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Silver binding to *Pseudomonas aeruginosa* azurin

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Summary. The interaction between azurin and silver ions was investigated, by means of ultraviolet, fluorescence and atomic absorption spectroscopies, as a function of the redox state of the protein. The Ag(I) ion has a very low affinity for oxidized azurin. Interestingly, the affinity is much higher for reduced azurin; in this case Ag(I) completely displaces the Cu(I) ion from the native binding site. The effect is very specific for silver ions since other ions, such as Hg(II), Ni(II) and Cd(II), do not produce the same effect. Treatment of reduced and oxidized azurin with excess Ag(I) (2–8-fold stoichiometric) shows that there is a second binding site for silver ions on the protein which can also bind Cu(II) and Hg(II) with comparable affinities.

Key words: Azurin – Copper proteins – Fluorescence – Silver ions

Introduction

Azurin from *Pseudomonas aeruginosa* is a copper protein involved in the bacterial electron transfer chain where it probably functions as an electron donor for *Pseudomonas* cytochrome oxidase, together with cytochrome c_{551}. It contains a so-called type-I copper centre (Malkin and Malmström 1970), characterized in the oxidized form by an intense blue colour (absorption maximum at 625 nm) and an unusually narrow hyperfine structure of the EPR spectrum (Gray and Solomon 1981). The structure of the protein has been determined by X-ray crystallography at 0.27-nm resolution (Adman and Jensen 1981). The copper ion is complexed in a flattened tetrahedral coordination site by two imidazole nitrogens (His-46, His-117) and two sulfur atoms from Cys-112 and Met-121 (Adman et al. 1982). The copper environment is highly hydrophobic (Finazzi-Agro' et al. 1970; Koenig and Brown 1973; Boden et al. 1974; Farver and Pecht 1981) and the metal not readily accessible, at a distance from the surface of 0.6–0.8 nm (Adman et al. 1978; Mims et al. 1984).

A single tryptophan (at position 48; Ryden and Lundgren 1976) confers to the molecule an unusual fluorescence spectrum with an emission maximum at 308 nm, which is not modified by the redox state of the copper. Removal of the metal ion is associated with a threefold increase in fluorescence yield without shifts of the emission maximum. The process is reversible since addition of Cu(II) to apoazurin quenches the fluorescence by about 70% (Finazzi-Agro' et al. 1970). Similarly, other ions quench the fluorescence of this single tryptophan; in the case of Ag(I) quenching is about 30%. The origin of this phenomenon is not clear at present (Finazzi-Agro' et al. 1973; Ugurbil and Bersohn 1977; Turoverov et al. 1985).

We have carried out a careful investigation of the binding of Ag(I) to azurin. Our results provide evidence for a dependence of the silver–copper interaction on the redox state of the protein and for the existence of a second metal-binding site.

Materials and methods

Azurin was purified from *Pseudomonas aeruginosa* (NCTC 6750) according to Parr et al. (1976). The ratio $A^{\mathrm{ox}}_{625\,\mathrm{nm}}/A_{280\,\mathrm{nm}}$ of purified samples was never less than 0.55. Reduction of the protein was obtained by addition of solid sodium dithionite and subsequent chromatography on a Sephadex G-25 column (1 cm × 10 cm) to wash away excess reductant and by-products. The concentration of reduced azurin was measured as the absorption of ferricyanide reoxidized sample using an $\varepsilon^{\mathrm{ox}}_{625\,\mathrm{nm}} = 3500\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ (Brill et al. 1968).

Ag(I) was added in the form of $AgNO_3$. All experiments were carried out in distilled water (pH 6.2) in order to avoid problems related to the great insolubility of the silver ion. $(CH_3COO)_2Hg$ and $(CH_3COO)_2Cd$ were dissolved in water; $(CH_3COO)_2Ni$ was diluted from a stock solution in water and alcohol (8:2, by vol.). Absorption spectroscopy was performed on a Cary 219 instrument at room temperature.

Fluorescence spectra were run on a G. K. Turner 210 spectrofluorimeter or FP-770 Jasco using excitation and emission band-

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widths of 7.5 nm or 10 nm. All measurements were carried out at 20° C with samples having an absorbance of about 0.1–0.2 at 280 nm.

Atomic absorption measurements were made on a Perkin-Elmer 5100/Z (acetylene/air flame); values given for each sample are the average of three measurements (with an associated error of 2%) obtained by subsequent dilutions of the sample. The smallest copper and silver concentrations measurable were around 0.02–0.05 mg/l. In the case of reduced azurin treated with Ag(I), the samples were washed on a Sephadex G-25 column (1 cm × 10 cm) before atomic absorption to remove excess free metal. The concentration of the silver derivatives obtained after gel filtration was estimated from the tryptophan peak at 292 nm which is only slightly affected by the silver addition (see below); the proper absorption coefficient (4200 M^{-1} cm^{-1}) was evaluated on oxidized native azurin samples.

All reagents were of analytical grade.

Results

Absorption spectroscopy

Addition of Ag(I) ions to reduced azurin in a 1:1 molar ratio immediately affects the usual ultraviolet spectrum (Fig. 1a), decreasing the absorbance around 280 nm (Fig. 1b); this effect will be referred to as type-A modification. Azurin after the stoichiometric addition of Ag(I) is not reoxidizable by ferricyanide.

Further addition of silver ions (up to eightfold molar excess) leads to an increase of absorption in the ultraviolet region and loss of a well defined maximum (type-B modification; Fig. 1c). This spectral modification is very similar, if not identical, to that reported by Ugurbil and Bersohn (1977) upon addition of excess Cu(II) to apoazurin from *Ps. fluorescens*. The addition of a great excess of $CuSO_4$ to the Ag(I)-treated sample causes a slow recovery of absorbance at 625 nm and thus of the blue color, but leaves unaltered the type-B modification in the ultraviolet region.

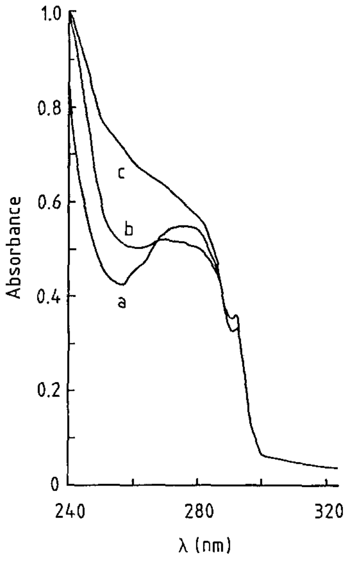

Fig. 1. Effect of Ag(I) on ultraviolet spectrum of reduced azurin. (**a–c**) Ultraviolet spectra of reduced azurin (**a**) before and (**b, c**) after addition of (**b**) a stoichiometric amount and (**c**) eightfold molar excess of Ag(I). Protein concentration was 72 μM in water at 20° C

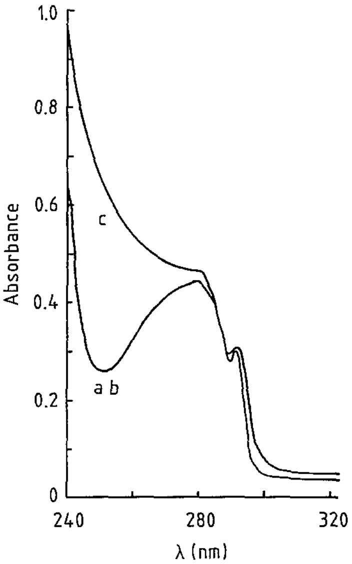

Fig. 2. Effect of Ag(I) on ultraviolet spectrum of oxidized azurin. (**a–c**) Ultraviolet spectra of oxidized azurin (**a**) before and (**b, c**) after addition of (**b**) a stoichiometric and (**c**) eightfold molar excess of Ag(I). Protein concentration was 68 μM in water at 20° C

In contrast, the addition of a stoichiometric amount of Ag(I) ions to oxidized azurin has no significant effect on the spectrum in the ultraviolet (Fig. 2a, b) or visible regions. Further additions up to eightfold molar excess produce a slow increase of the absorbance below 280 nm, eventually leading to type-B modification (Fig. 2c), and a slow decrease beyond 600 nm with loss of the blue protein color ($t_{1/2} = 60$ min).

Addition of Hg(II) ions to reduced azurin from stoichiometric up to eightfold molar excess produces only type-B spectral modification (not shown), but the sample is reoxidizable by ferricyanide.

Additions of Ni(II) and Cd(II) ions (up to eightfold stoichiometric amount of protein) do not modify the ultraviolet spectrum of reduced azurin at all.

Fluorescence spectroscopy

Fluorescence emission spectra of these various metal adducts were also recorded. Addition of a stoichiometric amount of Ag(I) ions to reduced azurin is associated with an increase of emission of about sevenfold; a further increase is obtained by increasing Ag(I) concentration up to sixfold molar excess (data not shown). The wavelength of maximum emission (308 nm) is not changed. In contrast, the fluorescence emission of reduced azurin is not affected by the addition of Hg(II), Ni(II) or Cd(II) ions (results not shown).

Addition of a slight excess of Ag(I) to oxidized azurin increases the intensity of emission threefold; further addition produces no other change. Fig. 3 shows the different behavior of reduced and oxidized azurin in the presence of twofold excess Ag(I).

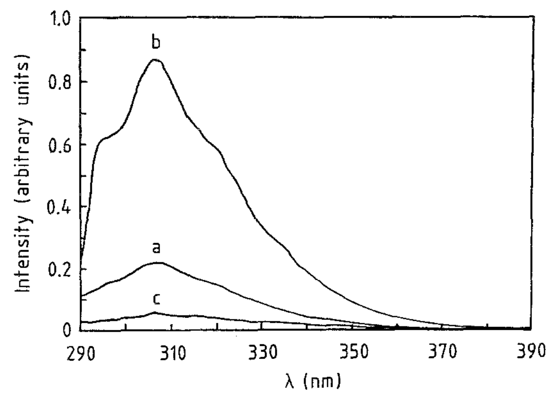

Fig. 3. Effect of Ag(I) on fluorescence spectra of reduced and oxidized azurin. (**a–c**) Fluorescence spectra of (**a**) oxidized and (**b**) reduced azurin (at the same concentration) after addition of twofold excess of Ag(I). Fluorescence intensities of the two samples before Ag(I) addition were identical (**c**). All spectra were recorded in water at 20° C. The excitation wavelength was 280 nm

Atomic absorption data

The stoichiometry of the Ag(I) adducts of reduced azurin was determined by atomic absorption on Ag(I)-treated samples after Sephadex G-25 chromatography; all samples displayed only a type-A spectrum in the ultraviolet region. The results reported in Table 1 show that reduced azurin plus Ag(I) lost at least 95% of the copper but contained a stoichiometric amount of silver.

Discussion

The effect of addition of Ag(I) to *Pseudomonas aeruginosa* azurin is clearly dependent (a) on the initial oxidation state of the copper and (b) on the molar ratio between Ag(I) and azurin. Addition of a stoichiometric amount of Ag(I) to reduced azurin induces a characteristic ultraviolet spectral change (type A; see Fig. 1b) and (more importantly) forbids protein reoxidation by ferricyanide. A spectroscopic contribution of the metal ion in the ultraviolet region mediated by the polypeptide chain has been proposed to explain the different absolute spectra of Cu(I), Cu(II) and apoazurin (Yamanaka et al. 1963; Ugurbil and Bersohn 1977). In line with this proposal, we assign type-A modification to the binding at the blue copper site of Ag(I), which in the case of reduced azurin is associated with the exclusion of Cu(I). The atomic absorption data (see Table 1) show unequivocally that, after addition of Ag(I), reduced azurin contains no copper but one equivalent of silver. Furthermore the —SH group of Cys-112 is not titratable with *p*-hydroxymercuribenzoate either in the reduced protein (as expected), or in the reduced plus Ag(I) protein (Tordi M. G., unpublished results). This is independent evidence that Ag(I), when added to the reduced species, displaces copper and occupies the metal-binding site of azurin.

Table 1. Copper and silver content of reduced azurin after addition of excess Ag(I)

Reduced azurin + Ag(I) (μM)	Cu (μM)	Ag (μM)
104	3	113
87	3	88
29	1	29

The table shows copper and silver concentrations of samples of reduced azurin after excess Ag(I) addition and Sephadex G-25 chromatography. The copper content of reduced azurin before silver addition, determined as control, was always consistent with protein concentration

The lack of an effect of stoichiometric Ag(I) on the ultraviolet spectrum of the oxidized protein suggests a much higher stability of the Cu(II)-protein complex compared to that of Cu(I). The fluorescence emission of reduced azurin increases considerably upon addition of Ag(I) in spite of the known quenching effect of this metal; this phenomenon is readily explained by the displacement of copper by silver, which is a weaker quenching agent. Significantly, the maximum of fluorescence remains in all cases at 308 nm, confirming that the characteristic hydrophobic environment of Trp-40 is not modified. This is indirect evidence that copper and silver maintain a similar distance from this chromophore.

The protein binding site for reduced copper has a high affinity for silver and only a large Cu(II) molar excess (20-fold) can restore the absorption at 625 nm. This effect is specific for Ag(I) because addition of stoichiometric amounts of Hg(II), Cd(II) and Ni(II) to reduced azurin is without effect (on the ultraviolet and fluorescence emission spectra as well on protein reoxidation). As a comparison, it may be remarked that the addition of Ag(I) to reduced stellacyanin does not significantly modify its ultraviolet spectrum (Tordi M. G., unpublished result). Thus the interaction between Ag(I) and reduced azurin seems rather specific. At least three factors may be significant in this respect: (a) Ag(I) and Cu(I) have identical coordination numbers (two in both cases, i.e. linear complexes); (b) they both have high affinity for —SH groups, and thus for Cys-112 (metal ligand); (c) their dimensions are not very different [Cu(I), 96 pm; Ag(I), 126 pm (West 1976)]. While addition of stoichiometric Ag(I) to Cu(II)-azurin is without effect, a large molar excess of Ag(I) changes the visible spectrum (625 nm) of oxidized azurin, which cannot be reoxidized by ferricyanide. This result suggests that large amounts of Ag(I) can displace Cu(II) from its site, in agreement with previous observations for spinach plastocyanin (Katoh and Takamya 1964).

Upon addition of Ag(I) in excess a different ultraviolet spectrum (type B) appears in oxidized as well as in reduced azurin (see Figs. 1c and 2c) which is practically equivalent to that found during reconstitution of *Ps. fluorescens* apoazurin with Cu(II) in large stoichiometric ratio (Ugurbil and Bersohn 1977). This effect has been ascribed to binding of a second Cu(II) ion to a site different from the primary coordination metal site. Similarly we propose that the type-B spectral modification induced by an excess of Ag(I) is due to the binding of this ion at a second site different from the physiological copper coordination site. We conclude, in agreement with previous work (Finazzi Agro' et al. 1973), that azurin has two sites for metal ions, the second site having a much lower affinity for metal ions than the first. Since an excess of Ag(I) ions, similarly to Cu(II) ions, has no further significant effect on the fluorescence emission of reduced azurin, the B site is likely to be rather removed from Trp-48. Moreover, addition of Hg(II) in stoichiometric amounts induces a type-B ultraviolet spectrum, in the absence of displacement of Cu(I) (since this protein is still oxidized by ferricyanide).

These results on metal binding to azurin lead to two relevant conclusions. (a) The metal-binding site normally coordinating copper is somewhat different in the reduced and oxidized protein, either because of coordination distances or because of accessibility, in agreement with EXAFS data of copper-ligand distances (Groeneveld et al. 1986). (b) A second metal binding site for Ag(I) and Hg(II), of lower affinity and specificity, is present on the macromolecule; it may be interesting to investigate whether this site is coincident with or related to the chromium-binding site identified by Farver and Pecht (1981).

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