Biochimie 93 (2011) 1610–1622

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Validation of the catalytic mechanism of *Escherichia coli* purine nucleoside phosphorylase by structural and kinetic studies

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ARTICLE INFO

Article history:
Received 5 November 2010
Accepted 26 May 2011
Available online 13 June 2011

Keywords:
Purine nucleoside phosphorylase
Mechanism of catalysis
X-ray diffraction
Conformational change
Active (binding) sites

ABSTRACT

The catalytic mechanism of *Escherichia coli* purine nucleoside phosphorylase (PNP) is revised using site-directed mutagenesis, kinetic studies and structure determinations.

The experimental evidence on the role of the particular catalytic amino acid during catalysis has not been available. Therefore, the active site mutants Arg24Ala, Asp204Ala, Asp204Asn, Arg217Ala and Asp204Ala/Arg217Ala were prepared and their kinetics and thermodynamic studies were carried out. The activity tests with natural substrates and 7-methylguanosine confirmed the earlier hypothesis, that catalysis involves protonation of the purine base at position N7 by Asp204, which is triggered by Arg217.

The crystal structures of the wild type in complexes with phosphate and sulphate, respectively, and of the Arg24Ala mutant in complex with phosphate/sulphate were determined. The structural data show that previously observed conformational change is a result of the phosphate binding and its interaction with Arg24.

As *E. coli* PNP is a promising candidate for the tumour-directed gene therapy, our results may also help to design efficient mutants useful in gene therapy.

1. Introduction

Purine nucleoside phosphorylase (PNP, purine nucleoside orthophosphate ribosyl transferase, EC 2.4.2.1) is the key enzyme in the purine salvage pathway [1]. It catalyses the reversible phosphorolytic cleavage of the glycosidic bond of purine nucleosides and some analogues:

purine nucleoside + orthophosphate ↔ purine base + α-D-pentose-1-phosphate

There are two main classes of PNPs with very low sequence similarity: high- (homohexameric) and low-molecular mass PNPs (homotrimeric). *Escherichia coli* PNP belongs to the homohexameric class, and structurally it can be considered a trimer of dimers. In addition to 6-oxo purine nucleosides it also accepts 6-amino and other substituents at position 6 of purine substrates, such as adenosine and 6-methyl-purine nucleoside. Differences in specificity between low- and high-molecular mass PNPs offer a basis for enzyme-activating prodrug gene therapy by enabling selective killing of tumour cells expressing the *E. coli* PNP gene [2,3]. The most promising is a non-toxic prodrug 6-methyl-purine nucleoside (MeP-R), which liberates highly toxic 6-methyl-purine (MeP). However, the method is limited by presence of *E. coli* PNP in patients' digestive systems and liberation of toxic 6-methyl-purine there. Therefore, to minimise toxicity caused by intestinal bacteria, *E. coli* PNP should be redesigned to allow cleavage of only the prodrug by the enzyme present in tumour cells. One such mutant, Met64Val is already available, as well as prodrugs designed to be good substrates of this mutant and be poorly cleaved by the wild type of *E. coli* PNP [2]. To design new mutants suitable for gene therapy, knowledge of a detailed mechanism of phosphorolysis would be helpful.

We proposed such a mechanism [4] and we verify it here by kinetic, binding and structural studies of *E. coli* PNP active site mutants. Since some prodrugs (5′-5′-dimethyl-MeP-R) tested in the above proposed gene therapy interact with amino acids forming a phosphate binding site, we have focused our investigations on a detailed study of phosphate interactions with the wild type enzyme and Arg24Ala mutant. This mutant was selected due to the fact that the Arg24 residue was implicated in the conformational change observed in the structure of *E. coli* PNP [4], and is the most

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doi:10.1016/j.biochi.2011.05.030

conserved in all homohexameric PNPs with solved 3D structures (see Supplemental Fig. S1).

The biologically active form of the *E. coli* purine nucleoside phosphorylase (PNP) is a homohexamer whose structure could be described as a trimer of dimers. In the apoenzyme all six subunits show similar structure with entry into the active site open [5]. By contrast, in the ternary complex with substrate analogues [4] in each dimer one site showed open active site conformation while in the other monomer in the dimer segmentation of the C-terminal helix H8 partially closes the entrance to the active site pocket. Solution studies suggest that conformational change already occurs in the binary complex of the enzyme with phosphate [6]. However, to date crystals of *E. coli* PNP complexed with various ligands were grown from crystallisation solutions containing ammonium sulphate [4,7,8]. Since a sulphate ion is a PNP inhibitor [6] and probably occupies at least some of the phosphate binding sites in the crystallised complexes, detailed interactions of phosphate with the enzyme and its role in conformational change could not be ambiguously interpreted. Herein, the crystal structure of the enzyme complexed with a phosphate ion, which was obtained from the crystal grown in the absence of ammonium sulphate, is presented for the first time. Therefore, the role of phosphate in conformational change observed in the structure of *E. coli* PNP may be discussed.

2. Material and methods

2.1. Chemicals

Inosine (Ino), guanosine (Guo) and adenosine (Ado) were purchased from Sigma and were of the highest available purity. Xanthine oxidase from butter milk (1 U/mg) was also obtained from Sigma. 7-methylguanosine was synthesised according to Jonnes' and Robins' method [9] involving methyl iodide as a methylating agent. This yields the preparation free from sulphate, which is a PNP inhibitor and therefore could bias the results.

2.2. Site-directed mutagenesis and sequencing

Plasmid pSE380 containing the *deoD* gene (pSE380*deoD*) was isolated from *E. coli*, strain HS533 (both gifts from Dr. Joanne L. Turnbull, Concordia University, Montreal, Canada) [10], subcloned into *E. coli* strain DH10B (Invitrogen) and subsequently isolated from it using Gen-Elute™ Plasmid MiniPrep kit (Sigma). Site-directed mutagenesis was employed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and sets of complementary primers for each amino acid substitution (underlined in sequence):

Arg24Ala (5^′-CCAGGCGACCCGCTGGCTGCGAAGTATATTGCTG-3^′, 5^′-CAGCAATATACTTCGCAGCCAGCGGGTCGCCTGG-3^′);
Asp204Ala (5^′-CCATCTGCACCGTATCTGCCCA-CATCCGCACTCACGAGC-3^′, 5^′-GCTCGT GAGTGCGGATGTGGGCAGATACGGTGCAGATGG-3^′); Asp204Asn (5^′-CCATCTGCACCGTATCTAACCACATCCGCACTCACGAGC-3^′, 5^′-GCTCG TGAGTGCGGATGTGGTTAGATACGGTGCAGATGG-3^′); Arg217Ala (5^′- GACCACTGCCGCTGAGGCTCAGACTACCTTCAACGAC-3^′, 5^′-GTCGTTG AAGGTAGTCTGAGCCTCAGCGGCAGTGGTC-3^′).

Primers were PAGE purified and purchased from Sigma. The reaction conditions for PCR amplification were as follows: 1 min hot start at 95 °C followed by 18 cycles of (1) denaturation at 95 °C for 50 s, (2) annealing at 60 °C for 50 s, and (3) elongation at 68 °C for 5 min 15 s. The final elongation step was done at 68 °C for 7 min. PCR conditions for mutations Arg24Ala and Arg217Ala were the same as above except that the annealing temperature was 63 °C. To remove the parental DNA template, PCR products were digested at 37 °C for 2 h using 10 U of *Dpn* I restriction enzyme (Stratagene) followed by transformation of *E. coli* XL-10 Gold Ultracompetent cells (provided by the manufacturer).

Positive transformants were selected on Luria-Bertani (Difco LB Broth-BD) agar plates containing 50 μg/ml ampicillin (Sigma). The inserted point mutations in the pSE380*deoD* sequence for each amino acid substitution were confirmed by sequence analysis using an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems) employing a deoD-f primer (5′-ATGGCTACCCCACACATTAATGCAG-3′) or a deoD-r (5′-CTCTTTATCGCCCAGCAGAACGG-3′).

2.3. Expression

Plasmids, pSE380 containing wild type and mutated *deoD* genes were subcloned into *E. coli*, BL21(*DE3*) strain (Invitrogen). Expression of PNP mutants was done according to Esipov et al. [11] In details, 30 ml of overnight *E. coli*, BL21(*DE3*) culture was added to 500 ml LB media containing 50 μg/ml ampicillin. Cells were grown at 37 °C to $OD_{600} = 0.6$ and expression was induced with 0.2 mM IPTG. Cells were additionally grown for 4 h at 37 °C and harvested at 4000 *g*, 4 °C.

2.4. Protein purification

Firstly, recombinant PNPs were isolated from *E. coli*, BL21(*DE3*) and purified using Q-Sepharose Fast Flow anion-exchange resin (GE Healthcare) as described in Lee et al. [10]. PNPs were further purified on a Sephacryl-S-200 Superfine gel filtration column (Pharmacia) (160 ml) and subsequently eluted in 10 mM Tris–HCl (pH 7.4) buffer containing 200 mM NaCl. Fractions containing PNPs were collected and concentrated by ultrafiltration at 4000 *g* using Vivaspin 2 (10000 MWCO) centrifugal concentrators (Sartorius Stedim Biotech). For crystallisation experiments PNP samples were desalted and transferred to a 10 mM citric buffer (pH 7.0) using a Sephadex G-25 Medium column (Amersham Biosciences).

2.5. Enzymatic procedures – activity measurements and kinetics of phosphorolysis

Activity of all mutants *vs.* various substrates was determined at pH 7.0, 50 mM Hepes/NaOH buffer at 25 °C. Concentration of phosphate was 50 mM and that of nucleosides is shown in Table 1.

A direct spectrophotometric method was used in the case of Ado, Guo [12] and m^7Guo [13], and a coupled assay with xanthine oxidase in the case of Ino [14]. 10-, 5-, 2- and 1 mm cuvettes were used for measurements. The reaction was started by the addition of

Table 1
Spectral data for maximal absorbance of PNP substrates (neutral forms, except of m^7Guo), concentrations of substrates used in activity determination, and data for observation wavelength and differences in extinction coefficients for these substrates and corresponding bases – products of the phosphorolytic reaction, used in activity determination.

Substrate	λ_{max} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	Concentration [μM]	λ_{obs} [nm]	$\Delta\varepsilon$ [$M^{-1}cm^{-1}$]
Ino	249	12,300	500	300	+9600[a]
Ado	260	14,900	400	265	−1690[b]
Guo	252	13,650	500	258	−4850[b]
m^7Guo (pH 7.0)	258	8500	400	260	−4600[c]

[a] The standard coupled xanthine oxidase procedure [14].
[b] Direct spectrophotometric assay [12].
[c] Direct spectrophotometric assay [13].