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Rack-induced bonding in blue copper proteins: spectroscopic properties and reduction potential of the azurin mutant Met-121 → Leu

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Site-directed mutagenesis has been used to prepare azurin in which the methionine-121 residue has been replaced by leucine. The oxidized mutant protein displays the strong blue color and characteristic EPR signal of a type 1 Cu(II) ion, showing that methionine is not an obligatory component of a blue copper site. The optical absorption maximum is shifted 5 nm towards longer wavelength and the extinction coefficient increased by about 10% compared to the wild-type protein. In addition, there are small changes in the EPR parameters, in particular the copper hyperfine splitting. The reduction potential is increased by 70 mV. The results show that a small change in primary structure without any alteration in the three strong ligands can perturb the Cu(II) site and shift the reduction potential, in accord with the concept of rack-induced bonding in blue copper proteins.

Azurin; Blue copper; EPR; Charge transfer; Reduction potential

1. INTRODUCTION

Gray and Malmström [1] in 1983 introduced the idea of rack-induced bonding in blue copper proteins. According to this concept, conformational energy of the peptide backbone structure forces the Cu(II) ion to adopt the special structure of a blue copper site. It was furthermore suggested that variations in the reduction potential of different blue proteins can be ascribed to changes in π back-bonding at the blue copper site induced by the protein structure. This suggestion receives support from the relationship observed [1] between ligand-field strengths and electron-transfer enthalpies for blue copper proteins.

Several possible structural causes of the variation in back-bonding have been considered [1–3]. Changes in the strength of the Cu-S(Cys) and Cu-S(Met) interactions are indicated by resonance-Raman measurements [2,3]. The π back-bonding may also be affected by NH-S hydrogen bonding [1]. In addition to the back-bonding mechanism, the reduction potential may be tuned by variations in the solvent exposure of the copper site, which would cause changes in the entropic term.

We have initiated a research program in site-directed mutagenesis to test further the concept of rack-induced bonding in blue copper proteins [4,5]. In this communication we describe the production in *E. coli* of a mutant Met-121 → Leu of *Pseudomonas* azurin, and we present comparisons of the optical and EPR spectra as well as the reduction potential between the mutant and the wild-type proteins. The results clearly demonstrate that a small change in primary structure can cause a considerable change in reduction potential without grossly perturbing the spectroscopic properties of the oxidized copper site, as predicted by the rack hypothesis.

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2. MATERIALS AND METHODS

2.1. *Azurin production*

The bacterial strains, plasmids, DNA techniques and protein purification have been described earlier [5]. After the second ion-exchange step the protein was transferred to a buffer containing 150 mM NaCl and 18 mM Na_2HPO_4, pH 7.2.

2.2. *Site-directed mutagenesis*

Two new restriction enzyme sites were introduced by site-directed mutagenesis in the azurin structural gene [4] on both sides of the codon for the Met-121 residue. An oligonucleotide linker was synthesized and ligated in the gene. Details of this casette-mutagenesis procedure will be described elsewhere.

2.3. *Optical and EPR-spectra*

Optical spectra were recorded between 260 and 800 nm in a Shimadzu 3000 spectrophotometer. EPR spectra were obtained on a Bruker ER 20D-SRC spectrometer equipped with an Oxford Instrument EPR-9 helium cryostat. Integrations were performed as described earlier [6] with Cu(II) in 2 M $NaClO_4$, pH 2, as reference.

2.4. *Reduction potential*

The reduction potentials of the wild-type and the mutant azurins were determined potentiometrically at 20°C in a modified spectrophotometer cuvette provided with a Pt electrode and with an Ag/AgCl electrode in 1 M KCl as reference [7]. The redox mediators used were $K_3Fe(CN)_6$, 1,4-benzoquinone, 2,3,4,5-tetramethyl-*p*-phenylenediamine and sodium ascorbate. The concentration of oxidized protein at each measured potential, E, was estimated from the absorbance around 630 nm. The reduction potentials $E^{\circ\prime}$ were estimated from Nernst plots.

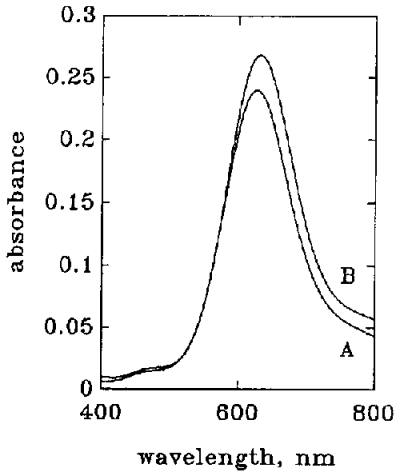

Fig.1. Visible absorption spectra of wild-type (A) and mutant (B) azurin (1-cm path). The absorbance scale for spectrum A is 0–0.2, whereas the scale for B is that given on the axis.

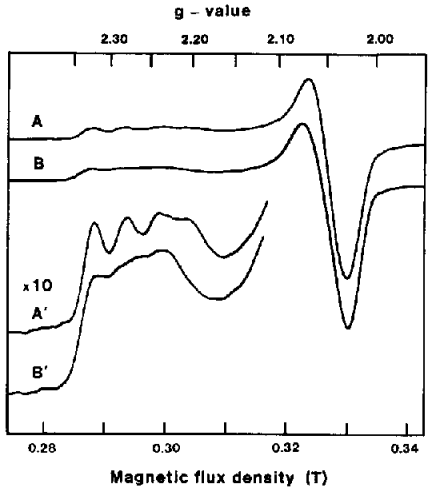

Fig.2. EPR spectra of wild-type (A, A′) and mutant (B, B′) azurin. According to integrations, the concentration of Cu(II) is 31 μM (wild type) and 45 μM (mutant), respectively. Conditions: microwave frequency, 9.375 MHz; power, 2 mW; modulation amplitude, 2.0 mT; temperature, 25 K; gain, 1.25×10^5 (A) and 1.00×10^5 (B).

3. RESULTS

The optical spectra of the wild-type and mutant proteins are compared in fig.1. The EPR spectra of the same proteins are shown in fig.2. There were no other EPR signals than those from type 1 Cu(II) seen in the two samples.

When the samples were concentrated by dialysis, it was found that the blue color of the mutant protein was partially lost, indicating that the blue site is less stable than in the wild-type, and this was confirmed by EPR analysis. The extinction coefficients at the wavelength of maximum absorption were estimated on the basis of the Cu(II) concentrations determined by integration of the EPR signals. The optical characteristics and reduction potential of the wild-type and mutant proteins are

Table 1

Optical characteristics and reduction potentials ($E^{\circ\prime}$) of wild-type and mutant azurin

Protein	Abs. max. (nm)	Ext. coeff. ($M^{-1}\cdot cm^{-1}$)	A_{max}/A_{280}	$E^{\circ\prime}$ (mV)
Wild type	628	5300	0.56	308
Mutant	633	6000	0.33	375

Table 2

EPR parameters for wild-type and mutant azurin

Protein	g_x	g_y	g_z	A_z (10^{-4} cm^{-1})
Wild type	2.035	2.052	2.261	57
Mutant	2.0353	2.058	2.274	39

summarized in table 1, and the EPR parameters are compared in table 2.

4. DISCUSSION

The mutant azurin has the typical strong blue color (fig.1 and table 1) and narrow EPR hyperfine splitting (fig.2 and table 2) associated with a type 1 Cu(II) site. This shows that a methionine sulfur is not necessary to create a blue copper site, as also evidenced by the fact that stellacyanin does not contain a methionine residue [8]. The ratio of the absorbance around 630 nm to that at 280 nm is lower in the mutant compared to the wild-type protein (table 1), indicating that Cu(II) is not occupying the blue site in all molecules of the mutant protein, because some is lost in the purification due to the lower stability of the mutant protein (see section 3). Thus, even if methionine is not a necessary part of a blue site, it may confer a greater stability, which could be the reason why methionine is a conserved residue in most blue proteins [9].

The EPR spectrum at high amplification shows that there is no Cu(II) bound nonspecifically to the mutant protein. The true extinction coefficient for Cu(II) in the blue site of the mutant protein can consequently be calculated on the basis of the concentration estimated from integration of EPR signal. Since the uncertainty in the integrations is about 10%, the higher value found for the mutant (table 1) is probably significant. There is also a definite shift in the wavelength of maximum absorption from 628 nm in the wild type to 633 nm in the mutant protein (fig.1). In addition, there is a significant change in the EPR parameters, particularly the copper hyperfine splitting (table 2). Thus, there is a small change in the Cu(II)-ligand interactions in the mutant compared to the wild-type protein.

The reduction potential of the Leu-121 azurin mutant is shifted upwards by about 70 mV compared to the wild-type Met-121 protein. This shows that the strong Cu-S(Met) interaction cannot be mainly responsible for the high potential in a blue copper protein [1–3]. Instead, the shift in the charge transfer transition to a lower energy by about 5 nm indicates that the substitution of a leucine residue for the methionine at position 121 indirectly causes a change in the strength of the Cu-S(Cys) interaction. The changed EPR parameters may also reflect alterations in Cu(II)-ligand interactions. To elucidate the basis for the increased potential, it becomes important to determine the change in the ligand field in the mutant and its possible correlation with Cu-ligand stretching frequencies determined in resonance-Raman experiments.

It is known that the entropic term, related to changes in solvation, is important in determining the reduction potential of azurin [10]. Thus, it is possible that the increased potential in the mutant is due to a less negative reaction entropy because of a change in the accessibility to the solvent. A study of the temperature dependence of the reduction potential is obviously necessary. It is, however, already clear that changes in protein conformation caused by a single amino acid substitution can result in subtle changes in Cu-ligand interactions, which in turn tune the reduction potential. The concept of a rack-induced bonding in blue copper proteins is consequently well borne out by the experimental observations reported here.

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