Structure and Dimerization of IreB, a Negative Regulator of Cephalosporin Resistance in *Enterococcus faecalis*

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Abstract

Enterococcus faecalis, a leading cause of hospital-acquired infections, exhibits intrinsic resistance to most cephalosporins, which are antibiotics in the beta-lactam family that target cell-wall biosynthesis. A comprehensive understanding of the underlying genetic and biochemical mechanisms of cephalosporin resistance in *E. faecalis* is lacking. We previously determined that a transmembrane serine/threonine kinase (IreK) and its cognate phosphatase (IreP) reciprocally regulate cephalosporin resistance in *E. faecalis,* dependent on the kinase activity of IreK. Other than IreK itself, thus far the only known substrate for reversible phosphorylation by IreK and IreP is IreB, a small protein of unknown function that is well conserved in low-GC Gram-positive bacteria. We previously showed that IreB acts as a negative regulator of cephalosporin resistance in *E. faecalis*. However, the biochemical mechanism by which IreB modulates cephalosporin resistance remains unknown. As a next step toward an understanding of the mechanism by which IreB regulates resistance, we initiated a structure–function study on IreB. The NMR solution structure of IreB was determined, revealing that IreB adopts a unique fold and forms a dimer *in vitro*. Dimerization of IreB was confirmed *in vivo*. Substitutions at the dimer interface impaired IreB function and stability *in vivo*, indicating that dimerization is functionally important for the biological activity of IreB. Hence, these studies provide new insights into the structure and function of a widely conserved protein of unknown function that is an important regulator of antimicrobial resistance in *E. faecalis*.

Introduction

The Gram-positive bacterium *Enterococcus faecalis* is a leading cause of hospital-acquired infections [1–3]. *E. faecalis* is intrinsically resistant to cephalosporins, and prior cephalosporin therapy is a well-known risk factor for the acquisition of enterococcal infections [4–6]. Cephalosporins are a subclass of the beta-lactam family of antibiotics that impair the final stages of peptidoglycan synthesis by inhibiting the D, D-transpeptidase activity of peptidoglycan biosynthetic proteins, known as penicillin-binding proteins, which cross-link peptidoglycan to maintain cell-wall integrity. Although the genetic and biochemical mechanisms of cephalosporin resistance in *E. faecalis* are still being unraveled, recent studies have revealed a critical role for the signal transduction system composed of a eukaryotic-like serine/threonine kinase (IreK) and its cognate phosphatase (IreP) that antagonistically regulate cephalosporin resistance [7,8].

IreK is a transmembrane serine/threonine kinase that has been hypothesized to sense cephalosporin-induced cell-wall damage, activate its kinase activity through autophosphorylation, and initiate a signaling pathway as part of an adaptive biological response that promotes cephalosporin resistance. IreP is thought to regulate the IreK signaling pathway by dephosphorylating IreK to keep kinase activity in check and by dephosphorylating downstream substrates of IreK, presumably when the stimulatory cell-wall damage

has been repaired. However, little is known about the direct substrates that are phosphorylated by IreK or about other downstream effectors in the IreK pathway that drive resistance. We identified one *E. faecalis* protein, IreB, which is reversibly phosphorylated by the IreK kinase and IreP phosphatase at residues Thr4 and Thr7 [9].

IreB is a cytosolic 10.5-kDa protein of unknown function that is highly conserved among low-GC Gram-positive bacteria (as are homologs of IreK). Bioinformatics analyses of IreB provides no clues about its function, as its only identifiable domain is a "domain of unknown function" (DUF965; PF06135) spanning nearly the entire length of the protein. DUF965 domains are essentially found only in homologs of IreB in other Gram-positive bacteria (which have not been functionally characterized). Our studies of *E. faecalis* mutants lacking *ireB* revealed that IreB acts downstream of IreK as a negative regulator of cephalosporin resistance in *E. faecalis* [9], although the precise biochemical function of IreB remains unknown. IreB can be modified by phosphorylation *in vivo*, and mutations that prevent modification at the known sites of phosphorylation (T4 and T7) influence the ability of IreB to modulate cephalosporin resistance [9]. Intriguingly, two-dimensional PAGE studies of an IreB mutant that cannot be phosphorylated at Thr4 or Thr7 revealed two isoforms with distinct isoelectric points, suggesting that IreB may be subject to modification at an additional as-yet-unknown site *in vivo* [9]. Although these findings collectively shed some light on the *in vivo* role of IreB in *E. faecalis*, the biochemical mechanism by which IreB exerts its effect on cephalosporin resistance remains unknown.

As a next step toward an understanding of the mechanism by which IreB negatively regulates enterococcal cephalosporin resistance, we initiated a structure–function study on IreB. The NMR solution structure of IreB was determined, revealing that IreB adopts a unique fold and forms a dimer *in vitro*. Dimerization of IreB was confirmed *in vivo*. Substitutions at the dimer interface impaired IreB function and stability *in vivo*, indicating that dimerization is functionally important for the biological activity of IreB. Hence, these studies provide new insights into the structure and function of a widely conserved protein of unknown function that is an important regulator of antimicrobial resistance in *E. faecalis*.

Results

NMR structure of IreB

To begin understanding the mechanisms by which IreB regulates cephalosporin resistance in *E. faecalis*, we used NMR to elucidate the IreB solution structure. IreB was determined to be a homodimer using specialized NMR techniques to distinguish intermonomer from intramonomer contacts [10]. A

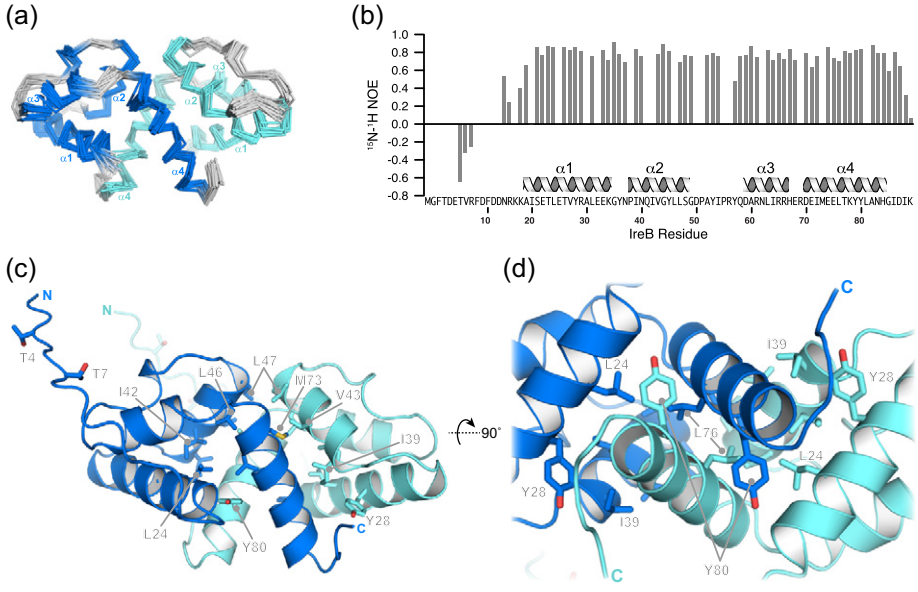

Fig. 1. NMR structure of IreB. (a) Ensemble of 20 final NMR conformers shown as Cα trace (PDB ID: 5US5). Residues 18–88 are shown for clarity. (b) Heteronuclear ^{15}N–^{1}H NOE values measured at 14.1 T. Residues with overlapping signals were omitted. (c) IreB adopts a novel dimeric fold. Each IreB protomer contains four α-helices, with the α4 helix partially domain-swapped. Side chains of selected interface residues are shown as sticks and labeled. (d) IreB dimer interface as viewed along the axis of 2-fold symmetry. Hydrophobic core residues that make intermolecular contacts involving the α4 helix are labeled.