Communication

Intramembrane Bis-Heme Motif for Transmembrane Electron Transport Conserved in a Yeast Iron Reductase and the Human NADPH Oxidase

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A plasma membrane iron reductase, required for cellular iron acquisition by Saccharomyces cerevisiae, and the human phagocytic NADPH oxidase, implicated in cellular defense, contain low potential plasma membrane bis cytochromes that share elements of structure and function. Four critical histidine residues in the FRE1 protein of the iron reductase were identified by site-directed mutagenesis. Individual mutation of each histidine to alanine eliminated the entire heme spectrum without affecting expression of the apoprotein, documenting the specificity of the requirement for the histidine residues. These critical residues are predicted to coordinate a bis-heme structure between transmembrane domains of the FRE1 protein. The histidine residues are conserved in the related gp91phox protein of the NADPH oxidase of human granulocytes, predicting the sites of heme coordination in that protein complex. Similarly spaced histidine residues have also been implicated in heme binding by organelle cytochromes with little overall sequence similarity to the plasma membrane bis cytochromes. This bis-heme motif may play a role in transmembrane electron transport by distinct families of polytopic bis cytochromes.

The human phagocyte NADPH oxidase is a heme-containing enzyme complex critical for defense against microorganisms (1). Defective functioning of this oxidase results in the susceptibility to infection that characterizes patients with chronic granulomatous disease. The genetic defects affecting the oxidase are currently the focus of corrective gene therapy trials (2, 3). The NADPH oxidase assembles from two membrane (gp91phox and p22phox) and three cytosolic (p67phox, p47phox, and p40phox) components in response to a signaling pathway mediated through the small GTP-binding protein Rac (1). The heme cofactor is required for enzymatic function: the transfer of electrons from cytosolic NADPH across the vacuolar membrane to molecular oxygen. The superoxide generated by this process in turn gives rise to toxic byproducts which are used in cellular defense against ingested bacteria and fungi (1).

Despite efforts by several laboratories, the sites within the oxidase where the essential heme cofactors are bound have not been defined (4–6). Heme copurifies with the cytochrome b subunits, gp91phox and p22phox (6). However, inherited mutations in either of these subunits are associated with the absence of detectable heme in the enzyme complex and destabilization of the proteins, thus frustrating efforts to identify the specific heme-liganding residues (7).

We undertook to address this problem via study of the FRE1 reductase of the yeast Saccharomyces cerevisiae. FRE1 is homologous to gp91phox (8) and encodes a plasma membrane-associated subunit of an iron reductase (9). FRE1 or FRE2 is required for iron uptake in yeast (10). FRE1 is required for the major (90%) extracellular ferric reductase activity under most conditions and appears to be a structural subunit of the extracellularly directed reductase activity (8). Chelated ferric iron outside the cell is reduced by this activity to ferrous iron and then transported into the cell by the high-affinity iron uptake system (11). Thus, this iron reductase of yeast, like the NADPH oxidase of humans, is involved in the transfer of electrons from a cytosolic donor across a membrane to an extracellular acceptor.

The yeast iron reductase, like the human NADPH oxidase, requires heme for its function. The requirement of heme for the plasma membrane ferric reductase of yeast is supported by the lack of activity in mutants deficient in heme biosynthesis (12). In addition, FRE1 expression levels correlate with the visible spectrum attributed to the b heme cofactor (13). The appearance of this visible spectrum is extremely similar to the spectrum of the purified NADPH oxidase b cytochrome (13). Two hemes are probably present in each complex of the NADPH oxidase, as indicated by quantitation of heme in the purified cytochrome (4, 14). Midpoint potential titration of the gp91phox Arg54→Ser mutant is also consistent with the presence of two hemes with potentials of −220 and −300 mV (5), and subsequent reanalysis of the data for the normal protein indicates the presence of two low potential hemes of −225 and −265 mV (5). Data from resonance Raman spectroscopy suggests a bis-imidazole coordination (15). The low potential hemes distinguish this enzyme from most other heme enzymes. In this regard, it is significant that redox titration of the heme spectrum of the yeast FRE1 reductase also reveals an extremely low midpoint potential of about −250 mV (13). The spectral and functional similarities of the yeast FRE1 reductase and the human NADPH oxidase suggested to us that study of the heme ligands in FRE1 might shed light on heme coordination in the human enzyme.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Strain 499Δ1Δ2 (MATa ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 Δfre1::LEU2...
An absorption coefficient of 121 raised in rabbits. Approximately 2 COOH) was synthesized, and polyclonal antipeptide antisera were PAGE, polyacrylamide gel electrophoresis. The analogous residues in gp91 contain multiple hydrophobic domains consistent with trans-membrane domains occurring within the amino-terminal two-thirds of the protein. A pair of histidine residues separated by 12 amino acids (His364 and His378). The analogous residues in gp91 membrane (20), and cytb6 (spinach), of the S. cerevisiae, the b cytochrome of the bc1 complex in the inner mitochondrial membrane (20), and cytb (spinach), of the b6f complex in the chloroplast membrane (21). Numbers shown to the left of each amino acid sequence correspond to the initial amino acid in the depicted sequence. B, hydroxylation analysis of FRE1 and gp91Hiss sequences was performed using the Kyte-Doolittle algorithm (22). The mutated histidines in FRE1, amino acid numbers 294, 308, 364, 378, and 462, are indicated by black triangles. The homologous residues in gp91Hiss, amino acid numbers 101, 115, 209, 222, and 338, are similarly indicated. The hydrophobic domains including these histidine residues are blackened.

The contribution of these four conserved histidine residues to the heme binding of FRE1 was therefore examined experimentally. To eliminate background reductase activity, the endogenous FRE1 and FRE2 genes were deleted from the haploid genome of a test strain of yeast (YPH499). Surface reductase activity conferred by transformation of this strain with a high copy-number plasmid carrying FRE1 could then be attributed entirely to expression from the plasmid. Site-directed mutagenesis of the FRE1 plasmid, individually altering histidine residues at amino acid positions 294, 308, 364, or 378 to alanine, reduced the ferric reductase activity of the transformants to levels indistinguishable from the vector control (Fig. 2A). The effect of these mutations upon heme binding was determined by evaluating the reduced-minus-oxidized absorption spectrum of the plasma membranes. Transformsants with the mutated FRE1 plasmids almost completely lacked the plasma membrane heme spectrum (Fig. 2B), and the heme content which was calculated from the amplitude of the y band at 428 nm was negligible (Fig. 2A). The very small amount of residual heme may be attributed to plasma membrane heme proteins other than FRE1 or FRE2 or to very low levels of mitochondrial cross-contamination. As a control, we chose to mutate another histidine in the FRE1 primary sequence. Instead of a random histidine, we selected a residue in a conserved region. This mutated histidine residue (FRE1 amino acid 462) occurs in the consensus HPPT motif (Hist-PRO-Phe-Tyr) which is situated in the hydrophilic carboxyl-terminal region and is thought to play a role in FAD-isoulooxazino binding (14). The reductase activity of the H462A transformant was 27% compared with the wild-

\( \Delta \text{fre2::HIS3} \) was derived from YPH499 (6) by deleting the FRE1 and FRE2. Construction of a deletion was achieved by inserting a unique 2.7-kilobase ClaI fragment containing the FRE1 gene was subcloned into pUC19, and the unique 2.7-kilobase ClaI fragment was replaced with a LEU2 cassette derived from YEp13. For deletion of FRE1, this plasmid, called PUMG2-LEU, was digested with BamHI and HindIII and used to transform YPH499 to leucine prototrophy. For deletion of FRE2, a line of amino acid sequences from HIS3 and flanked by sequences from FRE2 was generated using polymerase chain reaction and used to transform the FRE1:LEU2 strain to histidine prototrophy, as has been described (17). For expression of FRE1, the genomic BamHI to SacI fragment containing FRE1 was modified so that the SacI site was altered to a 5' site. This fragment was then inserted into the corresponding sites of the high copy number vector, YEp352, creating plasmid 352-FRE1. 352-FRE1 was subjected to site-directed mutagenesis (Chameleon, Stratagene) changing FRE1 amino acids 294, 308, 364, 378, and 462 from histidine to alanine and creating plasmids H294A, H308A, H364A, H378A, and H462A, respectively. The presence of the mutations in these plasmids was verified by DNA sequencing.

Enzyme Assays and Spectral Analysis—After induction, cells were harvested and assayed for ferric reductase activity as described (8). Aliquots were lysed with glass beads, and plasma membranes were prepared as described previously (13). Dithionite reduced-minus-oxidized spectra were measured on plasma membrane preparations which had been diluted to a protein concentration of 0.9 mg/ml. The contribution of heme in the plasma membrane preparations was determined from the amplitude of the y-heme peak in the spectrum at 428 nm using an absorption coefficient of 121 \( \mu \text{M} \cdot \text{cm}^{-1} \).

FRE1 Antibody and Protein Detection—A multiple antigenic peptide based on FRE1 amino acids 600 to 615 (NH2-DTNSDESTKGFDEE-NH2) was synthesized, and polyclonal antipeptide antisera was raised in rabbits. Approximately 2 \( \times \) 10^7 cells were suspended in 1.85 M urea, 1% mercaptoethanol, and the protein was recovered by precipitation with an equal volume of 50% trichloroacetic acid. The precipitate was spun down in a microcentrifuge and resuspended in 2-fold concentrated Laemmli SDS-PAGE sample buffer followed by neutralization with 0.1 volume of 1 M Tris base and heating at 37 °C for 5 min. For visualization of FRE1, lysates (approximately 4 \( \times \) 10^7 cell equivalents) were separated by SDS-10% PAGE, blotted onto nitrocellulose (in 30 glacial Tris base, 140 glacial glycerol), and probed with anti-peptide antisera diluted 1:5000. Detection was with anti-rabbit horseradish peroxidase and enhanced chemiluminescence (Amer sham). The abbreviations used are: Mes, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

RESULTS

Our initial objective was to identify the heme-liganding residues within the FRE1 predicted protein. FRE1, like gp91Hiss, contains multiple hydrophobic domains consistent with trans-membrane domains occurring within the amino-terminal two-thirds of the protein. A pair of histidine residues separated by 12 amino acids (His294 and His308) appears within one of these hydrophobic domains. This is followed by an intervening hydrophobic domain and another hydrophobic domain containing a pair of histidines, again separated by 13 amino acids (His364 and His375). The analogous residues in gp91Hiss can be recognized by their spacing and context (Fig. 1A, B, and C). A pair of histidine residues separated by 13 amino acids (His101 and His115) appears buried in a hydrophobic region, followed by an intervening hydrophobic domain and another hydrophobic domain containing two histidines separated by 12 amino acids (His209 and His222) (Fig. 1, A and B). A similar motif consisting of two pairs of spaced histidine residues has been linked to heme coordination in organelle b cytochromes of mitochondria (cytb6 of the bc1 complex) and chloroplasts (cytb6 of the b6f complex) (21, 23, 24) (Fig. 1A).
type allele; however, the heme spectrum, although reduced in amplitude, was not fundamentally altered (Fig. 2).

We considered that the failure to insert heme into FRE1 might destabilize the protein, making it difficult to distinguish mutations affecting heme liganding from mutations affecting protein stability. Therefore, we evaluated FRE1 protein expression in the histidine mutants. The mutations did not significantly affect the level of FRE1 protein as assessed by immunoblotting (Fig. 3), making it likely that abrogation of the heme spectrum was a specific consequence of the point mutation and not the result of protein destabilization.

**DISCUSSION**

The data presented here support a common model for the heme coordination in FRE1 and gp91

FIG. 2. FRE1 mutant alleles deficient in reductase activity and heme. A, ferric reductase from whole cells (open bars) and heme levels from plasma membranes (filled bars). Strain 499Δ1Δ2 was transformed with plasmids: lane 1, carrying the wild-type FRE1 allele; lane 2, lacking FRE1; lanes 3–6, containing FRE1 with the critical histidine mutations H294A, H308A, H364A, H378A; or lane 7, containing FRE1 with the HPFT histidine mutation H462A. The transformants were induced and ferric reductase activities were measured (12) (open bars). The mean values of three determinations, with standard deviations, are shown. The plasma membranes were purified from cell lysates, and the heme contents were estimated (filled bars). Representative experiments of three determinations are shown. B, spectra of purified plasma membranes. The transformants of strain 499Δ1Δ2 described in A were analyzed by purifying the plasma membranes and resuspending at a concentration of 0.9 mg/ml. Dithionite reduced minus oxidized spectra were determined (15). Representative experiments of three determinations are shown.

mutations affecting heme liganding from mutations affecting protein stability. Therefore, we evaluated FRE1 protein expression in the histidine mutants. The mutations did not significantly affect the level of FRE1 protein as assessed by immunoblotting (Fig. 3), making it likely that abrogation of the heme spectrum was a specific consequence of the point mutation and not the result of protein destabilization.

FIG. 3. FRE1 protein levels expressed by the wild-type and mutant FRE1 alleles. Strain 499Δ1Δ2 was transformed with plasmids lacking FRE1, carrying the wild-type FRE1 allele, the critical histidine mutations H294A, H308A, H364A, H378A, or HPFT histidine mutant H462A. The transformants were induced for ferric reductase activity. Cell lysates (alkali lysis) were separated by SDS-PAGE, blotted onto nitrocellulose filters, and probed with a FRE1 anti-peptide antibody followed by enhanced chemiluminescence. Lanes as in Fig. 2.

FIG. 4. Structural model of stacked intramembranous histidines coordinating two hemes. The plasma membrane is portrayed as two layers of circles with tails to indicate the phospholipid bilayer. Cylinders represent three consecutive transmembrane α helices. Imidazoles of heme-coordinating histidines are numbered according to their location in FRE1, and corresponding residues in gp91

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discorded by an intervening transmembrane domain. The 12–13 amino acids of primary sequence separating heme-liganding histidines corresponds to the separation between the two hemes. These regions appear α-helical in character, and, thus, the imidazoles separated by 12–13 amino acids are likely to face the same side of the helix. The hemes coordinated by these histidines are likely to be situated one above the other within the membrane and perpendicular to the plane of the membrane (Fig. 4). This orientation of the hemes near opposite sides of the lipid bilayer would be expected to facilitate the transmembrane electron transport that is critical for the function of these proteins. This model resembles the model described for heme coordination in a class of organelle b cytochromes (21, 23, 24).
An unexpected finding was that mutation of individual critical histidine residues in FRE1 abrogated the entire heme spectrum rather than leading to a 50% reduction. This result is difficult to reconcile with the bis-heme model described above. It is possible that disruption of the imidazole ligand of one of the hemes interferes with insertion of the second heme, due to cooperativity in the heme loading process. Alternatively, effects on protein folding or membrane insertion resulting from the second heme might result in loss of the second heme.

The similarity between the yeast FRE1 reductase and the human NADPH oxidase helps to elucidate features of both enzymes. The residues of FRE1 required for heme binding are conserved with gp91\textsuperscript{phox}, suggesting that the corresponding residues coordinate heme in the NADPH oxidase. However, the corresponding mutations in gp91\textsuperscript{phox} have been uninformative. Three of the four residues corresponding to the heme-liganding residues of FRE1 have been mutated in gp91\textsuperscript{phox} as experiments of nature, i.e. as naturally occurring mutations in chronic granulomatous disease patients (His\textsubscript{101} \rightarrow Arg, His\textsubscript{209} \rightarrow Tyr, His\textsubscript{322} \rightarrow Arg) (7). In cells taken from each of these patients, neither the heme spectrum attributable to flavocytochrome b\textsubscript{558} nor the mutant gp91\textsuperscript{phox} protein was detected (7). Thus, structure-function relationships in the NADPH oxidase could not be deduced from these naturally occurring mutations.

Although the FRE1 reductase functions primarily to reduce iron and the NADPH oxidase functions to reduce oxygen, the FRE1 reductase has recently been shown to be capable of inefficient oxygen reduction (9). Introduction of components of the NADPH oxidase into yeast therefore might provide a set of tools for studying the requirements for efficient superoxide generation. The requirements for heme insertion into the active membrane complex could also be studied in yeast. Defining and satisfying these requirements will be important for efforts to reconstitute the NADPH oxidase in patients with chronic granulomatous disease.

The shared bis-heme binding motif raises the question of the evolutionary and functional relationships between the cell surface membrane proteins gp91\textsuperscript{phox} and FRE1 and the organelle b cytochromes. At the level of primary amino acid sequence, little more than the spacing of the histidines in a generally hydrophobic context is conserved between these two groups of proteins. However, a more closely related protein family can be defined, including FRE1 and gp91\textsuperscript{phox}. The members of this family possess hydrophathy profiles with multiple (six to eight) hydrophobic domains at the amino terminus. The bis-heme binding motif occurs in this region. A hydrophilic carboxyterminal domain characterizes these proteins and is absent from the organelle b cytochromes. Within this hydrophilic tail are a number of conserved amino acid motifs that may relate to cofactor binding (NADPH and FAD) (19, 25) and that appear in the same order and with similar spacing. This protein family includes the homologs of the gp91\textsuperscript{phox} gene from various other species including pigs, mice, and even plants (26), and these genes presumably function as part of a superoxide generating complex in cellular defense. A computer search of the recently completed Saccharomyces Genome Data Base for homologs of FRE1 identified FRE2 and seven additional open reading frames that appear to belong to the same gene family (Table I).

TABLE I

| Gene (identifier) | FASTA score (versus FRE1) | Bis-heme HP(F/Y)(T/S) GP(F/Y)G CG(P/S) |
|-------------------|--------------------------|----------------------------------|
| FRE1 YLR214w      | 4593                     | 294 462 514 652                  |
| FRE2 YKL220c      | 565                      | 316 479 526 677                  |
| YNR060w           | 574                      | 309 472 519 685                  |
| YLL051c           | 572                      | 323 483 538 678                  |
| YOR381w           | 566                      | 318 479 526 677                  |
| YOR384w           | 566                      | 310 473 520 660                  |
| YOL152w           | 351                      | 197 369 411                      |
| YGL160w           | 254                      | 136 306 543                      |
| YLR047c           | 149                      | 152 320                          |
| CVTb PIR A00159   | 43                       | 52                               |

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