Introduction of a Cu_A site into the blue copper protein amicyanin from *Thiobacillus versutus*

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Received 13 April 1995

Abstract The C-terminal loop of the blue copper protein amicyanin, which contains three of the four active site ligands, has been replaced with a Cu_A binding loop. The purple protein produced has visible and EPR spectra identical to those of a Cu_A centre. Recent evidence strongly suggests that the Cu_A centre of cytochrome *c* oxidase and the A centre of nitrous oxide reductase are similar and are both binuclear. It therefore follows that the purple amicyanin mutant created here also possesses a binuclear Cu_A centre.

Key words: Copper protein; Site-directed mutagenesis; Amicyanin; Blue copper

1. Introduction

Cytochrome *c* oxidases are the terminal electron acceptors in aerobic respiratory chains and hence catalyse the reduction of dioxygen to water [1–5]. The aa_3-type cytochrome *c* oxidases possess two functional subunits. Subunit I contains the binuclear oxygen binding site which comprises a high-spin haem iron (cytochrome a_3) and a copper site referred to as Cu_B. This subunit also contains a low-spin haem site known as cytochrome *a*. Subunit II contains a copper site [6] which, along with cytochrome *a* of subunit I, is thought to be involved in the passage of electrons from the cytochrome *c* donor to the oxygen binding site. The copper site in subunit II is known as Cu_A and recent studies indicate that it is binuclear [6–13].

The suggestion that the Cu_A centre of cytochrome *c* oxidase may be binuclear has existed for many years [14]. However, this proposal was usually met with a large degree of scepticism, and only recent studies on nitrous oxide reductase (N_2OR) have led to the universal acceptance of this fact. EPR studies on N_2OR indicated that its A centre contains two copper atoms in a mixed valence [Cu(1.5)...Cu(1.5)] site [15]. Similarities between the EPR spectrum of the A centre of N_2OR and that of the Cu_A site of cytochrome *c* oxidase led to the conclusion that Cu_A is also binuclear [12,13]. The ligands to the two copper atoms in Cu_A are probably two cysteines, two histidines and a methionine [7]. Recent EXAFS studies on a soluble Cu_A domain of cytochrome *c* oxidase reveal that the two copper atoms are within 2.5 Å of each other [16]. The authors of this work proposed a model for the Cu_A site in which there is a Cu–Cu bond and in which the individual copper atoms are coordinated by a cysteine, a histidine and a third residue, which for one of the copper atoms is believed to be a methionine. An alternative binuclear model has recently been proposed [11] containing no bond between the copper atoms but in which the two cysteine residues act as bridging ligands. Resonance Raman studies, utilising the copper thiolate chromophore present in the Cu_A site of cytochrome *c* oxidase and the A centre of N_2OR, demonstrate that only one Cu-S(Cys) stretching vibration is detectable for these two sites [17]. This is thought to be due to the two Cys ligands being spectroscopically equivalent which is claimed to be consistent with the EXAFS model.

Type I blue copper proteins (cupredoxins) have a single copper atom at their active site which is usually coordinated by a cysteine, two histidines and a methionine, in a distorted tetrahedral arrangement [18]. Three of these ligands (the Cys, a His and the Met) are found close together in the C-terminal sequence. From sequence alignments this loop in the cupredoxins corresponds to the Cu_A binding region of subunit II of cytochrome *c* oxidase (COII). The main difference between the type I copper and Cu_A binding proteins appears to be the length of this ligand-containing loop, with the latter having a longer loop possessing an extra cysteine residue (Table 1). Mutagenesis studies have shown that a type I blue copper site and a Cu_A site can be introduced into subunit II of the *o*-quinol oxidase from *Escherichia coli*, which naturally lacks both of these sites [6], confirming that a cupredoxin-like domain is present in subunit II of both cytochrome *c* and *o*-quinol oxidases. In this article we show that a Cu_A site can be introduced into the blue copper protein amicyanin from *Thiobacillus versutus*.

2. Materials and methods

2.1. Construction of the Cu_A mutation

The sequence from Thr-94 to Phe-98 in wild-type amicyanin was replaced with the sequence Ala-Glu-Ile-Cys-Gly-Pro-Gly-His-Ser-Gly- (Table 1) using a modified version of the unique-site elimination mutagenesis protocol [19]. The sequence introduced is identical to that which was used to produce a Cu_A binding mutant in the *o*-quinol oxidase (Table 1)[6]. This mutation, as well as providing the proposed ligands, introduces glutamate and isoleucine/leucine residues between the two cysteines, and a glycine following the second cysteine. All of these amino acids are conserved in COII sequences [2].

2.2. Expression and purification

E.coli BL21 [20] was transformed with a pUC18 derivative harbouring the amicyanin construct containing the Cu_A mutation under the control of the *lac* promotor. The procedure used for expression and purification was a modified version of that described previously [21]. All of the pre-cultures contained $Cu(NO_3)_2$ to a concentration of 100 μM. The cells were allowed to grow at 37°C for only 3 h after induction. Longer incubation times between induction and harvesting were found to result in a decrease in the amount of protein present in the cells. The

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SSDI 0014-5793(95)00429-7

cells were resuspended in sucrose buffer (20% sucrose, 30 mM Tris and 1 mM EDTA, pH 8.0) and the periplasmic proteins were released by a single cycle of freezing and thawing. The final CM column, at pH 4.5, used in the wild-type procedure was replaced with purification on an FPLC Mono-Q column (Pharmacia) in 20 mM Tris buffer at pH 7.5. Elution of the protein was achieved using an ionic strength gradient created by 1 M NaCl.

The Cu_A domain of the caa_3-type cytochrome *c* oxidase from *Bacillus subtilis* was isolated as described previously [9].

SDS-PAGE and Western blotting were carried out using a Bio-Rad mini protein II system. Western blotting was carried out according to Sambrook et al. [22] using Hybond C super membrane (Amersham) and polyclonal antibodies raised in rabbit against amicyanin. SDS-PAGE was also performed on a Pharmacia PhastSystem.

2.3. Spectroscopic characterisation

For the spectroscopic measurements the Cu_A amicyanin mutant was exchanged, using ultrafiltration (Amicon, YM5 membrane), into 50 mM HEPES buffer, pH 7.0. The visible spectrum was obtained on a Shimadzu UV-2101PC spectrophotometer at 25°C. The X-band EPR spectrum was obtained on a Varian E-9 spectrometer with a home-made cryostat operating at 23 K. For the EPR measurements the protein solution was mixed with an equal volume of 87% glycerol.

3. Results and discussion

During the isolation procedure of the Cu_A amicyanin mutant a purple protein was observed which was obtained as a relatively pure fraction from the FPLC Mono-Q column. This protein gave a main band on an SDS-PAGE gel with a slightly larger molecular weight than wild-type amicyanin. This band comprised >90% of the sample and was detected with polyclonal antibodies raised against amicyanin.

The Cu_A mutation in amicyanin introduces an extra cysteine into the primary structure of the protein which could lead to the formation of dimers via disulfide bridges. To investigate the possibility of dimer formation the mutant was analysed by SDS-PAGE in a similar way as to that used by Kumer et al. [23] for *Parococcus denitrificans* amicyanin. A sample of the Cu_A mutant of amicyanin which was pre-treated in soley SDS-PAGE buffer, prior to electrophoresis, was run on a gel along with a sample pretreated in SDS-PAGE buffer and heated prior to electrophoresis, and a sample pretreated in SDS-PAGE buffer plus β-mercaptoethanol and heated prior to electrophoresis. In all cases the purple Cu_A amicyanin mutant gave a band corrsponding to a monomer. As a control, these experiments were repeated using wild-type *Thiobacillus versutus* amicyanin. This protein gave a monomer except when the sample was heated prior to electrophoresis in the absence of β-mercaptoethanol, when a dimer was observed. These results are identical to those published for amicyanin from *P. denitrificans* [23].

The visible spectrum of the purple Cu_A amicyanin mutant is shown in Fig. 1a and has peaks at 360, 483, 532 and a broad absorption at approximately 790 nm. The spectrum is almost identical to that of the native Cu_A domain of cytochrome *c* oxidase from *B. subtilis* (Fig. 1b) and is similar to those of the native Cu_A domain of cytochrome *c* oxidase from *Paracoccus denitrificans* [8], the Cu_A binding mutant of the *o*-quinol oxidase [6] and the A centre of N_2OR [17]. Resonance Raman studies on the Cu_A domain of cytochrome *c* oxidase from *B. subtilis* have shown that excitation of the three visible absorption bands above 400 nm produces the same set of RR frequencies. These bands are therefore assigned to different electronic transitions of the same Cu-S(Cys) chromophore.

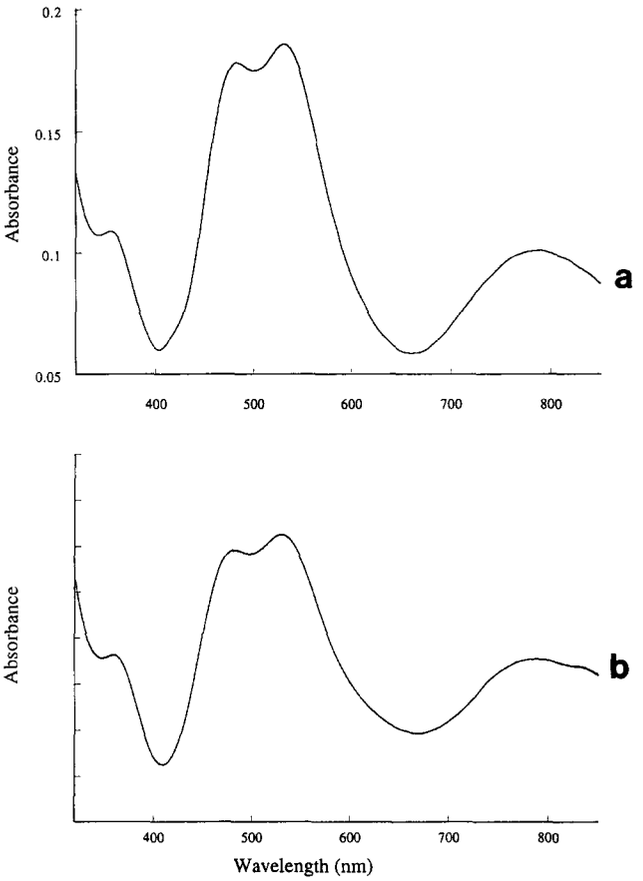

Fig. 1. Visible spectrum of (a) the Cu_A-containing amicyanin mutant (25°C) in 50 mM HEPES buffer at pH 7.0 and (b) the Cu_A domain of cytochrome *c* oxidase from *B. subtilis* (as in [9]).

Table 1
Partial amino acid sequence of *Thiobacillus versutus* amicyanin showing the C-terminal loop which contains three of the four active site ligands

Protein	Amino acid sequence												
Amicyanin	**Cys**	–	–	–	–	–	Thr	Pro	**His**	Pro	Phe	**Met**	99
COII	**Cys**	Ala	Glu	Leu	**Cys**	Gly	Pro	Ser	**His**	Ala	Leu	**Met**	227
N_2OR	**Cys**	Ser	Trp	Phe	**Cys**	His	Ala	Leu	**His**	Met	Glu	**Met**	628
CyoA	Ser	Ala	Ser	Tyr	Ser	Gly	Pro	Gly	Phe	Ser	Gly	**Met**	219
CyoA*	**Cys**	Ala	Glu	Ile	**Cys**	Gly	Pro	Gly	**His**	Ser	Gly	**Met**	219

Also shown are the homologous sequences from subunit II of cytochrome *c* oxidase from *Bacillus subtilis* (COII), nitrous oxide reductase from *Pseudomonas stutzeri* (N_2OR), cytochrome *o*-quinol oxidase from *Escherichia coli* (CyoA) and the Cu_A binding mutant of cytochrome *o*-quinol oxidase (CyoA*) [6]. In all cases the (proposed) copper ligands are in bold and the position of the final residue (methionine) shown, in the primary structure of the respective proteins, is indicated in the final column.

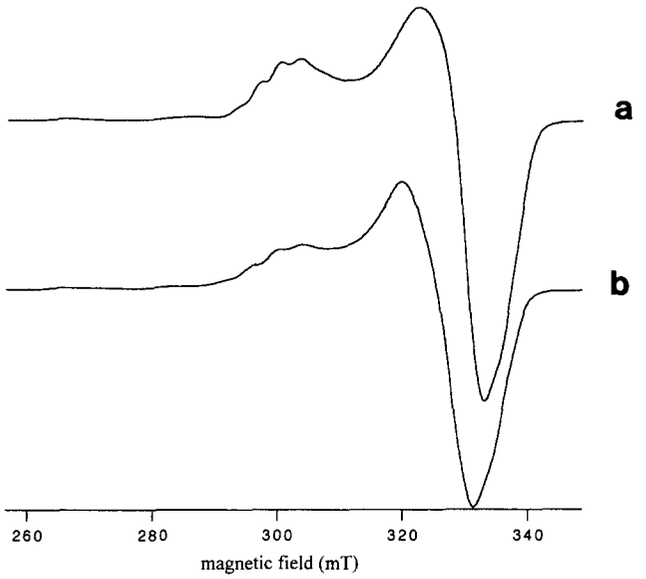

Fig. 2. X-band EPR spectrum at 23 K and using a power level at which the type II signal is saturated (20 mW) of (a) the Cu_A-containing amicyanin mutant in 25 mM HEPES (~40% glycerol) at pH 7.0 and (b) the Cu_A domain of cytochrome *c* oxidase from *B. subtilis* in 20 mM Tris buffer (10% glycerol) at pH 8.0.

The EPR spectrum of the Cu_A amicyanin mutant contains signals from two Cu(II) species which are of approximately equal intensity. One is a distinctive type II copper site which becomes saturated at high power levels. Similar signals are also found for the Cu_A-containing mutant of the soluble fragment of subunit II of cytochrome *o*-quinol oxidase [6], the soluble Cu_A domain of cytochrome *c* oxidase from *B. subtilis* [9] and also in another active site mutant od amicyanin [24]. In all cases this is assigned to adventitiously bound copper.

The second EPR signal is characteristic of a Cu_A centre and is shown in Fig. 2 along with the spectrum of the Cu_A-containing domain of cytochrome *c* oxidase from *B. subtilis*. The EPR parameters for these two spectra are shown in Table 2. The spectrum of the Cu_A amicyanin mutant is also similar to other published Cu_A EPR spectra [6,8]. The 7-line hyperfine structure, which is consistent with one unpaired electron interacting with two S = 3/2 nuclei, i.e. in a mixed valence binuclear site, is only fully resolved at X-band frequency for the A centre of N_2OR, but not completely for any of the Cu_A-containing proteins. The fine structure of the G_z signal in the case of the Cu_A mutant of amicyanin is consistent with a 7-line pattern which is more resolved than in the *B. subtilis* Cu_A spectrum, despite the smaller A_z value in the former.

Table 2
EPR parameters for the Cu_A amicyanin mutant (Cu_Aami) and the Cu_A domain of cytochrome *c* oxidase from *B. subtilis* (B2)

Protein	g_z	A_z (mT)	$g_{x,y}$
Cu_Aami	2.18	3.24	1.99–2.02
B2	2.18	3.82	1.99–2.03

In conclusion, the purple amicyanin presented here seems to bind copper as a Cu_A site. Since this site has been shown to be binuclear in the soluble Cu_A domains of cytochrome *c* oxidase and also in the Cu_A-containing mutant of the *o*-quinol oxidase it follows that the site created in amicyanin also binds two copper atoms. This work confirms the idea that the Cu_A domain of the oxidases possesses a cupredoxin-like fold. Work is currently underway to further characterise this amicyanin mutant.

Acknowledgements: This research was funded, in part, by the EC Science project SCI-CT90-0434 along with the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for Scientific Research (NWO), and under the auspices of the BIOMAC Graduate Research School of Leiden and Delft.

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