Water-Soluble, Recombinant Cu_A-Domain of the Cytochrome ba_3 Subunit II from *Thermus thermophilus*†

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ABSTRACT: Recently, the genes of cytochrome ba_3 from *Thermus thermophilus* [Keightley, J. A., et al. (1995) *J. Biol. Chem. 270*, 20345−20358], a homolog of the heme-copper oxidase family, have been cloned. We report here expression of a truncated gene, encoding the copper A (Cu_A) domain of cytochrome ba_3, that is regulated by a T7 RNA polymerase promoter in *Escherichia coli*. The Cu_A-containing domain is purified in high yields as a water-soluble, thermostable, purple-colored protein. Copper analysis by chemical assay, mass spectrometry, X-ray fluorescence, and EPR spin quantification show that this protein contains two copper ions bound in a mixed-valence state, indicating that the Cu_A site in cytochrome ba_3 is a binuclear center. The absorption spectrum of the Cu_A site, free of the heme interference in cytochrome ba_3, is similar to the spectra of other soluble fragments from the aa_3-type oxidase of *Paracoccus denitrificans* [Lappalainen, P., et al. (1993) *J. Biol. Chem. 268*, 26416−26421] and the caa_3-type oxidase of *Bacillus subtilis* [von Wachenfeldt, C., et al. (1994) *FEBS Lett. 340*, 109−113]. There are intense bands at 480 nm (3100 M^{-1} cm^{-1}) and 530 nm (3200 M^{-1} cm^{-1}), a band in the near-IR centered at 790 nm (1900 M^{-1} cm^{-1}), and a weaker band at 363 nm (1300 M^{-1} cm^{-1}). The visible CD spectrum shows a positive-going band at 460 nm and a negative-going band at 527 nm, the opposite signs of which may result from the binuclear nature of the site. The secondary structure prediction from the far-UV CD spectrum indicates that this domain is predominantly β-sheet, in agreement with the recent X-ray structure reported for the complete *P. denitrificans* cytochrome aa_3 molecule [Iwata, S., et al. (1995) *Nature 376*, 660−669] and the engineered, purple CyoA protein [Wilmanns, M., et al. (1996) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11955−11959]. However, the thermostability of the fragment described here ($T_m \approx 80$ °C) and the stable binding of copper over a broad pH range (pH 3−9) suggest this protein may be uniquely suitable for detailed physical-chemical study.

Cytochrome *c* oxidases catalyze the four-electron reduction of dioxygen to water in the terminal step of aerobic respiration in eukaryotic organisms and some bacteria [for reviews, see Babcock and Wikström (1992) and Trumpower and Gennis (1994)]. Electrons enter the complex from the outside of the energy-transducing membrane through four consecutive bimolecular electron transfers from ferrocytochrome *c*. The subsequent reduction of dioxygen is coupled to the uptake of four protons from the mitochondrial matrix in eukaryotes or the cytoplasm in bacteria. In addition to the pH gradient generated across the membrane due to dioxygen reduction, redox free energy is used to pump four additional protons per dioxygen reduced to the opposite side of the membrane. The proton gradient is then used by ATP synthase to produce ATP, a process originally described in Mitchell's chemiosmotic theory (Mitchell, 1961).

The simplest oxidases, found in bacteria, perform these functions with only two or three subunits; subunits I, II, and III of the more complex mammalian oxidases are homologous to these simple bacterial oxidases, indicating that these three subunits contain the functional core of the enzyme (Saraste, 1990; Keightley et al., 1995). The three-dimensional structures of the cytochromes aa_3 from *Paracoccus denitrificans* (Iwata et al., 1995) and bovine mitochondria (Tsukihara et al., 1995) confirm the suspected homology between bacterial and mitochondrial oxidases. Heme-copper oxidases are classified according to the types of bound hemes which are present (Trumpower & Gennis, 1994). Detailed electron transfer studies on the aa_3-type oxidases, which possess a cytochrome *a* and a cytochrome a_3-Cu_B redox center located on subunit I and a Cu_A site on subunit II, have shown that the Cu_A site is the initial electron acceptor from cytochrome *c* (Pan et al., 1993; Brzezinski et al., 1995). Protein labeling (Bisson et al., 1982) and mutagenesis studies (Lappalainen et al., 1995) have localized a portion of the cytochrome *c* binding site to the Cu_A domain of subunit II. Additionally, several acidic residues on subunit III of *P. denitrificans* aa_3 oxidase are correctly positioned to interact with the lysines of cytochrome *c*, suggesting that the complete cytochrome *c* binding crevice is formed by subunits I, II, and III (Iwata et al., 1995). The electron on Cu_A is then transferred to cytochrome *a*, which is in rapid redox

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equilibrium with Cu_A (Oliveberg & Malmström, 1991). This electron is subsequently transferred to the bimetallic cytochrome a_3-Cu_B active site, where dioxygen is bound and reduced [Oliveberg et al., 1989; see Wikström and Babcock (1992) for review].

The thermophilic eubacterium *Thermus thermophilus* utilizes at least two cytochrome *c* oxidases, a caa_3- and a ba_3-type, depending on the growth conditions and oxygen availability (Fee et al., 1986; Zimmermann et al., 1988; Keightley et al., 1995). The as-isolated cytochrome ba_3 protein is much simpler than the caa_3 oxidase (Fee et al., 1993); it lacks subunit III, has a shortened subunit II and a longer subunit I (Keightley et al., 1995), and utilizes a *b* heme in the low-spin heme location (Zimmermann et al., 1988). While most subunits II of heme-copper oxidases have two predicted transmembrane helices at the N-terminal region which anchor the monomer to the membrane, cytochrome ba_3 has only one (Keightley et al., 1995). The conserved Cu_A motif, containing two histidines, two cysteines and one methionine, is found toward the C-terminal end of subunit II. This part of the protein projects into the periplasmic space and apparently represents an independent folding domain. By analogy to the more thoroughly characterized aa_3-type oxidases, the Cu_A site is believed to function as the primary electron acceptor of electrons from cytochrome *c* (Keightley et al., 1995).

The traditional view of Cu_A described the site as a mononuclear redox center, similar to the blue copper proteins (Martin et al., 1988). Recently, the C-terminal portion of subunit II from the *P. denitrificans* aa_3 (Lappalainen et al., 1993) and the *Bacillus subtilis* caa_3 (von Wachenfeldt et al., 1994) oxidases has been expressed. The optical spectra of these soluble fragments, free of the heme spectral interference which dominates the spectra of oxidases, clearly demonstrates that the Cu_A site is quite dissimilar to more thoroughly characterized blue copper sites. Unlike the absorption spectra of the blue copper proteins, which are dominated by a ligand-to-metal charge transfer (LMCT) band near 600 nm [for review, see Solomon et al. (1992)], the Cu_A absorption spectrum has prominent bands at 480 and 530 nm and less intense bands centered at 360 and 790 nm.

A copper binding site similar to that of Cu_A is also found in nitrous oxide reductase (N_2OR),[1] which catalyzes the conversion of N_2O to N_2 in denitrifying bacteria. Antholine et al. (1988) were able to resolve a seven-line hyperfine pattern in the S-band EPR spectra of N_2OR, consistent with a two copper, mixed-valence site. They further proposed that the Cu_A site found in the cytochrome oxidases is binuclear. However, the EPR data for the bovine cytochrome *c* oxidase are ambigious, because direct observation of the Cu_A signal is hindered by interference from the low-spin cytochrome *a* signal. Soluble fragments of Cu_A have permitted EPR characterization of the Cu_A site without the complication of the heme signal and have yielded data which are suggestive of the N_2OR results. The subunit II domains expressed so far have, however, the complications either of containing a significant amount of type 2 copper (van der Oost et al., 1992; von Wachenfedt et al., 1994) or of being unstable to moderate changes in solution conditions (Lappalainen et al., 1993). For these reasons, we wanted to express a Cu_A domain from a thermostable bacterium in an attempt to find a system which would be more favorable for biophysical studies. For example, a more stable, soluble fragment would allow detailed electron transfer and electrochemical studies, thus providing data that could enhance the understanding of the functional role of Cu_A. For example, a more stable, soluble fragment would allow detailed electron transfer and electrochemical studies (cf. Slutter et al., 1996).

MATERIALS AND METHODS

Bacterial Strains. *Escherichia coli* strain DH10B from Gibco BRL (Gaithersburg, MD) [genotype: F^- *mcr*A ϕ(*mrr-hsd*RMS-*mcr*BC) j80d*lac*ZϕM15 ϕ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 ϕ(*ara*, *leu*)7697 *gal*U *gal*K l^- *rps*L *nup*G] (Grant et al., 1990) was used for the ligation and sequencing steps. *E. coli* strain BL21(DE3) from Novagen (Madison, WI) [genotype: F^- *omp*T *hsd*S_B(r_B^- m_B^-) *gal dcm* (DE3)] (Studier & Moffatt, 1986) was used for the expression of the Cu_A fragment. *T. thermophilus* HB8 (No. 27634) was obtained from American Type Culture Collection (Rockville, MD).

Construction of pETCu$_A$. *T. thermophilus* genomic DNA was prepared using preparative columns (Qiagen, Chatsworth, CA), and the gene fragment of the cytochrome ba_3 subunit II gene was amplified using PCR methods. Oligonucleotides used in the PCR reaction were synthesized at the Microchemical Facility at the California Institute of Technology using an Applied Biosystems 380B DNA synthesizer. The sense oligonucleotide primer, 5′-d(CTTCGTCTTCATCGCCCAT**ATG**GCCTACA)-3′, contained an *Nde*I site (underlined), the start codon (bold type), and the N-terminal portion of the Cu_A fragment. The antisense oligonucleotide primer, 5′-d(TTGCGCGCACCGGGATCCT**TCA**CTCCTTCA)-3′, contained a *Bam*HI site (underlined), the stop codon (bold type), and the C-terminal portion of the fragment. The PCR reaction was optimized using the PCR Optimizer Kit (Invitrogen, San Diego, CA). Buffer J (5× working concentration: 300 mM Tris-HCl, 75 mM $(NH_4)SO_4$, 10 mM $MgCl_2$, pH 9.5, at 22°C) yielded the largest amplification in 30 cycles and was used to prepare the inserts. The PCR fragment was extracted once with one volume of chloroform to remove any traces of mineral oil, washed twice with 2 mL of 3 M sodium acetate, pH 5.3, in a Centricon 100 (Amicon, Beverly, MD) to remove the *Taq* polymerase, nucleotides, and PCR buffer from the sample, and washed twice with doubly distilled H_2O. After digesting the PCR fragment and pET9a vector (Novagen, Madison, WI) with *Nde*I and *Bam*HI, each was purified on a NuSieve (FMC, Rockland, MA) low melting temperature gel. The fragments were cut from the gel, melted at 70 °C, and equilibrated at 45 °C, and the agarose was then digested with Gelase (Epicenter, Madison, WI). This preparation was ligated into the *Nde*I/*Bam*HI fragment of pET9a to give the vector pETCu$_A$. The construct was sequenced with *Taq* polymerase and an Applied Biosystems DNA sequencer by Harold Kochounian at the DNA Sequencing Facility, Kenneth Norris Jr. Comprehensive Cancer Center, at the University of Southern California.

Expression and Purification of the Soluble Cu$_A$ Domain. Ten milliliters of culture medium (LB and 50 mg/mL

[1] Abbreviations: BCA, bicinchoninic acid; BCS, bathocupreine sulfonate; Cu_A, copper A; ICP-MS, induction coupled plasma mass spectrometry; N_2OR, nitrous oxide reductase; TCA, trichloroacetic acid; TXRF, total-reflection X-ray fluoresence.

kanamycin) was innoculated from a freshly streaked plate of BL21(DE3) cells containing the pETCuA plasmid. After incubation overnight at 37 °C, this culture was used to inoculate a l L flask of LB and 50 mg/mL kanamycin. This culture was incubated at 37 °C, typically for about 2 h, until the A_{600} reached 0.4−0.6, and induced for 4−12 h using a final concentration of 0.4 mM IPTG. The cells were pelleted by centrifugation at 5000*g* for 5 min. (At this point, the cell pellet can be frozen for future use.) The pellet from l L of culture was resuspended in 25 mL of 50 mM Tris-HCl, pH 8.0, 4 mg/ml lysozyme, 40 units/mL DNase I, 3 units/mL RNase A, and 0.1% Triton X-100. PMSF to a final concentration of 2 mM to inhibit proteolysis was added, and the extract was incubated at 30 °C for at least 30 min. The cell debris was separated from the extract by centrifugation at 12000*g* for 15 min at 4 °C. One volume of 50 mM sodium acetate, pH 4.6, was added to decrease the pH and 15 μL/mL volume of 100 mM Cu(II)$(His)_2$ was added to form the Cu_A site. At this point, the solution turned purple and some precipitation occurred. Heat treatment at 65 °C for 10 min resulted in additional formation of color and additional precipitation of cell debris. This precipitate was pelleted by centrigufation at 12000*g* for 30 min. The pH of the supernatant was readjusted to 4.6 with 50% acetic acid and incubated an additional 30 min on ice, and additional precipitate was removed by a second 30-min centrifugation at 12000*g*. The supernatant, which has a distinct purple color, was loaded onto a CM-Sepharose gravity column that had been equilibrated with 50 mM sodium acetate, pH 4.6, at 4 °C, washed with several column volumes of equilibration buffer, and eluted with a 0−1 M NaCl gradient in 50 mM sodium acetate. Fractions containing the purple colored eluate were combined and dialyzed against 25 mM ammonium succinate, pH 4.6, at 4 °C prior to being lyophilized. The dried protein was stored until required or taken up in ~2 mL of doubly distilled H_2O per liter of original culture and stored frozen. Treated thusly, the chromophore appears to be stable indefinitely.

Protein Analyses. Protein concentrations were regularly measured using the BCA protein assay kit from Pierce (Rockford, IL). Quantitative amino acid analyses and N-terminal sequencing were carried out at the University of New Mexico Protein Chemistry Facility as described by Pastuszyn in Keightley et al. (1995). SDS−PAGE was carried out with minor modifications according to the method of Downer et al. (1976) using a Bio-Rad Mini-PROTEAN II electrophoresis cell (Hercules, CA). Nondenaturing gel electrophoresis was carried out using the same apparatus according to the method of Gabriel (1972). Thin layer isoelectric focusing was carried out using precast gels (FMC Isogel, pH range 3−7) in a Bio-Rad Bio-Phoresis horizontal electrophoresis cell. Protein samples were subjected to electrospray ionization mass spectrometry after removal of sodium ions by passage over a prepoured Pharmacia PD-10 column (Alameda, CA) equilibrated with 10 mM ammonium acetate. ESI-MS was carried out at the Scripps Research Institute Mass Spectrometry facility using a Perkin-Elmer SCIEX API III mass analyzer (Irvine, CA) with the orifice potential set at 100 V (Siuzdak, 1994). The GCG (Devereux et al., 1984) program, PEPTIDESORT, was used to calculate expected properties of the protein from its amino acid composition.

Copper Analyses. Copper was released from the protein by precipitation and heating at 40 °C for several minutes in 4.4% TCA. Protein was removed by centrifugation, and copper analyses were performed on the supernatant using the bathocupreine sulfonate (BCS) method, as described by Broman et al. (1962). More precise measurements of total copper were carried out by first denaturing the protein in a solution of 60% HCOOH, 30% 2-propanol, and 10% water (hereafter called denaturing solvent), adding a trace of H_2O_2, and then measuring the total Cu(II) by quantification of its EPR spectrum relative to a known standard at 77 K. Additional quantification of copper and other elements was obtained by induction coupled plasma mass spectrometry (ICP-MS) and by total-reflection X-ray fluoresence (TXRF) analysis (Pettersson & Wobrauschek, 1995). The ICP-MS spectra were recorded at the Scripps Institution of Oceanography, and the TXRF spectra were recorded at Chalmers Tekniska Högskola.

EPR Spectra. Spectra were recorded at liquid nitrogen temperatures using Bruker ER 200D-SRC X-band spectrometers either at the Scripps Research Institute in La Jolla or in Göteborg. Signals were quantified as described by Aasa and Vänngard (1975) using Cu(II) in 2 M $NaClO_4$ at pH 2 as reference. Cytochrome ba_3 was obtained by the method of Keightley et al. (1995) and was maintained in solution with a Tris-EDTA buffer containing 0.1% Triton X-100.

Optical Absorption and Circular Dichroism Spectra. Optical spectra of the Cu_A fragment were recorded on a Hewlett Packard 8452A Diode Array or SLM/AMINCO model DB3500 spectrophotometers in 1-cm cells. The azurin spectrum was taken on a Cary 4 UV−visible spectrophotometer. The protein concentrations used were 150 μM Cu_A domain or 100 μM azurin. Circular dichroism spectra were recorded on a Jasco J720 spectropolarimeter at 20 °C over the wavelength range 260−185 nm. A 1-mm path length cell was used, and the cell compartment was continuously flushed with N_2. The total absorbance of the protein (0.05 mg/mL) and buffer (0.5 mM potassium phosphate buffer, pH 7.4) in the sample never exceeded 0.6 over the wavelength range used. All spectra are the average of 10 recordings. The melting temperature of the fragment was measured by recording the change in CD signal at 218 nm using a Model 62A DS Aviv CD polarimeter equipped with a specialized thermoelectric device that permitted the sample temperature to be stepped, in this case, at intervals of 0.5 °C from 40 to 120 °C. The sample is retained in a sealed cuvette having a pathlength of 1 mm. Visible region CD data were collected at 20 °C over the wavelength range 310−700 nm in a 1-cm cell. The samples contained 0.25 mg/mL Cu_A in 0.5 mM potassium phosphate buffer, pH 7.4. The absorption of the far-UV CD samples was measured against air in the same 1-mm cell used to collect the CD data.

Secondary Structure Prediction. The computer program used to estimate the amount of secondary structure from CD data compares the CD data to that of 22 proteins with known structures (Hennesy & Johnson, 1981; Johnson, 1990). To improve the accuracy of this algorithm, the homologous blue copper proteins azurin and plastocyanin have been included in this set of reference spectra. The statistical method of variable selection (Weisberg, 1985) was used to select the reference proteins most resembling the sample proteins in secondary structure.

RESULTS

Expression and Purification of the Thermus Cytochrome ba_3 Cu_A Domain. The nucleotide sequence encoding the first 32 N-terminal residues of subunit II of cytochrome ba_3 (Keithtley et al., 1995) was removed by inserting a methionine start codon before Ala-32. This truncation disrupts a highly hydrophobic region (residues 18−38) that is presumed to form a helix anchoring subunit II to the membrane. Consequently, the remaining C-terminal portion of subunit II should contain only the soluble Cu_A domain. Including the start codon, the translated N-terminal amino acid sequence of the gene is MAYTLAT-extending to the C-terminus (-GTIVVKE). The expected soluble protein fragment is 136 amino acids long with a molecular weight of 14 936. Because there are no signal sequences designed into the gene sequence, expression is directed to the cytoplasm.

Upon induction with IPTG, the cells produce only the apo form of the protein, even when grown in the presence of ~1 mM Cu(II)$(His)_2$. However, after cell lysis, the addition of Cu(II), usually as the bishistidine complex although other complexes work as well, causes the cell extract to become purple.[2] The very simple purification procedure described in Materials and Methods typically yields ~30 mg of pure holo protein per liter of culture medium.

It is also possible to prepare purified apoprotein by omitting the addition of Cu(II), and this can be done even when Zn(II)$(His)_2$ is added to the cell lysate. Interestingly, ICP-MS analyses of purified apoprotein showed that no metals were bound to the final product. This is an unexpected result given that overexpression of blue copper proteins, azurin for example, tends to yield a mixture of apoprotein, zinc protein, and holoprotein. Holoprotein can be obtained from purified apoprotein by adding an excess of Cu(II)$(His)_2$ (or other Cu complexes); however, prior exposure to air can destroy the ability to bind Cu(II). The latter can be partially reversed by treatment with β-mercaptomethanol,[3] suggesting that the formation of a disulfide in the active site prevents Cu (II) binding.

Protein Characterization. SDS−PAGE analysis (Figure 1A) shows a single protein band of very high purity with an apparent M_r ~15 000. This is consistent with a predicted M_r of ~14 800 (see below). Similarly, Figure 1B shows an electrophoresis gel run under nondenaturing conditions. This gel is significantly overloaded and shows several minority bands. However, densitometry scans of several gels indicate that the protein preparations are >95% pure. A thin-layer isoelectric focusing gel (Figure 1C) illustrates that the cupration reaction may not always proceed to completion. The upper band (pI = 6.0) corresponds to fully cuprated holoprotein, while the lower (pI = 6.2) corresponds to the metal-free apoprotein; the program PEPTIDESORT predicts that the pI of the apoprotein should be 6.1. By following the preparative procedure described in Materials and Methods, and as evidenced by the absence of the apoprotein band in IEF gels (not shown), most of our preparations are free of apoprotein.

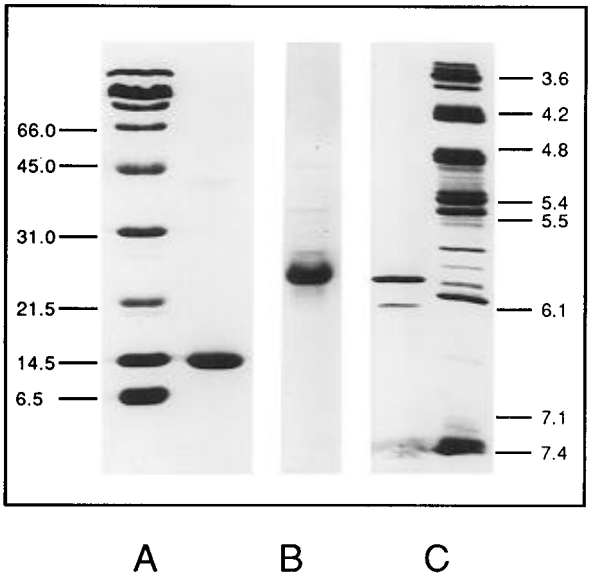

FIGURE 1: Electrophoretic properties of purified ba_3-Cu_A protein. (A) SDS−PAGE: Total acrylamide concentration was 15% and the ratio to bisacrylamide was 1:37.5. Protein was denatured in 0.35% SDS, 5% β-mercaptoethanol, 2% glycerol, and 6.25 mM Tris-HCl at 95 °C for 5 min. The gel was fixed and stained in a 45% methanol, 45% water, and 10% acetic acid solution containing 0.25% Coomassie Brilliant Blue R250 from Bio-Rad. The right lane contained 10 μg of purified Cu_A protein, while the left lane contained a total of 25 μg of the following molecular weight standards: serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500), lysozyme (14 400), and aprotinin (6500). (B) Nondenaturing PAGE: The stacking gel was 3.5% and the running gel was 15% acrylamide, and the bisacrylamide/acrylamide ratio was 1:37.5 in 20% glycerol and 50 mM Tris-HCl at pH 6.8; 20 μg of protein was used, and the gel was fixed and stained as described in panel A. (C) Thin layer gel isoelectric focusing, pH 3−7. In the right lane, standards and their isoelectric points are amylglucosidase (3.6), glucose oxidase (4.2), ovalbumin (4.8), β-lactoglobulin (A, 5.4; B, 5.5), carbonic anhydrase (6.1), and myoglobin (minor,7.0; major, 7.4); 10 μg of CuA protein was used in the left lane. The holo-Cu_A protein has a pI_{obs} of 6.0 while the apoprotein has a pI_{obs} = 6.2.

Our initial preparations of the Cu_A protein contained several different N-terminal sequences in varying amounts: TLATHTAGVIPA, THTAGVIPA, and GVIPA, indicating that proteolysis was occurring. After addition of PMSF during cell lysis, however, a homogeneous sample with the experimentally determined N-terminal sequence AYTLATH was obtained. The resulting protein should thus be 135 amino acids in length. Facile removal of the formyl-Met (Miller, 1987) and subsequent N-terminal proteolysis suggest this region of sequence may not be tightly packed in the soluble domain. Table 1 presents a comparison of the

[2] During overexpression of this fragment, purple-colored membranes were also found in the cell pellet, suggesting that the fragment still retained some hydrophobic residues. Because this hydrophobic patch may cause problems with self-association in solution, and our early work indicated that this region is sensitive to proteases and therefore less structured than the C-terminal portion, we constructed a second fragment with a larger portion of this region removed. The predicted N-terminal sequence of this fragment is MVIPAG, which is 10 residues shorter than the original construct and corresponds to the most degraded protein fragment which was isolated in the original protein preparation minus the first glycine residue. During purification, there are actually two purple colored fractions, one of which is N-terminally blocked, presumably with an N-formyl group (Miller, 1987) while the other has the N-terminal sequence VIPAG.

[3] The apoprotein was regenerated by reduction with β-mercaptoethanol followed by gel filtration on a PD-10 column equilibrated with 50 mM Tris-HCl, pH 8, and 10 mM $CuSO_4$. The colorless, reduced apoprotein immediately formed a distinct purple band upon entering the column. Initial attempts to regenerate the apoprotein without Cu(II) on the column were unsuccessful, as the time required to elute the protein from the column is sufficient for complete reoxidation.

Table 1: Expected and Observed Composition of the Cytochrome ba_3-Cu_A Soluble Domain

amino acid	expected[a]	observed[b]
Asp[c]	9	11.5
Glu[d]	17	17.2
Ser	2	2.5
Gly	13	14.0
His	4	3.6
Arg	5	5.1
Thr	12	12.1
Ala	10	10.6
Pro	11	10.6
Tyr	7	6.6
Val	17	14.6
Met	1	0.2[e]
Cys	2	ND[f]
Ile	9	7.0
Leu	5	5.7
Phe	6	6.0
Lys	4	4.3
Trp	1	ND
Total	135	(121.0)[g]

[a] Based on the gene sequence from Keightley et al. (1995). [b] Determined experimentally as described in Materials and Methods. The deviation in three different experiments was ~±10%. [c] Asn + Asp. [d] Gln + Glu. [e] In three separate analyses, this value ranged from 0.2 to 1.7. The value reported here is unique to this measurement. [f] Not determined. [g] Not corrected for nondetermined amino acids.

observed amino acid composition with that predicted from translation of the gene sequence; they are, within error, indistinguishable.

Further evidence that the cloned DNA fragment encodes the desired protein was obtained from ESI-MS experiments. The predicted mass of the apoprotein having the N-terminal sequence AYTLATH- is 14 804 Da. Figure 2A shows the mass spectrum of the apoprotein, prepared by dissolution of the holoprotein into the denaturing solvent (HCOOH/2-propanol/water). The principal component has a molecular mass of 14 804 Da. The very weak bands at higher mass may reflect the presence of Na^+ and or Cu^{2+} ions bound to the protein even under these strongly denaturing conditions. Figure 2B shows the mass spectrum of nondenatured holoprotein. The principal peak is at 14 928 Da and the "ladder" of peaks trailing off at higher mass represent holoprotein having 1, 2, 3, etc. Na^+ ions bound: 14 950, 14 972, 14 994 and 15 015 Da. The lowest mass peak is the apoprotein at 14 803 Da, followed by two minor peaks due to apoprotein having one and two Na^+ bound. The small peak at 14 865 is probably due to the apoprotein having a single Cu bound.

Copper Analyses. While the electrospray mass spectrum of Figure 2B is consistent with two Cu ions per protein molecule, this was confirmed by measuring Cu/protein ratios. Initially we encountered considerable difficulty in removing the Cu from the protein. For example, even when the protein is precipitated with TCA, as is often done in metalloprotein analyses (Massey, 1957), substantial amounts of Cu are retained in the precipitate. However, when heated at 40 °C for several minutes in 4.4% TCA, the protein releases most of its copper. Quantitative ICP-MS also gave unexpectedly low Cu/protein ratios, presumably because protein in the very dilute (~1 μM) aqueous solutions used in these measurements, adhered to the walls of the plastic tubes. Analytical data consistent with the ESI-MS result were obtained, however, using quantitative EPR spectroscopy after a dissolution of the protein into the denaturing solvent (see Materials and Methods). Four samples, judged from isoelectric focusing gels to be free of apoprotein, gave values of 2.00, 1.56, 1.88 and 1.88, mol of Cu per 14 800 g of protein; the concentration of protein was determined by either BCA or/and quantitative amino acid analyses; similarly, two samples analyzed for Cu by TXRF gave values of 1.71 and 1.62. All our analytical data (from BCS, ICP-MS, EPR, and TXRF) support the interpretation of the observed mass of the holoprotein (14 928 Da) as arising from the apoprotein plus two Cu ions: apoprotein (14 804) *plus* two Cu ($2 \times 63.5 = 127$) *minus* two protons from the cysteine residues (-2) *equals* 14 929 Da. Note that the error in these mass measurements is ±1 part in 10 000 (Siuzdak, 1994). Both ICP-MS and TXRF simultaneously measure a broad spectrum of elements, and significant amounts of other metals were not found in our samples.

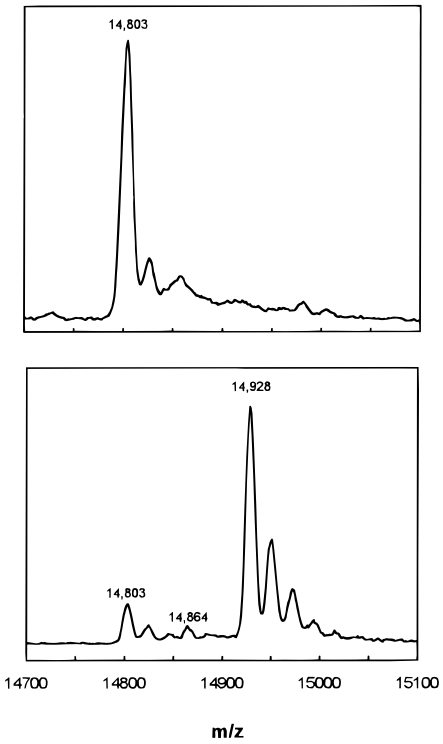

FIGURE 2: Electrospray ionization mass spectra of *Thermus* cytochrome ba_3-Cu_A protein. Panel A shows the mass spectrum of the apoprotein. The experiment was done by first removing Na^+ ions from the solution by passsage over a short gel filtration column equilibrated with 10 mM ammonium acetate (see Materials and Methods) and then dissolving the protein into 60% HCOOH and 30% 2-propanol to a final concentration of ~2 mg/mL. The purple color disappeared in a few seconds, after which the sample was immediately admitted to the spectrometer. Panel B shows the spectrum of the holoprotein. The solution ~2 mg of protein per mL in 10 mM ammonium acetate.

Absorption and Visible CD Spectra. Figure 3 compares the absorption and visible CD spectra of the Cu_A domain with that of azurin from *Pseudomonas aeruginosa*. The *Thermus* soluble fragment shows absorption bands at 363, 480, 530, and 790 nm. The second derivative spectrum (not shown) reveals an additional peak at ~590 nm, which can be seen as a shoulder in the absorption spectrum. Based on the Cu analyses and quantitative EPR (see below), the extinction coefficient at 790 nm is 1900 ± 200 $[2Cu]^{-1}$ cm^{-1}. The energies and extinction coefficients of these transitions are similar to those reported for other soluble Cu_A fragments

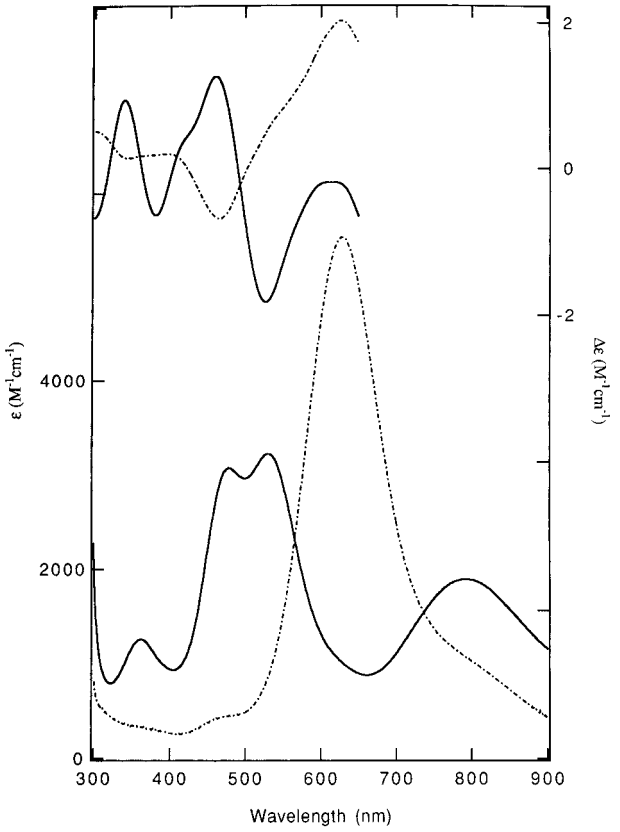

FIGURE 3: Optical absorption and circular dichroism spectra of the blue copper protein azurin from *P. aeruginosa* (dash-dot) and of the *Thermus* cytochrome ba_3-Cu_A protein (solid). The optical spectra are expressed as molar absorbance (e) and the CD spectra are presented as differential molar absorption coefficients ($\Delta\epsilon = \epsilon_l - \epsilon_r$).

Table 2: Absorption Data for Cu_A Soluble Domains and Engineered Cu_A Sites

protein	source	absorbance in nm			
Cu_A from aa_3[a]	*Paracoccus denitrificans*	363	480	530	808
Cu_A from caa_3[b]	*Bacillus subtilis*	365	480	530	790
Cu_A fromba_3[c]	*Thermus thermophilus*	360	480	530	790
Cu_A engineered into CyoA[d]	*Escherichia coli*	358	475	536	765
Cu_A engineered into azurin[e]	*Pseudomonas aeruginosa*	350	485	530	765
Cu_A engineered into amicyanin[f]	*Thiobacillus versutus*	360	483	532	790
Cu_A from N_2OR[g]	*Achromobacter cycloclastes*	350	481	534	780
Cu_A from N_2OR[h]	*Pseudomonas stutzeri*	350	480	540	780

[a] Lappalainen et al. (1993). [b] von Wachenfeldt et al. (1994). [c] This work. [d] Kelly et al. (1993). [e] Hay et al. (1996). [f] Dennison et al. (1995). [g] Hulse et al. (1990). [h] Riester et al. (1989).

from cytochrome oxidases, the Cu_A sites in two characterized N_2OR proteins, and an engineered purple center. However, there are subtle differences in the position of these bands, suggesting that the properties of the site depends somewhat on the overall protein environment; Table 2 summarizes these features.

The visible CD spectrum of the ba_3-Cu_A domain shows features at 340 nm (+0.946), 381 nm (−0.638), 460 nm (+1.27), and 527 nm (−1.81) where the numbers in parentheses are the differential molar absorption coefficients ($\Delta\epsilon = \epsilon_l - \epsilon_r$). There is also a strong shoulder at 425 nm and a negative going feature at ≈690 nm. However, the

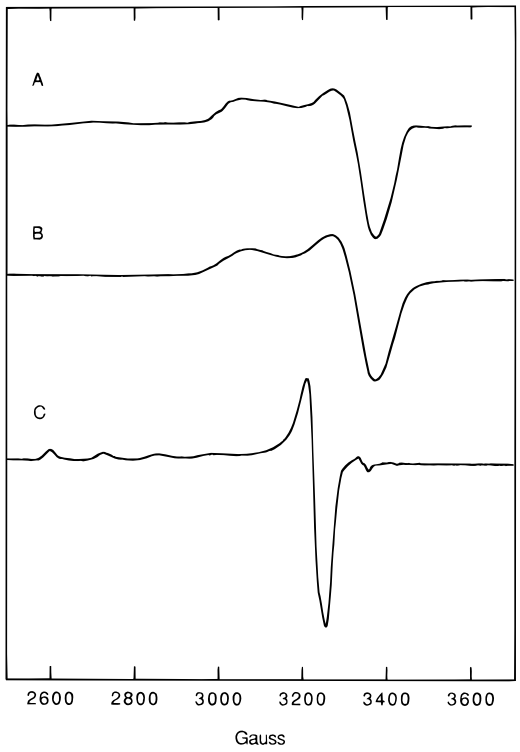

FIGURE 4: X-band EPR spectra of *Thermus* cytochrome ba_3 and the cytochrome ba_3-Cu_A domain. Trace A shows the spectrum of oxidized cytochrome ba_3 recorded at 80 K. Trace B shows the spectrum of the oxidized cytochrome ba_3-Cu_A protein (77 K), and trace C shows the latter after denaturation in 60% HCOOH/30% 2-propanol/10% H_2O (77 K). Spectrometer settings were typically as follows: frequency, 9.378 GHz; modulation amplitude, 10−12.5 G; modulation frequency, 100 kHz; power, 2 mW; recording time, 200 s; time constant, 200 ms. Spectra are presented to show qualitative features. See the text for details of quantitation.

latter is not shown, because the lamp available in the instrument only gives reliable signals up to ∼700 nm. It is noteworthy that the two strong absorption bands around 500 nm in the absorption spectrum have opposite signs in the CD spectrum (see below). The near-UV absorption spectrum shows a sharp peak at 276 nm with an observed extinction coefficient of 17 000 ± 400 M^{-1} cm^{-1}; because the value predicted from PEPTIDESORT is 14 770 M^{-1} cm^{-1}, it is possible the Cu_A center also has absorption bands in the near-UV region.

This is the first report of the CD spectrum of Cu_A, although the MCD spectrum has been described (Farrar et al., 1995), and it deserves some comment. The spectra of azurin are fairly well understood (Solomon et al., 1992; Han et al., 1993; Larsson et al., 1995a), while work has just begun on the origin of the various bands in the Cu_A spectra (Gray et al., work in progress). However, CNDO/S calculations of Larsson et al. (1995b) suggest that the two absorption bands of nearly equal intensity at 480 and 530 nm arise from an interaction between the two Cu ions and predict, as is observed, that the corresponding CD bands should have opposite signs.

EPR Spectra. The X-band EPR spectrum of native cytochrome ba_3 from *T. thermophilus* recorded at ∼80 K is shown in trace A of Figure 4. In this particular sample, there is a significant amount of the unknown impurity, evident at ∼2700, 3000, and 3500 G, as first described by Zimmermann et al. (1988) and seen in many of our preparations (J.A.F., unpublished observations). However, the majority signal

clearly arises from Cu_A. Trace B of Figure 4 shows the spectrum of the soluble Cu_A domain recorded at 77 K under similar instrument settings. Even if trace A is disturbed by the presence of the impurity, the closeness of the *g*-values of the majority signal with those of trace B allows one to conclude that the environment of the Cu_A center in the soluble domain is highly similar to that in the native protein. As reported elsewhere (Fee et al., 1995), the signal is axial having *g* values of 2.187 and ~2.00, and additional details will be published elsewhere (manuscript in preparation).

Double integration of the Cu_A signal and comparison to a standard Cu(II) solution yielded values of spin concentration, [$S = 1/2$]. This was done for 10 different preparations for which independent Cu analyses were available (BCS). The [$S = 1/2$] /[Cu] ratio ranged from 0.32 to 0.68 with an average of 0.54 ± 0.09. In two additional samples, the Cu_A spin concentration was determined as described above, and total Cu was obtained by dissolving the protein in the denaturing solvent, adding a trace of H_2O_2, and then twice integrating the resulting EPR spectrum (see Materials and Methods).[4] The spectrum of the denatured protein shown in trace C of Figure 4 arises largely from a single specie having $g_{\|} = 2.40$, $g_{\perp} = 2.08$, and $A_{\|} = 125$ G. Both samples gave values of [$S = 1/2$; CuA]/[$S = 1/2$; Cu_{total}] = 0.40.[5] These data show that only half of the Cu present in the protein is EPR detectable, a result predicted from the binuclear model of Kroneck and Antholine and co-workers (Antholine et al., 1992).

Far-UV CD Spectra and Secondary Structure Prediction. The far-UV CD spectrum for the *T. thermophilus* fragment is shown in Figure 5A. The estimated percentages of five forms of secondary structure calculated from this spectrum are summarized in Table 3; also listed for comparison are the calculated percentages of each type of secondary structural element for two other Cu_A domains as well as those known from high-resolution X-ray data for azurin (Nar et al., 1991) and plastocyanin (Colman et al., 1978). The two blue proteins, which share considerable sequence similarity with the Cu_A domains, have been included in the basis set of related proteins that was used to predict the fold of this domain. The results are generally consistent with the newly reported X-ray structures (Iwata et al., 1995; Tsukihara et al., 1995) and with previous deductions of secondary structure in subunits II (Wittung et al., 1994) and suggest that the *T. thermophilus* fragment is a structural homolog of the *P. denitrificans* subunit II, having a β-barrel fold with some additional α-helical structure.[6]

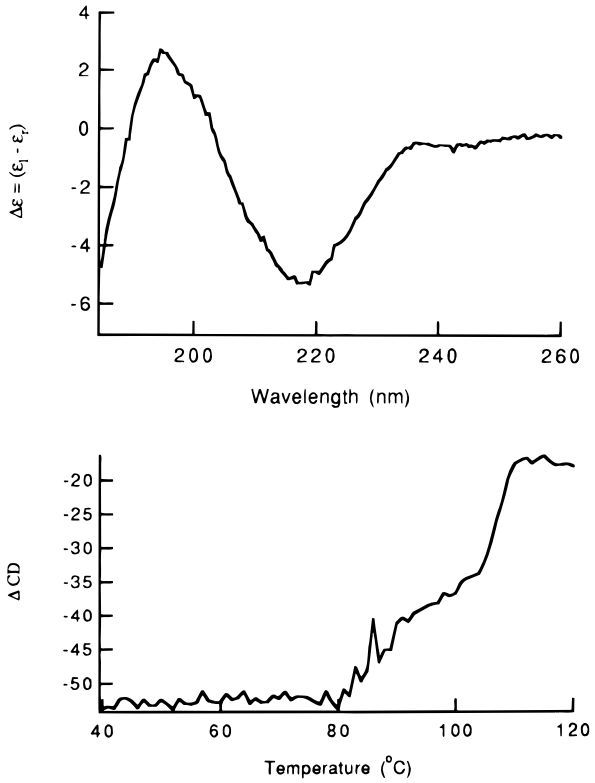

FIGURE 5: Far-UV CD spectrum spectra of the *Thermus* cytochrome ba_3-Cu_A domain (upper panel) and the effect of temperature on the CD at 218 nm (lower panel). The spectrum was obtained from a solution containing ~0.05 mg protein/mL in 0.5 mM potassium phosphate buffer at pH 7.4. The path length was 1 mm. The effect of temperature (lower panel) shows the change in dichroism at 218 nm as a function of solution temperature. The sample was maintained in the liquid state in a sealed cuvette, and ~4 h was required to complete the temperature scan. See the text for details.

Table 3: Secondary Structure of the *Thermus* Cu_A Domain from Circular Dichroism Spectra

protein	α-helix	antiparallel β-sheet	parallel β-sheet	β-turn	other	sum
azurin (X-ray)	10	33	11	22	24	100
plastocyanin (X-ray)	4	38	18	28	12	100
Paracoccus Cu_A	14 ± 6	31 ± 10	17 ± 6	27 ± 5	12 ± 8	101
Thermus Cu_A	17 ± 7	20 ± 11	11 ± 8	13 ± 5	39 ± 8	100
E. coli CyoA	10 ± 3	33 ± 4	11 ± 3	22 ± 2	25 ± 4	101

Thermal and pH Stability. As shown in Figure 5B, protein secondary structure is retained up to at least 80 °C with denaturation being complete at ~110 °C. We have not studied the effect of temperature on the integrity of the Cu_A site, but preliminary studies during the cupration reaction suggest it is formed quite rapidly even at 65 °C and is stable at this temperature for at least 1 h. The Cu_A center is particularly stable to acid pH ≥3, but protein precipitation

[4] This solvent also dissolves the cytochrome *c* oxidase from bovine heart mitochondria and releases the Cu as Cu(II). It was easy to demonstrate that the ratio of Cu to heme *a* is 1.5 in purified oxidase (R. Aasa, J. A. Fee, and B. G. Malmström, unpublished observations). This is consistent with earlier work that indicated the presence of additional Cu in the bovine oxidase (Öblad et al., 1979; Einarsdottir et al., 1985; Steffens et al., 1987).

[5] Because the expected result is 0.5 with an approximate error of ±10%, it is possible that some of the Cu_A protein in these samples was reduced.

[6] While this paper was nearing completion, Dr. Matthias Wilmanns provided us with coordinates of his model for the purple-CyoA protein, making it possible for us to briefly examine the model using molecular graphics. We have also compared the amino acid sequences of the soluble ba_3-Cu_A protein and the soluble CyoA [see also Mather et al. (1992) for the alignment of CyoA sequence with other CuA containing proteins]. After alignment of ba_3-Cu_A and CyoA sequences such that the metal liganding amino acids are in corresponding positions, it is evident that CyoA possesses a C-terminal extension of ~70 additional amino acids (34 of which are observable in the present X-ray data). Moreover, with this alignment, ba_3-Cu_A possesses an additional 33 amino acids at its N-terminus that are not represented in the purple CyoA structure. Because of the small size of the ba_3-Cu_A protein compared to CyoA, approximately 70 of the C-terminal residues of CyoA can have no counterpart in the structure of ba_3-Cu_A, and our analysis of the near-UV CD spectrum suggests that much of this segment probably exists in an α-helical form. The molecular graphics analysis indicates that the 57 C-terminal amino acids observable in the CyoA structure that cannot be part of the ba_3-Cu_A structure, make no contacts with the Cu_A center, and form a "cap" that fits onto the "β-barrel" which constitutes the core of the subunit II structure.

occurs at lower pH (∼2.5) with some loss of copper. Above pH 9 the protein loses its color with apparent oxidation of the active site cysteines to a disulfide; the purple site can be partially regenerated after reduction with thiols.[3]

Redox Properties. Preliminary electron transfer studies have been carried out with the protein (Slutter et al., 1996), and, in a study that will be reported elsewhere (manuscript in preparation), the redox potential at pH 8.1 is 250 mV vs NHE, similar to that observed for other Cu_A centers in cytochrome *c* oxidases (Wang et al., 1986). It is sufficient to state that the protein exhibits well-behaved one-electron, Nerstian behavior and is not oxidized to the 2 Cu(II) form at available potentials.

DISCUSSION

The idea that the "purple copper" center is widely distributed in nature was suggested by the pioneering work of Kroneck, Antholine, and Zumft and their co-workers (cf. Antholine et al., 1992), who originally discovered that nitrous oxide reductase contains a Cu_A center. The recent X-ray structures of *P. denitrificans* cytochrome aa_3 (Iwata et al., 1995), bovine cytochrome aa_3 (Tsukihara et al., 1995), and the engineered purple center in the CyoA fragment (Wilmanns et al., 1995) reveal that Cu_A is a binuclear site in which two cysteine thiolates bridge two Cu to form a four-membered ring. In addition, each Cu has one strong coordinate bond to an imidazole ring of histidine (Gurbiel et al., 1993) and a much weaker coordinate bond to either the sulfur of the conserved methionine or to the carbonyl oxygen of a nearby peptide bond. In all the structures, both Cu appear to be bound in a highly distorted geometry and are separated by ∼2.5 Å, which is consistent with EXAFS data [Blackburn et al., 1994; see also Bertagnoli and Kaim (1995)]. The observations reported here, and multifrequency EPR results (Fee et al., 1995), show that the cytochrome ba_3 from *T. thermophilus* also contains a Cu_A center. While this enzyme is currently the most divergent of the heme-copper oxidases (Lübben et al., 1994), comparative analysis of its amino acid sequence (Keightley et al., 1995) suggests that the overall three-dimensional structure of the ba_3 -Cu_A protein should be similar to that found in the recent X-ray structures,[6] and this is consistent with our analysis of the far-UV CD spectrum (see Figure 5 and Table 3).

The unique spectral features of Cu_A, which have puzzled investigators for more than three decades [see Peisach and Blumberg (1974), Antholine et al. (1992), Gurbiel et al. (1993), Larsson et al. (1995b), and Farrar et al. (1995) for references to early work] can now begin to be understood in terms of the cluster-like structure described above. Unlike a structurally similar $[(Cys)_2[2Fe\text{-}2S](Cys)_2]^{2-}$ center, which shows strong valence *localization* (Sands & Dunham, 1975), the Cu_A center unexpectedly behaves as a spin-delocalized, possibly mixed-valence structure in which the single unpaired electron is found with equal probability at either metal.[7] This property was first recognized in N_2OR (cf. Antholine et al., 1992) and was confirmed for cytochrome ba_3-Cu_A by Fee et al. (1995). Thus, spin (or valence) delocalization appears to be a fundamental property of the Cu_A center that distingushes it from the Fe/S clusters of ferredoxins.

That long-range electron transfer must be occurring in cytochrome *c* oxidase is evident from the recently published structures. For example, in the *P. denitrificans* oxidase structure (Iwata et al., 1995), the distances from the edge of the conjugated p-electron system of the Cu_A ligand, His-224, to the edge of heme *a* and heme a_3, are ∼12 and ∼15 Å, respectively, while the Cu_A ligand, His-224, is hydrogen bonded to the highly conserved Arg-473 in loop XI-XII of subunit I [cf. Table 2 of Keightley et al. (1995)]. In addition, several residues of this loop are in contact with the propionate groups of heme *a*, suggesting possible electron transfer path(s) from Cu_A to heme *a* [see Ramirez et al. (1995)]. Sequence analyses (Steffens et al., 1979), mutagensis (Kelley et al., 1993), and protein engineering (Dennison et al., 1995) studies indicate a possible evolutionary relationship between soluble blue copper proteins and the soluble, C-terminal domain of subunits II. This raises the question of why a binuclear purple site, rather than a mononuclear blue site, serves as the primary electron acceptor in cytochrome *c* oxidases. While mononuclear sites have redox potentials suitable for this role, preliminary electrochemical characterization (Slutter et al., unpublished results) suggests that Cu_A may have a significantly lower reorganization energy (cf. Marcus & Sutin, 1985; Langen et al., 1995) than that of mononuclear copper. Indeed, a theoretical analysis based on electronic spectroscopic properties indicates that this energy decreases from about 0.4 eV in a blue site to less than 0.2 eV in a purple site (Larsson et al., 1995b), which may be an important reason why nature has "chosen" a binuclear center over a mononuclear Cu center in this function. The availability of the well-defined and stable ba_3-Cu_A protein should permit detailed experimental studies of long-range electron transfer in cytochrome *c* oxidases.

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[7] This would correspond to a class III complex in the scheme of Robin and Day (1967). Interestingly, there is no direct experimental evidence for the formal Cu(II)/Cu(I) valence states, although this is a reasonable starting assumption.

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