Unique Environment of Trp48 in *Pseudomonas aeruginosa* Azurin As Probed by Site-Directed Mutagenesis and Dynamic Fluorescence Spectroscopy†

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ABSTRACT: Two mutants of the blue copper protein azurin from *Pseudomonas aeruginosa*, Ile7Ser and Phe110Ser, were prepared. The mutations were aimed at affecting the mobility and the fluorescence properties of Trp48, the only tryptophan residue present, which in the wild-type protein is located in a highly hydrophobic and rigid environment. EPR, UV–vis, and NMR spectroscopy show that the copper binding site and the overall structure of the wild-type protein are preserved and that structural effects occur only on a local scale. Steady-state fluorescence spectra of both mutants, particularly in the copper-free form, show that tryptophan fluorescence is dramatically affected by the introduction of a polar residue close to it. The emission maximum is red-shifted and dependent on the excitation wavelength. This indicates a loosening of the matrix around the indolyl side chain and an increase of the effective dielectric constant of the microenvironment. Time-resolved fluorescence spectroscopy also shows substantial changes in the fluorescence lifetimes and in the distribution of the lifetimes of the mutants; these variations are interpreted in terms of a change in solvation of the Trp48 side chain.

Azurin is a small blue copper protein (14.6 kDa) which is part of the electron-transfer system of denitrifying bacteria (Horio et al., 1961; Parr et al., 1976). The presence of one copper ion per molecule confers to the protein a strong absorption band centered at 620 nm (ϵ = 3000–6000 M^{-1} cm^{-1}) and a characteristic electron paramagnetic resonance (EPR) spectrum, with a small hyperfine coupling constant $\leq 70 \times 10^{-4}$ cm^{-1}. The spectroscopic features of the copper site of azurin correspond to those of a type-1 copper site [for a recent review, see Canters and Gilardi (1993)]. The three-dimensional structure of azurin from *Pseudomonas aeruginosa* is known from X-ray crystallography (Nar et al., 1991a). The folding topology consists of an α-helix and eight β-strands organized in a β-sandwich; two connecting loops contain the copper ligands. The β-sandwich is closely packed and forms a highly hydrophobic core.

The primary structure of azurin from *Pseudomonas aeruginosa* contains only one tryptophan residue (position 48), and its side chain is pointing toward the center of the hydrophobic core of the protein. This residue has been shown to have unique spectroscopic features: upon excitation at 280–300 nm, a fluorescence maximum is seen at 306–308 nm. This is by far the highest energy emission of tryptophan-containing compounds at room temperature. With a few exceptions (for instance, amicyanin and ferritin) buried tryptophan residues in proteins usually show emission maxima in the range 325–335 nm (Burstein et al., 1973). The emission of Trp48 also shows a fine structure and is strongly quenched by copper. The removal of copper increases the fluorescence quantum yield 10–20-fold without affecting the shape or the position of the spectrum. The origin of this unique blue-shifted emission, as well as the mechanism underlying the quenching by copper, has been the subject of strong debate over the last 20 years (Finazzi-Agrò et al., 1970; Szabo et al., 1983; Petrich et al., 1987; Hutnik & Szabo, 1989a,b; Hansen et al., 1990). The picture emerging from these studies is in line with the interpretation of the fluorescence spectra first given by Finazzi-Agrò and co-workers in 1970, who proposed that the fluorescence properties are consistent with a buried residue in a highly hydrophobic and rigid environment, as later confirmed by X-ray crystallography (Nar et al., 1991a).

The interpretation of the quenching effect observed on Trp48 when copper is present is still the subject of research. Petrich and co-workers (Petrich et al., 1987) interpreted this effect in terms of electron transfer from the excited state of tryptophan to copper(II), with formation of an excited-state tryptophan radical cation. Also in recent studies the quenching of the phosphorescence of azurin has been ascribed to a tryptophan-to-copper electron transfer (Strambini & Gabellieri, 1991; Klemens & McMillin, 1992).

Electron transfer from a tryptophan residue is indeed an interesting possibility. Kim and co-workers (Kim et al., 1991, 1992) have shown recently by site-directed mutagenesis that photolyase can repair DNA pyrimidine dimers by converting the energy of light into chemical energy by means of a specific tryptophan residue present in this enzyme, Trp277. The tryptophan in this case functions as electron donor to the site of the DNA damage. Long-range intramolecular electron transfer studies of azurin have shown the possibility of electron transfer from the radical anion generated on the disulfide bridge (Cys6–Cys23) by pulse radiolysis to the copper(II) (Farver & Pecht, 1989). A purported electron-transfer pathway between the disulfide bridge and the copper site involves Trp48, and parallel results obtained with azurins lacking the tryptophan residue in position 48 give evidence that the indole ring system may play a role either in enhancing

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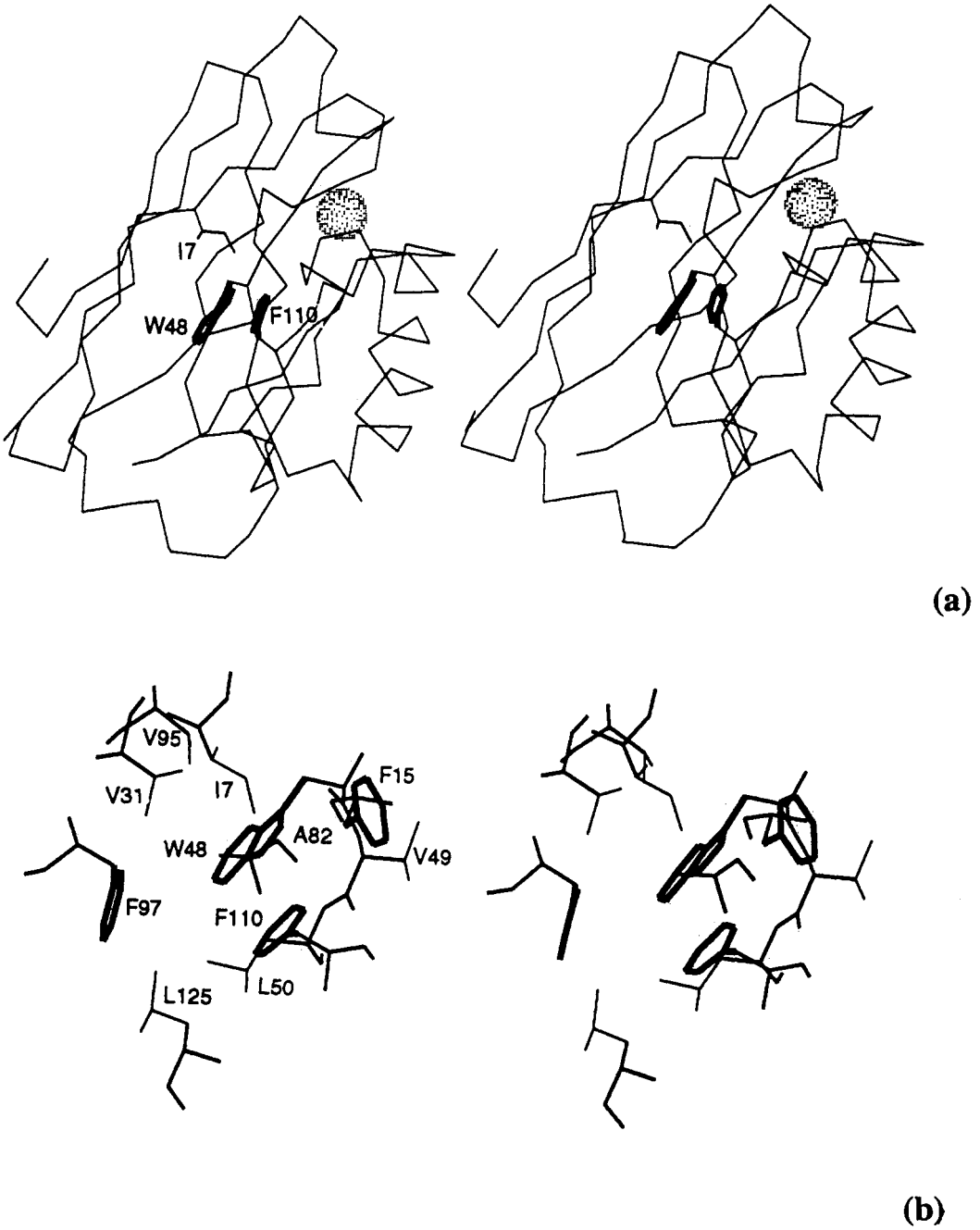

FIGURE 1: (a) Stereoview of the α-carbon backbone of wild-type azurin from *Pseudomonas aeruginosa*. The side chains of Ile7 (I7), Trp48 (W48), and Phe110 (F110) are indicated. The copper is represented by a dotted sphere. (b) Immediate environment of Trp48 (W48) in the hydrophobic core of *Pseudomonas aeruginosa* azurin, including the side chains of Ile7 (I7), Phe15 (F15), Val31 (V31), Val49 (V49), Leu50 (L50), Ala82 (A82), Val95 (V95), Phe97 (F97), Phe110 (F110), and Leu125 (L125) (Nar et al., 1991a).

the mixing of the electron donor and acceptor wave functions or in affecting the reorganization energy of the reaction (Farver & Pecht, 1992; Farver et al., 1992). Altogether these studies indicate that Trp48 is indeed a residue that may participate in electron transfer and that it is certainly a good model system for fluorescence spectroscopy studies.

In contrast to the electron-transfer hypothesis, resonance Raman spectroscopy data do not indicate the formation of a tryptophan radical cation intermediate, but point to a quenching mechanism that involves energy transfer (Sweeney et al., 1991).

We therefore undertook a study of the fluorescence properties of two azurin mutants that were modified at positions which might be expected to affect the environment of Trp48, i.e., positions that are close to Trp48 in the tertiary structure of the protein. The chosen residues were Ile7 and Phe110. Both of them take part in the formation of the hydrophobic core of azurin, and they are in close contact with Trp48 (Figure 1). The two residues were each mutated to a Ser (Ile7Ser and Phe110Ser), introducing perturbations in the protein core in terms of an increased polarity and in terms of a smaller volume occupied by the side chain. These variations can be expected to affect both the steady-state fluorescence and the time-resolved fluorescence properties of azurin, since they would influence the microenvironment and the dynamics of Trp48. This approach allows further insight into the structural basis of the unique spectroscopic properties of Trp48 in azurin, and it opens the possibility of checking how the dielectric constant of the matrix around the tryptophanyl residue may affect its fluorescence. Furthermore,

the study of fluorescence decay in these mutants compared with that in the wild-type azurin provides a model toward understanding the complex fluorescence decay profiles observed in many proteins.

MATERIALS AND METHODS

Site-Directed Mutagenesis. DNA cloning experiments were performed on the pGC4 plasmid, into which the azurin gene from *Pseudomonas aeruginosa* was cloned as previously reported (Canters, 1987; van de Kamp et al., 1990a). The *Eco*RI–*Sal*I and the *Eco*RI–*Sma*I fragments were ligated into the polylinker of M13mp18 for the mutagenesis of Ile7 and Phe110, respectively, and transformed into *Escherichia coli* JM101. Site-directed mutagenesis was performed with an *in vitro* system based on the method of Ecksten and coworkers (Sayers et al., 1988). The mutagenic oligonucleotide was annealed to the M13mp18 single-stranded DNA containing the azurin gene fragments described above and extended by T7 polymerase in the presence of T4 ligase to generate a mutant heteroduplex. The nucleotide mixture used in the extension by T7 polymerase contained a deoxycytidine 5′-α-thiotriphosphate (dCTPαS) thionucleotide (Amersham). The non-mutant strand was selectively removed through digestion by exonuclease III after nicking with *Nci*I restriction endonuclease. The latter enzyme is unable to cleave the phosphorothioate mutant DNA strand, which protects the DNA from digestion. The mutant strand was then used as a template to synthesize *in vitro* the double-stranded DNA by T7 polymerase and T4 ligase and was transformed into *E. coli* JM101 cells. A 16-nucleotide-long complementary oligodeoxyribonucleotide, CACCTGTCGGTCCCAT (Ser codon underlined), was used to substitute the Ile7 codon TAG for the Ser codon AGC, and ATGTACAGGAAGACGT (Ser codon underlined) was used to substitute the Phe110 codon AAG for the Ser codon TCC. The occurrence of the mutations was confirmed by single-stranded DNA sequencing, extended to the whole DNA fragment to check for unwanted additional mutations. Then the *Eco*RI–*Sal*I and the *Eco*RI–*Sma*I fragments were exchanged with the wt fragments in the pGC4 plasmid, and the resulting azurin genes were screened for the mutation by hybridization with the oligonucleotide used for the mutagenesis (Sambrook et al., 1989).

The plasmids containing the mutant azurin genes Ile7Ser and Phe110Ser were transformed into *E. coli* K12 strain JM101 (relevant genotype Δ*lac-pro*AB,F$^+$(*pro*AB, *lacI*^{q}, *lacZ*ΔM15)) (Yanisch-Perron et al., 1985) that was used for heterologous expression. Transformed bacterial cells were grown in a 42-L fermentor in Luria–Bertani (LB) medium supplemented with 50 mg/L ampicillin under aerobic conditions at 37 °C. Azurin expression was induced by addition of isopropyl β-D-thiogalactopyranoside up to a concentration of 100 μM.

Mutant Isolation and Purification. The procedure of the isolation and purification of the Ile7Ser and Phe110Ser mutants of *Pseudomonas aeruginosa* azurin is similar to that used for the wild-type protein (van de Kamp et al., 1990b). The 30-L cell culture was harvested and concentrated down to 2 L by using a Millipore filtration system. The cell suspension was then centrifuged at 3000*g* for 10 min. The bacterial pellet was resuspended in 1.5 L of 30 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 20% (w/v) sucrose and stirred for 15 min at room temperature. After centrifugation at 3000*g* for 15 min the supernatant sucrose fraction was kept at 4 °C, and the pellet was resuspended in a minimal volume of the same buffer. Quickly 2 L of ice-cold water was poured into the cell suspension to achieve osmotic shock, and the mixture was stirred vigorously at 4 °C for 15 min. The suspension was then centrifuged at 5000*g* for 10 min, and the supernatant was pooled with the sucrose fraction previously stored at 4 °C. At this stage cupric sulfate and potassium ferricyanide were added to final concentrations of 1 mM and 10 μM, respectively. The pH was lowered to 4.0 by stepwise additions of 1 M acetic acid, and the resulting solution was cleared by centrifugation. The supernatant was loaded onto a Pharmacia CM column (30 × 2.5 cm) equilibrated with 50 mM ammonium acetate buffer, pH 4.0. The column was eluted with 2 L of a pH 4.0 to 5.0 gradient (flow rate, 200 mL/h), and as the elution proceeded, the color of the mutant protein bound to the top of the column intensified to a dark blue band. After elution, the blue fractions were pooled and concentrated in an Amicon ultrafiltration system (Amicon Corp.). At this stage particular care was taken to separate the copper-containing mutant azurin from the zinc-containing species. This was achieved with the method described by van de Kamp et al. (1990b). The buffer was changed to 5 mM Tris-HCl, pH 8.0, and the sample was carefully reduced by addition of sodium dithionite. The resulting colorless solution was applied to a DEAE column (30 × 2.5 cm) equilibrated with the same buffer. The elution was performed with 4 L of a 0 to 25 mM NaCl gradient in the same buffer. Fractions containing the mutant azurin were detected by the blue color that developed after reoxidation with potassium ferricyanide and addition of a small excess of copper. A final CM column, developed under the same conditions as the one previously described, was used to improve the sample purity by separating small impurities and nonspecifically surface-bound copper. The purity of the protein samples was evaluated by measuring the A_{628}/A_{280} ratio by UV-vis spectrophotometry. The mutants were also checked by electrophoresis, carried out on a PhastSystem (Pharmacia, Sweden), and isoelectric focusing (IEF) on polyacrylamide gels with pH 3 to 9 gradients; wild-type azurin was used as a reference.

EPR Spectroscopy. Electron paramagnetic resonance (EPR) spectra were recorded on a Jeol JESRE2X spectrometer at 77 K. The protein concentration was 2 mM, and the sample was prepared in 20 mM MES buffer at pH 6.0 containing glycerol to a final concentration of 40%. Acquisition parameters: sweep rate, 12.5 mT; field modulation width, 0.79 mT. The magnetic field was calibrated with α,α-diphenyl-β-picrylhydrazyl (DPPH).

NMR Spectroscopy. Proton NMR spectra were recorded on a Bruker WM-300 spectrometer operating at 300-MHz proton frequency. Protein samples were prepared to a final concentration of 2 mM in 20 mM potassium phosphate buffer at pH 6.0 in 99.9% deuteriated water. Oxygen was eliminated from the solution by extensive bubbling of argon through the sample in the NMR tube. Azurin was then reduced with 0.1 M sodium dithionite in 0.1 M NaOD added in small aliquots (typically 5 μL); the sample was kept under an argon atmosphere. The HOD resonance was suppressed by presaturation. Spectra were collected at 298 K, using 8K of computer memory, deconvoluted by Gaussian multiplication, and Fourier transformed. Chemical shifts are referenced to 4,4-dimethyl-4-silapentane-1-sulfonate.

Fluorescence Measurements. All the fluorescence measurements were performed in 20 mM Hepes buffer at pH 8.0; the protein absorbance was kept lower than 0.4 at the excitation wavelength. Emission spectra were recorded with a single-photon counting spectrofluorimeter (Fluoromax Instruments S. A., Paris). The temperature of the samples was kept at

Table 1: EPR and UV–Vis Parameters of wt, Ile7Ser, and Phe110Ser Azurins from *Pseudomonas aeruginosa*[a]

azurin sample	EPR parameters			UV-vis parameters		
	$g_\perp$ (±0.001)	$g_\parallel$ (±0.002)	$A_\parallel$ (10^{-4} cm^{-1}) (±2 × 10^{-4} cm^{-1})	λ_{max} (nm)	Trp48 λ_{max} (nm)	purity (A_{628}/A_{280})
wild type	2.059	2.259	59	628	292	0.580
Ile7Ser	2.057	2.259	56	626	292	0.618
Phe110Ser	2.058	2.257	59	630	292	0.621

[a] The EPR measurements were performed at 77 K on a 2 mM protein solution in 20 mM MES buffer, pH 6.10. UV–vis spectra were obtained at room temperature in 20 mM Hepes buffer, pH 8.0.

298 K by an external circulating water bath. Excitation wavelengths are specified for the different experiments in the following section. The bandwidths of excitation and emission were varied in the range 1–4 nm to maximize the fluorescence signal and to keep the scattering as low as possible. The relative quantum yields were calculated by integration of corrected emission spectra, taking into account the differences in absorbance of the samples.

The time-resolved fluorescence of azurin has been studied by phase-shift and demodulation techniques (Gratton et al., 1984) using the synchrotron radiation of the Adone storage ring (Frascati, Italy) (Gratton et al., 1983). Excitation was at 295 nm (bandwidth, 8 nm), while emission was collected after the light was passed through a 305-nm cutoff filter. A solution of *p*-terphenyl in cyclohexane was used as reference with a lifetime, τ, of 1.05 ns. All experiments were performed at 298 K by using an external circulating water bath. The data were analyzed by minimizing the χ^2 with a routine based on the Marquardt algorithm using the Globals Unlimited software (Beechem & Gratton, 1988).

RESULTS AND DISCUSSION

Mutant Purification. Azurin mutants Ile7Ser and Phe-110Ser were expressed in *E. coli* with a relatively high yield of 20 mg of pure protein from 1 L of bacterial culture. The isoelectric point (p*I*) of the mutants was checked by isoelectric focusing electrophoresis: the p*I* of both mutants was found to be identical to that of the wt azurin from *Pseudomonas aeruginosa*, that is, p*I* 5.6 for the oxidized metal-containing (holo)mutants and p*I* 4.6 for the reduced holomutants. The pI of the mutants is the same as that of the wt protein, both in its oxidized and reduced states, and this is consistent with the presence of mutations that are located in the protein interior and that do not introduce changes in the surface charge of the protein. It also indicates that the 3D structure of the native protein is probably maintained.

Anion-exchange chromatography of the reduced mutants allowed the separation of a protein fraction that shares many characteristics with wt Zn azurin [previously called azurin* (van de Kamp et al., 1990b; Nar et al., 1992a)], that is, the lack of the typical absorption band at 628 nm, a p*I* of 5.4 (the metal-free forms have a p*I* of 5.8), and the lack of reduction after addition of sodium dithionite. These observations suggest that these fractions are the Zn-containing forms of the mutants Ile7Ser and Phe110Ser. These fractions were set aside, and the azurin mutants Ile7Ser and Phe110Ser were purified to a high degree in order to avoid the presence of contaminants that could affect the fluorescence measurements. The A_{628}/A_{280} ratio, which represents a purity criterion, was greater than 0.6 for both mutants (Table 1).

Spectroscopic Characterization. The parameters measured from the EPR spectra of the Ile7Ser and Phe110Ser mutants are reported in Table 1. The parameters of the mutants are very close to those of the wt protein, and the small variations

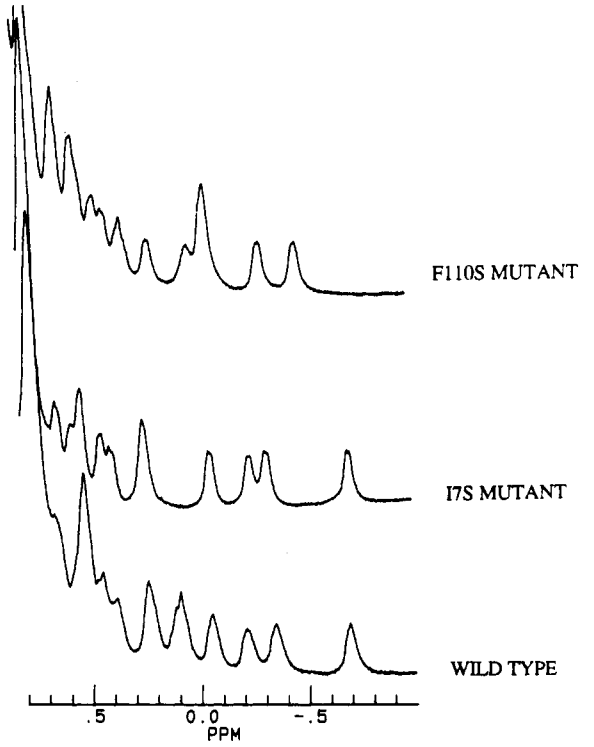

FIGURE 2: Upfield region of the 300 MHz proton NMR spectra of wild-type and Ile7Ser (I7S) and Phe110Ser (F110S) mutant reduced holoazurins. Samples contained 3 mM protein in 10 mM phosphate buffer, pH 6. Spectra were recorded at 298 K.

are within the error margins of the measurements, indicating that the copper is incorporated and ligated with the same geometry as that of the wild type, that is, the geometry of a distorted trigonal-pyramidal conformation.

The 300-MHz proton NMR spectra of the upfield region of wt, Ile7Ser, and Phe110Ser azurins are shown in Figure 2. The positions of the resonances in the upfield region of the azurin NMR spectrum are influenced by the ring current effect of the Trp48 side chain; substantial differences in the chemical shifts of these resonances are likely to be due to variations in the relative positions of the residues surrounding Trp48. A first glance at the NMR spectra of the mutants already confirms that the proteins are folded, as a well-defined upfield region indicates the presence of a distinct tertiary structure (Figure 2). In a more detailed analysis specific differences can be noted between the spectra of the mutants and the wt protein. The main difference between the wt and the Ile7Ser spectrum is the absence of the peak at 0.10 ppm. This peak has been assigned to the $C^{\delta 1}H_3$ of the Ile7 side chain in wt azurin (van de Kamp et al., 1992), and therefore its absence in the spectrum of the mutated protein is consistent with Ile7 being replaced by a Ser. The resonances of Val31 are also slightly shifted as a result of the mutation (Table 2), while the Val22 resonances are not significantly affected because their upfield-shifted positions are not due to the ring

Table 2: Proton Resonance Assignments of the Upfield NMR Spectral Region of wt, Ile7Ser, and Phe110Ser Azurins[a]

resonance assignment	chemical shift (ppm)		
	wt	Ile7Ser	Phe110Ser
Val22 $C^{\gamma}H_3$	0.37, –0.32	0.38, –0.31	0.39, –0.29
Val31 $C^{\gamma}H_3$	–0.22, –0.67	–0.24, –0.69	–0.05, –0.47
Leu102 $C^{\delta}H_3$	0.64, –0.03	0.61, –0.03	0.62, –0.03
Met121 $C^{\epsilon}H_3$	–0.05	–0.05	–0.05

[a] The chemical shift assignments of wt azurin are taken from van de Kamp et al. (1992); those of the mutants were made with the aid of 2D NMR spectra at 600 MHz (complete data will be reported elsewhere). Spectra were recorded at 298 K; protein final concentration was 2 mM in 20 mM potassium phosphate buffer at pH 6.0 in 99.9% deuteriated water. Chemical shift error is ±0.02 ppm, and values are expressed relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

current effect of Trp48, but to that of Phe29; moreover, this residue is not within the immediate environment of the mutated residue.

Considerable differences are present in the spectrum of Phe110Ser. The resonances at –0.67 and –0.22 ppm of the wt spectrum, which have been assigned to the $C^{\gamma}H_3$ proton of Val31, are shifted downfield, overlapping with the $C^{\epsilon}H_3$ resonance of Met121 at –0.05 ppm (Table 2). The $C^{\delta 1}H_3$ resonance of Ile7 at 0.10 ppm in the wt spectrum has probably shifted to around 0.0 ppm. In both mutants 2D NMR spectroscopy (data to be reported elsewhere) has shown that the $C^{\epsilon}H_3$ resonance of Met121 at –0.05 ppm and the $C^{\delta}H_3$ resonance of Leu102 at –0.03 ppm are not shifted; apparently they are not affected by the mutation.

The X-ray structure of wt azurin shows that Val31 is very close to Trp48 in the tertiary structure: one of the γ-carbons of Val31 is only 3.6 Å away from the δ2-carbon of Trp48 (Nar et al., 1991a). The downfield shift of the Val31 resonance can be explained as being due to a change in the position of Val31 with respect to Trp48. This is a result of the mutation at the position of Phe110 on the other side of the Trp48 side chain (Figure 2). Investigations are in progress for the identification of the resonance present at around 0.0 ppm in the Phe110Ser spectrum. Additional studies on structural changes are in progress by 2D–3D NMR spectroscopy and X-ray crystallography.

Figure 3 reports the UV spectral regions of the holo- and metal-free (apo)proteins. The spectra of the mutant proteins show differences in the shape of the shoulder at 292 nm. This shoulder has been assigned to the 0–0 ^1La (Strickland et al., 1973) electronic transition of Trp48, and its broadening, exhibited especially by the apoproteins, reflects the perturbation of the Trp48 environment generated by the mutation. The visible region does not show considerable differences from the wt, with an absorption maximum at 626 nm for Ile7Ser and at 630 nm for Phe110Ser (Table 1).

Steady-State Fluorescence Spectroscopy. The structural changes that generate the differences in the NMR spectra of the Ile7Ser and Phe110Ser mutants are also expected to have an influence on the fluorescence spectra of these proteins. Tryptophan is a very useful built-in fluorescence probe for structural variations in proteins, as the emission of this natural fluorophore is very sensitive to the immediate environment (Beechem & Brand, 1985). The steady-state fluorescence spectra of the Ile7Ser and Phe110Ser mutants excited at 285 nm are reported in Figure 4a, together with the spectrum of wt azurin, and the data are summarized in Table 3.

The fluorescence emission spectrum of wt azurin at 308 nm is the most blue shifted signal observed in proteins so far. The position of the maximum and the presence of a fine structure

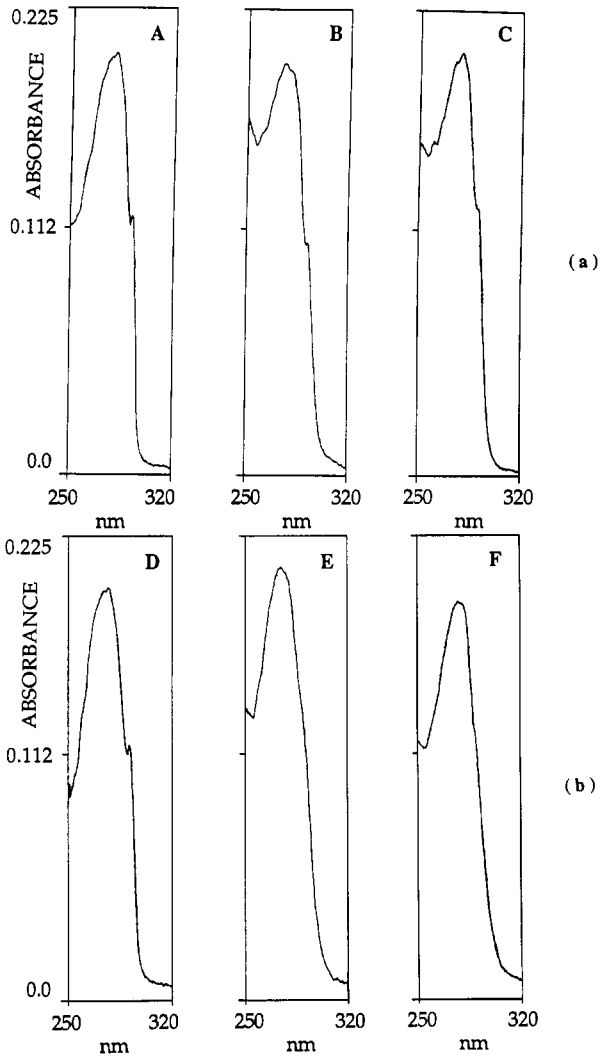

FIGURE 3: UV spectra of (a) wild-type (A), Ile7Ser (B), and Phe110Ser (C) holoazurins and (b) wild-type (D), Ile7Ser (E), and Phe110Ser (F) apoazurins. Data were collected at 298 K in 20 mM Hepes buffer at pH 8.0.

in the emission band have been explained as due to the high hydrophobicity and rigidity of the Trp48 environment (Finazzi-Agrò et al., 1970). These characteristics have been perturbed in the two mutants: the spectra of Figure 4a show a red-shifted maximum at 313 nm for Ile7Ser and at 312 nm for Phe110Ser. In both mutants the emission band is broader, as measured by the width at half-height (Δλ) reported in Table 3, and the fine structure, typical of wt azurin, is blurred. These effects are even more pronounced in the apo forms of the mutants (Figure 4b). The removal of the copper in wt azurin is accompanied by a very large increase in fluorescence quantum yield (Table 3), but no significant influence is observed on the maximum position and the shape of the emission band. In contrast, the apo forms of the Ile7Ser and Phe110Ser mutants also show an increase in quantum yield, but it is accompanied by a further red shift and a broadening of the emission band with respect to those of their holo forms. In fact, the emission maximum of apo-Phe110Ser is at 327 nm, while that of apo-Ile7Ser is at 341 nm, i.e., the typical emission of partially water solvated tryptophanyl side-chains (Burstein et al., 1973). It is important to note that the fluorescence spectra of the holoproteins were recovered upon re-addition of the copper to the apoproteins, demonstrating that the removal of the metal was reversible and not accompanied by denaturation. Furthermore, CD experiments

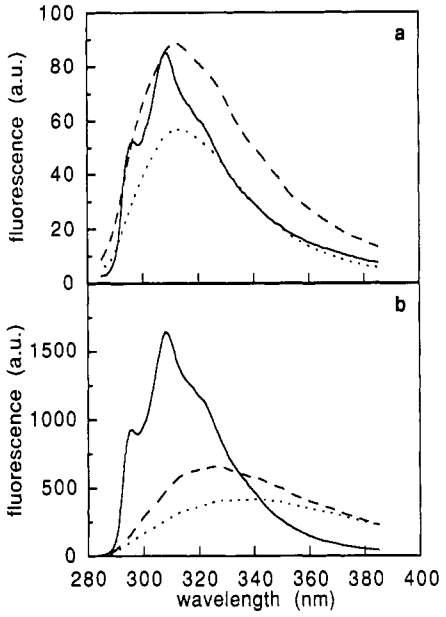

FIGURE 4: Corrected steady-state fluorescence spectra of holoazurins (a) and apoazurins (b): (—) wild type (WT), (···) Ile7Ser (I7S), and (– –) Phe110Ser (F110S). Data were collected at 298 K in 20 mM Hepes buffer at pH 8.0; the excitation wavelength was 285 nm.

(data not shown) demonstrated that the amount of secondary structure in these mutants is the same as that found in the respective wild-type holo- and apoproteins.

The red shift of the Ile7Ser and Phe110Ser spectra can be interpreted in terms of an increase in polarity and mobility in the immediate environment of Trp48, due to the introduction of a polar and smaller Ser residue, in the place of the apolar and bigger Ile and Phe side chains. The broader emission bands may reflect heterogeneity in the environment around Trp48. The dependence of the emission maximum on the excitation energy was also studied in order to ascertain whether the red-shifted spectra of the mutants were due to a solvent relaxation around the excited indolyl side chain (Demchenko, 1986). As reported in Figure 5, while the emission of the wt is qualitatively identical whatever the excitation wavelength used, the emission maximum of the mutants in their holo and apo forms depends almost linearly on the excitation wavelength. The effect is most evident for the Phe110Ser mutant.

Dynamic Fluorescence Spectroscopy. The possibility of measuring dynamic as well as static fluorescence has added a new dimension to the study of proteins by means of emission spectroscopy. The increased resolving power in the time domain has revealed an unforeseen complexity in the behavior of protein-bound tryptophans. In fact, many proteins containing a single tryptophan residue show more than one component in their fluorescence decay (Beechem & Brand, 1985). This behavior was at first interpreted as due to some kind of structural heterogeneity. A closer look at some of these proteins showed an even more complex behavior, since their fluorescence decay appeared to be best fitted not by a single lifetime, nor by a sum of two or more lifetimes, but by a continuous distribution of lifetimes.

The presence of a single tryptophan in a well-defined hydrophobic and rigid environment makes azurin a good model for investigations on the fluorescence decay behavior. The results obtained from these studies on holo and apo forms of wt and Ile7Ser and Phe110Ser mutant azurins are reported in Table 3.

For the sake of simplicity the fluorescence decay of the apoazurins will be discussed first. The fluorescence decay of wt recombinant apoazurin (Table 3) is clearly monoexponential, with a lifetime of 4.7 ns. This figure is consistent with most of the data reported by other laboratories on the native azurin (Szabo et al., 1983; Petrich et al., 1987). The decay of the wt apoazurin fluorescence was also tentatively fitted with Lorentzian or Gaussian distributions of lifetimes. However, these attempts always gave poorer quality of fitting, with higher χ^2 values. This indicates that the Trp48 signal decays with a discrete lifetime, typical of a residue in a homogeneous and rigid environment. A different situation was observed for the apo-Ile7Ser and apo-Phe110Ser mutants. Both samples showed a fluorescence decay that could better be fitted with a Lorentzian distribution of lifetimes ($\chi^2 \leq 2.2$). The lifetime distribution is similar in both mutants, with a spread larger than 2 ns (Figure 6). Moreover, apo-Ile7Ser also shows a mean lifetime of 3.5 ns, which is significantly lower than that observed for apo-wt (4.7 ns). Such a distribution of lifetimes is a novel observation for azurin, and it indicates that the dynamic properties of Trp48 are indeed affected by the mutations. In the wt protein, this residue is closely packed in the core of the protein, in contact with hydrophobic side chains that considerably restrict its mobility (Chen et al., 1988). Ile7 is a residue buried close to Trp48, occupying a mean volume of 169 Å^3 in the protein interior. The substitution of Ile7 with a Ser residue, which has a mean volume of 99 Å^3, leaves *circa* 70 Å^3 of free space, i.e., an empty cube with a side of 4.1 Å. In the structure of the wt protein Ile7 has almost no externally accessible surface area (≤2 Å^2; Chothia & Lesk, 1982). Changes in the Trp48 spectroscopic properties are therefore expected and consistent with the distribution of lifetimes with shorter values. The distribution of lifetimes in apo-Phe110Ser can also be explained in the same way: Phe110 occupies a mean volume of 203 Å^3, leaving 104 Å^3 of free space in the Phe110Ser mutant, an empty cube with a side of 4.7 Å. It is relevant to note that Phe110 is a totally buried residue, with no externally accessible surface area.

At variance with that of the copper-free form, the fluorescence decay of native holoazurin is complex. This decay can be fitted by two components whose origin is still debated (Szabo et al., 1983; Petrich et al., 1987; Hutnik & Szabo, 1989a,b; Hansen et al., 1990). The recombinant wt azurin shows the same behavior (Table 3). Our data are consistent with previous data on azurin naturally expressed in *Pseudomonas* (Hutnik & Szabo, 1989a,b; Hansen et al., 1990). The biexponential fluorescence decay has a short component of 200 ps and another one with a much longer lifetime (4.5 ns), which is approximately that of apoazurin (4.7 ns). In an attempt to explain this behavior, it has been suggested that the longer lifetime is due to a contamination of the holoprotein by an "apo-like" protein (Petrich et al., 1987). However, the long-lifetime component is always present, no matter what preparation procedure or further biochemical manipulation is done, such as addition of excess metal. Special care was taken to remove the zinc-containing protein, and the proteins were purified to an A_{628}/A_{280} purity ratio greater than 0.6. Isoelectric focusing experiments on our samples ruled out the presence of apoprotein. We consistently observed the presence of the longer-lifetime component in every mutant studied, in the ones reported here (Table 3) as well as in other ones (mutants M44K, H35L, H35F, and H35Q; data to be published). It appears unlikely that the same type of contaminant species is always present.

These observations indicate that the presence of two different lifetimes in holoazurin can be ascribed to a conformational

Table 3: Static and Dynamic Fluorescence Data of Apo and Holo Forms of wt and Ile7Ser and Phe110Ser Mutant Azurins[a]

	wt		I7S		F110S	
	holo	apo	holo	apo	holo	apo
			Static Fluorescence			
λ_{em} (nm)	308	308	313	341	312	327
$\Delta\lambda$ (nm)	45	45	51	88	59	83
Q_{rel}	1	16.8	0.83	9.57	1.39	13.12
			Dynamic Fluorescence			
τ (ns) (best fit)	$\tau_1 = 0.2$ $\tau_2 = 4.5$ $f_1 = 0.39$	$\tau = 4.7$	$\tau_1 = 0.2$ $\tau_2 = 2.4$ $f_1 = 0.75$	$C = 3.5$ $W = 2.4$	$C_1 = 0.2$ $W_1 = 0.1$ $f_1 = 0.78$ $C_2 = 4.1$ $W_2 = 0.2$	$C = 4.3$ $W = 2.1$
	$\chi^2 = 1.1$	$\chi^2 = 2.3$	$\chi^2 = 1.4$	$\chi^2 = 2.2$	$\chi^2 = 1.4$	$\chi^2 = 1.7$
red-edge effect	–	–	+	+	+	+

[a] λ_{em} is the maximum of the emission spectrum in nanometers; $\Delta\lambda$ is the width at half-height of the emission spectra in nanometers; Q_{rel} is the quantum yield relative to wt holoazurin; τ is the fluorescence lifetime in nanoseconds; C is the center of the Lorentzian distribution of lifetimes, and W is its width, in nanoseconds ($\Delta\tau_1 \approx 10$ ps; $\Delta\tau_2 \approx 50$ ps); f_1 is the fraction of fluorescence relative to $\tau_1(f_1 + f_2 = 1; \Delta f \approx 0.01)$. Data were collected at 298 K in 20 mM Hepes buffer at pH 8.0. Time-resolved fluorescence data were acquired by excitation at 295 nm and through a 305-nm cutoff filter; *p*-terphenyl in cyclohexane with a lifetime of 1.05 ns was used as a reference.

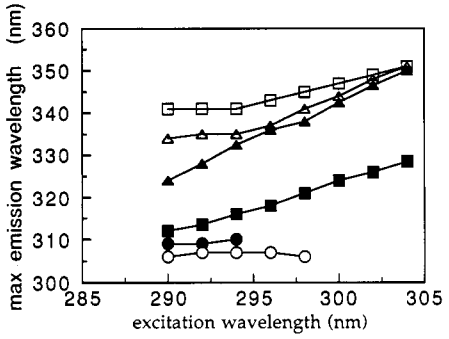

FIGURE 5: Maximum fluorescence emission versus excitation wavelength of azurins. Filled symbols refer to holoazurins, and open symbols refer to apoazurins: wt (circles), Ile7Ser (squares), and Phe110Ser (triangles) from *Pseudomonas aeruginosa* .

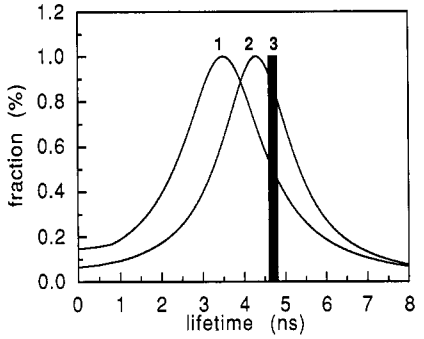

FIGURE 6: Lorentzian continuous distributions of fluorescence lifetimes for apo-Ile7Ser (1) and apo-Phe110Ser (2) azurin mutants. The single lifetime of apo-wt is reported as a bar (3). Time-resolved fluorescence experiments were performed by excitation at 295 nm at 298 K; data were analyzed with a Marquardt algorithm.

heterogeneity, as previously suggested by Hutnik and Szabo in 1989. This interpretation is consistent with observations made by X-ray crystallography of azurin. For example, two distinct conformations have been observed in the buried residue Ile7, whose side-chain electron density shows two alternative configurations, eclipsed and staggered with respect to the main chain (Nar et al., 1991b). No attempt was made by these authors to model this disorder in the structure. Moreover, X-ray crystallography of the wt apoazurin has shown the presence of two molecular forms that may represent two rigid conformational states characteristic of the protein structure before (form 2) and after (form 1) the copper incorporation (Nar et al., 1992b). Although the major differences between form 1 and form 2 involve the residues around the copper site, smaller conformational changes involve residues of the hydrophobic protein core [Figure 2 of Nar et al. (1992b)].

The presence of two distinct lifetimes is also observed in the fluorescence decay of holo-Ile7Ser and holo-Phe110Ser azurin mutants, but with some differences. The 200-ps decay is present as the minor component in the wt azurin ($f_1 = 0.39$), but it is predominant in both mutants ($f_1 \geq 0.75$). Also, the slower decaying component of Ile7Ser ($\tau_2 = 2.4$ ns) is considerably different from that of the wt holoprotein ($\tau_2 = 4.5$ ns). The origins of these differences might be interpreted in terms of different extents of Trp48 quenching in these mutants. An interesting observation for the Ile7Ser mutant is that while the decay of the apo form shows a distribution of lifetimes, the decay of the holo form could only be fitted with two discrete lifetimes. This seems to suggest that in this mutant the incorporation of copper causes a conformational change responsible for two sharp lifetimes, probably by increasing the rigidity of the conformers that no longer show a distribution of lifetimes. A similar behavior is observed for the Phe110Ser holo mutant, where the shorter lifetime also predominates; in this case the lifetimes are distributed around mean values of 0.2 and 4.1 ns, again indicating heterogeneity of the environment around the Trp48 side chain.

CONCLUSIONS

Site-directed mutagenesis of residues of the azurin's hydrophobic core allows the study of structural changes that occur as the effect of the mutation. Particular interest is focused on key positions where the evolution of blue copper proteins has preserved specific residues, such as Phe110. Naturally occurring mutations in the hydrophobic core tend to preserve the main characteristics of the buried residues, in particular their hydrophobicity. In this study a drastic variation was introduced in the hydrophobic core of azurin that changed both the volume and the polarity of two side chains. Generally changes in shape and size of buried residues in proteins with a β-sandwich topology are accommodated by shifts in the relative positions of the packed β-sheets; for example, the differences in the primary sequences of two closely related blue copper proteins, namely, azurin and plastocyanin, in the region where the two β-sheets pack together, are adjusted by a 3.8-Å shift of the sheets with respect to each other (Chothia & Lesk, 1982). Although to date X-ray structures of Ile7Ser

and Phe110Ser azurin mutants are not available, preliminary proton NMR spectra of the upfield region of these proteins provide useful information on the relative arrangement of the side chains around Trp48 in the Phe110Ser mutant. EPR data show that the geometry of the copper site of these mutants is not affected by the mutations; this could be expected since the mutated sites are relatively far from (Ile7) or just adjacent to (Phe110) the copper ligands.

The perturbations introduced by the polar and small Ser residue in positions 7 and 110 are reflected by the fluorescence properties of the Trp48 side chain. Conformational and environmental heterogeneity has been detected in both mutants, as indicated by their different fluorescence lifetimes and lifetime distributions. The distribution of lifetimes of these mutants is a novel observation for azurin. The considerable red shift of the fluorescence emission maximum observed for the holo and apo forms of the mutants is consistent with the presence of a more polar environment. This phenomenon is particularly marked in the apo form of Ile7Ser, which shows an emission in the range of solvated tryptophanyl side chains. The NMR data do not show the presence of immobilized water molecules around the Trp48 side chain, at least in the holo forms, but the difference of 70 and 104 Å^3 between the Ile and Ser and the Phe and Ser side-chains, respectively, certainly makes this a possibility. Additional NMR experiments and X-ray crystallography are in progress to test this hypothesis on copper-containing and copper-free samples.

Furthermore, the red shift of the fluorescence spectrum upon excitation is consistent with relaxation around the tryptophan of polar chemical groups (Demchenko et al., 1986). This relaxation could also be responsible for the heterogeneous decay of the copper-free mutants because each differently relaxed indolyl residue may have a slightly different fluorescence lifetime.

We therefore believe that the present experiments allow us to conclude that, at least in the case of apoazurin, the single tryptophan may decay either with a single lifetime or with a distribution of lifetimes depending on the interaction of the indole side chain with the surrounding groups.

This conclusion may help in (re)interpretation of the data on the complex fluorescence decay observed in many proteins.

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REFERENCES

Beechem, J. M., & Brand, L. (1985) *Annu. Rev. Biochem. 54*, 43–71.
Beechem, J. M., & Gratton, E. (1988) *Proc. SPIE-Int. Soc. Opt. Eng. 909*, 70–81.
Burstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263–279.
Canters, G. W. (1987) *FEBS Lett. 212*, 168–172.
Canters, G. W., & Gilardi, G. (1993) *FEBS Lett. 325*, 39–48.
Chen, L. X. Q., Engh, R. A., Brunger, A. T., Nguyen, D. T., Karplus, M., & Fleming, G. R. (1988) *Biochemistry 27*, 6908–6921.
Chothia, C., & Lesk, A. M. (1982) *J. Mol. Biol. 160*, 309–323.
Demchenko, A. P. (1986) Ultraviolet spectroscopy of proteins, in *Topics in fluorescence spectroscopy* (Lakowicz, J. R., Ed.) Springer-Verlag, Berlin.
Farver, O., & Pecht, I. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6968–6972.
Farver, O., & Pecht, I. (1992) *J. Am. Chem. Soc. 114*, 5764–5767.
Farver, O., Skov, L. K., van de Kamp, M., Canters, G. W., & Pecht, I. (1992) *Eur. J. Biochem. 210*, 399–403.
Finazzi-Agrò, A., Rotilio, G., Avigliano, L., Guerrieri, P., Boffi, V., & Mondovì, B. (1970) *Biochemistry 9*, 2009–2014.
Gratton, E., Jameson, D. M., Rosato, N., & Weber, G. (1983) *Rev. Sci. Instrum. 55*, 486–494.
Gratton, E., Jameson, D. M., & Hill, R. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 105–124.
Hansen, J. E., Longworth, J. W., & Fleming, G. R. (1990) *Biochemistry 29*, 7329–7338.
Horio, T., Higashi, T., Yamanaka, T., Matsubara, H., & Okunuki, K. (1961) *J. Biol. Chem. 236*, 944–951.
Hutnik, C. M., & Szabo, A. G. (1989a) *Biochemistry 28*, 3923–3934.
Hutnik, C. M., & Szabo, A. G. (1989b) *Biochemistry 28*, 3935–3939.
Kim, S. T., Heelis, P. F., Okamura, T., Hirata, Y., Mataga, N., & Sancar, A. (1991) *Biochemistry 30*, 11262–11270.
Kim, S. T., Li, Y. F., & Sancar, A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 900–904.
Klemens, F. K., & McMillin, D. R. (1992) *Photochem. Photobiol. 55*, 671–676.
Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1991a) *J. Mol. Biol. 221*, 765–772.
Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1991b) *J. Mol. Biol. 218*, 427–447.
Nar, H., Huber, R., Messerschmidt, A., Filippou, A. C., Barth, M., Jaquinod, M., van de Kamp, M., & Canters, G. W. (1992a) *Eur. J. Biochem. 205*, 1123–1129.
Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1992b) *FEBS Lett. 306*, 119–124.
Parr, S. R., Barber, D., Greenwood, C., Phillips, B. W., & Melling, J. (1976) *Biochem. J. 157*, 423–430.
Petrich, J. W., Longworth, J. W., & Fleming, G. R. (1987) *Biochemistry 26*, 2711–2722.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sayers, J. R., Schmidt, W., & Eckstein, F. (1988) *Nucleic Acids Res. 16*, 791–802.
Strambini, G. B., & Gabellieri, E. (1991) *J. Phys. Chem. 95*, 4352–4356.
Strickland, E. H., & Billups, C. (1973) *Biopolymers 12*, 1989–1995.
Sweeney, J. A., Harmon, P. A., Asher, S. A., Hutnik, C. M., & Szabo, A. G. (1991) *J. Am. Chem. Soc. 113*, 7531–7537.
Szabo, A. G., Stepanik, T. M., Wayner, D. M., & Young, N. M. (1983) *Biophys. J. 41*, 233–244.
van de Kamp, M., Silvestrini, M. C., Brunori, M., van Beeumen, J., Hali, F. C., & Canters, G. W. (1990a) *Eur. J. Biochem. 194*, 109–118.
van de Kamp, M., Hali, F. C., Rosato, N., Finazzi-Agrò, A., & Canters, G. W. (1990b) *Biochim. Biophys. Acta 1019*, 283–292.
van de Kamp, M., Canters, G. W., Wijmenga, S. S., Lommen, A., Hilbers, C. W., Nar, H., Messerschmidt, A., & Huber, R. (1992) *Biochemistry 31*, 10194–10207.
Yanisch-Perron, C., Vieria, J., & Messing, J. (1985) *Gene 33*, 103–119.