Metabolic pathways are tightly regulated to control the concentration of metabolites in the cell. While a number of different methods are used to achieve such regulation, perhaps the most fascinating is control of enzyme activity through allostery, in which binding of an effector molecule either increases or decreases the catalytic activity. Thus, the activity of allosteric enzymes is modulated by the chemical composition of their environment. In recent years, attempts have been made to design such effects in existing nonallosteric enzymes; this is potentially interesting for applications, such as chemical sensors, as this provides a mechanism for signal amplification. A potentially versatile approach to designed allosteric enzymes involves introduction of nonproteinogenic moieties into an enzyme; these moieties can then be used to control enzyme activity. For this purpose, DNA is particularly attractive; the highly specific base-pairing interactions can be used to modulate enzyme activity with an exceptional degree of control. A few approaches in which DNA hybridization has been used to control enzymatic activity have been reported to date; these include mechanical induction of conformational changes in enzymes or peptide ligands, rigidification of a DNA tether between an enzyme and a competitive inhibitor, and directed assembly of multi-enzyme systems. Here, we present a novel approach to DNA-controlled enzymatic activity; this involves a split enzyme system that can be reassembled into a catalytically active conformation by hybridization of the protein-fragment-conjugated oligonucleotides with a template DNA strand.

Split proteins have emerged as versatile and attractive tools for in vivo and in vitro sensing applications for protein–protein interactions, protein–DNA interactions, and small-molecule binding. Recently, split proteins have been combined with nonproteinogenic recognition moieties, including DNA oligomers. To date this has been limited to the functional, nontemplated, recombination of fluorescent and luminescent proteins. A similar approach has been reported for a split peroxidase DNAzyme.

We envisioned that a split enzyme equipped with oligonucleotides conjugated to both protein fragments would give rise to an artificial allosteric enzymatic system; upon binding of a complementary external DNA strand the enzyme is reassembled into an active conformation, thereby allowing catalysis to occur (Figure 1). In this manner, catalytic activity can be modulated by both the concentration and the sequence of the external DNA strand.

The design was based on the enzyme murine dihydrofolate reductase (mDHFR), which catalyzes the NADPH dependent reduction of dihydrofolate into tetrahydrofolate. mDHFR was selected because it can be dissected into two fragments, which can be recombinated to give a stable protein, as has been demonstrated for this and the homologous E. coli enzyme. However, a secondary covalent or noncovalent interaction between the two protein segments is required for catalytic activity to be restored. Based on these studies, mDHFR was disconnected between amino acids 105 and 106 on the genetic level.

A maleimide coupling strategy was selected for the preparation of the mDHFR fragment oligonucleotide conjugates. This strategy requires the mDHFR fragments to be equipped with a nucleophilic cysteine residue (Scheme 1). The two fragments of mDHFR, that is, N-terminal mDHFR, corresponding to residues 1–105 and C-terminal mDHFR, corresponding to the 106–186 fragment, were cloned and expressed independently. A C-terminal cysteine was added on the genetic level to N-terminal mDHFR along with an N-terminal His 6-tag for purification purposes. In the case of the C-terminal mDHFR fragment a C-terminal His 6-tag was included. The cysteine needed for conjugation was included to the N terminus as part of an MGC or MGCGGSGG extension, to give C-terminal mDHFR (1) and C-terminal mDHFR (2), respectively.

The mDHFR fragments were expressed in E. coli BL21 (DE3), purified under denaturing conditions on a nickel–agarose column, and analyzed by gel electrophoresis and ESI mass spectrometry. In the case of the C-terminal mDHFR fragments a mass corresponding to the loss of the N-terminal methionine...
was found; m/z 10694 Da for C-terminal mDHFR (1) (calcd M-Met: 10692 Da), and 11009 for C-terminal mDHFR (2) (calcd M-Met: 11008 Da).

16-Mer oligonucleotides were conjugated to the mDHFR fragments, as these would provide a strong thermodynamic driving force for the split enzyme reassembly upon hybridization with the template DNA.

The maleimido 5’- and 3’- modified oligonucleotides were prepared by treatment of the corresponding terminal amino-modified oligonucleotides with 3-maleimidopropionic acid N-hydroxysuccinimide ester (Scheme 1). The resulting maleimide functionalized oligonucleotides were purified by size-exclusion chromatography and analyzed by reversed-phase HPLC and MALDI-TOF. Isolated yields were typically around 90 %. The N-terminal mDHFR and C-terminal mDHFR fragments were conjugated to 5’- and 3’- oligonucleotides, respectively, by maleimide coupling (Scheme 1) under denaturing conditions to prevent precipitation. Conversions were estimated from PAGE to be around 70 % for the N-terminal mDHFR fragment and around 10 % for the C-terminal mDHFR (1) fragment. The conversion of the conjugation of the C-terminal mDHFR (2) fragment, which contains a GGSGG linker between the cysteine and the start of the mDHFR fragment, was considerably higher, that is, around 40 %. This indicates that the steric hindrance of the amino acids surrounding the reactive cysteine is important for the effectiveness of the coupling.

The resulting protein–DNA conjugates were purified from uncoupled mDHFR by anion-exchange chromatography on a HiTrap QFF column. Purification from uncoupled oligo-nucleotide was achieved by affinity chromatography on a nickel–nitriloacetic acid (Ni–NTA) column; this takes advantage of the His\textsubscript{6} tags on the protein fragments. Moreover, this last step also results in concentration of the protein–DNA conjugates. This purification procedure gave rise to highly pure protein–DNA conjugates, as was confirmed by PAGE and analytical size-exclusion chromatography, which demonstrated that the material was free of unconjugated DNA (see the Supporting Information).

The catalytic activity of the system was evaluated in the NADPH-dependent reduction of dihydrofolate into tetrahydrofolate, by using the standard DHFR assay.\textsuperscript{(28)} The system was assembled by using a rapid dilution protocol.\textsuperscript{(29)} Equimolar amounts of the DNA conjugates of N-terminal mDHFR and C-terminal mDHFR (2), and DNA template in 8 M urea were premixed and diluted into the reaction buffer, and this allowed for protein refolding and DNA hybridization to occur. The progress of the enzymatic reaction was monitored by following the consumption of NADPH with time by using UV–visible measurements at 340 nm. In the absence of template DNA, no catalytic activity was observed; the observed decrease in NADPH absorption coincided with that found for the uncatalyzed NADPH degradation. When including the fully complementary DNA template in the reaction, a rapid consumption of NADPH was observed, thereby demonstrating that the assembled system indeed is catalytically active (Figure 2). A lag phase is observed in the early stages of the reaction. Therefore, in order to allow quantitative comparison of reaction rates, the initial 15 % of the reaction was not considered. Compared to the wild-type mDHFR, the DNA-based system is only 2.5-fold slower (Table 1, entries 1, 2); this suggests that the conformation of the reassembled enzyme closely resembles that of the wild-type enzyme. These results clearly demonstrate that the secondary interactions provided by the DNA template result in the assembly of an active enzyme, and induce catalysis of the dihydrofolate reduction.
The catalytic activity was found to be dependent on the concentration of the DNA template (Table 1, entries 2–8). With less than 1 equivalent of template with respect to N-terminal mDHFR and C-terminal mDHFR (2) DNA conjugates, a lower activity is observed, as expected. Maximum activity was observed when 1 equivalent of template was used. Increasing the concentration of the DNA template to >1 equivalent also results in a progressively lower activity. This is attributed to the fact that with more than equimolar amounts of template present, a mixture of the active reassembled system and templates hybridized with only N-terminal mDHFR or C-terminal mDHFR (2) DNA conjugates, which are not active, are formed, thereby resulting in an overall decrease in activity. Moreover, experiments in which the refolding of full-length mDHFR was performed in the presence of free DNA showed lower activity compared to when the DNA was absent, and this indicates that an excess of DNA might also inhibit the enzyme.

The effect of mismatches in the DNA template on catalytic activity was investigated. By using 1 equivalent of template, a decrease in catalytic activity was found when the number of mismatches was increased. Compared to the full complementary template the activity was reduced by 50% when five mismatches were introduced into the template at positions 12, 14, 16, 20, and 27. The observed activities with one mismatch at position 16 or three mismatches at positions 16, 20, and 27, were found to be similar. This is due to the fact that the three mutations are distributed over the template with one and two mismatches in the sequences complementary to oligonucleotides 1 and 2, respectively. Hence, the observed decrease in activity is less than would be expected with all three mutations in the sequence complementary to oligonucleotide 1 or 2. Combined, these results show that destabilization of the DNA duplex results in a decrease in activity. This can be tentatively explained by assuming that a weaker duplex results in more structural flexibility in the protein part, and this results in less of the protein being in the catalytically active conformation.

With the DNA-based split-enzyme systems investigated here, a lag phase is observed before catalysis occurs efficiently. A tentative explanation is that, upon dilution with the reaction buffer, some time is required for the hybridization and folding of the system into a catalytically active conformation to occur. However, the lag phase was still observed when the system was preincubated either in the reaction buffer or in the buffer containing dihydrofolate for 2 h. Moreover, in these cases a significant decrease in activity was found. Most likely, in addition to the DNA-driven reassembly other interactions are required for obtaining the active conformation of the enzyme. Based on earlier work on the homologous E. coli enzyme, it is hypothesized that binding of both, dihydrofolate and NADPH, is also required for folding into an active conformation.

In conclusion, here we have shown that the catalytic activity of a DNA-conjugated split enzyme, that is, mDHFR, can be modulated by the concentration and sequence complementarity of a DNA template. This represents a novel and versatile approach to artificial allosteric enzymes. Particularly attractive of the present design is that in addition to DNA-controlled catalytic activity, it is envisioned that the concept can be adapted readily for small-molecule-dependent control over catalytic activity, by inclusion of DNA aptamer structures in the template DNA strand.

Experimental Section

General procedure for the reduction of dihydrofolate catalyzed by the split mDHFR system: A solution of equimolar amounts of DNA template, N-terminal mDHFR and C-terminal mDHFR-oligonucleotide conjugates in buffer (100 mM NaH2PO4, pH 4, 100 mM Tris-
HCl, 8 mM urea, as obtained from the final purification step from the uncoupled oligonucleotide on the Ni–NTA column) was prepared. NADPH (2 μL of 50 mM stock solution in MilliQ water) was added to a dihydrofolate (H2F) solution (100 μM) in Tris-HCl buffer (50 mM, pH 7.7, 5 mM MgCl2, 3.3 mM KCl, and 10 mM 1,4-dithiothreitol (DTT)). The amount of buffer used corresponds to the necessary volume to obtain a total reaction volume of 1 mL. The experiment is initiated by the addition of the corresponding volume of the premixed split mDHFR system solution to obtain a final concentration of 100 nM in the reaction mixture. Progress of the reaction was monitored by following the decrease of the NADPH absorption at 340 nm with UV–visible spectroscopy.

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