogenesis of diabetic complications. It is therefore an attractive therapeutic target for strategies to prevent blindness and nerve conduction deficits often associated with diabetes mellitus. While the enzyme presents itself as an attractive drug target in diabetic patients with chronic hyperglycemia, little is known about the physiological role of this enzyme or of related aldo-keto reductases in the prediabetic state or in euglycemia. Given that effective therapy is most likely to occur when intervention is begun at the earliest stage of diabetes (1), a balance must be struck between the benefits of enzyme inhibition in diabetic target tissues and potential complications resulting from blockade of the enzyme's beneficial functions. At the present time, virtually nothing is known about the physiological role fulfilled by aldose reductase.

In mammalian systems, a gene knock out approach is often used to establish gene function. However, this approach for the aldo-keto reductases has not provided insight into their physiological roles in target tissues. Functional compensation by redundant AKRs including AKRs 1A (aldehyde reductase), 1B7 (FR-1) and 1B8 (MVDP) most likely obscures phenotypes that would otherwise emerge following ablation of a single AKR gene. As an alternate strategy, we are examining the budding yeast *Saccharomyces cerevisiae* as a model system for a functional genomics study of AKRs. A distinct advantage of this system centers on the ability to readily ablate multiple targeted genes in a single strain. In addition to providing insights into functional redundancy, this system allows us to use a genetic approach to study possible effector pathways associated with one or more individual genes.

In previous studies, we carried out BLAST analysis to identify yeast open reading frames encoding AKRs with functional similarity to human aldose reductase, also known as AKR1B1 (2). Some of these putative AKRs were functionally validated by studies of their cognate recombinant proteins. In the present study, we report preliminary evidence showing that ablation of the yeast AKR genes most functionally similar to AKR1B1 results in a unique strain of *S. cerevisiae* that shows enhanced sensitivity to stress. Studies with oligonucleotide arrays show that the triple null strain has an altered transcription profile consistent with an attenuated stress response. These data indicate that AKR-null strains may provide new insights into signaling mechanisms involving this family of proteins.

Diversity of Yeast Aldo-Keto Reductases

Yeast open reading frames that encode proteins with structural similarity to human aldose reductase were identified using the BLAST search algorithm (www.stanford.edu/saccharomyces) to query the *Saccharomyces cerevisiae* genome sequence. As shown in Table 1, *S. cerevisiae* has six open reading frames that encode proteins with a high degree of sequence similarity (50-60%) and sequence identity (30-44%) to human aldose reductase. Four of the six identified ORFs corresponded to previously annotated genes. In the following section, we summarize new and previously published data for each of the putative AKR genes in *Saccharyomyces cerevisiae*.

YHR104W (*GRE3*)

The *GRE3* gene encodes a ~37 kDa polypeptide shown by Kuhn and coworkers to be an NADPH-dependent oxidoreductase with catalytic activity toward a wide variety of aldehydes including aldoses and substituted aromatic aldehydes such as *p*-nitrobenzaldehyde (3). Using enzyme purified from a recombinant expression system, we recently confirmed that Gre3p has many kinetic similarities to human aldose reductase (2).

GRE3 was so-named on the basis of its upregulation in concert with two other genes (Genes de Respuesta a Estres) following stress (osmotic, oxidative, heat shock) of yeast cultures (4). *GRE3* transcripts are found to be markedly upregulated at the diauxic shift as well. While Gre3p is a relatively efficient catalyst of xylose reduction (K_m~25 mM), hyperosmotically-stressed yeast cells do not show an increase in xylose reductase activity despite an apparent induction of *GRE3* transcripts. That *GRE3* transcription is positively regulated by Msn2p and Msn4p, two well known stress responsive transcription factors (5), also suggests that this gene could play a role in the stress response (4). Null strains for *GRE3* show no obvious phenotype when examined under normal conditions or under conditions of osmotic or oxidative stress (4,6).

YOR120W (*GCY1*)

GCY1 was initially identified as a Galactose-inducible Crystallin-like Yeast protein) in a genomic screen for genes induced by heme (7). Gene sequencing revealed an open reading frame (YOR120W) that encoded a protein with similarity to ρ-crystallin (AKR 1C10), a protein found in abundance in the frog lens (8). Although Bandlow and coworkers failed to observe stong evidence to substantiate a catalytic role for Gcy1p (7), we and others subsequently demonstrated that the protein is a respectable aldo-keto reductase with activity toward simple aldehydes such as DL-glyceraldehyde and *p*-nitrobenzaldehyde (2,9). Using X-ray crystallography, Wilson and coworkers have shown that the overall structure of Gcy1p is remarkably similar to human aldose reductase, although some structural features of the active site suggest different mechanisms might regulate the catalytic sequence of the human and yeast enzymes (10). Although *GCY1* is strongly induced by galactose, the enzyme is not able to use this aldose as a substrate. Null strains for *GCY1* show no obvious phenotype when grown under normal laboratory conditions (2).

YDR368W (*YPR1*)

In the course of a study of yeast reduction of α- and β-keto esters, Nakamura and coworkers isolated and characterized an NADPH-dependent reductase activity that showed a degree of enantioselectivity that could be exploited for synthetic chemisty applications (11). Amino acid sequencing studies established the colinearity of their purified reductase and YDR368Wp and thus Yeast Putative Reductase 1 *(YPR1*). Using enzymes purified from recombinant systems, we (2) and others (12) demonstrated the broad substrate specificity of Ypr1p. Among all yeast aldo-keto reductases, Ypr1p was the only enzyme sensitive to inhibition by sorbinil, a clinically-relevant aldose reductase inhibitor (2). Although *YPR1* is modestly induced by stress at the transcript (13) and protein (12) levels, null strains for YPR1 show no obvious phenotype when grown under normal culture conditions (2,12).

YBR149W (*ARA1*)

YBR149W (*ARA1*) encodes a subunit of arabinose dehydrogenase, a heterodimeric enzyme that catalyzes the oxidation of D-arabinose in the pathway leading to D-erthyroascorbic acid synthesis (14). Functioning predominantly in the direction of alcohol oxidation, Ara1p is one of the few aldo-keto reductases that acts as a carbonyl producer rather than carbonyl reducer. Not only does Ara1p differ in catalytic preference from the three yeast AKRs described above, but it also appears to be much less involved as a possible anti-stress gene. Transcriptional profiling studies have shown that *ARA1* transcripts are induced to a minimal extent following osmotic or oxidative stress in comparison to those derived from *GRE3, GCY1* and *YPR1* (13).

YJR096W

A polypeptide of 282 amino acids is encoded by the YJR096W open reading frame. The product of this gene has not been described in the literature, although evidence demonstrating the gene is transcriptionally active can be deduced from a various DNA microarray studies (see Microarray Global Viewer, http://www.transcriptome.ens.fr/ymgv/). For example, data from a study conducted by Causton and coworkers show that transcripts corresponding to YJR096W are induced under various stress conditions (15).

To further investigate whether YJR096Wp might fulfill a functional role in yeast metabolism, we examined the kinetic properties of the recombinant enzyme. In previous studies, we demonstrated that YJR096Wp can catalyze the NADPH-dependent reduction of various aldehydes such as DL-glyceraldehyde and D-xylose (2). As shown in Table 2, we have now determined kinetic constants for an extensive range of potential substrates. As can be seen from these data, YJR096Wp utilizes a broad variety of aldehyde containing compounds as substrates, including aromatic and aliphatic aldehydes as well as aldoses such as xylose. The best substrate examined is the substituted aromatic hydrocarbon, *p*-nitrobenzaldehyde, which is reduced with a catalytic efficiency approximately 3 orders of magnitude greater than all other compounds examined in this study. Catalysis was dependent on NADPH, as no activity could be detected when NADH was used as a cofactor. Likewise, we were unable to detect alcohol oxidation when YJR096Wp was assayed in the presence of NADP or NAD. These results suggest that this enzyme functions primarily as an aldehyde reductase.

YDL124W

YDL124W is another open reading frame that encodes a putative aldo-keto reductase. While no functional significance has yet been assigned this open reading frame, DNA microarray studies clearly demonstrate that the gene is transcriptionally active. Indeed, transcripts corresponding to this ORF were found to be elevated under some stress conditions (see Microarray Global Viewer, http://www.transcriptome.ens.fr/ymgv/). Following up from a previous study (2), we evaluated the catalytic potential of recombinant YDL124Wp by examining reduction reactions with a broad range of aldehyde-containing compounds listed in Table 2. Under conditions found to support activity of GRE3p, GCY1p and YPR1p, we failed to detect significant reaction rates when assays were conducted using aldose and aliphatic aldehydes, even when the enzyme was examined at a concentration likely to significantly exceed a reasonable physiological level (1-10 μM). However, relatively good reaction rates were observed using phenylglyoxal (apparent K_m~ 8 mM). Using this substrate, we were able to determine the apparent $K_{m\ NADPH}$ to be approximately 7 μM. Substitution of NADPH with NADH as the pyridine cofactor failed to support reductase activity. Further study will be required to identify additional compounds that could be physiologically significant substrates for YDL124Wp.

Comparative DNA sequence analysis of YJR096W and YDL124W in *Saccharomyces* species

Johnston and coworkers have recently established the genome sequence of several *Saccharomyces* species in an effort to identify functional elements by comparative DNA sequence analysis (16). Since the functions of YJR096W and YDL124W are not currently recognized, we hypothesized that an analysis of the encoded protein sequences deduced from various *Saccharomyces* species would suggest whether the respective protein products were of functional significance. As shown in Table 3, the yeast gene products that display robust aldo-keto reductase activity (GRE3p, GCY1p, YPR1p, YJR096Wp) are all highly conserved across *Saccharomyces* species (88-98% identity to their *Saccharomyces cerevisiae* orthologs). Although YDL124W displays only modest reductase activity *in vitro,* a high degree of conservation is seen with the gene product in *S. paradoxus* (92%) and *S. mikatae* (89% identity). The genomic region corresponding to the ortholog in *S. bayanus* requires further coverage before a homology calculation can be completed.

Catalytically competent mammalian AKRs all possess a catalytic tetrad consisting of aspartate^{43}, tyrosine^{48}, lysine^{77} and histidine^{110} (numbering according to the human aldose reductase primary sequence), with tyrosine^{48} and histidine^{110} playing key roles in the reaction mechanism of aldehyde reduction (17,18). As shown in figures 1 and 2, alignment of the human aldose reductase primary sequence with YJR096Wp and YDL124Wp derived from *Saccharomyces* species demonstrates that all four catalytic residues have been completely conserved. Indeed, all four catalytic residues are conserved in each of the six putative AKRs identified in *Saccharomyces cerevisiae* (2).

Phenotypic Characterization of Aldo-Keto Reductase Knock Out Strains

In an effort to determine the physiological function of the AKRs in *Saccharomyces cerevisiae*, both individually and as a family of enzymes, we constructed isogenic deletion strains lacking one or more AKR open reading frames. As noted in our previous studies, AKR null strains containing a single

AKR open reading frame deletion are without noticeable phenotype when screened for sensitivity to a variety of stress conditions such as osmotic and oxidative stress and heat shock (2,6). We hypothesized that yeast strains null for a single AKR open reading frame were phenotypically normal due to functional compensation by remaining AKR genes. Support for this hypothesis can be drawn from the broad overlapping substrate specificity observed with at least three of the six yeast AKRs (GRE3p, YPR1p, and GCY1p) (2). Accordingly, we devised a scheme whereby open reading frames corresponding to these three catalytically active AKRs were progressively deleted.

As shown previously, isogenic double AKR null strains (*gre3Δ ypr1Δ, gre3Δ gcy1Δ, ypr1Δgcy1Δ*) were all found to be without significant phenotype (6). However, the triple AKR null strain *gre3Δ ypr1Δ* gcy1*Δ* demonstrated a marked sensitivity to heat shock (6). To establish the effect of AKR gene deletion on yeast metabolism and stress response, we compared the transcriptional profile of wild type and the isogenic triple AKR null strain using Affymetrix oligonucleotide arrays.

DNA microarray studies

Given the marked sensitivity of the triple AKR null strain (*gre3Δ ypr1Δ* gcy1*Δ*) to heat shock stress, we compared the transcription profile of wild type and triple null strains in log phase growth under both normal conditions (30˚C) and following a mild heat shock (30 min, 37˚C). Each strain was cultured in triplicate under both growth conditions (12 microarray chips in total). DNA targets were produced from total RNA extracted from yeast cultures using standard conditions.

Data from the microarray chips (Affymetrix Genechip YGS98) were initially subjected to a scaling process using Affymetrix Microarray Suite 5.0 software. The resulting data were then imported into Silicon Genetics GeneSpring 4.2. Each ORF was normalized to a value of 1 using the mean of the values obtained from all 12 gene chips. As only gene sets that passed a comparatively more stringent significance test were entered into the results reported here, fewer induced gene transcripts are identified than were previously reported from this study (6).

A comparison of the transcription profile from wild type *Saccharomyces cerevisiae* grown under normal and heat shock conditions shows that heat shock results in the induction of a large number of genes. As shown in Figure 3, 346 genes showed a ≥ 2.5-fold increase in transcript level. In contrast, only 61 genes met the same criteria for induction following heat shock in the triple AKR null strain. Of the 61 heat shock-induced genes, the vast majority could be classified as heat shock responsive, as they were the same genes as those induced by heat shock in the wild type strain. Nevertheless, it is clear that the triple AKR null strain has a markedly reduced ability to mount a transcriptional response to heat shock stress.

Comparison of wild type and the triple AKR null strains grown under basal culture conditions also revealed an unexpected transcriptional profile. A large number (91) of genes appear to be activated even under basal culture conditions in the triple null strain as compared to wild type (Figure 4). All 91 genes found to be constitutively-induced in the triple AKR null strain were heat shock inducible in the wild type strain. However, of the 91 constitutively-induced genes in the triple AKR null strain, only 17 were further inducible by heat shock in the deletion strain (data not shown).

These data indicate that ablation of the three most catalytically competent aldo-keto reductase genes results in dysregulation of the heat shock response in *Saccharomyces cerevisiae*. Many genes that should be induced by heat shock are constitutively upregulated even under basal conditions. Furthermore, heat shock treatment of the triple AKR null strain fails to elicit the large majority of genes that should respond to this type of stress, as evidenced by the relatively small number of genes that are induced (~18% of wild type). This combination of dysregulated transcription control most likely is responsible for the heat shock sensitivity we observed in the triple AKR null strain.

Many investigators have shown that the stress response in yeast is mediated in part through the action of the transcription activators Msn2p and Msn4p (15,19). The ability of these transcription activators to affect gene expression is controlled in part through their subcellular distribution. To upregulate target genes, Msn2p and Msn4p, which are cytoplasmic proteins, must accumulate in the nucleus. Nuclear localization is reported to be affected by protein kinase A-mediated phosphorylation (5) or alternatively by interactions with factors that retain them in the cytoplasm (20). It is interesting to consider whether the altered transcription profile observed in the triple AKR null strain results from an alteration in the subcellular distribution of transcriptional activators such as Msn2p and Msn4p or other factors that contribute to the regulation of the stress response. Additional studies will be required to establish a mechanism linking the presence of the yeast AKRs and the stress response program in yeast.

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Table Caption List

Table 1. Putative Aldo-Keto Reductases in *Saccharomyces cerevisiae*

NOTE: HAR, human aldose reductase, AKR1B; Synonyms are gene names recognized by the yeast genetics community, "-" represents a gene synonym has not yet been designated.

SOURCE: BLAST results were collated through the YPD™ (21)

Table 2: Kinetic constants for yeast aldo-keto reductases

NOTE: Activity measurements with purified recombinant yeast aldo-keto reductases were carried out in a Tris-MES buffer at pH 8.0. Kinetic constants with recombinant human aldose reductase were reported elsewhere (17,22,23). NMA, no measurable activity; Trace indicates that detectable activity was below the level necessary to accurately determine kinetic constants.

Table 3. Homology of orthologous aldo-keto reductases in *Saccharomyces* species.

NOTE: Data are percent identity with the corresponding ortholog in *Saccharomyces cerevisiae*; "-" Data for the YDL124W ortholog in *S. bayanus* are not currently available. Genome sequences that made these comparisons possible were generously provided by P. Cliften, M. Johnston and the Washington University Genome Sequencing Center (personal communication).

Figure Caption List

Figure 1. Alignment of *Saccharomyces* orthologs of YJR096W.

Sequences were aligned using Vector NTI (Informax) and GeneDoc. Aligned residues demonstrating100% conservation with human aldose reductase are shown in black background; those demonstrating 80-99% conservation are shown in gray background. Key catalytic residues identified in mammalian AKRs are shown in boxes.

Figure 2. Alignment of *Saccharomyces* orthologs of YDL124W

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Figure 3. The triple AKR null strain has an attenuated heat shock response.

Heat shock resulted in induction of 346 genes in the wild type strain compared with 61 genes in the triple AKR null strain. The region of overlap shows that 53 genes in common were induced in both strains.

Figure 4. Constitutive upregulation of heat shock responsive genes in the triple AKR null strain.

Heat shock resulted in induction of 346 genes in the wild type strain.When compared with wild type under basal culture conditions, the triple AKR null strain demonstrated upregulation of 91 genes. The region of overlap shows that all 91upregulated genes in the null strain were heat shock inducible genes in the wild type.