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Structural basis for inhibition of 17β-hydroxysteroid dehydrogenases by phytoestrogens: The case of fungal 17β-HSDcl

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ABSTRACT

Phytoestrogens are plant-derived compounds that functionally and structurally mimic mammalian estrogens. Phytoestrogens have broad inhibitory activities toward several steroidogenic enzymes, such as the 17β-hydroxysteroid dehydrogenases (17β-HSDs), which modulate the biological potency of androgens and estrogens in mammals. However, to date, no crystallographic data are available to explain phytoestrogens binding to mammalian 17β-HSDs. NADP(H)-dependent 17β-HSD from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) has been the subject of extensive biochemical, kinetic and quantitative structure–activity relationship studies that have shown that the flavonols are the most potent inhibitors. In the present study, we investigated the structure–activity relationships of the ternary complexes between the holo form of 17β-HSDcl and the flavonols kaempferol and 3,7-dihydroxyflavone, in comparison with the isoflavones genistein and biochanin A. Crystallographic data are accompanied by kinetic analysis of the inhibition mechanisms for six flavonols (3-hydroxyflavone, 3,7-dihydroxyflavone, kaempferol, quercetin, fisetin, myricetin), one flavanone (naringenin), one flavone (luteolin), and two isoflavones (genistein, biochanin A). The kinetics analysis shows that the degree of hydroxylation of ring B significantly influences the overall inhibitory efficacy of the flavonols. A distinct binding mode defines the interactions between 17β-HSDcl and the flavones and isoflavones. Moreover, the complex with biochanin A reveals an unusual binding mode that appears to account for its greater inhibition of 17β-HSDcl with respect to genistein. Overall, these data provide a blueprint for identification of the distinct molecular determinants that underpin 17β-HSD inhibition by phytoestrogens.

1. Introduction

Phytoestrogens are plant-derived compounds that are functionally and structurally similar to mammalian estrogens, and they can initiate estrogen-receptor-mediated transcription by binding to estrogen receptors. These naturally occurring compounds belong to several chemically different classes, which include, among others, lignans, isoflavones, coumestans, flavanones, flavones, and flavonols [1].

As constituents of food, phytoestrogens have received considerable attention in terms of their potential effects on human health [2]. Emerging *in-vitro* and *in-vivo* studies have investigated the potential preventive actions that different phytoestrogen classes might have against specific pathologies [3,4]; e.g., flavonols against cardiovascular diseases [5], and isoflavones against various cancers [6]. More specifically, phytoestrogens can act at the molecular level in different ways. First, they can act as agonists or antagonists at estrogen receptors, pregnane X receptors, and constitutive androstane receptors. They can also increase the levels of sex hormone binding globulin, and inhibit tyrosine kinases, to prevent growth-factor-mediated stimulation of proliferation. At the pre-receptor level, they can modulate the activities of key enzymes in the biosynthesis and metabolism of steroid hormones [7]. The following physiological effects of phytoestrogens have also been reported: (i) protection against pro-inflammatory-factor–induced

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vascular endothelial barrier dysfunction, which is a major event in the pathogenesis of atherosclerosis [8]; (ii) regulation of bone remodeling and prevention of bone loss [9]; (iii) mitigation of postmenopausal depression, anxiety, cerebral ischemia, and cognitive dysfunction [10]; and (iv) neuroprotection through attenuation of the cytotoxic effects of the amyloid β-protein (25–35) [11].

An epidemiological study that was aimed at investigating potential correlations between phytoestrogen-rich diets and hormone-related forms of cancer [12] suggested a likely protective action of phytoestrogens against breast and prostate cancers [13,14]. On the contrary, concerns have been raised about the potential negative effects of phytoestrogens as endocrine-disrupting chemicals [15]. As phytoestrogens can modulate the activities of the endocrine system, understanding their interactions with the enzymes that are involved in steroidogenesis is still of great interest. Indeed, it has been reported that phytoestrogens show broad inhibitory activities towards several steroidogenic enzymes, and among others, toward different types of 17β-hydroxysteroid dehydrogenases (17β-HSDs) [16,17].

17β-HSDs modulate the biological potencies of androgens and estrogens through interconversions between the inactive 17-keto-steroids and their active 17β-hydroxy forms, which have higher affinities for their corresponding receptors. 17β-HSDs are therefore involved in the pre-receptor regulation of steroid hormone actions [18–22]. 17β-HSDs are widespread among all organisms, as they have been described for bacteria and fungi [23], and 14 different forms have been identified in vertebrates [24]. A constitutive NADP(H)-dependent 17β-HSD was isolated and purified from the filamentous fungus *Cochliobolus lunatus* (17β-HSDcl) [25], an opportunistic pathogen of both plants and humans. 17β-HSDcl was cloned and overexpressed in *Escherichia coli* [25], and it has been the subject of extensive biochemical and kinetic studies [25–28]. 17β-HSDcl is considered to be one of the model enzymes of the cP1 classical subfamily of the short-chain dehydrogenase/reductase protein superfamily, which are characterized by the Rossmann fold structural motif [26,29]. The functional profile of 17β-HSDcl has been defined through biochemical, kinetic, and quantitative structure–activity relationship studies, which have shown that flavones and flavonols are among the most effective inhibitors of 17β-HSDcl [7,30]. A crystallographic study on coumestrol-inhibited 17β-HSDcl revealed subtle conformational differences in the substrate-binding loop that appear to modulate the catalytic activity of 17β-HSDcl [29].

The aim of the present study was two-fold: (i) to determine the kinetics of the enzymatic inhibition mechanism of 17β-HSDcl by flavones and isoflavones; and (ii) to use X-ray crystallography to provide a structural interpretation of the observed structure–activity relationships of the inhibition of 17β-HSDcl by flavonoids. Kinetic inhibition of 17β-HSDcl by six flavonols, one flavanone, one flavone, and two isoflavones was studied. For the X-ray crystallographic studies, two flavonols were selected as among the most potent inhibitors of 17β-HSDcl on the basis of their different hydroxylation patterns, 3,7-dihydroxyflavone and kaempferol [7], with genistein and biochanin A also chosen as they are representative isoflavones and are weaker inhibitors of 17β-HSDcl [7].

2. Materials and methods

2.1. Chemicals

All of the chemicals used were acquired commercially (Carl Roth GmbH; Sigma-Aldrich; Fluka), while fisetin and myricetin were a kind gift from Dr. Stefan Martens (Department of Food Quality and Nutrition Department, IASMA Research and Innovation Centre, Fondazione Edmund Mach, S. Michele all'Adige, Italy). All of the chemicals used were at reagent or ultra-pure grades, and were used without further purification. Prior to their use in the inhibition assays and crystal-soaking experiments, 100 mM stock solutions of 4-oestrene-17β-ol-3-one and the flavonoids were prepared in pure dimethylsulfoxide.

2.2. Expression and purification of recombinant 17β-HSDcl

17β-HSDcl was expressed as a glutathione S-transferase fusion protein in *E. coli* BL21 cells. 17β-HSDcl purification was carried out as previously described, with cleavage of the glutathione S-transferase tag by thrombin [26]. Protein concentrations were determined using the Bradford method [31], with bovine serum albumin as standard. The purity of 17β-HSDcl was determined using SDS-PAGE on 12% polyacrylamide gels stained with Coomassie blue [32].

2.3. Inhibition assays and kinetics experiments

2.3.1. Inhibition of 17β-HSDcl by flavonoids

Initially, the flavonols 3-hydroxyflavone, 3,7-dihydroxyflavone, fisetin, and myricetin, the flavanone naringenin, the flavone luteolin, and the isoflavones genistein and biochanin A (Scheme 1) were tested for their inhibitory activities against 17β-HSDcl. The oxidation of 4-oestrene-17β-ol-3-one was monitored by the increase in absorbance at 340 nm, in the absence and presence of each individual flavonoid, using $NADP^+$ as the coenzyme. The reaction was allowed to initiate upon addition of the enzyme. According to the reaction mechanism involving 4-oestrene-17β-ol-3-one [29], its oxidation was initially monitored at only one concentration (50 or 100 μM 4-oestrene-17β-ol-3-one) and in the presence of large excess of coenzyme (2 mM $NADP^+$). The complete conversion of 4-oestrene-17β-ol-3-one to 4-oestrene-3,17-dione in the presence of the flavonols, flavanone, flavone, and isoflavones in the 10 μM to 100 μM range was then monitored. The aim of these measurements was both the determination of the inhibition constants, and to check for possible oxidation of the flavonoids tested. We therefore inspected the kinetics of myricetin action, and under identical conditions, also the effects of the two closely related flavonols kaempferol and quercetin. Further details of myricetin kinetics analysis are reported in the supplementary contents. All of the measurements were performed in 100 mM sodium phosphate buffer, pH 8.0, using a conventional spectrophotometer (Lambda 45; Perkin-Elmer).

2.3.2. Data analysis

Progress curves of 4-oestrene-17β-ol-3-one oxidation to 4-estrene-3,17-dione in the presence of $NADP^+$ and the different flavonoids were analyzed using the ENZO software [33], a numerical integration and fitting tool that is implemented through its web server http://enzo.cmm.ki.si. This server is designed for rapid and easy testing of different kinetic mechanisms, and it can translate graphically presented reaction schemes into sets of differential equations, with the subsequent estimation of the relevant kinetic parameters.

The putative reaction scheme used throughout the progress curves analysis assumed, according to the crystallographic results, that only one flavonoid molecule at time binds at the catalytic site of the holoenzyme.

Progress curves analysis revealed in all cases lower initial rates, i.e. the decreasing tangent's slopes of the curves at time zero, by increasing the flavonoid concentration. This behavior is consistent with an instantaneous type of inhibition. We always considered inhibition steps as fast events and therefore all the second order rate k_{on} constants (k_6 and k_8 in our case) were assumed