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Substrate-Selective Supramolecular Tandem Assays: Monitoring Enzyme Inhibition of Arginase and Diamine Oxidase by Fluorescent Dye Displacement from Calixarene and Cucurbituril Macrocycles

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Abstract: A combination of moderately selective host−guest binding with the impressive specificity of enzymatic transformations allows the real-time monitoring of enzymatic reactions in a homogeneous solution. The resulting enzyme assays ("supramolecular tandem assays") exploit the dynamic binding of a fluorescent dye with a macrocyclic host in competition with the binding of the substrate and product. Two examples of enzymatic reactions were investigated: the hydrolysis of arginine to ornithine catalyzed by arginase and the oxidation of cadaverine to 5-aminopentanal by diamine oxidase, in which the substrates have a higher affinity to the macrocycle than the products ("substrate-selective assays"). The depletion of the substrate allows the fluorescent dye to enter the macrocycle in the course of the enzymatic reaction, which leads to the desired fluorescence response. For arginase, *p*-sulfonatocalix[4]arene was used as the macrocycle, which displayed binding constants of 6400 M^{-1} with arginine, 550 M^{-1} with ornithine, and 60 000 M^{-1} with the selected fluorescent dye (1-aminomethyl-2,3-diazabicyclo[2.2.2]oct-2-ene); the dye shows a weaker fluorescence in its complexed state, which leads to a switch-off fluorescence response in the course of the enzymatic reaction. For diamine oxidase, cucurbit[7]uril (CB7) was used as the macrocycle, which showed binding constants of 4.5×10^6 M^{-1} with cadaverine, 1.1×10^5 M^{-1} with 1-aminopentane (as a model for the thermally unstable 1-aminopentanal), and 2.9×10^5 M^{-1} with the selected fluorescent dye (acridine orange, AO); AO shows a stronger fluorescence in its complexed state, which leads to a switch-on fluorescence response upon enzymatic oxidation. It is demonstrated that tandem assays can be successfully used to probe the inhibition of enzymes. Inhibition constants were estimated for the addition of known inhibitors, i.e., *S*-(2-boronoethyl)-L-cysteine and 2(*S*)-amino-6-boronohexanoic acid for arginase and potassium cyanide for diamine oxidase. Through the sequential coupling of a "product-selective" with a "substrate-selective" assay it was furthermore possible to monitor a multistep biochemical pathway, namely the decarboxylation of lysine to cadaverine by lysine decarboxylase followed by the oxidation of cadaverine by diamine oxidase. This "domino tandem assay" was performed in the same solution with a single reporter pair (CB7/AO).

Introduction

The monitoring of enzymatic processes is of fundamental importance for the understanding of biological phenomena.[1] Inspired by indicator displacement and synthetic pore strategies,[2−13] we have recently introduced a label-free method based on the competitive encapsulation of a fluorescent dye and an enzymatic product by macrocyclic hosts to monitor enzymatic reactions (Scheme 1).[14−16] In our previous examples, the investigated enzymes transformed a weak competitor (substrate) into a strong competitor (product) which displaced the fluores-

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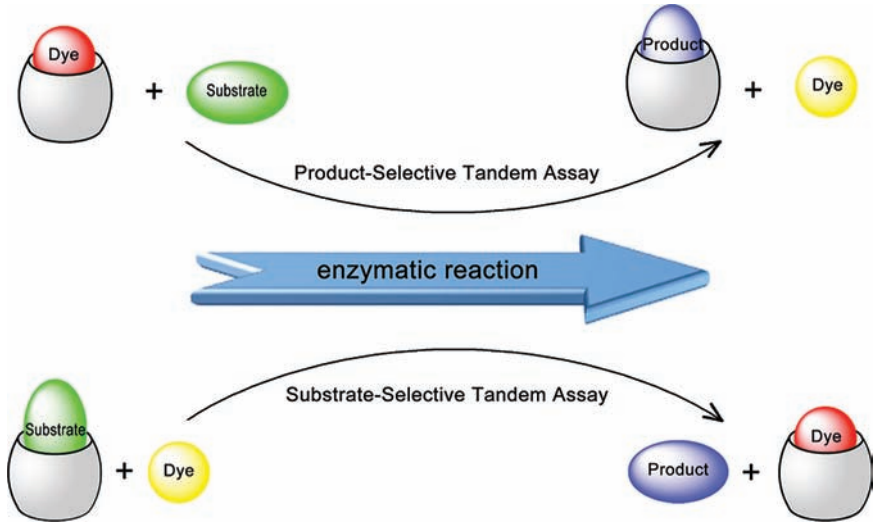
Scheme 1. Product- *versus* Substrate-Selective Tandem Assays for Monitoring Enzymatic Activity



cent dye from the macrocycle and effected a concomitant change in its fluorescent properties. This method can be described as a "product-selective supramolecular tandem assay", because the enzymatic product acts as the analyte responsible for the stronger competitive binding and associated fluorescence response (Scheme 1, top). We have further suggested that the macrocyclic hosts in these types of continuous enzyme assays serve as cheap substitutes for antibodies, which have become indispensable in enzyme assays.[17−20] We also demonstrated that the lower selectivity of macrocycles[14,15] (as opposed to highly specific antibodies) toward the binding of different, structurally related products[21,22] can be used as an advantage in the development of enzyme assays, because it allows access to several enzymes affecting closely related functional group interconversions.

We now find that macrocycles in combination with simple fluorescent dyes not only can substitute antibodies in product-selective enzyme assays but also allow for a different line of enzyme assays in which the *substrate* binds more tightly to the receptor and competes with the fluorescent dye. This establishes the concept of a "substrate-selective tandem assay" (Scheme 1, bottom) in which the enzymatic reaction occurs with the uncomplexed substrate. The latter is in a rapid dynamic supramolecular equilibrium with the host−substrate complex, allowing for a continuous fluorescence signaling of the ongoing enzymatic reaction. The possibility of using substrate-selective macrocycles substantially broadens the applicability of tandem assays and opens new opportunities, because comparable enzyme assays with continuous monitoring based on substrate-specific antibodies are nonfeasible. Biological receptors bind analytes through antibody−antigen interactions which, while highly specific and exceedingly strong, suffer from an unfavorably strong binding and slow release kinetics,[23] which would prevent a real-time response in a substrate-specific enzyme assay. In contrast, macrocyclic receptors bind analytes more weakly and reversibly through supramolecular interactions, which is essential for substrate-selective tandem assays to be performed.

Besides the conceptual advancement, our present study is original in that we expand the range of tandem assays to two new enzymes (arginase and diamine oxidase versus the previously studied amino acid decarboxylases) and two additional enzyme classes (a hydrolase (EC3) and an oxidoreductase (EC1) versus the previously documented examples of several lyases (EC4)). Moreover, we apply an additional reporter pair, namely cucurbit[7]uril (CB7)/acridine orange (AO)[24] and demonstrate for the first time the potential of tandem assays for inhibitor screening. Finally, we show that the working principle of tandem assays is not limited to enzymatic reactions in which the charge status in substrate versus product is altered, and where several orders of magnitude difference in binding constants apply. Instead, it can be extended to more subtle structural variations, such as the size or shape of substrate and product, which are also associated with much smaller variations in affinity to the macrocycle (factor of 10).

Results

Conceptual Approach. Once an enzymatic reaction of interest has been identified for which a supramolecular tandem assay should be set up according to Scheme 1, a suitable reporter pair needs to be identified. The macrocyclic component of the reporter pair must necessarily display a sufficiently large binding differentiation between the substrate and the product of the enzymatic reaction. As will be seen herein, even a rather small variation in binding constants by 1 order of magnitude can be sufficient to conduct the assay. Furthermore, it should display a sizable binding with either the substrate (for a substrate-selective assay) or with the product (for a product-selective assay); i.e., the respective binding constant should be sufficiently

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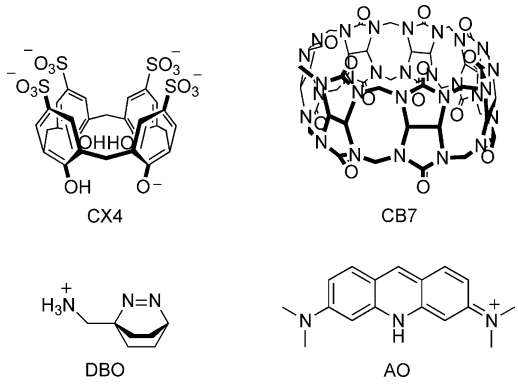
large to effect a sizable complexation within the substrate concentration range appropriate for the particular enzymatic conversion. Additionally, the fluorescent dye must be selected such that it shows a large change in fluorescence properties upon complexation under the conditions, particularly the pH, required for the enzymatic reaction. Also important, its concentration must be adjusted such that the dye competes with the substrate or product in binding to the macrocycle. For example, when the fluorescent dye displays very strong binding, it can be used in a more dilute form, presuming that its fluorescence is still instrumentally detectable. In order for the reporter pair to produce a sufficiently large fluorescence response, a sizable fraction of dye must become complexed or uncomplexed in the course of the enzymatic reaction. This degree of dye uptake or displacement can be conveniently varied through the concentration of added macrocycle, and it can be predicted from direct fluorescence titrations of the dye with the respective macrocycle. The concentration of macrocycle should ideally lie within the titration range where the fluorescence intensity of the dye is most sensitive to the addition of macrocycle, i.e., not in the plateau region of nearly quantitative dye complexation. Once a reporter pair and the concentrations of the individual components have been set up, the robustness of the system for a potential enzyme assay can be tested in the absence of the actual enzyme by carrying out competitive fluorescence titrations with the substrate and product of the enzymatic reaction. As an additional control experiment, to assess interactions of the fluorescent dye with the enzyme, the fluorescence of the dye or of the reporter pair can be monitored upon addition of enzyme, in the absence of substrate. While the procedure just outlined may sound quite complex and involved, it is actually quite intuitive, as will be shown in the following.



Binding Studies and Assay Working Principles. The first substrate-selective supramolecular tandem assay was developed for the enzyme arginase, which hydrolyzes the guanidinium group of arginine to yield the amino acid ornithine and urea as products. Arginase is involved in asthma,[25] immune response,[26] and sexual arousal,[27] such that arginase assays are presently of considerable current interest for use both in academic laboratory settings and in an industrial high-throughput screening format. Recall that the tandem assay principle requires a differential binding of the substrate and the product of the enzymatic reaction with the supramolecular receptor, in our case the macrocyclic host. From our previous study,[14] both *p*-sulfonatocalix[4]arene (CX4) and cucurbit[7]uril (CB7) were known to bind with poor to moderate selectivity to different amino acids at pH 6, but only the former showed the differentiation in binding between arginine [$K = (2800 \pm 100)$ M^{-1}] and ornithine [$K = (210 \pm 10)$ M^{-1}] required for a tandem assay for arginase. The preferential binding of arginine, the substrate, to CX4 is also the prerequisite for performing the tandem assay in the substrate-selective mode. Moreover, a suitable fluorescent dye (1-aminomethyl-2,3-diazabicyclo[2.2.2]oct-2-ene, DBO, $K = 60\,000 \pm 16\,000$ M^{-1}; see Supporting Information) was already known for this macrocycle,[28,29] thereby setting up the reporter pair for the desired tandem assay.[30]

In our present study, the CX4/DBO reporter pair was first employed to remeasure, by competitive fluorescence displacement titrations, the binding constants of the amino acids at the alkaline pH most suitable for the enzyme arginase.[31−33] The resulting values at pH 9.5 ($K = (6400 \pm 250)$ M^{-1}) for arginine and $K = (550 \pm 130)$ M^{-1} for ornithine) revealed essentially the same differentiation (a factor of 12 difference) as that at pH 6 (factor of 13)[14] with a slight increase in absolute binding constants by a factor of *ca.* 2.5. This can be accounted for by the different electrolyte concentrations employed previously for pH 6 (10 mM NH_4OAc)[14] and presently for pH 9.5 (no buffer), since inorganic cations are well-known to show a competitive binding to the calixarene, thereby lowering the observed binding constants with analytes.[34] It should be noted that the difference in binding constants for the arginine/ornithine pair is much smaller than that previously found for the amino acid/biogenic amine pairs because the binding with the calixarene macrocycle is based on a more subtle amino acid residue recognition (preferential binding of the larger arginine)[35−37] as opposed to the previously applicable recognition of charge status (largely favored binding of the bis-cationic diamines).[14] In general, CX4 is well-known to recognize positive charges but displays a poor, but for our purposes sufficient, selectivity toward other structural variations.[28,38,39]

The observed preferential binding for the substrate arginine should allow the setup of a substrate-selective tandem assay according to Scheme 2. Accordingly, arginase converts a

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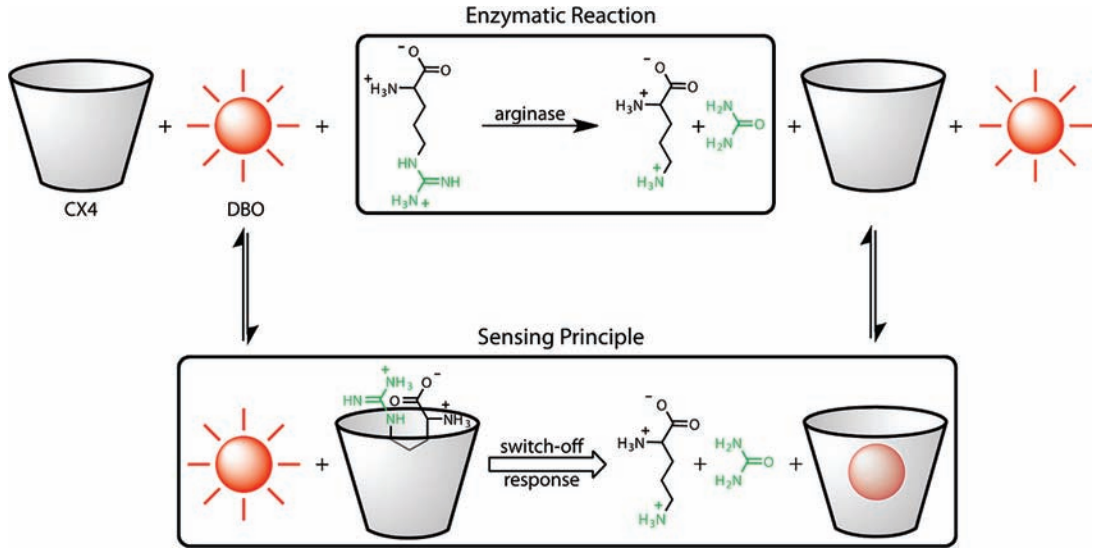
Scheme 2. Binding Equilibria in a Substrate-Selective Switch-Off Supramolecular Tandem Assay for Arginase[a]



[a] It should be noted that the dye, substrate, and product are in a rapid dynamic competitive equilibrium for encapsulation within the CX4 macrocycle.

relatively stronger competitor (arginine) to relatively weaker competitors (ornithine and urea), thereby allowing the dye to compete more efficiently in forming a complex with the macrocycle as the enzymatic reaction proceeds and depletes the substrate. The concentration of uncomplexed substrate is rapidly replenished through its dynamic chemical equilibrium with its corresponding macrocycle−substrate complex.[40] Overall, because the fluorescence of the dye is lower in the inclusion complex, the fluorescence of the system should diminish as a stronger competitor is enzymatically converted into a weaker one ("switch-off" response, see Scheme 2 and Figure 1).

We next turned our attention to the enzyme diamine oxidase, which converts its substrate cadaverine to 5-aminopentanal. Diamine oxidase plays important roles in cancer, tumor growth, and apoptosis;[41−43] it also regulates cellular polyamine levels and, as such, is implicated in cell growth and proliferation processes.[44−46] We have previously studied the binding of cadaverine to the CB7 macrocycle in its capacity as a product of the enzymatic decarboxylation of lysine.[14−16] The primary driving force in the encapsulation of alkylamines is the attractive interaction between their cationic ammonium sites and the electronegative oxygen atoms of the cucurbituril portal carbonyls, as evidenced by the higher binding affinity of the biscationic cadaverine to CB7 [$K = (4.5 \pm 1.3) \times 10^6$ M^{-1}; lit.: $K = 1.4 \times 10^7$ M^{-1} in 10 mM NH_4OAc buffer, pH 6] than the monocationic 1-aminopentane [amylamine, $K = (1.1 \pm 0.1) \times 10^5$ M^{-1}; see Figure 1b]. Both binding constants were determined by competitive displacement titrations in 10 mM $(NH_4)_2PO_4$ buffer, pH 7.2 (using the CB7/AO reporter pair, see below), and the observed variation in binding proclivity was comparable to the differential binding of the same set of guests with the smaller CB6 (factor of 70).[47,48] Note that amylamine serves as a model for 5-aminopentanal, which is the primary enzymatic product of the diamine oxidase reaction but which is thermally unstable under the reaction conditions.[49−51]

Our previous product-selective tandem assay studies utilizing CB7 employed the fluorescent dye Dapoxyl to monitor the reaction.[14] The fluorescence response of this dye upon CB7 encapsulation stems primarily from a complexation-induced pK_a shift;[52] it is consequently strongly pH dependent, which precludes its use at neutral pH and above. Since the optimum pH for diamine oxidase lies at pH 7.2, an alternative dye had to be utilized. Recently,[24] the fluorescent dye AO has been shown to undergo significant changes to its fluorescence properties upon encapsulation by CB7. A direct fluorescence titration of this dye with CB7 under the recommended conditions (pH 7.2, 10 mM $(NH_4)_2PO_4$ buffer, see Supporting Information) led to an approximately 6.5-fold increase in its fluorescence intensity and afforded a binding constant of $(2.9 \pm 0.1) \times 10^5$ M^{-1} (lit. 2.0×10^5 M^{-1} at pH 7).[24] Application of the CB7/AO reporter pair in a substrate-selective tandem assay for monitoring the enzymatic oxidation of cadaverine (Scheme 3) should consequently lead to a "switch-on" response in fluorescence intensity over time, as a strong competitor (cadaverine) is transformed into a weaker one (initially 5-aminopentanal).

Enzymatic Assays. Enzymatic activity of arginase (available as partially purified enzyme) was investigated by adapting the conditions of Greenberg.[33] When the enzyme (140 nM) was added to an arginine (0.1−10 mM) solution containing the CX4/DBO reporter pair (200 μM/100 μM), the enzymatic activity was immediately signaled by a continuous decrease in fluorescence intensity, the absolute magnitude of which depended on the absolute concentrations of substrate (Figure 2a). According

(40) For the investigated macrocycles, a rapid complexation kinetics has been experimentally confirmed through NMR measurements. These established a fast guest exchange (milliseconds or faster) between arginine and CX4, as well as between cadaverine and CB7, ensuring a fast response to the much slower (minutes to hours, Figure 2) enzymatic conversion.

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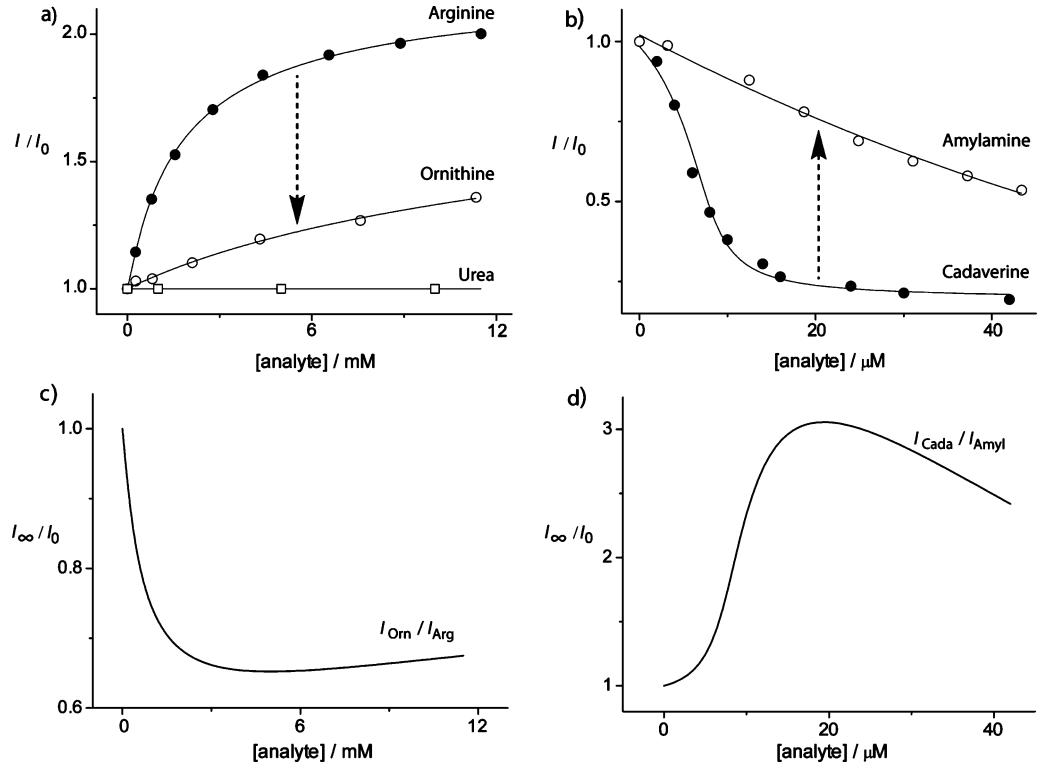
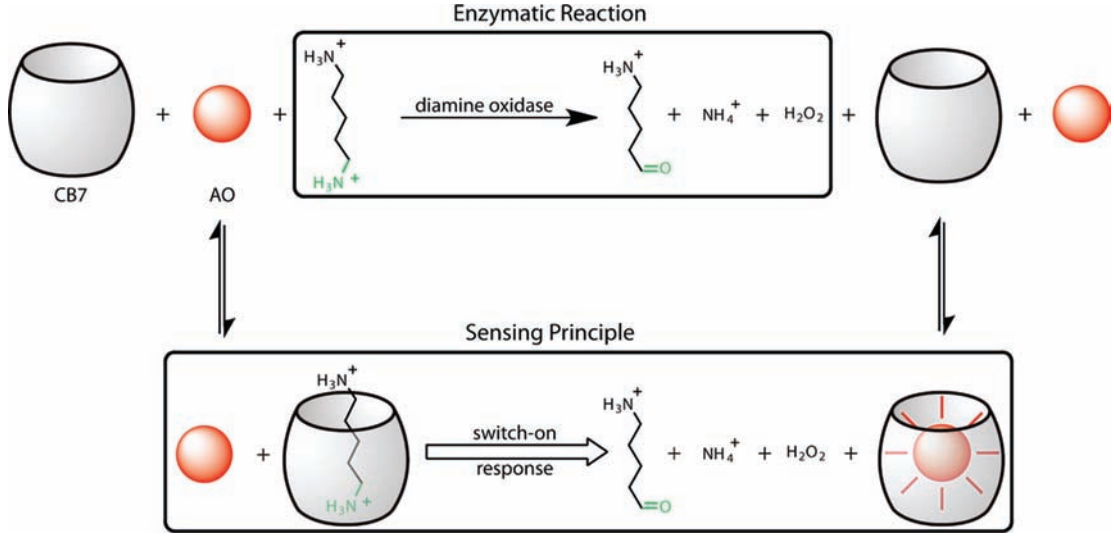


Figure 1. (a) Competitive fluorescence titration plots ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 450$ nm) of L-arginine (●), L-ornithine (○), and urea (□) in H_2O, pH 9.5, with the CX4/DBO reporter pair (200 μM/100 μM). (b) Competitive fluorescence titration plots ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 510$ nm) of cadaverine (●) and amylamine (○) in 10 mM $(NH_4)_2PO_4$ buffer, pH 7.2, with the CB7/AO reporter pair (8 μM/0.5 μM). The solid lines correspond to the fitted curves analyzed according to a competitive binding function; *cf.* Experimental Section. Plots (c) and (d) were obtained by dividing the fitted competitive binding curve of the substrate through that of the product, thus representing the expected fluorescence differentiation (I_∞/I_0) between substrate and product in a tandem assay. Values of I_∞/I_0 smaller and larger than 1 indicate a switch-off and switch-on fluorescence response, respectively, in the course of an enzyme assay, which is also qualitatively illustrated by the arrows in (a) and (b).

Scheme 3. Binding Equilibria in a Substrate-Selective Switch-On Supramolecular Tandem Assay for Diamine Oxidase[a]



[a] It should be noted that the dye, substrate, and product are in a rapid dynamic competitive equilibrium for encapsulation within the CB7 macrocycle.

to Scheme 2, arginase transforms the "strong" competitor arginine into the weaker competitor ornithine, which allows a greater fraction of the fluorescent dye (DBO) to compete with the enzymatic product and therefore to bind to the macrocycle (CX4). Thus, a significant decrease in fluorescence was observed (switch-off fluorescence response), which approached a plateau region as the enzymatic reaction neared completion.

The enzymatic activity of diamine oxidase (available as crude enzyme extract) was followed with the CB7/AO reporter pair (8 μM/0.5 μM) under conditions (10 mM $(NH_4)_2PO_4$ buffer, pH 7.2,) similar to the ones used by Aarsen and Kemp.[53] Monitoring the enzymatic oxidation of cadaverine led to the

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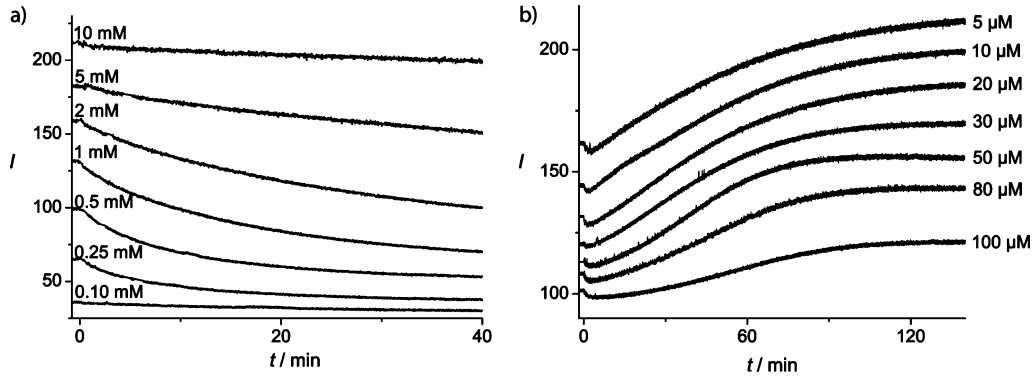

Figure 2. (a) Evolution of fluorescence intensity (λ_{ex} = 365 nm, λ_{em} = 450 nm) of the CX4/DBO (200 μM/100 μM) system during enzymatic hydrolysis of arginine by arginase at different substrate concentrations at 25 °C. The reaction was initiated by addition of arginase (140 nM) at t = 0 min. (b) Evolution of fluorescence intensity (λ_{ex} = 485 nm, λ_{em} = 510 nm) of the CB7/AO (8 μM/0.5 μM) system during enzymatic oxidation of cadaverine to 5-aminopentanal at different substrate concentrations at 37 °C. The reaction was initiated by addition of crude diamine oxidase extract (1 unit/ml) at t = 0 min. Note that the different initial fluorescence intensities are due to varying degrees of dye displacement at different substrate concentrations. Background fluorescence (in the absence of dye) accounts for not more than 10% of the total fluorescence intensity.

expected switch-on response in fluorescence intensity over time, as the strong competitor cadaverine is transformed into a weaker competitor, initially 5-aminopentanal and its cyclization product Δ^1-piperideine (2,3,4,5-tetrahydropyridine), and ultimately larger trimers.[49−51] Again, the appearance of a plateau region in the time-resolved fluorescence traces signaled the completion of the enzymatic reaction (notable at high substrate concentrations, Figure 2b).

Through comparison of the competitive titration plots of the substrates (in this case arginine and cadaverine) and products (ornithine and amylamine), it is possible to approximately predict the change in fluorescence response during the enzymatic transformations at various substrate concentrations (Figure 1) and to identify the substrate concentration range in which the largest fluorescence changes should occur. For this purpose, the *ratios* of fluorescence intensities observed at comparable substrate and product concentrations were plotted (Figure 1c and d). As can be seen, the titration plots predict a maximum fluorescence decrease by ca. 35% at 2 to 6 mM substrate concentration for the arginase assay (Figure 1c), which is even somewhat more pronounced in the actual enzyme assay (decrease by nearly 50%, Figure 2a), where it occurs in a similar concentration range (1−5 mM). For the diamine oxidase assay, the titration plots predict a maximum fluorescence enhancement of 3 near a 20 μM substrate concentration (Figure 1d), which in this case is not reached in the actual assay (maximum factor of 1.5 increase). Control experiments revealed that diamine oxidase, which was used as a crude hog kidney extract and consequently contained large amounts of unknown impurities,[54] caused a sizable reduction of the fluorescence enhancement of the CB7/AO reporter pair (see below), which is held responsible for this quantitative variation. Nevertheless, the concentration range in which the largest fluorescence differentiation is expected according to the competitive fluorescence titrations (15−30 μM, Figure 1d) agrees very well with the experimental results of the diamine oxidase assay runs (20−30 μM, Figure 2b).

The results for arginase and diamine oxidase confirm the viability of substrate-selective supramolecular tandem assays according to Schemes 2 and 3. The activity of both enzymes previously investigated by other assay methods[31−33,53,55−58] can now be sensitively detected and continuously monitored by fluorescence of an added dye in the presence of a suitable macrocycle. While the *qualitative* signaling of enzymatic activity is readily observable, its *quantification* from the recorded fluorescence traces is less straightforward, due to the complexity of the multiple and inter-related reaction equilibria and kinetics involved (see Schemes 2 and 3). In particular, the fluorescence traces (Figure 2) do not provide a direct measure of the absolute reaction rate. For example, the initial slopes of the fluorescence traces are steepest in an intermediary concentration range (around 0.5−2 mM for arginase and 20−30 μM for diamine oxidase) and are reached far below the respective K_M values of the enzymes (5.14 mM for arginase[59] and 1.28 mM for diamine oxidase[58]). This is a manifestation of the tandem assay principle, because at higher concentrations the excess substrate is still capable of displacing the fluorescent dye from the macrocycle, although the enzymatic reaction has already considerably progressed. However, the development of new enzyme assays is currently not driven by the challenge to extract *absolute* enzyme kinetics (which are already known for these two enzymes[27,58]) but to supply convenient tools for rapid screening of *relative* enzymatic activity in the presence of either a series of potentially pharmaceutically relevant inhibitors or a biotechnologically engineered library of enzyme mutants.[60] Therefore, we have investigated in further detail the utility of supramolecular tandem assays by studying the effects of inhibitors on the enzymatic transformations.

Monitoring of Inhibitory Activity. We have investigated known inhibitors of both enzymes in the corresponding substrate-selective tandem assays. For example, *S*-(2-boronoethyl)-L-cysteine (BEC) and 2-(*S*)-amino-6-boronohexanoic acid (ABH) are known inhibitors of arginase.[27] We studied arginase inhibition at substrate concentrations of 0.25−0.5 mM which presented a favorable balance between a rapid and sufficiently

(54) Note that purification of crude hog kidney diamine oxidase extracts can lead to more than a 1000-fold increase in its activity; *cf.* refs 55 and 56.

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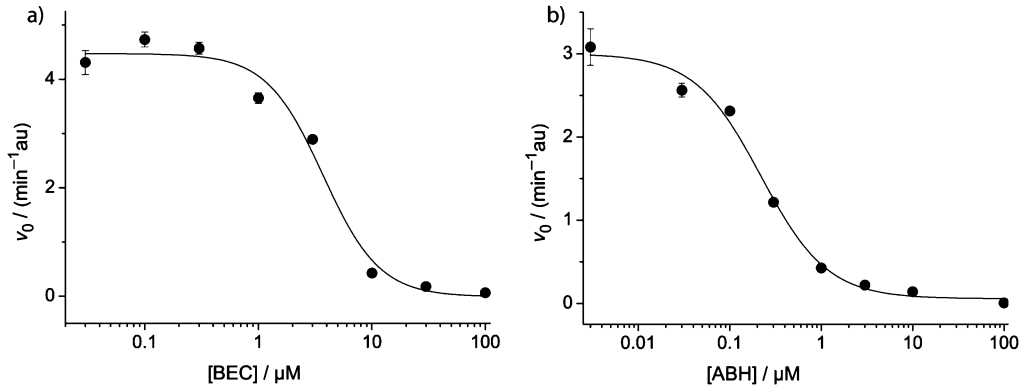


Figure 3. Determination of arginase inhibition by (a) BEC and (b) ABH at 25 °C and corresponding dose−response curves. The inhibition was determined in the presence of 140 nM arginase in H_2O (pH 9.5). 100 μM DBO and 200 μM CX4 were used as a reporter pair. Arginine concentrations were 0.5 mM for BEC and 0.25 mM for ABH.

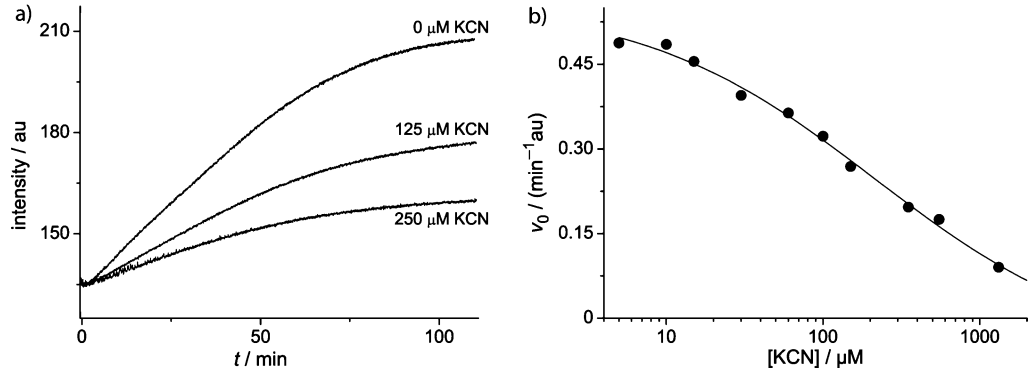
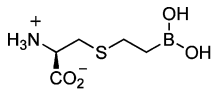
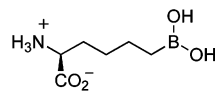


Figure 4. Determination of diamine oxidase inhibition by KCN (0−1320 μM) at 37 °C upon addition of 1 unit/mL diamine oxidase extract at $t = 0$ min. Monitored with the CB7/AO reporter pair (8 μM/0.5 μM) in 10 mM ammonium phosphate buffer at pH 7.2 with the cadaverine concentration held at 30 μM. (a) Selected continuous fluorescence traces ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 510$ nm) upon addition of enzyme (raw data) for the determination of the initial rates. (b) Dose−response curve.

sensitive fluorescence response (see Figure 2a). In fact, addition of either inhibitor in high concentration (100 μM) completely suppressed the fluorescence response, signaling efficient inhibition. We additionally assessed the extent of inhibition by recording fluorescence decays at intermediary inhibitor concentrations. The initial decrease in fluorescence intensity versus time was taken as a *relative* reaction rate to allow the analysis of the resulting dose−response curves (Figure 3a and b) with the Hill equation.[60] The IC_{50} values were 3.7 ± 0.7 μM for BEC and 0.22 ± 0.04 μM for ABH, which can be readily converted into the inhibition constants K_I by considering the enzyme concentration ($IC_{50} = K_I^{app} + {}^1/_2[E]$.)[60] The resulting values for K_I (3.6 ± 0.7 μM for BEC and 0.15 ± 0.04 μM for ABH) are in good agreement with previously reported values of 2.2 and 0.11 μM, respectively,[27] and nicely reflect the approximately 20 times more potent inhibitory activity of ABH.



BEC



ABH

Inhibition of diamine oxidase by cyanide was studied similarly with the substrate-selective tandem assay involving the CB7/AO reporter pair. A substrate concentration of 30 μM was selected, which produced a strong fluorescence response in the absence of inhibitor (see Figure 2b). In this case, the initial *increase* in fluorescence intensity versus time (Figure 4a) was taken as a *relative* reaction rate for the dose−response curve over a cyanide concentration range of 0−1320 μM (Figure 4b).[60] The resulting IC_{50} value for cyanide was 210 ± 110 μM, which compares very well with literature reports of inhibition constants, which are in the range 80−380 μM.[61−63] Incidentally, as can be seen from Figure 4a, the fluorescence intensities reach a plateau at a lower level in the presence of inhibitor, which is fully in agreement with the fact that cyanide acts as a mixed noncompetitive inhibitor of diamine oxidase,[61] thereby irreversibly deactivating the enzyme and resulting in only partial conversion. More potent inhibitors of diamine oxidase were also investigated and found to have a similar inhibition potential as reported in the literature; e.g., the IC_{50} value of semicarbazide was estimated to lie below 10 μM.[62,63] However, due to the crude nature of the enzyme extract, no detailed quantification was performed.

It is worthwhile in this context to consider the potential undesirable effects which an inhibitor could have in a screening based on a substrate-selective supramolecular tandem assay. The most relevant aspect is that an inhibitor might itself bind significantly to the macrocyclic host, which would inevitably reduce its ability to inhibit the enzyme. Fortunately, a significant

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binding of an inhibitor to the host would lead to immediate dye displacement, which would be readily identified by a sudden change in fluorescence intensities upon addition of the compound. In the case of the arginase assay, the fluorescence readout would be artificially *increased*, while, for the diamine oxidase assay, a *decreased* intensity would be observed. Such changes, which are in fact *opposite* to the fluorescence response expected for the enzymatic reactions themselves, were *not* observed for the presently investigated inhibitors,[64] such that this complication did not need to be further considered. Despite the potential interference of the macrocycle present in the assay mixture, a substrate-selective supramolecular tandem assay offers an intrinsic possibility to identify potential false negatives by considering all fluorescence intensity outliers, which show, upon addition of inhibitor, an immediate fluorescence change opposite to the expected direction.[67]

Effect of Macrocycle on the Enzymatic Reaction. The non-negligible binding of the enzymatic substrate with the macrocycle, which constitutes the working principle of any substrate-selective tandem assay, leads to a compulsory modulation of the enzyme kinetics because the free substrate concentration is effectively lowered due to partial complexation.[68] In fact, macrocycles,[69−71] including the presently employed CB7,[72] can even be used as (apparent) inhibitors of enzymatic reactions, e.g., of proteases, whenever they bind the substrate and their concentration is sufficiently high.[71−75] However, the required concentrations of macrocycles to successfully employ them in tandem assays (μM) are much lower than the concentrations previously employed to achieve sizable inhibition effects (mM), such that no large effect on the enzyme kinetics was *a priori* effected. To verify this conjecture, we have monitored the enzymatic transformations by alternative methods.

For arginase, owing to the high substrate concentrations employed in the assay, the reaction could be independently monitored by ^{1}H NMR (data not shown). Integration of the signals from the δ-CH_2 protons verified that the transformation in the absence of CX4 (by ^{1}H NMR) did indeed occur on the same time scale as in the supramolecular tandem assay in the presence of CX4 (by fluorescence). For diamine oxidase, an alternative, established enzyme-coupled assay was used which exploits the enzymatic (with peroxidase) oxidation of the dye *o*-dianisidine by hydrogen peroxide, the enzymatic byproduct (Scheme 3).[53] The depletion of the dye (which shows no detectable binding with CB7) was followed UV-spectrophotometrically at 440 nm to monitor the progress of the enzymatic reaction in the absence and presence of CB7 (see Supporting Information). While a strong retardation of the enzymatic reaction was observed at concentrations above 0.1 mM of CB7, lower concentrations such as those selected for the enzymatic assays (8 μM) displayed quite small effects, particularly on the initial rates relevant for inhibitor studies (see above). This can be rationalized by considering the percentage of bound substrate in each of these cases; for instance, when more than 30 μM, i.e., a large excess, of substrate is used with 8 μM CB7, a maximum of 26% of the substrate is complexed. However, when 0.1 mM of CB7 is utilized, the substrate is quantitatively (>99%) complexed which necessarily leads to a reduced conversion rate. Thus, although the presence of the macrocycle *does* affect the enzyme kinetics in a substrate-selective tandem assay, the conditions can be selected such that this interference becomes either insignificant or at least practically acceptable. Most important, applications in inhibitor screening are based on the effects of additives on *relative* enzyme kinetics, such that small, but constant, influences on the absolute rates are generally tolerable.

Monitoring Multistep Enzymatic Transformations by Domino Tandem Assays. We also tested the possibility whether the tandem assay principle would be suitable to monitor a multistep enzymatic reaction, namely the sequential transformation of lysine to cadaverine by lysine decarboxylase followed by the oxidation of cadaverine to 5-aminopentanal (*vide supra*) by diamine oxidase.

As a common reporter pair for such a "domino tandem assay" we selected CB7/AO, and as common working conditions for both enzymes we chose pH 7.2 and 37 °C. The initial decarboxylation of lysine to cadaverine results in a dramatic increase (ca. ×10 000) in affinity for the analyte toward the macrocycle (CB7).[14] This results in the dye (AO) being ejected from its macrocyclic host (CB7) as is shown in Scheme 4, accompanied by a drastic decrease of the overall fluorescence intensity of the system (Figure 5). The plateau region (~140 min) demonstrates the exhaustion of substrate as the conversion to cadaverine nears completion. After this point, addition of the second enzyme (second arrow), diamine oxidase, initiates the conversion of cadaverine to 5-aminopentanal and its cyclization products (*vide supra*). These products, in turn, have a lower binding affinity to CB7 than cadaverine resulting in an increase in the overall fluorescence intensity as the equilibrium shifts (Figure 5), allowing a greater amount of the dye (AO) to once again become encapsulated by CB7 (Scheme 4).

Noteworthy is that the fluorescence of the system does not fully recover to its initial intensity, which is ostensibly due to two factors: First, a non-negligible residual binding of the product 5-aminopentanal (Figure 1) or its cyclization products[49−51] applies, e.g., amylamine ($K = 1.1 \times 10^5$ M^{-1}, our model for 5-aminopentanal) competes with cadaverine ($K = 4.5 \times 10^6$ M^{-1}) more effectively for CB7 encapsulation than does lysine (870 M^{-1}),[14] thereby allowing a lower fraction of dye to re-enter the macrocyclic cavity. Second and more important, diamine oxidase is being added as a crude enzyme extract,

(64) KCN causes interferences at high millimolar concentrations due to competitive metal ion (K^+) binding to the carbonyl portals of CB7; *cf.* refs 65 and 66.
(65) Marquez, C.; Hudgins, R. R.; Nau, W. M. *J. Am. Chem. Soc.* **2004**, *126*, 5806–5816.
(66) Marquez, C.; Huang, F.; Nau, W. M. *IEEE Trans. Nanobiosci.* **2004**, *3*, 39–45.
(67) To further identify false negatives, a secondary screening is routinely performed in high-throughput applications. In the case of supramolecular tandem assays, this could be done in the simplest case by adding the reporter pair *after* incubation with the enzyme. Such an additional single-point determination would conveniently eliminate the influence of the inhibitor-macrocycle binding on the enzyme kinetics. Alternatively, a secondary screen with a different reporter pair could be performed.
(68) This establishes an important contrast to product-selective tandem assays, where a complexation of the enzymatic product would generally have no effect on the enzyme kinetics, or potentially a beneficial effect in cases where product inhibition applies.
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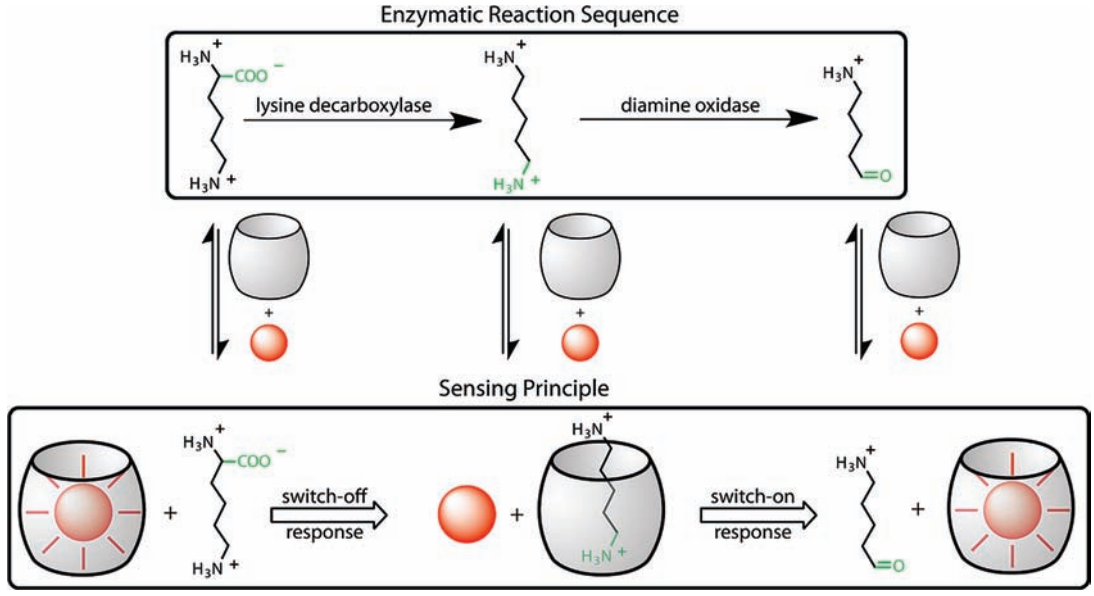
Scheme 4. Binding Equilibria in a "Domino" Tandem Assay, Combining a "Switch-Off" and "Switch-On" Fluorescence Response As Well As Both Product- And Substrate-Selective Binding



requiring the addition of a 140 times larger amount by weight than the partially purified lysine decarboxylase. This results in the addition of a large excess of unknown impurities[54] which lead to a reduction in fluorescence intensity, most likely by partial displacement of dye from the complex. In fact, we were independently able to estimate the extent of this second effect (dashed line in Figure 5) by adding the same amount of crude enzyme extract to a solution containing only the CB7/AO reporter pair (8 μM/0.5 μM) and lysine (30 μM) *without addition of lysine decarboxylase*, thereby mimicking the conditions after complete conversion (and assuming negligible binding of the product). Accordingly, impurities in the crude enzyme extract are largely responsible for the lower fluorescence intensity at the end of the domino tandem assay. Notwithstanding, both enzymatic reactions can be reliably followed by this unconventional approach.

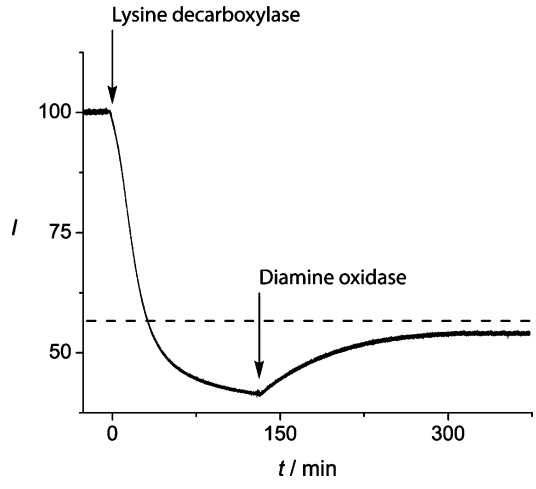


Figure 5. Evolution of fluorescence intensity (λ_{ex} = 485 nm, λ_{em} = 510 nm) of the CB7/AO (8 μM/0.5 μM) system during enzymatic decarboxylation of lysine (30 μM) initiated by lysine decarboxylase (40 μg/mL, t = 0 min), followed by oxidation of the intermediary cadaverine to 5-aminopentanal by diamine oxidase (5.6 mg/mL, $t \approx$ 140 min), at pH 7.2 and 37 °C. The dashed line represents the fluorescence intensity of the system when the crude diamine oxidase extract is added without addition of lysine decarboxylase.

Discussion

Enzymatic assays frequently require the use of fluorescently or radioactively labeled substrates or cofactors, or they depend on a recognition of the reaction products through subsequent binding to antibodies in competition with added fluorescently labeled antigens.[1,19] Also popular are assays in which the reaction products are converted into chromophoric or fluorescent secondary products, either catalyzed by another added enzyme (enzyme-coupled assays) or by chemical follow-up reactions with added (functional-group selective) reagents.[1] The various assay types require, in particular those involving radioactive labels, antibodies, and chemical follow-up reactions, multiple incubation steps or heterogeneous workup, which preclude, with few exceptions,[19] a direct and continuous monitoring of the reaction progress. For example, the established arginase assays require either multiple incubation steps, heating to 100 °C, a colorimetric detection of the enzymatic byproduct urea,[32,33] or the use of chromophoric derivatives like 1-nitro-3-guanidinobenzene.[31] The colorimetric enzyme-coupled assay[53] (see Results) and oxygen-consumption based assays[55,56] common for the determination of diamine oxidase concentration and activity are inherently less sensitive than fluorimetric assays, and only the latter allow for implementation into fluorescence microplate readers and up-scaling to high-throughput screening formats,[76,77] as well as the use of state-of-the-art detection techniques including time-resolved fluorescence.[77,78]

As documented by the kinetic traces in Figure 2 and the inhibition studies in Figures 3 and 4, the assays developed herein for arginase and diamine oxidase allow a real-time, direct, and sensitive monitoring of the progress and inhibition of the

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(78) Hennig, A.; Roth, D.; Enderle, T.; Nau, W. M. *ChemBioChem* **2006**, *7*, 733–737.

enzymatic reaction in homogeneous solution by fluorescence. They entirely bypass the use of antibodies, radioactive labels, covalently attached fluorescent probes, chromogenic or fluorogenic substrates or cofactors,[1,31] chemical follow-up reactions, multiple incubation steps or heating, and heterogeneous workup. Instead, they operate by simple addition of two additives (a macrocycle and a dye), which are either commercially available (CX4, CB7, AO) or readily synthesized (DBO).

We have referred to assays which exploit indicator displacement from macrocycles according to the working principles illustrated in Schemes 1−3 as "tandem assays". Tandem assays exploit a differential, reversible, and competitive intermolecular binding of three potential guests (substrate, product, and dye) with a synthetic receptor and therefore present a genuinely supramolecular approach to the design of enzyme assays. Principles of supramolecular chemistry have been previously utilized in enzyme assays, including the vesicles with synthetic pores pioneered by Matile and co-workers,[9−13] and case studies of tailor-made fluorescent chemosensors, which chelate the substrate or product of an enzymatic reaction.[79−82] In contrast to the known supramolecular approaches, tandem assays allow a continuous monitoring of the enzymatic reaction and bypass the need for the construction of specific fluorescent chemosensors, respectively. Most importantly, they can be simply devised by screening a library of reporter pairs composed of different macrocycles and common fluorescent dyes and testing them for differential binding and a fluorescence response (these are the two prerequisites for the development of any tandem assay), under the enzymatic reaction conditions.

The development of tandem assays for arginase and diamine oxidase presents a very good example of how powerful this approach can be. Although our own "library" is presently still vanishingly small with only four reporter pairs employed until now (CX4/DBO,[14,28,29] CB7/Dapoxyl,[14,52] CB7/AO,[24] and CB6/3-amino-9-ethylcarbazole[16]), it was nevertheless sufficiently large to find at least one suitable reporter pair. For example, CB7 does not show the required differential binding toward arginine and ornithine, but CX4 does, such that the CX4/DBO reporter pair was selected for the arginase assay. Conversely, the fluorescent dye Dapoxyl does not show a sufficient fluorescence response at pH 7, the preferred condition for the diamine oxidase assay, but AO does, such that the CB7/AO reporter pair was preferred in this case.

The tandem assay approach was originally inspired by antibody-based indicator displacement assays and introduced for enzymatic reactions, which afford *products that bind strongly* to the macrocycle (product-selective assays, Scheme 1, top). The presently designed tandem assays are substrate-selective, i.e., based on competitive complexation of the substrate as the stronger competitor. This conceptual step from "product-selective" to "substrate-selective" is nontrivial, as can be seen from a comparison between the potential of *synthetic* receptors (macrocycles) and their *biological* counterparts (antibodies).[19] The latter would bind the substrate too tightly (in some cases irreversibly on the pertinent time scale) and moreover release it too slowly to result in a real-time fluorescence response to the enzymatic conversion.[23] To become specific, antibodies typically display binding constants in the range 10^7-10^9 M^{-1} and "on rates" in the range 10^3-10^6 M^{-1} s^{-1},[19,23] which corresponds to "off rates" in the range $10^{-1}-10^{-6}$ s^{-1}. The release of a complexed substrate, which would be relevant in a substrate-specific antibody assay, would consequently take seconds to days ($1/k_{off}$), far too slow for a continuous monitoring of enzymatic reactions. Note also that the complexation of the substrate would itself require an additional incubation step. In fact, while exceptions are known, it is good practice to equilibrate (incubate) antibodies for typically 5−20 min in homogeneous assays.[19] This limits enzyme assays involving substrate-specific antibodies to indirect examples, in which the function of the antibody is essentially to assess conversion through a single-point measurement[83,84] and not to replenish the free substrate through a dynamic equilibrium.

For comparison, common macrocyclic receptors like cyclodextrins and calixarenes typically show binding constants in the range 10^2-10^6 $M^{-1}$and "on rates" in the range 10^6-10^9 M^{-1} s^{-1},[85−91] which corresponds to "off rates" in the range $1-10^7$ s^{-1}. The release rates of a substrate bound to a macrocycle is consequently faster (seconds to microseconds) in relation to the typical times of enzymatic reactions in enzyme assays (minutes to hours).[40] The reversibility of the guest−macrocycle complexation, ensured by the mM to μM binding constants in combination with a rapid exchange dynamics,[23,88] are consequently critical parameters of supramolecular tandem assays, which ensure the reporter pair to respond sufficiently rapidly and precisely to the depletion of substrate as affected by the enzymatic conversion.

Any sequestration of the substrate by a receptor will inevitably lower its apparent concentration and consequently result in a lowering of the absolute enzymatic reaction rate. This peculiarity of substrate-selective tandem assays[68] is not a primary concern in inhibition studies, where hits are based on clear-cut relative effects in a large series of investigated compounds. Additionally, we could show that adverse effects on the reaction rate can be minimized by working under conditions in which only a small fraction of substrate is complexed, while still allowing the reporter pair to "observe" the enzymatic reaction through its response to the chemical equilibrium changes. This can be ensured, in particular, by working at low macrocycle concentrations with an excess of substrate. For example, the diamine oxidase assay functions quite well by using a macrocycle concentration of 8 μM and substrate concentrations above 30 μM, conditions under which less than 26% of the substrate are complexed. This results in comparably small and tolerable

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effects on the absolute reaction rate, as was demonstrated through control experiments with an alternative assay.

The substrate/product differentiation in the previously reported tandem assays for decarboxylase assays[14−16] was due to an increase in the net positive charge of the competitor during enzymatic transformation, which resulted in a 100−10000-fold difference in binding constants of substrate and product with cation-receptor macrocycles (CX4, CB6, and CB7). The working principle for the diamine oxidase tandem assay is similar (charge-selective binding), except that in this case the substrate binds only ca. 40 times more strongly than the product. As demonstrated for arginase, however, the application of supramolecular tandem assays is not restricted to enzymatic reactions, in which the charge status of the substrate is changed. Note that arginine and ornithine are expected to have a very similar charge status between pH 6 and 9.5. Enzymatic reactions in which the size (or shape) of the competitor changes can be similarly explored, owing to the fact that binding to any macrocycle is also significantly size selective (hole-size selectivity) as well as functional-group selective.[28,38,39] Although in this case the differentiation with respect to the binding constants of substrate and product can be frequently quite small (factor of only 12 for the arginine/ornithine couple), the fluorescence response in the arginase tandem assay is sufficiently robust to reliably monitor the effects of added inhibitors and varying substrate concentration.

The results for arginase thus demonstrate that the assay principle can also be expanded to enzymatic reactions, which affect more subtle structural variations than a change in charge status, and even a factor of 10 difference in binding constants between substrate and product can be sufficient to ensure a sizable fluorescence response through the tandem assay working principle. In other words, even poorly selective macrocycles[28,38,39] can be successfully employed, which presents another illustrative example of the shift toward differential as opposed to highly selective receptors in the research area of molecular recognition.[92,93] As shown for the arginase assay, even a *high affinity* of the macrocycle is no principal requirement. The binding constant of CX4 with arginine, for example, is only on the order of 10^3 M^{-1}, that is, a weak or at best moderate binding. The binding constant does, however, determine the concentration range in which substrate conversion can be reliably monitored, and the latter also depends on the investigated enzyme.

In the course of our investigations on the enzymatic reactions involving amino acids, we became aware of the interrelationship of the various enzymes as well as the intermediary polyamines in fundamental biochemical reaction pathways during cell growth and differentiation.[44−46] Elevated levels of arginase and diamine oxidase activity, for example, are found in cancerous tissues. Enzymatic conversion of arginine by arginase yields ornithine which in turn undergoes decarboxylation to putrescine, which is finally oxidized by diamine oxidase.[58,94] Cadaverine adapts a similarly central position in a metabolic pathway involving decarboxylation of lysine as precursor and deamination of 1-aminopentanal as product.[95,96]

It was our idea to apply the supramolecular tandem assay principle to such a cascade of enzymatic reactions and monitor a multistep enzymatic reaction sequence by coupling product- with substrate-selective sensing. In essence, the first reaction could be signaled through the binding of a product with the macrocycle, which could then serve as the substrate for a second reaction. In an extreme situation, an analyte could go through a series of enzymatic transformations (or an entire metabolic pathway) that all affect its binding affinity to the macrocycle, and by combining the tandem assay principle with a sequential addition of the different enzymes, it should be possible to continually monitor these transformations in real time and in a "one-pot" fashion, potentially by using a single reporter pair. This "domino" tandem assay technique should be especially powerful when a metabolite undergoes transformations that alternate its affinity to the macrocycle, e.g., from weak to strong and back to weak.

The enzymatic reaction sequence from arginine (strong competitor) to ornithine (weak competitor) to putrescine (very strong competitor) to 1-aminobutanal (weaker competitor) fulfills this requirement, as well as the reaction sequence from lysine (weak competitor) to cadaverine (very strong competitor) to 1-aminopentanal (weaker competitor). Unfortunately, we lacked access to the enzyme ornithine decarboxylase and could therefore not examine the associated three-step enzymatic process, but the two enzymes involved in the reaction sequence involving cadaverine were available: lysine decarboxylase (in partially purified form) and diamine oxidase (as crude extract). The working principle of the corresponding domino tandem assay is illustrated in Scheme 4. As can be seen from Figure 5, the two assays can indeed be sequentially combined to afford first a fluorescence decrease due to decarboxylation and then, upon addition of diamine oxidase, a fluorescence increase due to oxidative deamination (on−off−on response). The fact that the fluorescence does not fully recover after the enzymatic conversion by diamine oxidase is attributed to the use of this enzyme as a crude enzyme extract, which results in partial dye displacement due to large amounts of biological "impurities" (dashed line in Figure 5). But regardless of this interference caused by the lack of selectivity, tandem assays are sufficiently robust to function also in more complex biological mixtures, as was previously demonstrated through comparative experiments with purified enzymes, crude extracts, and whole cells expressing a particular enzyme. This may be quite surprising, but the concentration of biological impurities contained in a certain enzyme preparation is expected to remain constant as the enzymatic reaction proceeds. The interference is consequently "static" in nature and does not influence the time-resolved change in fluorescence intensity, which presents the optical read-out of the assay and which is diagnostic and specific for the enzymatic reaction.

The domino tandem assay shows that it is possible to monitor, in real time, quite complex, but biologically highly relevant, metabolic pathways by a very simple method. Additionally and very interesting to note, similar enzymatic reaction cascades with alternating on−off−on optical signaling have been proposed to be promising for biocatalyst-stimulated logic gate operations in the context of biocomputing.[97,98] In any case, our results present a proof-of-principle; they demonstrate nicely the

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potential of the tandem assay principle and reveal the inherent advantage of employing differentially selective as opposed to highly specific receptors.

Conclusions

We have expanded the range of supramolecular tandem assays from product- to substrate-selective variants. We demonstrated that even small differences in binding constants of substrate and product are sufficient not only to monitor the activity of two important enzymes in real time in a homogeneous solution but also to study inhibition effects. Tandem assays are exceptionally adaptable and flexible, which is attractive in view of the ever-expanding range of substrates and enzymes. Instead of designing a specific chemosensor or raising a specific antibody, generally for the product of an enzymatic reaction, tandem assays can be simply devised by screening a library of reporter pairs. Even by combining all commercially available macrocycles and fluorescent dyes, or focusing on the large diversity of host−dye complexes already investigated,[2−4,14,16,24,28,29,52,99−113] a very large library could readily be built up. This approach has enormous potential for enzyme assay development, and the effort, time, and resources related to testing such a library should compete well with alternative strategies in assay development. Owing to the moderate but known selectivity-of different macrocycles, the search can also be rationally limited (e.g., to cation-receptor macrocycles for reactions involving positively charged substrates and products) and a once identified reporter pair can be with high probability transferred to enzymes affecting similar functional group interconversions; e.g., the CB7/Dapoxyl reporter pair was previously employed for not less than six amino acid decarboxylases.[14,15]

Experimental Section

DBO was synthesized according to a literature procedure[114] and purified by precipitation as the sulfate salt from diethyl ether. Acridine orange, cadaverine, putrescine, ornithine, and CX4 (all Fluka) as well as ornithine, dianisidine, urea, and potassium cyanide (all Sigma-Aldrich) were obtained in the highest purity available and used as received. Cucurbit[7]uril was synthesized in >95% purity, following established synthetic protocols.[66,115,116] The arginase inhibitors ABH and BEC were received as ammonium salts from Alexis Biochemicals (Lausen, Switzerland). Diamine oxidase (crude extract from hog kidney, 0.18 units/mg solid), peroxidase (type II from horseradish, 181 purpurogallin units/mg), and lysine decarboxylase (partially purified, 1.6 U/mg) were from Sigma-Aldrich. Arginase (partially purified, from cow liver, 100−200 units/mg solid) was from Fluka.

Absorption measurements were performed with a Varian Cary 4000 spectrophotometer. For bovine liver arginase an extinction coefficient of $1.1 \times 10^5\ M^{-1}\ cm^{-1}$ at 278 nm was used.[117] For fluorescence measurements, a Varian Eclipse fluorimeter (arginase assays: $\lambda_{ex} = 365$ nm, $\lambda_{em} = 450$ nm, diamine oxidase assays: $\lambda_{ex} = 485$ nm, $\lambda_{em} = 510$ nm) was used. Continuous assays for arginase (16 μg/mL, 140 nM) were performed unbuffered at pH 9.5 in the presence of 100 μM DBO and 200 μM CX4 in a variable-temperature cell holder at 25.0 ± 0.1 °C. Continuous assays for diamine oxidase (1 unit/ml) were measured in 10 mM $(NH_4)_2PO_4$ buffer, pH 7.2, with a mixture of 0.5 μM acridine orange, 8 μM CB7, and 30 μM of cadaverine in a variable-temperature cell holder at 37.0 ± 0.1 °C. Dose−response curves of the inhibitors BEC and ABH were obtained in a similar manner using 0.5 mM and 0.25 mM of arginine. Inhibition kinetic traces for KCN were obtained with 30 μM cadaverine for a series of KCN concentrations (0−1320 μM).

For analytical analysis of the titrations, we define $[D]_0$, $[C]_0$, and $[H]_0$ as the total concentrations of dye, competitor (substrate or product), and host (macrocycle). [D], [C], and [H] are the concentrations of uncomplexed dye, uncomplexed competitor, and uncomplexed host. [H•D] and [H•C] are the concentrations of the host−dye and host−competitor complex, and K_C and K_D are the association constants of the competitor and dye with the host.

The fluorescence intensity (I) in the course of the titration can be expressed as a linear combination of the fluorescence intensity of the uncomplexed dye (I_D) and that of the host−dye complex ($I_{H•D}$), weighted by their molar fractions according to eq 1. $I_{H•D}$ was extrapolated from host−guest titrations (in the absence of competitor) fitted according to a 1:1 binding model.[28,52,118]

$$I = \frac{[D]}{[D]_0} I_D + \frac{[H•D]}{[D]_0} I_{H•D} \quad (1)$$

Upon appropriate substitution one obtains eq 2, with the concentration of uncomplexed host as variable; the latter is defined by a cubic equation (eq 3).[119]

$$I = I_D + (I_{H•D} - I_D)\frac{K_D[H]}{1 + K_D[H]} \quad (2)$$

$$0 = a[H]^3 + b[H]^2 + c[H] - d, \text{ where}$$
$$a = K_C K_D,\ b = K_C + K_D + K_C K_D([D]_0 + [C]_0 - [H]_0),$$
$$c = K_C([C]_0 - [H]_0) + K_D([D]_0 - [H]_0) + 1, \text{ and } d = -[H]_0 \quad (3)$$

The fitting was implemented in OriginPro 7.5 (OriginLab Corporation, Northampton, MA), by using a subroutine to solve

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the cubic eq 3 with the Newton−Raphson method. The module is available from the authors upon request.

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Supporting Information Available: Host−dye titrations and spectrophotometric assays of diamine oxidase to study the effect of added macrocycle. This material is available free of charge via the Internet at http://pubs.acs.org.

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