Functional Display of Active Bovine Adrenodoxin on the Surface of *E. coli* by Chemical Incorporation of the [2Fe – 2S] Cluster

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The display of heterologous proteins on the surface of living cells bears promising options for a wide variety of biotechnological applications. Up to now, however, cellular surface display was merely restricted to simple polypeptide chains. Here we present for the first time the efficient display of a protein (bovine adrenodoxin) that contains an inorganic, prosthetic group in its active form on the surface of Escherichia coli. *For this purpose apo-adrenodoxin was transported to the cell surface and anchored within the outer membrane by the autotransporter pathway. Incorporation of the iron – sulfur cluster was achieved by a single-vial, one-step titration under anaerobic conditions. The biological function of surface-displayed holo-adrenodoxin could be established through adrenodoxin-dependent steroid conversion by two different cytochrome P450 enzymes and the number of functional molecules on the cell surface could be determined to be more than 10^5 per cell. Neither the expression of adrenodoxin nor the incorporation of the chemical iron – sulfur cluster reduced the viability of the bacterial cells.*

KEYWORDS:

adrenodoxin · autotransporter pathway · electron transport · metalloproteins · surface display

Introduction

Whole-cell biofactories and taylor-made enzymes from laboratory evolution are two examples of increasing importance in the application of protein surface display.[1] Different systems have been applied for the surface display of heterologous proteins in yeast,[2, 3] and gram-positive[4, 5] and gram-negative bacteria.[6] Besides other systems[7–14] autodisplay is a very elegant way to express a recombinant protein on the surface of a gram-negative bacterium.[15, 16] Autodisplay is based on the secretion mechanism of the autotransporter family of proteins.[17–19] These proteins are synthesized as polyprotein precursors that contain structural elements sufficient for secretion.[20] They cross the inner membrane by using a typical signal peptide at the N terminus. Once the precursor has arrived in the periplasm, the C-terminal part folds into the outer membrane as a porin-like structure, a so-called β barrel.[15, 21, 22] Through this pore, the passenger domain attached to the N terminus is translocated to the surface. There, it might be cleaved off—either autoproteolytically or by an additional protease—or remain anchored to the cell envelope by the transporter domain.[23] Replacing the natural passenger by a recombinant protein results in its proper surface translocation.[15, 16, 24–27]

For this purpose an artificial precursor must be constructed by genetic engineering; this precursor consists of a signal peptide, the recombinant passenger, the β barrel, and a linking region in between, which is needed to achieve full surface access.[21] The AIDA-I autotransporter, which is a part of the adhesin involved in diffuse adhesion, is present in some pathogenic strains of *Escherichia coli*[28] and was successfully used in this way for efficient surface display of various passenger domains.[15, 16, 27, 29] Up to now, however, the autotransporter-mediated surface display has been restricted to monomeric proteins that were devoid of any nonproteinaceous cofactors. This could be due to the fact that a polypeptide chain must be in a relaxed, unfolded conformation to be transported by the autotransporter pathway.

The ferredoxin from bovine adrenal cortex, termed adrenodoxin (Adx), belongs to the [2Fe – 2S] ferredoxins, a family of small acidic iron – sulfur proteins that can be found in bacteria, plants, and animals.[30] It plays an essential role in electron transport from adrenodoxin reductase (AdR) to mitochondrial cytochrome P450 enzymes, which are involved in the synthesis of steroid hormones (Scheme 1).[31] The iron – sulfur cluster of Adx is coordinated by four sulfur atoms from the side chains of four of its five cysteine residues.[32] Bovine adrenodoxin is encoded by a nuclear gene, synthesized in the cytoplasm and processed upon mitochondrial uptake. The mechanism of iron – sulfur cluster incorporation is still not clear, although during

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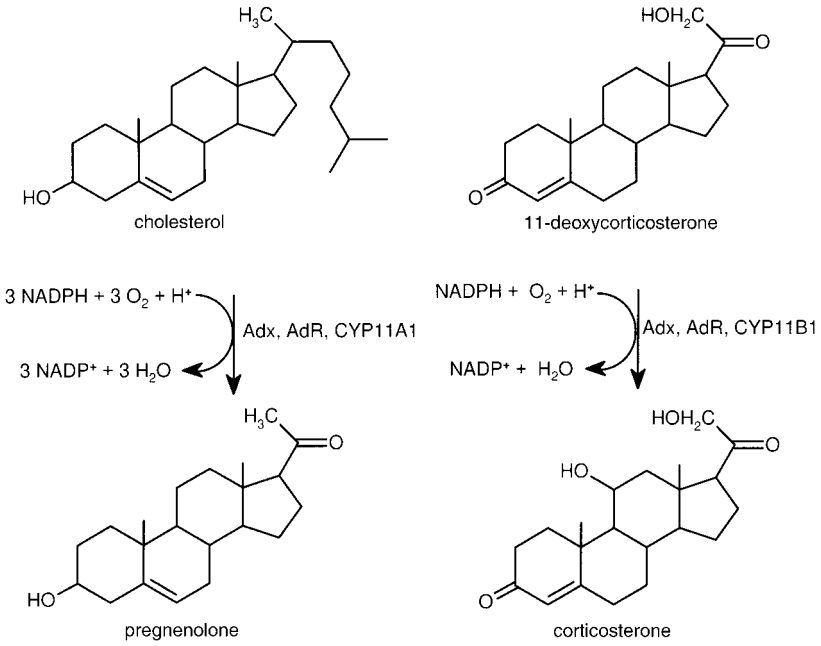

Scheme 1. *Adx-dependent reactions of the side-chain cleaving enzyme (CYP11A1) and 11β-hydroxylase (CYP11B1). The electron-transfer activity of surface-presented Adx was analyzed in reconstituted systems containing the natural final electron acceptors CYP11A1 and CYP11B1, which catalyze the indicated chemical reactions.*

heterologous expression in *E. coli*, it can be assembled in the cytoplasm as well as in the periplasm.[33]

In the present paper, we report on the construction of a fusion protein containing a signal peptide, a monomeric Adx, and the transporter domain of AIDA-I and on its efficient and stable surface display. At the cell surface, the iron – sulfur cluster could be chemically incorporated by a one step procedure, yielding functional Adx. We show, for the first time, that autodisplay can be applied to proteins that contain inorganic cofactors and that these proteins are freely accessible at the cell surface, even for large ligands or partner proteins such as cytochrome P450 enzymes. This investigation opens the door for further developments in the application of autodisplay or surface display in general, by extending the application of the evolutive design of catalytic biomolecules or new whole-cell factories to proteins containing prosthetic groups.

Results

Fusion protein construction

The coding region of bovine Adx was amplified with the polymerase chain reaction (PCR) to obtain an in-frame fusion with the gene segments needed for autodisplay. The PCR primers used added an *Xho*I site at the 5′ end and a *Kpn*I site at the 3′ end of the Adx encoding region. To avoid any hindrance with bacterial surface translocation, an Adx gene that was devoid of the coding region for the mitochondrial targeting sequence was used as the PCR template, as previously described.[33] The amino acid and the nucleotide sequence of the resulting PCR product, which was confirmed by dideoxy sequencing, is shown in Figure 1 a. For the construction of the recombinant fusion protein encoding gene, plasmid pJM7 was cleaved with *Xho*I and *Kpn*I. pJM7 is a pBR322-derived high copy number plasmid that directs the expression of a choleratoxin$_\beta$ – Aida-I fusion protein under control of the constitutive P_{TK} promoter.[15, 23] Cleavage with *Xho*I and *Kpn*I resulted in the deletion of the DNA region encoding choleratoxin$_\beta$ (CTB). Insertion of the cleaved Adx PCR fragment yielded plasmid pJJ004, which encoded a fusion protein consisting of the signal peptide of CTB, Adx, and the AIDA-I autotransporter region (AIDA$_\beta$), including a linker region that proved to be sufficient for full surface access in earlier studies[15] (Figure 1 B). The artificial construct still contains eight amino acids of mature CTB due to the ligation procedure. Based on the predicted molecular mass of 65.9 kDa, the fusion protein was termed FP66.

Expression of Adx – AIDA-1 fusion proteins

Most of the available *E. coli* host strains possess an outer-membrane protease (OmpT) that catalyzes the sequence-specific release of surface-exposed proteins.[34] As the linker used in our Adx – AIDA$_\beta$ fusion contains an OmpT protease specific cleavage site, it was necessary to use an *ompT*-negative strain for Adx surface display. In former studies *E. coli* UT5600 (*ompT*)[35] proved to be suitable to prevent cleavage of similarly constructed surface-exposed autotransporter fusion proteins.[23, 36] Therefore pJJ004 was transformed into *E. coli* UT5600 and the expression of FP66 was monitored by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of outer-membrane protein preparations. As shown in Figure 2 A, FP66 could be easily detected by Coomassie brilliant blue staining of the outer-membrane proteins. Expression was at almost the same level as expression of the natural outer-membrane proteins OmpA and OmpF/C. Neither the growth rate nor the optical density reached in the stationary phase of *E. coli* UT5600 grown in liquid medium was decreased by the expression of FP66 (not shown). Electrophoretic mobility of the Adx – AIDA$_\beta$ fusion protein was in perfect agreement with the predicted molecular mass of 65.9 kDa. Finally, the identity of FP66 was confirmed by Western blot analysis with an Adx-specific polyclonal rabbit antibody[37] (Figure 2 B).

Probing the surface display of Adx

Intact cells of Adx-expressing *E. coli* UT5600 pJJ004 were subjected to protease digestion with trypsin to clarify whether the passenger domain of FP66 was exposed to the surface and not directed to the periplasm. External protease accessibility has been a common tool to verify the surface translocation of autotransporters or other secreted proteins.[12, 15] The outer membrane is a natural barrier that does not allow the passage of proteins in the range of 20 kDa, such as trypsin, from the outside

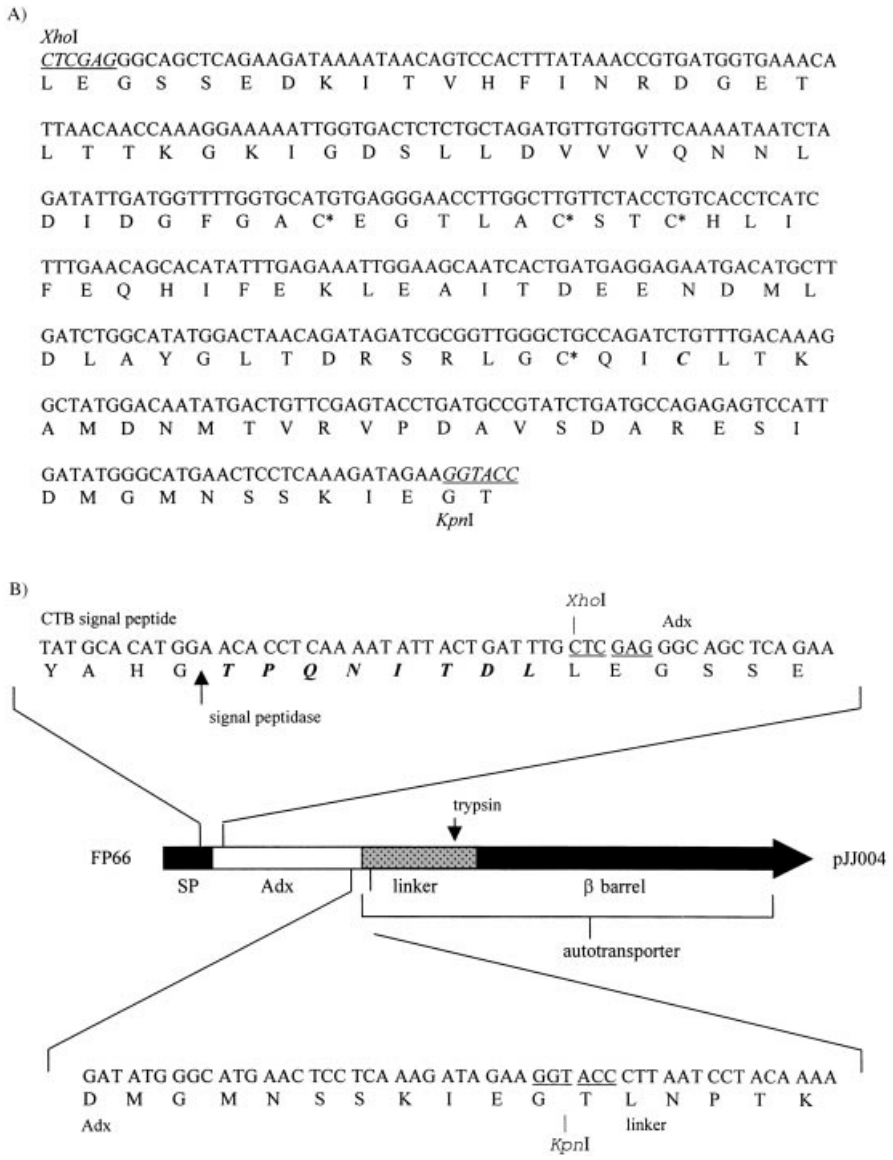

Figure 1. *A) Nucleotide and amino acid sequence of bovine adrenodoxin devoid of the mitochondrial target sequence, as used in this study. B) Structure of the fusion protein FP66 encoded by pJJ004. The environments of the fusion sites are given as sequences. The eight amino acids of CTB origin are written in italics. The signal peptidase and trypsin cleavage sites are indicated.*

to the periplasm. Therefore, proteolytic degradation of a protein by externally added proteases must be due to its surface exposure. Outer membranes were prepared as previously described[38] from trypsin-treated cells and analyzed by SDS-PAGE and Western blot analyses. In former studies it has been shown that membrane embedding of the AIDA-I autotransporter resulted in a trypsin-resistant core of 37.1 kDa.[15] This core is due to a trypsin cleavage site found in the linker region (Figure 1 B). Predicted trypsin cleavage sites that are located closer to the C terminus of the transporter are protected from trypsin access by membrane topology. SDS-PAGE and subsequent staining with Coomassie blue indicated that external trypsin addition to Adx-expressing *E. coli* UT5600 cells resulted in the disappearance of the full-size fusion protein and generated two lower molecular weight products (Figure 2 A). One of them corresponds to the 37.1 kDa trypsin-resistant autotransporter core (T1). The second digestion product (T2) has a larger molecular weight of around 45 kDa. Therefore it must result from trypsin cleavage within the Adx passenger domain, which is known to contain several consensus cleavage sites. The trypsin processing within the passenger domain may induce a conformation that sterically hinders subsequent trypsin access to the linker region and therefore prevents formation of T1. Obviously, in our experiments, this altered conformation only occurred after processing of a distinct cleavage site, which resulted in a noncleavable 45 kDa product (T2), as there was no further prominent digestion product detectable.

OmpA has a periplasmic moiety that is degraded by trypsin when the periplasm is accessible for the protease.[15] In our experiments, the size and the amount of OmpA was identical in trypsin-treated cells and cells not treated with trypsin (Figure 2 A). This indicates that the outer membrane remained intact in our experiments. In contrast, when trypsin was added after cell disruption and preparation of outer membranes, and the periplasmic side was thus made accessible, the size of the OmpA band was reduced by approximately 10 kDa (not shown).

Electron-transfer function of Adx displayed on the bacterial surface

Since the previous results clearly indicate that bovine Adx is transported to the bacterial surface by the autotransporter pathway, the next step was to investigate if the biomolecule displayed was active. The activity was monitored with the side-chain cleaving enzyme (CYP11A1) dependent conversion of cholesterol into pregnenolone or with the 11β-hydroxylase (CYP11B1) dependent conversion of deoxycorticosterone into corticosterone (Scheme 1). Both assays containing the native electron acceptors of Adx and whole cells of *E. coli* UT5600 pJJ004 showed no substrate conversion (Figure 3). This emphasized that a self-assembled holo-Adx containing the redox-active [2Fe – 2S] cluster is not formed after expression and transport to the cell surface. The absence of the iron – sulfur cluster was confirmed by electron spin resonance (ESR) measurements with whole cells (not shown). This finding fits the concept of the autotransporter secretion mechanism published earlier.[17, 21] Accordingly, proteins can only be transported by the autotransporter pathway as long as they maintain a relaxed, unfolded confirmation. Since the *E. coli* ferredoxin, which is a structural homologue of adrenodoxin, obtains the [2Fe – S] cluster in the

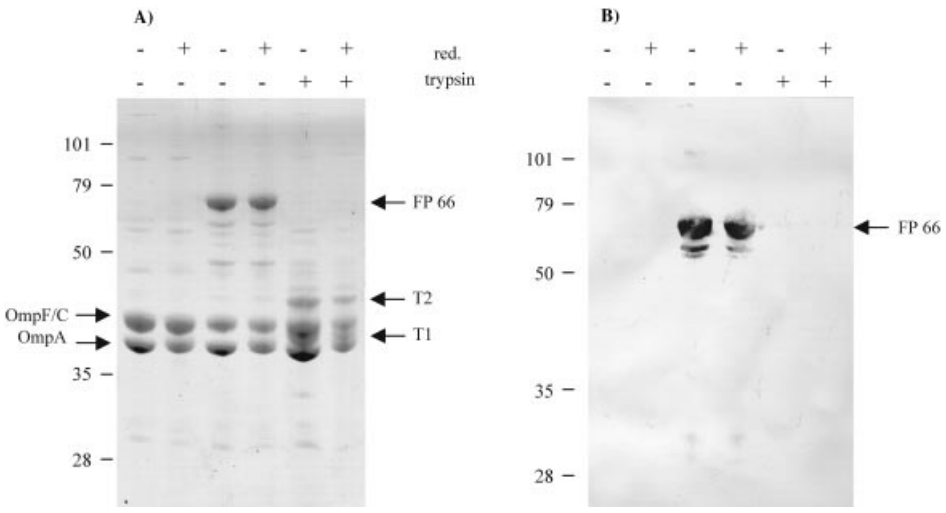

Figure 2. SDS-PAGE (A) and Western blot analysis (B) of outer-membrane preparations from E. coli *UT5600 (lanes 1, 2) and UT5600 pJJ004 (lanes 3 – 6). Molecular weight markers are indicated. red. + : sample buffer contained 2-mercaptoethanol; red. − : sample buffer did not contain 2-mercaptoethanol. trypsin + and − : whole cells were or were not, respectively, digested with trypsin before outer-membrane preparation. Natural outer-membrane proteins OmpF/C and OmpA are marked by arrows. T1 = the trypsin-resistant, membrane-embedded autotransporter core; T2 = the second trypsin digestion product.*

cytoplasm,[39] we also analyzed the cytoplasmic fraction and, as a control, the periplasmic fraction for Adx – autotransporter fusion proteins. Neither in the cytoplasmic nor in the periplasmic fraction was any Adx molecule detectable. This might indicate either that signal-peptide targeting initially prevents cluster incorporation or that cluster incorporation followed by transport intersection results in rapid proteolytic degradation in the cytoplasm.

Electron-transfer activity after reconstitution of the iron – sulfur cluster

As the expression of apo-Adx on the *E. coli* surface was very efficient, it seemed worthwhile to try posttransport integration of the [2Fe – 2S] cluster into surface-displayed Adx by chemical reconstitution.[40] Therefore whole cells of *E. coli* UT5600 pJJ004 were resuspended in tris(hydroxymethyl)aminomethane (Tris)/HCl buffer, incubated under an argon atmosphere, and supplemented with 1 mM mercaptoethanol and 0.2 mM ferrous ammoniumsulfate at different temperatures (22, 40, 60, and 70 °C). Subsequently, 100 mM LiS_2 was added dropwise to maintain the low overall sulfide concentrations and avoid precipitation of iron sulfides.[41] By this procedure iron – sulfur clusters were formed and immediately incorporated into apo-Adx displayed at the bacterial surface. The successful folding of the Adx peptides around [2Fe – 2S] clusters was indicated by a significant product formation in two cytochrome P450 enzyme tests (Figure 3). In these tests adrenodoxin reductase and CYP11A1 or CYP11B1, respectively, were added to whole cells after chemical reconstitution to yield the complete electron-transfer chain as present in bovine adrenals. Since the controls were negative and no additional Adx was added to the assays, the product formation must clearly be due to an electron-transfer function of surface-displayed Adx, which signifies a successful chemical reconstitution. These results show that Adx displayed on the *E. coli* surface by the autotransporter pathway is biologically active and is able to transfer electrons to the steroid-hydroxylating P450 enzymes CYP11A1 and CYP11B1.

The number of active Adx molecules after reconstitution on the surface of *E. coli* could be estimated by the use of the specific-substrate conversion rate in the CYP11A1 assay. For this purpose the enzyme assay was performed without *E. coli* cells but with different concentrations of purified holo-Adx. This allowed a calibration curve to be recorded. This curve describes the dependency of substrate conversion on the number of Adx molecules present in the assay. The calibration curve was used to estimate the apparent number of active Adx molecules on *E. coli* UT5600 pJJ004 under assay conditions. As pointed out in Table 1, reconstitution of the [2Fe – 2S] cluster was successful at all the temperature conditions applied. It seemed to be favored at the lower temperatures of 22 °C and 40 °C, whereas heat denaturation at 60 °C or 70 °C prior to refolding experiments resulted in significantly decreased amounts of assembled holo-Adx. Moreover, denaturating conditions of 70 °C dramatically decreased the number of viable bacterial cells. An apparent number of 1.8×10^5 functional Adx molecules per cell indicated optimal conditions for refolding of the peptide around the [2Fe – 2S] center at 22 °C. A preceding heat denaturation of surface-displayed apo-Adx was obviously not necessary for successful cluster incorporation.

Discussion

Surface display of active proteins on living cells provides several advantages in biotechnological applications. If such cells are used as whole-cell biocatalysts, the substrate to be processed does not need to cross a membrane barrier but has free access. Moreover, being connected to a carrier (the cell as a biological matrix), the surface-displayed biocatalyst can, in most cases, be purified, stabilized, and applied to industrial processes more conveniently than if it is a free molecule. The use of cellular surface display in the creation and screening of peptide or protein libraries in order to perform laboratory evolution has another benefit. By selecting the correct structure expressed at the surface, the corresponding gene, which serves as an intrinsic label, is coselected and can be used in further studies and applications. Therefore, the need for systems that allow the surface display of a very broad spectrum of different proteins is obvious, and this is gaining more and more importance in typical biochemical or bioorganic application fields, such as enzyme engineering or drug discovery.

In the present study, bovine adrenodoxin was expressed on the *E. coli* cell surface by the autotransporter pathway. The expression rate was in the same order of magnitude as the expression of the natural outer-membrane proteins OmpF/C or

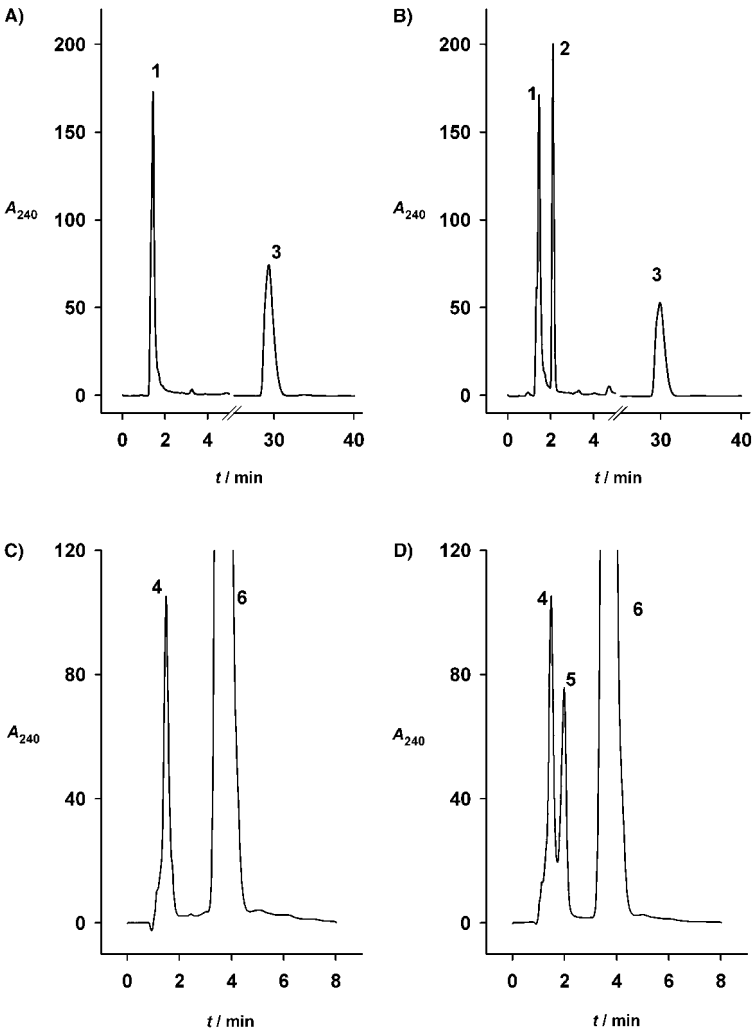

Figure 3. *HPLC chromatograms of CYP11A1- and CYP11B1-dependent substrate conversion. Chromatograms were obtained from extracted samples with* E. coli *cells containing pJJ004 before reconstitution (A, C) and after reconstitution (B, D) of the iron – sulfur cluster in surface-displayed adrenodoxin. The indicated substance peaks represent 1) cortisol (internal standard), 2) pregnenolone (product), and 3) cholesterone (substrate); 4) cortisol (internal standard), 5) corticosterone (product), and 6) deoxycorticosterone (substrate). Steroids were analyzed in A and B with an isocratic solvent system of acetonitrile/isopropanol (15:1) and for C and D with an isocratic solvent system of acetonitrile/isopropanol (1:1).*

OmpA without disturbing the outer-membrane integrity or reducing cell growth (Figure 2 A). Adx passenger molecules transported to the cell surface by the autotransporter initially did not contain an iron – sulfur cluster. This fits the concept of the autotransporter secretion mechanism.[15, 17, 20] Accordingly, the C terminus of these secreted proteins forms a porin-like structure, a so-called β barrel, in the outer membrane and proteins with stable and extended three-dimensional structures cannot pass this gate. Incorporation of the [2Fe – 2S] cluster into apo-Adx results in the acquisition of a stable structure[30] and is therefore not compatible to surface translocation. Apo-Adx expressed on the *E. coli* cell surface could be chemically supplemented with the [2Fe – 2S] cluster by a one-step reconstitution procedure under anaerobic conditions. Anaerobic conditions proofed to be best in an argon atmosphere (not shown). Whole cells expressing Adx could be efficiently used to transfer electrons from adrenodoxin reductase to P450 enzymes CYP11A1 and CYP11B1 (Figure 3). Activity could be quantified by determining Adx-dependent product formation of either pregnenolone or corticosterone by high-pressure liquid chromatography (HPLC). By calibrating Adx activity in the substrate-formation assay with purified holo-Adx, the apparent number of active Adx molecules could be determined as more than 10^5 molecules per cell. At first sight, this appears to be amazing, but a quantity of 200 000 copies per cell in the outer membrane has also been determined for the natural outer-membrane protein OmpA.[42] This indicates that the capacity of the *E. coli* outer membrane to insert proteins could be even beyond these numbers and may be an explanation for the fact that viability and membrane integrity was not affected in cells expressing the Adx – autotransporter fusion proteins.

At this point it can not be proved whether Adx expressed on the *E. coli* surface has an improved or reduced electron-transport efficiency in comparison to free holo-Adx or whether the affinity to its reaction partners, AdR and P450, has been altered in any respect. High expression of recombinant and chemically reconstituted, active Adx, however, had no influence on the viability of *E. coli*. Reconstitution at higher temperatures in order to get easier unfolding of the apo-Adx peptide chain did not result in a better [2Fe – 2S] cluster incorporation. Moreover, higher temperatures significantly decreased the number of viable cells. The best ratio between active Adx molecules expressed on the surface and viable *E. coli* cells was obtained under room temperature conditions (Table 1). This indicates that apo-Adx displayed on the surface forms a structure well-prepared for efficient [2Fe – 2S] cluster acceptance, even at nondenaturing conditions.

Table 1. *Active Adx molecules on the surface of viable* E. coli *cells after reconstitution at different temperatures.*

Reconstitution temperature	Cells applied per mL[a]	Cells per mL after reconstitution[a]	nmoles of Adx per mL[b]	nmoles of Adx per living cell	Adx molecules per living cell	Adx molecules per cell applied
22 °C[c]	1.4×10^{10}	2.0×10^{10}	0	0	0	0
22 °C	1.3×10^{10}	1.1×10^{10}	3.25	2.95×10^{-10}	1.8×10^5	1.5×10^5
40 °C	1.5×10^{10}	2.1×10^{10}	2.75	1.31×10^{-10}	0.8×10^5	1.1×10^5
60 °C	1.1×10^{10}	1.5×10^{10}	1.75	1.16×10^{-10}	0.7×10^5	0.9×10^5
70 °C	1.3×10^{10}	0.7×10^7	0.75	1.10×10^{-8}	6.6×10^7	0.3×10^5

[a] Mean of three independant determinations of colony number after plating adequate dilutions on LB_{amp}. [b] Determined by a calibration curve that was established for the enzymatic conversion of cholesterol into pregnenolone with purified Adx. [c] Control experiment. Cells incubated without reconstitution.

Conclusion

Our results show that the autotransporter pathway can be used for the surface display of proteins that require an inorganic prosthetic group for biological activity. The activity of the reconstituted Adx indicates that it is freely accessible at the cell surface, even for molecules as large as AdR (50 000 kDa) or CYP11A1 and CYP11B1 (52 000 kDa and 45 000 kDa, respectively), as direct contact between Adx and AdR or the P450 enzymes is a prerequisite for electron transfer[43] (Figure 4). The expression of more than 10^5 active molecules per cell without hampering cell viability is, to our knowledge, unique among bacterial surface display systems applied so far. It enables high Adx activity to be provided by whole cells as carriers or protectors. This has promising options for further applications. On one hand, the role of distinct amino acids in the electron transfer through Adx or in the interaction with its redox partners can be studied, either by random or by rational variation of the protein, without the need for purification of mutant enzymes. Random mutants, for example, can be analyzed for their electron-transfer efficiency by applying whole cells in the assay, and interesting mutants will reveal their variant amino acid composition directly through their gene sequence. On the other hand, the system developed here—efficient surface translocation of an unfolded apo-protein and chemical reconstitution of the prosthetic group—might also be applicable to proteins containing FAD, FMN, or heme groups. Particularly heme-containing P450 enzymes appear to be important, as they are involved in the syntheses of a wide variety of valuable products and also in the degradation of numerous toxic compounds.[44] With the intent of using enzyme-coated cells for these applications, autotransporter-mediated surface display of, for example, P450 enzymes could open a new dimension in the development of whole-cell factories. This is currently under investigation.

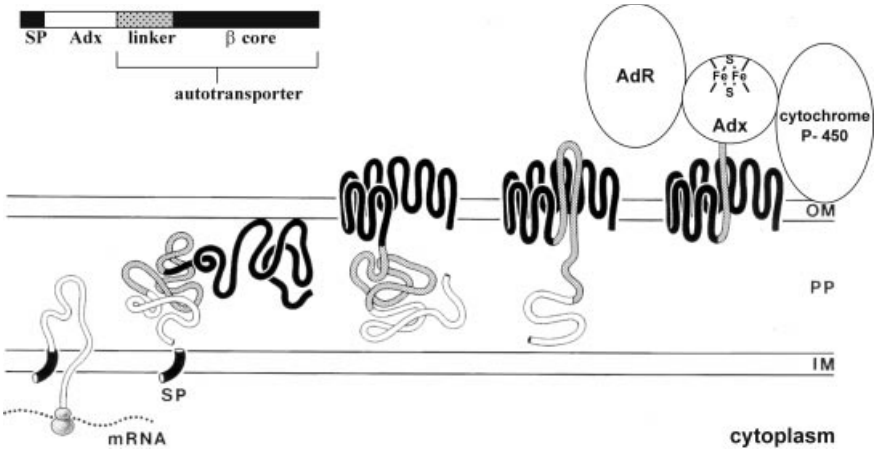

Figure 4. *Schematic representation of Adx surface display by the autotransporter pathway in* E. coli. *SP = signal peptide, IM = inner membrane, PP = periplasm, OM = outer membrane.*

Experimental section

Bacterial strains, plasmids, and culture conditions: *E. coli* UT5600 (F$^-$ *ara14 leuB6 azi-6 lacY1 proC14 tsx-67 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi1*, Δ*ompT-fepC266*) was used for the expression of autotransporter fusion proteins.[45] *E. coli* TOP10 (F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* ϕ*80lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ*(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG*) and the vector pCR2.1-TOPO, which were used for subcloning of PCR products, were obtained from Invitrogen (Groningen, the Netherlands). Plasmid pJM7,[15] which encodes the AIDA-I autotransporter, and plasmid pKKHCAdx,[33] which encodes bovine adrenodoxin, have been described elsewhere. Bacteria were routinely grown at 37 °C in Luria-Bertani (LB) broth containing 100 mg of ampicillin per liter. For Adx expression studies, ethylenediaminetetraacetate (EDTA) was added to a final concentration of 10 μM and β-mercaptoethanol was added to a final concentration of 10 mM.

Recombinant DNA techniques: For the Adx–autotransporter fusion, the Adx gene was amplified by PCR from plasmid pKKHCAdx with oligonucleotide primers JJ3 (5′-ccgctcgagggcagctcagaaga-taaaataacagtc-3′) and JJ4 (5′-ggggtaccttctatctttgaggagttcatg-3′). The PCR product was inserted into vector pCR2.1-TOPO and recleaved with *Xho*I and *Kpn*I. The restriction fragment was ligated to pJM7, restricted with the same enzymes. This yields an in-frame fusion of Adx with the AIDA-I autotransporter (Figure 1 B) under the control of the strong P_{TK} promoter.

Outer-membrane preparation: *E. coli* cells were grown overnight and the culture (1 mL) was used to inoculate LB medium (20 mL). Cells were cultured at 37 °C with vigorous shaking (200 rpm) for about 5 h until an OD_{578} of 0.7 was reached. After harvesting and washing with phosphate-buffered saline (PBS), outer membranes were prepared according to the rapid isolation method of Hantke.[38] For whole-cell protease treatment, *E. coli* cells were harvested, washed, and resuspended in PBS (5 mL). Trypsin was added to a final concentration of 50 mg L^{-1} and cells were incubated for 5 min at 37 °C. Digestion was stopped by washing the cells three times with PBS containing 10% fetal calf serum (FCS) and outer membranes were prepared as described above.

SDS-PAGE and Western blot analysis: Outer-membrane isolates were diluted (1:2) with sample buffer (100 mM Tris/HCl (pH 6.8) containing 4% SDS, 0.2% bromphenol blue, and 20% glycerol), either with or without 2-mercaptoethanol (reducing and nonreducing conditions, respectively). The samples were then boiled for 20 min and analyzed on 12.5% SDS-PAGE. Proteins were visualized with Coomassie brilliant blue with prestained molecular-weight protein markers (Bio-Rad, München, Germany). For Western blot analysis, gels were electroblotted onto polyvinylidene-difluoride (PVDF) membranes and blotted membranes were blocked in PBS with 3% FCS overnight. For immunodetection, membranes were incubated with the primary anti-Adx antibody, diluted (1:500) in PBS with 3% FCS for 3 hours. Prior to addition of the secondary antibody, immunoblots were rinsed three times with PBS. Antigen–antibody conjugates were visualized by reaction with horseradish peroxidase linked goat anti-rabbit IgG secondary antibody (Sigma, Deisenhofen, Germany), diluted (1:1000) in PBS. A color reaction was achieved by adding a

solution consisting of 4-chloro-1-naphthol (2 mL; 3 mg mL^{-1} in ethanol), PBS (25 mL), and H_2O_2 (10 μL; 30 %).

Reconstitution of the [2Fe – 2S] center in surface-displayed adrenodoxin: Cells were grown overnight, washed twice in PBS, and resuspended to a calculated final OD_{578} of 50. Refolding of adrenodoxin on the surface of the *E. coli* cells was achieved either by heat denaturation at 70, 60, and 40 °C followed by a slow temperature ramp to 22 °C, or was performed directly at ambient temperature. Simultaneous chemical reconstitution of the iron – sulfur cluster was performed under strictly anaerobic conditions in 50 mM Tris/HCl buffer (pH 7.4). The bacterial suspension (4 mL) was supplemented with 1 mM β-mercaptoethanol and 0.2 mM ferrous ammonium sulfate and was slowly titrated with 100 mL of a solution containing 100 mM Li_2S and 2 mM dithiothreitol.[46]

Protein purification: Recombinant Adx and AdR were purified as described.[33] The protein concentration was calculated with $\varepsilon_{414} = 9.8$ (mM cm)$^{-1}$ for Adx and $\varepsilon_{450} = 11.3$ (mM cm)$^{-1}$ for AdR. Purification of CYP11A1 and CYP11B1 from bovine adrenals was performed according to Akhrem et al.[47] with slight modifications.[33]

Enzyme activity assays: The biological electron-transfer function of adrenodoxin was detected in adrenodoxin-dependent reactions containing its natural effector enzymes, cytochromes CYP11A1 and CYP11B1. Tests were performed as previously described[48, 49] with modifications.

The cholesterol side-chain cleavage activity of cytochrome CYP11A1 was assayed in a reconstituted system catalyzing the conversion of cholesterol into pregnenolone. Assays were performed at 37 °C in 50 mM potassium phosphate (pH 7.4) with 0.1 % Tween 20 and also contained *E. coli* cells (100 μL), 0.5 μM adrenodoxin reductase, 0.4 μM CYP11A1, 400 μM cholesterol, and an NADPH regenerating system, which was composed of 600 μM glucose-6-phosphate, 4 units mL^{-1} of glucose-6-phosphate dehydrogenase, and 100 μM NADPH. After the reaction, the steroids were converted into their corresponding 3-one-4-ene forms by addition of 2 units mL^{-1} of cholesterol oxidase; these were then extracted and analyzed by reverse-phase HPLC.

Substrate conversion from deoxycorticosterone into corticosterone in cytochrome CYP11B1 assays were performed at 37 °C in 50 mM potassium phosphate (pH 7.4) with 0.1 % Tween 20 and also contained *E. coli* cells (100 μL), 0.5 μM adrenodoxin reductase, 0.2 μM CYP11B1, 400 μM deoxycorticosterone, and the NADPH regenerating system (as described above).

Extracted steroids were separated on a Jasco reverse-phase HPLC system of the LC800 Series with a 3.9 × 150 mm Waters Nova-Pak C_{18} column and an isocratic solvent system of acetonitrile/isopropanol (15:1) for the CYP11A1 test and with an isocratic solvent system of acetonitrile/isopropanol (1:1) for the CYP11B1 test. The amounts of reconstituted Adx on the cell surface were estimated by comparison of the steroids produced in cell-dependent reactions with reactions which contained defined amounts of purified holo-Adx.

Electron spin resonance measurements: ESR measurements were carried out on a Bruker ESP300E spectrometer at −163 °C. Cell samples in 50 mM potassium phosphate (pH 7.4) were dithionite-reduced under anaerobic conditions and frozen in liquid nitrogen.

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