DNA–Enzyme Conjugates

Covalent Hemin–DNA Adducts for Generating a Novel Class of Artificial Heme Enzymes**

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An important goal of nanobiotechnology is the generation of novel biomaterials with precise control at the nanometer scale.[1] DNA oligomers play an important role as structure-directing agents in the bottom-up fabrication of nanostructured functional devices, owing to their tremendous molecular recognition capabilities. The generation of semisynthetic DNA–protein conjugates makes it possible to combine the unique properties of DNA with the almost unlimited variety of functional components of proteins, which have evolved naturally to perform highly specific catalytic turnovers, energy conversions, or translocations.[2] However, to take advantage of the distinct functionalities of DNA–protein conjugates, one must face the challenge of developing synthetic strategies that permit control over both the stoichiometry and regioselectivity of DNA–protein coupling reactions.

Herein we report a novel approach toward well-defined and highly functional conjugates between DNA oligomers and enzymes that contain the hemin (iron protoporphyrin IX complex) prosthetic group. In the first step, covalent DNA–hemin adducts ($hemD_1$ and $hemD_2$; Scheme 1a) were synthesized on solid phase. After cleavage from the solid phase and purification, the hemD adducts were used to reconstitute apo-myoglobin (apo-Mb: myoglobin without its hemin prosthetic group).[3] Reconstitution of apo-Mb with $hemD_1$ and $hemD_2$ produced enzymatically active myoglobin that contained one or two DNA oligomers (MbD_1 and MbD_2, respectively; Scheme 1b) coupled to the enzyme in close proximity to the active site. To show that the DNA oligomers can be used as molecular handles for selective DNA-directed assembly, the MbD conjugates were hybridized to complementary capture oligomers immobilized on a solid support (Scheme 1c). Subsequent analysis of the enzymatic peroxidase activity revealed that the MbD conjugates are far more active than native myoglobin (Mb).

The oxygen storage protein Mb, which contains a non-covalently bound heme moiety, has been studied intensively over the past 30 years as a model system to investigate the functional role of the heme iron center,[4,5] long-range electron-transfer processes,[6] and the effects of metal exchanges or modifications of the heme side chains on the properties of the protein.[7,8] Although artificial Mbs have been prepared by site-directed mutagenesis,[9] the relative ease of heme removal and subsequent reconstitution of apo-Mb with functional porphyrin derivatives makes this system convenient for the design and study of artificial heme enzymes.[6] As an example, Hayashi and co-workers have shown that heme chemically modified with anionic groups at its propionate chains imparts Mb with enhanced peroxidase activity toward cationic substrates.[10]

Artificial Mb was chosen as an initial model system to investigate our concept of DNA conjugation (summarized in Scheme 1), as there is already a large body of knowledge about this protein. To this end, a 5′-alkylamino-modified 12-mer oligonucleotide (5′-amino-TCTCAACTCGTA) was synthesized with conventional phosphoramidite chemistry. Hemin was activated with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DiPEA) in DMF/CH_3CN, and the resulting solution was injected into a DNA synthesizer to carry out the coupling reaction with the 5′-amino group of the oligomer. Following incubation for 30 min and deprotection under mild conditions (*tert*-butylamine, MeOH, H_2O), the oligonucleotide was purified with reversed-phase HPLC.[11] The two major products formed were identified by MALDI MS as hemin coupled with either one or two DNA oligomers ($hemD_1$ and $hemD_2$, respectively; Scheme 1a).

Both $hemD_1$ and $hemD_2$ were used in the reconstitution of apo-Mb to yield the conjugates MbD_1 and MbD_2, respectively (Scheme 1b). Chromatographic analysis of the reconstitution

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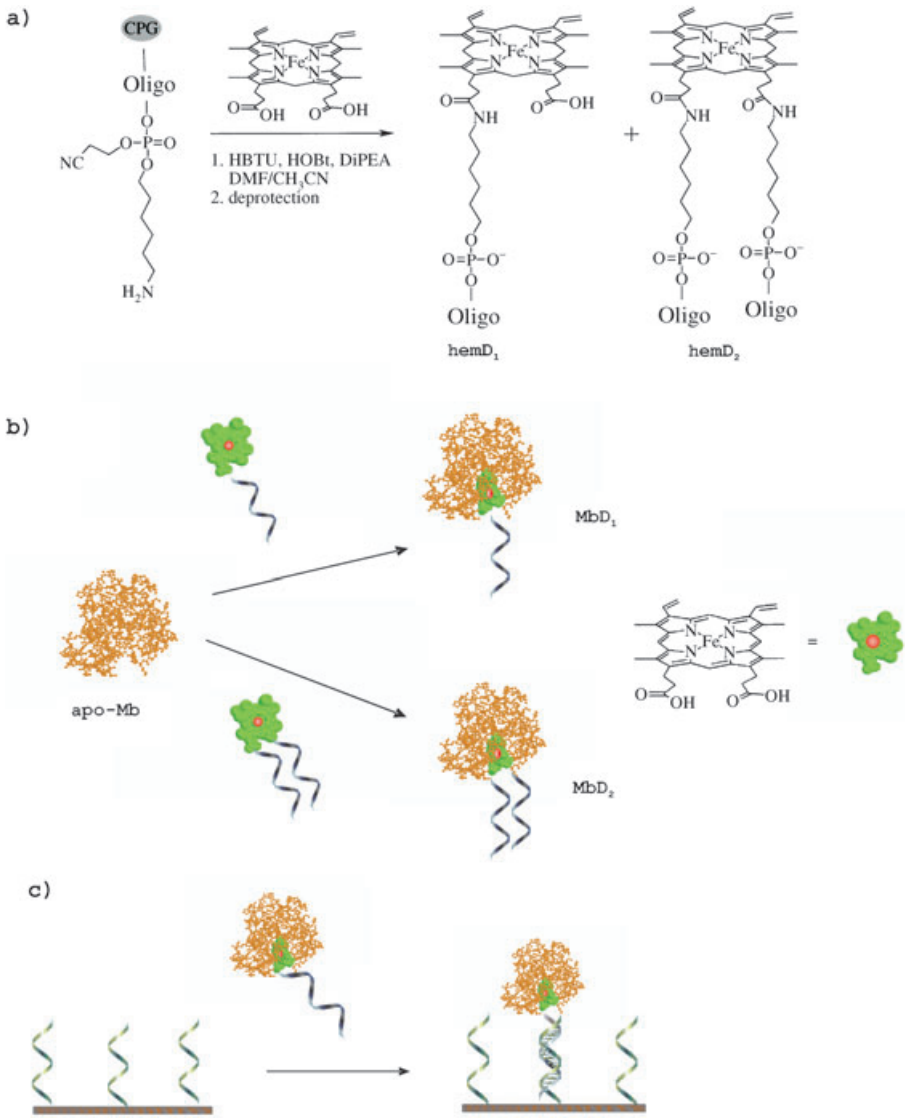

Scheme 1. Schematic representation of the synthesis and assembly of DNA–heme-enzyme conjugates; a) solid-phase synthesis of covalent DNA–hemin adducts $hemD_1$ and $hemD_2$, which contain one or two DNA oligomers, respectively; b) reconstitution of apo-Mb with hemin–DNA adducts leads to the formation of DNA–Mb conjugates MbD_1 and MbD_2; c) DNA-directed immobilization of the DNA–Mb conjugates. CPG = controlled pore glass; HOBt = 1-hydroxybenzotriazole.

by anion-exchange FPLC revealed that the uptake of $hemD_2$ was significantly slower than that of $hemD_1$ (36 and 24 h, respectively), which may be attributed to the greater steric bulk of $hemD_2$ which limits its diffusion into the heme-binding pocket of apo-Mb. Representative chromatograms of hemD purification (Figure 1) demonstrate the significant difference between the retention times of apo-Mb and the respective hemD and MbD conjugates, which enables fast and efficient purification. UV/Vis spectra of both MbD_1 and MbD_2 show an absorption maximum at 408 nm (sharp Soret band) and weak bands at 502, 543, and 632 nm (Q bands), which are in agreement with literature values for native Mb, as well as a broadened peak at 276 nm that arises from absorbance by proteins and DNA.[11]

To further characterize the semisynthetic MbD conjugates, the peroxidase activities of MbD_1 and MbD_2 were compared with that of native Mb.[12] To attain maximal assay sensitivity, Amplex UltraRed (Molecular Probes) was chosen as substrate, which, in the presence of H_2O_2, is converted into a fluorescent dye by peroxidase activity.[13] Figure 2a shows the increase in fluorescence from the enzymatic transformation of Amplex UltraRed by either native Mb, MbD_1, or MbD_2 (5 pmol in each case). It is evident that the activities of MbD_1 and MbD_2 are significantly higher than that of native Mb. Interestingly, MbD_2 shows an even higher activity than MbD_1 despite the greater potential for steric hindrance of the MbD_2 active site by the two bulky DNA moieties nearby. Although the reasons behind its enhanced activity remain unclear, MbD_2 was detectable in quantities as small as 0.1 pmol, whereas detection of native Mb reached a limit at 2 pmol. Further studies of the peroxidase activity, including investigations of other substrates, are currently underway.

To demonstrate that the DNA oligomers can indeed be used as recognition sites for selective binding to complementary oligonucleotides, we performed DNA-directed immobilization assays of MbD_1 and MbD_2 on solid phase (Scheme 1c). We have previously demonstrated that the DNA-directed immobilization of proteins proceeds with high efficiency and allows reversible, site-selective functionalization of solid substrates with proteins or other chemical functionalities.[14] Peroxidase activity was measured after MbD_1 and MbD_2 were immobilized to streptavidin-coated microplate wells functionalized with complementary biotinylated capture oligomers (5′biotin-TACGAGTTGAGA; Figure 2b). Again, the MbD_2 conjugates showed a significantly higher activity than MbD_1. Moreover, in control assays carried out in plate wells containing non-complementary capture oligomers, no enzymatic activity was detected. This indicates that the immobilization occurs exclusively by specific Watson–Crick base pairs formed between the Mb-bound DNA moieties and the capture sequences.

We have shown that novel artificial myoglobin proteins can be generated by reconstitution of apo-Mb with covalent DNA–hemin conjugates. Although we could clearly analyze the composition and chromatographic properties of these novel DNA–Mb conjugates, the elucidation of their particular catalytic properties reported herein has only just begun. Our future work will therefore focus not only on the peroxidase activity, but on characterizing the redox and electron-transfer properties of these conjugates as well.[17] Moreover, the highly specific binding properties of the DNA moiety will be studied in more detail by assembly of the DNA–Mb conjugates both

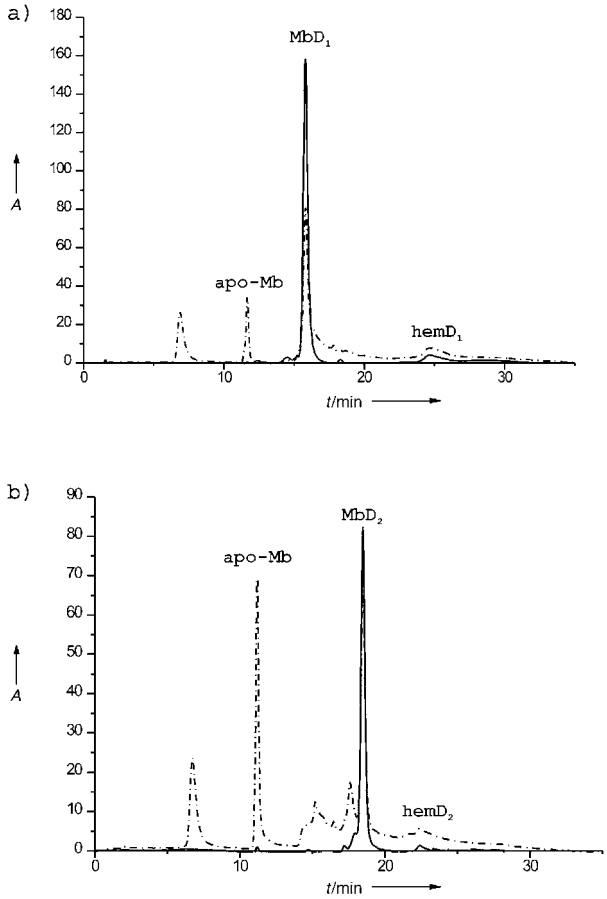

Figure 1. FPLC chromatograms of the reconstitution of apo-Mb with a) $hemD_1$ and with b) $hemD_2$; the dotted and solid lines indicate the absorbance measured at 280 and 405 nm, respectively.

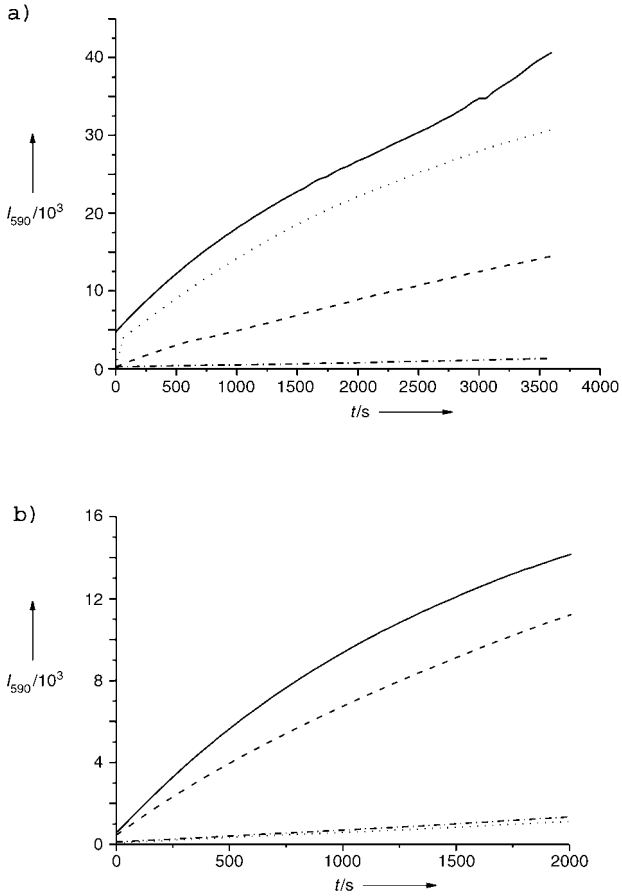

Figure 2. a) Peroxidase activity of 5 pmol native Mb (-----), MbD_1 (•••••), and MbD_2 (——) measured in solution with Amplex UltraRed as the substrate. A control reaction (–•–•) was carried out in the absence of peroxidase. b) Peroxidase activity of MbD_1 (-----) and MbD_2 (——) bound to microplate surfaces through DNA-directed immobilization. Control reactions with noncomplementary capture oligomers (••••• and –•–•) indicate the specificity of surface-capture events.

at the micrometer and nanometer length scales. Whereas the arrangements in the micrometer range should be useful for the generation of enzyme microarrays, the nanoscale assembly holds great potential for the fabrication of multifunctional protein constructs for applications in materials research[15] and life sciences.[2,16]

Notably, Mb served as a representative model system in this study for the seminal demonstration of our concept of generating well-defined DNA–heme-enzyme conjugates through the reconstitution of apo-enzymes with covalent hemin–DNA adducts. This method should transfer well to other heme enzymes, and we therefore anticipate that this approach will open the door to a large variety of novel redox catalysts. Through the programmable binding properties made possible by the specificity of DNA hybridization, such semisynthetic DNA–protein conjugates may be useful in a broad range of applications, from biocatalysts, sensors and transducer elements, to building blocks for micro- and nanostructured devices.

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[11] UV/Vis data for $hemD_1$, $hemD_2$, MbD_1, and MbD_2 as well as MALDI MS spectra and details on the solid-phase coupling of DNA with hemin, the reconstitution of apo-Mb, and the enzyme activity assays are available in the Supporting Information.

[12] It is known that Mb reacts with H_2O_2 to form ferryl species that correspond to compound II of usual peroxidases.[10] Therefore, Mb has the potential to catalyze the oxidation of various substrates in the presence of H_2O_2. Notably, the peroxidase activity of Mb is much lower than that of other peroxidases such as horseradish peroxidase.

[13] The structure of Amplex UltraRed (Invitrogen A36006) is proprietary and undisclosed. However, according to information supplied by the manufacturer, the nonfluorescent Amplex UltraRed reacts similarly to Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes) in the presence of H_2O_2 at a stoichiometric ratio of 1:1 to produce a brightly fluorescent and strongly absorbing reaction product. The chemical structure and reaction path of Amplex Red are shown in the Supporting Information (Figure S4).

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