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DNA-directed assembly of artificial multienzyme complexes

Joachim Müller, Christof M. Niemeyer *

Technische Universität Dortmund, Fakultät Chemie, Biologisch-Chemische Mikrostrukturtechnik, Otto-Hahn Str. 6, D-44227 Dortmund, Germany

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ABSTRACT

This study aims to establish model systems for the exploration of proximity effects, occurring in natural multienzyme complexes. DNA-directed assembly of covalent conjugates of DNA oligonucleotides and Glucose Oxidase (GOX) or Horseradish peroxidase (HRP) was used to generate supramolecular complexes, in which the two enzymes were arranged with defined spatial orientation. Electrophoretic studies indicated that the assembly efficiency significantly depends on positional and sterical factors of the two DNA–enzyme conjugates. Kinetic rate measurements of the coupled reaction of glucose oxidation and Amplex Red peroxidation were carried out with microplate-immobilized DNA–GOX–HRP complexes, and the influence of Catalase on this reaction was determined. The kinetic measurements revealed a significant increase in the reactivity of the complexes, in which GOX and HRP were immobilized in direct proximity on a complementary DNA carrier.

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Multienzyme complexes are large polypeptides with defined tertiary and quaternary structure, which comprise multiple catalytic centers [1]. These complexes are abundant in nature, and prominent examples include fatty acid synthase [2,3], tryptophan synthase [4,5], or non-ribosomal peptide and polyketide synthases [6,7]. Multienzyme complexes display distinct advantages over isolated enzymes during a sequential multistep transformation of a substrate. In particular, the close proximity of active sites leads to mechanistic advantages because reactions limited by the rate of diffusional transport are accelerated and the "substrate-channeling" of intermediate products also avoids side reactions [8]. Thus, creation and engineering of artificial multienzyme complexes for potential applications in biocatalysis represents a challenging goal. A feasible approach to develop artificial multienzyme complexes is based on protein engineering, often combined with methods of directed evolution [9–11]. Because this approach essentially requires large efforts in protein chemistry, we have previously suggested that semisynthetic conjugates of enzymes tagged with short DNA oligonucleotides might be used to rapidly assemble multienzyme complexes by virtue of complementary Watson–Crick base-pairing [12]. Such a DNA-directed assembly of DNA–enzyme conjugates would offer a high degree of modularity and spatial control, which is otherwise not accessible by conventional chemical cross-linking. Although many semisynthetic DNA–enzyme conjugates have meanwhile been developed [13], examples of DNA-assembled multienzymes are still scarce. One marked example concerns the DNA-directed assembly of a bienzyme complex comprised of luciferase and oxidoreductase, which catalyzed the consecutive reactions of flavinmononucleotide reduction and aldehyde oxidation [14]. Indeed, overall enzymatic activity of this complex depended on the absolute and relative spatial orientation of the two enzymes.

Because such studies are useful to explore proximity effects in biochemical pathways and they might also lead to the development of artificial multienzymes as novel biocatalysts, we here report on the DNA-directed assembly and kinetic analysis of a bienzyme system comprised of Glucose Oxidase (GOX) and Horseradish peroxidase (HRP). The GOX–HRP system combines the oxidation of glucose to gluconolactone and H_2O_2 by GOX with the subsequent transformation of H_2O_2 and the fluorogenic dye Amplex Red to form highly fluorescent resorufin by HRP (Fig. 1A). The GOX–HRP system has often been used as a reporter system in biosensing [15,16] or as a model to develop microfluidic [17] or logical [18] devices. However, to the best of our knowledge, no detailed investigation of spatial coupling of these two enzymes has been reported so far. We here employ the DNA-directed assembly of covalent DNA conjugates GOX and HRP to generate several supramolecular complexes (**1**–**4**, in Fig. 1B) on the surface of microtiterplates. The complexes were analyzed by measuring the kinetic rates for the two-step reaction shown in Fig. 1A. Moreover, the influence of Catalase on this reaction was determined. Our results show clear evidence of improved reactivity of complexes, in which GOX and HRP are immobilized in direct proximity on a complementary DNA carrier strand.

Materials and methods

Chemicals. Horseradish peroxidase (HRP; purity number of 3) and Glucose Oxidase (GOX, from *Asparagillus niger*) were purchased

* Corresponding author. Fax: + 49 231 755 7082.
E-mail address: christof.niemeyer@tu-dortmund.de (C.M. Niemeyer).

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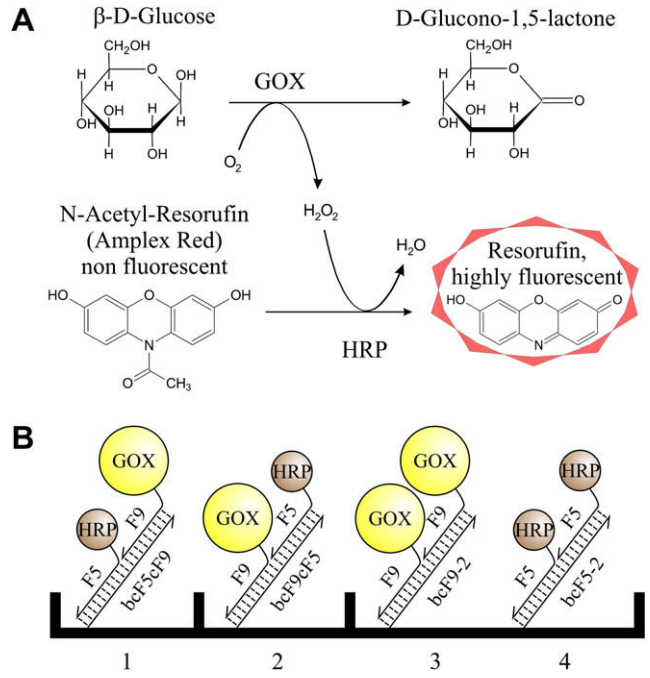

Fig. 1. Design of complexes. The mechanism of the coupled enzymes (A) includes the initial reaction of Glucose Oxidase (GOX) to oxidize Glucose to Glucono-1-lacton while producing H_2O_2. In the second step, Horseradish Peroxidase (HRP) reduces the H_2O_2 to water and the substrate *N*-Acetyl-resorufin (Ampex Red) is oxidized to form the highly fluorescent product Resorufin. Four different complexes were assembled by DNA-directed immobilization on microplate surfaces (B). Complex **1** and **2** contain the two enzymes in two different arrangements, while in control experiments, similar amounts of the enzymes were assembled in a pairwise homodimeric fashion (complex **3** and **4**), to account for differences in hybridization efficiency which result from positional and sterical influences (see text for details). Note that the arrows indicate the 3′–5′ direction of DNA and that enzymes are approximately drawn to scale. The oligonucleotide sequences are listed in Table 1.

from Sigma–Aldrich. Sulfo-succinimidyl-4-(*N*-maleimido-methyl)-cyclohexan-1-carboxylate (sSMCC) was obtained from Pierce, Amplex Red was purchased from Invitrogen. Oligonucleotides SHF5, SH-T6F5, SHF9, SH-T6F9, SH-3'-F9, bcF5cF9, bcF9cF5, bcF9-2 and bcF5-2 were from Sigma–Aldrich, sequences are listed in Table 1.

Synthesis of DNA–Enzyme-Conjugates. DNA–HRP-conjugates were synthesized from HRP and thiolated DNA-oligonucleotide SH-F5, as described earlier [19]. The same protocol was used for the synthesis of GOX-conjugates, using GOX and thiolated oligonucleotide SH-F9 (for sequences, see Table 1). In brief, the enzyme was activated with sSMCC, purifed by size-exclusion chromatography and mixed with a solution of the thiolated oligonucleotide.

Table 1
Used DNA Sequences

Abbreviation	Sequence
bcF5cF9	5′Biotin-CTT ATC GCT TTA TGA CCG GAC CCT TCA CGA TTG CCA CTT TCC AC
bcF9cF5	5′Biotin-CTT CAC GAT TGC CAC TTT CCA CCT TAT CGC TTT ATG ACC GGA CC
bcF9-2	5′Biotin-CTT CAC GAT TGC CAC TTT CCA CCT TCA CGA TTG CCA CTT TCC AC
bcF5-2	5′Biotin-CTT ATC GCT TTA TGA CCG GAC CCT TAT CGC TTT ATG ACC GGA CC
F5	5′-GGT CCG GTC ATA AAG CGA TAA G
F9	5′-GTG GAA AGT GGC AAT CGT GAA G
T6-F5	5′-TTT TTT GGT CCG GTC ATA AAG CGA TAA G
T6-F9	5′-TTT TTT GTG GAA AGT GGC AAT CGT GAA G

Subsequent to incubation for 3 h, the mixture was concentrated by ultrafiltration. Conjugates were purified by anion-exchange chromatography [19] and quantified spectrophotometrically (Cary 100 Bio), using $\varepsilon_{405} = 102\ mM^{-1}\ cm^{-1}$ for HRP [20] and $\varepsilon_{450} = 28.2\ mM^{-1}\ cm^{-1}$ [21] for GOX conjugates.

Gelelectrophoretic binding studies. Samples for gelelectrophoretic analyses (Fig. 2, see also Supplementary material S1) were prepared by mixing respective amounts of enzyme conjugates and complementary oligonucleotide carrier strands in a total volume of 10 µl in TBS buffer (150 mM NaCl, 20 mM Tris, pH 7.3). The samples were incubated for 30 min at room temperature. After adding 2 µl 6× tracking dye (Massruler Loading Dye #R0631, Fermentas), the samples were loaded on a precasted 4–20% Tris–HCl gradient polyacrylamidgel (Biorad). 10 µl 50 bp ladder (O'Range #0613, Fermentas) was used as marker. Electrophoresis was performed at 4 °C under constant 120 V for 2.5 h. The bands were visualized using the Sybr Gold staining kit (Molecular Probes).

Kinetic measurements. Supramolecular DNA–bienzyme complexes **1**–**4** (Fig. 1B) were assembled in wells of microplates, containing the complementary carrier strands. The microplates were prepared by initial coating with Streptavidin (STV) and subsequent binding of biotinylated capture oligomers bcF9cF5, bcF5cF9, or as control, an equimolar mixture of cF9-2 and cF5-2, using the previously described protocol [22]. Subsequent to immobilization, 150 fmol of the DNA–enzyme conjugates were allowed to bind for 90 min. The plate was washed with TETBS (150 mM NaCl, 20 mM Tris, 5 mM EDTA, 0.05% Tween 20, pH 7.3), and Kpi300 (50 mM Potassiumphosphat, 300 mM NaCl, pH 7.4) buffer. To start the reaction, 100 µl of substrate solution, containing 1 mM glucose, 20 µM Amplex Red in KPi300 and optional 1 µM Catalase were added to each well. The fluorescence emission was monitored continuously at 590 nm (λ_{ex} 530 nm) in a Microplate Reader (Synergy II, Biotek), and initial rates were determined using the associated Software Gen5 (Biotek). Typically, data were determined by at least four independent measurements.

Results and discussion

Synthesis and assembly of DNA–enzyme conjugates

To assemble the supramolecular DNA–enzyme complexes **1**–**4**, depicted in Fig. 1B, covalent conjugates of GOX and HRP and 5′-thiolated oligonucleotides F9 and F5, respectively, were synthesized as previously described [19]. Oligonucleotide sequences of F9, F5, and of the complementary carrier strands bcF5, bcF9, bcF5cF9, bcF9cF5, bcF9-2, and bcF5-2, were chosen due to their highly specific and uniform hybridization efficiency [23,24]. In addition to the conjugates GOX-F9 and HRP-F5, additional conjugates of GOX and HRP were synthesized from oligonucleotides comprising a T_6-spacer in between the alkylthiol group and the 5′-end of the coding sequence. These conjugates were termed as GOX-T6-F9 and HRP-T6-F5. To analyze influences of steric constraints, one additional conjugate of GOX was synthesized, in which the enzyme was linked to the 3′-end of the oligomer F9 (GOX-3′-F9).

To initially investigate the DNA-directed assembly of GOX- and HRP-conjugates, experiments in homogeneous solution were performed and analyzed by native polyacrylamide gelelectrophoresis (Fig. 2). To study effects of the spatial arrangement on the efficiency of DNA-directed assembly, GOX-F9 and GOX-3′-F9 conjugates were used, in which GOX was linked to either the 5′- or the 3′-end, respectively, of the oligonucleotide F9. As shown in Fig. 2, all three enzyme conjugates revealed a single band (lanes 1, 9, and 14), which indicated their integrity and purity. Addition of carrier oligomer led to slight changes in electrophoretic mobility and increased band intensities, due to higher DNA content of these

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