Transformation of the Cu_A Redox Site in Cytochrome *c* Oxidase into a Mononuclear Copper Center†

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ABSTRACT: Subunit II of the aa_3 type cytochrome *c* oxidase contains a binuclear copper center (Cu_A) which functions as the entry point for electrons donated by cytochrome *c*. We have introduced site-specific mutations in residues liganding the Cu_A center in the oxidase of the bacterium *Paracoccus denitrificans*; the purified, fully assembled enzyme complexes were analyzed by various techniques, including EPR, optical spectroscopy, and total-reflection X-ray fluorescence spectrometry, to determine metal to protein ratios. In the C216S mutant, the binuclear Cu_A site is transformed into a mononuclear copper center. In contrast to wild type, the C216S mutant does no longer exhibit the characteristic absorption band in the near-infrared region of the optical spectrum that has been assigned to Cu_A. These major changes in the Cu_A site of this mutant correlate with an almost complete loss in catalytic activity.

The Cu_A site is a redox center located in subunit II of cytochrome *c* oxidases of the mitochondrial aa_3 type. The crystal structures of the enzymes from the soil bacterium *Paracoccus denitrificans* and from beef heart mitochondria have been solved recently (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995). Furthermore, the three-dimensional structure of a protein fragment containing an engineered Cu_A binding domain (van der Oost *et al.*, 1992) has been determined (Wilmanns *et al.*, 1995). Confirming previous suggestions (Kroneck *et al.*, 1990; Antholine *et al.*, 1992), the structures reveal a binuclear copper center liganded by two cysteines, two histidines, a methionine, and a carbonyl oxygen from the peptide backbone.

The Cu_A site functions as the primary acceptor of electrons donated by cytochrome *c* which are then transferred via heme *a* to the heme a_3·Cu_B binuclear oxygen reduction site (Hill, 1993). It has been shown that, in a site-directed mutant of the Cu_A site in position M227, the electron transfer between Cu_A and heme *a* is significantly decreased (Zickermann *et al.*, 1995).

The periplasmic domain of subunit II of cytochrome *c* oxidase is significantly structurally similar to a group of small soluble mononuclear copper proteins which are involved in electron transfer (Steffens & Buse, 1979; Holm *et al.*, 1987; Iwata *et al.*, 1995). The copper atom in these proteins is liganded by two histidines, one cysteine, frequently one methionine, and in some cases a carbonyl oxygen from the peptide backbone. This structural motif is also found in more complex proteins like the so-called blue oxidases laccase and ascorbate oxidase, or some nitrite reductases (Adman, 1991; Kukimoto *et al.*, 1994). Due to their EPR[1] spectra, copper sites of this type are classified as type I (Peisach & Blumenberg, 1974).

Cu_A exhibits very unusual EPR spectroscopic properties, being only similar to that of the binuclear copper center A in nitrous oxide reductase, which was an indication to assume that Cu_A in cytochrome *c* oxidase is also binuclear (Kroneck *et al.*, 1988). In the near-IR region of the optical spectrum, a broad absorption centered around 830 nm has been assigned to originate predominantly from Cu_A [Wikström *et al.*, 1981; but see Hendler *et al.* (1994)].

The fact that a direct bond exists between both nuclei [Blackburn *et al.*, 1994; but see Bertagnolli and Kaim (1995)] and that the near-IR absorption is a charge transfer transition between the two copper atoms (Larsson *et al.*, 1995) has been discussed. Recently, new experimental evidence for a direct Cu−Cu bond has been obtained (Wallace-Williams *et al.*, 1996).

Although the three-dimensional structure of the Cu_A site has been revealed by three independent crystal structures (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995; Wilmanns *et al.*, 1995), the unusual binuclear character of this metal binding site and its function in the enzyme complex need further clarification.

We have constructed site-directed mutants of the cysteines and histidines in subunit II which are ligands to the two coppers and have characterized the purified enzyme complexes with various techniques, including the determination of metal to protein ratios by total-reflection X-ray fluorescence spectrometry (TXRF).

Here we focus on mutant H181N which exhibits only minor deviations from the wild type and on mutant C216S which has lost one copper atom and now contains a mononuclear copper center, leading to an almost complete loss of enzyme activity.

MATERIALS AND METHODS

Site-directed mutagenesis, expression of mutant enzyme in a deletion strain of P. denitrificans, and membrane

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[1] Abbreviations: EPR, electron paramagnetic resonance; TXRF, total-reflection X-ray fluorescence spectrometry; ICP-AES, inductively coupled plasma atomic emission spectroscopy.

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preparation were done as described earlier (Zickermann *et al.*, 1995).

Protein was purified by streptavidin affinity chromatography (Kleymann *et al.*, 1995; Ostermeier *et al.*, 1995). An engineered monoclonal antibody fragment (F_v) linked with the strep tag and directed against an epitope on the periplasmic domain of subunit II was used for affinity purification of an oxidase−F_v complex. Excess antibody fragment was removed by FPLC gel filtration.

Metal Determination. Metal to protein ratios were determined by total-reflection X-ray fluorescence spectrometry (TXRF) which allows the simultaneous multielement detection in surface and trace analysis (Prange & Schwenke, 1992). Using an X-ray beam, characteristic fluorescence radiation of the elements is excited and detected with a Si(Li) solid state detector adjusted close to the surface of the plane sample carrier. Because the X-ray beam has a very small angle of incidence, it is totally reflected and practically does not penetrate into the sample carrier. Secondary effects are thus avoided, and the fluorescence radiation is doubled in intensity because of excitation of the sample by the incident and by the reflected radiation. Light elements with an ordinal number below 14 are not detected because their fluorescence yield is very low. Consequently, the protein environment does not disturb the measurement.

We used an EXTRA II A spectrometer (Atomika Instruments, Oberschleissheim, Germany) with MoKα radiation and a measuring time of 1000 s. The buffer of the samples was exchanged to 100 mM Tris/acetate (pH 8), 0.2 g/L dodecyl maltoside, and 1 mM EDTA to avoid interference by chloride and phosphate in the sulfur determination. After addition of a Rb standard solution, 5 μL of an oxidase sample (10−60 μM) was pipetted onto a plane quartz glass carrier and dried to a thin film.

EPR spectra were recorded at various temperatures using a Bruker ER 200cw-spectrometer equipped with an Oxford Instruments ESR 910 cryostat and a PC-based data acquisition unit. EPR spectra were simulated with the program of Neese (1993), which is based on first-order approximation of the usual spin Hamiltonian for Zeeman and hyperfine interactions.

Visible spectra were recorded on a Kontron 941 spectrophotometer. Each spectrum was scanned five times and averaged.

Steady state kinetic measurements with reduced horse heart cytochrome *c* were carried out at 25 °C in 20 mM Tris/HCl (pH 8), 20 mM KCl, 1 mM EDTA, and 0.2 g/L dodecyl maltoside using a Kontron 941 photometer as described in Witt *et al.* (1995).

RESULTS

We have constructed the site-directed mutants H181N, C216S, C220S, and H224N of the Cu_A site of cytochrome *c* oxidase from *P. denitrificans*. On the basis of membrane spectra, expression levels of mutant oxidases ranged from 20 to 60% of that of wild type. The mutated enzymes and the wild type were purified as four-subunit complexes using the F_v−streptavidin tag (see Materials and Methods). On SDS gels (not shown), the mutant complexes are indistinguishable from a wild type preparation in terms of purity and subunit migration behavior, with the exception of the H224N mutant where an additional protein band is found between subunit II and III.

Table 1: Metal to Protein Ratios in Wild Type and Mutant Oxidases as Determined by TXRF[a]

	A				B		
sample	Cu:Fe	oxidase (nmol/mL)	Fe:Ox ratio	Cu:Ox ratio	oxidase (nmol/mL)	Fe:Ox ratio	Cu:Ox ratio
wild type	1.38	57.5	2.18	2.97	59	2.11	2.91
H181N	1.26	33.4	2.25	2.82	33.6	2.21	2.78
C216S	0.92	22.2	2.13	1.97	22.6	2.1	1.92
C220S	1.27	12.4	1.99	2.52	11.1	2.2	2.8
H224N	1.16	30.2	2.25	2.57	28.9	2.32	2.69

[a] As the basis for metal to protein stoichiometries, the oxidase concentration was calculated in part A by dividing the sulfur concentration of the sample by the sum of cysteine and methionine residues in the oxidase−F_v complex and in part B by dividing the sum of the copper and iron content in the sample by 5 and in the case of the C216S mutant by 4.

Metal to protein ratios (see Table 1) were measured by TXRF. To determine the oxidase concentration of the sample, the sulfur content was divided by the sum of cysteine and methionine residues in the oxidase−F_v complex (method A). Alternatively, the oxidase concentration may be derived from its metal content. For the wild type, a ratio of three Cu and two Fe per oxidase molecule has been accurately measured using ICP-AES (Steffens *et al.*, 1987, 1993) and has been confirmed by the crystal structures of cytochrome *c* oxidase and the soluble Cu_A binding domain (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995; Wilmanns *et al.*, 1995). Dividing the metal content of the sample ([Cu] + [Fe]) by 5 thus yields the oxidase concentration (method B).

In the case of the wild type and the H181N mutant, we obtain metal to protein ratios that are sufficently close to the expected 3:2 Cu:Fe per oxidase stoichiometry. In the case of the C216S mutant, we find a decrease in the copper content of the enzyme and a clear 2:2 Cu:Fe per oxidase stoichiometry. Consequently, the oxidase concentration in column B was obtained by dividing the metal content by 4 instead of 5. The H224N mutant shows a substoichometric decrease of the copper content and a ratio of copper to iron of 1.16. In the C220S mutant, we find a copper to iron ratio of 1.27, comparable to that of the H181N mutant, but the copper stoichiometry determined by method A is only 2.52 as in the H224N mutant (see also the Discussion).

We have recorded EPR spectra of the wild type and the mutants to determine the influence of the mutation on the electronic structure of the metal center (see Figures 1 and 2). In the H181N mutant (trace A in Figure 1), the *g* values are slightly shifted, and some hyperfine resolution features emerge, but the overall shape of the spectrum is similar to that of wild type. In contrast, the spectrum of the C216S mutant (Figure 2) deviates significantly from that of the wild type. A simulation reveals a four-line pattern of the hyperfine splitting, which is indicative of mononuclear copper complexes.

In the optical spectrum of the oxidized wild type (trace A in Figure 3) we observe the broad absorption band centered around 830 nm which has been assigned to Cu_A. In the H181N mutant, this absorption maximum is shifted to 815 nm. Strikingly, in the C216S mutant, the near-IR band is completely lost.

Steady state activity was measured with reduced horse heart cytochrome *c* in the range of 2.5−40 μM cytochrome

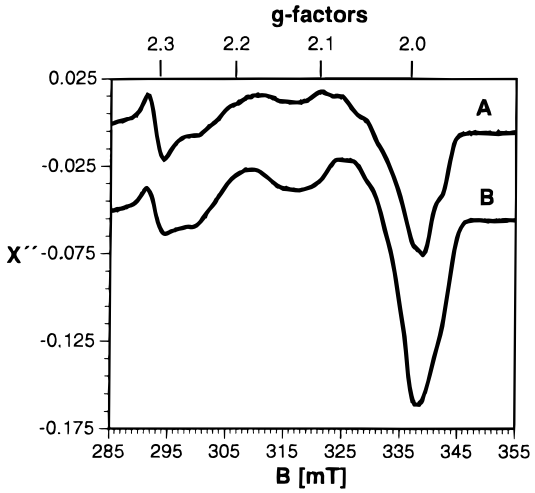

FIGURE 1: X-Band EPR spectra of the isolated H181N mutant (A) and the wild type (B) cytochrome *c* oxidase from *P. denitrificans*. Spectrometer settings were as follows: temperature of 19.3 K, microwave power of 2 mW, and microwave frequency of 9.4318 GHz (A) and 9.4310 GHz (B).

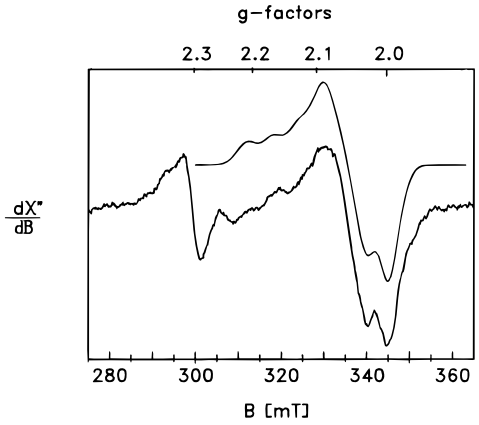

FIGURE 2: X-Band EPR spectrum of the isolated C216S mutant and simulation of the spectrum. Spectrometer settings were as follows: temperature of 90 K, microwave power of 10 mW, and microwave frequency of 9.6444 GHz. The spectrum was simulated (upper trace) with the following parameters: $g = 2.013$, 2.056, and 2.145 and $A = 80$, 80, and 180 MHz, Lorentzian line shape with isotropic line widths of 28 G. The simulation also took into account a small isotropic Lorentzian signal at $g = 2.003$, with a line width of 25 G.

c in the assay. For the H181N mutant, we find an activity of approximately 60% of that of the wild type while the activity of the C216S mutant is below 1%. The activity of the C220S and H224N mutants is in the range of 5−10% of that of the wild type.

DISCUSSION

The crystal structures of cytochrome *c* oxidase from *P. denitrificans* and bovine heart and of the soluble Cu_A binding domain have revealed that the following residues (given in the numbering of the enzyme from *P. denitrificans*) are involved in the ligation of the binuclear Cu_A site: H181, C216, C220, H224, M227, and the carbonyl function of the peptide bond of E218 (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995; Wilmanns *et al.*, 1995). We have constructed the site-directed mutants H181N, C216S, C220S, and H224N; the mutant M227I has been described earlier (Zickermann *et al.*, 1995).

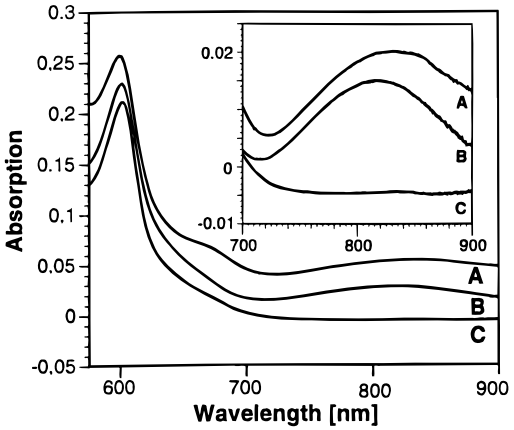

FIGURE 3: Absorption in the near-infrared region of the oxidase: (A) wild type, (B) H181N mutant, and (C) C216S mutant. Spectra of the oxidized samples were normalized to the same absorption in the 600 nm region (peak maximum minus 730 nm); the insert shows the 700−900 nm scans.

Exchanges in each of the five ligands amenable to mutagenesis lead to individual structural consequences. Two mutants with effects differing most widely from those of the other (H181N and C216S) will be discussed here in more detail.

Mutant and wild type cytochrome *c* oxidases have been purified using an engineered monoclonal antibody fragment for affinity chromatography. The antibody recognizes an epitope on the periplasmic domain of subunit II of the native enzyme (Ostermeier *et al.*, 1995). Since we obtain a fully assembled oxidase complex with a tightly bound F_v, we assume that the mutations do not affect the overall protein folding significantly.

We have used TXRF for the determination of metal to protein ratios in wild type and mutant cytochrome *c* oxidase (Table 1). The classical application of this method for multielement detection is process control in semiconductor manufacturing (Penka & Hub, 1989). Since TXRF requires only small amounts of sample and no extensive pretreatment, it is also well-suited for the analysis of biological samples.

Cytochrome *c* oxidase of the aa_3 type contains two heme irons and three copper ions (Cu_B and the binuclear Cu_A) (Steffens *et al.*, 1987, 1993). In the wild type and the H181N mutant, we find stoichiometries that are close to the expected value of 1.5 Cu:Fe, corresponding to 3 Cu and 2 Fe/complex. It is a frequent observation (Steffens *et al.*, 1987; Zickermann *et al.*, 1995) that the iron content of purified *P. denitrificans* oxidase, measured e.g. by inductively coupled plasma atomic emission spectroscopy, exceeds the theoretical value, while the copper content falls short of the expected value. Our data for the metal content of the wild type enzyme obtained by TXRF (Table 1) are in good agreement with these findings. There is also no significant deviation in the oxidase concentration as determined by the sulfur or metal content, indicating a pure and homogeneous preparation. The H224N mutant shows a substoichiometric decrease in its copper content which may be explained by partial loss of copper due to a weaker binding in the mutated site. The C220S mutant is the only case where the results from the two methods for the quantitation of oxidase concentration differ by more than 10%. This mutant could only be purified in small quantities, and the determination of sulfur was difficult due to the low sample concentration. Calculation method

A, relying exclusively on the sulfur content, leads to an underestimation of the metal:protein ratio (see Table 1). From the copper:iron ratio and the metal:protein ratios, method B, we assume that the C220S mutant contains a Cu_A site that is mostly binuclear.

In contrast, the copper content of the C216S mutant is significantly changed. We find a stoichiometry very close to 2:2 Cu:Fe per oxidase which indicates the transformation of the binuclear Cu_A site into a mononuclear copper center.

The EPR spectrum of the H181N mutant (Figure 1) shows some similarity to that of wild type. This is surprising because the $N_{\delta 1}$ of H181 is located a distance of only 2.1−2.2 Å from one of the copper atoms (Iwata *et al.*, 1995). An exchange of this important residue should therefore induce major changes in the electronic structure of the site. From a computer model of the mutated site based on the structure of the wild type, we assume that the amide group of the asparagine provides the oxygen, or alternatively, the nitrogen atom as a ligand within reasonable distance to the copper, compensating the histidine $N_{\delta 1}$ ligand to some extent.

The mutant C216S exhibits a completely different EPR spectrum (Figure 2). It can be simulated with a four-line pattern of the hyperfine splitting, indicating a mononuclear site which is in good agreement with the TXRF data (see below). While the g_z and A_z parameters for the simulation are in accordance with the classification as type I copper (Peisach & Blumberg, 1974), the low g_x value is somewhat below the typical range. Although the mutated site much more resembles a type I than a type II copper, it does not fit perfectly into this classification scheme which has been designed for naturally occurring copper proteins. Since we have generated a mononuclear site by mutation, the deviation from the classical type I sites found in blue copper proteins or in blue oxidases is not unexpected.

Like in the EPR, we observe significant differences between the two mutants when we compare their absorption in the near-infrared region of the spectrum (Figure 3). While in the spectrum of the H181N mutant the absorption is shifted but only slightly diminished, this band is lacking entirely in the spectrum of the C216S mutant.

From metal analysis and from spectroscopic investigations, we conclude that the extent in which the two individual mutations H181N and C216S affect the Cu_A site is drastically different. In its properties, the H181N mutant comes close to wild type, while in the C216S mutant, a completely different copper center is generated.

Site-directed mutants have also been obtained in the soluble fragment of the Cu_A binding domain from *P. denitrificans* (Farrar *et al.*, 1995). In contrast to our results with the complete four-subunit enzyme, these authors find that the mutation corresponding to H181N and mutation of M227 drastically affect the copper binding and folding of the fragment. We concluded that differences between results obtained with the enzyme complex and the soluble fragment may be explained by an increased exposition to solvent and a decreased stability of Cu_A in the soluble fragment (Zickermann *et al.*, 1995). However, the corresponding mutation to C216S in the fragment from *P. denitrificans* cytochrome *c* oxidase is also mononuclear.

Cu_A is the primary acceptor for electrons donated by cytochrome *c*. A perturbation of this site should therefore lead to a significant decrease or complete loss of enzyme activity because electrons for the oxygen reduction are no longer transferred to the catalytic site. In the H181N mutant, we still find an activity of 60% of the wild type while the C216S mutant is virtually inactive.

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amicyanin        EL-HVK.....GTYDYH----CTP-HPFMRGKVVVE
COX II           DVIHAW.....GVYFGQCSELCGINHAYMPIVVKAV
COX II C216S     DVIHAW.....GVYFGQSSELCGINHAYMPIVVKAV
                    |            |
                   181          216
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FIGURE 4: Alignment of the copper binding motifs of subunit II and a blue copper protein. The *P. denitrificans* amicyanin sequence (van Spanning *et al.*, 1990), the corresponding region of the *P. denitrificans* wild type cytochrome *c* oxidase subunit II (COX II) sequence (Steinrücke *et al.*, 1987), and the C216S mutant (this study) are compared. The loop region between β-strands 9 and 10 in wild type COX II (Iwata *et al.*, 1995) is underlined.

Recently, we have shown that, in the site-directed mutant M227I, the electron transfer from Cu_A to heme *a* is inhibited (Zickermann *et al.*, 1995). In this mutant, Cu_A is still binuclear but the paramagnetic electron is no longer delocalized, changing the [Cu(1.5)···Cu(1.5)] system into [Cu(I)···Cu(II)]. The EPR spectrum consequently resembles that of a mononuclear site. In the near-IR region of the optical spectrum, a diminished and shifted absorption band is found. This mutant shows a steady state activity of 10% of that of the wild type. The M227I mutant thus shares properties with both the H181N and the C216S mutant.

The comparison of these three different mutants confirms that the unusual form of the wild type EPR spectrum is due to the coupling between the two copper ions in the binuclear site as has already been stated earlier (Kroneck *et al.*, 1988, 1990; Antholine *et al.*, 1992).

From the spectra in the near-IR region, we conclude that the 830 nm band is due to an interaction between the two copper ions, since in mutant C216S with the mononuclear Cu_A site a complete loss of absorption in the near-IR is observed.

We find an obvious correlation between the change in the properties of the Cu_A site in the three mutants and the consequences for their enzyme activity. The H181N mutant which shows the smallest deviation from the wild type has the highest activity. In the M227I mutant, two copper atoms are bound but the coupling between them is lost, leading to an electronically pseudo-mononuclear site in the EPR and a residual activity of only 10% of that of the wild type. In the C216S mutant, we observe a copper site with genuine mononuclear character, paralleled by an almost complete loss of catalytic activity.

This observation clearly demonstrates that an unperturbed Cu_A site is essential for enzyme activity. Within the limits of steady state activity measurements, we find no indication for an alternative route of electron transfer between cytochrome *c* and heme *a*.

The three-dimensional structure of the periplasmic domain of subunit II of cytochrome *c* oxidase is related to the structure of blue copper proteins like plastocyanin or azurin (Holm *et al.*, 1987; Iwata *et al.*, 1995), soluble mononuclear type I copper proteins that function in electron transfer. Most of them have the same set of ligands as the Cu_A site except that one cysteine is missing.

Figure 4 shows an alignment of the metal binding sites from the blue copper protein amicyanin and subunit II of cytochrome *c* oxidase, both from *P. denitrificans*. In the primary sequence of subunit II, most ligands are clustered

on a loop between two β-strands while one, H181, is located further toward the N terminus. From a sequence comparison of the blue copper protein amicyanin and the C216S mutant, it is conceivable that mutation of the first cysteine in the Cu_A sequence may specifically generate a mononuclear copper site which differs from the blue copper protein predominantly in the length of the loop between the two β-strands.

It is interesting that the C216S mutation with its far-reaching consequences for the Cu_A site is tolerated and that the oxidase is expressed as a fully assembled and processed enzyme complex.

Considering the C216S mutated subunit II as kind of a blue copper protein with the addition of a membrane anchor, it is tempting to speculate that we observe an intermediate in the evolutionary process from a soluble mononuclear electron carrier to a binuclear electron input machinery now tightly associated with its membrane-intrinsic electron acceptor.

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