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Rational design of novel mutants of fungal 17β-hydroxysteroid dehydrogenase

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Abstract

Reduction of 17-ketosteroids is a biocatalytic process of economic significance for the production of steroid drugs. This reaction can be catalyzed by different microbial 17β-hydroxysteroid dehydrogenases (17β-HSD), like the 17β-HSD activity of *Saccharomyces cerevisiae*, *Pichia faranosa* and *Mycobacterium* sp., and by purified 3β,17β-HSD from *Pseudomonas testosteroni*. In addition to the bacterial 3β,17β-HSD the 17β-HSD of the filamentous fungus *Cochliobolus lunatus* is the only microbial 17β-HSD that has been expressed as a recombinant protein and fully characterized. On the basis of its modeled 3D structure, we selected several positions for the replacement of amino acids by site-directed mutagenesis to change substrate specificity, alter coenzyme requirements, and improve overall catalytic activity. Replacement of Val161 and Tyr212 in the substrate-binding region by Gly and Ala, respectively, increased the initial rates for the conversion of androstenedione to testosterone. Replacement of Tyr49 within the coenzyme binding site by Asp changed the coenzyme specificity of the enzyme. This latter mutant can convert the steroids not only in the presence of $NADP^+$ and NADPH, but also in the presence of NADH and NAD^+. The replacement of His164, located in the non-flexible part of the 'lid' covering the active center resulted in a conformation of the enzyme that possessed a higher catalytic activity.

Keywords: Biotransformation of steroids; 17β-Hydroxysteroid dehydrogenase; Fungi; *Cochliobolus lunatus*; Site-directed mutagenesis; Enzyme engineering

1. Introduction

Biotransformation of steroids is of great interest for the pharmaceutical industry (Zaks and Dodds, 1997, 1998; Kumar et al., 2001; Donova et al., 2005). A variety of steroid drugs are widely used as anti-inflammatory, diuretic, anabolic, contraceptive, anti-androgenic, progestational and anticancer agents, as well as in other applications. Pharmacological application of steroids requires both improvements in the yields of the desired metabolites, as well as the preparation of novel steroids, which are generally

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difficult to synthesize by chemical means (Mahato and Garai, 1997). Lead compounds for these important agents are derived from natural phytosterols that can be obtained from soya (β-sitosterol, campesterol and stigmasterol), conifers (β-sitosterol, campesterol and β-sitostanol) and rape seed (β-sitosterol, campesterol and brassicasterol) (Schmid et al., 2001). These natural raw sources are used for the production of androstenedione and androsta-1,4-dien-3,17-dione, which represent the basic substrates for the subsequent chemical and biotechnological production of steroid drugs (Schmid et al., 2001).

Specific microbial transformation steps have been incorporated into numerous synthetic pathways for steroid compounds to evaluate their potential as drugs and hormones (Mahato and Garai, 1997; Zaks and Dodds, 1997, 1998). Biocatalytic processes of economic significance include hydroxylation (mostly at the 11α, 11β and 16α positions; with *Rhizopus nigricans*, *Curvularia* sp., *Streptomyces* sp.), carbon–carbon bond cleavage (sterols to androsta-1,4-diene-3,17-dione, cholesterol to testosterone, sitosterol to androstenedione; with *Mycobacterium* sp.), dehydrogenation (hydrocortisone to prednisolone; at the Δ^1-position), isomerization (Δ^5-3-keto-steroid to Δ^4-3-keto-steroid, respectively; with *Pseudomonas testosteroni*) and reduction (17-ketosteroid reduction) (Smith, 1984; Präve et al., 1987; Mahato and Garai, 1997; Zaks and Dodds, 1997, 1998).

17-Ketosteroid reduction and 17β-hydroxysteroid oxidation are the final steps in androgen and estrogen biosynthesis, and they are catalyzed by 17β-hydroxysteroid dehydrogenases (17β-HSD). To date, 14 different 17β-HSD isoenzymes have been described in mammals (Adamski and Jakob, 2001; Mindnich et al., 2004; Lukacik et al., 2006). 17β-HSD activity has also been detected in bacteria (Lefebvre et al., 1979), filamentous fungi (Itagaki and Iwaya, 1988; Plemenitaš et al., 1988; Lanišnik et al., 1992; Lanišnik Rižner and Žakelj-Mavrič, 2000) and yeast (Charney and Herzog, 1967; Ghraf et al., 1978; Dlugonski and Wilmanska, 1998; Pajič et al., 1999; Lanišnik Rižner et al., 2001a). Indeed, the 17β-HSD activity of bacteria and yeast are being used in the biotransformation of steroids, such as for the reduction of androstenedione to testosterone with *Saccharomyces* sp. and *Mycobacterium* sp., and the use of purified 3β,17β-hydroxysteroid dehydrogenase from *P. testosteroni* (Smith, 1984; Donova et al., 2005).

Incorporation of enzymatically catalyzed reactions into steroid synthesis has many advantages. Enzymes are capable of accepting a wide array of complex molecules as substrates and they are distinctly selective, catalyzing reactions with enantio- and regioselectivities (Schmid et al., 2001). High selectivity also fosters reactions with few by-products, thereby making enzymes an environmentally friendly alternative to conventional chemical catalysts (Zaks and Dodds, 1998; Schmid et al., 2001; Donova et al., 2005). The ability to alter enzyme structure to cause a predicted change in function has become very interesting for pharmaceutical companies and a large number of enzymes with potential industrial significance have been produced. Such enzyme redesign involves changes in substrate or coenzyme specificity, inversion of stereochemistry, engineering new reaction mechanisms into the same active center and conversion of ligand-binding sites into catalytic centers (Penning and Jez, 2001; Hibbert et al., 2005)

In addition to the bacterial 3β,17β-HSD enzyme, 17β-HSD from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) is the only microbial 17β-HSD that has been expressed as the recombinant protein and fully characterized (Lanišnik Rižner et al., 1996, 1999, 2000, 2001b). This recombinant fungal 17β-HSD preferentially catalyzes the reduction of estrogens and androgens in the presence of NADPH (Lanišnik Rižner et al., 1996, 1999, 2001b). The previously defined homology-built model (Lanišnik Rižner et al., 2000) has been confirmed by preliminary X-ray analysis of 17β-HSDcl (Cassetta et al., 2005) (superimposed on a model with an r.m.s.d. of 0.88 Å) and has provided an ideal starting point for the redesign of this fungal enzyme that has potential biotechnological importance. The aim of the present study was to alter substrate specificity and coenzyme specificity (NADP(H) versus NAD(H)), and to facilitate accessibility to the active center.

2. Methods

2.1. Site-directed mutagenesis

Mutant proteins were constructed using a Quick Change Site-Directed Mutagenesis Kit, according to the manufacturer's instructions (Stratagene), the pGex-