Site-directed mutagenesis of azurin from *Pseudomonas aeruginosa* enhances the formation of an electron-transfer complex with a copper-containing nitrite reductase from *Alcaligenes faecalis* S-6

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Abstract **Kinetic analysis of electron transfer between azurin from *Pseudomonas aeruginosa* and copper-containing nitrite reductase (NIR) from *Alcaligenes faecalis* S-6 was carried out to investigate the specificity of electron transfer between copper-containing proteins. Apparent values of k_{cat} and K_m of NIR for azurin were 300-fold smaller and 172-fold larger than those for the physiological redox partner, pseudoazurin from *A. faecalis* S-6, respectively, suggesting that the electron transfer between azurin and NIR was less specific than that between pseudoazurin and NIR. One of the major differences in 3-D structure between these redox proteins, azurin and pseudoazurin, is the absence and presence of lysine residues near their type 1 copper sites, respectively. Three mutated azurins, D11K, P36K, and D11K/P36K, were constructed to evaluate the importance of lysine residues in the interaction with NIR. The redox potentials of D11K, P36K, and D11K/P36K azurins were higher than that of wild-type azurin by 48, 7, and 55 mV, respectively. As suggested by the increase in the redox potential, kinetic analysis of electron transfer revealed reduced ability of electron transfer in the mutated azurins. On the other hand, although each of the single mutations caused modest effects on the decrease in the K_m value, the simultaneous mutations of D11K and P36K caused significant decrease in the K_m value when compared to that for wild-type azurin. These results suggest that the introduction of two lysine residues into azurin facilitated docking to NIR.**

Key words: Azurin; Electron transfer; Nitrite reductase; Pseudoazurin; Site-directed mutagenesis

1. Introduction

Pseudoazurin from *Alcaligenes faecalis* S-6 is a redox protein belonging to the cupredoxin family, which contains a single type 1 (blue) copper atom and functions as an electron donor to a copper-containing nitrite reductase (NIR) [1–3]. X-ray analysis of pseudoazurin has revealed an 8-stranded β-barrel structure followed by two α-helices at the C-terminus, binding a type 1 copper atom on the top of the β-barrel [4,5]. The structure is surprisingly similar to that of another cupredoxin, azurin [6], although no significant identity in amino acid sequences of the two proteins was observed [7]. By using site-directed mutagenesis of pseudoazurin, we have previously shown that a 'ring' of lysine residues (Lys-10, Lys-38, Lys-57, and Lys-77) located at the edge of a hydrophobic region surrounding a type 1 copper site is involved in interaction with NIR [8]. In azurin, however, this ring of lysine residues is absent on the surface (Fig. 1). This suggests that these lysine residues in pseudoazurin are one of the major determinants of specificity between cupredoxins and NIRs. To understand the specificity between redox partners, we investigated electron transfer reaction between *Pseudomonas aeruginosa* azurin and *A. faecalis* NIR by steady-state kinetics. α-Carbon atoms of residues Asp-11 and Pro-36 of azurin are approximately located in positions analogous to those of Lys-10 and Lys-38 of pseudoazurin, respectively (Fig. 2). We constructed three mutated azurins in which Asp-11 and Pro-36 were independently and simultaneously exchanged for Lys by site-directed mutagenesis to evaluate the importance of placing lysine residues at these locations on the interaction of cupredoxins with *A. faecalis* NIR. We here report the electron transfer between *P. aeruginosa* azurin and *A. faecalis* NIR and the effect of introduction of positively charged residues into azurin on the interaction with *A. faecalis* NIR.

2. Materials and methods

2.1. Construction of azurin mutants

Escherichia coli strain JM105 [*Δ(lac pro) thi strA endA sbcB15 hsdR4* F′ *traD36 proAB lacIq lacZΔM15*] was used as a host for the production of wild-type and mutated azurins. pGC4 [9], an expression plasmid for the azurin gene from *Pseudomonas aeruginosa* CIT135, was kindly given by Prof. Dr. G.W. Canters. Polymerase chain reaction was carried out with pGC4 as a template using two oligonucleotides, 5′-GCCCAAGCTTACCTAGGAGGCTGCTCCA-TGCTA-3′ and 5′-TGAGCCCTGCAGGCGCCCATGAAAAAG-CCCGGC-3′, to introduce the *Hin*dIII and *Pst*I sites to just upstream of the region for translational initiation and just downstream of the termination codon, respectively. The amplified DNA fragment of 525 bp was digested with *Hin*dIII and *Pst*I and then cloned into M13 mp18. The mutated azurin genes D11K and P36K, in which Asp-11 and Pro-36, respectively, were replaced by Lys, and D11K/P36K, in which Asp-11 and Pro-36 were simultaneously replaced by Lys, were constructed as follows. To replace Asp-11 and Pro-36 by Lys, site-directed mutagenesis of azurin was carried out by the method of Kunkel [10] using oligonucleotides 5′-CAGGGTAACAAGCA-GATGCAG-3′ and 5′-CTGTCCCACAAGGGCAACCTG-3′, respectively. The nucleotide sequences of wild-type and mutated azurins were confirmed directly by the M13 dideoxy chain termination method [11,12]. Both wild-type and mutated azurin genes were subcloned into pUC19 and then introduced into *E. coli* according to the method of Norgard [13].

2.2. Purification of azurin, azurin mutants, and NIR

An *E. coli* transformant was precultured aerobically at 37°C for 8 h in 10 ml of 2×YT medium containing 50 μg of ampicillin/ml, and then inoculated into 1 liter of the same medium containing 0.5 mM $CuSO_4$ and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the *lac* promoter of pUC19. After 15 h of cultivation, the cells were harvested and cell fractionation was carried out to prepare the

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periplasmic fraction by the method of Cornelis et al. [14]. 1 M Tris-HCl buffer, pH 8.0, and 0.1 M $CuSO_4$ was added to the periplasmic fraction to give concentrations of 10 mM and 1 mM, respectively, and the resulting solution was applied to a DEAE-Toyopearl column (30×70 mm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0 (buffer A). Passing fractions with an intensely blue color were collected and concentrated with a Centriprep 10 tube (Amicon). The concentrated sample was applied to a HiLoad 16/60 Superdex 75 FPLC (Pharmacia Biotech) gel filtration column equilibrated with buffer A containing 150 mM NaCl and eluted with the same buffer. Purity of each protein was analyzed by SDS-PAGE [15]. *A. faecalis* NIR was produced in *E. coli* and purified as described previously [16].

2.3. Spectral analysis

Absorption spectra of wild-type and mutated azurins were measured with a DU7400 (Beckman) spectrophotometer. CD spectra were measured with a JASCO J-720 spectrophotometer. The redox potential of azurin was determined by redox titration using ascorbic acid and potassium ferricyanide as described previously [17].

2.4. Kinetic analysis

Kinetic analysis of electron transfer from wild-type or mutated azurins to NIR was carried out as described previously [16]. Each reaction mixture contains 20 mM potassium phosphate buffer, pH 7.0, 2 mM KNO_2 and reduced azurin with concentrations of 0.2–1.3 mM. By addition of NIR to give concentration of 3.3×10^{-7} M, the reaction was started and the increase in absorbance at 628 nm was monitored. Data were processed with the initial velocity program of Cleland [18]. Protein concentrations were determined by the method of Bradford [19].

3. Results and discussion

3.1. Comparison of the X-ray structures of azurin and pseudoazurin

Both the structures of pseudoazurin from *A. faecalis* S-6 and azurin from *P. aeruginosa* have been determined by X-ray crystallographic analysis [4–6]. Although identity in amino acid sequence between the two blue-copper proteins is very low (11%), the X-ray analyses revealed that these two proteins have a similar cupredoxin fold with an 8-stranded β-barrel and 70 α-carbons can be superimposed with rms deviation of 2.0 Å (Fig. 2). The largest structural difference is a large insertion which forms a 'back flap' region near a type 1 copper site consisting of residues 52–81 in azurin. We have previously shown that four lysine residues, Lys-10, Lys-38, Lys-57 and Lys-77, surrounding the Cu atom of pseudoazurin are involved in the interaction with NIR. Since involvement of the corresponding face of azurin in the electron transfer reactions with both cytochrome c_{551} and cytochrome cd_1-type NIR has also been reported [20], it is reasonable to think that this region of azurin could be involved in electron transfer to *A. faecalis* NIR. As shown in Fig. 1, however, the lysine residues in pseudoazurin important for NIR interaction are missing around the electron transfer site of azurin. Furthermore, the loops containing these important lysine residues are different in length in azurin. Superposition of the two cupredoxin structures shows that α-carbons of Asp-11, Pro-36, His-83, and Phe-111 roughly occupy the positions corresponding to those of Lys-10, Lys-38, Lys-57 and Lys-77 of pseudoazurin, respectively (Fig. 2). The absence of lysine residues on the surface of azurin molecule allows us to estimate the low electron transferring ability of azurin to *A. faecalis* NIR.

3.2. Site-directed mutagenesis and physicochemical measurements of azurin

To examine the hypothesis described above, we prepared azurin of *P. aeruginosa* as described in Section 2. In addition, to see if we could improve electron transfer between azurin and NIR by adding lysine residues to the surface of azurin, we constructed a series of mutated azurins, D11K, P36K, and D11K/P36K, in which Asp-11 and Pro-36 were replaced by lysines. We chose only two of the four amino acid residues of azurin described above for site-directed mutagenesis, because Asp-11 and Pro-36 are located on the surface of the azurin

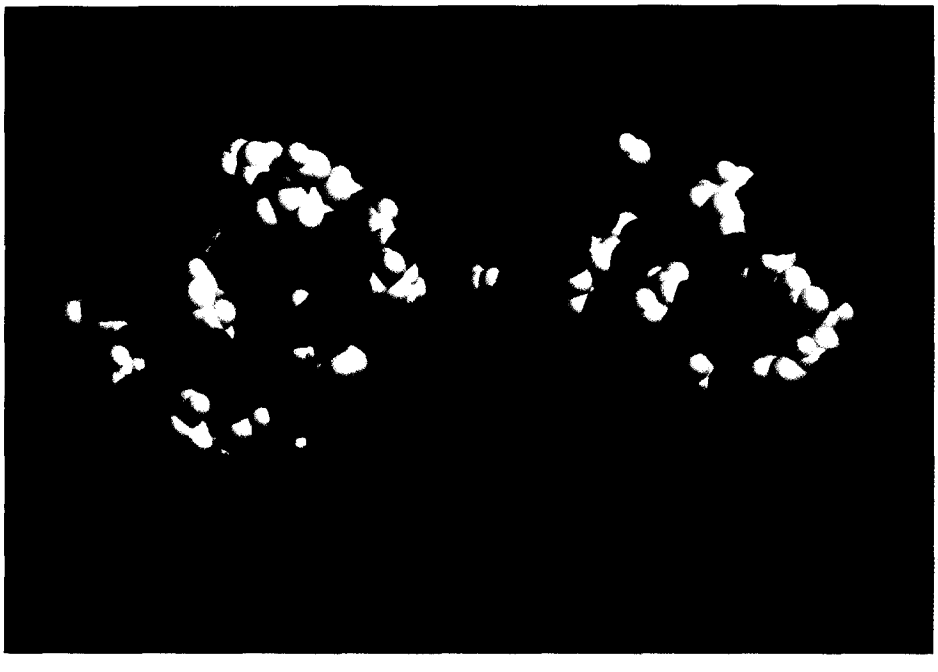

Fig. 1. Structures of azurin from *P. aeruginosa* (left) and pseudoazurin from *A. faecalis* S-6 (right) drawn with Molscript [28] and Raster3d [29]. The residues of both molecules are colored as follows: Lys and Arg, blue; Glu and Asp, red; His, cyan; amino acid residues with hydrophobic side chains (Ala, Val, Leu, Ile, Met, Phe, Tyr, Trp, Cys and Pro), green; Gly, Ser, Thr, Asn and Gln, white.

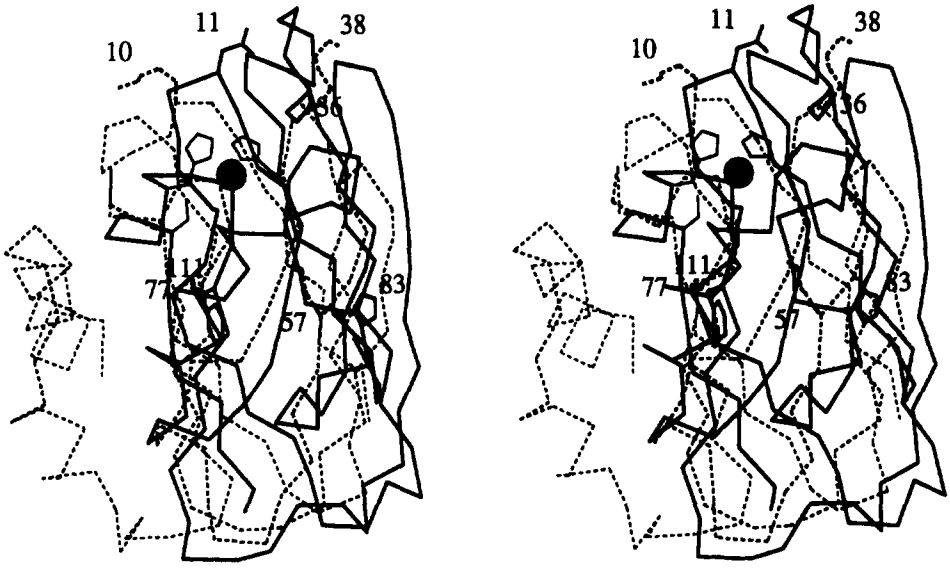

Fig. 2. Stereo view of Cα structures of azurin from *P. aeruginosa* (full lines) and pseudoazurin from *A. faecalis* S-6 (dashed lines) superimposed using the programs Clusterpose [30] and Clusterid (M.E.P. Murphy, unpublished).

molecule, while His-83 and Phe-111 are occluded by the 'back flap' region, and it is therefore unlikely that any residues introduced at those positions would extend their side chains to the molecular surface without a gross conformational change. All the mutant proteins were purified in the same way as wild-type azurin. The CD spectra of both wild-type and mutant azurins are almost identical (data not shown), suggesting that the overall structure is not greatly distorted by the mutations. Wild-type azurin showed an intense absorption peak at a wavelength of 628 nm in the optical spectrum (data not shown). Table 1 summarizes maximum absorption wavelengths (λ_{max}) and molar extinction coefficients (ε) obtained from the optical spectra of the mutated azurins. Since the optical spectra of all the mutated azurins were principally similar, although small differences in the spectral parameters were observed, we concluded that the environment around type 1 copper is not greatly altered by the mutations. Differences of ± 3 nm in maximum absorption are commonly seen for azurin mutants of surface residues [21].

Redox potentials of wild-type and mutated azurins were determined by redox titration in 0.1 M potassium phosphate buffer at pH 7.0. As shown in Table 1, the redox potentials of D11K, P36K, and D11K/P36K azurins are higher than that of wild-type azurin by 48, 7, and 55 mV, respectively. The observed change in the redox potentials can be explained by an increase in positive charge close to the copper site. Similar electrostatic effects have also been reported for other mutated azurins, Met-44 Lys and Met-64 Glu, which showed increases of 53 mV and 28 mV in redox potential, respectively [22,23].

3.3. Electron transfer between azurin and NIR

To investigate the specificity of the electron transfer reaction between wild-type or mutated azurins and a copper-containing NIR from *A. faecalis* S-6, electron transfer from reduced azurin to NIR was measured by following the increase in absorption at 628 nm due to the oxidation of azurin. Table 2 shows apparent k_{cat} and K_m values of NIR for azurins along with those for the physiological electron donor, *A. faecalis* pseudoazurin. Wild-type azurin could transfer electrons to *A. faecalis* NIR but at a decreased efficiency; the k_{cat} value of NIR for wild-type azurin is 1.3 s^{-1}, which is 300-fold smaller than that for pseudoazurin (396 s^{-1}). It was shown that donor-acceptor distance and difference in redox potentials between redox centers were the important factors which determine electron transfer rate [24]. Considering that the redox potential of wild-type azurin (313 mV) is higher than that of *A. faecalis* pseudoazurin (270 mV) determined in the same conditions [8], it seems reasonable that the electron transfer rate between azurin and NIR is slower than that between pseudoazurin and NIR. In addition, we assume that the 'back flap' in azurin may interfere with forming an efficient electron transfer complex with *A. faecalis* NIR. The K_m value of NIR for wild-type azurin is 8.6 mM, which is 172-fold larger than for pseudoazurin (0.05 mM). This indicates that azurin is far less specific than pseudoazurin as an electron donor to NIR from *A. faecalis* S-6.

When the kinetic parameters for D11K, P36K, and D11K/P36K azurins were compared with those for wild-type azurin, further decreases in the k_{cat} values were observed in the mu-

Table 1
Spectral parameters and redox potentials of azurin mutants

Azurin	λ_{max} (nm)	ε (mM^{-1} cm^{-1})	Redox potentials (mV)
Wild-type	628	5.7	313
D11K	625	5.8	362
P36K	630	5.2	320
D11K/P36K	628	6.2	368

Table 2
Apparent kinetic parameters of NIR for cupredoxins

Cupredoxin	k_{cat} (s^{-1})	K_m (mM)
Wild-type azurin	1.3	8.6
D11K azurin	0.3	4.8
P36K azurin	0.1	7.7
D11K/P36K azurin	0.3	1.5
Pseudoazurin	396	0.05

tants (0.3, 0.1, and 0.3 s^{-1}, respectively). These reduced abilities of electron transfer can be attributed to increases in redox potentials observed in the mutated azurins as described above. On the other hand, each replacement of D11K and P36K caused a slight decrease in the K_m value (4.8 and 7.7 mM, respectively). Simultaneous replacements of D11K and P36K, however, caused a significant (5.7-fold) decrease in the K_m value (1.5 mM) compared to that for wild-type azurin (8.6 mM). The second mutation does more than simply add to the first, which suggests some interaction between the two mutations. In any case, the introduction of two positively charged residues into the electron transfer surface of azurin clearly facilitates the interaction of azurin to NIR and is consistent with the idea that electrostatic interactions are important in the recognition of cupredoxins by *A. faecalis* NIR. Indeed, our recent site-directed mutagenesis of NIR shows that several glutamic and aspartic acid residues are important in recognition of pseudoazurin [25]. It should be noted that the K_m value of NIR for D11K/P36K azurin (1.5 mM) is distinctly smaller than that for wild-type azurin (8.6 mM) but still far larger than that for wild-type pseudoazurin (0.05 mM). This suggests that two complementary structures of the interaction surfaces are important for the efficient recognition/interaction process between pseudoazurin and NIR which cannot be modelled by simply introducing two positively charged residues on the surface of azurin.

Pseudoazurin functions as an electron donor to a copper-containing NIR in *A. faecalis* S-6, while the physiological redox partner for azurin is still unknown [26]. The absence of localization of charged residues on the molecular surface of azurin suggests that azurin forms an electron transferring complex with its redox partner in a different manner from that of pseudoazurin-NIR complex. The fact that the interactions that mediate the transfer of electrons between two structurally related cupredoxins and their redox partners are different is interesting in the sense of molecular evolution. It is interesting that for the known structure of cytochrome cd_1-type NIR from *Thiosphaera pantotropha*, a pseudoazurin also from *T. pantotropha* is proposed to interact in the same way as the copper-containing NIR [27]. It is likely that the important aspect is the close approach of electron transfer centers, and that the intervening surface residues facilitate specificity.

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