Characterization of a New Hemoprotein in the Yeast Saccharomyces cerevisiae*

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Geppo Sartori‡, Laura Aldegeri‡, Gabriella Mazzotta‡, Gerolamo Lanfranchi‡, Helene Tournu¶, Alistair J. P. Brown¶, and Giovanna Carignani‡‡

From the ‡Dipartimento di Chimica Biologica and ¶Dipartimento di Biologia, viale G. Colombo, 3, 35121 Padova, Italy and †Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom

The Saccharomyces cerevisiae gene YNL234w encodes a 426-amino acid-long protein that shares significant similarities with the globin family. Compared with known globins from unicellular organisms, the Ynl234wp polypeptide is characterized by an unusual structure. In this protein, a central putative heme-binding domain of about 140 amino acids is flanked by two sequences of about 160 and 120 amino acids, respectively, which share no similarity with known polypeptides. Northern analysis indicates that YNL234w transcription is very low in cells grown under normal aerobic conditions but is induced by oxygen-limited growth conditions and by other stress conditions such as glucose repression, heat shock, osmotic stress, and nitrogen starvation. However, the deletion of the gene had no detectable effect on yeast growth. The Ynl234wp polypeptide has been expressed in Escherichia coli, and the hemoprotein nature of the recombinant protein was demonstrated by heme staining after SDS/polyacrylamide gel electrophoresis and spectroscopic analysis. Our data indicate that purified recombinant Ynl234wp possesses a noncovalently bound heme molecule that is predominantly found in a low spin form.

MATERIALS AND METHODS

**Strains, Vectors, and Media**

Escherichia coli strains INVaF* (Invitrogen), used for amplification of the plasmids, and BL21 (DE3) (hsdS, gal [acl8851 ind1 Sani7 nin5 lacUV5-T7 gene1]), used for expression and purification of the recombinant protein, were grown in Luria-Bertani (LB) medium (1% Difco bacto tryptone, 0.5% Difco yeast extract, 0.5% NaCl).

S. cerevisiae strains W303–1B/A (MATa, ade2–1, his3–11, 15, leu2–3, 112, trpl–1, ura3–1, can1–100) (14), W3/H (MATa, hem1::HIS3) (15) and FY73 (MATa, his3Δ200, ura3–52) (16) were used for RNA preparations, and strain FY1679 (MATaMATα, ura3–52/ura3–52, his3Δ200/HIS3, leu2Δ1/LEU2, trplΔ83/TRP1) (16) was used for the gene disruption experiments.

Cosmid 14-5 was provided by P. Philippens (University of Basel). This cosmid contains a 38.8-kilobase pair DNA fragment from chromosome XIV of the S. cerevisiae strain FY1679.

**Analysis of Yeast Transcripts**

Growth Conditions

Effect of Hypoxia (see Fig. 3A)—Cells of strain W303–1B/A were grown aerobically at 28 °C in YPGal medium (1% yeast extract, 1% bactopeptone, 2% galactose, 20 mg/ml adenine) supplemented with 0.2% Tween 80 and 30 mg/l ergosterol (Sigma) up to a density of 2 × 10⁶ cells/ml. Then half of the cells were allowed to grow aerobically to a density of 2 × 10⁷ cells/ml and subsequently harvested for analysis (AIR), and the other half was transferred to an air-tight flask as de-
scribed in Ohaniance et al. (17) and shifted to anaerobic conditions by vigorous bubbling of ultrapure N2 for 30 min. The latter culture was shaken under N2 pressure until it reached a density of 2 x 10^7 cells/ml; then, before opening, the flasks were transferred to ice and chilled for 30 min, and the cells were harvested for analysis (N2).

**Effect of Heme Depletion** (Fig. 3B)—Cells of wild type W303-1B/A and of W3/H mutant (hem1) were grown in YPGal medium supplemented with 0.18 mg 5-aminolevulinic acid (Sigma) at 28 °C to a density of 2 x 10^7 cells/ml.

**Effect of Glucose Concentration**—Cells of strain FY73 were grown at 30 °C in YP medium (1% yeast extract, 1% bactopeptone) supplemented with 2% glycerol and 1% ethanol until the A_600_ of 0.8, whereupon one-third of the cells were harvested for analysis (Fig. 3C, lane 1). The remaining cells were washed in sterile distilled water, resuspended in an equal volume of YP containing 2% glucose (YPD), and grown for 60 min at 30 °C. Half of these cells were harvested for analysis (Fig. 3C, lane 2), whereas the remaining cells were harvested after a further 24 h of growth (Fig. 3C, lane 3).

**Nitrogen Starvation and Osmostress**—Cells of strain FY73 were grown at 30 °C in GYNB (0.67% yeast nitrogen base without amino acids, 4% glucose, 20 μg/ml uracil, 20 μg/ml histidine) and starved for 2 h at 30°C (nitrogen starvation; Fig. 3C, lane 5). NaCl was added (0.7 M final concentration) to the final third of cells, which were harvested after 60 min at 30 °C (osmostress; Fig. 3C, lane 6).

**Heat Shock**—Cells of strain FY73 were grown at 23 °C in GYNB to an A_600_ of 0.8, whereupon half of the cells were taken for analysis (control at 23 °C; Fig. 3C, lane 7). The other half was incubated at 36 °C for 30 min before harvesting (heat shock; lane 8).

**Northern Analysis**

Total RNA was prepared by a phenol extraction procedure as described previously (18) and subjected to electrophoresis in a formaldehyde-denaturing agarose gel as described in Sambrook et al. (19). Northern blot hybridization was performed either at 42 °C in 0.7 M NaCl, 0.05 M NaHPO_4_, 4 mM EDTA, 1% SDS, 50% formamide, pH 7.2 (Fig. 3, A and B) or at 65 °C in 0.5 M NaHPO_4_, 7% SDS, 1 mM EDTA, pH 7.2 (Fig. 3C). Filters were washed in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 15 min at 65 °C and then in 2 x SSC containing 0.1% SDS for 15 min at 65 °C. DNA fragments of the YNL234w and ACT1 genes were 32P-labeled to specific activities greater than 5 x 10^6 dpm/μg of DNA by random-priming, whereas the end-labeled oligonucleotide 5'-CCTCCGTTATTAGTATGCTTAG-20 was used to probe the 25 S RNA gene. Hybridization signals were quantified by direct two-dimensional phosphorimaging of Northern membranes using the Bio-Rad GS-525 Molecular Imageager system.

**YC11234wp Gene Disruption and Phenotypic Tests**

The YNL234w gene disruption was performed by the PCR-based method of Wach et al. (21). The diploid yeast strain FY1679 was transformed by the lithium acetate procedure (22) with a PCR-amplified DNA fragment containing the kanMX4 module (conferring resistance to geneticin) of plasmid pFA6-kanMX4 (21) flanked by two 40-base pair S. cerevisiae sequences corresponding to regions located immediately upstream and downstream of the YNL234w coding sequence. Haploid ynl234wp mutants were obtained by sporulation of heterozygous diploids, and selection of spores was performed on geneticin-containing medium. The correct chromosomal insertion of the kanMX4 module was verified by PCR amplification using primers specific for the disruption cassette and the chromosome XIV DNA regions flanking the YNL234w gene. For the phenotypic analysis, several dilutions of fresh stationary-phase cultures of mutant strains were spotted together with the corresponding wild-type strains on YP plates (1% yeast extract, 1% bactopeptone) containing different carbon sources (2% glucose, 2% galactose, 3% glycerol). The growth was followed for 5 days at three different temperatures (16, 28, and 36 °C). For the heat shock experiments, cells were grown in YPD medium at 25 °C until exponential phase, when half of the cells were exposed to 38 °C for 20 min in a water bath, whereas the remaining cells were left at 25 °C. Cells were then diluted, plated on YPD, and incubated at 25 °C for 5 days, when sizes of the colonies and percentage of survivors were determined.

**Cloning, Expression, and Purification of Recombinant YNL234wp**

A DNA fragment corresponding to the YNL234w coding sequence lacking the 87 last nucleotides at the 3' end has been amplified by PCR from DNA of cosmid 14–5 using primers A (5'-GCTGGTTGCAATGTCAGCAGA-3') and B (5'-ATTACCTTTCGTCAGCAG-3'). These introduce, respectively, an Ndel site at the ATG codon of the gene and a SalI site 29 codons upstream of the stop codon. DNA amplification was performed using a Phu (Stratagene) and Taq (Life Technologies, Inc.) DNA polymerase mix in a 1:5 ratio. The product of amplification was digested with Ndel and SalI restriction enzymes and cloned into the expression vector pET-20b(+) (Novagen). The resulting plasmid, pYNL234-His6, allows the synthesis in a bacterial T7 expression system (23) of a recombinant 46 kDa YNL234wp protein with a C-terminal tag of six histidines. The sequence of the cloned fragment was verified by automated dideoxy sequencing (ABI373 DNA sequencer, Applied Biosystem).

Cells of the E. coli strain BL21(DE3), transformed with the recombinant plasmid, were grown in LB medium containing 100 μg/ml ampicillin and 10 μM 5-aminolevulinic acid at 37 °C until an A_600_ of 0.5–0.6, when the temperature was shifted to 20 °C and transcription of the YNL234w coding sequence was induced by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside. Induction at the usual 37 °C temperature resulted in higher yields but also in a prevalent insoluble form of the protein. After 5–7 h of further incubation, cells were harvested and resuspended in 10 ml of SB (20 mM Tris-HCl, 0.5–0.6, 0.3 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, pH 8.0/8.9 of wet pellet and sonicated. Purification of the recombinant protein was performed according to the manufacturer's protocol by an affinity column containing nickel nitritotriacetic acid-agarose (Quiagen). Recombinant YNL234wp was eluted with 10 ml of SB containing 100 mM imidazole. Orange-colored fractions were collected and dialyzed against SB (without phenylmethylsulfonyl fluoride) at pH 7.5. The purity of the preparation was assessed by SDS/PAGE according to Laemmli (24), and protein concentration was determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard. For N-terminal sequencing, the recombinant polypeptide was electrophoretted after SDS-PAGE to the PVDF membrane (ProBlott, Applied Biosystems), visualized with Coomassie Blue, and analyzed by automated Edman degradation.

**Heme Staining**

Heme staining of purified YNL234wp recombinant protein was carried out with tetramethylbenzidine after SDS-PAGE was performed without the addition of sulfhydril reducing agents according to Thomas et al. (25). The proteins were loaded in different amounts because of the lower sensitivity of the staining method for human Hb.

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Spectrophotometric Assays

Spectrophotometric measurements were made using a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer at room temperature. Absolute spectra were recorded by scanning the purified protein preparation against the protein buffer (20 mM Tris-HCl, 0.3 mM NaCl, 10% glycerol, pH 7.5) before and after the addition either of an excess of potassium ferricyanide or of few grains of sodium dithionite. The CO difference spectra were recorded by scanning the purified protein preparation using a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer at room temperature. Absolute spectra were obtained by bubbling CO directly in the protein solution at pH 7.5.

RESULTS AND DISCUSSION

The Central Region of the Ynl234wp Polypeptide Shows the Characteristics of a Hemoglobin Domain

The 426-amino acid-long sequence of the polypeptide encoded by the YNL234wp gene was compared with protein data bases, and a weak but significant similarity was found with several hemoglobin chains (15). In fact, the amino acid sequence of the Ynl234wp central region matches fairly well to the amino acid sequence template established by Moens et al. (27) based on the alignment of nonvertebrate hemoglobin sequences. It matches also, but with a higher penalty score, to the bacterial Hb domains.

Transcription of the YNL234wp Gene Is Induced by Hypoxia and Other Stress Conditions

Expression of the YNL234wp gene in yeast cells was shown by Northern blot analysis. A weak band of about 1.4 kilobases, which is in agreement with the size of the gene, indicates that the YNL234wp gene is expressed at low levels in cells grown under normal aerobic conditions. Transcription of the gene, however, is significantly enhanced in cells grown under hypoxia (Fig. 3A) and under different stress conditions as glucose repression, nitrogen starvation, osmostress, and heat shock (Fig. 3C and Ref. 30). These data identify S. cerevisiae YNL234wp as a hypoxic and a stress-responsive gene. Hypoxic gene expression in yeast is regulated by two different systems. The first involves the heme molecule and, in the majority of the cases, the transcriptional regulators Hap1p and Rox1p; the second is heme-independent, but little is known about its mechanism (31, 32). Preliminary results indicate that expression of the YNL234wp gene is moderately increased in heme-depleted cells in comparison to wild-type cells (Fig. 3B). However, as the increase of YNL234wp transcription in the case of the absence of heme is moderate (about 3-fold) in comparison with the one observed in the case of hypoxia (about 20-fold), it is conceivable that regulation of YNL234wp transcription by oxygen is mediated both by the heme molecule and by a heme-independent mechanism. The mechanism of the general stress response in yeast has not been fully elucidated; however, it is known that, in response to diverse stress conditions, two homologous zinc finger proteins, Msn2p/Msn4p, activate gene transcription by binding to the stress response element STRE (33–35). The induction of YNL234wp transcription by several stress conditions is in agreement with the presence of two STRE sequences at positions −56 (5′-CCGGCC) and −158 (5′-AGGGG) from the first ATG codon. Examples of yeast genes whose transcription is controlled both by oxygen and by different stress conditions are the iso-2-cytochrome c gene CYC7 (36, 37) and the catalase gene CTT1 (33). Expression of the ROX3 gene, which encodes an essential protein that contributes to the global stress response (37, 38), is also induced by oxygen starvation and several stress conditions; however, the effect of oxygen is not mediated by heme, and the stress response is not activated through the STRE sequence. It has recently been shown that Rox3p is a component of the multiprotein complex “mediator,” which is part of the RNA polymerase II holoenzyme (39). We believe that a better understanding of the mechanisms that control YNL234wp expression, and in particular, of the relationships existing between oxygen starvation and other stress conditions in inducing its transcription could give some interesting insights into the function of this new putative hemoglobin in the yeast cell.
The YNL234w Gene Is Not Essential for Yeast Growth

The YNL234w gene was deleted in the yeast strain FY1679 using the PCR-based technique described by Wach et al. (21) (this work was performed under the Eurofan I Biotech Program). A preliminary analysis of the disrupted haploid strains was performed by growing them at different temperatures on solid media containing different carbon sources, but no significant phenotypic differences between the wild-type and the mutant strains were observed. The sensitivity of the mutant strains to heat shock treatment was tested by shifting exponentially growing cultures from 25 to 38 °C and then determining the percentage of survivors on YPD plates, but also in this case, a clear difference between the wild-type and the mutant strains was not observed. However, if the role of the YNL234w gene, as suggested by the transcription data, is to mitigate cellular damage in different stress conditions, more accurate analysis seems to be required to highlight a phenotype of the YNL234w-deleted strains.

Recombinant Ynl234wp Binds Noncovalently a Low Spin Form Heme Molecule

Expression and Purification of Recombinant Ynl234wp Protein

To facilitate the characterization of the Ynl234wp protein, which is normally poorly expressed in yeast, we decided to purify a heterologously expressed protein. A PCR-fragment corresponding to the nucleotide sequence encoding a Ynl234wp polypeptide lacking the 29 C-terminal residues was obtained by amplification of the genomic DNA of S. cerevisiae strain FY1679. The fragment was cloned in the isopropyl-β-D-thiogalactopyranoside-inducible pET-20b (+) E. coli expression vector, upstream of and in-frame with a short plasmidic sequence coding for eight amino acids (DKLAAALE) followed by six residues of histidine (His-tag). Nucleotide sequencing of the cloned fragment confirmed its identity with the wild-type sequence.

E. coli cells transformed with this plasmid were grown in liquid culture supplemented with the heme precursor 5-aminolevulinic acid to increase de novo heme synthesis (40). After induction with isopropyl-β-D-thiogalactopyranoside, a prominent protein band of the expected 46 kDa was visible in bacterial extracts. This was purified to near homogeneity by a single-step affinity chromatography utilizing the chelating ligand nitrilotriacetic acid charged with Ni²⁺ ions. The identity of the 46-kDa band present in the orange-colored fractions with the recombinant Ynl234wp polypeptide was confirmed by N-terminal sequencing, which revealed the expected TGEKI sequence.

Biochemical Characterization of the Ynl234wp Recombinant Protein

Heme Staining—The purified recombinant Ynl234wp protein was analyzed by benzidine heme staining after SDS-PAGE (25). This method allows detection of very low levels of heme-associated peroxidase activity. Furthermore, the denaturing conditions of electrophoresis led to a loss of heme from hemoproteins in which the prosthetic group is noncovalently bound and the free heme appears as a diffuse band with a mobility similar to that of the bromphenol blue dye. By this simple method, it is possible to distinguish between hemoproteins with covalently and noncovalently bound heme. Intense staining of Ynl234wp and the presence in the same electrophoretic lane of abundant free heme clearly indicate that this protein does bind heme and that the prosthetic group is noncovalently bound (Fig. 4).

Spectroscopic Analysis—The absolute visible spectrum of purified recombinant Ynl234wp protein at pH 7.5 is shown in Fig. 5A, trace 1. The spectrum shows the characteristics of a hemo-
protein, with an intense Soret band positioned at 411 nm, but the \(a\) and \(b\) peaks are not well resolved. This profile is not modified by the addition of potassium ferricyanide (data not shown), indicating that the hemoprotein is already in its oxidized form. After the addition of sodium dithionite, the optical spectrum is characterized by a Soret peak shifted to 426 nm, a minor \(b\) peak centered at 530 nm, and a major \(a\) peak at 559 nm (Fig. 5A, trace 2). These spectroscopic profiles are characteristic of hemichromes (41–43) and suggest that the major part of the protein, probably obtained in the form of high spin met form, has been converted to low spin hemichromes. This hypothesis is confirmed by the CO difference spectrum (Fig. 5B, trace 3) in which the trough present at the same wavelength of the \(a\) peak (559 nm) has the characteristics of low spin heme molecules; however, the intensity of the trough is not as pronounced as those observed for true low spin hemoproteins (43), suggesting that the heme molecule bound to Ynl234wp may be present as a mixture of low and high spin states, as already observed for the heterologously expressed Hb of the unicellular alga *Clamydomonas eugametos* (44) and for the Hb from the cyanobacterium *Nostoc commune* (45). The susceptibility to autoxidation and hemichrome formation varies with different Hbs, and the rates of the two processes are relatively rapid for some Hbs, as is the case of the myoglobin isolated from *Paramecium caudatum* (46).

The knowledge of the real redox state of the heme molecule bound to Ynl234wp, together with the role played by the two regions flanking the heme-binding domain, will be necessary elements to understand the function of this protein in the yeast cell. The results presented here arouse the interest in this new hemoprotein of *S. cerevisiae*, whose expression is induced in cells grown under hypoxia and other stresses and that is probably necessary to the cell under these severe conditions. The two regions flanking the heme-binding domain, which have no apparent homologs in the yeast cell, might actually represent new functions or, more likely, be docking domains responsible for the recruitment of proteins whose activation (or inactivation) is necessary to the cell grown under these particular conditions.
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