Regulation of *Escherichia coli* adenylate cyclase activity during hexose phosphate transport

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*In Escherichia coli*, cAMP levels vary with the carbon source used in the culture medium. These levels are dependent on the cellular concentration of phosphorylated EnzymeIIA^AglC, a component of the glucose-phosphotransferase system, which activates adenylate cyclase (AC). When cells are grown on glucose 6-phosphate (Glc6P), the cAMP level is particularly low. In this study, we investigated the mechanism leading to the low cAMP level when Glc6P is used as the carbon source, i.e. the mechanism preventing the activation of AC by phosphorylated EnzymeIIA^AglC. Glc6P is transported via the Uhp system which is inducible by extracellular Glc6P. The Uhp system comprises a permease UhpT and three proteins UhpA, UhpB and UhpC which are necessary for *uhpT* gene transcription. Controlled expression of UhpT in the absence of the regulatory proteins (UhpA, UhpB and UhpC) allowed us to demonstrate that (i) the Uhp regulatory proteins do not prevent the activation of AC by direct interaction with EnzymeIIA^AglC and (ii) an increase in the amount of UhpT synthesized (corresponding to an increase in the amount of Glc6P transported) correlates with a decrease in the cAMP level. We present data indicating that Glc6P per se or its degradation is unlikely to be responsible for the low cAMP level. It is concluded that the level of cAMP in the cell is determined by the flux of Glc6P through UhpT.

**Keywords:** *Escherichia coli*, adenylate cyclase, cyclic AMP, phosphotransferase system, glucose 6-phosphate transport

INTRODUCTION

In *Escherichia coli*, intracellular cAMP concentration is dependent on the carbon source used in the culture medium (Epstein et al., 1975; Joseph et al., 1982). The lowest concentration is obtained when cells are grown on glucose or glucose 6-phosphate (Glc6P). A model has been proposed which explains the low level of cAMP observed when cells grow with glucose as the carbon source. This model involves the phosphotransferase system (PTS) which regulates adenylate cyclase (AC) (Saier & Feucht, 1975; Postma et al., 1993). EnzymeIIA^AglC, a glucose-specific component of the PTS is phosphorylated via a phosphorylation cascade including Enzymel and HPr, the general components of the PTS. When phosphorylated, EnzymeIIA^AglC activates AC. During glucose transport, the intracellular concentration of phosphorylated EnzymeIIA^AglC decreases causing a decrease in intracellular cAMP concentration (Feucht & Saier, 1980; Postma et al., 1981). This model is supported by the fact that total cAMP in ΔacrR strains (lacking EnzymeIIA^AglC) is low and close to that of a wild-type strain grown on glucose. However, the mechanism leading to the activation of AC by EnzymeIIA^AglC remains to be explained.

Glc6P is transported into the cell via the Uhp system which is inducible by extracellular Glc6P (Dietz & Heppel, 1971a). The Uhp system consists of a permease UhpT (encoded by *uhpT*) which is transcriptionally regulated by three regulatory proteins, UhpA, UhpB and UhpC (encoded by *uhpA*, *uhpB* and *uhpC*, respectively) (Weston & Kadner, 1987). In the presence of external Glc6P, the membrane proteins UhpB and UhpC probably phosphorylate UhpA, promoting the transcription of the *uhpT* gene. UhpA and UhpB belong to the family of two
component regulatory systems (Weston & Kadner, 1988) and UhpC shows sequence similarity with UhpT (Island et al., 1992). It has been suggested that UhpB and UhpC interact during induction (Island & Kadner, 1993). Glc6P does not inhibit AC activity when measured in toluenized cells (Harwood & Peterkosky, 1975) or with purified AC (Yang & Epstein, 1983). The mechanism leading to a low level of cAMP when Glc6P is used as the carbon source is not yet understood. Expression of glucose-PTS proteins is not diminished in strains grown on Glc6P as shown by fusion experiments (De Reuse & Danchin, 1988), which indicates that the low cAMP level is not related to a low level of expression of EnzymeIIA^EIE. In this study, we analyse the effect of various hexose phosphates on cAMP production. This level is shown to be dependent on the presence of phosphorylated EnzymeIIA^EIE. We then investigate the activity of phosphorylated EnzymeIIA^EIE during growth on Glc6P. We show that activation of AC by phosphorylated EnzymeIIA^EIE is prevented when Glc6P is transported into the cell.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this work are listed in Table 1. The growth medium was either LB or M63 minimal medium (Miller, 1992) supplemented with the required amino acids (1 mM), thiamine (5 µg ml^{-1}) and a carbon source (0.4%). When required, ampicillin and tetra-
cycle were added at 100 μg ml⁻¹ and 10 μg ml⁻¹, respectively. P1 transductions were done as described by Miller (1992). Uhp-
constitutive strains were isolated on M63 plates supplemented
with fructose-1-phosphate (Fru1P) (Ferenci et al., 1971). Strains
lacking phosphoglucoisomerase (pgi) produced pink colonies
on glucose MacConkey plates and showed slow growth on
glucose M63 plates. Strain TP9535 lacking Glc6P dehydro-
genase (zwf) was checked for the lack of Glc6P dehydrogenase
activity according to the method of Fraenkel & Leissohn
(1967). Strain TP9525 which contains a deletion of the uhp
genes (AuhpABCT) was obtained as follows. The Tn10
transposon of strain TP9524 (which contains a deletion in the
uhpT gene, Table 1) was removed from the chromosome according
to the method of Bochnet et al. (1980) as modified by Maloy & Nunn
(1981). Tet' clones were analysed by PCR for the presence of the
uhpC gene. One of the clones showing a deletion of the uhpC
gene was further analysed by Southern blot hybridization
(Southern, 1975).

**Determination of total cAMP concentrations.** In *E. coli*, more
than 99% of the cAMP synthesized is excreted in the culture
medium (Matin & Matin, 1982). In addition, under our
experimental conditions, the excretion rate of cAMP is a linear
function of the intracellular concentration (Epstein et al., 1975;
Crasnier et al., 1992). For these reasons, total cAMP was
measured. A radioimmunological assay was performed as
described by Guidi-Rontani et al. (1981) with exponential phase
cells grown in M63. The amount of cAMP was expressed as
pmol (mg dry wt bacteria)⁻¹. Total cAMP (mg dry wt bacteria)⁻¹
was constant during the exponential phase of growth. Values
were calculated as the means of three determinations.

**Measurements of cAMP synthesis.** In *vivo* measurements of
cAMP synthesis were performed as described by Harman &
Botsford (1979). Bacteria were grown in M63 to mid-
exponential phase. Cells were centrifuged and resuspended in
fresh M63 medium supplemented with 0.1% casamino-acids
and chloramphenicol at 100 μg ml⁻¹. The cell suspension was
incubated at 37 °C, samples were taken at zero time and after 5, 10,
20 and 30 min. Total cAMP was determined as described above
and rates of cAMP synthesis were calculated from the slopes of
plots.

**Cloning of the uhpT gene.** A promoterless uhpT gene was
obtained by PCR using chromosomal DNA from strain TP2503.
The primers were chosen according to the sequence of the
uhpT gene (Island et al., 1992). The upstream primer (CCATCGA-
TTTACATAATGCATGCCTACGC) was located 41 nucleotides
upstream of the ribosome-binding site. The downstream primer
(CGGAATTCGTTTATGCCACTGTCAACTG) covered the
translation termination codon of uhpT. The amplified 1.5 kb
fragment was digested with CiaI and EcoRI (whose restriction
sites were introduced in the primers) and cloned into pBR322 to
obtain pDIA187. This plasmid placed the
control of the anti-tet promoter. Ap' transformants of strain
TP9525 which contains a deletion of the uhpT gene (AhupABCT)
was obtained as follows. The Tn10 transposon of strain TP9524
(which contains a deletion in the uhpT gene, Table 1) was removed
from the chromosome according to the method of Bochnet et al.
(1980) as modified by Maloy & Nunn (1981). Tet' clones were
analysed by PCR for the presence of the
uhpC gene. One of the clones showing a deletion of the uhpC
gene was further analysed by Southern blot hybridization
(Southern, 1975).

**RESULTS**

**Effect of hexose phosphates on cAMP levels**

Glc6P is transported into the cell by the UhpT permease
which also mediates the entry of a large variety of hexose
phosphates (Winkler, 1966). We investigated cAMP
production by the wild-type strain TP2503 growing with
different hexose phosphates. With Glc6P, total cAMP was
threefold lower than that obtained with pyruvate and
twofold lower than that obtained with glucose (Table 2),
in agreement with previous data (Epstein et al., 1975).

Growth on Fru6P or mannose 6-phosphate (Man6P)
resulted in an increased level of cAMP as compared to
that obtained with Glc6P, although strain TP2503 grew on
Fru6P, Man6P or Glc6P with the same doubling time.

However Fru6P and Man6P, which are transported into
the cell by UhpT, have been shown to be poor inducers
of the Uhp system. This prompted us to analyse the effect
of the hexose phosphates on cAMP production in a Uhp-
constitutive derivative of strain TP2503. The uhpA mu-

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**Fig. 1.** Plasmid pDIA188. Plasmid pDIA188 carries the uhpT
gene under the control of the tac promoter.
Table 2. cAMP levels in strains grown on different hexose phosphates

Values are means of three determinations ± SEM. ND, not determined; NG, no growth.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Total cAMP [pmol (mg dry wt)^-1]</th>
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<tbody>
<tr>
<td></td>
<td>TP2503 (wild-type)</td>
</tr>
<tr>
<td>Glucose</td>
<td>300 ± 100</td>
</tr>
<tr>
<td>Glc6P</td>
<td>150 ± 20</td>
</tr>
<tr>
<td>Fru6P</td>
<td>800 ± 150</td>
</tr>
<tr>
<td>Man6P</td>
<td>1000 ± 200</td>
</tr>
<tr>
<td>G1c1P</td>
<td>3200 ± 1000</td>
</tr>
<tr>
<td>Fru1P</td>
<td>NG</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1000 ± 200</td>
</tr>
</tbody>
</table>

Effect of Glc6P metabolism on cAMP levels

A zwf^ strain lacking Glc6P dehydrogenase (TP9535, Table 1) showed a low level of cAMP when grown on Glc6P [220 pmol (mg dry wt)^-1]. As a pg^ strain lacking phosphoglucone isomerase (TP9532, Table 1) which, as previously reported (Fraenkel & Levisohn, 1967), has increased doubling time when grown on Glc6P as compared to wild-type strains, also has a relatively low cAMP level [400 pmol (mg dry wt)^-1]. These relatively low cAMP levels in cells grown on Glc6P might be related to the concentration of Glc6P in the cell. Therefore, we measured the cAMP in a pg^ strain grown on Fru6P. In this strain the reversible transformation of Fru6P into Glc6P does not occur. Strains TP9529 (uhp^ pg^) and TP9532 (uhp^ pg^) grown on Fru6P showed the same growth rate and had similar low quantities of cAMP, i.e. 200 pmol (mg dry wt)^-1 with TP9532 versus 150 pmol (mg dry wt)^-1 with TP9529. These results suggest that Glc6P per se is not causing a decrease in the cAMP level. Addition of 0.1% deoxyglucose 6-phosphate (a non-metabolizable analogue of Glc6P) to cells of strain TP9529 (uhp^) growing on pyruvate decreased the cAMP level from 1000 to 300 pmol (mg dry wt)^-1. In these conditions, the addition of deoxyglucose 6-phosphate did not affect growth of strain TP9529.

Controlled expression of UhpT

To further analyse the role of UhpT in the regulation of the cAMP level, a plasmid was constructed containing the hexose phosphate permease gene (uhpT) under the control of the tac promoter (pDIA188, Fig. 1). Due to the presence of the lacP repressor gene on the plasmid, the expression of uhpT was dependent on the amount of IPTG in the culture medium. A strain containing a deletion of the entire uhp operon was constructed as described in Methods (TP9525, Table 1). This deletion was introduced into strain TP2100 (a lac derivative of TP2503). The resulting strain TP9527 (ΔuhpABCT Δlac) transformed with pDIA188 (Fig. 1) was grown in Glc6P M63 with different concentrations of IPTG. The cAMP levels and the doubling times of growth as a function of
the IPTG concentration are shown in Fig. 2. In the absence of IPTG, strain TP9527(pDIA188) grew with a doubling time of 3 h and produced an elevated amount of cAMP [about 3000 to 4000 pmol (mg dry wt)\(^{-1}\), Fig. 2a]. Increasing the IPTG concentration from 0 to 20 \(\mu M\) resulted in a decrease of the doubling time which correlated with a decrease of the cAMP level (Fig. 2a). The doubling time and the cAMP level of the wild-type strain were obtained when an IPTG concentration of 20 \(\mu M\) IPTG was used. Above 20 \(\mu M\) IPTG, the growth stopped after two doubling times (see Discussion). With glucose instead of Glc6P, there was no variation in the doubling time or the cAMP levels as a function of the IPTG concentration, even above 20 \(\mu M\) IPTG.

With TP9527(pDIA488) grown on Fru1P, 100 \(\mu M\) IPTG was required to obtain a growth rate and a cAMP level comparable to those of the \(\Delta hbp\) strain TP9529 grown on Glc6P (Fig. 2b).

**Activity of EnzymeIIA\(^{\text{Ac}}\) during hexose phosphate transport**

AC activity measured in resting cell suspensions incubated with casamino-acids (Methods) was not detectable in a \(\Delta crr\) strain (TP9503) grown on Glu6P while, in a wild-type strain (TP2503) grown on Glc6P or glucose, activity was 130 pmol cAMP min\(^{-1}\) (mg dry wt\(^{-1}\)) (Fig. 3). These data, and the fact that \(\beta\)-galactosidase activities with a \(cya\–lacZ\) protein fusion [encoded by pDIA483 (Crasnier et al., 1994)] were similar in strains grown on glucose or Glc6P (data not shown), suggest that the amounts of AC and phosphorylated EnzymeIIA\(^{\text{Ac}}\) available for AC activation are the same in resting cell suspensions obtained from cells grown either on glucose or on Glc6P. Addition of Glc (which is known to cause EnzymeIIA\(^{\text{Ac}}\) dephosphorylation) or Glc6P in the assay medium resulted in inhibition of activity (Fig. 3).

The ability of Glc6P to cause inducer exclusion through dephosphorylation of EnzymeIIA\(^{\text{Ac}}\) was analysed on M63 plates supplemented with Glc6P (see Methods). In contrast to that which was observed with glucose, both wild-type strain FB8 and FB8\(\Delta pts\) exhibited a blue phenotype on these plates, i.e. no inducer exclusion as in the wild-type strain, Glc6P does not act on AC via dephosphorylation of EnzymeIIA\(^{\text{Ac}}\). As a control, inducer exclusion was observed (white phenotype) with strain FB8\(\Delta pts\) transformed with plasmid pDIA4705 which carries the crr gene.
Effect of hexose phosphates on PTS-sugar transport

Glc6P has been shown to inhibit PTS-mediated transport (Kornberg, 1973; Lengeler & Steinberger, 1978). We analysed the effect of Glc6P on the uptake of α-[methyl-14C]glucoside, a non-metabolizable analogue of glucose. As reported by Lengeler & Steinberger (1978), an inhibitory effect of Glc6P on the uptake of α-[methyl-14C]glucoside was observed only when the Uhp system was induced. The ubpC strain TP9529 exhibited the same inhibition of α-methylglucoside uptake in the presence of Glc6P. Addition of Frub6P, Man6P or deoxyglucose 6-phosphate also resulted in an inhibition of α-[methyl-14C]glucoside transport in strain TP9529 (data not shown), indicating that Glc6P was not the only sugar phosphate that could cause the decreased α-[methyl-14C]glucoside transport rate. The same experiments were carried out with strain TP9532, a pgz− derivative of strain TP9529 (in this strain, Glc6P cannot be synthesized from Fru6P). The inhibitory effect of Frub6P on α-methylglucoside transport is shown in Fig. 4. The same inhibition was observed with both pgz+ and pgz− strains, suggesting that Glc6P per se was not responsible for the decreased α-[methyl-14C]glucoside transport rate.

We then investigated the effect of Glc6P transport on [14C]mannose uptake in a Δrr strain (lacking EnzymeIIAglc). A total deletion of the srr gene was transduced into the ubpC strain TP9529. The resulting strain TP9533 was able to transport mannose through its specific PTS. As shown in Fig. 5, mannose uptake was also inhibited by the addition of 1 mM Glc6P. This result indicated that Glc6P transport inhibited the PTS-dependent transport of mannose in the absence of EnzymeIIAglc.

DISCUSSION

Strains growing in the presence of Glc6P as sole carbon source show low levels of cAMP but the regulatory mechanism leading to the low level of cAMP is not known. We have shown here that Glc6P degradation is not directly involved in the regulation of the cAMP level. Transport of a non-metabolizable analogue of Glc6P (deoxyglucose 6-phosphate) decreased the cAMP level when added to cells growing on pyruvate cells. Strains lacking either Glc6P dehydrogenase (7wJ) or phospho-glucose isomerase (pgi) had a low level of cAMP when grown on Glc6P, close to that of a wild-type strain grown on Glc6P. In addition, Glc6P per se was not involved in the regulation of the cAMP level as apgz− strain showed low cAMP levels when grown in the presence of Fru6P. The fact that the cAMP level in a pgiz− strain grown on Glc6P was increased as compared to the same strain grown on Fru6P [400 versus 200 pmol (mg dry wt)−1] was most likely due to the slow growth observed with Glc6P as compared to Fru6P.

We also showed that a low level of cAMP was obtained only when UhpT, the Glc6P permease, is induced and Glc6P (or a UhpT substrate) transported as shown by the following results: (1) in the absence of Glc6P transport, a Uhp-constitutive strain grown on pyruvate showed a high level of cAMP (Table 2); (2) different substrates of the UhpT permease were able to decrease the cAMP level provided that the UhpT permease was expressed; and (3) increasing the amount of UhpT synthesized, by using a vector carrying the ubpT gene under the tac promoter (IPTG-inducible), led to a decrease in the cAMP level.
when the hexose phosphates transported by UhpT were added. In the absence of IPTG, slow growth was observed and an elevated amount of cAMP was measured as compared to a wild-type strain grown on Glc6P. In agreement with this data, Yang et al. (1979) reported that Glc6P-limited growth in a chemostat also resulted in a high cAMP level. With Glc6P, both growth rate and cAMP levels characteristic of a wild-type strain were obtained for 20 μM IPTG. Fürste et al. (1986) also showed that, with a similar expression vector, induction by IPTG is detectable in the range 10–100 μM. Above 20 μM IPTG, the growth stopped, as demonstrated previously by Kadner et al. (1992), showing that amplified UhpT transport inhibits growth. With Fru1P, the same response to IPTG was observed with a shift in the curve as compared to that obtained with Glc6P (five times more IPTG required to obtain both the same growth rate and cAMP level). In addition, the cAMP level of the ubpΔ strain grown on Fru1P was slightly increased as compared to that of the same strain grown on the other hexose phosphates (Table 2). These data could be related to the low affinity of Fru1P for UhpT as compared to that of Glc6P (Pogell et al., 1966; Maloney et al., 1990). Taken together, these results suggest that UhpA, UhpB and UhpC did not play any role in decreasing the cAMP level in cells growing in the presence of hexose phosphates, although, in wild-type cells, these proteins may have an indirect effect on the cAMP level by regulating the synthesis of UhpT. Thus the level of cAMP is dependent on the amount of Glc6P (or any other hexose phosphates) transported by the UhpT permease.

We propose that the regulation of the cAMP level when Glc6P was used as carbon source occurred during Glc6P transport. The UhpT transport system has been proposed to function in two modes (Maloney et al., 1990). In one mode, two Glc6P- anions move into the cell in exchange for the export of one Glc6P2-. This exchange is equivalent to the net entry of one Glc6P2- and two H+ and is thermodynamically equivalent to a 2H+:Glc6P2- symport with ApH acting as the sole driving force. It has been reported that a collapse of the proton electrochemical gradient leads to a decrease of AC activity (Peterkofsky & Gazdar, 1979). However, under our experimental conditions, a collapse of the gradient by transport via UhpT is unlikely to occur as proton pumping under aerobic steady-state conditions is too rapid for a collapse of the gradient to take place. The exchange system by UhpT can also operate by moving two H2PO4- anions out and one Glc6P2- in. Although it has been shown that phosphate is necessary for the activation of the AC–PTS complex (Peterkofsky, 1988), it is unlikely that Glc6P transport leads to a depletion of the phosphate pool which is tightly maintained in E. coli. However, the possibility remains that local phosphate concentrations at the membrane level are decreased during Glu6P transport, affecting the activation of AC by EnzymeIICβ.

Both functions of the PTS, i.e. glucose transport and activation of AC, are inhibited during Glc6P transport. To explain the inhibitory effect of Glc6P on PTS transport, it has been proposed that the PTS permeases, EnzymeIICβΔ or EnzymeIICβut, were inhibited by intracellular hexose phosphates (Lengeler & Steinberger, 1978). However, attempts to reproduce the inhibitory effect of Glc6P on the PTS permeases in vitro have failed (Jacobson et al., 1983). In strain TP2504 (lacking EnzymeIICβΔ) grown on Glc6P, a low cAMP level was measured [200 pmol (mg dry wt)-1], indicating that transport of Glc6P remained effective in maintaining a low cAMP level in the absence of the glucose permease. Therefore an inhibition of EnzymeIICβΔ by hexose phosphate (which could have affected EnzymeIICΔ regulation of AC) could not be related to the low cAMP level. Finally, the fact that EnzymeIICΔ was present in strains grown on Glc6P (as shown by measurement of AC activity) and was probably phosphorylated (as shown by the inducer exclusion experiment) suggested that AC cannot be activated by phosphorylated EnzymeIICΔ during Glc6P transport. Thus it is worth noting that the mechanism of 'catabolite repression' by hexose phosphate may be different from the one occurring with glucose. It must be emphasized that regulation of AC activity during Glc6P transport probably occurs at the membrane level. This is supported by data indicating that the soluble enzymes of the PTS exist as peripheral membrane constituents associated with the integral membrane Enzyme II complex (Saier et al., 1982) or with data showing a localization of Enzyme I to the inner surface of the cytoplasmic membrane (Ghosh et al., 1989).

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