Relationship of the Expression of the S20 and L34 Ribosomal Proteins to Polyamine Biosynthesis in *Escherichia coli*

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Polyamine biosynthesis in *Escherichia coli* is regulated transcriptionally and post-translationally. Antizyme and ribosomal proteins S20 and L34 participate in post-translational inhibition of the polyamine biosynthetic enzymes ornithine and arginine decarboxylase. The aim of the present study was to investigate the significance of S20 and L34 in polyamine regulation *in vivo*. *In vivo* overexpression of S20 and L34 lowered the activities of ornithine and arginine decarboxylases and decreased total polyamine production. The levels of cadaverine, a related diamine whose synthesis is not regulated by S20 and L34, did not decrease but increased. The diminished ornithine and arginine decarboxylase activities are shown to result from reversible post-translational inhibition since the enzymes could be reactivated to normal levels upon titration of the inhibitors. The effects were specific as overexpression of eight other ribosomal proteins had no influence. Overexpression of ornithine decarboxylase results in elevated polyamine production and it increases S20 and L34 levels but not those of other ribosomal proteins. Ornithine depletion decreases S20 and L34 to normal levels in the ornithine decarboxylase overproducing cells. Immunoprecipitation experiments coupled with immunoblots indicated that ornithine and arginine decarboxylases physically interact with S20 and L34. This study shows that ribosomal proteins S20 and L34 can inhibit ornithine and arginine decarboxylases and polyamine biosynthesis *in vivo*. It is concluded that, unlike other basic ribosomal proteins and polycationic compounds which inhibit the activities of these enzymes only *in vitro*, S20 and L34 are biologically relevant in the regulation of the polyamine biosynthetic pathway.

Keywords: Ornithine decarboxylase Arginine decarboxylase Antizyme Inhibitors Post-translational


**INTRODUCTION**

Our earlier studies have provided evidence that the activity of *Escherichia coli* ornithine decarboxylase (ODC; L-ornithine carboxy-lyase, E.C. 4.1.1.17) is inhibited by three protein inhibitors, two basic proteins and the antizyme (Heller et al., 1983a,b; Kyriakidis et al., 1978, 1983). The levels of all three inhibitors increase when polyamines are added to the *E. coli* growth medium (Panagiotidis et al., 1989) in a manner analogous to the induction of the mammalian ODC-antizyme by polyamines (Fong et al., 1976; Heller et al., 1976). We identified the two basic inhibitors as the ribosomal proteins S20 and L34 and showed that compared to a mixture of basic ribosomal *E. coli* proteins, these two ribosomal proteins inhibit highly purified ODC (Panagiotidis and Canellakis, 1984). Recently, we also reported the identification, cloning and sequencing of the *E. coli* antizyme gene (Canellakis et al., 1993). Sequence com-
parisons indicate that antizyme might also have a second function as a transcriptional regulator belonging to the two-component system family (Parkinson and Kofoid, 1992).

The physiological significance of the role of S20 and L34 ribosomal proteins in the regulation of ODC activity in particular, and polyamine biosynthesis in general, has been questioned by Kashiwagi and Igarashi (Kashiwagi and Igarashi, 1987, 1988) on the basis of their in vitro mixing experiments which indicated that other ribosomal proteins, e.g. L20, were more potent inhibitors of ODC than S20 or L34. Although assay of the ability of cell extracts to inhibit ODC activity served for the initial identification of the inhibitory fractions, it is apparent that persistence in this approach can not resolve the issue of the in vivo involvement of S20 and L34 in the regulation of polyamine biosynthesis. Titration of ODC by S20 and L34 is affected by a number of factors including purity of the preparations, variations in the fraction of denatured protein within each preparation, salt and nucleotide concentrations (Hölta et al., 1972, 1974), as well as by the presence of nucleic acids (Huang et al., 1984). This makes the interpretation of the results by Kashiwagi and Igarashi very difficult, since they performed their mixing experiments under conditions which might have not been optimal for ODC activity i.e. in the presence of 0.5 mM magnesium acetate and pH 8.5.

In this study we attempt to resolve the issue of the physiological significance of S20 and L34 on the regulation of ODC and arginine decarboxylase (ADC; L-arginine carboxylase, E.C. 4.1.1.19), by studying the effects of these proteins in vitro. We approached this problem by determining the consequences of S20 or L34 overproduction on (i) polyamine levels (ii) mRNA, protein and activity levels of ODC and ADC and (iii) the in vitro inhibited levels of ODC and ADC. As controls for the specificity of the observed effects, we also studied the effects of the overproduction of L20, as well as seven other ribosomal proteins, the S10, S15, S19, L2, L4, L22 and L23, most of which have isoelectric points above 11.5 (Kaltschmidt, 1971). We also attempted to probe the existence of ODC or ADC complexes with S20 and L34 in E. coli lysates by performing immuno-precipitation experiments. Our results emphasize the unique relationship between the S20 and L34 ribosomal proteins and polyamine biosynthesis.

MATERIALS AND METHODS

Materials

All electrophoresis reagents were purchased from Bio-Rad, nitrocellulose BA-85 (0.45 mm) was purchased from Schleicher and Schuell, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal) were from BRL, and the restriction enzymes were from BRL, Pharmacia Molecular Biologicals and from Boehringer. [5,6-3H]uridine (39 Ci/mmole), t-[1-14C]ornithine (58 mCi/mmole) and d,L-[1-14C]arginine (50 mCi/ mmole) were purchased from Moravek (Brea, CA), and t-[2,3-3H]ornithine (20 Ci/mmole) from New England Nuclear. Peroxidase-conjugated goat anti-rabbit IgG antibodies were obtained from Miles-Yeda; peroxidase-conjugated rabbit anti-sheep IgG antibodies were obtained from Kirkegaard and Perry Labs. Rabbit anti-sheep IgG immunoglobulins, and normal sheep and goat sera were obtained from Jackson Immuno-Research Labs and 125I-protein A (30 μCi/mg) from ICN.

Bacterial strains, plasmids and phages

The E. coli K12 strain MG1655 (λ−, F−) (Guyer et al., 1981) was used in most of this work. E. coli LL308 (F−, lacIq, ZAM15 Y+ pro+ /Δ[lac-pro] nadA recA supE thi) (Lindahl and Zengel, 1979) and its derivatives LL519, LL469, LL497, and LL405 carrying plasmids pLL36, pLL153, pLL161, and pLL133 with the genes of ribosomal proteins S10, L2 and L23, L22 and S19, and L4 (Zengel et al., 1980), respectively, were generously provided by Dr Lasse Lindahl. E. coli JM103 (Δ[lac-pro], thi, strA, supE, endA, sdeB, hsdR, F′traD36, proAB, lacIq, ZAM15) was used for the propagation of the M13mp18 and M13mp19 phages (Yanisch-Perron et al., 1985).

Plasmid pODC-1 (Boyle et al., 1984), carrying the structural gene of ODC, was provided by Dr Stephen Boyle; plasmid pGP2 (pS20) (Mackie, 1981) was provided by Dr George Mackie and plasmid pRB1A (Braun et al., 1985), containing the gene of the ribosomal protein L34 (rpmH), was a gift of Dr A. Wright. The 0.95 kb EcoRI fragment of plasmid pRB1A, which includes the L34 gene (Hansen et al., 1982), was subcloned in the EcoRI site of pBR322 and the resulting plasmid is designated as pL34. Plasmid A127S (Takata et al., 1984), containing the gene of ribosomal protein S15, was provided by Dr R. Takata, whereas Dr M. Springer provided plas-
mid pUA6 (Springer et al., 1985) carrying the gene of ribosomal protein L20. *E. coli* MG1655 was transformed with pBR322 or with the various recombinant plasmids and the transformants were designated as MG1655/pODC, MG1655/pS20, MG1655/pL34, etc., depending on the plasmid with which they were transformed.

**Hybridization probes, RNA labeling and RNA isolation**

Plasmid DNA was prepared by the alkaline lysis method, as described (Maniatis et al., 1989). The hybridization probes were obtained by cloning appropriate DNA fragments in M13mp18 and M13mp19 using standard procedures (Maniatis et al., 1989). The ODC probe was prepared by inserting a 1.3 kb *Smal*-HindIII fragment from pODC-1 (Boyle et al., 1984) into M13mp19. A control probe carrying the nonhybridizing strand of the DNA fragment was constructed by using M13mp18. The S20 probe was obtained by inserting a 550 bp HindIII-HindIII fragment (Mackie, 1981) from pS20 into M13mp18 and the control probe was obtained by inserting the nonhybridizing strand into M13mp19. The L34 probe was prepared by inserting the 950 bp EcoRI fragment of pR1A (Hansen et al., 1982) into M13mp19. Phages carrying the two different strands were selected. In some experiments the 560 bp ClaI-EcoRI fragment of pR1A (Hansen et al., 1982) was isolated and used as the probe. RNA labeling and DNA–RNA hybridizations were performed according to Zengel et al. (Zengel et al., 1980). Total *E. coli* RNA was isolated as previously described (Huang et al., 1990; Panagiotidis et al., 1994).

**Preparation of cell extracts**

Cells were grown in medium 56 (Monod et al., 1951; Panagiotidis et al., 1994) supplemented with 0.4% (w/v) glucose and 1 µg/ml thiamine, at 37 °C with vigorous shaking. When A₆₀₀ reached 0.5 the cultures were rapidly chilled by pouring over crushed ice and the cells were collected by centrifugation at 8000 g for 15 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. The 15,000 g supernatant fluids of the cell lysates were used for quantitation of protein levels of ODC, ADC, S20 and L34, as well as ODC and ADC activities, as described below. The ribosomal proteins were extracted from the 15,000 g supernatants of the cell lysates with 66% glacial acetic acid in the presence of 0.1 M MgCl₂, diazylized against 2% acetic acid, and lyophilized.

Protein concentrations were determined by the method of Bradford (Bradford, 1976).

**Enzyme activity assays**

ODC and ADC activities were measured in vitro, as previously described (Kyriakidis et al., 1978; Morris and Boeker, 1983; Panagiotidis et al., 1994). In vivo ODC activities were assayed in 100 µl of bacterial culture grown to an A₆₀₀ of 0.5, by incubating for 15 min at 37 °C with 10 µl (0.25 µCi) of 4.95 mM tris-[1-¹⁴C]ornithine in sterile 12 by 75 mm Falcon tubes. The reactions were terminated by adding 200 µl 10% trichloroacetic acid and ¹⁴CO₂ was trapped on 1/4 inch filter discs (Schleicher and Schuell) soaked with 25 µl NCS™ (Amersham), as in the in vitro assay.

Reactivation of ODC and ADC activities, respectively, from crude cell extracts was performed by adding increasing amounts of ADC and ODC, respectively, to the 15,000 g supernatants of cell lysates, as previously described (Panagiotidis et al., 1994).

**Preparation of ribosomes**

Ribosomes were prepared from *E. coli* grown as described above, either by the method of Zimmermann (Zimmermann, 1979) in which the cells are lysed with alumina grinding or by the method of Gourse et al. (Gourse et al., 1985) in which the cells are broken with lysozyme in the presence of a nonionic detergent. No differences were observed in the results obtained by these two methods. Unless otherwise specified, the ribosomal fractions refer to the 150,000 g unwashed ribosomal pellets and the post-ribosomal fractions to the 150,000 g supernatates.

**Electrophoresis and immunoblotting**

SDS-polyacrylamide slab gel electrophoresis was performed as described by Laemmli (Laemmli, 1970). The proteins were transferred electrophoretically from the polyacrylamide gels to nitrocellulose membranes (BA-85; Schleicher and Schuell) as described (Towbin et al., 1979). The membranes were quenched with PBSTA (4% bovine serum albumin, 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 three 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. We have already described the methodology used for the quantitation of individual proteins in cell extracts using immunoblotting (Panagiotidis et al., 1994).

ODC and ADC purification, as well as the production and purification of anti-ODC and anti-ADC antibodies have been described (Panagiotidis et al., 1994). Whole anti-S20 and anti-L34 antisera raised in sheep, were kindly provided by Dr G. Stößler. Ribosomal proteins S20 and L34 were purified from *E. coli* ribosomes as previously described (Panagiotidis and Canellakis, 1984).

Two dimensional gel electrophoretic analysis of the ribosomal proteins extracted from the 15,000 g supernatants of the cell lysates was performed according to Geyl et al. (Geyl et al., 1981). The proteins were visualized on the gels with the DPC-silver staining (Daichi Pure Chemicals Co, Ltd) following the manufacturers instructions.

**Immunoprecipitation**

ODC, ADC, S20 and L34 were immunoprecipitated from 15,000 g *E. coli* extracts by mixing these extracts with protein A-Sepharose (Pharmacia) on which the appropriate antibodies had been preadsorbed. Because sheep IgGs bind very poorly to protein A, rabbit anti-sheep IgG antibodies (Jackson Immunoresearch Labs) were used to bind the sheep anti-S20 and anti-L34 IgGs to this matrix. The immunoprecipitates were washed at least five times with ODC or ADC assay buffers and the washed pellets were suspended in SDS-polyacrylamide gel electrophoresis loading buffer and boiled for 5 min. After boiling, the matrices were spun down and the supernates were analyzed on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with the appropriate antibodies as described above.

**Polyamine identification and quantification**

The polyamines were identified and quantified in the 10% trichloroacetic acid extracts of both the cell pellets and the growth media as previously described (Panagiotidis et al., 1987). Acid hydrolysis of the extracts prior to HPLC did not change the elution profiles nor did it change the relative quantities of polyamines; this result indicates that the acetylated forms of polyamines were not formed under our experimental conditions. Furthermore, labeling of the cells with uniformly labeled ornithine (Panagiotidis et al., 1987) did not reveal the presence of any peaks other than those of putrescine and spermidine.

**RESULTS**

**ODC overproduction increases S20 and L34 levels**

To determine whether there is a unique relationship between polyamine biosynthesis and the ribosomal proteins S20 and L34 *in vivo*, we first asked whether ODC overproduction elicited any changes in their levels. Transformation of *E. coli* strain MG1655 with the multicopy plasmid pODC-1, carrying the ODC structural gene (MG1655/pODC), caused a 55-fold increase in the ODC mRNA levels and an 120-

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>ODC</th>
<th>S20</th>
<th>L34</th>
</tr>
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<tbody>
<tr>
<td>pBR322</td>
<td>1.0 ± 0.1 [1.0 ± 0.1]</td>
<td>1.0 ± 0.05 [1.0 ± 0.05]</td>
<td>1.0 ± 0.03 [1.0 ± 0.05]</td>
</tr>
<tr>
<td>pODC</td>
<td>55.0 ± 5.0 [120.0 ± 15.0]</td>
<td>2.1 ± 0.3 [1.7 ± 0.2]</td>
<td>1.4 ± 0.2 [2.2 ± 0.4]</td>
</tr>
<tr>
<td>pS20</td>
<td>0.95 ± 0.08 [0.95 ± 0.08]</td>
<td>21.0 ± 2 [2.5 ± 0.3]</td>
<td>0.8 ± 0.1 [0.8 ± 0.05]</td>
</tr>
<tr>
<td>pL34</td>
<td>0.85 ± 0.15 [0.85 ± 0.1]</td>
<td>1.0 ± 0.05 [1.0 ± 0.05]</td>
<td>35.0 ± 10.0 [5.0 ± 1.0]</td>
</tr>
</tbody>
</table>

The levels of mRNA and of protein were determined as described in "Materials and Methods". The nonbracketed numbers represent the relative values of the mRNA; the numbers in brackets represent the corresponding protein values. Both sets of numbers are multiples of the corresponding values for the control MG1655. Transformation with pBR322 did not affect the amounts of proteins or mRNAs of interest. The values represent the average of five independent experiments.
Table 2. Effect of S20, L34 and ODC overproduction on the in vitro and in vivo activities of ODC and on the in vivo activity of ADC

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>In vitro [μmol 14CO2/mg enzyme/h (%)*]</th>
<th>In vivo [μmol 14CO2/10^6 cells/h (%)*]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>27.4 ± 0.4 (100)</td>
<td>4030 ± 150 (100)</td>
</tr>
<tr>
<td>pODC</td>
<td>70.0 ± 3 (256)</td>
<td>11,300 ± 240 (280)</td>
</tr>
<tr>
<td>pS20</td>
<td>17.1 ± 0.5 (62.5)</td>
<td>3200 ± 120 (75)</td>
</tr>
<tr>
<td>pL34</td>
<td>14.8 ± 0.4 (54.1)</td>
<td>2800 ± 90 (70)</td>
</tr>
</tbody>
</table>

In vivo and in vitro ODC activities as well as in vitro ADC activity were assayed as described in "Materials and Methods". Transformation of E. coli with plasmid pBR322 did not affect ODC or ADC activities of control untransformed cells that are 1250 and 75 nmol 14CO2/mg E. coli protein/h, respectively. The average normal protein levels for ODC and ADC are 0.31 and 2.7 μg/mg of total E. coli protein respectively. *Numbers in parentheses express the values obtained as percentage of the corresponding pBR322 transforms. The results represent the average of five (for the in vitro activity assays) or three (for the in vivo assay) independent determinations.

fold overproduction of ODC protein (Table 1). This increase was associated with a more than 300-fold increase in the levels of the in vitro assayable ODC activity (Panagiotidis et al., 1994). The more pronounced increase in the activity compared with that in protein levels was the result of titration of ODC inhibitors by the overproduced protein (Panagiotidis et al., 1994), and resulted in a 2.8-fold increase of the ODC specific activity (Table 2). Surprisingly, the total polyamine production in the ODC overproducers is only two times higher than in control cells (Table 3). The difference is not an artifact due to the inavailability of ornithine; similar results were obtained when ODC activity was assayed in vivo in the presence of excess ornithine (Table 2). The in vivo assay measures the potential of the intact cells to decarboxylate ornithine and thus represents a measurement closer to the in vivo situation than the in vitro assay which measures enzymatic activities in cell lysates which contain artificial buffers and protein concentrations much lower than those within the cell. The presence of ample quantities of unmodified ornithine both intracellularly and in the growth media, as verified by HPLC analysis at the end of each in vivo assay (data not shown), indicated that there were no transport problems and that ornithine limitation was not the cause of the observed lower-than-expected in vivo ODC activity. The increase in the cadaverine production noted in the pODC transformant (Table 3) could be due to elevated lysine decarboxylase activity or to the nonspecific decarboxylation of lysine by the large intracellular amounts of ODC (Igarashi et al., 1986).

The levels of both S20 and L34 proteins almost doubled in the ODC overproducer (Table 1). These increased protein levels corre-

Table 3. Intracellular and extracellular putrescine, spermidine and cadaverine levels in E. coli MG1655 transformed with pBR322, pODC, pS20 and pL34 plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Putrescine</td>
<td>Spermidine</td>
<td>Cadaverine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Putrescine</td>
</tr>
<tr>
<td>pBR322</td>
<td>415 ± 20</td>
<td>35 ± 4</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>pODC</td>
<td>310 ± 40</td>
<td>15 ± 2</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>pS20</td>
<td>430 ± 40</td>
<td>50 ± 4</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>pL34</td>
<td>400 ± 35</td>
<td>45 ± 5</td>
<td>1.7 ± 0.2</td>
</tr>
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</table>

Polyamines were determined both in cell extracts and in the growth media as described in "Materials and Methods". Spermidine was not present in detectable quantities in the growth media. The results are expressed as nmols amine per 10^6 cells and are the mean of three separate determinations. *Numbers in parentheses express values obtained as percent of the corresponding pBR322 transformants. No acetylated forms of polyamines were detected, as described in "Materials and Methods". ND = not detectable.
S20 and L34 overexpression inhibits polyamine production

Transformation of *E. coli* MG1655 with pBR322-based plasmids bearing the structural genes of S20 (pS20) or L34 (pL34) resulted in increased mRNA levels and overproduction of these proteins (Table 1). In close agreement with the results obtained by Parsons and Mackie in pS20 (pGP2) transformed *E. coli* (Parsons and Mackie, 1983), the S20 mRNA increased by 21-fold while its protein levels increased by 2.5-fold. In the pL34 transformed *E. coli* we also found that a disproportional increase occurred in the L34 mRNA (35-fold increase) as compared to the increase in its protein level (5-fold increase).

The total polyamine production of the S20 and L34 overproducers was calculated by measuring the intracellular as well as extracellular putrescine and spermidine in these cells. It was found that in both cases polyamine production was reduced by approx. 25% (Table 3). This decrease primarily reflects the change in their extracellular concentration; the cells retained and even increased their intracellular polyamine levels. However, cadaverine, which accounts for only a small fraction of the total polyamines, was increased on a percent basis (Table 3). This effect is in keeping with our earlier finding that the biosynthetic lysine decarboxylase is not inhibited by the S20 and L34 ribosomal proteins (Canellakis *et al.*, 1985) and the fact that cadaverine is thought to be a compensatory polyamine whose levels increase upon polyamine depletion (Igarashi *et al.*, 1986).

S20 and L34 overexpression results in post-translational inhibition of ODC and ADC

The decreased polyamine production in the S20 and L34 transformants appears to be the result of post-translational inhibition of both ODC and ADC in these cells. This conclusion arises from the fact that whereas the mRNA or protein levels of ODC and ADC do not decrease significantly in these cells (Table 1 and Fig. 1), their activities were found to be significantly lower (Table 2). As a result, the specific activities of both ODC and ADC in these cells, expressed as μmol 14CO₂ produced/mg decarboxylase/hr, are decreased by 25-30% and 38-46%, respectively (Table 2). ODC activity was also found to be lower in the S20 and L34 overproducers, by 27% and 45% respectively.
even when it was measured in vivo (Table 2). The observed activity decreases are in good agreement with the reduction in polyamine production seen in the pS20 and pL34 transformants (Table 3). It should also be appreciated that these decreases in ODC and ADC activities are superimposed upon enzymes that normally function at a greatly inhibited level. We have previously shown that in control E. coli MG1655 growing under identical conditions, the ODC and ADC activities are inhibited by at least 65% and 50%, respectively (Panagiotidis et al., 1994).

The decreased ODC and ADC activities are the result of reversible, post-translational inhibition. This was demonstrated by using our in vitro reactivation system (Panagiotidis et al., 1994) where e.g. addition of excess ADC to cell extracts can reactivated ODC by binding to and titrating out common inhibitors. We found that both ODC and ADC could be reactivated from extracts of pS20 and pL34 transformants to a significantly greater extent than extracts from E. coli MG1655 transformed with control pBR322 plasmid (Fig. 2).

To examine whether the observed effects are specific and not due to nonspecific interactions between two basic ribosomal proteins and the ornithine and arginine decarboxylases, we studied the effects of eight other basic ribosomal proteins on the activities of ODC and ADC. These proteins were the S10 (pI = 7.9), S15 (pI > 12) and S19 (pI > 12) of the small ribosomal subunit and L2 (pI > 12), L4 (pI = 7.6), L20 (pI > 12), L22 (pI = 11.5) and L23 (pI = 9.6) of the large ribosomal subunit (pI values from Kalschmidt, 1971). Two-dimensional gel electrophoresis showed that these eight proteins were overproduced in E. coli transformed with plasmids carrying their structural genes, to an equal or even greater extent than S20 and L34 in the pS20 and pL34 transformed cells (Fig. 3). Table 4, however, shows that the overexpression of these other ribosomal proteins had little or no effect on the ODC and ADC specific activities, in contrast with the overproduction of S20 and L34 which caused significant inhibition.

Co-immunoprecipitation of S20 and L34 ribosomal proteins with ODC and ADC

Post-translational inhibition of ODC and ADC by S20 and L34 ribosomal proteins in vivo should involve physical interactions between these proteins. Since the activity levels of ODC and ADC in cell extracts have been found to be inhibited (Panagiotidis et al., 1994; and the present work), we assumed that the enzyme-inhibitor complexes survive, at least partially, cell lysis during extract preparation. To probe for the existence of such ODC and ADC complexes with S20 and L34 we performed immunoprecipitation experiments with the appropriate antibodies and analyzed the immune complexes by SDS-polyacrylamide gel electrophoresis and immunoblotting. If ODC, for example, interacts with S20, then S20 should coprecipitate with ODC immune complexes and it should be de-

![Fig. 2. Reactivation of ODC and ADC from extracts of S20 and L34 overproducers. ODC (A) and ADC (B) were reactivated from extracts of E. coli MG1655 transformed either with control plasmid pBR322 (○), or plasmids pS20 (○) and pL34 (▲), by adding the indicated amounts of highly purified ADC or ODC, respectively. ODC assays were performed using 2 μg total cellular protein, whereas the ADC assays used 16 μg. ODC activity (100%) in the control extract is 1250 nmol [14C]CO₂/mg cell protein/hr, whereas it is 870 and 710 nmol [14C]CO₂/mg cell protein/hr in extracts from the pS20 and pL34 transformants, respectively. The respective ADC activities are 75 (control pBR322), 47 (pS20) and 40 (pL34) nmol [14C]CO₂/mg cell protein/hr.](image-url)
Fig. 3. Two-dimensional polyacrylamide gel electrophoresis of the ribosomal proteins from the various transformants. Ribosomal proteins were extracted from the 15,000 g supernates of the cell lysates, and two dimensional gel electrophoresis was performed as described in "Materials and Methods". The control gel (A) is shown in its entirety, whereas only the pertinent portions of the other gels are shown. (A) control untransformed Escherichia coli MG1655; (B) pS20 transformant (S20 overproducer); (C) pLL161 transformant (L22 and S19 overproducer); (D) pLL153 transformant (L2 and L23 overproducer); (E) pLL133 transformant (L4 overproducer); (F) pLL36 transformant (S10 overproducer); (G) pUA6 transformant (L20 overproducer); (H) A127S transformant (S15 overproducer). IPTG (0.1 mM) had been added to the cultures of the pLL161, pLL153 and pLL133 transformants to induce the synthesis of the relevant proteins. In the A127S transformant gel analysis an unknown protein spot (?) appeared. The overproduction of the L34 protein in the pL34 transformants is not shown since protein L34 is not well separated in this gel system. The relevant ribosomal proteins are numbered and indicated with arrows.
detected by probing the immunoblots with anti-S20 antibodies. Our results indicate that both S20 and L34 coprecipitate with ODC and with ADC and the same is true for ODC and ADC in S20 and L34 immune complexes (Fig. 4). The results appear to be specific since no proteins are detected when nonimmune serum is used for the immunoprecipitations (Fig. 4), and no biosynthetic lysine decarboxylase, a related enzyme that is not inhibited by S20 and L34 (Canellakis et al., 1985), is detected in the S20 and L34 immunoprecipitates (data not shown).

Having demonstrated that ODC and ADC physically interact with S20 and L34 we were left with the question of whether these two ribosomal proteins could interact with the polyamine biosynthetic enzymes while being integral components of the ribosomes. Alternatively, it could be that ODC and ADC interact with ribosomal proteins that exist in the post-ribosomal fraction, and that these "free" S20 and L34 proteins are involved in the post-translational inhibition of ODC and ADC. To determine whether S20 and L34 are present in the post-ribosomal supernatant we prepared the appropriate fractions using two available cell lysis methods, in order to avoid method-specific artifacts. Immunoblot analysis showed that a

Table 4. Effect of ribosomal protein overexpression on ODC and ADC activities

<table>
<thead>
<tr>
<th>r-protein</th>
<th>ODC specific activity [µmol/mg enzyme/h (%)]</th>
<th>ADC specific activity</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>4030 ± 150 (100) 27.4 ± 0.4 (100)</td>
<td></td>
</tr>
<tr>
<td>S10</td>
<td>4150 ± 300 (103) 29.0 ± 0.3 (106)</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>4050 ± 200 (100) 27.8 ± 0.4 (102)</td>
<td></td>
</tr>
<tr>
<td>L2 and L23</td>
<td>4200 ± 180 (104) 26.8 ± 0.6 (98)</td>
<td></td>
</tr>
<tr>
<td>L22 and S19</td>
<td>4000 ± 160 (100) 26.2 ± 0.8 (96)</td>
<td></td>
</tr>
<tr>
<td>L20</td>
<td>3950 ± 200 (98)  27.1 ± 0.2 (99)</td>
<td></td>
</tr>
<tr>
<td>S15</td>
<td>4000 ± 300 (100) 27.1 ± 0.4 (99)</td>
<td></td>
</tr>
<tr>
<td>S20</td>
<td>3200 ± 120 (75)  16.2 ± 0.3 (59)</td>
<td></td>
</tr>
<tr>
<td>L34</td>
<td>2800 ± 90 (70)   14.8 ± 0.4 (54)</td>
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The specific activities of ODC and ADC (µmol 14CO₂/mg enzyme/h) were determined in E. coli transformed with plasmids carrying the genes of ribosomal proteins S10 (plasmid pLL36), L4 (pLL133), L2 and L23 (pLL153), L22 and S19 (pLL161), S15 (A1275S), L20 (pUA6), S20 (pS20), L34 (pL34) or none (pBR322). IPTG (0.1 mM) had been added to the cultures of the pLL133, pLL153 and pLL161 transformants, to induce the synthesis of the proteins whose genes are under the control of the tac promoter. The ODC and ADC specific activities of E. coli transformed with a control pBR322 plasmid and grown under identical conditions are also provided for comparison. *Numbers in parentheses express activities as percentages of the activity of these control cells.

fraction of the S20 and L34 proteins, especially in the MG1655/pODC, MG1655/pS20 and MG1655/pL34 strains which overproduce them, was located in the post-ribosomal supernatant, with the rest being present in the unwashed ribosomal fraction (Fig. 5). The excess S20 and L34 proteins present in the crude ribosomal fractions of the overproducers do not appear to be incorporated into the structural portions of the ribosomes since they could be extracted with 1 M NH₄Cl (data not shown).

FIG. 4. Co-immunoprecipitation of the S20 and L34 with ODC and ADC. ODC, ADC, S20 and L34 were immunoprecipitated from E. coli extracts, as described in "Materials and Methods." Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis, electroblotted on nitrocellulose filters and the filters were probed with antibodies that recognize ODC, ADC, S20 and L34. The antibodies used for probing the immunoblots are specified on top of each panel, whereas the antibodies used for immunoprecipitation are indicated at the top of each lane. The preimmune serum (PRE), that served as a specificity control, had been obtained from the rabbit used for anti-ODC antibody production prior to immunization. Only the pertinent regions of the immunoblots are shown. The heavy and light chains of the immunoglobulins used in the immunoprecipitation which are also recognized by the secondary antibodies do not affect the determination since they migrate in different positions (48 kDa and 28 kDa respectively) than the proteins of interest (ODC at 81 kDa, ADC at 75 and 72 kDa, S20 at 9.7 kDa and L34 at 5.4 kDa).

DISCUSSION

Our work over the last 15 years has provided evidence that regulation of polyamine biosynthesis in Escherichia coli by the end products of the biosynthetic pathway takes place at multiple levels. Polyamines negatively regulate the expression of their biosynthetic enzymes both at the transcriptional as well as the post-translational level (Kyriakidis et al., 1978; Canellakis et al., 1985; Huang et al., 1990; Panagiotidis et al., 1994). The transcriptional
effect is mediated by polyamine-inducible negative transcription factor(s) (Panagiotidis et al., 1994) whereas post-transcriptional regulation is mediated by inhibitor proteins which resemble eucaryotic antizymes in that they are also polyamine-inducible (Heller et al., 1983b; Kyriakidis et al., 1978, 1983; Panagiotidis et al., 1994). We have identified three antizyme-like proteins in E. coli, the ribosomal proteins S20 and L34 (Panagiotidis and Cancellakis, 1984) and antizyme which, after cloning its gene (Canellakis et al., 1993), was found to be homologous with transcription factors of the two-component system family (Parkinson and Kofoid, 1992). The significance of the involvement of S20 and L34 in regulating the activities of the polyamine biosynthetic enzymes ODC and ADC has been questioned by Kashiwagi and Igarashi on the basis of the ability of other basic ribosomal proteins to inhibit ODC activity in vitro (Kashiwagi and Igarashi, 1987, 1988). The apparent nonspecificity of ODC inhibition by basic proteins in vitro, led these authors to question the validity of the post-translational inhibition as a mode of regulation of polyamine biosynthesis. We recently resolved part of this controversy by showing that the polyamine biosynthetic enzymes ODC and ADC are reversibly inhibited in vivo, and that the inhibited enzyme levels rise when cells are exposed to polyamines (Panagiotidis et al., 1994). In the present work we further advance the issue of the in vitro relevance of the ribosomal proteins S20 and L34 in the regulation of polyamine biosynthesis.

The results presented in this work indicate that constitutive overexpression of S20 or L34 inhibits polyamine biosynthesis by affecting ODC and ADC activities. This post-translational inhibition is reversible and specific since overexpression of eight other basic ribosomal proteins did not inhibit the activities of these polyamine-biosynthetic enzymes. Interestingly, overexpression of ribosomal protein L20, which had been found to be a more potent inhibitor of ODC than S20 and L34 in vitro (Kashiwagi and Igarashi, 1987), was found to have no effect in vivo. The levels of cadaverine, a related diamine whose biosynthetic enzyme, lysine decarboxylase, is not inhibited by S20 and L34 (Canellakis et al., 1985) did not decrease but were actually increased in the S20 and L34 overproducing strains. It is interesting that cadaverine is thought to be a compensatory polyamine whose synthesis increases under conditions of polyamine depletion (Igarashi et al., 1986). The expression of S20 and L34 was found to increase in ODC overproducing cells. Despite overproducing ODC protein 120-fold the total polyamine production of these cells increases only 2-fold. These results are in agreement with observations made by others (Kashiwagi and Igarashi, 1988). The difference is not due to the presence of inactive ODC in inclusion bodies since the protein is both soluble and active in extracts. Nor is it due to limiting substrate, since similar results were obtained in the presence of excess ornithine, which was found to be transported into the cells. Therefore, there must be a level of ODC regulation in these cells which is lost upon cell lysis. The possibility exists that it is the increased levels of S20 and L34 in this strain that are involved in such a regulation. ADC protein and mRNA levels are reduced 5-fold in the ODC overproducers, compared with the 2.2-fold decrease that takes place upon cell exposure to high extracellular polyamine levels (Huang et al., 1990; Panagiotidis et al., 1994). This indicates that what is sensed by the cells is the increased polyamine production and not the absolute intracellular polyamine content, which is actually lower in MG1655/pODC. Inhibition of polyamine biosynthesis by addition of arginine to the growth medium, which represses ornithine biosynthesis (Glansdorff, 1987), resulted in increased ADC and decreased S20 and L34 levels proving that it is the

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Fig. 5. Overproduced S20 and L34 ribosomal proteins are located in the post-ribosomal as well as in the ribosomal fractions. Ribosomal and post-ribosomal fractions were prepared as described in "Materials and Methods". Equal amounts of protein (1.5 μg from each ribosomal fraction and 70 μg from each post-ribosomal fraction), were analyzed on SDS-polyacrylamide gels, blotted on nitrocellulose filters and probed either (A) with anti-S20 or (B) anti-L34 antibodies. The ribosomal and post-ribosomal fractions are indicated above the appropriate lanes, as well as the plasmids with which the cells are transformed.
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polyamine overproduction that is actually responsible for the observed effects in the pODC-transformed cells.

Finally, by performing immunoprecipitation experiments, we find that both ODC and ADC are physically associated with S20 and L34. Such an association is not observed with lysine decarboxylase, a related decarboxylase which is not regulated by S20 and L34 (Canellakis et al., 1985). A small fraction of S20 and L34 is found in the post-ribosomal supernatant. The non-ribosomal S20 and L34 levels are found to increase when these proteins are overproduced either in cells transformed with pS20, pL34 or pODC. This raises the possibility that this is the "free" fraction of S20 and L34 that is actually involved in ODC and ADC inhibition since previous work has shown that addition of nucleic acids (Huang et al., 1984) or ribosomes (Kashiwagi and Igarashi, 1987) can reverse this inhibition in vitro.

In conclusion, the present work confirms our previous observations on the unique role of the S20 and L34 ribosomal proteins in regulating polyamine biosynthesis. It also underscores the necessity for the performance of in vitro studies whenever disputes based on in vitro results arise.

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