Review

Fluorescence-based analysis of enzymes at the single-molecule level

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Enzymes, and proteins in general, consist of a dynamic ensemble of different conformations, which fluctuate around an average structure. Single-molecule experiments are a powerful tool to obtain information about these conformations and their contributions to the catalytic reaction. In contrast to classical ensemble measurements, which average over the whole population, single-molecule experiments are able to detect conformational heterogeneities, to identify transient or rare conformations, to follow the time series of conformational changes and to reveal parallel reaction pathways. A number of single-molecule studies with enzymes have proven this potential showing that the activity of individual enzymes varies between different molecules and that the catalytic rate constants fluctuate over time. From a practical point of view this review focuses on fluorescence-based methods that have been used to study enzymes at the single-molecule level. Since the first proof-of-principle experiments a wide range of different methods have been developed over the last 10 years and the methodology now needs to be applied to answer questions of biological relevance, for example about conformational changes induced by allosteric effectors or mutations.

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1 Introduction

The first experiment designed to detect the activity of single enzyme molecules dates back to the early 1960s [1]. Single molecules of the enzyme β-galactosidase were encapsulated together with a substrate in droplets of a water-in-oil emulsion. Upon cleavage of the substrate a fluorescent dye was generated that accumulated in the droplets over time and could be detected after several hours with a wide field fluorescence microscope. Thirty years after this pioneering work intense efforts in developing optical instrumentation and detection techniques made it possible to detect a single fluorescent dye in aqueous solution at room temperature [2]. This breakthrough then enabled not only the analysis of single enzyme molecules but also the detection of single enzymatic turnover reactions [3].

In the following years, further developments in single-molecule fluorescence detection and spectroscopy laid the basis for the broad application of single-molecule fluorescence approaches in biology, chemistry and physics. These developments together with an overview of current state of the art instrumentation are summarized in several excellent reviews [4–7]. For the study of biological systems, single-molecule fluorescence techniques have now evolved into indispensable tools. Besides

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Abbreviations: **APD**, avalanche photodiode; **CalB**, *Candida antarctica* lipase B; **COx**, cholesterol oxidase; **DHFR**, dihydrofolate reductase; **ET**, electron transfer; **FAD**, flavin adenine dinucleotide; **FMN**, flavin mononucleotide; **FRET**, Förster resonance energy transfer; **HRP**, horseradish peroxidase; **PDMS**, poly(dimethylsiloxane); **spFRET**, FRET between a single pair of chromophores; **TIRF**, total internal reflection fluorescence; **TLL**, *Thermomyces lanuginosa* lipase

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being the basis for a number of high-resolution imaging techniques [8–13], single-molecule approaches have provided detailed insights into a number of molecular processes. Their application to the study of molecular motors [14, 15], RNA polymerases [16], the ribosome [17, 18] and other DNA and RNA manipulating enzymes [19] has been reviewed recently and will not be the topic of this review.

This review focuses on single-molecule approaches to study the catalytic activity of enzymes, thereby focusing mainly on enzymes with low molecular weight substrates. Different experimental designs have been used providing different levels of insight into the reactivity of single enzymes. The main difference between these approaches is the time resolution of the measurement, which ranges from simple endpoint measurements after minutes or hours down to a sub-millisecond time resolution. Endpoint measurements can only yield static information, *e.g.*, whether all enzymes generated the same amount of product during the incubation time. In contrast, experiments spanning the same time window but with a time resolution in the millisecond range contain dynamic information, *e.g.*, whether the turnover rate is constant or varies over time. Since enzymes are intrinsically dynamic molecules the observation of dynamic processes on this time scale can provide a more detailed understanding of conformational changes that are related to the function of enzymes.

2 Fluorescent reporter systems

The analysis of single enzymes with fluorescence-based methods requires reporter systems that allow the read out of the behavior of the enzyme (Fig. 1). These reporter systems can be divided into two categories. The first category contains artificial substrates (fluorescent or fluorogenic substrates) that are converted by the enzyme thereby directly reporting on the catalytic reaction. The second category represents fluorescent dyes that are coupled to the enzyme at specific positions. These dyes change their properties as a function of the process that is monitored. This can either be the catalytic reaction or a conformational change. In contrast to artificial substrates, the same fluorescent dye is monitored over the duration of the measurement until it finally bleaches or dissociates from the enzyme.

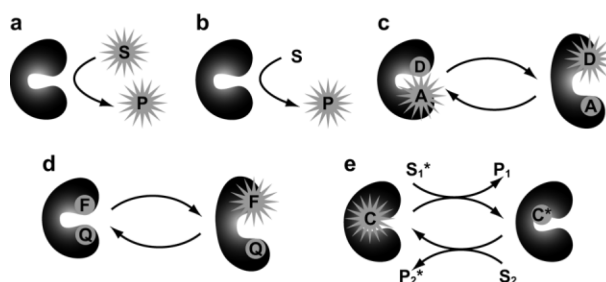

Figure 1. Fluorescent reporter systems. (a) A fluorescently labeled substrate molecule (S) binds to the enzyme and is converted into a fluorescent product (P). (b) A non-fluorescent substrate molecule (fluorogenic substrate) is converted into a fluorescent product. (c) Two fluorescent dyes forming a FRET pair are coupled to the enzyme at two positions. A conformational change alters the distance between the FRET donor (D) and the FRET acceptor (A) and, as a result, the FRET efficiency. (d) One fluorescent dye is coupled to the enzyme at a specific position, *e.g.*, in proximity to a tyrosine residue. A conformational change alters the distance between the fluorophore (F) and the tyrosine (Q), which quenches the fluorescence resulting from electron transfer. (e) The enzyme contains a fluorescent cofactor (C). The fluorescence of the cofactor is altered during one reaction cycle, *e.g.*, by reduction.

2.1 Fluorescent substrates

Fluorescent substrates consist of a fluorescent dye, which is coupled to a natural substrate at a position where it does not interfere with substrate recognition and turnover. For example, ATP labeled with the dye Cy3 has been used to investigate single ATP cleavage reactions by the molecular motor protein myosin [3]. Fluorescent substrates are rarely used. They only report on the binding and dissociation of the fluorophore but do not provide the information whether the substrate has been converted into product.

2.2 Fluorogenic substrates

The most commonly used fluorogenic substrates consist of a fluorescent dye and a component that is specifically recognized by the enzyme. The dye is linked to this component by the chemical bond that is normally cleaved by the enzyme. In contrast to fluorescent substrates, fluorogenic substrates only become detectable upon cleavage of this bond. While this chemical linkage is intact the fluorescence of the dye is either quenched or its spectral characteristics are significantly altered. Fluorogenic substrates are available for a number of hydrolyses such as proteases, lipases, phosphatases and glycosidases [20]. In addition, several fluorogenic substrates exist for oxidoreductases [21–23]. These non-fluorescent substrates become fluorescent upon reduction or oxidation.

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2.3 Förster resonance energy transfer

Förster resonance energy transfer (FRET) involves non-radiative energy transfer from a donor chromophore in its excited state to a ground state acceptor chromophore by a long-range dipole-dipole coupling mechanism [6, 24]. When exciting only the donor located at a sufficiently short distance from the acceptor, a significant amount of energy is transferred from the donor to the acceptor resulting in decreased donor and increased acceptor fluorescence. Being inversely proportional to the sixth power of the separation distance between the chromophores, FRET is a very sensitive reporter for distance changes between these chromophores on a length scale between 2 and 8 nm. FRET between a single pair of chromophores (spFRET) can therefore be applied as a reporter for conformational changes of enzymes that occur on this length scale. The usefulness of this principle was demonstrated for the first time by Ha *et al*. [25]. By labeling the enzyme staphylococcal nuclease with the dyes tetramethylrhodamine and Cy5, the labeled enzyme was found to display gradual fluctuations in donor and acceptor emission intensity. These fluctuations occurring on time scales between 10 ms and 1 s were considered to be the result of conformational changes in the protein. Despite this convincing proof of the power of FRET at the single-molecule level, the experiment also shows one of its biggest disadvantages. spFRET requires that two chromophores are attached to the enzyme. Ha *et al*. attached the donor site-specifically, while the acceptor was attached at a random position. To really obtain information about molecular distances a site-specific attachment of both chromophores is essential. This requires detailed structural information and, in many cases, the complicated preparation of doubly chromophore labeled enzymes. As an alternative, chromophoric cofactors [26] or chromophore-labeled substrates [25] have been used as one FRET partner. Independent of the labeling strategy used, another limitation of FRET is the unavoidable inactivation of the fluorophore (due to photobleaching etc.), which normally limits the measurement time to a few seconds.

2.4 Fluorescence quenching by photo-induced electron transfer

Fluorescence emission can be quenched when a transfer of electrons from the excited state chromophore to a quencher can occur. This electron transfer (ET) requires very short distances between the chromophore and the quencher (subnanometer length scale). In contrast to FRET, the ET efficiency has an inverse exponential dependence upon the distance. In proteins, ET from a chromophore often occurs with tryptophan or tyrosine residues. As a consequence, the analysis of conformational changes based on ET requires the attachment of only one chromophore. This reduces the efforts to produce a suitable chromophore-labeled enzyme significantly. In the case of an enzyme possessing a fluorescent cofactor, no labeling is required at all. This principle has been used to analyze the conformational dynamics of the enzyme flavin reductase from *E. coli* [27], which contains a fluorescent flavin cofactor. ET between this flavin and a nearby tyrosine was influenced by distance changes between the flavin and the tyrosine originating from conformational dynamics. ET not only results in a reduced fluorescence intensity but also shortens the lifetime of the excited state. To observe ET both parameters can be measured [6]. In the case of low quantum yield fluorophores such as flavin, lifetime measurements are more sensitive to environmental changes than intensity measurements and have been applied in the described example.

2.5 Fluorescent cofactors modified during the catalytic reaction

Fluorescent flavin cofactors of flavoenzymes represent not only a sensitive electron transfer probe. Flavoenzymes are oxidoreductases and the flavin undergoes reduction and oxidation in each reaction cycle. Both flavin cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are fluorescent in their oxidized and nonfluorescent in their reduced form. Hence, following the fluorescence of the flavin yields information about the catalytic reaction. The first single-molecule study using this principle was performed with the enzyme cholesterol oxidase from *Brevibacterium* [28] and is described in more detail below (see Section 3.2.2).

3 Approaches and detection schemes

Two principally different types of single-molecule experiments can be distinguished to study enzymatic reactions. The first type focuses on the analysis of the catalytic activity of a single-enzyme molecule normally with a relatively low time resolution in the second range or just as an endpoint measurement. The second type are highly time-resolved approaches with single fluorophore sensitivity that go into much more detail and monitor the time se-

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ries of single events such as conformational changes or turnover reactions.

3.1 Single enzyme approaches without single fluorophore sensitivity

In conventional ensemble assays the activity of enzymes is mostly determined from the increase of the concentration of product molecules over time. This principle can be transferred to the single-molecule level. Fluorogenic substrates provide sufficient sensitivity and the accumulation of fluorescent product molecules can be measured either only once after a certain reaction time (endpoint measurement) or repeatedly at certain intervals (time-resolved measurement). For both types of measurements each single enzyme molecule is confined in space together with a fluorogenic substrate. The confinement chemically isolates the individual enzymatic reactions and retains the product molecules in proximity to the enzyme. The volume containing the enzyme together with its substrate and the generated product molecules is normally between 10 and 500 fL. In such a small volume a single enzyme has a "concentration" between 3 and 150 pM. As a result, the generated product molecules quickly reach a detectable amount and substrate turnover by the enzyme dominates over other processes such as autohydrolysis.

A variety of methods exists to achieve the required confinement and many of them can be used for endpoint (Table 1) and time-resolved measurements (Table 2). These methods together with several examples will be summarized below.

3.1.1 Endpoint measurements

As already described above [1], one possibility to confine single enzymes is the encapsulation of single enzyme molecules together with their substrate in droplets of a water-in-oil emulsion. The usage of a huge excess of substrate ensures that the substrate concentration will not be significantly affected by the activity of a single enzyme. The reaction conditions in the droplets remain constant while the amount of fluorescent product molecules increases.

Emulsions can be produced by a variety of methods. Enzyme molecules dissolved in the aqueous phase are distributed into the generated droplets in a random fashion following a Poisson distribution. This means that with a calculated average number of one enzyme per droplet, 37% of the droplets contain no enzyme, 37% of the droplets contain one enzyme, 18% contain two enzymes and 6% even contain three enzymes. The number of enzymes in the droplets is adjusted with the enzyme concentration in the aqueous phase before producing the emulsion. Therefore, to avoid the difficulty of identifying the droplets with more than one enzyme the starting concentration is chosen such that the probability for multi-enzyme droplets is much lower than in the shown example. This strategy results in a large number of empty droplets, but since they will not become fluorescent they can be easily tolerated. The problem with finding the right concentration lies in the fact that the size of the emulsion droplets is not easily adjustable and that the size of the droplets is not constant. Rotman [1] partially solved this problem by only selecting droplets of the same size (14–15 µm) for his measurements. When doing so, the reaction rate can be calculated by comparing the fluorescence generated by the enzyme with a calibration curve. Since the volume of the droplets is known the number of product molecules can be calculated from these data, thereby immediately yielding the turnover number k_{cat}. An accurate calculation of k_{cat} from ensemble measurements can be difficult since it requires an exact determination of the enzyme concentration. Hence, already this relatively simple

Table 1. Summary of publications describing different approaches for endpoint measurements

Enzyme	Substrate	Method	References
β-Galactosidase	6-Hydroxyfluoran β-D-galactopyranoside	Emulsion droplets	[1]
Lactate dehydrogenase	Lactate + NAD	CE capillary	[29]
Alkaline phosphatase	2´-(2-Benzothiazolyl)-6´-hydroxybenzothiazole phosphate (AttoPhos)	CE capillary	[30]
β-Galactosidase	Resorufin β-D-galactopyranoside	CE capillary	[31]

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Table 2. Summary of publications describing different approaches for time-resolved measurements without single fluorophore resolution

Enzyme	Substrate	Reporter system	Method	Time resolution	References
Chymotrypsin	$(sucAAPF)_2$-rhodamine 110	Fluorogenic substrate	Emulsion droplets	8 min	[32]
Lactate dehydrogenase	Lactate + NAD	Fluorogenic substrate (NAD)	Microfabricated femtoliter wells in quartz glass	10 s	[33]
β-Galactosidase	Fluorescein di-β-D-galactopyranoside	Fluorogenic substrate	Microfabricated femtoliter wells in PDMS	1 s	[34]
β-Galactosidase	Resorufin β-D-galactopyranoside	Fluorogenic substrate	Femtoliter wells etched in optical fiber bundles	15 s	[35, 36]

experiment contains a strong argument for analyzing enzymes at the single-molecule level.

A completely different approach to analyze the activity of single enzyme molecules is to fill a very dilute solution of enzymes into a narrow capillary used for capillary electrophoresis [29–31]. These capillaries have a diameter of 10 or 20 µm and a length of up to 1 m. When adjusting the enzyme concentration such that the capillary contains 5–20 enzymes only, the average spacing between each enzyme molecule is on the order of several centimeters. With this spacing the diffusion zones of the enzymes will not overlap during the measurement. If the capillary contains a high concentration of fluorogenic substrate in addition, each enzyme will turn over the substrate leading to a local accumulation of fluorescent product molecules. After a certain incubation time a voltage is applied to the capillary and the product (and enzyme) molecules move along the capillary towards a fluorescence detector. Each product zone will be detected as a peak whose area is proportional to the amount of generated product molecules.

With this capillary assay it was shown for the first time that not all enzymes of a preparation have the same activity. The detected product concentrations varied by factors between 4 and 20 for the enzymes alkaline phosphatase, lactate dehydrogenase and β-galactosidase [29–31]. These differences in activity could be reproduced by repeating the experiment with exactly the same enzyme molecules. This can be done in the capillary in a very elegant way since the substrate and the enzyme molecules have a different electrophoretic mobility. An applied voltage separates the enzyme from the product and the incubation can be repeated. The reproducibly detected differences in activity suggest that these observations are not an artifact of the measurement but must be an intrinsic property of the enzymes that persists longer than the duration of the measurement. Whereas for alkaline phosphatase these differences could be partially explained with different glycosylation patterns [30], lactate dehydrogenase and β-galactosidase are non-glycosylated enzymes. Instead, the differences in activity were attributed to different conformations of the enzymes. Therefore, these experiments revealed for the first time that the catalytic activity of enzymes shows static disorder that originates from different enzyme conformations.

3.1.2 Time-resolved measurements

Although the capillary assay is powerful for endpoint measurements, it cannot easily be adapted for time-resolved measurements required to obtain dynamic information. In contrast, the initially described approach of compartmentalizing single enzymes in emulsion droplets can be easily performed in a time-resolved manner. The increase in fluorescence resulting from the enzymatic reaction can be monitored in many droplets in parallel with a wide field fluorescence microscope combined with a CCD camera. For example, the cleavage of the fluorogenic substrate $(sucAAPF)_2$-rhodamine 110 by the enzyme chymotrypsin was detected in intervals of 8 min within a total time of 80 min [32]. A statistical analysis of more than 1000 droplets with a diameter smaller than 1 µm at different time points showed a broadening of the distribution of the detected fluorescence intensities with increasing incubation time. This broadening of the distribution, which was not observed in a control experiment, was attributed to differences in the activity of the individual enzymes in the droplets. In addition, the data show that the increase in product molecules is not constant and that the activity of the enzymes is not only different between individual enzymes but also varies over time. Un-

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fortunately, a more detailed analysis of the data was prohibited by the slow movement of the droplets during the experiment.

The problems with different droplet sizes and the potential movement of the droplets were solved with the use of arrays of small wells with a diameter between 5 and 10 µm and a depth between 1 and 4 µm [33–36]. These arrays containing hundreds or thousands of identical wells with femtoliter volumes at fixed positions were fabricated with standard microfabrication techniques either in glass [33] or poly(dimethylsiloxane) (PDMS) [34]. Alternatively, wells can be directly generated in optical fiber bundles by etching the fiber material with acid; the cladding surrounding each individual fiber is more acid resistant [35, 36]. Wells prepared in any of these ways can be filled by pipetting a dilute enzyme solution containing an excess of substrate on the array followed by sealing the wells with either a cover slip or a flexible silicone sheet. Closing of the wells is essential to ensure a constant volume and to prevent evaporation or an exchange of solution between the wells during the measurement. Fluorescent product molecules generated in the wells are either excited with a powerful lamp or a laser, and detection is performed with a CCD camera allowing a field of view containing more than 100 wells. With this approach the time resolution could be reduced to below 1 min.

Following the first proof-of-principle experiments with lactate dehydrogenase in quartz glass wells [33] and with β-galactosidase in PDMS wells [34], a more detailed kinetic analysis was performed with β-galactosidase in optical fiber arrays [35, 36]. Wells with a volume of around 40–50 fL were filled with a solution containing between 1 and 4 pM enzyme and 100 µM of the substrate resorufin-β-D-galactopyranoside. With a k_{cat} between 730 [37] and 916/s [36], maximally 2.2% of substrate molecules are turned over per minute thereby allowing measurements for more than 30 min. The reaction producing the fluorescent dye resorufin was monitored in intervals of 15 or 30 s with an integration time of 2 s, resulting in an average of thousands of turnovers per measurement point. As a consequence, the method cannot report on functionally important conformational changes on the millisecond time scale that might affect the catalytic reaction since these should occur several times during each interval. However, the method still yielded information about differences in enzymatic activity that occurred on slower time scales.

The measurement of a large number of enzymes at different substrate concentrations has revealed differences in reaction velocities between individual enzymes [36]. Again, this distribution is attributed to different enzyme conformations, which interconvert on time scales slower than the time of the measurement (2 min). In a second study, the activity of β-galactosidase was monitored in the presence of an inhibitor [35] and the rates of inhibitor binding and release were determined. The experiments also revealed only a one-step jump from no activity to the highest state of activity. This result was unexpected, since β-galactosidase is a tetramer and enzyme molecules with different numbers of bound inhibitor molecules could exist. However, the absence of intermediate activity states suggests a cooperative inhibitor release from the four monomers. This cooperativity could not be shown unambiguously in previous ensemble experiments since these cannot distinguish between a population where all individuals have similar intermediate activity or a population containing a fraction of enzymes with full activity and a fraction with no activity.

3.2 Approaches with single fluorophore sensitivity

A principally different approach from that described above, which monitors increasing numbers of fluorophores over time, is the detection of individual fluorescent reporter molecules that change their properties during the time course of the enzymatic reaction. To detect individual fluorophores more sophisticated optical equipment is required. The excited volume needs to be as small as possible to reduce the fluorescence background originating from fluorescent impurities and from elastic (Rayleigh) and inelastic (Raman) scattering from solvent molecules. This can either be achieved with a confocal microscope or with a total internal reflection fluorescence (TIRF) wide field microscope, which reduces the excitation volume in the z-direction. In both cases the enzyme needs to be immobilized to prevent its diffusion and to ensure that all events are detected during the time course of the measurement. In the case of a confocal microscope, the laser is directed to a position on the surface where a single enzyme was immobilized and fluorescence time traces are recorded at this position with an avalanche photodiode (APD) detector. When using a TIRF microscope, an area of up to 1 mm^2 can be excited and detection is normally achieved with a CCD camera. Despite the potential of analyzing several individual molecules at the same time, TIRF-CCD set-ups are rarely used for experiments where dynamic effects should be analyzed because of the limited time resolution of the CCD camera (5 ms) compared to APDs with a time resolution below 1 ms. In contrast, confocal-APD-

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Table 3. Summary of publications describing different approaches with single fluorophore resolution to monitor conformational changes or single enzymatic turnover events

Enzyme	Substrate	Reporter system	Reference(s)
Lysozyme	*E. coli* cell wall particles	FRET	[38]
Dihydrofolate reductase	7,8-Dihydrofolate and/or NADPH	ET (attached dye)	[39, 40]
Dihydrofolate reductase	7,8-Dihydrofolate + NADPH	FRET	[41]
Adenylate kinase	No substrate present	FRET	[42]
p-Hydroxybenzoate hydroxylase	No substrate present	Fluorescent cofactor	[43]
Staphylococcal nuclease	+/– single stranded DNA	FRET	[25]
Flavin reductase	No substrate present	ET-dependent lifetime of fluorescent cofactor	[27]
Cholesterol oxidase	Cholesterol or 5-pregene-3β-20α-diol	Fluorescent cofactor	[28]
Dihydroorotate dehydrogenase A *(E. coli)*	Dihydroorotate + dichlorophenol indophenol	Fluorescent cofactor	[44]
Dihydroorotate dehydrogenase A (*Lactococcus lactis*)	Dihydroorotate + fumarate	Fluorescent cofactor	[45]
Nitrite reductase	Nitrite	FRET	[26]
Horseradish peroxidase	Dihydrorhodamine 6G	Fluorogenic substrate	[21, 49, 61]
Horseradish peroxidase	Dihydrorhodamine 123	Fluorogenic substrate	[22]
Lipase (CalB)	2',7'-Bis-(2-carboxy-ethyl)-5-(and-6)- carboxyfluorescein, acetoxymethyl ester	Fluorogenic substrate	[46, 53]
β-Galactosidase	Resorufin β-D-galactopyranoside	Fluorogenic substrate	[37]
Lipase (TLL)	Carboxyfluorescein diacetate	Fluorogenic substrate	[48]
Chymotrypsin	$(sucAAPF)_2$- rhodamine 110	Fluorogenic substrate	[47]
Haloperoxidase	Aminophenyl fluorescein	Fluorogenic substrate (secondary reaction)	[23]

based approaches have been used for a number of studies to determine conformational changes based on spFRET or ET as well as for experiments that analyze conformational changes indirectly by their influence on the catalytic reaction (Table 3).

3.2.1 Direct detection of conformational changes

Conformational changes are always correlated with changes in intramolecular distances and can, therefore, be detected with spFRET and ET reporter systems. Depending on the placement of the reporter system on the enzyme either specific local conformational changes or not predefined conformational fluctuations are monitored.

Conformational changes of the enzyme lysozyme were monitored using two site-specifically attached chromophores forming a FRET pair [38]. Lysozyme consists of two domains and the hinge-mediated relative motion of these two domains is responsible for opening and closing of the active site. It is considered that during the catalytic reaction the active site opens to allow the binding of the substrate. After closing of the active site, the catalytic reaction takes place followed by dissociation of the product. To study this motion, tetramethylrhodamine was attached to one domain and Texas Red to the other domain. Fluorescence time traces of both chromophores were recorded for single enzymes in the absence and presence of substrate. Auto-correlation and cross-correlation analysis showed that certain changes in donor and acceptor intensity occurred in the presence of substrate but not in its absence. These changes were attributed to

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the conformational change resulting from the hinge bending motion. In addition, other intensity changes were detected that occurred both in the presence and absence of substrate, suggesting that other conformational changes take place that are not directly related to the catalytic reaction. A more detailed analysis of the time traces allowed the determination of the rate constant for the substrate-related conformational change. The analysis showed that this conformational change occurs on the same time scale as the catalytic reaction, indicating that each conformational change is indeed directly related to a single substrate turnover.

A similar but much more detailed study to determine catalysis-related conformational changes was carried out with the enzyme dihydrofolate reductase (DHFR) [39–41]. DHFR undergoes multiple conformational changes during the catalytic reaction. ET studies showed the movement of a loop close to the active site, which was induced by inhibitor binding [39], the binding and dissociation of either dihydrofolate or NADPH and during the catalytic reaction itself [40]. Furthermore, a FRET reporter system showed catalysis-related conformational changes between the above-mentioned loop and a region distant from the active site [41] proving the involvement of more global conformational changes in the catalytic reaction.

In contrast to lysozyme and DHFR, where conformational changes were shown to be induced by the presence of substrate or inhibitor, other enzymes also show catalytically relevant conformational changes in the absence of substrate. Using spFRET, the movements of two loops of the enzyme adenylate kinase (ADK) were observed [42]. The conformation with both loops closed is considered to represent the substrate bound form and the enzyme with both loops in the open conformation corresponds to the substrate-free form. However, the closed conformation was also identified in the absence of substrate. The fact that this observation was confirmed with a series of NMR experiments and molecular dynamics simulations leads to the conclusion that substrate binding shifts the equilibrium towards the closed conformation. Similarly, the existence of the "substrate-bound" conformation in the absence of substrate was also detected for the flavin enzyme *p*-hydroxybenzoate hydroxylase [43]. For this enzyme, conformational changes in proximity of the FAD cofactor could be detected as the FAD is fluorescent in one conformation and quenched by a tyrosine in another conformation.

In addition to this qualitative information about conformational changes, the above examples also provided quantitative information about the time scales of the observed conformational changes. The conformational change of interest resulted in a large amplitude intensity change, and two different intensity states could be identified. Measuring the times of the durations of these states (dwell times) allowed the determination of the rate constants of the molecular processes. According to Kramer's reaction rate theory, a mono-exponential fit to the dwell time histogram yields the desired rate constant. However, this way of analyzing the data assumes that only one specific conformational change took place with its characteristic rate constant. This is probably an oversimplification and additional conformations might exist that lead to smaller changes in the observed signal [25, 38, 40, 42].

The above examples show that spFRET and ET measurements yield a variety of information about conformational changes. These measurements do not require the presence of substrate and, more importantly, are able to compare the conformational changes of the enzyme in the presence and absence of substrates or inhibitors. Yet it might be more interesting to directly determine the influence of conformational fluctuations on the catalytic reaction itself.

3.2.2 Detection of single enzymatic turnover events

The usage of a fluorescent reporter system that participates in the catalytic reaction and, as a result, changes its properties during the time course of the catalytic reaction allows the detection of individual catalytic turnover reactions and the corresponding times between two turnover reactions. The first study using this approach was performed with the enzyme cholesterol oxidase (COx) [28]. COx is a redox enzyme containing an FAD cofactor. The fluorescent FAD is reduced in the presence of the substrate in the first half-reaction to yield the non-fluorescent $FADH_2$, which is then converted back into FAD by oxygen in the second part of the reaction. One switching from a high fluorescence state to a low fluorescence state and back corresponds to one catalytic reaction cycle. However, for the data analysis both half reactions were treated separately. Histograms of the measured dwell times in the high fluorescence state (on-time) and the low fluorescence state (off-time) were generated and rate constants were determined from fits to the on-time and off-time histograms. The data also contained additional information that could be extracted with a detailed correlation analysis. First, the auto-correlation function of the intensity time traces could not be fitted with a mono-exponential function as would be expected for a process with a single rate constant. Second, a two-dimensional probability distribution of pairs of consecutive on-

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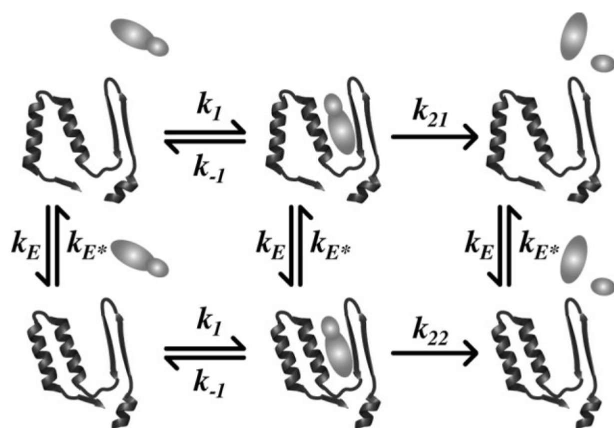

Figure 2. Kinetic scheme with interconverting conformers. The enzyme switches between two different conformations with the rate constants k_E and k_E*. Both conformations turn the substrate over into the product. Both reaction pathways follow Michaelis-Menten kinetics whereby the rate constants k_1 and k_{-1} for the formation of the enzyme substrate complex are identical and the rate constant for the turnover reaction is different for the two conformations (k_{21} and k_{22}). In this scheme, when the rate constants for the interconversion between the conformers are on a similar or a slower time scale than the rate constants for the turnover reaction, the scheme explains the observed "memory effect" and the heterogeneities in the rate constant for the turnover reaction.

times showed that a short on-time is more likely to be followed by another short on-time, and that a long on-time is more likely to be followed by another long on-time. This observation was explained with a kinetic scheme for the enzymatic reaction, which involved two conformations of the enzyme with different rate constants for the turnover reaction (Fig. 2). In summary, these experiments represent the first example where fluctuations in the catalytic rate constants (dynamic disorder) have been determined for a single enzyme directly. Subsequent experiments with other enzymes and other reporter systems now suggest that dynamic disorder originating from different enzyme conformations is a general property of enzymes.

Different conformations are also considered to be the origin of the heterogeneity observed in the reactivity of individual molecules of the enzyme dihydroorotate dehydrogenase from *E. coli*, which follows a similar catalytic mechanism as COx involving reduction and oxidation of the FMN cofactor [44]. However, in this case heterogeneities were only detected when comparing different enzyme molecules. This is most likely due to the fact that the length of the time traces was restricted to approximately ten turnovers because of FMN dissociation from the enzyme. For this reason, the enzyme does not sample all its conformational states during this short measurement time. Using a very similar approach, a homodimeric dihydroorotate dehydrogenase A from *Lactococcus lactis* was analyzed [45]. Although the dissociation of the cofactor again limited the length of the time traces, the experiment allowed the establishment of a kinetic model, which clearly supports negative cooperativity between the monomers.

The approach of monitoring the alternating properties of a cofactor was extended to non-fluorescent cofactors using the enzyme nitrite reductase from *Alcaligenes faecalis* (NiR) as the model system [26]. NiR is a homotrimeric enzyme with two copper cofactors per monomer. The reduction of nitrite involves ET from one copper to the second copper before the electron is finally transferred to nitrite to yield nitric oxide. The first copper, which accepts the electron from a physiological donor, absorbs light in the visible range in its oxidized but not in its reduced state. The attachment of a fluorophore in proximity to this copper established a FRET pair, which reported on the oxidation state of the copper during the time course of the catalytic reaction. Fluorescence time traces with several hundred turnovers could be recorded in this way at different nitrite concentrations. Time constants were extracted from auto-correlation analysis, and again a kinetic model of the enzymatic reaction could be established. Like for COx, the auto-correlation function could not be fitted with a mono-exponential function resulting from heterogeneities in the reaction rates. These heterogeneities were explained with structural variations in the surroundings of the copper cofactors that might influence their redox potentials.

The above experiments reporting on the redox reaction of cofactors already indicate that the number of turnovers detected determines how much information can be extracted from a turnover time trace of a single enzyme. Because of the bleaching of the chromophore or cofactor dissociation, time traces are limited to a maximum of several hundred turnovers. This problem can be solved with the use of fluorogenic substrates. With this approach new fluorescent product molecules are produced constantly as a result of the catalytic reaction. With a surface-immobilized enzyme, the generation of the product molecules can be monitored at the position of the immobilized enzyme for a very long time. Since the product molecules diffuse out of the detection volume quickly each detected burst ideally corresponds to one turnover reaction. A number of examples prove the power of this approach, and time traces of single enzymes have been measured for over 1 h [46, 47].

Fluorogenic substrates have been used for the enzymes horseradish peroxidase (HRP) [21], lipase B from *Candida antarctica* (CalB) [46], β-galactosidase [37], the lipase from *Thermomyces lanuginosa*

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(TLL) [48] and chymotrypsin [47]. In all these cases neither the dwell time histograms nor the autocorrelation functions could be fitted with a monoexponential function, and fluctuations in the rate constants spanning a broad range of time scales were detected. The only two examples so far that did not show direct proof of dynamic disorder are the haloperoxidase from *Curvularia verruculosa* [23] and a study with HRP encapsulated in a virus capsid [49]. For the encapsulated HRP, diffusion is considered to be the rate-limiting step so that fluctuations in the rate constants of the enzymatic reaction cannot be observed. In the case of the haloperoxidase, the reaction producing the fluorescent dye is a non-enzymatic secondary reaction and the rate-limiting step might again be diffusion or might be part of the secondary reaction.

All the above examples show that heterogeneities either between individual enzymes (static disorder) or in the reaction rates of a single enzyme (dynamic disorder) are the rule rather than an exception. Experiments yielding short time traces almost always show static disorder and have so far been restricted to the determination of the rate constants themselves. In contrast, long time traces contain additional information about dynamic disorder. Trajectories that are sufficiently long represent a statistically relevant sampling of all conformational states [50]. In contrast, when time traces are too short, rare conformations or long-lasting conformational states are underrepresented. Therefore, in an ideal case, in very long time traces static disorder between enzymes should disappear and a one time trace should contain the information about all the accessible conformational states of the enzyme.

4 Relating dynamic disorder to conformational dynamics

The most straightforward analysis of the single-molecule time traces is the determination of the average reaction rate (obtained from the number of turnovers detected in a certain time interval). When plotting the average reaction rate against the substrate concentration, a typical Michaelis-Menten saturation curve is obtained and the Michaelis-Menten parameters can be extracted [37, 51, 52]. The validity of the Michaelis-Menten equation in the presence of dynamic disorder (and the absence of static disorder) was shown theoretically [51, 52] and experimentally for the enzymes CalB [46] and β-galactosidase [37]. This is an important result but does not provide information about the presence of dynamic disorder.

To obtain information about dynamic disorder, experiments need to be performed under substrate saturation conditions [37]. Otherwise substrate diffusion to the active site is the rate-limiting step preventing the observation of dynamic disorder. In the time traces obtained from measurements with fluorescent cofactors or fluorogenic substrates, the conformational states and the resulting dynamic disorder are not directly detectable because only one bright and one dark state are monitored. Dynamic disorder is a hidden mechanism that requires statistical analysis.

The initial processing of the time traces can be performed in different ways (Fig. 3). In most cases the data are binned and a threshold is applied to determine the on-times and/or off-times from the time traces [37, 46, 47, 53]. The on-times and off-times are then plotted into histograms. In the case of dynamic disorder, these histograms cannot be fitted with a mono-exponential function as would be expected for a reaction with a single rate-limiting step. Being a scrambled histogram, no more information about dynamic disorder and the memory of the system can be obtained from these histograms. In contrast, a correlation analysis that involves successive on- or off-times provides much more information [28, 37, 47, 48, 53–60]. The two-dimensional histograms shown in Fig. 3 are just one example of this higher order correlation analysis. Methods based on the determination of on-/off-times are therefore a good basis for a more detailed analysis of the system. However, the binning reduces the time-resolution since conformational changes that are faster than the binning time are averaged out. Furthermore, binning might lead to artifacts as successive fast turnovers might be counted as only one event. Additional artifacts can be created when defining the threshold.

Correlation analysis can also be directly applied to photon arrival times, *e.g.*, to calculate the intensity auto-correlation function [21, 26, 28, 37, 61]. Dynamic disorder also leads to a non-exponential decay in the auto-correlation function (Fig. 3). Using this approach for analyzing the data avoids all kinds of artifacts from the binning and thresholding, and does not minimize the time resolution. However, especially at the sub-millisecond time scale other processes such as diffusion and triplet blinking contribute to the auto-correlation curve and cannot be separated from potential fast contributions from the enzymatic reaction.

With the exception of higher order correlation analysis, these initial processing steps provide only a phenomenological description and simply prove

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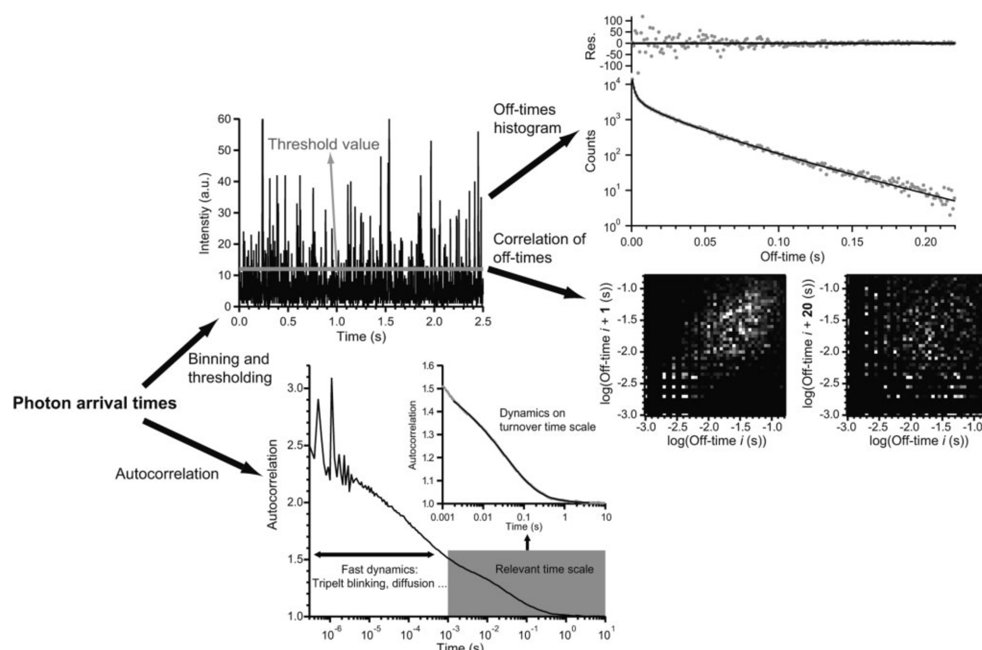

Figure 3. Data processing of single turnover time traces. A representative example of a time trace of photon arrival times [4, 6] measured under saturation conditions was taken from De Cremer *et al*. [47]. The data evaluation can be done in two principally different ways. The first method determines the off-times for every individual turnover event. This is done by first binning the data and then applying a floating threshold. Values above this threshold are counted as on-events and values below this threshold represent off-events. In the next step the time between two on-events is determined as an off-time. Having determined all the off-times along the time trace, these off-times are plotted into a histogram. In the histogram shown here the number of counts is plotted logarithmically. In this representation a process without dynamic disorder should yield a straight line. This is clearly not the case and the data (gray dots) were fitted with a stretched exponential function (black line) instead. In addition, off-times directly following each other (+1) and off-times separated by 20 turnovers (+20) were plotted into two-dimensional histograms (see De Cremer *et al*. [47] for more details). The diagonal feature in the histogram containing off-times directly following each other shows the correlation between consecutive off-times representing dynamic disorder. In contrast, when the off-times are separated by 20 turnovers the diagonal feature disappears. The second method relies on the calculation of the auto-correlation function directly from the photon arrival times. Again, a stretched exponential function was used to fit the data in the relevant time scale between 1 ms and 10 s.

that dynamic disorder is present. From an enzymological point of view one would like to know the biological relevance of dynamic disorder and the influence of different conformations on the enzymatic reaction. To address these questions a number of theoretical approaches have been developed. The number of theoretical papers now by far exceeds the number of enzyme substrate systems published [21, 26–28, 37, 46–48]. Different theoretical approaches have been reviewed recently [50, 62] and describing them in detail is far beyond the scope of this review. Only a short overview based on the most representative examples [54–60, 63–67] is given here. Coming back to the model with two conformations in Fig. 2, it needs to be considered that it represents the simplest model that is able to explain dynamic disorder. Proteins have rugged energy landscapes that define a huge number of different conformations [68–70]. Depending on the topology of the underlying energy landscape conformational changes might involve transitions over high-energy barriers. Alternatively, the energy landscape might be smooth under the conditions of the measurement and the system shows a diffusive behavior. In any case, the rate constant for the catalytic reaction is replaced by a random function of time. The fluctuating rate constant can either jump between discrete values (high energy barriers) or be described by a continuous function of time (diffusive behavior) [54, 56, 59, 63–66, 71].

Recently, a diffusive model for the enzyme flavin reductase was established [27, 66]. As in this example only local fluctuations around the flavin cofactor in the absence of substrate were measured, it remains to be established which approach is best suited for enzymatic reactions with dynamic disorder. In a closer collaboration between experimentalists and theoreticians the models will have to be applied to the data of many different enzymes, and parameters in the catalytic reaction (*e.g.*, the rate-limiting step) will have to be changed to test the validity of different models experimentally. Furthermore, only conformational changes perpendicular to the reaction coordinate of the enzymatic reaction have been included in most models. Since many enzymes undergo conformational changes during

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the catalytic reaction these models will have to be extended to include these processes [58, 67].

5 Conclusions

The analysis of enzymes at the single-molecule level has proven to provide additional information about enzymatic reactions and related conformational changes that cannot be obtained at the ensemble level, with the observation of dynamic disorder being the most relevant finding. Besides the observation of dynamic disorder, experiments with single enzymes have yielded novel information about enzyme inhibition [35] and inactivation [47] processes. However, especially with respect to dynamic disorder, experiments with single enzymes are just about to leave the proof-of-principle stage. So far, the model systems used have mostly been restricted to commercially available enzyme substrate systems. This is mainly due to the lack of fluorogenic substrates. The substrates used so far are highly artificial. Although no systematic studies have been performed, the nature of the fluorescent leaving group might influence the catalytic reaction. Furthermore, some substrates are prone to autohydrolysis and many fluorescein- and rhodamine 110-based substrates contain two cleavable bonds, complicating a detailed kinetic analysis. To circumvent these problems substrates based on ET have been developed, which resemble natural enzyme substrates more closely [72, 73]. Unfortunately, these substrates might still show considerable fluorescence preventing their use in single-molecule experiments due to background problems. Novel developments are not only required to obtain more suitable fluorogenic substrates, but also for FRET or ET measurements. Reporter systems are required that have a good brightness and sufficient photostability to allow for measurements over long times.

In many cases the quality of the data evaluation is limited by the amount of data generated from one experiment. To obtain better statistics, parallel detection of many enzymes with a sub-millisecond time resolution would be highly desirable. Looking at the experiments described here, the gap in time resolution between the parallel set-ups using small wells (Table 2) and the APD-based single turnover counting currently spans a few orders of magnitude. Bridging this gap with improved experimental set-ups will greatly improve the amount and quality of the data.

The possibility of analyzing more enzymes at the same time will also simplify measurements under different reaction conditions. The analysis of

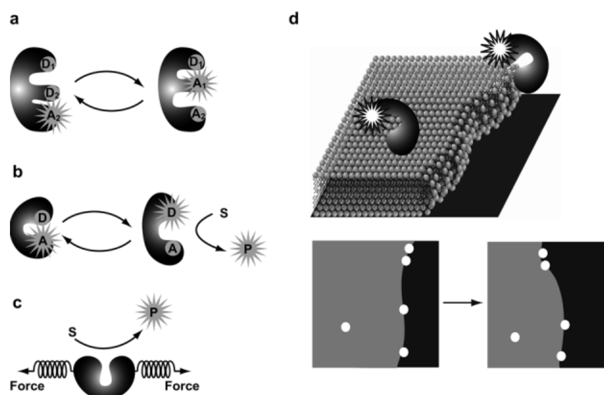

Figure 4. Outlook showing new single-molecule approaches. More detailed insight in molecular mechanisms of enzymatic reactions can be expected from extensions and combinations of different approaches. (a) More information about correlated conformational changes can be obtained from three-color FRET measurements. In three-color FRET-measurements a cascade of two transfer reactions is established. In this cascade the acceptor of one FRET pair represents the donor of the second FRET pair (A_1 and D_2 is the same chromophore). With this approach relative distances between the FRET pairs D_1–$A_1(D_2)$ and $(A_1)D_2$–A_2 can be measured simultaneously. (b) Information about catalytically relevant conformational changes can be obtained by attaching a FRET pair reporting about the expected conformational change while simultaneously monitoring the turnover of a fluorogenic substrate. (c) The combination of single-molecule fluorescence measurements with single-molecule force techniques allows the application of an external force to an enzyme molecule while simultaneously monitoring the turnover of fluorogenic substrate molecules. (d) The action of processive enzymes hydrolyzing 2-D substrates such as phospholipid bilayers can be studied in more detail with wide-field fluorescence microscopy. The approach makes use of a weakly fluorescently labeled bilayer (gray area) and fluorescently labeled phospholipase enzymes (white circles). As the bright fluorescence of the single enzyme molecules can be distinguished from the weak fluorescence of the layer, the diffusive movement of the enzymes as well as their catalytic action can be followed. Upon enzymatic cleavage of the phospholipids, the disappearance of the lipid layer can be monitored as an increase in the dark area while simultaneously monitoring the movement of the enzyme molecules. Since only enzymes docking at the edge hydrolyze the bilayer a different diffusive behavior is observed for enzymes docking to the bilayer's edge and for enzymes binding to the top of an intact layer.

mutants of an enzyme with altered catalytic properties or measurements with modified substrates as well as in the presence of inhibitors will provide more detailed information about the influence of different conformations on the catalytic reaction and allow the establishment of kinetic schemes. In this respect, experiments with single enzymes need to be combined more extensively with classical enzymology, structure determination and analysis methods as well as theoretical simulations since these approaches might provide complementary information.

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6 Future perspectives

Advances in single-molecule enzymology are not only expected from a combination with more classical biochemistry approaches but also from further developments in single-molecule analysis and manipulation (Fig. 4) [7]. For example, three-color FRET measurements can provide additional information about conformational changes. The simultaneous observation of the distance changes of a FRET pair in combination with the turnover of a fluorogenic substrate will report on specific conformational changes directly related to the catalytic reaction. An elegant approach to simultaneously monitor the movement and the enzymatic reaction of processive enzymes is the labeling of the substrate and the enzyme. This approach has recently been applied to a variant of TLL hydrolyzing a lipid bilayer [74]. The movement of enzymes on the bilayer was determined by tracking their successive positions and the catalytic reaction was observed by the disappearance of the fluorescently labeled bilayer. Finally, a combination of single-molecule fluorescence with single-molecule force measurements will allow for a defined manipulation of either an enzyme or a substrate molecule while monitoring the catalytic reaction. With this combination the testing of more complicated hypotheses, for example the contribution of mechanical strain to the lowering of the energy barrier of the reaction by the enzyme, will potentially become possible in the future.

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