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Substrate flexibility and reaction specificity of tropinone reductase-like short-chain dehydrogenases

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ABSTRACT

Annotations of protein or gene sequences from large scale sequencing projects are based on protein size, characteristic binding motifs, and conserved catalytic amino acids, but biochemical functions are often uncertain. In the large family of short-chain dehydrogenases/reductases (SDRs), functional predictions often fail. Putative tropinone reductases, named tropinone reductase-like (TRL), are SDRs annotated in many genomes of organisms that do not contain tropane alkaloids. SDRs *in vitro* often accept several substrates complicating functional assignments. *Cochlearia officinalis*, a Brassicaceae, contains tropane alkaloids, in contrast to the closely related *Arabidopsis thaliana*. TRLs from *Arabidopsis* and the tropinone reductase isolated from *Cochlearia* (CoTR) were investigated for their catalytic capacity. In contrast to CoTR, none of the *Arabidopsis* TRLs reduced tropinone *in vitro*. NAD(H) and NADP(H) preferences were relaxed in two TRLs, and protein homology models revealed flexibility of amino acid residues in the active site allowing binding of both cofactors. TRLs reduced various carbonyl compounds, among them terpene ketones. The reduction was stereospecific for most of TRLs investigated, and the corresponding terpene alcohol oxidation was stereoselective. Carbonyl compounds that were identified to serve as substrates were applied for modeling pharmacophores of each TRL. A database of commercially available compounds was screened using the pharmacophores. Compounds identified as potential substrates were confirmed by turnover *in vitro*. Thus pharmacophores may contribute to better predictability of biochemical functions of SDR enzymes.

1. Introduction

Tropinone reductases are short-chain dehydrogenases/reductases (SDRs) specific for tropane alkaloid biosynthesis. In many genomes of organisms that do not contain tropane alkaloids, tropinone reductase-like genes are annotated due to sequence similarities. The SDR superfamily comprises over 47,000 sequences in gene and protein databases [1], among them over 300 SDR crystal structures in the PDB [2]. The SDR nomenclature initiative [3] divides the members of the SDR superfamily into seven types, wherein classical SDRs with a length of about 250 amino acids form the largest group in eukaryota and bacteria. SDR enzymes typically show a low pairwise sequence identity between 20% and 30%, but they share the three-dimensional structure [4,5]. A single-domain Rossmann-fold consisting of a central β-sheet with seven segments, flanked by three to four α-helices on each side is a folding pattern typical for nucleotide binding enzymes. Classical SDR enzymes are mostly NAD(P)(H) dependent carbonyl-alcohol oxidoreductases (EC 1.1.1.-) [4]. Binding of the cofactors NAD(H) or the phosphorylated NADP(H) is determined by few amino acids in the N-terminal glycine-rich motif (TGXXXGXG) and by few amino acids at the C-terminal end of the second β-sheet [6]. Classical SDRs share a catalytic triad comprising serine, tyrosine and lysine. The triad was extended into a tetrad for many SDRs by a conserved asparagine, which takes part in a proton relay system through a backbone bound water molecule [5,7]. Serine stabilizes the substrate by a hydrogen bond, and tyrosine acts as catalytic acid, from which a proton is transferred to the substrate carbonyl. Lysine forms a hydrogen bond with the

Abbreviations: CoTR, tropinone reductase of *Cochlearia officinalis*; Cotr, tropinone reductase of *Cochlearia officinalis* (cDNA); DsTRI, tropine forming tropinone reductase of *Datura stramonium*; DsTRII, pseudotropine forming tropinone reductase of *Datura stramonium*; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum correlation; Rt, retention time; SDR, short-chain dehydrogenase/reductase; TMS, tetramethyl silane; TR, tropinone reductase; TRL, tropinone reductase-like short-chain dehydrogenase/reductase.

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nicotinamide-ribosyl moiety (2′OH) of the cofactor and thereby lowers the pK_a of the tyrosine hydroxyl, promoting the proton transfer [7,8]. The substrate binding sites at the C-terminus of SDRs accept a wide array of substrates from simple aliphatic alcohols and sugars to complex alkaloid precursors, steroids and xenobiotics [9]. Individual SDRs often accept several substrates, which renders functional predictions complicated.

Tropinone reductase-like SDRs (TRLs) belong to the classical SDRs and were combined in group SDR65C by the SDR nomenclature initiative [3]. SDR65C currently contains over 350 members (http://www.sdr-enzymes.org, table of the SDR65C family, accessed July 2013). Genes of plants and microorganisms are annotated as (putative) tropinone reductases based on a sequence similarity of >50% to proven tropinone reductases from Solanaceae and sequence motif comparisons using Hidden Markov models. Typically, tropinone reductases (TRs) catalyze the stereospecific reduction of the alkaloid metabolite tropinone in the tropane alkaloid biosynthesis of Solanaceae, such as *Atropa belladonna* or *Datura stramonium* (Fig. 1). The product of TRI (EC 1.1.1.206) is tropine (α-tropanol) necessary for tropane alkaloid formation. TRII (EC 1.1.1.236) forms the β-alcohol pseudotropine, a precursor for nortropane alkaloids like calystegines [10]. In the Brassicaceae *Cochlearia officinalis*, the tropane alkaloid cochlearine and several calystegines were identified [11,12]. A SDR encoded by *cotr* (EMBL ID: **AM748271**) reduced tropinone with catalytic specificities different from Solanaceae TRs. The tropinone reductase isolated from *C. officinalis* (CoTR) forms both alcohols, tropine and pseudotropine, and catalyzes their oxidation back to tropinone (Fig. 1). The CoTR is so far the only functional TR identified from Brassicaceae. In the UniProt database [13] 16 genes of *Arabidopsis thaliana* (Brassicaceae) were annotated as "putative tropinone reductase", "tropine dehydrogenase" or "tropinone reductase I". These genes were later named "tropinone reductase-like SDR", because *A. thaliana* does not contain any tropane or nortropane alkaloids, and tropinone reducing activity appeared implausible in the plant [11]. Most of those genes are arranged in tandem on chromosome 2, spanning over positions At2g29150 to At2g29370.

The uncertainty of denomination and of functional assignment of *A. thaliana* SDRs prompted us to investigate the catalytic capacities of TRLs. For functional examination, two TRLs with an amino acid identity of 79% to the proven tropinone reductase from *C. officinalis* were chosen. They are encoded at loci *at2g29350* (EMBL ID: **AY081642**) and *at2g29150* (EMBL ID: **DQ056552**). In addition, one TRL encoded at *at2g29330* (EMBL ID: **BT005864**) was investigated, which is more distant with 61% overall identity to CoTR. The enzymes are subsequently named by their loci in the *Arabidopsis* genome. They were examined for their catalytic activities towards several small carbonyl and hydroxyl groups containing compounds. Sequence similarity to known TRs enabled construction of homology models of the enzymes At2g29150, At2g29330, At2g29350 and CoTR and substrate docking. Experimentally proven substrate structures then afforded the frame for pharmacophore descriptions that allowed screening a large database of commercially available compounds for identification of further substrates for the TRLs.

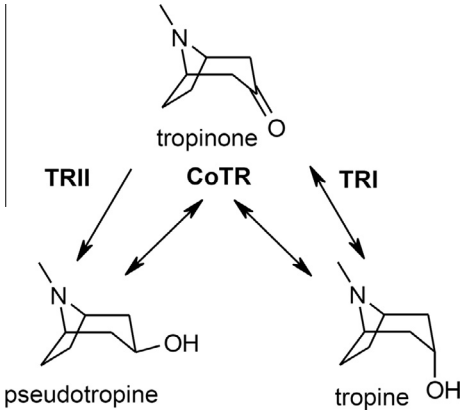

Fig. 1. Tropinone reduction: branch point in tropane and nortropane alkaloid biosynthesis. TRI, tropine forming tropinone reductase; TRII, pseudotropine forming tropinone reductase; CoTR, tropinone reductase from *Cochlearia officinalis*.

2. Results and discussion

2.1. Substrate binding site and tropinone reduction

First, tropinone-reducing capacity of the TRLs was examined by docking tropinone into the active site of protein models of the enzymes. Equally, tropinone was applied as substrate *in vitro*. In contrast to successful docking into homology models of the enzymes and a position of the carbonyl group adapted for reduction, none of *A. thaliana* TRLs reduced tropinone or oxidized tropine or pseudotropine *in vitro*. So far reduction of tropinone appears to be limited to tropinone reductases from Solanaceae and to CoTR from *C. officinalis*. In addition, a TRL protein called DnTR1 from *Dendrobium nobile* (Orchidaceae) reduced tropinone to tropine [14]. Crystallization and site-directed mutagenesis of TRI and TRII of *Datura stramonium* identified E156 in DsTRII and V168 at the equivalent position in DsTRI as the important amino acids for the stereospecific reduction of tropinone to either tropine or pseudotropine [15,16]. Compared to DsTRI and DsTRII the active sites of CoTR and the *Arabidopsis* TRL enzymes are more constricted. All Brassicaceae enzymes show a gap in the C-terminal region which forms the active site (position 221 in CoTR, Fig. 2), yet CoTR is a tropinone-reducing enzyme. The homology model of CoTR showed tropinone docked different from *Datura* TRs and in contact with Y209 [17]. In At2g29350, At2g29330 and At2g29150 hydrophobic phenylalanine or isoleucine residues are located at this position (Fig. 2, white on grey). As further space restriction, At2g29350 and At2g29150 contain a bulky tryptophane at the active site, where functional tropinone reductases contain a small glycine (black on yellow; Fig. 2). We conclude that bicyclic tropinone does not serve as substrate to TRL due to spatial hindrance of binding in spite of successful docking.

Tropinone reductases including CoTR are able to reduce ketones with structural similarity to tropinone (Fig. 3) like nortropinone, quinuclidin-3-one, and *N*-methyl- and *N*-propylpiperidin-4-one [17,19,20]. Except for At2g29330, the TRLs of this study did not reduce any of those compounds. The reducing activity of At2g29330 for tropinone-analogous substrates was too low for kinetic characterization.

In summary, these TRLs are no functional tropinone reductases. They neither reduce tropinone nor nitrogen-containing tropinone analogues, probably due to limited space in the binding pocket, although *in silico* docking of tropinone was possible.

2.2. Specificity of cofactor binding

The contrast between prediction of tropinone as substrate and the *in vitro* activity provoked to test how efficient substrates and cofactors for the TRLs overall may be predicted *in silico*. Kallberg et al. [6,21] developed an algorithm for prediction of cofactor specificity of classical SDRs, distinguishing NAD(H) and the phosphorylated forms. Basic amino acids like lysine (K31 in DsTRI) or arginine (R19 in DsTRII) in the glycine-rich motif (Table 1, Fig. 2) stabilize the 2′-phosphate-group of NADP(H) by ionic interactions. Arginine (R53 in DsTRI) at the first loop position after the second β-sheet