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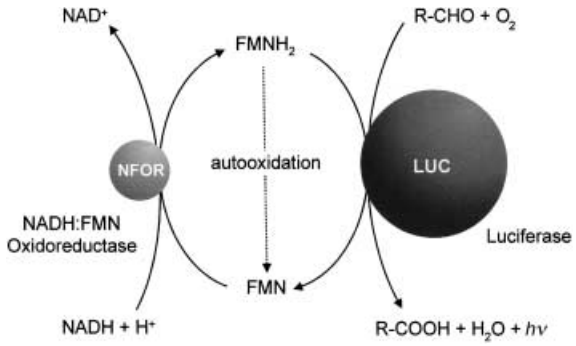

Figure 1. *Schematic representation of the bienzymic reaction cascade catalyzed by NAD(P)H:FMN Oxidoreductase (NFOR) and Luciferase (Luc). R = $CH_3(CH_2)_{10}$.*

DNA-Directed Assembly of Bienzymic Complexes from In Vivo Biotinylated NAD(P)H:FMN Oxidoreductase and Luciferase

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The DNA-directed assembly of proteins offers a promising route to the generation of spatially ordered multienzyme complexes (MECs), which are not accessible by conventional chemical cross-linking or genetic engineering.[1] MECs with several catalytic centers arranged in a spatially defined way are abundant in nature. Mechanistic advantages of MECs are revealed during the multistep catalytic transformation of a substrate since reactions limited by the rate of diffusional transport are accelerated by the immediate proximity of the catalytic centers. Furthermore, the "substrate-channeling" of intermediate products avoids side reactions. Artifical multienzymes would allow the development of novel catalytic systems for enzyme process technology that are capable of regenerating cofactors,[2] as well as multistep chemical transformations;[3–5] they are also useful for exploration of proximity effects in biochemical pathways.

Herein we report the initial steps towards the development of artificial multienzyme complexes through the DNA-directed assembly of two enzymes, NAD(P)H:FMN Oxidoreductase (NFOR) and Luciferase (Luc), which catalyze two consecutive reaction steps (Figure 1). NFOR reduces flavin mononucleotide (FMN) to $FMNH_2$ by using nicotinamide adenine dinucleotide (NADH) as an electron donor. $FMNH_2$ dissociates from NFOR and binds to Luc. In a second step, dodecanal is oxidized by Luc with the aid of molecular oxygen and emits blue-green light.[6] The spontaneous autooxidation of $FMNH_2$ may take place as a competing side reaction.

To facilitate the DNA-directed organization of NFOR and Luc, we chose a modular construction approach, based on the high-affinity biotin – streptavidin coupling system (Figure 2). To avoid damage of the enzymes through chemical biotinylation procedures, and to improve the regioselective coupling of the DNA and the protein moieties, we employed the in vivo biotinylated

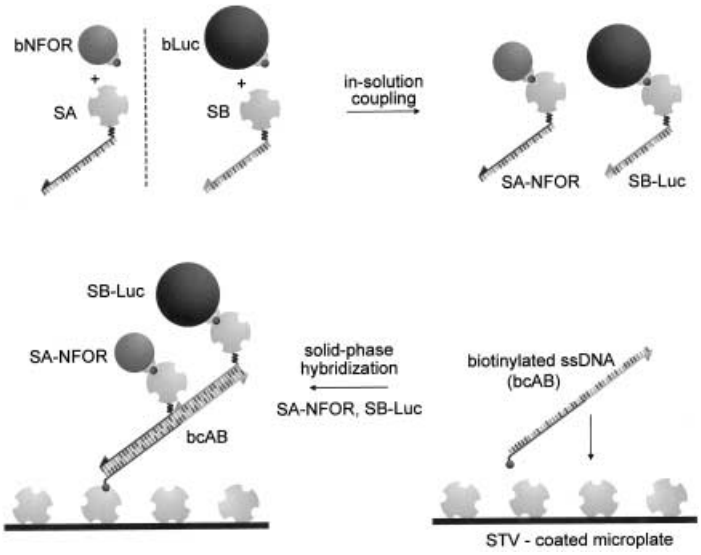

Figure 2. *Schematic representation of the DNA-directed organization of NFOR and Luc. A modular construction approach was chosen which used in-vivo biotinylated recombinant enzymes, bNFOR and bLuc, each with a single biotin group attached at the amino terminus. The biotinylated enzymes were coupled with covalent DNA – streptavidin (STV) conjugates, SA or SB, and the resulting enzyme – DNA conjugates (SA-NFOR and SB-Luc shown) self-assemble on a single-stranded DNA carrier containing complementary sequence stretches (for example, bcAB). The 3′-ends of the DNA fragments are indicated by arrow heads. Note that the schematic drawing of the SA – NFOR and SB – Luc is simplified, since conjugate species of other stoichiometry are also present in the assembly reaction.*

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recombinant enzymes bNFOR and bLuc, which each contain a single biotin group attached at the amino terminus.[7] The biotinylated enzymes were coupled with "biomolecular adapters", covalent conjugates comprised of the biotin-binding protein streptavidin (STV) and single-stranded DNA oligomers.[8] Two different DNA – STV conjugates, SA and SB, which contain individual oligonucleotide sequences, were used to prepare enzyme – DNA conjugates such as SA – NFOR and SB – Luc. The specificity of Watson – Crick base pairing leads the enzyme – DNA conjugates to self-assemble on a single-stranded DNA carrier (for example, bcAB in Figure 2) that contains complementary sequence stretches. The DNA carrier was bound to STV-coated microplates through a 5′-terminal biotin group prior to hybridization.

To produce in vivo biotinylated enzymes, the DNA plasmids which encode for the recombinant bNFOR and bLuc enzymes, pRSET-C-BCCP-oxidoreductase and pET-28C-BCCP-luciferase, respectively, were obtained from R. J. Stewart[7]. Downstream of the T7 promoter sequence, the plasmids contain the coding sequences for a 6 × His tag, the 249 base pair (bp) sequence of a portion of the *Escherischia coli* biotin carboxy carrier protein (BCCP), and the sequences for the enzymes (the oxidoreductase gene of *Vibrio fisheri*, or the α- and β-subunit of the luciferase gene of *Vibrio harveyi*). The 83 amino acid BCCP peptide on the N-terminal side of the fusion protein functions as the acceptor of a single biotin residue in the posttranslational modification of the protein by *E. coli* biotin ligase.[9] Overexpression of the fusion proteins and one-step purification by Ni-NTA (nickel – nitrilotriacetic acid) agarose affinity chromatography[7] yielded the recombinant enzymes with a high specific activity. The extent of in vivo biotinylation of the enzymes varied from culture to culture but was about 30 ± 10%.

Covalent conjugates of DNA and STV (SA and SB in Figure 2), with a single oligonucleotide moiety per STV, were synthesized from 5′-thiolated oligonucleotides and recombinant STV by chemical cross-linking, as previously described.[8] To study the formation of the DNA conjugates of NFOR and Luc a fixed amount of conjugate SB (12 pmol) was mixed with various molar amounts of the biotinylated enzymes, taking into account the extent of in vivo biotinylation of the particular enzyme batches. The samples were analyzed by nondenaturing acrylamide gel electrophoresis, staining of the DNA with the fluorescent dye SYBR-Gold (Molecular Probes), and densitometric analysis of the bands (Figure 3).

The coupling of the biotinylated enzyme to the DNA – STV conjugate led to the formation of conjugate species with reduced electrophoretic mobility. In the case of the 28 kDa protein NFOR, the two new bands that appeared as the amount of the biotinylated enzyme was increased were assigned as conjugates of varying stoichiometry, SB – $NFOR_1$ and SB – $NFOR_2$ (Figure 3). The three conjugates SB, SB – NFOR, and SB – $NFOR_2$, were present in a relative ratio of about 1:10:10 when an equimolar coupling ratio was used. At a coupling ratio of SB:NFOR = 1:2, a shoulder in the high molecular weight region indicated the formation of a third conjugate, probably the adduct SB – $NFOR_3$ (Figure 3C). Similarly, in the case of the 77 kDa protein Luc, the two new bands that appeared as the amount of

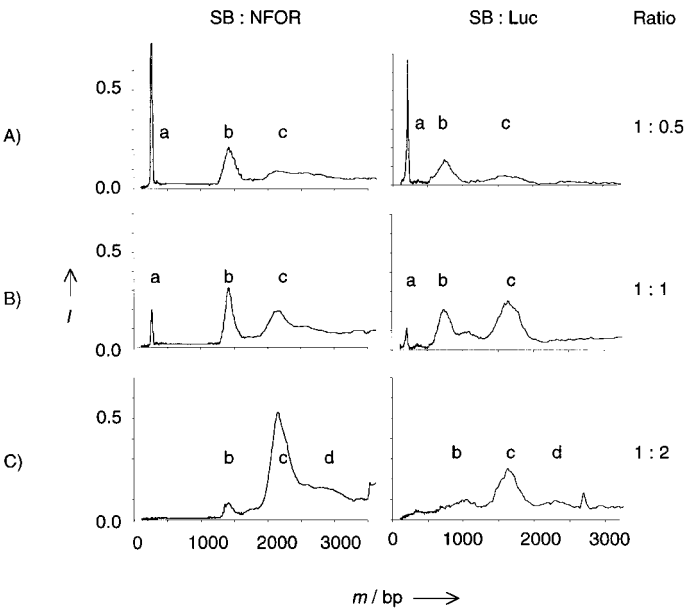

Figure 3. *Lineplot of the gel-electrophoretic analysis of the DNA – enzyme conjugates. Coupling of the DNA – STV conjugate SB with 0.5, 1, or 2 molar equivalents of in vivo biotinylated enzyme NADH:FMN Oxidoreductase (NFOR; left panel) or Luciferase (Luc; right panel) is shown. The bands with a lower electrophoretic mobility than the DNA – STV conjugate SB (position a) were assigned as SB – enzyme$_1$ (b), SB – enzyme$_2$ (c), and SB – enzyme$_3$ (d). The intensity (I) of the bands is plotted against the mobility (m) of the conjugates, as compared to a 123 base pair ladder DNA molecular weight marker (GIBCO); m is given as lengths in base pairs (bp).*

the biotinylated enzyme was increased were assigned as conjugates SB – Luc and SB – Luc_2. No significant amounts of the adduct SB – Luc_3 were observed at higher coupling ratios, probably for steric reasons (Figure 3C). The three conjugates SB, SB – Luc, and SB – Luc_2, were present in a relative ratio of about 1:8:12 when an equimolar coupling ratio was used. Similar results were obtained from coupling experiments with the DNA – STV conjugate SA (data not shown).

To study the DNA-directed assembly, fixed amounts of biotinylated NFOR and Luc were mixed with equimolar quantities of DNA – STV conjugate, SA or SB, taking into account the extent of in vivo biotinylation of a particular enzyme batch. The resulting conjugates, such as SA – NFOR or SB – Luc, were then allowed to bind to their corresponding oligomer complement, bcA or bcB, respectively. The complementary oligomers were previously immobilized on an STV-coated microplate by using the STV – biotin interaction.[10] Subsequent to hybridization, the enzymatic activity of the two enzymes was measured by using the two-step assay shown in Figure 1. In negative controls, the DNA – enzyme conjugates were incubated in microplate wells that contained the noncomplementary oligonucleotide bcD. No signficant enzymatic activity was observed in such controls, which confirmed that the signals listed in Tables 1 and 2 are caused by binding that occurs exclusively through specific DNA hybridization. The absolute signal intensities obtained from independent microplates were poorly reproducible (compare column 6 of Table 1 and column 2 of Table 2). However, very good reproducibility was observed for various experiments

Table 1. *Influence of the oligonucleotide sequences in the DNA-directed assembly of bienzyme complexes.*

	Enzyme activity [RLU][a]					
	1	2	3	4	5	6
carrier	none[c]	bcA	bcB	bcA/bcB	bcA/bcB	bcAB
conjugates	bNFOR	SA-NFOR	SB-NFOR	SB-NFOR	SA-NFOR	SA-NFOR
	bLuc	SA-Luc	SB-Luc	SA-Luc	SB-Luc	SB-Luc
amount of binding sites[b]						
1 pmol[b]	86 ± 4	208 ± 10	130 ± 18	161 ± 10	92 ± 9	252 ± 11
2 pmol[b]	148 ± 15	362 ± 26	238 ± 19	306 ± 15	183 ± 16	375 ± 37

[a] The numbers indicate maximum relative light units (RLU), a measure of the coupled NFOR – Luc enzymatic activity. [b] Amount of oligonucleotide binding sites for SA- and SB-enzyme conjugates immobilized in the microplate well. [c] Direct immobilization of biotinylated enzymes on STV-coated plates.

Table 2. *Influence of the spatial positioning of bienzyme complexes on the overall enzymatic activity.*

	Enzyme activity [RLU][a]			
	1	2	3	4
carrier	bcAB	bcAB	bcBA	bcBA
conjugates	SB-NFOR	SA-NFOR	SB-NFOR	SA-NFOR
	SA-Luc	SB-Luc	SA-Luc	SB-Luc
amount of binding sites [b]				
1 pmol[b]	412 ± 7	480 ± 33	447 ± 64	247 ± 16
2 pmol[b]	593 ± 49	743 ± 54	760 ± 29	420 ± 32

[a] The numbers indicate maximum relative light units (RLU), a measure of the coupled NFOR-Luc enzymatic activity. [b] Amount of oligonucleotide binding sites for SA and SB immobilized in the microplate well.

carried out on a single microplate (see deviations in Tables 1 and 2 for $n \geq 3$). This observation suggests that experimental errors were mainly caused by changes in the quality of the capture plates. Consequently, the two sets of data shown in Tables 1 and 2 were each obtained from individual microplates.

As indicated in Table 1, the signal intensities obtained by the DNA-directed immobilization were generally much higher than those obtained by immobilization of the biotinylated enzymes directly on the STV-coated microplates. The superior efficiency of the DNA-directed immobilization might be a result of the lean structure of the rigid double-helical DNA spacer and the reversibility of nucleic acid hybridization, which should provide a larger effective surface area and enable denser packing of the enzyme layer. In addition, the larger distance between the surface and the enzyme might allow for a higher enzymatic activity caused by a more homogeneous type of substrate transformation reaction.[10b]

The sequences of the DNA oligomers employed in the DNA-directed assembly process influence the hybridization efficiency of the enzyme conjugates. When both NFOR and Luc are immobilized through identical sequences (Table 1, columns 2, 3), the signal intensities obtained with capture oligomer bcB are 35% smaller than the values obtained for the bcA capture. These results precisely correlate with the sequence-specific hybridization efficiencies that result from variations in the thermodynamic duplex stability and the hybridization kinetics of these two sequences.[10a] Furthermore, the sequence-dependent hybridization efficiencies are affected by the size of the enzymes. When an equimolar mixture of bcA and bcB was immobilized to provide the capture oligomers, intermediate signal intensities were observed when the bulky enzyme Luc was coupled to SA (compare column 4 with columns 2 and 3 in Table 1). The signals were reduced by about 40% when the bulky Luc was immobilized by the less efficient sequence SB (compare columns 4 and 5).

To investigate whether a spatial proximity of the DNA-immobilized enzymes might affect the coupled enzymatic activity, we compared the random assembly that used a mixture of bcA and bcB with the spatially controlled assembly that utilised the longer carrier bcAB. This longer carrier contained two binding sites, one for SA and one for SB (Figure 4; Table 1, columns 5, 6). The enzymatic acitivities observed were more than twice as high in the case of the bcAB template as for the mixture of bcA and bcB, which suggests that the immediate spatial proximity of the two enzymes is beneficial for the overall activity of the bienzymic system. When the absolute amounts of enzymes immobilized were decreased from 2 to 1 pmol, the relative signal increase as compared to the respective data of the random assembly was enhanced from 200 to 270% (Figure 4). A lower surface coverage should lead to an increased distance of diffusion of the intermediate $FMNH_2$ between the two randomly assembled enzymes (Table 1, column 5). Therefore, the increase in relative signal with decrease in amount of immobilized enzyme further supports our hypothesis that the immediate spatial proximity (column 6) enhances the coupled enzymatic activity.

To further elucidate the effects of the spatial arrangement of the two enzymes, various permutations of the conjugates and the binding sites were studied (Table 2). Two different carriers, bcAB and bcBA, were used. These carriers contained either the efficient cA binding site (Table 2, columns 1, 2) or the less efficient cB binding site (Table 2, columns 3, 4) close to the surface, respectively. With carrier bcAB, higher signal intensities were obtained when the bulky Luc was immobilized through DNA – STV conjugate SB, despite its lesser binding efficiency (compare columns 1 and 2 in Table 2). This result suggests that steric hindrance significantly affects the formation of the bienzymic complexes. Experiments with carrier bcBA confirm this assumption. The highest signals were obtained when the small NFOR occupies the binding site in close proximity to the surface, and the bulky Luc is bound at a distance from the surface (compare columns 3 and 4 in Table 2). The steric effects obviously override the influences of the sequence-specific binding efficiencies. The latter, however, are still apparent from

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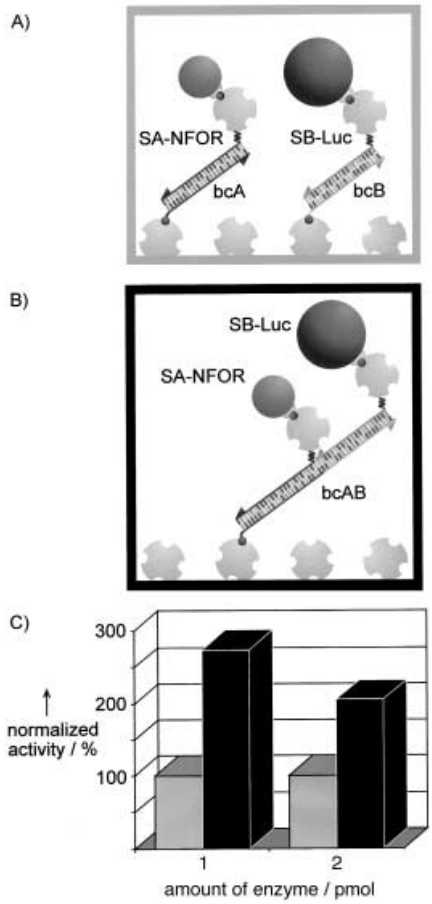

Figure 4. *Graphical representation of the effect of spatial proximity on the activity of bienzymic constructs. The heights of the histograms (C) correspond to the overall enzymatic activities obtained from conjugates (A) immobilized through random hybridization (grey bars) or (B) from assembly in direct proximity at a DNA carrier strand (dark bars). The data correspond to the coupled enzymatic activities listed in column 6 of Table 1, normalized as a percentage fraction of the activities shown in column 5 of Table 1. Note that in the case of low surface coverage (1 pmol of each of the two enzymes), the relative increase in activity is higher than in the case of a denser surface coverage (2 pmol).*

a comparison of the two cases in which Luc is either bound in proximity to, or at a distance from, the surface (columns 1 versus 4, and 2 versus 3, in Table 2).

In conclusion, we have shown that DNA-directed immobilization of proteins can be used for the efficient generation of artificial bienzymic complexes. This approach allows for the rational construction of spatially well-defined oligofunctional protein assemblies on the nanometer length scale. Further studies will concern the detailed investigation of the catalytic properties of DNA-linked NFOR/Luc bienzymes. In particular, kinetic measurements at substrate concentrations below the Michaelis – Menten constant K_M will provide deeper insights into substrate channeling and neighbourhood effects of multienzyme complexes. Potential applications of such supramolecular systems include their use as signal amplification devices in biosensors as well as novel catalysts for enzyme process technology capable of regenerating cofactors, or enzymes to perform multistep chemical transformations.

Experimental Section

Overexpression of the NFOR and Luc fusion proteins was carried out as described.[7] The individual activities of NFOR and Luc were determined spectrophotometrically by the consumption of NADH or by dodecanal oxidation-dependent generation of light.[7] Details on the structure of the DNA vectors, the expression procedure, yields, extent of biotinylation, and enzymatic activity of the fusion proteins are available in the Supporting Information. Synthesis and purification of covalent DNA – STV conjugates, SA and SB, were carried out from the corresponding thiolated oligonucleotides, 5′-thiol-TCC TGT GTG AAA TTG TTA TCC GCT′ (SA) and 5′-thiol-GTA ATC ATG GTC ATA GCT GTT-3′ (SB), respectively, and recombinant streptavidin (IBA, Göttingen), by a method similar to that already described.[8] Preparation of the oligonucleotide-coated capture plates was carried out as described in ref. [10a]. The sequences of the biotinylated capture oligomers were 5′-biotin-AGC GGA TAA CAA TTT CAC ACA GGA-3′ (bcA), 5′-biotin-AAC AGC TAT GAC CAT GAT TAC-3′ (bcB), 5′-biotin-AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC TAT GAC CAT GAT TAC-3′ (bcAB), 5′-biotin-AAC AGC TAT GAC CAT GAT TAC-AGC GGA TAA CAA TTT CAC ACA GGA-3′ (bcBA), and 5′-biotin-GGA TCC TCT AGA GTC GAC CTG-3′ (bcD).

DNA conjugates of bNFOR and bLuc were obtained from stock solutions of the enzymes (1 μM), mixed with similar amounts of the DNA – STV conjugates, SA or SB (1 μM in a buffer containing 10 mM Tris-HCl (tris(hydroxymethyl)aminomethane-HCl), 1 mM ethylenediaminetetraacetate, pH 7.5), and incubated for 15 min at room temperature. Equimolar amounts of the two DNA – enzyme conjugates, for example SA – Luc and SB – NFOR, were mixed and diluted with the Tris buffer (final enzyme concentration of 10 nM). This mixture (250 μL) was applied to microplate wells that contained DNA capture oligomers and incubated for 45 min at room temperature. Phosphate reaction buffer (225 μL, pH 6.3), which contained FMN (4 μM) and dodecanal (0.0001 % v/v), was then added. The reaction was started by addition of NADH (1 mM) in phospate buffer (25 μL). Light intensities were measured with a Victor Multilabel-Counter (Wallac) and the activity was reported as relative light units (RLU).

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