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Do enzymes sleep and work?

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Single-enzyme studies suggest that dynamic disorder is a general characteristic of enzyme catalysis.

Enzymes, the ubiquitous catalysts of Nature, are remarkable molecular constructs, which determine the patterns of chemical transformations. For more than a century scientists have been studying enzyme activity in bulk, aqueous solution, at the so-called ensemble level and a great wealth of information has been obtained. Using techniques by which the average activity of a large number of enzyme molecules (say $\sim 10^{15}$) is measured, many general principles have been discovered, including Michaelis–Menten kinetics, the occurrence of pH and temperature optima, allosteric interaction, *etc.* Numerous studies have also looked at the ensemble synergistic action of enzymes.¹ Enzymes in their natural environment, the cell, often interact with

numerous neighbouring proteins, and their catalytic activity generally depends upon their environment and their role in the catalytic cycle.² In ensemble measurements, only the average value of an observable parameter is detected, and no information is obtained about what precisely the contribution of individual molecules to the overall studied process is. Moreover, the concentration of intermediate steady states is often too small to be detected.

Pioneering experiments in 1961 by Rotman showed the possibility of detecting the activity of single β -D-galactosidase molecules in oil-dispersed water droplets.³ (Fig. 1) Enzymatic hydrolysis of the pro-fluorescent 6-hydroxyfluoran-13-D-galacto-pyranoside substrate yielded the fluorescent compound 6-hydroxyfluoran. By analyzing the increase in the fluorescence intensity of the individual droplets with the help of Poissonian statistics, the number of active enzymes in each droplet could be determined, which in several droplets amounted to exactly one.

The most interesting outcome of these very first single-enzyme experiments was the observation from thermal denaturation studies that thermal inactivation of the enzyme leads to a mixture of fully-active and completely inactive enzyme molecules; in stark contrast to studies at the ensemble level, in which only an average decrease in activity was measured.

Rotman's analysis included the assumption that each active enzyme molecule exhibits the same activity. It became apparent in 1995 that this is not necessarily the case for enzymes when it was shown that the activity of individual lactate dehydrogenase molecules can vary by a factor of four.⁴ The origin of such activity differences ('static disorder') may lie in the presence of several active conformations of the enzyme, which remain stable for extended periods of time.⁵ Similar results were obtained for single molecules of alkaline phosphatase for which it was shown that the activity range can span an order of magnitude.⁶ It was also observed that partial thermal

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degradation of a bulk sample of alkaline phosphatase divides the population into active and inactive molecules. Again, the individual activity of the surviving enzymes remained the same, very similar to the early results of Rotman with β -D-galactosidase.³

Moving on from *time-averaged ensemble measurements* to *time-averaged single enzyme studies* allows for the detection of static disorder, *i.e.* differences in activity of individual enzyme molecules. The

actual measurement, in most cases the fluorescence intensity of the formed product, is still an ensemble measurement in the sense that millions of product molecules are detected.

The detection of dynamic disorder, *i.e.* time-dependent fluctuations, requires the measurement of the single-enzyme kinetics in real-time, that is, turnover by turnover. In order to achieve this, it is necessary to measure the formed product at the individual chromophore level.⁷

The detection of a single chromophore in water has only been possible since the early 1990s, when advanced optical instrumentation became available.⁸ One of the first examples of truly real-time enzyme kinetics was the observation in real-time of the binding of fluorescently labelled ATP and ADP to single myosin molecules.⁹ Using a total internal reflection microscope, single fluorescently labelled myosin molecules were imaged and individual ATP



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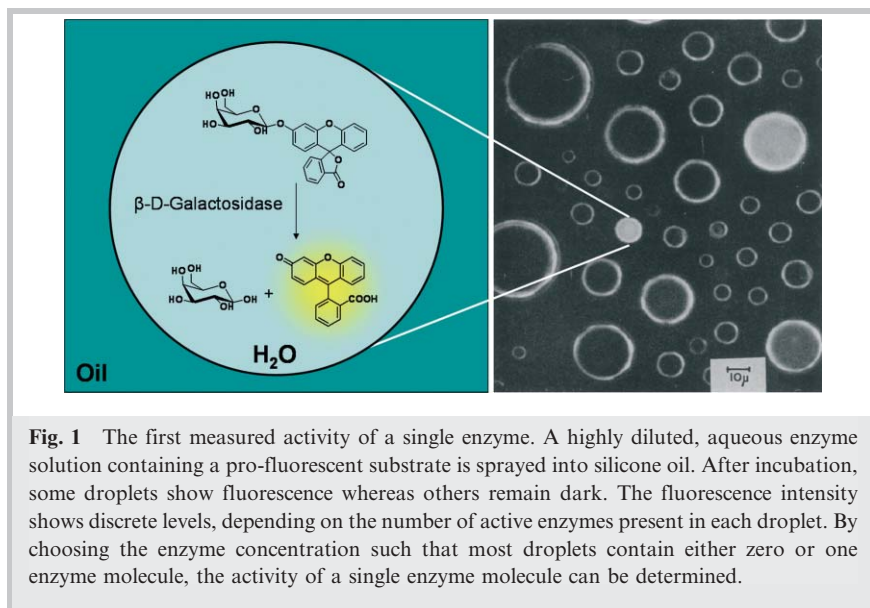


Fig. 1 The first measured activity of a single enzyme. A highly diluted, aqueous enzyme solution containing a pro-fluorescent substrate is sprayed into silicone oil. After incubation, some droplets show fluorescence whereas others remain dark. The fluorescence intensity shows discrete levels, depending on the number of active enzymes present in each droplet. By choosing the enzyme concentration such that most droplets contain either zero or one enzyme molecule, the activity of a single enzyme molecule can be determined.

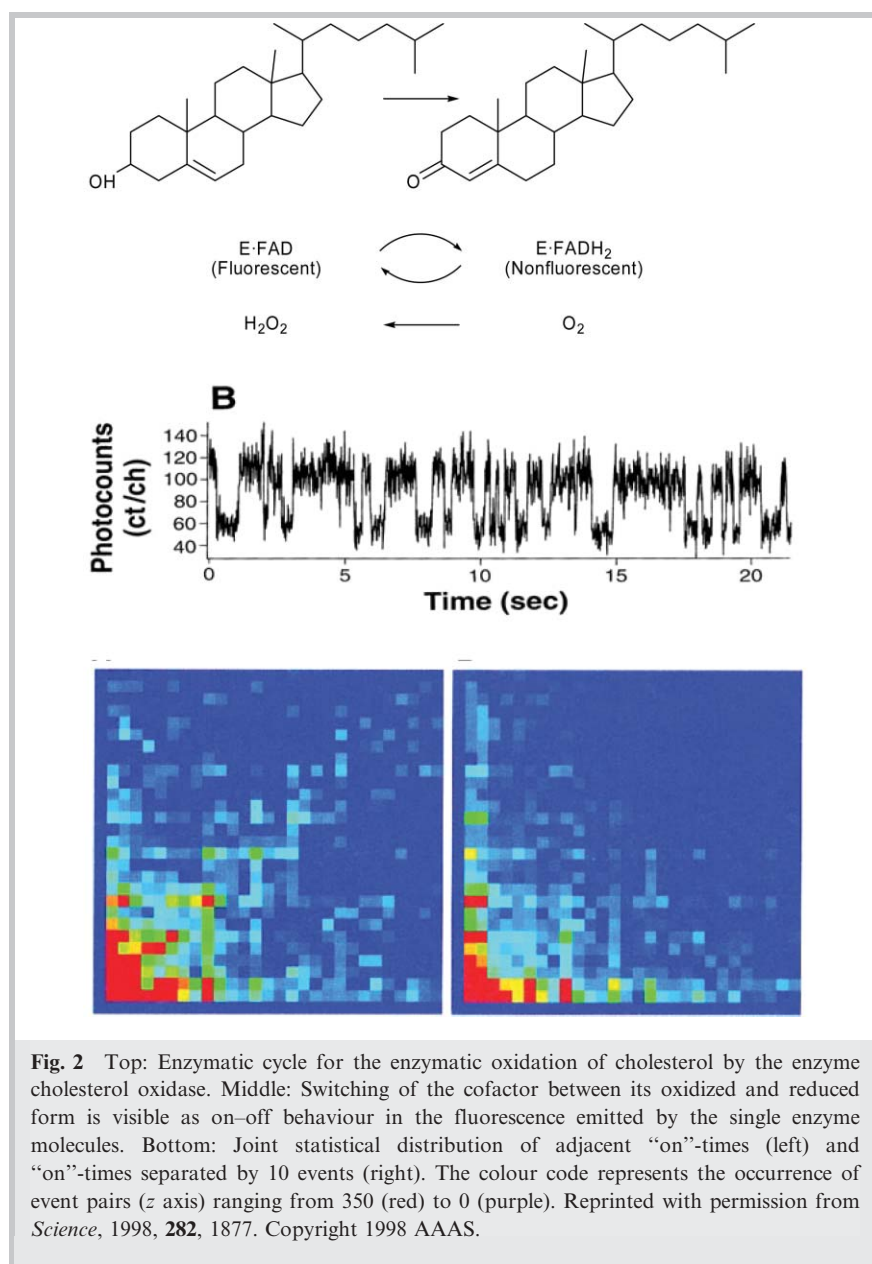
turnover reactions detected. As the substrate and the product could not be distinguished, only the rate of dissociation of the product was determined in this way. This rate of dissociation turned out to agree extremely well with the bulk turnover rate, suggesting that the binding events correspond to actual hydrolysis reactions. One of the best examples of real-time single-enzyme studies was performed by Xie *et al.*¹⁰ The oxidation of cholesterol by cholesterol oxidase is known to be accompanied by the reduction of a flavin adenine dinucleotide (FAD) in the enzyme, a cofactor which is naturally fluorescent in its oxidized form but not in its reduced form. The resulting FADH₂ is then reoxidized by O₂, yielding H₂O₂. By immobilizing the enzyme in an agarose gel in the focus of a confocal microscope, the fluorescence emission of the cofactor could be followed in time. A time-trace of the single FAD emission in the presence of cholesterol clearly exhibited on–off behaviour, see Fig. 2. Each on–off cycle corresponds to one enzymatic turnover, and the emission on- and off-times correspond to the “waiting times” for the FAD reduction and oxidation reactions, respectively. The possibility of measuring the turnovers in real-time and not only as a time-average led to the finding that the rate of the enzymatic reaction slowly fluctuates in time. One manifestation of this phenomenon is the fact that the consecutive enzymatic turnovers are not completely independent, leading to the

conclusion that the enzyme exhibits a so-called “memory effect”. A plot of the joint statistical distribution of adjacent “on” times of the cholesterol oxidase enzyme clearly shows a diagonal feature, whereas turnovers that are ten events apart have no correlation (Fig. 2). Fast turnovers are more often followed by fast turnovers and slow turnovers more often by slow turnovers. Recently, a re-examination of the data led to the conclusion that on top of the slow conformational motion, the conformational cycle of the enzyme during each turnover should also be taken into account to explain the strong correlations between adjacent turnover cycles.¹¹

The method described by Xie *et al.*,¹⁰ although very elegant, has some important drawbacks. By observing the cofactor, which is prone to photobleaching, the amount of data that can be collected from one enzyme is limited. Furthermore, the actual product formation and release are not observed. These problems can be avoided by using a pro-fluorescent substrate that is converted into a fluorescent product by the enzyme of interest. The first example of this approach was reported by Rigler and coworkers. They recorded the thermodynamic fluctuations in the activity of horseradish peroxidase enzyme at the single-turnover level.¹² Here, the pro-fluorescent substrate dihydrorhodamine-6G was used, which, after enzymatic oxidation yields the highly fluorescent rhodamine-6G fluorophore, allowing for the direct observation of the

individual substrate turnover reactions with a confocal microscope. As the product, once released from the enzyme, diffuses away from the focal volume very quickly, only the fluorescence of the enzyme–product complex is observed. This enables the separate analysis of the oxidation of the substrate and the dissociation of the enzyme–product complex. In classical Michaelis–Menten kinetics, these two distinct processes are regarded as one step. Interestingly, the measured data showed single-exponential product dissociation kinetics, but a large distribution of rates for the enzyme to form the enzyme–product complex.

As part of our studies on the design of new building blocks for the construction of nano-sized self-assembled systems, we have recently developed a new class of amphiphilic macromolecules, so-called giant amphiphiles, which consist of an enzyme head group (*e.g.* a lipase) connected to one or two hydrophobic polymeric tails.¹³ Within this project, we became interested in studying the activity of single lipase enzymes and polymeric derivatives of these biomolecules. We studied at the single enzyme level the kinetics of the lipase B from *Candida Antarctica* catalyzed hydrolysis of a non-fluorescent substrate, *viz.* BCECF-AM (Fig. 3), which, after hydrolysis, is converted into the fluorescent BCECF acid.¹⁴ The Cal B enzyme was adsorbed onto a hydrophobic cover slip and positioned in the focus of a confocal microscope (Fig. 3). Using such an approach we were able to measure the single-enzyme activity as a function of substrate concentration on one single enzyme molecule for very long periods of time, even up to 6 hours. Just as reported in the literature for cholesterol oxidase and horseradish peroxidase, substrate hydrolysis by Cal B displayed on–off behaviour. By setting a cut-off value for the background noise, the fluorescence peaks resulting from the BCECF product could be separated and their appearance followed as a function of time. The resulting “bar-code” traces were analysed by statistical methods (Fig. 4). Remarkably, from the statistical properties of the waiting time distributions, we observed that the single Cal B enzyme exhibits a breathing motion, moving between numerous conformational states of which only a very few



are catalytically active.¹⁵ These results indicated that this enzyme has bursts of top activity for ~ 30 ms and then is inactive for 970 ms (Fig. 4). This intriguing behaviour can be better imaged as an enzyme which effectively ‘sleeps’ for 97% of the time and is awake and active for only 3% of its day. Although such an image is an attractive concept the real situation is one in which the enzyme is slowly moving through a landscape of conformations, each with its own specific activity. However within this family of enzyme conformations only a few exhibit a high catalytic activity. The measured bulk activity is therefore an average of short periods of top-activity and periods

of inactivity, a finding which never could have been deduced from ensemble measurements. This breathing clearly demonstrates the fundamental importance of studying individual enzyme behaviour and the single molecule turnover, particularly when one wishes to find out the real reason why directed evolution results in more active enzymes.

In further studies we also increased the substrate concentration during the experiment and showed for the first time that the kinetics of an individual Cal B enzyme molecule exhibit Michaelis–Menten-like properties, saturating at a certain substrate concentration, very similar to the ensemble behaviour

(Fig. 5). The average k_{cat} and K_{m} values calculated from the saturation curve amount to 4 s^{-1} and $2.5 \times 10^{-7} \text{ M}$, respectively. Interestingly, the top activity derived from our single-enzyme measurements, the actual maximum rate, amounts to $k_{\text{fast}} = 125 \text{ s}^{-1}$, almost two orders of magnitude higher.¹⁵

The above experiments had several minor drawbacks in that the process of absorbing the enzyme to the surface is uncontrolled and only a limited number of enzymes remain active. To overcome this problem we have recently constructed an enzyme protein heterodimer consisting of a Lipase (TLL) and a commonly used coating protein (BSA), which acts as a “protein foot”.¹⁶ Deposition of this protein dimer resulted in all the bound enzymes remaining active and exhibiting comparable behaviour. Upon the addition of the pro-fluorescent substrate 5-(and-6)-carboxy-fluorescein diacetate, again a clustering of events was observed, which when further analysed revealed a clear memory effect. In contrast to the earlier work of Xie in which the memory observed in the glucose oxidase lasted only for a couple of events, for TLL–BSA, the lipase enzyme remembers considerably more, approximately 20 events. This does not mean that the TLL is cleverer than glucose oxidase, it simply carries out more catalytic conversions in the same time period, before it “forgets” and the geometry changes. These memory effects further support the concept of a landscape of conformations, which fits the model of a fluctuating enzyme.

As the results presented above indicate, exciting progress has been made in the field of single-molecule enzymology. There are however considerable questions that remain, such as: why are some enzymes or perhaps all enzymes only active a fraction of the time?; how could one restrict the conformational landscape such that the enzyme comes back more quickly to, or remains in the active conformation and why hasn’t Nature done this already? One could argue that having periods of inactivity is Nature’s approach for removing the energy generated during the catalytic reaction. Possibly, the conformational changes leading to the inactive conformations are actually *caused* by the necessity to dissipate the cumulated energy produced

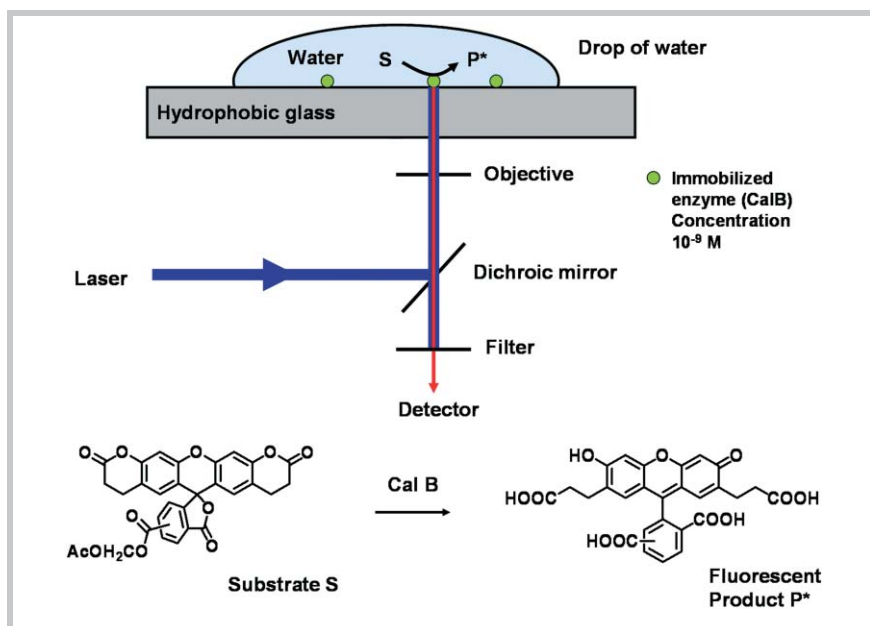


Fig. 3 Setup used for the detection of single turnovers of Cal B. A single molecule of the enzyme is adsorbed on a hydrophobic glass plate in the focus of a confocal microscope. Enzymatic hydrolysis of the substrate BCECF-AM (S) yields the fluorescent product BCECF acid (P*), which is detected by the confocal microscope.

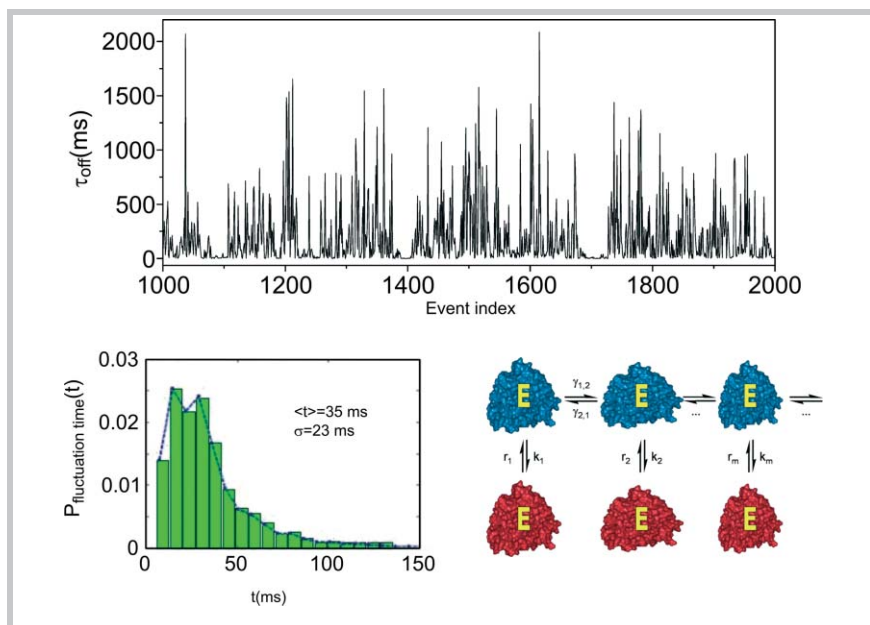


Fig. 4 Top: Plot of the durations of the waiting times between consecutive events as function of event index of Cal B-catalysed hydrolysis. The trace shows that events of a given duration come as clusters; short events are often followed by short events and long events are often followed by events of a similar duration. Bottom left: A histogram of the time durations of the clustered events reveals that the Cal B enzyme resides in its most active conformation for a period of 35 ms and then changes into a less active conformational state. Bottom right: Model for the enzyme (E) conformational-reactivity behaviour. The off-stage (blue) consists of a variety of coupled sub-states that interconvert. Each of the off sub-states is characterized by a different reaction rate. Once the enzyme has transformed the substrate into the fluorescent product the fluorescent on-state is seen (red). The product then diffuses away from the enzyme and the biomolecule returns to its relevant off sub-state.

during the enzymatic reaction.¹⁷ Investigating this question and other questions will require the simultaneous measurement of protein dynamics with single-pair Fluorescence Resonance Energy Transfer (FRET) and enzyme kinetics at the single-turnover level, as a function of substrate concentration. One elegant way of doing this would be the use of a non-fluorescent substrate, which upon the enzymatic conversion is transformed into a fluorescent product, which can act as an excitation energy donor to an acceptor located at a strategic position with respect to the active site on the enzyme. By following the emission from the acceptor and the donor simultaneously, enzyme activity can be directly correlated to slow conformational changes in the enzyme. Intermolecular single-pair FRET studies have been carried out before on different systems, *viz.* donor-labelled Staphylococcal nuclease and acceptor-labelled DNA substrate.¹⁸ In our case using a fluorescent product as donor would have the advantage of (i) diminishing the degree of bleaching of the chromophore which is attached to the enzyme, as it is only excited when a product is formed, and (ii) the ability to observe the enzymatic turnovers directly.

Enzymes in Nature are susceptible to all kinds of regulation mechanisms by external stimuli. They show substrate selectivity, act at an optimum pH and temperature, show inhibition, activation, denaturation and most importantly display allosteric behaviour. In addition to all of the above, they do their work in biological cells which are literally crowded with all kinds of molecules, balancing numerous simultaneously occurring processes. The newly developed single molecule techniques open the way to unravel in detail the pathways by which enzymes operate, complementing in this way the vast amount of data that has already been gathered from ensemble measurements. An excellent example of work in this direction is the recent study by van Oijen and coworkers on the behaviour of the multi-enzyme T7 replication fork.¹⁹ In this scanning probe approach the individual contributions and synergy between the multiple protein components in the replication system could be resolved. The fact that single enzyme experiments can now be carried out for long periods of time

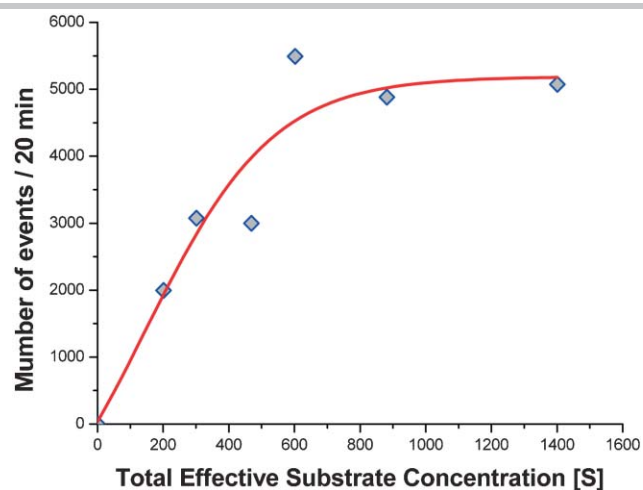


Fig. 5 Michaelis–Menten behaviour of a single Cal B enzyme molecule. Plot of the number of fluorescent events in a time-interval of 20 minutes as a function of the substrate concentration. Saturation is reached at approximately 600 nM. The red line is the fitted Michaelis–Menten curve for $k_{\text{cat}} = 4 \text{ s}^{-1}$ and $K_{\text{m}} = 2.5 \cdot 10^{-7} \text{ M}$.

is especially rewarding, since it will lead to more insight into how enzymatic reactions are coupled in space and in time. As scanning probe microscopy has revolutionized the field of material science, it is without doubt that single enzyme studies in real time will have a similar effect in the field of enzymology.

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Notes and references

- 1 R. P. de Vries, H. C. M. Kester, C. H. Poulsen, J. A. E. Benen and J. Visser, *Carbohydr. Res.*, 2000, **327**, 401.

- 2 S. J. Benkovic and S. Hammes-Schiffer, *Science*, 2003, **301**, 1196.
- 3 B. Rotman, *Proc. Natl. Acad. Sci. U. S. A.*, 1961, **47**, 1981.
- 4 Q. F. Xue and E. S. Yeung, *Nature*, 1995, **373**, 681.
- 5 W. H. Tan and E. S. Yeung, *Anal. Chem.*, 1997, **69**, 4242.
- 6 D. B. Craig, E. A. Arriaga, J. C. Y. Wong, H. Lu and N. J. Dovichi, *J. Am. Chem. Soc.*, 1996, **118**, 5245.
- 7 For a recent review on single-molecule fluorescence spectroscopy, see P. Tinnefeld and M. Sauer, *Angew. Chem., Int. Ed.*, 2005, **44**, 2642.
- 8 E. B. Shera, N. K. Seitzinger, L. M. Davis, R. A. Keller and S. A. Soper, *Chem. Phys. Lett.*, 1990, **174**, 553.
- 9 T. Funatsu, Y. Harada, M. Tokunaga, K. Saito and T. Yanagida, *Nature*, 1995, **374**, 555.
- 10 (a) H. P. Lu, L. Y. Xun and X. S. Xie, *Science*, 1998, **282**, 1877; (b) X. S. Xie and H. P. Lu, *J. Biol. Chem.*, 1999, **274**, 15967; (c) S. N. Xie, *Single Mol.*, 2001, **2**, 229; (d) H. P. Lu, *Curr. Pharm. Biotechnol.*, 2004, **5**, 261; (e) W. Min, B. P. English, G. Luo, B. J. Cherayil, S. C. Kou and X. S. Xie, *Acc. Chem. Res.*, 2005, **38**, 923; (f) B. P. English, W. Min, A. M. van Oijen, K. T. Lee, G. Luo, H. Sun, B. J. Cherayil, S. C. Kou and X. S. Xie, *Nat. Chem. Biol.*, 2006, **2**, 87.
- 11 H. P. Lerch, R. Rigler and A. S. Mikhailov, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 10807.
- 12 (a) L. Edman, Z. Foldes-Papp, S. Wennmalm and R. Rigler, *Chem. Phys.*, 1999, **247**, 11; (b) L. Edman and R. Rigler, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 8266.
- 13 (a) J. M. Hannink, J. J. L. M. Cornelissen, J. A. Farrera, P. Foubert, F. C. De Schryver, N. A. J. M. Sommerdijk and R. J. M. Nolte, *Angew. Chem., Int. Ed.*, 2001, **40**, 4732; (b) K. Velonia, A. E. Rowan and R. J. M. Nolte, *J. Am. Chem. Soc.*, 2002, **124**, 4224; (c) M. J. Boerakker, J. M. Hannink, P. H. H. Bomans, P. M. Frederik, R. J. M. Nolte, E. M. Meijer and N. A. J. M. Sommerdijk, *Angew. Chem., Int. Ed.*, 2002, **41**, 4239.
- 14 K. Velonia, O. Flomenbom, D. Loos, S. Masuo, M. Cotlet, Y. Engelborghs, J. Hofkens, A. E. Rowan, J. Klafter, R. J. M. Nolte and F. C. De Schryver, *Angew. Chem., Int. Ed.*, 2005, **44**, 560.
- 15 O. Flomenbom, K. Velonia, D. Loos, S. Masuo, M. Cotlet, Y. Engelborghs, J. Hofkens, A. E. Rowan, R. J. M. Nolte, M. Van der Auweraer, F. C. De Schryver and J. Klafter, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 2368.
- 16 N. S. Hatzakis, H. Engelkamp, K. Velonia, J. Hofkens, P. C. M. Christianen, A. Svendsen, S. A. Patkar, J. Vind, J. C. Maan, A. E. Rowan and R. J. M. Nolte, *Chem. Commun.*, 2006, in press (DOI: 10.1039/b516551b).
- 17 (a) R. D. Astumian, *Appl. Phys. A: Mater. Sci. Process.*, 2002, **75**, 193; (b) R. D. Astumian, *Science*, 1997, **276**, 917.
- 18 T. J. Ha, A. Y. Ting, J. Liang, W. B. Caldwell, A. A. Deniz, D. S. Chemla, P. G. Schultz and S. Weiss, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 893.
- 19 J.-B. Lee, R. K. Hite, S. M. Hamdan, X. S. Xie, C. C. Richardson and A. M. van Oijen, *Nature*, 2006, in press (DOI: 10.1038/Nature04317).