Glutathione-dependent redox status of frataxin-deficient cells in a yeast model of Friedreich’s ataxia

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Friedreich’s ataxia is a neurodegenerative disease caused by reduced expression of the mitochondrial protein frataxin. The main phenotypic features of frataxin-deficient human and yeast cells include iron accumulation in mitochondria, iron–sulphur cluster defects and high sensitivity to oxidative stress. Glutathione is a major protective agent against oxidative damage and glutathione-related systems participate in maintaining the cellular thiol/disulfide status and the reduced environment of the cell. Here, we present the first detailed biochemical study of the glutathione-dependent redox status of wild-type and frataxin-deficient cells in a yeast model of the disease. There were five times less total glutathione (GSH/GSSG) in frataxin-deficient cells, imbalanced GSH/GSSG pools and higher glutathione peroxidase activity. The pentose phosphate pathway was stimulated in frataxin-deficient cells, glucose-6-phosphate dehydrogenase activity was three times higher than in wild-type cells and this was coupled to a defect in the NADPH/NADP⁺ pool. Moreover, analysis of gene expression confirms the adaptative response of mutant cells to stress conditions and we bring evidence for a strong relation between the glutathione-dependent redox status of the cells and iron homeostasis. Dynamic studies show that intracellular glutathione levels reflect an adaptation of cells to iron stress conditions, and allow to distinguish constitutive stress observed in frataxin-deficient cells from the acute response of wild-type cells. In conclusion, our findings provide evidence for an impairment of glutathione homeostasis in a yeast model of Friedreich’s ataxia and identify glutathione as a valuable indicator of the redox status of frataxin-deficient cells.

INTRODUCTION

Friedreich’s ataxia (FA) is a neurodegenerative disease characterized by progressive neurodegeneration and cardiomyopathy. It is an inherited autosomal recessive disorder caused by reduced expression of the mitochondrial protein frataxin (1,2). The major phenotypic features of frataxin-deficient human and yeast cells include iron accumulation in mitochondria, iron–sulphur cluster defects and high sensitivity to oxidative stress (reviewed in 3). This raises the question of the status of the cellular defences against oxidative injury in FA cells. There are preliminary experiments suggesting that patients with FA suffer a disturbance of glutathione homeostasis and modifications of glutathione-dependent antioxidant defences (4–6).

Thiol (-SH)-containing antioxidants, including cystein and glutathione, make a large contribution to the protection against oxidative damage; they do so by reacting with reactive oxygen species (ROS). In yeast, the thiol-dependent response to oxidative stress consists of the parallel glutathione/glutaredoxin and thioredoxin pathways which are both required under aerobic and anaerobic conditions (reviewed in 7–10). Glutathione (L-γ-glutamyl-cysteinyl-glycine) (GSH/GSSG) is the most abundant low-molecular-weight non-protein thiol in eukaryotic cells (11,12). It is involved in a variety of cellular functions: protection against oxidative...
damage, the maintenance of the mitochondrial structure and membrane integrity, cell differentiation and development (13,14). Due to its low redox potential ($E^0 = -240$ mV) and its high cellular concentration (1–10 mM), glutathione is a major redox buffer in thiol-based redox systems (7). It is also a strong nucleophile and conjugates with ROS and with a range of electrophilic compounds and numerous xenobiotics (15). Its unusual $\gamma$-glutamyl peptide bond results in it having substantial protection to proteolytic degradation. GSH depletion results in a particular defect in the maturation of iron–sulphur proteins, leading to iron accumulation in the cells (16,17).

The reduced sulphhydryl group in glutathione (GSH), when oxidized, produces a disulphide, or ‘oxidized glutathione’ (GSSG), and functions as a source of reducing equivalents to reduce cellular disulphide bonds, often in conjunction with glutaredoxins (8,18). Oxidants interfere with a range of cellular components and cause accumulation of ROS and/or alter the GSH/GSSG ratio, and as a consequence changes in GSH concentration directly reflect intracellular redox changes. The levels of intracellular-reduced glutathione are maintained by strict regulation of a combination of glutathione-dependent enzymatic systems that balance its rate of synthesis and reduction. Glutathione reductases restore and maintain physiological GSH/GSSG balances under both stressed and unstressed conditions by reducing GSSG in a NADPH-dependent reaction. The response to oxidative stress also involves GSH biosynthesis enzymes, and peroxide-eliminating glutathione peroxidases and glutaredoxins (19). In addition, glutathione-S-transferases play a protective role by attaching GSH to biomolecules, targeting them for export from the cell (20).

Yeast also contains a thioredoxin system, including thioredoxin and thioredoxin reductase, which functions as a protection against ROS generated during respiratory metabolism (21–23). Thioredoxins, like glutaredoxins, are small heat-stable oxidoreductases containing two conserved cysteine residues in their active site. The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase, whereas glutaredoxin is reduced by glutathione with NADPH as the electron donor (8). The thioredoxin system involves both cytosolic and mitochondrial thioredoxin peroxidases (24,25). However, in Saccharomyces cerevisiae, the thioredoxin and GSH/glutaredoxin systems are not functionally redundant. Glutathione is involved in critical functions of redox regulation not shared with the thioredoxin pathway, and works on $\Delta gsh1$ mutants suggest that GSH is important for the maintenance of the mitochondrial genome (26,27). S. cerevisiae defective for GSH biosynthesis (lacking $GSH1$) or altered in their GSH redox state are unable to grow in the absence of GSH and are less tolerant than wild-type to a wide range of stress conditions induced by peroxides and the superoxide anion, and to the toxic products of lipid peroxidation; they also undergo apoptosis more than their parental cells (28–31). Depletion of mitochondrial and cytosolic glutathione is associated with increased generation of ROS, defects in iron–sulphur cluster assembly and rapid loss of mitochondrial function (16,17,19,26,32,33). Inversely, administration of exogenous glutathione is capable of rescuing cells from accelerated aging (34).

Abnormal glutathione homeostasis has been described in various neurodegenerative diseases, and during cell aging and apoptosis (14,35). Indeed, in conditions of oxidative stress, intracellular glutathione depletion may be attributed to several factors, including increased degradation, conjugation, oxidation, efflux and biosynthesis decrease (12,13,36,37). Yeast is a good eukaryotic model for studying some of these pathologies because genetic and biochemical tools are available for manipulations. Partly because the significance of glutathione in neurodegeneration is becoming increasing evident and because of potential applications as a clinical marker of oxidative stress (38), we studied glutathione-dependent redox status in a S. cerevisiae model of Friedreich’s ataxia, and of the wild-type.

We show that GSH/GSSG levels in frataxin-deficient cells are low, and this is associated with high activities of thiol-dependent enzymes including glutathione peroxidases. The HPLC analysis of NADPH/NADP$^+$ pools demonstrated a relation between the glutathione-redox status and a strong stimulation of the pentose phosphate pathway. The changes in thiol-dependent antioxidants were associated with modifications of gene transcription.

We also report the glutathione-dependent response to iron excess conditions. Dynamic studies show that intracellular glutathione levels in wild-type and mutant cells reflect adaptation to iron excess conditions. Addition of excess iron to cultures of the mutant cells resulted in GSH levels similar to those in wild-type cells. Our findings reveal that the hyper-sensitivity of frataxin-deficient cells to oxidative stress is associated with modifications of the glutathione-dependent antioxidant defences and possibly of glucose metabolism. They are also consistent with there being substantial physiological interaction between iron and glutathione pathways.

**RESULTS**

**Total glutathione levels (GSH + GSSG) are decreased in frataxin-deficient cells**

Total (GSH+GSSG) and oxidized (GSSG) glutathione levels were measured (Fig. 1). Wild-type cells (strain S150-2B) grown in minimum YNB medium under normal aerobic conditions have a redox ratio (GSH/GSSG) of $\sim 6/1$ (Fig. 1) indicating that most of the intracellular free glutathione is in a reduced (GSH) form; this prevents the toxic formation of mixed disulphur-protein products, as previously described (10,39). Frataxin-deficient cells contained less total glutathione, with 87.2 ± 19.2 nmoles glutathione/mg protein, rather than the 173.4 ± 23.5 nmoles glutathione/mg protein in wild-type cells (Fig. 1). In addition, the redox balance (GSH/GSSG) was shifted towards the oxidized form GSSG, reflecting a depletion of the reduced form of glutathione such that there were similar amounts of the reduced and oxidized form in the mutant cells (the GSH/GSSG ratio was 0.8). The GSH/GSSG ratio, therefore, indicates reducing conditions inside the cell, consistent with classical exposure stress conditions, in which oxidized GSSG levels are elevated in parallel with a decrease in intracellular GSH levels (10,29). Similar results were obtained when cells were grown on a rich YPD-medium and with strain YPH499, showing that the overall glutathione defect was not strain dependent (data not shown).

Inversely, total glutathione levels were very high in mitochondria isolated from frataxin-deficient cells (11.6 ± 1.2 nmoles
Glutathione/mg protein in wild-type cells and 40.3 ± 5.4 nmol glutathione/mg protein in Δyfh1 cells). Interestingly, the distribution of glutathione pools between cytosol and mitochondria was abnormal in the mutant. Most glutathione (93.3%) in wild-type cells is on the cytosol, but, in frataxin-deficient cells, 46.2% of total glutathione was found inside the isolated mitochondria. *S. cerevisiae* mitochondria cannot synthesize glutathione, so these findings suggest import of glutathione into the mitochondrial matrix from the cytosol in response to the oxidative stress associated with the frataxin deficiency.

**Glutathione peroxidase activity is enhanced in frataxin-deficient cells**

Glutathione peroxidases (Gpx) are the main peroxide detoxifying enzymes in *S. cerevisiae*, and act by catalysing the breakdown of H₂O₂ and larger hydroperoxides using GSH as a reductant (40). These enzymes are required for cellular protection against lipid peroxidation. Three gene products (Gpx1, Gpx2 and Gpx3) with significant homology (30, 41 and 40%, respectively) to mammalian peroxidases have been identified from the yeast genome (41–45). We assayed glutathione peroxidase activities in crude cell extracts and isolated mitochondria (Fig. 2). Gpx activity was much higher in frataxin-deficient cells than wild-type cells (specific activities of 995.9 ± 75.8 units/mg and 2424.3 ± 107.1 units/mg in wild-type and frataxin-deficient cells), which confirm the severe oxidative stress status of the mutant cells. The specific activity in isolated mitochondria was similarly higher in the mutant than wild-type cells (Fig. 2). The lower specific activity in isolated mitochondria than in cell extracts was presumably due to most of the Gpx being in the cytoplasm. Thus, the Gpx activity in cytosol is 2.3 times higher in Δyfh1 than wild-type cells and three times higher in mitochondria, consistent with the difference in total glutathione levels and with the thiol-dependent antioxidant defences being ‘switch-on’ in frataxin-deficient cells.

**The total reduced thiol groups are depleted in *S. cerevisiae* frataxin-deficient cells**

We assayed total reduced thiol groups in wild-type and mutant cells (Fig. 3). Total thiol groups include free glutathione, free thiol groups such as cysteine, and protein-bound thiol groups. Crude extracts of wild-type cells contained 203.1 ± 24.7 nmol of total thiols/mg of protein and those of frataxin-deficient cells 83.0 ± 11.6 nmol total thiols/mg protein. This difference is consistent with the severe stress status of the frataxin-deficient cells and their lower total glutathione content. Moreover, the respective amounts of reduced glutathione (calculated from Fig. 1) and total reduced thiol groups are in good agreement with glutathione being the main non-protein thiol molecule in the cell (85.4%). However, the amounts of reduced thiol groups in mitochondria were similar in mutant and wild-type cells (27.1 ± 2.7 nmol total thiols/mg protein, Fig. 3). These results could suggest a difference in the distribution of thiol groups between cytosol and mitochondria, or transport of these groups to the surface of the cell. Indeed, when cells or tissues are subjected to oxidative stress, export of GSSG has been observed and this efflux appears to be a part of the protection of cells and tissues against oxidative stress. To further explore this issue, extracellular glutathione was assayed; there was no significant difference between wild-type and frataxin-deficient cells, and
Wild-type & $\Delta yfh$ & \\
--- & --- & \\
NADPH (nmoles/mg protein) & 120.9 ± 8.27 & 29.3 ± 3.8 & \\
NADP$^+$ (nmoles/mg protein) & 65.1 ± 12.7 & 46.8 ± 13.4 & \\
Glucose 6-phosphate dehydrogenase specific activity (nmoles NADPH/min/mg protein) & 53.4 ± 5.9 & 155.5 ± 20.1 & \\

After acidic or alkaline extraction of the cellular extracts, NADPH and NADP$^+$ levels were assayed by reverse phase HPLC. Products were monitored spectrophotometrically at 260 nm and quantified by integration of the peak absorbance area, employing a calibration curve of increasing concentrations of NADPH and NADP$^+$. Glucose-6-phosphate dehydrogenase activities were assayed by following the increase of absorbance at 340 nm due to the conversion of NADP$^+$ to NADPH. Wild-type cells and frataxin-deficient cells were grown in YNB-glucose minimum medium and all values are means ± SD of at least three determinations.

There are no evidence of enhanced export of glutathione from mutant cells.

### The pentose phosphate pathway is strongly stimulated in S. cerevisiae frataxin-deficient cells

Most of the electron sources for the two glutathione/glutaredoxin and thioredoxin systems are provided by NADPH which, due to its low redox potential ($E^{\text{red}} = -315$ mV), can act as the primary hydrogen donor for both systems. Indeed, glutathione reductases maintain physiological GSH/GSSG balances by reducing GSSG in a NADPH-dependent reaction, and the oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase. We determined relative NADPH and NADP$^+$ concentrations in wild-type and mutant cells by reverse-phase HPLC experiments (Table 1).

Under our experimental conditions, wild-type cells contained 120.9 ± 8.3 nmoles NADPH/mg protein, and mutant cells 29.3 ± 12.7 nmoles NADPH/mg protein. These results are in good agreement with the higher glutathione peroxidase activity observed in mutant cells (Fig. 2): GSSG is reduced back to GSH by glutathione reductase using NADPH for reducing power.

The NADPH/NADP$^+$ ratios were 1.9 in wild-type cells and 0.6 in mutant cells, indicating a substantially lower pool of the antioxidant reduced NADPH in frataxin-deficient cells (Table 1). Depletion of reduced NADPH is characteristic of severe oxidative stress conditions, which, for whatever reason, cannot be counterbalanced by antioxidant systems in the cells. Glucose-6-phosphate dehydrogenase and, to a lesser extent, NADP$^+$-specific isocitrate dehydrogenase (IDP) are believed to be the major sources of NADPH used directly by NADPH and thioredoxin reductase. We determined the oxidized disulphide form of thioredoxin is reduced by NADPH in the cytosol of S. cerevisiae cells (46), so these findings suggest that the pentose phosphate pathway is strongly perturbed in frataxin-deficient cells.

Glucose-6-phosphate dehydrogenase is the key enzyme of the pentose phosphate pathway that is responsible for the generation of NADPH and plays a role in the protection against oxidative damage in eukaryotic cells (47,48). Specific G6PDH activity was significantly higher in frataxin-deficient (155.5 ± 20.1 nmoles NADPH/min/mg protein) than in wild-type (53.4 ± 5.9 nmoles NADPH/min/mg protein) cells. This indicates, as suggested by the NADPH/NADP$^+$ ratios, that the pentose phosphate pathway is stimulated in frataxin-deficient cells. Indeed, when NADPH is being rapidly converted to NADP$^+$, as in frataxin-deficient cells, the level of NADP$^+$ rises, allosterically stimulating G6PDH to accelerate NADPH biosynthesis. These observations are also in good agreement with previous work showing that G6PDH expression is enhanced by oxidative stress induced by agents that specifically deplete the intracellular glutathione pool (49,50).

### Regulation of genes involved in thiol-dependent ROS detoxification in frataxin-deficient cells

We used real-time quantitative PCR for transcriptional analysis of the genes implicated in the glutathione-dependent systems of ROS detoxification in wild-type and frataxin-deficient cells. The results (Table 2) are in good agreement with the biochemical data. The ratios of expression of the genes GSH1 and GSH2 in frataxin-deficient (1.15) and wild-type cells (1.32) were similar, consistent with the lower glutathione levels in mutant cells being a consequence of pool redistribution and not reduced biosynthesis. Transcription of the gene encoding the high-affinity glutathione transporter OPT1 was twice as high in frataxin-deficient cells, consistent with the decrease in total glutathione (Table 2). However, transcription of OPT1 has been reported to be repressed by exogenous GSH but not regulated in a $\Delta gsh1$ mutant deficient in glutathione synthesis (51). Our results present no evidence of a change in transcription of the glutaredoxin gene family GRX1-3, nor in that of the glutathione transferase GTTI-3 (Table 2).

Frataxin deficiency causes an oxidative stress and depletion of glutathione and NADPH pools and induction of Gpx and G6PDH activities. Accordingly, transcription of GPX2 and ZWF1 genes was twice as high as wild-type in $\Delta yfh$ mutant cells (Table 2). However, the gene GLR1 encoding the glutathione reductase that uses NADPH to reduce GSSG was unaffected in frataxin-deficient cells (Table 2). These various results show that the changes in enzymatic and thiol levels are due to transcriptional and post-transcriptional modifications, and reveal the particular metabolic patterns in frataxin-deficient cells.

### Glutathione redox status of frataxin-deficient S. cerevisiae cells is modified in the presence of excess iron

In S. cerevisiae, deletion of the frataxin homolog YFH1 results in a 10-fold increase in iron in mitochondria and increased ROS production (52,53). It has been suggested that this iron excess is responsible both for ROS production by the Fenton reaction and for secondary phenotypes such as aconitase deficiency and mitochondrial DNA loss. It has also been suggested that the glutathione-dependent redox status of the cells and iron homeostasis are linked. GSH depletion causes a specific defect in the maturation of iron–sulphur proteins, leading to iron accumulation in the mitochondria (17). In addition, deficiency in the mitochondrial glutaredoxin Grx5 associates defective activity of mitochondrial iron–sulphur proteins and cellular iron accumulation (16).

We grew cells in presence of various concentrations of radioactive $^{55}$Fe and followed iron accumulation (Fig. 4). As...
expected, iron accumulation was much higher in Δyfh1 than wild-type cells, and was proportional to the amount of iron added to the culture medium. At 100 μM iron, the highest concentration tested, corresponding to a 100-fold more than in standard YNB medium, mutant cells accumulated 614.5 ± 104.8 pmol Fe/OD$_{600nm}$ and wild-type cells accumulated 105.9 ± 27.7 pmol Fe/OD$_{600nm}$ (Fig. 4). Consequently, we studied the glutathione-dependent redox status of wild-type and mutant cells grown in the presence of a 100 μM iron.

The total glutathione (GSH+GSSG) concentration (Fig. 5A) in mutant cells cultivated in the presence of an excess iron was similar to that in wild-type cells, and nearly double than in mutant cells cultured on YNB medium. The presence of excess iron was associated with a decrease of the oxidized disulphide GSSG; 48.4 ± 2.3 nmoles GSSG/mg protein and 18 ± 5.7 nmoles GSSG/mg protein for Δyfh1 cultured in minimum and iron-supplemented YNB medium, respectively (data not shown). In the presence of excess iron, there was also a 10-fold increase of the GSH/GSSG ratio, suggesting a return to non-stress redox conditions. At the transcriptional level, the raise in total glutathione cannot be easily attributed to an increased synthesis, because at least GSH1 and GSH2 transcription was not affected by iron addition (Table 2). Interestingly, the expression of the high-affinity glutathione transporter encoding gene OPT1 was not modified and remained up regulated which suggested that cells still sense the need of external glutathione in the presence of iron. As shown in Figure 5B, glutathione peroxidase activities were decreased in the presence of an excess iron and were similar to the ones observed for wild-type cells. In agreement with reduced Gpx activity, GPX2 transcription was lowered in frataxin-deficient cells cultured with excess iron but the gene remained induced compared to wild-type cells (Table 2). The consequences of the presence of the iron chelator BPS (bathophenanthrolin disulphonic acid) were tested, but the glutathione-dependent redox status and Gpx activity were modified and remained up regulated which suggested that cells still sense the need of external glutathione in the presence of iron.

As shown in Figure 5C, G6PDH activity in mutant cells cultivated with an excess iron was 102.8 ± 3.9 nmoles NADPH/min/mg protein, lower than the 155.5 ± 20.1 for mutant cells grown in standard minimum medium. However, in presence of an excess iron, G6PDH activity of frataxin-deficient cells was

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’→3’)</th>
<th>Relative expression Δyfh1/wt YNB</th>
<th>YNB + Fe</th>
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<tr>
<td>GPX1</td>
<td>GGGAAAGTCTGGAAATAAAAATGATAAA TACTTCTGGTTGGAATTCAAA</td>
<td>1.18 ± 0.65</td>
<td>1.67 ± 0.73</td>
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<tr>
<td>GPX2</td>
<td>AAAAGCCAAAAGCAGGGTTTACT CAAAGGCTGATGTTTTCTTT</td>
<td>3.51 ± 0.39</td>
<td>2.61 ± 1.03</td>
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<tr>
<td>GPX3</td>
<td>CGGATGATTGTTGGCGTCGAG GATTTCGAGGATTGCTTC</td>
<td>0.75 ± 0.21</td>
<td>0.65 ± 0.18</td>
</tr>
<tr>
<td>GSH1</td>
<td>GACCAGATGGCGAAAACTGA CCGTGCACCTGTGATGCTATT</td>
<td>1.15 ± 0.29</td>
<td>1.31 ± 0.12</td>
</tr>
<tr>
<td>GSH2</td>
<td>GATAGCGATGAGGCCTTGATT CTCGCAAGCTCGTGATCA</td>
<td>1.32 ± 0.67</td>
<td>1.43 ± 0.50</td>
</tr>
<tr>
<td>GLR1</td>
<td>AGGTTTTTCGTCGACATT CCTTATGGGCCCACCATTTT</td>
<td>0.76 ± 0.21</td>
<td>0.95 ± 0.16</td>
</tr>
<tr>
<td>GRX1</td>
<td>AAAGAACCGTGCCAAACATC CAATTCACCAGTCTCCCTCAA</td>
<td>0.92 ± 0.45</td>
<td>1.59 ± 0.82</td>
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<tr>
<td>GRX2</td>
<td>GGCCAAAAGAAGGTTTGGTGT CCTCAATCTTGGAGGTTG</td>
<td>0.48 ± 0.15</td>
<td>0.55 ± 0.10</td>
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<tr>
<td>GRX3</td>
<td>TCAATTCCGACATCACAAT CTGTCTGCTATCGCTCT</td>
<td>1.80 ± 0.54</td>
<td>1.45 ± 0.33</td>
</tr>
<tr>
<td>GRX4</td>
<td>AGCTGACCTGTGATGTATT GCAGAATTTGTTGTGTTTC</td>
<td>0.74 ± 0.24</td>
<td>0.56 ± 0.18</td>
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<tr>
<td>GRX5</td>
<td>GAATGGACATCTCCACAA TCGGCCAACATTCACACAG</td>
<td>0.66 ± 0.21</td>
<td>0.48 ± 0.16</td>
</tr>
<tr>
<td>GGT1</td>
<td>CGAATTTGTTGGAGGTTGAC CCCATGAAAAAGGAAAGAC</td>
<td>0.75 ± 0.18</td>
<td>1.34 ± 0.34</td>
</tr>
<tr>
<td>GGT2</td>
<td>AACTGGCATACTGGACCTGGTTT GCAGATTCCACTCTTGTTC</td>
<td>1.21 ± 0.28</td>
<td>3.41 ± 0.67</td>
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<tr>
<td>GGT3</td>
<td>ACTATTTCCGTCGTAATAC ATGCGACGGAGAAAGAAGAAA</td>
<td>1.14 ± 0.447</td>
<td>0.86 ± 0.17</td>
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<tr>
<td>YCF1</td>
<td>GCAGTGGCGTGGAGACA GCAGTGATGGTCCAGATCTT</td>
<td>1.43 ± 0.37</td>
<td>1.68 ± 0.34</td>
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<tr>
<td>OPT1</td>
<td>GCAGTGGCGTGGAGACA GGCAGATGGTCCAGATCTT</td>
<td>2.17 ± 0.60</td>
<td>3.20 ± 0.93</td>
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<tr>
<td>ZWF1</td>
<td>CCGGCGCTTGAGGGTTTGC CCGGCGCTTGAGGGTTTGC</td>
<td>2.07 ± 0.88</td>
<td>3.14 ± 0.86</td>
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<tr>
<td>RPO21</td>
<td>GTTGGTATCGTACCTTTACCTCAT GATAAGACCTTCACGACC</td>
<td>nd</td>
<td>nd</td>
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</table>

Cells were cultivated on YNB-medium, supplemented or not with 100 μM iron citrate, to exponential phase. Relative expression was calculated using the equation described by (79). Sequences of primers used are depicted, the first is the forward and the second the reverse (nd, not determined).
still higher than the one observed in wild-type cells (Fig. 5C). As shown in Table 2, ZWF1 gene remains activated in the presence of iron, which confirms that the adaptative response to iron stress conditions can be attributed to metabolic choices of the cells. The major change in transcription in the presence of iron was the induction of mitochondrial glutathione-S-transferase GTT2, the main transferase involved in cadmium and oxidative stress resistance and adaptation (54,55). These results show that the glutathione-dependent redox status of mutant cells is strongly dependent on the abundance of iron in the environment and could reflect an adaptative response of the cells to iron-dependent stress conditions.

Iron-dependent glutathione redox status reflects an adaptative response to excess iron conditions

In response to stress conditions, yeast cells up regulate the synthesis of a number of protective molecules including components of both the GSH/glutaredoxin and the thioredoxin systems (19,29). This forms the basis of an inducible adaptative response, in which, for example, cells treated with a low dose of oxidant can adapt to become resistant to a subsequent stress and otherwise lethal treatment. In the following experiments, wild-type and mutant cells were cultured in minimum YNB medium until reaching the exponential phase of growth: aliquots were treated with excess iron, and other were transferred to an iron-depleted culture medium to create iron-deficient conditions. Thiol-dependent antioxidants, including glutathione concentrations and Gpx activities, were followed for up to 2 h.

There was no change in total glutathione pools or Gpx activity for wild-type or mutant cells when shifted, after filtration, to the same YNB medium (Figs. 6A and 7A). This control was very important to check that the filtration process did not constitute a stress for the cells, and that any changes in glutathione levels or enzymatic activities detected could be attributed to the changes in the culture conditions.

For wild-type cells, the presence of an excess iron in the culture medium resulted in a rapid decrease in the total glutathione concentration (Fig. 6B), from 173.4 ± 23.5 nmoles glutathione/mg protein at baseline to 84.3 ± 8.7 nmoles glutathione/mg protein after 1 min of treatment. Moreover, the calculated GSH/GSSG ratio was 6.0 at baseline and 6.3 after 1 min with no subsequent change. In parallel, as expected, the specific glutathione peroxidase activity increased after 1 min in the presence of an excess iron (Fig. 7B), followed by a return to normal conditions after 10 min. This suggests an acute response of wild-type cells to iron stress conditions, followed by an adaptation and equilibration after 5 min and a return to non-stress conditions. Repeat measurements, 120 min after the start of treatment (data not shown), did not reveal any further changes in the glutathione-dependent redox status of these cells, showing adaptation of wild-type cells to the new culture conditions was complete within 10 min.
In contrast, excess iron had little or no effect on glutathione levels or on GSH/GSSG ratios in Δyfh1 cells (Fig. 6B). However, Gpx specific activity decreased from 2424.3 ± 107.1 units/mg protein at baseline to 1645 ± 138.6 units/mg protein after 1 min, and returned to the initial value after 10 min (Fig. 7B). Thus, wild-type and mutant cells respond very differently to excess iron and show the fundamental difference between an acute and a constitutive response to iron excess conditions.

We found that iron deficiency constituted a stress for wild-type cells. Indeed, total glutathione levels increased after 1 min (Fig. 6C), then decreased to a value lower than baseline value observed for wild-type cells. This observation suggests that the cells have only a poor capacity to continue their adaptative response in conditions of severe iron deficiency. However, there was no significant change in Gpx activity in wild-type cells after exposure to an iron-depleted Bio101 culture medium (Fig. 7C). In contrast, Δyfh1 cells were not sensitive to iron deficiency (Figs. 6C and 7C), which confirms the opposite adaptative mechanisms of wild-type and constitutively stressed mutant cells when subjected to iron excess conditions.
DISCUSSION

We exploited the experimental advantages of S. cerevisiae to conduct a detailed study of the glutathione-dependent cellular defences of frataxin-deficient cells. The tripeptide glutathione is an important molecule in the protection of yeast cells against oxidative stress (9,10,19,28,30). In the first part of this work, we compared the glutathione-dependent redox status of wild-type and frataxin-deficient cells, and then we extended the study to explore the adaptive response of cells to iron-dependent stress conditions.

Frataxin-deficient cells are characterized by a deficiency of total glutathione levels, associated with a shift of the GSH/GSSG ratio towards the oxidized disulphide form GSSG (Fig. 1). This is consistent with the role of GSH as both a free radical scavenger and a cofactor for various antioxidant enzymes, including glutathione peroxidases, glutathione-S-transferases and glutaredoxins. The GSH/GSSG ratio reflects the reducing conditions inside the cell, so this observation agrees with classical exposure to stress conditions, where oxidized GSSG levels are elevated and intracellular GSH levels reduced. Total reduced thiol groups were also less abundant in the mutant than wild-type (Fig. 3), confirming the abnormal thiol-dependent redox status of the mutant cells. The expression levels of GSH1 and GSH2, encoding the enzymes involved in the glutathione biosynthesis pathway, are not modified in frataxin-deficient cells (Table 2). Therefore, the low total glutathione levels are not due to low rates of glutathione biosynthesis, but to a consumption of glutathione associated with stress conditions. Microarray data (not shown) do not reveal a change in ECM38 (encoding γ-glutamyl-transpeptidase). Also YCF1 (encoding vacuolar GSH transporter; Table 2) transcription is unaffected so increased glutathione degradation in vacuoles does not seem to be responsible for the low GSH concentration. The expression of GLRI coding for glutathione reductase which catalyses the recycling of GSH to GSSG was not affected by frataxin deficiency (Table 2), so the elevated GSH/GSSG ratio was most likely not due to an abnormality in the activity of this enzyme.

S. cerevisiae contains three glutathione peroxidases (Gpx1, Gpx2 and Gpx3) which catalyse the breakdown of H₂O₂ and larger hydroperoxides using GSH as a reductant (30,40–43). Two members of the glutaredoxins family, Grx1 and Grx2, are also active as glutathione peroxidases (56). A significant activation of the glutathione peroxidase pathway to ~150% of control activity, associated with high GSSG levels, has been described in lymphoblasts from FA patients (4). This is similar to the two to three times higher Gpx activity in mutants than wild-type S. cerevisiae cells (Fig. 2). Glutathione peroxidases reduce H₂O₂ and other organic hydroperoxides to the corresponding alcohol, using the reducing power provided by GSH. The higher Gpx activity is also consistent with observations that oxidized glutathione levels are significantly higher in mutants, with a GSH/GSSG ratio indicative of a stressed status of the cells. Gpx activity in mitochondria was also high but most of the enzymatic activity was in the cytosolic fraction. Our transcription data indicate that Gpx2 seems to be the only gene controlled by Yap1 to be induced in mutant cells (43). This absence of transcriptional response reflects the constitutive adaptation of Δyfh1 to oxidative stress conditions.

Glutathione is usually found in the cytosol, vacuole and other compartments including the endoplasmic reticulum, nucleus and mitochondria; it can be assimilated from the medium by yeast cell-surface transporters (57). Under our experimental conditions, glutathione was mainly found in the cytosol in wild-type cells. There was 4-fold more total glutathione in the mitochondria of mutant than wild-type cells (Fig. 1), indicating a different distribution of the glutathione. However, glutathione cannot be synthesized inside yeast mitochondria and must be imported to the mitochondrial matrix from the cytosol. Our results suggest an import of glutathione to the mitochondria under the stress conditions induced by frataxin deficiency. This hypothesis is supported by the observed induction of the OPT1, gene coding for glutathione transporter in response to a decrease of intracellular total glutathione levels (Table 2).

We also tested whether glutathione was exported from the cells. GSH can be found outside the cells, but generally in small amounts, and this extracellular glutathione is thought to function in detoxification processes and provide protection against oxidative injury. Indeed, in response to an oxidative challenge, oxidized GSSG, and protein-bound and extracellular glutathione are all elevated in parallel with the decrease of intracellular GSH levels (29). However, under our conditions, no glutathione excretion to the culture medium was detected within 48 h. Furthermore, measurements of extracellular glutathione in mutant cells provided no evidence of export of glutathione out the cells.

FA patients have abnormally low free glutathione concentration, high glutathione transferase (GST) activity, associated with a significant increase of glutathione bound to haemoglobin in erythrocytes (5,6), suggesting that protein glutathionylation is a response to stress conditions induced by frataxin deficiency. Indeed, cysteine is the most easily oxidized residue in proteins, resulting in intermolecular protein cross-linking and enzyme inactivation (58). Such irreversible oxidation events can be prevented by protein-S-thiolation in which protein SH groups react with low molecular weight thiols and particularly GSH (glutathionylation) (59–61). We postulate that the decrease of total thiols and glutathione levels in mutant cells may be a consequence of the consumption of glutathione by a process such as protein glutathionylation.

HPLC experiments (Table 1) show NADPH levels in mutant cells being one quarter that in the wild-type, associated with a shift of the NADPH/NADP⁺ ratio from 1.9 in wild-type cells to 0.6 in frataxin-deficient cells. Indeed, due to the significantly higher Gpx activity (Fig. 2), GSSG is presumably reduced back to GSH by glutathione reductase, using NADPH for a reducing power, so as to maintain the redox status of the cells. NADPH provides electrons for the reduction of both the oxidized disulphide form of thioredoxin, catalysed by thioredoxin reductase, and GSSG to GSH catalysed by glutathione reductase (8,62). The substantial imbalanced NADPH/ NADP⁺ ratio we observed confirms the severe oxidative stress status and the malfunction of the thiol-dependent antioxidant defences of mutant cells, suggesting alternative metabolic mechanisms are used to survive the stress associated with frataxin deficiency. Surprisingly, our results show a decrease of the total (NADPH+NADP⁺) levels, and this implies oxidative modification of NADPH, such as the
degradation into NADH catalysed by phosphatases. Supporting this hypothesis, the genes coding for glycerol-3-phosphatases, RHR2 and HOR2, are induced in frataxin-deficient cells (microarray data, not shown).

In S. cerevisiae cells, glucose-6-phosphate dehydrogenase and, to a lesser extent, NADP⁺-specific IDP are believed to be the major sources of the NADPH used by thiol-dependent peroxidases in the cytosol (46). In frataxin-deficient cells, specific G6PDH activity was significantly higher than wild-type (three times) and the expression of ZWF1, coding for G6PDH, was induced (Table 2). In addition, microarray data (not shown) indicate that the expression of IDP1,2,3 coding for IDPs is two or three times higher than in the wild-type. Consequently, the pentose phosphate pathway is stimulated in mutant cells, as by the NADPH/NADP⁺ ratios. Indeed, when a cell is rapidly converting NADPH to NADP⁺, as observed in frataxin-deficient cells, the level of NADP⁺ rises, allosterically stimulating G6PDH to accelerate NADPH biosynthesis. However, the strong induction of G6PDH cannot restore the NADPH/NADP⁺ pools sufficiently to be able to maintain the normal redox status of the cells. Our results are in good agreement with previous work showing that G6PDH expression is enhanced by oxidative stress (63). Moreover, it has been reported that this activation of the enzyme is associated with a decrease of the glutathione pool and that this increase is blocked by treatment with antioxidants that specifically replenish the intracellular GSH (49,50). G6PDH expression is inversely correlated to intracellular GSH levels and GSH replenishment may depend on G6PDH expression (49); this is consistent with the induction of ZWF1 we detected by the transcriptional analysis. Our results confirm the unusual metabolic choices of frataxin-deficient cells concerning glucose metabolism, which could be relevant to defects in glucose metabolism in FA patients (64,65).

Iron accumulation inside the mitochondria is one of the main phenotypes observed in frataxin-deficient cells and has been described as being responsible for the severe oxidative stress status of these cells due to Fenton chemistry processes (reviewed in 3). In addition, GSH depletion causes a specific defect in the maturation of iron–sulphur proteins and cellular iron accumulation (17). We studied the glutathione-dependent response of wild-type and frataxin-deficient cells to iron-dependent stress conditions.

When cultured in the presence of 100-times excess ferric citrate, total glutathione levels and both specific Gpx and G6PDH activities returned to values similar to those in wild-type cells (Fig. 5). It, therefore, appears that, in the presence of an excess iron, frataxin-deficient cells are able to restore the initial thiol-dependent antioxidant status. However, there were little changes at the transcriptional levels (Table 2). The increase of total glutathione levels cannot be attributed to an increased synthesis, because at least GSH1 and GSH2 transcription was not affected by iron addition (Table 2).

In yeast, high-affinity iron uptake is regulated at the transcriptional level by Aft1. Aft1-dependent activation of the ‘iron regulon’ is constitutive in Δyfh1 mutants such that the high affinity iron transport system of the cell membrane is strongly induced regardless of the iron content of the medium (52). In Δyfh1 mutants, the cytosolic iron concentration is lower than in wild-type cells grown in the same medium, but it accumulates in the mitochondria. Our results suggest that an excess iron in the culture may allow to restore a normal glutathione-dependent redox status, influencing metabolism.

We report the first dynamic study of the specific glutathione-dependent adaptive response to iron-dependent stress conditions. Wild-type and frataxin-deficient cells reacted differently to the stress induced by the addition of iron in the medium (Figs. 6 and 7). For wild-type cells, transfer to an iron-supplemented culture medium was followed by a significant decrease of glutathione levels within 1 min, followed by adaptation and equilibration within 5 min and a return to initial conditions. In parallel, specific Gpx activity increased then returned to the initial values after 10 min. Thus, there was a decrease in total glutathione associated with an increase in Gpx activity, which consumes GSH to form GSSG. In contrast, exposure of mutant cells to excess iron did not affect the glutathione levels or the Gpx and G6PDH activities; excess iron does not appear to act as an additional stress for mutant cells which already accumulate iron inside the mitochondria.

Wild-type cells were also stressed by transfer into iron-depleted conditions. The transfer of the cells to a Bio101 culture medium was followed by an increase of total glutathione levels, without a subsequent return to initial conditions; the cells are thus unable to continue their adaptive response in condition of severe iron deficiency. Once again, the glutathione-dependent redox status of mutant cells was not affected by iron deficiency. Indeed, mutant cells, whereas accumulating iron, might behave like iron-deficient cells. Our findings show that wild-type cells show an acute response to iron-stress conditions due to a change in the redox potential of the cellular environment, whereas mutant cells, which are constitutively stressed and are able to accumulate iron inside the mitochondria, had already responded to long-term iron-excess conditions. As suggested by studies in gsh1 mutants (17) and more recently by Wheeler and Grant (10), it appears likely that the regulation of GSH and iron metabolism is intrinsically linked. Indeed, GSH and glutaredoxin 3 and 4 are involved in iron sensing by the Aft1 transcriptional regulator of iron homeostasis (66,67).

In conclusion, we report evidence of an impairment of glutathione homeostasis in a yeast model of Friedrich’s ataxia, revealing this molecule as an indicator of the redox status of frataxin-deficient cells, in agreement with the role of free radicals in the pathophysiology of the disease. Our findings indicate that glutathione assays could be use as a routine tool to detect the oxidative stress status of frataxin-deficient cells. We also describe a strong link between the glutathione-dependent redox status of the cells and iron pathways, providing new insights into the complicated and controversial relationship between iron and oxidative stress in the study of the disease.

MATERIALS AND METHODS

Yeast strains, media and growth conditions

The S. cerevisiae strains used were S150-2B (MATa, his3Δ1, leu2-3112, trp1-289, ura3-52), S150-2BΔyfh1 (Δyfh1::TRP1),
YPH499 (cycloheximide-resistant, derived from YPH499 (MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, cyh2) and the yfh1 shuffle strain YPH499 [Δyfh1::TRP1 (pRS318-LEU2-CYH2-YFH1)] (68). In this strain, the yfh1 mutation is covered by pRS318, a plasmid containing the CEN and CYH2 and the YFH1 HindIII genomic fragment. The plasmid was removed prior to experiments by counter selection on medium containing 10 μg/ml cycloheximide, which is toxic in the presence of the CYH2 allele, under anaerobic conditions. The resulting mutant was named YPH499Δyfh1.

Unless otherwise stated, cells were grown at 30°C in minimal YNB (yeast nitrogen base, Bio 101, Inc., 2% D-glucose) plus the required amino acids. Other media used were complete YPD (1% yeast extract, 2% bacteriological peptone and 2% D-glucose) and YPR (1% yeast extract, 1% bacteriological peptone, 2% raffinose and 0.2% glucose). Minimal YNB (yeast nitrogen base without copper and iron, Bio 101, Inc., 2% glucose) medium supplemented with the required amino acids and 4 μM copper sulphate was used for cultures in iron-deficient medium. Iron-rich medium was supplemented with 100 μM iron citrate. For growth medium shift experiments, yeast strains were cultured to early log phase and the cells were collected by filtration. Cells were collected from the culture media at various times after resuspension in the new medium.

Preparation of crude cell homogenates and measurement of protein content

Yeast cells were cultured until reaching an OD600nm~0.7 and harvested by centrifugation. The pellets were resuspended in 50 mM potassium phosphate buffer pH 7.8 in the presence of protease inhibitors and the cells were disrupted using glass beads, and centrifuged for 30 min at 5000 g; and the supernatant was used as the crude cell extract.

Protein content of crude cell homogenates or isolated mitochondria was determined using the BCA (bicinchoninic acid) assay (69) and enzyme activities and thiol contents are reported in units/mg protein.

Isolation of yeast mitochondria

Mitochondria were isolated as described by Gasser and Schatz (70) with modifications. Yeast cells were cultured in 1.5 l of YPR medium and harvested by centrifugation. They were then incubated with 2 mg of Zymolyase-100T (MD) per gram of cells in spheroplast buffer (1.2 M sorbitol, 50 mM Tris pH 7.5, 10 mM DTT) at 30°C for 60 min. The spheroplasts were washed twice with the spheroplast buffer without DTT, suspended in ice-cold homogenization buffer (20 mM Hepes-KOH pH 7.4, 0.6 M sorbitol) to a concentration of 0.8 g of cells/ml and then homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, USA). The homogenate (45 ml) was diluted with an equal volume of the homogenization buffer and centrifuged at 1500 g for 5 min at 4°C. The supernatant was saved and the pellet was homogenized in 40 ml of homogenization buffer, and re-centrifuged as above. The two supernatants were combined and centrifuged again as before to remove residual cell debris. Mitochondria were collected from the supernatant by centrifugation (10 min, 9600 g, 4°C) and resuspended in a small volume (1–2 ml) of homogenization buffer.

Measurement of total reduced thiol content

Thiol content was determined by spectrophotometric quantification of the conversion of 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) into 5-thio-2-nitrobenzoic acid (TNB) at 412 nm (71). Standard curves were obtained using various GSH and/or cysteine concentrations. All data points in the figures and the values listed, expressed as nmol of total reduced thiols/mg of protein, are means of at least three independent determinations.

Determination of glutathione levels (GSH + GSSG)

Glutathione levels were determined using a modified procedure of the recycling enzymatic assay described by Tietze (72). Yeast strains were grown in minimum medium and cells were harvested at the appropriate growth phase by centrifugation. Extracellular glutathione was measured directly in the resulting supernatant. For the estimation of total intracellular glutathione, cell pellets were washed and resuspended in 50 mM potassium phosphate buffer pH 7.8 containing ice-cold 5% 5-sulphosalicylic acid, and broken with glass beads. The resulting mixture was clarified by centrifugation (30 min, 5000 g, 4°C) and the supernatant was used to determine total free glutathione. A typical reaction mixture contained the cell extract, 20 mM DTNB and 10 mM NADPH in 50 mM potassium phosphate buffer pH 7.8. The reaction was started by addition of glutathione reductase (1.5 units/ml) and the kinetics of conversion of DTNB into TNB were followed spectrophotometrically at 412 nm. Glutathione concentrations were calculated from standard curves obtained with various GSH and GSSG concentrations, using the rates of TNB formation, and are expressed as nmol of glutathione/mg of protein. All data points in the figures and the values listed are means of at least three determinations, and Student’s t-test was used to identify significant differences. For quantification of oxidized glutathione (GSSG), samples (including GSSG standards) were pre-treated with 5% (vol/vol) 2-vinylpyridine for 1 h at room temperature before analysis.

Measurement of glutathione peroxidase activity

Glutathione peroxidase (Gpx) activity in cell extracts and isolated mitochondria was assayed as previously described (40,41,43) with modifications, using tert-butyl hydroperoxide (t-BHP) as a substrate for the peroxidase. The assay was performed in 50 mM potassium phosphate buffer pH 7.0 containing 10 mM reduced GSH, 200 μM NADPH and 4 mM t-BHP. The reaction was started by addition of glutathione reductase to a final concentration of 0.45 units/ml, and the kinetics of NADPH oxidation were followed spectrophotometrically at 340 nm. Specific glutathione peroxidase activity was then calculated using a millimolar absorption coefficient ε340nm = 6200 M⁻¹ cm⁻¹. One unit of glutathione peroxidase activity was defined as the amount of enzyme which catalyses oxidation of 1 μmole of GSH into GSSG per minute. All data points in the figures and the values listed are means of three
or more determinations and all results are reported relative to the protein concentration of the cell extract.

Measurement of glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase (G6PD) activity was determined as described in references (73,74). G6PDH catalyses the conversion of glucose-6-phosphate to 6-phosphogluconolactone, which is rapidly hydrolyzed to 6-phosphogluconate, the substrate for 6-phosphogluconate dehydrogenase (6PGD), the second enzyme of the pentose phosphate pathway. Because 6PGD also produces NADPH, both 6PGD and total dehydrogenase activities (G6PD+6PGD) were measured separately as described by Tian et al. (74), to determine enzyme activity accurately. The conversion of NADP$^+$ to NADPH catalysed by the two dehydrogenase enzymes was measured spectrophotometrically by following the increase of absorbance at 340 nm due to the conversion of NADP$^+$ to NADPH. 6PGD enzymatic activity was measured first in a reaction mixture containing 50 mM potassium phosphate buffer pH 7.8 (containing 1 mM MgCl$_2$, 0.2 mM NADP$^+$ and 0.4 mM 6-phosphogluconate). The kinetics of NADPH production by the 6PGD reaction were measured, and glucose-6-phosphate (the specific substrate for G6PD) was then added to the cuvette to 1 mM final concentration, resulting in an increase of the rate of production of NADPH, corresponding to the total dehydrogenase activity. G6PD activity was then calculated by subtracting the activity of 6PGD from total dehydrogenase activity and the results are expressed as nmol of NADPH/mg protein.

Nucleotides extraction

Acid extraction was used for oxidized nicotinamide nucleotides (NADP$^+$) and alkaline extraction for reduced forms (NADPH), using a procedure modified from references (75,76). After protein extraction with 50% TCA and centrifugation for 5 min (6000 g), the resulting supernatants were treated either with 0.5 M HClO$_4$ (35% v/v in ethanol) for acid extraction, or with 0.5 M KOH (50% in ethanol) for alkaline extraction. The samples were homogenized, incubated for 10 min on ice and centrifuged for 15 min at 4°C (5000 g). For maximal recovery of reduced pyridine nucleotides, alkaline extracts were incubated for a further 10 min at 60°C, and then allowed to cool for 10 min in ice, as previously described in Klein et al. (75). Before assaying the nucleotide extracts, the supernatants were neutralized to pH 7.0 by carefully adding 10 N HCl to the alkaline extracts, and 1 M KOH to acid extracts, and thoroughly vortexing. The neutralized extracts were then kept at 0°C for at least 10 min before centrifugation for 5 min at 5000 g. The resulting samples were then injected on the same day into the HPLC system.

HPLC analysis of NADPH/NADP$^+$ pools

Nicotinamide nucleotides were analysed by Reverse Phase-HPLC (Dionex HPLC system interfaced with the Millenium software). Samples were injected onto a Kromasil ODS2 C18 column (length = 250 nm, internal diameter = 4 mm, particle size = 5 μm) (Interchim) at room temperature. The mobile phase used for the separation of these nucleotides consisted of two eluants: solvent A was 10 mM ammonium acetate buffer (pH 6.0) and solvent B was methanol. Compounds were separated by the following discontinuous gradient at a flow rate of 0.8 ml/min: a linear increase in solvent B to 25% over 20 min, followed by an increase to 30% in the next 2 min, and stable at 30% for 4 min; this was followed by a decrease from 30 to 0% over the next minute, and the initial conditions were then maintained for 20 min. The products were monitored spectrophotometrically at 260 and 340 nm and quantified by integration of the peak absorbance area, employing a calibration curve established with various known concentrations of NADPH and NADP$^+$.

Iron accumulation measurements

Iron accumulation by the cells was measured in microtitre plates, after growing the cells overnight in minimum medium with 1 to 100 μM $^{55}$Fe(II)-citrate (86 mCi/mg), as described previously (77).

RNA isolation and real-time quantitative PCR analysis

Total RNA was extracted from cells cultured in YNB media to an OD$_{600nm}$~0.7 using the hot phenol method as described previously (78) and purified using QIAIgen columns (RNeasy kit). cDNA was synthesized from 500 ng of RNA, using oligo-p(dT)$_{15}$ primer and the AMV reverse transcriptase according to the kit manufacturer’s instructions (First Strand cDNA Synthesis Kit for RT–PCR, Roche Diagnostics). PCRs were performed on a LightCycler® 480 System (Roche Diagnostics). PCRs were performed on a LightCycler® 480 System (Roche Diagnostics) in 384-well plates. Each reaction was carried out in 10 μl with 2 μl of cDNA from a 10X dilution of the RT reaction (equivalent to 5 ng of reverse transcribed RNA), 250 or 350 nM primer concentration depending on the primer pair and 0.8X Absolute™ QPCR SYBR® Green Mix (ABgene Inc.). Serial dilutions of genomic DNA (250–0.08 ng) were used to generate a quantitative PCR standard curve. The LightCycler protocol was: 15 min of 95°C hot-start enzyme activation; 40 cycles of 95°C denaturation for 15 s, 60°C annealing for 25 s and 72°C elongation for 10 s; and melting at 95°C for 5s, 70°C for 60 s, and then heating to 95°C. Water was used as the template for negative control amplifications included with each PCR run. All reactions were performed in duplicate in each 384 well plate. Data were analysed using the Roche LightCycler® 480 software and CP was calculated by the Second Derivate Maximum Method. The amount of the target mRNA was examined and CP was calculated by the Second Derivate Maximum Method. The relative expression ratio of a target gene was calculated as described by Pfaffl (79), based on real-time PCR efficiencies. Primer pairs are listed in Table 2 and were designed with Primer3 software to generate products of 82 to 150 bp. Results reported were obtained from at least three biological replicates and PCR runs were repeated at least twice.

Conflict of Interest statement. None declared.
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