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Identification of Essential Amino Acids within the Proposed Cu_A Binding Site in Subunit II of Cytochrome *c* Oxidase*

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Henry Speno, M. Reza Taheri, Derek Sieburth, and Craig T. Martin‡

From the Department of Chemistry and the Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003-4510

To explore the nature of proposed ligands to the Cu_A center in cytochrome *c* oxidase, site-directed mutagenesis has been initiated in subunit II of the enzyme. Mutations were introduced into the mitochondrial gene from the yeast *Saccharomyces cerevisiae* by high velocity microprojectile bombardment. A variety of single amino acid substitutions at each of the proposed cysteine and histidine ligands (His-161, Cys-196, Cys-200, and His-204 in the bovine numbering scheme), as well as at the conserved Met-207, all result in yeast which fails to grow on ethanol/glycerol medium. Similarly, all possible paired exchange Cys,His and Cys,Met mutants show the same phenotype. Furthermore, protein stability is severely reduced as evidenced by both the absence of an absorbance maximum at 600 nm in the spectra of mutant cells and the underaccumulation of subunit II, as observed by immunolabeling of mitochondrial extracts. In the same area of the protein, a variety of amino acid substitutions at one of the carboxylates previously implicated in binding cytochrome *c*, Glu-198, allow (reduced) growth on ethanol/glycerol medium, with normal intracellular levels of protein. These results suggest that a precise folding environment of the Cu_A site within subunit II is essential for assembly or stable accumulation of cytochrome *c* oxidase in yeast.

Cytochrome *c* oxidase accepts electrons from cytochrome *c* to reduce dioxygen to water in the final step of cellular respiration, according to:

$$4H^+ + O_2 + 4e^- \rightarrow 2H_2O$$

Coupled to this electron transfer, the enzyme pumps protons against an electrochemical gradient, and the energy stored in this gradient is subsequently utilized for the production of ATP (1, 2). Although eukaryotic cytochrome *c* oxidase contains up to 12 subunits (3, 4), subunits I, II, and III (encoded by the mitochondrial genome) are thought to form the core of a functional enzyme. Within this core reside the redox active metal centers.

The site of dioxygen reduction is a binuclear metal center comprised of cytochrome a_3 and Cu_B. In addition, two metal centers serve in electron transfer from cytochrome *c* to the oxygen binding site. Electrons from the one electron carrier cytochrome *c* enter cytochrome *c* oxidase through the intermembrane (cytosolic) face of the protein, via Cu_A (5) and/or cytochrome *a* (6, 7). The binuclear oxygen binding site then accepts electrons from Cu_A and/or cytochrome *a* and transfers them to bound dioxygen. Finally, coupled to one or more of these electron transfers, the enzyme pumps protons across the inner mitochondrial membrane (2).

Recent mutagenesis studies in *Escherichia coli* quinol oxidase and an aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* have identified six histidine residues in subunit I as ligands to cytochrome *a*, cytochrome a_3, and Cu_B (8–10). In contrast, subunit II has long been thought to provide ligands to Cu_A (11–14). Electron nuclear double resonance spectroscopic studies of isotopically substituted enzyme have defined two histidines and one cysteine as ligands to Cu_A (14–16). Evolutionary constraints further require that subunit II contributes at least one cysteine ligand to the Cu_A center (14). Specifically, sequence alignment of each of subunits I, II, and III against a diverse group of species reveals only two conserved cysteines, located at positions 196 and 200 (numbering according to the bovine enzyme) in a highly conserved region of subunit II, as shown in Fig. 1. The two conserved cysteines have been implicated as ligands to Cu_A by differential labeling studies (17). Within the C-terminal end of subunit II, there are only two conserved histidines, located at positions 161 and 204, and cross-linking studies have implicated the region of subunit II near position 160 (as well as near position 198) in the binding between cytochrome *c* oxidase and cytochrome *c* (18). Recent mutagenesis studies on C-terminal fragments of subunit II from *E. coli* quinol oxidase (19, 20) and from the aa_3-type cytochrome *c* oxidase from *Paracoccus denitrificans* (21–23) are consistent with these assignments.

The sequence of the C-terminal region of subunit II has been compared to that of the copper binding region of blue copper proteins (13) and, more recently, to nitrous oxide reductase (20, 24). While the former clearly coordinate a single copper atom, the latter is thought to contain a mixed valence binuclear copper site (25). Recent multifrequency EPR studies of cytochrome *c* oxidase suggest that the Cu_A center also contains a binuclear copper site (25–27). In any case, sequence alignments between all three classes of sites suggest potential common ligands to copper.

Modification of bovine cytochrome *c* oxidase with a water-soluble carbodiimide in the presence and absence of cytochrome *c* has identified specific negatively charged amino acids in subunit II which may be involved in the electrostatic interaction between cytochrome *c* oxidase and a positively charged face of cytochrome *c* (13). Similarly, a monoclonal antibody to subunit II inhibits cytochrome *c* binding and protects regions of subunit II from reaction with the carbodiimide (28). One of the protected carboxylates, Glu-198, is rigorously conserved and is located directly between the two conserved Cys

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‡ To whom correspondence and reprint requests should be addressed: Dept. of Chemistry and the Program in Molecular and Cellular Biology, University of Massachusetts, Box 34510, Amherst, MA 01003-4510. Tel.: 413-545-3299; Fax: 413-545-4490; E-mail: CMartin@Chem.UMass.edu.

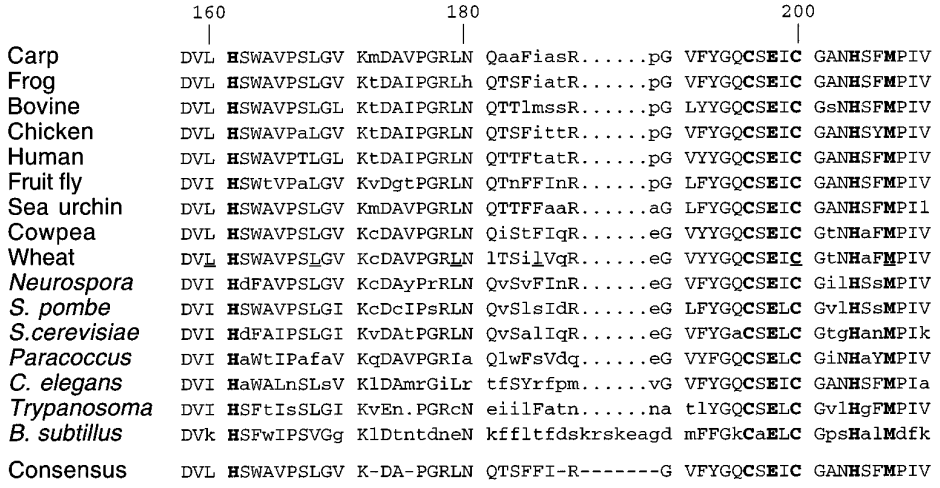

FIG. 1. **Sequence conservation in subunit II.** *Capitals* indicate either invariant or conservative substitutions. Amino acids targeted for substitution are shown in *bold*. Residues which have been shown to be post-transcriptionally edited are indicated by an *underline*.

residues at 196 and 200.

In the current study, site-directed mutagenesis of subunit II in the eukaryotic enzyme has been carried out in the yeast *Saccharomyces cerevisiae* to test directly the involvement of the four most likely ligands to Cu_A: His-161, Cys-196, Cys-200, and His-204, as well as the potential ligand Met-207. Single substitutions have been introduced at each site and include mutations intended either to retain or remove the ability to coordinate copper. Additionally, double mutations have been prepared which exchange proposed ligand functional groups (*e.g.* a mutation of Cys to His at one site combined with a mutation of His to Cys at another). Finally, to complement studies of the closely spaced Cys residues and to test the role of this region in the binding of the physiological redox partner cytochrome *c*, the centrally located Glu-198 has also been replaced by semiconservative, as well as more dramatic substitutions.

EXPERIMENTAL PROCEDURES

In Vitro Site-directed Mutagenesis—A bacterial vector (derived from pTZ18U) containing a 2.4-kilobase pair fragment encompassing the *cox2* gene (referred to as cox2/pTZ18U) was kindly provided by T. Fox. Mutagenesis primers were synthesized on a Cyclone Plus DNA synthesizer (Milligen/Biosearch) with the trityl group on and were purified by reverse phase chromatography. Site-directed mutagenesis was followed with only slight modifications by the method of Kunkel *et al.* (29).

Mitochondrial Transformation—Introduction of the mutant gene by mitochondrial transformation was accomplished using microprojectile bombardment with a PDS-1000 helium biolistic delivery system (Bio-Rad). Large scale plasmid purification was performed on either Qiagen or Promega Magic Maxiprep™ columns. Since mitochondrial transformants cannot be screened directly, 5 μg of plasmid pCGE137 (obtained from T. Fox), which contains a nuclear marker to complement the Ura^- phenotype, were coprecipitated with 15 μg of mutant cox2/pTZ18U DNA onto 0.5-μm tungsten particles (Johnson Matthey Electronics) using slight modifications[1] of the procedure described by Fox *et al.* (30). Treatment of *S. cerevisiae* strain TF189 rho^0 prior to transformation has also been described by Fox *et al.* (30). All yeast strains used were obtained from T. Fox.

Genetic Analyses and Tests for Functionality—Identification of mitochondrial transformants was accomplished by crossing haploid candidates to AB-4D/V25 as described by Fox *et al.* (30). Mitochondrial transformation results in a haploid strain containing a concatenated form of the mutant *cox2* plasmid (31); resulting strains typically show >95% retention of the plasmid. The transformed strains are subsequently referred to collectively as TF189 rho^-.

Enzyme function was tested by crossing TF189 rho^- with TF145 (*cox2*Δ); at the same time TF189 rho^- was crossed with AB-4D/V25 to ensure that TF189 rho^- cells did not loose their plasmid. A control strain PT424, carrying a wild type *cox2* plasmid, was used as a control for AB-4D/V25 and TF145. Haploid mutants which grow on ethanol/glycerol medium when crossed to TF145 were crossed with AB-4D/V25, and the resulting diploids were screened by DNA hybridization (Southern) analysis for the appropriate restriction site. For nonrespiring mutations, diploids were selected from a cross to the wild type haploid strain DL1 on minimal medium (SD, 0.7% bacto-yeast nitrogen base without amino acids (Difco), 2% glucose, 2% bacto-agar (Difco)); diploid colonies which failed to grow on an ethanol/glycerol medium were picked for further characterization.

DNA Hybridization (Southern) Analysis to Confirm Constructs—Genomic DNA isolation was followed (with slight modification) according to Sherman *et al.* (32). These crude genomic DNA extracts were then digested with *Hae*III to produce a 2.7-kilobase pair fragment containing the *cox2* gene. The DNA was then further digested with the restriction enzyme appropriate to the specific mutant (Table I), run on an agarose gel, transferred to Nytran (Schleicher & Schuell), and hybridized with a ^{32}P-end-labeled sequencing primer.

Polymerase Chain Reaction Amplification and DNA Sequencing—Crude genomic DNA was digested with *Hin*dIII and precipitated prior to amplification by the polymerase chain reaction. Briefly, approximately 2 μg of digested DNA were combined with 100 pmol of primers and 5 units of *Taq* DNA polymerase (Promega) using a mineral oil overlay where the denaturing and annealing (42 °C) steps were extended to 1 min. The amplified DNA was precipitated, extracted with chloroform, and sequenced according to Promega's *fmol*™ system, except that annealing was at 60 °C, and dideoxyguanosine 5′-triphosphate and dideoxycytidine 5′-triphosphate concentrations were at 600 μM.

Analysis of Subunit II Synthesis and Stability in Vivo—To verify the correct synthesis of the subunits for cytochrome *c* oxidase, a standard procedure for *in vivo* labeling of mitochondrial translation products with [^{35}S]Met (DuPont NEN) in the presence of the cytoplasmic translation inhibitor cycloheximide was followed (33). Protein from crude mitochondrial preparations was analyzed on a 12.5% SDS-polyacrylamide gel.

To monitor the *stable accumulation* of subunit II, an immunolabeling analysis was carried out on a mitochondrial extract prepared as described by Meunier *et al.* (33). Mitochondrial protein extracts containing equal amounts of total protein (34) were loaded onto a 12.5% SDS-polyacrylamide gel and electrophoresed at a constant voltage of 200 V. Protein was electrotransferred to nitrocellulose (Schleicher & Schuell, BA83) in 80 mM Tris base, 12.8 mM glycine, 20% methanol, incubated with either the monoclonal antibody to cytochrome *c* oxidase subunit II, CCO6, or the polyclonal antibody YR6-T (provided by T. Mason), and detected using a horseradish peroxidase-conjugated secondary antibody (Amersham Corp.).

Spectroscopic Characterization in Vivo—Information on the environment of the cytochromes (from the α-region of the heme spectrum) can be readily observed in whole cell suspensions at room temperature using visible absorption spectroscopy. Typically, 50-ml yeast cultures were grown in 1% Bacto-yeast extract (Difco), 2% Bacto-peptone (Difco), 2% galactose (Sigma) to saturation, harvested, and resuspended in 1.2 M sorbitol, 50 mM Tris, 10 mM EDTA, pH 7.4, yielding $\approx 1\text{–}2 \times 10^{10}$ cells/ml, providing a highly concentrated sample. In this case, the high degree of scattering enhances sensitivity in the detection of the cytochrome absorbances (35). For oxidation and reduction of a 750-μl suspension, 10 μl of 10 mg/ml antimycin A (Sigma) were added prior to the

[1] T. Fox, personal communication.

TABLE I
Summary of mutant constructs

In the gene sequences, the codon for the target amino acid is centered, and base changes are indicated in bold. All constructs, except those containing an asterisk in the last column, contain double base mutations within the codon, such that a primary reversion will require two base changes. Note that for Cys^{200} the construct denoted "Ser^{TCC}" is distinct from that termed "Ser" in that the former contains only a single base substitution within the codon. To facilitate screening, restriction sites have been engineered into (+) or out of (−) the site as indicated.

Target	Substitution	Restriction site	Sequence
Cys^{196}			GGG GCA **TGT** TCT GAG
	Ser	−*Dde*I	GGG GCA T**CA** T**CA** GAG
	Met	−*Dde*I	GGG GCA **ATG** T**CA** GAG
	Ala	−*Nla*III	GGG G**CA** **GC**T TCT GAG
	Asp	−*Nla*III	GGG G**CA** **GA**T TCT GAG
	His	+*Bmy*I	GGG GCA **CA**T TCT GAG
Cys^{200}			GAG TTG **TGT** GGG ACA
	Ser^{TCC}	+*Nci*I	GAG TTG T**CC** GGG ACA*
	Ser	+*Rsa*I	GAG TTG T**CA** GG**T** ACA
	Met	+*Rsa*I	GAG TTG **ATG** GG**T** ACA
	Ala	+*Rsa*I	GAG TTG **GC**T GG**T** ACA
	Asp	+*Fok*I	GAG TTG **GAT** GGG ACA
	His	+*Mae*III	GAG TT**A** **CA**T GGG ACA
His^{204}			ACA GGT **CAT** GCA AAT
	Asn	+*Bsm*I	ACA GG**G** **AA**T GCA AAT*
	Cys	+*Fok*I	ACA GG**A** **TG**T GCA AAT
His^{161}			GTT ATT **CAT** GAT TTT
	Asn	+*Bsp*HI	GTT ATT **AA**T GAT TTT*
	Cys	+*Bsp*HI	GTT ATT **TG**T GAT TTT
Glu^{198}			TGT TCT **GAG** TTG TGT
	Asp	−*Dde*I	TGT T**CT** **GAT** TTG TGT
	His	−*Dde*I	TGT T**CT** **CA**T TTG TGT
	Arg	−*Dde*I	TGT T**CT** **AGA** TTG TGT
	Gln	−*Dde*I	TGT T**CT** **CA**A TTG TGT*

sequential addition of 10 μl of 3% H_2O_2 and 10 μl of 0.1 M dithionite. A plastic cuvette was placed in the chamber such that the path length was 0.4 mm; no cuvette was placed in the reference beam. Absorbance data from a Hitachi U2000 spectrophotometer were collected through a serial connection to a computer.

To assay for total heme content by the pyridine hemochromogen assay (36), spheroplasts were prepared by lyticase (Sigma) treatment of yeast cells. Then 1.5 ml of spheroplasts were collected by centrifugation and resuspended in 750 μl of 2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8. This suspension was centrifuged, and the supernatant was retained. To 475 μl of supernatant, a pinch (≈1 mg) of solid dithionite and 250 μl of pyridine were added, and the solution was mixed by gentle inversion. The solution was centrifuged again for clarification and 25 μl of 2 M NaOH, 2 mM EDTA were added to the supernatant. This process yields a solution containing the pyridine adducts of all hemes in the sample and allows for the quantitative distinction of hemes *a*, *b*, and *c* (37).

RESULTS

Sequence conservation and spectroscopic analyses point to specific cysteine and histidine residues within subunit II as potential ligands to the Cu_A site. In order to directly probe the role of these conserved amino acids, 14 single amino acid substitutions were introduced into subunit II, targeting His-161, Cys-196, Cys-200, and His-204 as described in Table I and summarized in Fig. 2. Substitutions for His include Cys and Asn, both polar amino acids and potential replacement ligands to copper. Substitutions for Cys include the polar amino acids His, Met, Ser, and Asp, as well as Ala, which simply removes the cysteine sulfhydryl group. In subsequent studies, four double mutant constructs were prepared which exchange individual Cys and His residues, as shown in Fig. 2. In addition, sequence comparisons and extended x-ray absorption fine structure spectroscopy studies have prompted the proposal that Met-207 may coordinate copper (38, 39). To test this proposal, Met-207 was replaced by Cys and double mutants were constructed to exchange Met and Cys residues. Finally, Glu-198, which lies in the primary sequence of subunit II between the two targeted cysteines and has been implicated in the

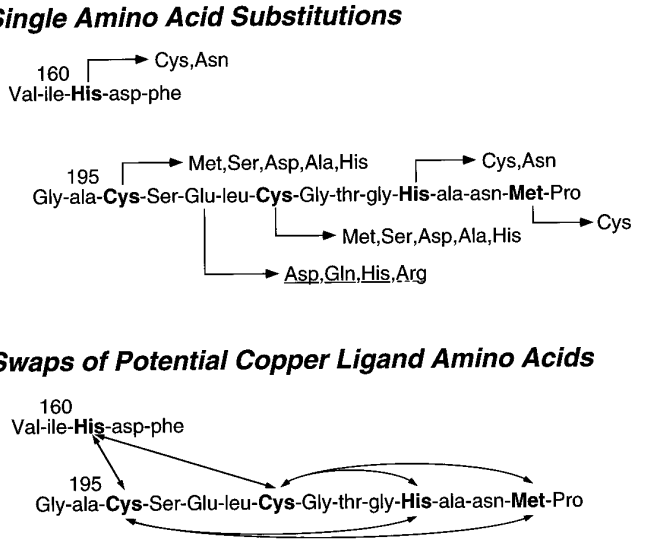

FIG. 2. **Summary of mutagenesis results.** All mutations at His^{161}, Cys^{196}, Cys^{200}, His^{204}, and Met^{207}, including mutations which exchange Cys and His or which exchange Cys and Met, result in cells which are unable to respire. Respiration is detectable only in cells containing the mutations of Glu-198 to Asp, Gln, His, and to a lesser extent Arg (*underlined above*). Immune detection using a monoclonal antibody to subunit II shows an underaccumulation of subunit II in all Cys, His, and Met mutants. Cys^{196} → Met and Cys^{200} → Met showed similar results using a polyclonal antibody. Pyridine hemochromogen extract analyses were performed on the following: Cys^{196} → Ser, Met, Asp, His; Cys^{200} → Ser^{TCC}, Met, His; His^{204} → Cys; and His^{161} → Cys; and the C196H/H204C double mutant. In all cases, accumulation of heme *a* was not observed. Translation products from [^{35}S]Met labeling are not impaired. Examined only for mutants: Cys^{196} → Ser, Met, Ala; Cys^{200} → Ser^{TCC}, Met, Ala, Asp; His^{204} → Cys; and His^{161} → Cys; and C196H/H204C.

binding of cytochrome *c*, was substituted with single amino acid mutations, as indicated in Table I and Fig. 2. Two types of mutations were intended, those that would be relatively conservative (Gln and Asp) and those expected to be disruptive to cytochrome *c* binding (His and Arg).

As described under "Experimental Procedures," mutations in the gene encoding subunit II of yeast cytochrome *c* oxidase were constructed according to standard procedures in a bacterial mutagenesis system. When possible, restriction sites have been either introduced or deleted at or near the mutation to facilitate subsequent analyses, and mutants were constructed so as to require a minimum of a 2-base reversion to restore the wild type amino acid. Verified mutant constructs were then introduced into *rho*^{0} yeast mitochondria via high velocity microprojectile bombardment (30). Haploid strains containing transformed mitochondrial DNA were identified initially by marker rescue and, in some cases, by DNA hybridization analysis of restriction patterns and by DNA sequencing. To simply verify the presence of the cox2 gene (mutant or wild type) by marker rescue, candidate cells were crossed to strain AB-4D/V25, possessing a complete mitochondrial genome, but containing a nonsense mutation near the N-terminal end of the gene for subunit II. Since all mutant constructs in the current study have modifications only near the C terminus, homologous recombination in the diploid can result in a wild type phenotype, verified by ability to grow on ethanol/glycerol media. Finally, haploids containing the modified gene were then crossed to either wild type strains or to strain AB-4D/V25, as appropriate, to construct the diploid strain expressing the subunit II mutant in a complete mitochondrial genomic environment.

Tests for Function of Cytochrome c Oxidase—In order to test for respiratory function in the mutant cytochrome *c* oxidase proteins *in vivo*, a haploid construct verified to contain a spe-

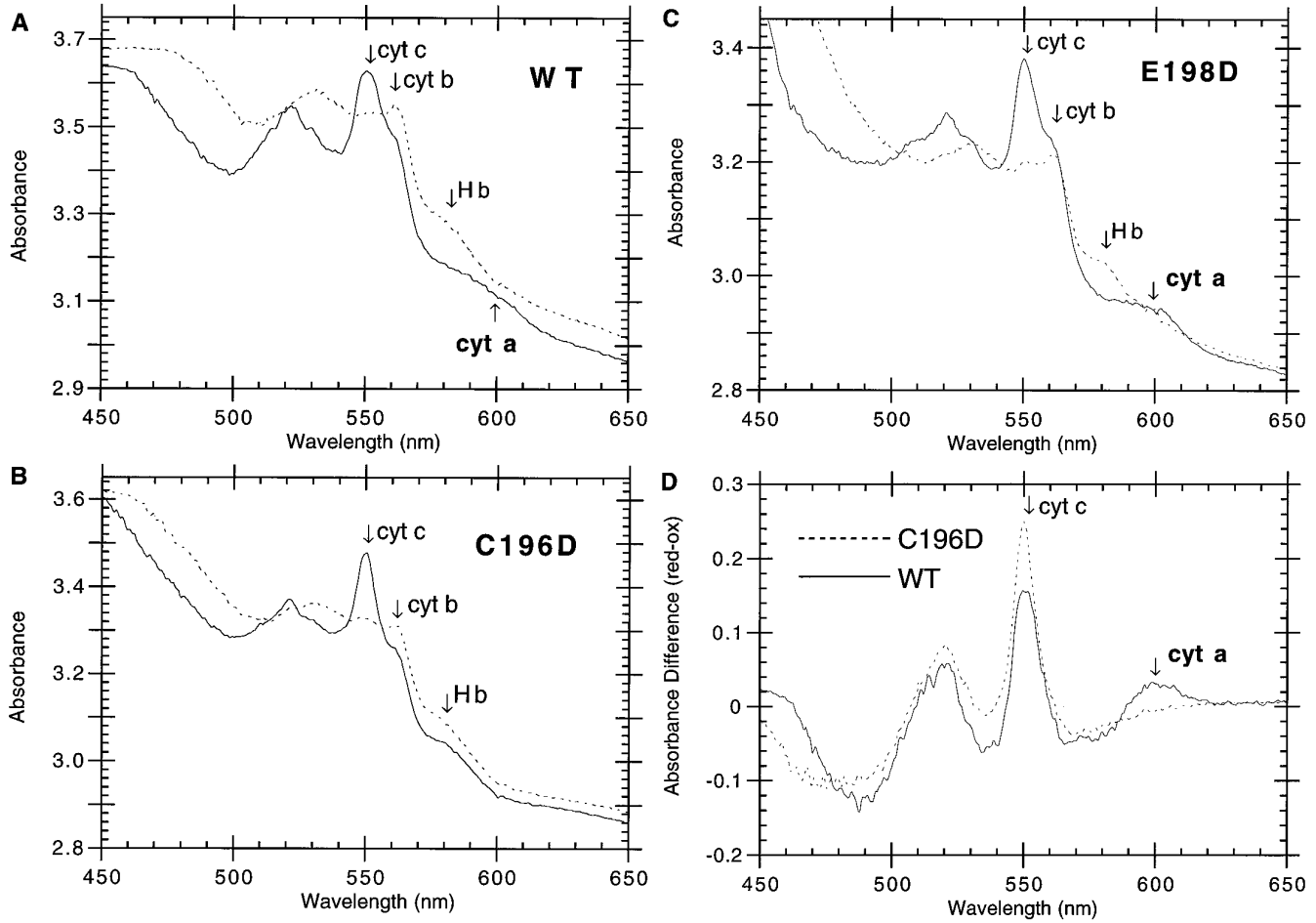

FIG. 3. ***In vivo* visible absorption spectroscopy.** Absorption spectra of concentrated suspensions of yeast cells were recorded at room temperature, in order to determine the presence of assembled cytochrome *a*. *A*, spectra of reduced and oxidized cell suspensions. To fully oxidize the samples, antimycin A was added prior to the addition of the oxidant H_2O_2 (*dashed line*); an excess of the reductant dithionite was then added to provide spectra of the reduced samples (*solid line*). *B*, similar spectra for a representative mutant, C196D. Other nonrespiring mutants showed similar spectra. *C*, spectra of a representative mutant, E198D, which shows reduced growth on ethanol/glycerol medium. *D*, reduced minus oxidized difference spectra of wild type and mutant cells clearly demonstrate the absence of an absorbance for cytochrome *a* in the mutant.

cific mutation in *cox2* was crossed to the *mit*$^-$ strain TF145 (*cox2*Δ), which lacks the *cox2* gene, but contains an otherwise competent mitochondrial genome. The ability of the resulting diploid to grow on ethanol/glycerol plates indicates a functional subunit II. Under this assay, all of the substitutions replacing the putative Cu_A ligands fail to grow on ethanol/glycerol medium at 30 °C. This is true even for the double mutant constructs which attempt to maintain a dithiolate, dihistidyl coordination at the Cu_A center. These results suggest that the specific arrangement of ligands to Cu_A is critical to copper coordination and/or to enzyme stability.

The absolutely conserved glutamic acid residue at position 198 lies immediately between the two Cys residues, within a proposed peptide loop. Modifications of Glu-198 to Asp, Gln, or His allow respiratory growth at 30 °C, indicating that a negative charge at this position is not essential for function. However, modification of Glu-198 to Arg allows only very weak respiratory growth, suggesting that the introduction of a large side chain and/or a positive charge interferes with function. Examining these effects more closely, in growth on ethanol/glycerol plates, the colony sizes of the His mutant are substantially reduced from those of wild type (although much larger than those of the Arg mutant), while the colony sizes of the Asp and Gln mutants were slightly reduced from wild type. To further characterize the effects of these mutations, the effect of growth temperature was examined. Mutations to Asp and Gln allow respiration at 15, 30, and 37 °C, while mutation to the potentially charged amino acids His and Arg allows respiration at 30 and 37 °C, but not at 15 °C.

Tests for Protein Assembly—To characterize mutant protein from constructs containing a nonfunctional mutation, TF189 *rho*$^-$ haploid cells were crossed to the wild type strain DL1, and nonrespiring diploids were selected. Since a novel restriction site was originally introduced or deleted along with each amino acid change, DNA hybridization of restriction digests allows for the confirmation of the successful recombinants. The observed change in the restriction fragment pattern of the diploid construct strongly suggests that the intended substitution is present. Finally, correct incorporation of the desired mutation was confirmed for every construct by directly sequencing the polymerase chain reaction amplified DNA fragment.

The visible absorption spectrum of wild type whole cells, shown in Fig. 3*A*, reveals three features corresponding to the reduced alpha bands of cytochromes *a*, *b*, and *c*, centered near 600, 560, and 550 nm, respectively. In order to more clearly distinguish changes in these features in mutant strains, reduced minus oxidized difference spectra were obtained. To obtain the oxidized spectrum, electron transfer from the cytochrome bc_1 complex was first inhibited by the addition of antimycin A, and cells were then oxidized by addition of H_2O_2. To obtain the spectrum of the reduced enzyme, dithionite was then added to cells, and a second spectrum was measured (the

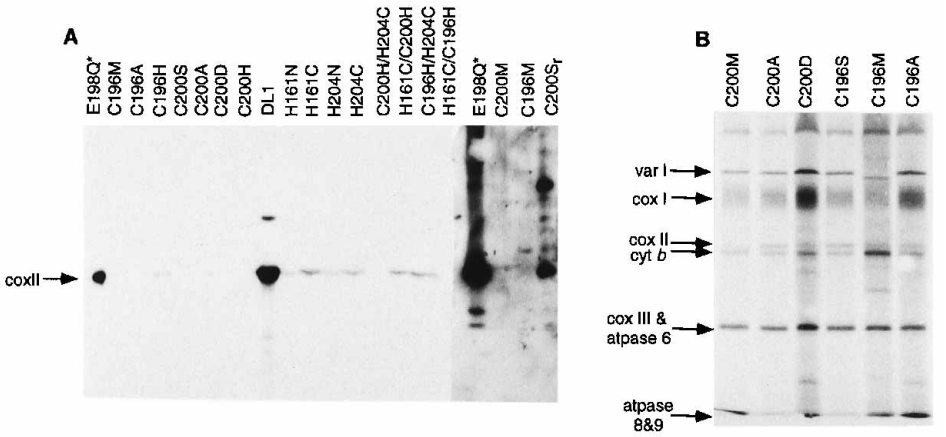

FIG. 4. **Immunolabeling and [^{35}S]Met labeling of mitochondrial translation products.** *A*, for wild type and various mutant constructs, a monoclonal antibody was used to detect accumulation of subunit II of cytochrome *c* oxidase in mitochondrial extracts (see text) of yeast grown to saturation in galactose medium. To verify that the mutation did not disrupt the monoclonal epitope, a polyclonal antibody was also used as a probe (*last four lanes*). *E198Q** represents purified enzyme from the E198Q mutant. *C200S$_r$* and *DL1* represent wild type yeast strains. *B*, to assess the level of synthesis of subunit II, mitochondrial translation products were selectively labeled with [^{35}S]Met in the presence of the cytoplasmic translation inhibitor, cycloheximide. In each case, mitochondrial extracts were run on a 12.5% SDS-polyacrylamide gel.

order of treatment was not found to be critical). Whole cell spectra of all yeast strains containing mutations to proposed His and Cys ligands to Cu_A show the absence of an absorbance at 600 nm and the presence of a new peak (see below) around 580 nm (*e.g.* Fig. 3*B*). The difference spectra of reduced minus oxidized whole cells shown in Fig. 3*D* clearly identify the presence of the 600-nm absorbance in wild type cells and the lack of such an absorbance at 600 nm in the mutants targeting Cu_A ligands. In contrast, in mutants substituting Glu-198 by Asp, Gln, His, and Arg, the 600-nm absorbance is present (an example is shown in Fig. 3*C*). The obvious lack of a 600-nm band for the nonfunctional mutants demonstrates that cytochrome *c* oxidase is not assembling properly.

The absorption band at 580 nm (denoted *Hb* in Fig. 3*A*) observed in the spectra of the oxidized mutants also occurs in H_2O_2-oxidized wild type cells and has been attributed to yeast hemoglobin (40, 41). It appears in the spectra of all respiration-deficient strains, including those that lack subunit II completely (data not shown), those containing a nuclear mutation which disrupts respiration (42), and in strains with a mutation in the cytochrome bc_1 complex (43).

To assess the stable accumulation of hemes (specifically heme *a*), pyridine hemochromogen assays were performed on whole cell extracts from 10 of the mutants. The pyridine hemochromogen assay extracts the porphyrins and replaces the *in vivo* axial ligands of the hemes with the strong field ligand pyridine, resulting in a well defined environment independent of the original protein environment. In this assay, the absorption maxima for cytochromes *b* and *c* coincide near 550 nm, while heme *a* is characterized by an absorption at 587 nm. None of the respiration-deficient mutants examined has a detectable absorption maximum at 587 nm (data not shown).

Tests for Protein Expression and Intracellular Stability—The above results indicate that in the respiration-deficient mutants, fully assembled cytochrome *c* oxidase is not accumulating to normal cellular levels. In order to determine whether the cytochrome oxidase mitochondrial subunits are efficiently expressed in each construct, *in vivo* [^{35}S]Met labeling of mitochondrial translation products was carried out. The results presented in Fig. 4*B* show that subunits I, II, and III of cytochrome *c* oxidase are correctly synthesized in the constructs examined, including mutant constructs which show no accumulation of cytochrome *a*.

To determine whether these expressed subunits are stably accumulating in the cell, crude mitochondrial extracts were probed with antibodies specific to subunit II. Using a monoclonal antibody, all mutant constructs which do not respire show a substantial underaccumulation of subunit II compared to wild type. As shown in Fig. 4*A*, following a 1-h digestion of the cell wall with lyticase in the absence of the protease inhibitor phenylmethylsulfonyl fluoride, levels of subunit II are greatly reduced for the mutant cells compared to wild type. A shorter lyticase treatment (10 min) in the presence or absence of phenylmethylsulfonyl fluoride (data not shown) yields higher levels of subunit II (but still reduced 10-fold relative to wild type), suggesting that the mutant proteins are less stable. Similar analyses of C196M and C200M using *polyclonal* antibodies to subunit II confirm this lack of accumulation and further verify that the lack of immunolabeling is not the result of direct modification of the antigenic determinant for the monoclonal antibody to subunit II. These results further support a model in which the precise structure of the Cu_A site is required for the stability of the overall enzyme complex.

DISCUSSION

An understanding of the structural environment around the Cu_A center in cytochrome *c* oxidase has remained elusive, despite the availability of a wealth of spectroscopic and sequence conservation data. Spectroscopic evidence has unambiguously identified at least one cysteine sulfur ligand to the copper ion(s) within the site, with strong arguments for a second cysteine ligand (14). Sequence analyses place the cysteines within a highly conserved region near the C terminus of subunit II, shown below (numbering in this manuscript follows that of the bovine enzyme).

Bovine: His^{161}-...-Gln-Cys^{196}-Ser-Glu^{198}-Ile-Cys^{200}-Gly-Ser-Asn-His^{204}-Ser-Phe-Met^{207}

Yeast: His^{174}-...-Ala-Cys^{221}-Ser-Glu^{223}-Leu-Cys^{225}-Gly-Thr-Gly-His^{228}-Ala-Asn-Met_{232}

Similarly, coordination by at least one His imidazole has been demonstrated (14, 15), and spectroscopic arguments for two are very compelling (16). In the current work, site-directed mutagenesis in yeast (the same system used in the spectroscopic studies of isotopically substituted protein) has been used to probe these four proposed ligand residues within subunit II. In

addition, the importance of the conserved Glu-198, which lies directly between the two Cys residues, has been directly probed. The results provide constraints on the protein structure at this critical metal site.

Mutations at the Cu_A Center Disrupt Protein Folding—To probe their role in copper coordination and enzyme function, each of Cys-196 and Cys-200 has been replaced by a variety of amino acids. Single-site substitutions of Cys by His, Met, Ser, Ala, and Asp were intended to provide an alternate coordination of copper, or to disrupt copper coordination while maintaining a minimal structural perturbation. Similarly, each of His-161 and His-204 has been replaced by potential copper ligands Cys and Asn. Finally, the potential copper ligand Met-207 has been replaced by Cys. Results presented here indicate that, for each mutation of these potential Cu_A ligands, the protein is either not assembling properly or is unstable within the cell. Labeling of mitochondrial translation products with [^{35}S]Met verifies the translation of subunit II in amounts comparable to those observed in wild type; however, immunolabeling indicates a dramatic underaccumulation of the subunit II polypeptide. Optical measurements *in vivo* indicate the lack of formation of a native-like heme environment around cytochrome *a*, verifying the conclusion that stably folded enzyme fails to accumulate in these constructs. Taken together, these results indicate that, although the expression of subunit II has not been affected by these mutations, the accumulation of a stably folded cytochrome *c* oxidase has been substantially reduced for direct mutations to the Cu_A site.

Mutants Targeting Glu-198 Retain Some Function—Various lines of evidence identify Cu_A as the primary site of entry of electrons accepted from cytochrome *c* (5, 44, 45). Labeling studies using the water-soluble 1-ethyl-3-[3-[^{14}C](trimethylamino)]-propyl carbodiimide have implicated Glu-198, closely situated in the primary sequence between the two conserved cysteines, as a surface-exposed participant in the binding site for cytochrome *c* (13, 28). Structural studies of cytochrome *c* and its physiological partner cytochrome *c* peroxidase show interactions between positively charged amino acids on the surface of cytochrome *c* and negatively charged amino acids on cytochrome *c* peroxidase (46). Mutation of Asp-37 on the surface of cytochrome *c* peroxidase and within the binding patch for cytochrome *c* results in an order of magnitude decrease in the rate of electron transfer from cytochrome *c* (47). The result that the mutation of Glu-198 to Asp or to neutral amino acids in cytochrome *c* oxidase weakens (but does not abolish) respiration, is consistent with previous proposals placing Glu-198 (and therefore Cu_A) at the cytochrome *c* binding site. These data are also consistent with recent studies in the enzyme from *P. denitrificans* in which the mutation E198Q (246 in the *Paracoccus* numbering) reduces the rate of electron transfer from cytochrome *c* less than 2-fold in the water-soluble Cu_A-containing fragment (23) and reduces the steady-state K_m/k_{cat} about 10-fold in the steady-state assay in the intact enzyme (22).

Implications for the Structure of the Cu_A Site—Models for the conserved Cys-containing sequence have folded this region either as α-helical, with the two Cys ligands to Cu_A on one face of the helix and Glu-198 on the other (14, 48), or more likely, as a loop joining two sheet structures, as in the blue copper family of proteins (20, 49, 50). Either model places Glu-198 on the surface of subunit II, in close proximity to the Cu_A center. Consistent with the current results, neutral and even positively charged substitutions of Glu-198 on the surface of the subunit would not completely disrupt protein folding. In contrast, for the nearby Cys and His amino acids that are proposed to coordinate directly to copper within the protein interior, even fairly conservative amino acid substitutions (*e.g.* Cys → Ser) disrupt completely the functioning of the enzyme. It appears that the structure and/or function of the Cu_A site is highly dependent on a precise coordination environment.

The Cu_A center has been compared to the blue copper class of isolated copper sites (13, 50) and, more recently, to a binuclear center found in nitrous oxide reductase (25, 51, 52). In similar studies of blue copper proteins, primary ligands to the copper have been independently mutated (53–55). Many substitutions which are not tolerated in the Cu_A center (*e.g.* Cys → Asp) nevertheless allow stable folding of the blue copper proteins. The sensitivity of the Cu_A center to such semiconservative substitutions may be expected for a more complex binuclear structure. Finally, mutagenesis has been carried out in a peptide fragment engineered to restore a Cu_A fold into a homologous protein (19, 20). As for azurin, most of these mutations result in a folded structure which binds copper, although typically more weakly than wild type. The mutations of proposed ligands to the native eukaryotic Cu_A site presented here do not allow accumulation of folded protein. This most likely results from effects on cytochrome *c* oxidase assembly or stability in the cell.

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Note Added in Proof—The crystal structures of cytochrome *c* oxidase from bovine heart (Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) *Science* **269,** 1069–1074) and from the bacterium *P. denitrificans* (Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature* **376,** 660–669) have recently been solved at 2.8 Å resolution. The structures confirm that the Cu_A center is binuclear and that the coppers are coordinated by Cys-196, Cys-200, His-161, His-204, Met-207, and the backbone carbonyl of Glu-198 (bovine numbering). The side chain carboxylate of Glu-198 lies buried at the interface between subunits I and II, far from the surface of the protein, and coordinates a Mg(II) ion at the subunit interface. The Mg(II) ion is located along a potential electron transfer path from Cu_A to the heme edge of cytochrome a_3.

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