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The role of His117 in the redox reactions of azurin from *Pseudomonas aeruginosa*

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Abstract The electron-transfer properties of H117G- and wild-type azurin were compared by applying both as electron acceptors in the conversion of 4-ethylphenol by 4-ethylphenol methylenehydroxylase (4-EPMH). The reactivity of H117G-azurin was determined in the absence and presence of imidazoles, which can substitute the missing fourth ligand. In the absence of imidazoles, H117G-azurin reacted directly with 4-ethylphenol, this reaction was abolished in the presence of imidazoles. The enzymatic reduction of H117G-azurin by 4-EPMH was 40 times slower than that of wild-type azurin. The rate of this reaction was enhanced by some imidazoles, diminished by others. In all cases the reduction of H117G-azurin was irreversible. These results demonstrate that His117 is vital for electron transfer and effectively protects the copper site against aspecific reactions.

Key words: Azurin; Histidine 117; Imidazole; 4-Ethylphenol methylene hydroxylase; Redox reaction

1. Introduction

The blue copper proteins (for reviews see refs. [1] and [2]) form a well-defined family of small proteins that contain one copper atom with a characteristic ligand configuration consisting of one cysteine-sulfur, two histidine-nitrogens and a fourth ligand that is usually a methionine-sulfur. Their unusual structure endows them with very distinctive spectroscopic properties, notably a strong absorbance band in the 600 nm region and a quite small hyperfine splitting of the EPR signal. Site-directed mutagenesis has been successfully applied to gain a better insight in the way in which the typical type-1 structure of these proteins affects their spectroscopic features, redox potential, and electron transfer properties (reviewed in ref. [3]). The azurin H117G-mutant is particularly interesting in this respect, since His117, as a ligand to the copper, not only affects the spectroscopic properties, but is assumed to play a key role in electron transfer as well [3].

Azurin has been implicated as the physiological electron acceptor for the *Pseudomonas putida* enzyme 4-ethylphenol methylenehydroxylase (4-EPMH[1]) which catalyses the conversion of 4-ethylphenol to 1-(4′-hydroxyphenyl)-ethanol, which, subsequently, is dehydrogenated by the same enzyme to 4-hydroxyacetophenon [4].

In this paper we describe the results of studies of the reaction of 4-EPMH, with H117G-azurin as the electron acceptor, both in the absence and presence of imidazoles.

2. Materials and methods

4-EPMH was purified from *P. putida* as described in ref. [5]. *Pseudomonas aeruginosa* azurin was purified according to ref. [6]. The concentration was determined spectrophotometrically at 628 nm, using an absorbance coefficient of 5700 $M^{-1} \cdot cm^{-1}$ [7]. The H117G mutant of azurin was obtained in the apo-form according to ref. [8]. The concentration of H117G-azurin was determined from the absorbance at 280 nm ($\varepsilon = 9800\ M^{-1} \cdot cm^{-1}$ [6]).

Optical spectra and kinetics were measured on a Hewlett-Packard 8452A diode array-spectrophotometer. The reduction of azurin was monitored by measuring the absorbance difference between 630 and 500 nm as a function of time. Apparent second order rate constants were determined either from first-order fits of the complete reduction traces, using the fitting program of the spectrophotometer, or, alternatively, from the initial rates, in which case the reduced-minus-oxidised absorbance difference coefficients (at 630–500 nm) were estimated from the total absorbance change induced by the reaction.

3. Results and discussion

The absence of histidine 117 in H117G-azurin results in dramatic changes in the optical and EPR spectroscopic features [8,9]. The small EPR hyperfine splitting ($A_{\|} = 61$ G), typical for type-1 copper centers, and the characteristic absorbance band at 628 nm are absent, instead of which a larger splitting constant ($A_{\|} = 139$ G), such as is usually associated with type-2 copper sites, and a band centered at 420 nm are observed. Addition of an imidazole to this mutant, however, generates a compound that is spectroscopically almost indistinguishable from the wild-type protein, indicating that externally added imidazole can serve as a substitute for the lacking histidine. Compounds, other than imidazoles, will also serve as ligands, with varying effects on the spectroscopic properties [9]. Several of those compounds, i.e. imidazole, 1-methylimidazole (1-MeIm), 2-methylimidazole (2-MeIm) and 4-methylimidazole (4-MeIm), as well as some others, i.e. 2-aminoimidazole (2-AmIm), 4-nitroimidazole (4-NiIm), 2-mercapto-1-methylimidazole (2-Mc-1-MeIm) and acetate were added by us as ligands to H117G-azurin.

Apo-H117G-azurin (25 μM) was incubated with a 4-fold excess of $Cu(NO_3)_2$ until no further absorbance increase was observed (at 630–500 nm). The protein was then incubated with a 40-fold excess of the ligand (final concentration

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Abbreviations: 4-EPMH, 4-ethylphenol methylenehydroxylase; Im, imidazole; 1-MeIm, 1-methylimidazole; 2-MeIm, 2-methylimidazole; 4-MeIm, 4-methylimidazole; 2-AmIm, 2-aminoimidazole; 4-NiIm, 4-nitroimidazole; 1-MeIm, 1-Methylimidazole; 2-Mc-1-MeIm, 2-mercapto-1-methylimidazole.

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Table 1
Estimated absorbance coefficients at 420 and 628 nm of H117G-azurin in the presence and absence of imidazoles

Ligand	ε_{628} $mM^{-1}\cdot cm^{-1}$	ε_{420} $mM^{-1}\cdot cm^{-1}$
H_2O	1.4	2.5
Im	5.0	0.5
1-MeIm	5.3	0.6
2-MeIm	4.5	1.2
4-MeIm	4.7	0.5
4-NiIm	4.8	1.5
acetate	4.7[a]	0.6
H117[b]	5.7	0.2

Absorbance coefficients were estimated assuming a value of 9.8 $mM^{-1}\cdot s^{-1}$ for ε_{280}. [a]Absorbance of the peak at 640 nm. [b]Wild-type azurin.

Table 2
Reactivity of H117G-azurin with 4-ethylphenol and 4-EPMH in the presence or absence of imidazoles

Ligand	k_{4-EP} $(M^{-1}\cdot s^{-1})$	k_{4-EPMH} $(M^{-1}\cdot s^{-1})$
H_2O	20.0	$1.19\cdot10^5$
4-MeIm	–	$0.37\cdot10^5$
2-MeIm	4.0	$1.24\cdot10^5$
1-MeIm	–	$2.39\cdot10^5$
4-NiIm	4.7	$0.45\cdot10^5$
Im	–	$5.38\cdot10^5$
Acetate[a]	4.3	$0.51\cdot10^5$
H117[b]	–	$4.8\cdot10^6$

The numbers in the table represent the apparent second order rate constants. The rate constants for 4-ethylphenol were calculated from the initial rates. The rate constants for 4-EPMH were calculated from the pseudo-first order rate constants. Conditions: 25 μM H117G-azurin; 100 μM $Cu(NO_3)_2$; 1 mM imidazole; 120 nM 4-EPMH; 200 μM 4-ethylphenol; 10 mM Mes (pH 6.0). [a]40 mM KAc (pH 6.0), instead of 10 mM Mes (pH 6.0). [b]Wild-type azurin.

1 mM) and the absorbance increase monitored. In Fig. 1 the optical absorbance spectra of H117G-azurin before and after addition of imidazole are shown. Nearly identical spectra were obtained with 1-MeIm or 4-MeIm as ligands. Similar observations were also made with 2-MeIm and 4-NiIm, except that with these compounds, at the concentrations applied (1 mM), the 420 nm peaks did not completely vanish (Table 1). With 2-AmIm a total bleaching of the absorbance in the visible region, probably due to autoreduction, occurred before the conversion of 420 to 630 nm was complete. Addition of 2-Mc-1-MeIm caused the disappearance of the 420 nm band with the concomitant formation of a low, broad and asymmetric band centred at 606 nm. An identical spectrum was obtained in the absence of azurin, indicating that 2-Mc-1-MeIm draws the copper ion out of the protein.

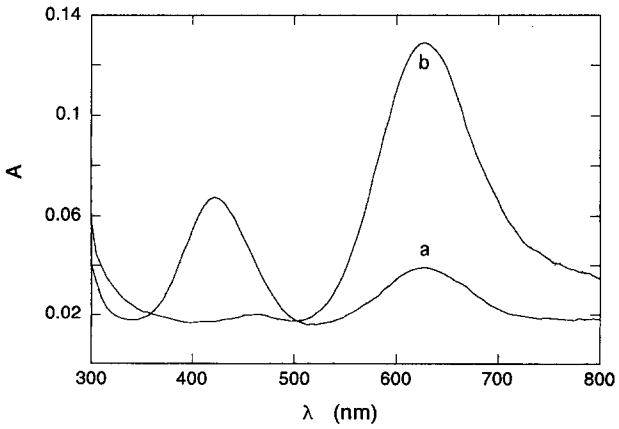

Fig. 1. Optical absorbance spectra of H117G-azurin in the absence (a) and presence (b) of imidazole. Conditions: 25 μM H117G-azurin; 100 μM $Cu(NO_3)_2$; 1 mM imidazole; 10 mM Mes (pH 6.0).

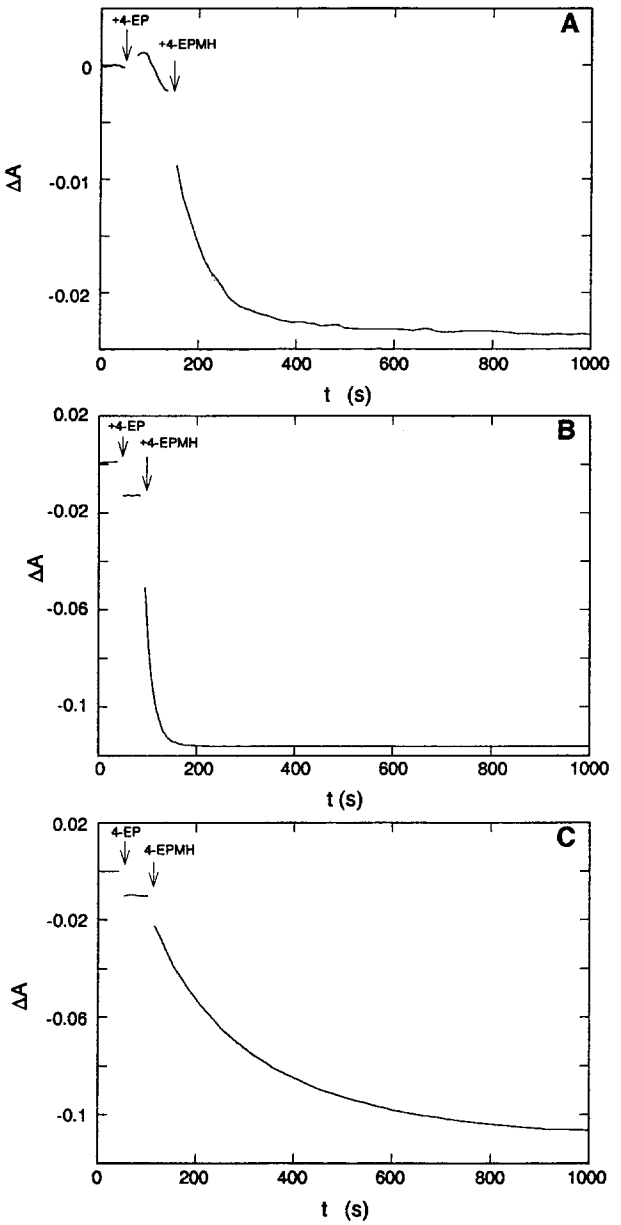

Fig. 2. Absorbance changes induced by the addition of 4-ethylphenol and 4-EPMH to H117G-azurin in the absence (a) and presence of imidazole (b) or 4-methylimidazole (c). Times of addition of 4-ethylphenol and 4-EPMH are indicated by arrows. The reactions were monitored by measuring the absorbance difference between 630 and 500 nm. Conditions: 25 μM H117G-azurin; 100 μM $Cu(NO_3)_2$; 1mM imidazole (or 4-methylimidazole); 200 μM 4-ethylphenol; 120 nM 4-EPMH; 10 mM Mes (pH 6.0).

The observation that H117G-azurin exhibits an absorbance peak at 640 nm in the presence of acetate shows, in line with ref. [9], that the presence of a histidine-imidazole, or even of a nitrogen atom, at the fourth ligand position of the copper is

no prerequisite for the typical spectral properties of blue copper proteins. Absorbance spectra resembling that of wild-type azurin have also been reported for mutants lacking either histidine 46 or methionine 121 [10,11] but not for a mutant lacking cysteine 112 [12], underscoring the fact that it is primarily the cysteine-sulphur that determines the spectral signature of type-1 copper.

The importance of histidine 117 for the electron-transfer properties of azurin was investigated by employing the H117G-mutant as an electron acceptor for 4-EPMH, both in the presence and absence of imidazoles. The reaction of the mutant was in all cases much slower than that of wild-type azurin. Fig. 2 shows the absorbance changes at 630-minus-500 nm induced by the consecutive addition of 4-ethylphenol and 4-EPMH to H117G-azurin in the absence or presence of imidazoles. Unlike the wild-type protein, H117G-azurin was reduced by 4-ethylphenol directly (Fig. 2A); however, the reduction rate was strongly enhanced by the addition of a catalytic amount of 4-EPMH. The direct reaction of the mutant with 4-ethylphenol was abolished in the presence of imidazoles (Fig. 2B,C); the effect of imidazoles on the rate of the 4-EPMH-catalyzed reaction varied with the identity of the imidazole. Unsubstituted imidazole enhanced the reaction rate (Fig. 2B), whereas 4-methylimidazole induced a rate decrease (Fig. 2C). Table 2 summarises the results, obtained with these and other imidazoles. The pH-optima for the specificity constant of 4-EPMH for both wild-type azurin, and H117G-azurin in the presence of 4-methylimidazole, were at pH 6. In line with previous observations with dithionite and ascorbate [9], the reduction of H117G-azurin was irreversible.

The reactivity of H117G-azurin with 4-ethylphenol illustrates the greater accessibility of the copper atom in the mutant and the importance of His117 in preventing aspecific reactions. The reaction with 4-ethylphenol is completely abolished in the presence of imidazoles. The remaining activity observed with 2-MeIm or 4-NiIm is proportional to the fraction of H117G that still exhibits the 420 nm band with 1 mM of these ligands, which appear to have greater dissociation constants than the other imidazoles.

The effect of imidazoles on the reaction of H117G-azurin with 4-EPMH depends on the ligand used. Whereas in some cases the rate increases, other imidazoles have an inhibitory effect. Surprisingly, 4-MeIm, which most resembles the amino acid side chain of histidine, induces a decrease in the reaction rate. Ligation of copper to N1 instead of to N3, which serves as the copper ligand in wild-type azurin, could be the cause for this phenomenon. The highest activity was found with unsubstituted imidazole, which induced a 5-fold enhancement of the reduction rate as compared to unliganded H117G-azurin, and which yielded a 15-fold higher activity than did 4-MeIm.

The low activity of the mutant compared to that of wild-type azurin (11% in the case of imidazole as the external ligand) is testimony for the crucial role of His117 in electron transfer. The fairly great variation in rate attained with different imidazoles, which all have the same spectroscopic signature in optical absorbance and EPR, illustrate how subtle structural changes of the copper site affect the electron-transfer properties.

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