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Probing the structure and mobility of *Pseudomonas aeruginosa* azurin by circular dichroism and dynamic fluorescence anisotropy

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Abstract

The UV dynamic fluorescence and CD of several *Pseudomonas aeruginosa* azurins bearing single amino acid mutation have been studied. Two classes of mutants were examined. In the first class, two hydrophobic residues in the core of the protein, Ile 7 and Phe 110, nearest to the azurin single tryptophan Trp 48, were substituted by a serine (mutants I7S and F110S). In the second class, two residues in the outer sphere of the copper ligand field were changed, obtaining the following mutants: M44K, H35F, H35L, and H35Q. All these proteins showed two fluorescence lifetimes in the copper-containing form, but only one in the copper-free form. The lifetime of the latter derivatives was different from either those of the metal-bound samples, definitely ruling out the presence of apo-like species in the holo protein. Copper-free I7S and F110S showed a more complex fluorescence decay profile requiring a distribution of lifetimes rather than a single lifetime. Holo F110S was also better fitted, in the limit of confidence, with two distributions rather than a pair of lifetimes. Time-resolved anisotropy of these two mutants as well as of wild-type (wt) protein showed two components (rotational times for wt $\leq$ 200 ps and 7 ns, respectively). These components were not affected significantly by copper removal in the case of wt protein. Instead, the short rotational component of the mutants dropped dramatically to values near zero, indicating a much greater mobility of the tryptophanyl residue in the mutant apo azurins. These data were supported by CD measurements showing a small effect of the copper presence in the region below 250 nm, i.e., in the secondary structure, but almost a collapse of the aromatic asymmetry at 270–295 nm related to a relaxation of the structural constraint around the tryptophan. Altogether these data show that copper does not play a structural role in wt azurin, whereas it is crucial in the stabilization of I7S and F110S mutants. Furthermore, although the metal site geometry is rigidly kept in wt apo-azurin, it regains the native form only in the presence of the metal in the "core" mutants. This finding is important for the theory of entatic states in metalloproteins (Williams RJP, 1995, *Eur J Biochem 234*:363–381).

Keywords: entatic state; fluorescence lifetime distribution; site-directed mutagenesis; solvent relaxation; tryptophan

Spectroscopic techniques applied to proteins in solution are essential tools in the investigation of the structure and dynamics of these macromolecules under different chemico-physical conditions. Absorption spectra, electron paramagnetic resonance, steady-state and dynamic fluorescence, and CD are nowadays thoroughly exploited methods, often used in combination, in order to gain information about biological systems. In particular, the measurement of optical activity provides an important and unique characterization of the secondary and tertiary structure of biopolymers (Towell & Manning, 1994). It is well known that aromatic side chains of amino acids, which generally may show a CD signal in the near UV (250–300 nm), may also give a strong contribution to the far UV (200–250 nm) region of the spectrum (Manning & Woody, 1989; Woody, 1978; Woody, 1994). Recently, it has been demonstrated (Vuilleumier et al., 1993) that a single point mutation involving an aromatic amino acid in a protein sequence may affect the CD signal in the far UV spectrum. This is a very interesting finding because the correlation between CD spectra and the secondary structure of proteins rests largely on empirical observations. Therefore, the subtraction of spurious contributions from aromatic residues may give a better estimate of the secondary structure itself. On the other hand, it has been shown that fluorescence spectros-

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copy is a suitable technique to investigate the local properties of protein domains when they contain phenylalanine, tyrosine, and especially tryptophan residues (Mei et al., 1992; Silva et al., 1993; Ferreira et al., 1994). Thus, fluorescence and CD measurements are complementary methods to explain to what extent a single aromatic residue and the polypeptidyl chain may influence each other's properties. The detailed knowledge of structural, kinetic, and spectroscopic features of azurin, a small blue copper protein (14.6 kDa) component of the electron transfer system of denitrifying bacteria (Horio et al., 1961; Parr et al., 1976), makes this molecule an interesting model for protein structural–functional studies and a test case for the application of sophisticated spectroscopic techniques. The peculiar spectroscopic features of *Pseudomonas aeruginosa* azurin have been described (Finazzi-Agró et al., 1970, 1973). The single tryptophanyl residue present in this protein, Trp 48, has a highly structured CD spectrum in the near UV as well as a unique steady-state fluorescence signal which is blue-shifted (λ_{max} = 308 nm). These features were confirmed later by X-ray crystallography, showing that the tryptophan is almost immobilized in a highly hydrophobic cavity in the protein core (Adman et al., 1978). Furthermore, the possible relationship between Trp 48 and the likewise spectroscopically peculiar copper ion were extensively investigated both in terms of reciprocal influence and in terms of electron transfer mechanism.

In a previous paper (Gilardi et al., 1994), we have shown that *site-directed* mutagenesis can be used to obtain mutants in which the environment of Trp 48 is drastically perturbed. Dynamic fluorescence measurements of these mutants, Ile 7 Ser (I7S) and Phe 110 Ser (F110S), showed that local conformational changes have occurred. In particular, the changes in lifetime have been ascribed both to the different interaction of Trp 48 with surrounding groups and to the dipolar relaxation of the solvent molecules, which, upon the removal of the copper ion, gain access to the hydrophobic core of the mutated protein.

In the present study, rotational correlation lifetimes and the CD spectrum from 200 to 700 nm have been measured for both the holo and apo wild type (wt) and mutants F110S and I7S in order to check the rotational freedom of Trp 48. We have also studied some mutants involving residues close to the copper site, namely His 35 Leu (H35L), His 35 Gln (H35Q), His 35 Phe (H35F), Met 44 Lys (M44K), to check the specificity of the spectroscopic features observed. With the exception of M44K, all the site-directed mutants concern residues that are buried in the protein fold (see Fig. 1) and thus have been preserved during evolution. These residues are thought to stabilize the tertiary structure of the protein by their close packing (Chothia & Lesk, 1982).

Results

CD measurements

The CD spectra of the wt and mutant holo enzymes in the far UV are reported in Figure 2A and B. These spectra show a strong absorption around 220 nm, which is characteristic of β-structure (Strickland, 1974). Only in the case of F110S was a significant negative deviation from the spectrum of the wt sample observed, at 230 nm. Figure 3 shows the CD spectra of the copper-free proteins. The mutants F110S and I7S, which involve residues located in the protein core, show a decreased CD signal at 220 nm (Fig. 3A). No significant changes with respect to the holo protein were observed for the wt and for mutants involving residues

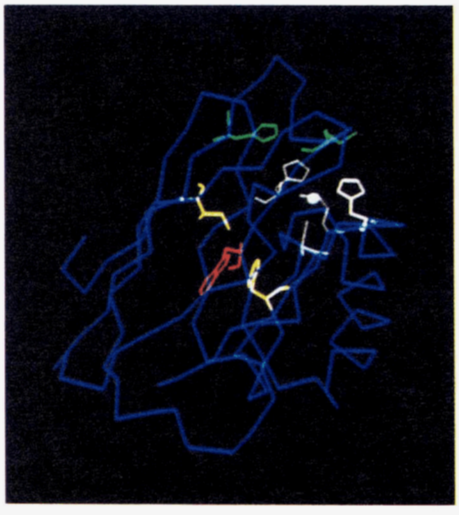

Fig. 1. Alpha-carbon backbone of wt azurin (blue line) from *P. aeruginosa*. Side chains of the residues Ile 7 (yellow, left), Phe 110 (yellow, right), Trp 48 (red), His 35 (green, left), Met 44 (green, right) are showed. The copper ion is indicated as a white dot. Also in white are the four copper ligands.

far away from the hydrophobic core of the enzyme (Fig. 3A,B). F110S, I7S, and to a lesser extent H35F mutants show distinct differences in the fine structure of the CD spectra in the aromatic region (250–300 nm) both in their holo and apo protein forms (Figs. 4A,B, 5A,B). For the "core" mutants, the difference from wt protein was much larger when copper was removed. As reasonably expected, the addition (H35F) or the removal (F110S) of a phe-

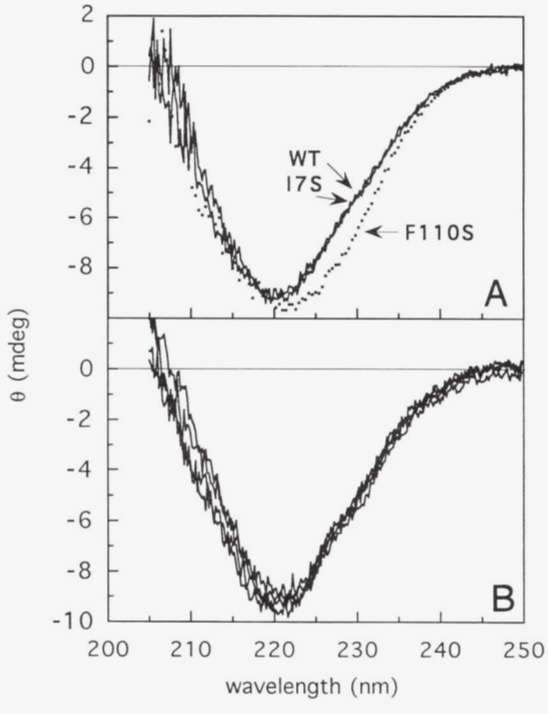

Fig. 2. Far-UV CD spectra of (**A**) holo wt, I7S, F110S samples and (**B**) holo H35F, H35L, H35Q, and M44K.

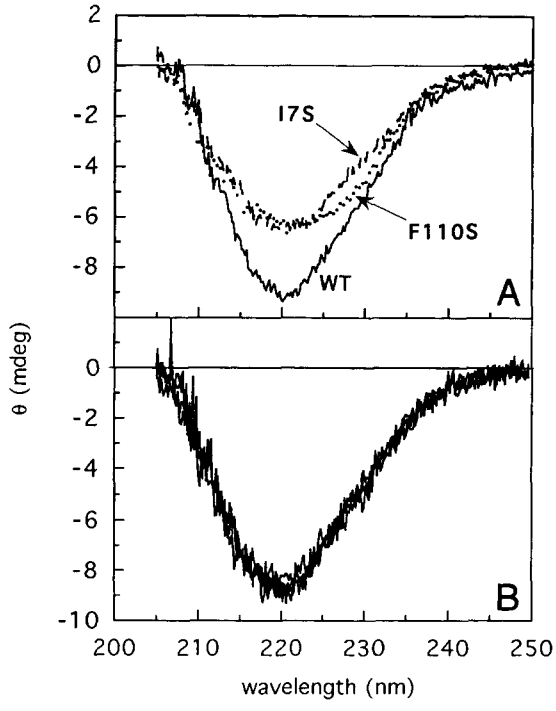

Fig. 3. Far-UV CD spectra of (**A**) apo wt, I7S, F110S samples and (**B**) apo H35F, H35L, H35Q, and M44K.

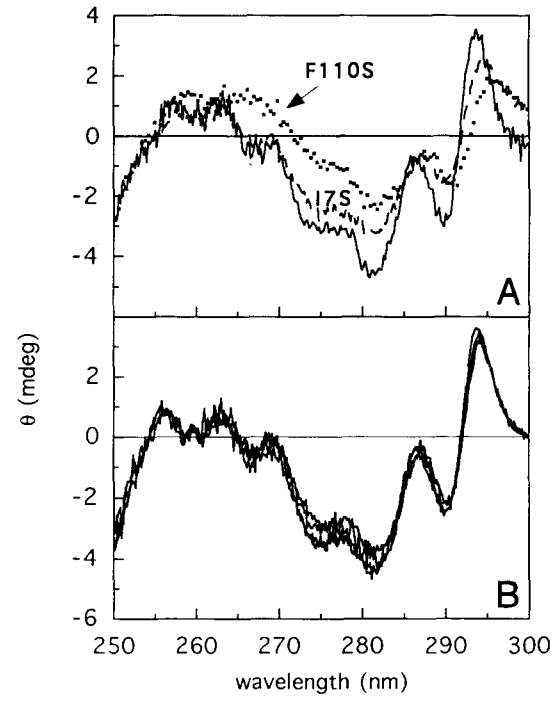

Fig. 5. Near-UV CD spectra of (**A**) apo wt (solid line), I7S (dashed line), F110S (dotted line) samples and (**B**) apo H35F, H35L, H35Q, and M44K.

nylalanine residue affects the peaks attributable to this residue (i.e., 262, 270, and 275 nm). It also should be noted that, in the mutants F110S and I7S, the bands typical of Trp 48 (282, 290, and 292 nm) are modified, indicating an increased indolyl side-chain mobility or an increased polarity of its microenvironment. The CD spectra in the visible region showed that the copper-associated transitions are more or less affected in all the mutant holo samples (Fig. 6A,B). F110S, I7S, and M44K have a greater effect on the absorption band centered at 625 nm, whereas other mutants mainly affect the bands at 465 and 620 nm. Interestingly, the intensity of CD band at 625 nm and the redox potential of each sample are related linearly (Fig. 6).

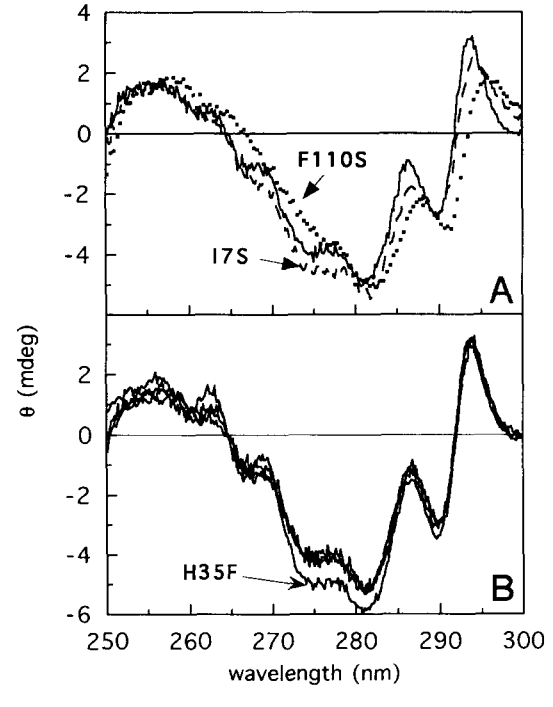

Fig. 4. Near-UV CD spectra of (**A**) holo wt (solid line), I7S (dashed line), F110S (dotted line) samples and (**B**) holo H35F, H35L, H35Q, and M44K.

Dynamic fluorescence measurements

The phase and modulation data of the fluorescence decay as a function of frequency are reported in Figure 7, together with the best fit obtained for each individual sample (solid lines). The shape of the data for holo proteins suggests that their fluorescence decays have a higher degree of complexity with respect to those of the metal-free samples. The corresponding fluorescence lifetimes obtained on the basis of the minimum chi-squared values are reported in Table 1. Indeed, as already reported, none of the holo sample fluorescence decays could be fitted with a single exponential function, but required two components of different lifetime and fraction (Gilardi et al., 1994). Although the short lifetime component, τ_1, is almost identical in all the holo samples, significant changes are observed in the longer fluorescence lifetime values, τ_2, as well as on their relative fractions. The removal of copper was always accompanied by the collapse of the two lifetimes into a single exponential component. As already reported (Gilardi et al., 1994), the decays of the mutants I7S and F110S in the apo form could be fitted only by a distribution of lifetimes. The mutant F110S might require a distribution also for the holo sample. It is important to note that four mutants in their holo form have $\tau_2 < 4$ ns, whereas much higher values were observed in the corresponding apo sam-

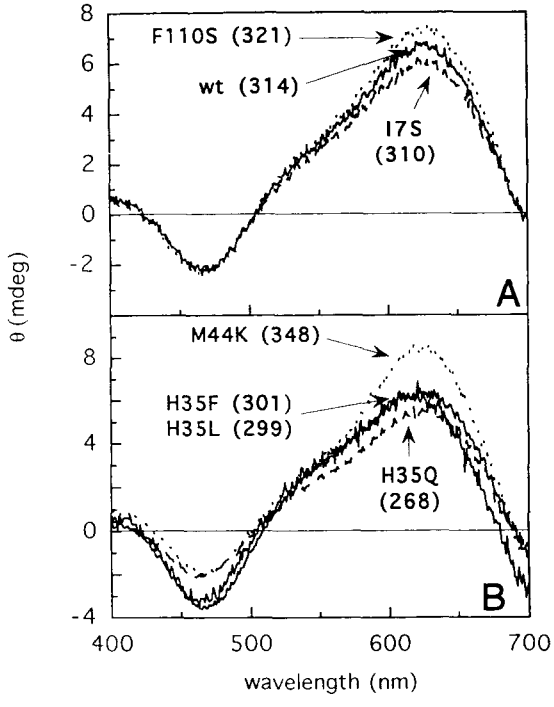

Fig. 6. Visible CD spectra of (**A**) holo wt, I7S, F110S samples and (**B**) holo H35F, H35L, H35Q, M44K. The number in brackets represent the redox potential of each sample expressed in mV (see Materials and methods).

Table 1. *Dynamic fluorescence parameters*[a]

Holo samples	τ_1	w_1	F_1	τ_2	w_2	χ^2_a	χ^2_b
WT	0.22		0.39	4.51		1.2	3.2
I7S	0.20		0.75	2.40		1.1	47.3
F110S	0.22	0.10	0.78	4.12	0.20	1.0	1.7
H35L	0.24		0.66	3.96		1.0	10.9
H35Q	0.24		0.57	3.79		1.2	41.8
H35F	0.24		0.78	3.90		0.9	14.4
M44K	0.23		0.70	4.32		1.0	4.7
Apo samples				τ	w	χ^2	
WT				4.70		1.3	
I7S				3.52	2.40	1.2	
F110S				4.30	2.10	1.2	
H35L				4.55		0.9	
H35Q				4.59		1.0	
H35F				4.59		1.0	
M44K				4.87		1.1	

[a] τ, τ_1, τ_2, fluorescence lifetimes (i.e., centers of lorentzian distributions if w_1 or w_2 are reported) in ns ($\Delta\tau_1 \approx 20$ ps; $\Delta\tau_2 \approx 50$ ps). w_1, w_2, widths of lorentzian distributions in ns ($\Delta w \approx 0.04$ ns). F_1, fractional intensity relative to τ_1 ($F_1 + F_2 = 1$; $\Delta F_1 \approx 0.01$). χ^2_a, reduced chi-squared values relative to the best fits reported in Figure 7 (solid lines). χ^2_b, reduced chi-squared value obtained fixing τ_2 to the value of the apo protein.

ples. This result seems to exclude, at least in these cases, the hypothesis that an apo-like contaminant was present in the holo proteins (Petrich et al., 1987). In order to test this hypothesis, each set of data for copper-containing samples was also fitted using a long lifetime component, τ_2, set equal to the apo protein lifetime. The chi-squared values obtained in this way are reported in Table 2 and are clearly higher than the values obtained for the best fit. The mobility of the Trp 48 was also studied when a bulky residue in its neighborhood was substituted with a smaller one. In particular, we measured the anisotropy decay fluorescence of F110S and I7S. The results are reported in Table 2, together with the values measured for the wt protein. The slow rotation ($\Phi_2 \approx 6$–7 ns) observed in each sample is in good agreement with the value reported in the literature (Petrich et al., 1987), and is compatible with the tumbling of the whole protein molecule. A smaller value of Φ_2 (≈3 ns) was observed for apo F110S and I7S, possibly indicating a superimposed segmental mobility of protein domains. The fast rota-

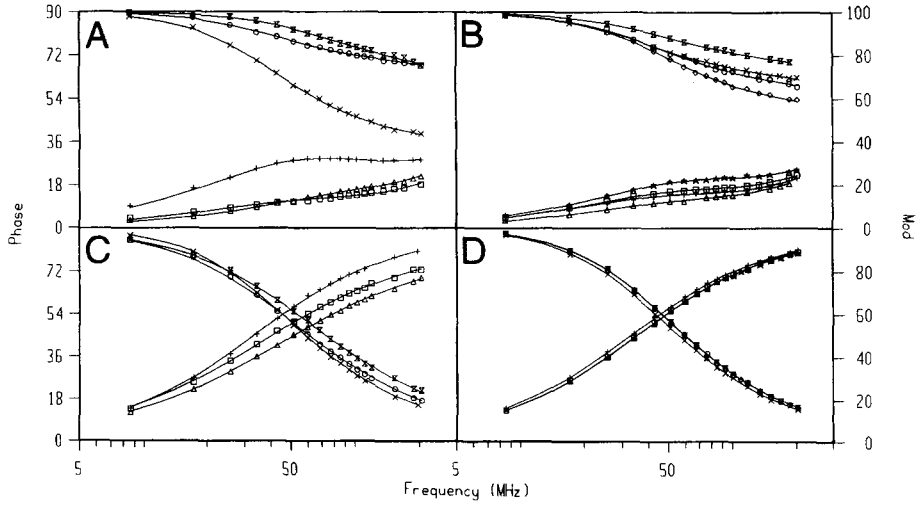

Fig. 7. Dynamic fluorescence measurements for the (**A,B**) holo and (**C,D**) apo protein samples. **A,C:** Phase and modulation data of wt (+, ×) F110S (□, ○) and I7S (△, ⧖). **B,D:** Phase and modulation data of (+, ×) M44K, H35L (□, ○), H35F (△, ⧖), and H35Q (☆, ◇).

Table 2. *Dynamic fluorescence anisotropy parameters*

	ϕ_1 (ns)	ϕ_2 (ns)	F_1 (%)	Wobbling cone amplitude	$\langle d \rangle$ (Å) Mean distance	χ^2
Holo samples						
WT	0.190	6.71	0.61	43°	4.08	1.3
F110S	0.080	6.42	0.59	43°	4.03	1.2
I7S	0.180	6.87	0.52	39°	3.96	1.3
Apo samples						
WT	0.140	7.01	0.67	47°	—	1.2
F110S	0.039	3.20	0.65	—	—	1.1
I7S	<0.010	3.34	0.52	—	—	1.3

[a] ϕ_1, ϕ_2, rotational correlation lifetimes ($\Delta\phi_1 \approx 20$ ps; $\Delta\phi_2 \approx 0.5$ ns). $\langle d \rangle$, arithmetical mean of the three distances between Trp 48 center of mass and carbon atoms 31Cγ2, 50Cβ, and 125 Cγ1. χ^2, reduced chi-squared value.

tional lifetime Φ_1 is significantly different in the case of holo F110S, which is the only sample having a distributed fluorescence decay (Table 1). This result might be explained on the basis of the presence of a few solvent molecules in the otherwise hydrophobic protein core around the indolyl side chain (Hammann et al., 1996).

The short rotational lifetime drops to very small values in the mutated samples upon copper removal, indicating that Trp 48 can move faster due to a less rigid environment. From the rotational correlation lifetimes and related fractions, the maximum allowed "wobbling cone" of Trp 48 was evaluated (see Materials and methods), and its amplitude for the holo and apo wt, holo I7S and holo F110S samples is reported in Table 2. The above model cannot be applied to apo I7S and apo F110S because the molecule does not behave anymore like a rigid sphere with a rotational correlation time of about 6.5 ns. This finding was strenghtened by calculating the "mean distance," ($\langle$d$\rangle$), between Trp 48 and its closest surrounding atoms (31Cγ2, 50Cβ, 125 Cγ1), averaging each distance obtained from the X-ray structure for the holo samples (Nar et al., 1991; Nar et al., 1992). These data are also reported in Table 2. For the apo wt sample, the calculated allowed wobbling cone amplitude is larger in comparison to the relative holo protein, suggesting a slight widening of the Trp 48 hydrophobic cavity.

NMR measurements

The proton spin-lattice relaxation time, T_1, was measured on the Trp 48 side-chain resonances in the case of wt azurin, and of I7S and F110S samples. The value of T_1 decreased in both mutants from 1.12 ± 0.02 s (wt) to 1.01 ± 0.02 s (I7S) and 0.78 ± 0.02 s (F110S). In order to check if these modifications were strictly related to the specific amino acids mutated in the neighborhood of Trp 48, the same parameter was also measured (under the same conditions) on the Ile 81 resonances. As a matter of fact, no significant differences among the different proteins were observed.

Discussion

Small globular proteins containing significant amount of secondary structure are well suited for studying general rules governing the overall folding and stability of polypeptides (Kim & Baldwin, 1982). The ideal situation would be when the crystal structure of the protein is also known and there exists the possibility of expressing large quantities of this protein and of its mutants in heterologous systems.

This is indeed the case of *P. aeruginosa* azurin. This protein has a few further interesting properties. It belongs to the class of blue copper proteins where the metal ion gets very unusual features in terms of optical and EPR spectroscopy (Finazzi Agró et al., 1970). Besides that, azurin is rather unique in the fluorescence properties of the single tryptophanyl residue embedded deeply in the core of the β-barrel forming the structure of the protein. This tryptophan shows a most unusual fluorescence emission, near-UV CD spectra, and fluorescence decay. Several papers have dealt with these properties (Finazzi Agró et al., 1973; Szabo et al., 1983; Petrich et al., 1987). More recently, thanks to site-directed mutagenesis, we have shown that our previous interpretation of the optical spectroscopic data was correct. In this paper, we have extended the observations to time-resolved fluorescence anisotropy and to far- and near-UV CD measurements in order to better understand which phenomenon is taking place upon changing the environment of the indolyl side chain. We also studied several azurin mutants at different sites in order to check the specificity of the mutations nearest to the tryptophan and to explore the relationship between tryptophan and copper.

As shown in Table 1, all the copper-free mutant azurins, much alike wt, show a single lifetime decay of tryptophanyl residue. The presence of denaturated protein was excluded by complete reconstitution of the holo-forms by addition of copper to apoprotein as previously described (Hutnik & Szabo, 1989a). The values of lifetimes are also very similar, with the significant exception of the I7S mutant and possibly of the mutant F110S. Both mutations perturb the microenvironment of the indolyl side chain, increasing the polarity. Interestingly, in both cases, a Lorentzian distribution of lifetimes is required to fit the data (Table 1; Gilardi et al., 1994). The presence of copper in every case studied splits the emission decay of the single tryptophan into two components, a shorter one, which always falls in the range 200–240 ps, and a longer one, with much larger variability (from 2.40 to 4.50 ns). Furthermore, the fraction of fast-decaying fluorescence varies from about 0.4 in the wt to about 0.8 for the mutants F110S and H35F. No clear explanation has been yet put forward for the mechanism of fluorescence quenching by copper, despite extensive work (Petrich et al., 1987; Sweeney et al., 1991). In any case, the presence of metal seems to induce the formation of two different emitting species. This conformational heterogeneity was also observed in several metalloazurin derivatives (Hutnik & Szabo, 1989b) and was therefore ascribed to an indirect influence of the metal center. The simplistic explanation that the longer lifetime might be associated with an apo-like azurin molecule was ruled out by Hutnik and Szabo (1989a) on the basis of a refined purification procedure and of the comparison between the fluorescence decays of holo and copper-reconstituted samples. The results reported in Table 1 are indeed compatible with this hypothesis. In particular, we have shown that (1) all the mutants and the wt protein show a longer living fraction, although of different lifetime and weight, and (2) the removal of the metal makes all the lifetimes alike. Furthermore, whenever an apo-like component was included in the holo fit, the chi-squared value increased, confirming that the τ_2 lifetime reflects an intrinsic heterogeneity of the holo proteins' fluorescence decay.

Among the copper-containing species, only F110S showed a hint of lifetime distribution, in the limit of analysis confidence. This may suggest that, execpt for F110S, the bound metal makes the population of molecules in solution more homogeneous.

The relationship between the presence of copper and the mobility of indolyl side chain was also indicated by the fluorescence anisotropy (Table 2). It shows that holo-azurin fluorescence anisotropy could be analyzed in terms of two rotational lifetimes, a longer one, which could be reasonably attributed to the tumbling of the whole molecule. Instead, the shorter rotational lifetime is only compatible with local movement of the peptidyl units around Trp 48 and is restricted by the presence of neighbor side chains. In fact, the two mutants around this residue, i.e., F110S and I7S, showed no difference in the longer relaxation time, whereas the shorter one appears to be decreased only in the F110S mutant. This effect might be ascribed to the penetration of a few water molecules in the cavity of Trp 48 when Phe 110 is substituted by a serine residue. Interestingly enough, the "wobbling cone" amplitudes calculated from the fluorescence anisotropy decay data are consistent with the average distance of Trp 48 from the closest residues, calculated for the holo samples using the X-ray structure (Hammann et al., 1996). This result is also consistent with NMR measurements, which show how the effect on the relaxation time of Trp 48 is related specifically to the mutation.

The removal of copper does not dramatically affect the dynamic fluorescence anisotropy parameters of wt azurin, but a striking decrease of the short relaxation time was observed in the mutant proteins, again suggesting a far greater mobility of tryptophan in these samples. Moreover, the slower component, ϕ_2, is decreased by more than 50% and is no more compatible with the rotation of a rigid sphere of the same size of azurin. These data are instead consistent with the appearance of an internal mobility due to the relative motion of rigid domains, connected by flexible structures. The greater internal mobility is therefore enhancing the structural heterogeneity of the protein in the nanosecond time range. This effect may explain the distributions of lifetimes observed in the I7S and F110S apo proteins (Table 1).

The interpretations of the fluorescence data were corroborated by CD measurements. In fact, whereas the far-UV CD spectra do not show any gross change between wt and mutant proteins, indicating that the secondary structure is retained in the mutants in the presence or absence of copper, the near-UV CD spectra of the mutants I7S and F110S showed a dramatic decrease of chirality, which was mostly evident in the copper-free samples. This further indicates that, upon removal of copper, the constraint imposed by the protein on the tryptophanyl side chain is almost completely released at variance with the wt apoprotein (Fig. 3).

On the contrary, mutations of residues far from Trp 48 but near to the copper site affect only the visible CD spectra, i.e., those related to the symmetry of the copper ion crystal field. The intensity of the prominent band at 625 nm correlated well with the redox potential of each sample, as expected for a charge transfer transition between the S of Cys 112 and the copper (Solomon et al., 1980, 1993). Interestingly enough, the presence of one more (H35F) or one less absence of one (F110S) phenylalanine residue was clearly detectable in the far- and near-UV spectra.

In conclusion, we have shown that a single point mutation that makes more polar the environment of Trp 48 in azurin (Fig. 1) induces the following changes in the spectroscopic properties of this residue. (1) Emission spectra are red-shifted from a minimum of 308 nm to a maximum value of 350 nm in the apo forms (Gilardi et al., 1994). (2) Heterogeneity exists in the emission properties, i.e., the presence of a distribution of lifetimes rather than a single lifetime. This property is much more evident in the copper-free samples, indicating a looser conformation around the tryptophan. (3) A much faster rotational relaxation time results, indicating a greater mobility of the indolyl side chain, confirmed by the release of CD signal and the behavior of the relaxation time.

Last but not least, we have shown that, whereas copper always affects in a subtle way the emission properties of Trp 48, its presence is only needed to keep the native tertiary structure in I7S and F110S mutants. In other words, although the wt azurin generates an "entatic" state of the bound copper, the latter becomes equally energized by an induced fit mechanism in the "core" mutants (Williams, 1995).

Materials and methods

Sample preparation (mutants and apo-proteins)

Site-directed mutagenesis was performed with an in vitro system based on the method of Ecksten and co-workers (Sayers et al., 1988). DNA cloning experiments were performed on the pGC4 plasmid in which the azurin gene from *P. aeruginosa* was cloned as reported previously (Canters, 1987; van de Kamp et al., 1990). Details of the site-directed mutagenesis procedure, protein expression, isolation, and purification have been described elsewhere (Gilardi et al., 1994). The purity of the protein samples was evaluated by UV-vis spectrophotometry, by measuring the ratio A_{625}/A_{280} that was always greater than 0.58 for all proteins, indicating a high degree of purity.

The apoproteins were prepared in the presence of 100 mM KCN and 1 mM EDTA in 50 mM Tris-HCl buffer, pH 8.5.

The redox potentials of the wt and mutant azurins were determined by redox titrations of reduced azurin with $K_3Fe(CN)_6$ in 0.1 M phosphate buffer, pH 7.0, 298 K. Sample preparation, measurements, and data analysis were carried out as described by van de Kamp et al. (1990).

CD measurements

CD spectra were recorded using a Jasco-J600 spectropolarimeter. The sample holder was thermostated at 20 °C using an external bath circulator. Measurements were taken using quartz cuvettes of different pathlength, namely 0.1 cm for the peptide bond region (200–250 nm) and 0.5 cm for the aromatic (250–300 nm) and visible (400–700 nm) regions. Samples concentrations were always in the range 1–10 μM.

Fluorescence measurements

Steady-state fluorescence experiments were performed using a photon-counting spectrofluorometer (Fluoromax Instruments S.A., Paris). Fluorescence lifetimes have been measured using the synchrotron radiation facility of the Adone storage ring (Frascati, Italy). The excitation wavelength was 295 nm while emission was collected through a WG305 cut-off filter. Phase shift and demodulation techniques have been used to collect time-resolved fluorescence data at several modulation frequencies, from about 5 to 210 MHz. The Marquardt algorithm of the Globals Unlimited software (Beechem & Gratton, 1988) was used for data analysis and the best fits were selected on the basis of the smallest chi-squared value. All measurements were obtained using *p*-terphenyl in ethanol as a standard reference ($\tau \approx 1.05$ ns). Elimination of polarization effects on lifetime measurements was achieved using a polarizer set at 35.5° with respect the vertical direction along the

excitation pathway (Gratton et al., 1986). Rotational correlation lifetimes were calculated from the anisotropy decay measurements, taking into account the relative fluorescence lifetimes. In particular, the tryptophanyl residue was considered as a label attached to a spherical rotating molecule and therefore a double exponential fit was used (Steiner, 1983):

$$r(t) = r_0[F_1 e^{-t(1/\Phi_1 + 1/\Phi_2)} + F_2 e^{-t/\Phi_2}].$$

The amplitude of the allowed rotation was calculated following the model of the "wobbling cone" (Lipari & Szabo, 1980; Steiner, 1983):

$$\theta = \arccos((\sqrt{1 + 8\sqrt{A_\infty} - 1})/2)$$

which requires the knowledge of the residual anisotropy at infinite time, r_∞ (i.e., the product between r_0 and A_∞). When the faster correlation lifetime is very short compared to the slower protein motion, the parameter A_∞ can be approximated by the fraction of the longer component, F_2 (Silva et al., 1993).

NMR relaxation times

NMR spin-lattice (T_1) relaxation times were measured on the proton resonances at 300 MHz with a conventional inversion recovery pulse sequence (180–τ–90–collect). Series of 15 spectra were collected with different delays, τ, and the signal intensities were fitted to the function:

$$I = I_0[1 - 2\exp(-\tau/T_1)] \ ,$$

where I is the intensity of the signal at the delay τ and I_0 is the intensity of the fully relaxed signal.

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