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Advanced purification strategy for CueR, a cysteine containing copper(I) and DNA binding protein

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ABSTRACT

Metal ion regulation is essential for living organisms. In prokaryotes metal ion dependent transcriptional factors, the so-called metalloregulatory proteins play a fundamental role in controlling the concentration of metal ions. These proteins recognize metal ions with an outstanding selectivity. A detailed understanding of their function may be exploited in potential health, environmental and analytical applications. Members of the MerR protein family sense a broad range of mostly late transition and heavy metal ions through their cysteine thiolates. The air sensitivity of latter groups makes the expression and purification of such proteins challenging. Here we describe a method for the purification of the copper-regulatory CueR protein under optimized conditions. In order to avoid protein precipitation and/or eventual aggregation and to get rid of the co-purifying *Escherichia coli* elongation factor, our procedure consisted of four steps supplemented by DNA digestion. Subsequent anion exchange on Sepharose FF Q 16/10, affinity chromatography on Heparin FF 16/10, second anion exchange on Source 30 Q 16/13 and gel filtration on Superdex 75 26/60 resulted in large amounts of pure CueR protein without any affinity tag. Structure and functionality tests performed with mass spectrometry, circular dichroism spectroscopy and electrophoretic gel mobility shift assays approved the success of the purification procedure.
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1. Introduction

Metal ions bound to proteins play important roles in biochemical processes either by the stabilization of the structure of these proteins or by participating in enzymatic reactions. For instance, transition metal ions are often necessary for the optimal function of transcription factors, however, the increased amounts of the otherwise essential metal ions may cause toxic effects in living organisms [1,2]. Accordingly, the concentration of these metal ions must be under a strict metal ion sensitive and selective control in the cell [2–9]. Understanding the details of bacterial metal ion regulatory mechanisms may forward the design of molecules that selectively bind specific metal ions.

Metalloregulatory proteins represent a sub-class of transcriptional regulators that respond to the change of metal ion concentration or availability by balancing the expression of cellular metal uptake and efflux/detoxification systems [6,7]. MerR proteins are one of the ten metal ion regulatory protein families that are distinguished in bacteria [7]. Representative examples are e.g. the Hg^{II}-ion binding MerR (the name of the family originates from this protein) and the Cu^{I}-ion regulatory CueR proteins [9]. A characteristics of the MerR family members is the similarity observed in the first ~100 amino acids of their sequences [10]. The N-terminal DNA binding domain contains a helix-turn-helix-β-hairpin motif followed by a long dimerization helix forming an antiparallel coiled-coil structure [11]. Significant differences are, however, found in the C-terminal effector (metal ion) binding domains (Fig. 1) allowing dimeric MerR proteins to distinguish between various metal ions.

The dimer of MerR apo-protein binds to a 19–20 bp (base pair) segment of the promoter region of DNA between the −35 and −10

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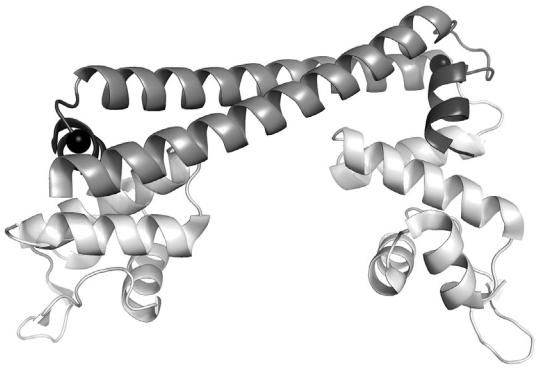

Fig. 1. The crystal structure and the amino acid sequence of the Cu^{I}-binding CueR (PDB id: 1Q05 [11]). Grey-scale coding applied for the various domains: the DNA-binding domains are marked with white, dimerization helices with light grey, metal-binding domains with dark grey and Cu^{I}-ions with black spheres. (The sequence of a fragment with unresolved structure is italicized.)

sites. The regulatory mechanism is based on the conformational change of the protein upon metal ion binding, which influences the DNA structure and initiates the RNA polymerase action. As a consequence, a series of proteins are expressed, which participate in the removal of the unwanted metal ions from the cell.

Crystal structures of CueR with Cu^{I}-, Ag^{I}- and Au^{I}-ions reflect that all of these ions are bound in the effector binding domain close to the C-terminus [11]. A loop is formed around the metal centers via the coordination of two cysteine thiolates, which restricts the metal ion into a linear coordination geometry. According to *in vitro* experiments, CueR gives a transcriptional-activation response to single-charged, but not to double-charged metal ions [11]. Recently, the crystal structures of the metal ion free modified protein and the Ag^{I}-bound form, both co-crystallized with DNA, have been published providing more insight into the influence of metal ion binding on the structure of the DNA [12]. Based on the combination of experimental studies with CueR model peptides and quantum chemical calculations we proposed the participation of a protonated Cys thiol in the metal ion binding domain of CueR and the operation of a protonation switch in the mechanism of the protein [13]. However, the potential role of the protonation/deprotonation of Cys112 in the function of CueR has not been proved, yet. It is also unexplored how CueR so successfully rejects soft divalent metal ions. A further open question is whether the CCHH fragment close to the C-terminus, which has otherwise no direct influence on the transcriptional activity of CueR [14], plays any role in the operation of the protein. In order to better understand the mechanism of the selective metal ion recognition and regulation of CueR we aim at expressing and purifying the wild type CueR from *Escherichia coli* for subsequent structural and activity investigations. Although a published method for the purification of CueR is available in the literature [15], based on the protocol applied for ZntR, a related MerR homologue [16], we faced difficulties to adapt it. Therefore, in this paper we describe an alternative procedure for the purification of CueR eliminating the precipitation step of the protein, which may provide general guidelines for working with air-sensitive DNA binding proteins.

2. Materials and methods

2.1. Strains and media

E. coli DH10B F^{-} *endA1 recA1 galU galK deoR nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC)* λ^{-} [11,17] was applied as cloning host for recombinant DNA work and *E. coli* BL21 (DE3) F^{-} *ompT gal [dcm] [lon] hsdS*$_B$ [18] for the overexpression of CueR protein. Bacteria were grown in LB medium [19] containing ampicillin (100 μg/ml) at 37 °C.

2.2. Plasmid construction

The gene of the wild type CueR in a pET24a (Kan^{R}) plasmid was kindly provided by prof. Alfonso Mondragon (Department of Molecular Biosciences, Northwestern University, Evanston, Illinois, USA). The DNA segment, encoding CueR, was recloned into a pET21a (Amp^{R}) plasmid (Novagen). The gene was amplified by PCR using T7 sequencing primers (T7 forward primer: 5′-TAATACGACTCACTATAGGG-3′ and T7 reverse primer: 5′-GCTAGTTATTGCTCAGCGG-3′) then cloned between NdeI and BamHI sites to create pET21a-CueR. A stop codon prior to the BamHI cleavage site assured the expression of the protein without any additional amino acids encoded by the plasmid.

2.3. Protein purification

E. coli BL21 (DE3) bacteria expressing the wild type CueR from the pET21a-CueR expression vector were first grown in 50 ml LB/Amp+ medium (including 0.1 mg/ml ampicillin at final concentration) at 37 °C for ~ 4 h until OD_{600} = 0.6–1.0 was reached. This pre-culture was sedimented by centrifugation at 4 °C and 18,000*g* for 10 min. The cells were re-suspended in 50 ml fresh LB/Amp+ medium, and 6.5 ml of this culture was used to inoculate 650 ml LB/Amp+ medium. When OD_{600} of 0.4–0.6 was attained, the expression of CueR was induced by the addition of IPTG to a final concentration of 0.1 mM. The cultures were incubated overnight at 20 °C to avoid aggregation otherwise observed at 37 °C. The cells were harvested by centrifugation at 4 °C and then suspended in 20 mM Tris/HCl buffer, pH 7.5 to a total volume of 40 ml. $Na_2S_2O_4$ was added to the sample before and after cell lysis to a final concentration of 2 mM. The cells were lysed by sonication and the extract was centrifuged at 4 °C and 18,000*g* for 20 min. Nucleic acids in the supernatant were digested at *RT* for 1.5 h in the presence of DNase I (25 μg/mL) and $MgCl_2$ (2 mM). The sample was diluted with 20 mM Tris/HCl, pH 7.5 to a conductivity of 2.3 mS/cm and filtered through a 0.45 μm GHP Acrodisc® GF 25 mm Syringe Filter (Life Sciences).

After the preparatory procedures, the CueR protein was purified in four chromatographic steps (Fig. 2) in the order of anion exchange, affinity chromatography, a second anion exchange and a final gel filtration. Between each purification step the pooled fractions were ultrafiltrated three times in a Millipore 5124 Amicon Stirred Cell Model 8400, 400 ml (N_2 gas, PLBC 3000 membrane) with the binding buffer used during the following purification step.

First, the filtered solution was loaded onto a HiLoad Sepharose Fast Flow Q 16/10 column, which had been equilibrated with 5 column volume (CV) of 20 mM Tris/HCl, pH 7.5 (Buffer A). The bound proteins were eluted with a linear gradient of 20 mM Tris/HCl, 1 M NaCl, pH 7.5 (Buffer B) from 15% to 60% in 6 CV. The CueR containing fractions were collected and diluted with 20 mM Tris/HCl, pH 7.5 to a conductivity of 7 mS/cm.

In the second step the sample was filtered and loaded onto a HiPerp Heparin FF 16/10 column, preequilibrated with a 20 mM Tris/HCl, 50 mM NaCl, pH 7.5 buffer. The bound protein was eluted

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