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Purification and characterization of a non-reconstitutable azurin, obtained by heterologous expression of the *Pseudomonas aeruginosa azu* gene in *Escherichia coli*

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The azurin-encoding *azu* gene from *Pseudomonas aeruginosa* was cloned and expressed in *Escherichia coli*. A purification procedure was developed to isolate the azurin obtained from the *E. coli* cells. No differences were observed between azurins isolated from *P. aeruginosa* and *E. coli*. A non-reconstitutable azurin-like protein, azurin*, with a spectral ratio (A_{625}/A_{280}) less than 0.01 could be separated from holo-azurin with a spectral ratio of 0.58 ($\pm$0.01). The properties of azurin* were examined by electrophoretic (SDS-PAGE and IEF) and spectroscopic (UV/vis, ^{1}H-NMR, static and dynamic fluorescence) techniques, and compared to the properties of holo-azurin and apo-azurin. Azurin* resembles apo-azurin (same pK_a^* values of His-35 and His-117, same fluorescence characteristics). However, it has lost the ability to bind Cu-ions. It is tentatively concluded that azurin* is a chemically modified form of azurin, the modification possibly being due to oxidation of the ligand residue Cys-112 or the formation of a chemical bond between the ligand residues Cys-112 and His-117. In agreement with previous results from Hutnik and Szabo (Biochemistry (1989) 28, 3923–3934), fluorescence experiments show that the heterogeneous fluorescence decay observed for holo-azurin is not due to the presence of azurin*, but most likely originates from conformational heterogeneity of the holo-azurin.

Introduction

The blue copper protein azurin (Azu) (M_r = 14600) from *Pseudomonas aeruginosa* has only one Trp (residue number 48), located in the hydrophobic interior of the protein near the Cu-centre [1,2]. It endows the protein with unique fluorescent properties, among them the occurrence of a static fluorescence emission maximum at very short wavelength (308 nm) [3–5]. This fluorescence has been the subject of several time-resolved fluorescence studies. Especially intriguing is the apparent multi-exponential decay of the fluorescence [6–10]. For a long time it has been uncertain whether this behaviour was an intrinsic property of Azu, or whether it was due to the presence of a possibly azurin-like contaminating protein that appeared difficult to remove [6,8,9,11]. By carefully paying attention to the homogeneity of their Azu samples Hutnik and Szabo [9] succeeded in eliminating the contamination. Fluorescence studies on the purified holo-Azu and several metallo-derivatives confirmed that the multi-exponential decay behaviour of Azu originates from conformational heterogeneity and not from the presence of the contaminating protein [9,10]. Yet the nature of the latter substance remained enigmatic.

Recently, the Azu encoding *azu* gene of *P. aeruginosa* has been cloned in this laboratory [12–14]. The enhanced level of expression when *E. coli* is used as a host, in combination with a newly developed purification procedure, opened up the possibility to isolate the contaminating protein in sufficiently high amounts to permit its characterization. The present report describes

Abbreviations: Azu, azurin; IEF, isoelectric focussing; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobasepair(s); LB, Luria-Bertani (medium); NMR, nuclear magnetic resonance (spectroscopy); ppm, parts per million; RIGE, rocket immuno gel electrophoresis; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TC, titration curve.

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this isolation and characterization of the contaminating protein (which was named azurin* (Azu*)) by electrophoretic and spectroscopic methods. Fluorescence measurements on holo-Azu and Azu* show that the fluorescence decays of both proteins are multi-exponential.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli K12 strains JM101 (relevant genotype: *Δlac-proAB,* F^+(*proAB, lacI*^{q}, *lacZ*ΔM15)) [15] and KMBL1164 (relevant genotype: *Δlac-pro*, F^-) [16] were used for cloning and heterologous expression of the *P. aeruginosa azu* gene. Cells were grown on Luria-Bertani (LB) medium, supplied with the required antibiotics [17], at 37°C under aerobic conditions. Plasmids pUC8 and pGC1 have been described elsewhere [12,13,18]. Recombinant DNA techniques were used according to standard protocols [17].

Cell growth and purification procedure

Azu was purified from *E. coli* KMBL1164 cells which had been transformed with plasmid pGC4 containing the highly expressed *azu* gene (see Results). 1 litre LB medium supplied with ampicillin (100 mg/ml) in a 2 litre flask was inoculated 1 : 100 from a 8-h-old culture. Cells were grown for 16 h at 37°C under shaking (200 rpm), and subsequently collected by centrifugation ($3000 \times g$ for 10 min), which resulted in 5 g cell paste. The purification procedure comprised five steps.

Step 1: Osmotic shock. An osmotic shock was applied to 5 g cell paste as follows. Cells were suspended in 100 ml of a 20% (weight/volume) sucrose, 30 mM Tris-HCl, 1 mM EDTA buffer (pH 8.3) [19]. After 15 min stirring at 25°C cells were centrifuged ($5000 \times g$ for 15 min). From here on all manipulations were performed at 4°C. Pellets were quickly resuspended in 250 ml ice-cold metal-ion free water, and stirred for 15 min. After centrifugation ($5000 \times g$ for 15 min) periplasmic proteins including Azu remained in the supernatant.

Step 2: Fractionated precipitation. Cupric sulphate and potassium ferricyanide were added to the periplasmic fraction up to a concentration of 1 and 0.1 mM, respectively, in order to obtain stable oxidized Cu(II) Azu. Subsequently the pH was decreased to 3.9 by adding concentrated HOAc. The Azu-containing solution was cleared from precipitated proteins by centrifugation ($5000 \times g$ for 10 min).

Step 3: Cationic exchange chromatography. The Azu-containing supernatant was applied to a 2.5×25 cm Whatman CM23 column which was equilibrated with 300 ml of a 50 mM NH_4OAc buffer solution (pH 3.9). Azu bound to the top of the column, and was eluted by 2 litre of a 50 mM NH_4OAc pH gradient of 3.9–6.0 (flow rate 200 ml/h). Blue fractions were pooled, and the spectral ratio A_{625}/A_{280} was determined by UV/vis spectroscopy. At this stage the value of this ratio was usually between 0.3 and 0.4.

Step 4: Anionic exchange chromatography. The protein mixture was reduced with a slight excess of sodium dithionite. By repeated concentration and dilution under N_2 with the use of a 200 ml Amicon ultrafiltration cell (Amicon Corp., USA) the buffer was changed to 5 mM Tris-HCl pH 8.5. After concentration to 10 ml the reduced solution was applied to a 2.5×60 cm Whatman DE52 column, which was equilibrated with 1 litre of the same Tris buffer. Azu bound on top of the column. Elution was performed with 4 litre of a salt gradient of 0–25 mM NaCl in the Tris buffer (flow rate 120 ml/h). This step resulted in the complete separation of reduced holo-Azu and a contaminating protein (see Results). The contaminating protein eluted from the column in front of reduced holo-Azu. Holo-Azu-containing fractions were detected by their colouring blue upon addition of potassium ferricyanide. After pooling the holo-Azu-containing fractions complete oxidation was achieved by the addition of a small excess potassium ferricyanide. The fractions that contained the contaminating protein were detected by monitoring the A_{280}, and were subsequently pooled. Addition of potassium ferricyanide did not result in the appearance of any blue colour.

Step 5: Cationic exchange chromatography. Small remaining impurities were removed from the holo-Azu fraction and from the fraction that contained the contaminating protein by repeating step 3 for each fraction. Proteins were eluted with a pH stepgradient starting at pH 4.1 (flow rate 100 ml/h), the pH being increased every 250 ml by 0.05 unit until the protein started to elute. In the case of the oxidized holo-Azu blue fractions were pooled after elution. When eluting the contaminating protein the A_{280} of all collected fractions was monitored and fractions for which A_{280} was > 0.01 were pooled.

After this purification step the spectral ratio A_{625}/A_{280} for oxidized holo-Azu was typically 0.58 (± 0.01), while the same ratio for the contaminating protein was below 0.01. Yields were similar for both proteins and amounted to 50 mg.

Gel electrophoresis

Rocket immuno gel electrophoresis (RIGE) [20] was performed with crude cell lysates and polyclonal antibodies raised against Azu purified from *P. aeruginosa*. For all other gel electrophoresis experiments the Pharmacia PhastSystem (Pharmacia, Sweden) was used. Discontinuous denaturing SDS-PAGE was performed with 20% polyacrylamide gels. Titration curve (TC) analysis and IEF were performed with polyacrylamide gels in which a pH gradient was established of 3–10. p*I* values were determined from a comparison with p*I* marker proteins from Pharmacia.

^{1}H-NMR spectroscopy

^{1}H-NMR samples in D_2O were prepared by repeated concentration and dilution using an Amicon ultrafiltration cell. Holo-Azu was reduced with a 0.1 M sodium dithionite solution in 0.1 M NaOD during this preparation. The final protein concentration of the NMR samples was 3–4 mM. K_2DPO_4/KD_2PO_4 buffer was added to a final concentration of 20 mM. A small amount of TMA (tetramethylammonium nitrate) was added for chemical shift and temperature calibration. Argon was passed through the reduced holo-Azu samples in the NMR tube to prevent reoxidation by oxygen.

The pH of the samples was adjusted by the addition of small amounts of NaOD or DCl solutions (0.01–1.0 M in D_2O). pH titrations were started at high pH. Care was taken that the ionic strength of the sample during the titration varied only slightly. pH measurements were performed using a Radiometer PHM-84 research pH-meter equipped with a Broadley-James combination electrode with calomel reference. Meter readings were not corrected for the deuterium isotope effect, nor were the calculated pK_a values. Uncorrected pH and pK_a values are denoted by an asterisk.

^{1}H-NMR spectra were recorded on a Bruker WM-300 spectrometer at 298 and 315 K. The HDO resonance was suppressed by presaturation. Free induction decays were accumulated in 8K memory, zero filled to 16K data points, deconvoluted by Gaussian multiplication and Fourier transformed. When integration of peaks was needed to determine pK_a* values deconvolution was omitted. Temperature calibration was achieved by recording the signal of TMA and measuring the position of the HDO resonance with respect to the TMA resonance. Chemical shifts are quoted in ppm downfield from sodium 2,2-dimethyl-silapentane-5-sulfonate (DSS).

For the resonances which are in fast exchange pK_a* values were determined from a computer analysis of the variation of the chemical shift versus pH*. pK_a* values for resonances which are in slow exchange were determined from a computer analysis of the variation of relative peak intensity versus pH*. Relative peak intensities were determined with the help of the standard Bruker integration program.

Protein handling

Oxidation of holo-Azu was performed by adding small amounts of a 0.1 M potassium ferricyanide solution, while reduction was achieved by the addition of small amounts of a 0.1 M sodium dithionite solution in 0.1 M sodium hydroxide. Apo-Azu was prepared by a modified version of the potassium cyanide dialysis procedure according to Groeneveld et al. [21]. 100 ml of a 0.1 M potassium cyanide solution in 0.15 M Tris-HCl (pH 8.3) containing the required amount of reduced holo-Azu was stirred overnight at 4°C. Cyanide was removed by ultrafiltration and the produced apo-Azu was transferred to the required buffer by repeated concentration and dilution. This procedure resulted in > 95% pure apo-Azu. In a similar way Azu* was treated with potassium cyanide to remove possibly bound metal ions. The binding of Hg(II) ions to Azu* was studied by the addition of equimolar amounts of mercury nitrate ($Hg(NO_3)_2$) to KCN treated Azu* directly in the NMR tube. Reconstitution experiments with Azu* and apo-Azu were performed in 50 mM ammonium acetate buffer (pH 5–6) with cupric sulphate at 4°C and at room temperature. Denatured holo-Azu was obtained by heating holo-Azu at 80°C for 15 min in 100 mM Tris-HCl buffer pH 9.0.

Fluorescence measurements

Fluorescence spectra were recorded on a Jobin Yvon 3D spectrofluorometer. The multifrequency phase-shift and demodulation measurements [22] were performed using the apparatus built at the synchrotron radiation facility of the ADONE storage ring, PULS Laboratory, Frascati, Italy. The experimental set up has been described previously [23]. For data acquisition commercially available electronics (ISS, La Spezia, Italy) were used. The samples were excited at 295 nm with a bandwidth of 4 nm. The absorbance was about 0.2 at the excitation wavelength. Fluorescence light was collected after passage through a 305 nm cut-off filter to remove scattered light. All the fluorescence experiments were carried out at 25°C. Phase and modulation data were analyzed either in terms of a single- and double-exponential decay [24] or with a model based on a continuous distribution of lifetimes having a lorentzian shape [25]. In this latter case the centers (C) and the widths (W) of the lorentzian functions were obtained by minimizing the χ^2 values with a routine based on the simplex method, running on an IBM personal computer. The software was provided by ISS, Champaign, IL.

Results

In this section results are subsequently presented on a) the expression of the *azu* gene, b) the isolation and purification procedure of Azu and Azu*, c) the electrophoretic and d) spectroscopic characterization of Azu*, and finally e) the fluorescent properties of Azu*. The spectroscopic characterization of Azu* mainly rests on specific ^{1}H-NMR assignments.

Expression of the azu gene in E. coli

The *azu* gene from *P. aeruginosa* is located on a 1.3 kb *Pst*I fragment [12–14,26]. This fragment has previously been cloned into the *Pst*I site of pBR322 (plasmid pGC1) on which vector the *azu* gene is poorly expressed in *E. coli* (Fig. 1) [12,13]. To obtain higher

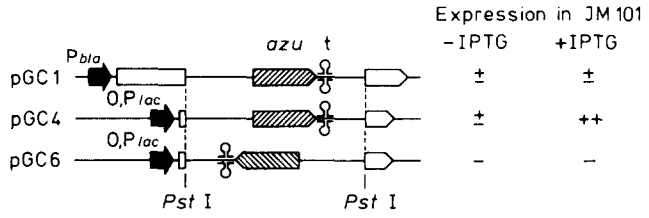

Fig. 1. Organization scheme and expression of the *P. aeruginosa azu* gene on different plasmids. The level of expression (−: not detectable; ± = low; + +: very high) was determined by RIGE analysis of crude lysates of *E. coli* JM101 cells that were transformed with these plasmids and grown in the presence (0.1 mM) or absence of IPTG until an absorbance at 610 nm of 0.4 was attained. P*bla*: promoter of the *bla* gene; O,P*lac*: operator and promoter regions of the *lac* promoter; t: bidirectional Rho-independent terminator.

expression the *Pst*I fragment was cloned behind the *lac* promoter in the unique *Pst*I site of pUC8, resulting in pGC4. Upon transformation to *E. coli* JM101 cells and growth in the presence of 0.1 mM IPTG, large amounts of Azu were produced, as judged by RIGE and SDS-PAGE. To investigate whether this expression was due to transcription originating from the *lac* promoter or from one or both promoters located upstream of the *azu* gene on the *Pst*I fragment [14,26], *E. coli* JM101 cells transformed with pGC4 were grown in the absence of IPTG. Expression was reduced, but had not totally disappeared (Fig. 1).

Because this residual expression might be caused by incomplete repression of the *lac* promoter due to insufficient amounts of *lac* repressor in the cell, the orientation of the *Pst*I fragment was reversed, resulting in pGC6 (Fig. 1). No Azu could be detected in cell extracts of JM101 transformed with pGC6, grown either in the absence or presence of IPTG (Fig. 1). It was concluded that the observed expression of the *azu* gene on pGC4 arises from transcription initiated at the *lac* promoter.

Isolation and purification

Azu was isolated from *E. coli* KMBL1164 cells transformed with pGC4, grown on LB medium at 37°C in the absence of IPTG. The purification was performed as described in Materials and Methods. It resulted in the separation of very pure holo-Azu from an inactive non-reconstitutable form of Azu (see below). The critical step for this separation appeared to be step 4. The spectral ratio A_{625}/A_{280} of the Azu-containing solution, which was obtained by pooling blue fractions after cationic exchange chromatography (step 3), was between 0.3 and 0.4. This ratio could not be increased either by oxidation with potassium ferricyanide, or by reconstitution with Cu(II) ions supplied as cupric sulphate. As the reported value of this ratio is 0.53 for pure Azu [9,27], a still sizeable impurity had to be present. However, denaturing SDS-PAGE with 20% polyacrylamide gels showed only one band migrating at the position of correctly processed Azu (M_r = 14600) (not shown). In contrast, IEF showed that the same sample gave rise to three bands, two heavy bands originating from proteins with a p*I* of 5.6 and 5.4, and a much fainter band from a protein with a p*I* of 4.6 (Fig. 2, lane b). TC analysis showed that the proteins which correspond to the heavy bands should be separable either at high pH with anionic or at low pH with cationic exchange chromatography, provided that the applied solution would be reduced first. Anionic exchange chromatography was preferred, because the reduced holo-Azu oxidizes back when applied to a cation exchange column. Thus step 4 comprised DE52 chromatography of the reduced Azu solution.

After the last purification step (step 5) the spectral ratio A_{625}/A_{280} of holo-Azu was 0.58 (±0.01), while the same ratio for the contaminating protein was below 0.01. A value of 0.58 for A_{625}/A_{280} is therefore considered as a better purity criterion for the holo-Azu than the literature value of 0.53 [9,27].

Electrophoretic characterization of the contaminating protein

In view of the many literature reports on the interference by an apo-Azu-like contamination in fluorescence measurements on Azu [6,8,9,11], it was considered important to further characterize the contaminating protein. IEF showed that it has a p*I* of 5.4, while oxidized holo-Azu has a p*I* of 5.6 (Fig. 2, lanes b-d). Neither the addition of sodium dithionite nor a subsequent treatment with potassium cyanide at alkaline pH changes the p*I* of the contaminating protein (Fig. 2, lanes f and h). In contrast, reduction or reduction plus

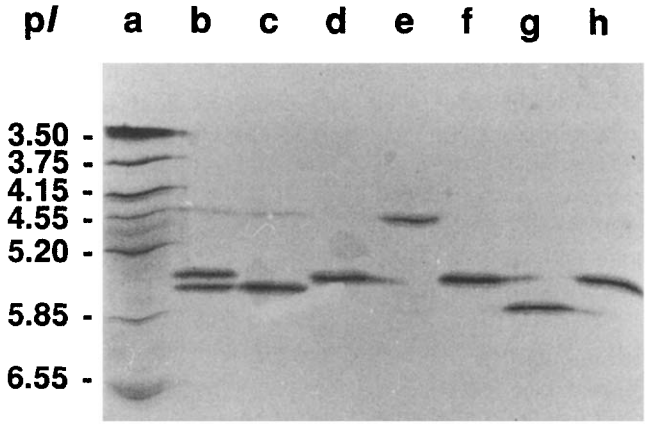

Fig. 2. IEF gel electrophoresis of the following samples: lane a: p*I* marker proteins (corresponding p*I* values are noted at the left); lane b: sample obtained after cationic exchange chromatography (step 3 of purification procedure), A_{625}/A_{280} = 0.35; lane c: purified oxidized holo-azurin, A_{625}/A_{280} = 0.59; lane d: purified contaminating protein (= azurin*), A_{625}/A_{280} = 0.01; lane e: reduced holo-azurin; lane f: azurin*, treated with sodium dithionite; lane g: apo-azurin; lane h: azurin*, treated with potassium cyanide at pH 8.3 after treatment with sodium dithionite.

subsequent KCN treatment of holo-Azu causes changes in p*I*. The p*I* values of reduced holo-Azu and apo-Azu (4.6 and 5.8 respectively; Fig. 2, lanes e and g) are different from the p*I* of the contaminating protein, showing that the contamination is dissimilar from these Azu forms. The contaminating protein differs also from denatured Azu, which has a p*I* of 6.2 (not shown). Apparently, the faint band that appeared at p*I* 4.6 when pure oxidized holo-Azu was applied to the IEF gel (Fig. 2, lane c) represents a minor amount of holo-Azu, that got reduced upon application to the gel. This is supported by the observation that KCN treatment of holo-Azu after reduction causes the disappearance of this band (Fig. 2, lane g).

In conclusion, the contaminating protein is neither oxidized nor reduced holo-Azu, nor apo-Azu, nor denatured holo-Azu. It can not be reconstituted with cupric sulphate and it can not be oxidized by potassium ferricyanide. However, it comigrates with holo-Azu on a denaturing SDS-polyacrylamide gel. It was checked that all these properties, as well as the properties of holo-Azu, were the same, irrespective of whether the proteins were isolated from *P. aeruginosa* [9] or from *E. coli* cells.

Spectroscopic characterization of the contaminating protein

To obtain more information about the contaminating protein 300 MHz ^{1}H-NMR spectra were recorded at 298 K as a function of pH* (10–4.5), and at 315 K at two different pH* values (8.50 and 5.00). Fig. 3 shows a spectrum of the contaminating protein together with spectra of reduced holo-Azu and apo-Azu, recorded at 315 K pH* 8.50. The spectrum of the contaminating protein shows a high degree of similarity with both the reduced and the apo-Azu spectra. For this reason the contaminating protein has been denoted by Azu*.

Notably, in the spectrum of apo-Azu nearly all NH protons in the downfield region of the spectrum have disappeared (Fig. 3). This H/D exchange is much faster for apo-Azu than for reduced holo-Azu (see also [28]), because the structural rigidity of the apo-protein is less than that of the holo-protein. The decreased rigidity of apo-Azu is also reflected in a lesser thermal stability compared to holo-Azu [29,30]. The amount of H/D exchange for Azu*, on the other hand, is of the same order as for reduced holo-Azu (Fig. 3). This means that the protein is more rigid than apo-Azu. Experiments in which the temperature was elevated to 353 K indicated that Azu* is even more stable than reduced holo-Azu: while holo-Azu started to denature at this temperature, Azu* retained its native configuration (not shown). The remainder of this section now is concentrated on the detailed analysis and assignment of ^{1}H-NMR resonances from residues belonging to the immediate environment of the Cu-site and Trp-48.

The ^{1}H-NMR spectra of reduced holo-Azu and apo-

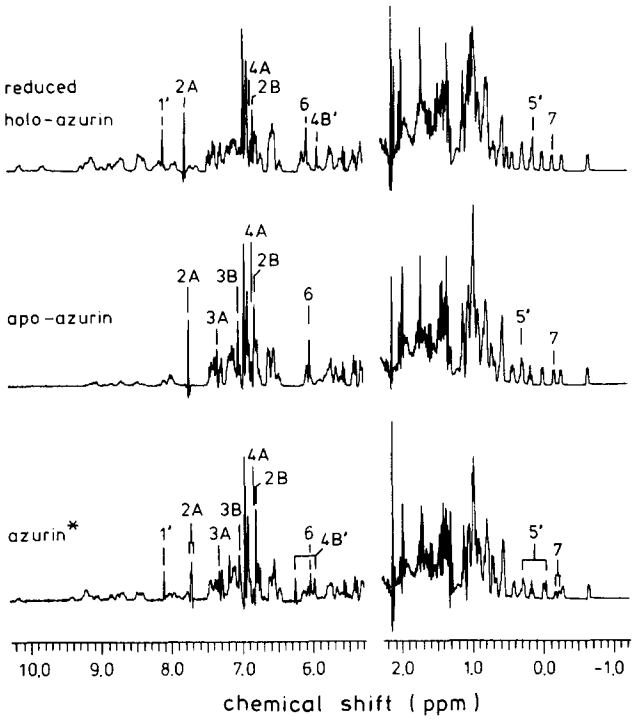

Fig. 3. 300 MHz ^{1}H-NMR spectra of 4 mM samples of reduced holo-azurin, apo-azurin and azurin* in 20 mM potassium phosphate buffer (pH* 8.5) in D_2O recorded at 315 K. Assignments of coded resonances are compiled in Table I.

Azu have been partially assigned [28,31–34]. In passing it is pointed out that the ^{1}H-NMR spectra of reduced holo-Azu isolated from *P. aeruginosa* [31] and *E. coli* appear to be identical. By studying the pH dependence of the NMR spectra of apo-Azu and Azu*, and by comparison with the spectra of reduced holo-Azu, it was possible to make tentative assignments for peaks arising from the histidines 35, 46, 83 and 117 in the spectra of apo-Azu and Azu*. (His-46 and 117 are Cu-ligands in holo-Azu). Additional resonances were assigned to Met-121, Trp-48 and Val-31. They are of interest (a) because Met-121 coordinates to Cu in holo-Azu, and (b) because Trp-48 and Val-31 are sensitive reporters of small structural changes. This is because the Val-31 γ-CH_3 groups are located above the Trp-48 ring [1,2] and experience the strong shielding effect of the aromatic π-system. As a result small changes in the position of Val-31 relative to Trp-48 result in relatively large changes in the chemical shift of the Val-31 γ-CH_3 resonances. The putative assignments are compiled in Table I and discussed in more detail below. Titration curves for reduced and apo-Azu, as well as for Azu*, are shown in Fig. 4. Close inspection of the experimental data reveals that Azu* may occur in the form of two species, Azu*^{a} and Azu*^{b}, with very slight spectroscopic differences. This is commented on further on.

Assignments of resonances to specific histidine residues in the spectra of apo-Azu and Azu* were based

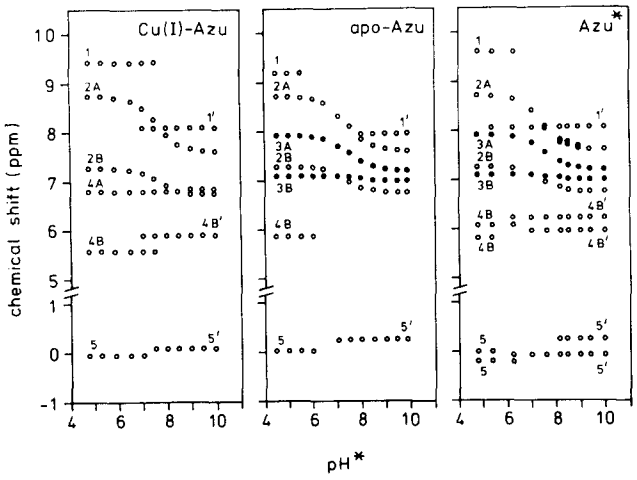

Fig. 4. pH* titration curves for singlet resonances assigned to His-35 C2H (1), His-83 C2H (2A) and C4H (2B), His-117 C2H (3A) and C4H (3B), His-46 C2H (4A) and C4H (4B), and Met-121 ϵ-CH_3 (5) in the 300 MHz ^{1}H-NMR spectra of reduced holo-azurin, apo-azurin and azurin* recorded at 298 K.

(a) on the singlet nature of these resonances, (b) on a comparison of their chemical shift positions with their positions in the spectra of reduced holo-Azu and/or apo-Azu, (c) on the proton exchange regime that applies to them, and (d) on a comparison of their pK_a* values (when applicable) with the pK_a* values observed for holo- and/or apo-Azu.

In the spectra of reduced holo-Azu the His-35 C2H resonances labelled 1 (visible at low pH) and 1′ (visible at high pH) are in slow exchange at 298 K [28,31–33]. This behaviour is also observed for the singlet resonances 1 and 1′ in the same region of the spectra of apo-Azu and Azu* (Fig. 4). Therefore, they are also assigned to the His-35 C2H proton. At 315 K the His-35 C2H resonances of apo-Azu are in intermediate exchange, which explains the absence of resonance 1′ in the apo-Azu spectrum in Fig. 3. Both in apo-Azu and Azu* His-35 titrates with a pK_a* of about 6.4, which is considerable lower than the pK_a* of His-35 in reduced holo-Azu (7.2). The decrease of this pK_a* for apo-Azu is due to the absence of Cu in the protein. The same decrease for Azu*, therefore, points to the absence of Cu in this protein, too.

The chemical shift positions, the proton exchange regime and the pK_a* values for the resonances 2A and 2B in the spectra of reduced holo-Azu, apo-Azu and Azu* are similar to each other (Fig. 4). For reduced holo-Azu they have been assigned to the His83 C2H and C4H protons respectively [28,31]. The resonances 2A and 2B in the apo-Azu and Azu* spectra were therefore assigned similarly.

Hill et al. noted that apart from the His-35 and His83 resonances a singlet resonance (3A) titrated (pK_a* $\approx$ 7.7) in the apo-Azu spectra under a fast exchange regime, which they assigned to His-117 [32]. In contrast to holo-Azu, His-117 is titratable in the apo-protein. A similar resonance (3A) is observed in the Azu* spectra (Fig. 4). Thus also in Azu* His-117 is titratable, which again points to the absence of metal coordination. The singlet resonances 3B that are observed in the apo-Azu and Azu* spectra but not in the reduced holo-Azu spectra titrate with a similar pK_a* (Fig. 4). Although the difference in chemical shift position at low and high pH is somewhat small for a histidine proton, the resonances 3B are tentatively assigned to the second His-117 proton. From the general observation that the chemical shift of histidine C2H protons exhibit a much larger pH dependence than the corresponding C4H proton resonances, it is inferred that the resonances labelled 3A arise from the His-117 C2H protons while the resonances 3B arise from the His-117 C4H protons of apo-Azu and Azu*. (In Fig. 3 the His-117 C2H and C4H resonances are not indicated in the reduced holo-Azu spectrum because they overlap with other resonances [31,34].)

In the low pH spectra of apo-Azu and Azu* the singlet resonances 4B are present at about the same position as resonance 4B in the spectra of reduced holo-Azu (Fig. 4). The latter resonance has been assigned to the His-46 C4H proton [31]. Therefore, resonances 4B in the spectra of apo-Azu and Azu* were assigned to the same proton. Resonance 4A in the spectrum of reduced holo-Azu has been assigned to the His-46 C2H proton [31]. Corresponding resonances of apo-Azu and Azu* could not be detected in the 298 K spectra, probably due to overlap, but they are visible in the spectra recorded at 315 K (Fig. 3, Table I). In Azu* the slow exchange of resonance 4B (visible at low pH) with the correlated resonance 4B′ (visible at high pH) reflects the slow protonation of His-35 as in reduced holo-Azu [33]. This implies that His-46 does not titrate in Azu*, unlike His-117. Although in the apo-Azu spectra the correlated resonance 4B′ could not be observed at high pH (possibly due to overlap) this conclusion presumably holds also for apo-Azu because the intensity of resonance 4B decreases in the same way with increasing pH as in the spectra of reduced holo-Azu and Azu*. The fact that His-46 is located in the interior of the protein [1,2] might explain why in apo-Azu and Azu* this residue does not titrate. That such a behaviour is not unprecedented is illustrated by the case of His-35. In holo-Azu from *P. aeruginosa* His-35 has a small solvent accessible surface area of 8 Å^2 [35] and exchanges protons with the solvent at an unusually slow rate, therefore [28]. In the azurins from *Alcaligenes denitrificans* and *A. faecalis* His-35 even does not titrate at all [36,37]. In holo-Azu His-46 has no solvent accessible surface area [35] and is tied up by a hydrogen bonding network [1,2]. Both features may contribute to His-46 being non-titratable in Azu* and in apo-Azu.

At 0.00 ppm in the low pH spectra of apo-Azu and Azu* a singlet resonance (5) is present which is in slow exchange with a 0.25 ppm downfield shifted resonance (5′) at high pH, with a pK_a^* similar to the pK_a^* of His-35 (Fig. 4). In the spectra of reduced holo-Azu similar resonances 5 and 5′ show the same behaviour. They have been assigned to the ϵ-CH_3 group of Met-121 [31,33]. The same resonances in the spectra of apo-Azu and Azu* are, therefore, also assigned to Met-121.

For apo-Azu and Azu* resonances 6 and 7 were assigned to the Trp-48 C2H and Val-31 γ-CH_3 protons because they occur at about the same positions as the Trp-48 C2H and Val-31 γ-CH_3 resonances in the spectra of reduced holo-Azu [31].

Interestingly, in the Azu* spectra a number of resonances has doubled, including resonances assigned to the His-83 C2H (2A), His-117 C4H (3B), His-46 C4H (4B), Met-121 ϵ-CH_3 (5) and Val-31 γ-CH_3 (7) protons (Figs. 3 and 4, Table I). Apparently, Azu* may occur in the form of two different species (denoted by Azu*[a] and Azu*[b]) with slightly different conformations. Differences are roughly confined to a region encompassing the surroundings of the active site and Trp-48; resonances assigned to the His-35 C2H (1) and His-83 C4H

TABLE I

Assignments of a number of resonances observed in the 300 MHz ^{1}H-NMR spectra of reduced holo-azurin, apo-azurin and azurin at 298 K*

For resonances in the Azu* spectra that are doubled two entries are given, corresponding to the Azu*[a] and Azu*[b] species (see text); otherwise a single entry is given. (Errors in the chemical shift values are 0.01 ppm. Errors in pK_a^* values are < 0.05, unless indicated otherwise in brackets.)

No.	Residue	Proton	Protein	Chemical shift (ppm)		Exchange regime		pK_a^*	Ref.
				pH* 5.0	pH* 10.0	slow	fast		
1	His35	C2H	holo-Azu	9.41	8.07	+		7.2 (0.1)	28,31–33
			apo-Azu	9.20	7.95	+		6.5 (0.3)	28
			Azu*	9.60	8.07	+		6.3 (0.2)	
2A	His83	C2H	holo-Azu	8.72	7.61		+	7.65	28,31,32
			apo-Azu	8.72	7.61		+	7.35	28
			Azu*[a]	8.72	7.61		+	7.49	
			Azu*[b]	8.72	7.61		+	7.42	
2B	His83	C4H	holo-Azu	7.27	6.74		+	7.64	28,31,32
			apo-Azu	7.28	6.75		+	7.37	28
			Azu*	7.28	6.74		+	7.44	
3A	His117	C2H	holo-Azu	6.89	6.89				31,34
			apo-Azu	7.92	7.19		+	7.56	32
			Azu*[a]	no[a)]	no				
			Azu*[b]	7.93	7.18		+	7.69	
3B	His117	C4H	holo-Azu	6.86	6.86				31,34
			apo-Azu	7.10	7.00		+	7.7 (0.2)	
			Azu*[a]	7.10	6.99	+		7.3 (0.3)	
			Azu*[b]	7.10	6.99		+	7.8 (0.2)	
4A	His46	C2H	holo-Azu	6.80	6.77		+	7.2 (0.1)	31,34
			apo-Azu	no	6.80[b)]				
			Azu*[a]	no	no				
			Azu*[b]	no	6.82[b)]				
4B	His46	C4H	holo-Azu	5.57	5.89	+		7.2 (0.1)	31,34
			apo-Azu	5.81	no	+			
			Azu*[a]	6.07	6.22	+		6.3 (0.2)	
			Azu*[b]	5.82	5.94	+		6.3 (0.2)	
5	Met121	ϵ-CH_3	holo-Azu	−0.06	0.08	+		7.2 (0.1)	31,33
			apo-Azu	0.00	0.24	+[c)]	+[c)]	6.5 (0.3)	
			Azu*[a]	−0.19	−0.08	+		6.3 (0.2)	
			Azu*[b]	0.00	0.26	+		6.3 (0.2)	
6	Trp48	C2H	holo-Azu	6.05	6.02				31
			apo-Azu	6.00	6.00				
			Azu*	6.00	6.00				
7	Val31	γ-CH_3	holo-Azu	−0.24	−0.22				31
			apo-Azu	−0.26	−0.26				
			Azu*[a]	−0.31	−0.31				
			Azu*[b]	−0.26	−0.26				

[a)] No: not observed.
[b)] Observed only at 315 K.
[c)] Intermediate exchange regime.

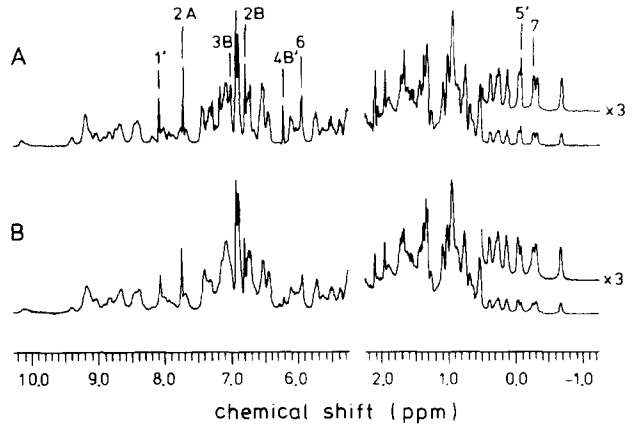

Fig. 5. 300 MHz ^{1}H-NMR spectra of azurin*[a] (A) and KCN treated azurin*[a] to which a stoichiometric amount of $Hg(NO_3)_2$ was added (B), recorded at pH* 8.5 and 315 K. Putative assignments for coded resonances are compiled in Table I. Note the effect of the addition of Hg(II) ions on the resonances assigned to the His-117 C4H (3B), His 46 C4H (4B'), Met-121 ϵ-CH_3 (5') and Val-31 γ-CH_3 (7) protons.

(2B) protons did not show this doubling. As judged by their NMR spectra some other preparations appeared to contain only Azu*[a] (Fig. 5). The His-117 C2H resonance (3A) could not be detected in the Azu*[a] spectra, while a slow exchange regime applied to the His-117 C4H (3B) resonances in Azu*[a] (Table I). His-117 in Azu*[a] still could be protonated but the corresponding pK_a* value is 0.5 pH unit lower than in apo-Azu and Azu*[b] (Table I).

Treatment of Azu*[a] with potassium cyanide at alkaline pH had no effect on the ^{1}H-NMR spectrum. The metal binding capability of Azu*[a] was investigated by the addition of equimolar amounts of Hg(II) ions to KCN treated Azu*[a]. Addition of a stoichiometric amount of mercury nitrate to a 4 mM Azu*[a] ^{1}H-NMR sample resulted in a disappearance or shift of resonances assigned to the His-117 C4H (3B), His-46 C4H (4B) and Met-121 ϵ-CH_3 (5) protons (Fig. 5), indicating the binding of Hg(II) ions at or near the 'active' site of Azu*[a].

*Fluorescence properties of holo-Azu and Azu**

The static fluorescence spectrum of Azu* is very similar to the spectra of oxidized holo- and apo-Azu [3–5,8] with an emission maximum at 308 nm and a shoulder at 295 nm. The relative intensity is about 10-times that of Cu(II) Azu. However, while the fluorescence of apo-Azu was reduced tenfold by addition of Cu(II) or Hg(II) ions (see also [3,8]), the fluorescence intensity of Azu* was only reduced by about 20% by Hg(II), and not at all by Cu(II). Treatment of Azu* with potassium cyanide to remove metals that might be present did not affect the fluorescence, nor did it change the effect of Cu(II) or Hg(II) ions on the fluorescence intensity.

The fluorescence decay of apo-Azu could be fitted by a single exponential. On the other hand the decays of oxidized holo-Azu and of Azu* are clearly not mono-exponential and have to be fitted by at least two exponential functions or by a continuous distribution of lifetimes (Table II).

Discussion

Heterologous expression and purification

To obtain holo-Azu and Azu* use was made of heterologous expression of the *P. aeruginosa azu* gene in *E. coli*. Cloning of a 1.3 kb *Pst*I fragment, that contains the *azu* gene [12,14], downstream of the *lac* promoter in the *Pst*I site of plasmid pUC8 resulted in a high expression level. It was not necessary to decrease the length of the 0.5 kb long region upstream of the *azu* gene, in line with the absence of transcription termination signals in this region [14]. Protein yields were comparable to those obtained by Karlsson et al. who also developed a heterologous expression system for the *P. aeruginosa azu* gene but deleted part of the region upstream of the *azu* gene [27].

It is interesting to compare the present purification procedure with the one developed by Karlsson et al. [27] and to see why these authors apparently were not troubled by the occurrence of an Azu-like contamination in their holo-Azu preparations. The main difference with the present purification procedure lies in step 3 where a solution containing oxidized holo-Azu is applied to the cationic exchange CM-column, while Karlsson et al. bind the Azu in the apo-form to the

TABLE II

Fluorescence decay parameters of azurins

τ, τ_1, τ_2, fluorescence lifetimes in nanoseconds (errors in τ are 0.1 ns). f_1, fraction of fluorescence corresponding with τ_1 (or with C_1, W_1); fraction of fluorescence corresponding with τ_2 is given by $1-f_1$ (errors in f are 0.05). C_1, C_2, W_1, W_2, centers and widths of lorentzians in ns (errors in C and W are 0.1 ns). χ^2, reduced chi-square.

Protein	One lifetime		Two lifetimes				Two lorentzian continuous distributions					
	τ	χ^2	τ_1	τ_2	f_1	χ^2	C_1	W_1	C_2	W_2	f_1	χ^2
Apo-azurin	4.9	4.0	0.7	4.9	< 0.01	5.3	< 0.1	0.5	4.9	< 0.1	< 0.01	5.5
Cu(II) azurin	1.1	3800	0.2	4.7	0.39	1.8	0.2	0.1	4.8	< 0.1	0.40	1.5
Azurin*	4.6	52	< 0.1	4.9	0.01	2.7	< 0.1	0.1	4.0	0.1	0.02	3.9

CM-column [27]. From the IEF results obtained in the present study it can be seen that in the latter case apo-Azu will separate from Azu*. Nevertheless, judging by the reported spectral ratio A_{625}/A_{280} of 0.53 [27] (as opposed to 0.58 in the present work), possibly not all of the Azu* was removed in the holo-Azu preparations of Karlsson et al.

Structural characterization

The properties of Azu* were investigated by electrophoretic and spectroscopic techniques. Molecular weight and ^{1}H-NMR characteristics appear compatible with Azu* having the same primary structure as holo-Azu. Comparison of the ^{1}H-NMR spectra of reduced holo-Azu, apo-Azu and Azu* indicate that the structure of Azu* resembles the structure of apo-Azu, although, unlike apo-Azu, it can not be reconstituted with Cu(II) ions. Similarities include the pK_a* of His-35 and the titratability of His-117. Also the fluorescence intensity of Azu*, like that of apo-Azu [3,8], is much higher than of holo-Azu. These observations point to the absence of any metal-ion in the 'active' centre of Azu*. Further support for this conclusion comes from the lack of any effect of a treatment with potassium cyanide on the electrophoretic and spectroscopic properties of Azu*. The broadening or shift of the Azu* resonances assigned to His-46, His-117 and Met-121 upon addition of Hg(II) ions cannot be ascribed to a similar binding of Hg(II) as in apo-Azu, because the decrease of the fluorescence intensity upon Hg(II) addition is much less for Azu* than for apo-Azu. The observed effects can be explained by the binding of Hg(II) near the 'active site', or only to some ligand residues. We tentatively conclude that a subtle modification of one or more Cu-ligand residues (as hinted at before [9]) has made it impossible for Azu* to bind Cu, and affects the binding of Hg(II).

The reason why the active site of Azu* apparently is vulnerable to chemical modification is not understood. Also the exact nature of the modifications is not clear yet. It is, however, worthwhile considering here some possibilities. The observation of two Azu* species by ^{1}H-NMR spectroscopy suggests that the different forms may have slightly differently modified Cu-ligands. Possible modifications are the oxidation of the sulphur of Cys-112, and the formation of a covalent Cys-112/His-117 link. Such a His/Cys modification has been reported for the *Neurospora crassa* copper protein tyrosinase, in which Cys-94 is linked via a thioether bridge to the C2 position of His96 [38]. The occurrence of this type of modification in Azu*^{a} is suggested by the observation that in the ^{1}H-NMR spectra of Azu*^{a} no His-117 C2H resonance could be identified, while His-117 could still be protonated. It is noted that the absence of a noticeable effect on the chemical shift of the His-117 C4H resonance of Azu*^{a} compared to Azu*^{b} and apo-Azu might argue against the purported His/Cys link. On the other hand the pK_a* value and the exchange regime of His-117 in Azu*^{a} are different from Azu*^{b} and apo-Azu (Table I). Definite conclusions must await further stuctural analyses.

Although Azu* may function as a pro-Azu that has to be activated, as has been proposed for the tyrosinase from *Neurospora crassa* mentioned before [38], we favour the hypothesis that it is a by-product of the purification procedure.

Fluorescence properties

The presently reported results on the fluorescence of holo- and apo-Azu are in line with previously obtained results. The fluorescence decay of holo-Azu has been analysed before in terms of a sum of two exponentials with different lifetimes [6–8]. More recently, Hutnik and Szabo [9] have shown that a fit of the fluorescence decay of holo-Azu with three decays gives better results. In our case the analysis of the fluorescence decay of holo-Azu by three lifetime components (not shown) gives a χ^2 similar to that obtained for the fit with two continuous Lorentzian distributions of lifetimes (Table II). As the floating parameters are five in both cases, there is no statistical evidence in favour of one of the two models. We prefer the results of the continuous distribution model as we consider it closer to the physics of proteins [25,39]. However, the main conclusion from the fluorescence experiments is that they confirm that the fluorescence decay of holo-Azu, after removal of the contaminating Azu*, is heterogeneous [9].

Furthermore, it was found that the fluorescence decay of pure apo-Azu could be fitted by a single lifetime function (Table II), as reported before [6,8,9,11]. Finally, it was found that the fluorescence decay of Azu* had to be fitted by at least two lifetime-components or a continuous distribution of lifetimes (Table II). The data demonstrate that Azu* cannot be responsible for the multi-exponential fluorescence decay observed for holo-Azu, which most likely is due to conformational heterogeneity [9].

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