DOI: 10.1002/chem.200601806

Tryptophan-to-Dye Fluorescence Energy Transfer Applied to Oxygen Sensing by Using Type-3 Copper Proteins

Gerhild Zauner,[a] Emanuela Lonardi,[a] Luigi Bubacco,[b] Thijs J. Aartsma,[c] Gerard W. Canters,[a] and Armand W. J. W. Tepper*[a]

Abstract: A fluorescence-based system to sense oxygen in solution is described. The method exploits the sensitivity of the endogenous fluorescence of type-3 copper proteins towards the presence of oxygen by translating the near-UV emission of the protein to label fluorescence in the visible range through a FRET mechanism. The main protein in this study, a recombinant tyrosinase from the soil bacterium *Streptomyces antibioticus*, has been covalently labeled with a variety of fluorescent dye molecules with emission maxima spanning the whole visible wavelength range. In all cases, the emission of the label varied considerably between O_2-bound and O_2-free protein with a contrast exceeding that of the Trp emission for some labels. It is shown that different constructs may be simultaneously observed using a single excitation wavelength. Next to the described application in oxygen sensing, the method may be applicable to any protein showing variations in tryptophan fluorescence, for example as a function of ligand binding or catalysis.

Keywords: copper · fluorescence sensors · oxygen · proteins · tryptophan

Introduction

Oxygen plays a universal role in biological systems. Consequently, there is a continuous demand for improved techniques to monitor oxygen concentrations in vitro and in vivo. Many methods in this field are based on solid-state sensors which require direct contact of a (bulky) electrode with the sample. The Clark-type electrode is the prototypical example. Drawbacks of the electrode are its size, its consumption of oxygen during the measurement, and the interference by compounds other than oxygen.[1] To circumvent these limitations, several systems to measure O_2 by optical means have been developed. Many of them are based on the quenching of luminescence of an inorganic reporter or of polymers doped with organic dyes.[1,2] These compounds can be unstable in water, for which reason they may require encapsulation in a polymeric matrix.[1,2] This limits the time resolution of these systems because of the slow diffusion of oxygen inside the polymer. A system making use of delayed fluorescence of Pd protoporphyrin IX, a compound endogenously synthesized in mitochondria, has been applied to sense O_2 in vivo.[3] Yet, examples of protein-based systems to sense O_2 are scarce, despite the potential advantages of such systems in terms of biocompatibility, selectivity and stability. Here we report on a new principle to measure $[O_2]$. It is based on the "translation" of endogenous protein emission into the fluorescence of a dye molecule conjugated to the protein. It is shown that several commonly used fluorescent dyes attached to a protein may be excited through FRET from endogenous protein tryptophan residues.

The method makes use of so-called "type-3" copper proteins which selectively and reversibly bind molecular oxygen with dissociation constants in the range of physiological oxygen concentrations (μM range). Hemocyanins (Hc) are oxygen carriers found in arthropods and molluscs, while the tyrosinases (Ty) are monooxygenases that catalyze the hydroxylation of phenols and the oxidation of the diphenolic products to the quinones, which are precursors in melanin synthesis. The type-3 sites in these proteins consist of two copper ions each coordinated by three His residues

[a] G. Zauner, E. Lonardi, Prof. Dr. G. W. Canters, Dr. Ing. A. W. J. W. Tepper
Leiden Institute of Chemistry, Leiden University
2300 RA Leiden (The Netherlands)
Fax: (+31) 71-527-4349
E-mail: w.tepper@chem.leidenuniv.nl

[b] Prof. Dr. L. Bubacco
Department of Biology, University of Padua
Via Trieste 75, 30121 Padua (Italy)

[c] Prof. Dr. T. J. Aartsma
Leiden Institute of Physics, Leiden University
2300 RA, Leiden (The Netherlands)

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

(Figure 1). Molecular oxygen binds to the reduced and colorless [Cu^{I}–Cu^{I})] type-3 centre to yield the oxygenated [Cu^{I}-O_2-Cu^{I})] species in which oxygen is bound in a Cu_2 bridging "side-on" geometry. The oxy form is characterized by a strong optical transition around 345 nm ($\varepsilon_{345}=12$ to 25 $mM^{-1}\,cm^{-1}$) and a weak d–d transition at higher wavelength[4] ($\approx$600 nm; $\varepsilon < 1$ $mM^{-1}\,cm^{-1}$, Figure 2a).

Most proteins contain the aromatic residues tyrosine and tryptophan, which fluoresce in the near-UV upon excitation around 280 nm. For proteins that contain both residues, it is often found that the emission is dominated by the contribution of the Trp residues, which emit in the range of 335–345 nm. The intensity and bandshape of the emission depend, among other factors, on the polarity of the microenvironement.[5] Consequently, Trp emission has been applied to study protein folding, enzymatic catalysis, ligand binding and protein association. The Trp fluorescence of type-3 copper proteins is rather special. In the oxygenated form, a large fraction of the endogenous emission of type-3 proteins is quenched by fluorescence resonance energy transfer (FRET or Förster energy transfer) to the 345 nm absorption.[6,7] In the oxygen-free (de-oxy) form this band and, therefore, the quenching is absent (Figure 2a). The difference in Trp emission intensity between oxygenated and oxygen-free protein may reach more than a factor of ten.[7] It follows that the Trp fluorescence is a sensitive reporter of the amount of oxygen bound to the type-3 centers, which is a direct measure for the O_2 concentration in solution. Thus, the type-3 proteins would make ideal oxygen sensors. Unfortunately they cannot be used in samples containing other compounds fluorescing in the near-UV because the background emission may swamp the emission of the type-3 protein. Furthermore, near-UV fluorescence is often difficult to detect in scattering media, such as biological samples.

To make type-3 proteins suitable for O_2 sensing, the strategy illustrated in Figure 1 was devised. A label is attached to the protein, that fluoresces in the visible range and exhibits one or more optical absorption bands which overlap with the Trp emission. In the oxygen-free state, while the Trps in the protein are excited by UV light, FRET will occur from the Trps to the label and the latter will start fluorescing in the visible range of the spectrum. As soon as O_2 binds, a second FRET decay channel for the Trp will be opened, namely to the 345 and 570 nm absorption bands of the oxygenated active site of the protein. Consequently, the energy channeled to the label diminishes and the label fluorescence

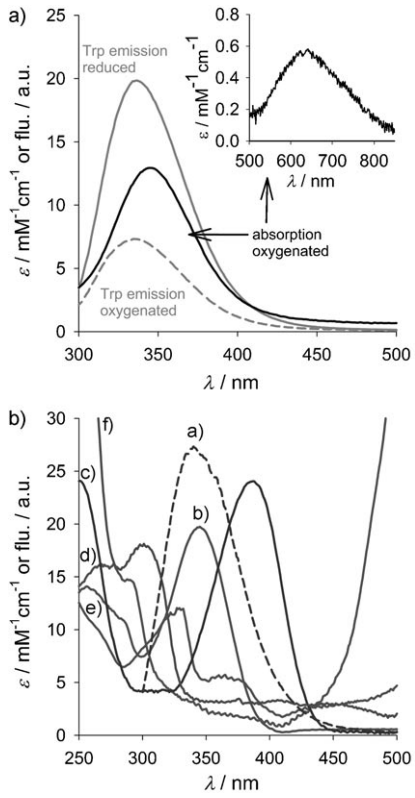

Figure 2. a) Absorption spectrum of oxygenated Ty corrected for the contribution of the reduced protein between 300 and 500 nm (main panel, black line) and between 500 and 850 nm (inset), and fluorescence emission spectra ($\lambda_{ex}=280$ nm) of reduced (solid grey line) and oxygenated Ty (dashed grey line). b) Absorption spectra of the used dyes and their overlap with the Ty Trp emission (a). b) Alexa350; c) Atto390; d) Cy3; e) Cy5; f) Atto655.

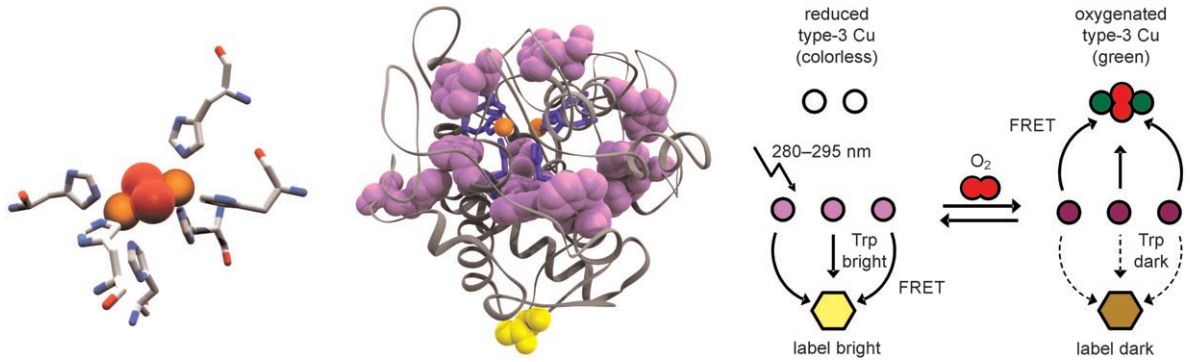

Figure 1. Structure of the oxygenated type-3 center (left) and of *Streptomyces antibioticus* tyrosinase (right) showing the type-3 Cu atoms (orange spheres), the coordinating His residues (blue sticks), the 12 Trp residues (violet) and Thr1 (yellow), corresponding to the attachment point of the label. The structure has been obtained from homology modeling using the structure of *Streptomyces castaneoglobisporus* tyrosinase (PDB 1WX3; 82 % sequence identity; 91 % sequence similarity). The principle of the FRET based O_2 sensing is shown at the right (see text for details).

drops. Due to the R^{-6} distance dependence of FRET efficiency (R denoting the distance between the donor and acceptor),[5] the label will only communicate with Trps which are on the same protein molecule as the label and the interference by other compounds fluorescing in the near-UV will be minimal.

Results

The FRET method: To illustrate the principle outlined in Figure 1, two type-3 copper proteins have been selected: Hc from the arthropod *Carcinus aestuarii* (Mediterranean crab) and a recombinant Ty from the soil bacterium *Streptomyces antibioticus*. Both proteins were labeled at the N-terminus of the enzyme.[8] Figure 2b shows the absorption spectra between 250 and 500 nm of the five fluorophors selected for this study (Alexa 350, Atto390, Cy3, Cy5, Atto655), as well as their overlap with the Ty Trp emission. For the Ty, the spectral overlap between the dye absorptions and the Trp emission have been collected in Table 1 together with the corresponding Förster radii, R_0.[5] We note that the dyes emitting in the visible wavelength (Cy3, Cy5, Atto655) range yield similar Förster radii as the "dedicated" near-UV dyes Alexa 350 and Atto390 due to the very high molar absorption of the former dyes.

Table 1. Effect of oxygen binding on the emission characteristics of tyrosinase and hemocyanin.

Dye	λ_{em} [nm]	SR[a] Ty	SR[a] Hc	J_{Trp} Ty[b] [$nm^4 M^{-1} cm^{-1} \times 10^{-13}$]	$R_{0,Trp}$ Ty[c] [Å]
Trp	339	2.7	2.2	13.0	23
Alexa350	440	1.8	2.4	16.5	24
Atto390	470	2.2	2.1	21.1	25
Cy3	566	2.8	2.2	5.9	20
Cy5	665	4.2	2.3	5.8	20
Atto655	684	4.3	2.1	7.2	21

[a] SR denotes the dye emission switching ratio (F_{red}/F_{oxy}) observed with excitation at 280 nm. [b] Calculated spectral overlap integrals between the Trp emission and the absorption of the oxygenated protein (for Trp) or between the Trp emission and the absorption of the dye (for the labels). [c] Förster radii R_0 were calculated as described in the Experimental Section.

The calculated R_0 values are in the range of distances between the Trps and the N-terminus (16–32 Å; Figure S1 and Table S1, Supporting Information), predicting that FRET between Trp and the label will occur for all dyes. Indeed, label fluorescence upon excitation at 280 nm was observed in all cases (Figure 3a). The excitation spectra of the conjugates (λ_{em} at label maximum) showed a band at 280 nm with a shape similar to the protein absorption, while this peak was missing in the excitation spectra of the free dyes (Figure S2, Supporting Information). This confirmed that the label is excited through FRET from the Trps to the label.

In all cases, an increase in label emission was observed after deoxygenating the sample solution (Figure 3a). This

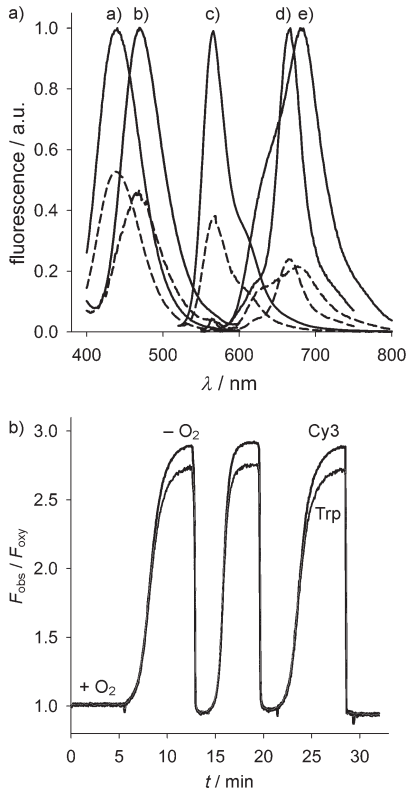

Figure 3. a) Label emission of different Ty-label conjugates of the oxygen-free, reduced, protein (solid lines) and of the oxygenated form (dashed lines) upon excitation at 280 nm. a) Alexa350; b) Atto390; c) Cy3; d) Cy5; e) Atto655. b) Reversible oxygenation and deoxygenation of a solution containing a mixture of unlabelled Hc (95%) and Hc conjugated with Cy3 (5%).

was fully reversible as illustrated in Figure 3b showing the Trp and label emission intensities as a function of time for a sample containing a mixture of unlabelled Hc (95%) and Hc-Cy3 (5%) while being repeatedly deoxygenated and oxygenated. The Cy3 fluorescence is specific for the labeled protein, while the Trp emission derives mainly (> 95%) from unlabelled Hc. Similar observations were made for other labels, and when Ty was used instead of Hc. They illustrate that the Trp and the label emissions vary in the same way and that the presence of the label does not affect the K_d for O_2 binding. The fluorescence intensity of Atto390 attached to bovine serum albumin (BSA) was not dependent on $[O_2]$ (not shown). The emission of all constructs was stable for hours upon continuous illumination at 280 nm.

The constructs could be easily detected at nanomolar concentration with a standard fluorimeter. Next to a good sensitivity, a high contrast in label fluorescence between O_2-free and O_2-bound protein is crucial for practical sensing applications. For the Hc, the dye switching ratio SR ($=F_{red}/F_{oxy}$), is similar to the SR value for the Trp fluorescence of the native protein (Table 1). For Ty, the SR values vary between the labels. Förster theory predicts that the switching ratio of a label may vary depending on the balance between the various factors determine the quenching efficiency. A quantita-

tive analysis of the quenching requires the evaluation of terms, like the distance, the orientation and the spectral overlap between the type-3 centre, the label and each of the several Trp residues, as well as the quantum yields of the label and of each of the twelve Trp residues.

In explaining the variations in SR observed for Ty, it is reasonable to assume that the involved distances, orientations and Trp quantum yields are constant for a given system labeled with different dyes. Thus, the variations in label contrast between O_2-free and O_2-bound tyrosinase have to be sought in variations in spectral overlap. The relatively high SR value measured for the red-emitting dyes Cy5 and Atto 655 is probably related to the presence of the weak d–d absorption of the oxygenated protein (see the inset in Figure 2a), which provides an additional quenching pathway for the excited label.[9] This mechanism can be quite efficient due to the relatively high wavelength of the absorption and the λ^4 dependence of FRET. This quenching is independent of Trp fluorescence and should also be operative when the label is excited directly. Indeed, upon a change in the redox state of the type-3 center, the fluorescence of these two dyes (Cy5 and Atto 655) changes by a factor of about two when the labels are excited in their main absorption band. Upon excitation of the Trp residues, this mechanism enhances the contrast in label emission and leads to switching ratios higher than those observed for the Trp fluorescence itself. Conjugates displaying very high switching ratios may be designed by carefully considering the position of the label with respect to the Trp residues and that of the type-3 copper center.

Oxygen sensing: The *Carcinus aestuarii* Hc consists of a mixture of hexamers and dodecamers, which are self-assembled from three different types of subunits.[13] Hcs, in the native multimeric forms, display cooperative O_2 binding due to allosteric interactions between the constituent subunits of the Hc. A titration of Hc carrying the Atto390 label with O_2 monitored by following the Trp and label fluorescence (Figure 4) showed that this cooperativity is lost. The titration data were fit to a modified form of the Hill equation:

$$F_{[O2]} = F_{red} - \frac{(F_{red} - F_{oxy}) \cdot [O_2]^n}{[O_2]^n + K_d} \quad (1)$$

where $F_{[O2]}$ is the observed fluorescence at given $[O_2]$ and F_{red} and F_{oxy} denote the emission intensities of the reduced and oxygen-bound protein, respectively. K_d is the dissociation constant and n denotes the Hill constant, which is a measure of cooperativity of the binding process (for non-cooperative binding $n=1$). The fitting resulted in a K_d of 22 μM and an n value of 1.09. The latter is much lower than the n values of 2.6 and 3.1 reported for intact hexameric and dodecameric *Carcinus aestuarii* Hc, respectively, and is diagnostic of almost total loss of cooperativity. It is known that arthropod Hcs dissociate into the subunits at high pH and in the absence of divalent metal ions. Since both conditions were met during the labeling procedure, it is likely that our Hc samples contain a mixture of dissociated labeled subunits. The Ty, in contrast, is monomeric and does not show cooperative O_2 binding in the native or labeled forms.

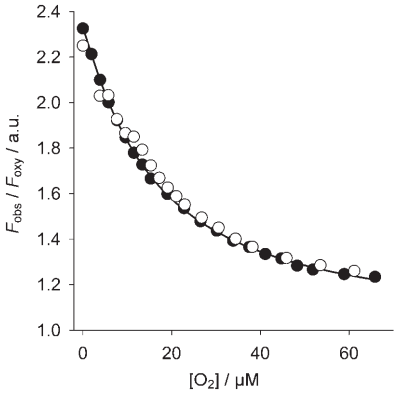

Figure 4. Titration of Hc-Atto390 with O_2 monitored by the Trp (○) and dye (●) emission upon excitation at 280 nm. The solid line represents the best fit to the data [Eq. (1)] with $K_d = 22$ μM and $n = 1.09$. The Trp fluorescence has been normalized to the Atto390 fluorescence to facilitate comparison.

The data presented above show that labels with widely different emission wavelengths may be utilized, which opens the possibility to observe two or more constructs in the same sample using a single excitation wavelength. Figure 5 illustrates an experiment involving Hc-Atto390 and Ty-Atto655 in a sample where $[O_2]$ was gradually lowered by deoxygenating the solution. The O_2 dissociation rate is in the range of 9–20 s^{-1} for arthropod Hc[10] and is $\approx 300\ s^{-1}$ for the Ty[11] meaning that the proteins can be considered to be in equilibrium with the O_2 in solution on the timescale of the experiment.

The $[O_2]$ was determined as a function of time from the Hc-Atto390 fluorescence with Equation (1) and the K_d and Hill constant for O_2 binding determined in an independent O_2 titration (Figure 4). The Ty-Atto655 fluorescence was plotted as a function of the determined $[O_2]$ at each time-point. The resulting Ty-Cy5 O_2 binding curve could be accurately fit to the Hill equation, yielding a K_d of 15 μM ($n = 1.0$), which is within error of the literature dissociation constant of 16 μM.[6] The experiment illustrates the accuracy of the $[O_2]$ determination by monitoring the Hc-Atto390 fluorescence.

The specificity of the method is illustrated by a titration of a mixture of Hc-Alexa350 and Ty-Cy5 with iodide in an air-saturated solution (Figure 6). Iodide displaces oxygen from the Ty active site[12] but not from *Carcinus aestuarii* Hc.[13] The difference in iodide binding between Hc and Ty is reflected in the dye fluorescence: an increase in label fluorescence is observed for Ty-Cy5 upon adding I^-, while the Hc-Alexa350 fluorescence changes minimally. The small fluorescence decrease of the latter can be ascribed to the quenching of the label and/or the Trp fluorescence by iodide.[5,13] The Ty-Cy5 fluorescence intensity versus $[I^-]$ could be accurately fit to Equation (1) (with $[O_2]$ replaced

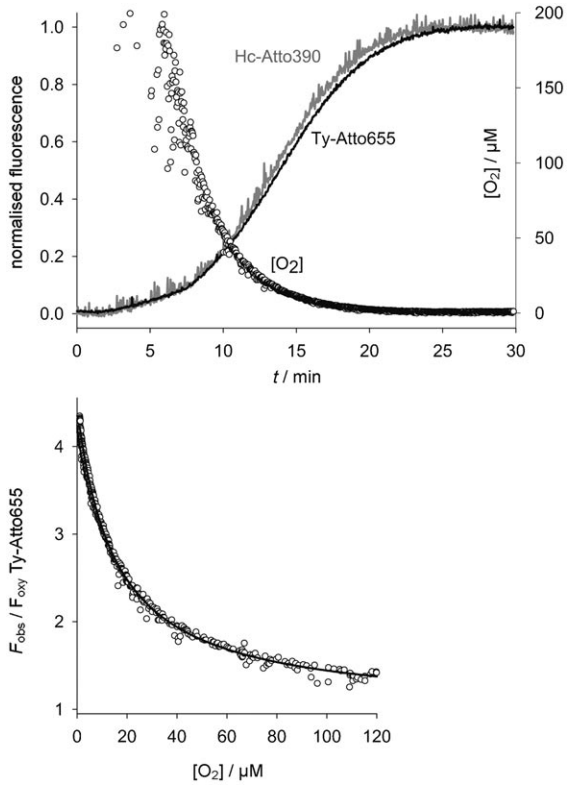

Figure 5. Label fluorescence as a function of time for a mixture of Ty-Atto655 (black trace) and Hc-Atto390 (grey trace) upon deoxygenation (initial $[O_2] = \approx 0.6$ mM; $\lambda_{ex} = 280$ nm). The experimental curves (left panel) have been normalized between the start and end values to facilitate comparison. The raw data of the Hc-Atto390 traces were used to calculate $[O_2]$ at each timepoint (left panel, open black circles, right axis) using Equation (1) with $K_d = 22$ µM and $n = 1.09$. The observed Ty-Atto655 fluorescence (normalized to the intensity observed for the fully oxygenated protein) was then plotted against the calculated $[O_2]$ at each time-point (right panel). The data were fit to Equation (1) (solid line; $K_d = 15$ µM, $n = 1.0$).

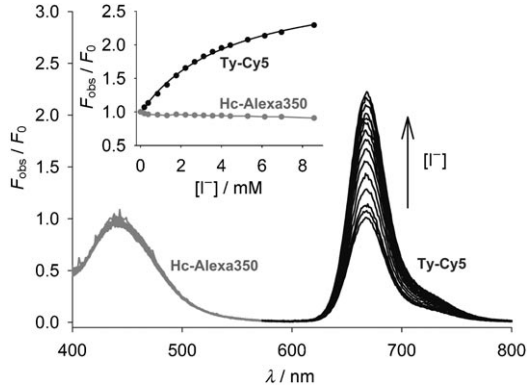

Figure 6. Titration of a mixture of Hc-Alexa350 and Ty-Cy5 with iodide monitored by following the emission between 400 and 800 nm upon excitation at 280 nm. The spectra in the main panel were normalized to one intensity for the fluorescence observed in the absence of iodide. The inset shows the fluorescence intensities of each dye vs $[I^-]$. The solid line through the Ty-Cy5 data represents the best fit to Equation (1) ($K_d = 4.4 \pm 0.3$ mM, $n = 1.00$).

by $[I^-]$), yielding an apparent K_d of 4.4 ± 0.3 mM ($n = 1.00$). The difference between the experimental K_d and the literature value of 3.0 ± 0.3 mM[6] may be related to its dependence on the $[O_2]$ in the sample, which may have varied between the two experiments.

The inner-filter effect, where a portion of the excitation light is absorbed by the sample before reaching the fluorophore, is a potential problem with any system that makes use of UV excitation. Yet, since the absorbance is proportional to the pathlength, the inner-filter effect is negligible for thin samples. Thus, the system may be of use in cell biology where O_2 may be monitored on a (sub)cellular level using fluorescence microscopy provided that precautions are taken to minimize the inner-filter effect. We also note that the inner-filter effect does not affect the contrast in label emission between O_2-bound and O_2-free protein as long as the background UV absorption is constant. Furthermore, surface assembly of the oxygen sensing enzyme in combination with selective surface excitation (e.g. total internal reflection) could be advantageous when studying highly absorbing samples. If UV excitation is undesired, tryptophan may be excited through a two-photon process using common laser lines.[14] Alternatively, the constructs containing a label emitting in the red part of the visible spectrum may be excited at the absorption maximum of the dye (e.g. 645 nm for Cy5) at the expense of some contrast in emission between the O_2-free and O_2-bound states.

Discussion

The described method exploits the sensitivity of the endogenous tryptophan fluorescence of type-3 copper proteins towards the presence of oxygen by translating the Trp emission in the near-UV to label fluorescence in the visible. The most important characteristics of the method can be summed up as follows: 1) It allows freedom of choice of the fluorescent dye molecule to tune the emission wavelength to specific applications. 2) The label contrast between O_2-free and O_2-bound protein can be made to exceed the contrast observed for the Trp fluorescence. 3) Sensor response times down to the millisecond range are feasible. 4) Hcs have evolved to bind O_2, while minimizing interactions with other (biological) compounds, thereby ensuring selectivity. 5) It allows two or more conjugates to be monitored at the same time. The latter may be applied, for instance, to monitor $[O_2]$ in different (microscopic) sample compartments by targeting type-3 protein conjugates carrying different labels to specific locations in the sample. For instance, $[O_2]$ could be monitored at the surface of living cells by conjugating the labeled type-3 protein with an antibody specific for a certain epitope on the cell surface, for example. Combining this scheme with fluorescence microscopy methods would possibly enable measurement of O_2 consumption at sub-cellular levels and msec timescales and to monitor, for example, metabolic activity. Although the method has been described

for its application in oxygen sensing, it can be utilized, in principle, for all systems displaying Trp fluorescence changes.

Experimental Section

Materials: Tyrosinase (Tyr) from *Streptomyces antibioticus* was expressed and purified as described previously.[16] Hemocyanin (Hc) from the arthropod *Carcinus aestuarii* was purified as described elsewhere.[17] Atto 655 and Atto 390 NHS-ester were purchased from ATTO-TEC Biolabeling and Ultraanalytics (Siegen, Germany). Cy5 and Cy3 NHS-ester were purchased from Amersham Biosciences (Freiburg, Germany). Alexa 350 NHS-ester was purchased from Molecular Probes (Leiden, The Netherlands). The dye stock solutions were prepared by dissolving the powder in water-free dimethyl sulfoxide (DMSO) to a concentration of roughly 50 mM.

Enzyme labeling: Both enzymes Tyr and Hc were labeled on the N-terminus[8] with one of the NHS-reactive fluorophors in a roughly 10 times molar excess over the protein concentration. The constructs were purified using PD-10 gel-filtration columns purchased from Amersham Pharmacia Biotech. The Dye over Protein (DOL) ratio has been quantified as suggested by the manufacturers taking $\varepsilon_{665}=120\ \mathrm{mM^{-1}\,cm^{-1}}$ for Atto655, $\varepsilon_{390}=24\ \mathrm{mM^{-1}\,cm^{-1}}$ for Atto390, $\varepsilon_{645}=250\ \mathrm{mM^{-1}\,cm^{-1}}$ for Cy5, $\varepsilon_{550}=150\ \mathrm{mM^{-1}\,cm^{-1}}$ for Cy3 and $\varepsilon_{346}=19\ \mathrm{mM^{-1}\,cm^{-1}}$ for Alexa350. The concentration of purified tyrosinase was determined optically at 280 nm using $\varepsilon_{280}=82\ \mathrm{mM^{-1}\,cm^{-1}}$. The DOL values were kept below 1 to increase the selectivity fro the N-terminus of the protein.

Absorbance and fluorescence measurements: Absorption spectra were measured using a Cary-50 Spectrophotometer with a slit-width equivalent to a bandwidth of 5 nm. Fluorescence measurements were taken on a Cary Eclipse Spectrophotometer with an emission band-pass of 10 nm and an excitation band-pass of 5 nm. Suitable optical filters on both the excitation and emission paths were utilized to minimize the effects from second order diffraction of the monochromator gratings. To avoid the inner-filter effect, the optical absorbance of the sample was kept below 0.05 at the excitation and emission wavelengths. For all measurements a 10×10 mm airtight quartz cuvette (Hellma Benelux bv, Rijswijk, Netherlands) was utilized. All measurements were performed using a 100 mM phosphate buffer at pH 6.8 at room temperature. Amine reactive dyes in the form of a succinimidylester were reacted with the primary amine group of Tris prior to measurements on the isolated fluorophors. The excitation and emission wavelengths of the utilized dyes were as follows: Atto 655: λ_{ex} 665 nm, λ_{em} 685 nm; Atto 390: λ_{ex} 390 nm, λ_{em} 480 nm; Cy5: λ_{ex} 645 nm, λ_{em} 665 nm; Cy3: λ_{ex} 550 nm, λ_{em} 570 nm; Alexa 350: λ_{ex} 346 nm, λ_{em} 455 nm. Oxygenated Ty was prepared by reduction of the oxidized protein with hydroxylamine as described previously.[6] For the anaerobic fluorescence measurements, the buffer was deoxygenated in the cuvette prior to measurements by equilibrating with high quality argon (<1 ppm O_2) for 1 hour before the labeled protein was added. An argon flow over the sample was maintained during the measurements. For the oxygen titrations, the cuvette was completely filled with deoxygenated sample solution, after which µL amounts of an air-saturated buffer ($[O_2]=1.3$ mM) were injected.

Förster radius calculations: The Förster radius, R_0, was calculated from the equation $R_0=0.211(J\kappa^2 n^{-4}\Phi_D)^{1/6}$ (Å) as described earlier.[5] In the formula κ^2 is an orientation factor, n is the refractive index, Φ_D is the fluorescence quantum yield of the donor, and J is the 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 and $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor at wavelength λ, with λ expressed in nanometers. Experimental protein absorption spectra and fluorescence spectrum of the dyes 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.[5] A value of 0.07 was used for the Trp quantum yield Φ_D.[7]

Acknowledgements

This work is supported by the Stichting voor Fundamenteel Onderzoek der Materie (FOM) with financial aid from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO).

[1] Y. Amao, *Microchim. Acta* **2003**, *143*, 1–12.
[2] P. Hartmann, W. Ziegler, G. Holst, D. W. Lubbers, *Sens. Actuators B* **1997**, *38*, 110–115.
[3] E. G. Mik, J. Stap, M. Sinaasappel, J. F. Beek, J. A. Aten, T. G. van Leeuwen, C. Ince, *Nature Methods* **2006**, *3*, 939–945.
[4] E. I. Solomon, U. M. Sundaram, T. E. Machonkin, *Chem. Rev.* **1996**, *96*, 2563–2605.
[5] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Publishers, New York, **1996**.
[6] A. W. J. W. Tepper, L. Bubacco, G. W. Canters, *J. Biol. Chem.* **2004**, *279*, 13425–13434.
[7] W. Erker, R. Hubler, H. Decker, *Eur. Biophys. J.* **2004**, *33*, 386–395.
[8] S. Kuznetsova, G. Zauner, R. Schmauder, O. A. Mayboroda, A. M. Deelder, T. J. Aartsma, G. W. Canters, *Anal. Biochem.* **2006**, *350*, 52–60.
[9] W. Erker, A. Schoen, T. Basche, H. Decker, *Biochem. Biophys. Res. Commun.* **2004**, *324*, 893–900.
[10] A. Messerschmidt, P. T. W. K. Huber, *Handbook of Metalloproteins, Vol. 2*, Wiley, New York, **2001**.
[11] S. Hirota, T. Kawahara, E. Lonardi, E. de Waal, N. Funasaki, G. W. Canters, *J. Am. Chem. Soc.* **2005**, *127*, 17966–17967.
[12] A. W. J. W. Tepper, L. Bubacco, G. W. Canters, *J. Biol. Chem.* **2002**, *277*, 30436–30444.
[13] P. Di Muro, M. Beltramini, P. Nikolov, I. Petkova, B. Salvato, F. Ricchelli, *Z. Naturforsch. C: Biosci.* **2002**, *57*, 1084–1091.
[14] M. Lippitz, W. Erker, H. Decker, K. E. van Holde, T. Basche, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2772–2777.
[15] G. Gaietta, T. J. Deerinck, S. R. Adams, J. Bouwer, O. Tour, D. W. Laird, G. E. Sosinsky, R. Y. Tsien, M. H. Ellisman, *Science* **2002**, *296*, 503–507.
[16] L. Bubacco, E. Vijgenboom, C. Gobin, A. W. J. W. Tepper, J. Salgado, G. W. Canters, *J. Mol. Catal. B* **2000**, *8*, 27–35.
[17] L. Bubacco, R. S. Magliozzo, M. Beltramini, B. Salvato, J. Peisach, *Biochemistry* **1992**, *31*, 9294–9303.

Received: December 15, 2006
Revised: March 29, 2007
Published online: June 19, 2007

