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In Vitro Reconstitution of Catabolite Repression in *Escherichia coli*[*][S]

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A widely accepted model for catabolite repression posits that phospho-IIA^{Glc} of the bacterial phosphotransferase system activates adenylyl cyclase (AC) activity. For many years, attempts to observe such regulatory properties of AC *in vitro* have been unsuccessful. To further study the regulation, AC was produced fused to the transmembrane segments of the serine chemoreceptor Tsr. Cells harboring Tsr-AC and normal AC, expressed from the *cya* promoter on a low copy number vector, exhibit similar behavior with respect to elevation of cAMP levels resulting from deletion of *crp*, expressing the catabolite regulatory protein. Membrane-bound Tsr-AC exhibits activity comparable with the native form of AC. Tsr-AC binds IIA^{Glc} specifically, regardless of its phosphorylation state, but not the two general phosphotransferase system proteins, enzyme I and HPr; IIA^{Glc} binding is localized to the C-terminal region of AC. Binding to membranes of either dephospho- or phospho-IIA^{Glc} has no effect on AC activity. However, in the presence of an *Escherichia coli* extract, P-IIA^{Glc}, but not IIA^{Glc}, stimulates AC activity. Based on these findings of a direct interaction of IIA^{Glc} with AC, but activity regulation only in the presence of *E. coli* extract, a revised model for AC activity regulation is proposed.

In *Escherichia coli*, cAMP, produced by adenylyl cyclase (AC),[5] is an important regulatory molecule, essential for controlling the expression of numerous operons. The cellular levels of cAMP are regulated mainly via effects on AC activity. It has been firmly established that the phosphoenolpyruvate:sugar phosphotransferase system (PTS) plays an important role in the regulation mechanism. Thus, in wild-type but not in PTS mutant cells, exposure to glucose results in decreased cellular cAMP levels; this decrease accounts for the phenomenon of catabolite repression (1).

A popular, but never proven, model for the regulation of AC activity is that the phosphorylated form of IIA^{Glc} of the PTS stimulates AC activity; thus, glucose transport is presumed to lead to dephosphorylation of IIA^{Glc} resulting in a de-activation of AC. It has also been observed that strains of *E. coli* deficient in the cAMP-binding protein, CRP, produce extraordinarily large amounts of cAMP (2). This CRP-dependent regulation of cAMP levels depends on the presence of IIA^{Glc} (3). It has been proposed that the CRP-cAMP complex promotes expression of a phosphatase that converts P-IIA^{Glc} to dephospho-IIA^{Glc} (4). Consequently, in the absence of CRP, a greater proportion of the pool of IIA^{Glc} is in the phospho-form and the AC is more fully activated.

One approach to allow a further understanding of the mechanism by which AC is regulated has involved the use of permeable cells. In this case, exposure of the permeable cells to glucose results in inhibition of AC activity (5). In this system, it was discovered that P_i is essential for high activity of AC as well as for the capability of the cells to be inhibited by glucose. Because P_i was also shown to stimulate PTS activity in this system, P_i may somehow be involved in the coupling of the interaction of PTS proteins to AC.

A number of studies designed to further elucidate the mechanism of regulation of AC have suggested that a simple activation of AC by phospho-IIA^{Glc} is insufficient to account for the regulation mechanism. Liberman *et al.* (6) demonstrated that unphosphorylated IIA^{Glc} resulted in a P_i-dependent inhibition of AC in permeable cells. These data suggested that dephosphorylated IIA^{Glc} can interact with and influence the activity of AC.

Reddy *et al.* (7) attempted the reconstitution of the regulatory properties of AC *in vitro*. Although the partially purified AC or diluted crude extracts showed no effect of added PTS proteins, relatively concentrated extracts showed a variety of effects characteristic of AC activity in permeable cells. These experiments suggested that at least one other uncharacterized factor was required for the effective coupling of PTS proteins to AC. It has been repeatedly speculated elsewhere (8, 9) that other unknown factors could be involved in the regulation of AC activity.

In order to further clarify the nature of regulation of AC, a form of AC (Tsr-AC) was engineered in which the enzyme was attached to the membrane. In this way, pull-down experiments might be used to define factors that interact directly with and affect the activity of AC. For the first time, it was possible to show a specific interaction of AC with IIA^{Glc}. The studies reported here demonstrate that Tsr-AC is active, that it interacts with both forms of IIA^{Glc}, and that regulation by P-IIA^{Glc} can be reconstituted only when crude extract is included. These data allow for the formulation of a new model for the regulation of AC activity involving interaction of the regulatory domain of AC with both a regulatory factor and IIA^{Glc}, whereby the state of phosphorylation of IIA^{Glc} determines the level of AC activity.

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[5] The abbreviations used are: AC, adenylyl cyclase; PTS, phosphoenolpyruvate:sugar phosphotransferase system; EI, the enzyme I phosphocarrier protein of the PTS; IIA^{Glc}, the enzyme IIA component of the glucose-specific PTS; Tsr-AC, the fusion protein containing the two transmembrane segments of Tsr fused to AC; Tsr-ACN, the fusion protein containing the N-terminal region of Tsr fused to the N-terminal region of AC; CRP, the cAMP receptor protein; RF, a factor, as yet uncharacterized, capable of regulating AC activity; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; CE, crude extract.

TABLE 1
Strains and plasmids used

Strain or plasmid	Genotype or description	Source or Ref.
Strains		
GI698	$F^-\lambda^-$ *lacI*^{q} *lacPL8 ampC*::P_{trp}cI	30
GI698Δ*pts*	*PtsHIcrr*::kan	31
XL1-Blue	General cloning vector	Stratagene
MG1655	Wild-type *E. coli* K12	32
DC646	λ lysogen used for stable transformation of constructs encoding the λ promoter	D. Court, NCI, National Institutes of Health, Frederick, MD
CA8306	Δ*cya854*	E. Brickman, Harvard University
CA8445	Δ*cya854*Δ*crp45*	E. Brickman, Harvard University
Plasmids		
pRE1	Expression vector under control of λ P_L promoter, Amp^r	15
pBluescript		Stratagene
pPR100	Expression vector encoding AC	15
pDIA100	Encodes AC under control of its own promoter	A. Danchin, Pasteur Institute, Paris, France
pJFG5	Encodes Tsr, serine chemoreceptor	C. Manoil, University of Washington (33)
pWSK29	Low copy number plasmid	S. Kushner, University of Georgia (14)
pDIA(*tsr-cya*)	Encodes Tsr-AC fusion	This study
pPR100(*tsr-cya*)	Encodes Tsr-AC fusion	This study
pLP1663	Encodes AC (low copy)	This study
pLP1664	Encodes Tsr-AC fusion (low copy)	This study
pPR100(*tsr-cyaN*)	Encodes fusion of Tsr with N-terminal half of AC	This study
pPR100(His tag)	Encodes AC with His tag	This study

EXPERIMENTAL PROCEDURES

Plasmids and Strains

All plasmids and strains used are described in Table 1.

Media

Rich medium was used for growth and expression of proteins encoded by pRE1-based vectors: 1× M9 salts, 2% Difco Bacto-Casamino acids, 1% glycerol, 10 mM $MgSO_4$, 0.1 mM $CaCl_2$, 100 μg/ml ampicillin; MLM medium (10), also used for expression of proteins encoded by pRE1-based vectors has been described; Luria-Bertani (LB) medium (11) was used for general cloning.

Other Materials

Ready-to-Go PCR Beads and Microspin G-25 columns were from Amersham Biosciences. Restriction endonucleases (NdeI, EcoRI, SalI, and BamHI) were purchased from New England Biolabs. Precast SDS-acrylamide gels and molecular weight markers (SeeBlue Plus2) were from Invitrogen. Oligonucleotides for sequencing and PCRs were synthesized by Vicky Guo (NHLBI, National Institutes of Health) using a model 394 DNA/RNA synthesizer (Applied Biosystems) or were purchased as high pressure liquid chromatography-purified preparations from Qiagen. Tryptophan was purchased from Invitrogen. Enzyme I (EI), HPr, and IIA^{Glc} were purified as described previously (12). BD Talon His tag purification resin was from BD Biosciences.

Methods

Transformation of appropriate host cells with plasmids was accomplished by electroporation using an *E. coli* Pulser (Bio-Rad).

Membrane Preparation—Transformants in GI698 cells were cultured in 1 liter of Rich medium supplemented with ampicillin (100 μg/ml) at 30 °C. When the culture reached an absorbance at 600 nm of 0.4, tryptophan (100 μg/ml) was added, and growth was continued overnight. The cells were harvested and resuspended in 20 mM Tris·HCl, pH 8.0, 200 mM NaCl. The cell suspension was passed twice through a French press at 15,000 p.s.i. The broken cells were centrifuged at 10,000 rpm for 10 min, and the supernatant solution was then centrifuged for 90 min at 40,000 rpm. The pellet was resuspended and washed with 1 M urea in Tris buffer by centrifuging again at 40,000 rpm. The washed pellet was finally resuspended in 1 ml of Tris buffer. The protein concentrations of the membrane preparations were ~20 mg/ml. Protein concentrations were determined by the BCA (bicinchoninic acid) protein assay (Pierce).

Calculation of Binding Constants—The interaction of Tsr-AC (126,292 Da) with IIA^{Glc} (18,251 Da) was assumed to be between the monomeric forms of both proteins. As described in the text, various concentrations of IIA^{Glc} were incubated with membranes enriched in either Tsr-AC or Tsr-ACN (control membranes). The samples were processed, using SDS-PAGE, as described in the text (see Fig. 4). Gel scanning was used to quantitate the binding to Tsr-AC and the control membranes as well as the unbound fraction. The specific binding was calculated by subtracting the binding to Tsr-ACN from that bound to Tsr-AC. The formula K_D = (IIA^{Glc} unbound or free IIA^{Glc}) × (AC unbound or free Tsr-AC)/(IIA^{Glc}-AC complex) was used to calculate the K_D.

Adenylyl Cyclase Activity Assay of Membrane Preparations—The assay was modified from a method described previously (5). In a reaction volume of 50 μl containing Bicine buffer, pH 8.5 (25 mM), $MgCl_2$ (10 mM), ATP (1 mM), dithiothreitol (2 mM), Na_2HPO_4 (20 mM), and 200 mM NaCl, ~2.5 μg of membranes were added, and incubation was carried out at room temperature. Samples (25 μl) were withdrawn at the indicated times into 50 μl of 1 N perchloric acid. After centrifugation, the supernatant solutions were assayed for cAMP. cAMP concentrations were determined using the cAMP enzyme immunoassay kit from Amersham Biosciences.

Plasmid Constructs

pDIA(tsr-cya)—A BamHI fragment from pDIA100 encoding the region around the *cya* start site was mutagenized to create an NdeI site at the translation initiation codon of *cya*. This 530-bp fragment was first subcloned into the BamHI site of pBluescript. This clone was used as the template for PCR-based mutagenesis by the method of Higuchi (13). Two PCRs were carried out using a mixture of mutagenic primer 6134 (5′-GGG TAG CAA ATC AGG CGA TAC **CAT ATG** TAC CTC TAT ATT GAG ACT CTG-3′) (NdeI site in boldface) and primer 6142, upstream of the BamHI site in pBluescript (5′-AAT TAA CCC TCA CTA AAG GG-3′) in one and a mixture of mutagenic primer 6135 (5′-CAG AGT CTC AAT ATA GAG GTA **CAT ATG** GTA TCG CCT GAT TTG CTA CCC-3′) and primer 6143, downstream of the BamHI

site in pBluescript (5′-GTA AAA CGA CGG CCA GT-3′). The two PCR products were mixed and used as the template for a third PCR using primers 6142 and 6143. The resultant PCR product was digested with BamHI and then recloned into pBluescript digested with BamHI.

Plasmid pJFG5, encoding the *tsr* gene, was used as the template for a PCR designed to clone the fragment encoding residues 1–259 of Tsr with newly created NdeI sites at these residues. The primers 5′-CCA CAG GAA AGA GAA **CAT ATG** TTA AAA CGT ATC-3′ (NdeI site in boldface; codon for residue 1 underlined) and 5′-CTC TCC CTG **CAT ATG** GCG CAA ACT-3′ (NdeI site in boldface; codon for residue 259 underlined) were used for the amplification. The product was digested with NdeI and cloned into the NdeI site of the recombinant pBluescript described above. The BamHI fragment of this new construct was moved back into pDIA100 resulting in a derivative of pDIA100 in which an N-terminal fragment of *tsr* was fused to the beginning of the *cya* gene. The steps in the construction are schematized in supplemental Fig. 1.

pWSK29 Derivatives Encoding cya Genes—In order to obtain low level expression, *cya* genes were moved into the low copy number vector pWSK29 (14). As outlined in supplemental Fig. 1, the EcoRI-SalI fragments encoding the *cya* gene and its upstream promoter derived from the plasmid pDIA100 or the *tsr-cya* gene and its upstream promoter from the plasmid pDIA(*tsr-cya*) were moved into pWSK29 resulting in pLP1663 and pLP1664, respectively.

pPR100(tsr-cya)—pPR100 is an expression vector for AC (15). The fragment encoding residues 1–259 of Tsr described above was, after digestion with NdeI, cloned into the NdeI site at the N terminus of the *cya* gene of pPR100. As shown in supplemental Fig. 2, this expression vector encodes a 130-kDa Tsr-AC fusion protein.

All constructs were verified by DNA sequencing (16). The sequence of *cya* in our constructs matches that reported for *E. coli* K12 by Blattner *et al.* (32) (GenBank™ accession number M87049).

pRE1(tsr-cyaN)—The expression vector encoding the N-terminal half of AC was constructed by a PCR using pPR100 (15) as a template and primer 2753 (upstream of the multiple cloning site of pRE1 (15); 5′-AAC CAC ACC TAT GGT GTA TGC A-3′) and primer 6403, encoding a double translation stop after Ala-425 (5′-ATA TAT **GTC GAC** *CTA TTA* CGC GGC ATA CAG CTT ACG CGT CAG-3′; the underlined region corresponds to a new sequence not found in the *cya* gene, the italicized regions are the two stop codons, and the boldface region is the new SalI site). The PCR product was digested with NdeI and SalI and cloned into pRE1 digested with the same enzymes. Then the fragment encoding residues 1–259 of Tsr with the newly created NdeI sites at these residues, described above, was cloned in-frame into the NdeI site. This expression vector encodes a Tsr fusion containing the first 425 amino acid residues of AC (Tsr-ACN).

pPR100(His Tag)—An expression vector encoding AC with an N-terminal His tag for facile purification of the enzyme was constructed by inserting a linker into the NdeI site of pPR100. The linker encodes the sequence Met-Gly-$(Ser)_2$-$(His)_6$-$(Ser)_2$-Gly-Leu-Val-Pro-Arg-Gly-Ser-His, which resides directly in front of the normal initiator methionine residue. The underlined sequence corresponds to a thrombin cleavage site (cleavage occurs between the Arg and Gly residue).

Purification of Soluble AC—GI698/pPR100(His tag) cells were cultured in 1 liter of rich medium supplemented with ampicillin (100 μg/ml) at 30 °C. When the culture reached an absorbance at 600 nm of 0.4, tryptophan (100 μg/ml) was added, and growth was continued overnight. The cells were harvested and resuspended in 20 mM Tris·HCl, pH 8.0, 200 mM NaCl. The cell suspension was passed twice through a French press at 15,000 p.s.i. The broken cells were centrifuged at 10,000 rpm for 10 min. The supernatant solution was purified by affinity chromatography using Talon metal affinity resin. After washing out the unbound proteins, the His tag AC was eluted with 25 mM Tris, pH 8.0, 200 mM NaCl, 100 mM imidazole. The eluate was then further purified and desalted by gel filtration chromatography (Superose 12, 1.6 × 50 cm; Amersham Biosciences) in 25 mM Tris, pH 8.0, 200 mM NaCl. The purified protein was about 1 mg/ml.

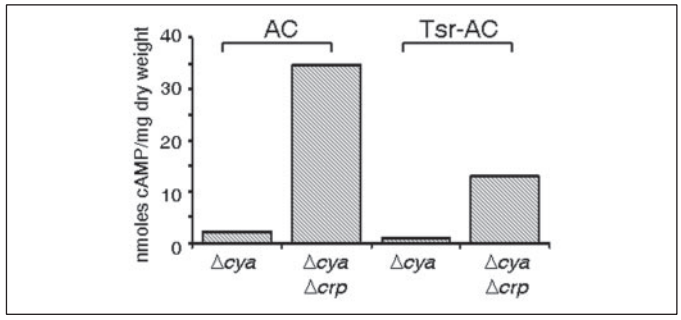

FIGURE 1. **Formation of cAMP by CA8306 (Δ*cya854*) and CA8445 (Δ*cya854* Δ*crp45*) transformed with pLP1663 and pLP1664.** pLP1663 encoding AC and pLP1664 encoding Tsr-AC (see supplemental Fig. 1) were transformed by electroporation (see "Experimental Procedures") into strains CA8306 (Δ*cya*) and CA8445 (Δ*cya* Δ*crp*) (see "Experimental Procedures"). The transformants were grown at 37 °C in LB medium plus ampicillin (100 μg/ml), and the growth was followed by measuring the absorbance at 600 nm of the cultures. At approximately $A_{600} = 0.4$, samples of the cultures were withdrawn and placed in a boiling water bath for 3 min. After removal of insoluble material, the samples were assayed for cAMP (see "Experimental Procedures"). For calculation of dry weight, an A_{600} of 1 is taken as equivalent to 1.75×10^9 cells/ml, and the volume of a single cell is taken as 10^{-12} cm^3.

Crude Extract—The crude extract (CE) was the source of RF. Strain MG1655 or GI698Δ*pts* was grown overnight in 100 ml of LB medium. The culture was centrifuged, and the cells were suspended in 2 ml of 20 mM Tris·HCl, 200 mM NaCl and then disrupted by passage twice through a French press at 10,000 × *g*. Cell debris was removed by centrifugation at 10,000 rpm for 10 min. The protein concentration of CE was ~25 mg/ml.

RESULTS AND DISCUSSION

Expression and Properties of Membrane-tethered AC

pDIA(tsr-cya)—As a first step in developing the methodology, it was necessary to determine whether the tethering of AC to the membrane resulted in a protein that was still capable of regulation in the same way that normal AC is regulated. Consequently, the first construct that was created was the fusion of a *tsr* fragment (encoding the two transmembrane segments of the *E. coli* serine chemoreceptor) with the *cya* gene in pDIA100, a pBR322-based vector (see supplemental Fig. 1). In this construct, the expression of the Tsr-AC fusion protein is under the control of the normal *cya* promoter; the low level expression of the gene in this construct avoids the toxicity associated with high levels of cAMP (17, 18). In order to achieve an even lower level of expression, the fragments encoding both AC and Tsr-AC were recloned into the low copy number plasmid pWSK29 (14) (see supplemental Fig. 1).

The plasmids encoding full-length AC (pLP1663) and the Tsr-AC fusion protein (pLP1664) were used to transform strains of *E. coli* deleted for the genes encoding AC (Δ*cya*) or both AC and CRP (Δ*cya* Δ*crp*). The transformants, grown at 37 °C in LB medium, all had similar growth curves (data not shown).

It has been reported that cAMP levels increase dramatically in Δ*crp* strains (2); because this increase in cAMP levels requires the presence of IIA^{Glc} (3, 19), this phenomenon has been accepted as an indicator of the capability of an AC to be regulated. The data in Fig. 1 demonstrate the capability of AC encoded by the two plasmids to be affected by the absence of CRP. cAMP levels are about 15-fold higher in the Δ*crp* strain

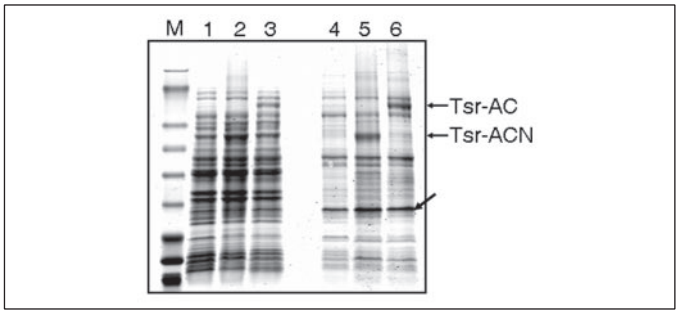

FIGURE 2. **Hyperexpression of Tsr-ACN and Tsr-AC.** Three expression vectors (pRE1, the control vector; pPR100(*tsr-cya*N), encoding Tsr-ACN; and pPR100(*tsr-cya*), encoding Tsr-AC) were electroporated into GI698 (30), a host suitable for controlled expression of the plasmids. The cultures were grown in rich medium and induced with tryptophan as described (30). Whole cells (*lanes 1–3*) and membrane preparations (*lanes 4–6*) from the induced cells were examined for protein expression by SDS-PAGE (NOVEX 4–20% Tris-glycine gradient gel). The gel was stained with Coomassie Blue. Lane identifications are as follows: *lane M*, SeeBlue Plus2 molecular weight markers (Invitrogen); *lanes 1* and *4*, pRE1; *lanes 2* and *5*, pPR100(*tsr-cya*N); *lanes 3* and *6*, pPR100(*tsr-cya*). The band labeled *Tsr-ACN* corresponds to expressed Tsr-ACN (~70 kDa), and the band labeled *Tsr-AC* corresponds to the expressed Tsr-AC fusion protein (~130 kDa). The *dark arrow points* to a reference membrane protein.

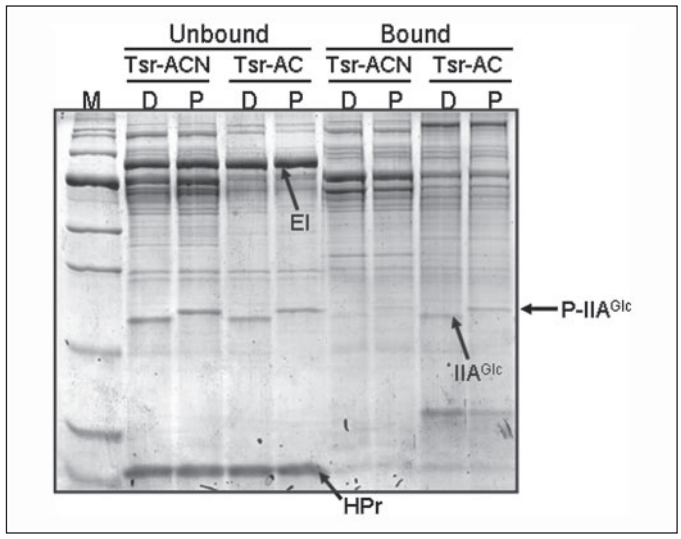

FIGURE 3. **Specific binding of IIA^{Glc} to membrane-bound Tsr-AC.** Membranes (250 μg of protein) containing expressed Tsr-AC or Tsr-ACN (see Fig. 2) were washed by mixing with 20 mM Tris·HCl, pH 8.0, followed by centrifugation at 50,000 rpm for 10 min. The pelleted membranes were resuspended and incubated at room temperature for 10 min in a medium containing 20 mM Tris·HCl, pH 8.0, 2 mM dithiothreitol, 2 mM $MgCl_2$, HPr (4.5 μg), Enzyme I (3 μg), and IIA^{Glc} (1.5 μg) (total volume, 50 μl). Mixtures designated *D* were supplemented with glucose (5 mM) to ensure complete dephosphorylation of the added IIA^{Glc}, and those designated *P* were supplemented with P-enolpyruvate (5 mM) to ensure complete phosphorylation of the added IIA^{Glc}. The mixtures were then centrifuged (50,000 rpm for 10 min). The supernatant solution (unbound fraction) was removed, and the pellet was rinsed with 20 mM Tris·HCl, pH 8.0, and then extracted with 50 μl of 0.5 M urea (bound fraction). Samples of all the fractions were run on a 13% SDS-PAGE. The gel was stained with Coomassie Blue. *M*, molecular weight markers (Technical Frontier Co., Japan).

when AC is either made as the normal protein expressed from pLP1663 (Fig. 1, *left half*) or as the Tsr-AC fusion expressed from pLP1664 (Fig. 1, *right half*). The actual level of cAMP accumulated in the Tsr-AC fusion strain is lower than that in the strain expressing the normal form of AC. Because Tsr-AC is highly active, as shown in the following studies, this difference in cAMP accumulation probably reflects the limitation in incorporation of the fusion protein into the cytoplasmic membrane. In any event, the important finding is that the Tsr-AC fusion protein is subject to a similar CRP-dependent regulation *in vivo* as is the normal form of AC.

pPR100(tsr-cya)—By having established that tethering of AC to the membrane does not eliminate its capacity to be regulated, the next step in this study was to hyperexpress the tethered enzyme. The construction of the plasmid used for this purpose is described in supplemental Fig. 2. The previously described expression vector, pPR100 (15), which is capable of overexpressing AC under the control of the λ promoter, was used as the starting material for the construct. The N-terminal region, encoding the two transmembrane segments of Tsr, the serine chemoreceptor, was amplified by PCR and then cloned into the NdeI site of pPR100, resulting in pPR100(*tsr-cya*). This expression vector encodes the Tsr-AC fusion protein (~1,100 residues long, see supplemental Fig. 2).

It has been suggested that AC contains two domains (20, 21) as follows: the N-terminal half of the protein contains the catalytic region and the C-terminal half of the protein contains the regulatory region. Consequently, as a control for subsequent studies, a vector hyperexpressing a Tsr-AC fusion containing only the N-terminal half of AC (Tsr-ACN) was also constructed (see "Experimental Procedures").

Fig. 2 demonstrates the expression of the two tethered forms of AC. Transformed cells harboring pRE1 (the control expression vector), pPR100(*tsr-cyaN*) (the expression vector for tethered ACN), and pPR100(*tsr-cya*) (the expression vector for tethered full-length AC) were induced for protein expression, and the induced cells were then processed for preparation of membrane vesicles. SDS gel analysis of the whole cells (Fig. 2, *lanes 1–3*) showed clear bands corresponding to Tsr-ACN (*lane 2*) and full-length Tsr-AC (*lane 3*).

When membrane preparations from the three induced cells are examined (Fig. 2, *lanes 4–6*), considerable levels of Tsr-ACN (*lane 5*) and Tsr-AC (*lane 6*) are observed. Comparison of the amount of the two expressed proteins relative to that of a reference integral membrane protein (Fig. 2, denoted by *heavy arrow*) in the whole cell extracts and the membrane preparations suggests essentially complete recovery of the expressed proteins in the membranes.

The membrane preparation enriched with the Tsr-AC fusion was compared with control membranes (pRE1) for AC activity (see "Experimental Procedures"). Although there was essentially no detectable activity in the control membranes, the specific activity of AC in the membranes containing Tsr-AC was ~4 nmol/min/mg of membrane protein. Scanning and quantitation of a gel with Tsr-AC-enriched membranes indicated that the protein corresponding to Tsr-AC accounts for about 10% of the total protein (see Fig. 4, *lane 6*); using those figures, the specific activity of AC in the membranes is 40 nmol/min/mg of Tsr-AC protein. With different membrane preparations, the AC activity has varied between 20 and 40 nmol/min/mg of AC. It is also noteworthy that the tethered form of AC is stable to storage. The AC activity of a membrane preparation stored at −80 °C for 3 years was unchanged. In contrast, soluble AC tends to aggregate on storage.

Binding of IIA^{Glc} to Membranes

A critical test of the properties of tethered AC was to determine whether the protein had the capability to interact with its putative regulator IIA^{Glc}. Membranes containing expressed Tsr-AC were compared with control membranes (Tsr-ACN) for interaction with purified IIA^{Glc} (Fig. 3). In one set of reactions, all the components necessary for glucose phosphorylation were included resulting in accumulation of the IIA^{Glc} in the dephospho-form (Fig. 3, reaction mixtures labeled *D*). In another set of reaction mixtures, P-enolpyruvate but not glucose was included, resulting in accumulation of the IIA^{Glc} in the phospho-form (Fig. 3, reaction mixtures labeled *P*). After removal of the unbound fraction, the membranes were extracted with urea to release the bound fraction. The data indicate that considerably more of both the dephospho- and phos-

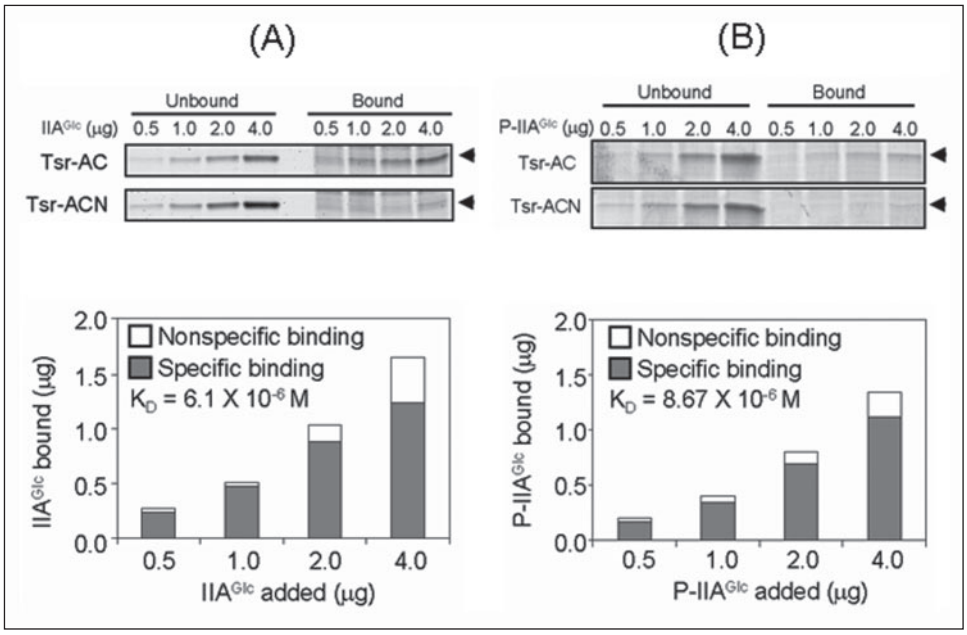

FIGURE 4. **Concentration-dependent binding of IIA^{Glc} to membranes enriched with Tsr-ACN or Tsr-AC.** Incubation mixtures were prepared and processed as described in the legend to Fig. 3. *A*, corresponding to conditions under which IIA^{Glc} is maintained in the dephospho-form, incubation mixtures (50 μl) were supplemented with glucose. *B*, corresponding to conditions under which IIA^{Glc} is maintained in the phospho-form, incubation mixtures (60 μl) were supplemented with EI, HPr, and P-enolpyruvate. Incubations contained the amounts of IIA^{Glc} indicated in the figure. The gels, after processing through SDS-PAGE, were stained with Coomassie Blue. Only the portions of the gels corresponding to the IIA^{Glc} region are shown in the *upper panels*. Calculation of binding constants is shown in the *lower panels*. SDS-polyacrylamide gels shown in the *upper panels* were scanned with a UMAX PowerLook III scanner (UMAX Technologies, Inc., Dallas, TX), and proteins were quantitated using a standard curve of IIA^{Glc} concentration as a reference. The amount of Tsr-AC in the reaction mixtures was calculated to be about 40 μg. The K_D value (see "Experimental Procedures") at each concentration of IIA^{Glc} was calculated based on the specific binding by subtracting the amount of IIA^{Glc} bound to Tsr-ACN from that bound to Tsr-AC. The calculated K_D values ($\times 10^{-6}$ M) for dephospho-IIA^{Glc} (*left panel*) were as follows: 0.5 μg, 5.492; 1 μg, 5.359; 2 μg, 5.1085; and 4 μg, 8.2917. Average value = 6.06 $\pm$ 1.49. The comparable values for P-IIA^{Glc} (*right panel*) were as follows: 0.5 μg, 8.7583; 1 μg, 8.4669; 2 μg, 7.759; and 4 μg, 9.7119. Average value = 8.67 $\pm$ 0.81.

pho-forms of IIA^{Glc} were bound to the Tsr-AC membranes than to the Tsr-ACN membranes. The level of IIA^{Glc} bound to the Tsr-ACN membranes was similar to that bound to the control pRE1 membranes (data not shown). Although higher concentrations of EI and HPr compared with IIA^{Glc} were included in the reaction mixtures, only the binding of IIA^{Glc}, regardless of its phosphorylation state, to Tsr-AC membranes was observed. Furthermore, the band intensities corresponding to IIA^{Glc} in the unbound fraction decreased in reaction mixtures supplemented with Tsr-AC compared with those with Tsr-ACN. These data clearly demonstrate that both phospho- and dephospho-IIA^{Glc} can specifically interact with the C-terminal domain of AC in the absence of other cytoplasmic proteins.

Because in wild-type *E. coli* the cellular concentration of AC is low (less than 20 molecules/cell) (22) and that of IIA^{Glc} is substantially higher ($\sim 10^{-5}$ M) (23), it is reasonable to assume that the physiological state of AC is as a complex with IIA^{Glc}. The state of phosphorylation of IIA^{Glc} in the complex should reflect that in the cytoplasm.

The concentration dependence for interaction of IIA^{Glc} and P-IIA^{Glc} with Tsr-AC-enriched and control (Tsr-ACN-enriched) membranes was studied (Fig. 4). Through the range of 0.5–4 μg of IIA^{Glc} in a 50–60-μl reaction volume, the interaction appeared to be saturable and specific. The calculated K_D value for IIA^{Glc} is 6.1×10^{-6} M and that for P-IIA^{Glc} is 8.67×10^{-6} M. The absence of significant specific binding to ACN indicates that the site of interaction of IIA^{Glc} with AC is at the C-terminal half of the protein, consistent with previous suggestions that the C-terminal part of the protein is the regulatory domain (20, 21). It is worth pointing out that this binding affinity is in the same range as that reported for other regulatory interactions involving IIA^{Glc}. The K_D value for the interaction of IIA^{Glc} with FrsA is 1.8×10^{-7} M (24), with lactose permease, 1 μM (10) and with glycerol kinase, 9 μM in the absence of zinc and 0.6 μM in the presence of zinc (25). Phosphotransfer interactions involving IIA^{Glc} with HPr (26) or IIB^{Glc} (27) are not as tight.

The binding data of Fig. 4 were used to calculate the stoichiometry of the protein-protein interaction (28); double-reciprocal plot analysis of the total amount of IIA^{Glc} specifically bound (bound to Tsr-AC minus bound to Tsr-ACN) against the total amount of IIA^{Glc} added minus the nonspecifically bound IIA^{Glc} (bound to Tsr-ACN) was carried out. The 1/*y* intercept is equivalent to the reciprocal of the total amount of IIA^{Glc} bound under saturating conditions. The molar stoichiometry was calculated from the molar amount of Tsr-AC present in the assay and the *y* intercept. According to this calculation, the stoichiometry was about 0.8 for dephospho- and about 0.9 for phospho-IIA^{Glc} per Tsr-AC, implying a 1:1 interaction.

Regulation of AC Activity by P-IIA^{Glc} Depends on Additional Factor(s)

Initial tests of the effect of added IIA^{Glc} in either phosphorylation state indicated that there was no effect on Tsr-AC activity, regardless of the molar ratio of IIA^{Glc} to Tsr-AC added to the reaction mixture (data not shown). These findings suggest that a model for regulation of AC by P-IIA^{Glc} alone is not correct and that there might be another factor(s) regulating activity of the IIA^{Glc}-AC complex, dependent on the phosphorylation state of IIA^{Glc}. Consequently, we embarked on a search for other regulatory factors. As part of this strategy, we tested the effect of adding a crude extract (CE) of *E. coli* to reaction mixtures for synthesis of cAMP by Tsr-AC or Tsr-ACN membranes (Fig. 5). Addition of ~500 μg of CE to either type of membrane preparation resulted in about 90% inhibition of AC activity. When we replaced the substrate ATP with cAMP to test its stability under the assay conditions used, we found that the amount of CE used led to ~90% loss of the added cAMP in the absence of any added membrane preparation. Thus, it is likely that the

apparent inhibition of AC activity by CE is because of the action of cAMP phosphodiesterase (29). It should be noted that 500 μg of CE itself did not show detectable AC activity regardless of the addition of either form of IIA^{Glc}, and furthermore, the cAMP hydrolyzing activity of CE was not affected by either form of IIA^{Glc} (data not shown).

A further series of experiments were carried out, searching for an additional factor(s) in CE that would promote a response to IIA^{Glc} (see Fig. 6). Incubation mixtures containing Tsr-ACN, Tsr-AC, or partially purified soluble AC, all supplemented with CE, were tested for the effect of added IIA^{Glc} or P-IIA^{Glc}. Although Tsr-ACN was insensitive to the addition of either form of IIA^{Glc}, the other enzyme forms behaved differently. Tsr-AC showed no response to IIA^{Glc} but was stimulated to more than 300% of the control value by P-IIA^{Glc}. A similar P-IIA^{Glc}-specific response was observed with soluble AC; in this case, the stimulation was to ~250% of the control value. The reasonable interpretation of these findings is that there is a factor(s) in CE that can interact with either the regulatory domain of AC or with IIA^{Glc} (or perhaps both proteins) that results in a stimulation of AC activity when IIA^{Glc} is in the phospho-form (see Fig. 7).

We considered and eliminated the possibility that that the activation of AC by P-IIA^{Glc} was accompanied by its dephosphorylation. It should be noted that P-IIA^{Glc} migrates slower on the SDS-PAGE than does IIA^{Glc} (see Fig. 3). Because incubation mixtures incubated with P-IIA^{Glc} under the conditions described for Fig. 6 show only that form of the protein recovered from the bound fraction, this system does not result in dephosphorylation of P-IIA^{Glc}.

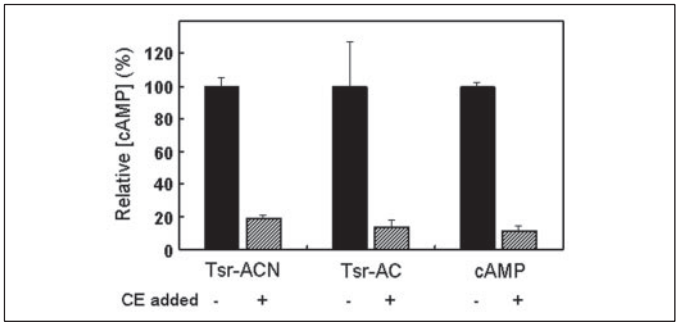

FIGURE 5. **An AC inhibitory factor in *E. coli* extracts.** AC activity assays (see "Experimental Procedures") were carried out for 60 min at room temperature, using either Tsr-ACN-enriched membranes or Tsr-AC-enriched membranes; the amount of both AC fusion proteins was estimated to be 0.2 μg. 100% activity for Tsr-ACN corresponds to 40 nmol/min/mg cAMP produced and that for Tsr-AC corresponds to 20 nmol/min/mg cAMP produced. The *error bars* denote the S.D. Each assay was carried out a minimum of three times. Where indicated, assays were supplemented with 500 μg of CE (see "Experimental Procedures"). In the section labeled cAMP, incubations were carried out with cAMP (400 fmol) instead of ATP.

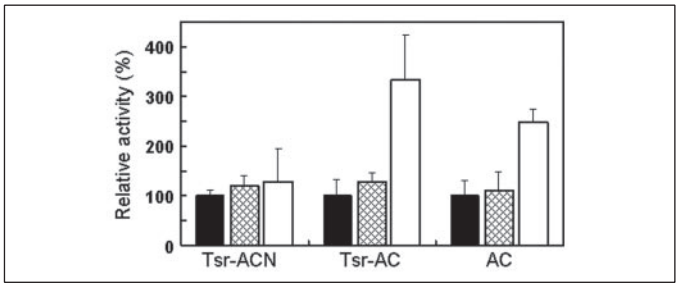

FIGURE 6. **Phospho-IIA^{Glc} activates AC in the presence of an *E. coli* extract.** AC activity assays (see "Experimental Procedures") were carried out for 60 min at room temperature, supplemented with P-enolpyruvate (2 mM), and EI (3 μg), using either Tsr-ACN-enriched membranes, Tsr-AC-enriched membranes, or partially purified soluble AC; the amount of AC was estimated to be 0.2 μg. CE was added at 500 μg of protein, and IIA^{Glc} at 20 μg. For phosphorylation of IIA^{Glc}, assays were supplemented with HPr (2 μg). 100% activity for Tsr-ACN corresponds to 8 nmol/min/mg cAMP produced and that for Tsr-AC corresponds to 3 nmol/min/mg cAMP produced. For soluble AC, 100% activity corresponds to 2 nmol/min/mg cAMP formed. The *error bars* denote the S.E. Each assay was carried out a minimum of three times. *Solid bars*, control activity; *cross-hatched bars*, dephospho-IIA^{Glc} added; *open bars*, P-IIA^{Glc} added.

A Model for AC Regulation

The data presented here allow us to formulate a model for the regulation of AC activity that is consistent with previous observations, including the phenotype of PTS mutants (6, 8, 19) (Fig. 7). The model proposes that the physiological form of AC is a complex of the regulatory C-terminal domain with RF and P-IIA^{Glc}, leading to a high activity form of the enzyme. As indicated in the figure, the IIA^{Glc}-dependent regulation is phosphorylation state-dependent. Thus, a mutant deficient in IIA^{Glc} would be expected to have low AC activity.

Concluding Remarks

This study describes a novel approach to the study of AC activity regulation and interaction with IIA^{Glc}. Noteworthy findings are that Tsr-AC is quite active and stable. In whole cells, tethered AC and cytoplasmic AC exhibit similar regulatory properties. Washed membrane preparations devoid of extraneous cytoplasmic factors are able to bind IIA^{Glc}; this is the first demonstration of a direct interaction of IIA^{Glc} with AC. AC activity in isolated membranes is not affected by either phospho- or dephospho-IIA^{Glc}. However, there is a factor(s) in *E. coli* extracts that interacts with AC, IIA^{Glc}, or the AC-IIA^{Glc} complex; the complex of three proteins (AC, IIA^{Glc}, and RF) renders AC subject to a phosphorylation state-dependent regulation. The results of this study, using tethered AC, provide proof that P-IIA^{Glc} directly interacts with and stimulates AC *in vivo*. The essential *in vivo* environment is accomplished by the addition of CE; a factor(s) in CE is essential for the stimulatory effect of P-IIA^{Glc}, and this is in keeping with previous speculations (8, 9) concerning the complexity of the catabolite repression mechanism. The identity of the nature of the factor(s) in *E. coli* extracts required for the regulation of AC activity is essential for a further understanding of the mechanism; attempts to accomplish this are under way.

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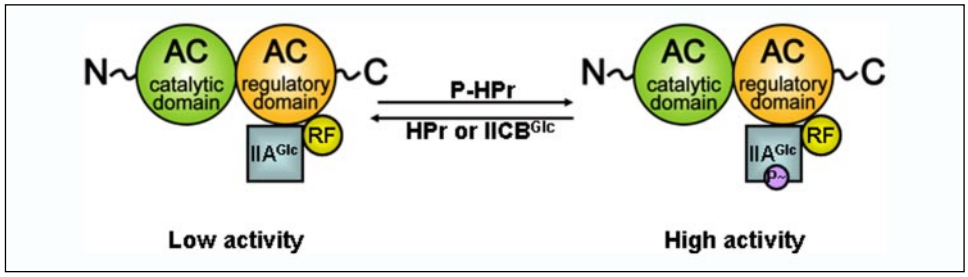

FIGURE 7. **A model for the regulation of AC activity.** The two globes represent the two-domain structure of AC; *RF* corresponds to the uncharacterized regulatory factor present in crude *E. coli* extracts. It is proposed that the physiological form of AC is composed of a complex including RF and IIA^{Glc} bound to the C-terminal domain of AC. Depending on the metabolic state of the cell, IIA^{Glc} exists in either the dephospho- or phospho-form. As indicated in the diagram, the state of phosphorylation of IIA^{Glc} determines the level of AC activity.

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