An amicyanin C-terminal loop mutant where the active-site histidine donor cannot be protonated

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ORIGINAL ARTICLE

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An amicyanin C-terminal loop mutant where the active-site histidine donor cannot be protonated

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Abstract A novel blue copper protein was constructed by replacing the C-terminal loop of amicyanin (*Paracoccus versutus*) by the homologous loop of rusticyanin. The C-terminal loop of both amicyanin and rusticyanin contains three (His, Cys, Met) of the four copper ligands. The amicyanin mutant exhibits all spectroscopic properties normally encountered for blue copper sites. The midpoint potential (369 mV) is the highest reported value for an amicyanin mutant. Cyclic voltammetry and NMR studies of the reduced form indicate that, in contrast to wild-type amicyanin and all amicyanin mutants described so far, the C-terminal histidine ligand does not protonate in the accessible pH range (pK_a<4.5).

Keywords Mutagenesis · Blue copper proteins · Cyclic voltammetry · Rusticyanin · Amicyanin

Introduction

Type 1 blue copper proteins function as mobile electron carriers in the redox chains of many organisms [1, 2]. They contain a redox-active copper site where the copper is coordinated by the N^{δ} atoms of two histidines, the S^{γ} atom of a cysteine and the S^{δ} atom of a methionine at a longer distance (see Fig. 1). One of the histidine ligands is located between the fourth and the fifth β-strand of the protein, while the remaining three ligands are found together in a C-terminally located surface-exposed loop between the seventh and eighth (last) β-strand. In the blue copper protein amicyanin, the exposed histidine ligand, located in the C-terminal loop, can be protonated in the reduced state. Owing to this protonation the reduction potential exhibits a huge rise when the pH is lowered below the pK_a. NMR studies showed that the protonation also coincides with a drop in the electron self-exchange rate [3] and, therefore, the protonated state is sometimes referred to as redox inactive. These properties of amicyanin are shared by other blue copper proteins, e.g. pseudoazurin and plastocyanin. For the latter it was proposed that in vivo this might have a function in controlling the redox activity in photosynthesis [4] since a high photosynthetic rate means a drop in pH and therefore a drop of activity for plastocyanin.

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Materials and methods

The EPR spectra were analyzed with the program KOPER [15].

Electrochemical measurements were carried out at room temperature, using an Autolab potentiostat (Eco Chemie, Hol-

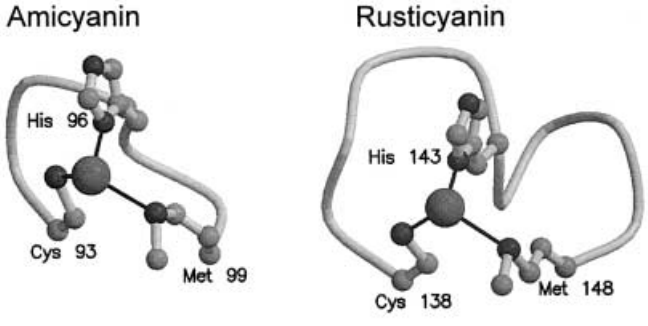

Fig. 1 C-terminal loop structure representations and residue sequence of amicyanin (*Paracoccus versutus*) and rusticyanin (*Thiobacillus ferrooxidans*) based on atomic coordinates in the Protein Data Bank (entries 1AAC and 1RCY). Amicyanin: **Cys** Thr Pro **His** Pro Phe **Met**, Rusticyanin: **Cys** Gln Ile Pro Gly **His** Ala Ala Thr Gly **Met**

land) with a mercaptopyridine-treated gold working electrode, a calomel standard electrode and a platinum counter electrode. The sample concentration was 1–2 mg ml^{-1}. Measurements were carried out at pH 9 (10 mM TES [*N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid]), pH 8 (10 mM TES), pH 7 (10 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)]) and pH 6.5 (10 mM MES [2-(*N*-morpholino)ethanesulfonic acid]).

^{1}H NMR spectra were recorded on a Bruker DMX600 spectrometer. For sample preparation and procedures, see [3, 5, 18]. The protein concentration in the samples for the k_{ese} measurements and for the pH titration was approximately 6 mM. All samples were dissolved in 99.95% D_2O. The quoted pH values have not been corrected for the isotope effect. Singlet selection pulse programs (Hahne and CPMG) were used to minimize the number of peaks in the region where the $C^{\varepsilon 1}$ and $C^{\delta 2}$ protons of the histidines were expected. k_{ese} measurements were carried out at 298 K, pH 8.14.

Results and discussion

Wild-type amicyanin from *Paracoccus versutus* has a pK_a of 6.8. In recent studies it was possible with loop-directed mutagenesis to lower this to 5.4 [5]. The aim of the present study was to design a loop mutant of amicyanin with an even lower pK_a, ideally below detection. *Thiobacillus ferrooxidans*, is an acidophilic bacterium with an optimum pH for growth of 2 [6, 7]. In agreement with the growth conditions of this organism, rusticyanin is stable at pH 2, the C-terminal histidine is not protonated at this pH and the protein is still redox active. We have introduced the C-terminal loop of rusticyanin from *T. ferrooxidans* into amicyanin to check whether it is possible to transfer these properties from rusticyanin into amicyanin.

Mutation and protein isolation

To construct the AmiRus mutant, the C-terminal loop of rusticyanin was transferred to amicyanin (see Fig. 1). The mutation from $C^{93}TPH^{96}PFM^{99}$ to $C^{93}GIPGH^{98}AATGM^{103}$ was performed by a method based on two consecutive PCR reactions. The mutation was verified by DNA sequencing. The plasmid pUC18 was used as a cloning vector, with the gene coding for the mutants under control of the *lac* promoter. The gene encoding AmiRus was transformed into JM109; expression and isolation were performed as described for wild-type amicyanin [8].

EPR and electronic spectra

The number of residues between the coordinating ligands was increased from 4 (amicyanin) to 8 (AmiRus) by the mutation (see Fig. 1). Despite the drastic structural changes in the active site, a mutant with a stable copper chromophore was obtained, as was apparent from electronic and EPR spectroscopy. The spectroscopic properties of AmiRus are intermediate between those of the two wild-type proteins (see Table 1). The optical spectra of wild-type amicyanin and AmiRus are shown in Fig. 2. AmiRus has a small increase of the absorption band around 460 nm with respect to amicyanin, but this is smaller than for rusticyanin (see Table 1). The increased ratio of the two absorption bands (460/600 nm) may indicate an increased rhombicity of the site [9, 10, 11].

The EPR parameters [15] also indicate an increased rhombicity of the AmiRus site compared to amicyanin, but the effect is again smaller than for rusticyanin (see Fig. 3 and Table 1) [10, 11]. The hyperfine coupling constants are small and in the range expected for type 1 copper proteins [5, 16].

Table 1 Properties of wild-type amicyanin, wild-type rusticyanin and the mutant AmiRus

Name	wt Ami[a]	wt Rus[b]	AmiRus[c]
A_{460nm}/A_{600nm}	0.10	0.48	0.27
g_x	2.049	2.019	2.028
g_y	–	2.064	2.045
g_z	2.239	2.229	2.210
A_x (mT)	–	6.5	6.6
A_y (mT)	–	2.0	–
A_z (mT)	5.3	4.5	3.8
pK_a (I=0.05) (His protons)	7.2	Not titrable	Not titrable
E_{mid} (mV)	260	680	369
Electron selfexchange rate (M^{-1} s^{-1})	1.2×10^5	1.0×10^4	$<1.0\times10^4$

[a] *P. versutus* [5]
[b] *T. ferrooxidans* [12, 13, 14]
[c] This work

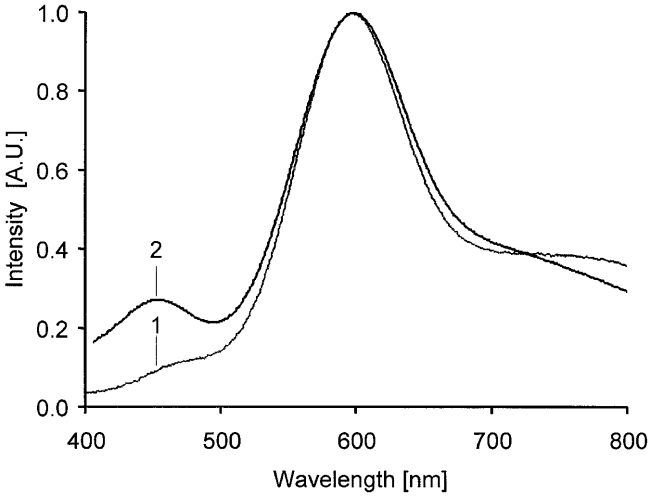

Fig. 2 UV/vis spectra of amicyanin (*1*) and AmiRus (*2*). UV/vis spectra were recorded on a Shimadzu UV-2101PC spectrophotometer at 25 °C in 10 mM HEPES (pH 7)

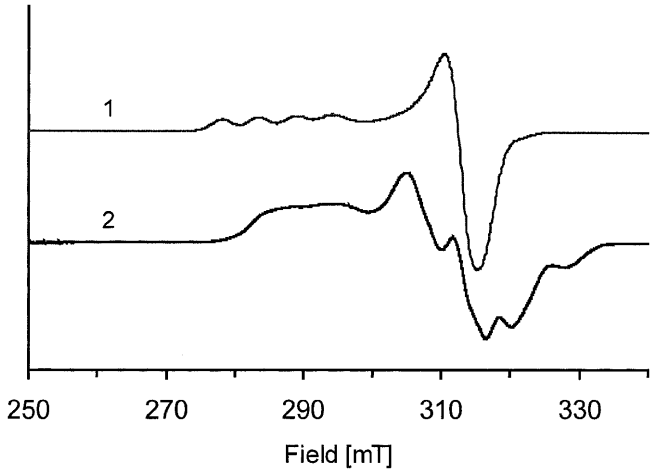

Fig. 3 X-band EPR spectra of amicyanin (*1*) and AmiRus (*2*), recorded at 77 K on a JEOL ESPRIT 330 at approximately 9 GHz with DPPH as an external reference. The protein was dissolved in 10 mM HEPES (pH 7) with 40% glycerol. The spectra were simulated with the program KOPER

Cyclic voltammetry

The mutant AmiRus has a midpoint potential (E°) of 369 mV at pH 7, as determined by diffusion-controlled cyclic voltammetry. This is about 100 mV higher than the value for amicyanin but 300 mV below that of rusticyanin (see Table 1). The midpoint potential of wild-type amicyanin increases when the pH is lowered [17]. It is known that this pH dependence is caused by the protonation of His96 [3, 5, 17] In contrast, the potential of AmiRus is constant (±3 mV) between pH 6.5 and pH 9 (see Table 2). In the same range, amicyanin shows a variation of approximately 30 mV [17]. These results for AmiRus indicate that there is no protonation of the C-terminal histidine at pH>6.5. Therefore the pK_a value of AmiRus is below 5.5, since a rise in mipoint potential should be observable at least one pH unit above the pK_a [17]. Owing to peak broadening it was not possible to determine the midpoint potential with cyclic voltammetry below pH 6.5.

^{1}H NMR spectroscopy

With ^{1}H NMR it often is possible to detect the histidine protonation of the reduced protein directly by measurement of the chemical shifts of the $C^{\varepsilon 1}$ and $C^{\delta 2}$ protons as a function of pH [3, 5, 18]. However, for the reduced AmiRus mutant, none of the five observed singlets in the aromatic region showed a large downfield shift upon lowering the pH from 8.5 to 4.7. Also, none of the singlets showed a considerable decrease in intensity (at pH<4.5 all peaks broaden and decrease), and no new singlets formed on lowering the pH. These results indicate that the pK_a of the C-terminal histidine is <4.5. Destabilization of AmiRus prevented the measurements of NMR spectra below pH 4.5. This confirms the result of the cyclic voltammetric measurements and suggests that the pK_a for AmiRus is below 4.5. This is the first mutant for which such a low pK_a has been observed.

^{1}H NMR may also be used to determine the electron self-exchange rate (k_{ese}) by measuring the increase in the T_2 relaxation of protons close to the copper ion after addition of small amounts of the oxidized protein [5, 18]. For AmiRus, no such increase was observed until 20% of the sample was oxidized and, therefore, only a limiting value for k_{ese} could be established ($<1.0\times10^{-4}$ M^{-1} s^{-1}).

Table 2 Cyclic voltammetry of AmiRus

pH	6.5	7.0	8.0	9.0
E° (mV)[a]	371	369	366	368
Peak separation (mV)	91	71	65	69

[a]Midpoint potential, vs. SHE; all measurements at 22 °C

Conclusion

With both cyclic voltammetry and ^{1}H NMR spectroscopy, no histidine protonation for the AmiRus mutant could be observed. Cyclic voltammetry shows clearly that the pK_a of this mutant is below 5.5, and based on pH-dependent ^{1}H NMR spectroscopy a pK_a<4.5 can be suggested. This is the first amicyanin mutant that exhibits this behavior. Further studies will be performed to confirm and elaborate this result. The midpoint potential of AmiRus has increased to 369 mV, resulting in the highest E° reported for an amicyanin mutant.

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