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Distinct structural features of Rex-family repressors to sense redox levels in anaerobes and aerobes ☆

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ABSTRACT

The Rex-family repressors sense redox levels by alternative binding to NADH or NAD^+. Unlike other Rex proteins that regulate aerobic respiration, RSP controls ethanol fermentation in the obligate anaerobe *Thermoanaerobacter ethanolicus* $JW200^T$. It is also found in other anaerobic microorganisms. Here we present the crystal structures of *apo*-RSP, RSP/NADH and RSP/NAD^+/DNA, which are the first structures of Rex-family members from an obligate anaerobe. RSP functions as a homodimer. It assumes an open conformation when bound to the operator DNA and a closed conformation when not DNA-bound. The DNA binds to the N-terminal winged-helix domain and the dinucleotide, either reduced or oxidized, binds to the C-terminal Rossmann-fold domain. The two distinct orientations of nicotinamide ring, *anti* in NADH and *syn* in NAD^+, give rise to two sets of protein–ligand interactions. Consequently, NADH binding makes RSP into a closed conformation, which does not bind to DNA. Both the conserved residues and the DNA specificity of RSP show a number of variations from those of the aerobic Rex, reflecting different structural bases for redox-sensing by the anaerobic and aerobic Rex-family members.

1. Introduction

Negative control of gene expression involves repressors, which prevent transcription by occupying the cognitive operator regions of DNA. In response to environmental changes that signal the need for specific gene products, the repressors undergo structural transformations and dissociate from the operators, allowing RNA polymerases to access the coding regions of the genes. Most repressors sense the signals by allosteric interactions with inducers. A popular example is the Lac repressor of *Escherichia coli*, which functions as a dimer of dimers (Bell and Lewis, 2001). Each monomer comprises a DNA-binding domain and a core domain, the latter further divided into N and C subdomains. Upon binding of allolactose or isopropyl β-D-thiogalactoside (IPTG), the interactions between N subdomains are disrupted and the signal propagates to the DNA-binding domains, resulting in an induced conformation.

Another example is the Rex-family repressors. They are negative regulatory proteins that control the expression of genes in aerobic respiration and related metabolic pathways (Brekasis and Paget, 2003). Rex functions as a dimer by incorporating either NAD^+ or NADH in the C-terminal Rossmann-fold domain (Nakamura et al., 2007; Sickmier et al., 2005; Wang et al., 2008). The nicotinamide of NAD^+ is bound with the *syn* orientation, which allows Rex an open conformation for binding to the Rop operator by the N-terminal winged-helix domain. Substitution of NAD^+ by NADH, which has a higher affinity and binds to Rex with the *anti* orientation, results in a closed conformation that prevents Rex from Rop binding (McLaughlin et al., 2010). In this way, Rex directly senses the NADH and NAD^+ concentrations as a redox state indicator, and actively regulates the metabolic pathways at the transcription level. It is different, and probably more efficient, than other redox-sensing mechanisms that involve, for example, intermolecular disulfide bond formation (Barford, 2004). The switching between reduced and oxidized forms of the same cofactor also presents a distinct mechanism from that of an inducer.

☆ *PDB codes:* RSP: 3WG9; RSP/NADH: 3WGH; RSP/NAD^+/DNA: 3WGI.

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Rex is found in many mesophilic and thermophilic Firmicutes from various genera including *Bacillus*, *Streptomyces*, *Streptococcus*, *Staphylococcus* and *Thermoanaerobacter*, but also in thermophiles from other phyla such as *Thermus* and *Thermotoga*. In *Bacillus subtilis*, the protein YdiH is a Rex-like repressor of a respiratory oxidase, two dehydrogenases, and a transporter (Gyan et al., 2006). In *Staphylococcus aureus*, Rex is a central regulator of anaerobic metabolism (Pagels et al., 2010). Rex is also involved in oxidative stress and biofilm formation in *Streptococcus mutans* (Bitoun et al., 2012), as well as hydrogen production by *Thermotoga maritima* (Ravcheev et al., 2012). The Rex structures of aerobic group from *Thermus*, *Bacillus* and *Streptococcus* (known as T-Rex, B-Rex and S-Rex) have been determined, either alone or with bound cofactor and/or DNA (McLaughlin et al., 2010; Nakamura et al., 2007; Sickmier et al., 2005; Wang et al., 2008) (PDB ID: 1XCB, 2DT5, 2VT2, 2VT3, 3IKT, 3IKV, 3IL2, 3KEO, 3KEQ and 3KET; Table S1). Although the above structures have consistently explained how Rex regulates energy metabolism and aerobic respiration, the Rex-like proteins and the operator DNA in anaerobic bacteria show significant differences from those in aerobic bacteria (Ravcheev et al., 2012).

In our earlier work, we cloned, expressed and characterized a Rex ortholog termed RSP (for redox sensing protein) from *Thermoanaerobacter ethanolicus*. It binds to palindromic sequences of 5′-ATTGTTANNNNNNTAACAAT-3′ in the transcription regulation regions (TRR) of genes *adhA*, *adhB* and *adhE*, which encode key alcohol dehydrogenases in the ethanol fermentation pathway of *Thermoanaerobacter* (Pei et al., 2010). The binding of RSP can repress the transcription of these genes and affect the production of ethanol (Pei et al., 2011) (Fig. 1). We have shown that NADH or high concentration of NAD^+ inhibited the formation of RSP/DNA complexes; that RSP alone inhibited *in vitro* transcription of *adhB*, which was subsequently recovered by adding NADH; that NAD^+ interfered with the effects of NADH on RSP; and that the specificity and length of TRR sequence were significant (Pei et al., 2011). To further investigate the mechanism by which RSP regulates these genes, we expressed and purified the protein for crystallographic studies. Here we present the X-ray structures of RSP in an *apo*-form, in binary complex with NADH, and in ternary complex with NAD^+ and DNA. Comparison of the protein–ligand and protein–DNA interactions with those in the other Rex structures provides a comprehensive picture of ligand-dependent conformational variations and the origin of DNA sequence specificity in anaerobic microbes.

2. Materials and methods

2.1. Preparation of DNA oligonucleotides and RSP protein

The RSP gene was obtained from the genomic DNA of *T. ethanolicus* by using the primers 5′-CCCCCATGGAGAGCAAAAAGACTATAGTATC-3′ (forward) and 5′-CCCCTCGAGCCCATCTATTTTAGCAGTTTC-3′ (reverse), and subsequently cloned into pET28a vector at *Nco*I/*Xho*I sites to make the plasmid pET28a-RSP. Gene expression in *E. coli* BL21 (DE3) cells harboring pET28a-RSP was induced by adding IPTG. After 6 h of expression at 30 °C, cells were harvested. The cell pellets were re-suspended with 50 mM Tris–HCl, pH 7.8 and disrupted by sonication. The supernatant was applied to DEAE Sepharose FF pre-equilibrated with the same buffer and eluted by using NaCl gradient. All fractions were analyzed by using SDS–PAGE and the fractions with RSP protein were pooled and brought to 60% saturated $(NH_4)_2SO_4$ at 4 °C. The pellets were dissolved in 150 mM NaCl, 25 mM Tris–HCl, pH 7.5 and dialyzed against the same buffer to remove $(NH_4)_2SO_4$. The purified RSP protein was finally concentrated to 10 mg ml^{-1} for crystallization.

Partial palindromic sticky-end DNA oligonucleotides of various lengths (21–24 bases in 1-base increments) were designed according to the consensus sequences of RSP operator sites and synthesized by Sangon Co. (Shanghai, China) for use in co-crystallization. Only the 24-base oligonucleotides of 5′-TAG**ATTGTTAAATGAATAACAAT**C-3′ and 5′-TAG**ATTGTTATTCATTTAACAAT**C-3′ (RSP operator concensus site in bold) yielded diffraction data of sufficient quality for structure determination. Double-stranded DNA were prepared by annealing complementary oligonucleotides (2.1 mM each) in 50 mM NaCl, 1 mM EDTA and 10 mM Tris pH 8.0, heating the reaction to 95 °C for 5 min and allowing it to cool slowly from 95 °C to 25 °C in 45 min.

2.2. Crystallization and data collection

The RSP protein was firstly crystallized as an *apo*-form by using the PEG Ion 2 kit (Hampton Research) and sitting-drop vapor diffusion method. 1 μl of the protein solution (10 mg ml^{-1} in 150 mM NaCl and 25 mM Tris–HCl, pH 7.5) was mixed with 1 μl of reservoir, equilibrating with the 300 μl of the reservoir at room temperature. The optimal crystallization condition was 20% w/v polyethylene glycol (PEG) 3350, 0.4 M $(NH_4)_2SO_4$ and 0.1 M Bis-Tris, pH 5.5. The protein was also co-crystallized with NADH by using 5 mM NADH, whereas the optimized condition was 14.4%

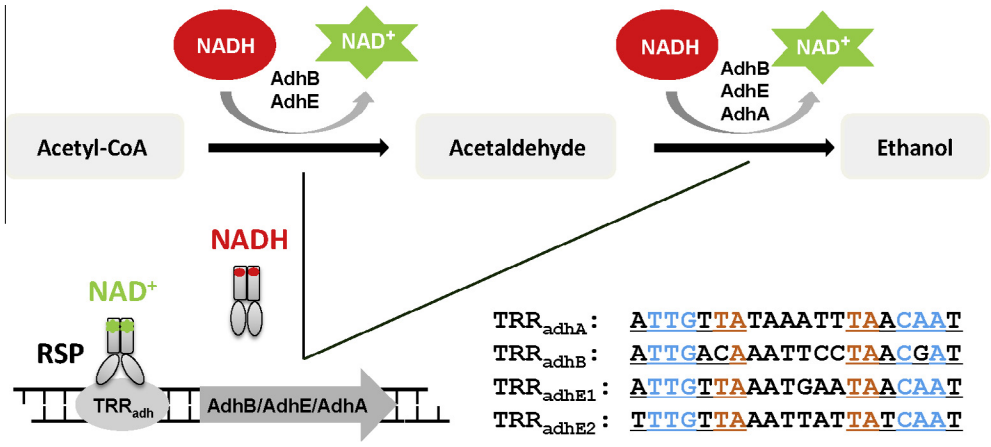

Fig.1. Metabolic regulation by RSP. The genes *adhA*, *adhB* and *adhE* involved in ethanol production from acetyl-CoA are repressed by RSP binding to their operator regions, termed TRR. When NAD^+ is replaced by NADH, RSP adopts a closed form and cannot bind TRR. The palindrome or partial palindrome sequences are underlined. The blue-colored bases and the complementary bases of the orange-colored ones are recognized by RSP. When plenty of NADH is present, it binds to RSP and results in gene induction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)