POST-TRANSLATIONAL AND TRANSCRIPTIONAL REGULATION OF POLYAMINE BIOSYNTHESIS IN ESCHERICHIA COLI

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Abstract—1. Ornithine and arginine decarboxylases (ODC and ADC) of Escherichia coli are inhibited post-translationally by antizyme and ribosomal proteins S20 and L34.
2. The inhibition of either enzyme is relieved when excess of the other decarboxylase is added.
3. Using this approach, in vitro as well as in vivo, we demonstrate that the extent of the post-translational inhibition of ODC and ADC in E. coli is at least 65 and 50%, respectively.
4. The inhibited enzyme levels increase even further upon exposure of cells to polyamines.
5. The post-translational mode of regulation can counteract a 4-fold increase of ODC protein in the cell.
6. The negative transcriptional regulation of ODC and ADC expression by polyamines is mediated by transcription factors and not by direct polyamine effects on the promoters of their genes.
7. Three proteins interacting with the ODC promoter region were found by southwestern blot analysis.

INTRODUCTION

In E. coli, putrescine can be synthesized by two pathways; through the direct decarboxylation of ornithine to putrescine by ornithine decarboxylase (ODC; L-ornithine carboxylate-lyase, EC 4.1.1.17) and through the decarboxylation of arginine to agmatine by arginine decarboxylase (ADC; L-arginine carboxylate-lyase, EC 4.1.1.19), followed by the hydrolysis of the guanidino group of agmatine by agmatine ureohydrolase (AUH; agmatinase; EC 3.5.3.7). There are two sets of ornithine and arginine decarboxylases, the biosynthetic and the biodegradative (Morris and Pardee, 1966). The latter are induced by low pH and by the presence of the respective amino acid in the growth medium, in contrast with the former, which are constitutively expressed (for review, see Tabor and Tabor, 1985).

The activity of the biosynthetic ornithine decarboxylase is modulated by a number of positive and negative effectors. These include low molecular weight compounds like GTP, which activates ODC (Höltta et al., 1972), and guanosine 5'-diphosphate-3'-diphosphate, which inhibits its activity (Höltta et al., 1974). ODC activity is also inhibited by polyamines, either directly by negative feedback (Morris et al., 1970), or indirectly, by polyamine-inducible protein inhibitors that have been named antizymes (Canellakis et al., 1979; Kyriakidis et al., 1978). The latter mechanism of regulation for ODC activity also has been identified in mammalian (Fong et al., 1976; Fujita et al., 1984; Kitani and Fujisawa, 1984) and plant cells (Kyriakidis, 1983; Panagiotidis and Kyriakidis, 1985). Three such proteins have been identified in E. coli. These include antizyme, which was originally recognized as an inhibitor of ornithine decarboxylase (Kyriakidis et al., 1978), and two basic inhibitory proteins with antizyme-like properties (Heller et al., 1983) that were identified as being the ribosomal proteins S20 and L34 (Panagiotidis and Canellakis,
The antizyme gene was recently cloned and sequenced (Canellakis et al., 1993). Polyamines also negatively modulate ODC and ADC expression at the transcriptional level (Huang et al., 1990). Additionally, there are reports that ODC, as well as the enzymes of the second polyamine biosynthetic pathway (ADC and AUH), are negatively controlled by cyclic-AMP at the transcriptional level (Moore and Boyle, 1991; Satishchandran and Boyle, 1984; Wright and Boyle, 1982; Wright et al., 1986).

In the present study we attempt to resolve two issues: (i) whether post-translational inhibition of polyamine biosynthetic enzymes has in vivo relevance or that the isolated inhibitors represent artifacts of the in vitro assay. This was approached by determining whether inhibited forms of ODC and ADC exist in cells and whether polyamines further increase the fractional amounts of the cellular enzymes that are inhibited. Furthermore, we attempted to probe the “tightness” of the post-translational mode of regulation. (ii) Whether polyamines exert their negative transcriptional effects directly, e.g. by altering the conformation of promoter DNA, or indirectly, through negative transcription factors.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and culture conditions**

The *E. coli* K12 strain MG1655 (λ−, F−) (Guyer et al., 1981), its speA, B speC derivative KL527 (Panagiotidou et al., 1987) and *E. coli* HB101 (F−, hsdM recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, met-1, supE44, λ−) (Boyer and Rouland-Dussoix, 1969) were used throughout this work. Plasmids pODC-1 (a pBR322-based plasmid containing the ODC structural gene) (Boyle et al., 1984), pCOD (containing the promoter of the ODC gene and approx. 30% of the coding region, fused to a promoterless tetracycline resistance gene) (Wright et al., 1986) and pKAS (ADC, AUH, metK genes) (Boyle et al., 1984), were kindly provided by Dr S. M. Boyle, Virginia Tech. The 4.9 kb Bal I-EcoR I fragment of plasmid pKAS, containing the ADC and metK genes (Boyle et al., 1984), was subcloned in the Hinc II-EcoR I site of plasmid pGEM-4 to yield plasmid pHPC-1 (Huang et al., 1990).

Cells were grown either in minimal medium 56 (M56) (5.3 g KH₂PO₄, 8.7 g Na₂HPO₄, 0.2 g MgSO₄, 2 g (NH₄)₂SO₄, 0.1 g Ca(NO₃)₂ and 0.05 g FeSO₄ per l.) (Monod et al., 1951) supplemented with 0.4% glucose and 1 μg/ml thiamine, or in LB Broth (Miller, 1972) at 37°C under vigorous shaking.

**Preparation of cell extracts and enzyme assays**

Cultures were rapidly chilled by pouring over crushed ice and the cells were collected by centrifugation at 8000 g for 10 min. The cell pellets were washed twice with ice-cold saline, suspended in 5 ml ice-cold ODC assay buffer (50 mM Tris-HCl pH 8.2, 0.1 mM EDTA, 50 μM pyridoxal 5′-phosphate, 1 mM dithiothreitol) and disrupted by sonication in an ice bath (3 times, 1 min each). The 15,000 g supernatant fluids of the cell lysates were used for all subsequent experiments.

We had previously noted large variations during the determination of the in vitro activity of ODC in crude *E. coli* extracts, probably due to its sensitivity to the different amounts of activating and inhibitor nucleotides that are present in *E. coli* extracts (Höltta et al., 1972; Höltta et al., 1974). Most of this variability can be prevented if the ODC assays are performed in the presence of 1 mM GTP, a concentration that gives maximal activation of ODC and provides a uniform environment. With this modification, ODC activities were measured in vitro, as previously described (Kyriakidis et al., 1978). ADC activity was measured as described (Morris and Boeker, 1983).

When the reactivation of acidic antizyme-, S20-, or L34-inhibited ODC or ADC by ADC and ODC, respectively, was assayed, increasing amounts of purified competitor enzyme were added to an enzyme-inhibitor mixture, and the extracts were preincubated at 4°C for 2 hr before initiating the enzymatic reaction by addition of the substrate. The length of each reaction was 30 min at 37°C. Control experiments showed that purified ODC did not decarboxylate arginine nor did ADC decarboxylate ornithine under the conditions of the experiment. Furthermore, the addition of increasing amounts of the one decarboxylase had no effect on the activity of the other when no inhibitor had been added.

Reactivation of ODC and ADC activities from crude cell extracts was performed essentially as described above, with the difference that increasing amounts of ADC and ODC, respectively, were added to the 15,000 g supernates of cell lysates.

Protein concentrations were determined by the method of Bradford (Bradford, 1976).
Purification of ODC, ADC, acidic antizyme, S20 and L34

ODC and ADC proteins were purified from apparent homogeneity from the ODC or ADC overproducing E. coli, strains MG1655/pODC and HB101/pKA5, respectively. In the ODC purification protocol, which was similar to that previously described (Applebaum et al., 1977), the steps included streptomycin precipitation of the nucleic acids, 39–51% ammonium sulfate fractionation, heat treatment at 62°C for 10 min, and chromatography on phenyl-Sepharose, hydroxyapatite, Sephacryl S-300 and finally, on DEAE-Biogel A. ADC was purified following a protocol essentially identical to that described (Wu and Morris, 1973). Antizyme and the ribosomal proteins S20 and L34 were purified as previously described (Kyriakidis et al., 1983). The purity of the individual preparations was determined by silver staining (Morissey, 1981) after electrophoretic analysis of 1–5 μg purified protein on denaturing polyacrylamide gels (Laemmli, 1970).

Antibody production and purification

Antibodies to the purified ODC and ADC proteins were prepared by injecting 8-week old female New Zealand White rabbits subcutaneously with 150 μg of pure enzyme protein in complete Freund's adjuvant. 100 μg boosts were given subcutaneously in incomplete Freund's adjuvant at weeks 4, 6 and 9, and the rabbits were bled from the ear vein at weeks 8 and 11.

The IgGs were partially purified by 50% ammonium sulfate precipitation and chromatography on Whatman DE-52 columns equilibrated with 15 mM sodium phosphate pH 6.8, 1 mM EDTA, 3 mM β-mercaptoethanol and 10% glycerol. The flow through fractions of the DE-52 columns, containing the IgGs, were concentrated by using Amicon YM10 filters and stored at −20°C. The anti-ODC IgGs were further purified by affinity chromatography on an ODC-Sepharose column.

Electrophoresis and immunoblotting

SDS–polyacrylamide slab gel electrophoresis was performed essentially as described by Laemmli (Laemmli, 1970) on polyacrylamide minigels. The transfer of the proteins from the polyacrylamide gels to nitrocellulose membranes (BA-85, Schleicher and Schuell) was performed as described (Towbin et al., 1979). The membranes were quenched with PBSTA (4% BSA, 0.1% Tween 20 in PBS) either overnight at 4°C or for 2 hr at 37°C, and they were subsequently incubated with an appropriate dilution of antibody in PBSTA for 2 hr at 37°C. After the filters were washed 3 times with 0.1% Tween 20 in PBS they were incubated either with peroxidase-conjugated goat anti-rabbit IgG antibodies (Jackson Immunoresearch Labs) diluted 1/2000 in PBSTA or with 0.1 μCi/ml 125I-protein A (30 μCi/mg, ICN) in PBSTA.

The X-ray films or the negatives of the photographic prints of the western immunoblots were scanned with a Zeineh soft laser scanning densitometer (model SL-TRF) connected to an Apple IIe computer and analyzed using a program created by Mr Robert Dreyer of this department. Standard curves prepared with known amounts of protein provided a linear correlation between the densitometer measurement and the amount of protein in a given band. The concentrations of the ODC and ADC proteins were quantitated by comparing the intensity of the immunostained bands with the intensity given by a series of graded amounts of the respective purified proteins within the same gel.

Southwestern blot analysis of the E. coli proteins was performed as described (Miskimins et al., 1985), after electrophoresis of total cell proteins on denaturing 8–18% gradient polyacrylamide gels. The probe was an end-labelled PsI-Hind III fragment of the ODC gene, carrying the ODC gene promoter.

RNA preparation and Northern blots

Total E. coli mRNA was isolated from cultures grown in M56 to an A600 of 0.5. The cells were chilled rapidly by pouring the cultures over an equal volume of crushed frozen M56 containing 50 mM sodium azide and shaking for 30 sec, in a flask that was immersed in a dry-ice-ethanol bath. The cells were immediately harvested by centrifugation at 8000 g for 5 min at 4°C, the pellet was suspended in 50 mM Tris–HCl pH 8.0 and the cells were lysed by adding the suspension to an equal volume of boiling SDS buffer (Zengel et al., 1980). The mixture was phenol extracted, ethanol precipitated and the pellet was then dissolved in 4 M guanidinium thiocyanate and centrifuged through a cushion of CsCl to free RNA from contaminating DNA (Huang et al., 1990). After the RNA pellet was dissolved in 10 mM Tris–HCl pH 8.0, 1 mM EDTA, the concentration was determined spectrophotometrically.
and equal amounts were analyzed by denaturing electrophoresis on agarose gels as described (Maniatis et al., 1989). Following transfer of the RNAs from the gels to nitrocellulose filters, the filters were hybridized with DNA or RNA probes specific for ODC or ADC as described (Huang et al., 1990).

RESULTS

Model system for the reactivation of antizyme-, S20-, or L34-inhibited ODC and ADC by ADC and ODC, respectively

We have previously shown that both ODC and ADC are inhibited by the same group of E. coli proteins that includes antizyme and the ribosomal proteins S20 and L34 (Heller et al., 1983; Kyriakidis et al., 1978; Kyriakidis et al., 1983; Panagiotidis and Canellakis, 1984). We have developed a model system to examine whether the inhibited form of one enzyme could be reactivated by adding an excess of the other enzyme. A similar method has been used successfully by Hayashi’s group (Murakami et al., 1985) in studying of the post-translational control of the mammalian ODC by antizyme; in that case the competitor enzyme was ODC inactivated with the suicide inhibitor z-difluoromethylornithine.

To determine the feasibility of the approach and the effectiveness of the in vitro reactivation system we tested it first using purified proteins. ODC was premixed either with partially purified antizyme or highly purified ribosomal proteins S20 and L34. The amounts of the added proteins were such as to produce an 80–90% inhibition of ODC activity. Addition of increasing amounts of highly purified ADC resulted in the reactivation of ODC inhibited by any of the three protein inhibitors used, although in the case of antizyme only 50% of the inhibition was relieved (Fig. 1A). ODC was also effective in reactivating ADC that had been inhibited by any of the three proteins (Fig. 1B).

When the same concentrations of either ODC or ADC were added in reactions lacking the inhibitor protein they produced no significant change on each other’s activity. This indicates that: (i) these two enzymes do not decarboxylate each other’s substrate and (ii) the observed effects are not due to increased protein concentrations. Addition of other proteins, such as bovine serum albumin or ovalbumin, produced no reactivation of the inhibited ODC or ADC (data not shown).

Extracts of E. coli contain inhibitor-bound forms of ODC and ADC

Having demonstrated in the model system described above that our method is effective for reactivating inhibited forms of ODC and ADC, we used it to determine whether inhibitor-bound forms of these enzymes exist in E. coli extracts. This assay was similar to the one used in the purified system, the difference being that ODC or ADC was added to cell extracts instead of mixtures of purified components. Addition of ADC resulted in an almost 3-fold reactivation of ODC, whereas addition of ODC doubled ADC activity [Fig. 2, (O)]. Addition of control proteins such as bovine serum albumin or

Fig. 1. Reactivation of ODC or ADC inhibited by antizyme or the ribosomal proteins S20 or L34 by addition of ADC or ODC, respectively. Highly purified preparations of ODC (1 ng) or ADC (50 ng) were inhibited by the addition of partially purified antizyme (5 ng, approx. 60% pure) (□) or highly purified ribosomal proteins S20 (120 ng) (●) or L34 (90 ng) (△). The inhibitions were reversed by the addition of the indicated amounts of highly purified ADC (A) or ODC (B).
Growth of *E. coli* in the presence of polyamines increases the proportion of inhibitor-bound forms of ODC and ADC

Using our reactivation system, we tested directly whether polyamines increase the inhibitor-bound levels of ODC and ADC in cells. ODC activity, from extracts of polyamine-exposed cells, was increased to as much as 500% of control value by the addition of excess ADC, while ADC was reactivated up to 360% [Fig. 2, (●)]. These numbers indicate that the proportions of inhibitor-bound ODC and ADC in these extracts were significantly higher than those of control cells that had not been exposed to exogenous polyamines.

To further demonstrate that the activities of ODC and ADC were inhibited to a higher degree in polyamine-exposed cells we quantitated the protein as well as the activity levels for these two enzymes. Together, these two values allow calculation of a specific activity that is expressed as units of activity per mg enzyme protein per hour of incubation. This specific activity should decrease when the levels of the post-translationally inhibited enzyme rise. In accordance with our previous results (Huang *et al.*, 1990) we found that growth in the presence of polyamines resulted in decreased ODC and ADC protein levels (Table 1). The activities of both ODC and ADC also decreased but significantly more than protein levels. As a result, the specific activities of both ODC and ADC (units/mg ODC or ADC protein/hr) were 30 and 55% lower, respectively, in extracts from cells exposed to polyamines compared to control cells (Table 1). These lower specific activities were the result of a higher proportion of inhibitor-bound, inactive ODC and ADC forms that could be reactivated by the addition of excess ADC and ODC, respectively (Fig. 2).

Feedback inhibition by polyamines may also be involved *in vivo* in regulating enzyme activity, especially of ODC (Morris *et al.*, 1970). This does not appear, however, to be a factor in our determinations since we obtained the same results even when we used dialyzed extracts. It should also be noted that these results are independent of the growth rate of *E. coli* MG1655 which is not affected by the presence of polyamines in the growth medium (Huang *et al.*, 1990).
Table 1. Effect of polyamines on the protein and activity levels of ODC and ADC

<table>
<thead>
<tr>
<th>Enzyme protein (µg/mg cell protein)</th>
<th>ODC</th>
<th>ADC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-PA</td>
<td>+PA</td>
</tr>
<tr>
<td>0.31 ± 0.06</td>
<td>0.15 ± 0.02</td>
<td>0.48</td>
</tr>
<tr>
<td>Enzyme activity (nmol ¹⁴CO₂/mg cell protein/hr)</td>
<td>1250 ± 100</td>
<td>400 ± 50</td>
</tr>
<tr>
<td>Specific activity (µmol ¹⁴CO₂/mg enzyme/hr)</td>
<td>4030 ± 150</td>
<td>2670 ± 100</td>
</tr>
</tbody>
</table>

Escherichia coli MG1655 was grown to an A₆₀₀ = 0.5 in M56, in the absence (-PA) or in the presence (+PA) of 1 mM putrescine and 1 mM spermidine. ODC and ADC activities, as well as their protein levels, were quantitated as described in “Materials and Methods”. Specific activities are expressed as µmol ¹⁴CO₂ liberated/mg enzyme/protein/hr of incubation. The values represent the mean of five separate experiments.

Reactivation of inhibited ODC and ADC by in vivo overexpression

One potential problem with our in vitro reactivation system is that it can not differentiate between enzyme–inhibitor complexes formed in the physiological environment of the cell and those formed during cell lysis and extract preparation. In an effort to overcome this limitation, we undertook the in vivo counterpart of our reactivation system. In this case E. coli overproducing either ODC or ADC were created by transforming cells with multicopy plasmids carrying the genes of the respective enzymes. The overproduced proteins were expected to titrate out the common inhibitors. Indeed, this was the case, since the 120-fold ODC protein overproduction in pODC-1 transformed cells resulted in a 300-400-fold increase in ODC activity (Table 2). As a result, ODC specific activity increased 3-fold in these cells. Although ADC protein levels decreased in the ODC overproducer, presumably as a result of increased polyamine production in this strain (unpublished results), its specific activity increased more than 2-fold (Table 2).

ADC overproduction by transformation of E. coli with either plasmid pKA5 (which contains the ADC, AUH and metK genes) (Boyle et al., 1984) or pHPC-1 (which contains the ADC and metK genes) (Huang et al., 1990) also resulted in increased specific activities for both ODC and ADC (Table 2). Plasmid pHPC-1 was used as a means of increasing the ADC levels without affecting polyamine concentrations, which may interfere with the expression or activity levels of ODC and ADC. These data are in agreement with the results obtained with the in vitro reactivation system and suggest that a large proportion of the intracellular ODC and ADC are reversibly inhibited by factors common to both enzymes.

The presence of the increased polyamine levels, or agmatine in the case of plasmid

Table 2. Consequences of low and high level ODC or ADC overproduction on their protein and activity levels

<table>
<thead>
<tr>
<th>Effector plasmid</th>
<th>ODC</th>
<th>ADC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (µg/mg cell protein)</td>
<td>Activity (nmol ¹⁴CO₂/mg protein/hr)</td>
</tr>
<tr>
<td>pBR322</td>
<td>0.34 ± 0.04</td>
<td>1200 ± 150</td>
</tr>
<tr>
<td>pODC-1</td>
<td>37.2 ± 2.00</td>
<td>387,500 ± 4000</td>
</tr>
<tr>
<td>pKA5</td>
<td>0.06 ± 0.004</td>
<td>560 ± 50</td>
</tr>
<tr>
<td>pHPC-1</td>
<td>0.23 ± 0.03</td>
<td>1870 ± 100</td>
</tr>
<tr>
<td>pCOD</td>
<td>1.25 ± 0.2</td>
<td>1500 ± 140</td>
</tr>
</tbody>
</table>

Escherichia coli MG1655 transformed with the indicated plasmids were grown to A₆₀₀ = 0.5 in M56, at 37°C. ODC and ADC were assayed as described in “Materials and Methods”. Transformation of the specA,B,C deletion mutant strain KLS27 with plasmid pCOD did not confer ODC activity or polyamine production in this mutant. Protein quantitations were performed by comparing the intensity of the immunostained bands with that of graded amounts of purified proteins on the same western blot, as described in “Materials and Methods”. The values represent the mean of three separate experiments.
pHPC-1, in the cell extracts from the ODC and ADC overproducers does not appear to be a factor in our determinations since experiments performed with dialyzed extracts produced similar results. Addition of ODC or ADC to extracts prepared from ODC or ADC overproducers did not activate ODC and ADC activities (results not shown). This was expected since the overproduced enzymes had already titrated out the inhibitors.

Tightness of the post-translational mode of regulation

ODC protein levels increase 4-fold when E. coli MG1655 is transformed with the plasmid pCOD (Wright et al., 1986), which carries the ODC promoter and the 5'-end of the ODC structural gene (Fig. 3 and Table 2). The simplest explanation for this increase is that it results from transcriptional activation of the chromosomal ODC gene copy, possibly due to titration of negative transcription factors by the multiple copies of the plasmid-borne ODC promoter (see below). ADC protein (and mRNA) levels in the pCOD-transformed cells were slightly lower than in control cells (Fig. 3 and Table 2). This may indicate that different factors are involved in the negative transcriptional regulation of the ODC and ADC genes.

Surprisingly, the 400% increase in ODC protein in cells transformed with pCOD is associated with only a 20% increase in enzymatic activity, resulting in a 65% reduction of the specific activity (Table 2). ADC protein and activity levels also decreased in these cells so that ADC specific activity decreased by 14% (Table 2). The decrease in ODC specific activity clearly is the result of post-translational inhibition since addition of ADC to extracts from pCOD-transformed cells resulted in a 9-fold reactivation of ODC activity (Fig. 4). This value is significantly higher than the 3-fold increase observed in extracts from control untransformed or pBR322-transformed cells. These results suggest that the post-translational regulation of the polyamine biosynthetic enzymes is tight enough to compensate for, and even to exceed, such increases in ODC protein.

The negative transcriptional regulation of ODC by polyamines is mediated by protein factors

Transcriptional repression plays a significant role in the negative-feedback regulation of polyamine biosynthesis, with increased polyamine concentrations resulting in decreased transcription from the ODC and ADC genes (Huang et al., 1990) and reduction of the ODC and ADC protein levels (Table 1). The question remained whether these transcriptional effects were due to direct interaction of polyamines with the DNA of these genes, or if the effect was mediated by polyamine-inducible negative transcription factors.

To answer this question we compared the effects of polyamines in wild type E. coli transformed with either pBR322 or pODC-1 (Boyle et al., 1984), carrying the ODC gene. If polyamines exert their effects directly at the DNA level, by binding and altering its

![Fig. 4. In vitro reactivation of ODC from extracts of wild-type E. coli or cells transformed with plasmids pBR322 or pCOD. The indicated amounts of highly purified ADC were added to extracts (2 µg) prepared from E. coli MG1655 (○) or from MG1655 transformed with plasmids pBR322 (▲) or pCOD (●). The extracts were prepared from cells grown under identical conditions in M56 until the A600 of each culture was 0.5, and the assays were performed as described in "Materials and Methods". ODC activities (100%) of MG1655 or MG1655/pBR322 are identical at 1250 nmol 14CO2/mg cell protein/hr whereas that of MG1655/pCOD is 1500 nmol 14CO2/mg cell protein/hr.](image-url)
conformation, then the ODC gene copy number per cell should not affect this interaction since the small amount of anionic charges brought into the cell by the 60–70 copies of plasmid pODC-1 (data not shown) should not affect significantly the concentration of polyamines. Therefore, polyamines should be able to inhibit transcription of the ODC gene even in cells transformed with pODC-1. This, however, was not the case. Polyamine concentrations as high as 10 mM putrescine plus 10 mM spermidine failed to reduce ODC protein or mRNA levels in E. coli carrying plasmid pODC-1 (Fig. 5). In contrast, polyamine concentrations of 1 mM putrescine and 1 mM spermidine caused a 52% decrease in ODC protein levels and a 75% reduction of activity in control cells (Table 1). Therefore, polyamines appear to exert their effect in trans, by activating and/or inducing the synthesis of some negative transcription factor(s). The loss of negative transcriptional regulation by polyamines in E. coli transformed with plasmid pODC-1 is consistent with the idea that inhibitory transcription factor(s) were titrated out by the high copy number of the ODC gene in these cells.

Further support for the above idea came when the levels of ODC mRNA, protein and activity were measured in E. coli transformed with plasmid pCOD, which contains a 1.2 kilobase DNA insert carrying the promoter and the 5'-end of the ODC gene (Wright et al., 1986). As we mentioned above, pCOD-transformed cells contained 4-times more ODC protein than control cells (Table 2). This increase is due to an equivalent increase in the ODC mRNA whose levels are 3–5-fold higher than those of control cells (data not shown). Cells transformed with pCOD also accumulate high levels of a smaller ODC mRNA which originates from the plasmid-borne truncated ODC gene (data not shown).

One method for identifying DNA binding proteins with specificity for particular DNA sequences is southwestern blotting (Miskimins et al., 1985). Following electrophoresis, the proteins were transferred electrophoretically to nitrocellulose filters and then probed with a 32P-labelled DNA restriction fragment (PstI-Hind III) carrying the ODC gene promoter. Three proteins, with apparent molecular weights of 22,000, 12,000 and 10,000 Da hybridized with the ODC promoter (Fig. 6). The hybridization is specific since it is not affected by a 100-fold excess of nonspecific competitor DNA, but is competed by a 10-fold molar excess of homologous nonradioactive DNA probe (Fig. 6). The intensity of the hybridization signal increased, modestly but reproducibly, for all three proteins in extracts from cells grown in the presence of polyamines. The intensity of the signal did not increase when the hybridization was performed in the presence of 1 mM spermidine (data not shown), suggesting that the
increased binding of these proteins is not simply the result of polyamine-induced protein or DNA conformational changes.

**DISCUSSION**

Inhibition of ODC and ADC by addition of polyamines in *E. coli* growth media was observed over two decades ago and it was thought to be the result of both activity inhibition and repression of enzyme synthesis (Tabor and Tabor, 1969). In the process of investigating ODC activity regulation we isolated protein inhibitors that we termed ODC-antizymes and we proposed that polyamine-inducible antizyme proteins are responsible for the post-translational inhibition of ODC activity (Fong et al., 1976; Heller et al., 1976). Polyamine-inducible ODC and ADC inhibitors were also found to exist in *E. coli* (Kyriakidis et al., 1978) and we subsequently identified them as antizyme and the ribosomal proteins S20 and L34 (Panagiotidis and Canellakis, 1984; Canellakis et al., 1985). We have also demonstrated that the repressive polyamine effect on ODC and ADC synthesis in *E. coli* is exerted on the transcriptional level (Huang et al., 1990).

In the first part of the present work we attempt to detect the presence of inhibited forms of ODC and ADC in *E. coli* extracts and to determine the fraction of each enzyme that is inhibited in cells grown in the absence or presence of polyamines. These results were expected to clarify the extent of the post-translational inhibition of these enzymes, the existence of which has been questioned by one group of investigators on the basis of their in vitro mixing experiments under conditions which may not have been optimal for the activities of either ODC or its inhibitors (Kashiwagi and Igarashi, 1987; Kashiwagi and Igarashi, 1988). The approach we used involved addition of increasing amounts of ODC in extracts containing inhibited forms of ADC and vice versa. The excess amount of one enzyme was expected to trap common inhibitors and therefore allow reactivation of the other enzyme, provided, of course, that the affinities of the two enzymes for the inhibitors were not widely different. A similar approach has been used by Hayashi’s group to demonstrate the presence of ODC-antizyme complexes in extracts from mammalian cells (Murakami et al., 1985).

The feasibility of the method was tested using purified enzymes and inhibitors and it was found that addition of one decarboxylase would reactivate the other almost to full extent. Application of the method either by adding decarboxylases to cell extracts, or by in vivo ODC or ADC overproduction provided evidence that at least 65% of the ODC activity and 50% of the ADC activity of *E. coli* are inhibited post-translationally by inhibitors that are common for both enzymes. Consequently, it appears that since only a small fraction of the total ODC and ADC activity is active under normal conditions, post-translational regulation of these enzymes contributes significantly to the regulation of polyamine biosynthesis. That these enzymes are partially inhibited in *E. coli* is not surprising, considering that normally, *E. coli* has high intracellular concentrations of polyamines (Tabor and Tabor, 1985); the *E. coli* MG1655 strain that we used in this work has basal intracellular concentrations of 15 mM putrescine and 3 mM spermidine when grown under identical conditions (Panagiotidis et al., 1987). Therefore, it may be assumed that, under normal conditions, the basal high intracellular polyamine concentrations help maintain the activities of ornithine and arginine decarboxylase at inhibited levels. Higher intracellular concentrations of polyamines, in polyamine-exposed bacteria (Tabor and Tabor, 1969), increase the inhibited forms of ODC and ADC even further (Fig. 2), in agreement with our original polyamine-

![Fig. 6. Southwestern blot analysis of proteins interacting with the ODC gene promoter. The 15,000 g extracts from cells grown in M56 in the absence (Lanes 1 and 3) or presence (Lanes 2 and 4) of 1 mM putrescine and 1 mM spermidine were analyzed on SDS-polyacrylamide gels (50 µg per lane), the proteins were transferred to nitrocellulose and the filters were hybridized using the radiolabelled ODC gene promoter as a probe, as described in “Materials and Methods”. The hybridization was performed in the presence of either a 100-fold molar excess of pBR322 DNA as nonspecific competitor (Lanes 1 and 2) or a 10-fold molar excess of nonradio-labelled Patl-Hind III fragment of the ODC gene, carrying the ODC gene promoter (Lanes 3 and 4). Addition of 100-fold excess of nonspecific DNA competitor does not affect hybridization (data not shown).](#)
inducible antizyme theory (Fong et al., 1976; Heller et al., 1976). The post-translational mode of regulation is also tight enough to compensate for a 4-fold ODC protein increase in pCOD-transformed cells.

In the second part of the paper we attempt to identify whether the negative transcriptional regulation of ODC and ADC (Huang et al., 1990) is due to direct effects of polyamines on DNA structure (Feuerstein and Marton, 1988), or whether negative transcription factor(s) are involved. Our findings indicate that the latter suggestion is correct, since introduction of multiple copies of plasmid-borne ODC promoter results in transcriptional activation of the chromosomal ODC gene. This is consistent with the idea that negative transcription factor(s) are titrated out by the excess of promoter DNA. Furthermore, exposure of cells carrying multiple copies of the ODC gene to high polyamine concentrations had no effect on the levels of both ODC protein and mRNA. This supports the notion that the polyamine effect is not exerted at the DNA level since, if that was the case, the increased gene copy number should not have made a difference when excess polyamines were present. Southwestern blot analysis identified three proteins that interact with the ODC gene promoter. The levels of these proteins were found to increase, albeit modestly, when cells were exposed to polyamines, raising the possibility that they are transcription factors mediating the negative polyamine effect.

In conclusion, we demonstrate that the post-translational mode of regulation of ODC and ADC does exist and tightly regulates its activities, in concert with negative transcriptional regulation by polyamine-inducible DNA-binding proteins.

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