Published in final edited form as:
Biochim Biophys Acta. 2007 March ; 1773(3): 321–329.

Functional studies of aldo-keto reductases in *Saccharomyces cerevisiae**

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SUMMARY

We utilized the budding yeast *Saccharomyces cerevisiae* as a model to systematically explore physiological roles for yeast and mammalian aldo-keto reductases. Six open reading frames encoding putative aldo-keto reductases were identified when the yeast genome was queried against the sequence for human aldose reductase, the prototypical mammalian aldo-keto reductase. Recombinant proteins produced from five of these yeast open reading frames demonstrated NADPH-dependent reductase activity with a variety of aldehyde and ketone substrates. A triple aldo-keto reductase null mutant strain demonstrated a glucose-dependent heat shock phenotype which could be rescued by ectopic expression of human aldose reductase. Catalytically-inactive mutants of human or yeast aldo-keto reductases failed to effect a rescue of the heat shock phenotype, suggesting that the phenotype results from either an accumulation of one or more unmetabolized aldo-keto reductase substrates or a synthetic deficiency of aldo-keto reductase products generated in response to heat shock stress. These results suggest that multiple aldo-keto reductases fulfill functionally redundant roles in the stress response in yeast.

Keywords

aldo-keto reductase; aldose reductase; *Saccharomyces cerevisiae*; mutagenesis; heat shock

1. Introduction

Aldo-keto reductases (AKR)1 comprise a superfamily of structurally-similar proteins that catalyze the NADPH-dependent conversion of various carbonyl compounds into their corresponding alcohol products. In humans, the AKRs are implicated in diseases involving disposition of excess aldoses. Aldose reductase (AR), a prototypical AKR, catalyzes the conversion of glucose into sorbitol, which represents the first step in the polyol pathway. In humans with diabetes mellitus, increased activity of the polyol pathway due to chronic hyperglycemia has been associated with complications such as cataract and retinopathy [1], neuropathy [2], and nephropathy [3]. Beneficial roles for AKRs have been difficult to establish because tissues typically contain many similar enzymes with overlapping substrate and

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inhibitor specificities. Genetics-based approaches to explore physiological function using the mouse model have also been inconclusive, primarily due to probable redundancies between aldose reductase and similar aldo-keto reductases such as MVDP [major vas deferens protein, ref. 4] and FR-1 [fibroblast growth factor regulated-1, ref. 5]. Disruption of the aldose reductase gene in mice demonstrate a relatively benign phenotype resembling that of diabetes insipidis, which may be related to altered osmotic regulation in the kidneys of mutant animals [6,7].

The purpose of the current study was to utilize a simpler and more manageable eukaryotic model system to explore AKR gene function. By BLAST analysis of the *Saccharomyces cerevisiae* genome, we previously identified six open reading frames (YHR104w, YOR120W, YDR368W, YBR149W, YJR096W, YDL124W) which encode proteins with high predicted sequence similarity to human aldose reductase [8]. Three of these ORFs (YHR104W, YDR368W, YOR120W) have been previously shown to encode proteins with robust activity as NADPH-dependent carbonyl reductases [9–11], whereas one (YBR149W) functions primarily in the direction of alcohol (arabinose) oxidation [12]. In preliminary studies, we previously demonstrated that a triple null strain produced by disruption of ORFs encoding the three known AKRs exhibits a heat shock phenotype [13]. In the current study, we sought to evaluate the remaining two ORFs identified in our BLAST analysis (YJR096W and YDR368W) with respect to the kinetic properties of their encoded proteins as well as to probe for phenotypes of a mutant strain deficient in the five targeted aldo-keto reductases.

2. Materials and Methods

Expression and purification of recombinant yeast AKRs

Slightly different methods were used for production of recombinant yeast AKRs. The ORF for YJR096W (AKR5F), obtained from The Institute for Genome Research (TIGR, Rockville, MD), was subcloned into the prokaryotic expression plasmid pET23D (Novagen, Madison, WI) for expression of the recombinant protein in *Escherichia coli* strain BL21. The ORFs for YDL124W (AKR5G) and YOR120W (AKR3A1), obtained by PCR amplification from genomic DNA purified from *S. cerevisiae* strain S288c, were cloned into pET16B and pET23D (Novagen), respectively. Coding sequences in all plasmids were confirmed against the genome sequence of wild type *S. cerevisiae* strain S288c [14] prior to expression studies.

Recombinant proteins were purified from cultures grown in baffled shake flasks. In all cases, log phase cultures were grown for 4–16 hours following treatment with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression of recombinant proteins. Cells were harvested by centrifugation (15,000 x g, 15 min, 4 °C) and were lysed after treatment with DNase (Sigma, St. Louis, MO) and lysozyme as described previously [15]. Extraction buffers contained the protease inhibitors (each at 1 μg/ml) antipain, bestatin, chymotrypsin, leupeptin, and pepstatin (Roche, Indianapolis, IN).

All extraction and purification steps were carried out at 4°C. As a group, yeast AKRs were readily prepared as recombinant proteins in E. coli host cultures. All except AKR5G (Ydl124w) were recovered as buffer-soluble proteins following extraction from host cells. In preliminary studies of AKR5G, expression of the encoded protein with an amino terminal histidine tag (7 residues in length) was associated with poor aldo-keto reductase activity [8]. For this reason, all yeast AKRs used in this study were expressed without fusion with affinity epitopes.

Methods and conditions for purification of each of the individual recombinant yeast AKRs are given below. In all cases, our methods routinely yielded in excess of 30 mg of highly purified protein from each liter of culture.

AKR3A1 (Yor120w)—Initial purification of AKR3A1 from host cell lysates was achieved by IEC using a carboxymethyl exchange resin (Macro-Prep CM, Bio-Rad). Lysate material from host cultures was first extensively dialyzed against a solution of 20 mM (2-[N-morpholinoethanesulfonic acid, MES), pH 6.0, containing 1 mM each of DTT and EDTA. After a brief centrifugation to remove insoluble materials, the dialyzed lysate was applied at 1 ml/min to a 2.5 x 10 cm column packed with the Macro-Prep CM resin. Unadsorbed material was flushed from the column with approximately 80 ml loading buffer. Bound materials were then eluted with a linear gradient of NaCl (0–0.5 M), contained in the loading buffer, over a volume of 600 ml. The eluate was continuously monitored by absorbance at 280 nm and was collected into 10 ml fractions for measurement of NADPH-dependent reductase activity using 5 mM DL-glyceraldehyde as substrate. Column fractions with reductase activity were pooled and dialyzed against 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 1 mM EDTA. Yor120w was further purified by dye-affinity chromatography using Affi-Gel Blue® resin (Bio-Rad, Hercules, CA). After loading to the column (2.5 x 5 cm) and a thorough wash with the loading buffer, bound material was eluted with a linear gradient of NaCl (0–1.4 M) contained in 400 ml loading buffer supplemented with 1mM each of DTT and EDTA. The eluate was continuously monitored by absorbance at 280 nm and was collected into 10 ml fractions. Fractions containing reductase activity were pooled and concentrated by ultrafiltration to approximately 1 mg/ml. The purified material was stored at 4°C and used within 2 weeks or stored at −70°C.

Expression and purification of the Y55F mutant of AKR3A1 was carried out exactly as for the wild type protein. Purification of the mutant protein was monitored by the presence of a highly enriched~36 kDa protein band on SDS-PAGE analysis of purification fractions. Materials extracted from induced cultures of the *E. coli* strain BL21 host transformed with a nonrecombinant (empty) pET expression plasmid were analyzed in a like manner.

AKR5F (Yjr096w)—Purification of recombinant AKR5F (Yjr096w) was achieved by anion exchange chromatography using DEAE-Sepharose (Amersham Biosciences, Piscataway, NJ). After overnight dialysis against 50 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 1 mM DTT, the cell-free extract was centrifuged briefly and the resulting supernatant applied to a 2.5x10 cm column of the ion exchange chromatography (IEC) resin. Bound proteins were eluted with a linear gradient of NaCl (0–0.4 M) contained in 600 ml of equilibrating buffer. Presence of AKR5F in column fractions was estimated by measuring NADPH-dependent reductase activity using 2 mM *p*-nitrobenzaldehyde as the aldehyde substrate. Proteins in column fractions were also visualized after separation by SDS-PAGE and Coomassie staining. Fractions containing reductase activity were pooled, dialyzed into 10 mM potassium phosphate buffer, pH 7.0, and applied to a 2.5 x 5 cm chromatography column packed with Affi-Gel Blue® (Bio-Rad) previously equilibrated with the same buffer. After a wash to remove unadsorbed material, bound proteins were eluted with a linear gradient of sodium chloride (0–2.5 M) contained in the equilibration buffer. Reductase assays on column fractions identified an activity peak that was coincident with a peak defined by A_{280}. Fractions containing reductase activity were pooled, concentrated to ~ 1 mg/ml, and stored at 4 °C.

AKR5G (Ydl124w)—Preliminary studies showed that AKR5G expressed as a His-tagged fusion protein had very low AKR activity [16, data not shown]. In the present study, the enzyme was expressed without an amino-terminal His-tag epitope. The majority of recombinant AKR5G was found in the insoluble cell pellet following DNAse-lysozyme extraction (not shown), and was recovered by treatment for 30 minutes at 4 °C with a cracking buffer consisting of 10 mM sodium phosphate, pH 7.2, 6 M urea, 142 mM β-mercaptoethanol, and protease inhibitors essentially as described previously [17]. Materials solubilized in cracking buffer were dialyzed (M_r 12,000–14,000 cut-off tubing) against 100 volumes of DEAE column buffer (10 mM TrisHCl, pH 7.4, 0.5 mM DTT, 0.5 mM EDTA. After centrifugation to remove

insoluble materials, lysates were applied at 1 ml/min to a chromatography column (2.5 x 10 cm) packed with DEAE ion exchange resin previously equilibrated with column buffer noted above. After flushing out unadsorbed materials with a 500 ml buffer wash, bound proteins were eluted with a linear gradient (0–200 mM) of sodium chloride prepared in column buffer. Eluate fractions (10 mL) were assayed for the presence of NADPH-dependent aldo-keto reductase activity using 5 mM DL-glyceraldehyde as a carbonyl substrate. Fractions with reductase activity were pooled and further purified by dye affinity chromatography essentially as described above for purification of AKR5F, with the exception that bound materials were eluted first with a step increase from 0 to 1.5 M NaCl in the column buffer (200 mL) followed by a linear NaCl gradient (1.5–2.5 M) contained in 300 ml column buffer. Fractions containing reductase activity were pooled, dialyzed against 40 volumes of 10 mM potassium phosphate, pH 7.2, containing 0.5 mM DTT and 0.5 mM EDTA. Purified materials were stored at 4 °C for up to 2 weeks or for indefinite periods at −70 °C.

Enzyme assays

Reductase activity was measured at 23 °C using a Cary 1E spectrophotometer interfaced with a peltier temperature-controlling device. Assay mixtures of 1.0 ml contained 0.15 mM NADPH and aldehyde substrate in a triple buffer system consisting of 25 mM 2-(*N*-morpholino) ethanesulfonic acid, 25 mM potassium phosphate, and 90 mM Tris, at a pH titrated to correspond to the pH optimum for the enzyme being tested (see below). Reactions were initiated by addition of enzyme, and initial rates calculated from the decrease in absorbance at 340 nm upon oxidation of NADPH. To determine steady state kinetic constants, initial velocities observed as a function of substrate concentration (ranging from approximately 0.1- to 5-times K_m) were fitted to the Michaelis-Menten equation using Graphpad Prizm (San Diego, CA).

Strains, Gene Deletion, and Growth Conditions

Parental wild-type yeast strain (BY4742) and isogenic single deletion mutants, which were made available through the Yeast Deletion Project (ref PMID: 12140549) see http://www.stanford.edu/group/yeastdeletionproject/deletions3.html), were obtained from Research Genetics (Carlsbad, CA). A list of yeast strains used in this study is summarized in Table I. Generation of AKR gene deletion mutants was performed by the PCR-mediated gene disruption approach [18]. Targeting primers for disruption of open reading frames used in conjunction with several different selectable markers carried in the pRS4XX series of plasmids are shown in Table II. PCR amplimers for targeted gene disruption were used to transform recipient cells using the lithium acetate method. After growth of transformants on selective media, deletion of the targeted gene sequences was routinely confirmed by PCR using primer sets directed toward sites internal as well as flanking the targeted gene of interest (Table III).

Cloning and mutagenesis of AKR3A1 (Gcy1) and AKR1B1 (human aldose reductase) for expression in yeast

While in the context of pBLUESCRIPT, codons for tyrosine-48 of AKR1B1 (human AR) [19], and the homologous site (tyrosine-55) of AKR3A1 (Gcy1), were mutated to phenylalanine using a QuikChange site-directed mutagenesis kit (Stratagene). For preparation of yeast expression constructs, the mutant genes were released by endonuclease treatment (*Spe*I and *Hin*dIII for AKR1B1, and *Bam*HI and *Pst*I for AKR3A1) and were ligated into the corresponding sites of the episomal GPD425 expression vector as described above. DNA sequencing across ligation sites and open reading frames was carried out to confirm the fidelity of all plasmid constructs.

3. Results

Characterization of yeast aldo-keto reductases

Yeast AKRs may be identified according to a gene name, an ORF association, and according to a systematic AKR nomenclature [20]. A cross-referenced list of genes, ORFs, and AKR designations is shown in Table IV. By BLAST search, we previously identified six ORFs (YDR368W, YHR104W, YOR120W, YBR149W, YJR096W, YDR124W) in the *S. cerevisiae* genome sequence which showed clear amino acid sequence similarity (30–44% identity) to human aldose reductase (AKR1B1) [8]. Alignment of the deduced sequences against the primary structure of human aldose reductase indicates that amino acid side chains known to facilitate catalysis in mammalian AKRs are strictly conserved across these yeast proteins [19,21,22]. As a group, these proteins share approximately 30–40% sequence identity, excepting AKR3A1 (Gcy1) and AKR3A2 (Ypr1), which are approximately 65% identical. To evaluate the ability of the encoded proteins to function as an NADPH-dependent aldo-keto reductase, ORFs for putative AKRs were cloned into expression plasmids and over-expressed in *Escherichia coli* host cultures. Studies of AKR3C (Ara1) were not undertaken because, unlike AR, this enzyme functions preferentially in the direction of NADP-dependent *aldose* oxidation [12]. Various structural and kinetic properties of some yeast AKRs examined in our study, including, AKR3A2 (Ypr1) and AKR2B6 (Gre3), were reported previously by us [8] and other investigators [9–11,23] using recombinant and native enzymes purified from yeast cultures, respectively. In the present work, we carried out a systematic evaluation of the remaining putative AKRs including AKR5F (Yjr096w), AKR5G (Ydl124w) and AKR3A1 (Gcy1) so as to further our understanding of these proteins as functional aldo-keto reductases.

Since only limited data was previously published on the putative reductase activities of these putative AKRs [8,11,11,16], we initiated a screen for aldo-keto reductase activity using 150 μM NADPH as cofactor and either DL-glyceraldehyde or *p*-nitrobenzaldehyde as aldehyde substrate. These enzymes all showed robust activity, with optima at pH 7.5-8.5 for AKR5F and pH 6.0–7.5 for AKR3A1 (Figure 2). Reductase activity for AKR5G showed no distinct optimum over a pH range of pH 5 to pH 8.5, but dropped sharply above pH 8.5 (Figure 1). The reductase activity level of each protein was not above background when assays were conducted using DL-glyceraldehyde or *p*-nitrobenzaldehyde as the aldehyde substrate and NADH instead of NADPH. Using reaction conditions optimized for each enzyme, catalytic constants were determined with a variety of aldehyde substrates. As shown in Table V, the catalytic efficiency of AKR3A1 was generally higher than AKR5F or AKR5G with almost all substrates examined. No measurable activity was observed when each of these enzymes was assayed using aldose substrates such as D-glucose and D-xylose. As a group, the enzymes showed highest catalytic efficiencies with aromatic aldehydes such as benzaldehydes and phenylglyoxal.

Alignment of amino acid sequences demonstrates that a tyrosine at the position corresponding to tyrosine-48 in human aldose reductase is conserved among all yeast AKRs examined in this study [8]. We hypothesized that substitution of phenylalanine for tyrosine-55 would result in loss of AKR activity in AKR3A1 (Gcy1), similar to that observed with aldose reductase [19] and 3α-hydroxysteroid dehydrogenase [24]. As with wild type AKR3A1, the Y55F mutant was expressed to high levels in IPTG-induced cultures and was obtained in virtually homogeneous form by a two-step column purification procedure. Although reductase activity in the Y55F mutant was virtually undetectable, purification of the mutant was easily followed by the appearance of a protein band of approximately M_r 36000 on SDS-PAGE of material from column fractions (Figure 2). High reductase activity levels were found in the corresponding fractions from preparations starting with wild type AKR3A1. A protein band corresponding in size and abundance to AKR3A1 was not observed in whole cell extracts or in purification fractions using material from induced *E.coli* expression cultures transformed with an empty pET21d expression plasmid (not shown).

Consistent with results from other aldo-keto reductases, the substitution of Phe for Tyr-55 caused a dramatic reduction in catalytic efficiency. Using DL-glyceraldehyde as a typical carbonyl substrate, the catalytic efficiency of wild type AKR3A1 was reduced from 553 $mmol^{-1}min^{-1}$ to 0.16 $mmol^{-1}min^{-1}$ for the Y55F mutant. Virtually all of this change results from a reduction in k_{cat}, as there was a relatively small change in K_m DL-glyceraldehyde. The K_m was 0.97±0.1 mM for wild type and 1.5±0.1 mM for the Y55F mutant.

Phenotypic Characteristics of AKR deletion mutants

Based on this and previous work, it is clear that two of the yeast AKRS examined, AKR5F (Yjr096w)and AKR5G (Ydl124w), have a relatively poor ability to catalyze reduction of carbonyls represented in our battery of substrates. Given that AKR3A1 (Gcy1), AKR3A2 (Ypr1) and AKR2B6 (Gre3) demonstrate more robust activity as aldo-keto reductases, we hypothesized that these enzymes would be excellent candidates to test for functional conservation with mammalian aldo-keto reductases [8]. Accordingly, *GCY1*, *YPR1*, and *GRE3* were disrupted in a series of mutant strains to test the hypothesis that human aldose reductase can functionally compensate for one or more of the yeast AKR genes.

Single gene deletion mutants of *YPR1*, *GRE3* or *GCY1* do not display an obvious phenotype [25–27]. This is not surprising since these genes may be functionally redundant. We examined double and triple AKR gene deletion mutants with a variety of phenotypic screens including growth rate at normal (30°C) or elevated temperatures (37°C) in YPD, cell morphology/ budding pattern, growth sensitivity to various stress inducible agents, growth auxotrophy for different carbon sources and nutrients, and growth sensitivity to screening drugs selected to probe major signaling transduction pathways and nucleic acid metabolism (Table VI). In preliminary studies, we observed that mutants missing any two of the three AKR genes were not distinguishable from the wild type strain, but deletion of all three AKR genes (*ypr1Δ/ gre3Δ/gcy1Δ;* strain ARD10) resulted in a marked heat shock sensitivity [13]. In the current study, we show that further disruption of ORFs that encode the two additional putative AKRs resulted in an enhancement of the heat shock phenotype (Figure 3A).

Transformation of strain ARD10 with a plasmid carrying *GCY1*, but not *gcy1* Y55F, partially rescued the heat shock sensitivity. (Figure 3B). The human aldose reductase cDNA expressed from the GPD promoter partially rescued heat shock sensitivity, but not if it carried a mutation of the active site tyrosine-48 residue (Y48F) to create a catalytically inactive enzyme (Figure 3B). These results indicate that either human or yeast AKRs is capable of rescuing the heat shock phenotype in triple AKR deficient mutant strains, suggesting some degree of functional conservation among AKRs in yeast and humans.

4. Discussion

The budding yeast *Saccharomyces cerevisiae* has six ORFs that encode putative aldo-keto reductases (Table IV). Products of YHR104W (Gre3) and YDR368W (Ypr1) were initially identified as stress response proteins on the basis of their upregulation following growth in the presence of hypertonic media [26,28]. The product of YOR120W (Gcy1) was initially identified as a galactose-inducible crystallin-like yeast protein [27]. Subsequent studies demonstrated that each of these gene products had properties typical of classical aldo-keto reductase, i.e., catalytic activity with various classes of carbonyl-containing substrates, preferentially in the direction of carbonyl reduction, using NADPH as the reducing coenzyme [9–11]. Current evidence suggests that YBR149W (Ara1) encodes a dehydrogenase that functions preferentially in the direction of arabinose oxidation rather than reduction [12]. The functions of the remaining ORFs identified in our BLAST analysis, including YDL124W and YJR096W, have not been extensively studied. Ishihara recently reported evidence that a yeast protein, identical to that encoded by YDL124W, catalyzes the NADPH-dependent reduction

of aromatic α-keto amides and α-keto esters [29]. Recent studies also indicate that the proteins encoded by YJR096W and YDL124W and may play a role in arabinose and xylose metabolism [30].

In preliminary studies, we showed that Gre3, Ypr1, Gcy1, and human AR, purified as recombinant proteins, have NADPH-dependent aldo-keto reductase activity using aldehyde substrates such as DL-glyceraldehyde and *p*-nitrobenzaldehyde [8]. This suggested to us that the functions of AKR genes between yeasts and humans may be similar. Our observation that ectopic expression of human aldose reductase rescues the heat shock phenotype of the triple AKR null strain suggests that human and yeast AKRs fulfill similar catalytic functions that are necessary for recovery from stress associated with heat shock. Since the catalytically-inactive mutants of human aldose reductase or Gcy1 failed to show a rescue effect, it is possible that the heat shock phenotype results from either an accumulation of one or more unmetabolized AKR substrates or a synthetic deficiency of AKR products generated in response to stress or culture conditions. Further studies are ongoing to identify pathways and processes that are influenced by the action of AKRs.

Previous studies have shown that 4-hydroxynonenal (4-HNE), a lipid-derived aldehyde produced during oxidative stress, is a high affinity substrate for aldose reductase in mammalian cells [31]. In addition, aldose reductase gene expression is induced in response to osmotic stress [32,33]. Accordingly, we expected that deletion of the three cognate AKR genes would induce phenotypes representative of oxidative and osmotic stress. However, this is not the case, as essentially no difference was observed in the growth of this mutant when compared to wild type yeast under various oxidative and osmotic stress conditions. We hypothesize that this outcome may result from at least two possibilities. First, lipid-derived reactive aldehydes, such as 4-HNE, are not produced in yeast due to low amounts of polyunsaturated fatty acids, the obligate precursors to lipid aldehydes generated by oxidative stress [34]. Although the heat shock phenotype was glucose-dependent, we consider it unlikely that any of the three disrupted AKRs play roles in the response to osmotic stress through production of an osmolyte such as sorbitol. Unlike human AR, none of the yeast AKRs are capable of converting glucose to sorbitol, an active osmolyte known to be involved in renal response to hypertonicity [35]. However, at least two of the yeast AKRs, namely *GRE3* and *YPR1*, were identified as osmoregulated genes in previous studies [26,26,28]. Similar to findings reported here, previous investigators observed no measurable difference from wild type control when *gre3* or *ypr1* mutants were subjected to osmotic stress [26,36].

Although AKR3A2 (Ypr1) and AKR2B6 (Gre3) have been studied in the context of their activity as NADPH-dependent carbonyl reductases, very little is known regarding the catalytic activities of other yeast enzymes such as AKR3A1 (Gcy1), AKR5F (Yjr096w) and AKR5G (Ydl124w). Our results demonstrate each of these gene products has easily measured activity as an NADPH-dependent aldehyde reductase. Human and yeast AKRs appear to have similar activities when assays were conducted with substrates such as aliphatic and aromatic aldehydes. These results confirm that yeast, like mammalian tissues, contain multiple AR-like enzymes with activity against a broad range of aromatic and aliphatic aldehydes [37].

Crystallography studies of mammalian aldose reductases indicate that the proteins adopt a $(\beta/\alpha)_8$ barrel structural fold with the active site buried deep within the hydrophobic barrel core. Functional and structural studies with human aldose reductase (AKR1B1) suggest the importance of Asp-48, Lys-77, Tyr-48 and His-110 as key residues in the catalytic sequence [19,38,39]. Comparison with AKR1B1 shows that all of these key residues are conserved at homologous sites throughout the yeast AKRs. Similar to that observed with human aldose reductase [19], mutagenesis of the presumptive hydrogen donor to create the Y55F mutant of AKR3A1 (Gcy1) resulted in essentially complete loss of AKR activity. Structural studies have

shown that, unlike the case with Tyr-48 in human aldose reductase [19,40], the orientation of the Tyr-55 side chain is not favorably positioned to function as an acid in the reduction reaction [41]. Further study will be required to establish whether AKR3A1 utilizes a different catalytic mechanism than hypothesized for mammalian AKRs.

Since phenotypic abnormalities did not appear until ORFs corresponding to at least three yeast AKRs were interrupted, we hypothesize that these enzymes fulfill functionally redundant roles even though their expression may fall under different transcriptional control. Some differences were noted between human and yeast enzymes with respect to catalytic efficiency with some physiologically relevant substrates such as glucose. However, the observation that human aldose reductase can rescue the heat shock phenotype of the triple AKR null mutant suggests that human and yeast AKRs share overlapping physiological roles. Future studies will be needed to identify pathways and structures that may be affected by deficiencies in AKR gene expression in *S cerevisiae*. Such studies may provide valuable clues in our ongoing studies to discover physiological roles of this enzyme family in human tissues.

Acknowledgements

We wish to thank Dr. Mark Johnston for advice and help with the manuscript, his associate Linda Riles for her technical assistance with targeted gene disruption procedures, and Loryn Rikimaru, B.S.N. Murthy, Kent Morris and Michael Young for their assistance with cloning and expression of recombinant yeast proteins. This work was supported in part by NIH grants EY05856, EY02687, DK20579 and by awards to the Department of Ophthalmology and Visual Sciences from Research to Prevent Blindness, Inc. Support from the Kilo Diabetes and Vascular Research Foundation is also gratefully acknowledged.

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The abbreviations used are

AKR
: aldo-keto reductase

AR
: aldose reductase

GPD
: glyceraldehyde 3-phosphate dehydrogenase

IEC
: ion exchange chromatography

ORF
: open reading frame

YPD
: yeast peptone dextrose, synthetic complete

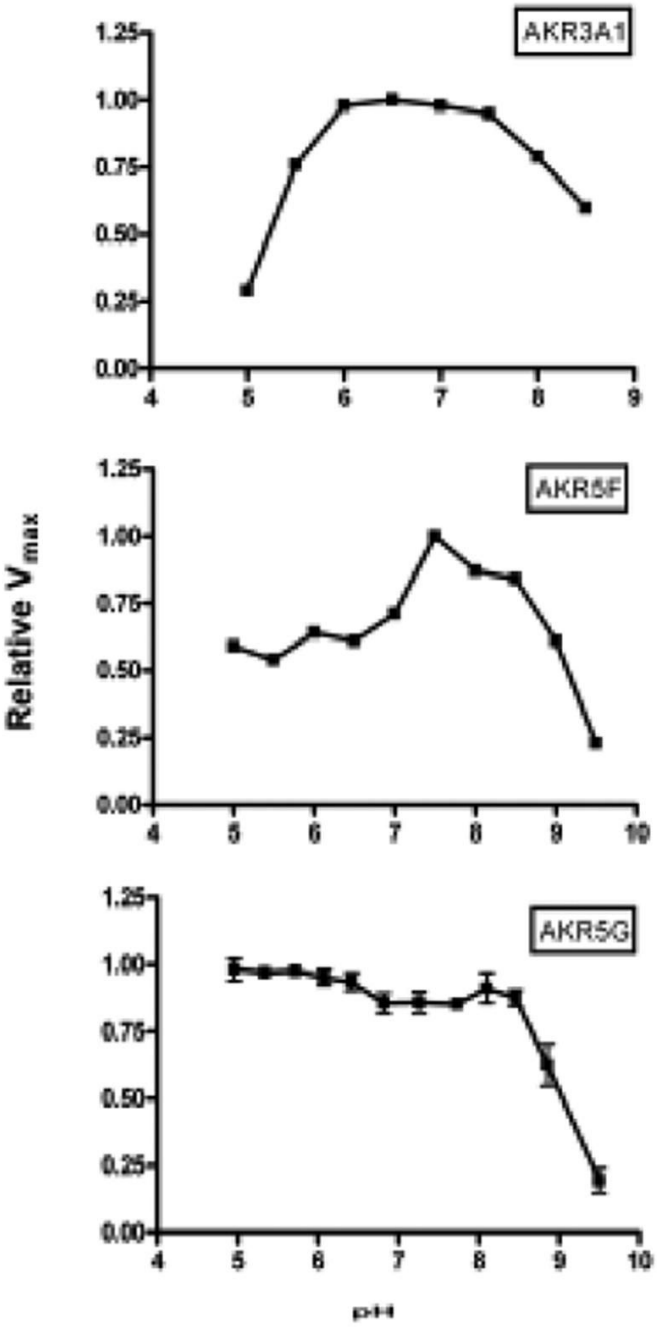

Fig. 1.

pH Optima of yeast AKRs. Reductase activities were measured in a 1.0 ml assay solution containing enzyme, aldehyde substrate and 0.15 mM NADPH in a triple buffer system titrated to the indicated pH values.

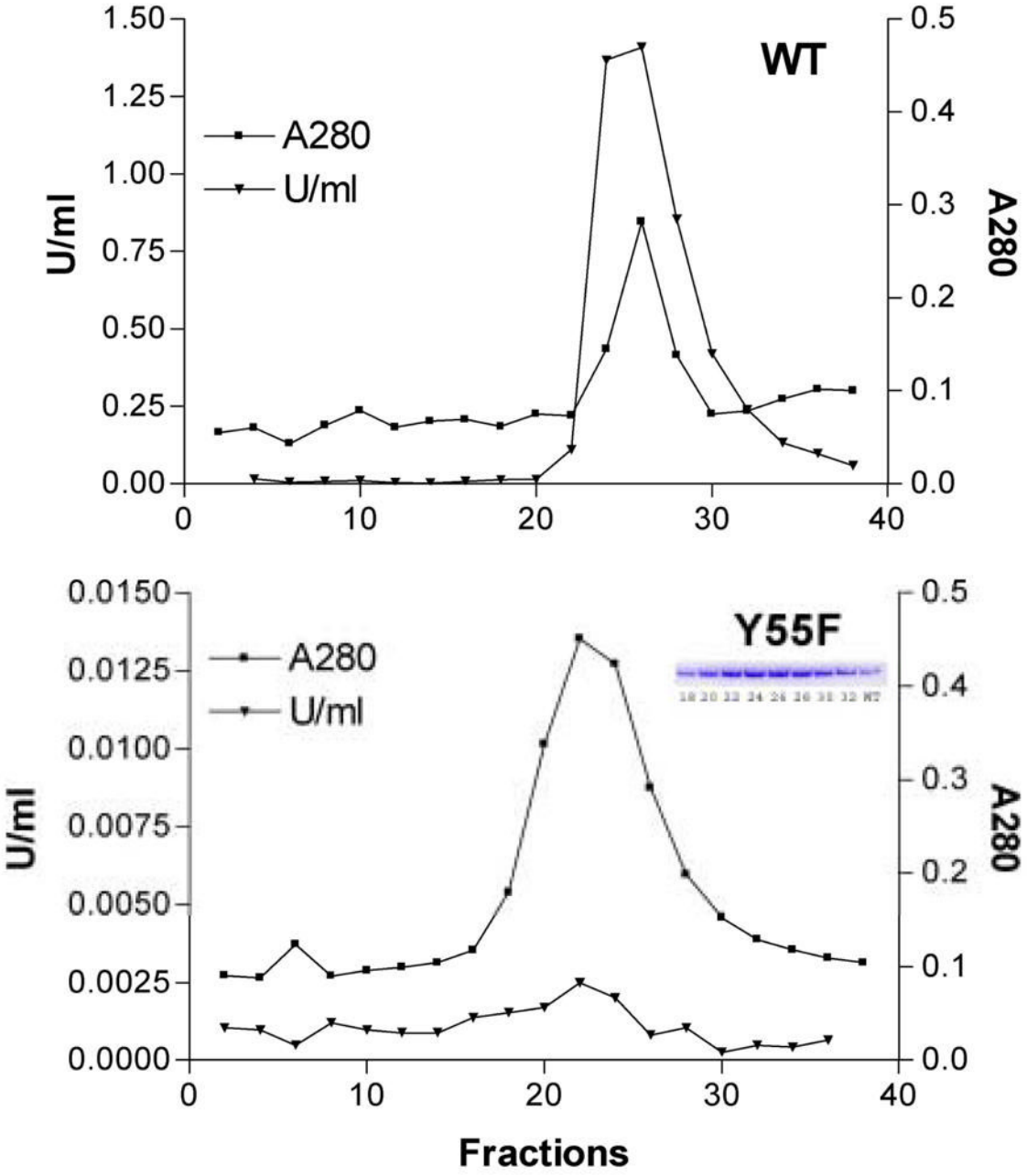

Fig. 2.

Purification of AKR3A1 (Gcy1). Wild type (WT) and mutant (Y55F) AKR3A1 were purified by dye-affinity chromatography using Affi-Gel Blue (Bio-Rad). Column fractions were measured for protein by absorbance at 280 nm (▪) and for NADPH-linked reductase activity using 5 mM DL-glyceraldehyde as substrate (▾). Note 100-fold difference in scale on the ordinate axes showing enzyme activities. (Inset: SDS-PAGE of material from the indicated column fractions; WT, wild type AKR3A1).

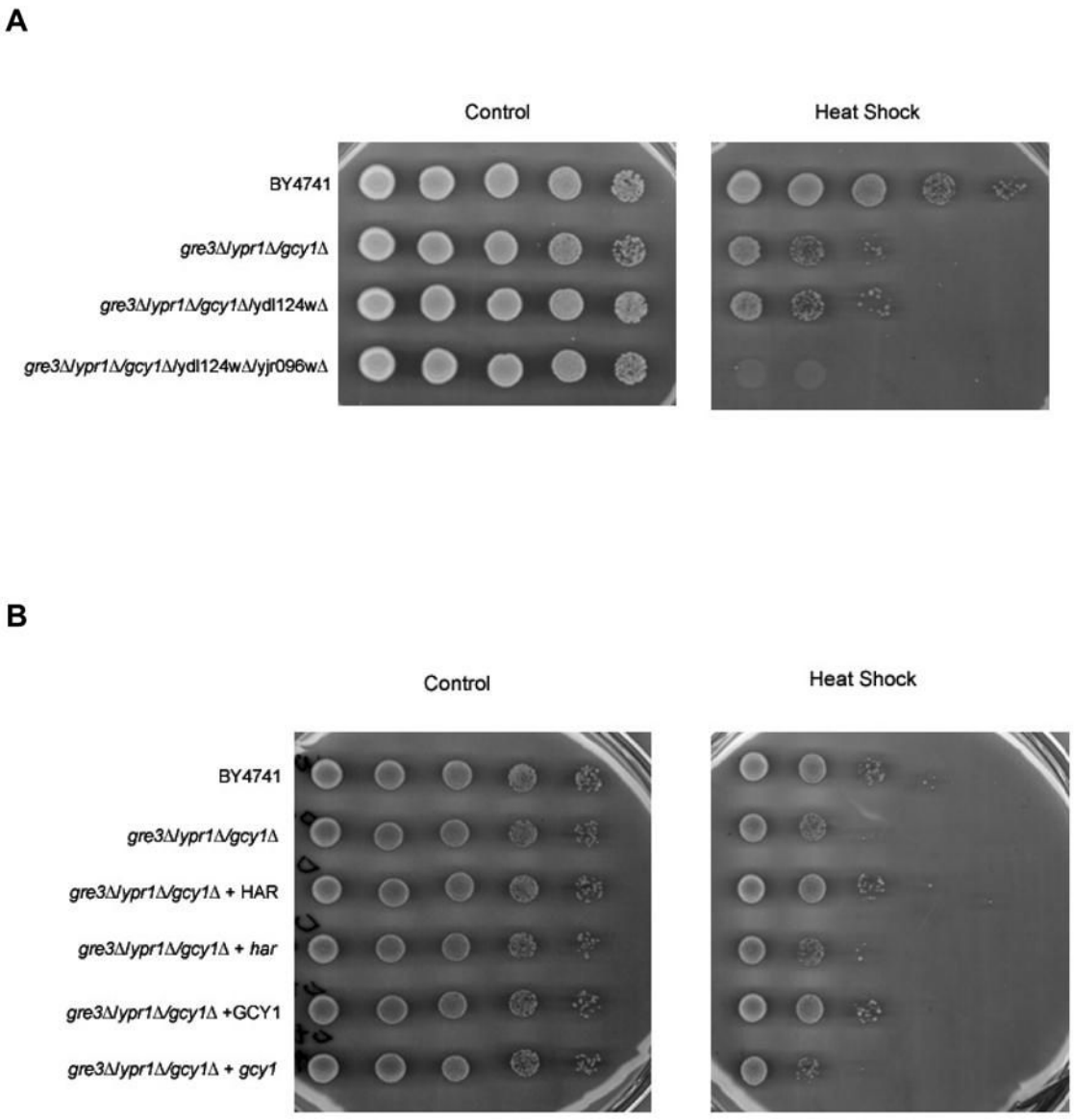

Fig. 3.

Heat shock phenotype of AKR mutant strains. After heat shock treatment, cell suspensions were serially diluted into water and spotted on selective plates (−Leu). After incubation for ~72 h at 30°, plates were imaged on a flat bed scanner to document recovery of growth. *(A)* Growth recovery following heat shock in the wild type parental strain (BY4741) and isogenic strains containing 3 (ARD10), 4 (ARD11), and 5 (ARD12) disrupted AKR genes. Genotypes of relevant strains are ARD10 (BY4742 *gre3Δ::HIS3 ypr1Δ::kanMX4 gcy1::URA3*), ARD11 (BY4742 *gre3Δ::HIS3 ypr1Δ::kanMX4 gcy1::URA3 ydl124wΔ::LEU2*), and ARD12 (BY4742 *gre3Δ::HIS3 ypr1Δ::kanMX4 gcy1::URA3 ydl124wΔ::LEU2 yjr096wΔ::ble*^{r}). *(B)* Heat shock rescue experiments. Strains included the wild type parental strain (BY4741), and the triple AKR null strain ARD10 (BY4742 *gre3Δ::HIS3 ypr1Δ::kanMX4 gcy1::URA3*). Heat shock rescue experiments were carried out by transforming strains BY4741 and ARD10 with expression plasmids encoding either human aldose reductase (HAR, AKR1B1) or Gcy1 (AKR3A1). Dependence of phenotypic rescue on reductase activity was assessed by

transforming heat shock sensitive strains with functionally inactive point mutants of human aldose reductase (*har*) or Gcy1 (*gcy1*).

Table I

Strains used in this study

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
ARD10	BY4742 *qre3Δ::HIS3 ypr1Δ::kanMX4 qcy1::URA3*	This study
ARD11	BY4742 *qre3Δ::HIS3 ypr1Δ::kanMX4 qcy1::URA3 ydl124w Δ::LEU2*	This study
ARD12	BY4742 *qre3Δ::HIS3 ypr1Δ::kanMX4 qcy1::URA3 ydl124w Δ::LEU2 yjr096w Δ::ble*^{r}	This study

Table II

Oligonucleotides used for construction of deletion strains

Targeted Gene/ORF	Primer sequence 5′ to 3′	Template
GCY1	Sense: ATATGCCTATCAGGCATTCACCCGTGTGACGAATCGCACA//AGATTGTACTGAGAGTGCAC Anti-sense: TGCCTTATACACCACCTATTCTGGCATCTGCGGGATTTCG//CTGTGCGGTATTTCACACCG	pRS406 (*URA3*)
YDL124W	Sense 5′ACACATGTAAACGGTTGTGTTACCCTAAAGAAACGGAGGTCAGTTAACAACACTTTTACAGATGCAGCTGAAGCTTCGTACGC3′ Anti-sense 5′AAGTCGATTAATAAAGTTATACAAAACAAATATGACTCGTACATAAATTATCCGGTATTTAGCATAGGCCACTAGTGGATCTG3′	pUG73(*LEU2*)
YJR096W	Sense 5′ATATAAGAGCATAAGCAACTGATCTTACTTTAGTAATTAACTTAGCATACCTAGCCCGAAGGACAGCTGAAGCTTCGTACGC3′ Anti-sense 5′AAAAAGCTACATGTAATATTAATACTTTGTGAAACGTAAACTAATTATATGTTCTATATACGTGCATAGGCCACTAGTGGATCTG	pUG66(*ble*^{r})

Table III

Verification oligonucleotides.

Gene/ORF	Confirmation primers
YPR1	A: 5′-CATTCTCACTGATGCTATTGTC-3′ B: 5′-ATCAATGTGTCTGTATCCAGC-3′ C: 5′-ATGGCACTGTTGACATCG-3′ D: 5′-GATGACGACGGTCCGAA-3′ B′ (HIS3): 5′-ACGCACTCTCACTACG-3′ C′: (HIS3): 5′-CCTAGCGATAGAGCACT-3′
GRE3	A: 5′-CTCCTGGTACACTGAAGTAT-3′ B: 5′-TGAGACCTTCTTGATTACAT-3′ C: 5′-GTGCTTGCGACTACGG-3′ D: 5′-TCAACCATACAAGAGATGAA-3′ B′: (*KanMX*) 5′-TGTACGGGCGACAGTCACAT-3′ C′: (*KanMX*) 5′-CCTCGACATCATCTGCCCAGAT-3′
GCY1	A: 5′-CCTATCAGGCATTCACC-3′ B: 5′-GACGTGCCAGCTAATAAC-3′ C: 5′-ATGGCTACCGACACATTG-3′ D: 5′-CTTGCCTTATACACCACC-3′ B′: (*URA3*) 5′-AGTTATCCTTGGATTTGG-3′ C′: (*URA3*) 5′-ATCTCATGGATGATATCC-3′
YDL124W	A: 5′-CGAAAGCGCGGAGAATTAA-3′ B: 5′-TGCCTTCCCAACTTCTGGATA-3′ C: 5′-TTGGTAGAAGCATACTCGCCA-3′ D: 5′-TAGCAACACATGATGCGCGT-3′ B′: (*LEU2*) 5′-AGTTATCCTTGGATTTGG-3′ [see ref.42] C′: (*LEU2*) 5′-ATCTCATGGATGATATCC-3′ [see ref.42]
YJR096W	A: 5′-TGAAGTAGTTATCATACATATTCCGTCG-3′ B: 5′-GAATCAAAAGAAGATCGATGTATTGC-3′ C: 5′-GGTCAACCAAATCGAGATATCACCT-3′ D: 5′-GGTAAGACTAGTAACGTCACATCGAAG-3′ B′: (*ble*^{r}) 5′-GGATGTATGGGCTAAATG-3′ [see ref.42] C′: (*ble*^{r}) 5′-CCTCGACATCATCTGCCC-3′ [see ref.42]

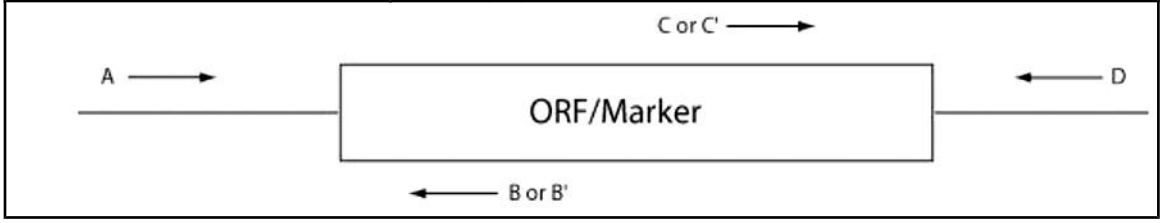

Table IV

Aldo-keto reductases in *Saccharyomyces cerevisiae*

ORF Designation	YHR104W	YOR120W	YDR368W	YBR149W	YJR096W	YDL124W
Gene Name	*GRE3*	*GCY1*	*YPR1*	*ARA1*		
AKR Designation[a]	AKR2B6	AKR3A1	AKR3A2	AKR3C	AKR5F	AKR5G
Reported Function	Aldo-keto reductase	Aldo-keto reductase	Xylose reductase	Arabinose Dehydrogenase	Xylose/arabinose reductase	Xylose/arabinose reductase
References	[26,30,36,43]	[27,30,41]	[10,25]	[12]	[30]	[30]

[a]Nomenclature for the AKR Superfamily is available at http://www.med.upenn.edu/akr/

Table V

Kinetic constants for yeast aldo-keto reductases

	AKR3A1	AKR5F	AKR5G
	Yor120w Gcy1	Yjr096w	Ydl124w
DL-Glyceraldehyde			
k_{cat} (min^{-1})	539	6.6	4.1
K_m (mM)	0.97 ± 0.1	54 ± 2	0.24 ± 0.03
k_{cat}/K_m ($min^{-1}mM^{-1}$)	556	0.12	17
p-Nitrobenzaldehyde			
k_{cat} (min^{-1})	71	88	3.3
K_m (mM)	0.13 ± 0.002	0.5 ± 0.06	0.03 ± 0.004
k_{cat}/K_m ($min^{-1}mM^{-1}$)	546	176	110
Benzaldehyde			
k_{cat} (min^{-1})	58	4	4
K_m (mM)	5.2 ± 0.6	47 ± 6	0.24 ± 0.03
k_{cat}/K_m ($min^{-1}mM^{-1}$)	11	0.09	17
Phenylglyoxal			
k_{cat} (min^{-1})	111	2.4	985
K_m (mM)	8.7 ± 0.19	35 ± 0.1	4.2 ± 0.3
k_{cat}/K_m ($min^{-1}mM^{-1}$)	13	0.07	234
Acrolein			
k_{cat} (min^{-1})	30	1.0	3.4
K_m (mM)	50 ± 12.8	22 ± 5.2	125 ± 88
k_{cat}/K_m ($min^{-1}mM^{-1}$)	0.6	0.05	0.03
Butyraldehyde			
k_{cat} (min^{-1})	81	0.5	14
K_m (mM)	54 ± 2.8	18 ± 8	210 ± 145
k_{cat}/K_m ($min^{-1}mM^{-1}$)	1.5	0.03	0.07
D-glucose			
k_{cat} (min^{-1})	NMA	NMA	NMA
D-Xylose			
k_{cat} (min^{-1})	NMA	NMA	NMA
NADPH			
k_{cat} (min^{-1})	438	123	7.2
K_m (mM)	0.012 ± 0.003	0.37 ± 0.1	0.023 ± .005
k_{cat}/K_m ($min^{-1}mM^{-1}$)	36500	332	313

Activity measurements with Yjr096w were performed at pH 8.0 and at pH 6.5 for Ydl124w and Gcy1. In all cases, NADPH was held constant at 0.15 mM. Apparent K_m and k_{cat} values were determined by fitting initial rate data to a general Michaelis-Menten equation using Graphpad Prizm (San Diego). For each enzyme-substrate pair, n≥10. NMA, no measurable activity. Apparent K_m for NADPH was determined in the presence of fixed concentrations of aldehyde substrate including 0.4 mM *p*-nitrobenzaldehyde for AKR3A1 (Yor120w), 2 mM *p*-nitrobenzaldehyde for AKR5F (Yjr096w), and 25 mM phenylglyoxal for AKR5G (Ydl124w).

Table VI

Phenotypic screens for yeast AKR mutants

Phenotype	Screening agent/parameter	References
Heat shock	Loss of viability following heat shock up to 55°C	[44,45]
Oxidative stress	YPD + 0.88 mM hydrogen peroxide	[46,47]
	YPD + 1.5 mM diamide	[48]
	YPD + 10 μM paraquat	[49]
Osmotic stress	YPD + 1 M sorbitol	
	YPD + 1 M NaCl	
Temperature sensitivity	Growth at 37°C, YPD	
Growth/morphology	Growth rate (doubling time)	
	Viability (exclusion of propidium iodide)	
	Budding pattern (light microscopy)	
	Calcofluor sensitivity (YPD + 0.1% calcofluor)	[50]
Carbon auxotrophy	YP + 2% glucose	
	YP + 2% galactose	
	YP + 2% raffinose	
	YP + 2% maltose	
	YP + 2% sucrose	
	YP + 3% glycerol	
	YP + 2% potassium Acetate	
Nitrogen starvation	Loss of growth on nitrogen-free medium	[45]
Signal transduction	YPD + 10 mM caffeine	[51]
	YPD + 0.1 μg/ml staurosporine	[52]
Nucleic acid metabolism	YPD + 0.05% methyl methanesulfonate	[53]
	YPD + 100 mM hydroxyurea	[54]
	YPD + 60 μg/ml 6-azauracil	[55]