Metal Binding to *Pseudomonas aeruginosa* Azurin: a Kinetic Investigation

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The interaction between azurin from *Pseudomonas aeruginosa* and Ag(I), Cu(II), Hg(II), was investigated as a function of protein state, i.e. apo-, reduced and oxidised azurin. Two different metal binding sites, characterized by two different spectroscopic absorbancies, were detected: one is accessible to Ag(I) and Cu(II) but not to Hg(II); the other one binds Ag(I) and Hg(II) but not copper. When added in stoichiometric amount, Ag(I) shows high affinity for the redox center of apo-azurin, to which it probably binds by the -SH group of Cys112; it can displace Cu(I) from reducedazurin, while it does not bind to the redox center of oxidizedazurin. Kinetic experiments show that Ag(I) binding to the reducedform is four times faster than binding to the apo-form. This result suggests that metal binding requires a conformational rearrangement of the active site of the azurin.

Interaction of Ag(I) or Hg(II) ions to the second metal binding site, induces typical changes of UV spectrum and quenching of fluorescence emission.

Introduction

Pseudomonas aeruginosa azurin (Az-Pae) is a low molecular weight (M_r 14000) blue copper protein involved in the electron transport of the bacterial respiratory chain (Antonini *et al.*, 1970, Brunori *et al.*, 1974). The copper ion is located in a hydrophobic surrounding at 70 nm far from the molecule surface (Finazzi-Agrò *et al.*, 1970, Nar *et al.*, 1991). The three-dimensional structure of the redox center has been determined by crystallography (Malmstrom and Leckner, 1998) and the coordination site of the copper ion (classified as type 1) is comprised by two histidines (His46, His117), one cysteine (Cys112) and, more weakly, one methionine (Met121) and one glycine (Gly45) arranged in a distorted tetrahedral conformation (Adman and Jensen, 1981, Nar *et al.,* 1991). Recently, the unfolding of native protein followed by CD transitions showed a slow change associated with the reduction of Cu^{2}+ by the thiol group of the ligand Cys 112 (Leckner *et al.,* 1998). However, the distance between copper and Cys112 is independent of the redox state of the protein, while the distance between copper and Met121 increases in the oxidized state (Groeneveld *et al.*, 1986).

Az-Pae contains a single tryptophan residue at position 48 (Ryden *et al.*, 1976), whose fluorescence is heavily quenched by the copper ion (Finazzi-Agrò *et al.,* 1973, Szabo *et al.*, 1983). Time-resolved fluorescence study in holo-Cu(I), holo-Cu(II) and apo-forms showed conformational heterogeneity and tryptophan mobility (Kroes *et al.*, 1998). Removal of the metal from the redox center is associated with an approximately 20 fold increase of the fluorescence emission of Trp48, which has its maximum at 308 nm due to hydrophobicity and rigidity of the environment (Finazzi-Agrò *et al.,* 1970, Nar *et al.*, 1991). The fluorescence of apo-azurin is quenched with different efficiency by the addition of cations such as Cu(II), Ag(I), Hg(II), Co(II), Ni(II), Cd(II) (Finazzi-Agrò *et al.*, 1973, Klemens and McMillin 1992). Metal binding experiments have been extensively performed to identify the existence of a second metal binding site on the molecule and to study the relationship between the structure of the redox centes and its functional properties. Taking advantage of the unique spectroscopic properties

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of holo and apo azurin, we have tried to address these questions and in this paper we investigate the interactions of Ag(I), Hg(II), and Cu(II) with different forms of azurin (apo, reduced and oxidized). Our results indicate the physiological metal ion Cu(II) and the Hg(II) bind to different sites, respectively represented by the Cys112 in the redox center of the protein and by another site on the protein molecule, while the small Ag(I) ion can bind both sites with different affinities depending of the redox state of the azurin.

Material and Methods

Azurin was purified from *Pseudomonas aeruginosa* according to Parr *et al.* (1977). The ratio between the extinction coefficients $\varepsilon_{(625\ nm)}$ / $\varepsilon_{(280\ nm)}$ of the oxidized form protein was never less than 0.55. Azurin, reduced by addition of solid sodium dithionite, was set free from excess reductant by chromatography through a Sephadex G-25 column (1cm × 10 cm). The concentration of reduced azurin was assessed after reoxidation with ferricyanide using an $\varepsilon_{ox(625\ nm)} = 3500\ M^{-1}\ cm^{-1}$ (Brill *et al.*, 1968). Reduced samples of azurin were used immediately after preparation.

Apo-azurin was prepared by dialysis at 4 °C against 0.1 M KCN in 0.2 M phosphate buffer, pH 7.4; when the protein was completely colorless it was dialyzed against distilled water to remove KCN (Yamanaka *et al.*, 1963). Addition of Cu(II) reconstitutes the holoprotein with complete restoration of its spectral properties. Apo-azurin concentration was calculated using an $\varepsilon_{(280\ nm)} = 5930\ M^{-1}\ cm^{-1}$ or reconstituting the protein with a large excess of $CuSO_4$. Ag(I) was added in the form of $AgNO_3$, and, due to its low solubility, all experiments were carried out in water obtained from Millipore filtration apparatus (pH 6.2). Hg(II) was added in the form of $Hg(CH_3COO)_2$.

Optical spectra were recorded with a Cary 219 spectrophotometer at room temperature. Fluorescence spectra were recorded with a Perkin Elmer L50 spectofluorimeter using excitation and emission band-widths of 7.5 or 10 nm. Kinetic experiments were performed with a manual stopped-flow apparatus (Hi-tech SFA11) with a dead time of 0.5 s. All experiments were carried out at room temperature with samples having an absorbance of about 0.1–0.2 at 280 nm. Atomic absorption measurements were carried out on a Perkin-Elmer 5100/Z (acetylene/ air flame): values given for each sample are the average of three measurements (with the associated error of 2%) obtained by subsequent dilutions of the sample (detection limit 0.02–0.05 mg/l). In the case of protein treated with metals, before atomic absorption, all samples were passed through a Sephadex G-25 column (1 × 10 cm) to remove excess free metal. After gel filtration, the concentration of metal adduct was calculated from the Trp maximum at 292 nm, which is not affected by the metal addition; the proper absorption coefficient (4200 M^{-1} cm^{-1}) was evaluated by oxidized native azurin.

Results and Discussion

Addition of Ag(I), Hg(II) and Cu(II) ions to apo-, reduced – and oxidized azurin from *Pseudomonas aeruginosa*, respectively, induces peculiar modifications in the absorption and fluorescence spectra.

Apoprotein

Ag(I) binds apoazurin with very high affinity affecting the near ultraviolet absorption and fluorescence emission. Titration of the apoprotein with $AgNO_3$ induces changes in the absorption spectrum around 250 nm, ending in a characteristic spectral shape (A-type) when a stoichiometric amount of Ag(I) is added (Fig. 1). In this condition, atomic absorption measurements on the metal adduct, after gel filtration on Sephadex G25, confirm the presence of one Ag(I) ion bound per protein molecule (Table I).

Increasing Ag(I) concentration induces an additional change of absorbance in the ultraviolet, reaching its maximum (B-type spectrum) in corresponding to a 2:1 stoichiometric ratio for the ligand (insert to Fig. 1). Atomic absorption measurements performed after gel filtration of a sample treated with excess Ag(I) indicate the presence of two metal ions, which are firmly bound to the protein molecule (Table I). Titration of apoazurin with Ag(I) also induces quenching of the intrinsic fluorescence as already reported (Finazzi-Agrò *et al.*, 1973). Addition of an equivalent amount of metal ion to the protein quenches the fluorescence by approximately 30%. Higher Ag(I) concentration induces further quenching

Table I. Atomic metal determination on various forms of azurin.

Azurin form	Metal ion added (μM)	Spectra type	Atomic metal determination*		
			Cu	Ag	Hg
Apo-azurin	Ag(I)				
(76 μM)	76	A		75	
	160	B		152	
	280	B		165	
	Hg(II)	B			80
	160				
Ag(I)-azurin	Cu(II)				
(54 μM)	54	B	47	55	
Oxidized azurin	Ag(I)				
36 (μM)	36	B	28	33	
	Hg(II)				
	36	B	34		41
Reduced-azurin	Ag(I)				
30 (μM)	30	A	3	29	
	87	B	3	56	
	104	B	1	60	
	Hg(II)				
	36	B			31

* After gel filtration on Sephadex G 25 (see text).

and, in the presence of excess ligand, the fluorescence emission is reduced to approximately 60% of the apoprotein emission (Fig. 2A). The time course of fluorescence quenching induced by Ag(I) is biphasic. The fast phase can only be estimated at relatively low concentration of ligand, due to limitation in time resolution of the mixing apparatus (approximately 0.5 s). In these conditions (i.e. Ag(I) = 10–100 μM), neglecting the contribution of the slow phase, the initial slope of the time course may be assumed to represent the pseudo first order rate of binding to the fast site (protein concentration ~ 1 μM). The dependence of this on the ligand concentration, within this range, yields a value $l' = 2.2 \times 10^4$ M^{-1} s^{-1} for its bimolecular rate constant. At higher Ag(I) concentrations (250–1000 μM), binding to the first site is completed within the dead time of mixing and only the slow phase is observed with a rate constant $l'_{slow} = 3.5 \times 10^3$ M^{-1} s^{-1} (insert of Fig. 2A). These data clearly indicate the presence of two different Ag(I) binding sites on apoazurin which are characterized by different optical properties and distinct affinity for the metal, but no information is available about their localization on the protein.

In order to better characterize the Ag(I) binding sites of apoazurin, a sample of the protein with two Ag(I) ions bound per molecule (B-type UV spectrum) was treated with an excess of the physiological metal Cu(II). After a 24-h dialysis, the typical blue color of the native protein was developed but the absorption in the ultraviolet remained unmodified (B-type). Atomic absorption measurements carried out on this azurin derivative indicates the presence of one Cu(II) and one Ag(I) per molecule (Table I). We interpret this finding as an evidence that the large excess of Cu(II) can slowly displace the Ag(I) ion bound from the active site of apoazurin reconstituting the type 1 configuration of the redox center, but leaving in place the second Ag(I) which is bound in an another site with no affinity for Cu(II). On the other hand, when the sulphydryl group of cysteine (Cys112), which is known to be involved in binding Cu(II) in the redox center (Adman and Jensen, 1981), is blocked by *p*-hydroxymercuribenzoate, only one Ag(I) per molecule can bind to the apoprotein. In fact, treating apoazurin with *p*-hydroxymercuribenzoate induces a fluorescence quenching of about 12%, and the subsequent addi-

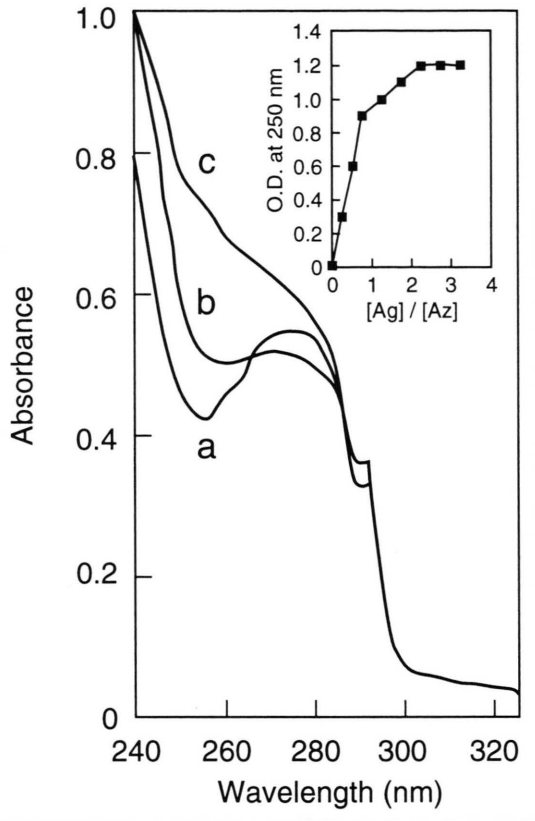

Fig. 1. Effect of Ag(I) on the UV spectrum of apo-azurin.
UV spectrum of apo-azurin (70 μM) in water at 20 °C, before (a) and after addition of a stoichiometric (b) or excess amount (c) of Ag(I).
In the insert the effect of Ag(I) at different concentration on the absorbance of apo-azurin at 250 nm.

tion of Ag(I) increases the quenching by only 10%, much less than when the metal is added to apoazurin (40%), and corresponding to the quenching effect of Ag(I) binding to the second site.

In the case of Hg(II), atomic absorption measurement, performed after gel filtration of a sample treated with a two-fold excess of Hg(II) indicate the presence of only one mercury bound per protein molecule (Table I). Moreover, Hg(II) addition to apoazurin induces only a B-type modification of the spectrum in the UV region, probably due to the binding of metal into a site which may be tentatively identified for the similarity of U. V. spectrum (B-type) with the second binding site for Ag(I). As shown in Fig. 2B, Hg(II) strongly quenches the intrinsic fluorescence of apoprotein when added in stoichiometric amount. The time course for Hg(II) binding is monophasic with a kinetic constant of approximately 7×10^5 M^{-1} s^{-1}. Due to the rapidity of the reaction with respect to the dead time of mixing, most of the time course is lost and the rate constant can only be evaluated at very low concentration of the ligand, i.e. under condition of pseudo first order in excess of the protein (see inset to Fig. 2B).

Oxidized form

Oxidized azurin contains Cu(II) in the redox center and therefore its fluorescence emission is strongly quenched (Finazzi-Agrò *et al.*, 1973). Addition of a stoichiometric amount of Ag(I) to this derivative leaves the absorption spectrum unchanged both in the visible region and only little modifications in the UV are induced. Addition of an eight-fold excess of silver ion leads to a B-type spectrum in the UV region and a negligible fluorescence enhancement at short time ($t_{1/2}$ 60 min.); after several hours of incubation under these conditions, a loss of absorbance at 625 nm and a significant fluorescence enhancement are observed, accompanied by a partial removal of Cu(II) from the active site (Table I). These findings suggest initial binding of Ag(I) to the second site of the protein (B-type spectral modification) followed by a slow displacement of Cu(II) from the redox center.

When high Hg(II) concentration is added to the oxidized derivative of holoazurin (eight-fold excess) only the B-type spectral modification in the UV region is observed, with no loss of the blue color and a negligible change in intrinsic fluorescence. This is what is to be expected if Hg(II) can bind at the second site but cannot modify the type 1 copper site. Atomic absorption under these conditions confirms the presence of one Hg(II) and one Cu(II) ion per protein molecule (Table I).

Reduced form

Addition of a stoichiometric amount of Ag(I) to reduced azurin induces an A-type UV spectral feature similar to that observed with apoprotein. Under these conditions, azurin cannot be transformed into its blue colored oxidized form and,

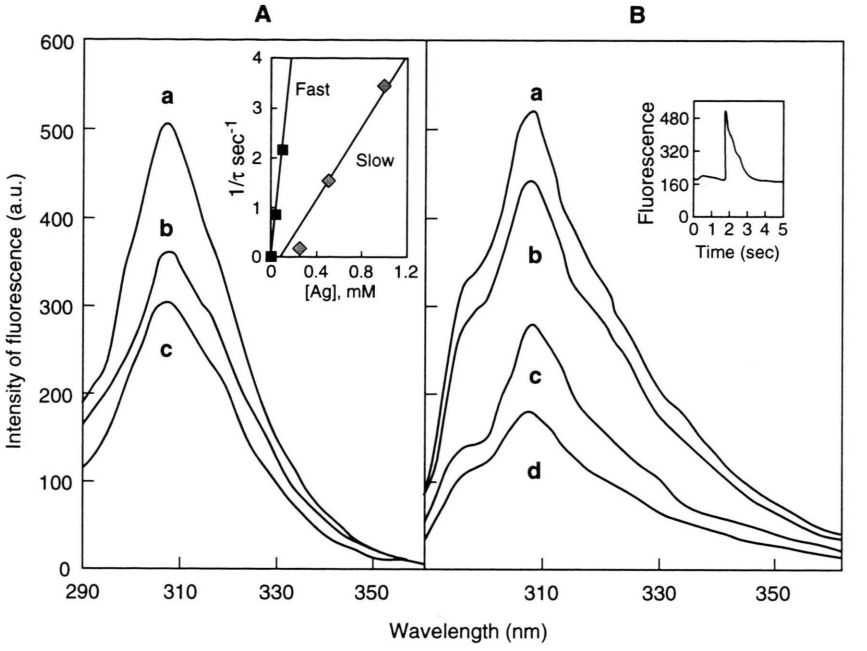

Fig. 2. Effect of Ag(I) (A) and of Hg(II) (B) on the fluorescence emission spectrum of apo-azurin.
2A. Fluorescence spectrum of apo-azurin 1.1 μM before (a) and after addition of 1.1 μM (b) or 8.8 μM (c) of Ag(I). Excitation wavelength 280 nm; temperature 20 °C.
The dependence of the pseudo-first order rate costant of Ag (I) on the ligand concentration is shown in the insert for two phases (see text).
2B. Fluorescence spectrum of apoazurin 1 μM (a); + Hg(I) 0.125 μM (b); + Hg(I) 0.625 μM (c); + Hg(I) 1 μM (d). The insert shows the time course of fluorescence quenching of apo-azurin 1 μM upon addition of Hg(II) (0.625 μM, under pseudo first order condition).

accordingly, atomic absorption shows that copper has been completely removed from the redox center (Table I). In fact, the displaced Cu(I) can be found by EPR in solution in the form of Cu(II) once oxidized by atmospheric oxygen (Zolla *et al.*, 1984). The half time for Cu(I) displacement from redox center by a stoichiometric amount of Ag(I), and its oxidation in solution has been evaluated to be approximately 3 min. On the contrary, during this time range no removal of copper was observed upon addition of Ag(I) to the oxidized form of azurin. Furthermore the -SH group of Cys112 is not titratable with *p*-hydroxymercuribenzoate either in the reduced protein (as expected), or in reduced plus Ag(I) protein, confirming the Ag(I) binding in the redox center.

The fluorescence of reduced azurin, which is usually strongly quenched by copper, upon addition of a stoichiometric amount of Ag(I) increases to a level corresponding to that observed for apoprotein saturated with one equivalent of Ag(I) without modification of the maximal fluorescence. The rate of the exchange reaction depends on the Ag(I) concentration as shown in Fig. 3. The linearity of the dependence allows to evaluate an apparent rate constant for the binding of Ag(I) to the redox site of azurin when Cu(I) occupies the latter. This can be estimated as 8×10^4 M^{-1} s^{-1}, four times higher than Ag(I) binding to apoazurin.

Addition of Hg(II) ions to reduced azurin induces only B-type UV modification, the protein is still reoxidable and the extremely low fluorescence does not increase. All these data support the hypothesis of an exclusive binding of Hg(II) to the second site without affecting the integrity of the type 1 binding site.

In conclusion, the results obtained in this study indicate the existence of two different metal binding sites in the azurin. The first site is located in the redox center of the protein, where Ag(I) binds

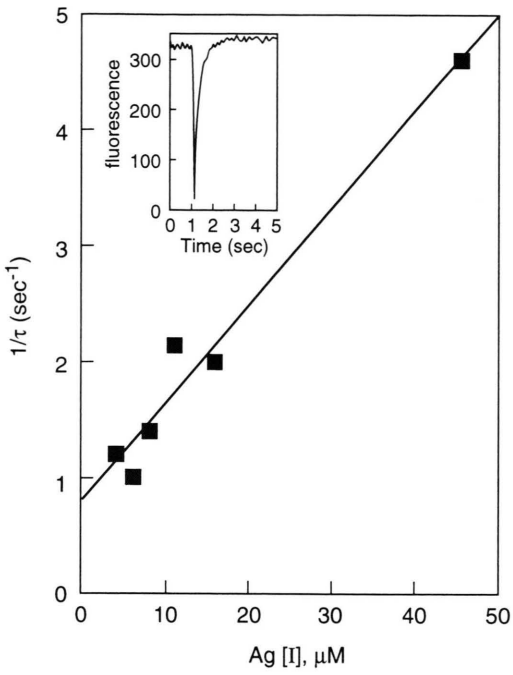

Fig. 3. Ag (I) effect on the rate of copper displacement from the redox center of reduced azurin.
The experiment was performed by monitoring the time course of fluorescence dequenching observed upon addition of different concentrations of Ag(I) to reduced azurin 2.2 μM in water at 20 °C.
The inset shows the time course of dequencing fluorescence observed upon addition of 25 μM Ag(I).
Measuring wavelength = 310 nm. Excitation 280 nm.

probably to the -SH group of Cys112. The interaction with the metal produces A-type absorption spectrum and quenching of fluorescence emission of Trp48 without modifying its hydrophobic surrounding. Interestingly, the A-type absorption spectrum is very similar to the spectra obtained from azurins where Ile7 and Phe110 residues (located in the hydrophobic environment of Trp48) were mutated (Gilardi *et al.*, 1994). If copper, as in the case of holoazurin occupies the redox center, Ag(I) displaces the copper atom only when it is in reduced state. The different susceptibility of reduced and oxidized azurin to Ag(I) has to be attributed to the higher stability of the Cu(II)-protein complex compared to Cu(I)-protein complex, since the secondary structure of the redox center in both reduced and oxidized azurin have been found to be similar (Groeneveld *et al.*, 1986; Baker, 1988; Van De Kamp *et al.*, 1992). On the other hand, the Ag(I) binding to the redox site of reduced azurin is faster than binding to the apoprotein, suggesting a significant structural difference of the redox site of apo- and reduced-form, despite the found similarity between the two structures (Nar *et al.*, 1992; Nar *et al.*, 1992). In this way, our experimental evidence is not supported by chrystallographic data and for this reason no structural explanations are available but they could suggest the existence of different conformers of the apo- reduced and oxidized azurin in solution. To this regard, folding experiments showed that the coordination structure of copper is different in oxidized and reduced unfolded states: linear or trigonal geometry for CuI, whereas CuII prefers to be tetragonal (Winkler *et al.*, 1997).

The possible second binding site could be the disulfide bridge which connects Cys3 and Cys26 at the end of an exterior antiparallel β loop of the azurin but, against this hypothesis, there is the notion that modification of the disulfide bridge heavily affects the structure of the protein and induces a strong shift of the fluorescence emission (Bonander *et al.*, 1996). The second metal binding site is located outside the type 1 redox center and it is characterized by a spectroscopic contribution in the UV-region (B-type spectrum) as observed upon addition of an excess of Ag(I) or a stoichiometric amount of Hg(II). This UV spectral modification is equivalent to what is observed after treating *Pseudomonas fluorescens* azurin with a large excess of Cu(II) (Ugurbil and Bersohn, 1977) and is similar to that induced by binding of chromium as reported by Farver and Pecht (Farver and Pecht, 1981). Binding of Ag(I) and Hg(II) to this site affects the fluorescence emission of the protein supporting its localization not far from the Trp48. Extending the data previously reported (Finazzi-Agrò *et al.*, 1973) and based on the well known affinity properties of Hg(II) and Ag(I) for a sulfur ligand, the second binding site could be Met121 which is not essential for type 1 coordination of Cu (Karlsson *et al.*, 1991). Met121 is located close to a tunnel connecting the bulk phase with the type 1 site and it is proposed to protect it from unspecific bindings (Karlsson *et al.*, 1991). This is in agreement with our data showing that Hg(II) binding does not modify the presence of Cu in the reduced or oxidized azurin and Ag(I) does not displace Cu in oxidized azurin.

Another possible second binding site could be the disulfide bridge which connects Cys3 and Cys26 at the end of an exterior antiparallel β-loop of the azurin but, against this hypothesis, there is the notion that modification of the disulfide bridge heavily affects the structure of the protein and induces a strong shift of the fluorescence emission (Bonander *et al.*, 1996).

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