Supplementary Materials for

The role of DNA nanostructures in the catalytic properties of an allosterically regulated protease

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1. Michaelis-Menten model for serine proteases

The catalytic activity of all serine proteases is described by the following three-steps mechanism (53):

\[ E + S \xrightleftharpoons{\frac{k_1}{k_f}} K \xrightarrow{\frac{k_2}{k_3}} E \cdot P \]

where \( E \) is the enzyme
\( S \) is the substrate
\( ES \) is the enzyme-substrate complex
\( EP' \) is the acylated intermediate (enzyme-product adduct)
\( P \) is the product
\( k_1 \) is the rate constant for substrate binding
\( k_f \) is the rate constant for substrate dissociation
\( k_2 \) is the rate constant for substrate acylation (normally the rate limiting step)
\( k_3 \) is the rate constant for substrate deacylation

The steady-state rate of the reaction obeys a MM equation:

\[ v = \frac{k_{cat}[E]_0[S]}{K_M + [S]} \quad \text{Eq. 1} \]

however, in this case, the kinetic parameters of the reactions are related in a complex way to the rate coefficients of the different steps, according to

\[ K_M = \frac{k_3(k_{-1}+k_2)}{k_1(k_2+k_3)} = \frac{k_{cat}}{k_1} \left( \frac{k_{-1}}{k_2} + 1 \right) \quad \text{Eq. 2} \]

\[ k_{cat} = \frac{k_2k_3}{k_2+k_3} \quad \text{Eq. 3} \]

In most practical cases, one can assume that the concentration of \( EP' \) does not accumulate in time, meaning that \( k_3 \gg k_2 \), and the kinetic scheme above simplifies to a classical two steps-mechanism leading to

\[ k_{cat} = k_2 \quad \text{Eq. 4} \]

and

\[ K_M = \frac{k_{-1}+k_2}{k_1} \quad \text{Eq. 5} \]
2. Substrate inhibition or inhibition by excess substrate

\[ v = \frac{v'_{\text{max}} [S]}{K'_{M} + [S] \left( 1 + \frac{[S]}{K_i} \right)} \]  

Eq. 6

where \( K_i \) is the dissociation constant of the SES complex. Here, one should consider that the kinetic parameters obtained, although they satisfy the usual definition, are not exactly the same parameters of a classical MM equation (50).
3. Kinetic analysis applying the transition-state model

Assuming a simple MM mechanism, we applied the transition state theory as described by A. Fersht (47) to gain a better insight into the relationship between the kinetic parameters obtained in our experiments (i.e. \(k_{\text{cat}}, K_M\) and \(k_{\text{cat}}/K_M\)) and the reaction rates observed for the various substrates and enzyme constructs. According to this model, the transition state of the reaction (ES‡) occupies a maximum of energy through which the substrate must pass to reach the products. The difference in energy between the unreacted E and S species and their association into the ES complex is the free Gibbs energy of binding between the two molecules (\(\Delta G_{ES}\)). For a MM mechanism, this value of energy is related to the \(K_M\) of the reaction. Similarly, the activation energy that separates the ES species from the transition state (\(\Delta G^\ddagger\)) is related to the \(k_{\text{cat}}\) of the reaction. Finally, the \(k_{\text{cat}}/K_M\) in a simple MM mechanism can be viewed as a pseudo second-order rate coefficient for the reaction that goes from the unreacted E and S species to the ES‡ and is thus linked to the difference in energy between the unreacted species and the transition state (\(\Delta G_{TS}^\ddagger\)). A simple relation therefore enables to calculate relative changes in energy from the ratio of the associated kinetic parameters, as described below:

\[
\Delta \Delta G_{ES} = -RT \ln \left( \frac{K_M}{K'_M} \right) \quad \text{Eq. 7}
\]

\[
\Delta \Delta G^\ddagger = -RT \ln \left( \frac{k_{\text{cat}}}{k'_{\text{cat}}} \right) \quad \text{Eq. 8}
\]

\[
\Delta \Delta G_{TS}^\ddagger = -RT \ln \left( \frac{k_{\text{cat}}/K_M}{k'_{\text{cat}}/K'_M} \right) \quad \text{Eq. 9}
\]

Here, \(k_{\text{cat}}, K_M\) and \(k_{\text{cat}}/K_M\), as well as, \(k'_{\text{cat}}, K'_M\) and \(k'_{\text{cat}}/K'_M\) refer to the kinetic parameters obtained for the same enzymatic reaction, however in two different conditions. In this way, it is possible to compare the cleavage of two distinct substrates by the same enzyme or the cleavage of the same substrate by a DNA-bound or DNA-unbound enzyme. By depicting an arbitrary energy diagram of the reaction for a condition taken as reference and observing how the energy levels change in a different condition, one can gather useful information on the effect of such external variations on the rate of the reaction.

We distinguished two possible scenarios: \([S] < K_M\) and \([S] > K_M\). In the former case (sub-saturating substrate concentrations), the ES complex will be poorly populated and will lay above the E+S level, whereas in the latter case, the opposite will be true (saturating substrate concentrations). This distinction is necessary to understand how the change in the kinetic parameters of the reaction reflects into a change of reaction velocity. Indeed, depending on the substrate concentration, the following relationships will be valid in a MM regime:

\[
[S] < K_M \rightarrow v \approx \frac{k_{\text{cat}} [E] a[S]}{K_M} \quad \text{Eq. 10}
\]

\[
[S] > K_M \rightarrow v \approx k_{\text{cat}} [E]_0 \quad \text{Eq. 11}
\]

Thus, in one case, the values of \(k_{\text{cat}}/K_M\) will be of major importance and the change in \(\Delta G_{TS}^\ddagger\) will dictate the change in the velocity of the reaction. In the other case, the \(k_{\text{cat}}\) of the compared reactions will play a major role and the change in \(\Delta G^\ddagger\) will mostly affect the reaction rate.
4. Mono- vs. bivalent binding of TBA-modified DNA origami to thrombin

A binding yield evaluated through AFM imaging may be affected by surface-related effects and mechanical forces applied during sample preparation and scanning. For this reason, we evaluated a large set of images from independent sample preparations, for a total of more than 7000 counted structures. We also performed ensemble in-solution FRET spectroscopy studies to verify that, in our experimental conditions, the aptamers effectively fold in the G4-conformation that is necessary for recognition of the exosite regions of thrombin (fig. S4 and S5). According to our AFM data, binding of thrombin to TBA1 (when scaffolded inside the rect DNA origami) was observed in only 5% of the structures, whereas the loading efficiency raised up to more than 20% in TBA2 modified cavities (fig. S2A and S2B). As TBA1 has a $K_D$ more than 100-fold higher than TBA2 (35), this result well matches with the theoretical expectations. We mostly observed mono-loaded chambers, meaning that one single molecule of thrombin was trapped within the DNA origami frame, independently of the number of aptamers. Doubly-loaded cavities (containing two thrombin molecules), although possible and clearly distinguishable by HR-AFM, were mostly negligible (less than 1.5%, fig. S2C). Along this line, we observed that the presence of both aptamers within the DNA cavity led to a number of binding events (44%) which is more than the sum of the contributions from the individual aptamers and almost two-fold the total amount of binding events observed for TBA1 (5%) and TBA2 (20%) structures together. These data suggest that, besides monovalent (1:1) aptamer/protein binding, a considerable amount of bivalent (2:1) interactions is taking place when both aptamers are present within the cavity, meaning that both aptamers are simultaneously binding to the same protein target. We reasoned that for every bivalent complex, the binding (+) or unbinding (-) of an aptamer ligand to its corresponding recognition site on thrombin may lead to four possible states (fig. S2B). A first state in which, despite both aptamers are linked to the DNA cage, no binding occurs at both sites (TBA1-/2-); a second and third state, characterized by the binding of only one of the two ligands, either TBA1 or TBA2 (respectively referred to as TBA1+/2- and TBA1-/2+); and a forth state in which both aptamers are bound (TBA1+/2+). Assuming that the binding affinity of each aptamer is independent on the vicinal presence of the other aptamer, the totality of the binding events in the doubly-modified design (44%) is thus distributed as follows: 5% in the TBA1+/2- state, 20% in the TBA1+/2+ state and the remaining 19% is related to the simultaneous binding of both aptamers, leaving 56% of unbound structures (corresponding, respectively to the TBA1+/2+ and TBA1+/2- states).
5. The effect of multiple enzyme species on thrombin catalysis

In order to better understand the impact of a mixture of free thrombin and DNA-bound thrombin on our kinetic analysis, we followed two approaches: (i) we attempted to increase the binding yield of the complex for its further isolation at high concentrations; (ii) we simulated the kinetic behavior of the system in presence of both the free and DNA-bound thrombin. This may give a theoretical means to face this issue when practical limitations cannot be easily overcome.

5.1 Purification of DNA-enzyme complexes

Ideally, high concentrations (above the $K_D$ of the complex) should be employed to isolate a non-covalent DNA-enzyme complex. The first problem arises from the fact that the value of $K_D$ is unknown, therefore we assumed this to be (at least) equal to the $K_D$ of the stronger aptamer (ca. 1 nM). Preparation of DNA-bound thrombin complexes was done in different conditions: using an excess of thrombin (50-fold to 500-fold) on a pre-purified DNA origami sample, adjusting the incubation time (from 2 h to overnight) and/or incubation temperature (24 °C or 37°C). The excess of protein was removed by PEG-precipitation and the extent of thrombin-loaded structures was estimated by manual counting of AFM images. Despite this purification procedure may indeed affect the equilibrium position, favoring dissociation of the complex, the use of relatively high concentrations should minimize the problem. On the other hand, excessive up-scaling (> ca. 200 nM) of protein-modified DNA origami was – in our hands – seriously limited by the occurrence of aggregation phenomena. We finally reached a compromise between high concentrations and sufficient solubility, and analyzed a total of 1095 rect1/2flex structures and 843 rect structures obtained from 10 successful independent preparations. The results indicate ca. 38% loaded structures in rect1/2flex and only about 4% unspecific binding in rect origami, which lack the TBA ligands. Together with our previous AFM data, performed on ca. 7000 counted structures, we conclude that – in our conditions – we can reach a yield of about 40% to a maximum of 44% DNA-enzyme complexes.

Assuming that the DNA-enzyme complex has a $K_D = 1$ nM (given by the strong affinity of the TBA2 aptamer to thrombin), the following equation will relate the concentrations of the three possible species at the equilibrium:

$$K_D = \frac{[E][DNA]}{[E_{DNA}]} = 1 = \frac{(1.2-x)(1-x)}{x}$$

Eq. 1

where [E], [DNA] and [E_{DNA}] indicate the equilibrium concentration of the free enzyme, unloaded DNA-origami cages and enzyme-loaded cages, respectively. Thus, a solution containing a 1.2 nM initial concentration of thrombin and 1 nM initial concentration of DNA-origami, as we used in our enzymatic assays, will lead at the equilibrium to a mixture containing about 0.77 nM free enzyme, 0.57 nM unloaded DNA cages and 0.43 nM DNA-enzyme complex, obtained by solving the quadratic expression in Eq. 1. These values match very well with the data obtained by AFM imaging (that is, ca. 60% unbound and 40% DNA-bound enzyme species), and let us conclude that our kinetic assays actually refer to a mixture of free and DNA-bound enzyme species. We thus suppose that the impact of DNA-scaffolded enzymes on the kinetics of thrombin-catalyzed amidase is largely underestimated.

5.2 Kinetic linkage of thrombin in presence of DNA

In our experimental conditions, binding of thrombin inside the DNA origami cage is mediated by a non-covalent bond and an equilibrium is probably established between the DNA-bound and the DNA-unbound enzyme species that severely complicates the analysis of the data. In addition, thrombin itself may exist in different forms, whose relative occurrence is allosterically regulated by external effectors (such as sodium or phosphate ions). A method is therefore
needed to analyze more complex reactions involving different enzyme species in a pseudo-equilibrium regime. Each enzyme species is capable to perform the catalytic cycle, however each cycle is characterized by its own rate coefficients. The result is a network of coupled enzymatic reactions that occur simultaneously with different rates. A major contribution to this field comes from Di Cera and coauthors in their formulation of the so-called kinetic linkage scheme of thrombin (54). The main idea behind this theoretical approach is to describe the steady-state rate of the reaction in a rigorous mathematical fashion, considering all possible kinetic fluxes that link the interacting species. The original scheme proposed by Di Cera et. al. (54) for the kinetic linkage of thrombin was adapted to our system considering two enzyme species: the free enzyme (E) and the DNA-bound enzyme (EDNA) (see fig. S39). The exact mathematical solution of this kinetic scheme results in the expression of the steady-state velocity of the enzymatic reaction as a ratio of two polynomials. Each polynomial has a degree \( n \) in substrate concentration, with \( n \) equals to the number of different enzyme species in solution (here \( n = 2 \)). A plot of the initial velocity vs. substrate concentration should in principle obey a non-MM equation; in practice, a MM behavior is observed in several cases and is the consequence of one of two possible reasons: (i) a fast pseudo-equilibrium regime among the free and the effector-bound species (here, the DNA-bound enzyme) or (ii) a particular mathematical relationship among the rate coefficients, (here, \( k_4 k_2 = k_{-1} k_5 \)). In either of these conditions, the enzyme apparently obeys a Michaelis-Menten mechanism, obscuring the actual existence of kinetically linked pathways.

We tested the applicability of this model for interpretation of our kinetic data; however, we did not perform a fit of our experimental curves to this model, as this would result in an over-parametrized problem (14 rate coefficients) with low information content. We instead performed various kinetic simulations using known values reported in the literature (53, 55) for the rate coefficients of thrombin in buffer conditions similar to those used in our experiments and made some further simplifications to the model to catch the essential message. In our simulations, the condition \( k_4 k_2 = k_{-1} k_5 \) is always satisfied, as \( k_4 = A k_{-1} \) and \( k_5 = A k_{-1} \), with \( A = \) multiplicative factor (100, 1 or 0.01). This ensures that our kinetic linkage scheme always results into an apparent MM kinetics, independently of the pseudo-equilibrium regime between the enzyme species. In this way, we can better simulate our experimental conditions, even in case the two species, i.e. the free E and the DNA-bound form EDNA, are not effectively in equilibrium. The results of our simulations are reported in fig. S39-S41.

The conditions for our simulations are reported below:

**Simulation conditions (valid for all simulations #1 to #12):**

- two initial enzyme species in solution, E and EDNA
- each enzyme species can perform a catalytic cycle
- initial concentrations of enzyme and “DNA effector” are
  - \([E]_0 = 1.2 \text{ nM}\)
  - \([\text{DNA}]_0 = 1 \text{ nM}\)
  to simulate our experimental conditions.
- all enzyme intermediates are in a pseudo-equilibrium regime according to a \( K_D \), which is identical (but variable) between the two kinetic pathways.
- for the free-thrombin (E) pathway, we chose the rate coefficients reported in the literature for similar buffer conditions (53, 55)
  - \( k_1 = 6.000 \text{ min}^{-1} \mu \text{M}^{-1} \)
\[ k_1 = 16,000 \text{ min}^{-1} \]
\[ k_2 = 100 \text{ min}^{-1} \]
\[ k_3 = 500 \text{ min}^{-1} \]

this would result in a \( k_{\text{cat}} = \text{ca. 80 min}^{-1} \) and a \( K_M = 2 \text{ \mu M} \), which well resemble the values obtained in our assays for thrombin-only samples.

- the dissociation constant for the \( E_{\text{DNA}} \) species is set to
  - \( K_D = 1.0 \text{ nM} \) (simulations #1 to #3)
  - \( K_D = 10 \text{ nM} \) (simulations #4 to #6)
  - \( K_D = 0.1 \text{ nM} \) (simulations #7 to #9)
  - \( K_D = 1 \text{ \mu M} \) (simulations #10 to #12)

to test how the pseudo-equilibrium between the species affects the velocity of the reaction.

for the reaction of the \( E_{\text{DNA}} \) species with the substrate (\( E_{\text{DNA}} \) pathway), we proceeded as follows:

**Simulation #1 to #3**
- \( k_7 = k_8 = k_9 = 10,000 \text{ min}^{-1} \text{ \mu M}^{-1} \)
- \( k_3 = k_9 = 10 \text{ min}^{-1} \)
- \( k_8 = \frac{(k_1 k_8 k_4 k_7)}{(k_1 k_7 k_4)} = 3 \text{ min}^{-1} \) to satisfy the thermodynamic cycle
- In these conditions, \( K_D = 1 \text{ nM} \) (valid for simulation #1, #2 and #3).
- at the equilibrium, \([E] = 64\% \) and \([E_{\text{DNA}}] = 36\% \)

- \( k_4 \) to \( k_6 \) are identical to their corresponding analogs (\( k_1 \) to \( k_3 \)) in the E cycle, thus both enzyme species perform equally (simulation #1)
- \( k_4 \) to \( k_6 \) are 100-fold their corresponding analogs in the E cycle, meaning that the \( E_{\text{DNA}} \) pathway is faster (simulation #2).
- \( k_4 \) to \( k_6 \) are 0.01-fold their corresponding analogs in the E cycle, meaning that the \( E_{\text{DNA}} \) pathway is slower (simulation #3).

**Simulation #4 to #6**
- \( k_7 = k_8 = k_9 = 10,000 \text{ min}^{-1} \text{ \mu M}^{-1} \)
- \( k_3 = k_9 = 100 \text{ min}^{-1} \)
- \( k_8 = \frac{(k_1 k_8 k_4 k_7)}{(k_1 k_7 k_4)} \) to satisfy the thermodynamic cycle
- In these conditions, \( K_D = 10 \text{ nM} \) (valid for simulation #4, #5 and #6).
- at the equilibrium, \([E] = 92\% \) and \([E_{\text{DNA}}] = 8\% \)

- \( k_4 \) to \( k_6 \) equal to \( k_1 \) to \( k_3 \) (equal rates for both kinetic pathways).
- \( k_4 \) to \( k_6 \) 100-fold the values of \( k_1 \) to \( k_3 \) (\( E_{\text{DNA}} \) pathway is faster).
- \( k_4 \) to \( k_6 \) 0.01-fold the values of \( k_1 \) to \( k_3 \) (\( E_{\text{DNA}} \) pathway is slower)

**Simulation #7 to #9**
- \( k_7 = k_8 = k_9 = 10,000 \text{ min}^{-1} \text{ \mu M}^{-1} \)
- \( k_3 = k_9 = 1 \text{ min}^{-1} \)
- \( k_8 = \frac{(k_1 k_8 k_4 k_7)}{(k_1 k_7 k_4)} \) to satisfy the thermodynamic cycle
- In these conditions, \( K_D = 0.1 \text{ nM} \) (valid for simulation #7, #8 and #9).
- at the equilibrium, \([E] = 33\% \) and \([E_{\text{DNA}}] = 67\% \)

- \( k_4 \) to \( k_6 \) equal to \( k_1 \) to \( k_3 \) (equal rates for both kinetic pathways).
• $k_4$ to $k_6$ 100-fold the values of $k_1$ to $k_3$ ($E_{DNA}$ pathway is faster)
• $k_4$ to $k_6$ 0.01-fold the values of $k_1$ to $k_3$ ($E_{DNA}$ pathway is slower)

**simulation #10 to #12**
• $k_7 = k_8 = k_9 = 10,000 \text{ min}^{-1} \mu\text{M}^{-1}$
• $k_{-7} = k_{-9} = 10,000 \text{ min}^{-1}$
• $k_8 = (k_1 k_8 k_4 k_{-7}) / (k_{-1} k_7 k_3)$ to satisfy the thermodynamic cycle
  
  In these conditions, $K_D = 1 \mu\text{M}$ (valid for simulation #10, #11 and #12).
  
  at the equilibrium, $[E] = 100\%$ and $[E_{DNA}] = 0\%$

• $k_4$ to $k_6$ equal to $k_1$ to $k_3$ (equal rates for both kinetic pathways).
• $k_4$ to $k_6$ 100-fold the values of $k_1$ to $k_3$ ($E_{DNA}$ pathway is faster)
• $k_4$ to $k_6$ 0.01-fold the values of $k_1$ to $k_3$ ($E_{DNA}$ pathway is slower)

All kinetic simulations were performed using the DynaFit program available free-of-charge at http://www.biokin.com. A representative script for simulation #1 is reported below. The script has been accordingly changed to run the other simulations.

**SIMULATION SCRIPT**

simulate a family of initial rate vs. substrate curves for the kinetic linkage of thrombin (1 effector) according to (54)

```plaintext
  [task]
  task = simulate
  data = rates
  approximation = king-altman

  [mechanism]
  reaction S  --> P
  modifiers DNA
  
  E + S  <=> ES : k1 k-1
  ES --> EP : k2
  EP --> E + P : k3
  
  E(DNA) + S  <=> ES(DNA) : k4 k-4
  ES(DNA) --> EP(DNA) : k5
  EP(DNA) --> E(DNA) + P : k6
  
  E + DNA  <=> E(DNA) : k7 k-7
  ES + DNA  <=> ES(DNA) : k8 k-8
  EP + DNA  <=> EP(DNA) : k9 k-9

  ;we suppose that at the beginning of the reaction the free enzyme species in solution is E

  [constants]
  k1 = 6000, k-1 = 16000
  k2 = 100
  k3 = 500
```
The expression of the steady-state rate has been derived upon application of the King-Altman method and is reported below:

**Rate equation**

\[ v = \frac{[E]_0 N}{D} = \frac{d[P]}{d t} = + k_1 [E] P + k_6 [EP(DNA)] \]

\[ N = n_1 [S]^2 + n_2 [S][DNA] + n_4 [S][DNA]^2 + n_5 [S]^2[DNA] + n_6 [S][DNA]^3 + n_7 [S]^3[DNA]^2 \]

\[ D = d_1 + d_2 [S] + d_3 [S]^2 + d_4 [DNA] + d_5 [DNA]^2 + d_6 [S][DNA] + d_7 [DNA]^2 + d_8 [S][DNA]^2 + d_9 [S]^2[DNA] + d_{10} [S][DNA]^3 + d_{11} [S]^2[DNA]^2 \]

**Coefficients**

\[ n_1 = n_S = \]

\[ \begin{align*}
  k_1 ( \ k_2 & \ k_3 \ k_4 \ k_5 \ k_6 \ k_7 \ k_8 \ k_9 \ k_10 \ k_11 \ k_12 \ k_13 \ k_14 \ k_15 \ k_16 \ k_17 \ k_18 \ k_19 \ k_20 \ k_21 \ k_22 \ k_23 \ k_24 \ k_25 \ k_26 \ k_27 \ k_28 \ k_29 \ k_30 \ k_31 \ k_32 \ k_33 \ k_34 \ k_35 \ k_36 \ k_37 \ k_38 \ k_39 \ k_40 \ k_41 \ k_42 \ k_43 \ k_44 \ k_45 \ k_46 \ k_47 \ k_48 \ k_49 \ k_50 \ k_51 \ k_52 \ k_53 \ k_54 \ k_55 \ k_56 \ k_57 \ k_58 \ k_59 \ k_60 \ k_61 \ k_62 \ k_63 \ k_64 \ k_65 \ k_66 \ k_67 \ k_68 \ k_69 \ k_70 \ k_71 \ k_72 \ k_73 \ k_74 \ k_75 \ k_76 \ k_77 \ k_78 \ k_79 \ k_80 \ k_81 \ k_82 \ k_83 \ k_84 \ k_85 \ k_86 \ k_87 \ k_88 \ k_89 \ k_90 \ k_91 \ k_92 \ k_93 \ k_94 \ k_95 \ k_96 \ k_97 \ k_98 \ k_99 \ k_{100} \ ) 
\end{align*} \]

\[ n_2 = n_{SS} = \\
  k_1 \ k_2 \ k_3 \ k_4 \ ( \ k_5 \ k_6 \ k_7 \ k_8 \ k_9 \ ) 
\]

\[ n_3 = n_{SDNA} = \\
  k_1 \ k_2 \ k_3 \ k_4 \ k_5 \ k_6 \ k_7 \ k_8 \ k_9 \ k_10 \ k_11 \ k_12 \ k_13 \ k_14 \ k_15 \ k_16 \ k_17 \ k_18 \ k_19 \ k_20 \ k_21 \ k_22 \ k_23 \ k_24 \ k_25 \ k_26 \ k_27 \ k_28 \ k_29 \ k_30 \ k_31 \ k_32 \ k_33 \ k_34 \ k_35 \ k_36 \ k_37 \ k_38 \ k_39 \ k_40 \ k_41 \ k_42 \ k_43 \ k_44 \ k_45 \ k_46 \ k_47 \ k_48 \ k_49 \ k_50 \ k_51 \ k_52 \ k_53 \ k_54 \ k_55 \ k_56 \ k_57 \ k_58 \ k_59 \ k_60 \ k_61 \ k_62 \ k_63 \ k_64 \ k_65 \ k_66 \ k_67 \ k_68 \ k_69 \ k_70 \ k_71 \ k_72 \ k_73 \ k_74 \ k_75 \ k_76 \ k_77 \ k_78 \ k_79 \ k_80 \ k_81 \ k_82 \ k_83 \ k_84 \ k_85 \ k_86 \ k_87 \ k_88 \ k_89 \ k_90 \ k_91 \ k_92 \ k_93 \ k_94 \ k_95 \ k_96 \ k_97 \ k_98 \ k_99 \ k_{100} \ ) 
\]

\[ n_4 = n_{SDNADNA} = \\
  k_1 \ k_2 \ k_3 \ k_4 \ k_5 \ k_6 \ k_7 \ k_8 \ k_9 \ k_{10} \ k_{11} \ k_{12} \ k_{13} \ k_{14} \ k_{15} \ k_{16} \ k_{17} \ k_{18} \ k_{19} \ k_{20} \ k_{21} \ k_{22} \ k_{23} \ k_{24} \ k_{25} \ k_{26} \ k_{27} \ k_{28} \ k_{29} \ k_{30} \ k_{31} \ k_{32} \ k_{33} \ k_{34} \ k_{35} \ k_{36} \ k_{37} \ k_{38} \ k_{39} \ k_{40} \ k_{41} \ k_{42} \ k_{43} \ k_{44} \ k_{45} \ k_{46} \ k_{47} \ k_{48} \ k_{49} \ k_{50} \ k_{51} \ k_{52} \ k_{53} \ k_{54} \ k_{55} \ k_{56} \ k_{57} \ k_{58} \ k_{59} \ k_{60} \ k_{61} \ k_{62} \ k_{63} \ k_{64} \ k_{65} \ k_{66} \ k_{67} \ k_{68} \ k_{69} \ k_{70} \ k_{71} \ k_{72} \ k_{73} \ k_{74} \ k_{75} \ k_{76} \ k_{77} \ k_{78} \ k_{79} \ k_{80} \ k_{81} \ k_{82} \ k_{83} \ k_{84} \ k_{85} \ k_{86} \ k_{87} \ k_{88} \ k_{89} \ k_{90} \ k_{91} \ k_{92} \ k_{93} \ k_{94} \ k_{95} \ k_{96} \ k_{97} \ k_{98} \ k_{99} \ k_{100} \ ) 
\]
concentrations, reaching a max value $V_{\text{max}}$ of about 0.08 µM min$^{-1}$
In the velocity curves should be visible independently of their relative concentrations, which is of

$k_{3} k_{7}$
$k_{d} k_{6} k_{8} k_{9}$
$k_{n} n_{d}$

$k_{4} k_{5} k_{6} k_{7} k_{8} k_{9}$

Interpretation of the kinetic simulations data

Our kinetic simulations show that, independently of the $K_{D}$, that is, of the fraction of E that is

$k_{6} k_{7}$
$k_{8} k_{9}$

$d_{1} = d$

$k_{3} k_{7}$
$k_{d} k_{6} k_{8} k_{9} + k_{1} k_{6} k_{8} + k_{1} k_{4} k_{9} + k_{1} k_{4} k_{6} + k_{1} k_{5} k_{9} + k_{1} k_{5} k_{6} + k_{2}

$d_{2} = d_{s} =

$k_{1} k_{8} k_{9} + k_{1} k_{8} k_{6} + k_{1} k_{8} k_{5} + k_{2} k_{1} k_{8} k_{9} + k_{2} k_{1} k_{8} k_{6} + k_{2} k_{1} k_{8} k_{5} + k_{2} k_{1} k_{8} k_{4}

$d_{4} = d_{DNA} =

$k_{1} k_{6} k_{7} k_{8} k_{9} + k_{1} k_{6} k_{4} k_{6} k_{7} k_{9} + k_{1} k_{6} k_{5} k_{6} k_{7} k_{9} + k_{2} k_{6} k_{7} k_{8} k_{9} + k_{2} k_{6} k_{4} k_{6} k_{7} k_{9}$

$d_{5} = d_{DNADNA} =

$k_{4} k_{6} k_{5} k_{7} k_{8} k_{9} + k_{1} k_{6} k_{7} k_{8} k_{9} + k_{1} k_{6} k_{4} k_{6} k_{7} k_{9} + k_{2} k_{6} k_{6} k_{7} k_{9} + k_{2} k_{6} k_{4} k_{6} k_{7} k_{9}$

$d_{6} = d_{SDNA} =

$k_{1} k_{6} k_{5} k_{6} k_{7} - 8 + k_{1} k_{6} k_{5} k_{7} k_{9} + k_{1} k_{6} k_{4} k_{6} k_{7} k_{9} + k_{1} k_{6} k_{5} k_{6} k_{7} k_{9}

$d_{7} = d_{DNADNA} =

$k_{6} k_{7} k_{8} k_{9} (k_{4} + k_{5})$

$d_{8} = d_{DNA} =

$k_{2} k_{6} k_{7} k_{8} k_{9} + k_{1} k_{6} k_{6} k_{7} k_{9} + k_{1} k_{4} k_{6} k_{7} k_{9} + k_{1} k_{5} k_{6} k_{7} k_{9} + k_{1} k_{4} k_{7} k_{6} k_{7} k_{9} + k_{1} k_{4} k_{6} k_{7} k_{9} + k_{1} k_{4} k_{6} k_{7} k_{9} + k_{1} k_{4} k_{6} k_{7} k_{9}$

$d_{9} = d_{SDDNA} =

$k_{1} k_{6} (k_{6} k_{8} k_{9} + k_{5} k_{8} k_{9} k_{2} k_{6} k_{6} k_{9} + k_{3} k_{8} k_{9} + k_{3} k_{6} k_{8} k_{9} + k_{2} k_{5} k_{8} k_{9} + k_{1} k_{5} k_{8} k_{9} + k_{1} k_{5} k_{8} k_{9} + k_{1} k_{5} k_{7} k_{8} k_{9} + k_{1} k_{5} k_{7} k_{8} k_{9} + k_{1} k_{5} k_{7} k_{8} k_{9}$

$d_{10} = d_{SDDNA} =

$k_{4} k_{7} k_{8} k_{9} (k_{6} + k_{5})$

$d_{11} = d_{SDDNA} =

$k_{1} k_{4} k_{5} k_{6} k_{9} (k_{6} + k_{5})$

Interpretation of the kinetic simulations data

Our kinetic simulations show that, independently of the $K_{D}$, that is, of the fraction of E that is bound to DNA, but as long as both enzyme species have the same rate coefficients ($k_{4}$ to $k_{6} = k_{1}$ to $k_{3}$; simulations #1, #4, #7 and #10), the reaction rate does not change at all substrate concentrations, reaching a max value $V_{\text{max}}$ of about 0.08 µM min$^{-1}$ in all cases. This means that, if the two species have the same kinetic performance with the same substrate, no difference in the velocity curves should be visible independently of their relative concentrations, which is of course intuitive. Thus, our experimental results are the consequence of (at least two) enzyme species characterized by different rate coefficients. Let’s now consider the situation in which the $K_{D} = 1$ nM. As described above, this means that in the conditions used in our enzymatic
assays, both the free and the DNA-bound species will be present at the equilibrium in ca. a 3:2 ratio ([E] = 64%, [EDNA] = 36%). Our simulations indicate that, with a $K_D = 1$ nM, the rate of the reaction is enormously affected by the rate coefficients of the single pathways. Specifically, a 50-fold factor increase in $V_{\text{max}}$ (from 0.08 µM min$^{-1}$ in sim. #1 to 4 µM min$^{-1}$ in sim. #2) is obtained by using rate coefficients for the $E_{\text{DNA}}$ pathway that are 100-fold higher than their counterparts in the free-enzyme pathway. The same ratio of rate coefficients, however, in favor of the free enzyme pathway does not lead to the same enhancement of reaction rate and instead results in a slight decrease of $V_{\text{max}}$ (0.04 µM min$^{-1}$ in sim. #3).

Basically, the reaction rate is largely dominated by the faster enzyme, even if this is present in a lower amount. This effect can be easily understood considering that the linkage described in our simulations is of kinetic (and not thermodynamic) nature and indicates the rate of the reaction at the steady-state regime, i.e. at the very beginning of the enzymatic reaction. Thus, if the DNA pathway is faster than the free-enzyme pathway, the initial rate will greatly increase; on the contrary, if the $E_{\text{DNA}}$ is slower than the free-enzyme, its impact on the initial rate of the reaction will be minimal. Of course, the fraction of enzyme species that catalyzes the faster route will also affect the steady-state rate of the reaction. Indeed, the contribution given by the $E_{\text{DNA}}$ pathway is lower for a higher $K_D = 10$ nM (10-fold increase in $V_{\text{max}}$ sim. #5 and almost no change in sim. #6) and becomes instead hugely predominant in a system characterized by a much lower $K_D = 0.1$ nM (100-fold increase in $V_{\text{max}}$ sim. #8 and 10-fold decrease in $V_{\text{max}}$ sim. #9). Finally, sim. #10 to #12 indicate the scenario occurring when $K_D = 1$ µM. In this case, and considering our initial concentrations of enzyme and DNA, the pseudo-equilibrium concentration of the $E_{\text{DNA}}$ species will be negligible and the kinetics will be of course solely defined by the free enzyme species.

To conclude, the different kinetic behavior observed between the substrates might be reasonably due to allosteric effects and can be simulated by choosing suitable rate coefficients for the hydrolysis of the S(0)/S(-1) and S(+1) substrates by the E species (fig. S41A and B). The presence of DNA creates an alternative kinetic route that competes with the free enzyme pathway. Assuming that the DNA improves the performance of the enzyme (for some reason), a higher flux into this path will be beneficial for the reaction, in all cases. Thus, all substrates will perform better in presence of DNA, leading to an increase of the steady-state rate of the reaction at almost all substrate concentrations. Such an increase is however different for the three substrates, with S(+1) being far a better substrate than S(0) and S(-1) in presence of DNA. This can be accounted for by a different enhancement of the rate coefficients of the $E_{\text{DNA}}$ pathway when compared to their corresponding values for the E path. We simulated a set of rate coefficients, such that the $E_{\text{DNA}}$ species performs 3-fold better than the E form when reacting with S(0) and S(-1) and 10-fold better than the E form when reacting with S(+1). In this sense, DNA amplifies the kinetic differences between the substrates, resulting even in the inversion of their relative performances.

Of course, our simulations are not intended to provide an estimation of the rate coefficients of the kinetic linkage but have been used only as a learning tool to test the impact of DNA and DNA-related phenomena on the kinetics of the entire system.
Figure S1. Schematic model of the TBAs within the DNA origami cavity. (A) The TBA1 and TBA2 aptamers are anchored at the opposite sides of the DNA origami void through a 16 bp duplex. This is formed by the elongation of the aptamer sequence with a 16 bases long single-stranded stretch (light and dark blue sequences) which is complementary to a handle extending from the DNA surface (yellow strand). Considering a helical pitch of 0.34 nm/bp and an approximate size of ca. 2 nm height for the compact G-quadruplex conformation, the two aptamers are spaced about 5 nm apart, leaving enough room for one single thrombin molecule. (B) Molecular models of the two aptamers and corresponding sequences, indicated according to their direction within the DNA construct. The TBA1 and TBA2 structures are taken from previously reported crystallographic data (PDB codes are, respectively, 4DIH and 4I7Y).
Figure S2. AFM characterization of thrombin binding to the DNA-origami rectangular frame. (A) The DNA origami frames, modified with one (TBA1 or TBA2) or both aptamers (TBA1/2) were loaded with 5 equimolar excess of thrombin for 1 h at room temperature, purified by PEG precipitation (resuspension overnight at room temperature) and immediately analyzed by AFM. A control sample (no TBA) lacking both aptamers in the central cavity was used as reference. Close inspection of the AFM images and manual counting allowed to determine the population of binding events ($p$) for every construct (indicated as percentage values in the AFM images). Scale bars are 100 nm. (B) Assuming that the binding affinity of each aptamer is independent on the presence of the vicinal aptamer, the totality of binding events (44%; $n = 2859$) in the doubly-modified design (TBA1/2) is distributed among four possible states as follows: 5% ($n = 1988$) in the TBA1+/2- state, 20% in the TBA1-/2+ state ($n = 2039$) and the remaining 19% is related to the simultaneous binding of both aptamers (TBA1+/2+), leaving 56% of unbound structures (TBA1-/2-). (C) The number of doubly-loaded chambers (i.e. bearing two thrombin molecules in their inner cavity), although clearly visible by AFM, was almost negligible (ca. 1.5%). Scale bars are 100 nm. More discussion in Suppl. Text 4.
Figure S3. TEM characterization of thrombin binding to the DNA-origami box structure. The DNA origami boxes, modified with both aptamers were loaded with 5 equimolar excess of thrombin for 1 h at room temperature, purified by PEG precipitation (resuspension overnight at room temperature) and immediately analyzed by TEM. A control sample (A) without the addition of thrombin was used as reference. Enlarged views of selected structures are shown in the right-side panels. (B) DNA origami structures bearing both TBAs clearly show the presence of thrombin at the expected position (see enlarged insets on the right-side hand). However, the small size of the protein and presumably the manipulation of the sample before and during imaging make difficult to estimate the amount of protein bound within the DNA cages by observation of the raw TEM micrographs. Scale bars are 100 nm.
Figure S4. Native PAGE analysis of the TBA motifs. The aptamer-bearing sequences (in light and dark blue, respectively) were labelled with a donor-acceptor pair (FAM/TAMRA) positioned directly up- and downstream the G4-motifs (TBA1 and TBA2, in lanes 1 and 2, respectively). Comparison of the fluorescein emission intensities of the doubly-labelled constructs with the donor-only modified analogs (lanes 3 and 4, respectively) revealed a strong decrease in the fluorescence signal, associated to a high FRET efficiency and thus indicative of correct folding of the G4 structures in the experimental conditions used. Full hybridization of the TBA-sequences with complementary strands (violet and orange strands) and formation of extended duplexes also led to decrease in the fluorescence emission intensity (lanes 5 and 6), although to a smaller extent. Gel running conditions: 25% acrylamide in TBEmg 1x buffer at 80 V, for 2h at 4°C. The gel was scanned with a Typhoon FLA 9000 at different wavelengths to monitor the presence of fluorescein (green) and TAMRA (orange) modified oligonucleotides and finally stained in ethidium bromide. Lane L contains a 10 bp ladder.
Figure S5. AGE analysis of the DNA-origami tethered TBA motifs. Aptamers (TBA1 and TBA2, in light and dark blue respectively) were embedded into the cavity of the DNA origami frame (rect) through hybridization to complementary handles (yellow) extending from the origami walls. Successful formation of the G4 motifs and their integration within the DNA nanostructure was proven by two-colors gel electrophoretic analysis. As in fig. S4, FAM-TAMRA and FAM-only labelled constructs were used to monitor correct folding of the aptamers in the experimental conditions used (lanes 1 and 2 for TBA1, 4 and 5 for TBA2 and 7, 8 for TBA1/2). Co-migration of fluorescently labelled bands with DNA origami bands (last panel, ethidium bromide staining) confirmed full integration of the aptamers into the DNA nanostructures. Aptamer sequences, appended with short toehold segments, were efficiently displaced from the origami structures upon addition of fully complementary strands (violet and orange strands), visible from the disappearance of both the FAM and TAMRA fluorescence signals (lanes 3, 6 and 9). Furthermore, the hybridization specificity of the TBA sequences to the extended handles was successfully shown in lane 10. Gel running parameters: 0.75% agarose in TBEMg 1x at 80 V, for 2.5 h at 4°C. Lane L contains a 1 kbp DNA ladder. DNA origami samples migrate between 1.5 kbp and 2.0 kbp.
**Figure S6. Thrombin fluorescent labeling.** 150 µL of thrombin stock solution (≈ 8.7 µM) were mixed with 1 µL NHS-A488 dye (ThermoFisher) in thrombin storage buffer solution. The samples were incubated at room temperature for 1h, protected from light irradiation and the excess of dye was removed using dye-removal columns (ThermoFisher) according to the manufacturer instructions. 2 µL sample were mixed with 2 µL of SDS loading buffer (containing DTT) and 6 µL thrombin storage buffer and heated for 10 min @ 70°C. 10 µL of sample was loaded on the gel at different concentrations (lanes 1 to 3). Lane 4 contains the unlabeled thrombin as reference. No change in band patterns is observable between the unlabeled and labelled protein, suggesting that no significant autodigestion took place during the labelling procedure. Thrombin was successfully labelled, as visible by the single band appearing upon gel illumination in the FAM channel. This band at ca. 32 kDa corresponds to the B chain fragment of alpha-human thrombin. The A chain fragment (MW ca. 6 kDa) is presumably too short to be retained in the gel at these conditions. Gel and running conditions: 12 % SDS PAGE with 4.5% stacking gel, 60 min at 200 V, ice cooled in 1x SDS running buffer. Lane M contains a protein marker (BioRad #161-0363).
Figure S7. AGE analysis of DNA origami structures loaded with thrombin. 20 uL of DNA origami cages, either with or without inner TBA modifications, were treated with 2.2 uL of A488-modified thrombin for 2 hours at room temperature. Co-migration of fluorescently-labelled thrombin bands with DNA origami bands bearing both aptamers (lanes 3 and 6) indicates successful loading of the enzyme into the DNA cages. No fluorescent signal appeared when treating rect and box origami structures (lacking the aptamers) with thrombin, indicating that the binding of the enzyme into the DNA origami cages is specifically associated to the presence of the TBAs (lanes 2 and 5). Lanes 1 and 4 contain, respectively, the p7249 and p8064 scaffold used for assembly of the two DNA origami structures. Gel running parameters: 1% agarose in TBEMg 1x at 80 V, for 2 h at 4°C. Lane L contains a 1 kbp DNA ladder. DNA origami samples migrate between 1.5 kbp and 2.0 kbp.
Figure S8. Schematic representation of the design used in the flex and rig configurations. (A) The elongated TBA sequences (TBA1 and TBA2, in light and dark blue respectively) are embedded into the cavity of the DNA origami frame through hybridization to complementary handles (yellow) extending from the origami walls (flex configuration). (B) In the rig configuration, the elongated TBA sequences insert into the DNA origami structure, thus limiting the rotational freedom of the TBA motifs and their relative orientation within the cavity.
Figure S9. Thrombin stability test. 10 µL of thrombin stock solution (≈ 8.7 µM, Cayman Chemical, #13188) were mixed with 2 µL 10x TEMg12.5 and 8 µL of ddH₂O. The samples were incubated at room temperature for the indicated time. The native sample (without DTT, lane 5) shows the expected MW of about 38 kDa. Samples treated with DTT (lanes 1 to 4) show the expected band pattern, with the larger fragment migrating slightly faster than the native protein (MW ca 32 kDa) and the shortest fragment (MW ca. 6 kDa) visible at the very bottom of the gel (indicated by an arrow). No change in band patterns is observable during time, suggesting that no significant autodigestion is taking place. Gel and running conditions: 12 % SDS PAGE with 4.5% stacking gel, 60 min at 200 V, ice cooled in 1x SDS running buffer. Lane M contains a protein marker (BioRad #161-0363). Samples were heated for 5 minutes at 95°C prior to loading.
Figure S10. Thrombin linearity test. (A) 50 µL of substrate S(0) at 5 µM concentration were mixed with 50 µL thrombin at various concentrations (from 0 nM to 5 nM) and the cleavage of the substrate was recorded at 37°C by measuring the fluorescence signal at 527 nm for a period of 80 min. (B) The initial rate of substrate hydrolysis is linear over the range of enzyme concentrations analyzed, ensuring that no protein aggregation or degradation is taking place in the experimental conditions used in our assays.
Figure S11. Standard curve for the substrate S(0). Different concentrations of a FAM-only S(0) substrate in the range between 0 and 25 µM in TEMg 1x were prepared and – upon excitation at 482 nm - the fluorescence emission signal at 527 nm was measured. The FAM fluorescence signal was then plotted against the peptide concentration and fitted with a non-linear function. The fit was used to convert relative fluorescence units (RFU) into product concentration for quantification of the enzymatic activity assays.
Figure S12. Exemplary progress curves for S(0). The reaction mixture contained the thrombin enzyme at 1.2 nM concentration (thrombin-only; A), the substrate S(0) at 0 to 25 µM concentration and a third component at 1 nM concentration. This latter was either one (TBA1 or TBA2; B and C) or both aptamers (TBA1/2; D), present in solution as freely diffusing species. Control samples were prepared to test the effect of the ionic strength (phosphate buffer at 14.5 µM; E), or the occurrence of unspecific binding to DNA, either in form of short single-stranded DNA of random sequence (ssDNA = 2 nM; F) or aptamer-free DNA origami (rect or box = 1 nM; G and H, respectively). An equimolar amount of DNA-scaffolded aptamers was used to evaluate the effect of specific scaffolding through flexible linkers (rect1 flex, rect2 flex and rect1/2 flex; I to K), the impact of structure integrity (rect1/2 melt; L) or rigidity (rect1/2 rig; M) on the kinetics of the reaction. Finally, the role of DNA origami shape on the kinetics of proteolysis was tested with a 3D box (open at one side) (box1/2 rig; N). The experiments were performed in TEMg 1x buffer. Here, the substrate used was FAM-GGfPR|SGGGK(BHQ1)KG-OH (calculated net charge of S(0) at pH 7 is 0). Insets show a zoomed-in image of the early linear phase. Enlarged version of this figure is given below for convenience.
Figure S13. **Standard curve for the substrate S(-1).** Different concentrations of a FAM-only S(-1) substrate in the range between 0 and 25 µM in TEMg 1x were prepared and – upon excitation at 482 nm - the fluorescence emission signal at 527 nm was measured. The FAM fluorescence signal was then plotted against the peptide concentration and fitted with a non-linear function. The fit was used to convert relative fluorescence units (RFU) into product concentration for quantification of the enzymatic activity assays.
Figure S14. Exemplary progress curves for S(-1). Same legend as in fig. S8. Here, the substrate used was FAM-GGfPR|SGGGK(BHQ1)KD-OH (where “|” indicates the position of peptide cleavage). The calculated net charge of the FRET peptide S(-1) at pH 7 is -1. Enlarged version of this figure is given below for convenience.
Figure S15. Standard curve for the substrate S(+1). Different concentrations of a FAM-only S(+1) substrate in the range between 0 and 25 µM in TEMg 1x were prepared and – upon excitation at 482 nm - the fluorescence emission signal at 527 nm was measured. The FAM fluorescence signal was then plotted against the peptide concentration and fitted with a non-linear function. The fit was used to convert relative fluorescence units (RFU) into product concentration for quantification of the enzymatic activity assays.
Figure S16. Exemplary progress curves for S(+1). Same legend as in fig. S8. Here, the substrate used was FAM-GGfPR|SGGGK(BHQ1)KK-OH (where “|” indicates the position of peptide cleavage). The calculated net charge of the FRET peptide S(+1) at pH 7 is +1. Enlarged version of this figure is given below for convenience.
Figure S17. Kinetic profiles (initial rate vs substrate concentration) for S(-1) (A) and S(+1) (B). The constructs used for the kinetic assays are the same employed for substrate S(0), as described in the legend to Figure 2 of the main manuscript.
Figure S18. Background fluorescence signal from substrate-only control. (A) Upon excitation at 482 nm, the fluorescence emission signal at 527 nm of a solution containing exclusively S(+1) in TEMg 1x buffer was measured during time for 80 min (i.e. in absence of thrombin). Despite the spatial proximity of the FAM and BHQ1 fluorophores in the peptide substrate should result in FAM quenching, the emission signal from the FAM fluorophore in the uncleaved peptide is not totally extinguished and slightly increases with peptide concentration, keeping however relatively stable over time. Thus, the relative change of fluorescence intensity during time due to the catalytic hydrolysis of the substrate will not be affected, as well as the determination of the initial rates. (B) The mean value of FAM concentration vs substrate concentration, in absence of peptide cleavage, shows that the background fluorescence of the substrate increases with substrate concentration. This background signal is however typically less than 1% of the FAM intensity recorded during peptide hydrolysis and can be therefore neglected, keeping the validity of the standard curve unaltered.
Figure S19. Specificity constants \((k_{\text{cat}}/K_M)\) for the thrombin-catalyzed hydrolysis of the three substrates in different conditions. The specificity constants of substrate S(-1) and S(0) are relatively similar and follow the same increase upon perturbation of the system with DNA-scaffolds (A and B). The specificity constant of substrate S(+1) instead is almost unaffected by the surrounding DNA environment and assumes a large value in all constructs (C).
Figure S20. Dissociation constants ($K_i$) of the inhibited enzyme/substrate complex. Inhibition by excess substrate was observed for all substrates, S(-1), S(0) and S(+1) (A-C) when hydrolyzed by DNA-scaffolded thrombin. Only substrate S(+1) showed slight inhibition in the other enzyme constructs (C) and minimal values of $K_i$ were reported for this substrate in box-like cavities.
Figure S21. Transition-state analysis of the thrombin-catalyzed hydrolysis of S(-1) and S(+1) with respect to S(0). The transition-state theory for a simple MM mechanism was applied to determine the changes in the energy levels of the enzyme-substrate complex (ES) and its transition state (ES‡) with respect to a reference reaction (here, the thrombin/S(0), black box). Arbitrary levels were chosen for the reference reaction, with ES occupying a higher (A) or lower (B) energy level, when [S] < K_M or [S] > K_M, respectively. The variation in the free energy change between the unreacted E + S species and the ES complex (ΔΔG_ES), the change in activation energy that separates the ES complex from ES‡ (ΔΔG^‡), and the change in activation energy that separates E + S from ES‡ (ΔΔG^‡TS) were calculated respectively from the K_M, k_cat and k_cat/K_M values of the thr/S(-1) or thr/S(+1) reaction with respect to the thr/S(0) reference, as described in the Suppl. Text 3. The energy level of the E + S species is assumed to be identical in all cases, as only energy changes rather than absolute values are relevant. Numerical values of the calculated energy barriers are reported in the table, with positive and negative values indicating, respectively, an increase or a decrease in the energy barrier considered.
Figure S22. Transition-state analysis of the box$^{1/2}_{\text{rig}}$-catalyzed hydrolysis of S(-1) and S(+1) with respect to S(0). The transition-state theory for a simple MM mechanism was applied to determine the changes in the energy levels of the enzyme-substrate complex (ES) and its transition state (ES$^\dagger$) with respect to a reference reaction (the box$^{1/2}_{\text{rig}}$/S(0), black box). Two regimes of substrate concentrations were considered: $[S] < K_M$ (A) and $[S] > K_M$ (B) and the same procedure described in the legend to fig. S21 was applied. The energy level of the E + S species is assumed to be identical in all cases, as only energy changes rather than absolute values are relevant. Numerical values of the calculated energy barriers are reported in the table, with positive and negative values indicating, respectively, an increase or a decrease in the energy barrier considered. Please note that here the reference reaction is box$^{1/2}_{\text{rig}}$/S(0), in order to focus the attention on the effect of substrate net charge in presence of DNA.
Figure S23. Structure of thrombin interacting with the TBA1 aptamer at the exosite I. The crystal structure of thrombin with the TBA1 aptamer bound to exosite I is shown (36). The protein is colored in yellow, Arg 73 and Arg 75 are in red. Lys 60F and Arg 35 at the vicinity of the exosite I are colored in light blue. The aptamer is shown in CPK representation. The residues involved in the interactions with the two most populated clusters for peptide S(-1) and in the most populated cluster for peptide S(0) are not involved in the interaction with the aptamer (fig. S25 and S26). The only residue from the ones mentioned above that is compromised in the interaction with the aptamer is residue Arg 75, which is interacting with S(0) in the second most populated cluster as shown in fig. S26. Therefore, since most of the main interactions of the peptides with the region involving exosite I and its vicinity are not inhibited by the presence of the aptamer, we believe that the peptides can still interact with that region in the scenario where the aptamers are used. Substrate S(+1) instead does not interact with the exosite I of thrombin and is therefore not expected to hinder aptamer interaction at this region of the protein (fig. S24). We note that, for clarity in the visualization of the DNA-origami model based on the position of its particles along the z-axis, residues that were outside the box after wrapping are not shown. Similarly, coarse-grained water and ion particles are not displayed.
Figure S24. MD simulations of the S(+1) peptide/thrombin complex. (A-C) Representative structures extracted from the three main clusters observed during the GaMD simulations. The relative populations of the most abundant clusters are shown. The protein is colored in yellow, the peptide, except the terminal amino acid, is colored in gray and rendered as transparent. The positively charged amino acids at the exosite I are colored in red. The terminal Lys of the peptide is highlighted and labelled. (D) S(+1) establishes conserved interactions with the exosite I through one of the fluorophores, HBQ1. This interaction is probably driven by the combination of the electrostatic attraction between the positive region at the exosite I and the partial negative charges at the nitro group in the fluorophore as well as hydrophobic interactions between the aromatic rings of HBQ1 and the hydrophobic residues of the exosite I. The close proximity of the nitro group on the HBQ1 fluorophore to Lys 149E at the exosite I (2.8 Å) indicates electrostatic interactions. Additionally, the hydrophobic interaction is facilitated by Pro 152 near the phenyl rings of the HBQ1 fluorophore. The values shown for the distances correspond to the most populated cluster observed during the GaMD (E). The HBQ1 fluorophore is depicted in gray, while the rest of the peptide is rendered as gray and transparent, the protein is shown in yellow (transparent in panel D). The positively charged residues of the exosite I are shown in red. Hydrogen atoms of Pro 152 and HBQ1 are omitted for simplicity.
Figure S25. MD simulations of the S(-1) peptide/thrombin complex. (A-B) The relative populations of the most abundant clusters are shown. The protein is colored in yellow, the peptide, except for the terminal amino acid, is shown in transparent gray. The positively charged amino acids at the exosite I are indicated in red. Two residues interacting with the C-terminal Asp are colored in green. These are better visible in the enlarged views below, which depict the interactions occurring in the main (C, D) and second (E, F) most populated clusters. The main interactions established between S(-1) and the exosite I of thrombin are of electrostatic nature and involve the negatively charged carboxylate group of the C-terminal Asp of S(-1) and the positively charged side chains of Lys 60F and Arg 35, with interaction distances of 2.8 Å and angles of 146° and 156° (D). To a less extent, Arg 73 at the exosite I also interacts with the C-terminal Asp of the peptide (E). The peptide is shown in transparent gray, the protein in yellow (transparent in panels D and E). The positively charged residues of the exosite I are shown in red (transparent in panels D and E).
Figure S26. MD simulations of the S(0) peptide/thrombin complex. (A-B) The relative populations of the most abundant clusters (A and D) are shown. (B) Like S(-1), S(0) interacts with Arg 35; however, at a larger distance (4.9 Å). Similar to S(-1), the neutral peptide also exhibits interactions with other residues of the exosite I, as shown for the second most populated cluster (D) where the C-terminal Gly residue of the peptide interacts with Arg 75 (C). The protein is shown in yellow, the peptide, except for the terminal amino acid, is depicted in transparent gray. The positive amino acids at the exosite I are indicated in red.
Figure S27. Representative TEM imaging of thrombin/S(-1) end-point reactions in presence of rect DNA origami structures. Enzymatic reactions were performed as described in the Methods section (main manuscript) using a solution of rect/thrombin incubated with varying amounts of S(-1) substrate (A-L: 0, 1.25, 2.5, 3.75, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM). After the 80 minutes-long kinetic analysis, a small aliquot of each reaction mixture was analyzed by TEM. The data show that the origami structures preserve their shape at all substrate concentrations and no aggregation occur. Insets show a zoomed-in region of the micrograph. Scale bars are 200 nm.
Figure S28. Representative TEM imaging of thrombin/S(0) end-point reactions in presence of rect DNA origami structures. Enzymatic reactions were performed as described in the Methods section (main manuscript) using a solution of rect/thrombin incubated with varying amounts of S(0) substrate (A-L: 0, 1.25, 2.5, 3.75, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM). After the 80 minutes-long kinetic analysis, a small aliquot of each reaction mixture was analyzed by TEM. The data show that the origami structures preserve their shape at all substrate concentrations and no aggregation occur. Insets show a zoomed-in region of the micrograph. Scale bars are 200 nm.
Figure S29. Representative TEM imaging of thrombin/S(+1) end-point reactions in presence of rect DNA origami structures. Enzymatic reactions were performed as described in the Methods section (main manuscript) using a solution of rect/thrombin incubated with varying amounts of S(+1) substrate (A-L: 0, 1.25, 2.5, 3.75, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM). After the 80 minutes-long kinetic analysis, a small aliquot of each reaction mixture was analyzed by TEM. The data show that the origami/enzyme structures start to aggregate above 10 µM substrate concentration. Insets show a zoomed-in region of the micrograph. Scale bars are 200 nm.
Figure S30. AGE analysis of rect DNA origami structures/substrate interactions. 20 µL of DNA origami rect cages (lacking the inner TBAs) were treated with different concentrations of FAM-labelled substrates, ranging from 0 to 25 µM, for 1 hour at 37°C (from A to C and D, respectively S(-1), S(0) and S(+1)). The pH of the reaction mixture was adjusted to 7.6 (A to C) or 10 (for S(+1) only, D), to test the effect of substrate net charge on the unspecific electrostatic interaction with the negatively charged DNA origami. The substrates migrate quite slowly and remain nearby the loading pockets of the gel. The direction of substrate migration is dependent on the net charge of the substrate, with S(-1) migrating towards the positive pole (as DNA), while S(0) and S(+1) mostly move towards the opposite direction. Unspecific electrostatic interactions between the DNA origami and the substrates can be inferred by the intensity of the DNA bands (after EtBr staining), which is approximatively constant at all substrate concentrations in presence of S(-1) and S(0) (A and B) and visibly decreases when in presence of S(+1) (C). This effect is accompanied by the appearance of aggregates in the gel pockets, especially at high substrate concentrations (C). Unspecific interactions with S(+1) almost completely disappear at pH 10 (D), when the net charge of S(+1) should be lowered to almost a neutral value. Gel running parameters: 1% agarose in TBEMg 1x at 80 V, for 1 h at 4°C.
Figure S31. Representative TEM imaging of thrombin/S(+1) at different time points in presence of rect DNA origami structures. Enzymatic reactions were performed as described above using a solution of rect/thrombin incubated with 15 µM of substrate S(+1) at different time points (A-I: 0 min [before addition of substrate], 0 min [after addition of substrate], 2 min, 5 min, 10 min, 20 min, 40 min, 60 min and 80 min). The data show that the origami/enzyme structures preserve their structure until 40 min (F), thus ensuring that within this time period, the reaction rates observed are not affected by aggregation events and that the kinetic parameters obtained from the initial linear phase are still reliable. Insets show a zoomed-in region of the micrograph. Scale bars are 200 nm.
Figure S32. Progress curves for S(+1) in presence of DNA origami rect. A particular aspect of the product conc. vs. time curves in presence of DNA origami structures and S(+1) is their sigmoidal shape for substrate concentrations above a certain minimal threshold value (A, top panel). Calculation of the first derivative of such progress curves enables to appreciate how the reaction rate changes over time for different substrate concentrations (A, bottom panel). Within the initial phase of the reaction (about 10 min), the rate of substrate hydrolysis increases with substrate concentration in a linear fashion (inset in A). Although this may justify the application of the Michaelis-Menten treatment in the initial phase of the reaction (the one used indeed for extrapolation of the kinetic parameters of the reaction), the aspect of the progress curves in the second phase of the reaction and for substrate concentrations above ca. 3 µM is indicative of a strong deviation from a standard behavior (yellow shaded region in B). Here, maximal rates become much larger than the rates observed at the beginning of the reaction (grey shaded area in B) and this discrepancy appears to be dependent on substrate concentration with a peak value at about 10 µM to 15 µM (0.24 µM min⁻¹ against ca. 0.11 µM min⁻¹). Maximal rates are also reached proportionally later in time for higher substrate concentrations (A, bottom panel). Enzymatic assay conditions: 1.2 nM thrombin, S(+1) from 0 to 25 µM, 1nM DNA origami without aptamers (rect) in TEMg 1x at 37°C. The data shown are the average result of three replicates.
Figure S33. Progress curves for S(+1) in presence of DNA origami rect1/2rig. Hysteresis observed for S(+1) in presence of rect1/2rig. Similar conclusions as in fig. S32. Enzymatic assay conditions: 1.2 nM thrombin, S(+1) from 0 to 25 µM, 1nM DNA origami with aptamers (rect1/2rig) in TEMg 1x at 37°C. The data shown are the average result of three replicates.
Effect of DNA origami concentration on the shape of the progress curves for S(+1). Effect of DNA origami concentration (from 0 to 10 nM) on the velocity graphs for a sample containing 1.2 nM thrombin and 10 µM substrate S(+1) in TEMg 1x at 37°C (A, top panel). The results show that even a minimal fraction of DNA origami drastically affects the reaction rate (A, bottom panel). Initial rates grow with the DNA concentration until a maximal value of about 0.15 µM min⁻¹ at 2.0 nM DNA (blue full circles in B). This suggests that – although the DNA origami definitely increases the reaction rate – this effect is not linear. As observed for the substrate S(+1) at constant DNA origami concentration (fig. S32-S33), also here a DNA origami threshold exists (0.3 nM) above which the time-response of the enzyme is not linear, resulting in the initial rate being lower than the maximal reaction rate (blue empty circles in B). Quite interestingly, the rapidity of this rate change is higher for lower DNA origami concentrations and levels down at higher DNA origami concentrations, resulting in a progressive transformation of the velocity profiles from a hyperbolic (0 – 0.3 nM) to a sigmoidal shape (0.5 – 5.0 nM) and then back to a canonical exponential growth shape (10 nM). Also, the time at which this rate change occurs (max of the first derivative in A, bottom panel) increases with increasing DNA concentration. The full view is therefore quite complex and underlines the presence of DNA-dependent counteracting effects that enter in play at different concentration regimes.
**Figure S35. Effect of pH on the thrombin-catalyzed cleavage of S(+1).** Enzymatic reactions were performed as described in the Methods section (main manuscript) using a solution of thrombin and 15 µM substrate S(+1) at different pH values, between 5 and 7.6 (green bars in A). For comparison, we report the value obtained for the same reaction in presence of 1 nM DNA origami in the standard buffer used in all our assays (rect, orange bar in A). The DNA origami clearly accelerates the enzymatic reaction for solutions that are initially at the same pH value (cfr. green and orange bars at a nominal pH 7.6). (B) The enzymatic cleavage of S(+1) was analyzed at higher pH values to test whether a change in the net charge of the substrate, specifically a less positive one, would result in a kinetic signature similar to that exhibited by substrate S(0). Two enzymatic assays were performed employing either a free thrombin (B) or the same amount of enzyme previously incubated with a TBA-modified DNA origami (rect1/2rig, C). The results show that increasing the pH of the solution, substrate S(+1) approaches the kinetic features shown by S(0), confirming the hypothesis that the net charge of the peptide plays a crucial role in the reaction mechanism. Enzymatic assays at higher pH values could not be performed due to the occurrence of salt precipitates.
Figure S36. Effect of Mg ions concentration on the initial rate of thrombin-catalyzed hydrolysis.
The enzymatic activity assays were performed as described in the Materials and Methods (main
manuscript). Solutions contained thrombin, either in absence (black bars) or preincubated with rect^{1/2}\text{rig} (brown bars) and different magnesium ions concentrations (between 7 and 50 mM). Upon addition of
10 μM substrate (from A to C; S(-1), S(0) and S(+1)), the initial rates at various Mg concentrations were
recorded and plotted. Here again the general charge-dependent trend in both variables, the substrate and
the DNA, is visible. First of all, S(-1) and S(0) show similar profiles. Moreover, from S(-1) to S(+1) the
initial rates in thrombin-only samples decrease, confirming that the charge of the peptide is critical in
defining the rate of the reaction and that a positive C-terminus is negatively affecting the enzyme
performance. This trend is inverted in presence of DNA origami. S(-1) and S(0) exhibit a slow
hydrolysis, while the S(+1) becomes the fastest. This trend is maintained along the entire range of Mg
buffer screened; however, it decreases for increasing Mg concentration and becomes almost not relevant
at all at 50 mM magnesium ions. We presume that at such high levels of magnesium concentration, the
DNA origami structures may aggregate, cancelling their positive effect.
Figure S37. Thrombin/TBA binding assays. Thrombin binding to TBA1 (A), TBA2 (C) or both (E, G) was measured with the switchSENSE technology. All experiments were performed on a heliX® instrument (Dynamic Biosensors GmbH, Martinsried, DE) with standard Adapter switchSENSE chips (ADP-48-2-0) and using the static measurement mode. The experimental workflow was set-up using the proprietary heliOS software (v 1.3.1). Scrambled sequences (TBAsc1 and TBAsc2) were used as control (B, D, F, H). Experiments were performed by mixing 500 nM aptamers (or control sequences) with 400 nM of the relevant adapter strand harboring a red or a green dye, for 1 h in TE40 (10 mM Tris-HCl, 40 mM NaCl, 0.05% Tween20, 50 μM EDTA, 50 μM EGTA). Pre-mixed solutions were then hybridized to the corresponding anchor strand covalently attached to the surface of the chip. Kinetic measurements were done in TE140 running buffer with a flow rate of 500 μL/min at 25 °C using 100 nM thrombin. The fluorescence traces were analyzed with the heliOS software (v 1.3.1, Dynamic Biosensors GmbH, DE) by fitting association and dissociation curves with a single-exponential fit model (Table S3).
Figure S38. Thrombin/TBA binding assays in presence of excess ssDNA. Thrombin binding to TBA1 (A) and TBA2 (C) was measured with the switchSENSE technology, as described in fig. S37. Scrambled sequences (TBAsc1 and TBAsc2) were used as control (B, D). Kinetic measurements were done in TE140 running buffer with a flow rate of 500 µL/min at 25 °C using 100 nM thrombin in presence of increasing concentrations (from 0 to 1 µM) of a 48mer DNA oligo that is not complementary to any sequence of the assay (5’-ATC AGC GTT CGA TGC TTC CGA CTA ATC AGC TTA CGA CTA -3’). Large excess of ssDNA does not affect the kinetics of thrombin/aptamers binding. The fluorescence traces were analyzed with the heliOS software (v 1.3.1, Dynamic Biosensors GmbH, DE) by fitting association and dissociation curves with a single-exponential fit model (Table S3).
Figure S39. The kinetic linkage scheme of thrombin in presence of a DNA effector ($K_D = 1$ nM). The system under investigation was modeled as a network of reactions consisting of two kinetic cycles: one catalyzed by the free enzyme (E) and the other catalyzed by the corresponding DNA-bound form (EDNA). Each enzyme species can perform a catalytic reaction, according to a serine protease mechanism that accounts for acylation (ES and ES$_{DNA}$) and deacylation (EP$'$ and EP$'_{DNA}$) of the enzyme before liberation of the product (P). The two kinetic cycles are coupled through the association and dissociation of the TBA-modified DNA cage to the enzyme. The values of the rate coefficients for the thrombin-only pathway ($k_1$ to $k_3$) were taken from the literature (53,55) and the rate coefficients of the two pathways were chosen such to result into an apparent MM behavior. The partition between the two kinetic fluxes and the performance of the EDNA pathway have been modeled, respectively, by the $K_D$ of the EDNA complex ($k_7$ to $k_9$) and the velocity of the reaction catalyzed by EDNA ($k_4$ to $k_6$). The steady-state rate of the reaction is largely dominated by the faster reaction, being either the one catalyzed by the EDNA species (sim. #2) or the free E (sim. #3). The fraction of the two species at the equilibrium has a lower impact on the steady-state rate of the reaction. Details of the model and simulations are reported in the Suppl. Text 5.
Figure S40. The kinetic linkage scheme of thrombin in presence of a DNA effector ($K_D = 10$ nM, 0.1 nM and 1 µM). Same legend as in Fig. S39, using different sets of rate coefficients for the association/dissociation of the $E_{DNA}$ complex ($k_7$ to $k_9$) and for the reaction catalyzed by $E_{DNA}$ ($k_4$ to $k_6$). The steady-state rate of the reaction is dominated by the faster enzyme, even if this is not the most abundant species. See Suppl. Text 5 for the details of the simulations.
Figure S41. Kinetic simulations of thrombin-catalyzed hydrolysis of different substrates in absence and presence of DNA. Simulations were run as described in the Suppl. Text 5. Rate coefficients for thrombin-only pathways (A and B) were $k_1 = 6,000 \text{ min}^{-1} \text{ M}^{-1}$; $k_{-1} = 16,000 \text{ min}^{-1}$; $k_2 = 80 \text{ min}^{-1}$ and $k_3 = 400 \text{ min}^{-1}$ for S(0) and, respectively, $5,000 \text{ min}^{-1} \text{ M}^{-1}$, $12,000 \text{ min}^{-1}$; $60 \text{ min}^{-1}$ and $300 \text{ min}^{-1}$ for S(+1). Simulations in presence of DNA (C and D) were done by imposing a $K_D = 1 \text{ nM}$. The steady-state rate of the reaction increases if the $E_{DNA}$ species is faster, even if not the most abundant species. The flux into the DNA channel was enhanced by increasing the rate coefficients of the $E_{DNA}$ pathway with respect to the same rate coefficients of the $E$ pathway (n-fold increase in enzyme rate is indicated by the increasing darkness of the boxes). Specifically, the rate coefficients of the $E_{DNA}$ path increased of a 3-fold factor for substrate S(0), leading to $k_4 = 18,000 \text{ min}^{-1} \text{ M}^{-1}$; $k_{-4} = 48,000 \text{ min}^{-1}$; $k_5 = 240 \text{ min}^{-1}$ and $k_6 = 1200 \text{ min}^{-1}$ and a 10-fold factor for substrate S(+1), leading respectively to $50,000 \text{ min}^{-1} \text{ M}^{-1}$; $k_{-1} = 120,000 \text{ min}^{-1}$; $k_2 = 600 \text{ min}^{-1}$ and $k_3 = 3000 \text{ min}^{-1}$. Please note that these values are only for indicative purposes and are not meant to resemble the true values of rate coefficients examined.
Table S1. Kinetic parameters for the thrombin-catalyzed hydrolysis of substrate S(-1). Nomenclature of the samples as described in the main text. Fit of the initial rate vs. substrate concentration plots was done using OriginPro 2017G using the built-in Michaelis-Menten equation with or without a substrate inhibition process. This led to the extraction of the kinetic parameters of the reaction, namely, the turnover rate ($k_{cat}$), the Michaelis-Menten constant ($K_M$), the specificity constant ($k_{cat}/K_M$) and – if relevant - the dissociation constant of the inhibited enzyme-substrate complex ($K_i$). Errors were calculated by the built-in Levenberg-Marquardt iterations algorithm (or the orthogonal regression distance) provided by the software. All data are the results of at least three replicates.

| Thr/S(-1)     | $k_{cat}$ [min$^{-1}$] | $K_M$ [$\mu$M] | $k_{cat}/K_M$ [min$^{-1}$] [$\mu$M$^{-1}$] | $K_i$ [$\mu$M] |
|---------------|------------------------|----------------|---------------------------------|---------------|
| thrombin only |                        |                |                                 |               |
| -             | 220 ± 14               | 9.5 ± 0.9      | 23 ± 3                          | -             |
| w/o DNA       |                        |                |                                 |               |
| TBA1          | 176 ± 11               | 7.1 ± 0.7      | 25 ± 3                          | -             |
| TBA2          | 204 ± 15               | 9 ± 1          | 22 ± 3                          | -             |
| TBA1/2        | 200 ± 13               | 9.5 ± 0.9      | 21 ± 2                          | -             |
| phosph        | 210 ± 11               | 9.1 ± 0.7      | 23 ± 2                          | -             |
| DNA-unbound   |                        |                |                                 |               |
| ssDNA         | 155 ± 8                | 6.0 ± 0.5      | 26 ± 3                          | -             |
| rect          | 208 ± 37               | 5 ± 1          | 42 ± 15                         | -             |
| box           | 176 ± 11               | 5.2 ± 0.6      | 34 ± 4                          | -             |
| DNA-bound     |                        |                |                                 |               |
| rect$^{1\text{flex}}$ | 150 ± 6               | 2.2 ± 0.2      | 69 ± 8                          | -             |
| rect$^{2\text{flex}}$ | 125 ± 8               | 1.8 ± 0.3      | 70 ± 12                         | 43 ± 9        |
| rect$^{1/2\text{flex}}$ | 142 ± 10              | 1.9 ± 0.3      | 73 ± 14                         | 42 ± 10       |
| rect$^{1/2\text{melt}}$ | 164 ± 11              | 2.1 ± 0.3      | 77 ± 13                         | 44 ± 10       |
| rect$^{1/2\text{rig}}$ | 172 ± 16              | 2.2 ± 0.5      | 77 ± 18                         | 32 ± 8        |
| box$^{1/2\text{rig}}$ | 141 ± 9               | 2.0 ± 0.3      | 70 ± 12                         | 63 ± 17       |
Table S2. Kinetic parameters for the thrombin-catalyzed hydrolysis of substrate \( S(+1) \). Nomenclature of the samples as described in the main text. Fit of the initial rate vs. substrate concentration plots was done using OriginPro 2017G using the built-in Michaelis-Menten equation with or without a substrate inhibition process. This led to the extraction of the kinetic parameters of the reaction, namely, the turnover rate \((k_{\text{cat}})\), the Michaelis-Menten constant \((K_M)\), the specificity constant \((k_{\text{cat}}/K_M)\) and – if relevant - the dissociation constant of the inhibited enzyme-substrate complex \((K_i)\). Errors were calculated by the built-in Levenberg-Marquardt iterations algorithm (or the orthogonal regression distance) provided by the software. All data are the results of at least three replicates.

| Sample          | Thr/S(+1) | \( k_{\text{cat}} \) [min\(^{-1}\)] | \( K_M \) [\( \mu \text{M} \)] | \( k_{\text{cat}}/K_M \) [min\(^{-1}\)\( \mu \text{M}^{-1} \)] | \( K_i \) [\( \mu \text{M} \)] |
|-----------------|-----------|---------------------------------|--------------------------------|-------------------------------------------------|---------------------------------|
| thrombin only   | -         | 42 ± 1                          | 0.46 ± 0.08                    | 91 ± 16                                         | 82 ± 15                         |
| w/o DNA         |           |                                 |                                |                                                 |                                 |
| TBA1            |           | 45 ± 1                          | 0.44 ± 0.06                    | 104 ± 13                                        | 92 ± 12                         |
| TBA2            |           | 48 ± 1                          | 0.55 ± 0.07                    | 87 ± 10                                         | 92 ± 13                         |
| TBA1/2          |           | 45 ± 1                          | 0.65 ± 0.06                    | 69 ± 6                                          | 93 ± 11                         |
| phosph          |           | 42 ± 1                          | 0.5 ± 0.08                     | 83 ± 13                                         | 86 ± 15                         |
| DNA-unbound     |           |                                 |                                |                                                 |                                 |
| ssDNA           |           | 56 ± 1                          | 0.51 ± 0.06                    | 110 ± 13                                        | 104 ± 15                        |
| rect            |           | 132 ± 5                         | 1.6 ± 0.2                      | 84 ± 10                                         | 89 ± 20                         |
| box             |           | 144 ± 1                         | 2.8 ± 0.2                      | 52 ± 4                                          | 42 ± 5                          |
| DNA-bound       |           |                                 |                                |                                                 |                                 |
| rect\(^1\)\(\text{flex}\) |       | 154 ± 3                         | 1.28 ± 0.08                    | 120 ± 8                                         | 109 ± 15                        |
| rect\(^2\)\(\text{flex}\) |       | 184 ± 6                         | 2.0 ± 0.2                      | 91 ± 8                                          | 124 ± 28                        |
| rect\(^1/2\)\(\text{flex}\) |       | 187 ± 9                         | 2.4 ± 0.3                      | 78 ± 10                                         | 115 ± 37                        |
| rect\(^1/2\)\(\text{melt}\) |       | 227 ± 14                        | 2.7 ± 0.4                      | 84 ± 13                                         | 59 ± 14                         |
| rect\(^1/2\)\(\text{rig}\) |       | 236 ± 16                        | 2.2 ± 0.4                      | 105 ± 19                                        | 70 ± 22                         |
| box\(^1/2\)\(\text{rig}\) |       | 451 ± 32                        | 6.5 ± 0.8                      | 69 ± 9                                          | 28 ± 5                          |
Table S3. Association ($k_a$) and dissociation ($k_d$) rate coefficients and equilibrium dissociation constant ($K_D = k_d/k_a$) for the aptamer/thrombin binding reaction. Thrombin binding to TBA1, TBA2 or both was measured with the switchSENSE technology. All experiments were performed on a heliX+ instrument (Dynamic Biosensors GmbH, Martinsried, DE) with standard Adapter switchSENSE chips (ADP-48-2-0) and using the static measurement mode. Kinetic experiments were performed using 100 nM thrombin in TE140 running buffer (10 mM Tris-HCl, 140 mM NaCl, 0.05 % Tween20, 50 µM EDTA, 50 µM EGTA) and monitoring the association and dissociation of the protein to the TBA anchored onto the chip with a flow rate of 500 µL/min at 25 °C. The same experiments were performed in presence of increasing concentrations of ssDNA (0 nM; 63 nM, 125 nM, 250 nM, 500 nM, 1 µM) constituted by a 48mer DNA oligo not complementary to any of the sequences used in the assay. More experimental details can be found in the Methods section (main manuscript).

|           | $k_a$ [M$^{-1}$s$^{-1}$] x $10^{-6}$ | $k_d$ [s$^{-1}$] x $10^3$ | $K_D$ [nM] |
|-----------|-----------------------------------|--------------------------|------------|
| TBA1      | 9.04 ± 0.66                       | 19.7 ± 0.3               | 2.18 ± 0.16|
| TBA2      | 3.99 ± 0.20                       | 5.26 ± 0.11              | 1.32 ± 0.07|
| TBA1 + TBA2 | 9.52 ± 2.22                   | 26.9 ± 2.3               | 2.82 ± 0.70|
| TBA1 + TBA2 + ssDNA | 14.3 ± 3.8                | 32.9 ± 1.6               | 2.29 ± 0.62|
| TBA1 + TBA2 + ssDNA | 3.13 ± 0.07            | 3.79 ± 0.06              | 1.21 ± 0.03|
| TBA1 + TBA2 + ssDNA | 6.61 ± 1.36             | 26.4 ± 1.0               | 4.00 ± 0.84|
| TBA1 + TBA2 + ssDNA | 2.70 ± 0.06            | 6.48 ± 0.05              | 2.40 ± 0.06|
Data S1. (separate file)
DNA sequences for the DNA origami structures.

Data S2. (separate file)
DNA sequences used for the SwitchSENSE binding assays.
In bold are the aptamer or scramble sequences (note that only this part of the full-length oligo folds into the desired TBA). These sequences are elongated with a 48-nt long stretch that is complementary to a portion of the adapter strand. The adapter strand contains two stretches: one stretch hybridizes to the sequence of interest, the other stretch hybridizes to an anchor strand, covalently attached to the surface of the chip, thus ensuring that the sequence of interest is bound to the surface. TBAsc1 and TBAsc2 sequences are designed to prevent the formation of a G-quadruplex, while keeping the same G:C ratio.

Movie S1. (separate file)
Ga MD simulations of the thrombin bound to peptide substrate S(-1). The trajectories shown do not include the equilibration time and the first 10 ns of simulation. Thrombin is shown in surf representation and colored as yellow (transparent). The residues from the exosite I are highlighted in red. The peptide is colored as gray in each case except for the C-terminal aminoacid.

Movie S2. (separate file)
Ga MD simulations of the thrombin bound to peptide substrate S(0). The trajectories shown do not include the equilibration time and the first 10 ns of simulation. Thrombin is shown in surf representation and colored as yellow (transparent). The residues from the exosite I are highlighted in red. The peptide is colored as gray in each case except for the C-terminal aminoacid.

Movie S3. (separate file)
Ga MD simulations of the thrombin bound to peptide substrate S(+1). The trajectories shown do not include the equilibration time and the first 10 ns of simulation. Thrombin is shown in surf representation and colored as yellow (transparent). The residues from the exosite I are highlighted in red. The peptide is colored as gray in each case except for the C-terminal aminoacid.

Movie S4. (separate file)
MD simulations for the DNA-origami rectangular frame structure hosting one thrombin molecule in its inner cavity. Thrombin is linked to the DNA origami frame by the TBA1 and TBA2. Regions around the plane of the initial DNA origami sheet are indicated in white. Regions above and below the initial DNA-origami plane are indicated, respectively, in red and blue.
REFERENCES AND NOTES

1. K. S. Rabe, J. Müller, M. Skoupi, C. M. Niemeyer, Cascades in compartments: En route to machine-assisted biotechnology. Angew. Chem. Int. Ed. 56, 13574–13589 (2017).

2. C. Teller, I. Willner, Organizing protein-DNA hybrids as nanostructures with programmed functionalities. Trends Biotechnol. 28, 619–628 (2010).

3. W. Engelen, B. M. Janssen, M. Merkx, DNA-based control of protein activity. Chem. Commun. 52, 3598–3610 (2016).

4. J. B. Trads, T. Torring, K. V. Gothelf, Site-selective conjugation of native proteins with DNA. Acc. Chem. Res. 50, 1367–1374 (2017).

5. Y. Hu, C. M. Niemeyer, From DNA nanotechnology to material systems engineering. Adv. Mater. 31, 1806294 (2019).

6. A. Jaekel, P. Stegemann, B. Sacca, Manipulating enzymes properties with DNA nanostructures. Molecules 24 (2019).

7. K. Zhou, J. Dong, Y. Zhou, J. Dong, M. Wang, Q. Wang, Toward precise manipulation of DNA–protein hybrid nanoarchitectures. Small 15, 1804044 (2019).

8. J. Fu, Z. Wang, X. H. Liang, S. W. Oh, E. St. Iago-McRae, T. Zhang, DNA-scaffolded proximity assembly and confinement of multienzyme reactions. Top. Curr. Chem. 378, 38 (2020).

9. X. Lv, S. Cui, Y. Gu, J. Li, G. du, L. Liu, Enzyme assembly for compartmentalized metabolic flux control. Metabolites 10 (2020).

10. D. Zhao, Y. Kong, S. Zhao, H. Xing, Engineering functional DNA-protein conjugates for biosensing, biomedical, and nanoassembly applications. Top. Curr. Chem. 378, 41 (2020).

11. C. M. Niemeyer, J. Koehler, C. Wuerdemann, DNA-directed assembly of bienzymic complexes from in vivo biotinylated NAD(P)H:FMN oxidoreductase and luciferase. Chembiochem 3, 242–245 (2002).

2. V. V. Demidov, N. V. Dokholyan, C. Witte-Hoffmann, P. Chalasani, H.W. Yiu, F. Ding, Y. Yu, C. R. Cantor, N. E. Broude, Fast complementation of split fluorescent protein triggered by DNA hybridization. Proc. Natl. Acad. Sci. U.S.A. 103, 2052–2056 (2006).

13. L. Fruk, J. Muller, C. M. Niemeyer, Kinetic analysis of semisynthetic peroxidase enzymes containing a covalent DNA-heme adduct as the cofactor. Chemistry 12, 7448–7457 (2006).

14. M. Glettenberg, C. M. Niemeyer, Tuning of peroxidase activity by covalently tethered DNA oligonucleotides. Bioconj. Chem. 20, 969–975 (2009).

15. Y. H. Lao, K. Peck, L. C. Chen, Enhancement of aptamer microarray sensitivity through spacer optimization and avidity effect. Anal. Chem. 81, 1747–1754 (2009).
16. Y. N. Gao, C. C. Roberts, J. Zhu, J.-L. Lin, C. A. Chang, I. Wheeldon, Tuning enzyme kinetics through designed intermolecular interactions far from the active site. ACS Catal. 5, 2149–2153 (2015).

17. J. Fu, Y. R. Yang, S. Dhakal, Z. Zhao, M. Liu, T. Zhang, N. G. Walter, H. Yan, Assembly of multienzyme complexes on DNA nanostructures. Nat. Protoc. 11, 2243–2273 (2016).

18. G. Ke, M. Liu, S. Jiang, X. Qi, Y. R. Yang, S. Wootten, F. Zhang, Z. Zhu, Y. Liu, C. J. Yang, H. Yan, Directional regulation of enzyme pathways through the control of substrate channeling on a DNA origami scaffold. Angew. Chem. Int. Ed. Engl. 55, 7483–7486 (2016).

19. Z. Zhao, J. Fu, S. Dhakal, A. Johnson-Buck, M. Liu, T. Zhang, N. W. Woodbury, Y. Liu, N. G. Walter, H. Yan, Nanocaged enzymes with enhanced catalytic activity and increased stability against protease digestion. Nat. Commun. 7 (2016).

20. G. Grossi, M. D. E. Jepsen, J. Kjems, E. S. Andersen, Control of enzyme reactions by a reconfigurable DNA nanovault. Nat. Commun. 8, 992 (2017).

21. W. P. Klein, R. P. Thomsen, K. B. Turner, S. A. Walper, J. Vranish, J. Kjems, M. G. Ancona, I. L. Medintz, Enhanced catalysis from multienzyme cascades assembled on a DNA origami triangle. ACS Nano 13, 13677–13689 (2019).

22. J. Muller, C. M. Niemeyer, DNA-directed assembly of artificial multienzyme complexes. Biochem. Biophys. Res. Commun. 377, 62–67 (2008).

23. O. I. Wilner, S. Shimron, Y. Weizmann, Z. G. Wang, I. Willner, Self-assembly of enzymes on DNA scaffolds: En route to biocatalytic cascades and the synthesis of metallic nanowires. Nano Lett. 9, 2040–2043 (2009).

24. O. I. Wilner, Y. Weizmann, R. Gill, O. Lioubashevski, R. Freeman, I. Willner, Enzyme cascades activated on topologically programmed DNA scaffolds. Nat. Nanotechnol. 4, 249–254 (2009).

25. J. Fu, M. Liu, Y. Liu, N. W. Woodbury, H. Yan, Interenzyme substrate diffusion for an enzyme cascade organized on spatially addressable DNA nanostructures. J. Am. Chem. Soc. 134, 5516–5519 (2012).

26. V. Linko, M. Eerikainen, M. A. Kostiainen, A modular DNA origami-based enzyme cascade nanoreactor. Chem. Commun. 51, 5351–5354 (2015).

27. O. Idan, H. Hess, Origins of activity enhancement in enzyme cascades on scaffolds. ACS Nano 7, 8658–8665 (2013).

28. Y. Zhang, S. Tsitkov, H. Hess, Proximity does not contribute to activity enhancement in the glucose oxidase-horseradish peroxidase cascade. Nat. Commun. 7, 13982 (2016).

29. J. A. Huntington, Molecular recognition mechanisms of thrombin. J. Thromb. Haemost. 3, 1861–1872 (2005).
30. W. Bode, D. Turk, A. Karshikov, The refined 1.9-A x-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human alpha-thrombin: Structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure-function relationships. *Protein Sci.* **1**, 426–471 (1992).

31. G. Chahal, M. Thorpe, L. Hellman, The importance of exosite interactions for substrate cleavage by human thrombin. *PLOS ONE* **10**, e0129511 (2015).

32. L. C. Bock, L. C. Griffin, J. A. Latham, E. H. Vermaas, J. J. Toole, Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* **355**, 564–566 (1992).

33. R. F. Macaya, P. Schultze, F. W. Smith, J. A. Roe, J. Feigon, Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3745–3749 (1993).

34. K. Padmanabhan, K. P. Padmanabhan, J. D. Ferrara, J. E. Sadler, A. Tulinsky, The structure of alpha-thrombin inhibited by a 15-mer single-stranded DNA aptamer. *J. Biol. Chem.* **268**, 17651–17654 (1993).

35. D. M. Tasset, M. F. Kubik, W. Steiner, Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. *J. Mol. Biol.* **272**, 688–698 (1997).

36. I. Russo Krauss, A. Merlino, C. Giancola, A. Randazzo, L. Mazzarella, F. Sica, Thrombin-aptamer recognition: A revealed ambiguity. *Nucleic Acids Res.* **39**, 7858–7867 (2011).

37. A. Aghebat Rafat, S. Sagredo, M. Thalhammer, F. C. Simmel, Barcoded DNA origami structures for multiplexed optimization and enrichment of DNA-based protein-binding cavities. *Nat. Chem.* **12**, 852–859 (2020).

38. X. Chen, B. Jia, Z. Lu, L. Liao, H. Yu, Z. Li, Aptamer-integrated scaffolds for biologically functional DNA origami structures. *ACS Appl. Mater. Interfaces* **13**, 39711–39718 (2021).

39. J. A. Huntington, Thrombin plasticity. *Biochim. Biophys. Acta Proteins Proteom.* **1824**, 246–252 (2012).

40. E. Di Cera, Thrombin. *Mol. Aspects Med.* **29**, 203–254 (2008).

41. M. Pozsgay, G. Szabó, S. Bajusz, R. Simonsson, R. Gáspár, P. Elödi, Study of the specificity of thrombin with tripeptidyl-p-nitroanilide substrates. *Eur. J. Biochem.* **115**, 491–495 (1981).

42. R. R. Cook, B. J. McRae, J. C. Powers, Kinetics of hydrolysis of peptide thioester derivatives of arginine by human and bovine thrombins. *Arch. Biochem. Biophys.* **234**, 82–88 (1984).

43. D. T. Rijkers, S. J. Wielders, G. I. Tesser, H. C. Hemker, Design and synthesis of thrombin substrates with modified kinetic parameters. *Thromb. Res.* **79**, 491–499 (1995).

44. E. Di Cera, Thrombin: A paradigm for enzymes allosterically activated by monovalent cations. *C. R. Biol.* **327**, 1065–1076 (2004).
45. E. C. Schoneweiss, B. Sacca, The collective behavior of spring-like motifs tethered to a DNA origami nanostructure. *Nanoscale* **9**, 4486–4496 (2017).

46. M. Erkelenz, R. Kosinski, O. Sritharan, H. Giesler, B. Sacca, S. Schlücker, Site-specific facet protection of gold nanoparticles inside a 3D DNA origami box: A tool for molecular plasmonics. *Chem. Commun.* **57**, 3151–3153 (2021).

47. A. Fersht, *Structure and Mechanism in Protein Science* (World Scientific, 2017), in Series in Structural Biology, vol. 9.

48. J. A. Huntington, How Na+ activates thrombin –A review of the functional and structural data. *Biol. Chem.* **389**, 1025–1035 (2008).

49. W. Bode, I. Mayr, U. Baumann, R. Huber, S. R. Stone, J. Hofsteenge, The refined 1.9 A crystal structure of human alpha-thrombin: Interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* **8**, 3467–3475 (1989).

50. A. Cornish-Bowden, *Fundamentals of Enzyme Kinetics* (Wiley-Blackwell, 2012).

51. C. Frieden, Slow transitions and hysteretic behavior in enzymes. *Annu. Rev. Biochem.* **48**, 471–489 (1979).

52. K. E. Neet, G. R. Ainslie Jr., Hysteretic enzymes. *Methods Enzymol.* **64**, 192–226 (1980).

53. E. Di Cera, R. De Cristofaro, D. J. Albright, J. W. Fenton, Linkage between proton binding and amidase activity in human .alpha.-thrombin: Effect of ions and temperature. *Biochemistry* **30**, 7913–7924 (1991).

54. E. Di Cera, Q. D. Dang, Y. Ayala, A. Vindigni, Linkage at steady state: Allosteric transitions of thrombin. *Methods Enzymol.* **259**, 127–144 (1995).

55. C. M. Wells, E. Di Cera, Thrombin is a Na+ -activated enzyme. *Biochemistry* **31**, 11721–11730 (1992).

56. M. R. Machado, E. E. Barrera, F. Klein, M. Sóñora, S. Silva, S. Pantano, The SIRAH 2.0 Force Field: Altius, Fortius, Citius. *J. Chem. Theor. Comput.* **15**, 2719–2733 (2019).

57. M. J. Abraham, T. Murtola, R. Schulz, S. Páll, J. C. Smith, B. Hess, E. Lindahl, GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1-2**, 19–25 (2015).

58. W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926–935 (1983).

59. Y. T. Pang, Y. Miao, Y. Wang, J. A. McCammon, Gaussian accelerated molecular dynamics in NAMD. *J. Chem. Theor. Comput.* **13**, 9–19 (2017).

60. T. Darden, D. York, L. Pedersen, Particle mesh Ewald: AnN-log(N) method for Ewald sums in large systems. *J. Chem. Phys.* **98**, 10089–10092 (1993).
61. J. C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kalé, K. Schulten, Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781–1802 (2005).

62. J. C. Phillips, D. J. Hardy, J. D. C. Maia, J. E. Stone, J. V. Ribeiro, R. C. Bernardi, R. Buch, G. Fiorin, J. Hénin, W. Jiang, R. McGreevy, M. C. R. Melo, B. K. Radak, R. D. Skeel, A. Singharoy, Y. Wang, B. Roux, A. Aksimentiev, Z. Luthey-Schulten, L. V. Kalé, K. Schulten, C. Chipot, E. Tajkhorshid. Scalable molecular dynamics on CPU and GPU architectures with NAMD. *J. Chem. Phys.* **153**, 044130 (2020).

63. K. Vanommeslaeghe, A. D. MacKerell Jr., CHARMM additive and polarizable force fields for biophysics and computer-aided drug design. *Biochim. Biophys. Acta* **1850**, 861–871 (2015).

64. J. Huang, S. Rauscher, G. Nawrocki, T. Ran, M. Feig, B. L. de Groot, H. Grubmüller, A. D. MacKerell Jr., CHARMM36m: An improved force field for folded and intrinsically disordered proteins. *Nat. Methods* **14**, 71–73 (2016).

65. V. Zoete, M. A. Cuendet, A. Grosdidier, O. Michielin, SwissParam: A fast force field generation tool for small organic molecules. *J. Comput. Chem.* **32**, 2359–2368 (2011).

66. J. D. Yesselman, D. J. Price, J. L. Knight, C. L. Brooks III, MATCH: An atom-typing toolset for molecular mechanics force fields. *J. Comput. Chem.* **33**, 189–202 (2012).

67. M. Krol, T. Borowski, I. Roterman, B. Piekarska, B. Stopa, J. Rybarska, L. Konieczny, Force-field parametrization and molecular dynamics simulations of Congo red. *J. Comput. Aided Mol. Des.* **18**, 41–53 (2004).

68. W. Humphrey, A. Dalke, K. Schulten, VMD: Visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38 (1996).