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Crp of *Streptomyces coelicolor* is the third transcription factor of the large CRP-FNR superfamily able to bind cAMP

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Abstract

The chromosomal inactivation of the unique transcription factor of *Streptomyces coelicolor* that displays a cyclic-nucleotide-binding domain, Crp^{Sco}, led to a germination-defective phenotype similar to the mutant of the adenylate cyclase gene (*cya*) unable to produce cAMP. By means of cAMP affinity chromatography we demonstrate the specific cAMP-binding ability of Crp^{Sco}, which definitely demonstrate that a Cya/cAMP/Crp system is used to trigger germination in *S. coelicolor*. However, electromobility shift assays with the purified Crp^{Sco}–cAMP complex and the CRP-like *cis*-acting element of its own promoter failed. Moreover, we were unable to complement an *Escherichia coli crp* mutant in trans with Crp^{Sco}. The fact that Vfr from *Pseudomonas aeruginosa* and GlxR from *Corynebacterium glutamicum* could complement such an *E. coli* mutant suggests that the way Crp^{Sco} interacts with DNA should mechanistically differ from its most similar members. This hypothesis was further supported by homology modelling of Crp^{Sco} that confirmed an unusual organisation of the DNA-binding domain compared to the situation observed in Crp^{Eco}.

Keywords: cAMP; Germination; CRP-FNR family; Cyclic-nucleotide-binding domain; *Streptomyces*

Streptomycetes are Gram-positive soil bacteria with a complex life cycle that includes germination, vegetative mycelial growth, aerial mycelium formation, and spore morphogenesis [1]. In order to restore vegetative growth once conditions become favourable, bacterial spores must be able to sense environmental changes that trigger the germination process. In the initial stages of germination, undefined signals trigger influx of water into spores, resulting in an increase in size and decreased phase brightness followed by germ-tube emergence [2]. Recently, we focused on the study of the unique CRP-FNR member of *Streptomyces coelicolor*, encoded by *orf* SCO3571 or the *crp* gene (noted Crp^{Sco} protein) [3]. Its chromosomal inactivation led to a germination-defective mutant of which the phenotype is similar to the mutant of the adenylate cyclase gene (*cya*) unable to produce cAMP [3,4]. These similar phenotypes for both mutants and the observation of a peak of cAMP accumulation during germination [4] suggest that cAMP should be regarded as a molecular signal that initiates germination events. However, before concluding that a Cya/cAMP/Crp^{Sco} system is used to trigger germination in *S. coelicolor*, clear evidence for an interaction of Crp^{Sco} with cAMP still needs to be demonstrated. Furthermore, the cAMP-binding ability of Crp^{Sco} became somehow more doubtful as a recent phylogenetic

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analysis of CRP-FNR members classified this transcription factor (TF) into the heme-containing CooA subfamily [5]. This subclassification suggests that the molecular event responsible for the DNA binding of the transcription factor is the binding of a carbon monoxide (CO) molecule via a prosthetic group [5].

We report here a refined in silico analysis that strongly suggests the classification of Crp^{Sco} into the cAMP-binding subfamily. We provide experimental proof for this by in vitro-binding assays and by isolation of cAMP-binding proteins in vivo. We further show that Crp^{Sco} does not bind to a suspected *cis*-acting element suggesting that the mechanism of Crp^{Sco} function must differ from its most similar homologues. These data are supported by homology modelling and discussed in detail.

Materials and methods

Bacterial strains, culture conditions, and preparation crude extracts. *Escherichia coli* strains XL1 blue, and BL21(DE3), were grown in liquid or solid LB medium at 37 °C. The *E. coli crp* mutant LDN-3 Δcrp-45 Spc^r, which has been constructed by P1-transduction of the Δcrp-45 deletion from a CA-8404 derivative into JWL184-1, was used for complementation assays [6,7]. *S. coelicolor* A3(2) M145 ($SCP1^-$ $SCP2^-$ prototroph) and *Streptomyces lividans* TK24 were grown in YEME liquid medium [1].

Expression and purification of His-tagged Crp^{Sco} in E. coli. The *crp* gene of *S. coelicolor* was amplified from genomic DNA by PCR with *Pfu* DNA polymerase. The oligonucleotides used for *crp* amplification were 5′-CATATGGACGACGTTCTGCGGCGCAA-3′ and 5′-TTAAAGCTTGCGGGAGCGCTTGGCCAGTC-3′ (restriction sites underlined). The PCR product was cloned into pGEM-T-Easy and sequenced. The resulting plasmid, pCIP261, was digested with *Nde*I and *Hin*dIII, and the isolated 675 bp *crp* fragment was ligated with the *E. coli* expression vector pET22b. The resulting plasmid, pCIP262, which encodes a Crp^{Sco} protein with a C-terminal hexahistidine tag (Crp^{Sco}-His_6), was transformed into *E. coli* BL21(DE3). Expression was induced with 1 mM IPTG after growth to an A_{600} of 0.6 in 500 ml LB medium at 37 °C. Incubation was continued for 3 h and cells were harvested by centrifugation and resuspended in 200 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 250 mM NaCl. Cells were disrupted with a French press and cell debris was subsequently removed by centrifugation. Benzonase was added to the supernatant (1 μl for 100 ml of supernatant) and left at 4 °C overnight. Crp^{Sco}-His_6 was purified from soluble cell extract on a nickel-nitrilotriacetic acid (Ni^{2+}–NTA) chelating column. Crp^{Sco}-His_6 eluted mainly at 250 mM imidazole from the column. The fractions containing Crp^{Sco}-His_6 were pooled and dialysed overnight against 100 mM sodium phosphate buffer (pH 7.0) containing 250 mM NaCl.

Expression and purification of His-tagged XlnR in E. coli. The *xlnR* gene of *Streptomyces* sp. EC3 was amplified from genomic DNA by PCR with *Pfu* DNA polymerase. The oligonucleotides used were 5′-GGGTTAGCATATGCCTGCTACCGACGACCGTCGGCCCAAGTACC-3′ and 5′-CCGCTAAGCTTGGCCGGGTCTCCGGGCGCTGTGGCGTCGG-3′ (restriction sites underlined). Cloning procedures, sequencing, and overexpressing vectors used, and production and purification conditions were as described above for Crp^{Sco} with the following differences: maximal production of XlnR was obtained after an overnight induction with IPTG, and XlnR-His_6 eluted mainly at 120 mM imidazole.

Anti-Crp^{Sco} antibodies and Western blot analysis. A polyclonal anti-Crp^{Sco} antiserum was generated by immunizing New Zealand white rabbits with purified Crp^{Sco}-His_6. The crude serum was used in immunoblotting at a final dilution of 1:3000. Twenty-five micrograms of total cytoplasmic proteins was separated by SDS–PAGE (12%) and electroblotted onto a nitrocellulose membrane. Immunoblot detection of anti-Crp^{Sco} antibodies was carried out with goat alkaline phosphatase-conjugated anti-rabbit antibodies, in combination with a color reaction of 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (Bio-Rad; immunoblot alkaline phosphate assay system).

cAMP-binding assay. cAMP was from Acros Organics. cAMP affinity matrix was purchased from Sigma. cAMP was immobilised on beaded agarose via the C8 residue of the base, with a spacer of 9 atoms. An equimolar quantity of purified Crp^{Sco}-His_6 was incubated in batch with 200 μl of C-8 cAMP–agarose, previously equilibrated in buffer A (100 mM sodium phosphate buffer, pH 7, 50 mM NaCl), at 25 °C during 30 min on a rotary mixer. After washing 8 times with 500 μl buffer A, bound proteins were eluted in two times by 30 min incubation in 500 μl buffer A supplemented with cAMP 30 mM at 25 °C. Fractions were analysed by 12% SDS–PAGE. For the cAMP-binding assay with soluble protein extract of *S. coelicolor*, the salt concentration of the buffer A was increased to 150 mM NaCl (buffer B) to diminish non-specific interactions between proteins and the matrix. We used 300 μl cAMP–agarose incubated 2 times with 750 μl *S. coelicolor* protein extract (6 mg/ml) from a 2 day culture in YEME. Determination of protein concentrations was performed using the 2-bicinchoninic acid assay (BCA protein assay; Pierce).

Tryptic digest and MS characterisation. The affinity purified proteins were separated by SDS–PAGE and stained with Coomassie blue R250. The identification of proteins was performed as described previously by Vanrobaeys et al. [8].

Bioinformatics and molecular modelling. The amino acids sequences of the CRP-FNR members were obtained using the Sequence Retrieval System (SRS) available at the Expert Protein Analysis System (ExPASy) Molecular Biology Server: http://www.expasy.org/, Expasy Home page. Multiple alignments were developed with MultiAlin [9] (available at http://prodes.toulouse.inra.fr/multalin).

The 3D model of Crp^{Sco} was built with the program ESyPred3D [10] based on homology and comparative modelling, using Crp^{Eco} (1I5Z PDB code) as reference. The Crp^{Sco} residue D15 was superimposed to the Crp^{Eco} T7 (Fig. 1). One deletion and one insertion were detected in order to match at best the two sequences. The deletion occurred in the turn from P86 to P88, superimposed to the Crp^{Eco} turn from E78 to E81. The insertion lay in the turn from G168 to V172 related to Crp^{Eco} residues from D161 to Q164. The subsequent geometry optimisation was performed on a dimer including two molecules of cAMP and water molecules. The initial conformation was built mostly like the dimer structure 1I5Z of Crp^{Eco}. Both Crp^{Sco} and Crp^{Eco} dimers containing two cAMP and crystallisation water molecules were optimised at the molecular mechanics level (MM) in order to compare equivalently resulting structures. The optimisation was performed at the MM level using the program Discover [11] with the Amber force field [12,13] and a relative dielectric constant equal to 2.

Overexpression and purification of Crp^{Sco} in Streptomyces lividans TK24. The *crp* gene of *S. coelicolor* was amplified from pCIP261 with *Pfu* DNA polymerase and oligonucleotides 5′-CCAGATCTGTGGACGACGTTCTGCGGCGC-3′ and 5′-CTGCAGTCAGCGGGAGCGCTTGGCCAGTCG-3′ (restriction sites underlined). The PCR product was cloned into pGEM-T-Easy and sequenced. The resulting plasmid pCIP266 was digested with *Bgl*II and *Pst*I, and the isolated 675 bp *crp* fragment was ligated with the Vpro p145.10 vector [14] digested by the same restriction enzymes, giving rise to pCIP267. Protoplasts of *S. lividans* TK24 were transformed by pCIP267. SDS–PAGE revealed that the production was maximal after 90 h of growth at 28 °C. Mycelium from a 100 ml culture was pelleted, washed, resuspended in 4 ml of 20 mM Tris–HCl, pH 7.5, and disrupted by

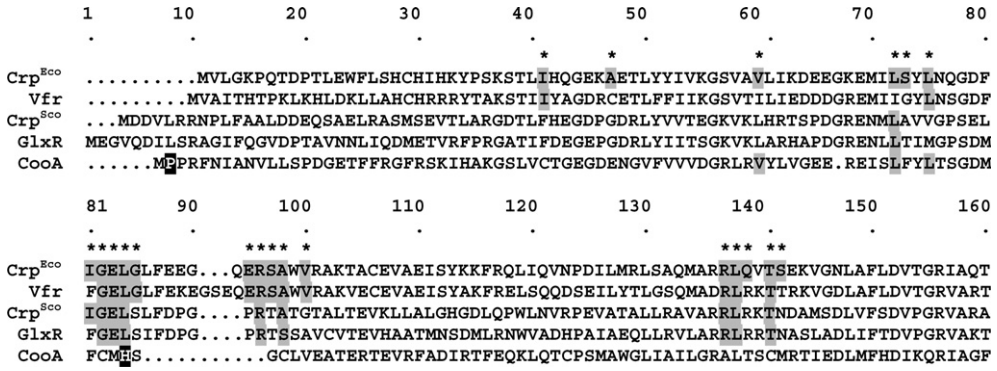

Fig. 1. Positional conservation of amino acids involved in cAMP binding. The multiple alignment of the various effector-binding domains was constructed with MultiAlin. Crp^{Eco}, cAMP receptor protein of *E. coli*; Vfr, virulence factor regulator of *Pseudomonas aeruginosa*; Crp^{Sco}, cAMP receptor protein of *Streptomyces coelicolor*; GlxR, regulator of glyoxylate bypass of *C. glutamicum*; and CooA, CO-dependent transcriptional activator of *Rhodospirillum rubrum*. Positional identities of residues involved in cAMP binding in Crp^{Eco} (marked with asterisks) are highlighted in grey. Residues involved in heme binding in CooA are highlighted in black.

sonication in a Branson ultrasonic disintegrator at a 12 μm amplitude for three 30-s bursts. The soluble cell fraction was obtained by centrifugation (16,000*g*, 30 min) of the lysate. Being genetically very close to *S. coelicolor*, one should also expect that some proteins of *S. lividans* TK24 interact with the cAMP matrix as observed previously. In order to avoid the purification of these proteins, we added ion exchange chromatography on a HiTrap QHP column (5 ml) as a first step of purification before the cAMP affinity chromatography step. The column was equilibrated with 20 mM Tris–HCl, pH 7.5. The adsorbed Crp^{Sco} was eluted with a linear NaCl gradient (50 ml) from 0.3 to 0.75 M. The fractions were analysed by 15% SDS–PAGE. Fractions containing Crp^{Sco} were pooled and dialysed overnight against buffer B (for buffer composition, see the cAMP-binding assay procedure). The protein was further purified on a cAMP–agarose column of 5 ml. The column was equilibrated with buffer B. The fractions were loaded on the column. The column was washed with 40 ml buffer B and the adsorbed Crp^{Sco} was eluted with 15 ml buffer B supplemented with 30 mM cAMP. The fractions were analysed by 15% SDS–PAGE. About 2 mg of nearly pure (>95%) Crp^{Sco} was obtained from a 100 ml culture. For gel shift assays we used fractions of Crp^{Sco} eluted from the cAMP–agarose column with 1 M NaCl in order to obtain the TF not complexed to its ligand. The fractions containing Crp^{Sco} were pooled and dialysed overnight against 100 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl.

Electromobility gel shift assays. Electromobility gel shift assays (EMSA) was performed as described previously [3,15]. The fluorescent double-stranded oligonucleotide representing the *cis*-acting element upstream *crp* (5′-Cy5-CATCCTTGTGACAGATCACACTGTTT-3′) was used as the DNA probe (underlined nucleotides represent the signature recognised by CRP-like members).

E. coli crp mutant complementation assays. The *crp* genes of *S. coelicolor* and *E. coli* were cloned behind the constitutive *lacZ* promoter into the pSU2718 derivative pF76 by replacing the *Afl*III–*Hin*dIII fragment with the respective *crp* fragment, giving pFT171 (crp^{Sco+}) and pFT172 (crp^{Eco+}). The *crp* genes were amplified by PCR from chromosomal DNA of *S. coelicolor* A3(2) and *E. coli* DH5α using appropriate oligonucleotides (crpSco1, AGTACACGTGGAC GACGTTCTGCGGCGCAACC; crpSco2, ATATAAGCTTGGTCA GCGGGAGCGCTTGGC; crpEco1, GTACACGTGGTGCTTGG CAAACCGC; crpEco2, ATATAAGCTTGATTAACGAGTGCCG TAAACG; restriction sites are underlined). Plasmids pFT76 (control), pFT171, and pFT172 were each transformed into the *E. coli crp* deletion mutant LDN-3 and analysed on MacConkey agar plates supplemented with either 25 mM lactose or maltose for their capacity to restore sugar fermentation. *E. coli* DH5α was used as wild type control.

Results and discussion

Comparative study of amino acids involved in cAMP binding

Crp of *E. coli* (Crp^{Eco}) and virulence factor regulator (Vfr) of *Pseudomonas aeruginosa* are actually the only two members of the large CRP-FNR superfamily that exhibit a cAMP-binding ability [16,17]. We checked whether residues involved in cAMP binding in Crp^{Eco} and Vfr were also present in Crp^{Sco} (Fig. 1). Crp^{Eco} and Vfr share 67% of positional identity, and Crp^{Sco} displays 48% identity with both of them. In contrast, in CooA only 24%, 19%, and 14% of the analysed residues are identical to Crp^{Eco}, Vfr, and Crp^{Sco}, respectively. For other CRP-FNR proteins not able to bind cAMP, the levels of positional identity were between 5% and 24% (data not shown), indicating that the level of conservation of residues involved in cAMP is much higher in Crp^{Sco} than in all other representative members of the cAMP-non-binding subfamilies. Moreover, the residues involved in heme binding in CooA [18] are not present in Crp^{Sco} (Fig. 1) which comforts the idea of an inappropriate clustering among CooA members [7]. We included GlxR of *Corynebacterium glutamicum* in our multiple alignment (Fig. 1), which also belongs to the CooA subgroup. Clear evidences for cAMP–GlxR interactions are still lacking but the involvement of cAMP as effector has been strongly suggested, as the binding of purified GlxR to its target promoter could be demonstrated only in the presence of cAMP [19]. These data support the feeling of Korner et al. [5] that the CooA subfamily is one of the less well defined and a relocation of members into other CRP-FNR subfamilies may be necessary.

Modelisation of the cAMP-binding pocket

We built the 3D model of Crp^{Sco} using Crp^{Eco} dimers containing two cAMP and crystallisation water

molecules as reference. In Crp^{Eco}, seven direct interactions are found with cAMP: five between each cAMP and V49, E72, R82, S83, T127 of the monomer to which it is linked, and two with L124 and S128 of the other monomer. In Crp^{Sco}, seven direct interactions were also found: five from each monomer involving L57, L69, E80, R89 and the HN backbone of T90, and two with residues R132 and N135 of the second monomer (Fig. 2). The involvement of R132 of the second monomer in the linking of cAMP in Crp^{Sco} should be responsible for a much stronger binding than the one resulting from L124 in Crp^{Eco}.

Indirect interactions were also predicted. In Crp^{Eco}, the residue Q125 of one monomer indirectly interacts with the cAMP of the other through a water molecule. The residue R123 is not in direct contact with cAMP but clearly orients E72. As for E81, it is oriented toward the solvent and does not seem to be directly related with cAMP. In Crp^{Sco} an indirect contact with T134 occurs through a water molecule. R130 is linked with E80 through a salt bridge, like R132-E72 in Crp^{Eco}. Thus, on the simple basis of the complex Crp^{Sco}–cAMP optimised geometry, the residues of the cAMP-binding pocket should be capable of cAMP binding in a quite similar manner than the one observed in Crp^{Eco}.

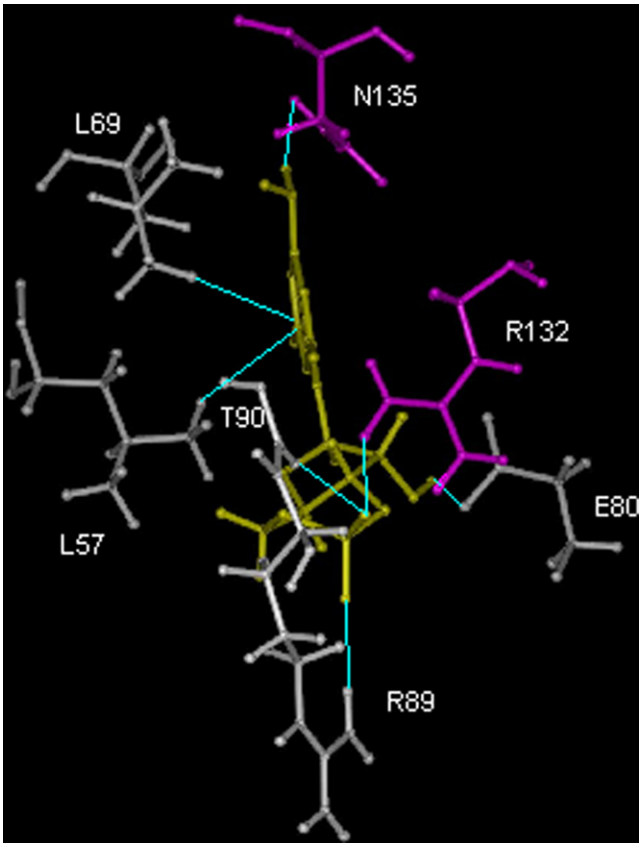

Fig. 2. Direct interactions within the cAMP-binding pocket of Crp^{Sco}. cAMP (yellow) surrounded by its direct residue environment (white for the first monomer, magenta for the second). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

Crp^{Sco} is able to bind cAMP

To demonstrate the interaction of Crp^{Sco} with cAMP as suggested by the above-presented in silico data, we applied purified his-tagged Crp^{Sco} (Crp^{Sco}-His_6) to a cAMP–agarose matrix. The protein remained bound to the matrix until it was eluted with free cAMP (Fig. 3A). Preincubation of Crp^{Sco}-His_6 with equimolar amounts of cAMP abolished binding to cAMP agarose, confirming a specific interaction via cAMP (Fig. 3B). As a negative control, we used the purified XlnR protein, a member of the GntR superfamily [20], for which no cyclic-nucleotide-binding properties have been reported so far. XlnR-His_6 was not retained by the cAMP matrix and was completely removed from the reaction mixture during the first wash step (Fig. 3C).

In a complementary approach, we were able to isolate four proteins from a crude *S. coelicolor* cell extract by cAMP affinity chromatography (Fig. 4A). The four proteins corresponded to molecular weights of approximately 20, 25, 37, and 40 kDa. Western blot analyses revealed that the 25 kDa band was Crp^{Sco} (Fig. 4B), which indicates that cAMP binding to Crp^{Sco} occurs in vivo. Using the same cAMP-binding assays, Brodbeck et al. [21] only isolated a nucleoside diphosphate (NDP) kinase from total extracts of *S. coelicolor* MT1110. We did not capture this protein, but the culture conditions were different. Moreover, Brodbeck et al. used a high salt concentration (1 M NaCl) in washes, so it is possible that the other proteins we captured were eluted during the washing steps. We confirmed this hypothesis as overexpressed Crp^{Sco} could be partially eluted from the cAMP affinity column by addition of 1 M NaCl (data not shown).

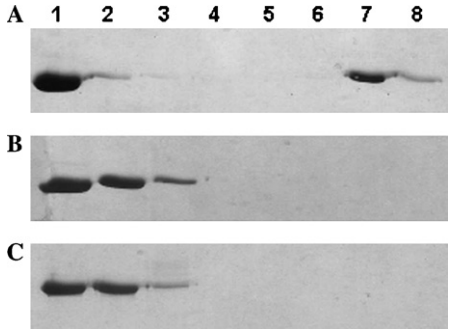

Fig. 3. The cAMP-binding ability of Crp^{Sco}-His_6. Twelve percent SDS–PAGE of the cAMP-binding assays with purified Crp^{Sco}-His_6 (A), with Crp^{Sco}-His_6 pre-incubated with cAMP in equimolar quantity before the cAMP-binding assay (B), and as a negative control with purified XlnR-His_6 (C). Lane 1, starting sample; lane 2, fraction unbounded to the cAMP matrix; lanes 3–6, fractions eluted during washing steps; and lanes 7 and 8, fractions eluted with 30 mM cAMP.

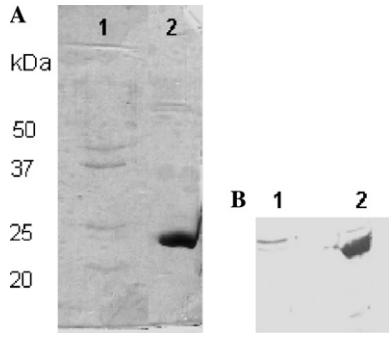

Fig. 4. Isolation of cAMP-binding proteins from the total soluble extract of *S. coelicolor* and identification of Crp^{Sco}. Twelve percent SDS–PAGE representing cAMP-binding proteins isolated from the crude extract of *S. coelicolor* (A) and Western blotting with Crp^{Sco} antibodies (B). Fraction eluted with 30 mM cAMP (lane 1) and purified Crp^{Sco}-His_6 (lane 2).

Identification of other cAMP-binding proteins in S. coelicolor

Tryptic peptides of the three other putative cAMP-binding proteins isolated were analysed by mass spectroscopy. The identification of the 40 kDa protein failed. The 20 kDa protein was identified as ORF SCO3907, encoding a putative single-strand DNA-binding protein (SSB) of 19,906 Da, with a C-terminus that contains glycine-rich repeats. SSB proteins play essential roles in many processes related to DNA metabolism such as DNA replication, repair, and homologous genetic recombination. Blast search revealed 61% identity with mycobacterial SSB proteins [22]. The latter have clearly been shown to be able to interact with nucleic acids, and with proteins involved in DNA metabolism, through their C-terminal glycine-rich domain. As no specific interactions with cyclic-nucleotides have been reported so far, it is difficult to state whether the isolation of this protein was due to a direct binding with the cAMP matrix or via protein–protein interaction with one of the three other proteins isolated from the crude extracts.

Two proteins were identified from the 37 kDa band: (i) SCO0741, which encodes a putative oxidoreductase with a molecular mass of 37,949 Da, and (ii) SCO1087, a putative aldolase with a molecular mass of 39,107 Da. SCO0741 displays 71% identity with mycobacterial AdhE2 proteins, and 70% with FadH, a NAD/mycothiol-dependent formaldehyde dehydrogenase of *Amycolatopsis methanolica*. Both proteins belong to the zinc-dependent alcohol dehydrogenase family. Proteins of this family use NAD^+ as coenzyme and two zinc ions as cofactors [23]. The capture of a NAD^+-binding protein using a cAMP matrix was also reported previously by Laukens et al. [24] and Weber et al. [25], as they isolated a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from tobacco cells and from various mammalian tissue extracts, respectively. If a preincubation with NAD^+ was performed, GAPDH was not retained on the cAMP matrix [24]. Interaction of GAPDH with the matrix appears to be specific for the nucleotide moiety [25]. In addition, it was shown in yeast that cAMP is able to inhibit the GAPDH activity, but the inhibition appeared to be relatively weak [26]. It is suggested that GAPDH binds NAD^+ and cAMP at the same site [24–26] which could explain the capture of SCO0741 by the cAMP–agarose matrix.

Regarding the second protein identified into the 37 kDa band, SCO1087 has an average 45% identity with L-threonine aldolase from many different bacterial strains [27]. These enzymes bind pyridoxal phosphate (PLP) as coenzyme. To our knowledge, this is the first time that a PLP-binding enzyme has been isolated using a cAMP matrix.

DNA-binding ability of Crp^{Sco}

Although the ligand-binding capability of Crp^{Sco} has now been demonstrated, it still remains to be established whether the protein–cAMP interaction is necessary to confer DNA binding. We previously demonstrated, using EMSA, that a protein present in crude extracts of *S. coelicolor* was able to delay the CRP-like *cis*-acting element found in the *crp* promoter [3]. However, EMSAs with purified Crp^{Sco}-His_6 performed in the presence and absence of cAMP were unsuccessful. With the idea that the histidine tag adjacent to the DNA-binding domain might interfere with DNA binding, we established the purification of native Crp^{Sco} produced in *S. lividans* and purified by cAMP affinity chromatography (Fig. 5). However, EMSAs performed with this protein, either with or without addition of cAMP, revealed the same result (Fig. 6A). We previously identified eight CRP-like *cis*-acting elements in the genome of *S. coelicolor* and presented them as possible target sites for Crp^{Sco} [3]. So far, all tested CRP-like DNA sequences were unable to interact with Crp^{Sco} (data not shown). The diversity of the conditions tested tends to indicate that Crp^{Sco} either shows an important difficulty to bind by itself CRP-like *cis*-acting elements or that it is just required for the transcriptional activation of another transcription factor.

Other data further support that Crp^{Sco} may not interact with the CRP-like site of its own promoter. In fact, in vivo complementation assays were performed to demonstrate whether Crp^{Sco} was mechanistically similar to Crp^{Eco}, Vfr or GlxR regarding the recognition of CRP-like binding sites. We were unable to complement the maltose- and lactose-negative phenotype of an *E. coli crp* mutant in trans with Crp^{Sco} (Fig. 6B), although we could demonstrate by Western blot that Crp^{Sco} was expressed (data not shown). In contrast, the cloned *glxR* or *vfr* genes could complement a *E. coli crp* deletion mutant [19,28]. This unsuccessful complementation sug-

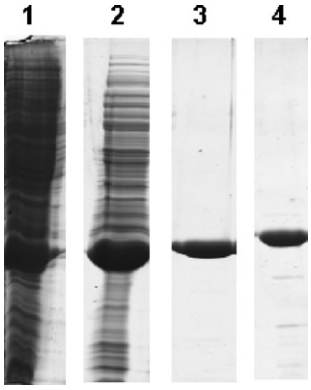

Fig. 5. cAMP-binding approach to purify overexpressed Crp^{Sco} in *S. lividans* TK24. Fifteen percent SDS–PAGE with crude extract of *S. lividans* overexpressing Crp^{Sco} (lane 1), the fraction containing Crp^{Sco} after the first step of purification (ion exchange chromatography, lane 2), the purified Crp^{Sco} protein after the cAMP affinity chromatography (lane 3), and Crp^{Sco}-His_6 previously purified (lane 4). The scanning of the protein profiles showed that overexpressed Crp^{Sco} represented 14% of the starting extract, 30% of the fraction was recovered after the ion exchange chromatography step, and 97% of the fraction was recovered after cAMP affinity chromatography. Under the conditions used, we collected 2 mg of pure Crp^{Sco} from a 100 ml culture.

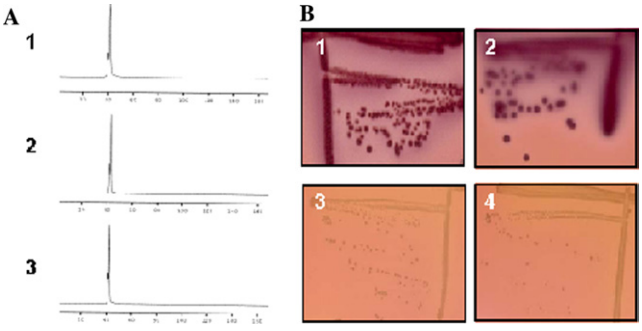

Fig. 6. DNA-binding inability of Crp^{Sco} on the CRP-like *cis*-acting elements. (A) EMSA with 8×10^{-6} M purified Crp^{Sco} (lane 1, free DNA-probe; lane 2, DNA probe and pure Crp^{Sco} without cAMP; and lane 3, DNA probe and pure Crp^{Sco} with 2×10^{-2} M cAMP). The time after detection of the fluorescent DNA-probe is indicated in minutes in the abscissa. (B) Complementation assay of the *E. coli crp* mutant LDN-3. The figure shows growth on MacConkey maltose plates, on which maltose-fermenting colonies appear red and maltose-negative colonies are white. 1, *E. coli* DH5α; 2, LDN-3(pFT172 crp^{Eco+}); 3, LDN-3(pFT171 crp^{Sco+}); and 4, LDN-3(pFT76 no *crp* gene present). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

gests that the way Crp^{Sco} interacts with DNA should mechanistically differ from its most similar members and that the recognised *cis*-acting sequence should differ from the defined CRP-like consensus.

As in vitro and in vivo experiments suggested a specific DNA-binding ability for Crp^{Sco} we checked whether its modelised DNA-binding domain presented a 3D organisation similar to Crp^{Eco}. A superimposition of Crp^{Sco} and Crp^{Eco} was performed using only the backbone atoms of the residues composing the helix–

Fig. 7. Modelisation of the DNA-binding domain of Crp^{Sco}. Superimposition of the Crp^{Sco} and Crp^{Eco} in their optimised geometry. C_α ribbon view of the Crp^{Sco} (white) and Crp^{Eco} (yellow) with the helix–turn–helix motif highlighted in cyan-red-blue for Crp^{Sco}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

turn–helix (HTH) motif thought to be involved in DNA binding (Fig. 7). Apparently, these HTH motifs are fitting very well with one another but the relative position of the rest of the DNA-binding domain differs a lot from what is observed in Crp^{Eco}. Moreover, the average distance between the effector-binding domain and the DNA-binding domain is considerably increased in Crp^{Sco} (data not shown). This positional rearrangement of the two domains must impose a different orientation and presentation of the HTH motif in the dimeric form, ultimately reflected by the accommodation of another *cis*-acting element as the CRP-like one.

The expression level of Crp^{Sco} being variable during the life cycle (data not shown) suggests that the *cis*-element of its promoter must be recognised by another regulatory protein. Such a putative candidate could be the Rex protein, a novel sensor of the $NADH/NAD^+$ redox poise [29]. Rex shares strikingly similar properties with those of Crp-like transcription factors although it does not belong to the CRP-FNR superfamily. In fact, Rex binds to the *cis*-elements of the *cyd* and *nua* operons, which possess the DNA palindromic pattern for CRP-FNR members. Furthermore, Brekasis and Paget [29] also showed that NAD^+ competes with NADH for Rex DNA binding, and it is now known that sites able to bind NAD^+ have also affinity for cAMP [24–

26]. According to these relevant observations, EMSAs with purified Rex protein, with or without Crp^{Sco} and cAMP as a putative partner(s) for cooperative binding, strongly need to be investigated on the CRP-like *cis*-acting element upstream *crp*.

Conclusions

The data reported in this work lead to four conclusions: (i) They correlate the previously obtained in vivo results of adenylate cyclase [4] and *crp* [3] null mutants strengthening the idea that cAMP is a key biomolecule responsible for correct spore germination in *S. coelicolor*, in which a Cya/cAMP/Crp system centrally triggers the developmental process. (ii) They demonstrate that Crp^{Sco} is, after Crp^{Eco} and Vfr of *P. aeruginosa* [17], the third member of the large CRP-FNR superfamily that binds cAMP. (iii) The cAMP–Crp^{Sco} interaction was strongly suggested from our refined in silico analysis that is based on a position-specific analysis, which appears to be more reliable than prediction based multiple alignments of full-length sequences as already reported for LacI–GalR members [30]. Based on our analysis, we predict that GlxR from *C. glutamicum* and homologues of mycobacterial and other actinomycetes species will soon join the CRP subgroup. (iv) Unsuccessful EMSAs and complementation assays as well as modelisation studies provide compelling evidence that Crp^{Sco} is unable to bind CRP-like *cis*-acting elements. It remains to be demonstrated whether Crp^{Sco} binds other *cis*-acting elements or whether the predicted unusual three-dimensional feature of its DNA-binding domain impedes any DNA–protein interaction.

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