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22-NBD-cholesterol as a novel fluorescent substrate for cholesterol-converting oxidoreductases

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ABSTRACT

Docking simulations and experimental data indicate that 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (22-NBD-cholesterol), a common fluorescent sterol analog, binds into active sites of bovine cytochrome P450scc and microbial cholesterol dehydrogenases (CHDHs) and then undergoes regiospecific oxidations by these enzymes. The P450scc-dependent system was established to realize *N*-dealkylation activity toward 22-NBD-cholesterol, resulting in 7-nitrobenz[c][1,2,5]oxadiazole-4-amine (NBD-NH_2) formation as a dominant fluorescent product. Basing on LC–MS data of the probes derivatized with hydroxylamine or cholesterol oxidase, both pregnenolone and 20-formyl-pregn-5-en-3β-ol were deduced to be steroidal co-products of NBD-NH_2, indicating intricate character of the reaction. Products of CHDH-mediated conversions of 22-NBD-cholesterol were defined as 3-oxo-4-en and 3-oxo-5-en derivatives of the steroid. Moreover, the 3-oxo-4-en derivative was also found to be formed after 22-NBD-cholesterol incubation with pathogenic bacterium *Pseudomonas aeruginosa*, indicating a possible application of the reaction for a selective and sensitive detection of some microbes. The 3-keto-4-en derivative of 22-NBD-cholesterol may be also suitable as a new fluorescent probe for steroid hormone-binding enzymes or receptors.

1. Introduction

Cholesterol (cholest-5-en-3β-ol) is an important lipid component of plasma membranes of most vertebrates. It is also a biosynthetic precursor of bile acids, vitamin D and steroid hormones, which are active participants of cell signaling and other regulation processes, including cell homeostasis, proliferation and apoptosis. Moreover, steroid hormones are involved in control of whole organism functions, such as sexual differentiation, reproduction, fertility and blood pressure [1,2]. Biosynthesis of steroid hormones is performed by two groups of oxidoreductases, namely, cytochromes P450 and hydroxysteroid-dehydrogenases. The first rate-limiting step of mammalian steroidogenesis is a conversion of cholesterol into pregnenolone catalyzed by P450scc (CYP11A1). This enzyme consists of a single polypeptide [3,4] and acts as a terminal oxidase of mitochondrial monooxygenative system, which includes additionally two electron-transferring proteins – adrenodoxin (Adx) and NADPH-dependent adrenodoxin-reductase (AdR). Pregnenolone has no hormonal activity. Like other 3β-hydroxy-5-ene, pregnanes and androstanes, it should be first converted into the corresponding 3-keto-4-en derivative by the $NAD(P)^+$-dependent 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase (3β-HSD) to form the active steroid hormone [2]. On the other hand, cholesterol-metabolizing oxidoreductases also play essential roles in the cholesterol assimilation by some microbes (*e.g.*, genera *Pseudomonas*, *Nocardia*, *Mycobacterium*, *etc.*). Notably, that the pathogenicity and persistence of *Mycobacterium tuberculosis* – a pathogen causing about 2 million deaths annually – are closely associated with the utilization of host cell cholesterol. An initial step of the microbial degradation of the cholesterol and other 3β-hydroxy-5-ene steroids is their conversion into corresponding 3-keto-4-en derivatives. This step is catalyzed by either cholesterol oxidases (CHOXs, EC 1.1.3.6) or NAD^+-dependent cholesterol dehydrogenases (CHDHs, EC 1.1.1.145), more often referred in the literature as "microbial 3β-HSD" [5–11].

22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (22-NBD-cholesterol) is a fluorescent cholesterol analog, which has been used widely for investigations of the

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sterol traffic in cells, structural organization of lipid membranes and some cholesterol-binding proteins (sterol carrier protein-2, steroidogenic acute regulatory protein, *etc.*) [12,13]. To the best of our knowledge, the enzymatic conversion of 22-NBD-cholesterol into the corresponding 3-O-esters has been reported only [1,14,15]. However, there are practically no publications about interaction of the fluorescent steroid with cholesterol-converting oxidoreductases of the aforesaid pathways. Previously, we announced the ability of the bacterial CHOXs to convert 22-NBD-cholesterol into its 3-keto-4-one derivative [16].

In this article we show the results of computer simulations supported by further experimental data, demonstrating that the fluorescent steroid is a substrate of two cholesterol-metabolizing oxidoreductases, namely, the mammalian P450scc and the bacterial CHDH. We establish structures of the corresponding fluorescent products of these reactions and also propose possible applications of the newly discovered processes.

2. Materials and methods

2.1. Enzymes and chemicals

The following reagents were used: 22-NBD-cholesterol (Molecular Probes), cholesterol, pregnenolone, progesterone, hydroxylamine hydrochloride, NADPH and NAD^+ (Sigma), Polysorbate 80 and sodium cholate (Fluka). CHOX from *Brevibacterium sterolicum* and CHDH from *Nocardia* sp. ($\geq$18 U/mg solid) were obtained from Sigma. P450scc, adrenodoxin (Adx) and adrenodoxin-reductase (AdR) were purified from bovine adrenal cortex as described previously [4]. Spectral purity indexes for P450scc, Adx and AdR were A_{391}/A_{280} 0.83, A_{414}/A_{280} 0.83 and A_{272}/A_{455} 8.5, respectively. The P450scc preparation was more than 90% in high-spin form and contained less than 5% inactive P420 fraction.

2.2. Homology search and molecular docking simulations

The homology search was carried out by BLAST 2.2.22+ program using BLASTP algorithm [17]. The docking simulations were conducted with Autodock 4.0; the Graphical User Interface "AutoDockTools" (The Scripps Research Institute) was used to prepare, run, and analyze the docking simulations [18,19] with the 3D-structure of bovine P450scc (PDB ID: 3MZS) [20]. Gasteiger partial charges [21] were calculated and assigned to the atoms of heme and amino acid residues. The docking space was defined as a 60 Å × 60 Å × 60 Å box with its center close to redox-active heme of the protein. Small molecules were created and prepared using HyperChem 7.01 (Hypercube). The Lamarckian genetic algorithm with default parameters was applied for rigid docking calculations. The binding energy values were calculated automatically by Autodock.

2.3. 22-NBD-cholesterol conversion by P450scc enzymatic system

The stock solution of 22-NBD-cholesterol in propanol-2 was added to 50 mM potassium phosphate buffer (pH 7.4) with 0.02% sodium cholate, 0.1% Polysorbate 80 and then sonicated. The initial concentration of the fluorescent steroid was 2–20 μM. Then P450scc, Adx and AdR were added from stock solutions up to 0.2 μM, 2 μM and 0.2 μM, respectively, and mixtures were pre-incubated for 5 min at 30 °C. NADPH was then added from a stock solution up to 250 μM and incubation proceeded at 30 °C for 30 min. Control experiments were conducted without either P450scc or Adx and AdR as well as with hydrogen peroxide instead of NADPH. The conversions were stopped by ethyl acetate extraction. Each sample (1 ml) was extracted twice with 4 ml of ethyl acetate; corresponding organic phases were combined, evaporated to dryness and then dissolved in 0.5 ml of ethanol.

2.4. 22-NBD-cholesterol conversion by CHDH

The stock solution of the 22-NBD-cholesterol in propanol-2 was added to 50 mM potassium phosphate buffer (pH 7.4) with 0.05% sodium cholate, 0.2% Polysorbate 80 and then sonicated for 0.5 min at 35 kHz, resulting in 2–100 μM initial concentrations of the fluorescent steroid. NAD^+ was added up to 500 μM. Unless otherwise stated, enzymatic oxidation was initiated by the addition of the CHDH up to 0.04 U/ml. The conversion went at 30 °C for various time periods and stopped by ethyl acetate extraction. Each sample (1 ml) was extracted twice with 4 ml of ethyl acetate. The corresponding organic phases were combined, evaporated to dryness and then dissolved in 0.5 ml of ethanol. Further processing of the probes was carried out as described earlier (see Section 2.3).

2.5. 22-NBD-cholesterol incubation with growing opportunistic bacteria

Opportunistic strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were taken from the collection of the Republican Research and Practical Center for Epidemiology and Microbiology (Republic of Belarus). The bacteria were cultivated for 24 h at 37 °C on Petri dishes as described elsewhere [22] with 22-NBD-cholesterol added up to 10 μM into molten medium (45 °C) prior to the cultivation. After the cultivation corresponding zones (medium + bacteria) were cut out and placed into different tubes containing 10 ml ethanol. Then the tubes were sonicated (15 min at 35 kHz) and centrifuged (10 min at 3000 × *g*). The supernatant aliquots (1 ml) were transferred into vials and analyzed by HPLC with fluorescent detection (see Section 2.8).

2.6. Spectrofluorimetry

Fluorescence measurements were conducted with the spectrofluorimeter SM2203 (Solar). The slits of excitation and emission were set at 5 nm for all the fluorescence measurements. The fluorometric registration of the CHDH-mediated conversion of 22-NBD-cholesterol was performed using excitation at 350 nm (close to the NADH absorption maximum); fluorescence was registered at 470 nm and 530 nm (close to emission maxima of NADH and 22-NBD-cholesterol, respectively).

2.7. TLC

TLC was performed on silica aluminum foils (Fluka) with fluorescent indicator (254 nm). TLC plates were developed with benzene:acetone mixture (4:1 (v:v)). Spots of substances were monitored visually under 365 nm UV-light. Individual spots were cut out and extracted by ethanol in the necessary cases.

2.8. HPLC

HPLC was conducted using either the LC-10AT (Shimadzu) system with photodiode array detector SPD-M10A (operated at 200–600 nm range) or the LC10-AD (Shimadzu) system with fluorometric detector RF-10Axl (medium sensitivity, wavelengths of excitation and emission 466 and 530 nm, respectively). In both cases the column was LiChroCART C18 (250 mm × 4 mm, 5 μm) (Merck) with the elution conducted at a flow rate of 1 ml/min using either eluent A comprising acetonitrile:water:propanol-2 (84:16:5 (v:v:v)) or eluent B comprising acetonitrile:water:propanol-2 (80:20:5 (v:v:v)).