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Second-site, intragenic alterations in the gene encoding subunit II of cytochrome *c* oxidase from yeast can suppress two different missense mutations

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Abstract Cytochrome *c* oxidase, a multi-subunit enzyme complex, accepts electrons from cytochrome *c* and transfers them to molecular oxygen to form water. Subunit II (Cox2p) of the enzyme complex provides the initial entry site for the electrons from cytochrome *c*. We report here the characterization of a yeast strain bearing a mutation in the gene encoding Cox2p which abolishes the activity of the enzyme complex. The alteration, at residue 163 in the yeast polypeptide, substitutes isoleucine for threonine and leads to loss of Cox2p and loss of the ability to carry out cellular respiration. We have also characterized 55 independent revertants of the mutant which have recovered the ability to respire. Of these revertants, 37 recover the ability to respire due to a compensatory alteration at residue 163, which produces either a wild-type threonine codon or one for valine or serine. The other 18 revertants recover function due to secondary changes at four different codons within the gene encoding Cox2p. Some of these second-site, intragenic revertants occur at sites significantly distant from the position of the original mutation. In addition, alterations at two of these sites have previously been shown to suppress a completely different missense mutation in the gene.

Keywords Cytochrome oxidase · Respiration deficiency · Yeast · Mitochondria

Introduction

Cytochrome *c* oxidase (CcO) is the terminal member of the electron transport chain, responsible for the transfer of electrons from cytochrome *c* (Cc) to molecular oxy-

gen. In addition, the enzyme complex uses the energy of electron transport to establish the proton gradient which drives synthesis of ATP. The enzyme is composed of a variable number of subunits, ranging from two in some bacteria to 13 in the bovine enzyme (Capaldi 1990). In most eukaryotes, the three largest subunits are encoded on mitochondrial DNA (*COX1*, *COX2*, and *COX3*) and form the catalytic core of the enzyme. Subunits I and II (Cox1p and Cox2p) carry all the metal ions associated with electron transfer and are sufficient for both electron transfer and proton pumping (Haltia et al. 1991). Subunit III (Cox3p) may be involved in assembly of the enzyme complex and has recently been shown, in *Paracoccus denitrificans*, to prevent suicide inactivation of the enzyme, by maintaining the structure of the CuB site in subunit I (Haltia et al. 1989; Bratton et al. 1999). X-ray crystal structures for both the bovine enzyme and the enzyme from *P. denitrificans* have been determined (Iwata et al. 1995; Tsukihara et al. 1996; Ostermeier et al. 1997).

Electrons enter the CcO complex through a binuclear copper site (CuA) found in Cox2p. Electrostatic interactions mediate the binding of Cc to Cox2p (Millet et al. 1983; Zhen et al. 1999). From this site of initial entry, electrons pass to other metal centers found in Cox1p, first to heme *a* and then to a dinuclear heme *a*₃/CuB center. Transfer of electrons through Cox1p is coupled to the movement of protons through the enzyme complex (Michel et al. 1998).

Site-specific mutations have been created in the genes for Cox1p and Cox2p in several species of bacteria and in yeast. These studies were directed at understanding the binding of Cc to Cox2p, pathways of proton movement through Cox1p, and the importance of residues which function as ligands to the metal centers in the two subunits (Speno et al. 1995; Mitchell et al. 1996; Zufferey et al. 1998; Wang et al. 1999; Zhen et al. 1999). Site-directed mutagenesis of Cox2p of both the *Rhodobacter sphaeroides* and *P. denitrificans* enzymes has shown that a number of negatively charged carboxylates on Cox2p interact with several positively charged lysine residues in

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Cc to promote binding of the two proteins (Witt et al. 1995; Zhen et al. 1999). The indole ring of a conserved tryptophan (position 143 in the *R. sphaeroides* subunit) mediates the transfer of electrons from Cc to the CuA site (Wang et al. 1999; Zhen et al. 1999). A great deal of new information about the catalytic mechanisms of the enzyme complex has been obtained from the combination of mutagenesis studies and biophysical studies. However, one limitation of analysis of site-specific mutations is that only residues previously implicated as important are selected for mutagenesis.

We have undertaken the analysis of a particular mutant strain of yeast which arose after manganese mutagenesis, rather than being derived by site-directed mutation. The mutation was shown to lie within *COX2*, which encodes for Cox2p in yeast (Weiss-Brummer et al. 1979). We have identified a single nucleotide change which abolishes the function of this protein, due to the alteration of a threonine residue to isoleucine at position 163 in the yeast polypeptide. This residue is immediately adjacent to an aspartic acid residue which is conserved in all of the species we examined. Further, mutation of this conserved aspartic acid to asparagine in the *R. sphaeroides* enzyme significantly affects the interaction of Cox2p with Cc from horse heart and cytochrome c_2 from *R. sphaeroides* (Zhen et al. 1999). In most organisms, valine is found at the position equivalent to T163 in the yeast enzyme. However, threonine is also found next to the conserved aspartic acid in the subunits from *P. denitrificans* and *R. sphaeroides*. In an effort to determine the role played by this particular residue in the protein and to determine whether other amino acids at this position are compatible with function, we isolated a large number of revertants from the T163I mutant yeast strain. We identified a total of 55 intragenic revertants of the T163I mutant, 25 of which restored a threonine codon, either ACT or CTT, while 12 carried a codon for either valine or serine at position 163. A total of 18 other revertants were also identified which carried the original T163I mutation, but which had now sustained a second change in the *COX2* gene that restored function to the polypeptide. These second-site suppressors were found at a number of different positions in Cox2p and many of these sites are somewhat distant from the position of T163 as shown in the X-ray crystal structures (Iwata et al. 1995; Tsukihara et al. 1996; Ostermeier et al. 1997). Wild-type, mutant, and revertant strains were characterized with respect to their growth rates on non-fermentable carbon sources, their CcO activity, and the amount of Cox2p which they produced.

Materials and methods

Strains and media

The strains of *Saccharomyces cerevisiae* used in this study were: 777/3A (*MAT α ade1 pet9* [ρ^+]; Kotylak and Slonimski 1977), m5351 (*MAT α ade1 pet9* [*cox2*]; Weiss-Brummer et al. 1979), and

DL2 ρ^0 (*MAT α lys 2* [ρ^0]; Folley and Fox 1991). The media used were previously described by Mazourek et al. 1999).

Isolation and genetic analysis of revertants

Independent revertants of the mutant strain were selected by first constructing a diploid of m5351 by crossing to DL2 ρ^0 , growing single diploid colonies under non-selective conditions, and then plating on selective medium as previously described by Overholtzer et al. (1996). Localization of suppressor mutations to mitochondrial DNA was accomplished by sporulation of the diploids and determination of the segregation pattern of respiration (Mazourek et al. 1999).

Biochemical and molecular analysis of mutant and revertant strains

Crude extracts of yeast cells were prepared as described by Hoffman and Winston (1987) and the entire *COX2* gene was amplified using the polymerase chain reaction and primers flanking the gene (Wilson and Cameron 1994). Aliquots were isopropanol-precipitated and sequenced using a dsDNA cycle sequencing kit (Life Technologies), using a battery of primers covering the entire *COX2* gene. Cox2p was detected by running cell extracts on 12% Tris-HCl Ready Gels (Biorad), incubation with a monoclonal antibody to Cox2p (4B12-A5; Molecular Probes) at a concentration of 1 μ g/ml, and detection with a Phototope-Star Western blot detection kit (New England Biolabs). CcO activity was measured by isolation of mitochondria (Yaffe 1991) and measurement of the reduction of cytochrome *c* spectrophotometrically (Mason et al. 1973).

Sequence alignment and molecular modeling

A total of 20 Cox2p amino acid sequences were retrieved from GenBank and aligned using CLUSTAL W as provided by the Biology Workbench (<http://workbench.sdsc.edu/>). The sequences aligned are from *S. cerevisiae* (yeast), *Bos taurus* (beef heart), *P. denitrificans*, *Rhodobacter sphaeroides*, *Drosophila melanogaster*, *Albinaria coerulea* (land snail), *Ornithorhynchus anatinus* (duck-billed platypus), *Lumbricus terrestris* (earthworm), *Metridium senile* (sea anemone), *Daphnia pulex*, *Prophyra purpurea* (red alga), *Rattus norvegicus*, *Cyprinus carpio* (common carp), *Ascaris suum* (roundworm), *Aedes aegypti* (mosquito), *Caenorhabditis elegans*, *Gallus gallus* (chicken), *Zea mays*, *Triticum aestivum* (wheat), and *Pisum sativum* (pea). Alignment scores for yeast/*Paracoccus denitrificans* and for yeast/beef heart pairs were carried out using the ALIGN (Meyers and Miller 1988) and LFASTA (Pearson and Lipman 1988) programs, also available at the Biology Workbench site. Coordinates from the Protein Data Base (PDB) were down-loaded and modeled using software obtained from Molecular Images.

Results

Characterization of the *cox2* mutant strain m5351

The respiration-deficient yeast strain m5351 was previously isolated and the genetic defect mapped to the *COX2* gene (Weiss-Brummer et al. 1979). The mutant *cox2* gene from this strain was amplified by the polymerase chain reaction and sequenced as described in Materials and methods. Loss of respiration was shown to be due to a single nucleotide change in codon 163 (ACT to ATT) which resulted in the substitution of an isoleucine codon for a threonine codon. As previously reported, the mutation in this strain resulted in loss of

the cytochrome $a-a_3$ absorption band and no Cox2p was detectable upon gel electrophoresis of labeled mitochondrial proteins (Weiss-Brummer et al. 1979).

Isolation of revertants of m5351

Independent diploid revertants of m5351 were isolated as previously described by Overholtzer et al. (1996), after growth in liquid medium containing glucose and plating the cells on medium containing the non-fermentable carbon sources ethanol and glycerol (YPEG). Portions of each culture were grown at 18 °C and 28 °C and the plates were monitored over a period of 10 days for the appearance of colonies. On any given day, only one colony from each plate was chosen for analysis, in order to insure that independent revertants were analyzed. Of the revertant strains, 36 were derived from cultures where more than one isolate was identified, although each arose on a different day. However, of those 36, 21 had sustained genetic alterations different from those of other colonies derived from the same culture. For example, three different revertants were identified from culture 19, on the plate incubated at 28 °C. The colony identified on day 4 carried a D187N second-site suppressor. The colony identified on day 7 bore a Y146H second-site suppressor and the one identified on day 10 carried a valine codon at position 163. The results indicate that colonies derived from the same culture, but identified on different days, are not likely to be siblings of one other, but represent independent genetic events.

DNA sequence analysis of revertant strains

Crude lysates were prepared from each revertant strain and the *cox2* gene was amplified from each as described above. The region of DNA surrounding the site of the original mutation was then sequenced for each revertant strain. As shown in Table 1, 25 of the revertants carried a threonine codon, ACT or CTT, at position 163. Seven revertants now carry a GTT valine codon at posi-

tion 163, while five now bear an AGT serine codon. While m5351 with an isoleucine codon at this site is unable to respire, strains with a wild-type threonine, or with valine or serine codons, can now grow on non-fermentable carbon sources. Since valine is permissible at this site while isoleucine is not, the size of the R-group adjacent to the conserved aspartic acid at 164 is apparently of significant importance.

A total of 18 intragenic revertants still carried the mutant isoleucine codon at position 163. We sequenced through other portions of the *cox2* gene in these strains until we identified a second codon which had been altered. The number of isolates of each type of revertant is given in Table 1. We identified 15 strains with second-site suppressors at codon 222, where serine was replaced with either tyrosine or phenylalanine. In addition, three other intragenic revertants were identified with the following changes: Y146H, D187N, and A189T. The entire *cox2* gene was sequenced for one representative of each of these classes of revertant to confirm that the second-site alteration we had identified was the only other change within the *cox2* gene.

Genetic analysis of second-site suppressors

While we were confident that alterations at position 163 were responsible for the recovery of function in our same-site revertants, we felt it important to confirm genetically that, in the case of these putative second-site, intragenic suppressors, restoration of respiratory function was due to alterations in mitochondrial DNA. One representative diploid revertant from each class of second-site suppressor was sporulated and the phenotypes of the resulting spores determined. The ability to respire segregated in a manner consistent with a mitochondrial suppressor, as previously described by Mazourek et al. (1999).

Phenotypic characterization of the revertant strains

While all our revertant strains were capable of respiration to some degree, we were interested to compare their growth rates on plates. Since the revertants were diploids, isogenic derivatives of 777/3A (the original parent strain of m5351) and m5351 were constructed by crossing to DL2 ρ^0 . All the diploid strains were streaked on YPEG plates and grown at 18 °C, 28 °C, and 37 °C. As shown in Fig. 1, the growth rates of these strains on plates were not significantly different from the wild type at any temperature tested. However, the mutant failed to grow on non-fermentable carbon sources at any of the three temperatures tested.

Since growth on plates is a fairly crude measure of CcO activity, we also isolated mitochondria from each of the strains (Yaffe 1991) and measured cytochrome *c* reduction spectrophotometrically (Mason et al. 1973). For each strain, the activity ($\mu\text{mol Cc reduced mg}^{-1}$

Table 1 Wild-type, mutant, and revertant strains of *Saccharomyces cerevisiae*. Specific oxidase activity ($\mu\text{mol cytochrome } c \text{ reduced min}^{-1} \text{ mg}^{-1}$ total mitochondrial protein) as a percentage of the wild type. – Not applicable

Strain	Number of isolates	Substitution	% Oxidase activity
777/3A	–	Wild type	100.0
m5351	–	T163I	0.7
Same-site threonine	25	I163T	90.0
Same-site valine	7	I163V	80.0
Same-site serine	5	I163S	56.0
Second-site 146	1	Y146H	28.0
Second-site 187	1	D187N	24.5
Second-site 189	1	A189T	25.0
Second-site 222	13	S222Y	8.5
Second-site 222	2	S222F	10.0

protein min^{-1}) was determined; and the percent activity with respect to the wild type is given in Table 1. Even though the S222Y/F revertants grew almost like the wild type on plates, their CcO-specific activity was reduced significantly (to about 10% of the wild type). Other second-site suppressor strains and strains carrying valine or serine codons at position 163 grew as well as the wild type and their CcO activities ranged from about 25% to 80%.

Western blot analysis of Cox2p in wild-type, mutant and revertant strains

CcO activity was significantly reduced (more than 75%) in a number of the revertant strains. To determine whether steady-state levels of Cox2p were affected in these strains, approximately equal amounts of protein from isolated mitochondria of each strain were sepa-

rated on acrylamide gels, transferred to nylon membranes, and the membranes incubated with Cox2p-specific primary antibody. Cox2p was then detected as described in Materials and methods; and the results are shown in Fig. 2. As previously reported, m5351 (lane 2) produced very little detectable Cox2p (Weiss-Brummer et al. 1979). Revertant strains with CcO activity greater than 50% of the wild type showed amounts of Cox2p approximately equal to that seen in the wild type (lanes 4–6), while strains with less than 50% of wild-type CcO activity all had reduced amounts of Cox2p (lanes 3, 7–10). The S222F/Y revertants showed the least amount of protein and the lowest CcO activity.

Discussion

Phenotype of the m5351 mutant strain

The mutant strain m5351 displays almost no CcO activity, which results in loss of the ability to grow on non-fermentable carbon sources. The mutation replaces a threonine codon at position 163 in the yeast polypeptide with an isoleucine codon, which substitutes a hydrophobic residue for a polar one. In addition, isoleucine is slightly larger than threonine. An alignment of GenBank amino acid sequences for Cox2p, using CLUSTAL W (Thompson et al. 1994), confirms that most organisms carry a valine codon at this position (data not shown). Threonine is found only in *S. cerevisiae*, *R. sphaeroides*, *P. denitrificans*, *Candida glabrata*, and in the common carp (see Materials and methods for species used in the alignment). This residue occurs within a conserved sequence motif, $^{160}\text{L L D/E V/T D}^{164}$. The aspartic acid residue at position 164 of this motif (yeast numbering) has been implicated in binding to Cc, since alteration of this residue in the *R. sphaeroides* subunit to glutamine produces a CcO complex with wild-type spectral characteristics, but with reduced turnover number for both horse heart Cc and *R. sphaeroides* Cc₂ (Zhen et al. 1999).

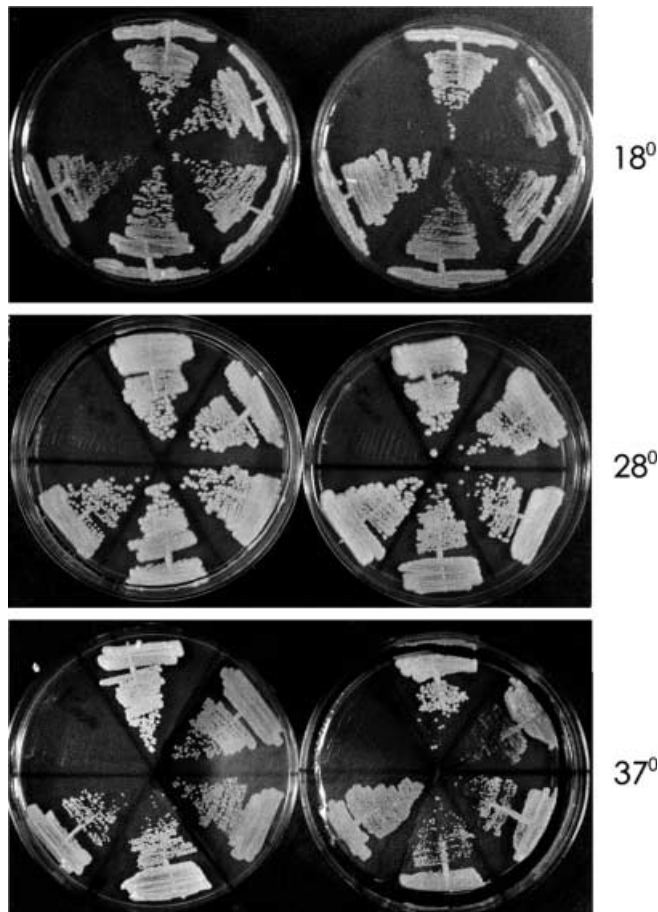


Fig. 1 Growth rates of strains on the non-fermentable carbon sources, ethanol and glycerol. Strains were grown for 5 days at the indicated temperatures (degrees Celsius). Counter-clockwise from the top, the strains are as follows. Left plate: wild-type, T163I mutant; D187N pseudo-wild-type; same site revertants I163S and I163V. Right plate: wild-type; T163I mutant; second-site revertants A189T, S222F, Y146H, and S222Y. The pseudo-wild-type strain carries a CTT threonine codon instead of a wild-type ACT threonine codon

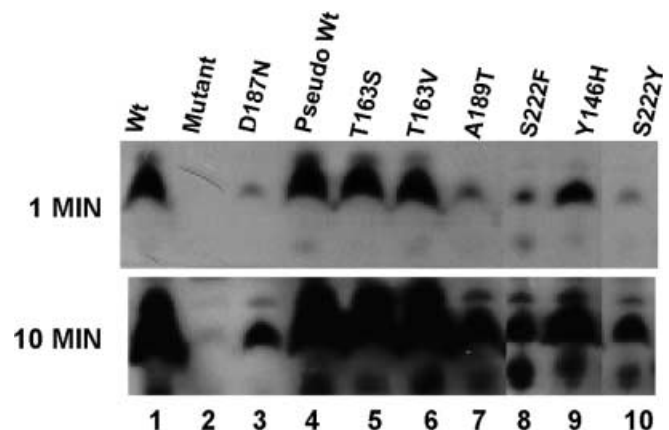


Fig. 2 Western blot analysis. Wild-type (*Wt*), mutant, and revertant cell extracts were separated on gels and probed with the anti-Cox2p antibody, 4B12-A5. The film was exposed for 1 min (*top panel*) and 10 min (*bottom panel*)

The position of the alteration at residue 163 in the Cox2 polypeptide is shown in red and marked with an arrow in Fig. 3. This model of the globular domain of Cox2p, which interacts directly with Cc, is derived from the crystal structure of the beef heart subunit (1OCC in PDB). We chose to model the position of the alteration in the yeast subunit on the bovine enzyme rather than the *P. denitrificans* enzyme, since two different alignment programs (LFASTA and ALIGN) gave higher alignment scores for the yeast/bovine Cox2p pair than for the yeast/*P. denitrificans* pair (Meyers and Miller 1988; Pearson and Lipman 1988). As shown in Fig. 3, residue 163 is located above the copper-binding domain of the protein, and the distance between residue 163 and the two copper atoms is 13-Å, as given in Table 2.

The T163I mutation appears to exert its effect by preventing assembly of the subunit into the oxidase complex or by destabilizing the polypeptide, resulting in rapid turnover and low or undetectable levels of protein. The results of the in vivo labeling experiments of Weiss-Brummer et al. (1979) and our Western blot analysis (see Fig. 2) are consistent with this hypothesis, as little or no Cox2p is detected using either approach. Initially, we were surprised that the T163I mutation resulted in the loss of essentially all Cox2p, since mutation of the adjacent aspartic acid at 164 to glutamine in the *R. sphaeroides* subunit results in CcO which is wild-type with regard to optical and electron paramagnetic resonance spectra, CO binding, and metal content, and which has an expression level of 0.5–1.0 compared to the wild type (Zhen et al. 1999). However, the mutated *R. sph-*

aeroides CcO was purified from an over-expression strain which, when used to express Cc₂, resulted in levels of protein 5- to 10-fold higher than the wild type (Brandner et al. 1989). Such over-expression might result in smaller differences between wild-type and mutant protein levels. It seems unlikely that the reduction in steady-state levels of protein seen in our experiments is the consequence of loss of antibody recognition of the mutant protein, because other amino acid substitutions at this site have no apparent effect on antibody-binding (see lanes 5, 6 of Fig 2) and the antibody binds to Cox2p, which carries the mutant I163 codon in the context of second-site, intragenic suppressors (lanes 3, 7–10).

Same-site revertants

Among our same-site revertants which incurred a second nucleotide change in codon 163, only two other amino acid codons were detected, valine and serine. Both of these amino acids are smaller than isoleucine, although one is polar and the other is hydrophobic, suggesting that size is more important than hydrophobicity. Strains bearing these codons have high levels of CcO activity (greater than 50% compared to the wild type) and these strains exhibit high steady-state levels of Cox2p, as shown by Western blotting (Fig. 2). Therefore, the presence of either valine or serine restores normal stability to the protein.

Of nine possible codons which can be derived from the mutant ATT isoleucine codon by a single nucleotide

Fig. 3 Model of Cox1p (magenta) and Cox2p (cyan), showing the locations of the mutation in m5351 (red) and the second-site suppressors of that mutation (yellow)



Table 2 Distance (Å) between residues, as determined from the X-ray crystal structures of Cox2p from *Bos taurus* and *Paracoccus denitrificans* [1OCC and 1AR1 in the Protein Data Base, respectively] using Molecular Images software. Residue numbers are from yeast with the corresponding numbers from *B. taurus* and *P. denitrificans* given in parentheses

<i>B. taurus</i>		<i>P. denitrificans</i>	
Residue pair	Distance	Residue pair	Distance
163 (138) to Cu1	14.0	163 (158) to Cu1	13.6
163 (158) to Cu2	14.9	163 (158) to Cu2	14.5
163 (138) to 146 (121)	5.2	163 (158) to 146 (137)	5.0
163 (138) to 187 (162)	16.4	163 (158) to 187 (182)	15.9
163 (138) to 189 (164)	14.4	163 (158) to 189 (184)	14.2
163 (138) to 222 (197)	16.0	163 (158) to 222 (217)	15.8

change, two encode for threonine and one each for valine, phenylalanine, serine, asparagine, methionine, tryptophan, and isoleucine. We recovered 25 same-site revertants with threonine codons (both wild-type ACT and CTT), seven with valine codons, and five with serine codons. However, within our collection of 55 revertant strains, none with methionine, tryptophan, phenylalanine, or asparagine codons was ever detected. Three of the four possible codons which we did not detect encode for amino acids with significantly larger molecular volumes than that of threonine. If, as suggested above, the size of the residue is important with respect to the stability of the protein, the larger sizes of methionine, tryptophan, and phenylalanine may explain why revertants bearing those codons were never isolated.

Second-site, intragenic revertants

The positions of the second-site intragenic alterations are also shown in Fig. 3, in yellow. These include Y146H, D187N, A189T, and S222F/Y. Each of the second-site revertants was identified only once, with the exception of the alteration at S222, where 13 revertants carrying a tyrosine codon and two with a phenylalanine codon were identified. The distance between the original mutated residue and each of these second-site alterations was determined from the X-ray crystal structures of both the bovine subunit and that from *P. denitrificans*, using Molecular Images modeling software; and those values are given in Table 2. The second-site alteration at residue 146 substitutes histidine for tyrosine. Residues at the mutant site, 163, and at this revertant site, 146, are reasonably close to one another in the X-ray crystal structure (approximately 5 Å apart) as can be seen in Fig. 3 and in Table 2. In the *P. denitrificans* subunit, the mutant site 163 (158 in *P. denitrificans*) is at the end of a 3/10 helix which connects β strands 3 and 4, while 146 (137 in *P. denitrificans*) is at the end of β strand 3 (Ostermeier et al. 1997). It has previously been shown for the *P. denitrificans* polypeptide that replacement of leucine by glutamine at the position corresponding to 146 (137) reduces the K_{on} rate for electron entry by 75%

and decreases K_{cat} by 2.5-fold, although the affinity of the enzyme for Cc remains unchanged (Witt et al. 1998). Based on these results, the investigators suggested that the initial encounter between CcO and Cc is mediated by electrostatic attractions, including a contribution from D164 adjacent to T163. More precise positioning of the two proteins then depends on short-range hydrophobic interactions, including an important hydrophobic contribution by the *P. denitrificans* L137 residue.

Witt et al. (1998) noted that the hydrophobic residues which they mutated in the *P. denitrificans* enzyme, including L137 (Y146 in the yeast), were either identical or conservatively substituted in the mitochondrial forms of the enzyme. Therefore, they argued that their results demonstrating the importance of a hydrophobic residue at position 137/146 were also valid for these mitochondrial oxidases. Tyrosine is found at this position in 19 of the 20 species we used in our CLUSTAL W alignment, including *R. sphaeroides*, with the only exception being *P. denitrificans*. Our revertant analysis suggests that in the context of the original mutation, T163I, replacement of the equivalent of L137 with histidine is compatible with enzyme stability and function. While a very large number of hydrophobicity scales have been developed (many with differentiating orders, from most to least hydrophobic) in all these scales, leucine is significantly more hydrophobic than either tyrosine or histidine (Cornette et al. 1987; Li et al. 1997). The positions of tyrosine and histidine in these scales are variable, with some suggesting tyrosine is more hydrophobic and others suggesting the reverse. Regardless of the scale used, it is clear that each Cox2p from the other species we examined in our CLUSTAL W analysis carries a significantly less hydrophobic amino-acid residue at this position than does the polypeptide from *P. denitrificans*. Therefore, the importance of this residue in the precise alignment of Cox2p and Cc in these other organisms is less obvious than for the *P. denitrificans* polypeptide.

The other second-site intragenic alterations are clustered in the vicinity of the two copper atoms found in the Cu_A site. As shown on the bovine structure in Fig. 3, S222F/Y is closest to the coppers, about 6 Å distant, while A189T is furthest away from the coppers at about 10 Å. However, as shown in Table 2, all three of the revertant sites are at least 14 Å from the site of the original mutation at codon 163. These results are consistent with those reported by Meunier and Rich (1998), from which they conclude that second-site reversion analysis does not necessarily identify residues which are close to one another in a three-dimensional protein structure. Meunier and Rich (1998) analyzed a collection of yeast strains with mutations in *COX1* and *COX2*, and a number of revertants of those mutant strains. For mutants which have optically detectable heme *a* and which therefore are “activity” mutants, some reversions occurred up to 22 Å distant from the original mutation. For those mutants which have no detectable heme *a*, and which are therefore categorized as “assembly” mutants, the reversions were as much as 30 Å distant (Meunier

and Rich 1998). For T163I, some of our second-site reversions were more than 16 Å distant from the original mutant site. Also, like Meunier and Rich (1998), we found that the second-site alterations which compensated for the T163I mutation were located near the subunit I/II interface, as shown in Fig. 3.

We identified one revertant strain carrying each of the following second-site changes: D187N and A189T. The second-site alteration at 189 replaces alanine with threonine. Threonine is slightly larger than alanine and is somewhat more polar. Interestingly, in six of the 20 Cox2p sequences used in our alignment, threonine is found at the equivalent position, with all the remainder bearing alanine codons. Position 187 is adjacent to a short conserved sequence, ¹⁸³D V I H¹⁸⁶, although the aspartic acid codon found at 187 in yeast is a serine or alanine codon in the 19 other sequences we examined. Aspartic acid and asparagine are very similar in size but differ in that aspartic acid is charged at physiological pH.

We have shown previously that an alteration of this same residue, 187 (in this case from aspartic acid to glycine), can also suppress another missense mutation in *cox2* (Mazourek et al. 1999). Strain m5801 bears a single nucleotide change in codon 228, which converts a glycine codon to valine. For the 20 species used in our alignment, 18 bear an asparagine codon at this site, *R. sphaeroides* carries a serine codon, and yeast displays a codon for glycine. Replacement of this glycine by valine in the yeast subunit leads to a significant increase in the molecular volume of the side chain, with concomitant loss of almost all CcO activity. Both m5351 and m5801 are restored to respiration proficiency by a change at D187, despite the fact that the original mutations are in residues distant from one another (163–228 = 18.8 Å) and from residue 187 (16.4 Å and 12.7 Å). The fact that alteration of the same residue, D187, can suppress two different missense mutations in *COX2* suggests that the mechanism of suppression is general; that is, alteration of D187 to glycine or asparagine can compensate for distortion of the polypeptide caused by a number of different amino-acid substitutions within the polypeptide.

The final second-site alteration is S222F/Y. We recovered the S222Y alteration in 13 independent revertants and the S222F change in two independent revertants. In both cases, a small polar residue is replaced by a significantly larger residue and, in the case of phenylalanine, a much more hydrophobic one. S222 is completely conserved in the 20 species we examined; and yet our revertant strains are able to respire, even if they carry amino-acid substitutions at this site, at least in the context of the T163I mutation. These S222F/Y second-site suppressors are of particular interest. First, as mentioned above, the serine is conserved in all the species we examined. Second, S222 is postulated to be a part of the extended metal environment of the Cu_A site. The Cu_A site is proposed to consist of: (1) two copper atoms and a magnesium ion, (2) the ligands which bind

directly to those metals, and (3) those residues within 3.5 Å of either the metals or their ligands (Karlin et al. 1998). S222, which corresponds to S197 in the bovine polypeptide modeled in this paper (1OCC in PDB), is a second-shell residue positioned where it can potentially interact with E223 II in Cox2p (E198 II in 1OCC) and with D369 I in Cox1p (same residue number in 1OCC). Both E223 II and D369 I are ligands to the magnesium ion which is part of the Cu_A metal center (Karlin et al. 1998); and S222 (197 in 1OCC) is close enough to both of these to form hydrogen bonds. Finally, as for D187, we have previously shown that an alteration at the 222 site can suppress the G228V mutation in m5801 (Mazourek et al. 1999). In the case of the S222 second-site suppressors, we found precisely the same amino-acid substitutions, tyrosine or phenylalanine, suppressing m5801 (G228V) and m5351 (T163V); and we identified multiple revertants of each mutant carrying suppressors at this site. Since the same alterations can suppress both missense mutations, the effects must rely on overall general structure rather than on specific interactions between the amino acids involved. Indeed, as shown in Table 2, residue 222 is more than 15 Å distant from residue 163. Because S222 is so highly conserved, it will be of interest to determine whether the S222F/Y alterations have phenotypes in the absence of the mutant codons, T163I and G228V. We are in the process of constructing, by site-directed mutagenesis, *COX2* genes bearing each of those mutations (S222F and S222Y) in an otherwise wild-type background.

For each of these second-site suppressors, there is no obvious pattern that would explain why the particular change would compensate for the original mutation at 163. We see substitution of amino acids of similar size but with different polarity, of polar for non-polar, and of large and hydrophobic for polar. However, as shown in Fig. 2, while the T163I mutation leads to loss of Cox2p, each second-site alteration leads to at least moderate accumulation of Cox2p (Fig. 2, lanes 3, 7–10). The T163I mutation must distort the globular domain of Cox2p sufficiently, such that the protein cannot be assembled into the enzyme complex or is simply degraded very rapidly. As suggested above for the second-site alteration at D187, each of the second-site alterations must, in a non-specific manner, relieve the distortion caused by the original mutation and restore stability to the polypeptide.

Taken together, our results lead to several conclusions. First, analysis of “random” mutations can reveal unexpected information about gene function. In our case, we identified an amino-acid substitution which abolished gene function in a residue unlikely to have selected for site-directed mutagenesis. Second, replacement of threonine 163 by isoleucine in Cox2p leads to a dramatic reduction in the steady-state levels of the protein. Third, replacement of threonine by both serine and valine is compatible with some level of protein function and restores essentially wild-type levels of the protein. Fourth, in agreement with Meunier and Rich

(1998), second-site alterations that restore protein function can occur at a significant distance from the original mutation. These second-site alterations restore stability to the polypeptide, occur at a number of different sites, and include a whole variety of types of substitutions. Finally, we have shown that alteration of the same residues, D187 and S222, can suppress two different missense mutations within *COX2*. In the case of S222, replacement of the completely conserved serine with precisely the same amino acids restores stability and activity for strains with different missense mutations.

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