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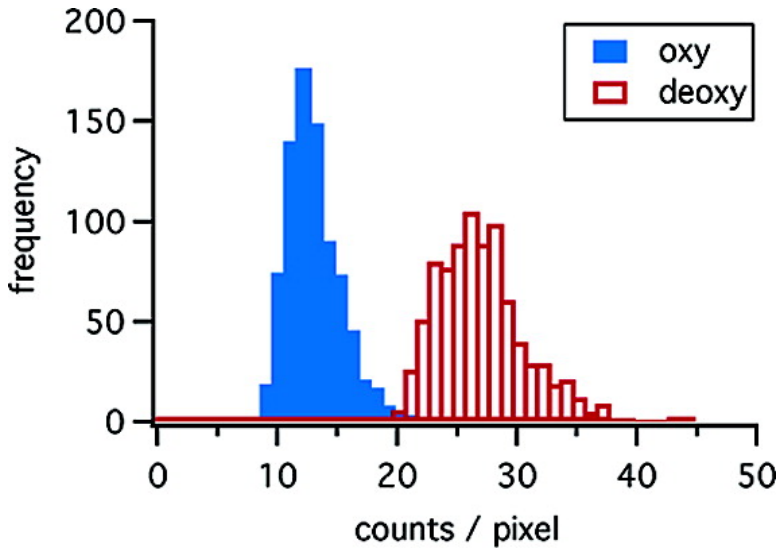
J. Am. Chem. Soc., **2005**, 127 (42), 14532-14533• DOI: 10.1021/ja053720k • Publication Date (Web): 28 September 2005



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Published on Web 09/28/2005

Detection of Single Oxygen Molecules with Fluorescence-Labeled Hemocyanins

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Laser-induced fluorescence detection of single molecules in solution has proven to be a powerful tool in molecular biology and analytical chemistry. Proposals for DNA sequencing based on single-molecule detection rely on the registration and characterization of fluorescent bursts from single dye-labeled nucleotides passing the laser-illuminated probe volume.[1] In such a scheme, the presence or absence of a single analyte molecule can be registered with high precision. Other detection schemes rely on a change in emission intensity of a single luminescent probe caused by interaction with an analyte. A prominent example is oxygen-induced quenching of the triplet state of a single molecule,[2] which typically results in fluctuations of the fluorescence intensity. Indeed, emission from single or a few molecules, which interacted with many oxygen molecules, was proposed for quantitative oxygen sensing.[3] Here, we introduce a method to detect individual oxygen molecules by fluorescence microscopy of single hemocyanins, which bind oxygen with high affinity. Former attempts to image single hemocyanins by their intrinsic tryptophan emission were severely limited by photobleaching.[4] In a new approach, a spectrometric signature of the oxygenated protein is transferred to an attached fluorescence label, which can be readily detected at the single-molecule level. The technique presented also opens new perspectives for the investigation of cooperative oxygen binding in respiratory proteins.

In our study, we used a well characterized hemocyanin, the respiratory protein of the tarantula *Eurypelma californicum*, as a model system.[5] It consists of 24 subunits, arranged in four hexamers. Each subunit is able to bind one molecule of oxygen between two copper ions in a side-on conformation.[6,7] The oxygen−copper complex is characterized by two charge-transfer absorption bands at 340 and 570 nm.[8] Both bands can be used as a linear signal to monitor the amount of bound O_2 and are absent when no O_2 is bound.[9] Therefore, hemocyanins are well suited for the kind of investigations described here. To conduct single-molecule spectroscopy, the absorption signal was transformed into a fluorescence signal by a dye label. The dye, TAMRA-SE, was covalently attached by its amino-reactive *N*-hydroxysuccinimidyl ester to lysine residues of the protein. The fluorescence quantum yield of the label decreases by roughly a factor of 2 when O_2 is bound by the hemocyanin.[10] Due to the spectral overlap and the close proximity, the quenching process is attributed to FRET (Fluorescence Resonance Energy Transfer), where the dye is acting as the donor and the oxygenated active site of the protein as the acceptor. The Förster radius ($R_0 \approx 3.0$ nm) is in the order of half of the subunit size (7 nm × 5 nm), suggesting that a dye molecule monitors the O_2 binding of the subunit where it is attached.[6,10] Labeling was performed with a molar ratio of 0.9 dyes per 24-mer hemocyanin in order to minimize double labeling. Ensemble oxygen binding curves in solution could be reliably measured with the labeled hemocyanins.[10] To study isolated subunits, the oligomers were dissociated following standard procedures from the literature.[5]

The dyes' emission intensity depends on the excitation power (P) and the oxygen content of the solution. To quantify these effects, we introduced the ratio

$$R = \frac{\mathrm{FI}_{\mathrm{deoxy}}}{\mathrm{FI}_{\mathrm{oxy}}}$$

of the total fluorescence intensities of the dye, FI, without oxygen (deoxy) and when oxygen is present (oxy). The notation (oxy) refers to solutions equilibrated with air. Under these conditions, 24-mer tarantula hemocyanin as well as its subunits is completely oxygenated.[11] In Figure 1a, the ratio R is plotted versus the excitation power. For dye without protein, the ratio R starts with a value of 1 and drops below unity with increasing laser power. This decrease occurs because at higher intensities more and more molecules are pumped into the triplet state. However, O_2 shortens the triplet off-times in the oxygenated sample and brings the dye molecules back into the fluorescence cycle.[2,3] Consequently, when O_2 is present, the dye becomes brighter than without. When the dye is attached to hemocyanin, the low intensity value of the ratio R is about 2 (Figure 1a). This is caused by an additional process, FRET, which quenches the fluorescence of the oxygenated sample. With increasing laser power, off-times due to the triplet state become significant. Again, oxygen-induced triplet quenching increases the dyes' brightness in the oxygenated sample. Therefore, the ratio R drops, when the excitation power increases. To have R sufficiently large in the following experiments the excitation power was kept as low as possible.

For single-molecule microscopy, labeled proteins were deoxygenated/reoxygenated in an atmos-bag and immobilized by adsorption on a glass cover slide. Images were recorded with a confocal microscope at the glass buffer interface (10 ms/pixel). Typically, about 50 molecules were detected per image, and 4−15 images were recorded for each excitation power and each oxygenation state. Images were smoothed by convolution with a Gaussian kernel (5 × 5 pixel) to determine x- and y-positions of the molecules. However, to determine the brightnesses of the molecules, in a first approach, the untreated images were used. To characterize the dye at the single-molecule level, a new ratio R' was defined based on the average brightness μ (counts per pixel) of the molecules in the deoxygenated and oxygenated state, respectively:

$$R' = \frac{\mu_{\mathrm{deoxy}}}{\mu_{\mathrm{oxy}}}$$

The ratio R' versus excitation power is plotted in Figure 1b. It starts with a value of 1.6, which is similar to the values obtained in the ensemble experiments. The signal drops below unity with increasing laser power. Again, this reflects the competition of fluorescence quenching and increase due to FRET and triplet quenching, respectively. Imaging of single dye molecules without

10.1021/ja053720k CCC: $30.25

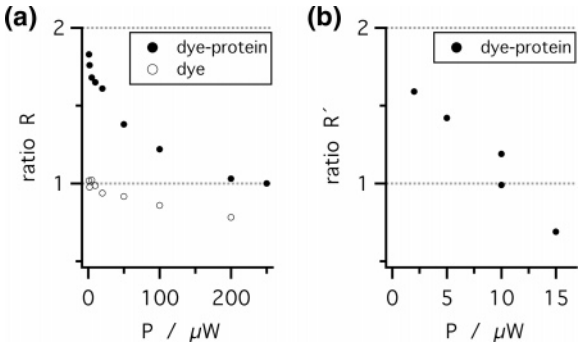

Figure 1. Ratios of TAMRA fluorescence intensity (deoxygenated/oxygenated samples) in dependence on the excitation power: (a) at the ensemble level in solution (ratio *R*), and (b) for single molecules immobilized on a surface (ratio *R*′). Protein stands for 24-mer tarantula hemocyanin.

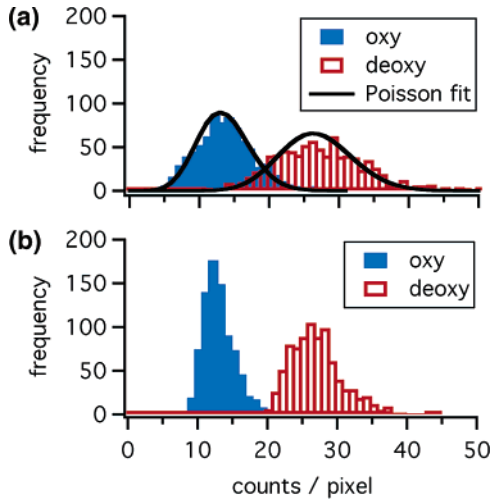

Figure 2. Brightness histograms of single TAMRA−hemocyanin subunits for oxygenated and deoxygenated samples. Analysis without filtering (a) and with Gaussian filter (b).

hemocyanin was not possible because TAMRA does not adsorb (for sufficiently long times) at the glass surface.

It has been shown that due to shot noise the brightness histogram of a single immobilized particle is Poissonian distributed.[12] In our analysis, the brightnesses of many molecules were determined at their center position. When averaging over many particles, one also expects a Poisson distribution. In the case of labeled 24-mer hemocyanins, the brightness histograms were broader probably because of intersubunit interactions. In contrast, a Poissonian shape could be observed for histograms of single subunits both in the deoxygenated and oxygenated state (Figure 2a). The distributions show a moderate overlap, meaning that at this stage of analysis it is possible to assign for many but not for all of the TAMRA−hemocyanins whether oxygen is bound or not.

In a second approach to image analysis, the brightnesses were also picked from the images smoothed with a 2D Gaussian. Filtering resulted in a weighted brightness average over 25 pixel. Using this method, the distributions became narrower. Actually, in the case of single subunits, the histograms are more narrow than a Poisson distribution (Figure 2b). In consequence, the overlap between the distributions of oxygenated and deoxygenated samples vanishes and the brightness histograms are completely separated. Due to the brightness of the dye, it is possible to assign whether a single O_2 molecule is bound or not by an individual subunit. This approach results in an averaging over 250 ms, which is longer than the retention period of oxygen bound by hemocyanin subunits of about 0.1 s.[13] Therefore, although only one oxygen molecule can be bound to a single subunit at a time, it need not be the same during image acquisition. In air, the mean off-time (no oxygen bound, bright state) of tarantula hemocyanin subunits is in the order of 1 ms, which is 10 times shorter than our integration time per pixel. In consequence, in air, hemocyanin subunits appear always oxygenated when measured with the current time resolution of our setup.

In summary, we have shown that single oxygen molecules can be detected by optically addressing single fluorescent-labeled subunits of hemocyanin. Although not yet fully explored and limited by oxygen-induced photobleaching, a potential application could be the development of very small and sensitive oxygen sensors. Devices based on tarantula hemocyanin subunits could span a range of about 1−50 kPa oxygen partial pressure above the solution. Hemocyanins from other animals possess different oxygen affinities, offering a multitude of sensor ranges.[5] In addition, our results pave the road toward detection of oxygen binding curves of single respiratory proteins. This promises to be an important step in understanding the cooperativity of oxygen binding.

Acknowledgment. We thank H. Decker (Molecular Biophysics, Mainz) for purified 24-mer tarantula hemocyanin.

Supporting Information Available: Methods; example images of single deoxygenated and oxygenated hemocyanin molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA053720K