

Benchmarks

Ni⁺-Affinity Purification of Untagged cAMP Receptor Protein

BioTechniques 33:728-730 (October 2002)

The cAMP receptor protein (CRP, also known as CAP) functions as a transcriptional activator at more than 100 different *E. coli* operons (3). CRP is arguably one of the most well-understood transcriptional activators. At simple CRP-dependent promoters, where CRP is the only activator, transcription activation by CRP involves direct contact with RNA polymerase at two distinct sites. These contacts are made by two patches of surface-exposed residues on CRP known as activating region 1 and activating region 2. Activating region 1 contacts the carboxyl-terminal domain, and activating region 2 contacts the amino-terminal domain of the RNA polymerase α subunit (3). Activating region 1 is located in the DNA-binding domain of CRP, very near the DNA moiety of the CRP-DNA complex, and consists of residues 156-164 (1,3,4,9,15). Activating region 2 is located in the cAMP-binding domain of CRP and consists of residues His19, His21, and Lys101 (3,8,10,11). While activating region 1 is required for all simple CRP-dependent promoters, activating region 2 is only required when CRP binds adjacent to RNA polymerase (2,3). CRP also activates transcription at many complex promoters that require at least one additional activator protein for maximal expression; however, the mechanism of activation by CRP at such promoters is not as well understood.

The structure of CRP has been determined numerous times, in the absence and presence of DNA and of its ligand cAMP (12,13). Purification of CRP is most often accomplished by cAMP affinity chromatography (14), typically using a cAMP-agarose resin [such as adenosine 3':5'-cyclic monophosphate agarose; Sigma, St. Louis, MO, USA (14)]. While this method utilizes affinity purification and therefore results in purification of CRP in a single chromatography step, the disadvantage of the method is the high cost of the cAMP-agarose resin. CRP has also

been previously purified as a His6-CRP fusion protein using immobilized metal-affinity chromatography (IMAC) (5,7). Here we report the finding that untagged CRP, with no added histidine residues, can be purified to apparent homogeneity using IMAC. The lower cost and widespread use of IMAC resins make this an attractive alternative to cAMP-affinity chromatography. Further, the finding that CRP can be purified by IMAC without the addition of a His6-tag means that any CRP overexpression plasmid can be used with this method and that post-purification protease digestions are not required to obtain untagged CRP.

Our finding that untagged CRP could be purified using IMAC arose during the purification of a His6-CRP fusion protein for our studies of the transcription regulation of the *E. coli* L-rhamnose operons (5,6). We cloned the *crp* gene into the *Nde*I and *Bam*HI sites of pET15b (Novagen, Madison, WI, USA) such that it would encode a His6-CRP fusion protein with a thrombin cleavage site (pET15b-His6-*crp*). Strain BL21(DE3) carrying pET15b-His6-*crp* was grown in 50 mL TY broth (8 g tryptone, 5 g yeast extract, and 85 mM NaCl) with 200 μ g/mL ampicillin to an A₆₀₀ of 0.4–0.6, 1 mM IPTG was added, and the cells were allowed to continue growing for an additional 3 h. The cells were harvested, resuspended in 2 mL of buffer containing 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, and lysed by sonication. The fusion protein was then purified using IMAC with a 3-mL column of Ni⁺-charged Chelex[®] 20 resin (Sigma). Elutions were performed with an imidazole step gradient, with steps of 60, 100, 200, and 400 mM, with His6-CRP eluting at 400 mM imidazole. Using this method, we obtained CRP that was apparently homogeneous (based on Coomassie Blue[®]-stained SDS-PAGE gels) with a yield of greater than 4 mg purified CRP from 50 mL *E. coli* culture (data not shown).

To ensure that the His6-tag did not interfere with the activity of CRP, we treated the purified fusion protein with thrombin (20 U/mg CRP, incubated at 4°C for 24 h; Sigma) to remove the His6-tag. We then attempted to purify the cleaved CRP away from the un-

cleaved His6-CRP fusion protein, again using Ni⁺-charged Chelex 20 resin. At this step, the cleaved fusion protein was not found in the flow-through as expected, but rather behaved as though it were binding to the resin. To test this hypothesis, we re-cloned the *crp* gene into pET15b, but this time we used the *Nco*I and *Bam*HI restriction sites; therefore, the resulting plasmid (pET15b-*crp*) would express a fully wild-type CRP with no added histidine residues (untagged CRP).

We attempted to purify the untagged CRP using the same growth and purification conditions described above. As shown in Figure 1A, the untagged CRP bound to the Ni⁺-affinity resin and could be eluted with imidazole. Interestingly, the untagged CRP eluted from the column at a lower imidazole concentration than His6-CRP (200 mM imidazole, and on further refinement of the procedure, 150 mM imidazole), suggesting that the absence of the His6-tag weakened the binding of CRP to the resin. In spite of this apparently weaker binding by untagged CRP compared with His6-CRP, the flow-through and low imidazole wash fractions were remarkably free of CRP, and the purified

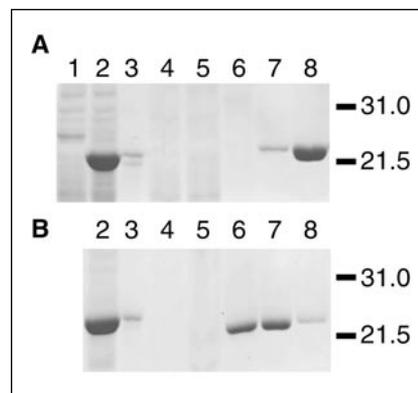


Figure 1. Purification of untagged CRP using Ni⁺-charged Chelex 20 resin. Protein extracts or purified fractions were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Blue. Buffer was 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and an imidazole concentration of 5 mM, unless otherwise indicated. Lane 1, BL21(DE3)/pET15b vector without insert; lane 2, BL21(DE3)/pET15b-*crp* or pET15b-*crp*His19Ala sonication supernatant; lane 3, BL21(DE3)/pET15b-*crp* or pET15b-*crp*His19Ala sonication pellet; lane 4, flow through; lane 5, wash; lane 6, 60 mM imidazole wash; lane 7, 100 mM imidazole wash; lane 8, 150 mM imidazole wash. (A) Wild-type CRP. (B) CRP His19Ala. Molecular weights (right) in kDa.

protein was remarkably free of contaminating proteins. We have used this purified protein in electrophoretic mobility shift assays, DNase I footprint assays, and in vitro transcription assays and found it to be fully functional (unpublished results).

We hypothesized that histidine residues in untagged CRP might be responsible for the binding to the IMAC resin. Inspection of the CRP amino acid sequence showed a total of six histidine residues, all of which were at least partly surface-exposed on the CRP crystal structure (12). Interestingly, three of the histidine residues (at positions 17, 19, and 21) were located very near to one another, in a "stripe" along a surface of CRP approximately opposite the DNA binding surface. This observation was particularly interesting given that two of these three histidine residues (at positions 19 and 21) are especially surface-exposed and are located within activating region 2, one of the surfaces that CRP uses to contact RNA polymerase. To test the hypothesis that the histidine residues in activating region 2 might allow the tight binding of untagged CRP to the IMAC resin, we subcloned a mutant *crp* gene that encoded Ala at residue 19 into pET15b (pET15b-*crp*-His19Ala), with no His6-tag (CRP His19Ala). His 19 is in the center of the stripe of histidine residues on CRP and

might therefore be predicted to have the greatest affect on CRP binding to the IMAC resin. We then overexpressed and attempted purification of CRP His19Ala using the same growth and purification conditions as above, except we used a 10-mL culture and a 1-mL column of Ni⁺-charged Chelex 20 resin (Figure 1B). While wild-type CRP primarily eluted at 150 mM imidazole, CRP His19Ala began to elute at 60 mM imidazole and was almost entirely eluted by 100 mM imidazole. Elution of this CRP derivative at a lower imidazole concentration suggests that the histidine at residue 19 in wild-type CRP may contribute to the ability of untagged CRP to bind to the IMAC resin. We are not aware of any previous reports implicating CRP His19 in metal ion binding.

Our hypothesis at this point was that histidine residues in untagged CRP, probably including His19, made specific interactions with the nickel in the IMAC purifications. It was also possible, however, that untagged CRP was able to interact with the resin itself, rather than the nickel. To test this hypothesis, we attempted purification of wild-type untagged CRP using two additional IMAC resins, Ni⁺-NTA-Agarose (Qiagen, Valencia, CA, USA) and Talon Metal Affinity resin (BD Biosciences Clontech, Palo Alto, CA, USA). Talon Metal Affinity resin uses cobalt rather than nickel as the metal for the purification; however, both resins can be used for the purification of His6-tagged proteins. The growth and purification conditions were the same as described above, except that the Ni⁺-NTA-Agarose purification used 40 mL culture and a 2-mL column volume, while the Talon Metal Affinity resin used 10 mL culture and a 1-mL column volume. Untagged CRP could be purified on each of these resins, although the imidazole concentration at which the protein eluted was lower in both cases (approximately 60 mM) than it was for the Ni⁺-charged Chelex 20 resin (Figure 2). This result suggests that at least a portion of the affinity of untagged CRP for these resins is likely to be due to an interaction with the metal. However, the apparent lower affinity for the Ni⁺-NTA-Agarose and Talon Metal Affinity resins suggests that there is also a contribution of the resin itself

to the purification of untagged CRP.

In summary, we have found that untagged CRP can be purified using IMAC. Untagged CRP apparently bound most tightly to Sigma's Chelex 20 resin. However, each of the IMAC resins we tested provided substantial purification of CRP. Further, it is likely that the imidazole elution steps could be modified to allow purification of CRP to near homogeneity on any of the resins tested. This method is an improvement over the widely used cAMP-affinity purification of CRP due to the lower cost of the resin as well as the widespread use of IMAC. The utility of this method for CRP derivatives other than His19Ala remains to be tested; however, it is possible that this method will be especially useful for cAMP binding mutants of CRP that cannot be purified by cAMP-affinity purification.

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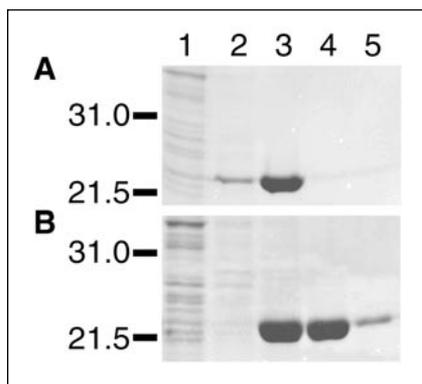


Figure 2. Purification of wild-type untagged CRP using two different IMAC resins. Overexpression used BL21(DE3)/pET15b-*crp*. All buffers contained 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and an imidazole concentration of 5 mM, unless otherwise indicated. Lane 1, flow through; lane 2, wash; lane 3, 60 mM imidazole wash; lane 4, 100 mM imidazole wash; lane 5, 200 mM imidazole wash. (A) Talon Metal Affinity resin. (B) Ni⁺-NTA-Agarose resin. Molecular weights (left) in kDa.

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This work was supported by Public Health Service grant no. GM55099 from the National Institute of General Medical Sciences to S.M.E. Address correspondence to Dr. Susan M. Egan, Dept. of Molecular Biosciences, 8031 Haworth Hall, 1200 Sunny-side Ave., University of Kansas, Lawrence, KS 66045-7534, USA. e-mail: sme@ku.edu

Received 24 May 2002; accepted 26 June 2002.

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Assay for Intracellular Calcium Using a Codon-Optimized Aequorin

BioTechniques 33:730-734 (October 2002)

The use of the photoprotein aequorin to measure intracellular calcium mobilization has become widespread because of the ability to express recombinant aequorin in a diverse range of cellular systems such as bacteria, yeast, slime molds, plants, and mammalian cells (1,9). Applications include the ability to engineer cell lines expressing apoaequorin to report receptor-mediated changes in intracellular calcium levels (5). A particularly important application is in the identification of cDNAs that encode orphan receptors [i.e., the receptors of unknown function and ligand preference identified as expressed sequence tag fragments or from the human genome initiative (3,4)]. Aequorin has also been used to monitor calcium flux in subcellular organelles, such as mitochondria, nucleus, endoplasmic reticulum, subplasmalemma region, golgi apparatus, and mitochondrial inter-membrane space, by modifying the amino acid sequence to include defined targeting signals (2).

Modification of a gene to possess codons that are favored by a particular host can result in enhanced levels of recombinant protein expression (6,10). To determine whether this might also apply to apoaequorin, a codon-optimized apoaequorin gene was constructed using overlapping and complementary oligonucleotides, essentially as described in Reference 6. All codons of the wild-type apoaequorin gene were replaced with those found in highly expressed human genes (6). The primary amino acid sequence was not altered in the codon-optimized apoaequorin variant. The resulting apoaequorin gene was inserted into the *EcoRI* site of pcDNA3 (Invitrogen, Paisley, UK) to create plasmid hucytaeqpcDNA3 (cytoplasmic aequorin). The effect of codon optimization on apoaequorin protein expression was assessed in human embryonic kidney cells (HEK293) transiently transfected with codon-optimized or wild-type aequorin constructs

in which a human hemagglutinin (HA) epitope tag YPYDVPDYA had been introduced at the amino terminus by PCR amplification (7). Transient transfection was performed by the calcium phosphate method (8). Briefly, cells were seeded 24 h before transfection in 6-cm tissue culture dishes at a density of 2×10^6 cells/well. Calcium chloride (62 μ L; 2 M) was mixed with 2 μ g plasmid DNA, and the DNA mixture was made up to a final volume of 500 μ L with double-distilled water. Into this mixture, 500 μ L $2 \times$ HBS (50 mM HEPES, pH 7.1, 10 mM potassium chloride, 12 mM glucose, 280 mM sodium chloride, 1.5 mM NaHPO_4) were added dropwise while bubbling air through the mixture. To compare the various aequorin constructs, an internal transfection control was included at a 1:10 ratio (0.2 μ g pEGFP) (BD Biosciences Clontech, Cowley, UK). Transfection efficiency was determined by FACS[®] analysis of co-transfected pEGFP using a FACScalibur[™] (BD Biosciences Clontech). Fluorescence was detected in FL-1 with a 560 dichroic short pass filter and a 530/30 bandpass filter (data not shown). Immunoblotting analysis of cytoplasmic protein fractions from the transiently transfected HEK293 cells using anti-HA antibody (3F10) showed a significant increase in apoaequorin expression with the codon-optimized variant (hucytaeqpcDNA3) (Figure 1b). Interestingly, we found there was an apparent lack of detection of the 7 amino acid (YDVPDYA) HA-1 epitope. This shorter version, as found on the original apoaequorin construct (Molecular Probes Europe, Leiden, The Netherlands), was not recognized by the 3F10 antibody (Figure 1a, lane 2). The addition of a codon-optimized epitope tag (HA epitope YPYDVPDYA) to the amino terminus of the wild-type apoaequorin sequence did not produce elevated expression equivalent to a fully optimized apoaequorin sequence, which suggests that codon optimization of the entire coding sequence, or a significant portion, may be required for maximal translational efficiency and protein expression.

To establish whether the increase in apoaequorin expression observed with the codon-optimized variant would re-