Characterization of ligand response properties of the CRP protein from *Pseudomonas putida*

JIANG Feng, TIAN ZheXian & WANG YiPing*

State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing 100871, China

Received April 18, 2012; accepted June 18, 2012; published online July 16, 2012

cAMP receptor protein (CRP) plays profound roles in many bacteria as a global regulator. In *Escherichia coli*, CRP_{*E. coli*} modulates the expression of many operons involved in carbon catabolism, in response to the fluctuation of intracellular cAMP level caused by carbon catabolism. A *crp* homologue gene has been identified in the genome of *Pseudomonas putida*, however, little is known about its cellular function. In this work, we investigated ligand response properties of this CRP protein (CRP_{*P. putida*}). The results showed that in the presence of exogenous cAMP or cGMP, CRP_{*P. putida*} can activate the *lac* promoter in *E. coli cya crp* mutant. *In vitro* isothermal titration calorimetry (ITC) assays indicated that CRP_{*P. putida*} could bind cAMP as well as cGMP. Its affinity to cAMP is much higher than CRP_{*E. coli*}. Sequence alignment of the CRP proteins suggested that the Thr132 of CRP_{*P. putida*} (analogous to Ser128 of CRP_{*E. coli*}) could be the key determinant for all ligand responsive properties observed above. When Thr132 of CRP_{*P. putida*} is mutated to Serine, two phenomena were observed: (i) its affinity to cAMP or cGMP was reduced to a level similar to CRP_{*E. coli*}; (ii) its transcriptional activation activity on *E. coli lac* promoter was diminished. The potential physiological implications of these ligand binding properties are discussed.

***Pseudomonas putida*, CRP, cAMP, high affinity, cGMP**

Citation: Jiang F, Tian Z X, Wang Y P. Characterization of ligand response properties of the CRP protein from *Pseudomonas putida*. Chin Sci Bull, 2012, 57: 3878–3885, doi: 10.1007/s11434-012-5360-3

cAMP receptor protein (CRP), a key member of CRP-FNR family, is widely distributed in prokaryotes, acting as a global regulator [1]. CRP proteins exert regulatory effect in the process of carbon metabolism, nitrogen assimilation, photosynthesis and virulence factor regulation [2–4]. The CRP protein of *Escherichia coli* (CRP_{*E. coli*}) is probably the best-characterized protein in the CRP-FNR family. It regulates the transcription of more than 200 genes in *E. coli* [5]. Upon cAMP binding, CRP_{*E. coli*} undergoes certain conformational changes, which in turn facilitate recognition of specific DNA target site. When it is the sole activator, DNA target site bound CRP_{*E. coli*} interacts with RNA polymerase holoenzyme (RNAP), and recruits RNAP to the target promoter, which in turn initiates gene transcription [6]. CRP_{*E. coli*} functions as a dimer of approximately 47 kD. Each monomer can bind two molecules of cAMP [7]. It consists of 209

amino acid residues and two major structural domains. The N-terminal domain (residues 1–133) contains a cAMP-binding hydrophobic pocket and the C-terminal domain (residues 139–209) contains the DNA recognition motif [8]. The consensus sequence of CRP_{*E. coli*} binding site is AAATGTGAN6TCACATTT, a 22-base motif containing an inverted repeat [9].

In *E. coli*, as the allosteric ligand of CRP protein, cAMP is synthesized by the adenylate cyclase encoded by *cyaA*. Intracellular cAMP concentration is tightly controlled and fluctuates significantly in response to quality change of carbon source. This control is achieved by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). When cells are grown on the PTS carbon source such as glucose, cAMP level stays low and CRP_{*E. coli*} exerts little activity. Intracellular cAMP level increases dramatically when cells are grown on the non-PTS carbohydrates, and subsequently the activity of CRP_{*E. coli*} is triggered by cAMP

*Corresponding author (email: wangyp@pku.edu.cn)

binding. Genes related to carbohydrate metabolism such as lactose, galactose and arabinose are activated by CRP_{E. coli}-cAMP [10]. Meanwhile, key operons like *glnALG* and *glnHPQ* which encode crucial proteins in nitrogen acquisition and ammonium assimilation, are proven to be regulated by the complex as well [4,11]. High through-put genomic analysis confirmed that the CRP_{E. coli}-cAMP complex participates mostly in the regulation of genes which involved in the transport and metabolism of carbon and nitrogen source [5]. Although CRP_{E. coli} can bind cGMP, no evidences show that cGMP-binding could induce CRP_{E. coli}'s conformational change required for DNA binding and transcriptional regulation [12].

The genus *Pseudomonas* comprises a group of strains that have profound environmental or pathogenic relevance. *Pseudomonas putida* is a widely studied pseudomonad for its potential application in bioremediation and biodegradation. Its genome encodes a CRP ortholog (PP_0424) that shares 62% identity and 80% similarity with the *E. coli* counterpart [13]. These two proteins share the similar cAMP binding pocket for ligand recognition and helix-turn-helix (HTH) motif for DNA binding. However, their physiological roles seem to be quite different [14]. In *P. putida* strain KT2440 (strain mt-2 deprived of mega- plasmid pWW0), CRP_{P. putida} plays a role in the utilization of several dipeptide as nutrients and has little influence on the carbon source metabolism [14]. In *P. putida* strain DOT-T1E, a *crp* mutant was unable to grow in the medium with ammonium as sole nitrogen source [15]. Therefore, the physiological role of CRP_{P. putida} is probably related to the nitrogen metabolism rather than the carbon metabolism. Intracellular cAMP in *P. putida* is produced by the enzyme encoded by the *cyaA* ortholog (PP_5222, CyaA_{P. putida}) [14]. The mutual dependence of CyaA_{P. putida} and CRP_{P. putida} for full regulatory function was established recently [14]. Following recent discovery of the existence of cGMP in bacteria [16], evidences have elucidated that members of CRP family protein respond to cGMP or c-di-GMP [17–19], suggesting cyclic nucleotide other than cAMP might have important roles in binding CRP homologues.

In this study, we characterized the signal responsive properties of CRP_{P. putida}, and significant deviation was observed compared to CRP_{E. coli}. Further determination of key residues for signal responsive properties was also carried out.

1 Materials and methods

1.1 Strains, plasmids and media

The bacterial strains and plasmids used in this work are listed in Table 1. The *crp cyaA* double mutant strains, BD7000 and BD8000, were constructed from *E. coli* MG1655 or BL21(DE3) respectively by P1 transduction. The donor strains (JW3778-3 and JW5702-4) were purchased from the Coli Genetic Stock Center. Briefly, lysates

were first generated by growing P1vir phage on the JW3778-3 and JW5702-4. The resulting lysate of JW3778-3 was used to incubate with MG1655 or BL21(DE3) to construct the MG1655 $\Delta cyaA$ or BL21(DE3) $\Delta cyaA$ mutant, then the lysate of JW5702-4 was applied subsequently to MG1655 $\Delta cyaA$ or BL21(DE3) $\Delta cyaA$ strain to get the final BD7000 or BD8000.

For routine growth, bacteria were grown at 37°C (for *E. coli*) or 30°C (for *P. putida*) in Luria-Bertani (LB) broth. For β -galactosidase assay, bacteria were grown in minimal M9 medium with 0.4% glucose as carbon source and 1 mmol L⁻¹ IPTG was added for expression of the CRP proteins. For the protein preparation, strains were grown in M9 medium supplemented with 1% glucose and 0.4% casamino acid (Difco) at 30°C.

1.2 Recombinant DNA techniques

Preparation of chromosomal and plasmid DNA, PCR amplification, restriction enzyme digestions, ligations and transformations were performed as described [23]. The site-directed mutagenesis of CRP was done by using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). In brief, two complementary primers (listed below) containing the desired mutation were applied for PCR amplification using pKU9401 as template. Subsequently, the PCR mixture was digested with *DpnI* and transformed into TOP10 competent cell (Invitrogen). The resulting plasmids containing the DNA fragments with specific mutation were verified by sequencing. The primers used are: p1, 5'-CGCC-TGCGCAACACTTCACGCAAGGTTGGCGATT-3'; p2: 5'-AATCGCCAACCTTGCGTGAAGTGTTCGCGCAGGCG-3' (for CRP_{P. putida} T132S construct); p3: 5'-GAGCTAGGCCT-GTTCGAGGATGGCCAGCAGCGCAGTGCCTGG-3'; p4: 5'-CCAGGCACTGCGCTGCTGGCCATCCTCGAACAG-GCCTAGCTC-3' (for CRP_{P. putida} lex construct).

1.3 Enzyme assays

The β -galactosidase activity assay was carried out as described by Miller [24].

1.4 Protein, antibody preparation and Western blot

To purify CRP and the mutant proteins, *crp* and derivative mutant genes were PCR amplified and cloned into the expression vector pET28a (Novagen) between *Nde* I and *Hind* III sites. Confirmed by sequencing, the resulting plasmids (pKU9800, pKU9801, pKU9802 and pKU9810) were introduced into expression strain BD8000. To express the protein, the strain was pre-grown overnight in M9 liquid medium. The overnight culture was diluted 1:100 into fresh M9 medium and grown at 30°C. At A₆₀₀ of 0.5, IPTG was added to a final concentration of 0.5 mmol L⁻¹. After 6 h induction, cells were collected by centrifugation. The pellet

Table 1 Bacterial strains and plasmids

Strains or plasmids	Characteristics	Source
Strains		
<i>Escherichia coli</i>		
MG1655	F ⁻ <i>lambda</i> ⁻ <i>ilvG</i> - <i>rfb</i> -50 <i>rph</i> -1	Lab stock
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>nupG</i> <i>recA1</i> <i>araD</i> 139 Δ (<i>ara</i> - <i>leu</i>)7697 <i>galE</i> 15 <i>galK</i> 16 <i>rpsL</i> (Str ^r) <i>endA</i> 1	Invitrogen
BL21(DE3)	F ⁻ <i>ompT</i> <i>hsdSB</i> (rB ⁻ mB ⁻) <i>gal dcm</i> (DE3)	Novagen
JW3778-3	F ⁻ Δ (<i>araD</i> - <i>araB</i>)567 Δ <i>lacZ</i> 4787(: <i>trnB</i> -3) & <i>lambda</i> - <i>rph</i> -1 Δ <i>cya</i> A751: <i>kan</i> Δ (<i>rhaD</i> - <i>rhaB</i>)568 <i>hsdR</i> 514	[20]
JW5702-4	F ⁻ Δ (<i>araD</i> - <i>araB</i>)567 Δ <i>lacZ</i> 4787(: <i>trnB</i> -3) & <i>lambda</i> - Δ <i>crp</i> -765: <i>kan</i> <i>rph</i> -1 Δ (<i>rhaD</i> - <i>rhaB</i>)568 <i>hsdR</i> 514	[20]
BD7000	MG1655 Δ <i>crp</i> Δ <i>cyaA</i>	This work
BD8000	BL21(DE3) Δ <i>crp</i> Δ <i>cyaA</i>	This work
<i>Pseudomonas putida</i>		
<i>mt</i> -2	Wild type	[21]
Plasmids		
pVTRA'	pSC101 derivative, <i>lacI</i> ^P - <i>Ptac</i> expression vector, Cm ^r	[22]
pET28a	Histag overexpression vector, Kan ^r	Novagen
pBluescript-SK	<i>ColE1</i> , <i>lacZ'</i> , Ap ^r	Stratagene
pKU9401	pBluescript containing <i>P. putida</i> <i>crp</i> gene, Ap ^r	This work
pKU9500	pVTRA' derivative encoding CRP _{<i>E. coli</i>} , Cm ^r	This work
pKU9600	pVTRA' derivative encoding CRP _{<i>P. putida</i>} , Cm ^r	This work
pKU9601	pVTRA' derivative encoding CRP _{<i>P. putida</i>} T132S, Cm ^r	This work
pKU9800	pET28a derivative expressing His-tagged CRP _{<i>P. putida</i>} , Kan ^r	This work
pKU9801	pET28a derivative expressing His-tagged CRP _{<i>P. putida</i>} T132S, Kan ^r	This work
pKU9802	pET28a derivative expressing His-tagged CRP _{<i>P. putida</i>} Δ lex (the three additional residues in cAMP binding pocket were deleted) Kan ^r	This work
pKU9810	pET28a derivative expressing His-tagged CRP _{<i>E. coli</i>} , Kan ^r	This work

was resuspended in buffer A (50 mmol L⁻¹ Tris, pH 7.4, 500 mmol L⁻¹ NaCl, 20 mmol L⁻¹ imidazole, 1 mmol L⁻¹ DTT) and disrupted by sonication (approx. 200 W for 5 min on ice of 10 s ON and 10 s OFF mode). Cell debris was removed by centrifugation and the supernatant was passed through a HisTrap Nickel affinity column (GE Healthcare). After washing with buffer A, the proteins were eluted by buffer A supplemented with 400 mmol L⁻¹ imidazole. The eluted fractions were then applied to a Superdex 75 gel filtration column (GE Healthcare). Protein purity was verified by SDS-PAGE followed by Coomassie blue staining. The purification procedures of CRP_{*E. coli*}, CRP_{*P. putida*} and the mutants were identical as described above. The polyclonal antibody of CRP_{*E. coli*} was from lab storage. The polyclonal antibody of CRP_{*P. putida*} was obtained from the rabbit serum after 45 d of immunization by 4 times' purified CRP_{*P. putida*} protein injection. The secondary antibody (Goat anti Rabbit IgG HRP) was purchased from Santa Cruz Biotechnology, Inc. Western blot was performed according to the standard procedure [23]. Quantification of the intensity of protein bands was done by using Quantity One software (Bio-Rad).

1.5 Isothermal titration calorimetry

The protein solution was extensively dialyzed against Tris buffer (50 mmol L⁻¹ Tris, pH 7.4, 500 mmol L⁻¹ NaCl) and the nucleotide was solved directly in Tris buffer. The ITC measurements were carried out using a MicroCal iTC200

calorimeter (GE Healthcare) at 25°C. In brief, 19 injections of 2 μ L nucleotide solutions (2 mmol L⁻¹) were introduced at 2-min intervals into the sample cell containing 200 μ L CRP or the mutants (0.1–0.2 mmol L⁻¹) with constant stirring at 1000 r min⁻¹, and the heat changes accompanying the additions were recorded. The data were fitted with the one-site model (except CRP_{*E. coli*} with cAMP, which was fitted with the two-site model) to calculate the association constant (K_a) using the MicroCal ORIGIN 7.0 software.

2 Results

2.1 *In vivo* assay reveals that CRP_{*P. putida*} has versatile ligand dependency and sensitivity

In order to investigate the role of *P. putida* CRP as a transcription factor, plasmid expressing CRP_{*P. putida*} was introduced into the *crp cya* double mutant *E. coli* strain BD7000. *In vivo* activity of the chromosomal *lac* promoter was monitored by β -galactosidase assay, when cells were grown in M9 medium supplemented with 1 mmol L⁻¹ cAMP or cGMP. As shown in Figure 1(a) and Figure 2, CRP_{*P. putida*} could activate the *lac* promoter in response to exogenous cAMP or cGMP in *E. coli*, while CRP_{*E. coli*} responded only to cAMP. Surprisingly, substantial *lac* promoter activity was observed in the strain expressing CRP_{*P. putida*} even under the condition without any exogenous ligand was supplied in the medium. This result indicated that CRP_{*P. putida*} might

Figure 1 Constantly expressed CRP proteins activate *lac* promoter in response to titrated exogenous cAMP in *E. coli*. (a) *E. coli* strain BD7000 expressing CRP_{*E. coli*}, CRP_{*P. putida*} or CRP_{*P. putida*} T132S were grown in M9 medium supplemented with 1, 0.5, 0.1, 0.01 and 0 mmol L⁻¹ exogenous cAMP. β-galactosidase activity was measured at the mid-log growth phase. Circle, CRP_{*E. coli*}; square, CRP_{*P. putida*}; triangle, CRP_{*P. putida*} T132S. (b) The upper portion shows Western blot analysis of the strains described above using CRP_{*P. putida*} or CRP_{*E. coli*} polyclonal antibody. The amounts of loading were normalized based on the concentration of the total protein. Lanes 1–5: 1, 0.5, 0.1, 0.01, 0 mmol L⁻¹ exogenous cAMP added respectively; the bottom portion illustrates the relative intensity of protein bands.

Figure 2 Exogenous cGMP triggers the activity of CRP_{*P. putida*} but not CRP_{*E. coli*} or CRP_{*P. putida*} T132S for activating *lac* promoter in *E. coli*. *E. coli* strain BD7000 was grown in M9 medium in the presence (black bar) or absence (white bar) of 1 mmol L⁻¹ cGMP. EcCRP, CRP_{*E. coli*}; PpCRP, CRP_{*P. putida*}.

exert its activity independent of cAMP or cGMP.

In vivo activity assay with titrated exogenous cAMP further demonstrated that much less cAMP was required for CRP_{*P. putida*} to activate the *lac* promoter. In strains containing CRP_{*P. putida*}, *lac* promoter activity was saturated promptly in

the presence of less than 0.1 mmol L⁻¹ cAMP. In contrast, in strains expressing CRP_{*E. coli*}, the promoter activity increased gradually and could not be maximized even in the presence of 1 mmol L⁻¹ cAMP (Figure 1(a)). In order to rule out the possibility of difference in intracellular protein level, Western blots were performed to examine the expressing level of proteins (Figure 1(b)). The levels of CRP proteins in every strain tested above were almost the same, suggesting that it was molecular property but not expression level that gave rise to the differences between these two proteins. Taken together, these results revealed that CRP_{*P. putida*} could act as a transcriptional regulator and possessed versatile ligand dependency and sensitivity than its *E. coli* counterpart.

2.2 CRP_{*P. putida*} displays ultra-high affinity with cAMP *in vitro*

For the purpose of validating the *in vivo* results described above, *in vitro* ITC analysis was performed to calculate the binding parameters of CRP proteins towards cAMP and cGMP. The purified CRP_{*P. putida*} solution was mixed with either titrated cAMP or cGMP at 25°C (see Materials and methods), and the heat change upon binding was measured (Table 2). As shown in Figure 3(a), CRP_{*P. putida*} has a strong affinity to cAMP, with an approximately 1:1 stoichiometry and an estimated association constant (K_a) of $(1.29 \pm 0.18) \times 10^7$ (mol L⁻¹)⁻¹. In contrast, CRP_{*E. coli*} bound to cAMP with a much lower association constant of $(2.3 \pm 0.22) \times 10^5$ (mol L⁻¹)⁻¹ (Figure 3(c)), which was similar with the data previously reported [8,25]. These results indicated that CRP_{*P. putida*} was much more sensitive to cAMP signal than CRP_{*E. coli*}, which was consistent with the observation in the *in vivo* assay above.

Since cGMP could induce the activity of CRP_{*P. putida*} in the *in vivo* assay above, the binding stoichiometry and affinity of CRP proteins to cGMP was determined by *in vitro* ITC analysis as well. As shown in Figure 3(b), the interaction between CRP_{*P. putida*} and cGMP fitted one-site binding model and the association constant was $(1.01 \pm 0.06) \times 10^5$ (mol L⁻¹)⁻¹, 100 times lower than the interaction between CRP_{*P. putida*} and cAMP. The association constant of CRP_{*E. coli*} and cGMP was $(1.77 \pm 0.13) \times 10^4$ (mol L⁻¹)⁻¹ (Figure 3(d)). Taken *in vitro* and *in vivo* assay data together, cAMP, but not cGMP, was the preferable signal ligand for CRP_{*P. putida*} to conduct its biological function.

2.3 Thr132 is crucial for both high cAMP affinity and cGMP binding of CRP_{*P. putida*}

As a first step to identify the determinant residues that caused the distinct property of CRP_{*P. putida*}, sequence alignment was made to compare the differences between CRP_{*P. putida*} and CRP_{*E. coli*}, particularly around the positions involved in direct cAMP or DNA binding (Figure 4). Two major dissimilarities were found in these two proteins: (i) CRP_{*P. putida*}

Table 2 ITC isothermal parameters of CRP_{P. putida}, CRP_{P. putida} T132S and CRP_{P. putida} lex binding to cAMP or cGMP

	Ligand	N^a	K_a ((mol L ⁻¹) ⁻¹)	ΔH (cal mol ⁻¹)	ΔS cal mol ⁻¹ deg ⁻¹
CRP _{P. putida}	cAMP	0.826±0.002	$(1.29 \pm 0.18) \times 10^7$	-2886±12.45	22.9
	cGMP	0.845±0.006	$(1.01 \pm 0.06) \times 10^5$	-6226±63.42	2.01
CRP _{P. putida} T132S	cAMP	0.556±0.011	$(2.36 \pm 0.50) \times 10^5$	-3244±90.67	13.7
	cGMP	0.743±0.012	$(3.70 \pm 0.25) \times 10^4$	-4730±110.5	5.04
CRP _{P. putida} lex	cAMP	0.709±0.004	$(2.69 \pm 1.02) \times 10^7$	-5192±63.19	16.6
	cGMP	0.724±0.013	$(1.76 \pm 0.29) \times 10^5$	-6906±183.2	0.832

a) N represents the number of ligand binding site on each protein calculated by ITC assay.

Figure 3 ITC analysis of cAMP and cGMP binding to CRP_{E. coli} or CRP_{P. putida}. (a) ITC assay of 0.2 mmol L⁻¹ CRP_{P. putida} with 2 mmol L⁻¹ cAMP in Tris buffer; (b) 0.2 mmol L⁻¹ CRP_{P. putida} with 2 mmol L⁻¹ cGMP; (c) 0.1 mmol L⁻¹ CRP_{E. coli} with 2 mmol L⁻¹ cAMP; (d) 0.2 mmol L⁻¹ CRP_{E. coli} with 10 mmol L⁻¹ cGMP. Data from (a), (b) and (d) are best fitted with one-site binding model, data from (c) are best fitted with two-site binding model.

contained three more residues (Pro79, Val80 and Glu83) in the cAMP binding pocket region; (ii) Thr132 of CRP_{P. putida} was replaced by Ser at the corresponding position of 128 in CRP_{E. coli}. In order to verify the contribution of these residues, site-directed mutagenesis was carried out and the resulting mutant protein was subjected to ITC analysis. As shown in Figure 5(c), (d) and Table 2, deletion of the three additional residues (designated as CRP_{P. putida} Iex) had no influence on the ligand responsiveness. In contrast, the CRP_{P. putida} T132S mutant showed incredible low cAMP affinity, almost two orders of magnitude's decrease (Figure 5(a), Table 2); The cGMP association constant of CRP_{P. putida} T132S dropped to the level below the physiological significance (Figure 5(b), Table 2). Both constants above were almost the same as CRP_{E. coli}, which was $(2.3 \pm 0.22) \times 10^5$ or $(1.77 \pm 0.13) \times 10^4$ (mol L⁻¹)⁻¹ in the control experiments with cAMP or cGMP, respectively (Figure 3(c) and (d)). In consistence with *in vitro* assay, when CRP_{P. putida} T132S was introduced into the *cya crp* double mutant *E. coli* strain, the *lac* promoter activity remained at very low level along with titrated exogenous cAMP and exhibited unresponsiveness to cGMP (Figures 1(a) and 2). All these data above suggested that the ligand binding properties of CRP_{P. putida} T132S were nearly identical to those of CRP_{E. coli} [25], and the residue Thr132 was the key factor that influence the ligand binding properties of CRP_{P. putida}.

3 Discussion

In this study, we demonstrated that CRP proteins from *P. putida* possess versatile ligand binding properties. CRP_{P. putida} has almost 100-fold higher cAMP affinity than CRP_{E. coli}. This implies that CRP_{P. putida} requires less intracellular cAMP than CRP_{E. coli} to maintain its function. It is reported that unlike *E. coli*, in which the intracellular cAMP concentration fluctuates dramatically [27], cAMP level in *Pseudomonas* stays extremely low and stable [28,29]. This phenomenon might due to the weak adenylate cyclase activity

[14] or the effect of more than one active phosphodiesterases in *Pseudomonas* [30]. Therefore, the ligand binding affinity of CRP_{P. putida} may adapt to much lower concentration of intracellular cAMP.

The question about how many ligand binding sites are present in one CRP subunit has not been completely determined to date [25,31,32]. In any case, it is evident that large cAMP-induced conformational change is required for CRP_{E. coli}'s binding to target DNA site [33]. The structural modeling of CRP_{P. putida} using CRP_{E. coli} as template indicates slight structural difference between these proteins within the cAMP and DNA binding domain. Our observations based on the proteolytic digestion pattern of CRP_{P. putida} with or without ligand suggest that CRP_{P. putida} undergoes a conformational transition upon cAMP binding. It is probably that similar conformational change (just like CRP_{E. coli}) occurs to cAMP-bound CRP_{P. putida}, which dramatically facilitates the protein-DNA interaction. Anyhow, it is evident that CRP_{P. putida} displays higher affinity with cAMP in one-site model *in vitro* and activates promoter activity *in vivo* in the presence of cAMP.

The responsiveness of CRP_{P. putida} towards cGMP is intriguing as well. As discussed above, cGMP could trigger the activity of CRP_{P. putida}. Actually, cGMP is previously considered to be capable of activate VFR, a CRP homolog in *Pseudomonas aeruginosa* [34]. Recent work argues that cGMP is unlikely to play a biological role in VFR binding [35]. Consequently, we believe the situation should be the same in CRP_{P. putida}, as conditions containing such a high cGMP concentration can hardly be found in the environment. The capability of binding cGMP is probably a by-product of CRP_{P. putida}'s evolutionary shift to high cAMP affinity. Another consequence of this shift would be the presence of CRP_{P. putida} activity in the absence of cNMP (Figures 1(a) and 2). The similar property is also discovered in VFR, which regulated the *lasR* promoter through cAMP-dependent and -independent mechanisms [35].

While the manuscript was submitted, a paper was published ahead online and showed that a MBP-CRP_{P. putida}

Figure 4 Sequence alignment of CRP protein from *E. coli* and *P. putida*. Star, residues participated in cAMP binding [2]; diamond, residues involved in stabilizing the cAMP binding pocket [2]; triangle, residues which directly contact with nucleotides in the binding site [26]; underlined, residues participated in the CRP-RNAP interaction [8]. Open circle, three additional residues in the cAMP binding pocket of CRP_{P. putida} compared with CRP_{E. coli}. EcCRP, CRP_{E. coli}; PpCRP, CRP_{P. putida}.

Figure 5 ITC analysis of cAMP and cGMP binding to CRP_{*P. putida*} mutants. (a) 0.2 mmol L⁻¹ CRP_{*P. putida*} T132S with 1 mmol L⁻¹ cAMP; (b) 0.2 mmol L⁻¹ CRP_{*P. putida*} T132S with 2 mmol L⁻¹ cGMP; (c) 0.2 mmol L⁻¹ CRP_{*P. putida*} Iex with 2 mmol L⁻¹ cAMP; (d) 0.2 mmol L⁻¹ CRP_{*P. putida*} Iex with 2 mmol L⁻¹ cGMP. All data are best fitted with one-site binding model.

fusion protein possesses high binding affinity for cAMP *in vitro* [36]. In addition to confirming the CRP_{*P. putida*}'s ultra-high cAMP responsiveness, this work has demonstrated the role of a key residue associated with ligand binding and promoter activation. The results obtained from the CRP_{*P. putida*} T132S mutant reveal that the Thr132 residue is responsible for all the signal sensing properties discussed above. Previous work has proved that the S128T mutation of CRP_{*E. coli*} at the equivalent position could make the protein sensitive to cGMP without changing the cAMP binding affinity [37]. It might be speculated that the Thr132 residue

has altered CRP_{*P. putida*}'s cAMP binding pocket a better space structure to accommodate cGMP for activation. The substitution of Thr132 to Ser should have caused somewhat modest conformational change, because of which remarkable loss of cAMP and cGMP affinities are taken place. Our preliminary data with multiple sequence alignments show that in genus *Pseudomonas*, nearly 90% residues at the position 132 are Thr; whereas in *Enterobacteria*, 90% remain Ser at this equivalent site. These interesting observations might be coincidental to meet the reality that the distinct ligand binding properties of CRP proteins originate from the

diverse amino acid residues at this position. In conclusion, CRP_{P. putida} demonstrates a high level of cAMP affinity, and Thr132 residue of CRP_{P. putida} plays important role in such ligand binding properties. A detailed study is expected to understand the reason of why it needs such unusual signal sensing properties.

We are grateful to Profs. V. de Lorenzo and J. L. Ramos for *P. putida* strains and plasmids. This work was supported by the National Basic Research Program of China (2010CB126503), and the National Natural Science Foundation of China (30830005 and 39925017).

- Kolb A, Busby S, Buc H, et al. Transcriptional regulation by cAMP and its receptor protein. *Annu Rev Biochem*, 1993, 62: 749–795
- West S E, Sample A K, Runyen-Janecky L J. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *J Bacteriol*, 1994, 176: 7532–7542
- Korner H, Sofia H J, Zumft W G. Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: Exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol Rev*, 2003, 27: 559–592
- Mao X J, Huo Y X, Buck M, et al. Interplay between CRP-cAMP and PII-Ntr systems forms novel regulatory network between carbon metabolism and nitrogen assimilation in *Escherichia coli*. *Nucleic Acids Res*, 2007, 35: 1432–1440
- Grainger D C, Hurd D, Harrison M, et al. Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. *Proc Natl Acad Sci USA*, 2005, 102: 17693–17698
- Ptashne M, Gann A. Transcriptional activation by recruitment. *Nature*, 1997, 386: 569–577
- Passner J M, Steitz T A. The structure of a CAP-DNA complex having two cAMP molecules bound to each monomer. *Proc Natl Acad Sci USA*, 1997, 94: 2843–2847
- Harman J G. Allosteric regulation of the cAMP receptor protein. *Biochim Biophys Acta*, 2001, 1547: 1–17
- De Crombrughe B, Busby S, Buc H. Cyclic AMP receptor protein: Role in transcription activation. *Science*, 1984, 224: 831–838
- Busby S, West D, Lawes M, et al. Transcription activation by the *Escherichia coli* cyclic AMP receptor protein. Receptors bound in tandem at promoters can interact synergistically. *J Mol Biol*, 1994, 241: 341–352
- Tian Z X, Li Q S, Buck M, et al. The CRP-cAMP complex and downregulation of the *glnAp2* promoter provides a novel regulatory linkage between carbon metabolism and nitrogen assimilation in *Escherichia coli*. *Mol Microbiol*, 2001, 41: 911–924
- Popovych N, Tzeng S R, Tonelli M, et al. Structural basis for cAMP-mediated allosteric control of the catabolite activator protein. *Proc Natl Acad Sci USA*, 2009, 106: 6927–6932
- Weinel C, Nelson K E, Tummler B. Global features of the *Pseudomonas putida* KT2440 genome sequence. *Environ Microbiol*, 2002, 4: 809–818
- Milanesio P, Arce-Rodriguez A, Munoz A, et al. Regulatory exaptation of the catabolite repression protein (Crp)-cAMP system in *Pseudomonas putida*. *Environ Microbiol*, 2011, 13: 324–339
- Daniels C, Godoy P, Duque E, et al. Global regulation of food supply by *Pseudomonas putida* DOT-T1E. *J Bacteriol*, 2010, 192: 2169–2181
- Linder J U. cGMP production in bacteria. *Mol Cell Biochem*, 2010, 334: 215–219
- Leduc J L, Roberts G P. Cyclic di-GMP allosterically inhibits the CRP-like protein (Clp) of *Xanthomonas axonopodis* pv. citri. *J Bacteriol*, 2009, 191: 7121–7122
- Tao F, He Y W, Wu D H, et al. The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. *J Bacteriol*, 2010, 192: 1020–1029
- Marden J N, Dong Q, Roychowdhury S, et al. Cyclic GMP controls *Rhodospirillum centenum* cyst development. *Mol Microbiol*, 2011, 79: 600–615
- Baba T, Ara T, Hasegawa M, et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol*, 2006, 2: 2006–2008
- Nakazawa T. Travels of a *Pseudomonas*, from Japan around the world. *Environ Microbiol*, 2002, 4: 782–786
- Li Y, Sun Y C, Yan H Q, et al. Alternative split sites for fragment complementation, and glyphosate function as extra ligand and stabilizer for the AroA enzyme complexes. *Chin Sci Bull*, 2011, 56: 514–520
- Sambrook J, Russell D. *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2001
- Miller J H. *Experiments in Molecular Genetics*. New York: Cold Spring Harbor Laboratory Press, 1972
- Gorshkova I, Moore J L, Mckenney K H, et al. Thermodynamics of cyclic nucleotide binding to the cAMP receptor protein and its T127L mutant. *J Biol Chem*, 1995, 270: 21679–21683
- Parkinson G, Wilson C, Gunasekera A, et al. Structure of the CAP-DNA complex at 2.5 angstroms resolution: A complete picture of the protein-DNA interface. *J Mol Biol*, 1996, 260: 395–408
- Hogema B M, Arents J C, Inada T, et al. Catabolite repression by glucose 6-phosphate, gluconate and lactose in *Escherichia coli*. *Mol Microbiol*, 1997, 24: 857–867
- Siegel L S, Hylemon P B, Phibbs P V Jr. Cyclic adenosine 3',5'-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3',5'-monophosphate phosphodiesterase in *Pseudomonas* and *Bacteroides*. *J Bacteriol*, 1977, 129: 87–96
- Phillips A T, Mulfinger L M. Cyclic adenosine 3',5'-monophosphate levels in *Pseudomonas putida* and *Pseudomonas aeruginosa* during induction and carbon catabolite repression of histidase synthesis. *J Bacteriol*, 1981, 145: 1286–1292
- Fuchs E L, Brutinel E D, Klem E R, et al. *In vitro* and *in vivo* characterization of the *Pseudomonas aeruginosa* cyclic AMP (cAMP) phosphodiesterase CpdA, required for cAMP homeostasis and virulence factor regulation. *J Bacteriol*, 2010, 192: 2779–2790
- Weber I T, Steitz T A. Structure of a complex of catabolite gene activator protein and cyclic AMP refined at 2.5 Å resolution. *J Mol Biol*, 1987, 198: 311–326
- Lin S H, Lee J C. Communications between the high-affinity cyclic nucleotide binding sites in *E. coli* cyclic AMP receptor protein: Effect of single site mutations. *Biochemistry*, 2002, 41: 11857–11867
- Sharma H, Yu S, Kong J, et al. Structure of apo-CAP reveals that large conformational changes are necessary for DNA binding. *Proc Natl Acad Sci USA*, 2009, 106: 16604–16609
- Beatson S A, Whitchurch C B, Sargent J L, et al. Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa*. *J Bacteriol*, 2002, 184: 3605–3613
- Fuchs E L, Brutinel E D, Jones A K, et al. The *Pseudomonas aeruginosa* Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. *J Bacteriol*, 2010, 192: 3553–3564
- Arce-Rodriguez A, Durante-Rodriguez G, Platero R, et al. The Crp regulator of *Pseudomonas putida*: Evidence of an unusually high affinity for its physiological effector, cAMP. *Environ Microbiol*, 2012, 14: 702–713
- Lee E J, Glasgow J, Leu S F, et al. Mutagenesis of the cyclic AMP receptor protein of *Escherichia coli*: Targeting positions 83, 127 and 128 of the cyclic nucleotide binding pocket. *Nucleic Acids Res*, 1994, 22: 2894–2901