A FRET-based method to study the activity of electron or oxygen transfer proteins and redox enzymes

Zauner, G.

Chapter II

A FRET-based method for fluorescence detection of the protein redox state[#]

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Abstract

A method for fluorescence detection of a protein's redox state based on resonance energy transfer from an attached fluorescence label to the prosthetic group of the redox protein is described and tested for proteins containing three types of prosthetic groups: a type-I copper site (azurin, amicyanin, plastocyanin and pseudoazurin), a heme group (cytochrome c550) and a flavin mononucleotide (flavodoxin). This method permits to reliably distinguish between reduced and oxidized proteins and to perform potentiometric titrations at submicromolar concentrations.

Introduction

Redox proteins and enzymes participate in key biological processes such as respiration, photosynthesis etc. Redox *proteins* act as electron carriers in electron transfer chains, while redox *enzymes* catalyze redox reactions by which one substance is converted into another. Most redox proteins and enzymes contain one or several prosthetic groups that determine their redox properties. To study the mechanism of redox enzymes and the function of electron transfer chains it is important to be able to monitor the redox state of the prosthetic groups.

One of the indicators of the redox state of a prosthetic group is its optical absorption spectrum. Many prosthetic groups absorb in the UV or visible region and their spectra change with their redox state. However, the use of absorption spectra for monitoring a protein's redox state may become problematic when the protein concentration is low or when the sample contains several components with overlapping absorption spectra. Here we describe a method for sensing the changes in protein absorption spectra based on Förster Resonance Energy Transfer (FRET).

FRET is widely used in biology as a sensitive method for measuring inter- and intramolecular distances ("spectroscopic ruler") (1;2). The excitation energy from a fluorescent donor molecule is transferred by a radiationless process to an acceptor chromophore, resulting in a decrease of donor fluorescence. The probability of this transfer (FRET efficiency, E) drops off as the inverse sixth power of the distance between donor and acceptor, R:

$$E=R_0^6/(R_0^6+R^6), \qquad \text{Eq.2.1}$$

where R_0 is the Förster radius, a parameter depending on the spectral properties of donor and acceptor and their relative orientation (2). Thus, if R_0 is known, FRET efficiency can be used to estimate the donor-acceptor distance, R.

In most cases fluorescent labels are used both as donor and acceptor for FRET. However, a protein's prosthetic group itself can also act as an acceptor. Rischel *et al*, attached the Alexa488 dye to yeast cytochrome c and used FRET from the dye to the heme to monitor the protein denaturation by Fluorescence Correlation Spectroscopy (3). Energy transfer from Cascade Blue to the heme of horse heart cytochrome c was used by Taylor *et al* for evaluating the distance from the dye to the heme (4). A similar method was applied in a myoglobin-detecting biosensor by using Cascade Blue-labeled antibody against myoglobin (5). Barker *et al* observed the effect of nitric oxide binding to cytochrome c' by the changes in the efficiency of FRET from the Oregon Green fluorescent label to the heme (6).

According to Förster, R_0 depends on the spectral overlap between the donor fluorescence and acceptor absorbance. When the protein prosthetic group is a FRET acceptor and its absorption spectrum changes with its redox state, R_0 will also depend on the redox state of the prosthetic group. With constant R, equation Eq.2.1 can be used to estimate R_0 (and hence the redox state of the protein) from the measured FRET efficiency.

Several conditions have to be fulfilled for this method to work:

- The absorption spectra of the protein prosthetic group should be different in the oxidized and the reduced state;
- The spectral overlap between the label fluorescence and the prosthetic group absorption should differ for the oxidized and reduced prosthetic group;
- The distance between the donor and the prosthetic group and their relative orientation should be within a range dictated by the characteristics of the FRET mechanism.

In the present work we present a proof of principle for the method of fluorescence detection of protein redox states with the Cy5 fluorescence label as a donor for redox proteins with three different types of prosthetic groups: *Pseudomonas aeruginosa* azurin, amicyanin from *Paracoccus versutus*, plastocyanin from *Dryopteris crassirhizoma* and *Alcaligenes faecalis* pseudoazurin, all four containing a type-1 Cu site; the hemoprotein cytochrome c550 from *Paracoccus versutus*; and the flavin-containing flavodoxin II from *Azotobacter vinelandii*. It will be shown that this method permits to reliably distinguish between reduced and oxidized proteins and to perform potentiometric titrations at submicromolar concentrations.

Materials and methods

Materials

Wild type azurin from *Pseudomonas aeruginosa* was overexpressed in *E. coli* and purified as previously described (7). Cytochrome c550 from *Paracoccus versutus* was expressed and purified as earlier described (8). Flavodoxin II C69A/V100C from *Azotobacter vinelandii* ATCC 478 was purified as described previously (9). Amicyanin from *Paracoccus versutus*, plastocyanin from *Dryopteris crassirhizoma* and *Alcaligenes faecalis* pseudoazurin were expressed and purified as described elsewhere (10-12).

Cy5 maleimide and NHS-ester were purchased from Amersham Biosciences (Freiburg, Germany). The stock solutions of the dyes were prepared by dissolving them in water-free dimethylsulfoxide to a concentration of roughly 30 mM.

All purification steps during protein labeling were performed using Centrispin 10 size-exclusion chromatography spin columns with a 5kDa cutoff (Princeton Separations; Adelphia, NJ, USA) according to the manufacturer's instructions.

Protein labeling

Flavodoxin II C69A/V100C was labeled on the mutated cysteine residue (Cys100) with Cy5 maleimide, whereas all other proteins were labeled at amino groups using Cy5 NHS-ester.

For amino labeling, Cy5 NHS-ester was added in 10 times molar excess to the 100 μM proteins in HEPES 20 mM, pH 8.3 and incubated for 2 hours at room temperature. These conditions are recommended by the manufacturers for N-terminal labeling. The unbound label was then removed by two consecutive size-exclusion chromatography steps.

For cysteine labeling, 100 μM C69A/V100C flavodoxin in HEPES 20 mM pH 7.0 was first incubated with 10 times molar excess of dithiotreitol (DTT) for 1 hour at room temperature to break the possible disulfide bridges between the introduced cysteines. After incubation, excess dithiotreitol was removed by a single step of size-exclusion chromatography. Then Cy5 maleimide was added to the protein in about 10–fold molar excess and left for 1 hour at room temperature before removing the unbound label as above.

The protein labeling ratio (dye/protein molecule) was estimated from the absorption spectra of labeled proteins, using ε_{645}=250 mM^{-1} cm^{-1} for Cy5 (13), ε_{280} =9.8 mM^{-1} cm^{-1} for azurin (14), ε_{410} =134 mM^{-1} cm^{-1} for cytochrome c550 (15) and ε_{452} =11.3 mM^{-1} cm^{-1} for flavodoxin (9).

Fluorescence and absorption spectroscopy

Absorption spectra were measured using a Perkin Elmer Instruments Lambda 800 spectrophotometer with a slit width equivalent to a bandwidth of 2 nm. Fluorescence spectra and time courses were measured with an LS 55 commercial fluorimeter (Perkin Elmer, USA), with a red sensitive photomultiplier (R928, Hamamatsu, Japan), set to 8 nm band pass. Cy5 fluorescence was excited at 645 nm, fluorescence intensity at 665 nm was used for the analysis of the FRET efficiency.

Fluorescence time courses

Fluorescence time courses were measured in a 5x5 mm quartz fluorescence cuvette (Perkin Elmer) in 20 mM HEPES, pH 7 or pH 8.3. The protein concentration was 1-10 μM. Protein reduction and oxidation during measurement was performed by adding reductants (dithiotreitol or ascorbate) and oxidant (sodium ferricyanide) from concentrated stock solutions directly into the cuvette to a final concentration of 1-3 mM.

Redox titrations

Potentiometric redox titrations were performed in 20 mM HEPES, pH 7 or pH 8.3 using a home made spectrophotometric cuvette for potentiometric titrations as described by Dutton (16) with 10 mm optical pathlength. A saturated calomel electrode was used as a reference electrode. A gold rod electrode (BAS Electrochemistry) was used as a measuring electrode for azurin and cytochrome titrations. For the C69A/V100C flavodoxin titration we used a platinum measuring electrode to avoid possible interaction of the surface cysteine with the gold electrode.

Potassium ferricyanide and dithiotreitol (azurin and cytochrome) or sodium dithionite (flavodoxin) were used to change the potential of the solution. When dithionite was used as a reductant, the buffer was deoxygenated in the potentiometric cuvette prior to measurements by passing Ar through it for 3 hours. After that the protein was added and deoxygenation was continued for 30 minutes. An Ar flow over the sample was also maintained during the measurements. In the flavodoxin titration 12 μM benzylviologen was added to the sample at the start of the titration as a mediator to facilitate protein reduction by sodium dithionite.

Förster radius calculations

R_0 was calculated as previously described(17) from the equation $R_0=0.211(J\kappa^2 n^{-4}\Phi_D)^{1/6}$ (Å). Here κ^2 is an orientation factor, n –refractive index, Φ_D –fluorescence quantum yield of the donor and J–spectral overlap integral, defined as $J= \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda / \int F_D(\lambda)d\lambda$, where $F_D(\lambda)$ is the fluorescence intensity of the donor, $\varepsilon_A(\lambda)$ - the extinction coefficient of the acceptor at wavelength λ with λ expressed in nanometers. Experimental protein absorption spectra and the Cy5 fluorescence spectrum supplied by the manufacturer (Amersham Biosciences) were used for the calculations. The refractive index was assumed to be 1.4 and the orientation factor κ^2 was taken to be 2/3 which corresponds to random orientations of both donor and acceptor (2;17). Incomplete isotropic averaging might affect the value of κ and, thus, the estimated Förster radius. It might also influence the comparison of the quenching efficiency among different dyes with the same protein since the transition dipoles of the different dyes might be oriented differently with respect to the acceptor within the protein framework. In the course of our experiments we have found no indications, however, for incomplete orientational averaging and therefore we followed the customary practice of using $\kappa^2 = 2/3$ throughout our study. Φ_D for Cy5 was taken to be 0.27 (18).

The distance (R) from Cy5 to the accepting prosthetic group was estimated as R= (d +1) nm ± 0.5 nm, where d is the distance from the attachment point of the dye (N-terminus for azurin, lysines for cytochrome and C_α of the Cys100 for the C69A/V100C flavodoxin). The distance d was estimated from the protein crystal structures. Adding 1 nm to the calculated distance d accounts for the approximate length of the linker chain.

It is conceivable that the solution structures might differ from the XRD structures and therefore the estimate of R, which is based on the latter, might differ from the value in solution. NMR data on the proteins used in this study contain no indications for gross differences between solution and XRD structures and we are confident therefore to use the XRD-based values with the above indicated margin of ± 0.5 nm.

Mass spectrometry

Electrospray ionization (ESI) mass spectrometry analyses of the intact protein were carried out on a MicroTOF instrument (Bruker Daltonics, Bremen). Protein samples (5–10 pmol/μl) dissolved in 0.2% formic acid and 50% methanol were continuously infused into the ESI source at a flow rate of 180 μl/hour. Spectra were recorded in the positive ion mode and the standard m/z range of 200–3000 was monitored. Molecular masses of proteins were calculated using a maximum entropy deconvolution algorithm incorporated as part of the DataAnalysis software supplied with the mass spectrometer.

For matrix-assisted laser desorption (MALDI) analysis of the trypsin-digested labeled proteins about 100 pM protein was resuspended in 100 μL of 25 mM ammonium bicarbonate (pH = 8.0). To this 2 μL of trypsin (1 μg/μL) solution was added. The reaction was carried out at 37^0 C for 16 h. After digestion, the peptides were desalted

using Poros 50 R2, packed in a pipette tip. Peptides were eluted in 60% acetonitrile/0.01% TFA and measured by MALDI-MS (Ultraflex II, Bruker Daltonics, Bremen) using α-cyano-4-hydroxycinnamic acid as a matrix.

Results and Discussion

Optimization of the labeling conditions

The labeling conditions were optimized to ensure that the dye -to-protein ratio is less than one. While a small dye-to-protein ratio only affects the sensitivity of the experiment, a ratio larger than 1 makes the estimation of donor-acceptor distances from the attached label to the prosthetic group of the protein unreliable. Moreover, it has been shown that proteins labeled with multiple amino reactive Cy5 labels show a decrease in fluorescence due to mutual resonance energy transfer and because of the formation of nonfluorescent dimers (19). The labeling ratios were estimated from the absorption spectra and found to be 20-90% depending on the protein and the experiment. The absorption spectrum of Cy5 was found to be unaffected by binding to any of the proteins used in this study in accordance with a previous report (19) and excludes the Cy5 dimer formation (20).

For azurin and cytochrome c550 electrospray ionization mass spectrometry was performed to check the number of label molecules per protein. For C69A/V100C flavodoxin mutant this was deemed unnecessary since there is only a single cysteine available for the Cy5-maleimide binding. For azurin and cytochrome c550 only peaks arising from unlabeled and singly labeled proteins were observed, showing that no protein molecules with multiple labels were present in the sample (see Figure 2.1 for azurin, for the cytochrome c550 the results are not shown).

To identify the exact location of the label the MALDI analysis of the trypsin digests of azurin and cytochrome c550 was made. In the MALDI mass spectrum of the Cy5-labeled azurin the Cy5 adduct of the fragment corresponding to residues 1-27 was found (see the inset to Figure 2.1). No Cy5 adducts of other peptides were seen in the spectrum. This observation combined with the presence of one label per protein at most (Figure 2.1) indicates that Cy5 NHS-ester reacts with the N-terminus of azurin only.

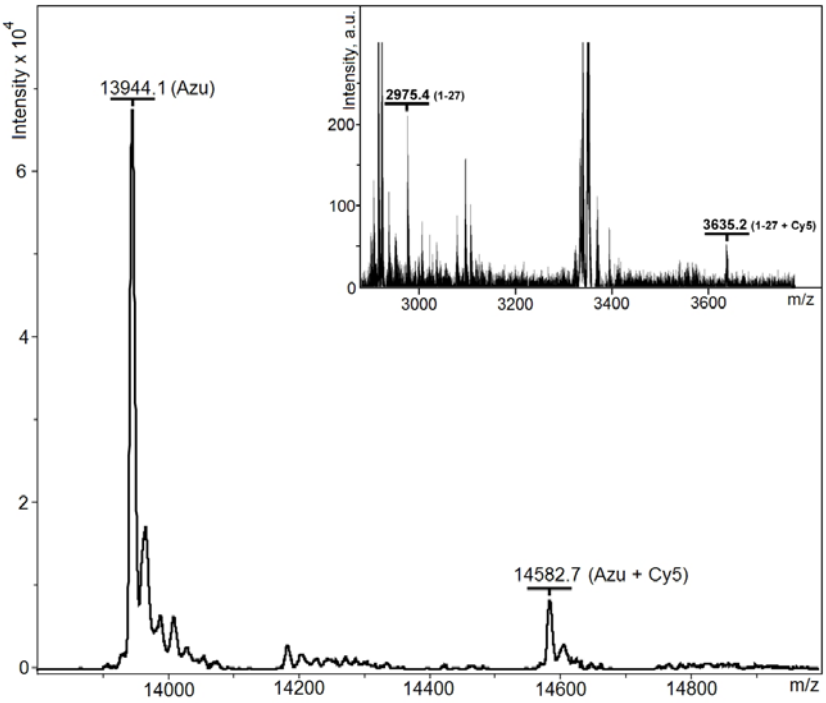

Figure 2.1. Deconvoluted ESI mass spectra of Cy5 labeled azurin, showing two species: native protein (13944) and protein with singe label (14582). The inset shows the trypsin digest of azurin Cy5 with the N-terminal (1-27) fragment and the sodium adduct of the same fragment with fluorescent label (1-27 + Cy5).

The MALDI analysis of the trypsin digest of cytochrome c550 from *Paracoccus versutus* shows that the N-terminus of the protein is pyroglutamated and thus inaccessible for labeling with Cy5 NHS-ester. Although the whole protein ESI mass spectrum shows a significant amount of cytochrome c550 labeled with Cy5, no labeled peptides could be identified in the MALDI mass spectrum of the trypsin digest of cytochrome c550 labeled with Cy5 NHS-ester (results not shown). This is probably caused by the fact that the label has an approximately equal probability of binding to any of the 13 exposed lysines of the cytochrome c550 so that the peaks arising from every of the corresponding labeled peptides are too small to be detected.

Fluorescence determination of protein redox states: azurin and other blue copper proteins

Azurin from *Pseudomonas aeruginosa* is a small (14kDa) electron transfer protein containing a type-1 Cu centre. Its physiological role has not been established with certainty, although it was shown to react *in vitro* with several redox proteins such as cytochrome c551 and various dehydrogenases (14). In its oxidized form it has a prominent absorption band at 630 nm (ε=5.7 mM^{-1} cm^{-1}) (14) that disappears upon reduction. This absorption band has a good spectral overlap with Cy5 fluorescence (Figure 2.2A), giving a Förster radius of 3.8 nm. The donor-acceptor distance for Cy5 attached to the N-terminus of *Pseudomonas aeruginosa* azurin as estimated from its crystal structure (4AZU.pdb, (14)) is 3.4 ± 0.5 nm, and should ensure high efficiency of resonance energy transfer from the fluorophore to the oxidized type-1 Cu centre (Figure 2.2B). As the reduced protein does not absorb above 500 nm, the FRET from Cy5 to the reduced type-1 Cu centre is not possible and reduced labeled protein can be expected to be significantly more fluorescent than the oxidized protein.

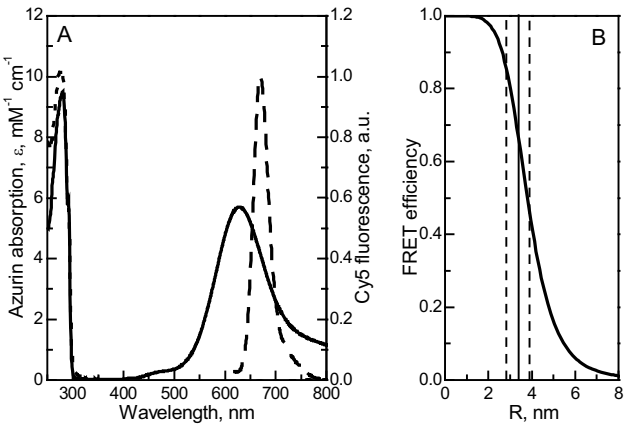

Figure 2.2. Azurin absorption spectra and estimated resonance energy transfer efficiency between Cy5 and the type-1 Cu site of azurin. A. Absorption spectra of oxidized (solid line) and reduced (dotted line) azurin from *Pseudomonas aeruginosa* and fluorescence spectrum of Cy5 (dashed line). B. Estimated FRET efficiency from Cy5 attached to N-terminus of the azurin to the oxidized type-1 Cu site. Solid vertical line marks the estimated donor-acceptor distance, dotted vertical lines – its estimated uncertainty.

It has been shown previously that the fluorescence intensity of Cy5-labeled azurin increases and decreases upon addition of excess reductant and oxidant, respectively. Cy5 attached to azurin where the Cu atom in the type-1 centre had been replaced by Zn, did not show any changes in fluorescence upon reduction or oxidation (21).

The absorption band at 590-630 nm present in the Cu(II) state and absent in the Cu(I) state is a common feature for all the type-1 Cu centres. It can, thus, be expected that other blue copper proteins, labeled with Cy5, will also show a significant resonance energy transfer from the fluorophore to the Cu centre in the oxidized but not in the reduced state. Figure 2.3 shows the changes in fluorescence intensity of several blue copper proteins with a Cy5-labeled N-terminus upon oxidation and reduction. *Pseudomonas aeruginosa* azurin, amicyanin from *Paracoccus versutus*, plastocyanin from *Dryopteris crassirhizoma* and *Alcaligenes faecalis* pseudoazurin all show a significant decrease in fluorescence intensity upon oxidation, while on protein reduction fluorescence goes back to almost the initial value (Figure 2.3B, C, D and E). The

effect does not depend on whether at the start of the experiment the protein is oxidized (Figure 2.3E) or reduced (Figure 2.3B, C, D). As all the studied blue copper proteins have similar absorption spectra as well as similar shape and size (9-15 kDa), the Förster radii for Cy5 – type-1 Cu(II) resonance energy transfer and the donor-acceptor distances are also expected to be similar.

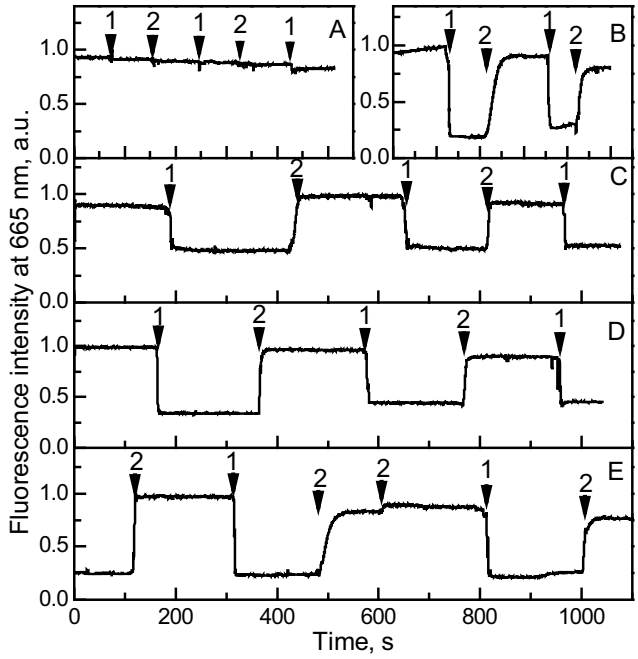

Figure 2.3. Changes in fluorescence intensity of blue copper proteins with Cy5-labeled N-terminus upon oxidation and reduction. A: *Pseudomonas aeruginosa* azurin with Cu replaced by Zn; B: *Pseudomonas aeruginosa* azurin; C: amicyanin from *Paracoccus versutus*; D: plastocyanin from fern *Dryopteris crassirhizoma*; E: pseudoazurin from *Alcaligenes faecalis*. The fluorescence intensity has been normalized to its maximal value to simplify the comparison. Arrows indicate the additions of excess oxidant (1) (potassium ferricyanide) or reductant (2) (dithiotreitol in A-D, ascorbate in E). Slower fluorescence rise in some steps in B and E is probably caused by a smaller excess of added DTT over ferricyanide already present in the sample as compared to other steps.

The data in Figure 2.3 show that azurin, amicyanin, plastocyanin and pseudoazurin labeled on the N-terminus with Cy5 are about 80 % less fluorescent in the oxidized than in the reduced state. This value is also in good agreement with the expected

energy transfer efficiency of 65 ± 20 % from Cy5 attached to the N-terminus of the type-1 Cu centre for oxidized azurin (Figure 2.2B). The fluorescence of free Cy5 (results not shown) and of Cy5 attached to a redox-inactive azurin in which the Cu has been removed (not shown) or replaced by Zn (Figure 2.3A) does not change upon oxidation or reduction. This indicates that the fluorescence quenching/enhancement upon oxidation/reduction is not due to chemical changes in the dye.

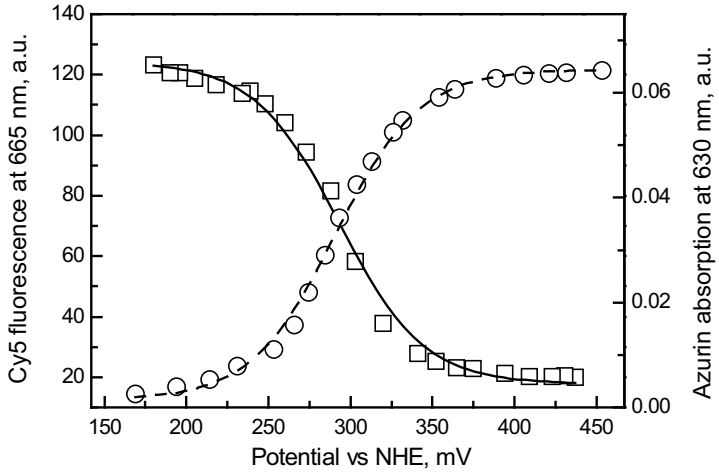

Figure 2.4. Potentiometric titrations of azurin from *Pseudomonas aeruginosa* is monitored by absorption and fluorescence. Squares: intensity of the fluorescence at 665 nm of Cy5 attached to the protein; solid line, Nernst fit of fluorescence intensity, giving a midpoint potential of 293 ± 2 mV vs NHE. Circles, azurin absorption at 630 nm; dashed line: Nernst fit of the absorption at 630 nm, giving a midpoint potential of 291 ± 2 mV vs NHE. Both titrations were performed in 20 mM HEPES, pH 8.3, protein concentration was 20 μM for the absorption and 0.5 μM for the fluorescence titration.

To check whether fluorescence intensity can be used to estimate the degree of protein oxidation or reduction in the sample, a potentiometric titration of azurin labeled with Cy5 on the N-terminus has been performed. Figure 2.4 shows a potentiometric titration of azurin monitored by the absorption at 630 nm and the titration of azurin labeled on the N-terminus with Cy5, monitored by Cy5 fluorescence at 665 nm. It can be seen that the fluorescence intensity of the attached dye goes up as the absorption

of the type-1 Cu(II) site at 630 nm decreases. The midpoint potentials obtained from the fits of both titration curves to the Nernst equation coincide (293 ± 2 mV vs NHE for the fluorescence and 291 ± 2 mV vs NHE for the absorption titration) and are in good agreement with the previously reported value of 292 mV vs NHE for the midpoint potential of *Pseudomonas aeruginosa* at pH 8 (22).

It should be noticed that the protein concentration used for the fluorescence titration is 40 times smaller than the one used for the absorption titration. It shows that the fluorescence method for monitoring the protein redox state has a significantly higher sensitivity compared to the absorption method.

Fluorescence determination of protein redox states: cytochrome c550

Cytochrome c550 from *Paracoccus versutus* is a 14.7 kDa heme-containing electron carrier protein present in the methylamine oxidising chain of this bacterium where it acts as an electron donor for the membrane-bound cytochrome c oxidase (15). It belongs to the class I of c-type cytochromes and contains a covalently-bound heme located asymmetrically near the protein surface, which is low-spin both in the oxidized and reduced forms. Reduced cytochrome c550 shows an intense absorption band at 416 nm (Soret band), a sharp peak at 550 nm (α band) and a smaller band at 522 nm (β band). In the oxidized form of the protein the Soret band is shifted and decreases in intensity, while α and β bands merge into a single broad absorption peak. Oxidized cytochrome also absorbs in the region of 570-750 nm, where the absorption of reduced cytochrome is significantly lower (Figure 2.5A). Although the spectral differences between the cytochrome absorptions in the oxidized and the reduced forms are significant, the spectra contain many isosbestic points, the distances between which are smaller than the width of the fluorescence bands of most commercially available fluorescent labels. Thus, for the labels fluorescing in the region where there are big differences in absorption between the oxidized and reduced

cytochrome, the effective change in spectral overlap with the fluorescence of the label is not so big. We chose Cy5 as a fluorescent donor, as the overlap between its fluorescence and the cytochrome absorption shows a small but stable increase on cytochrome oxidation (Figure 2.5A). The estimated Förster radii for FRET from Cy5 to the heme are 2.6 nm for the oxidized and 2.0 nm for the reduced cytochrome (Figure 2.5B). As the MALDI analysis of the labeled cytochrome c550 after trypsinolysis indicates that Cy5 has an equal probability to attach to any of the exposed lysines, the donor-acceptor distance from Cy5 to the heme is estimated from the crystal structure (23) as an average over all the possible attachment points and equals 2.8 ± 0.8 nm. For this donor-acceptor distance the estimated difference between the maximal and minimum fluorescence is about 30% (Figure 2.5B).

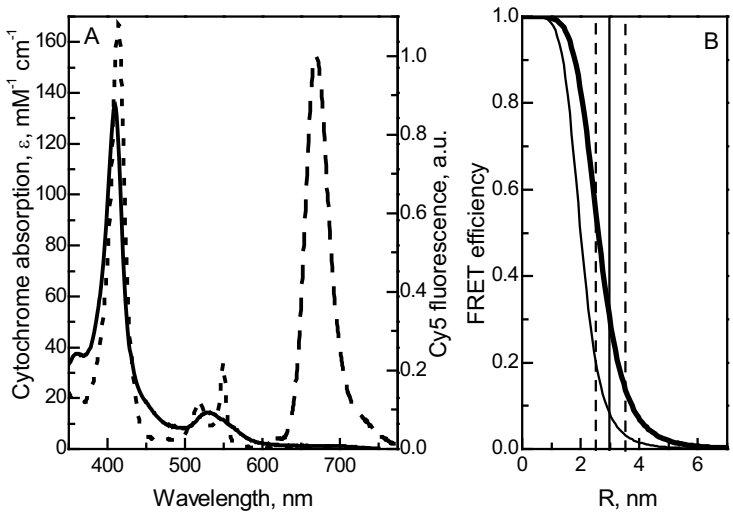

Figure 2.5. Cytochrome absorption spectra and estimated resonance energy transfer efficiency between Cy5 and the heme of the cytochrome. A. Absorption spectra of oxidized (solid line) and reduced (dotted line) cytochrome c550 from *Paracoccus versutus* and fluorescence spectrum of Cy5 (dashed line). B. Estimated FRET efficiency from Cy5 attached to N-terminus of cytochrome c550 to oxidized (thick line) and reduced (thin line) heme. Solid vertical line marks the estimated donor-acceptor distance, dotted vertical lines – its estimated uncertainty.

Figure 2.6 shows potentiometric titrations of cytochrome c550 based on the absorption at 550 nm and of cytochrome labeled with Cy5 NHS-ester based on the fluorescence at 665 nm. The Nernst fit of the absorption titration gives a midpoint potential of 300 ± 1 mV vs NHE, the fit of the titration by fluorescence gives a midpoint of 286 ± 4 mV vs NHE. The small discrepancy between the two values may be due to small variations between the lowest and highest fluorescence intensities leading to imprecise measurement of the midpoint potential on the basis of fluorescence. Both values for the midpoint potentials observed in this study are slightly higher than the previously reported value of 255 mV vs NHE (15).

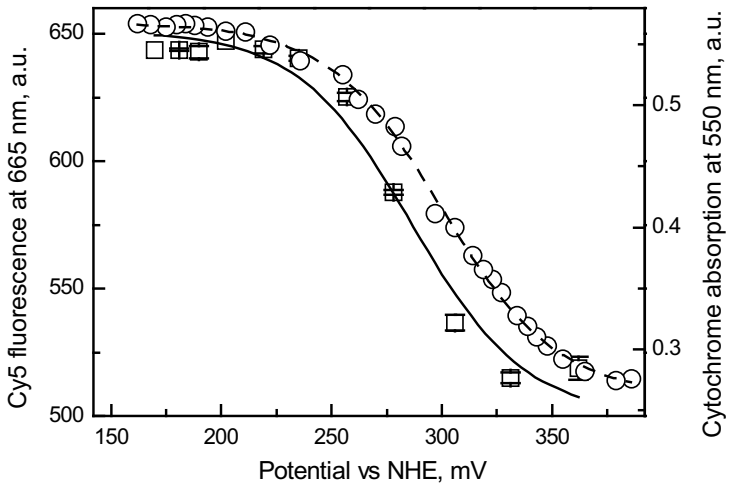

Figure 2.6. Potentiometric titrations of cytochrome c550 from *Paracoccus versutus* by absorption and fluorescence. Squares: intensity of the fluorescence of Cy5 attached to the protein; solid line, Nernst fit of fluorescence intensity at 665 nm, giving a midpoint potential of 286 ± 4 mV vs NHE. Circles, cytochrome absorption at 550 nm; dashed line: Nernst fit of the absorption at 550 nm, giving a midpoint potential of 300 ± 1 mV vs NHE. Both titrations were performed in 20 mM HEPES, pH 7, protein concentration was 20 μM for the absorption and 1 μM for the fluorescence titration.

Fluorescence determination of protein redox states: flavodoxin

Flavodoxins are electron transfer proteins, containing flavin mononucleotide (FMN) as a prosthetic group. FMN can exist in three possible redox states: oxidized (quinone), one-electron reduced (semiquinone) and two-electron reduced (hydroquinone). While in most cases flavodoxin expression is induced by iron deficiency, in *Azotobacter vinelandii* flavodoxin is expressed constitutively (24) and is likely to be an electron donor for the nitrogenase (9). *Azotobacter vinelandii* flavodoxins were reported to be unusually stable in the semiquinone form compared to other flavodoxins (9;25), facilitating the study of the one-electron reduced state of this protein.

Figure 2.7A shows the absorption spectra of oxidized and singly reduced flavodoxin II from *Azotobacter vinelandii* ATCC 478. In the semiquinone state a broad absorption peak appears between 580 and 620 nm that extends to 700 nm, which is not present in either the fully oxidized or the fully reduced state while the quinone form still has a weak absorption above 550 nm (Figure 2.7A). This makes Cy5 a suitable donor to distinguish between the oxidized and one-electron reduced flavodoxin using FRET efficiency (Figure 2.7B). The estimated Förster radii for FRET from Cy5 are 3.2 nm for the one-electron reduced flavodoxin and 1.1 nm for the fully oxidized state.

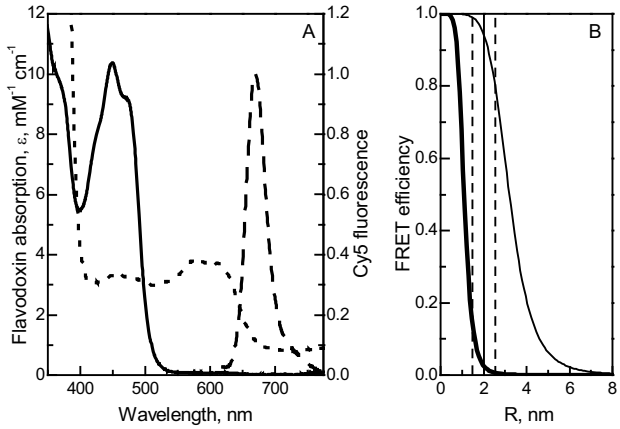

Figure 2.7. Flavodoxin absorption spectra and estimated resonance energy transfer efficiency between Cy5 and the flavin of the flavodoxin. A. Absorption spectra of fully oxidized (solid line) and singly reduced (semiquinone state, dotted line) C69A/V100C flavodoxin II from *Azotobacter vinelandii* and fluorescence spectrum of Cy5 (dashed line). B. Estimated FRET efficiency from Cy5 attached to Cys100 of C69A/V100C flavodoxin to oxidized (thick line) and semiquinone (thin line) flavin. Solid vertical line marks the estimated donor-acceptor distance, dotted vertical lines – its estimated uncertainty.

We used the C69A/V100C flavodoxin mutant, in which the natural exposed Cys69 is replaced by Ala and a new cysteine is introduced in position 100. Cys100 is only 9 Å from the flavin (26) and thus the donor-acceptor distance for the Cy5 attached to Cys100 can be roughly estimated as 2 ± 0.5 nm.

Potentiometric titrations of C69A/V100C flavodoxin by absorption at 577 nm and fluorescence of the Cy5 label attached to Cys100 at pH 7 are shown in Figure 2.8. The data show that the fluorescence intensity decreases as the absorption at 580-620 nm goes up. The Nernst fits of absorption and fluorescence titrations give identical midpoint potentials (-120 ± 9 mV vs NHE for the fluorescence and -126 ± 5 mV vs NHE for absorption). This value is in the interval of -45 ± 10 mV (pH 6) and -179 ± 10 mV (pH 8.5) vs NHE determined for the quinone/semiquinone potential of the C69A flavodoxin mutant by EPR titration (27).

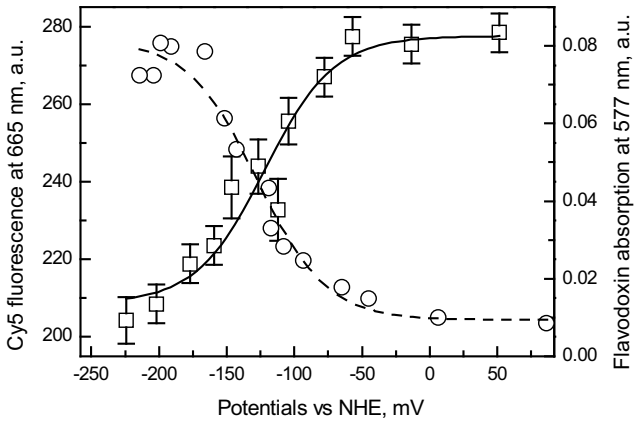

Figure 2.8. Potentiometric titrations of flavodoxin II from *Azotobacter vinelandii* by absorption and fluorescence. Squares, averaged intensity of the fluorescence of Cy5 attached to the protein; solid line: Nernst fit of the fluorescence intensity at 665 nm, giving a midpoint potential of -120 ± 9 mV vs NHE. The error bars represent the standard deviation of the measured fluorescence intensity. Circles, flavodoxin absorption at 577 nm; dashed line, Nernst fit of the absorption at 577 nm, giving the midpoint potential of -126 ± 5 mV vs NHE. Both titrations were performed in 20 mM HEPES, pH 7, protein concentration was 20 μM for the absorption and 8 μM for the fluorescence titration.

The difference between the maximal and minimal fluorescence is 25%, which is smaller than the value of 81 to 100% estimated from the Förster radii for Cy5-flavin FRET pair and the donor-acceptor distance assuming the orientation factor $\kappa^2=2/3$. This may have several causes. First, the distance between the donor and acceptor is rather small and thus the energy transfer can be affected by direct interaction between the fluorescent label and the flavin. Fluorescence quenching caused by Cy5 dimer formation (19;20) or from static quenching from interaction between Cy5 and Cy5.5 (28) have been reported, although in our case the absorption spectra of both flavin and the dye are not affected by the labeling (results not shown) and thus the formation of a complex between the dye and the flavin is not likely.

Second, quenching by photoinduced electron transfer to the dye is observed for some dyes in close contact with nucleotide bases or aromatic aminoacids (29;30). Cyanine

dyes were reported to be able to donate electrons to electron acceptors in solution with varying efficiency, depending on the electron acceptor (31), but if it occurs at all in our case it will be weakened considerably due to the much larger distance over which the electron has to be transferred. Nevertheless, it can not be completely excluded that the fluorescence of Cy5 is quenched by oxidized flavin through an electron transfer mechanism, while the semiquinone form of FMN cannot accept an electron from the dye due to the low midpoint potential of the semiquinone/hydroquinone pair (27). Thirdly, other mechanisms than Förster energy transfer (such as the Dexter mechanism) may operate when the donor and acceptor are close (32). Finally, the preferred orientation and the position of the label can be affected by interaction (e.g. stacking) with the flavin, affecting both the donor-acceptor distance and the orientation factor.

Nevertheless, in this case as well, the fluorescence intensity of the attached Cy5 permits to distinguish reliably between the oxidized and one-electron reduced states of flavodoxin and to measure the quinone/semiquinone midpoint potential.

Conclusions

The present paper gives a proof of principle for the fluorescence detection of a protein's redox state based on resonance energy transfer from an attached fluorescent label to the prosthetic group of the redox protein. This method permits not only to distinguish between the fully oxidized and fully reduced state of the protein but to estimate the degree of protein reduction or oxidation in the sample at submicromolar concentration. It can be potentially applied to any prosthetic group in a redox protein that changes its absorption spectrum upon reduction/oxidation, provided that a fluorescent label with a suitable fluorescence spectrum and a proper label attachment point can be chosen. While easily applied to small electron transfer proteins, as we

have demonstrated, this method can also prove useful to study redox proteins and enzymes with several prosthetic groups to selectively monitor the redox state of one of them during electron transfer. Efforts are going on to test the applicability of the method for monitoring the kinetics of redox reactions as well.

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