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Novel biochemical properties of a CRP/FNR family transcription factor from *Mycobacterium tuberculosis*

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Abstract

Cyclic AMP (cAMP) receptor protein (CRP)/fumarate nitrate reductase regulator (FNR) family proteins are actively associated with defense against low oxygen stress, starvation and extreme temperature conditions. They are DNA-binding proteins and regulate target genes carrying the regulatory CRP/FNR cognate nucleotide sequence elements. Recombinant protein encoded by the *Mycobacterium tuberculosis* ORF *Rv3676*, a putative CRP/FNR regulator, was purified from *Escherichia coli* and was found to exist as dimer, devoid of any metal cation cofactor. Purified rRv3676 exhibited cAMP binding in a concentration-dependent manner. At lower concentrations of cAMP (6–10 μM) rRv3676 shows positive cooperativity; at 10 μM cAMP the protein exists in the most open conformation. rRv3676 could bind specifically to the putative CRP/FNR nucleotide sequence elements as evident from electrophoretic mobility shift assay.

Keywords: Hypoxia; Starvation; Cyclic AMP; *Rv3676*; Transcription factor; Gene regulation

Introduction

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis, is responsible for ~3 million deaths every year (World Health Organization, 2006). *M.tb* can exist in latent phase (Ulrichs and Kaufmann, 2006), where it can survive in a very hostile environment, which includes low nutrition and hypoxia, for long periods. CRP/FNR (cAMP receptor protein/fumarate and nitrate reductase regulator) is one of the members of a family of transcriptional regulators. With over 370 family members, these DNA-binding proteins predominantly function as positive transcriptional regulators and are known to be associated with defense against oxygen stress and starvation, and at the same time respond to other environmental signals. The distinctive features of CRP/FNR superfamily proteins include the presence of a nucleotide-binding domain and a helix-turn-helix DNA-binding motif at the N- and C-terminal, respectively. The prototype cAMP-binding domain (Schultz et al., 1991) is a versatile structure that has evolved to accommodate different functional specificities in response to a broad range of signals (Green et al., 2001; Korner et al., 2003).

M.tb H37Rv ORF *Rv3676* codes for a putative CRP/FNR protein (Cole et al., 1998), which is required for

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virulence in mice and controls transcription of specific genes (Rickman et al., 2005). Recently, Bai et al. (2005) reported the characterization of Rv3676 protein using computational and experimental methods (Bai et al., 2005). We recently described the crystallization and preliminary X-ray diffraction data for this CRP/FNR regulator (Akif et al., 2006). Phylogenetically, *M.tb Rv3676* is nearest to the CooA branch represented by the CO sensor protein of *Rhodospirillum rubrum* (Korner et al., 2003). However, the relative positions of the regulatory and the DNA-binding domain are strikingly different in that the recognition helix of CooA is rotated 180° away from the position occupied in CRP-cAMP. Further differences between CooA and CRP include an extended N-terminus providing a ligand to the heme of the opposite subunit, an 11-amino-acid extension in the regulatory domain (positions 72–82) to accommodate the heme and a different composition of the hinge region toward the C-terminus, which causes the displacement of the DNA-binding domain (Lanzilotta et al., 2000). Sequence alignment suggests that these 11 residues are not fully conserved in *Rv3676*.

Escherichia coli CRP and FNR regulate transcription globally in response to glucose starvation and anaerobic conditions, respectively (Kolb et al., 1993). *E. coli* FNR is structurally related to CRP except for the presence of four conserved cysteine residues at the N-terminal extension, which form part of an iron–sulfur cluster and a redox-sensing domain of FNR. This iron–sulfur cluster is absent in *M.tb* Rv3676 similar to other members of the same family from other systems such as e.g. *Pseudomonas stutzeri* (Vollack et al., 1999) and *Bradyrhizobium japonicum* (Mesa et al., 2003). Although these proteins do not have an iron–sulfur cluster, they are the regulators of oxygen tension sensu stricto.

The earlier report by Bai et al. (2005) focused on CRP regulon prediction and the experimental validation of the same and provided the first direct evidence for cAMP binding to a transcription factor in *M.tb*, thereby suggesting a role for cAMP-mediated signal transduction in this bacterium. We now describe the purification and comprehensive characterization of a CRP/FNR regulator from *M.tb* in terms of oligomeric state, cAMP and DNA binding. Our results point to some new unusual properties of Rv3676 protein, which could have physiological relevance.

Materials and methods

Bacterial strains and plasmids

E. coli DH5α and *E. coli* BL21DE3 bacterial strains were used for cloning and expression purposes, respectively. DNA manipulations were carried out in pET23a plasmid vector using standard techniques. Integrity of the plasmid constructs was confirmed by DNA sequencing.

Cloning, expression and purification of recombinant *M.tb* Rv3676

M.tb ORF *Rv3676* was PCR amplified from *M.tb* H37Rv genomic DNA using forward (GGATAT**CATATG**GTGGACGAGATCCTGGCCAGGG) and reverse (CG**CTCGAG**CCTCGCTCGGCGGGCCAGTC) primers with restriction enzyme sites for cloning (shown in bold). The amplicon was cloned into the corresponding sites of pET23a, and recombinant Rv3676 protein was purified as a 6 × His-tagged fusion protein from *E. coli* BL21 (DE3) cells as described earlier (Akif et al., 2006). Protein concentration was estimated using the dye-binding method (Bradford, 1976). To determine suitable storage conditions, aliquots of recombinant Rv3676 (rRv3676) were dialyzed in different buffers, namely phosphate-buffered saline (PBS), 10 mM Tris and 10 mM HEPES. Storage temperature was also optimized, and the conditions under which rRv3676 was most stable were selected.

Analytical size exclusion chromatography

The oligomeric state of native recombinant protein was determined by analytical size exclusion chromatography using a Superose 6 fast protein liquid chromatographic column (BIORAD) at room temperature with PBS as running buffer. A standard curve was prepared according to the instruction manual using standard molecular weight markers. The void volume was determined using Blue Dextran 2000. The elution parameter K_{av} was calculated as follows: $K_{av} = (V_e - V_0)/V_s$, where V_e is the elution volume for the protein, V_0 the column void volume, and V_s the total stationary phase volume. K_{av} was plotted against log molecular weight.

Spectral analyses

To detect the presence, if any, of any associated cofactor, absorption was measured between 200 and 800 nm using a Perkin-Elmer spectrophotometer. Fluorescence spectrometric measurements and ligand-binding assays were carried out using a Perkin-Elmer LS50B luminescence spectrometer and a sample volume of 200 μl with 0.3 cm path length. Tryptophan fluorescence was measured at an excitation wavelength of 295 nm. The slit widths for excitation and emission were 10 and 20 nm, respectively. Emission spectra were recorded between 310 and 500 nm. All spectra measurements were corrected by subtracting the corresponding buffer