Click chemistry is used to construct a novel lipase–BSA hetero-dimer, in which the latter protein acts as a foot enabling the anchoring of the enzyme onto the surface for single enzyme studies.

Single enzyme experiments provide new means to obtain information regarding the properties and behaviour of enzymes. Although the first investigations in this direction were performed in the early sixties,1 it is only with the advances in fluorescent microscopy and scanning probe technologies that major progress has been made.2 Recent studies have shown that it is possible to probe the conformational motions in enzymes and to obtain unique aspects of the catalytic activity of single enzyme molecules, which are otherwise masked in ensemble-averaged measurements.3

Recently4 we reported on the direct and real time observation of a single-enzyme-catalyzed reaction employing the technique of confocal fluorescent microscopy (CFM). Using the lipase B from Candida Antarctica, immobilized on a hydrophobic glass surface, we were able to monitor single enzyme turnovers of the hydrolysis of a pro-fluorescent substrate for long periods of time (up to 3 hours). This study allowed us to unravel hidden characteristics in the catalytic behaviour of this lipase, which were explained using a model in which the single enzyme molecule exhibits distinctive catalytic activities arising from a number of slowly interconverting conformations.5

The main limitation of the above mentioned studies is the random orientation of the enzyme molecules physisorbed on the surface. As a result of this, enzymes may be adsorbed with active sites facing the surface making them inaccessible for substrate molecules or even denaturation of the enzyme molecules may occur. In order to overcome these limitations various techniques have been developed5 such as immobilization of the enzyme in a BSA layer,5c the use of an agarose gel2b and furthermore specific anchoring of the enzyme onto the surface for single enzyme experiments.6

In order to further extend the scope of our studies and to overcome difficulties arising from non-specific adsorption of the enzyme molecules onto the glass surface, we have developed a novel general protocol for studying single enzymes using a so-called ‘protein foot’. The latter is based on bovine serum albumin (BSA), which is a 67 kDa carrier protein often used for stabilization of enzymes, proteins and antibodies.6 Due to its strong adsorption to both hydrophilic and hydrophobic surfaces, BSA is also used as a biocompatible surface coating agent.7 For our purpose the BSA protein was coupled to Thermomyces Lanuginosa Lipase (TLL)8 by the Cu(I) catalyzed [3 + 2] Huisgen cycloaddition reaction,9 (commonly referred to as ‘click chemistry’) to give a unique protein hetero-dimer. TLL is a well characterized 30 kDa fungal lipase which is widely used as a workhorse enzyme due to its stability at high temperatures.

A TLL mutant10,11 with only one free solvent accessible lysine (Lys 46) was thought to be the ideal candidate for our experiments. We utilized this single reactive lysine group for the specific coupling of TLL to BSA. The lower affinity12 of TLL for hydrophobic surfaces, as compared to that of CALB, used in our previous experiments, should enable BSA to be preferentially adsorbed on the hydrophobic surface, acting as a protein foot. This would in turn allow the lipase to remain in solution and retain its native conformation, thereby helping overcome problems that arise from non-specific adsorption such as denaturation and low reproducibility.

The acetylene functionalized TLL (1) (Scheme 1) was obtained by coupling 4-pentynoic acid to the free lysine function of the enzyme using standard peptide coupling conditions.13 Confirmation that the acetylene function was attached to TLL was obtained by a reaction of the enzyme with an azide substituted coumarin dye (2) (see Supporting Information). Reaction of the free, solvent accessible thiol14 of BSA with 3-azidopropyl-1-maleimide (3) afforded the complementary azide functionalized BSA (4). Coupling of the acetylene functionalized TLL and the azide modified BSA was performed in 20 mM phosphate buffer (pH 7.2) by mixing equimolar quantities of the BSA and TLL components. The reaction, with CuSO₄/ascorbic acid was allowed to proceed for three days at room temperature and was followed by separation of the hetero-dimer (5) from unreacted BSA and TLL using gel filtration chromatography (yield of product > 80%). The formation and purity of the hetero-dimer were ascertained by FPLC analysis and further confirmed by SDS PAGE (see Supporting Information).
Catalytic activity studies on the TLL-BSA hetero-dimer and the mutant TLL were performed in solution using the pro-fluorescent substrate 5-(and-6)-carboxyfluorescein diacetate (5(6)-CFDA)-mixed isomers. The unfunctionalized TLL displayed an activity of 8 nmol/min in the hydrolysis of the CFDA ester (see Supporting Information). For the TLL covalently connected to BSA the relative activity, under identical conditions, was found to be twice as fast as the activity of the unmodified enzyme (~16 nmol/min). This increase in activity is tentatively attributed to the opening of the lid of this lipase by the conjugated hydrophobic BSA, a mechanism analogous to that of the normal enzyme.15

The enzymatic activity of individual hetero-dimer molecules was examined by single molecule experiments (Fig. 1). To this end the BSA molecules in the hetero-dimers 5 were labelled with Alexa-Fluor488 dye molecules16 allowing the direct visualization of the hetero-dimers by CFM. A solution (10^{-9} M) of thus modified Alexa488-5 in water was deposited on a hydrophobic cover slip. After allowing the hetero-dimer to adsorb, the solution was removed and the location of the labelled single hetero-dimer molecules was determined by CFM (Fig. 1A). Rinsing the cover slip repeatedly with water did not alter the CFM image, which indicates that adsorption of 5 to the surface was indeed strong. A solution of the pro-fluorescent substrate CFDA (10^{-6} M) was subsequently added, and a single molecule of 5 was positioned in the focus of the CFM. Fig. 1B shows that the Alexa488 labels attached to 5 became bleached within 50 seconds. At the same time, peaks of fluorescence emission started to appear (Fig. 1C). These peaks are the result of the enzymatic formation of the fluorescent product within the active site of the enzyme, followed by rapid diffusion into the bulk solution. Each peak is the result of a single enzyme turnover cycle.4 The time between each fluorescent peak corresponds to a single turnover event. The analysis of these peaks allows the direct measurement of the exact kinetics of a single TLL enzyme.

Fig. 1 Fluorescence emission experiments on individual Alexa488-labeled TLL-BSA hetero-dimers. A) Confocal Fluorescence Microscopy micrograph of Alexa488-labeled TLL-BSA molecules. B) Bleaching of the Alexa488 dye molecules attached to the BSA of the TLL-BSA dimer. The bleaching took place in the presence of substrate molecules. C) Time trace measured at the location marked in white in Fig. 1A, showing the local formation of fluorescent molecules. D) Time trace measured at the location marked in red in Fig. 1A, showing the background noise level.

Positioning the laser spot at a location where no 5 is present, i.e. the spot marked with a red circle in Fig. 1A, allowed for independent measurement of the background fluorescence signal (Fig. 1D). This was found to be similar to the background signal measured in between the peaks in Fig. 1C.

From the time traces an average turnover number of 17 s^{-1} per single hetero-dimer on the surface was determined. Furthermore, all visible enzyme molecules were now on average equally active, highlighting the favourable effect of anchoring the lipase onto the glass surface with the help of the BSA foot avoiding non-specific absorption. Further analysis of the waiting times of the individual turnovers showed that consecutive turnovers were not completely random. Fig. 2 shows the two-dimensional histograms of the time durations of the turnover cycles n events apart (n = 1, n = 15 and n = 100). For n = 1, that is events directly following each other, a
The fluctuating enzyme model, in which the enzyme adopts a variety of interconverting conformations, each one exhibiting different catalytic activity.\(^4\)

A clear diagonal feature is present, which can be explained as a memory effect.\(^2\) Long turnovers are more often followed by long turnovers and short turnovers are more often followed by short turnovers. Interestingly, even after 15 events, this memory effect is still present as there is still some correlation visible for \(n = 15\). For \(n = 100\), a correlation is no longer observed. The distribution of the turnover times was found to be in agreement with our recently proposed fluctuating enzyme model for the single enzyme behaviour of CALB.\(^4\) In this model the enzyme moves slowly through several conformations, each exhibiting its own activity and corresponding turnover times.\(^4\) The memory is lost once the enzyme changes conformation. It is likely that this model of enzyme fluctuations also applies to other enzymes\(^3,4\) and may be of more general nature (Fig. 3).

In conclusion we have shown that the copper catalyzed Huisgen [2 + 3] cycloaddition reaction can be efficiently used to prepare protein dimers. The resulting TLL-BSA hetero-dimer was shown to possess a higher activity than TLL, which is tentatively attributed to the “interfacial activation” of the lipase caused by the presence of the hydrophobic BSA. Furthermore, all studied dimers were active and had similar long term activity.\(^18\) This observation suggests that the above described anchoring approach is an ideal method for constructing active enzyme surfaces.

The initial single enzyme studies described strongly support the recently proposed fluctuating enzyme model.\(^3,4\) The anchored TLL enzyme remains active for more than 40,000 turnovers. It slowly fluctuates between different conformations, each with its own catalytic activity. Our approach allows the study of interesting characteristic features of the catalytic behaviour of enzyme molecules at the single molecule level, such as denaturation and the effect of temperature. These studies are currently underway in our laboratory.

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### Notes and references

11. Mutant TLL (TLL-1823) possessing only one free solvent accessible lysine was provided by Novo enzymes.
16. As we reported earlier (ref. 4a), labelling enzymes with Alexa fluor\(^488\) renders focusing of the laser beam easier. In this case the labelling occurs selectively on BSA due to the absence of lysines on TLL. In this way the native conformation and catalytic activity of TLL remain intact.
17. The non-specific hydrolysis of CFDA ester in the presence of BSA was studied using CEM. BSA was labelled with Alexa fluor\(^488\) and was immobilized on the hydrophobic glass under otherwise identical conditions. The observed hydrolysis was negligible (see supporting information).
18. The initial activity of the dimer on the surface was found to be 80 times higher than in solution. However the substrate concentration in the single enzyme studies was above saturation level, which could not be accomplished in solution.