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MINIREVIEW

The Catabolite Repressor/Activator (Cra) Protein of Enteric Bacteria

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Bacteria are frequently faced with metabolic questions which, if answered appropriately, can enhance their reproductive and survival capabilities. They must decide how much of a specific nutrient they will take up and metabolize, which of the various equivalent nutrients that may be available they will select, and how they will respond to changes in relative nutrient concentrations as a function of time. They must also coordinate the acquisition of one essential class of nutrients with those of all other such classes. Thus, *Escherichia coli* will select glucose over lactose, will limit the rate of glucose uptake to correspond to its needs even though it possesses a greater uptake capacity, and will further restrict its carbon uptake in the absence of a required source of nitrogen, phosphorus, or sulfur. We are just now coming to appreciate the molecular details of the processes by which these regulatory interactions are achieved.

In the 1970s and 1980s, the molecular details of catabolite repression in *E. coli*, mediated by cyclic AMP (cAMP) and its cognate receptor, the cAMP receptor protein (CRP), were elucidated (2, 26). It came to be accepted that cAMP provided the principal mechanism of catabolite repression in this enteric bacterium. As key details of the process became known, many investigators became convinced that the essence of the phenomenon had already been solved and that an understanding of this regulatory mechanism would quickly result in elucidation of other essential bacterial regulatory mechanisms. Consequently, interest, research, and funding for bacterial regulatory phenomena of this type began to subside.

As early as 1978, evidence had appeared suggesting that cAMP-independent mechanisms of catabolite repression were operative in *E. coli* (7, 15). Similarly, experiments conducted with *Bacillus subtilis* and other bacteria led to the suggestion that these organisms might possess multiple cAMP-independent mechanisms of catabolite repression (8, 25). Moreover, evidence began to accumulate suggesting that the mechanisms of catabolite repression in evolutionarily divergent bacteria are not the same (17, 37, 38). These facts led to the conclusion that our knowledge of catabolite repression, based on an understanding of the cAMP-CRP-mediated regulatory process in *E. coli*, represented just the tip of the iceberg (35).

The catabolite repressor/activator (Cra) protein of enteric bacteria was initially characterized as the fructose repressor, FruR. Mutants defective in the *cra* gene (previously designated *fruR*) exhibited a pleiotropic phenotype, being unable to grow with gluconeogenic substrates as the sole carbon source (5, 13). It became clear that the product of the *cra* gene controlled the transcriptional expression of numerous genes concerned with carbon and energy metabolism (4, 12, 14, 39). We summarize here the evidence suggesting that the Cra protein, a member of the LacI-GalR family, recognizes an imperfect palindromic DNA sequence to which it binds asymmetrically. If this Cra operator precedes the RNA polymerase binding site, it activates transcription of the downstream operon, but if it overlaps or follows the RNA polymerase binding site, it represses transcription. The effects of Cra on transcription are counteracted by micromolar concentrations of fructose-1-phosphate and millimolar concentrations of fructose-1,6-bisphosphate, which promote catabolite repression of Cra-activated operons and catabolite activation of Cra-repressed operons. Cra apparently controls the direction of carbon flow in *E. coli* and consequently influences the rates of utilization of dozens of exogenous carbon sources.

CENTRAL IMPORTANCE OF THE FRUCTOSE-SPECIFIC PHOSPHOTRANSFERASE SYSTEM (PTS)

Several independent lines of evidence suggest that fructose may have been important in the early evolution of carbohydrate metabolic pathways in bacteria. First, fructose is the only sugar that feeds directly into the centrally important Embden-Meyerhof glycolytic pathway without isomerization or epimerization. Second, the metabolism of fructose can be initiated by two distinct routes, and in *E. coli* both routes are mediated by the phosphotransferase system (PTS) (Fig. 1). Third, the fructose PTS of *E. coli* is unique in possessing its own HPr-like protein domain, FPr, encoded within the fructose (*fru*) catabolic operon. Fourth, bacteria in many evolutionarily divergent genera (e.g., *Azospirillum*, *Fusobacterium*, *Lactobacillus*, *Listeria*, *Pseudomonas*, *Rhodobacter*, *Streptomyces*, etc.) possess the high-affinity fructose-specific PTS, but they apparently cannot phosphorylate other sugars via the PTS (16, 22, 23, 32, 33, 42, 44). Fifth, in *Haemophilus influenzae*, the first bacterium for which a completely sequenced genome became available (10), genes only for a fructose-specific PTS permease are found. Sixth, in *E. coli* three silent gene clusters (designated *frv*, *frw*, and *frx*) uniquely encode silent "backup" fructose PTSs of unknown physiological function (31). Finally, the *fru* operon of enteric bacteria is regulated at the transcriptional level primarily by Cra, although the cAMP-CRP complex plays a secondary role (9). In the absence of cAMP or its receptor protein, fructose is one of the few sugars that can be utilized efficiently. It seems that Cra has assumed a role in regulating the flow of carbon via metabolic pathways in part because of the primeval role of glycolysis as a fructose-catabolic pathway (3, 33, 42).

ISOLATION AND PROPERTIES OF *cra* MUTANTS

Mutants of enteric bacteria lacking the Cra protein were first isolated by selecting for strains that synthesized the protein products of the fructose (*fru*) catabolic operon at high consti-

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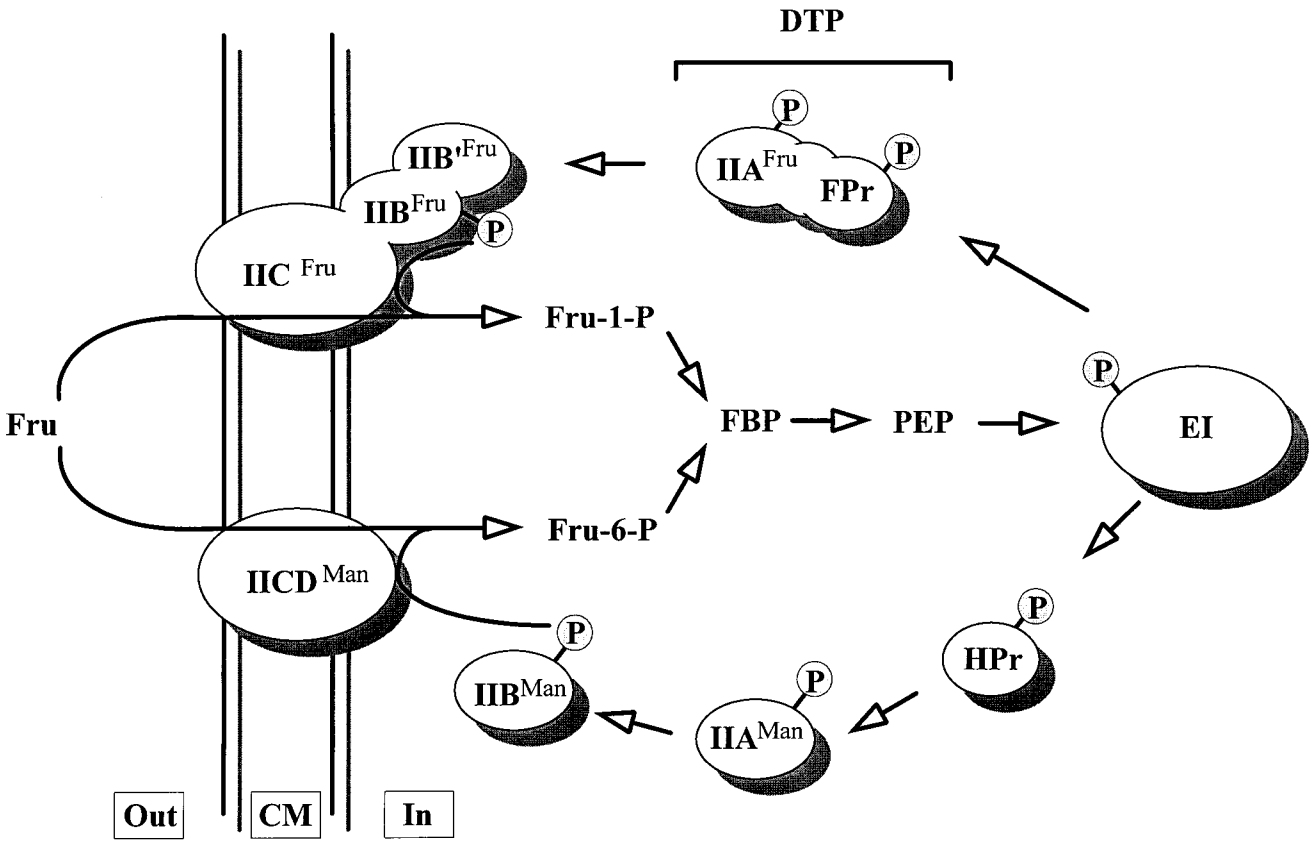

FIG. 1. The sequence of phosphoryl transfer events in the PTS-catalyzed phosphorylation of fructose in *E. coli*. PEP, the end product of glycolysis, is the immediate phosphoryl donor for the PTS phosphotransfer chain. This chain is initiated by autophosphorylation of Enzyme I (EI), which then transfers phosphate (P) either to the small, heat-stable phosphocarrier protein of the PTS, HPr (bottom scheme), or to its fructose-inducible paralog, FPr, a domain within the diphosphoryl transfer protein (DTP) which also contains the IIA^{Fru} domain (top scheme). HPr~P transfers the phosphoryl moiety to the IIA^{Man} constituent of the mannose Enzyme II complex and then to the IIB^{Man} protein in preparation for fructose phosphorylation on the 6-hydroxyl, catalyzed by the $IICD^{Man}$ permease complex (bottom scheme). FPr~P in DTP first transfers its phosphoryl moiety to the IIA^{Fru} moiety of DTP in preparation for IIB′BC phosphorylation and subsequent transport and phosphorylation of fructose on the 1-hydroxyl (top scheme). While II^{Fru} phosphorylates fructose with high affinity and specificity, II^{Man} phosphorylates fructose with low affinity and specificity. Both fructose phosphates are converted to the common intermediate of glycolysis, fructose 1,6-bisphosphate (FBP), which can be further metabolized to yield PEP.

tutive levels. *ptsH* mutants of *E. coli* and *Salmonella typhimurium* lack the small phosphocarrier protein of the PTS, HPr, and consequently cannot utilize most PTS sugars (glucose, mannitol, *N*-acetyl glucosamine, etc.). However, they can utilize fructose at nearly wild-type rates (40, 41) because of the presence of the fructose-inducible diphosphoryl transfer protein, DTP, which contains an HPr-like domain, FPr, that can substitute for HPr (Fig. 1; 12, 40). Secondary-site phenotypic revertants of *ptsH* mutants which regained the ability to utilize PTS sugars in a *ptsH* genetic background synthesized the products of the fructose operon constitutively, and they proved to be defective for Cra function (5, 13, 40). In addition to their fructose constitutive phenotype, these mutants lacked the ability to utilize acetate, pyruvate, alanine, citrate, malate, and all other gluconeogenic substrates (5). One gluconeogenic enzyme, phosphoenolpyruvate (PEP) synthase, was shown to be synthesized at depressed levels (13, 14). These facts led to the realization that the Cra protein must function directly or indirectly in some capacity other than that of controlling expression of the *fru* operon. As the effects of *cra* null mutations could not be overcome by addition of cAMP, it appeared that the mechanism of Cra action was independent of the cAMP-CRP control system (5). A novel regulatory mechanism seemed to be operative.

In subsequent studies, a *cra*::Tn*10* insertional mutation was shown to affect the levels of key carbohydrate catabolic enzymes. Thus, several glycolytic enzymes (phosphofructokinase, Enzyme I of the PTS, and several Enzymes II of the PTS) were synthesized in elevated amounts, but the three key gluconeogenic enzymes, PEP synthase, PEP carboxykinase, and fructose-1,6-bisphosphatase, were present in *cra* mutants at levels that were depressed relative to those in wild-type cells (Table 1). *cra* mutants also exhibited depressed levels of the Krebs cycle enzyme isocitrate dehydrogenase, and of the enzymes of the glyoxalate shunt, isocitrate lyase and malate synthase (4). Finally, certain terminal electron acceptors such as the cytochrome *d* complex, dimethylsulfoxide reductase, and nitrite reductase were apparently present in depressed amounts (Table 1; 14a, 28, 39).

The effects of glucose on carbon catabolic gene expression in wild-type *E. coli* and *S. typhimurium* cells proved to be similar to those of null mutations in the *cra* genes of these organisms (4, 12). It therefore seemed reasonable to propose that Cra mediates responses to carbon availability by transcriptionally activating the expression of genes that are normally subject to catabolite repression (e.g., *ppsA*, *pckA*, and *aceBA*; Table 1) while repressing the expression of genes that are subject to catabolite activation (e.g., *fru*, *pts*, and *edd* genes; Table 1). It

TABLE 1. Cra protein transcriptional regulation of genes encoding enzymes of carbon and energy metabolism in enteric bacteria

Enzyme	Gene or operon	Fold effect as determined by measuring[a]:	
		Enzyme activity	β-Galactosidase in gene-*lacZ* fusion
Positively regulated by Cra			
PEP synthase[b]	*ppsA*	20	10
PEP carboxykinase[b]	*pckA*	4	≥5
Malate synthase; isocitrate lyase[b]	*aceBA*	4	4
Isocitrate dehydrogenase[b]	*icd*	3	ND[c]
Fructose-1,6-bisphosphatase	*fdp*	2	ND
Cytochrome *d* oxidase	*cyd*	≥5	3
Negatively regulated by Cra			
Fructose catabolic enzymes[b]	*fruBKA*	~20	ND
HPr, Enzyme I[b]	*ptsHI*	3	ND
Entner-Doudoroff enzymes[b]	*edd-eda*	ND	3
Phosphofructokinase	*pfk*	2.5	ND
Mannitol catabolic enzymes[b]	*mtlADR*	6	2
Erythrose-4-phosphate dehydrogenase[b]	*gapB*	ND	3.5
Pyruvate kinase[b]	*pykF*	ND	3

[a] Activities of enzymes were assayed in isogenic wild-type and *cra* mutant strains grown in LB medium as reported by Chin et al. (4) and by Feldheim et al. (9). Cytochrome *d* oxidase was assayed by R. B. Gennis, University of Illinois, Urbana (see reference 39). All gene-*lacZ* fusions studied were transcriptional fusions (4, 27), except for the *pykF-lacZ* (1) and the *cyd-lacZ* (28) fusions, which were translational fusions. Growth conditions were as described in the cited references.

[b] Cra binding site(s) in the control region of the operon encoding this enzyme was identified in in vitro studies.

[c] ND, not determined.

became clear that Cra did not merely function in the control of *fru* operon expression but that it was also a global regulatory protein that served as a catabolite activator of some genes and a catabolite repressor of other genes (hence, its designation "catabolite repressor/activator" [Cra]).

SEQUENCE ANALYSES OF THE *cra* GENE

The sequence of the cloned *cra* gene (20, 21, 45) revealed that it is included within a monocistronic operon which maps at 2 to 3 min on the *S. typhimurium* or *E. coli* chromosome. The deduced amino acid sequence of Cra revealed homologies to several recognized transcriptional repressors, such as the lactose (LacI), galactose (GalR), and purine (PurR) repressors of *E. coli* and the so-called catabolite control proteins A and B (CcpA and CcpB) of *Bacillus* species (20, 21, 24, 37, 45, 46). These proteins all possess N-terminal helix-turn-helix domains that are involved in DNA binding. Their C-terminal ligand-binding domains are homologous to periplasmic sugar receptors, which exhibit specificity for sugars such as galactose, ribose, and arabinose (11, 43). The C-terminal domains of most of the repressor proteins bind carbohydrates or carbohydrate derivatives. Instead of the N-terminal DNA-binding domains present in the repressor proteins, the homologous periplasmic sugar-binding proteins possess N-terminal, cleavable leader sequences that target these receptors across the cytoplasmic membrane to the periplasm of the gram-negative bacterial cell (30). The two subfamilies of this receptor-repressor superfamily evidently arose by gene splicing and fusion events that occurred very early during their evolution (36).

Over 25 sequenced transcriptional regulatory proteins of dissimilar function, make up the current LacI-GalR family. Phylogenetic tree construction revealed that, with only a few exceptions, each branch of the tree bears a single protein, and almost all branches radiate from positions that are near the center of the circular tree (24). The appearance of the tree led to the suggestion that at a specific time in evolutionary history, perhaps at a time when primordial bacteria were acquiring multiple regulatory mechanisms to control the preferential catabolism of exogenous substrates, gene duplication events, all occurring within a relatively short period of evolutionary time, gave rise to the majority of the proteins of the family. Only occasionally did subsequent gene duplication and divergence events give rise to new members of the family with novel functions. Thus, Cra-mediated catabolite repression/activation probably arose at a time when bacteria were first imposing distinct transcriptional control mechanisms upon their carbohydrate catabolic and anabolic capabilities (24).

IN VITRO CHARACTERIZATION OF THE MECHANISM OF Cra ACTION

In vitro DNA-binding approaches have been used to examine the specificity and function of purified Cra (1, 6, 27, 29, 34). DNA fragments of various lengths (between 200 and 600 bp) that included the control regions of Cra target operons were prepared by using PCR. By a variety of approaches including gel retardation, targeted sequence alteration, and various footprinting techniques, Cra was shown to bind to the control regions of 10 of the operons listed in Table 1.

Six of the well-characterized Cra-regulated operons (Table 1) are repressed by Cra. The Cra-binding sites are located downstream of, or overlapping the promoters in all instances in which Cra functions in repression. Since Cra is displaced from its DNA-binding sites by micromolar amounts of fructose-1-phosphate or millimolar amounts of fructose-1,6-bisphosphate, the repressive effects are reversed by sugar catabolites. These six operons are consequently subject to catabolite activation.

The remaining four well-characterized operons (also in Table 1) are activated by Cra. In these operons, the Cra-binding sites are located upstream of the promoters. Since ligand binding to Cra displaces the protein from the DNA, these operons are subject to catabolite repression. Catabolite activation is thus generally observed whenever Cra represses transcription, while catabolite repression is observed whenever the protein activates transcription.

On the basis of 11 clearly identified Cra operators (27), a consensus sequence for Cra binding has been derived (Fig. 2,

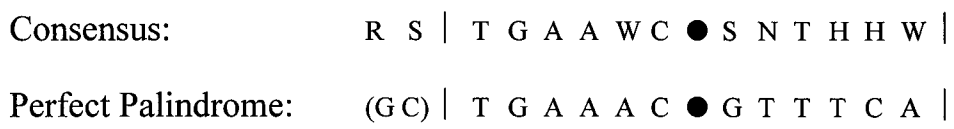

FIG. 2. The consensus sequence for Cra binding (top) and the idealized palindrome corresponding to this sequence (bottom). R, A or G; S, C or G; W, A or T; H, A or C or T; N, any nucleotide. The frequency of occurrence of nucleotides at each position as well as the actual binding sequence in each of the 11 operators studied is provided in reference 27. The solid circles indicate the center of the palindrome, and the vertical lines indicate the two boundaries of the palindrome.

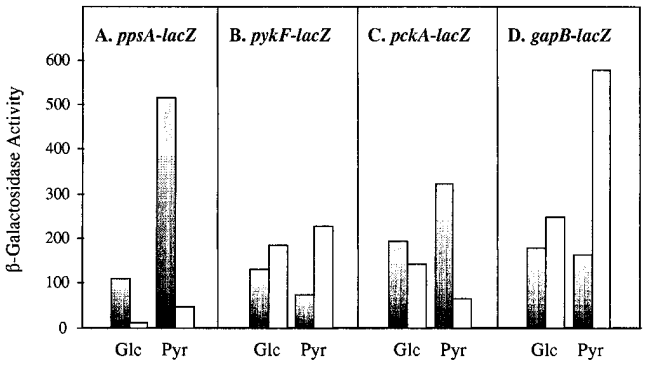

FIG. 3. Effects of carbon source on the expression of four *E. coli* genes in wild-type (shaded bars) and *cra* null mutant (open bars) *E. coli* cells. Experimental procedures were as described in references 1 and 27, with cells grown in minimal medium in the presence of 0.5% Casamino Acids. The *lacZ* fusions studied were to the following genes: (A) *ppsA* (PEP synthase), (B) *pykF* (pyruvate kinase), (C) *pckA* (PEP carboxykinase), and (D) *gapB* (erythrose-4-phosphate dehydrogenase). The carbon source used for growth and present in excess was either glucose (Glc) or pyruvate (Pyr).

top). This consensus sequence is an incomplete palindrome in which the left half-site of the palindrome is considerably better conserved than the right half-site (Fig. 2, bottom). A perfect palindrome occurs in only one operator, O_1 in the *E. coli* or *S. typhimurium fru* operon. In this regard it is interesting (a) that Cra binds to this operator with very high affinity, (b) that the *fru* operon is the only one known to possess two distinct Cra operators, and (c) that the fold repression of the *fru* operon by Cra is greater than that for any other known operon subject to Cra control. These unique features are consistent with the possibility that Cra evolved first as the *fru* operon repressor and only secondarily acquired pleiotropic functions in the regulation of other carbohydrate catabolic operons.

As noted above, the Cra-binding consensus sequence is asymmetric, with substantially greater conservation occurring in the left half-site than in the right half-site. This fact is in agreement with experimental data establishing that Cra binds asymmetrically to the imperfect palindrome, with stronger interactions occurring with the left half-site than with the right half-site (6). The degenerate nature of the Cra consensus sequence (27) may reflect the pleiotropic nature of Cra function and the need for a spectrum of DNA sites to which Cra binds with various affinities.

IN VIVO CHARACTERIZATION OF Cra ACTION

The consequences of Cra action have been determined both by measuring the enzymatic activities of Cra target gene products and by measuring the activities of reporter gene products in target gene-reporter gene fusions. Both approaches have led to essentially the same conclusions with respect to the directions and the magnitudes of Cra action (Table 1).

Data illustrating the dependency of gene expression on Cra under glycolytic and gluconeogenic conditions are presented in Fig. 3 for four representative genes. *ppsA* (Fig. 3A) and *pckA* (Fig. 3C) are subject to positive control by Cra, while *pykF* (Fig. 3B) and *gapB* (Fig. 3D) are subject to negative control. The graphs depict the responses of wild-type (shaded bars) and *cra* mutant (open bars) *E. coli* cells to two carbon sources: glucose (a glycolytic substrate) and pyruvate (a gluconeogenic substrate). Cra exerts an activating effect on *ppsA* expression regardless of the carbon source used. However, this activating effect is much more substantial when cells are grown with pyruvate (a gluconeogenic substrate) than when they are grown with glucose (a glycolytic substrate). In the absence of Cra, activity is very low. Presumably, catabolites such as fructose-1,6-bisphosphate and fructose-1-phosphate, generated from a glycolytic substrate, diminish the activating effect of Cra when grown with this sugar (catabolite repression). In Fig. 3B, the corresponding effects on *pykF* gene expression are reproduced. In this case Cra exerts a repressing effect, and the β-galactosidase activity in the presence of glucose is therefore higher than when cells are grown with pyruvate. Thus, Cra mediates catabolite activation (1).

The operator-promoter regions of the *ppsA* and *pykF* genes lack detectable CRP-binding sequences, and in these two genes, Cra plays a dominant and clear-cut role in transcriptional regulation in response to carbon source availability. However, in the *pckA* and *gapB* control regions, CRP-binding sites are also found. The situation is therefore more complex, as wild-type cells will exhibit responses to carbon source availability mediated by both Cra and CRP. Mutants lacking Cra are presumably responsive only to the presence or absence of the cAMP-CRP complex. In the *pckA* gene control region, two high-affinity CRP-binding sites are found, of which it is believed that one activates gene expression and the other represses (14b, 27). In the absence of Cra, glucose-grown cells have higher PEP carboxykinase activity than do pyruvate-grown cells (Fig. 3C), suggesting that the cAMP-CRP complex exerts a net negative effect on *pckA* gene expression. By contrast, in the wild-type cell, expression is higher in pyruvate-grown cells than in glucose-grown cells, showing that Cra represses and that the effect of Cra binding predominates over that of CRP binding. Finally, when *gapB* gene expression is examined (Fig. 3D), glucose exerts the normal catabolite-repressing effect in the absence of Cra, suggesting that the cAMP-CRP complex activates *gapB* gene expression. This effect is largely counteracted by Cra which strongly represses *gapB* expression when cells are grown in the presence of pyruvate but not when they are grown in the presence of glucose. As summarized in Table 2, the net results are that CRP and Cra exert opposing effects on *pckA* and *gapB* gene expression and that the effects of these two regulatory proteins nearly cancel each other under the specific physiological conditions used.

The consequences of a *cra* null mutation on the utilization of dozens of carbon sources has recently been documented (27). Of 84 tested, 36 exogenous carbon sources used by *E. coli* resulted in oxidative responses to a Cra deficiency. Generally, sugars and sugar derivatives were oxidized more readily in the *cra* mutant compared within wild-type cells, while gluconeogenic substances were oxidized less readily. This observation led to the generalized conclusion that Cra controls the direc-

TABLE 2. Cra and CRP action on selected target genes

Gene	Enzyme encoded	Consequences of protein binding[a]		Net result
		Cra	CRP	
ppsA	PEP synthase	+	No effect[b]	Catabolite repression
pykF	Pyruvate kinase	−	No effect[b]	Catabolite activation
pckA	PEP carboxykinase	+	−	Catabolite repression
gapB	Erythrose-4-phosphate dehydrogenase	−	+	Catabolite activation

[a] +, transcriptional activation due to protein binding; −, transcriptional inhibition due to protein binding.

[b] No effect of CRP was expected, since a CRP-binding sequence in the control region of this gene could not be identified.

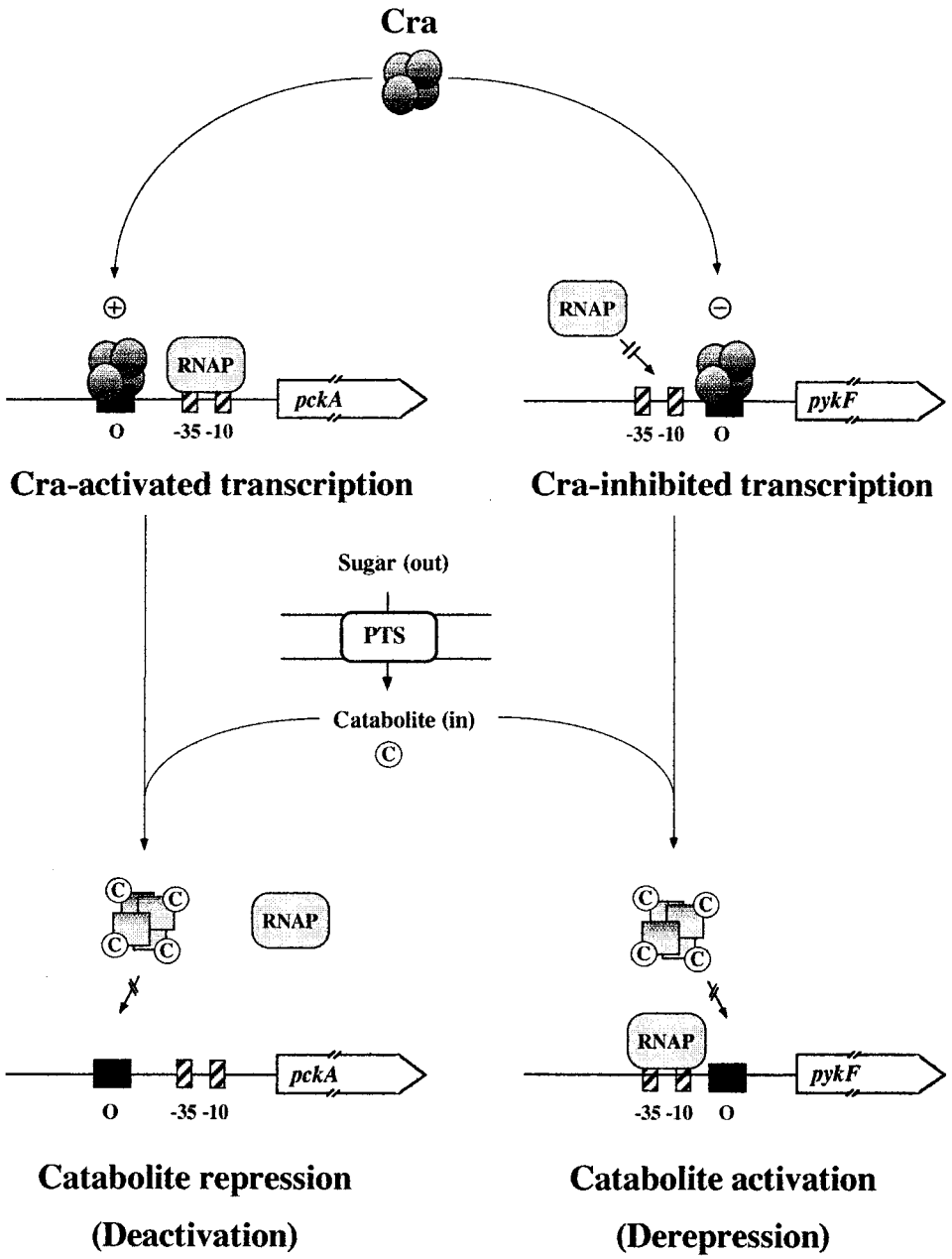

FIG. 4. Model illustrating the sensory transduction pathway for Cra-mediated catabolite repression and catabolite activation in *E. coli*. In the unliganded form, Cra binds to the operator (O) regions of target operons. When the Cra operator is upstream of the RNA polymerase (RNAP) binding site, activation of transcription is observed. When the operator overlaps or is downstream of the RNA polymerase binding site, inhibition of transcription is observed. The presence of an exogenous sugar (glucose, fructose, etc.) results in uptake via the phosphotransferase system, producing sugar phosphates, and cytoplasmic glycolytic catabolites (C) accumulate. These catabolites (fructose-1-phosphate and fructose-1,6-bisphosphate) bind to the tetrameric Cra protein, causing it to dissociate from the DNA. Dissociation reverses the activating effect of Cra, as in the case of the *pckA* gene of *E. coli*, which encodes PEP carboxykinase (catabolite repression, left), and reverses the inhibiting effect of Cra, as in the case of the *pykF* gene of *E. coli*, which encodes pyruvate kinase (catabolite activation, right).

tion of carbon flux and primarily functions to select a fermentative pathway or an oxidative pathway of carbon metabolism, depending on physiological conditions.

CONCLUSIONS AND PERSPECTIVES

Figure 4 presents a model for the mechanism of action of the pleiotropic regulator of carbon flux, Cra. Cra generally represses transcription of genes encoding glycolytic (sugar-catabolizing) enzymes (i.e., key enzymes in the Embden-Meyerhof and Entner-Doudoroff pathways) but activates transcription of genes encoding biosynthetic and oxidative enzymes (i.e., key enzymes in the Krebs cycle, the glyoxalate shunt, the gluconeogenic pathway, and electron transfer). Those genes that are activated by Cra are consequently subject to catabolite repression (e.g., *pckA*; Fig. 4, left side), while genes that are repressed by Cra are subject to catabolite activation (e.g., *pykF*; Fig. 4, right side). This metabolic effect is due to the fact that sugar catabolites bind to Cra and displace it from the operator sites in target operons. These catabolites are present in high con-

centrations during growth in the presence of sugars but in low concentrations during growth in the presence of gluconeogenic substances.

The work described in this minireview summarizes what is known concerning the molecular mechanism of action of Cra and the physiological consequences of this action. Recent studies have shown that the picture described here is probably an oversimplification. For example, the *cyd* operon, encoding the proteins of cytochrome *d* oxidase, is regulated severalfold by Cra although it does not exhibit a Cra-binding site in its control region. Preliminary studies have revealed that the effect of Cra action on *cyd* operon expression is dependent on the Fnr transcriptional regulator and antagonized by the ArcA transcriptional regulator (18, 19, 28). Furthermore, recent data have shown that under certain physiological conditions Cra modulates the activity of adenylate cyclase, the cAMP biosynthetic enzyme (6a). It is therefore clear that Cra does not act alone to exert its multifarious effects but interacts with other pleiotropic regulators to create a network of transcriptional effects that serve to coordinate the effects of various bacterial sensing devices. Such a network allows finely tuned responsiveness to multiple environmental and internal signals and coordinates the various activities of the cell at the transcriptional level. The details of such complex interactions have yet to be elucidated.

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