In Vitro Reconstitution of Catabolite Repression in Escherichia coli

A widely accepted model for catabolite repression posits that phospho-IIAGlc of the bacterial phosphotransferase system activates adenyl 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 IIAGlc specifically, regardless of its phosphorylation state, but not the two general phosphotransferase system proteins, enzyme I and HPr; IIAGlc 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-IIAGlc, but not IIAGlc, stimulates AC activity. Based on these findings of a direct interaction of IIAGlc 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 adenyl cyclase (AC), 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 IIAGlc of the PTS stimulates AC activity; thus, glucose transport is presumed to lead to dephosphorylation of IIAGlc 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 IIAGlc (3). It has been proposed that the CRP-cAMP complex promotes expression of a phosphatase that converts P-IIAGlc 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, is essential for high activity of AC as well as for the capability of the cells to be inhibited by glucose. Because P, was also shown to stimulate PTS activity in this system, P, 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-IIAGlc is insufficient to account for the regulation mechanism. Liberman et al. (6) demonstrated that unphosphorylated IIAGlc resulted in a P, dependent inhibition of AC in permeable cells. These data suggested that dephosphorylated IIAGlc 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 IIAGlc. The studies reported here demonstrate that Tsr-AC is active, that it interacts with both forms of IIAGlc, and that regulation by P-IIAGlc 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 IIAGlc, whereby the state of phosphorylation of IIAGlc determines the level of AC activity.
**TABLE 1**

| Strain or plasmid | Genotype or description | Source or Ref. |
|-------------------|-------------------------|----------------|
| **Strains**       |                         |                |
| GI698             | F’ λ lacIP lacI857 ampC ryB             | 30             |
| GI698Δpco         |                                        | 31             |
| XL1-Blue          | General cloning vector         | JM11003        |
| MG1655            | Wild-type E. coli K12          | 32             |
| DC646             | λ lysogen used for stable transformation of constructs encoding the Ω promoter | D. Court, NCI, National Institutes of Health, Frederick, MD |
| CA8306            | Δcya854                      | E. Brickman, Harvard University |
| CA8445            | Δcya854Δcrp45                 | E. Brickman, Harvard University |
| **Plasmids**      |                         |                |
| pREP1             | Expression vector under control of Ω promoter, Amp’ | 15 |
| pBluescript       | Expression vector encoding AC   | Stratagene     |
| pRI100            | Encodes AC under control of its own promoter | A. Danchin, Pasteur Institute, Paris, France |
| pDIA100           | Encodes AC                    | C. Manoil, University of Washington (33) |
| pLFG5             | Encodes Tsr, serine chemoreceptor | S. Kushner, University of Georgia (14) |
| pWSK29            | Low copy number plasmid       |                |
| pDIA (tsr-cya)    | Encodes Tsr-AC fusion         |                |
| pDIA(ts-cya)      | Encodes Tsr-AC fusion         | This study     |
| pL-P1663          | Encodes AC (low copy)         | This study     |
| pL-P1664          | Encodes Tsr-AC fusion (low copy) | This study |
| pPR100(ts-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 MgSO4, 0.1 mM CaCl2, 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. Trypsophan was purchased from Invitrogen. Enzyme I (EI), HPr, and IIAGlc 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 mL 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 IIAGlc (18,251 Da) was assumed to be between the monomeric forms of both proteins. As described in the text, various concentrations of IIAGlc 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 quantify 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-AC from that bound to Tsr-ACN. The formula $K_D = \frac{[\text{IIAGlc bound or free IIAGlc}]}{[\text{AC}] / [\text{IIAGlc AC}]}$ 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), MgCl2 (10 mM), ATP (1 mM), dithiothreitol (2 mM), Na2HPO4 (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(ts-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 ATG 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 AGG GG-3’) in one and a mixture of mutagenic primer 6135 (5’ - CAG AGT CAC AAT ATG GAG GAT CAT ATG GTA TCG CCT GAT TTG CTA CCC-3’) and primer 6143, downstream of the BamHI
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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 Ts 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 GCC 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-Sall 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(tsrs-cya) were moved into pWSK29 resulting in pLP1663 and pLP1664, respectively.

pPR100(tsrs-cya)—pPR100 is an expression vector for AC (15). The fragment encoding residues 1–259 of Ts 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(tsrs-cya)N—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 GTA TTA CCG GGC ATA CAG CTT-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 Ts 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 of 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.

Crude Extract—The crude extract (CE) was the source of RF. Strain MG1655 or GI698Δcpts 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(tsrs-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 (ΔcyA) or both AC and CRP (ΔcyA Δcrp). 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 Δcrp strains (2); because this increase in cAMP levels requires the presence of IIAluc (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 Δcrp strain.
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(ts)-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(ts)-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(ts)-cyaN (the expression vector for tethered ACN), and pPR100(ts)-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 diphospho-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 phosphory-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 diphospho- and...
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FIGURE 4. Concentration-dependent binding of IIAGlc 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 IIAGlc is maintained in the dephospho-form, incubation mixtures (50 μl) were supplemented with glucose. B, corresponding to conditions under which IIAGlc is maintained in the phospho-form, incubation mixtures (60 μl) were supplemented with E1, HPr, and P-enolpyruvate. Incubations contained the amounts of IIAGlc 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 IIAGlc 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 IIAGlc concentration as a reference. The amount of Tsr-AC in the reaction mixtures was calculated to be about 40 μg. The KD value (see “Experimental Procedures”) at each concentration of IIAGlc was calculated based on the specific binding by subtracting the amount of IIAGlc bound to Tsr-ACN from that bound to Tsr-AC. The calculated KD values (10^-6 M) for dephospho-IIAGlc (left panel) were as follows: 0.5 μg, 5.49; 1 μg, 5.359; 2 μg, 5.1085; and 4 μg, 8.2917. Average value = 6.06 ± 1.49. The comparable values for P-IIAGlc (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 ± 0.81.

Because in wild-type E. coli the cellular concentration of AC is low (less than 20 molecules/cell) (22) and that of IIAGlc is substantially higher (50–60-μM) (23), it is reasonable to assume that the physiological state of AC is as complex with IIAGlc. The state of phosphorylation of IIAGlc in the complex should reflect that in the cytoplasm.

The concentration dependence for interaction of IIAGlc and P-IIAGlc with Tsr-AC-enriched and control (Tsr-ACN-enriched) membranes was studied (Fig. 4). Through the range of 0.5–4 μg of IIAGlc in a 50–60-μl reaction volume, the interaction appeared to be saturable and specific. The calculated KD value for IIAGlc is 6.1 × 10^-6 M and that for P-IIAGlc is 8.67 × 10^-6 M. The absence of significant specific binding to ACN indicates that the site of interaction of IIAGlc 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 IIAGlc. The KD value for the interaction of IIAGlc 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 IIAGlc with HPr (26) or IIBGlc (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 IIAGlc specifically bound (bound to Tsr-AC minus bound to Tsr-ACN) against the total amount of IIAGlc added minus the nonspecifically bound IIAGlc (bound to Tsr-ACN) was carried out. The 1/y intercept is equivalent to the reciprocal of the total amount of IIAGlc 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-IIAGlc per Tsr-AC, implying a 1:1 interaction.

Regulation of AC Activity by P-IIAGlc Depends on Additional Factor(s)

Initial tests of the effect of added IIAGlc in either phosphorylation state indicated that there was no effect on Tsr-AC activity, regardless of the molar ratio of IIAGlc to Tsr-AC added to the reaction mixture (data not shown). These findings suggest that a model for regulation of AC by P-IIAGlc alone is not correct and that there might be another factor(s) regulating activity of the IIAGlc-AC complex, dependent on the phosphorylation state of IIAGlc. 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 IIAGlc, and furthermore, the cAMP hydrolyzing activity of CE was not affected by either form of IIAGlc (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 IIAGlc (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 IIAGlc or P-IIAGlc. Although Tsr-ACN was insensitive to the addition of either form of IIAGlc, the other enzyme forms behaved differently. Tsr-AC showed no response to IIAGlc but was stimulated to more than 30% of the control value by P-IIAGlc. A similar P-IIAGlc-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 IIAGlc (or perhaps both proteins) that results in a stimulation of AC activity when IIAGlc is in the phospho-form (see Fig. 7).

We considered and eliminated the possibility that the activation of AC by P-IIAGlc was accompanied by its dephosphorylation. It should be noted that P-IIAGlc migrates slower on the SDS-PAGE than does IIAGlc (see Fig. 3). Because incubation mixtures incubated with P-IIAGlc 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-IIAGlc.

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-IIAGlc, leading to a high activity form of the enzyme. As indicated in the figure, the IIAGlc-dependent regulation is phosphorylation state-dependent. Thus, a mutant deficient in IIAGlc 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 IIAGlc. 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 IIAGlc; this is the first demonstration of a direct interaction of IIAGlc with AC. AC activity in isolated membranes is not affected by either phospho- or dephospho-IIAGlc. However, there is a factor(s) in E. coli extracts that interacts with AC, IIAGlc, or the AC-IIAGlc complex; the complex of three proteins (AC, IIAGlc, and RF) renders AC subject to phosphorylation state-dependent regulation. The results of this study, using tethered AC, provide proof that P-IIAGlc 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-IIAGlc, 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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