FEBS Letters 589 (2015) 2683–2689

journal homepage: www.FEBSLetters.org

NMR structural characterization of the N-terminal active domain of the gyrase B subunit from *Pseudomonas aeruginosa* and its complex with an inhibitor

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ARTICLE INFO

Article history:
Received 29 April 2015
Revised 8 July 2015
Accepted 26 July 2015
Available online 10 August 2015

Edited by Christian Griesinger

Keywords:
NMR
Drug discovery
Topoisomerase
Gyrase B
Structure-based drug design

ABSTRACT

The N-terminal ATP binding domain of the DNA gyrase B subunit is a validated drug target for antibacterial drug discovery. Structural information for this domain (pGyrB) from *Pseudomonas aeruginosa* is still missing. In this study, the interaction between pGyrB and a *bis*-pyridylurea inhibitor was characterized using several biophysical methods. We further carried out structural analysis of pGyrB using NMR spectroscopy. The secondary structures of free and inhibitor bound pGyrB were obtained based on backbone chemical shift assignment. Chemical shift perturbation and NOE experiments demonstrated that the inhibitor binds to the ATP binding pocket. The results of this study will be helpful for drug development targeting *P. aeruginosa*.
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1. Introduction

The bacterial genome encodes two types of topoisomerases, I and II which differ in the mechanism of DNA strand breakage [1]. The type II topoisomerases consist of two types, DNA gyrase and topoisomerase IV (TopoIV). These enzymes play essential roles in DNA replication by managing the topological states of DNA in the cell [2,3]. In prokaryotes, type II topoisomerases consist of two subunits and are functional in tetrameric form, which is different from the eukaryotic type II topoisomerases that exist as homodimers [3]. Prokaryotic DNA gyrase contains two gyrase A (GyrA) and gyrase B (GyrB) subunits respectively to form the heterotetramer. For *Escherichia coli* (*E. coli*), GyrA is a 97kDa protein that is involved in DNA binding and GyrB, it is a 90kDa protein with an N-terminal ATP binding domain [4].

Interfering with bacterial DNA replication by targeting type II topoisomerases has been shown to be an efficient strategy to develop antibacterial agents [5]. Successful examples include the fluoroquinolone class of antibiotics [6]. Many other novel and potent inhibitors have been developed in recent years [7]. The N-terminal domain of the type II topoisomerases contains the ATP binding pocket and has been of great interest in drug development because this domain exhibited high sequence homology among pathogenic bacteria and low homology with eukaryotes [5,7]. Structure-based drug design has been demonstrated to be a powerful tool in developing inhibitors targeting both GyrB and TopoIV ATP binding domains. Several classes of inhibitors have been discovered using this approach [8–11].

The structures of the N-terminal ATP binding domains of both GyrB and E subunit of TopoIV (ParE) from *E. coli* have been reported [12,13]. The structures of GyrB/ParE and inhibitor complexes demonstrated that most of the inhibitors are binding with the ATP binding pocket [9]. Although the structure of this domain is similar among the type II topoisomerases, a single residue difference among different topoisomerases can result in different inhibitor potency [2]. Understanding protein-inhibitor interactions will provide useful information in the drug development process. NMR spectroscopy has been proven to be a useful tool in drug development [14]. Despite the extensive X-ray studies of GyrBs and ParEs, few NMR studies have been conducted for the N-terminal domain of GyrB and ParE from bacteria except for the

Author contributions: C.K., J.C., J.H. and T.H.K designed the experiments. Y.L, Y.X.W., Z.Y.P., Y.L.W., M.Y.L., H.Q.N., B.L, A.W.H., J.C., J.H., T.H.K. and C.K. conducted and analyzed the experiments. C.K., Y.L., J.C., J.H. and T.H.K. finalized the manuscript.

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http://dx.doi.org/10.1016/j.febslet.2015.07.044
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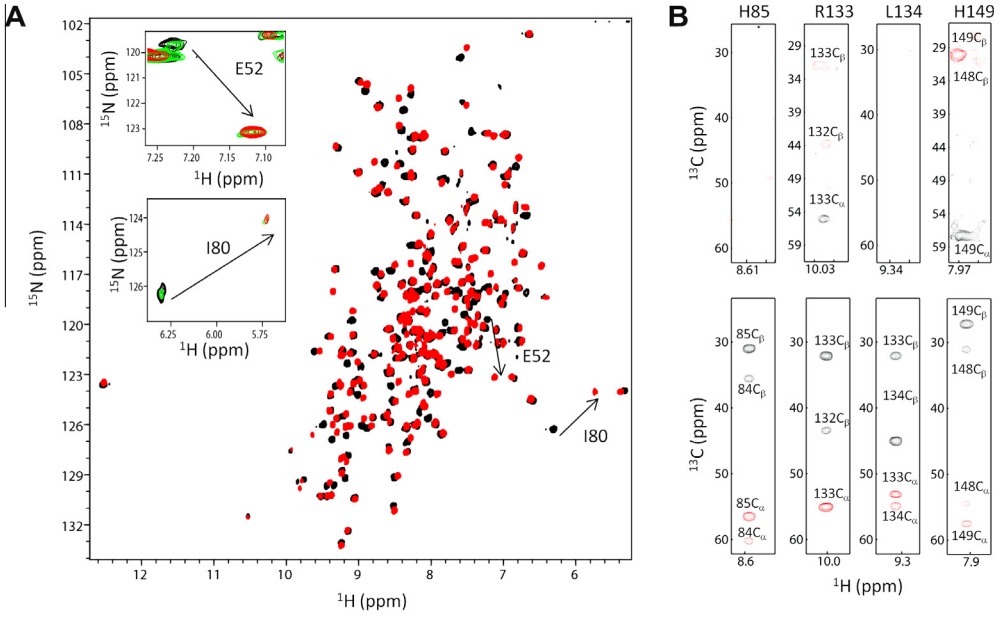

Fig. 1. NMR spectra of pGyrB. (A) ^{1}H–^{15}N-TROSY spectra of pGyrB. The NMR spectra of pGyrB in the absence (black) and presence (red) of the inhibitor were collected and superimposed, and two peaks that undergo significant shifts upon complex formation are highlighted. Inside spectra are selected regions of spectra with inhibitor/protein ratios of 0 (black), 0.5 (green) and 1 (red), respectively. The interaction is undergoing slow exchange. (B) 3D-HNCACB of pGyrB in the absence and presence of the inhibitor. Select strips of HNCACB spectrum for several residues are shown. Upper and lower panels are free and inhibitor-bound pGyrB, respectively. The peaks are labeled with residue number and atom types.

assignments of the P24 fragment of *Staphylococcus aureus* and the N-terminal 24kDa fragment of GyrB from *E. coli* [15,16].

In this study, we obtained the N-terminal 24kDa domain of the GyrB from *Pseudomonas aeruginosa* (*P. aeruginosa*) (referred as pGyrB) for NMR studies. As the structure of this domain is not available, structural information for this domain will be useful for structure-based drug design because *P. aeruginosa* is an important pathogenic species. We managed to obtain the backbone assignments for both free and inhibitor-bound forms of pGyrB. The secondary structure and dynamic property of pGyrB in solution were analyzed and the *bis*-pyridylurea inhibitor was shown to bind to the ATP binding pocket.

2. Materials and methods

2.1. Sample preparation

The cDNA encoding the pGyrB was amplified by polymerase chain reaction using genomic DNA of *P. aeruginosa* as a template and cloned into NdeI and XhoI sites of pET29b. The resulting plasmid can express residues 1–222 of GyrB and extra 8 residues (LEHHHHHH) at the C-terminus. To express pGyrB from *E. coli*, the plasmid was transformed in *E. coli* (BL21DE3) competent cells. The recombinant protein was expressed and purified using affinity purification and gel filtration chromatography [17,18]. Briefly, several colonies were picked up from the plate and inoculated in 20 mL of M9 medium. The overnight culture at 37 °C was then transferred into 1 L of M9 medium. The recombinant protein was induced for 18 h at 18 °C by adding β-D-1-thiogalactopyranoside (IPTG) to 1 mM. The *E. coli* cells were harvested by centrifugation and the recombinant protein was purified in a buffer that contained 20 mM sodium phosphate, pH 6.5, 80 mM KCl, 2 mM DTT and 0.5 mM EDTA. A triple-labeled sample (^{13}C, ^{15}N and ^{2}H) was prepared by growing *E. coli* in a M9 medium that contained 1 g/L $^{15}NH_4Cl$, 2 g/L ^{2}H-^{13}C-glucose and D_2O (99.9%). Purified protein was concentrated to 0.5–0.8 mM for further studies.

2.2. Backbone resonance assignment

Uniformly ^{15}N- and ^{13}C/^{15}N/^{2}H-labeled proteins were used in NMR data acquisition. Two- (2D) and three-dimensional (3D) experiments and transverse relaxation-optimized spectroscopy (TROSY) [19,20]-based experiments including HSQC, HNCACB, HNCOCACB, HNCOCA, HNCA, HNCACO and HNCO were collected and processed. For pGyrB and inhibitor complex, protein was first purified and inhibitor was then added into the solution to a protein: inhibitor molar ratio of 1:1.2. Inhibitor was synthesized and purified as described [21]. All the experiments were conducted at 25 °C on a Bruker Avance 700 spectrometer equipped with a cryoprobe. All the spectra were processed using NMRPipe [22] or Topspin 2.1 and analyzed using NMRView [23] and CARA (http://www.mol.biol.ethz.ch/groups/wuthrich_group). The secondary structure was analyzed using TALOS+ based on the backbone chemical shifts [24].

2.3. Protein-inhibitor interactions

^{1}H–^{15}N-HSQC spectra of pGyrB in the absence and presence of the inhibitor were compared and chemical shift perturbations (CSP) were monitored [25]. The combined chemical shift changes ($\Delta\delta$) were calculated using the following equation. $\Delta\delta = ((\Delta\delta_{HN})^2 + (\Delta\delta_N/5)^2)^{0.5}$, where $\Delta\delta_{HN}$ is the chemical shift changes upon inhibitor binding in the amide proton dimension and $\Delta\delta_N$ is the chemical shift changes in the amide dimension [25]. To obtain protein-inhibitor inter-molecular NOEs, a NOESY-TROSY experiment with a mixing time of 100 ms was recorded using a sample that contained 0.5 mM of ^{13}C/^{15}N/^{2}H-labeled pGyrB and 1 mM of inhibitor.

2.4. Effect of inhibitor on protein thermal stability

Thermal shift experiment was carried out on a Roche LC480 PCR machine. Each assay well contained 10 μM pGyrB, 20× spyro

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