Tools for discovery of inhibitors of the 1-deoxy-D-xylulose 5-phosphate (DXP) synthase and DXP reductoisomerase: an approach with enzymes from the pathogenic bacterium

Pseudomonas aeruginosa

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Abstract

Two Pseudomonas aeruginosa genes encoding the enzymes 1-deoxy-D-xylulose 5-phosphate (DXP) synthase and DXP reductoisomerase, both involved in the mevalonate-independent biosynthesis of isoprenoids, have been expressed as recombinant enzymes in Escherichia coli. The purified P. aeruginosa DXP reductoisomerase was inhibited by submicromolar concentrations of the antibiotics fosmidomycin and FR-900098 in a well established method. A novel and convenient spectrophotometric assay was developed to determine activity and inhibition of P. aeruginosa DXP synthase. Fluoropyruvate is described as a first inhibitor of DXP synthase. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Pseudomonas aeruginosa is an important bacterial pathogen in humans. Hospitalized patients like burn victims, individuals with cancer and those with cystic fibrosis are particularly at risk of infection. Because antibiotics are often ineffective against this pathogen, infections are difficult to treat [1]. New antibiotics with higher efficiency and fewer side effects are urgently needed.

In earlier studies it was shown that the antibiotic fosmidomycin, originally isolated from Streptomyces lavendulae, efficiently inhibits the growth of P. aeruginosa [2] and was well tolerated in volunteers [3]. However, the molecular target of fosmidomycin was unknown. Recently, it was demonstrated that fosmidomycin is an efficient inhibitor of 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase from Plasmodium falciparum and Escherichia coli [4,5], one of the enzymes involved in the biosynthesis of isoprenoids through the mevalonate-independent DXP pathway which is present in many eubacteria, algae, chloroplasts of higher plants, and malaria parasites [4,6,7]. In many of the respective bacteria including P. aeruginosa the DXP pathway represents the only source of isopentenyl pyrophosphate (IPP), the universal precursor of isoprenoids [8].

The biochemical reactions of the DXP pathway have not been elucidated completely yet. The initial step, the formation of DXP by condensation of pyruvate and D-glyceraldehyde 3-phosphate, is catalyzed by DXP synthase [9–12] in a thiamine-dependent way (Fig. 1). In a second step, DXP is converted by DXP reductoisomerase into 2-C-methyl-D-erythritol 4-phosphate (MEP) in the presence of NADPH. According to recent work [13,14], a cytidyl residue is added to MEP leading to 4-(cytidine 5’-diphospho)-2-C-methyl-D-erythritol (CDP-ME) by the enzyme MEP cytidyltransferase. This compound is then transformed into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate in a two-step reaction including phosphorylation of CDP-ME at position C2 followed by formation of 2,4-
cyclodiphosphate under elimination of CMP [15,16]. Further biochemical steps towards the terminal compound IPP are still unknown.

2. Materials and methods

2.1. Cloning of the P. aeruginosa genes encoding DXP synthase and DXP reductoisomerase

The DXP synthase gene (1884 bp) was amplified by PCR from genomic DNA of strain ATCC 27853 using the following primers (Interactiva, Germany) based on the genomic DNA sequence contig 54 (7/15/99) from the Pseudomonas Genome Project (www.pseudomonas.com):

PaDXSfor 5'-GGT ACC GAA TTC ATG CCC AAG ACG CTC CAT GAG-3' including an EcoRI site (underlined) and PaDXSrev 5'-GGT ACC AGA TCT CTG CCG GTC GAG ACG CTG GCG-3' including a BglII site (underlined). PCR was carried out in a total volume of 20 µl of PCR buffer (Promega) including 125 µM of each deoxynucleoside triphosphate, 10 pmol of each primer, 5% dimethyl sulfoxide and 1 U of Taq polymerase (Promega) in 30 cycles (95°C for 30 s, 60°C for 30 s, and 72°C for 120 s). The DXP reductoisomerase gene (1191 bp) was cloned in a similar way using the primers PaDXRfor 5'-AGA TCT ATG AGT CGA CCG CAG CGG ATC-3' (BglII site underlined) and PaDXRrev 5'-AGA TCT TAATGGCCGCCGTGCGTGCGGTCAACCA3' (HindIII site underlined) for PCR.

2.2. Plasmid construction for expression of the recombinant DXP synthase and DXP reductoisomerase in E. coli

The plasmid pDS-Pa-DXS was constructed by cleavage of the resulting PCR fragment with EcoRI and BglII and ligation into a derivative of plasmid pQE-9 (Qiagen) containing these two restriction sites in appropriate positions in the multiple cloning site (pDS-Eco-neu, E. Beck, unpublished). Plasmid pQE-Pa-DXR was constructed by digesting the respective PCR fragment with BglII and HindIII and ligation into the vector pQE-30 (Qiagen) that had been cleaved with BamHI and HindIII.

2.3. Expression and purification of DXP synthase and DXP reductoisomerase

E. coli 'TOP10F' (Invitrogen) cells transformed with pDS-Pa-DXS or pQE-Pa-DXR were grown at 37°C in standard I medium (Merck) supplemented with ampicillin (150 µg ml⁻¹) and protein expression was induced at OD₆0₀ of 0.6 with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h. The bacteria were harvested by centrifugation, resuspended in buffer A (100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 2 mM 2-mercaptoethanol) and broken by ultrasonic treatment. Both histidine-tagged recombinant proteins were purified from the soluble fraction by affinity chromatography on Talon Superflow resin (Clontech, Heidelberg, Germany) with an HPLC device (Gynotech, Germering, Germany) under similar conditions. The columns were washed each with 10 mM imidazole in buffer A and the proteins eluted with 100 mM imidazole in buffer A. Protein concentrations were determined according to the method of Bradford [17] using bovine serum albumin as a standard.

2.4. Assay of DXP reductoisomerase

The DXP reductoisomerase assay was carried out as described [4]. The conversion of DXP to MEP by the purified recombinant DXP reductoisomerase from P. aeruginosa was monitored spectrophotometrically. A standard reaction mixture contained 1 µg DXP reductoisomerase, 40 mM Tris-HCl (pH 7.5), 1 mM MnCl₂, 1 mM DXP, and 0.3 mM NADPH in a total volume of 600 µl.

2.5. Assay of DXP synthase

To establish a convenient spectrophotometric assay to measure DXP synthase activity, a coupled enzyme assay was developed. In this assay DXP generated by DXP synthase is further converted to MEP in a NADPH-dependent reaction by an excess of DXP reductoisomerase from E. coli that was cloned and purified in a similar way as described [18]. The NADPH consumption was monitored spectrophotometrically at 365 nm. A standard reaction mixture contained 40 mM Tris-HCl (pH 7.5), 0.3 mM NADPH, 1 mM MgCl₂, 0.3 mM thiamine pyrophosphate, 6 mM dl-glyceraldehyde 3-phosphate, 3 mM pyruvate, 1 mM 2-mercaptoethanol, 100 mM NaCl, 27 µg (= 1 U) of E. coli DXP reductoisomerase and 3 µg of DXP synthase from P. aeruginosa in a total volume of 600 µl. For inhibition studies the reaction mixtures were preincubated with different concentrations of inhibitor for 5 min at 37°C and started by addition of pyruvate.

Fig. 1. Outline of the DXP pathway for the biosynthesis of IPP. TPP, thiamine pyrophosphate; P, phosphate; PP, pyrophosphate.
3. Results and discussion

3.1. Identification of the P. aeruginosa genes encoding DXP synthase and DXP reductoisomerase

The genes of DXP synthase and DXP reductoisomerase from P. aeruginosa were identified by comparing the respective sequences from E. coli with the P. aeruginosa sequence database using the tblastn program (www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html). The amino acid sequences of DXP synthase and DXP reductoisomerase from P. aeruginosa revealed over 57% and 61% identity, respectively, with the corresponding E. coli enzymes. The genes were subsequently identified by PCR in six different P. aeruginosa strains including four clinical isolates and the strains ATCC 27853 and ATCC 9027. The amplification products derived from all strains tested appeared to be identical in size (Fig. 2). The sequence data have been submitted to GenBank with the assigned accession number AF282878 for DXP synthase and AF282879 for DXP reductoisomerase.

3.2. Inhibition of DXP synthase

Using a coupled enzyme assay the activity of the purified recombinant DXP synthase from P. aeruginosa (Fig. 3) was monitored spectrophotometrically. The pH optimum of this assay was in range of pH 7.5–8.0 (data not shown). Searching for inhibitors of DXP synthase, fluoropyruvate was identified as a first inhibitor. The DXP synthase from P. aeruginosa was inhibited with an IC50 of 400 μM (Fig. 4). For comparison, the IC50 of the respective enzyme from E. coli was 80 μM. The DXP synthase from E. coli was cloned into pQE9 vector and was purified in a similar way as described for the DXP synthase from P. aeruginosa. The activity of DXP reductoisomerase was not affected by fluoropyruvate (data not shown). Fluoropyruvate is supposed to bind covalently to the active site of the DXP synthase in a comparable way as demonstrated for the pyruvate dehydrogenase component (E1) in the pyruvate dehydrogenase complex [19].

3.3. Inhibition of DXP reductoisomerase

The activity of the DXP reductoisomerase from P. aeruginosa (Fig. 3) was monitored by NADPH consumption of the reaction in an established assay. Replacement of NADPH by NADH resulted in a decrease of the activity to about 1% of the original reaction rate (data not shown) as described for the E. coli enzyme [18].

The DXP reductoisomerase from P. aeruginosa was inhibited by the antibiotics fosmidomycin and FR-900098 with an IC50 of 150 nM for both drugs (Fig. 5).
Fig. 5. Inhibition of recombinant P. aeruginosa DXP reductoisomerase by fosmidomycin (○) and FR-900098 (■). Relative activity was calculated by regarding maximum activity (approximately 11.9 U mg⁻¹ protein) at given conditions as 100%.

In a previous study the P. falciparum DXP reductoisomerase was inhibited 4 times more efficiently with FR-900098 than with fosmidomycin [4]. Fosmidomycin was supposed to be a mixed-type inhibitor (competitive and noncompetitive) of the DXP reductoisomerase from E. coli in an earlier study [5].

Taken together with earlier data demonstrating that fosmidomycin was active in protecting mice challenged with P. aeruginosa [2], the DXP pathway must be considered a valid target for the chemotherapy of P. aeruginosa infections. Using the convenient assay for DXP synthase described in this study together with the well established assay for DXP reductoisomerase we provide tools for easy and fast screening of potential inhibitors. In future work, the development of specific and potent antibiotics targeting both DXP synthase and reductoisomerase may lead to synergistic drug combinations with increased therapeutic value.

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