Electron Transfer between Cytochrome *c* and the Isolated Cu_A Domain: Identification of Substrate-Binding Residues in Cytochrome *c* Oxidase†

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ABSTRACT: Subunit II of cytochrome *c* oxidase has a C-terminal domain that is exposed to aqueous solution on membrane surface and contains a copper center called Cu_A. The central part of the cytochrome *c* binding site is thought to reside in this domain. We have expressed the subunit II fragment of the *Paracoccus denitrificans* cytochrome *c* oxidase in a soluble form and studied its interaction with cytochrome *c* by stopped-flow spectroscopy. The oxidation of cytochrome *c* by the Cu_A domain follows monophasic kinetics, indicating the presence of a single kinetically competent binding site. In low ionic strength medium, the domain oxidizes *Paracoccus* cytochrome *c*-550 and horse mitochondrial cytochrome *c* at the rates of 1.5×10^6 and 3×10^5 M^{-1} s^{-1}, respectively. The reaction rates are strongly dependent on ionic strength, which must reflect electrostatic interactions within the complex. The K_D for the complex between the bacterial cytochrome *c* and the domain is 1.6 μM; i.e., it is similar to that between the mitochondrial cytochrome *c* and the intact oxidase, suggesting that both contain the same catalytically competent binding site. Using site-directed mutagenesis, we have identified five conserved residues of the Cu_A domain that are involved in the cytochrome *c* binding. Mutations of glutamine 148, glutamate 154, aspartate 206, aspartate 221, or glutamate 246 lead to a 35−85% decrease in the rate of cytochrome *c* oxidation. The simultaneous substitution of three invariant carboxylic acids (aspartate 206, aspartate 221, and glutamate 246) leads to a 95% decrease in the reaction rate. Conversely, the reaction can be enhanced by removing a positive charge (lysine 219) from the Cu_A domain.

Cytochrome *c* oxidase is the terminal enzyme in the mitochondrial and many bacterial respiratory chains. It reduces dioxygen to water and functions as a redox-linked proton pump (Babcock & Wikström, 1992). Mammalian cytochrome oxidases contain three subunits that are encoded by mitochondrial DNA and form the functional core of the enzyme, and up to 10 subunits that are encoded by nuclear DNA. Bacterial oxidases are structurally simpler, containing typically only the three core subunits of the eukaryotic enzyme (Saraste, 1990; Garcia-Horsman et al., 1994). Cytochrome *c* oxidase (cytochrome aa_3) has three redox centers, Cu_A, cytochrome *a*, and the binuclear cytochrome a_3-Cu_B site where oxygen is reduced to water. Cytochrome *a* and the binuclear center are located in the membrane-buried subunit I (Hosler et al., 1993; Garcia-Horsman et al., 1994), whereas Cu_A resides in subunit II (Van der Oost et al., 1992; Lappalainen et al., 1993) and has been shown to be the primary acceptor for electrons coming from cytochrome *c* (Hill, 1993). Cu_A is a dinuclear site which contains two coppers in a mixed-valence configuration (Antholine et al., 1992; Malmström & Aasa, 1993; Kelly et al., 1993; Lappalainen et al., 1993).

Some aerobic bacteria possess terminal oxidases that belong to the cytochrome oxidase superfamily but use quinols instead of cytochrome *c* as the electron donor (Saraste, 1990; Hosler et al., 1993; Calhoun et al.; 1994; Garcia-Horsman et al., 1994). These enzymes are otherwise structurally and functionally very similar to cytochrome *c* oxidases, but they have systematically lost the Cu_A center during evolution (Castresana et al., 1994).

Cytochrome *c* oxidases react with reduced cytochrome *c* with rates around 10^7 M^{-1} s^{-1}. The formation of the complex between cytochrome *c* and the oxidase is believed to be the rate-limiting step in this reaction (Antalis & Palmer, 1982), as the electron transfer between cytochrome *c* and Cu_A has been shown to be extremely rapid [about 70 000 s^{-1}; see Hill (1994)]. The fast reaction phase is followed by a much slower phase with a rate of 1−3 s^{-1}. The origin of this biphasic behavior is still not understood. Ferguson-Miller et al. (1976) and Garber and Margoliash (1990) have suggested that the two kinetic phases could be due to two distinct binding sites for cytochrome *c*. In contrast, Antalis and Palmer (1982) have proposed that a two-step electron transfer from a single binding site can explain the biphasic kinetics. Brzezinski and Malmström (1986) have further suggested that the biphasic kinetics could arise from two different conformational stages of the proton-pumping enzyme in which the primary electron acceptor has different reduction potentials.

The reaction between cytochrome oxidase and cytochrome *c* is strongly dependent on ionic strength, indicating that electrostatic interactions stabilize the complex (Antalis & Palmer, 1982). Several attempts have been made to deter-

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mine the number and location of the cytochrome *c* binding sites on the enzyme. Analytical gel filtration experiments have typically given a stoichiometry of two binding sites per oxidase monomer (Rieder & Bosshard, 1978; Taha & Ferguson-Miller, 1992). Direct binding studies have demonstrated interaction between cytochrome *c* and the high-affinity binding site with a K_D less than 100 nM (Ferguson-Miller et al., 1976). Conversely, spectroscopic and transient-state kinetic methods indicate the presence of only one catalytically active binding site per monomer, with a K_D of approximately 1 μM (Antalis & Palmer, 1982; Michel & Bosshard, 1984).

Subunit II of cytochrome *c* oxidase contains several well-conserved carboxylic acids which are not found in the homologous subunit of quinol oxidases. It is thought that these residues are involved in electrostatic interactions with a ring of conserved lysines that surrounds the heme edge in cytochrome *c* (Capaldi, 1990; Saraste, 1990). The location of the cytochrome *c* binding site has been mapped using several different methods including chemical modification (Seiter et al., 1979; Bisson et al., 1982; Millett et al., 1983), cross-linking (Erecinska, 1977; Briggs & Capaldi, 1978), and monoclonal antibodies (Taha & Ferguson-Miller, 1992). These studies indicate that the high-affinity binding site is mostly located in subunit II and that some nuclear subunits may constitute a part of it in the mitochondrial enzyme. These results are supported by kinetic studies of electron transfer, which have shown that the Cu_A center is the primary electron acceptor in cytochrome oxidase (Kobayashi et al., 1989; Nilsson, 1992; Pan et al., 1993; Brzezinski et al., 1995; Hill, 1994).

Several different *c*-type cytochromes are expressed in *Paracoccus denitrificans* (Bosma, 1989). The reactions between *Paracoccus* cytochrome aa_3 and different *c*-type cytochromes have been studied by Davies and co-workers. Using a series of cytochrome *c* deficient mutants, these authors showed that the soluble cytochrome *c*-550 is the major electron donor for cytochrome aa_3 (Bolgiano et al., 1989). The *Paracoccus* cytochrome aa_3 is also able to oxidize the bovine heart cytochrome *c* with high rates, suggesting that the structures of substrate-binding sites in the *Paracoccus* and mitochondrial cytochrome *c* oxidases are conserved (Bolgiano et al., 1988).

We have been able to express the Cu_A-binding C-terminal part of subunit II from the *Paracoccus* cytochrome *c* oxidase in a soluble form (Lappalainen et al., 1993). In the present work, we have investigated the electron-transfer reaction between this isolated Cu_A domain and the bacterial cytochrome *c*-550 as well as the horse heart cytochrome *c*. Furthermore, we have substituted the putative cytochrome *c* binding residues of subunit II and studied the effects of these mutations on the reaction kinetics in order to map the cytochrome *c* binding site.

MATERIAL AND METHODS

Preparation of Wild-Type and Mutant Paracoccus Cu_A Domains. The expression of the wild-type Cu_A domain in *Escherichia coli* BL21(DE3) was carried out as described before (Lappalainen et al., 1993). Mutants of the Cu_A domain were constructed with the overlap extension method (Higuchi et al., 1988) using the wild-type pET.PD1 plasmid (Lappalainen et al., 1993) as a template for polymerase chain reaction (PCR)[1] using AmpliTaq (Perkin-Elmer-Cetus Instruments). The DNA constructs were sequenced by the chain-termination method, and the clones containing undesired mutations were discarded. The mutant proteins were expressed in *E. coli* BL21(DE3) cells similarly to the wild-type protein. The protein refolding and purification was performed as described by Lappalainen et al. (1993).

Preparation of Paracoccus Cytochrome c-550. Cytochrome *c*-550 was purified from the *Paracoccus denitrificans* strain carrying the pEG400-*cycA* plasmid that leads to an approximately 10-fold increase in *c*-550 expression (J.-W. De Gier and J. Van der Oost, unpublished). The plasmid-carrying cells were first precultured under aerobic conditions for 30 h at 30 °C in TB medium (Ausubel et al., 1994) containing rifampicin (60 mg/L) and streptomycin (50 mg/L). These precultures were used to inoculate minimal media containing succinate (25 mM), potassium nitrate (100 mM), and streptomycin (50 mg/L). The cells were grown for 40 h under anaerobic conditions at 30 °C, harvested by centrifugation, and suspended to standard Tris buffer (20 mM Tris·HCl, pH 8.2) containing phenylmethanesulfonyl fluoride (0.15 mM), deoxyribonuclease (50 μg/mL), and magnesium sulfate (1 mM). The cells from 3 L of culture were broken with a French press at 4 °C, and the suspension was centrifuged twice for 60 min at 40 000 rpm in a Beckman Ti-45 rotor.

The clear supernatant was applied to a Q-Sepharose fast flow anion-exchange column (60-mL bed volume) equilibrated with the standard Tris buffer. At the flow rate of 5 mL/min, the proteins were eluted with a linear sodium chloride gradient from 0 to 0.5 M during 100 min. The cytochrome *c*-550 containing fractions eluted at 0.3 M NaCl. They were pooled, concentrated by ultrafiltration, and stored at −20 °C. The purity of the cytochrome *c*-550 preparation was 80−90% on the basis of proteins seen in the electrophoresis gels stained with Coomassie Blue, and no other chromophores could be detected in the absorbance spectrum.

Rapid Kinetic Measurements. Reduced cytochrome *c* samples were prepared by addition of a slight exess of ascorbic acid to the oxidized protein. Ascorbic acid was subsequently removed by gel filtration in Sephadex G-25 columns (PD-10, Pharmacia) equilibrated with 20 mM Bis-Tris (pH 7.0). The concentrations of the reduced cytochrome *c* and the oxidized Cu_A domain were determined using the absorption coefficients 30 mM^{-1} cm^{-1} at 550 nm and 3.0 mM^{-1} cm^{-1} at 480 nm, respectively. Because of the relatively fast autooxidation of *Paracoccus* *c*-550, the samples were protected from the light and kept on ice prior to use. The cytochrome *c*-550 samples were always used for experiments within 1 h from reduction and desalting.

The measurements were routinely carried out at 20 °C in 20 mM Bis-Tris (pH 7.0) containing 25 mM KCl. Experiments similar to those shown in Figure 1 were also performed in a 40 mM phosphate buffer (pH 7.0). The rates and amplitudes were identical to the ones measured in 20 mM Bis-Tris (pH 7.0), indicating that the buffer components have no effect on the reaction kinetics. The rapid kinetic experiments were conducted in a stopped-flow apparatus equipped with an Applied Photophysics Bio-Sequential DX17MV stopped-flow spectrophotometer using a 1-cm path

[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; PCR, polymerase chain reaction.

length optical cell. In each experiment, 500 data points were recorded over the course of the reaction. The observed changes in absorbance were fitted to a single-exponential process with software supplied by Applied Photophysics which uses a modernized and robust implementation of the Marquardt algorithm broadly based on the routine Curfit (Bevington, 1969). The dependence of the observed first-order rate constants on cytochrome *c*-550 concentration was analyzed using the linear regression routine found in the program Curfit v3.0 (Erithicus Software).

Miscellaneous. Cytochrome *c* (type IV, from horse heart) was obtained from Sigma and used without further purification. Optical absorbance spectra were recorded with a Perkin-Elmer-Cetus Instruments Lambda 2 spectrophotometer. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate in 15% gels was performed according to Laemmli (1970).

RESULTS

Reaction with Cytochrome c. In this work, we have studied the reaction between reduced cytochrome *c* and oxidized Cu_A domain. All experiments were carried out at pH 7 because the center within the isolated domain is modified at alkaline pH. At neutral pH, however, it is spectroscopically identical to the Cu_A center in the intact cytochrome *c* oxidase complex (Lappalainen et al., 1993; Farrar et al., 1995). The oxidation of cytochrome *c* and the reduction of Cu_A were monitored by the decrease of absorbance at 550 and 800 nm, respectively. A fast and simultaneous decrease of absorbance could be detected at these wavelengths when 10 μM Cu_A domain was mixed with 20 μM cytochrome *c* (Figure 1). At both wavelengths, the half-life of the reaction is approximately 0.2 s, and it is complete in ca. 0.8 s.

The absorbance changes can be fitted to single exponentials, indicating the presence of only one kinetically active cytochrome *c* binding site. However, the amplitudes of the reaction appear not to be consistent with a 1:1 stoichiometry of electron transfer. Since this observation could be distorted by the very small absorbance changes observed at 800 nm and by the fact that the Cu_A domain contributes to the absorbance at 550 nm, the stoichiometry of electron transfer was determined by equilibrium titration. The titrations at 550 and 800 nm showed that, for each mole of oxidized cytochrome *c*, 1 mol of Cu_A was reduced (data not shown). This is in good agreement with the earlier results suggesting that the dinuclear Cu_A site has a mixed-valence [Cu(1.5)—Cu(1.5)] configuration and therefore functions as a one-electron redox center [see Malmström and Aasa (1993)]. Furthermore, the equilibrium titration demonstrated that the relative redox potential of the Cu_A center is 10−15 mV lower compared to the one of horse heart cytochrome c [see also Lappalainen et al. (1993)]. Note that, due to the weak amplitudes at 800 nm, only the oxidation of cytochrome *c* was followed in further experiments.

Substrate Specifity of the Paracoccus Cu_A Domain. In order to investigate the substrate specifity of the Cu_A domain, we studied its reaction with both *Paracoccus* cytochrome *c*-550 and horse cytochrome *c*. The concentration of the Cu_A domain was constant (2 μM) in all experiments, whereas the cytochrome *c* concentration varied between 4 and 20 μM. The effect of ionic strength for the reaction rates was studied

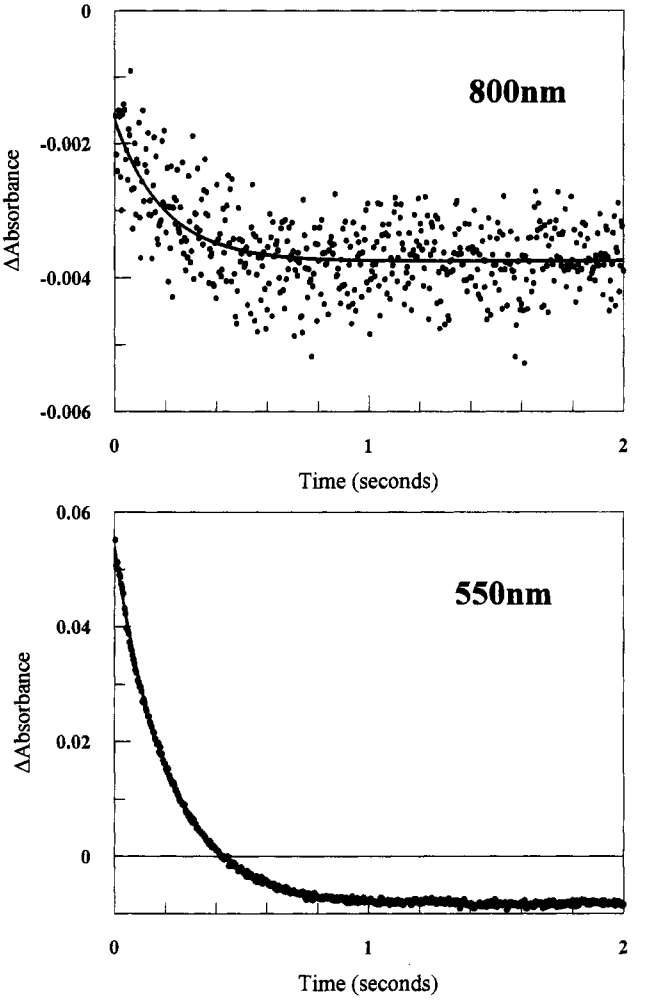

FIGURE 1: Typical time courses of the absorbance changes in the reaction between the reduced cytochrome *c* and the oxidized Cu_A domain. The reactions were carried out in 20 mM Bis-Tris (pH 7.0) containing 25 mM KCl at 20 °C. The concentrations of the horse heart cytochrome *c* and the *Paracoccus* Cu_A domain were 20 and 10 μM, respectively, after mixing. The reduction of Cu_A was followed at 800 nm (top panel), and the oxidation of cytochrome *c* was monitored at 550 nm (lower panel).

by carrying out the experiments in buffer solutions containing 25−300 mM KCl (Table 1). The fastest rates were observed at low ionic strength (25 mM KCl). Under these conditions, the oxidation of cytochrome *c*-550 has a rate of 1.5×10^6 M^{-1} s^{-1}, whereas the oxidation of horse heart cytochrome *c* takes place at a rate of 3×10^5 M^{-1} s^{-1}. The rates gradually decrease at higher salt concentrations (Table 1).

In Figure 2, the logarithms of k_{on} rates are plotted against the square root of ionic strength. The linear decrease of the rates in these Brönsted plots indicates that the electrostatic interactions are important for the reaction between cytochrome *c* and the Cu_A domain. The lower rates and the steeper slope in Brönsted plot in the case of the horse heart cytochrome *c* suggest that its reaction with the *Paracoccus* Cu_A domain is less specific than the one between cytochrome *c*-550 and the domain (Figure 2). The k_{on} and k_{off} rates as well as the apparent dissociation constants are listed in Table 1.

Cytochrome c Binding Residues. Figure 3 shows the amino acid sequence of the soluble *Paracoccus* Cu_A domain. In addition to the major copper ligands (cysteines 244 and 248 and histidines 209 and 252), cytochrome *c* oxidases have

Table 1: Effect of Ionic Strength on Rate Constants[a]

[KCl] (mM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)
	(a) *Paracoccus* c-550		
25	1.46×10^6	2.27	1.55
50	7.2×10^5	2.89	4.01
100	5.14×10^5	1.07	2.08
200	2.00×10^5	0.55	2.75
300	1.43×10^5	0.25	2.36
	(b) Horse Heart Cytochrome *c*		
25	3.00×10^5	3.23	10.8
50	9.57×10^4	0.69	7.17
100	3.75×10^4	0.15	4.00
200	1.54×10^4	0.06	3.74
300	1.01×10^4	0.04	3.96

[a] The reactions were carried out at 20 °C in 20 mM Bis-Tris (pH 7) containing 25, 50, 100, 200, or 300 mM KCl. The concentration of Cu_A domain was 2 μM after mixing, whereas the cytochrome *c* concentration was varied between 4 and 20 μM. The oxidation of cytochrome *c* was monitored at 550 nm. The observed rate constants at each cytochrome *c* concentration are the mean of at least four individual determinations. The rate constants k_{on} and k_{off} were obtained from the slopes and intercepts, respectively, of the second-order plot.

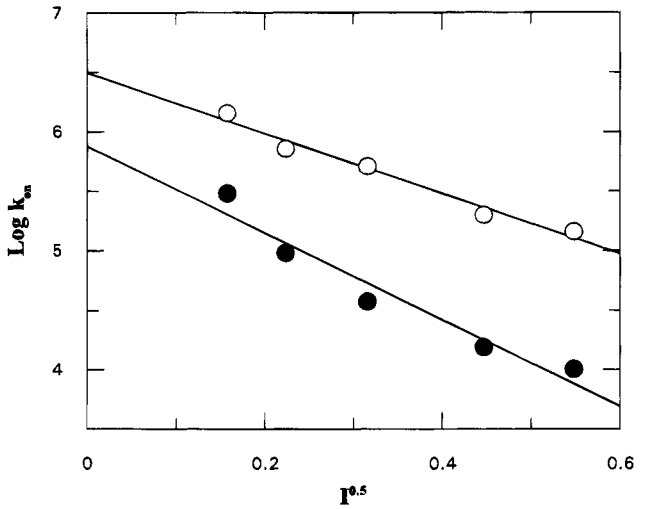

FIGURE 2: Dependence of the k_{on} rate constant on ionic strength. The rate constants were obtained from Table 1. The open symbols (○) show the k_{on} rate constants of the reaction with the *Paracoccus cytochrome c*-550, and the closed symbols (●) show the k_{on} rates of the reaction with the horse heart cytochrome *c*.

only five totally conserved residues within this domain that are not present in quinol oxidases. These are two aspartates (206 and 221), one glutamate (246), one lysine (219), and one glutamine (148). Chemical modification experiments have also indicated that a conserved although not invariant glutamate (residue 154 in the *Paracoccus* sequence) could be involved in cytochrome *c* binding (Millett et al., 1983).

The residues Q148, E154, D206, K219, D221, and E246 were individually mutagenized in order to determine their roles in the interaction with cytochrome *c*. The mutant proteins were expressed, refolded, and purified as the wild-type protein (Lappalainen et al., 1993). All the mutant proteins eluted from a Superdex-75 gel-filtration column in the same volume as the monomeric wild-type protein, indicating that they are also monomers. No significant perturbation could be detected in the absorption spectra of the mutants, showing that the amino acid substitutions affect neither the Cu_A center nor the overall fold of the proteins (Figure 4).

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                                    148     154
P.den MVFRSQEMPN DPDLVIKAIG HQWYWSYEYP NDGVAFDALM
Cox                          Q  W    Y
Qox                             W    Y
                                                206
P.den LEKEALADAG YSEDEYLLAT DNPVVVPVGK KVLVQVTATD
Cox                           P                   D
Qox                           P
       #           219 221                      # 246
P.den VIHAWTIPAF AVKQDAVPGR IAQLWFSVDQ EGVYFGQCSE
Cox    V H     P      K D                       C E
Qox    V       P

       #   #
P.den LCGINHAYMP IVVKAVSQEK YEAWLAGAKE EFAA*
Cox     CG  H  M               W
Qox      G     M               W
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FIGURE 3: Strategy of site-directed mutagenesis. Shown is the amino acid sequence of *Paracoccus denitrificans* Cu_A domain. The residues that are conserved in cytochrome *c* oxidases (Cox) and quinol oxidases (Qox) are shown below the sequence. The Cu_A ligands are indicated with pound signs (#). The residues mutated in this work are indicated with the numbers above the sequence.

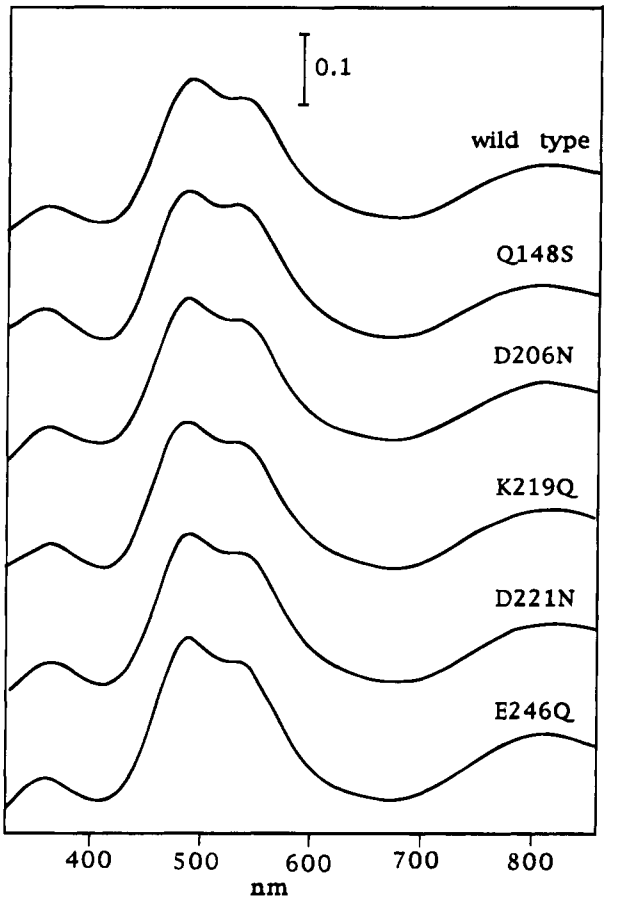

FIGURE 4: Optical spectra of the wild-type *Paracoccus* Cu_A domain and the mutant proteins. The spectra were recorded in 20 mM Bis-Tris (pH 7.0) using 100 μM protein samples. The vertical bar shows the absorbance scale.

The reactions between the mutant proteins and cytochrome *c*-550 were studied by stopped-flow spectrophotometry at low ionic strength. The data are shown in Figure 5. The most striking effect is seen when aspartate 206 is substituted with an asparagine. This D206N mutation leads to a 6-fold decrease in the k_{on} rate. Mutations Q148S, E154Q, D221N, and E246Q result in a 35−60% decrease in the rates. Conversely, the substitution of lysine 219 with a glutamine (K219Q) leads to an approximately 60% increase in the k_{on} rate. The simultaneous substitution of the three invariant

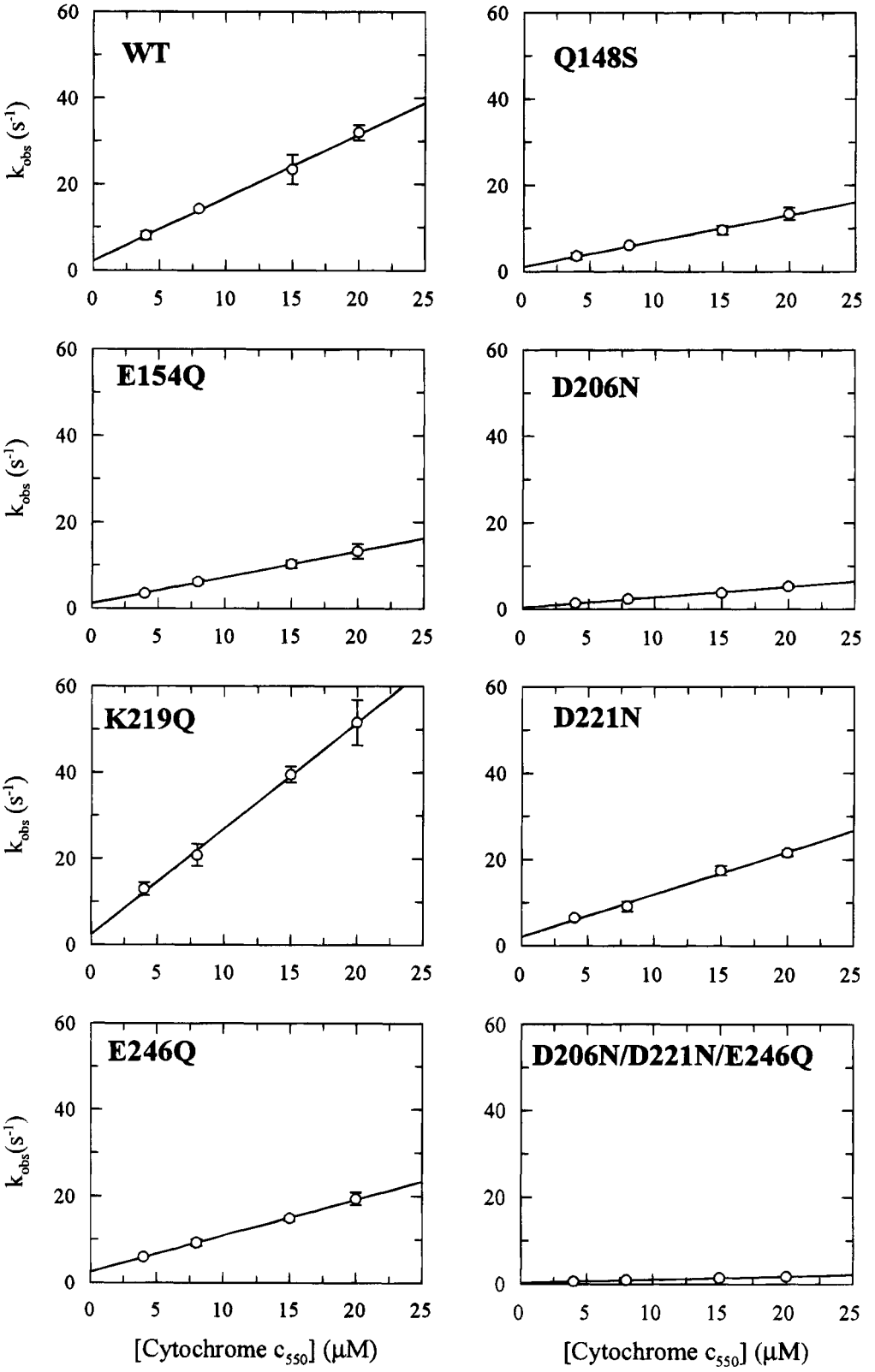

FIGURE 5: Reaction of mutant Cu_A domains with *Paracoccus* c-550. The reactions were carried out in 20 mM Bis-Tris (pH 7.0) containing 25 mM KCl at 20 °C. The concentration of Cu_A domains was 2 μM. Cytochrome *c* oxidation was monitored at 550 nm, and the observed rate constants at each cytochrome *c*-550 concentration are the mean of at least four individual determinations.

carboxylic acids (D206, D221 and E246) results in a 95% decrease in the k_{on} rate (Table 2). The dissociation constants for the Cu_A domain:cytochrome *c*-550 complexes were calculated from the kinetic data. Their values are between 1 and 4 μM for the mutants and 1.6 μM for the wild type protein (Table 2).

DISCUSSION

Cytochrome c Binding Site Resides within the Cu_A Domain. Our experiments show that the isolated Cu_A-binding domain of the *Paracoccus* cytochrome aa_3 can oxidize cytochrome *c in vitro*. The oxidation of *Paracoccus* cytochrome *c*-550

Table 2: Effects of Site-Directed Mutations on Rate Constants

Cu_A domain	k_{on} (M^{-1} s^{-1})[a]	k_{off} (s^{-1})[a]	K_D (μM)
wild type	1.46×10^6	2.27	1.55
Q148S	5.98×10^5	1.15	1.91
E154Q	6.06×10^5	1.22	2.01
D206N	2.42×10^5	0.39	1.61
K219Q	2.45×10^6	2.44	1.00
D221N	9.84×10^5	2.12	2.15
E248Q	8.33×10^5	2.61	3.13
D206N, D221N, E246Q	7.47×10^4	0.31	4.15

[a] The rate constants k_{on} and k_{off} were obtained from the slopes and intercepts, respectively, of the second-order plots.

occurs at the rate of 1.5×10^6 M^{-1} s^{-1} at low ionic strength. This value is approximately 10-fold lower than observed earlier with the intact mitochondrial oxidase. Using the cyanide-inhibited bovine oxidase, Antalis and Palmer (1982) found the rate of 3×10^7 M^{-1} s^{-1}, whereas rates of approximately 10^7 M^{-1} s^{-1} have been measured for the reaction between the active beef heart oxidase and cytochrome *c* [e.g., Andreasson (1975)].

The dissociation constants calculated from the kinetic data (Table 1) are very close to those that have been obtained by a similar method with the intact bovine enzyme [see Antalis and Palmer (1982)]. This suggests that the catalytically relevant cytochrome *c* binding site is mostly, if not completely, located within the C-terminal fragment of subunit II. However, previous experiments carried out using carboxyl-modifying reagents indicate that several nuclear-encoded subunits constitute part of the interaction domain in the mitochondrial oxidase (Bisson & Montecucco, 1982; Bisson et al., 1982; Kadenbach & Stroh, 1984; Capaldi, 1990). In contrast to the intact oxidase, no biphasic behavior could be observed in any salt concentrations in the reaction between the isolated Cu_A domain and cytochrome *c*. This is in line with proposals of Antalis and Palmer (1982) and Brzezinski and Malmström (1986) that the biphasic kinetics may arise from changes within the subunit I rather than from two distinct cytochrome *c* binding sites. However, our experiments do not exclude the presence of a second low-affinity site constructed by proteins other than subunit II in the eukaryotic enzyme complex.

The effect of ionic strength on the reaction rates in our experiments is very similar to that observed with the intact bovine oxidase (Antalis & Palmer, 1982). This suggests that similar electrostatic forces are involved in the interaction of the intact oxidase and isolated Cu_A domain with cytochrome *c*. However, the relative insensitivity of the calculated K_D's to ionic strength and neutralization of carboxylate residues (Tables 1 and 2) might suggest that the electron-transfer complex is not primarily stabilized by ionic interactions. Consequently, the carboxylic acid residues in the Cu_A domain might be actually required for correct orientation of the two proteins so that the electron-transfer complex can form.

The reaction between the *Paracoccus* Cu_A domain and cytochrome *c*-550 is about 5-fold faster that the reaction between the domain and horse heart cytochrome *c* (Table 1). The Brönsted plot (Figure 2) also shows that the oxidation of the latter is more sensitive to ionic strength. These results indicate that the binding of the mitochondrial cytochrome *c* to the *Paracoccus* Cu_A domain is less specific than the binding of the bacterial cytochrome *c*-550. In accordance with this, Bolgiano and co-workers (1988) have noticed that, although the *Paracoccus* oxidase reacts with bovine heart cytochrome *c* faster than with its own substrate at low ionic strength, the rates observed with *Paracoccus* cytochrome *c*-550 are faster at higher ionic strength (0.25 M). Comparison of the 3D structures of the horse heart cytochrome *c* and the *Paracoccus* cytochrome *c*-550 shows that the amino acids surrounding the heme crevice are almost identical (Timkovich & Dickerson, 1976; Qi et al., 1994). Minor structural differences of these residues may be responsible for the observed substrate specifity which is reflected by the different dissociation constants of the complexes between the Cu_A domain and the two *c*-type cytochromes (Table 1).

Cytochrome c Binding Residues. The labeling/protection experiments by Millett et al. (1983) have identified four potential cytochrome *c* binding residues in the bovine oxidase. One of them corresponds to the invariant E246, whereas the three others are less conserved residues. Only three carboxylic acids of subunit II are totally conserved in all cytochrome *c* oxidases. In addition to these, subunit II has six other invariant residues that are not conserved in quinol oxidases and therefore are probably important for cytochrome *c* oxidizing activity (Figure 3). Two cysteines and two histidines have been assigned to be the major copper ligands (Kelly et al., 1993; Farrar et al., 1995), whereas the function of the conserved glutamine and lysine are not known.

The oxidation of cytochrome *c*-550 by the mutant proteins shows that all three totally conserved carboxylic acids as well as glutamine 148 are involved in cytochrome *c* binding. Also the less conserved glutamate 154 of the *Paracoccus* oxidase appears to participate in the substrate binding (Figure 5). The strongest decrease in the oxidation rate is found with the mutant D206N. This aspartate is also the only conserved carboxylic acid in a short homologous sequence stretch that is shared by subunit II and another Cu_A-containing protein, nitrous oxide reductase (Zumft et al., 1992).

The simultaneous substitution of the three invariant carboxylates leads to a k_{on} rate that is only 5% of the wild-type rate (Table 2). The effect of these three mutations is cumulative since the calculated combination of the individual effects would lead to 6.5% of the wild-type activity.

The function of the conserved K219 is difficult to explain without further structural information on the Cu_A domain. However, because it is highly conserved, it is likely to have an important role either in the interaction with cytochrome *c* or in the electron transfer. Our results show that neutralizing this positive charge enhances the oxidation of cytochrome *c*. This is probably due to the increased stability of the complex between the reduced cytochrome *c* and the oxidized domain (K_D decreases from 1.6 to 1.0 μM, Table 2), again reflecting the importance of charge interactions for initial binding.

As a conclusion, our data confirm that the Cu_A domain is the electron entry site in cytochrome *c* oxidase. It probably contains the entire kinetically competent substrate binding site of the enzyme. The oxidization of cytochrome *c* by the isolated Cu_A domain follows monophasic kinetics, showing that this domain contains only one active binding site. The reaction of the *Paracoccus* Cu_A domain with its own cytochrome *c* substrate is faster and less dependent on ionic strength than the reaction with the horse cytochrome *c*,

reflecting substrate specifity. We have shown by site-directed mutagenesis that at least glutamine 148, glutamate 154, aspartate 206, aspartate 221, and glutamate 246 are involved in cytochrome *c* binding and that the electron-transfer rate can be enhanced by removing the positive charge of lysine 219. A triple mutant shows that the effects of the mutations on the interaction with cytochrome *c* are additive.

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