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Sensitive detection of the redox state of copper proteins using fluorescence

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Abstract The blue copper protein azurin from *Pseudomonas aeruginosa* has been covalently labelled with the fluorescing dye Cy5. The optical spectrum of the azurin changes markedly with its redox state. These changes are reflected in the fluorescence intensity of the dye through fluorescence resonance energy transfer (FRET). This provides a sensitive way to monitor biological redox events. The method shown to work in the nanomolar range of protein concentrations, can be easily extended into the sub-nanomolar regime and holds promise for single-molecule detection.

Keywords Energy transfer · FRET · Enzymes · Cu protein · Azurin · Fluorescence quenching · Fluorescent probes · Redox chemistry · Single molecule

Abbreviations FRET: Fluorescence resonant energy transfer · DMSO: Dimethylsulfoxide · DTT: Dithiothreitol · PBS solution: Phosphate buffered saline solution

Introduction

Fluorescence detection is a popular method for visualising and monitoring the activity and function of biomacromolecules. Often, dual-wavelength fluorescence detection of a donor–acceptor pair is used, where fluorescence resonance energy transfer (FRET) allows registration of conformational dynamics that is very

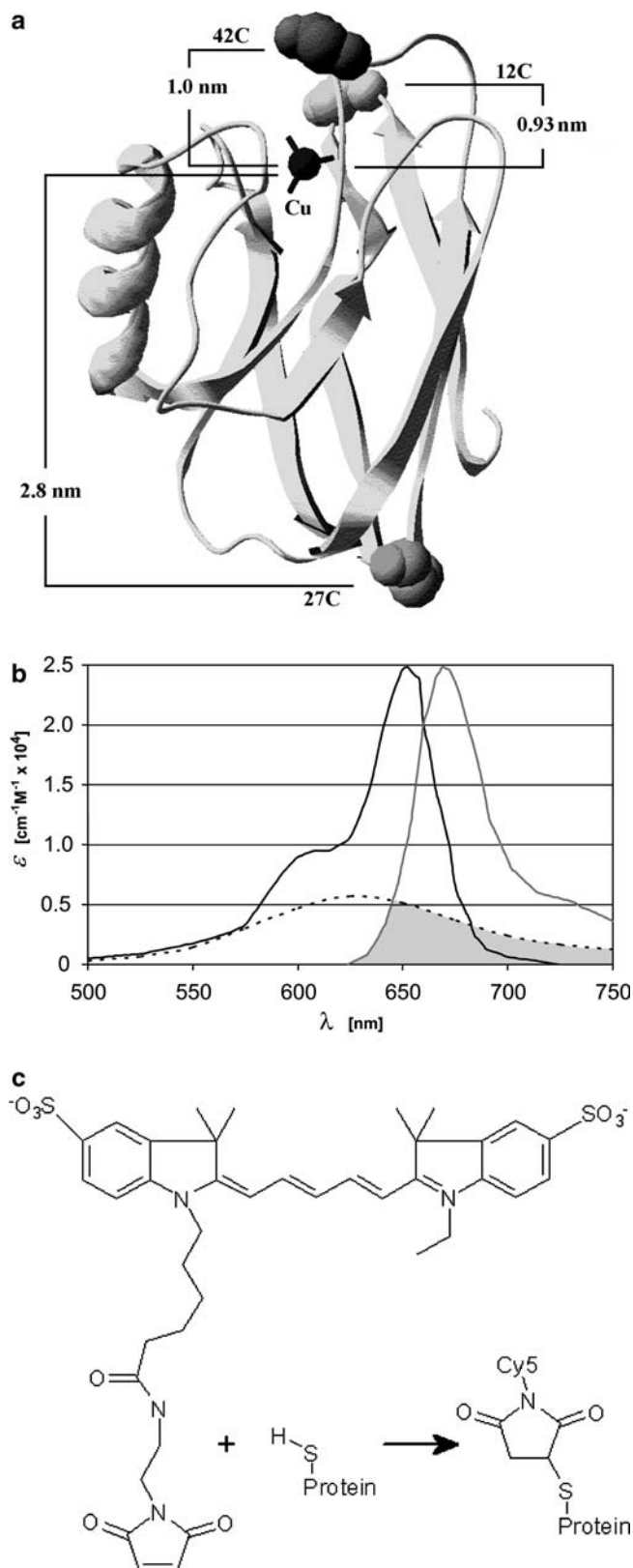
sensitive to donor–acceptor distance and relative orientation [1, 2]. Here we show the use of FRET in a novel way, in which the intrinsic redox-co-factor of a Cu protein functions as the energy acceptor. The donor is a fluorescent dye covalently attached to a specific residue on the protein surface. The present method takes advantage of the fact that the optical characteristics of the co-factor, in this case the redox-active Cu site of azurin from *Pseudomonas aeruginosa*, vary with a change of its redox state. FRET, then, is the mechanism whereby a change in redox state of the co-factor translates into a change in fluorescence intensity of the label. The objective is to use this method for sensitive fluorescence detection of electron transfer events and of enzymatic turn-over, which eventually could be applied at the single-molecule level.

The model system we use is azurin, a 14-kDa large, extensively studied protein carrying a single copper ion as its redox-active centre (Fig. 1a). Originally it was thought to be involved in the nitrate respiratory chain of the organism [3–5], but recent research has made it likely that azurin in *P. aeruginosa* fulfils a role in the oxidative stress response [6]. In its oxidised (Cu^{2+}) form the protein displays a strong ($\epsilon = 5.6 \text{ mM}^{-1} \text{ cm}^{-1}$) absorption in the 550–650 nm range (see Fig. 1b), which corresponds with a $\pi\text{-}\pi^*$ transition in the MO-scheme of the Cu site, involving mainly the $d_{x^2-y^2}$ -orbital on the Cu and a 3p-orbital on the Cys112 sulfur. This absorption disappears when the Cu site is reduced because in the reduced (Cu^+) form the Cu has a d^{10} electronic configuration and the optical absorption spectrum has no bands in the 300–600 nm range.

This pronounced change of the absorption spectrum will strongly modulate the fluorescence properties of a FRET donor–acceptor pair, with the Cu site as the energy acceptor and a dye-label, suitably linked to the protein, as the fluorescent donor. (Note that there is no conformational change involved here, like distance or relative orientation.) The idea is that with the Cu in the oxidised state the fluorescence of the dye is strongly quenched as a result of energy transfer to the $\pi\text{-}\pi^*$ ex-

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cited state of the Cu site (which is non-fluorescing itself), whereas with the Cu in the reduced state the fluorescence is essentially uninhibited since the $\pi-\pi^*$ state is absent.



Fig. 1 **a** Ribbon representation of the azurin structure [12]. The copper site is indicated in *black* (Cu); space-filling representations of the engineered cysteines are indicated (12C: Gln12Cys, 27C: Lys27Cys and 42C : Asn42Cys). Coordinates were taken from 4AZU.pdb [9]. Distances between the C_α carbon atom of the engineered cysteine to the Cu centre of azurin are indicated. Note that the length of the amino acid side chain, the spacer length and the dye size (totalling ~ 1 nm) still have to be added to obtain the distance between Cy5 and the Cu atom. **b** Room temperature absorption (*black*) and emission spectrum (*grey*) of Cy5, and absorption spectrum of oxidised azurin (*dashed*). The vertical extinction scale corresponds with the absorption spectra; the vertical scale for the emission spectrum is in arbitrary units. The azurin spectrum has been expanded in the vertical direction by a factor of 10. The *grey area* indicates the region of spectral overlap. **c** The protein is fluorescently labelled by a maleimide derivative of Cy5 which is covalently linked to a cysteine residue through oxidative coupling

Thus, the fluorescent dye acts as a *passive* “beacon” which is off in the oxidised, and on in the reduced state of the protein.

Materials and methods

Cloning

Preparation and purification of the mutants N42C and K27C azurin was done according to published procedures [9]. Preparation and purification of the Q12C mutant will be described elsewhere (S. Alagaratnam, unpublished results).

Labelling

Cy5 maleimid (Amersham Biosciences; Freiburg, Germany) was dissolved in water-free dimethylsulfoxide (DMSO) to a concentration of roughly 30 mM. All purification steps were performed using centri-spin 10 size-exclusion chromatography spin columns with a 5-kDa cut-off (Princeton Separations; Adelphia, NJ, USA) according to the manufacturer’s instructions. The reaction of cystein residues with Cy5 is highly specific, and follows a well-established protocol. The genetically introduced cysteine is the only accessible protein: the native cystein-112 is ligated to the Cu ion, while cystein-3 and cystein-26 are forming a very stable disulfide-bridge under the labelling conditions. Labelling efficiencies were in the range of 0.5–1 dye-molecule/protein as estimated from the absorption spectra using the extinction coefficients of the 280-nm protein absorption and of the Cy5 label.

Labelling of K27C form

Apo-protein solution ($\sim 16 \mu\text{M}$) was incubated at room temperature for 1 h with 3 mM dithiothreitol (DTT). This step was necessary to break up dimers which might

have formed via the introduced cysteine [10]. Removal of DTT and buffer exchange to 20 mM Tris pH 7.0, 100 mM NaCl was achieved by size-exclusion chromatography, after which the sample was incubated with 1 mM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) to minimise the formation of disulfide bridges. Subsequently CuNO_3 was added up to 50 μM (roughly four times molar excess over azurin). After 10 min at room temperature Cy5 was added up to ten times molar excess. After 1 h free dye was removed by two consecutive size-exclusion chromatography steps and the sample was transferred into phosphate buffered saline (PBS) solution (150 mM NaCl, 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4), which is known for its low fluorescent background.

Labelling of Q12C and N42C

The copper form of the proteins was treated with 1 mM DTT to remove potential dimers as described above, and transferred into labelling buffer. After 20 min a five-times excess of Cy5 maleimid was added. After 1 h incubation at room temperature the labelled protein was purified as above.

All zinc forms were directly transferred into PBS and a five-to-ten-times excess of Cy5 maleimid was added. After 1 h the protein was purified in a manner similar to the copper form.

Fluorescence correlation spectroscopy

Labelling was verified by fluorescence correlation spectroscopy (FCS) [11]. The diffusion correlation time observed in this experiment is characteristic of a 14-kDa protein and not of single Cy5 molecules confirming the covalent attachment of the label to the protein. FCS also indicated that the concentration of free dye is less than 5% (limited by the sensitivity of the method).

Measurements

Fluorescence was measured with a LS 50B or LS 55 commercial fluorimeter (Perkin Elmer, USA), with a red sensitive photomultiplier (R928, Hamamatsu, Japan), set to 5-nm band pass. DTT or $\text{K}_3\text{Fe}(\text{CN})_6$ (both from Sigma-Aldrich; Steinheim, Germany) was added from stock solutions of 100 and 10 mM in H_2O .

Results and discussion

For site-specific fluorescent labelling with the dye Cy5 ($\epsilon = 250 \text{ mM}^{-1} \text{ cm}^{-1}$) we have selected three cysteine mutants of azurin, Q12C, K27C and N42C, with cysteines at positions 12, 27 or 42 in the aminoacid chain, respectively (Fig. 1a). They are at different distances

from the copper site (as measured from the C_α carbon atom) as shown in Fig. 1a. In Fig. 1b we show the absorption and fluorescence spectra of Cy5 and the absorption spectrum of oxidised azurin (ten times). The overlap of the latter with the fluorescence spectrum of Cy5 is also indicated.

The protein-dye constructs were used to investigate the influence of the redox state of azurin on the fluorescence of the attached fluorophore. Cy5-labelled azurin was either oxidised by adding $\text{K}_3\text{Fe}(\text{CN})_6$ or reduced by adding DTT, and the fluorescence was recorded as a function of time. The results are shown in Fig. 2 for the Asn42Cys (N42C) azurin variant as an example. At $t = 0$ (see Fig. 2a) the azurin is in the oxidised form. Addition

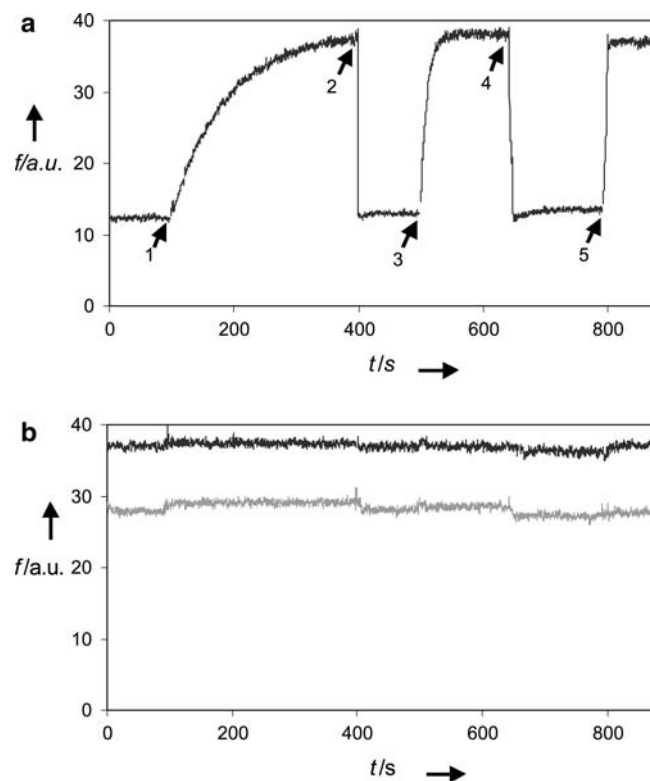


Fig. 2 Room temperature fluorescence intensity (vertical scale, arbitrary units) as a function of time (s). **a** Solution containing 3.4 nM of Cy5-labelled Asn42Cys azurin in 1:10 diluted PBS buffer, pH 7.4. At the start of the experiment ($t = 0$) the protein is in the oxidised form. At $t = 90, 480$ and 790 s (arrows labelled 1, 3 and 5) aliquots of a 100-mM solution of DTT in water were added. The summed concentrations of added reactant after the additions amounted to 1.5, 6 and 14.5 mM, respectively. At $t = 400$ and 625 s (arrows labelled 2 and 4) aliquots of a 100-mM solution of $\text{K}_3\text{Fe}(\text{CN})_6$ in water were added. The summed concentrations of this reactant after the additions amounted to 2.5 and 7.4 mM, respectively; excitation wave length: 645 nm; emission was collected at 665 nm. **b** Similar experiment on a 1.1-nM solution of the Zn form of the Cy5-labelled N42C azurin variant (lower trace) and a solution containing 1.4 nM of Cy5, only (upper trace). Buffer 1:10 diluted PBS, pH 7.4. Additions of reductant and oxidant were made at approximately the same time points as in (a). The small stepwise intensity changes observed for the lower trace are due to a slight contamination of the Zn azurin with the Cu form and slight dilution effects

Table 1 Quenching ratios observed for the three Cy5-labelled azurin variants listed in the first column and calculated distances between the Cu and the C_α positions of the engineered cystein residues

Azurin variant	C _α -Cu distance, nm	Quenching ratio
Q12C	0.9	0.57
K27C	2.8	0.54
N42C	1.0	0.78

Distances were taken from pdb files 4AZU & 5AZU [9]. The actual distance, R , from the Cy5 fluorophore to the Cu may differ by as much as 0.5–1 nm from the C_α-Cu distance. The quenching ratio, QR , is defined as $QR = (F_r - F_o)/F_r$ with F_r and F_o being the fluorescence intensities of the reduced and oxidised Cy5-labelled azurin

of an excess of DTT at $t = 100$ s causes a threefold increase in the fluorescence while subsequent addition of an excess of the oxidant brings the fluorescence back to the original level. Further additions of reductant and oxidant show that the switching is reversible. It is clear that in the beginning (100–400 s) the reduction rate is much slower than the oxidation rate (at $t = 400$ s), but that the reduction becomes faster at later stages. This is because the increasing amount of ferri/ferrocyanide, added at subsequent oxidation steps, acts as a mediator for the reductant.

As a control we have repeated the experiment with Cy5-labelled redox-inactive zinc azurin and with a solution containing only the Cy5 label. The results are shown in Fig. 2b. It is clear that the switching of the fluorescence as observed in Fig. 2a, is absent. For the other two azurin variants (K27C and Q12C) similar results were obtained. The data are summarised in Table 1.

The assumed quenching mechanism, FRET, is a strongly distance-dependent process. The quenching ratio, QR , as a function of distance R between the Cy5 label and the Cu site, is given by [1]:

$$QR = \frac{F_r - F_o}{F_r} = \frac{R_0^6}{R_0^6 + R^6} \quad (1)$$

F_r and F_o denote the fluorescence intensity of the labelled azurin in the reduced and oxidised form. R_0 is a characteristic distance that depends on the refractive index, n , the spectral overlap between donor and acceptor bands, $J(\lambda)$, the fluorescence quantum yield of the donor, Q_D , and the relative orientation of the optical transition moments of donor (Cy5) and acceptor (Cu centre) as reflected by the orientation factor κ^2 [1]. The latter may vary between 0 and 4 and amounts to 2/3 for two freely rotating dipoles. With $Q_D = 0.27$ [7], $\kappa^2 = 2/3$, $n = 1.4$ and $J(\lambda) = 7.3 \times 10^{-14} \text{ M}^{-1} \text{ cm}^3$ we obtain a Förster radius R_0 of 3.8 nm [1]. The actual value of R_0 may differ by as much as 20–30% from this value depending on κ^2 and the conformation of the label with respect to the protein. The purpose of the calculation is not to obtain a precise value of R_0 , but to show that for the combination of donor and acceptor chosen here, R_0 is of

a similar size as azurin, which has dimensions of 2.5×3×4 nm. Thus, a bound Cy5 label will exhibit a sizeable quenching ratio in agreement with the experimentally observed effects (Table 1 and Fig. 2). It is worthwhile to point out that the quenching ratio gives a rough estimate of the label-to-quencher distance ($QR = 0.6–0.8$ with $R = 0.9–1.0$ nm; $QR = 0.5$ with $R = 2.8$ nm), but for a precise estimate the values of κ would be needed.

In summary, these experiments demonstrate that the fluorescence of a dye coupled to a protein can be strongly affected by a change in oxidation state of the protein. This documents a very sensitive way to monitor changes in the redox state of a protein. The protein concentrations used in the present experiments amount to a few nM. Considering the S/N observed in Fig. 2a, the concentrations can be easily lowered by two or more orders of magnitude without compromising the S/N ratio when time averaging techniques would be employed, in addition. The method is not only applicable to proteins that contain a redox-active type 1 Cu site, but also to other proteins with co-factors that exhibit comparable changes in the absorption spectrum upon a change of redox state or another biochemical variable. Experiments in the lower picomolar range are within reach, which opens up opportunities for investigating molecules which are only available in minute quantities. Since Cy5 is a common dye for single-molecule fluorescence detection [8], the method presented here has the potential to study redox events in enzymes and proteins at the single-molecule level. Experiments are underway to explore these possibilities.

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