The phenotypic alterations of the mutant strain are observed with severe impairment of the mitochondrial respiratory chain. The mutant strain accumulates the peripheral arm of complex I and depicts decreased levels of complexes III and IV, consistent with severe impairment of the mitochondrial respiratory chain. The phenotypic alterations of the mutant strain are observed at the permissive growth temperature and exacerbated upon increase of the temperature. Surprisingly, glucose-6-phosphate dehydrogenase activities were similar in the wild-type and mutant strains, whereas mitochondrial activities for succinate dehydrogenase and alternative NADH dehydrogenases were increased in the mutant strain, suggesting that the VARS<sup>−/−</sup> mutation does not affect overall cytosolic protein synthesis. Expression of the wild-type vars gene rescues all of the mutant phenotypes, indicating that the VARS<sup>−/−</sup> mutation is a loss-of-function mutation that results in a combined respiratory chain deficiency.

Key words: aminoacyl-tRNA synthetase, mitochondrial, Neurospora crassa, oxidative phosphorylation (OXPHOS) complex, respiratory chain.

INTRODUCTION

Mitochondria are vital organelles that provide energy to the cells mainly through OXPHOS (oxidative phosphorylation). Indeed a myriad of human pathologies have been associated with mitochondrial dysfunction. OXPHOS comprises four multi-subunit enzyme complexes (I–IV) that catalyse the reduction of oxygen with the concomitant formation of a proton gradient subsequently used by complex V to generate ATP [1]. Biogenesis of the OXPHOS system involves the co-ordinated expression of mitochondrial- and nuclear-encoded subunits, mitochondrial import of nuclear-encoded polypeptides and their assembly into mature complexes. Among the plethora of proteins required for proper functioning of the OXPHOS system are those belonging to the mitochondrial translation machinery. In mammals, 22 tRNAs and two rRNAs of mitochondrial origin, along with more than 100 proteins of nuclear origin allow the synthesis of 13 OXPHOS polypeptides encoded by the mtDNA (mitochondrial DNA).

Thus it comes as no surprise that defects in proteins required for mitochondrial biogenesis, dynamics or function will lead to defects in OXPHOS and, consequently, to the development of highly heterogeneous diseases, namely metabolic disorders and neurological diseases such as Leigh Syndrome [2–4].

Among the mitochondrial translation machinery components are ARSs (aminoacyl-tRNA synthetases) that serve an essential role in protein synthesis and are thus ubiquitously expressed from bacteria to humans [5]. Accurate recognition of amino acids and tRNAs by these enzymes is vital for the fidelity of protein synthesis, and indeed at least one ARS is known for each amino acid. More so, many ARSs display editing activities to correct aminocacylation errors, thus preventing mistranslation or genetic code ambiguity [6,7].

In humans, three ARSs are bifunctional, charging tRNAs in both the cytosol and mitochondria, being essential for protein synthesis in both places [8]. Interestingly, whereas few mutations have been described for cytosolic ARSs, there have been increasing reports of mutations in mitochondrial ARSs [9].

Mutations in the GARS (glycyl-tRNA synthetase), KARS (lysyl-tRNA synthetase), AARS (alaninyl-tRNA synthetase) and YARS (tyrosyl-tRNA synthetase) tRNA synthetases have been reported in autosomal-dominant forms of the neurological Charcot–Marie–Tooth disease, although without evidence for mitochondrial involvement [8,10–12]. In contrast, mutations in SARS2 (serinyl-tRNA synthetase 2, mitochondrial) [13], RARS2 (argininyl-tRNA synthetase 2, mitochondrial) [14], HARS2 (histidyl-tRNA synthetase 2, mitochondrial) [15] and DARS2 (asparagyl-tRNA synthetase 2, mitochondrial) [16] have been associated with a number of early-onset autosomal-recessive diseases with variable defects in mitochondrial protein synthesis and OXPHOS enzymes.

In fungi, including Neurospora crassa and Saccharomyces cerevisiae, mutations in these enzymes have also been described associated with temperature-sensitive phenotypes and defective protein synthesis [17,18]. In Neurospora, temperature-sensitive mutations have been described for a variety of pathways [19] involving mitochondrial ribosome assembly [20] and mitochondrial import proteins [21], as well as mitochondrial ARSs [17,22], among others.

Abbreviations used: AARS, alanyl-tRNA synthetase; ACOX, alternative oxidase; ARS, aminoacyl-tRNA synthetase; BN, Blue Native; CCHL, cytochrome c haem lyase; GARS, glycyl-tRNA synthetase; HARS, hexaaminneruthenium; KARS, lysyl-tRNA synthetase; OXPHOS, oxidative phosphorylation; RIS, Rieske iron–sulfur; TMPD, N,N,N,N-tetramethyl-p-phenylenediamine; VARS, valyl-tRNA synthetase; WT, wild-type; YARS, tyrosyl-tRNA synthetase.

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The filamentous fungus *N. crassa* presents a highly branched respiratory chain with several alternative enzymes capable of either oxidizing NADH or reducing oxygen, bypassing the canonical respiratory chain complexes. Alternative NAD(P)H dehydrogenases are single polypeptide enzymes that oxidize cytosolic (external enzymes) or matrix (internal enzyme) NAD(P)H in a rotenone-insensitive manner [23]. The AOX (alternative oxidase), a potassium-cyanelle-insensitive enzyme that transfers electrons directly from ubiquinol to oxygen, is induced by stresses that compromise the canonical respiratory chain [24].

In the present paper, we report a point mutation in the bifunctional VARS (valyl-tRNA synthetase) of *N. crassa*, which leads to combined respiratory chain deficiency associated with a temperature-sensitive phenotype. Characterization of a mutant strain harbouring the aspartate-to-glycine substitution revealed a temperature-sensitive phenotype. Characterization of a mutant leads to combined respiratory chain deficiency associated with the mutant. This mutation alters a conserved aspartate residue sequenced, revealing an A-to-G transition at nucleotide 2328 in amplified from the wild-type and mutant strain, and subsequently that the altered gene is located in linkage group I close to the locus temperature-sensitive mutation, the mutated gene could be allelic to a previously characterized parental mutant strain. This observation raised the possibility that between mutant and WT crosses yielded Mendelian segregation that it was not the expected strain. However, genotype analysis of the strain by PCR revealed heterokaryotic complex IV mutant (FGSC#13677) and the WT IV mutants was isolated from the progeny of a cross between a A mutant strain displaying phenotypic characteristics of complex –

**EXPERIMENTAL**

**N. crassa** strains and manipulations

The *N. crassa* WT (wild-type) 74-OR23-1A and 74-OR8-1a strains were grown and handled according to standard procedures [25]. The strains FGSC#13677a (cox-4), FGSC#4278A (cyt-20) and FGSC#6103A (his-3) were obtained from the FGSC (Fungal Genetics Stock Center) [26]. Double mutants were obtained through genetic crosses as described previously [27]. *N. crassa* transformation was carried out according to standard procedures (FGSC; http://www.fgsc.net/neurosporaprotoocols/How%20to%20Prepare%20Spheroplasts.pdf).

**Isolation of the vars0−6 mutant**

A mutant strain displaying phenotypic characteristics of complex IV mutants was isolated from the progeny of a cross between a heterokaryotic complex IV mutant (FGSC#13677) and the WT strain. However, genotype analysis of the strain by PCR revealed that it was not the expected cox-4 mutation. Progeny of crosses between mutant and WT crosses yielded Mendelian segregation of the mutant phenotype, associated with the mating type of the parental mutant strain. This observation raised the possibility that the mutated gene could be allelic to a previously characterized temperature-sensitive mutation, cyt-20[17], which is linked to the mating type locus. Indeed, failure to obtain WT progeny from a cross between the mutant and cyt-20 (Supplementary Figure S1 at http://www.BiochemJ.org/bj/448/bj4480297add.htm) indicated that the altered gene is located in linkage group I close to the mating type and cyt-20 loci. The NCU01965 gene (cyt-20) was amplified from the wild-type and mutant strain, and subsequently sequenced, revealing an A-to-G transition at nucleotide 2328 in the mutant. This mutation alters a conserved aspartate residue to glycine in the VARS protein and, as such, the mutant was designated vars0−6.

**Protein synthesis assay**

Protein synthesis in *N. crassa* cultures was performed as described previously [28]. Briefly, cells grown overnight were labelled for 30 min in the absence or presence of cycloheximide to inhibit cytosolic protein synthesis, with 23.4 μCi/50 ml [35S]methionine and [35S]cysteine (EXPRE[S]S[S] Protein Labeling Mix, PerkinElmer). Upon labelling, cells were fractionated into mitochondria and post-mitochondrial supernatants. Equal amounts of protein were separated by SDS/PAGE or BN-PAGE, transferred on to a membrane and radioactive proteins were subsequently detected by fluorography upon exposure to a film for several days. To confirm equal loading of proteins the membranes were stained with Ponceau S and scanned.

**Oxygen consumption and enzymatic activities**

Preparation of *N. crassa* mitochondria was carried out as described previously [29]. Oxygen uptake in mitochondria was measured polarographically at 25°C with a Clark-type oxygen electrode (Hansatech) in a total volume of 1 ml of reaction medium containing 0.3 M sucrose, 10 mM potassium phosphate (pH 7.2), 5 mM MgCl2, 1 mM EGTA, 10 mM KCl, 4 μM carbonyl cyanide m-chlorophenylhydrazone, 0.02% BSA and 0.25–0.5 mg of protein. The assays were started with the addition of 1 mM NADH, 10 mM malate plus 10 mM pyruvate, 5 mM succinate or 2 mM ascorbate plus 0.5 mM TMPD (N,N,N,N-tetramethyl-p-phenylene-diamine). When assaying mutant mitochondria, 5 GMP was added to the reaction mixture at a final concentration of 1 μM [30]. Rotenone, antimycin A, n-propyl gallate and potassium cyanide were added to final concentrations of 15 μM, 0.4 μM, 50 μM and 1 mM respectively. NADH:HR (hexaammineruthenium) III reductase activity was measured photometrically (ε340 = 6.22 M–1 cm–1) in 50 mM Tris/HisCl (pH 8.0) in the presence of 1 mM potassium cyanide, 120 μM NADH and 2 mM HAR.

**Electrophoretic techniques**

Mitochondria were thawed on ice and centrifuged at 10000 g for 10 min. The pellet was suspended in the solubilization buffer containing 50 mM NaCl, 50 mM imidazole/HisCl (pH 7.0), 10% glycerol and 5 mM 6-aminocaproic acid. Mitochondria were solubilized with digitonin using a 4 g/g detergent/protein ratio by adding a freshly prepared 10% detergent solution. The samples were incubated for 30 min on ice followed by centrifugation at 10000 g for 30 min. Each lane was loaded with the extract from mitochondria containing 150 μg of protein before solubilization. For BN-PAGE, linear 4–13% gradient gels overlaid with a 3% stacking gel were used. Lanes from the first-dimension BN-PAGE were excised and resolved in a second-dimension SDS/PAGE (12% gel) upon incubation in a 1% SDS plus 1% mercaptoethanol solution for 2 h at room temperature (22°C)[31]. In-gel activity assays were performed as described elsewhere [32].

**Rescue of the vars0−6 mutant phenotype**

The vars gene was amplified by PCR from genomic DNA using the specific primers 5’-GAGGCCGCTTATAAACAATCA-3’ and 5’-CGTACTACCAACCTCTGAGC-3’. The 3.8 kb PCR product was cloned into pCRII TOPO, excised from the recombinant vector with EcoRI, blunt-ended with Klenow and cloned into the Smal site of pMF272 (FGSC), which contains sequences designed to promote integration of cloned DNA at the his-3 locus of *N. crassa*. The vars mutant containing the his-3 mutation (vars0−6 his-3 strain) was transformed with the plasmid, and resulting transformants were selected for integration at the his-3 locus by plating on medium lacking histidine. Colonies were picked and purified by two rounds of selection on medium lacking histidine. Several transformants were examined by spot growth, BN-PAGE
Valyl tRNA synthetase deficiency

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Figure 1 Identification of a mutation in VARS catalytic domain

Top panel: schematic representation of VARS protein domains. The predicted mitochondrial pre-sequence (MPS) and mutations described in N. crassa are shown. Bottom panel: protein sequence alignment of VARS orthologues. Identical amino acid residues present in at least eight sequences are shown on a grey background. The amino acid number is illustrated for the N. crassa VARS protein and the arrow indicates the site of the D750G mutation. The GenBank® accession numbers for the proteins are: BAE78255.1 for Escherichia coli (Ec), P28350.1 for N. crassa (Nc), XP_747286.1 for Aspergillus fumigatus (Af), P07806.2 for S. cerevisiae (Sc), P07806.2 for S. cerevisiae (Sc), EEO46054.1 for Candida albicans (Ca), AAW41811.1 for Cryptococcus neoformans (Cn), AEE29190.1 for Arabidopsis thaliana (At), NP_720346.3 for Mus musculus (Mm), NP_001161206.1 for Homo sapiens (Hs), CCI64076.1 for Caenorhabditis elegans (Ce) and 1GAX-A for Thermus thermophilus (Tt).

and SDS/PAGE. One strain that complemented all of the varsD−G phenotypes was designated as the rescued varsD−G strain.

Miscellaneous techniques

PCR and general cloning procedures were carried out according to standard protocols [33]. The techniques used for protein determination [34], SDS/PAGE [35], blotting and incubation of blots with antisera [36], detection of anti-rabbit alkaline-phosphatase-conjugated secondary antibodies [37] and glucose-6-phosphate dehydrogenase activity determination [38] were performed as described previously. The antibody against human VARS was a gift from Aviva Systems Biology (ARP46151_P050). Visible absorption spectra of mitochondrial cytochromes and their concentration were determined as described by Bertrand and Pittenger [39]. The absorption coefficients used were \( \varepsilon_{aa} = 16 \text{ mM}^{-1} \cdot \text{cm}^{-1} \), \( \varepsilon_{bb} = 20 \text{ mM}^{-1} \cdot \text{cm}^{-1} \) and \( \varepsilon_{cc} = 19 \text{ mM}^{-1} \cdot \text{cm}^{-1} \). Statistical analysis of cytochrome content was performed using SPSS 13.0 using the non-parametric Mann–Whitney test, for comparisons between two groups. \( P < 0.001 \) were considered statistically significant.

RESULTS

The VARS mutant varsD−G is temperature-sensitive

A mutant strain depicting a slow-growth phenotype, with a mutation putatively allelic to the previously characterized temperature-sensitive mutation cyt-20, was isolated as described in the Experimental section. The NCU01965 gene (cyt-20) was amplified from the WT and mutant strains and subsequently sequenced, revealing an A-to-G transition at nucleotide 2328 in the mutant. This mutation leads to an alteration of a highly conserved aspartate residue to glycine in the bifunctional VARS. This enzyme belongs to class I of tRNA synthetases that display an active domain and an anticodon recognition domain, and a known ability to correct misactivated non-cognate amino acids through their editing activity. The D750G mutation is located at the end of the catalytic domain of VARS in a highly conserved region, as shown in Figure 1.

The mutant strain, designated varsD−G, depicted slower growth than the WT in liquid medium, but produced as much conidia as the WT (in solid medium). Although depicting slower growth at 26°C, the mutant strain was unable to grow at 37°C. The WT grew similarly at both temperatures, indicating that varsD−G is a temperature-sensitive mutant (Figure 2). To determine whether varsD−G displayed an overall sensitivity to stress, we analysed its growth response to several oxidative stress conditions. For this purpose, serial dilutions of conidial suspensions were spotted on to paraquat-, phytosphingosine-, staurosporine- and H2O2-containing media. Aside from an increased resistance to H2O2 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/448/bj4480297add.htm), the varsD−G mutant ability to withstand all other sources of oxidative stress was similar to that of the WT, in contrast with the observed temperature sensitivity.

Deficiency of the canonical respiratory chain function in varsD−G

In N. crassa the vars gene encodes a bifunctional VARS protein and thus we expected the mutant varsD−G to display both mitochondrial and cytosolic deficiencies. We monitored
Figure 2  *vars*D<sup>−</sup>G is temperature-sensitive

Approximately 300 conidia of the indicated strains were plated in GFS medium and incubated for 4 days at either 26 or 37 °C.

Figure 3  Steady-state levels of VARS are decreased in the *vars*D<sup>−</sup>G mutant

The WT, *vars*D<sup>−</sup>G and rescued *vars*D<sup>−</sup>G strains were grown at 26 °C for 16 h followed by 3 h incubation at either 26 °C or 37 °C. Mitochondria and post-mitochondrial supernatants (PMS) isolated from these strains were separated by SDS/PAGE and analysed by Western blotting using antibodies raised against human VARS (ARP46151_P050, Aviva Systems Biology). The mitochondrial 13.4L and the cytosolic FKBP22 (FK506-binding protein 22) proteins were used as loading controls.

VARS levels in mitochondria and post-mitochondrial supernatants isolated from WT and *vars*D<sup>−</sup>G and found that the steady-state levels were decreased in the mutant, although the dual localization did not shift. As expected, the decrease was more evident upon incubation at the restrictive temperature (Figure 3).

To assess the effects of decreased VARS in mitochondrial and cytosolic protein synthesis, metabolic labelling studies were performed with the different strains. As shown in Figure 4, a decrease in mitochondrial protein synthesis is clearly observed in *vars*D<sup>−</sup>G when compared with the WT (mitochondrial proteins with cycloheximide, Figure 4A), whereas the nuclear-encoded mitochondrial proteins appeared only slightly decreased in *vars*D<sup>−</sup>G. Overall cytosolic protein synthesis was not affected in *vars*D<sup>−</sup>G despite a number of bands depicting a decreased signal in the mutant strain (Figure 4).

To evaluate the functionality of the mitochondrial respiratory chain in the mutant strain, we first analysed its cytochrome content under growth at the permissive temperature. The *vars*D<sup>−</sup>G mutant depicted a significant decrease in cytochromes aa<sub>3</sub> and cytochrome b in comparison with the WT, whereas an increase in cytochrome c content was observed (Figure 5). These results...
indicate a disruption of the mitochondrial respiratory chain and suggest decreases in complexes III and IV.

To assess the effects of var mutations in OXPHOS, we measured oxygen consumption rates by WT and var<sup>D−G</sup> intact mitochondria. Under pyruvate/malate respiration, mutant mitochondria exhibited a decrease in rotenone-sensitive oxygen consumption, albeit depicting an increased respiratory rate when compared with the WT. This suggested that var<sup>D−G</sup> mitochondria display a specific decrease in complex I activity and an increase in the internal alternative dehydrogenase enzyme. Complex I deficiency was further evaluated by measuring NADH:Har III reductase activity, confirming a decreased amount of complex I in var<sup>D−G</sup> mitochondria. Moreover, var<sup>D−G</sup> mitochondria presented increased activity of the external alternative dehydrogenases, as determined through NADH oxidation rates (Table 1).

We next evaluated cytochrome <i>c</i> oxidase specific activity using ascorbate/TMPD as a substrate and found it to be diminished in var<sup>D−G</sup>, which corroborates its lower cytochrome <i>a</i><sub>3</sub> content. More so, the respiratory activities of var<sup>D−G</sup> mitochondria could only be completely inhibited upon combined addition of potassium cyanide (complex IV inhibitor) and propylgallate (AOX inhibitor), in contrast with WT activities that were fully inhibited by potassium cyanide, indicating an induction of the AOX in var<sup>D−G</sup>.

Interestingly, the oxygen consumption rates in the presence of the complex II substrate succinate were increased in the mutant strain when compared with WT (Table 1), suggesting that this fully nuclear-encoded respiratory complex is not affected in the mutant. Given that VARS is a bifunctional enzyme in <i>Neurospora</i>, we found it appropriate to investigate whether the mutation affects other cytosolic activities. For this purpose, we measured the activity of glucose-6-phosphate dehydrogenase and found that both the WT and the var<sup>D−G</sup> mutant presented similar glucose-6-phosphate dehydrogenase activity rates (Table 1).

Overall, these results indicate a combined deficiency of the canonical mitochondrial respiratory chain due to the VARS<sup>D−G</sup> mutation. Accordingly, we postulate that the observed induction of the alternative respiratory chain in the var<sup>D−G</sup> mutant could occur as a compensatory response to a deficiency in canonical respiration.

### Table 1 Enzymatic activities of mitochondria from the var<sup>D−G</sup> mutant strain

<table>
<thead>
<tr>
<th>Assay</th>
<th>WT</th>
<th>var&lt;sup&gt;D−G&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>NADH:Har (arbitrary units)</td>
<td>5.21 ± 0.95</td>
<td>2.88 ± 0.92</td>
</tr>
<tr>
<td>Malate:O&lt;sub&gt;2&lt;/sub&gt; (nmol of O&lt;sub&gt;2&lt;/sub&gt;/min per mg)</td>
<td>20.1 ± 7.4 (48.7%)</td>
<td>37.3 ± 8.6 (7.8%)</td>
</tr>
<tr>
<td>NADH oxidase (nmol of O&lt;sub&gt;2&lt;/sub&gt;/min per mg)</td>
<td>84.8 ± 19.3</td>
<td>163.9 ± 65.2</td>
</tr>
<tr>
<td>Ascorbate/TMPD:O&lt;sub&gt;2&lt;/sub&gt; (nmol of O&lt;sub&gt;2&lt;/sub&gt;/min per mg)</td>
<td>348.4 ± 66.7</td>
<td>223.0 ± 66.9</td>
</tr>
<tr>
<td>Succinate:O&lt;sub&gt;2&lt;/sub&gt; (nmol of O&lt;sub&gt;2&lt;/sub&gt;/min per mg)</td>
<td>24.0 ± 6.6</td>
<td>52.2 ± 11.5</td>
</tr>
<tr>
<td>G-6-P dehydrogenase (units/mg)</td>
<td>0.120 ± 0.006</td>
<td>0.122 ± 0.015</td>
</tr>
</tbody>
</table>

Of mitochondrial proteins. As shown in Figure 6, the steady-state levels of complex I subunits, the complex III RIS (Rieske iron–sulfur) subunit and the complex IV COXIII subunit were decreased in the mutant strain when compared with the WT. On the other hand, the F1β subunit of complex V and the CCHL (cytochrome <i>c</i> haem lyase) proteins were both increased in the var<sup>D−G</sup>. The AOX is highly induced in var<sup>D−G</sup> and only faintly visible in WT mitochondria (Figure 6), in agreement with the respiratory activities described above.

Our data indicates that the VARS<sup>D−G</sup> mutation interferes with the steady-state levels of mitochondrial proteins that do not correlate with a reduction in protein synthesis.

**Figure 6** Altered steady-state levels of mitochondrial proteins in var<sup>D−G</sup>

Mitochondria isolated from WT and var<sup>D−G</sup> were loaded in different amounts as indicated (μg), separated by SDS/PAGE and analysed by Western blotting. Antisera against the following proteins were used: complex I peripheral arm subunits 78 kDa, 29.9 kDa, 30.4 kDa, 21.3 kDa and 21.3a kDa, and membrane arm subunits 20.9 kDa, 14 kDa and 12.3 kDa, the RIS subunit of complex III, the COXIII subunit of complex IV, the F1β complex V subunit, the CCHL and the AOX.

**vars<sup>D−G</sup> depicts hampered assembly of respiratory chain complexes**

Having established that the VARS<sup>D−G</sup> mutation affects respiratory chain activity we next set out to investigate their effects on the steady-state levels of the OXPHOS complexes. For this, mitochondria were solubilized with digitonin, resolved by BN-PAGE and stained for either NADH dehydrogenase or cytochrome <i>c</i> oxidase (Figure 7). Steady-state levels of complex I were found to be reduced in the var<sup>D−G</sup> mutant when compared with the WT, as determined by the NADH:NBT (Nitro Blue Tetrazolium) in-gel activity (Figure 6B). Strikingly, we detected an accumulation of the peripheral arm intermediate of complex I in the mutant strain, which also displays NADH oxidation activity and is not detectable in WT mitochondria (Figure 7B). In accordance with the observed reduced steady-state levels of RIS and COXIII subunits (Figure 6), the amounts of complex III and complex IV were also found to be decreased in the mutant (Figures 7A and 7C). The content of complex V was not significantly altered in var<sup>D−G</sup> when compared with the WT (Figure 7A and Supplementary Figure S3 at http://www.BiochemJ.org/bj/448/bj4480297add.htm), despite
Figure 7  Hampered assembly of the varsD−G mitochondrial respiratory chain

BN-PAGE of digitonin-solubilized mitochondria from the indicated strains was stained with Coomassie Blue (A), for NADH dehydrogenase activity (B) and for cytochrome c oxidase activity (C). Mitochondria from radiolabelled cells in the absence or presence of cycloheximide (CHX) were solubilized with digitonin, separated by BN-PAGE and analysed by fluorography (D). Relevant OXPHOS complexes and supercomplexes are marked on the left-hand side. PA, peripheral arm of complex I.

the fact that two of its structural polypeptides are mitochondrial-encoded.

To determine whether newly synthesized translation products efficiently assembled into respiratory complexes, radiolabelled mitochondria were solubilized with digitonin and subjected to BN-PAGE followed by fluorography (Figure 7D). As observed, we detected a clear impairment in respiratory chain assembly in varsD−G when compared with the WT. In fact, after 30 min of labelling the majority of the respiratory complexes are easily detected in WT mitochondria, whereas varsD−G mitochondria are almost devoid of newly synthesized respiratory complexes, depicting only the complex V dimer. Moreover, in the presence of cycloheximide, some incorporation of the mitochondrial translation products is observed in the mutant strain, suggesting that varsD−G mitochondria accumulate assembly intermediates that can progress in the assembly pathway in the presence of newly synthesized mitochondrial proteins.

In WT mitochondria, the respiratory chain complexes associate into supercomplexes [40,41]. Thus we also evaluated the supramolecular organization of the mitochondrial respiratory chain in varsD−G. Supercomplexes comprising complex I and complex III were detectable in varsD−G mitochondria in lower amounts than in the WT, whereas complex IV-containing supercomplexes were almost absent (Figures 7B and 7C). Decreased amounts of the respiratory chain complexes and supercomplexes were further confirmed through Western blot analysis of BN-PAGE gels (Supplementary Figure S4 at http://www.BiochemJ.org/bj/448/bj4480297add.htm). Further evaluation of the supramolecular organization of the OXPHOS system was accomplished through immunoblotting analysis of second-dimension BN/SDS/PAGE (Figure 8). Complex I, identified with specific antisera against the peripheral arm 21.3c kDa polypeptide and the membrane arm 14 kDa subunit, was found in its monomeric form and associated in supercomplexes in both the WT and the varsD−G mutant strains. However, the steady-state levels of the supercomplexes were significantly lower in the mutant strain. This analysis also confirmed the accumulation of the peripheral arm of complex I in varsD−G as detected using the antibody against the 21.3c kDa subunit, as well as an additional subcomplex containing the 14 kDa subunit of complex I, suggesting that complex I assembly may be disrupted due to a delay in mitochondrial protein synthesis. However, we cannot discard the possibility that the observed subcomplexes of complex I result from instability of the assembled enzyme upon detergent solubilization. A specific antibody against the COREII subunit of complex III detected its supercomplexes in both strains, although also in lower amounts in the mutant strain. Furthermore, as depicted in Figure 8, COREII was detected as a continuous streak of low molecular mass in the varsD−G mutant, suggesting a disturbed assembly or instability of complex III.

The results of the present study indicate that the decreased activity of the canonical respiratory chain is due to defective assembly of the respiratory chain complexes (Table 1).

WT vars gene rescues varsD−G phenotypes

To demonstrate that the phenotypes observed in the varsD−G mutant are indeed caused by the Asp750 to glycine mutation in VARS, we expressed the WT vars gene in the mutant background. For this purpose, a varsD−Ghis−3 double mutant was isolated and subsequently transformed with a recombinant plasmid containing the vars gene under the control of the ccc-1 promoter (see the Experimental section; Supplementary Figure S5 at http://www.BiochemJ.org/bj/448/bj4480297add.htm). Transformants complementing the his−3 mutation were selected.
by varSD so, our data demonstrates that phenotypic deficiencies depicted a loss-of-function rather than a gain-of-function mutation. More mutant strain phenotypes. that expression of the WT VARSD was able to rescue all of the other ARS mutations [42,43], the VARS bundle domain through interactions with the main-chain nitrogen arm and 14 kDa membrane arm) and the complex III (CORE II) subunits as indicated on the left-hand side. Mitochondrial ARSs are emerging as important players in the development of highly heterogeneous human diseases [9] that do not always mimic mitochondrial tRNA defects [44]. Mutations in several human mitochondrial ARSs have indeed been reported to interfere with mitochondrial protein synthesis although most of the studies did not address this in detail [9].

DISCUSSION

Mitochondrial ARSs are emerging as important players in the development of highly heterogeneous human diseases [9] that do not always mimic mitochondrial tRNA defects [44]. Mutations in several human mitochondrial ARSs have indeed been reported to interfere with mitochondrial protein synthesis although most of the studies did not address this in detail [9].

Figure 8 Mitochondrial supercomplexes in the varsD−G mutant

Mitochondrial proteins from WT (A) and the varsD−G mutant (B) were solubilized with digitonin, separated by BN-PAGE and subsequently analysed by two-dimensional BN/SDS/PAGE. The 2D gels were blotted and probed with a mixture of antisera against complex I (21.3c kDa peripheral arm and 14 kDa membrane arm) and the complex III (CORE II) subunits as indicated on the left-hand side.

The expression of the WT vars gene was able to rescue the slower growth and thermosensitive phenotypes of varsD−G (Figure 2). More so, the rescued strain depicted steady-state levels of VARS and mitochondrial proteins comparable with those of the WT strain and no AOX expression (Figure 3 and Supplementary Figure S6 at http://www.BiochemJ.org/bj/448/bj4480297add.htm). The rates of mitochondrial and cytosolic protein syntheses are also comparable with those of the WT strain (Figure 4), rescuing the assembly of the OXPHOS complexes (Figure 7D).

An increase in complexes I, III and IV without accumulation of assembly intermediates could also be observed in the rescued strain upon analysis of the steady-state levels of the OXPHOS system by BN-PAGE gels (Figure 9).

These results indicate that, unlike what has been described for other ARS mutations [42,43], the VARS bundle domain substitution results in a loss-of-function rather than a gain-of-function mutation. More so, our data demonstrates that phenotypic deficiencies depicted by varsD−G can be attributed to a defective VARS protein, given that expression of the WT VARS was able to rescue all of the mutant strain phenotypes.

Figure 9 varsD−G is rescued by expression of the WT vars gene

Mitochondrial proteins from the indicated strains were resolved by BN-PAGE upon digitonin solubilization. The gel was stained for NADH dehydrogenase activity followed by cytochrome c oxidase activity. Three independent rescued varsD−G transformants are depicted. Relevant OXPHOS complexes and supercomplexes are indicated on the left-hand side. PA, peripheral arm of complex I.

To date, mutations in cytoplasmic [10,11], mitochondrial [13–16,45–48] and one bifunctional [8] ARSs have been associated with disease. Initially it appeared that the nervous system was particularly sensitive to the disruption of normal ARS function, as several mutations in ARSs were all associated with central or peripheral neuropathies. However, more recently, additional mutations in mitochondrial ARSs have been associated with distinct phenotypes that included specific mitochondrial deficiencies not yet thoroughly characterized.

In the present paper we report a mutation in the bifunctional VARS protein from N. crassa that leads to a deficiency in the canonical respiratory chain. Specifically, our results indicate that the D750G mutation results in decreased mitochondrial protein synthesis consistent with the observed defects in respiratory chain complex assembly and function, with concomitant accumulation of assembly intermediates. These subcomplexes can either be assembly intermediates or dissociation products that accumulate upon detergent-solubilization of the membranes. These observations are coherent with the fact that the mitochondrial genome codes for structural subunits of complexes I, III, IV and V. The lack of alterations observed in complex V in the varsD−G mutant may be due to the fact that its mitochondrial-encoded subunits, ATP6, ATP8 and ATP9, enclose few valine residues, none of which are encoded by the uncommon mitochondrial codon GUG. The varsD−G mutant, a slow-growing strain with a temperature-sensitive phenotype, displayed defects in mitochondrial respiratory chain activity, even at the permissive temperature. These results suggest that a decrease in VARS bundle domain activity may occur as a result of decreased enzyme stability, which is exacerbated upon increasing the temperature to 37°C. The region of the D750G mutation is highly conserved from prokaryotes to eukaryotes (Figure 1B). An homology model based on the structure of VARS from Thermus thermophilus [49] allowed us to scrutinize the role of D750G and to predict the structural consequences of the mutation. In the model, the side chain of the strictly conserved Asp750 is engaged in N-capping of an 'anticodon-loop binding' helix bundle domain through interactions with the main-chain nitrogen
atoms of Tyr\textsuperscript{895} and Ile\textsuperscript{896}. The substitution of the side chain of Asp\textsuperscript{150} with the shorter glycine side chain will disrupt these interactions and ultimately result in an increased local flexibility and instability of the enzyme. This might result in altered enzyme activity. Moreover, the aspartate residue is located in the so-called stem contact-fold domain of VARS [50], which contacts the phosphate backbone of the tRNA and, as such, alterations in the conformation of this domain could interfere with the positioning of the tRNA at the enzyme-binding sites (Supplementary Figure S7 at http://www.BiochemJ.org/bj/448/bj4480297add.htm).

Despite the described bifunctional activity of VARS, the results of the present study indicate that the VARS\textsuperscript{G–G} mutation does not hinder cytosolic protein synthesis drastically (Figure 4), since the mutant strain displayed: (i) increased activity of complex II, the OXPHOS complex fully encoded in the nucleus, (ii) increased activities of alternative NAD(P)H dehydrogenases and AOX nuclear-encoded enzymes, (iii) normal activity of the cytosolic glucose-6-phosphate dehydrogenase and (iv) accumulation of the peripheral arm of complex I whose subunits are all nuclear-encoded.

The discrepancy of effects observed for cytosolic and mitochondrial VARS\textsuperscript{G–G} may be associated with the well-documented fact that mitochondrial synthetases are less efficient than their cytosolic counterparts [51,52], and thus a decrease in protein function will be more detrimental for mitochondria than to the cytosol. On the other hand, a different N. crassa vars mutant (cyt-20) was reported to depict normal mitochondrial and cytosolic functions despite a 100-fold and a 10-fold decrease in mitochondrial and cytosolic VARS aminoacylation activities respectively. The decrease in VARS activity was aggravated when the strain was grown at 37°C leading to clear defects [17], suggesting that VARS is not a limiting factor in protein synthesis, affecting translation only below a threshold.

We cannot discard the possibility that the different cytosolic and mitochondrial VARS\textsuperscript{G–G} effects result from altered interactions with the cognate tRNA or protein partners, which differ in the two compartments.

To date, no mutations in either cytoplasmic or mitochondrial VARS have been described associated with the development of human disorders. However, expression of an editing-deficient VARS into mammalian cells led to disruption of cell morphology and initiation of an apoptotic response due to amino acid misincorporation [42]. Moreover, mutations in the editing domain of AARSs have previously been described as the cause of several mitochondrial diseases [53,54], suggesting that VARS alterations could also lead to disease development. More so, mutations in mitochondrial tRNA\textsuperscript{G} have been linked to dominant mutations in YARS [10], KARS [12] and AARS [11,54], yet the pathogenic mechanisms remain unclear. In particular, it is still ambiguous whether the mutations result in a pathological gain-of-function, a partial loss-of-function or both. Motley et al. [43] addressed this issue by overexpressing a functional GARS protein in mice models of Charcot–Marie–Tooth disease associated with GARS mutations. They concluded that a predominant gain-of-function is the cause of the peripheral neuropathy in these models since the WT GARS protein was not able to rescue the mice phenotypes [43]. Furthermore, studies in Drosophila with YARS mutations showed that significant loss of aminoacylation activity is neither necessary nor sufficient to cause peripheral neuropathy [58]. Nonetheless, a loss of GARS function was associated with the reduced viability of the mice models, but not with the neurodegeneration effect [43].

The results of the present study suggest a different scenario. Indeed, the VARS\textsuperscript{G–G} mutation leads to a partial loss-of-function that results in deficient protein synthesis and ultimately in mitochondrial respiratory chain dysfunction. More so, all of the defects were rescued by expression of the WT vars gene.

Overall, the results of the present study points to N. crassa as a good model to study disease-causing mutations in the different synthetases as it may provide evidence of their pathogenicity and significant insight into the associated mechanisms of disease. In addition, our results indicate that mutations in mitochondrial ARSs should be considered when combined respiratory chain deficiencies are observed in human diseases.

**AUTHOR CONTRIBUTION**

Margarida Duarte designed and performed the experiments, analysed data and wrote the paper. Arnaldo Videira analysed data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Defective valyl-tRNA synthetase hampers the mitochondrial respiratory chain in *Neurospora crassa*

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**Figure S1** Temperature sensitivity of progeny from the cyt-20×*varsD−G* cross

Progeny spores were activated, plated in GFS medium and incubated either at 26°C for 4 days (A) or at 26°C for 1 day and then shifted to 37°C for 3 days (B). The spores stopped growing immediately after transfer to 37°C, indicating that none of the spores had a WT phenotype.

**Figure S2** *varsD−G* exhibits increased tolerance to H2O2

Serial dilutions of conidial suspensions of WT and mutant strains were spotted in GFS medium containing 2 mM H2O2 as indicated, and incubated at 26°C for 4 days.

**Figure S3** Similar levels of complex V in WT and *varsD−G* mutant

Mitochondria from the indicated strains were solubilized with digitonin and subsequently separated by BN-PAGE. Different lanes were stained with (A) Coomassie Blue, (B) NADH-NBT activity or (C) ATP hydrolase activity (complex V). Relevant OXPHOS complexes and supercomplexes are marked on the left-hand side.

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Figure S4  OXPHOS complexes and supercomplexes in WT and varsD−G mutant

Mitochondria from the indicated strains were solubilized with digitonin, separated by BN-PAGE and transferred on to a PVDF membrane. Different lanes were immunoblotted with antibodies against the (A) 21.3c kDa subunit of complex I, (B) the CORE II subunit of complex III, (C) the COXIII subunit of complex IV or (D) the F1β subunit of complex V. Relevant OXPHOS complexes and supercomplexes are marked on the left- and right-hand sides. PA, peripheral arm of complex I.

Figure S5  varsD−Ghis−3 double mutant

A his−3 strain was crossed with the varsD−G mutant to isolate a double mutant. The indicated strains were streaked in GFS plates containing 200 μg/ml histidine and incubated at 26 °C for 4 days. The slow-growth phenotype of the varsD−Ghis−3 mutant is also shown by the double mutant, but not by the his−3 strain.
Valyl tRNA synthetase deficiency

Figure S6  Steady-state levels of mitochondrial proteins in rescued vars\textsuperscript{-}\textsuperscript{-}G strains

Mitochondria isolated from the indicated strains were separated by SDS/PAGE and analysed by Western blotting using antisera against the complex I subunits 78 kDa and 14 kDa and the AOX. The vars\textsuperscript{-}\textsuperscript{-}Ghis\textsuperscript{-}3 double mutant was used for transformation and three independent transformants are depicted (rescued vars\textsuperscript{-}\textsuperscript{-}G).

Figure S7  Model of N. crassa VARS protein

Cartoon representation of the three-dimensional structure of the fungus VARS in complex with tRNA (blue), showing the localization of the mutated residue described in the present study (ball-and-stick) and its polar interaction network (Glu\textsuperscript{148} and Tyr\textsuperscript{666} shown as sticks). The model was made with the SwissModel server using Thermus thermophilus VARS as the template (PDB code 1GAX).