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Research paper

Truncation of N-terminal regions of *Digitalis lanata* progesterone 5β-reductase alters catalytic efficiency and substrate preference

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ABSTRACT

N-Terminal truncated forms of progesterone 5β-reductase (P5βR) were synthesized taking a full-length cDNA encoding for *Digitalis lanata* P5βR with a hexa-histidine tag attached at the C-terminus (r*Dl*P5βRc) as the starting point. Four pETite-c-His/*Dl*P5βR constructs coding for P5βR derivatives truncated in the N-terminal region, termed r*Dl*P5βRcn-10, r*Dl*P5βRcn-20, r*Dl*P5βRcn-30, and r*Dl*P5βRcn-40 were obtained by site-directed mutagenesis. The cDNAs coding for full-length r*Dl*P5βRc, r*Dl*P5βRcn-10 and r*Dl*P5βRcn-20 were over-expressed in *Escherichia coli* and the respective enzymes were soluble and catalytically active (progesterone and 2-cyclohexen-1-one as substrates). GST-tagged recombinant *Dl*P5βR (r*Dl*P5βR-GST) and r*Dl*P5βR-GSTr, with the GST-tag removed by protease treatment were produced as well and served as controls. The K_m values and substrate preferences considerably differed between the various *Dl*P5βR derivatives. As for the C-terminal His-tagged r*Dl*P5βR the catalytic efficiency for progesterone was highest for the full-length r*Dl*P5βRc whereas the N-terminal truncated forms preferred 2-cyclohexen-1-one as the substrate. Affinity tags and artifacts resulting from the cloning strategy used may alter substrate specificity. Therefore enzyme properties determined with recombinant proteins should not be used to infer *in vivo* scenarios and should be considered for each particular case.

1. Introduction

Recombinant enzymes are widely used as surrogates of genuine enzymes and the kinetic parameters and substrate preferences found for a particular recombinant enzyme are used to deduce the characteristics and roles of the respective genuine protein. Recombinant enzymes often differ from their natural counterparts as they possess additional amino acids in their flanking regions as a result of the cloning strategy applied. Affinity tags were added to facilitate protein purification after heterologous expression [1]. Moreover, peptide tags may have been added to simplify identification of recombinant enzymes by immunological techniques. Protein crystallization can be facilitated after the addition of suitable tags [2] or impaired [3]. Tagging is sometimes regarded as marginal since it is assumed that a small peptide attached to a protein has little effect on its crystal structure [4]. However, even amino acid substitutions peripheral to the active center may influence the charge properties and the protonation states of catalytic residues and thus influence the kinetic properties of an enzyme [5].

One of the most prominent tags is the His-tag added to either the N- or the C-terminus of a protein. The location of a hexa-histidine tag may affect not only the solubility but also the expression levels of recombinant proteins. Busso et al. (2003) [6] found that both N- and C-terminal His-tags can have a negative effect on protein solubility. In another study it was demonstrated that the presence of a C-terminal His-tag but not of an N-terminal His-tag has an effect on the release of iron from recombinant human serum transferrin [7]. N-terminal truncated mutants can lose binding activities as was demonstrated in several cases [8,9]. The catalytic properties of His-tagged recombinant enzymes and their wild-type forms may differ. The rate of catalysis, as well as substrate preferences or stereo- and regio-selectivity can be modified [10,11]. Several methods are available for cleaving off affinity tags before protein characterization. These procedures are time consuming and the cleavage may impair the enzyme under consideration in terms of its efficient expression, stability or other issues (see above). Progesterone 5β-reductase (P5βR), the enzyme

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investigated here, is a member of the short-chain dehydrogenase/reductase (SDR) super family of proteins and is encoded by *VEP1* in *Arabidopsis thaliana* and the homologues of *VEP1* found in many organisms. Members of the large and diverse group of SDRs are found in all domains of the tree of life [12] and many of them are thought to catalyze fundamental metabolic processes. Two isoenzymes (P5βR, P5βR2) have been identified so far [13,14]. Both can be produced as heterologous proteins in *Escherichia coli*. The recombinant *Dl*P5βR isolated by Herl et al. (2006a) [15] was a truncated, His-tagged form of the native *Digitalis lanata* P5βR. P5βR2 was expressed as a fusion protein with maltose-binding protein at the N-terminus [14]. The native P5βR of *Digitalis purpurea* as well as several recombinant forms of various plant origins have been characterized with regard to their substrate preferences [15–20]. All recombinant P5βRs isolated and characterized so far have been described as substrate-promiscuous enone reductases [19,20] (Fig. 1) whereas the substrate preference of P5βR2 has not yet been investigated. In this study we examined the influence of N-truncation of the *D. lanata* P5βR on enzymatic activity and substrate preference and give further evidence that kinetic constants determined with recombinant or tagged proteins may not be sufficient to reliably infer the physiological role of the native enzyme.

2. Material and methods

2.1. Biological material

D. lanata plants were grown in the greenhouse of the Department of Biology, FAU Erlangen-Nürnberg (latitude 49.600, longitude 11.015). Flowering was prohibited by keeping the plants at or above 15 °C. This way individual plants can be kept in the rosette stage over several years.

2.2. RNA extraction, cDNA synthesis and PCR

Fresh, young leaves of greenhouse plants were used for RNA isolation. Immediately after harvest, the leaves were frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. RNA was isolated using an innuPREP Plant RNA Kit (Analytik Jena AG, Jena, Germany) following the manufacturer's instructions. P5βR cDNA was synthesized employing SuperScript™ III First-Strand Synthesis for reverse transcription PCR (RT-PCR) Kit (Invitrogen, Karlsruhe, Germany). RT-PCR was carried out in a Personal Cycler 20 (Biometra GmbH, Göttingen, Germany) according to the instructions of the manufacturer. Briefly, 0.2 μg cDNA and 2 mM of each of the primers DirGATE and 1188brev were used (Table 1). Each reaction vessel contained in a final volume of 50 μL: 2.5 units peqGold Taq DNA-Polymerase (Peqlab GmbH, Erlangen, Germany), 1× reaction buffer S (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 0.1% Tween 20, 15 mM $MgCl_2$) and 0.5 mM of each dNTP. Nucleic acid amplification was completed after 30 PCR cycles (initial denaturation at 94 °C for 5 min; cycles: denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, elongation at 72 °C for 2 min; final elongation at 72 °C for 10 min). PCR products were analyzed by agarose gel electrophoresis [15]. Gels were stained with ethidium bromide and DNA visualized by illumination at UV_{365}nm. For size determination SmartLadder marker (Eurogentec GmbH, Köln, Germany) was used.

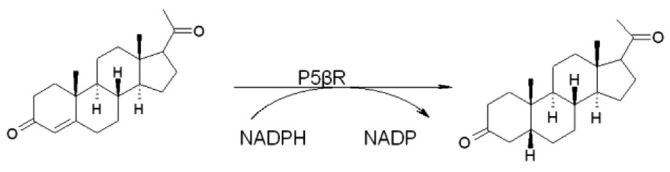

Fig. 1. Enantio-selective C=C double-bond reduction catalyzed by progesterone 5β-reductases.

Table 1
Primers used for constructing truncated plasmid vectors.

Name	Sequence 5′-3′	T_M
Mut10_For	GCTGCA AAGAAA AGGTTG	52 °C
Mut20_For	GCACAGCCAAAGCATTC	52 °C
Mut30_For	ATAGTTGGGGTAACCGG	52 °C
Mut40_For	AGCCTGGCGGAG	42 °C
Mut_Rev	CATATGTATATCTCCTTCTTATAGTTAAAC	48 °C
pETitedir	GAAGGAGATATACATATGAGCTGGTGGTGGG	58 °C
pETiterev	GTGATGGTGGTGATGATGAGGAACAATCTTGTAAGCTTTTG	60 °C
DirGATE	CACCATGAGYTGGTGGTGGGCT	55 °C
1188brev	AACCATGTCAAGGAACAATC	56 °C

A = adenine, C = cytosine, G = guanine, T = thymine, Y = pyrimidine.

2.3. Primer design and sub-cloning

For sub-cloning the DNA bands of expected size (about 1200 bp) were extracted with the Double Pure Kombi Kit (Bio & Sell e.K., Feucht, Germany) and ligated into the pCR2.1-TOPO cloning vector for subsequent transformation of *E. coli* Top10 cells (Invitrogen GmbH, Carlsbad, USA) following the instructions of the manufacturer. Plasmid DNA was isolated using the PeqGold plasmid miniprep Kit (Peqlab GmbH, Erlangen, Germany) and then sequenced by GATC Biotech AG (Konstanz, Germany).

Target cDNA was amplified with specific primers including 15–18 nt of overlap with the ends of the pETite vector encoding a C-terminal 6× His-tag. The primers were designed (Table 1) according to the Expresso T7 Cloning and Expression System manual (Lucigen, Middleton, WI, USA). The following steps, e.g., PCR amplification, agarose gel electrophoresis and sequencing were carried out as described earlier [21].

2.4. Deletion mutagenesis PCR

Phusion® Site-Directed Mutagenesis Kit (Finnzymes, Espoo, Finland) was used to create N-terminal truncated mutant proteins following the instructions provided by the manufacturer. Primers (Table 1) were deduced from the *D. lanata P5βR* (GenBank™ AY585867). The N-terminus was cut off in segments of 10 amino acids each. Ligation mixtures contained in a volume of 10.5 μL: 25 ng of the PCR product from the mutagenesis reaction, 1× Quick ligation buffer and 0.5 μL Quick T4 DNA Ligase (Finnzymes, Espoo, Finland). Circularization of mutated vector-insert constructs by ligation with Quick T4 DNA Ligase was achieved by incubation at 25 °C for 5 min. Then the wild-type P5βR-derived template DNA was selectively digested with restriction enzymes (2 h at 37 °C) by adding 20 units *Dpn*I (Fermentas GmbH, St. Leon-Rot, Germany) to the PCR mixture. Chemically competent *E. coli* BL21 (DE3) cells (Lucigen, Middleton, WI, USA) were transformed with 5 μL of the ligation mixture.

Transformed cells were selected on agar plates containing kanamycin (30 mg mL^{-1}). Colonies were picked and propagated overnight in 3 mL LB medium. The plasmid DNA was isolated using the PeqGOLD plasmid miniprep Kit (Peqlab GmbH, Erlangen, Germany). The quality and quantity of the DNA was evaluated with a NanoDrop 2000 (Thermo Scientific, Wilmington, USA) at a wavelength $\lambda = 260$ nm (and 280 nm). The samples were sequenced by Eurofins MWG (Eurofins MWG GmbH, Ebersberg, Germany) or GATC Biotech (GATC Biotech AG, Konstanz, Germany). The obtained sequences were aligned using ClustalW software package.