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Suppression of human cytochrome P450 aromatase activity by monoclonal and recombinant antibody fragments and identification of a stable antigenic complex

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Abstract

Human cytochrome P450 aromatase (P450arom) is responsible for biosynthesis of estrogens from androgens. Monoclonal antibody MAb3-2C2 to P450arom specifically binds to a conformational epitope and suppresses the enzyme activity in a dose-dependent manner. The crystal structure of the Fab fragment of MAb3-2C2 has been used to engineer a recombinant single chain antibody fragment (scFv) and a homodimeric variable domain of the light chain (VL_2). These recombinant antibody fragments have been expressed in *Escherichia coli* and purified. Here, we show that the recombinant scFv suppresses P450arom activity with an IC_{50} value similar to that of natural MAb3-2C2 F(ab′)$_2$. The recombinant VL_2 also exhibits dose-dependent suppression of the P450arom activity, but at a reduced level, demonstrating that the homodimer is unable to fully mimic the complementarity determining region (CDR) of a variable heavy chain (VH)–VL heterodimer. We prepare and purify a stable complex of P450arom with MAb3-2C2 F(ab′)$_2$ and show that the complex migrates and precipitates as a single molecular assembly. Efforts to crystallize P450arom for structure-function studies have yielded small single crystals. Our results suggest that formation of stable complexes with fragments of the monoclonal antibody could provide an alternative method for crystallization of P450arom.

Keywords: Human cytochrome P450 aromatase (P450arom); Single chain antibody fragment (scFv); Activity–suppressing antibody

1. Introduction

Estrogens play an essential role in physiology and pathology throughout human development. The three most important enzymes responsible for tissue-specific biosynthesis of estrogens are cytochrome P450 aromatase (P450arom), type 1 17β-hydroxysteroid dehydrogenase (17HSD1) and estrone/dehydroepiandrosterone sulfatase (ES). This three-enzyme system is primarily responsible for biosynthesis of the female hormone in breast, ovary, placenta, endometrium and other tissues [1–6]. P450arom, in the presence of NADPH-linked P450arom-reductase, is the only known enzyme capable of aromatizing androgens to produce estrogens. The body stores estrogens in the form of sulfate esters, and ES catalyzes the hydrolysis of these esters to release unconjugated estrone. 17HSD1 is responsible for the stereospecific reduction of inactive E1 to yield the most active female hormone, 17β-estradiol (E2). Several tumor models suggest a critical role of the three-enzyme system in autocrine and intracrine biosynthesis of E2 within breast tumor cells [2,4].

The functional human enzyme P450arom is a monomeric polypeptide chain of 503 amino acid residues (molecular mass 55 kDa). P450arom is mainly localized in the

Abbreviations: P450arom, human cytochrome P450 aromatase; ES, human estrone/dehydroepiandrosterone sulfatase; 17HSD1, human 17β-hydroxysteroid dehydrogenase type 1; MAb, monoclonal antibody; VH, variable domain of MAb heavy chain; VL, variable domain of MAb light chain; Fv, variable domain of MAb consisting of VH and VL; scFv, single chain Fv; CDR, complementarity determining region; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; E1, estrone; E2, 17β-estradiol; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; DDM , *n*-dodecyl-β-D-maltopyranoside; EDTA, ethylene diamine tetraaceticacid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; BOG, *n*-octyl-β-D-glucopyranoside; DLPC, dilauroylphosphatidylcholine; N-terminus, amino terminus; C terminus, carboxyl terminus; ELISA, enzyme-linked immunosorbent assay; LB, Luria broth

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endoplasmic reticulum, where it is anchored to the membrane at the amino (N)-terminus by a transmembrane helix [7]. However, it is possible that other membrane spanning or associating regions also have roles in the anchoring of the molecule to the membrane, as the elucidation of the three-dimensional structure of ES recently suggested [6]. The enzyme shares a number of structural features common to all members of the cytochrome P450 superfamily. The carboxyl (C)-terminus has a heme-binding region, which contains a conserved cysteine residue that serves as the fifth coordinating ligand to the heme iron. This is responsible for the characteristic absorption of P450s at 403 nm. P450arom is extremely specific for androstenedione and testosterone as substrates. It binds these substrates with nanomolar affinities and catalyzes the aromatization of the A-rings, thereby converting them to estrogens. Aromatization results in the loss of the methyl group at the C19 position of the substrate. P450arom catalysis requires coupling with a second enzyme, NADPH-reductase, which uses the co-enzyme NADPH to supply the all-important electrons required for hydroxylation and aromatization reactions [8].

The monoclonal antibody IgG MAb3-2C2 was used for purification of the active protein by immunoaffinity chromatography and for further study because of its superior ability to suppress P450arom activity in microsomal fractions of human placenta [9–11]. Evaluation of the aromatase suppression assays, Western blots, and enzyme-linked immunosorbent assays (ELISA) suggested that this monoclonal antibody binds to P450arom at the three-dimensional epitope essential for its catalytic function [9–12]. To understand the interaction in detail, Fab and $F(ab')_2$ fragments were prepared by digesting the antibody with immobilized papain. Single crystals of Fab were grown and the three-dimensional structure of the fragment was determined [13]. Modeling of its interaction with the proposed three-dimensional structure of P450arom suggests that the complementarity determining regions (CDRs) may bind the enzyme at or near the substrate-access channel, thus explaining the activity-suppression property of the antibody [13].

In the present study, we prepare small antibody fragments of MAb3-2C2 that retain the suppression activity and make complexes of these fragments with P450arom. These complexes can be used as crystallization tools to aid in the structure determination of the antigen. The use of antibody fragments for crystallization and structure determination has been successful in several cases such as HIV-1 reverse transcriptase [14], HIV-1 capsid protein p24 [15] and cytochrome c oxidase [16]. In order to develop fragments smaller than Fab we obtained the clones of variable domains of heavy and light chains, VH and VL, respectively, of the antibody and proceeded with preparing a single construct clone expressing CDR containing VH and VL domains (Fv). The Fv fragment that retains its antigen-recognition and activity-suppression properties, in all likelihood, is better suited for crystallization than the full-length IgG. Owing to the compact nature of the tertiary structure of Fv and its high solubility, preparation of the P450arom–Fv complex could prove to be a prudent way to crystallize the enzyme, as has been previously demonstrated in other enzyme systems. This approach also has the potential of providing insight into antibody–antigen interactions which could lead to the synthesis of a therapeutic and/or diagnostic polypeptide with optimal affinity and specificity for P450arom.

2. Materials and methods

2.1. Cloning of light and heavy chains of MAb3-2C2 and single chain Fv

The light chain and the heavy chain cDNA were cloned directly from the hybridoma producing monoclonal antibody MAb3-2C2. By using primer sequences based on regions of conserved sequence adjacent to mouse immunoglobulin light and heavy chain complementarity determining regions, 250–500 bp sequences were amplified by polymerase chain reaction (PCR) and were cloned into pCR^{TM} 2.1 (3.9 kb) vectors.

The clones of the variable light chain and the heavy chain were further subcloned into a Bluescript vector. The leader sequence from the N-terminal region and the constant regions from the carboxyl C terminus were excised from the cDNA sequence by using appropriate primers. Also, restriction enzyme sites 5′ *Not*I (N-terminus) and 3′ *Sac*I (C-terminus) were introduced in the sequence. The positive clones were selected by Bluewhite screening in DH5α host cells. Mutations for correcting the reading frame errors in the variable light chain was done by the PCR overlap technique [17]. The molecular weight of all positive clones were checked by agarose gel electrophoresis. Sequencing of the positive clones were done at the Roswell Park Cancer Institute, Biopolymer facility.

These clones were then subcloned into the pET 22 + *b* vector (Novagen) at the *Nco*I/*Hin*dIII site with a pelB leader sequence at the N-terminus (21 amino acids). We used our X-ray structure of Fab [13] for designing the single chain Fv (scFv1) and linking the two variable domains. The sequences were linked by the PCR overlap technique [17] with the primers containing the 5′ *Nco*I and 3′ *Hin*dIII sites. For linking the two variable regions, we substituted nine amino acids from the N-terminus conserved region of the VL clone with glycines and serines to yield the amino acid sequence gly–gly–ser–gly–ser–gly–gly–gly–gly. Appropriate 3 bp codons coding for these glycines and serines were added in the primers, designed to hybridize to the 5′ end of VL DNA sequence and 3′ end of VH DNA sequence. We also cloned them into the same vector at *Nde*I/*Not*I without a pelB leader sequence. We developed two clones of scFv1, with and without the pelB leader sequence having the same nine amino acid linker.

We constructed an additional scFv (scFv2) by adding two extra glycine residues in the linker yielding an 11-amino acid

long sequence (gly–gly–gly–gly–ser–gly–ser–gly–gly–gly–gly). A His-tag and a non-His tag clones were generated by using different restriction enzymes in the vector.

2.2. Expression and purification of the VH, VL and scFv clones

The *Escherichia coli* host strain BL21-DE3 was used for expression of VH, VL, scFv clones. Cells (1 l culture) harboring the VL plasmids were cultured in the shaker incubator at 37 °C in LB medium containing carbenicillin (500 μg/ml). The expression was induced at the mid-log phase (OD ∼ 0.6) by isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Cells were then sonicated in sonication buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA, 6 mM β-mercaptoethanol). The homogenate was centrifuged at 10,000 rpm for 20 min. The cell pellet was resuspended and solubilized in 6 M urea in PBS buffer containing 10% glycerol and 1 mM *n*-dodecyl-β-D-maltopyranoside (DDM), stirred for 30 min and then subjected to ultracentrifugation at 30,000 rpm for 30 min. The supernatant was dialyzed against PBS buffer (10 mM mono- and di-basic potassium phosphate (KPO4), 150 mM NaCl, pH 7.4) with 10% glycerol.

The dialyzed fraction was spun and concentrated in an amicon using a YM-10 membrane and applied to a P-100 size exclusion column equilibrated with 100 mM KPO4 buffer and glycerol. The fractions from the column were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel. The protein concentration was measured by the Bradford method.

A 2 l culture of scFv1/scFv2 harboring cells was grown and the periplasmic fraction was isolated by osmotic shock. The protein from periplasmic fraction was extracted by salting out with 80% saturated ammonium sulfate. Proteins secreted into the growth medium were recovered by ammonium sulfate precipitation as well. The cell pellet from the growth medium was re-suspended in 200 ml binding buffer (50 mM Tris–HCl, pH 8.0) and 2 ml of a 10 mg/ml lysozyme solution (final concentration −100 μg/ml) was added to it. The cell lysate was incubated at 30 °C for 15 min, sonicated and centrifuged at 12,000 rpm for 15 min. The pellets were resuspended in approximately 400 ml of binding buffer. The centrifugation was repeated once more and the pellet was resuspended in 200 ml of 6 M urea in the binding buffer. The solution was stirred on ice for 1 h and centrifuged at 20,000 rpm for 20 min. The supernatant was subjected to Ni-affinity column chromatography (Novagen). The precipitates from the ammonium sulfate precipitation of the periplasmic and media fractions were resolubilized in the binding buffer, concentrated and loaded onto the Ni-affinity column. The Ni-column was prepared by charging the metal chelating linker on the agarose with 50 mM $NiSO_4$. The column was washed previously with 50 mM immidazole and the protein was eluted with 300 mM immidazole. The eluate was applied on to a G-25 desalt column equilibrated with PBS buffer and DTT. The desalted fraction eluted as a single peak and was centrifuged at 40,000 rpm for 20 min.

2.3. Sequencing of N-terminal amino acids

The samples for sequencing were prepared by running SDS-PAGE with protein samples in 12% Tris–HCl pre-cast gels and transferring them to polyvinylidene difluoride membranes by blotting. The bands of interests were cut from the membrane, dried and then sent for sequencing. The sequencing was done by Edman degradation by a commercial company specializing in protein sequencing (Proseq Inc., Boxford, MA). Unambiguous determination of 10 N-terminal amino acid sequences is usually made by this method.

2.4. P450arom activity assay

A typical incubation for aromtase contained placental microsomes (4 μg of protein, 370.6 pmol/(min mg)) premixed with scFv preparations (typically protein 0–22 μg/ml purified from the inclusion body and 0–5 μg/ml purified from the periplasm) in presence of substrate ([1β-^{3}H,4-^{14}C] androstenedione, specific activity 2.7×10^{3} dpm of ^{3}H and 0.5% bovine serum albumin (BSA) in 1 ml of 100 mM KPO_4 buffer, pH 7.4. Following preincubation at 37 °C for 10 min, the aromatase reaction was started by addition of 0.1 ml of 0.5 mM NADPH in 100 mM KPO_4 buffer, pH 7.4. After shaking for 20 min at 37 °C, the reaction was terminated by the addition of 0.4 ml of 20% trichloroacetic acid and 1.0 ml of 5% charcoal. After continued shaking at 37 °C for another 30 min, the mixture was centrifuged and the supernatant was filtered through a cotton plugged disposable pasteur pipette. The ^{3}H water in the eluate was assessed according to the 1β elimination mechanism (75% release into water) [12].

For $F(ab')_2$ suppression, 4 μg of human placental microsomes (specific activity = 375.3 pmol/(min mg)) was premixed with 0–7.11 μg/ml of $F(ab')_2$ with other conditions same as previously described.

2.5. Preparation and purification of $F(ab')_2$

$F(ab')_2$ was purified as the second product of Fab purification from digestion of MAb3-2C2 IgG. IgG (58 mg protein in 2.5 ml of 0.15 M phosphate buffer, pH 7.0) was mixed with 8 ml of packed activated solid phase papain gel and 17 mM cysteine and 4 mM EDTA were added. The volume was adjusted to 16.5 ml with 0.15 M phosphate buffer and incubated at 37 °C for 6 h while shaking. Papain gel was then separated from the mixture by centrifugation ($2000 \times g$, 5 min at 4 °C) and washed twice with 0.15 M KCl. The supernatant was concentrated to 10 ml by amicon with a YM-30 membrane and the buffer was also changed to PBS. The supernatant in binding buffer was applied to a protein A column (1.5 cm × 6 cm, equilibrated with binding buffer). A mixture of $F(ab')_2$ and Fab was collected as the column flow-through.

The column wash containing binding buffer and the column pass were combined and concentrated, and then applied to a S-200 high resolution (HR) column (1.5 cm × 95 cm, equilibrated with PBS buffer). The $F(ab')_2$ containing fraction from the column was concentrated to 6 ml and was again gel filtered using the same column to completely remove Fab contaminants [18].

2.6. Complexation of $F(ab')_2$ with P450arom

P450arom was mixed with $F(ab')_2$ in a molar ratio of 2:1 in a solution of 100 mM KPO4 in Buffer A (10 mM KPO4, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.5 μM androstenedione) containing 1 mM DDM. The protein mixture was then incubated overnight in the cold room and applied to a MAb3-2C2 column equilibrated with Buffer A and 0.15% Emulgen. The pass and the washing fractions from the MAb3-2C2 column were then passed through a hydroxylapatite column for replacing Emulgen with 1 mM DDM. The column pass-through contained free $F(ab')_2$. The $F(ab')_2$-P450arom complex was eluted from the column by 200 mM phosphate buffer containing 1 mM DDM and was concentrated using a YM-30 membrane. P450arom was also complexed with VL_2 in a molar ratio of 1:1 in a similar way during a pilot experiment.

2.7. Reconstituted P450arom activity assay

(a) $F(ab')_2$–P450arom complex

The activity of P450arom complexed with VL_2 and $F(ab')_2$ was measured by reconstituted aromatase assay [19]. P450arom containing samples (purified P450arom: 0.20 μg, $F(ab')_2$–P450arom complex: 0.09 μg) were reconstituted with 2 μg NADPH-cytP450 reductase and 20 μg DLPC. Reconstituted aromatase was pre-incubated with 0.24 μM [1β-^{3}H,4-^{14}C] androstenedione, 0.0045% Emulgen in 1 ml 100 mM KPO4 buffer pH 7.4 at 37 °C for 1 min. Then aromatization reaction was started by the addition of NADPH (0.5 mM). After the reaction mixture was incubated for 10 min at 37 °C, 0.2 ml of 20% TCA was added to terminate the reaction.

(b) VL_2–P450arom complex

P450arom containing samples (purified P450arom: 0.20 μg, VL_2–P450arom complex: 0.41 μg) were reconstituted with 2 μg NADPH-cytP450 reductase and 20 μg DLPC. The procedure was the same as described above for the $F(ab')_2$ –P450arom complex.

2.8. Reduction of detergent (DDM) concentration in P450arom–antibody complexes

Experiments to reduce the DDM concentration in $F(ab')_2$- and VL_2-complexes of P450arom were done by ultrafiltration, lowering the detergent concentration in steps to about 0.02 mM.

2.9. Polyacrylamide gel electrophoresis (PAGE)

These experiments were conducted at denaturing and reducing conditions, in the presence of 3% SDS and 5% β-mercaptoethanol using 10–14% pre-cast gels. The native PAGE experiments were done with a low 0.02% SDS in absence of β-mercaptoethanol. All gels were developed using the silver-staining method.

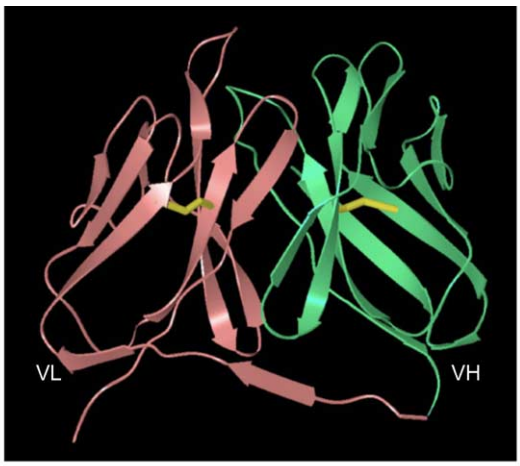
3. Results

3.1. Cloning of VH, VL and scFv

The variable and constant regions containing the hypervariable complementarity determining regions of light and heavy chain were cloned directly from the hybridoma producing MAb3-2C2 monoclonal antibody. The clones (KB1 and HF) were isolated according to their size by gel electrophoresis and were sequenced. These clones were then further subcloned so that the sequence contained only the CDRs and the conserved regions joining the CDRs. VH and VL clones were 357 and 300 bp long, respectively. The corresponding protein sequences from the sequenced DNA of both VH and VL were compared with the sequence obtained from the crystal structure of Fab. The comparison of the amino acid sequence of the VL clone showed some discrepancies, which were attributed to a frame shift mutation at amino acid 98 and one base substitution at amino acid 23 yielding a Tyr to Cys mutation. An insertion of the nucleotide cytosine after the codon GAG (GAGCT to GAGCCT) corresponding to the amino acid 97 rectified the frame shift mutation. A single base substitution (TAC

Table 1
Primers used for subcloning VL,VH and scFV

Primers for subcloning VL:
Start 5′-GGCCCATGGACATTGTGCTGACACAGTCTCCT-GCT-3′
Stop 5′-GGCAAGCTTCTAGGTCAAAGGCTCCCTAATG-TGCTGACA-3′
Primers for subcloning VH
Start 5′-GGCCCATGGATGTGCAGCTTCAGGAGTCGG-GACCT-3′
Stop 5′-GGCCTCGAGCTATGAGGAGACGGTGACCGT-GGTCC-3′
Primers for subcloning ScFv
VH start 5′-GGCCCATGGATGTGCAGCTTCAGGA-GTGGGACCT-3′
VL stop 5′-GGCAAGCTTCTAGGTCAAAGGCTCCCTAAT-GTGCTGACA-3′
VH-3′ primer 5′-GCCGCCACTGCCACTGCCTCCGCCT-CCTGAGGAGACGGTGACCGTGGT-3′
VL-3′ primer 5′-GGAGGCGGAGGCAGTGGCAGTGGCGGC-TCCTTAGCTGTTCTCTGGGG-3′



(a)

```
                10                  20                  30                  40
DVQLQESGPGLVKPSQSLSLTCTVTGYSISSDYAWNWIRQ
                50                  60                  70                  80
FPGNKLEWMGYISYSGSTSYNPSLKSRISITRDTSKNQFF
                90                  100                 110                 120
LQLSSVITEDTATYYCARGYYGSSHSPVWGAGTTVTVSSG
                130                 140                 150                 160
GGGSGSGGSLAVSLGQRATISCRASKSVSTSGYGYMHWNQ
                170                 180                 190                 200
QKPGQPPRLLIYLVSNLESGVPARFSGSGSGTDFTLNIHP
                210                 219
VEEEDAATYYCQHIREPLT
```

(b)

Fig. 1. (a) A model structure of the engineered scFv1 derived from the crystal structure of Fab. (b) The amino acid sequence of scFv1. The sequence in bold is the linker peptide. Underlined regions are the hypervariable regions (CDRs).

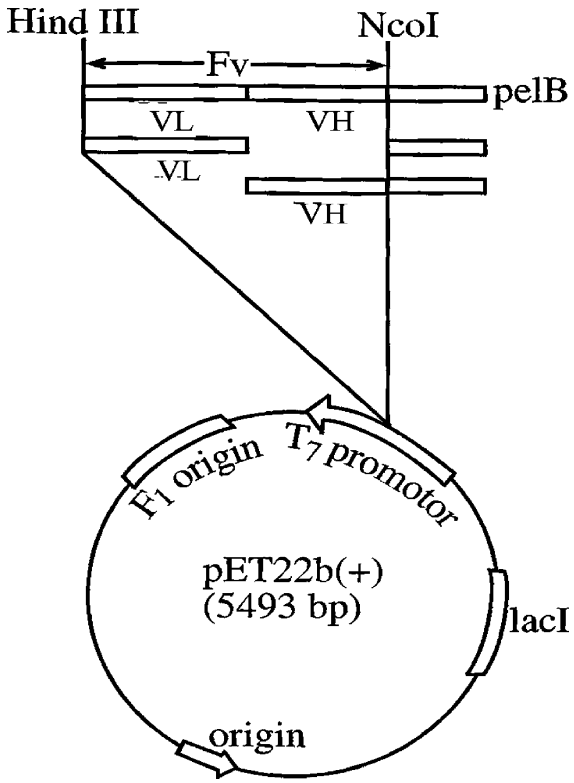


Fig. 2. A schematic diagram showing subcloning of antibody fragments VH,VL and scFV in pET22b(+).

to TGC) corrected a tyrosine at residue 23 of the protein sequence back to a conserved cysteine involved in disulfide bonding. The C terminus of VH was linked to the N-terminus of VL into a single chain Fv. The crystal structure of Fab suggested that the first beta strand (of VL) (Fig. 1a) not involved in CDR-mediated interactions could be converted into a linker peptide by making substitutions such that first eleven amino acids from the N-terminus conserved region of VL were all glycines and serines. The linker sequence was thus gly–gly–ser–gly–ser–gly–gly–gly–gly (Fig. 1b). A second clone, scFv2, was generated by adding two glycine residues to the linker. A N-terminal pelB leader sequence was also included in all these constructs in order for recombinant proteins to be secreted into the periplasm of *E. coli* cells [20,21]. A summary of all the cloning work is shown in Fig. 2. The primers for cloning are shown in Table 1.

3.2. Expression and purification of VH, VL and scFv

VH and VL were isolated from the inclusion-body fraction of the culture and were refolded by urea solubilization. VL was soluble in aqueous media, traveled as a homodimer (VL_2), and eluted as a 22 kDa fraction from a P-100 gel-filtration column (Fig. 3a and b). One liter of the culture typically produced about 1 mg of pure VL_2. The amount obtained as dimeric form of VH was less, approx. 0.5 mg from 1 l cell culture. Most of it solubilized by aggregation and was found in the void volume of the gel filtration column run. Although the presence of scFv1 could be verified in the periplasmic fraction using a control and by its ability to suppress P450arom activity, the expression level in the periplasmic fraction was very low. Like VH, both scFv1/scFv2 and VL were found to be present mainly in the inclusion body fraction. The protein was purified by Ni-affinity column chromatography. A silver-stained SDS-PAGE showed a prominent protein band corresponding to a molecular mass of ~23–24 kDa (scFv1) (Fig. 4a). However, the expression and overall yield of scFv1 was significantly lower than scFv2 (Fig. 4b). Consequently, most of the further experiments were conducted with the scFv2 material. The yield of scFv2 from the inclusion body after the Ni-affinity column purification was approximately 0.35 mg of protein per 1 l of cell culture. The purification was conducted in the presence of the detergent *n*-octyl-β-D-glucopyranoside (BOG), which probably helped in the solubilization of scFv's. A gel filtration column (P100) chromatography demonstrated that scFv2 traveled as a monomer of molecular weight of about 24 kDa (Fig. 5a). The corresponding SDS-PAGE is shown in Fig. 5b. Finally, the identities of expressed VL and scFv2 were confirmed by obtaining the amino acid sequences of

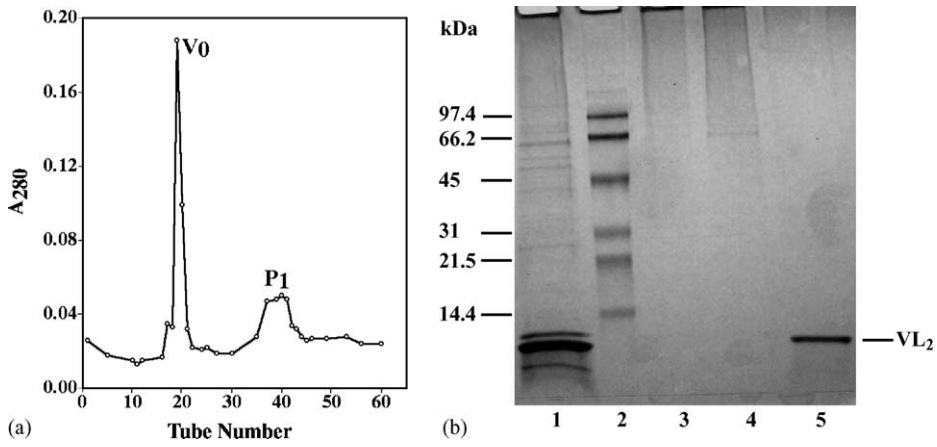


Fig. 3. (a) A P-100 gel filtration chromatogram showing void volume (Vo) and VL_2 (P1) peaks. (b) Silver-stained SDS-PAGE results showing lane 1: solubilized inclusion body, pre-P-100 column, lane 2: standards, lanes 3 and 4: void volume (Vo) and lane 5: purified VL_2 (peak P1).

10 N-terminal residues by Edman degradation. These data also showed that the neither protein retained the pelB leader sequence.

3.3. Suppression of P450arom activity by antibody fragments

The ability of the antibody fragments VL_2, scFv1, scFv2 and $F(ab')_2$ to suppress P450arom activity was measured using both purified and microsomal P450arom. Final measurements were taken with the purified enzyme. P450arom preparations were incubated with these antibody fragments to allow specific interaction between antibody fragments and the enzyme. Recombinant fragments VL_2 and scFv2 were found to suppress P450arom activity in a dose-dependent manner, similar to that observed for natural IgG (Fig. 6). The IC_{50} values (antibody necessary to suppress 50% of P450arom activity) measured for scFv2 and VL_2 were 1.2 and 87 μg/ml, respectively. The IC_{50} value of scFv2 is, thus, similar to that of $F(ab')_2$ or IgG previously determined to be about 1.0 μg/ml [10] and is considerably better than 42 μg/ml measured for scFv1 (data not shown).

3.4. Purification and crystallization of P450arom

Immunoaffinity chromatography that relies on the high specificity and affinity of MAb3-2C2 IgG for P450arom [11,22] was used to produce typically 3–5 mg of pure enzyme from one full-term human placenta.

All crystallization trials were conducted with enzyme preparations in the presence of ~1 μM concentrations of the natural substrate, androstenedione (K_D < 1 nM in absence of the reductase). A major hindrance towards successful

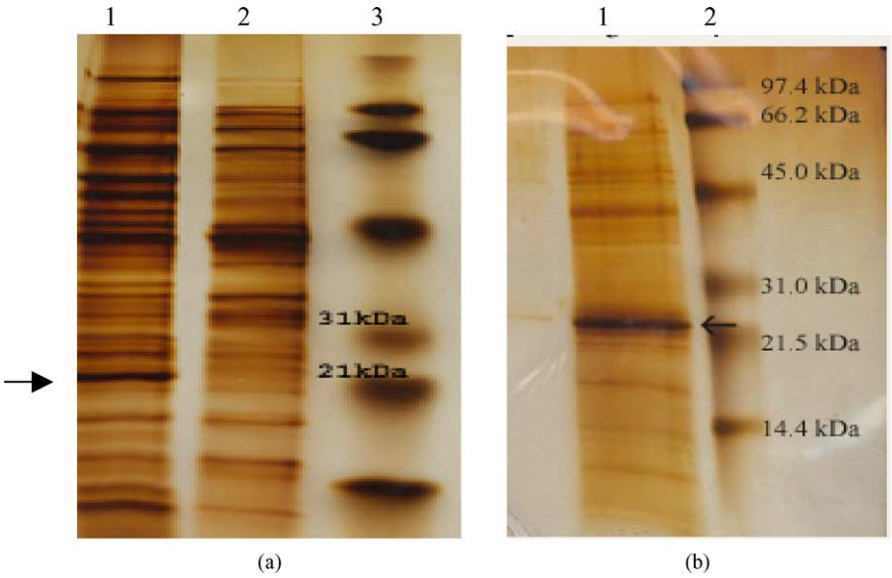


Fig. 4. (a) Silver-stained SDS-PAGE of scFv1, lane 1: around 24 kDa. Lane 2: periplasmic fraction. (b) Silver-stained SDS-PAGE of scFv2, lane 1.

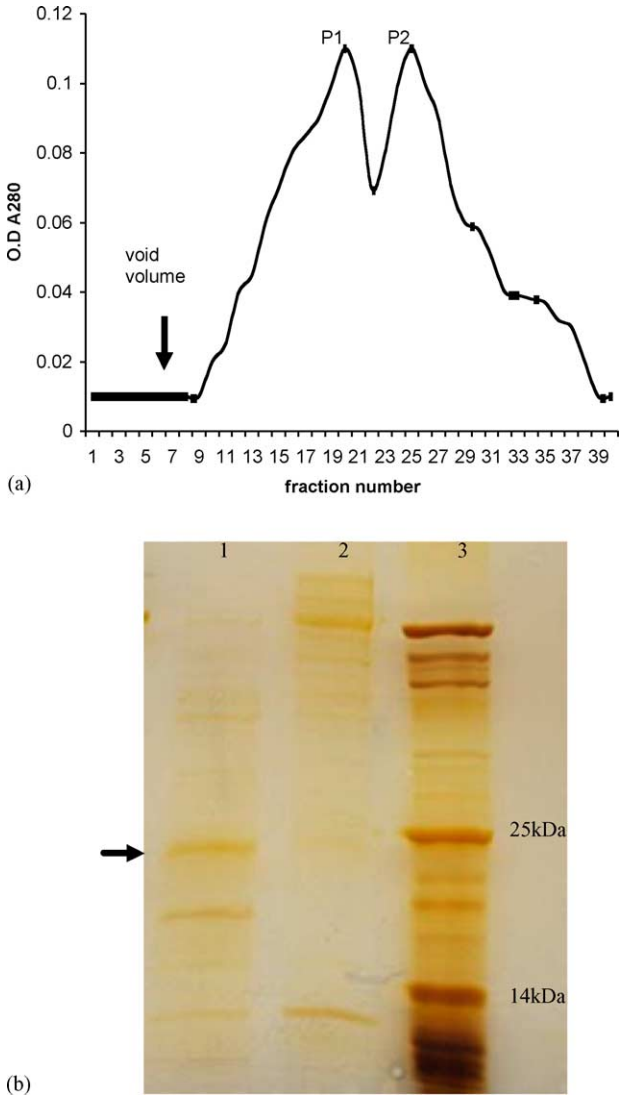


Fig. 5. (a) A P-100 gel filtration chromatogram showing the void volume and scFv2 (P2) peak positions. (b) Silver-stained SDS-PAGE of the peak fractions in (a). Lane 1: P2, showing the scFv2 as the most prominent band at about 24 kDa; lane 2: peak 1 (P1); lane 3: standards.

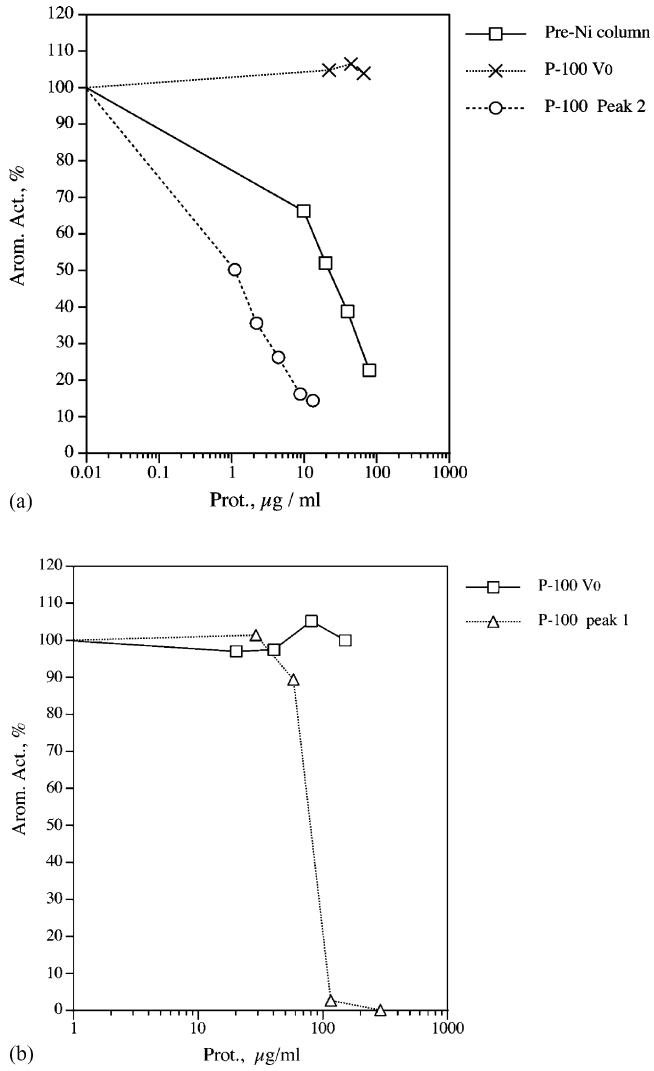


Fig. 6. Suppression of P450arom activity by antibody fragments. IC_{50} values computed from the suppression data 1.2 and 87.0 μg/ml for (a) scFv2 and (b) VL_2, respectively.

crystallization of P450arom was instability of the enzyme at 4 °C in a detergent solution suitable for crystallization. Emulgen 913, due to its heterogeneity and high molecular mass, is unsuitable for crystallization purposes. Initial crystallization trials were conducted with the detergent BOG at a concentration ranging from 0.02 to 4.0%. Subsequently, it was discovered that the enzyme was unstable in BOG at 4 °C (only 0.3% P450arom activity retained after 20 days in 2.0% BOG). After conducting extensive detergent screens with varying alkyl chain lengths and head groups, DDM at a 1 mM concentration was found to retain 83.2% of initial activity (51 nmol/(min mg)) at 4 °C after 100 days (Fig. 7a). Additional ELISA tests demonstrated that DDM stabilized P450arom at 4 °C for an extended period of time (data not shown).

The use of DDM and refinement of conditions have resulted in the growth of reddish needle-shaped crystals (Fig. 7b) from a solution containing 10 mg/ml of pure P450arom in 12% polyethyleneglycol 550, 0.1 M bicine, pH 9.0, containing 20% glycerol, 1 mM DDM and 0.1% NaN_3. Subsequent SDS-PAGE analysis (Fig. 7c) of thoroughly washed crystals confirms the presence of P450arom in the crystals. These single crystals are, however, still small (∼0.05 mm) for X-ray diffraction.

3.5. Complexation of P450arom with antibody fragments

Activity and suppression data for various steps of preparation and purification of the P450arom–$F(ab')_2$ complex are shown in Table 2. Typically, incubation of P450arom and $F(ab')_2$ in a 2:1 molar ratio exhibited 70–80% suppression of the P450arom activity. The Mab3-2C2 affinity column

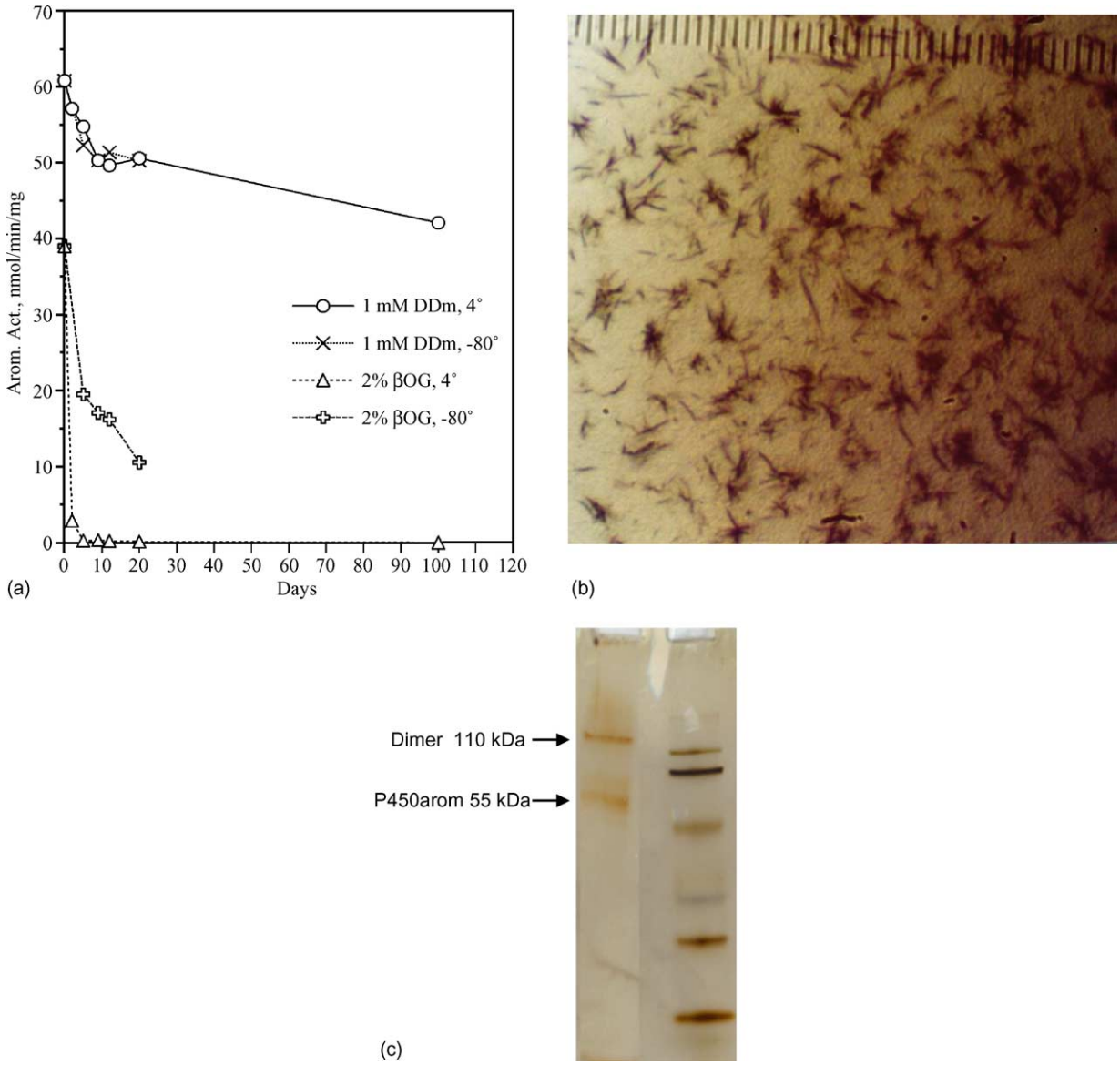


Fig. 7. (a) Time stability of aromatase activity in DDM and BOG at 4 °C. (b) A shower of small (∼0.05 mm) needle-shaped red single crystals of P450arom, grown from 12% polyethyleneglycol 550, 1 mM DDM in 0.1 M bicine, pH 9.0. (c) SDS-PAGE analysis of redissolved crystals.

trapped any residual free P450arom allowing the complex to pass-through, whereas the hydroxylapatite column bound the complex (because of its affinity for P450arom) and free $F(ab')_2$ passed through. When fractions of free P450arom and $F(ab')_2$ were combined, similar suppression of the enzyme activity was observed (Table 2, row 6).

Sufficient amounts of VL_2 were refolded from the inclusion body (∼1 mg l^{-1} of culture) and homodimerized

Table 2
Isolation of the [P450arom–$F(ab')_2$] complex

	Total P450arom (mg)	Specific activity (nmol/(min mg))	Specific activity (% of highest)
1 P450arom + mixture	0.750	28.3	35
2 Mab3-2C2 column eluate (free P450arom)	0.025	78.9	98
3 HA column pass-through (free $F(ab')_2$)	0.034	5.6	7
4 HA column eluate ([P450arom–$F(ab')_2$ complex])	0.154	16.7	21
5 P450arom used for complex formation	–	81.0	100
6 P450arom (#5) + HA column pass-through (#3)	–	33.1	41

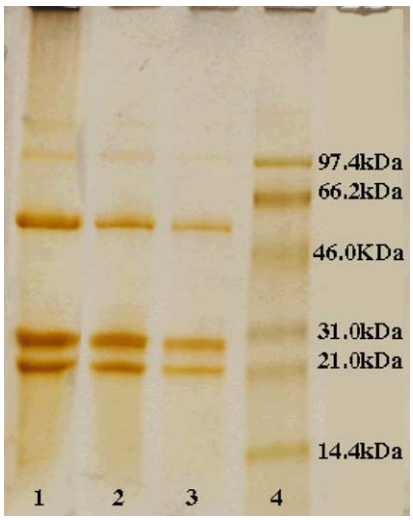


Fig. 8. A SDS-PAGE of P450arom–F(ab′)$_2$ complex. Lane 1: starting material 15 mg/ml, lane 2: supernatant, lane 3: microcrystaline precipitate. P450arom ∼55 kDa, heavy and light chains 21–26 kDa.

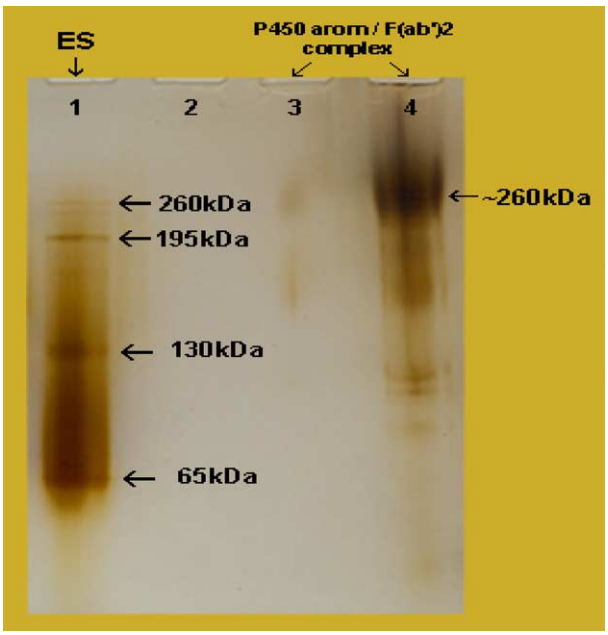


Fig. 9. Silver-stained non-denaturing SDS-PAGE of P450arom–F(ab′)$_2$ complex in lane 4. ES: estrone sulfatase used as a standard.

by dialysis. This homodimer of the variable domain of the light chain, the smallest in size of all the antibody fragments containing the CDRs, is soluble in aqueous medium. Suppression data shows that the IC_{50} value of VL_2 ranges between 80 and 90 μg/ml, similar to scFv1. Thus, this complex also provides an alternative to the crystallization of P450arom–F(ab′)$_2$.

The activity of P450arom complexed with VL_2 and F(ab′)$_2$ were measured by the reconstituted enzyme assay. Reconstitution assay done with the VL_2–P450arom complex showed a reduction of P450arom activity from 81.0 to 61.0 nmol/(min mg). Because of lack of sufficient quantities of pure scFv's, preparation of P450arom–scFv complexes are yet to be attempted.

The necessity of detergents for solubility of the enzyme was previously demonstrated [11]. Interestingly, however, P450arom complexes of both antibody fragments demonstrated reduced need for detergents. When the DDM concentrations in these complexes were reduced to as low as 0.02 mM, no precipitation of proteins was observed when examined with SDS-PAGE and protein assay. Efforts to crystallize the P450arom–F(ab′)$_2$ complex have begun. Fig. 8 is a SDS PAGE of the complex (lane 1), washed microcrystalline precipitate (lane 3) and supernatant (lane 2) from a crystallization droplet. All of the lanes display similar three-band patterns corresponding to P450arom (55 kDa), the F(ab′)$_2$-heavy chain (26 kDa) and the F(ab′)$_2$-light chain (23 kDa). Fig. 9 represents a native, non-denaturing and non-reducing PAGE conducted with the complex, against different oligomeric states of human ES, another microsomal, membrane-bound enzyme. The major band around the estimated molecular mass of 260 kDa provides direct evidence that the P450arom–F(ab′)$_2$ complex moves as a single assembly, as expected.

4. Discussion

4.1. Engineering of scFv and VL2

The design objective for the linker peptide was to keep the length to a minimum so that the inter-molecular cross-dimerization, leading to the formation of multimeric aggregates [23] could be prevented. This was achieved by mutating the side chains of the first beta-strand of VL, which does not participate in the CDR-mediated interactions, to all gly/ser. Disruption in the conformation of the first beta strand of VL and a linker having a less-than optimum length would have led to the disruption of folding of VH or VL or both domains. However, modeling of such a linkage between VH and VL domains using the crystal structure of Fab [13] suggested the feasibility of this design idea. Results showing successful expression and refolding of scFv in a soluble form provide the validation of design considerations. The solubilized and refolded scFv2 was shown to be primarily monomeric. In contrast, previous works had shown that a linking peptide of less than five residue-long tended to yield trimeric scFv ([24] and references therein). Our design utilized the first eleven residues including the first β-strand of VL, which does not participate in CDR-mediated interactions, as a linker peptide by converting all its residues to serines and glycines, and added only two extra glycines in constructing scFv2. Furthermore, the demonstration of a dose-dependent suppression of P450arom activity by the recombinant scFv, with an IC_{50} value similar to that of MAb3-2C2, suggests preservation of folding and conformation of the hypervariable region

of the recombinant protein. This, however, remains to be demonstrated by the crystal structure analysis.

Another interesting result with regard to the expression of recombinant antibody fragments is the purification of refolded, soluble homodimers of the VL domain. Although the expression levels of VL were consistently high, nearly all of the expressed protein was found, as expected, in the inclusion body. Nevertheless, VL could be refolded easily from the inclusion body by urea treatment and dialysis. The refolded, solubilized VL was found to be homodimeric, which could be easily purified by the one-step size-exclusion chromatography. By contrast, recombinant VH, although detected at high levels in the inclusion body, could not be solubilized by urea denaturation and renaturation. This behavior of variable domains of the heavy and the light chains, observed by others as well [24], is most likely due to incompatibility of the hydrophobic beta-sheet residues to pack against the same surface of another molecule of VH.

4.2. The P450arom–F(ab′)$_2$ complex

The incubated mixture in 2:1 molar ratio of P450arom to F(ab′)$_2$ after being passed through a MAb3-2C2 column contained mainly the complex, which subsequently eluted from the hydroxylapatite column as a single peak. The specific activity data shown in Table 2 demonstrate that the Mab3-2C2 column trapped the unbound fully active P450arom, while the pass-through after being eluted from the hydroxylapatite column had a suppressed activity, indicating the presence of the complex. The SDS-PAGE analysis of the complex under reducing conditions (Fig. 8, lane 1) showed the presence of the truncated heavy chain (∼28 kDa), the light chain (∼26 kDa) and P450arom (∼55 kDa). This result, along with data shown in Table 2, demonstrates the existence of a stable complex between P450arom and F(ab′)$_2$. Furthermore, a native PAGE analysis under non-reducing and non-denaturing conditions (Fig. 9) demonstrated that the complex traveled as an oligomer of about 260 kDa, in comparison with side-by-side run of human estrone sulfatase, which forms various oligomeric states. This result is consistent with an expected molecular mass of two P450arom molecules bound to one F(ab′)$_2$.

Initial attempts to produce diffraction-quality crystals of the complex have yielded microcrystalline precipitate in the crystallization droplets. Thoroughly washed and subjected to SDS-PAGE analysis, the precipitate displayed the same three bands (Fig. 8, lane 3) as shown by the complex solution (lane 1) and the supernatant of the crystallization droplet (lane 2). Furthermore, in all three lanes the band intensities have similar ratios, suggesting that likely content of the microcrystalline precipitate is the antibody–antigen complex.

4.3. Possible applications of antibody fragments

One possible application would be in crystallization of P450arom for structure-function studies. It has been suggested that instability of certain loop regions near the substrate entry path could have prevented crystallization of this membrane-bound enzyme despite years of attempts [12]. Modeling studies have shown that these loops are likely to be the epitope for Mab3-2C2, thereby explaining its activity-suppressing ability [13]. Complex formation with the antibody fragments could, therefore, add to the stability and integrity of the enzyme, thereby enhancing its crystallizability. Complexes with soluble antibody fragments would enhance the overall solubility of the enzyme preparation reducing its detergent need—a condition more conducive to crystallization.

The antibody and/or its fragments may have diagnostic and therapeutic applications, for detection of P450arom expression in tumor tissues and perhaps even for suppression of the enzyme activity. However, since the enzyme is endoplasmic reticulum membrane-bound, suppression of enzyme activity would require crossing of the plasma membrane by antibody fragments, limiting its possible applicability. Nonetheless, investigation of the molecular details of antibody–antigen interactions may lead to design of epitope-targeted pharmacophores that would specifically block the substrate entry channel, unlike active site directed inhibitors. Additionally, such molecular details could also be exploited in designing peptide-mimics of the epitope. These peptides would then be capable of triggering anti-P450arom immune response, providing a possible means for vaccination against estrogen biosynthesis by P450arom in high-risk population.

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