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Single-Molecule Spectroscopy Studies of Molecular Dynamics in Chemical and Biological Systems*

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Single-molecule chemical kinetics of interfacial electron transfer from excited cresyl violet molecules to the conduction band or energetically accessible surface electronic states of indium-tin-oxide (ITO) was studied under ambient conditions. We revealed that the physical origin of multiexponential kinetics of electron transfer in this system is the inhomogeneity of molecular interactions on the semiconductor surface of ITO. Spontaneous and photo-induced spectral fluctuations of single sulforhodamine 101 molecules at the glass-polymer interface were investigated and attributed to transitions between metastable minima in the molecular potential-energy surface. Similar conformational fluctuations are also observed for the active site of the cholesterol oxidase, flavin adenine dinucleotide (FAD), which results in dynamic disorder in the enzymatic reaction of cholesterol oxidase.

The applications of Single Molecule Spectroscopy (SMS) to studies of chemical and biological systems have provided new information which is not obtainable from ensemble-averaged measurements [1–27]. SMS is particularly powerful in investigating inhomogeneous molecular systems, such as interfacial chemical systems and proteins. In this article, we review our work on chemical and spectral dynamics of a few chemical systems and an enzymatic reaction system.

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Multiexponential kinetics of interfacial electron transfer is often observed in dye-sensitization systems [28−35]. However, the origin of the multiexponential kinetics in these systems is not well understood and is extremely difficult to identify from ensemble-averaged experiments [33]. To investigate the physical origins of the multiexponential kinetics in interfacial electron transfer, we examined photosensitization [28, 29] of single cresyl violet molecules on an indium-tin-oxide (ITO) semiconductor surface [34]. In this system, the electron is ejected from the excited cresyl violet molecule to the conduction band or energetically accessible surface electronic states of the ITO. The rate of the electron transfer is measured by the fluorescence decay of excited dye molecules using time correlated single photon counting [3].

Fig. 1A displays the fluorescence decay of a single cresyl violet molecule on ITO, which exhibits single-exponential kinetics with a time constant of 480 ps. The decay is attributed to interfacial electron transfer [3]. In this system, electron transfer kinetics is single exponential for all the single molecules examined. However, individual molecules exhibit different interfacial electron transfer rates due to their different local environments. Fig. 1B shows the wide distribution of lifetimes (in the range of 100 to 900 ps) for 40 cresyl violet molecules that undergo interfacial electron transfer. Based on this result, kinetics measured on large ensembles of molecules would have at least 20 exponentials, a situation that is impossible to resolve in conventional ensemble-average measurements. Interfacial electron transfer in the sub-picosecond time-scale [28, 32, 33, 35] is also possible in this system. However, we cannot measure lifetimes shorter than 100 ps because of the 160-ps (fwhm) instrument response time [3]. Although the distribution of the electron transfer rates we observed might not be a complete distribution, our observations do reveal that the multiexponential behavior of this system originates from the inhomogeneity of site-specific molecular interactions. We anticipate that inhomogeneity is also the general origin of multiexponential kinetics for interfacial electron transfer in other dye-sensitization systems.

Spectral fluctuations of single dye molecules at an interface is particularly important in investigating molecular motion and interactions. We observed spectral fluctuations at a rather slow time-scale, millisecond to hundred-second, in monitoring the emission spectra of individual sulphorhodamine 101 molecules at a glass-PMMA (poly-methyl methacrylate) interface [4]. Fig. 2A shows the emission spectra of a single sulphorhodamine 101 molecule taken sequentially with 170-ms data-collection times. Our statistical analyses showed two time-scales of the spectral fluctuations, corresponding to inter- and intramolecular conformational changes over two distinctly different types of barrier heights (see Fig. 2B, solid curve). The small barriers are associated with fast thermal fluctuations, and the large (gross) barriers are associated with photo-induced and slow thermal fluctua-

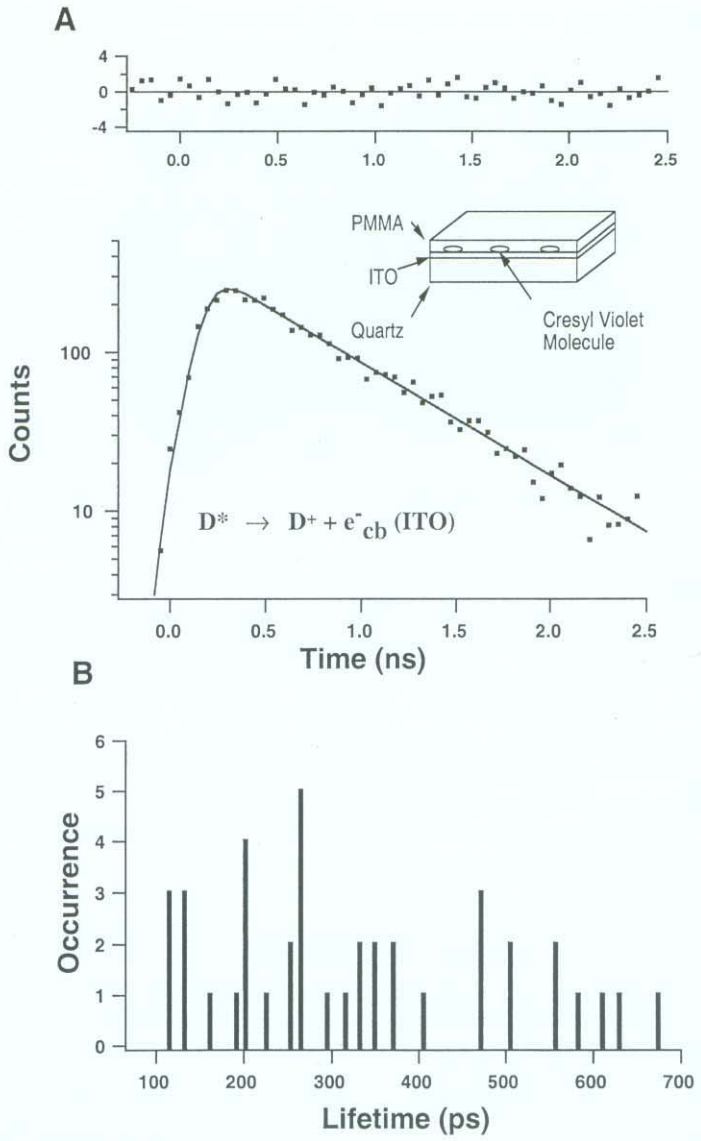

Fig. 1. (A) The fluorescence decay of a single cresyl violet molecule dispersed on an indium-tin-oxide (ITO) film, measured by time correlated photon counting. The solid line is the fitted decay (single exponential of 480±5 ps with $\chi^2 = 1.14$) convoluted with the instrumental response function (fwhm 160 ps). Weighted residuals are shown at the top. The single exponential decay is due to interfacial electron transfer from the excited molecule (D*) to the conduction band or energetically accessible surface electronic states of ITO. (B) Distribution of single-molecule emission lifetimes for 40 different molecules. A broad distribution of site-specific electron transfer kinetics is observed. (Reprinted with permission: see J. Phys. Chem. **B 101** (1997) 2753.)

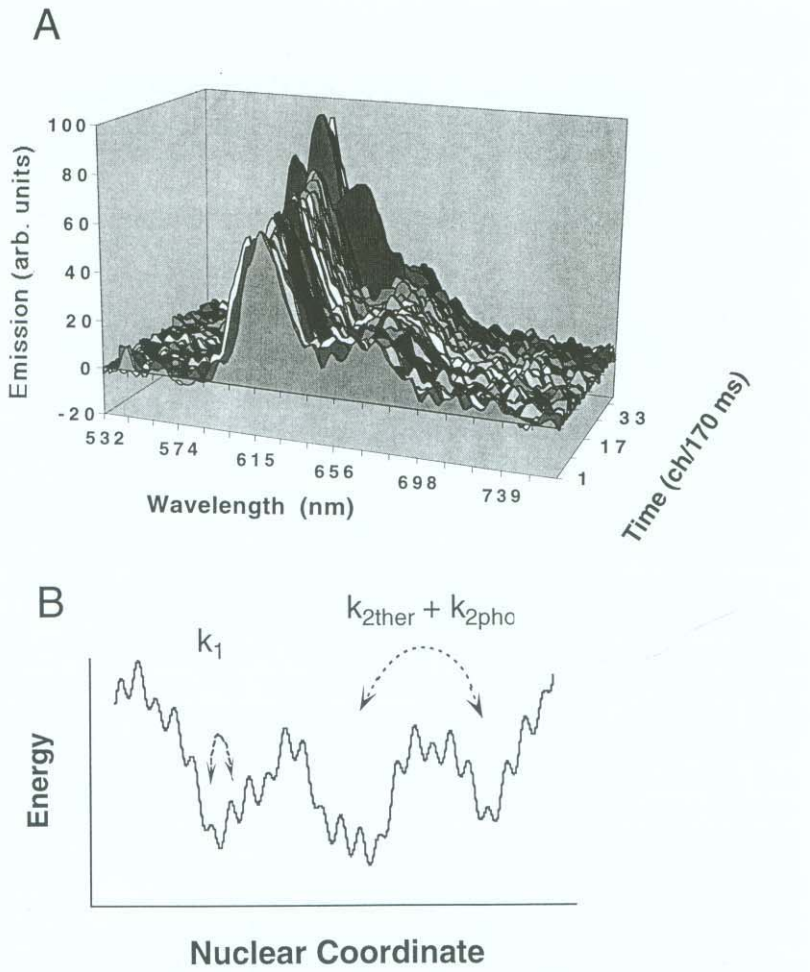

Fig. 2. The emission spectra of individual molecules were recorded with a high emission collection efficiency (10%) using a combination of a spectrograph and a back-illuminated CCD camera configured for a spectral resolution of 1 nm with no dead time in data collection. (A) Emission spectra of a single sulforhodamine 101 molecule taken sequentially with 170-ms data-collection times before the final photobleaching. Significant spectral shifts are evident. (B) A schematic of the potential energy surface of the nuclear coordinates. Our analyses of the spectral trajectories indicate the existence of two distinctly different types of barrier heights. The small barriers are associated with fast thermal fluctuation (k_1), and the gross barriers are associated with the photoinduced (k_{2pho}) and slow thermal (k_{2ther}) fluctuations. (Reprinted with permission: Nature **385** (1997) 143.)

tions. This experiment provided new insights into the general features of the potential energy surface and the dynamical properties of a single molecule in a particular environment.

Cholesterol oxidase (COx) is a flavoprotein that catalyzes the oxidation of cholesterol by oxygen [36]. The monosubunit flavoprotein contains a chromophore, flavin adenine dinucleotide (FAD), which is naturally fluorescent in its oxidized form but not in its reduced form. Fig. 3A shows a fluorescence image of single COx molecules in their oxidized form with 442-nm excitation. The single molecules of COx were confined in agarose gel of 99% water [5] with free rotation but no observable translational diffusion. With excess amounts of cholesterol and oxygen molecules that undergo essentially free translational diffusion within the gel, the single FAD emission exhibits on-and-off intensity blinking behavior (Fig. 3B). That is, the fluorescence turns on and off as the redox state of FAD toggles between the oxidized and reduced states, respectively, during each enzymatic turnover. The fluorescence on-times, corresponding to the time it takes for the reduction of FAD to occur, are stochastic on a single molecule basis. Without cholesterol molecules, there is essentially no intensity blinking. Moreover, the turnover rate is independent of excitation intensity but dependent on the substrate concentration.

Being able to monitor the enzymatic turnovers in real time and determine the rates of chemical reactions of single molecules, we can evaluate the static disorder [5, 17, 18] and dynamic disorder [5, 37] of the enzymatic reactions. The static disorder refers to the static heterogeneity of the rates among individual molecules during the course of the measurements and the dynamic disorder refers to the fluctuation of the rate of an individual molecule. A four-fold variation of the cholesterol oxidation rate is seen among many molecules when the reduction of FAD is the rate-limiting step [5], reflecting the static disorder.

The dynamic disorder refers to the variation of the rate of a single molecule, which is reflected by the autocorrelation function r(m) of the on-times (Fig. 3C), m being the index number of the turnovers. If there is no fluctuation of the rate of chemical reaction, then $r(m) = 0$ for $m > 0$. The fact that the r(m) is an exponential decay indicates that the rate fluctuates at the time-scale of enzymatic turnovers. Simulation indicated that the standard deviation of the fluctuating rate can be as large as 70% of the mean rate [5]. Every molecule in the system shows similar behavior. This dynamic disorder behavior, also known as non-Markovian behavior, is attributed to slow fluctuations of protein conformations [5], and is otherwise hidden in ensemble-averaged measurements.

The single molecule studies described in this article provide new insights into the chemical and biological systems. Single molecule work will continue to yield new information of molecular interactions and their influence to chemical reactions at a level of great detail.

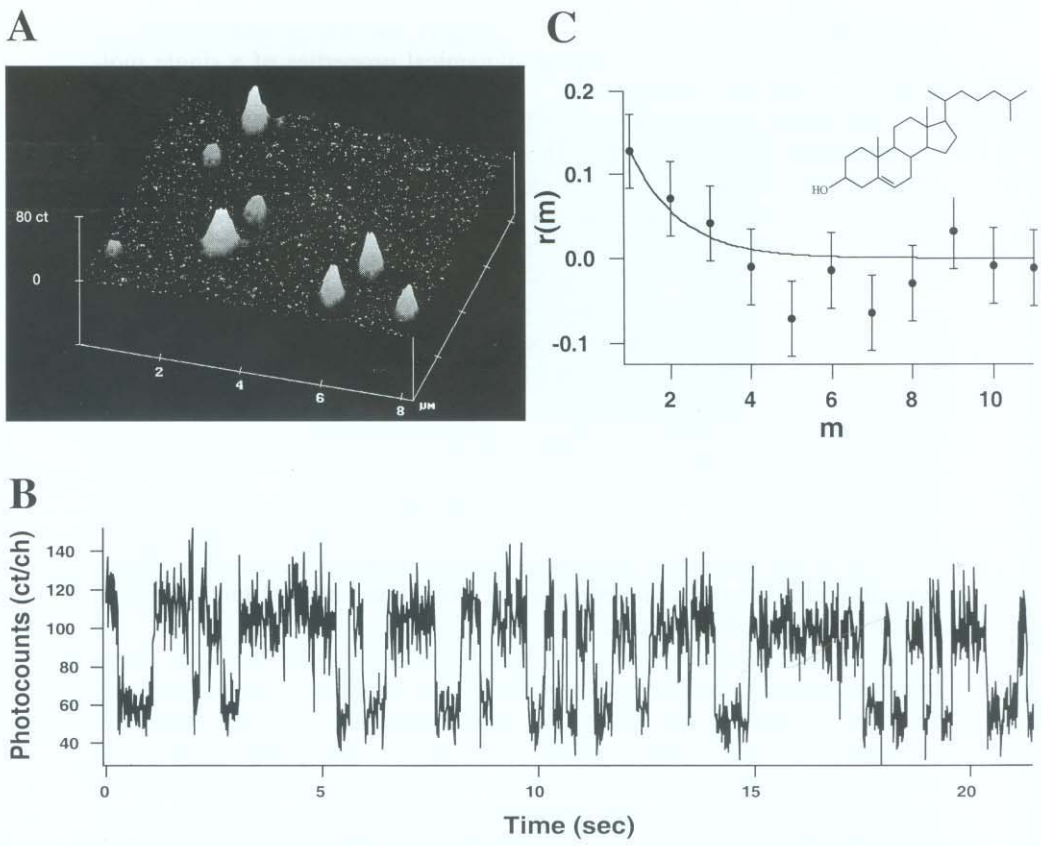

Fig. 3. (A) Fluorescence image (8 μm × 8 μm) of single COx molecules immobilized in a 10 μm film of agarose gel of 99% buffer solution. This image was taken in 4 minutes with an inverted fluorescence microscope by raster-scanning the sample with a focused laser beam of 500 nW at 442 nm. Each individual peak is attributed to a single COx molecule. (B) Real-time observation of enzymatic turnovers of a single COx molecule catalyzing oxidation of cholesterol molecules. This panel shows a portion of an emission intensity trajectory containing more than 500 turnovers. The trajectory was recorded with a cholesterol concentration of 0.2 mM and saturated oxygen concentration of 0.25 mM. The emission exhibits stochastic blinking behavior as the FAD toggles between oxidized (fluorescent) and reduced (not fluorescent) states, each on-off cycle corresponding to an enzymatic turnover. (C) r(m), the autocorrelation function of the on-times, for the COx molecule with 2 mM of cholesterol. The solid line is a single exponential fitting with a correlation time of 0.6±0.3 s for the fluctuating rate constant of the reduction of FAD, knowing the averaged turnover time is 500 ms.

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