A new application of the yeast two-hybrid system in protein engineering

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Cytochromes P450 are involved in the biosynthesis of steroid hormones in mitochondria of the adrenal gland. The electrons required for these reactions are provided via a redox chain consisting of adrenodoxin reductase (AdR) and adrenodoxin (Adx). A prerequisite for a fast and efficient electron transfer as well as high catalytic activity is the formation of functional complexes between the different redox partners. To improve the protein–protein interactions by directed evolution, we developed a new in vivo selection system. This high-throughput screening method is based on the yeast two-hybrid system. It enables a background-free screening for increased protein–protein interactions between stable and functional species including cofactor-containing proteins (FAD, [2Fe–2S], heme). The method was successfully applied for the directed evolution of Adx and selected variants were analyzed biochemically and biophysically. All analyzed proteins exhibit typical characteristics of [2Fe–2S]-cluster-type ferredoxins. Adx-dependent substrate conversion assays with different cytochromes demonstrated that the improved ability of the mutants to form complexes results in an enhanced catalytic efficiency of the cytochrome P450 system.

Keywords: cytochromes P450/directed evolution/ferredoxins/high-throughput screening method/two-hybrid system

Introduction

Adrenodoxin (Adx) is an iron–sulfur protein that belongs to the broad family of the [2Fe–2S]-type ferredoxins found in plants, animals and bacteria. Its primary function as a soluble electron carrier between the NADPH-dependent adrenodoxin reductase (AdR) and several cytochromes P450 makes it an irreplaceable component of the steroid hormone biosynthesis in the adrenal mitochondria of vertebrates. Bovine Adx participates in the biosynthesis of steroid hormones by mediating the electron transfer to the heme iron of two steroid hydroxylating cytochromes P450, CYP11A1 and CYP11B1, which are localized in the inner mitochondrial membrane of the adrenal cortex (Grinberg et al., 2000). Adx displays a compact (α+β) fold typical for [2Fe–2S]-ferredoxins (Müller et al., 1998; Pikuleva et al., 2000) and is organized into a large core domain and a smaller interaction domain. The interaction domain includes an acidic region that was shown to be mainly responsible for the recognition of the redox partners AdR and cytochrome CYP11A1 (Vickery, 1997). Additional sites involved in redox partner binding on the core domain have been identified in the resolved crystal structure of a cross-linked 1:1 complex of Adx and AdR (Müller et al., 2001a) and by site-directed mutagenesis studies (Hannemann et al., 2001).

Directed molecular evolution of functional proteins has emerged as an alternative to traditional forms of protein engineering, such as structure-based site-directed mutagenesis (Kunkel, 1985). Directed evolution involves multiple cycles of random gene mutagenesis and/or DNA recombination followed by screening or selection. The advantage of this technique is that knowledge of structural data, relationship between sequence, structure and mechanism is not required for a fast generation of a huge number of mutants. This approach has been applied to improve the properties of very different types of enzymes, for example the enantioselectivity of lipases (Reetz and Jaeger, 2000), the hydroxylation activity and selectivity of cytochrome P450 enzymes (Budde et al., 2006) and the substrate specificity of aldolases (Fong et al., 2000). The success of any attempt to apply directed evolution of proteins depends not only on an efficient random mutagenesis or DNA recombination method but also on an efficient and rapid high-throughput screening system (HTS) (Arnold, 2001; Reetz, 2002).

While directed evolution has been frequently applied to improve inter-protein complex formations such as antibody–antigen interactions (Boder et al., 2000), there are, to the best of our knowledge, no reports on the evolution of functional cofactor-containing catalytic protein complexes. In this study, we established a HTS that enables the selection for increased binding between cofactor-containing proteins. The approach is based on the yeast two-hybrid system (THS) (Fields and Song, 1989) and was successfully applied for the molecular evolution of Adx. Biochemical and biophysical analyses of the created Adx variants demonstrated that the improved interaction of Adx to its redox partners results in an increase of the catalytic efficiency of the investigated cytochrome P450 system.

Material and methods

Saccharomyces cerevisiae: Strain, transformation and THS colony lift assay

All experiments in which yeast cells were involved were performed using the Saccharomyces cerevisiae strain Y190. Transformation of the yeast cells, the colony lift assay and the extraction of yeast proteins for immunoblot analysis were performed according to the instructions of BD Bioscience Clontech (Clontech, 2001).

Bacterial strains

Escherichia coli strain BI21 (DE3) pLysS (Novagen) was used for Adx protein expression. All other experiments (construction of mutant libraries, site-directed mutagenesis,
plasmid preparation) were performed with the *E. coli* strain TOP10F* (Invitrogen).

**SDS-Page and immunoblot analysis**

Yeast cells carrying the respective plasmid coding for the activation domain (AD) or the binding domain (BD) were grown at 30°C in 250 ml of the suitable SD media (Clontech, 2001) to an optical density of OD600nm = 1.0. Protein extraction was performed with the ‘Yeast Protein Extraction Reagent (Y-PER TM)’ (Pierce) according to the manual. After the electrophoretical separation of the yeast proteins by SDS-PAGE, the protein extracts were blotted onto a nitrocellulose membrane. The following western-blot analyses were performed according to the ‘ECL Plus’ kit manual (Amersham Bioscience), using the primary antibodies ‘GAL4-AD’ and ‘Gal4-TA’ proteins by SDS-PAGE, the protein extracts were blotted onto a nitrocellulose membrane. The following western-blot analyses were performed according to the ‘ECL Plus’ kit manual (Amersham Bioscience), using the primary antibodies ‘GAL4-AD’ and ‘Gal4-TA’ (BD Bioscience Clontech). The secondary antibody was ‘Anti-mouse IgG’ (Dako).

**Generation of Adx mutant libraries by error prone PCR and Staggered Extended Process**

The error prone PCR (epPCR) was performed with the ‘GeneMorph™ PCR Mutagenesis Kit’ (Stratagene) according to the manual, using 200 nmol of the primers ‘Nadx4Nco’ (5'-CGC ATC CAT GGA TAA AAT AAC ATG CC-3'), *Nco* I site used for cloning underlined) and ‘Buffy’ (5'-AAC TTG CGG GTG TTT TCA GTA TCT A-3'), which is complementary to a sequence 50 bp upstream of the *Nco* HI site in the plasmid pACT2 itself.

For the recombination of Adx mutants, the ‘Staggered Extended Process’ (STEP) method was used (Zhao et al., 1998). For this technique, the ‘Taq DNA-Polymerase’ and the corresponding buffer (Qbiogene), as well as the ‘PCR-dNTP-Mix’ (Bioline) were used together with the primer combination ‘Nadx4Nco’ and ‘Buffy’. The PCR program was as follows: (i) 95°C for 30 s, (ii) 95°C for 30 s, (iii) 50°C for 1 min and (iv) repeat steps (ii) and (iii) 99 times.

**DNA recovery from yeast cells**

The recovery of the AdxC-dDNA after successful screening was performed via yeast-colony-PCR using ‘Taq-DNA-Polymerase’ and the corresponding buffer (Qbiogene), as well as the ‘PCR-dNTP-Mix’ (Bioline) were used together with the primer combination ‘Nadx4Nco’ and ‘Buffy’. The PCR program was as follows: (i) 95°C for 30 s, (ii) 95°C for 30 s, (iii) 50°C for 1 min and (iv) repeat steps (ii) and (iii) 99 times.

**Site-directed mutagenesis**

The introduction of site-directed mutations into Adx was performed according to the Stratagene manual using the ‘Pfu DNA-Polymerase’ and the corresponding buffer (Promega) and the ‘PCR-dNTP-Mix’ (Bioline). The used pair of primers was ‘ABA110T’ (5'-GTA CCT GAT ACC GTG TCT GAT GCC CCG-3') and ‘ABA110Trev’ (5'-GAC ATC AGA CAC GGT ATC AGG TAC-3'), for the substitution of Adx amino acid 110 (underlined), ‘D113Yfor’ (5'-GCC GTG TCT TAT GCC AGA GAG-3') and ‘D113Yrev’ (5'-CTC TCT GCC GAC GGC-3') for the substitution of Adx amino acid 113 (underlined), as well as ‘ABS125P’ (5'-GGC ATG AAC TCC CCA AAG ATA GAA-3') and ‘ABS125Prev’ (5'-TTT TAT ATT TTT GGA GTT CAT GCC-3') for the substitution of Adx amino acid 125 (underlined).

**Protein purification**

The cDNAs of the Adx variants were cloned into the expression vector pET3d (Novagen) using the *Nco* I and *Bam* HI restriction sites. The expression of the Adx proteins in the *E. coli* strain BL21 (DE3) pLysS (Novagen) as well as the purification of the proteins was performed according to Uhlmann et al., (1992). Recombinant bovine proteins, i.e. AdR, CYP11A1 and CYP11B1, were expressed and purified as described elsewhere (Ikushiro et al., 1989; Lepeshev and Usanov, 1998).

**Surface plasmon resonance measurements**

*In vitro* formation of the Adx/AdR and Adx/CYP11A1 complexes was assayed on a Biacore 2000 system using CM5 chips with a carboxymethylated dextran surface as previously described (Zöllner et al., 2002). Adx species were covalently coupled (200 resonance units) and the binding analysis was carried out by injecting different AdR and CYP11A1 concentrations of the oxidized proteins, in the range from 0.1 to 1 μM with a flow rate of 10 μl/min and recording the refractive index changes. All protein-containing solutions were prepared in Biacore HBS-EP buffer (0.01 M HEPES buffer pH 7.4, 0.15 M NaCl, 3 mM EDTA with 0.005% Surfactant P20). Analysis of the binding curves and the determination of *Kd*-values was performed using the Biacore evaluation software 3.1.

**Adx-dependent substrate conversion**

The conversion of cholesterol to pregnenolone by CYP11A1 and conversion of 11-deoxycorticosterone to corticosterone by CYP11B1 was determined with a reconstituted system as described with slight modifications (Beekert et al., 1994; Sugano et al., 1989), using the purified recombinant proteins Adx, AdR, CYP11A1 and adrenal CYP11B1. The reaction buffer was 50 mM HEPES (pH 7.4) including 0.05% Tween 20. The final concentration of the components was 0.4 μM CYP11A1 or 0.8 μM CYP11B1, 0.5 μM AdR, 400 μM cholesterol or 100 μM 11-deoxycorticosterone, 5 μM glucose-6-phosphate, 1 μM MgCl₂, 1 U glucose-6-phosphate-dehydrogenase, 100 μM NADPH and 0.4 U cholesterol oxidase. Cortisol was used as internal standard. The concentration of the Adx variants was within a range from 0.2 to 30 μM. The reaction mixture was incubated for 10 min at 37°C under mild agitation. The steroids were extracted with chloroform, dried and resuspended in 200 μl acetonitrile. Reversed phase high performance liquid chromatography (HPLC) was carried out using a C18 column (Waters) and an isocratic solvent-system consisting of acetonitrile/isopropanol (30:1) for CYP11A1 and acetonitrile/water (60:40) for CYP11B1 reactions.

**Results**

**Set-up and evaluation of the screening system**

In this study, we used the original yeast THS (Fields and Song, 1989) for the development of a new HTS for enhanced protein–protein interactions, especially of proteins that...
contain cofactors. The overall goal was to discover new protein variants that exhibit an enhanced complex formation as well as an increased catalytic efficiency. Here, we choose the mitochondrial steroid hydroxylating P450 electron chain as the test system since it has been shown that an increased interaction between the different components directly affects the Adx-dependent substrate conversion (Grinberg et al., 2000).

While developing the new technique, we considered the length of the ADH1-promoter to be crucial for the expression of the THS fusion proteins, as observed by Legrain (Legrain et al., 1994). As shown in Fig. 1, the longer ADH1-promoter of pODB8 resulted in a high expression (HE) of the BD-protein compared with pBridge (LE, low expression), indicated by the immunoblot analysis, where the signal on the ‘Hyperfilm™ ECL’ was clearly verifiable using the plasmid pODB8, whereas no signal could be detected using the BD-plasmid pBridge. The figure has been composed out of two different X-ray films.

The THS assays with CYP11A1 revealed that the affinity of Adx wt to CYP11A1 was not sufficient to cause a positive signal in the colony lift assay, neither with high-level-expressed nor with low-level-expressed CYP11A1. However, Adx T54S and Adx S112W, which have exhibited an improved affinity to CYP11A1 in former studies, did not induce a signal in the colony lift assay, in contrast to Adx 4-108 and Adx S112W (Table II). Taking the results of Adx 4-108 and Adx S112W into account, the affinity improvement measured in vitro with optical difference spectroscopy has to

Table I. Effect of different expression levels of the BD-fused protein AdR on the detectable interaction between certain Adx variants and AdR, using the THS colony lift assay

<table>
<thead>
<tr>
<th>Adx variants</th>
<th>Relative interaction with AdR according to the literature</th>
<th>AdR (HE)</th>
<th>AdR (LE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adx wt</td>
<td>1.00</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Adx 4-108</td>
<td>1.24</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adx 1-115</td>
<td>1.24</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adx T54S</td>
<td>0.91</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adx T76E</td>
<td>11.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adx T49Δ</td>
<td>3.00</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adx S112W</td>
<td>n.d.</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

n.d., not determined.

The subsequent colony lift assay has been performed three times with independent yeast co-transformations for every protein pair, whereas only plates have been used with at least 100 colonies. From these transformations, only colony lift assays that displayed at least 95% blue-colored colonies were considered to show a significant interaction (Tables I and II). The results confirmed that the sensitivity of the THS signal increases with the size of the promoter used in the expression vectors pBridge and pODB8.

To validate the system, different Adx variants in pACT2 were chosen according to prior in vitro affinity studies. Compared to Adx wild-type (wt), the variants displayed improved binding to AdR and CYP11A1 (Adx T54S), increased interaction with CYP11A1 (Adx 4-108, Adx 1-115 and Adx S112W), and decreased affinity to both partner proteins (Adx T76E, Adx T49Δ) (Grinberg et al., 2000; Hannemann et al., 2001; Schifferl et al., 2001; Zöllner et al., 2002).

Table I. Effect of different expression levels of the BD-fused protein AdR on the detectable interaction between certain Adx variants and AdR, using the THS colony lift assay

<table>
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<td>Adx T54S</td>
<td>0.91</td>
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</tr>
<tr>
<td>Adx T76E</td>
<td>11.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adx T49Δ</td>
<td>3.00</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adx S112W</td>
<td>n.d.</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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The subsequent colony lift assay has been performed three times with independent yeast co-transformations for every protein pair, whereas only plates have been used with at least 100 colonies. From these transformations, only colony lift assays that displayed at least 95% blue colonies were identified as positive interaction pairs (+). Previously published data of the tested variants are shown for comparison (Grinberg et al., 2000; Schifferl et al., 2001; Zöllner et al., 2002). Relative interaction parameters were used, where the interaction between Adx wt and AdR has been set to one. Higher values show a decreased interaction to AdR, lower values an improvement of the interaction.

Fig. 1. Expression levels of the BD-protein containing cells with the two BD-plasmids pODB8 and pBridge. (A) Expression level of the DNA-BD without fusion partner. (B) Expression of the fusion proteins consisting of BD + AdR and BD + CYP11A1, respectively. Detection of the proteins has been performed using ‘ECL plus western detection reagents’ on ‘HyperfilmTM ECL’ (Amersham Biosciences): primary antibody ‘GAL4-DNA BD’ (BD Bioscience Clontech) and the secondary antibody ‘Anti-mouse IgG’ (Dako). The figure has been composed out of two different X-ray films.
Adx C46D4-108 were expressed within the same amounts and we investigated the effects on the interaction signal for both fusion-proteins containing Adx 4-108 or not show any interaction with its redox partners in the THS.

We constructed a plasmid with the truncated form Adx C46D4-108 to construct a plasmid with the truncated form Adx C46D4-108 to investigate this point, we used Adx as model and analyzed the assembly of the iron–sulfur cluster and thereby the cofactor is actually assembled under THS conditions. This question is not trivial, since the proteins of the THS are produced in the cytoplasm and transported into the nucleus. To investigate this point, we used Adx as model and analyzed the assembly of the iron–sulfur cluster and thereby the correct folding of Adx in yeast cells. The cDNA of the mutant Adx C46D in which the [2Fe–2S]-cluster cannot be correctly formed (Uhlmann et al., 1992) was inserted into the subsequent colony lift assay has been performed three times with independent yeast co-transformations for every protein pair, whereas only plates have been used with at least 100 colonies. From these transformations, only colony lift assays that displayed at least 95% blue colonies were identified as positive interaction pairs (+). Previously published data of the tested variants are shown for comparison (Grinberg et al., 2000; Schiffler et al., 2001; Zöllner et al., 2002). Relative interaction parameters were used, where the interaction between Adx wt and CYP11A1 has been set to one. Higher values show a decreased interaction to CYP11A1, lower values an improvement of the interaction.

Since Adx4-108 produced a THS-signal in cells containing the plasmid pACT2, Immunological detection of the proteins has been performed with ‘ECL plus western detection reagents’ using ‘Hyperfilm™ ECL’ (Amersham Biosciences): primary antibody ‘GAL4-AD’ (BD Bioscience Clontech) and secondary antibody ‘Anti-mouse IgG’ (Dako). The band at 31 kDa represents the fusion-protein consisting of AD and Adx, the band at 19 kDa contains only AD. The band in between is a degradation product of the fusion protein.

**Table II.** Effect of different expression levels of the BD-fused protein CYP11A1 on the detectable signal between certain Adx variants and CYP11A1, using the colony lift assay

<table>
<thead>
<tr>
<th>Adx variants</th>
<th>Relative interaction with CYP11A1 according to literature data</th>
<th>CYP11A1 (HE)</th>
<th>CYP11A1 (LE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adx wt</td>
<td>1.00</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adx4-108</td>
<td>0.32</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adx T54S</td>
<td>0.28</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adx T49Δ</td>
<td>14.60</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adx S112W</td>
<td>0.12</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The subsequent colony lift assay has been performed three times with independent yeast co-transformations for every protein pair, whereas only plates have been used with at least 100 colonies. From these transformations, only colony lift assays that displayed at least 95% blue colonies were identified as positive interaction pairs (+). Previously published data of the tested variants are shown for comparison (Grinberg et al., 2000; Schiffler et al., 2001; Zöllner et al., 2002). Relative interaction parameters were used, where the interaction between Adx wt and CYP11A1 has been set to one. Higher values show a decreased interaction to CYP11A1, lower values an improvement of the interaction.

be at least 3-fold compared to Adx wt to produce a detectable signal in the THS colony filter assay. Furthermore, only the affinity of Adx S112W to CYP11A1 is intense enough that an interaction could be visualized even with the LE-CYP11A1 (Table II).

Summarizing, the experimental set-up of the yeast THS can be used to identify Adx variants that display an improved interaction to both redox partners, AdR and CYP11A1. Additionally, using THS-plasmids with promoters of different strength, the starting selection conditions in our screening system can be regulated in such a way that only affinity-improved proteins can be selected without background of false positives.

**Assembly of the iron–sulfur cluster under THS conditions**

A crucial point in using the THS for interaction screening of cofactor-containing proteins is the question whether the cofactor is actually assembled under THS conditions. This question is not trivial, since the proteins of the THS are produced in the cytoplasm and transported into the nucleus. To investigate this point, we used Adx as model and analyzed the assembly of the iron–sulfur cluster and thereby the correct folding of Adx in yeast cells. The cDNA of the mutant Adx C46D in which the [2Fe–2S]-cluster cannot be correctly formed (Uhlmann et al., 1992) was inserted into the subsequent colony lift assay has been performed three times with independent yeast co-transformations for every protein pair, whereas only plates have been used with at least 100 colonies. From these transformations, only colony lift assays that displayed at least 95% blue colonies were identified as positive interaction pairs (+). Previously published data of the tested variants are shown for comparison (Grinberg et al., 2000; Schiffler et al., 2001; Zöllner et al., 2002). Relative interaction parameters were used, where the interaction between Adx wt and CYP11A1 has been set to one. Higher values show a decreased interaction to CYP11A1, lower values an improvement of the interaction.

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**Directed evolution of Adx**

To apply the THS screening method in a directed evolution study, we generated a mature bovine Adx mutant library using epPCR. The obtained Adx genes were then cloned into the THS vector pACT2. Sequencing of randomly selected clones out of the 6000 cDNAs containing library revealed a mutation rate of 0.89 mutations/Adx. This library was used for a yeast co-transformation with the LE-AdR-plasmid. A total of 25 000 yeast colonies have been investigated in the subsequent colony lift assay and three positive colonies have been identified during the screening procedure. All three variants have been identified as Adx A110T/D113Y/S125P (Adx TYP). No false positive colony has been detected in this screen, a result that shows the low error-rate of our selection method. With the aim to investigate which of the three substitutions was responsible for the increased affinity to AdR, we generated all possible single and double mutations of this variant by site-directed mutagenesis. These mutants were tested with the THS colony lift filter assay. Table III shows that all Adx variants displayed a positive signal in the interaction with HE-AdR, whereas an interaction with LE-AdR was only detectable in yeast cells containing mutants with the substitution of aspartate to tryptophane at amino acid position 113. This shows that only the mutation D113Y improved the complex formation between Adx and AdR.
epPCR-library together with the cDNAs of Adx D113Y of this library containing 5000 cDNAs, we used the StEP technique (Zhao et al., 1998). For the construction of this library containing 5000 cDNAs, we used the epPCR-library together with the cDNAs of Adx D113Y (Adx Y), Adx T54S and Adx T54S/D113Y, since these mutants showed no improved affinity to CYP11A1, but an increased binding with AdR (Tables I and II). A total of 20 000 yeast colonies, co-transformed with HE-CYP11A1 plasmid and the pACT2-StEP-library, were investigated and one positive colony was detected, which was identified as the double mutant Adx T54S/D113Y (Adx SY). This variant showed an increased affinity to CYP11A1 compared to mutants containing only the single substitutions T54S and D113Y.

Since we were looking for Adx species with an increased affinity to both redox partners, we screened the library also for Adx variants with an improved affinity to CYP11A1. We analyzed 25 000 yeast colonies co-transformed with the HE-CYP11A1 and the pACT2-epPCR-library of Adx in the colony lift assay. No improved variant was found, which might have been caused by the fact that mutants needed to exhibit at least a 3-fold higher affinity to CYP11A1 than the wt in order to produce a signal (Table II). Therefore, we created a second library via DNA recombination using the StEP technique (Zhao et al., 1998). For the construction of this library containing 5000 cDNAs, we used the epPCR-library together with the cDNAs of Adx D113Y (Adx Y), Adx T54S and Adx T54S/D113Y, since these mutants showed no improved affinity to CYP11A1, but an increased binding with AdR (Tables I and II). A total of 20 000 yeast colonies, co-transformed with HE-CYP11A1 plasmid and the pACT2-StEP-library, were investigated and one positive colony was detected, which was identified as the double mutant Adx T54S/D113Y (Adx SY). This variant showed an increased affinity to CYP11A1 compared to mutants containing only the single substitutions T54S and D113Y.

### Yeast two-hybrid system

**Table III.** Test for responsible mutation(s) of the triple Adx mutant Adx TYP using the colony lift assay

<table>
<thead>
<tr>
<th>Adx variants</th>
<th>AdR (HE)</th>
<th>AdR (LE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adx TYP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adx T</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Adx Y</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adx P</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Adx TY</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adx TP</td>
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<td>–</td>
</tr>
<tr>
<td>Adx YP</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

All possible combinations of mutations have been studied to analyze the detectable interaction to high (HE) and low (LE) expressed AdR, using the vectors pOD8B and pBridge, respectively. A total of 200 yeast colonies of three independent transformations have been investigated.

### Optical biosensor measurements

In order to verify the results of the directed evolution by in vitro studies, we performed an optical biosensor measurement with the purified recombinant proteins of the selected variants. The cDNAs of the Adx mutants Adx Y and Adx SY were cloned into the expression vector pET3d (Novagen) using restriction sites NcoI and BamHI. E. coli strain B21 (DE3) pLysS (Fa. Novagen) was transformed with these pET3d constructs. Adx proteins, as well as AdR and CYP11A1, were expressed and purified. The proteins were used for surface plasmon resonance measurements (SPR) on a Biacore 2000 system. After injecting different concentrations of AdR and CYP11A1 (100 nM to 1 μM), the $K_d$-values, shown in Table IV, were determined. Both mutants displayed a lower $K_d$-value compared to Adx wt for the Adx/AdR-complex formation, whereas mutant Adx Y showed a more pronounced effect (Adx Y displayed 65% and Adx SY 80% of the Adx wt value). For the Adx/CYP11A1-complex, the $K_d$ of Adx Y was 30% higher and for Adx SY 400% lower than the $K_d$ of Adx wt.

### Substrate conversion measurements

To check the effects of the mutations on the Adx-dependent substrate conversion of the natural redox partner cytochromes P450, CYP11A1 and CYP11B1, HPLC separations of steroids extracted from enzyme assays were carried out with a reconstituted system consisting of AdR, Adx and the respective cytochrome P450 (Sugano et al., 1989; Uhlmann et al., 1994). The results displayed in Table V point out that Adx Y leads to a 2-fold and Adx SY to a 1.5-fold increased catalytic efficiency ($k_{cat}/K_m$) of the Adx-dependent CYP11A1 substrate conversion compared to a system containing Adx wt. A similar trend was observed in conversion assays containing another mitochondrial P450, CYP11B1. The Adx-dependent catalytic efficiency of both enzymes increased 2-fold when using the Adx mutants, Adx Y and Adx SY.

### Discussion

Over the past few years, various advances have been made in the improvement of biocatalysts suitable for biotechnological applications. Among these developments, directed evolution has been shown to be a useful method to change and improve characteristics of enzymes. In this study, we have developed a new screening method for the selection of improved protein–protein interactions, which subsequently lead to an enhanced catalytic efficiency of mitochondrial cytochrome P450 electron transfer chains. The screening method is based on the yeast THS, which already showed its potential as a selection technique in directed evolution studies (Baker et al., 2002; Sengupta et al., 2004; Thomson-Ziegler et al., 2004). However, in all the described applications of the THS, the screening did not result in improved functional proteins. The search for improved catalytic efficiency of β-lactamase only lead to the detection of variants with decreased activity (Sengupta et al., 2004) and also the screening for improved interaction of Toxoplasma gondii ferredoxin-NADP$^+$ reductase originated an affinity improved

### Table IV. Comparison of the in vitro affinities ($K_d$-values) of Adx wt and the selected Adx mutants Adx Y and Adx SY to their redox partners AdR and CYP11A1

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Adx wt</td>
<td>0.86 ± 0.05</td>
<td>3.19 ± 0.26</td>
</tr>
<tr>
<td>Adx Y</td>
<td>0.62 ± 0.04</td>
<td>4.22 ± 0.34</td>
</tr>
<tr>
<td>Adx SY</td>
<td>0.72 ± 0.05</td>
<td>0.74 ± 0.06</td>
</tr>
</tbody>
</table>

Formation of the Adx/AdR-complex and Adx/CYP11A1-complex has been measured using a Biacore 2000 system using CM5-chips with a carboxy-methylated surface (Biacore). For this study, purified bovine recombinant proteins of the Adx variants, as well as AdR and CYP11A1 were used. The values have been determined from three independent measurements ($n = 3$).
protein that completely lost its enzymatic activity (Thomson-Ziegler et al., 2004). In contrast to these studies, the application of our selection system resulted in two functional adrenodoxin variants, which displayed an improved affinity to their redox partner proteins (Table IV) and as a consequence thereof resulted in a decreased $K_M$-value, which increased the catalytic efficiency of the tested mitochondrial cytochrome P450 electron transfer chains (Table V).

Bovine adrenodoxin, the target protein for our directed evolution study, was already the subject of many investigations that analyzed the effects of Adx mutations on the interaction to its redox partners and the effects on the Adx-dependent substrate conversion catalyzed by the cytochromes P450. Many Adx variants were generated by site-directed mutagenesis based on computer models, structural data and by comparing Adx to other ferredoxins. Among these variants, we selected a representative variety of Adx proteins that displayed different binding behavior to their redox partners in former studies (Grinberg et al., 2000; Hannemann et al., 2001; Schiffer et al., 2001; Zöllner et al., 2002), to set up and evaluate our new screening system (Tables I and II). For the visualization of the THS results, we performed our selection with the THS colony lift assay. This test uses 5-bromo-4-chloro-3-indolyl-$\beta$-d-galactopyranoside, which is cleaved by the THS reporter $\beta$-galactosidase, yielding galactose and 5-bromo-4-chloro-3-hydroxyindole, which is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. Accordingly, yeast colonies that contain interacting THS-proteins are colored blue. This reporter signal depends on the expression level of the THS-proteins, which can be adjusted using different promoters in the THS-plasmids. We harnessed this dependence to decrease the background of non-improved proteins by adjusting the expression level of the THS-proteins containing the redox partners of Adx, using the high-expression plasmid pODB8 and the low-expression plasmid pBridge. By applying this approach, we were able to differentiate between increased and non-increased interactions (Tables I and II). These results verified the dependence of the sensitivity of the colony lift assay with the expression level of the tested THS fusion proteins. Therefore, our new adjustable system enables a screening on different protein–protein interaction, which allows, in contrast to previous applications of the THS in directed evolution approaches, to improve the protein–protein interaction in several evolutionary cycles. Interestingly, the developed screening system was sensitive enough to differentiate the influence of various mutations on redox partner interaction and thus to identify which of the replacements in the Adx molecule did exert the crucial effect for its improved interaction with AdR (Table III).

Since screening for affinity-improved variants of Adx is purposeful only when the fused redox protein is synthesized in the yeast cells as an active holoprotein, we had to prove the correct folding and assembly of the [2Fe–2S]-cluster. Therefore, experiments with mutant Adx C46D4-108, which is not able to integrate the redox center of the ferredoxin, were carried out. In THS colony filter lift assays, Adx C46D4-108 did not produce blue colonies with neither AdR nor CYP11A1. This shows that the correct folding of Adx and incorporation of the [2Fe–2S]-cluster are indispensable for proper functioning of the protein, and is also a necessity for a positive signal in this assay. Furthermore, this shows that the new selection technique can be used for the directed evolution of proteins with prosthetic groups, which is an advantage over other screening methods that cannot be applied with holoenzymes, e.g. ribosome display (Hanes and Plückthun, 1997), RNA display (Roberts and Szostak, 1997) or phage display (Smith, 1985).

We applied our screening method for the directed evolution of mature bovine Adx species with an improved affinity to both redox partners, AdR and CYP11A1. Taking into account that Adx has overlapping but not identical binding regions for its redox partners (Müller et al., 2001b; Vickery, 1997), we cannot assure that an improved binding to one partner will also increase the affinity to the other. The results of the directed evolution process of Adx display the possibility to improve the protein–protein interactions to both partner proteins. The first round of the directed evolution of Adx resulted in the selected variant Adx Y, which showed an improved binding only to AdR, whereas the affinity to CYP11A1 was not improved (Table II). In the subsequent directed evolution step, we used this variant together with Adx T54S and Adx 1-115 as well as the epPCR-library as templates to create a mutant library, which we screened to find mutants with additional increased binding to CYP11A1. The screening of this library resulted in the identification of Adx SY, which displayed an improved affinity to AdR as well as to CYP11A1. This successful strategy to improve the affinity to the redox partners sequentially might be a new starting point for further investigations.

The protein–protein interactions between the selected mutants and AdR as well as CYP11A1 have been validated by biosensor measurements with the Biacore 2000 system. These measurements, performed with highly purified proteins, confirmed the improved interaction between the selected mutants and their redox partners (Table IV),

### Table V. Comparison of the $k_{cat}$, $K_M$ and $k_{cat}/K_M$ values of Adx wt, Adx Y and Adx SY in the Adx-dependent substrate conversion of cholesterol to pregnenolone catalyzed by CYP11A, and of 11-deoxycorticosterone to corticosterone catalyzed by CYP11B1

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ $[s^{-1}] \times 10^{-3}$</th>
<th>$K_M$ $[\mu M]$</th>
<th>$k_{cat}/K_M$ $[s^{-1} \cdot M^{-1}] \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adx wt</td>
<td>64.7 ± 0.3</td>
<td>0.26 ± 0.03</td>
<td>249 ± 30</td>
</tr>
<tr>
<td>Adx Y</td>
<td>76.2 ± 3.2</td>
<td>0.15 ± 0.01</td>
<td>508 ± 55</td>
</tr>
<tr>
<td>Adx SY</td>
<td>67.7 ± 1.5</td>
<td>0.17 ± 0.02</td>
<td>398 ± 51</td>
</tr>
<tr>
<td>CYP11A1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP11B1</td>
<td></td>
<td></td>
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</tbody>
</table>
Compared to Adx wt, the SPR measurement displayed an improved binding to AdR for Adx Y of 35% and for Adx SY of 15%, and Adx SY shows a 4-fold higher affinity to CYP11A1 (Table IV). These data confirmed the results of the newly developed screening system and the directed evolution of Adx.

The aim of the directed evolution of Adx was to enhance the catalytic efficiency of the mitochondrial cytochrome P450 system. Therefore, we tested the effects of the selected variants Adx Y and Adx SY on the Adx-dependent substrate conversion of CYP11A1 and CYP11B1 in the next set of experiments. An earlier study concluded that there is no direct correlation between substrate conversion and Adx binding to CYP11A1, although more than 50% of the Adx variants with an improved affinity also induced a higher substrate conversion (Grinberg et al., 2000). As shown in Table V, our results indicate that the improved ability of the mutants to form complexes results in an increased catalytic efficiency. This might be due to the improved $k_{cat}$-values, which are significantly changed in contrast to the $K_{M}$-values, which displayed only slight modifications. The correlation is not proportional, but it displays the adaptability of our idea of ‘indirect screening for enhanced catalytic efficiency’ via direct selection of improved protein–protein interaction in mitochondrial P450 systems.

In contrast to the previously published results that focused only on the binding behavior of Adx with CYP11A1 and the associated substrate conversion, our investigations indicate that an improved interaction between Adx and AdR can be sufficient to increase the overall catalytic efficiency (Table V).

Both selected mutations, T54S and D113Y, are localized in regions on the surface of Adx, which has been described to be involved in the recognition of redox partners. Residue 54 is positioned in the vicinity of the loop structure covering the iron–sulfur-cluster that has been identified as an essential binding region (Hannemann et al., 2001; Heinz et al., 2005; Zölßner et al., 2002) and residue 113 is located in the flexible C-terminal part of the protein that influences the redox partner binding (Uhlmann et al., 1994). C-terminal mutations that improved the interaction to the redox partners, e.g. Adx Y, Adx SY, also resulted in an improved catalytic efficiency of the cytochrome P450 system. The reason for this might be that the flexible C-terminus of Adx wt interferes with interacting surfaces on the redox partners as suggested by crystal structures (Pikuleva et al., 2000). Adx Y, Adx SY, and Adx SY possess a truncated C-terminus and thus lack the flexible region. We suggest that the new mutation D113Y fixes the C-terminus with hydrophobic interaction to the rest of Adx. Therefore, the flexibility of this region is decreased and results in an improved interaction of Adx variants containing this mutation (Adx Y, Adx SY), which might also be responsible for the obtained increased catalytic efficiency of the P450 system. This assumption of intramolecular hydrophobic interaction is supported by the observation that a truncation of Adx D113Y to 113 amino acids did not further improve the affinity to the redox partners (data not shown).

Summarizing the results, the new screening method enables a background-free HTS (25 000 colonies per day can be screened) for improved protein–protein interactions between stable and functional protein species including cofactor-containing proteins. Further, this screening-technique can be applied on the mitochondrial P450 electron transfer chain as a kind of indirect selection method for enhanced catalytic efficiency. Taking this into account, we were successful in the development of a new tool for directed evolution for mitochondrial P450 systems, as well as proteins in general.

Acknowledgements

The authors thank A. Zölßner for his support with the Biacore measurements, as well as K. Bonnas, W. Klose and W. Reine for their help in cloning the THS constructs and for the purification of the used proteins. This work was supported by a grant from the BMBF (0312641A) and by the Fonds der Chemischen Industrie.

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Received September 4, 2006; revised December 7, 2006; accepted December 15, 2006

Edited by Bent Mannervik