Volume 258, number 2, 266–268 FEB 07878 December 1989

Modification of the electron-transfer sites of *Pseudomonas aeruginosa* azurin by site-directed mutagenesis

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Received 9 October 1989

Site-directed mutagenesis of the structural gene for azurin from *Pseudomonas aeruginosa* has been used to prepare azurins in which amino acid residues in two separate electron-transfer sites have been changed His-35-Lys and Glu-91-Gln at one site and Phe-114-Ala at the other The charge-transfer band and the EPR spectrum are the same as in the wild-type protein in the first two mutants, whereas in the Phe-114-Ala azurin, the optical band is shifted downwards by 7 nm and the copper hyperfine splitting is decreased by 4 10^{-4}/cm This protein also shows an increase of 20–40 mV in the reduction potential compared to the other azurins The potentials of all four azurins decrease with increasing pH in phosphate but not in zwitterionic buffers with high ionic strength The rate constant for electron exchange with cytochrome c_{551} is unchanged compared to the wild-type protein in the Phe-114-Ala azurin, but is increased in the other two mutant proteins The results suggest that Glu-91 is not important for the interaction with cytochrome c_{551} and that His-35 plays no critical role in the electron transfer to the copper site

Azurin, Cytochrome c_{551}, Electron transfer, EPR spectra, Reduction potential

1. INTRODUCTION

Azurin is a bacterial electron-transfer protein containing a single blue or type I Cu(II) ion [1]. It is thought to mediate electron transfer between cytochrome c_{551} and cytochrome oxidase/nitrite reductase. Two separate electron-transfer sites have been identified [2,3]. One involves the surface residue Glu-91 and the nearby His-35 residue, and it is believed to participate in the reaction with cytochrome c_{551}. The second site is a hydrophobic patch located close to the ligand residue His-117, and this site is considered to be important in the self-exchange reaction [4,5].

In this communication we report the production of three mutant azurins in which amino acid residues in the two electron-transfer sites have been changed: His-35-Lys, Glu-91-Gln and Phe-114-Ala. The proteins have been characterized by optical and EPR spectra, and the reduction potential has been determined at pH 7.0 and 6.0. In addition, the kinetics of the electron-transfer reaction with cytochrome c_{551} has been studied. The results show that the blue site remains unperturbed except in the Phe-114-Ala mutant, which displays a decrease in the copper hyperfine splitting and a slight increase in reduction potential. The second-order rate constant for the reaction with cytochrome c_{551} is increased in the two proteins which have replacements in the proposed electron-transfer pathway.

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Abbreviations Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, IPTG, isopropyl-β-D-thiogalactopyranoside, Mes, 4-morpholinoethanesulfonic acid

2. MATERIALS AND METHODS

2 1 *Site-directed mutagenesis and preparation of azurins*

The mutants were constructed with the use of the 'Oligonucleotide-directed in vitro mutagenesis system' (Amersham)

The bacterial strains, plasmids, DNA techniques and protein purification have been described in [6], except that the cells were grown in a 10 liter TB medium culture in a fermentor (Chemap-Fermentor, 20 l, series 3000) The culture was induced when the absorbance at 550 nm was approx 4 0 by the addition of IPTG to a final concentration of 0 3 mM, and the cells were harvested 4 h after induction Wet cells, 265 g, were obtained from the culture, from which 1 g azurin was purified [6] After the gel filtration step, the protein was transferred to a 100 mM potassium phosphate buffer, pH 7 0

2 2 *Optical and EPR spectra*

Optical and EPR spectra were recorded as in [7] The molar absorption coefficients at the wavelength of maximum absorption were estimated on the basis of the Cu(II) concentrations determined by integration of the EPR signals

2 3 *Reduction potential*

The reduction potentials were determined as in [7], but at 25°C and with the redox mediators $K_3Fe(CN)_6$, 1,4-benzoquinone, *N,N*-dimethyl-*p*-phenylenediamine sulfate and 2,6-dichlorophenol-indophenol (sodium salt) The buffers used were 100 mM phosphate, pH 6 0 and pH 7 0, or 10 mM Mes, pH 6 0, and 10 mM Hepes, pH 7 0, both containing 330 mM K_2SO_4 The fully oxidized protein was reduced stepwise by $Na_2S_2O_4$ The reduction potentials $E°$ were estimated from Nernst plots as described in [8], with an inaccuracy of ±5 mV

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00145793/89/$3 50

Table 1

Optical characteristics and second-order rate constant (k_{12}) for electron transfer from cytochrome c_{551} for wild-type and mutant azurins

Protein	Absorption maximum (nm)	k_{12} (10^6/M per s)
Wild-type	628	6
His-35-Lys	628	9
Glu-91-Gln	628	10
Phe-114-Ala	621	6

Table 2

Reduction potentials ($E°$) of wild-type and mutant azurins under different conditions

Protein	$E°$ (mV)			
	Mes pH 6 0	Hepes pH 7 0	Phosphate pH 6 0	Phosphate pH 7 0
Wild-type	341	341	338	311
His-35-Lys	336	330	334	306
Glu-91-Gln	332	328	324	306
Phe-114-Ala	360	357	359	350

2 4 *Electron transfer*

The electron transfer rate between reduced cytochrome c_{551} (1 μM) and oxidized azurin (5–40 μM) was measued at 25°C in a Durrum stopped-flow apparatus [9] Pseudo first-order rate constants were obtained by fitting recorded data to exponential functions using standard Marquardt least-square minimization The second-order rate constants were calculated from the linear dependence of the first-order rate constants on the azurin concentration, giving an inaccuracy of ± 10%

3. RESULTS

Optical characteristics of the wild-type and mutant azurins are compared in table 1. The molar absorption coefficients at the absorption maximum of the four proteins were identical within the experimental uncertainty. Table 1 also lists the second-order rate constants (k_{12}) for the electron-transfer reaction between the different azurins and reduced cytochrome c_{551}. The EPR spectra of the azurins were all identical except for the Phe-114-Ala mutant, as illustrated in fig.1. This mutant

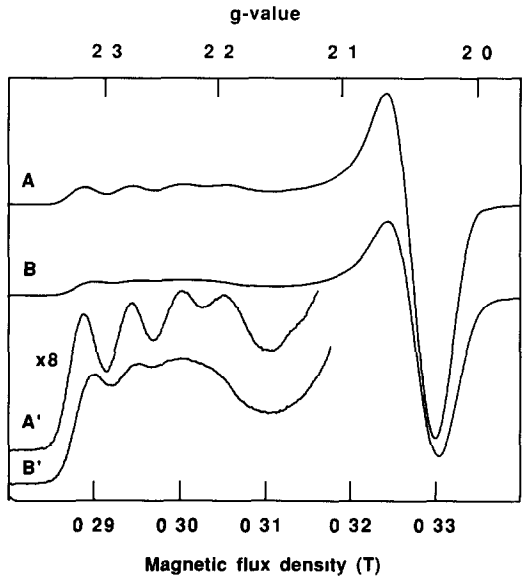

Fig.1 EPR spectra of wild-type (A,A') and Phe-114-Ala (B,B') azurin For all azurins the $g_\perp$ and $g_\parallel$ were 2.05 and 2.26, respectively, and $A_\parallel$ was 56 10^{-4}/cm for all except for Phe-114-Ala for which it was 52 10^{-4}/cm Conditions· microwave frequency, 9 379 GHz, power, 20 mW; modulation amplitude, 2 0 mT; temperature, 77 K

was considerably slower in picking up Cu(II) than the other proteins [6].

The reduction potentials of the four azurins under different conditions are summarized in table 2. Since the measurements in phosphate buffers gave different potentials at pH 7.0 and 6.0, determinations were also made in zwitterionic buffers with a constant ionic strength.

4. DISCUSSION

The spectral characteristics of the Glu-91-Gln mutant are not changed compared to the wild-type protein (table 1, fig.1). This is not surprising, because Glu-91 is on the protein surface about 10 Å from the copper site.

In contrast, His-35 lies with its imidazole ring close to ligand residue His-46 and has been suggested to control the redox properties of the protein [1]. However, the spectroscopic properties are unchanged when His-35 is replaced with lysine, and protonation of His-35 (by lowering the pH at high ionic strength in a zwitterionic buffer) or its replacement with lysine changes the reduction potential very little, if any. Thus, the role of this conserved residue remains uncertain.

Phe-114 is proximal to ligand residue His-117, and in the Phe-114-Ala mutant the copper site is definitely perturbed with a shift in the charge-transfer band to shorter wavelength by 7 nm (table 1) and a decrease in the copper hyperfine splitting by $4 \cdot 10^{-4}$/cm (fig.1). In addition, the reduction potential of this mutant is increased by 20–40 mV depending on the conditions used (table 2).

We have previously described [7] a mutant Met-121-Leu, in which the reduction potential is increased even more compared to wild-type azurin than in Phe-114-Ala. In the Met-121 case, the charge-transfer band is shifted towards a longer wavelength, however, showing that there is no obvious correlation between the position of this band and the reduction potential as discussed in [10]. Generally, however, spectroscopic changes are small, particularly in the position of the main charge-transfer band and g-values, suggesting very minor changes in the bonding. Only $A_\parallel$ shows a substantial relative change, possibly because of varia-

tions in the Fermi contact term, but the data set is still too small to allow theorisation about these effects.

The potentials measured in phosphate buffers are higher at pH 6.0 than at pH 7.0 (table 2). This is apparently not primarily due to a pH effect, since the difference disappears in zwitterionic buffers at high ionic strength. The reduction potential of the Phe-114-Ala azurin is, however, still the highest among the four proteins.

The two mutant azurins which display a significant increase in k_{12} (table 1), both involve changes in amino acid residues which are presumed to be important for electron transfer from cytochrome c_{551} [3,11]. Thus, our experiments support this identification. The following equation holds for a bimolecular electron exchange reaction:

$$k_{12} = K\ k_{et} \quad (1)$$

where K is the formation constant for the precursor complex between acceptor and donor and k_{et} the first-order rate constant for the electron transfer within this complex. An increase in k_{12} must consequently be caused by an increase in either K or k_{et} (or both). Since the driving force is the same as in the wild-type azurin, one might expect that it is K that has increased. It is, however, possible that it is instead k_{et} which has increased because of an increase in electronic coupling. In any case, it must be concluded that the negative charge of Glu-91 is not important for the formation of the precursor complex with cytochrome c_{551}.

It has been suggested that the π electron system of His-35 plays an important role in the electronic coupling between the electron donor and the copper site [11]. The fact that k_{12} is actually increased in the His-35-Lys azurin shows, however, that this residue cannot be critical for a high value of k_{et}.

In the Phe-114-Ala azurin, the driving force for electron transfer is increased compared to the wild-type protein. One might consequently expect an increase in k_{et}, but k_{12} is actually unchanged. Calculations show that the increase would be too small to be detected if the reorganization energy associated with the electron transfer is in the order of 0.5 eV.

Acknowledgements This investigation has been supported by grants from the Swedish Natural Science Research Council Miss Margareta Nordling is gratefully acknowledged for assistance with gene technology work Dr Curtis Hoganson has provided assistance and advice concerning the redox measurements and Dr Roland Aasa concerning the EPR measurements. Mr Goran Karlsson is acknowledged for valuable discussions and Mrs Ann-Britt Skånberg for technical assistance

REFERENCES

[1] Adman, E T (1985) in Metalloproteins, part I (Harrison, P ed) pp 1–42, Chemie, Weinheim.
[2] Lappin, A G , Segal, M G , Weatherburn, D.C , Henderson, R A and Sykes, A G (1979) J Am Chem Soc 101, 2302–2306
[3] Farver, O , Blatt, Y and Pecht, I (1982) Biochemistry 21, 3556–3561
[4] Adman, E.T (1979) Biochim Biophys. Acta 549, 107–144
[5] Groeneveld, C M and Canters, G W (1988) J Biol Chem 163, 167–173
[6] Karlsson, B G., Pascher, T , Nordling, M , Arvidsson, R H.G and Lundberg, L G (1989) FEBS Lett 246, 211–217.
[7] Karlsson, B G., Aasa, R , Malmstrom, B G and Lundberg, L G. (1989) FEBS Lett 253, 99–101
[8] Tanigucht, V T., Sailasuta-Scott, N , Anson, F C and Gray, H.B (1980) Pure Appl Chem 52, 2275–2281
[9] Wilson, M T , Greenwood, C , Brunori, M and Antonini, E (1975) Biochem. J 145, 449–457
[10] Gray, H B and Malmstrom, B G (1983) Comments Inorg Chem 2, 203–209.
[11] Farver, O and Pecht, I (1988) Prog Clin Biol Res 274, 269–283