Functional analysis of the hemK gene product involvement in protoporphyrinogen oxidase activity in yeast

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Abstract

The Escherichia coli hemK gene has been described as being involved in protoporphyrinogen oxidase activity; however, there is no biochemical evidence for this. In the context of characterizing the mechanisms of protoporphyrinogen oxidation in the yeast Saccharomyces cerevisiae, we investigated the yeast homolog of HemK, which is encoded by the ORF YNL063w, to find out whether it has any protoporphyrinogen oxidase activity and/or whether it modulates protoporphyrinogen oxidase activity. Phenotype analysis and enzyme activity measurements indicated that the yeast HemK homolog is not involved in protoporphyrinogen oxidase activity. Complementation assays in which the yeast HemK homolog is overproduced do not restore wild-type phenotypes in a yeast strain with deficient protoporphyrinogen oxidase activity. Protein sequence analysis of HemK-related proteins revealed consensus motif for S-adenosyl-methionine-dependent methyltransferase.

Keywords: Heme biosynthesis; Protoporphyrinogen oxidase; HemK; HemG; Yeast; Escherichia coli

1. Introduction

Cyclic tetrapyrroles are universal cell components and their metal derivatives are involved in fundamental processes, such as respiration, photosynthesis and oxygen transport [1]. Protoporphyrin IX, the last common intermediate in the heme and chlorophyll biosynthesis pathways, results from the oxidation of protoporphyrinogen IX by the enzyme protoporphyrinogen oxidase (EC 1.3.3.4). Protoporphyrinogen oxidase deficiency has been described in bacteria and yeast as well as in man [2], where deficient protoporphyrinogen oxidase activity is responsible for the inherited disease variegate porphyria. In plants and mammals, diphenyl ether-type herbicides, which inhibit protoporphyrinogen oxidase, induce massive accumulation of misslocalized protoporphyrin IX produced by photooxidation of protoporphyrinogen, which promotes peroxidation reactions and leads to cell death [3,4]. Sasarman et al. [5] characterized a mutant strain of Escherichia coli that accumulates porphyrins, including protoporphyrin IX, and forms small colonies on rich me-
dia. Since it has normal ferrochelatase activity, this mutant has been assumed to be deficient in protoporphyrinogen oxidase activity [6]. The gene affected in the mutant was designated hemG [7]. The hemG gene encoding a 181-amino acid protein with typical flavodoxine motif, restored normal growth in the hemG mutant and cells transformed with a multicopy plasmid carrying hemG displayed higher in vitro protoporphyrinogen oxidase activity than a wild-type strain. The enzyme activity is recovered in the membrane fraction of transformed cells [7], but the structure-function relationships of HemG are still only partially understood. A functional complementation assay has made it possible for the hemG mutant to be widely used to clone protoporphyrinogen oxidase cDNAs from human and mice and also from plants [2].

The product of the hemK gene may be a potential partner for HemG. A hemK mutant has been isolated and like hemG, was shown to be able to induce the reversion of ferrochelatase-deficient photosensitivity phenotype, resulting from protoporphyrin IX accumulation by a ΔvisA (ΔhemH) mutant [8]. This led Nakayashiki et al. [8] to suggest that HemK may also be involved in protoporphyrinogen oxidase activity. The hemK gene forms part of the hemA-prfA-hemK operon and encodes a 225 amino acid polypeptide. There is still no biochemical proof that HemK is involved in protoporphyrinogen oxidase activity. One striking difference between HemG and HemK is that HemK has significant similarity with many bacterial and eukaryotic gene products, whereas HemG does not. Since HemK may be involved in protoporphyrinogen oxidation, several databases entries describe it as ‘similar to bacterial protoporphyrinogen oxidase’ or even ‘protoporphyrinogen oxidase’.

Within the context of our studies on the catalytic mechanisms of protoporphyrinogen oxidase and its inhibition by diphenylethers, and porphyrin accumulation in the facultative aerobe Saccharomyces cerevisiae, we were interested in studying a possible role of the yeast homolog of HemK in protoporphyrinogen oxidase activity. A yeast hem14-1 mutant has been isolated which has no cytochromes, accumulates protoporphyrin and is devoided of protoporphyrinogen oxidase activity [9]. The structural gene for protoporphyrinogen oxidase, HEM14, was isolated by functional complementation of the hem14-1 mutant [10]. The HEM14 gene encodes a 539-amino acid protein somewhat similar to the coproporphyrinogen-protoporphyrinogen oxidase hemY gene product of Bacillus subtilis [11] and its mammalian and plant homologs. In order to find out whether the product of the ORF YNL063w, which is similar to the hemK gene of E. coli, is also involved in the protoporphyrinogen oxidase activity, we investigated the possible role in heme synthesis of this protein in yeast. We report data which suggest that the yeast homolog of bacterial HemK is not in fact involved in protoporphyrinogen oxidation.

2. Materials and methods

2.1. Strains and media

The S. cerevisiae strains used in this study were FY116 (Matα, ura3-52, his3Δ200, leu2Δ1, trp1Δ63) referred to wild-type strain in this paper, YNL063w (Matα, ura3-52, his3Δ200, leu2Δ1, trp1Δ63, YNL063w(4750):::kanMX4) (Eurofan II) referred to ΔYNL063w strain and S150-2BΔhem14 (Matα, his3, leu2, trp1, ura3, hem14::kanMX4) referred to Δhem14 mutant strain [10]. The cells were grown in a complete medium containing 1% yeast extract, 1% Bacto-peptone, 2% glucose (YPGlu) or 2% glycerol (YPGly). The E. coli strain was DH5α (φ80lacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17 (rK-, mK+), supE44, relA1, deoR, Δ(lacZYA-argF)U169).

Protein sequences encoded by hemK homologs were obtained from the Swissprot or Genpept databases via the Sequence Retrieval Service at Infobio- gen (CNRS, France). Sequence alignment was performed using CLUSTAL W 1.74 software [12].

2.2. DNA techniques

The 960-bp YNL063w fragment was amplified by PCR using the following oligonucleotides: 5′-GGA-TCATGCGCTGGAATATCTACATCATTTG-3′ and 5′-CCATGGTATTTGCAATTAGCTTTTGTGC-C-3′ (BamHI sites are underlined). The amplified product was cloned in pGEM-T vector (Promega) and subcloned at the BamHI sites of the YPGE2
vector [13] allowing the expression of the cloned product in yeast under the control of the strong promoter of the phosphoglycerate kinase gene. DNA cloning was carried out as described by Maniatis et al. [14]. Yeast transformation was achieved using the standard lithium acetate procedure.

2.3. RNA analysis

Yeast cells from 10 ml of culture (OD$_{600} = 1$) were collected by centrifugation for 10 min at 3000×g and resuspended in 440 µl of 50 mM Na-acetate pH 5.3, 10 mM EDTA and 1% SDS. The suspension was shaken vigorously and 440 µl of phenol at pH 5.3 was added. The mixture was incubated for 4 min at 65°C and then frozen in liquid nitrogen. After thawing, the suspension was centrifuged and the aqueous phase was collected and subjected to phenol-chloroform extraction. Total RNA was precipitated with 2.5 vols. of pure ethanol. The RNAs were then fractionated on a 1% denaturing agarose gel and then blotted onto a nylon membrane under vacuum. Standard procedures were used for hybridization with $^{32}$P-labeled probes.

2.4. Enzyme assays

Cytochrome content and/or porphyrin accumulation were monitored by low temperature (−196°C) absorption spectra of whole cells recorded as described by Labbe and Chaix [15]. Cell-free extracts of yeast, soluble fraction and membrane fraction were obtained as described by Camadro et al. [16]. Protein concentration was determined by the Bradford method [17]. The ferrireductase activity was assayed on colonies by the nitroprusside-plate test [18] and on whole cells spectrophotometrically, with 180 mM ferri citrate as substrate and 5% (w/v) glucose in 50 mM citrate buffer, pH 6.5 [19]. Protoporphyrinogen oxidase was assayed at 30°C by measuring the rate of appearance of protoporphyrin fluorescence [20,21]. The incubation mixture was 0.1 M potassium phosphate buffer, pH 7.2, saturated with air, containing 2 µM protoporphyrinogen IX, 3 mM palmitic acid (in dimethyl sulfoxide 0.5% (v/v) final concentration), 5 mM DTT, 1 mM EDTA, and 0.3 mg ml$^{-1}$ (final concentration) Tween 80 to maximize the fluorescence signal from the protoporphyrin IX. Protoporphyrinogen was prepared by reducing protoporphyrin IX dissolved in KOH/EtOH (0.04 N, 20%) with 3% sodium amalgam [22].

3. Results

3.1. Expression analysis

In 1995, Nakayashiki et al. [8] postulated that the hemK gene product of E. coli was involved in protoporphyrinogen oxidase activity. Since that time, bacterial, yeast and plant homologs of HemK (Fig. 1) have been listed as possible protoporphyrinogen oxidases in sequence databases. We were interested in the S. cerevisiae HemK homolog and wanted to find out if it has any functional role in protoporphyrinogen oxidase activity.

We analyzed the expression of the yeast homolog of hemK encoded by the ORF YNL063w. Northern blotting was performed using the amplified PCR product of YNL063w as a probe. Fig. 2 shows that two bands hybridized with the probe in the wild-type strain (1) and in the Δhem14 (3) strain, but none are detected in the strain disrupted for the ORF YNL063w (2). These results show that the ORF YNL063w is expressed. Two populations of RNA are transcribed which could result from two different transcription initiation sites or/and two different sites of transcription termination. As expected, when the

Table 1

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Wild-type</th>
<th>ΔYNL063w</th>
<th>Δhem14</th>
</tr>
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<tbody>
<tr>
<td>Crude extract</td>
<td>61</td>
<td>45</td>
<td>ND</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>21</td>
<td>27</td>
<td>ND</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>325</td>
<td>318</td>
<td>ND</td>
</tr>
</tbody>
</table>

Activities are expressed in pmol min$^{-1}$ (mg of total protein)$^{-1}$. ND, non-detectable.
Fig. 1. Sequence alignment of HemK proteins (CLUSTAL W 1.74 software [12]). It has been suggested that Motif I (underlined) may be a SAM binding site and that motif IV may be involved in the catalytic domain of SAM-dependent methyl transferases. Identical amino acids are highlighted in gray. Divergent N- and C-terminal sequences are not shown. Genbank accession numbers are given in parentheses. Ec1, *Escherichia coli* (U18555); Hi, *Haemophilus influenzae* (U32830); Mp, *Mycoplasma pneumoniae* (P75419); Mg, *Mycoplasma genitalium* (F64228); Ng, *Neisseria gonorrhoeae* (U95845); Li, *Lawsonia intracellularis* (U45241); Bs, *Bacillus subtilis* (Z49782); Ct, *Chlamidia trachomatis* (AE001277); Aa, *Aquifex aeolicus* (AE000673); Hp, *Helicobacter pylori* (AE00555); Sc, *Saccharomyces cerevisiae* (U12141).
ORF YNL063w is disrupted, there is no transcription of YNL063w. RNA quantity was checked by hybridization with an actin probe (data not shown).

3.2. Phenotype analysis

Phenotype analysis was used to determine whether the product of the ORF YNL063w is involved in protoporphyrinogen oxidase activity and in the heme biosynthesis pathway. Like any heme-deficient yeast strain, the hem14 mutant cannot grow on non-fermentable carbon sources (ethanol, glycerol or lactate) [10]. We therefore checked whether the ΔYNL063w strain is able to grow on glycerol. Fig. 3A shows the growth of the wild-type strain (FY), the Δhem14 mutant and the ΔYNL063w strain on a 2% glycerol agar plate. Serial dilutions of the cultures were plated and none of the ΔYNL063w dilutions showed any sign of delayed growth compared to the wild-type strain. The Δhem14 mutant did not grow on 2% glycerol. Another specific phenotypic trait of heme-deficient yeast strains is the lack of ferrireductase activity. Ferrireductase is a flavohemoprotein located at the plasma membrane of yeast cells involved in the reductive assimilation of iron. The nitroprusside-plate assay, in which the blue coloration of the clone provides a qualitative estimation of the ferrireductase activity of the cell [23], did not allow detection of any difference in the blue color of the wild-type and the ΔYNL063w mutant. The Δhem14 mutant has no ferrireductase activity and, therefore, formed white colonies (Fig. 3B). Quantitative measurements of the ferrireductase activity in the WT and ΔYNL063w strains were similar (data not shown).

3.3. Protoporphyrinogen oxidase activity

The potential accumulation of porphyrins in the ΔYNL063w strain was investigated by low temperature absorption spectra of whole cells (Fig. 4). No porphyrin accumulation was detected in the ΔYNL063w strain, which had a cytochrome-absorption spectrum similar to that of the wild-type strain. This suggests that the two strains have similar hemo-proteins. Porphyrins accumulated in the Δhem14 cells, which had an absorption spectrum typical of a protoporphyrinogen oxidase-deficient strain.

We then measured the protoporphyrinogen oxidase activity in both strains. Specific activities were measured for the crude extract, the soluble fraction and the membrane fraction of the ΔYNL063w strain and of the wild-type strain (Table 1). There was no detectable difference between them. In addition, the protoporphyrinogen oxidase activity in both strains could be inhibited by the diphenyl ether herbicide aicfluorfen-methyl (data not shown).

3.4. Complementation assay

The ORF YNL063w was cloned into the vector YPGE2 under the control of the strong constitutive promoter of phosphoglycerate kinase. The construct was introduced into the yeast Δhem14 mutant strain. The transformants were plated on 2% glycerol and on nitroprusside. Fig. 5 shows that there was no detectable growth of the transformant overproducing.
YNL063w protein on 2% glycerol (A) and no ferri-reductase activity (B). These results suggests that the overproduction of the product of YNL063w in a Δhem14 mutant is unable to restore protoporphyrinogen oxidase activity.

3.5. Sequence analysis

A BLAST search using the amino acid sequence of the product of YNL063w identifies numerous bacterial and eukaryotic proteins with significant similarities (Fig. 1). Twenty-one proteins are very similar and can be aligned using CLUSTAL W 1.74 software [12] to highlight the specific features of the sequences. One striking feature which is conserved in all the sequences is the protein motif NPPY. This motif is one of the two highly conserved motifs of DNA N6-adenine methyl transferase (N6MTase). The NPPY motif is known as motif IV (Fig. 1). A second motif is highly conserved in N6MTases, motif I GXG, and is also conserved in all the sequences shown in Fig. 1.

However, only one protein in the alignment has a known biochemical function. PapM in Streptomyces pristinaespiralis encodes an amino-phenylalanine methyltransferase involved in the antibiotic pristinamycin biosynthesis pathway. The BLAST search reports this sequence with an E-value of 10^−4 between residues 80 and 192 (matrix BLOSUM62).

4. Discussion

We report data concerning the homolog of the bacterial protein HemK in S. cerevisiae which is encoded by the ORF YNL063w. In E. coli, hemK is described as being involved in protoporphyrinogen oxidase activity [8]. But no biochemical evidence has yet been obtained to support this assumption. Our investigations of heme biosynthesis in yeast, and in particular of protoporphyrinogen oxidation, queried the question whether the yeast HemK homolog protein is in fact involved in protoporphyrinogen oxidation. Biochemical analysis and complementation assays provide evidence that the protein encoded by YNL063w is not involved in protoporphyrinogen oxidation. We have shown that no change in growth was observed in the strain disrupted for YNL063w compared to the wild-type.

Fig. 4. Low-temperature absorption spectra of yeast cells (480–640 nm) from (1) Δhem14 mutant strain, (2) ΔYNL063w strain and (3) wild-type strain (WT).

Fig. 5. Functional complementation assay of the Δhem14 mutant by overproduction of the protein encoded by the ORF YNL063w. (1) wild-type strain (WT), (2) Δhem14 mutant strain and (3) Δhem14 mutant strain transformed by a multicopy plasmid containing the cloned YNL063w ORF allowing overexpression of this gene. Cells seeded at dilution of 500, 50 and 5 cells per drop on 2% YPGly medium (A) and on YPGlu medium containing 10 mM nitroprusside (B).
strain and that yeast deficient in protoporphyrinogen oxidase activity was not rescued by the overproduction of the product of YNL063w. In addition, the protoporphyrinogen oxidase activity of ΔYNL063w was the same as that of the wild-type strain (Table 1). Nakayashiki et al. [8] showed that a hemA, hemK double mutant of E. coli is heme-deficient on the basis of a catalase-minus phenotype, even when supplemented with 5-aminolevulinate (ALA), the committed precursor of heme synthesis. However, the hemK mutant was selected on the basis of the reversion of a protoporphyrin IX-accumulation phenotype. The data presented in this paper showed that a ΔYNL063w yeast strain is heme prototroph. Taken together, these data suggest that HemK may rather be involved in ALA or protoporphyrin IX transport (or efflux) from the cells.

Two database entries (Chlamydia trachomatis and Escherichia coli Ec2) describe HemK homologs as possible ‘adenine-specific methyltransferases’. These enzymes catalyze the transfer of a methyl group from 5-adenosyl-L-methionine (SAM) to the N6 position of a specific adenine in their cognate DNA sequence. They are important components of repair-modification and mismatch repair systems in prokaryotes. Analysis of the sequence alignments of the HemK homolog group including YNL063w showed that consensus motifs of N6MTase were conserved. One consensus motif in the sequence of these enzymes, motif I (GXG), is involved in the binding of the SAM and another, motif IV (NPPY) is part of the catalytic domain [24]. To date, however, DNA methylation has not been detected in the genome of S. cerevisiae [25]. We can then raise the question of the function of this protein. The PupM sequence of Streptomyces pristinaespiralis, shown in Fig. 1 as an HemK homolog, has been shown to encode a 1-aminophenylalanine N-methyltransferase which transfers a methyl group from SAM to either 4-aminophenylalanine or 4-methylamino-1-phenylalanine [26]. This enzyme is involved in the biosynthesis pathway of the antibiotic pristinamycin. It is noteworthy that other proteins, such as double-strand RNA adenosine deaminase from Xenopus [27] and human mRNA N6-adenosine methyl transferase [28] contains consensus motifs of DNA N6-adenine methyltransferase. However, in the case of double-strand RNA adenosine deaminase, the binding domain of SAM is not well conserved, which is not surprising since the deaminases are not thought to bind SAM [27]. To date, three SAM-dependent methyltransferases have been crystallized, a C5-cytosine DNA methyltransferase, MH/Hal, [29], an N6-adenine DNA methyltransferase MTaqI [30] and a catechol methyltransferase [31]. These three enzymes all have similar catalytic domains harboring the catalytic and SAM-binding sites, which suggests that SAM-dependent methyltransferase catalytic domains may more generally have common structural features [32]. These data can reasonably be interpreted as suggesting that a homolog of HemK in yeast could have SAM-dependent methyltransferase activity.

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References

porphyrinogen oxidase (PPO) of *Escherichia coli* K-12. DNA Res. 2, 1–8.


