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Single-molecule dynamics of conformational changes in flavin adenine dinucleotide

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Abstract

Dynamic conformational changes of the prosthetic group, flavin adenine dinucleotide (FAD), in flavoprotein NADH peroxidase (Npx), in thioredoxin reductase (TrxR), and in free solution were monitored with total internal reflection fluorescence microscopy. FAD bound loosely in the proteins changed from the stacked conformation to the unstacked conformation upon laser excitation. On the other hand, our results show that FAD in free solution not only underwent conformational changes but also reacted with each other to form a dimer.

Keywords: Photochemistry; Protein conformation; Flavin; Single molecule

1. Introduction

Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are essential cofactors for the biological activity of many proteins. They play an important role in catalysis and electron transfer. Flavoproteins are often critical to electron transport pathways in living systems like photosynthesis, respiration, nitrogen fixation, DNA repair, metabolism, and signal transduction [1–12]. They are an interesting family of enzymes for studying the dynamic behavior of biomacromolecules. It is well known that the flavin cofactors make these enzymes suitable for the direct observation of conformational dynamics in catalysis. These prosthetic groups are not only auto-fluorescent, but the redox-active groups are also located in the integral part of the active site. Fluorescence anisotropy and time-resolved fluorescence have been used to study the dynamics of the active site of various flavoproteins [13,14].

Flavin cofactors are non-covalently bound to flavoproteins. However, some are bound tightly while some are bound rather loosely to the protein. It is known that one of the flavoprotein called thioredoxin reductase (TrxR) has tightly bound FAD molecules as cofactors [15]. In other flavoenzymes like ferredoxin NADPH-reductase (FNR), the prosthetic groups are less tightly held [16]. Will the tightness of the binding affect the dynamics of conformational changes? Will the equilibrium of the conformational change shift upon the exposure to laser light? By direct observation of the dynamics of single-protein and single-cofactor molecules, one could address the above questions.

It has been proposed that an intramolecular ground-state complex between the isoalloxazine ring and the adenine moiety exists primarily in aqueous solution, resulting in the formation of a non-fluorescent complex [17]. The quantum yield of FAD (0.03) was found to be nine times lower than that of FMN (0.27) in steady-state fluorescence experiments. However, after enzymatic digestion of the diphosphate bridge of the FAD molecule, the fluorescent intensity of FAD was found to be the same as that of FMN. For this reason, FAD was assumed to exist in two conformations. One of the conformations is a “closed” or stacked conformation in which the isoalloxazine and adenine rings interact through π–π interactions in a stacked geometry that resulted in efficient fluorescence quenching. Another conformation is an “open” or unstacked, extended conformation that is responsible for the

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FAD fluorescence. In solution, at least 80% of FAD is in the closed conformation. Although it is generally accepted that the open and closed conformations of the FAD molecules exist, little is known about the conformational dynamics in solution, or the biological implications of such a change. [18,19].

In this study, we demonstrate a sensitive, fast and simple method for recording the real-time dynamics of conformation changes of the cofactors in free solution and when bound to proteins. We report the dynamics of NADH peroxidase (Npx), TrxR, FMN and FAD in free solution at various pH with the aid of total internal reflection fluorescence microscopy.

2. Experimental section

2.1. Buffer solutions

Phosphate buffer solutions of pH 7.5–10.0 were prepared with ultra-pure 18 MΩ water. All the solutions were photobleached under a mercury lamp overnight and were filtered through a 0.2 μm filter prior to use.

2.2. Preparation of samples

NADH peroxidase (Npx), thioredoxin reductase (TrxR), flavin adenine dinucleotide (FAD, Fig. 1), and flavin mononucleotide (FMN) were obtained from Sigma Chemical Co. (St. Louis, MO). All samples were used without further purification. Npx and TrxR were derived from *Streptococuus faecalis* ATCC 11700 and *Escherichia coli*, respectively. FAD and FMN were of purity not less than 95%. All samples were prepared fresh just prior to the single-molecule imaging experiments. Npx was diluted to 30 nM with appropriate buffer solutions. TrxR, FAD and FMN were diluted to 47, 50 and 10 nM, respectively with pH 10.0 phosphate buffer solution.

2.3. Evanescent wave excitation geometry

The instrumental setup was similar to that previously described [20–23]. A volume of 5 μL of sample solution was sandwiched between a No. 1 (170 μm thick and 22 mm square) Corning glass cover slip (Corning, NY) and the hypotenuse face of a right-angle fused-silica prism (Edmund Scientific, Barrington, NJ). A laser beam was focused and directed through the prism to the prism–sample interface. The angle of incidence was about 66°. The laser beam was totally internally reflected at the prism/solution interface, and an evanescent field of ∼100 nm thick was created. Fluorescent molecules within this field can be excited and imaged.

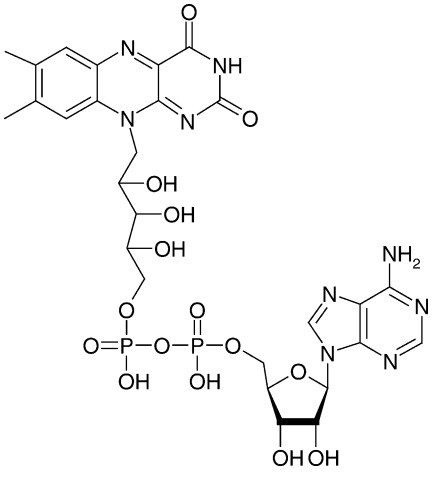

Fig. 1. Structure of $FADH_2$.

2.4. Single-molecule detection system

The excitation source was an argon-ion laser (Innova-90, Coherent, Santa Clara, CA) operated at 488 nm. Extraneous light and plasma lines from the laser were eliminated with an equilateral prism and a pinhole prior to its entrance to the observation region. The total laser power just before the prism was about 15 mW. The power density at the 80 μm × 60 μm excitation area is 300 W/cm^2.

2.5. Microscope and ICCD camera

The microscope objective was a Zeiss 40× Plan-Neofluar (oil, 1.3 NA). The objective was coupled to the cover slip with immersion oil (type FF, $n = 1.46$, Cargille, Cedar Grove, NJ). Images of the irradiated region through the objective were recorded by an electron amplifying CCD camera (Cascade, Roper Scientific, Trenton, NJ). The detector element (camera) was kept at −35 °C. A 488 nm holographic notch filter (Kaiser Optical System, Ann Arbor, MI) with an optical density of >6 was placed between the objective and the CCD camera. The digitization rate of the CCD camera was 1 MHz (16 bits). The DAC (digital-analog converter) setting was set at 3689. The frame-transfer CCD camera was operated in the external synchronization mode. Exposure timing for the CCD camera and the laser shutter was synchronized by a shutter driver/timer (Uniblitz ST132, Vincent Associates, Rochester, NY).

2.6. Image acquisition and analysis

The CCD exposure frequency was 2 Hz (0.5 s/frame) for all the sample images. The exposure time for each frame was 20 ms for Npx and TrxR, and 5 and 4 ms for free FAD and FMN, respectively, unless otherwise specified. Sequences of frames were acquired for each sample via V^{++} software (Roper Scientific, Trenton, NJ). All frames were analyzed off-line.

3. Results and discussion

3.1. Npx and TrxR

Like other proteins, Npx tended to be adsorbed onto the surface of fused-silica due to its hydrophobic nature when

Fig. 2. Native fluorescence images of Npx in pH 7.5 50 mM phosphate buffer. Excitation was at 488 nm and 300 W/cm^2. Exposure time was 20 ms at 2 Hz. Each spot registers a fluorescent molecule.

the pH is close to or below the p*I*. An image of the adsorbed molecules is shown in Fig. 2. The spot sizes do not reflect the physical sizes of the molecules. Rather, they represent the integrated trajectory of *x*–*y* motion during exposure. Almost all of the molecules adsorbed onto the fused-silica surface at pH 7.5 while about half of them adsorbed at pH 8.5. However, at higher pH (8.8–10.0), the protein molecules no longer adsorbed to the surface but diffused around. They appeared and disappeared at random locations on successive image frames as they moved in and out of the evanescent filed layer (EFL). At high pH, both the protein molecules and the fused-silica surface are negatively charged due to deprotonation of the protein and the silanol groups on the surface. So, electrostatic repulsion between the protein molecules and the surface minimized the adsorption events.

The number of molecules observed within the evanescent field layer at various pH is shown in Fig. 3. Fewer and fewer protein molecules diffused into the EFL as pH increased due to the stronger electrostatic repulsion between the protein molecules and the fused-silica surface. Interestingly, at pH > 8.8, the spot number from the images increased from frame to frame as shown in Fig. 4. The increase in spot number leveled off after a few frames. However, this phenomenon did not occur for another flavoprotein, thioredoxin reductase (TrxR). In fact, the number of TrxR molecules decreased from frame to frame due to photobleaching of the protein molecules (Fig. 5).

The increase in spot number in the Npx images from frame to frame can be explained by the assertion that the co-factor FAD in the protein molecule underwent a dynamic of conformational change from the "closed" to the "open" conformation when they were exposed to light. Since the "open" conformation is nine times more fluorescent than the closed one, all of the observed spots were attributed to FAD with an open structure. That is, the fluorescent intensity of a closed structure was too weak to be recorded under the same conditions. This phenomenon did not occur in the enzyme TrxR because there should not be any conformational change for FAD in TrxR. The flavin-cofactor is less tightly bound to Npx compared to TrxR. Also, the flavin in TrxR is bound in an almost completely extended conformation, as indicated by X-ray crystallography.

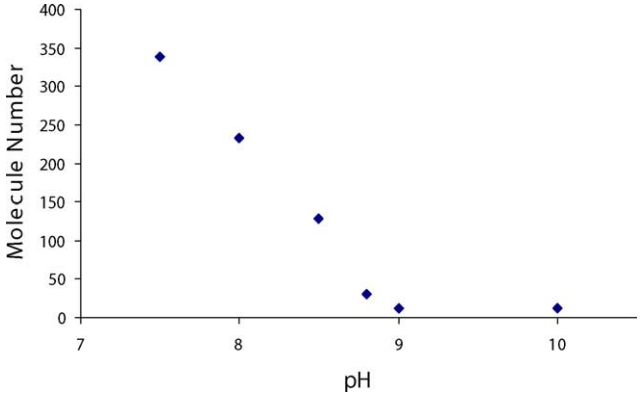

Fig. 3. Plot of number of molecules of Npx observed in the first frame at various pH. Other conditions are as in Fig. 2.

3.2. FAD and FMN

Images of the native fluorescent prosthetic groups, FAD and FMN were taken at pH 10.0, where adsorption is negligible. The observed fluorescence intensity of FAD molecules was found to be higher than those of Npx and TrxR molecules. Similar to Npx, the number of FAD molecules in the images increased from frame to frame upon exposure to laser light, as shown in Fig. 6. Furthermore, it is evident from Figs. 7 and 8 that the rate of increase depended on the laser exposure time and the concentration of the FAD molecules. Under identical concentrations, the longer the exposure time or the higher the laser power, the more the number of molecule spots and the higher the rate of increase (Fig. 7). Additionally, under identical exposure times and laser powers, the higher the concentration, the higher the rate of increase in molecule spots (Fig. 8). Over the range of conditions studied, the rate of increase in observed molecule number was proportional to the square of the exposure time and the square of the FAD concentration.

Figs. 7 and 8 indicate that a photochemical reaction is involved. A second order reaction describes the dependence on the number of supplied photons and the number of FAD molecules. This implies that not only did the opening of the closed FAD conformation require a photon, but also another photoproduct was formed. A possible mechanism is that upon

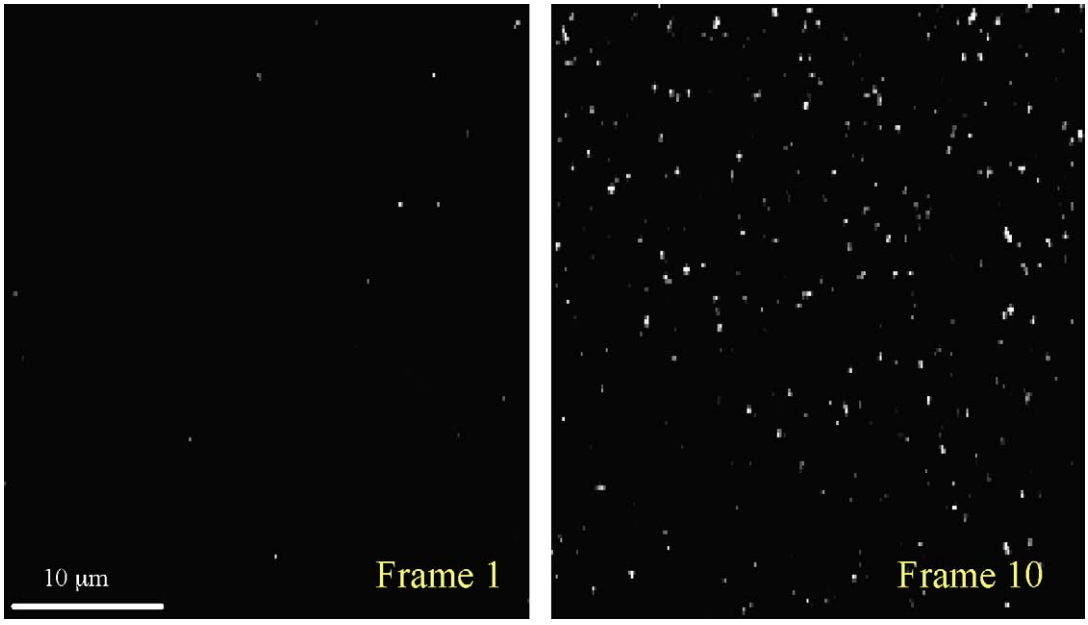

Fig. 4. Native fluorescence images of Npx at pH 10.0 for two irradiation periods. Other conditions are as in Fig. 2.

absorbing one photon each, FAD molecules were excited to an open conformation. Then, these excited molecules reacted with other FAD molecules to form a stable dimer that had an even higher quantum yield.

Native fluorescence images of FMN at pH 10.0 were also recorded. Under the same conditions, the fluorescence intensity of FMN molecules was weaker than those of the dimeric product of FAD described above. Also, there was no increase in the molecule numbers monitored from frame to frame. This indicated that there was no conformational change and no photoreaction for FMN. The presence of the adenine ring in FAD was likely responsible for the dimer formation, instead of the isoalloxazine ring.

In order to compare single-molecule fluorescence observations from FAD with ensemble-average measurements, 2 mL solutions of FAD with concentrations of 1–20 μM in a 1 cm cuvette were exposed to a 488 nm laser at ~15 mW/cm^2. The top half of the rectangular cuvette was exposed to the laser while the bottom half was not irradiated. The solution was constantly stirred with a micromagnetic bar to ensure all molecules in the solution were evenly exposed. Emission spectra were taken at regular intervals by a fluorimeter (Perkin-Elmer Luminescence Spectrometer LS50B). The relative fluorescence intensity excited at 488 nm at the maximum emission wavelength, 515 nm, was plotted against the total exposure time as shown in Fig. 9. We found that the

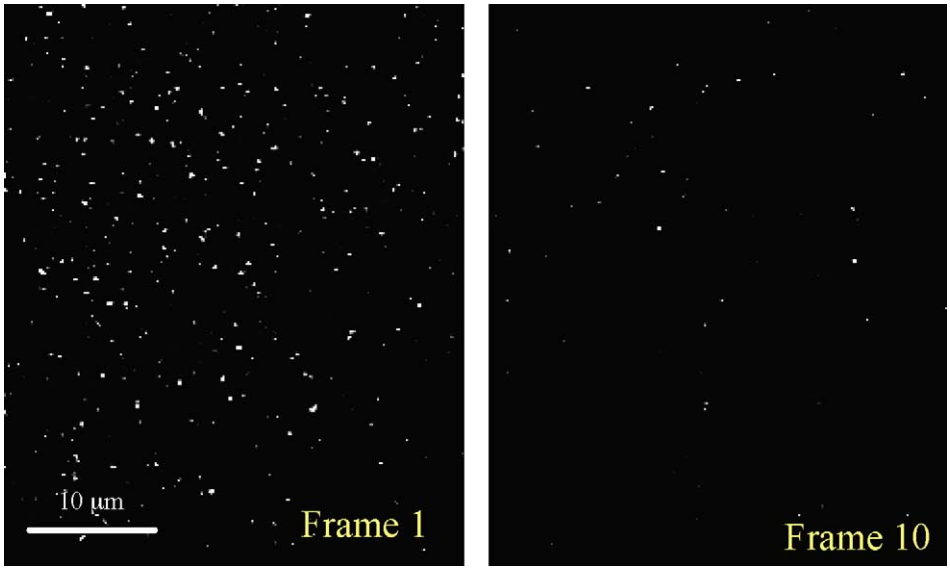

Fig. 5. Native fluorescence images of TrxR at pH 10.0 for two irradiation periods. Other conditions are as in Fig. 2.

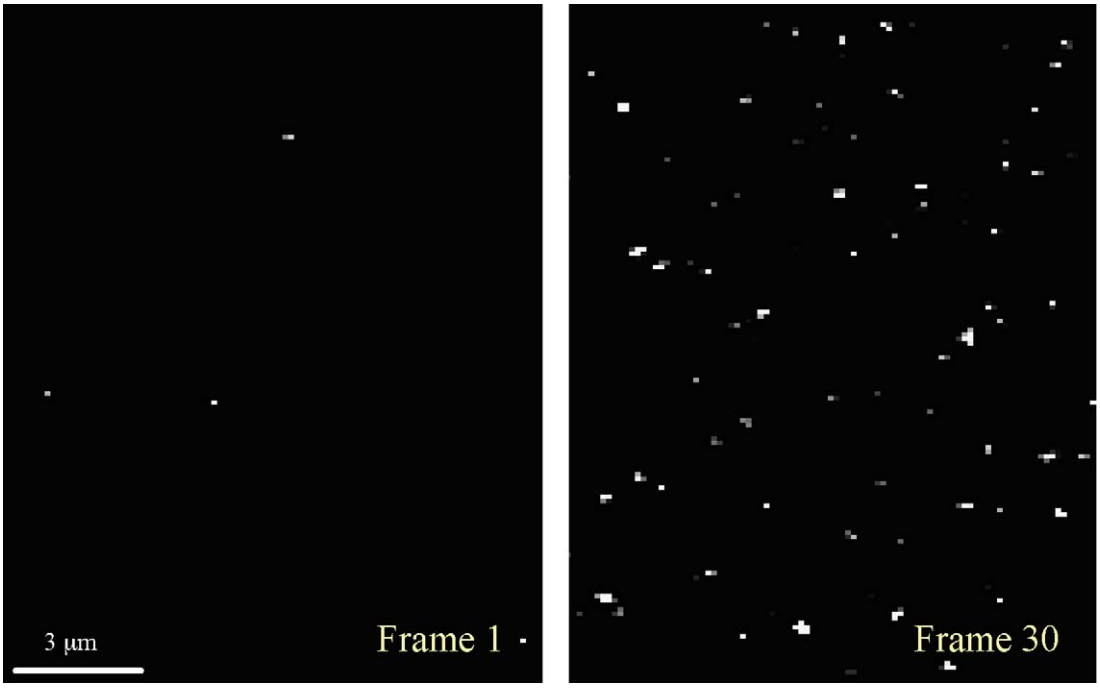

Fig. 6. Native fluorescence images of FAD at pH 10.0 for two irradiation periods. Other conditions are as in Fig. 2. The number of molecules recorded increased with laser irradiation.

fluorescence intensity from FAD increased with total laser exposure and leveled off at times longer than 15 min. The emission spectrum of FAD did not change throughout the prolonged laser exposure. The fluorescence intensities also did not change upon removing the cuvette from the laser source and storing at room temperature at dark condition. So, the photoproduct was stable physiochemically.

Fig. 9 shows that the relative intensity increase did not follow a simple relationship with FAD concentration and total exposure time. From 1 to 4 μM, doubling the concentration caused 2.5- to 2.8-fold increases in fluorescence intensity. At higher concentrations, the intensity increased at a lower rate. This did not agree with the results for SMD as discussed above (quadratic dependence). This discrepancy can be attributed to the facts that molecules in bulk solution at concentrations in the μM range (in order to be detectable) underwent self-absorption and that molecules were being photobleached continuously. The absorbance of a 20 μM FAD solution was 0.066 and 0.179 at 488 and 440 nm (absorption maximum), respectively. Having such a high value of absorbance, FAD was affected by self-absorption and in turn caused a lower apparent fluorescence intensity.

To evaluate the contribution of self-absorption, a thinner cell (2 mm pathlength) for laser irradiation and spectrometric measurement was used instead of the 10 mm cell. The fluorescence intensity of FAD increased initially linearly over a large range of concentrations, 0.75–30 μM. Based on the above absorption data, self-absorption is negligible with the shorter pathlength. All these solutions were exposed to 488 nm laser for 3 min continuously without stirring. The laser beam was

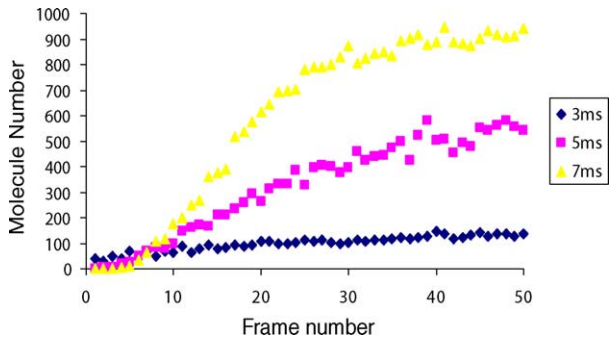

Fig. 7. Molecule numbers of FAD (50 nM) at pH 10.0 as a function of irradiation (different exposure times). The excitation wavelength was 488 nm at 300 W/cm^2. Data was acquired at 3 ms (♦), 5 ms (■) and 7 ms (▲) exposure times at 2 Hz.

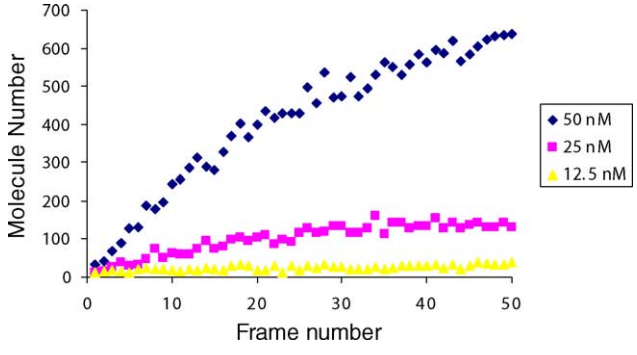

Fig. 8. Molecule numbers of FAD at various concentrations at pH 10.0 as a function of irradiation. FAD concentration was 50 nM (♦), 25 nM (■) and 12.5 nM (▲). The excitation wavelength was 488 nm at 300 W/cm^2. Data was acquired at 5 ms exposure time at 2 Hz.

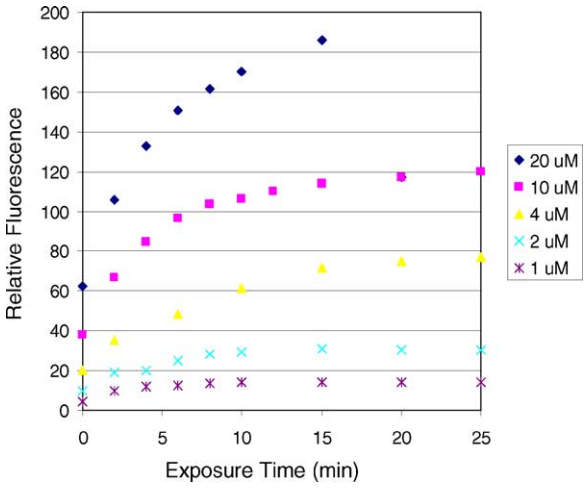

Fig. 9. Bulk fluorescence experiments for FAD after irradiation with a 1 cm^2 488 nm laser at 15 mW for various durations. The concentrations ranged from 1 to 20 μM.

Table 1
Relative emission intensity of FAD solution at various concentrations before and after 488 nm laser exposure

Concentration	0.75 μM	1.5 μM	3 μM	6 μM	12 μM	30 μM
$t = 0$	3.5	7.3	11.7	23.3	45.8	105.4
$t = 3$ min	3.4	6.8	11.9	23.5	42.9	100.0

expanded to cover the entire solution simultaneously. The changes in fluorescence emission intensity (as depicted in Table 1) were different from those discussed above. The emission intensity decreased slightly for low concentrations, increased slightly at medium concentrations, and dropped again for higher concentrations. This behavior can be attributed to photobleaching of the photoproduct. Unlike the above experiments in a 1 cm cuvette, the lack of excess solution confined all the molecules in the laser beam region. Even though the highly fluorescent photoproduct was formed, it was not able to move away from the irradiated region and was easily photobleached within the irradiation time of 3 min. The net result is that the intensity increased due to photoproduct formation and decreased from photobleaching, thereby counteracting each other and no obvious changes in emission could be seen. We note that these details about the reaction were not observable in bulk fluorescence measurements. But, in single-molecule measurements, self-absorption is eliminated because of the much lower concentration. Photobleaching is also less of a problem because molecules are recorded and thus counted well before photobleaching. Finally, molecule counting provides proper kinetics information because it is independent of the intensity of the spots.

4. Conclusions

We demonstrated a sensitive, fast and simple technique for monitoring the real-time dynamics of the photoproduct formation between FAD molecules. Insight is obtained regarding how tightly the FAD cofactor is bound to a flavoprotein. If FAD is bound tightly, conformational changes are restricted and the number of molecules that could be observed should remain constant. However, if the FAD molecules are loosely bound to the protein, conformational changes from the closed structure to an open structure would be allowed and hence brighter spots could be detected due to the higher quantum yield of the open structure. The observed kinetics point to the formation of a dimer on excitation. Our results indicate that single-molecule experiments are necessary to eliminate artifacts arising from self-absorption or fluorescence quenching.

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References

[1] C. Bauer, S. Elsen, L.R. Swem, D.L. Swem, S. Masuda, Redox and light regulation of gene expression in photosynthetic prokaryotes, Phil. Trans. Roy. Soc. Lond., Ser. B: Biol. Sci. 358 (2003) 147–154.
[2] S. Braatsch, M. Gomelsky, S. Kuphal, G. Klug, A single falvoprotein, AppA, integrates both redox and light signals in *Rhodobacter sphaeroides*, Mol. Microbiol. 45 (2002) 827–836.
[3] H. Amino, A. Osanai, H. Miyadera, N. Shinjyo, E. Tomitsuka, H. Taka, R. Mineki, K. Murayama, S. Takamiya, T. Aoki, H. Miyoshi, K. Sakamoto, S. Kojima, K. Kita, Isolation and characterization of the stage-specific cytochrome *b* small subunit (CybS) of *Ascaris suum* complex II from the aerobic respiratory chain of larval mitochondria, Mol. Biochem. Parasitol. 128 (2003) 175–186.
[4] M. Hetzel, M. Brock, T. Selmer, A.J. Pierik, B.T. Golding, W. Buckel, Acryloyl-CoA reductase from *Clostridium propionicum*. An enzyme complex of pripionyl-CoA dehydrogenase and electron-transferring flavoprotein, Eur. J. Biochem. 270 (2003) 902–910.
[5] C. Cosseau, A.M. Garnerone, J. Batut, The FixM flavoprotein modulates inhibition by AICAR or 5'AMP of respiratory and nitrogen fixation gene expression in *Sinorhizobium meliloti*, Mol. Plant–Microbe Interact. 15 (2002) 598–607.
[6] M.H. Tsai, M.H.J. Saier, Phylogenetic characterization of the ubiquitous electron transfer flavoprotein families ETF-alpha and ETF-beta, Res. Microbiol. 146 (1995) 397–404.
[7] R.J. Stanley, A.W. MacFarlane, in: Abstracts, Proceedings of the 36th Middle Atlantic Regional Meeting of the American Chemical Society, Princeton, NJ, 2003.
[8] A.W.I. MacFarlane, R. Stanley, *Cis*-syn thymidine dimer repair by DNA photolyase in real time, J. Biochem. 42 (2003) 8558–8568.
[9] N. Mano, N. Asakawa, J. Goto, Highly selective molecular recognition of biologically active substances using liquid phase separation, Chromatography 24 (2003) 9–34.
[10] A. Curcoy, R.K.J. Olsen, A. Ribes, V. Trenchs, M.A. Vilaseca, J. Campistol, J.H. Osorio, B.S. Andresen, N. Gregersen, Late-onset form of b-electron transfer flavoprotein deficiency, Mol. Genet. Metab. 78 (2003) 247–249.

[11] Z.M. Kravchuk, M.O. Druzhina, D.M. Grodzins'kii, O. Dmitriev, P. Dopovidi, Single transduction in cells of *Allium cepa* during "oxidative burst", Natsional'noi Akademii Nauk Ukraini (2003) 167–171.
[12] C. Cande, F. Cecconi, P. Dessen, G. Kroemer, Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death, J. Cell Sci. 115 (2002) 4727–4734.
[13] P.A.W. van den Berg, A.J.W.G. Visser, Tracking molecular dynamics of flavoproteins with time-resolved fluorescence spectroscopy, in: New Trends in Fluorescence Spectroscopy: Applications to Chemical and Life Sciences, Springer, Berlin, 2001, pp. 457–485.
[14] P.A.W. van den Berg, S.B. Mulrooney, B. Gobets, I.H.M. van Stokkum, A. van Hoek, C.H.J. Williams, A.J.W.G. Visser, Exploring the conformational equilibrium of *E. coli* thioredoxin reductase: characterization of two catalytically important states by ultrafast flavin fluorescence spectroscopy, Protein Sci. 10 (2001) 2037–2049.
[15] G. Waksman, T.S.R. Krishna, C.H. Williams Jr., J. Kuriyan, Crystal structure of *Escherichia coli* thioredoxin reductase refined at 2 Å resolution. Implications for a large conformational change during catalysis, J. Mol. Biol. 236 (1994) 800–816.
[16] L. Serre, F.M.D. Vellieux, M. Medina, C. Gomez-Moreno, J.C. Fontecilla-Camps, M. Frey, X-ray structure of the ferredoxin:$NADP^+$ reductase from the cyanobacterium Anabaena PCC 7119 at 1.8 Å resolution, and crystallographic studies of $NADP^+$ binding at 2.25 Å resolution, J. Mol. Biol. 263 (1996) 20–39.
[17] G. Weber, Fluorescence of riboflavin and flavin adenine dinucleotide, Biochem. J. 47 (1950) 114–121.
[18] P.A.W. van den Berg, K.A. Feenstra, A.E. Mark, H.J.C. Berendsen, A.J.W.G. Visser, Dynamic conformations of flavin adenine dinucleotide: simulated molecular dynamics of the flavin cofactor related to the time-resolved fluorescence characteristics, J. Phys. Chem. B106 (2002) 8858–8869.
[19] P.A.W. van den Berg, J. Widengren, M.A. Hink, R. Rigler, A.J.W.G. Visser, Fluorescence correlation spectroscopy of flavins and flavoenzymes: photochemical and photophysical aspects, Spectrochim. Acta, Part A 57 (2001) 2135–3144.
[20] X. Xu, E.S. Yeung, Direct measurement of single-molecule diffusion and photodecomposition in free solution, Science 276 (1997) 1106–1109.
[21] X.-H. Xu, E.S. Yeung, Long-range electrostatic trapping of single protein molecules at a liquid/solid interface, Science 281 (1998) 1650–1653.
[22] S.H. Kang, M.R. Shortreed, E.S. Yeung, Real-time dynamics of single-DNA molecules undergoing adsorption and desorption at liquid-solid interfaces, Anal. Chem. 73 (2001) 1091–1099.
[23] S.H. Kang, E.S. Yeung, Dynamics of single-protein molecules at a liquid/solid interface: Implications in capillary electrophoresis and chromatography, Anal. Chem. 74 (2002) 6334–6339.