Amino Acids (1997) 13: 299–309

Amino
Acids

Ornithine decarboxylase in *Thermus thermophilus*: An RNA-associated enzyme

A. A. Pantazaki[1], **M. Liakopoulou-Kyriakides**[2], and **D. A. Kyriakidis**[1]

[1] Laboratory of Biochemistry, Faculty of Chemistry and [2] Department of Chemical Engineering, Section of Chemistry, Aristotle University of Thessaloniki, Greece

Accepted May 16, 1997

Summary. Ornithine decarboxylase (ODC) of *Thermus thermophilus* is associated with the nucleoid protein fraction. Analysis of this fraction by agarose gel electrophoresis and immunostaining revealed that ODC was bound to two groups of RNA-protein complexes. These two complexes of 1.5 and 0.6 kb in size disappeared from the gel by RNase A treatment or migrated to small molecular weight complexes by proteinase K treatment. Phenol extraction of either the nucleoid fraction or the eluted RNA-protein complexes from the agarose gel, shows that both contain the 0.56 kb RNA. Both RNA-protein complexes contain the ODC protein (55 kDa) but their protein composition differs in at least six proteins. Extraction of the nucleoid fraction with H_2SO_4, indicates that ODC was present in the acid-soluble fraction, showing that it is a non-histone protein tightly bound to 0.56 kb RNA. The purified ODC by various columns (~140-fold), is close to homogeneity and still carries the 0.56 kb RNA further explaining all the difficulties in the purification of this enzyme.

Keywords: Amino acids – Ornithine decarboxylase – *Thermus thermophilus* – RNA complex

Introduction

The existence of polyamines as well as some novel polyamines such as thermine and thermospermine in the archaebacterium *Thermus thermophilus* has been reported (Oshima, 1983), but nothing is known about the biosynthetic pathway of these molecules. In the thermophilic organisms these novel polyamines thermine and thermospermine are in higher amounts than the normal polyamines. In the extremely thermophilic bacterium *Clostridium thermohydrosulfuricum* only the enzyme which converts ornithine to putrescine, ornithine decarboxylase (EC 4.1.1.17, ODC), has been studied so far (Poso and Paulin, 1983; Paulin and Poso, 1983).

ODC has been purified to homogeneity from different sources, its gene has been cloned (Hayashi and Canellakis, 1989) and comparison studies either on the protein or the gene level have been reported (Hayashi and Canellakis, 1989). In some eucaryotic cells, such as normal rat liver (Murphy and Brosman, 1976; Bartholens, 1983), chick embryos (Snyder and Kreuz, 1970), and *T. pyriformis* (Sklaviadis et al., 1985) ODC has been found in the nucleus. In germinated barley, corn, pea and bean seeds ODC is located both in the cytosol and in the nucleus tightly bound to chromatin (Panagiotidis et al., 1982; Foudouli and Kyriakidis, 1989).

In this paper we report that the ODC of *T. thermophilus* is associated with the nucleoid fraction tightly bound to RNA. The size of RNA was determined and it was found that after many steps of purification of ODC, leading almost to homogeneity, ODC still carries the 0.56kb RNA.

Materials and methods

Materials

L-(l-^{14}C) Ornithine (sp. activity 50mCi/mmol was purchased from Moravek Biochemicals, Inc., California (Dr E. S. Canellakis's gift). Bacto-tryptone and yeast extract were obtained from Difco (Detroit, MI). Agarose was purchased from Bio-Rad Laboratories (California, USA). ODC antibody was a generous gift from Dr C. Panagiotidis.

Growth of cells

Th. thermophilus, strain HB8, was used in all experiments. Microorganisms were grown at 75°C in a medium containing 0.3%(w/v) yeast extract, 0.5%(w/v) bactotryptone, 0.2%(w/v) NaCl, 0.1%(w/v) D-glucose, 2μM $FeCl_3$, 0.2mM $CaCl_2$ and 1mM $MgCl_2$. The pH was adjusted to 7.0 by concentrated KOH. Growth was monitored by measuring the absorbance at 600nm in a Perkin – Elmer spectrophotometer. The bacteria were harvested at the end of the logarithmic phase by centrifugation at 6,000g for 10min. Cells were washed twice with 0.9%(w/v) NaCl. The final yield was about 5g of wet cells per liter of culture medium.

Disruption of cells

Cells (10g) were suspended in 4ml of 10mM Tris-HCl buffer (pH 8.1), 20% sucrose (w/v) and 100mM NaCl. The suspension was incubated with lysozyme (10mg/ml) at room temperature for 3min. Then 5ml of a solution containing 10% Brij-58, 0.4% deoxycholate, 10mM EDTA was added and the mixture was kept at 0°C for 15min. The suspension of lysed cells was centrifuged at 10,000g for 10min as described (Yamazaki et al., 1984).

Isolation of nucleoids and nucleoid proteins

Nucleoids were isolated by sucrose density gradient (10%–50% w/v) described by Yamazaki et al. (1984).

Nucleoid proteins, which are the acid-soluble proteins, were extracted with 0.4M H_2SO_4. The acid-soluble proteins were further precipitated by two volumes of cold ethanol, whereas the acid-insoluble proteins were suspended in distilled water, neutralised by NaOH and dialysed extensively against water.

RNA isolation

Nucleoids prepared as described above were digested with 2 units/ml of DNase I for 30 min at 37°C. The RNA was prepared by extracting the previous mixture three times with phenol/chloroform/isoamyl alcohol and one time with chloroform/isoamyl alcohol. The obtained aqueous phase was precipitated with 3 vol. of cold ethanol at −20°C. The RNA was precipitated by centrifugation at 10,000 g for 20 min.

Antibody production – Immunoprecipitation

ODC of *E. coli* was purified to homogeneity as previously described (Moris and Boeker, 1983). The *E. coli* ODC antibody was produced as described (Huang et al., 1983).

The ODC-nucleic acid complex was immunoprecipitated from the nucleoid fraction with the ODC-antibody bound to protein A-Sepharose. The protein A-Sepharose beads were equilibrated with buffer A (10 mM Tris-HCl pH 7.4, 100 mM NaCl and 2.5 mM $MgCl_2$) containing 0.5% Triton X- 100 and the ODC-antibody dialysed in the same buffer. The ODC-antibody was bound to protein A-Sepharose as described (Choi and Dreyfuss, 1984) in the presence of 1 mM vanadyl ribonucleotide complex. The beads were either extracted with SDS/PAGE sample buffer for protein analysis or with TEL buffer (200 mM Tris-HCl pH 7.4, 25 mM EDTA, 100 mM LiCl) containing 1% SDS for RNA analysis. The RNA was precipitated as was mentioned above.

Agarose gel electrophoresis and immunoblotting

Agarose gel electrophoresis and capillary transfer of DNA to nitrocellulose filters was performed as described (Towbin et al., 1979). The nitrocellulose filter was dried, exposed under UV light for 10 min and immunostained as previously described (Towbin et al., 1979).

ODC assays

ODC activity was assayed as described (Kyriakidis et al., 1978). Incubations were performed at 65°C for 30 min in 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol and 50 mM pyridoxal-5′-phosphate. One unit of ODC activity is defined as the amount of enzyme that releases 1 nmol $^{14}CO_2$ in 1 h under the above described conditions.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide slab gel electrophoresis was performed by the standard method (Laemmli, 1970).

Protein estimation

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

Results

1. Sucrose gradient analysis of nucleoid-associated ODC

Nucleoids were isolated by centrifugation through a linear 10%–50%(w/v) sucrose density gradient from gently lysed cells of *T. thermophilus*. Part of the fractions were dialysed against ODC assay buffer and tested for ODC activity. Figure 1a shows that all of the ODC activity is associated with the nucleoids

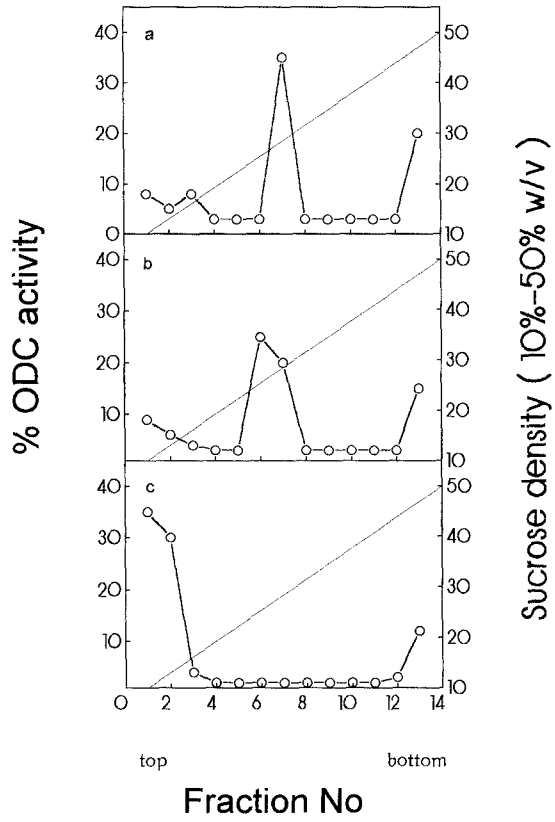

Fig. 1. Association of ODC activity with nucleoids. Nucleoids were isolated by centrifugation through a linear (10%–50% w/v) sucrose density gradient. The fractions were diluted with buffer B and centrifuged at 10,000 g for 20 min. The pellets were suspended in 0.1 ml ODC assay buffer and tested for ODC activity. Distribution of ODC activity after lysis of *T. thermophilus* with lysozyme **a**) for 1 min, **b**) for 3 min and **c**) for 1 min plus DNase (1 unit/ml) and RNase (1 unit/ml)

which appeared between 25%–35% of the sucrose density gradient. The distribution of ODC changes upon longer incubation of *T. thermophilus* with lysozyme or with lysozyme and DNase or RNase (Fig. 1b,c). When both DNase and RNase were used even at this low concentration (1 unit/ml), the ODC activity moved to the lower molecular weight complexes at the top of the sucrose gradient (Fig. 1c).

2. *Agarose gel electrophoresis of nucleoid-associated ODC*

The nucleoid fractions with ODC activity as analysed in Fig. 1, (fractions 6–10), were combined, diluted with equal volume of buffer B (10 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 60 mM KCl and 10 mM β-mercaptoethanol) and centrifuged at 10,000 g for 20 min. The pellet was suspended in loading buffer and electrophoresed on 1% agarose gel. Part of the gel was stained with EtBr (Fig. 2). Another part was transferred on a nitrocellulose membrane and stained

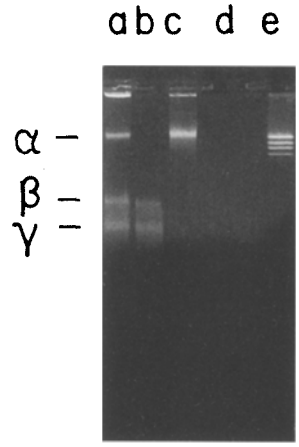

Fig. 2. Electrophoresis of ODC-RNA complexed fragments on 1% agarose gel. **a** Control, nucleoid fraction of Fig. 1 (fractions 6–10); **b** nucleoid fraction plus 2 units/ml of DNase I; **c** nucleoid fraction plus 0.5 unit/ml of RNase A; **d** nucleoid fraction plus DNase I and RNase A as in c; **e** molecular weight markers of λ DNA/Hind III fragments: 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 0.564 and 0.12 kb. All samples were incubated at 37°C for 30 min

with ODC-antibodies. A third part was cut in 0.5 cm horizontal bands and assayed for ODC activity. Three bands of around 24, 1.5, 0.6 kb appeared upon EtBr staining (Fig. 2, lane a, bands α, β and γ). A fourth band of 0.1 kb appeared occasionally as a degradation product of the nucleoid fraction (see Fig. 4, band δ). Bands β and γ coincided with the two ODC-antibody stained bands and presented ODC activity as well (data not shown). Bands β and γ moved to a lower molecular weight when the loaded fraction was treated with 2 units/ml DNase (Fig. 2, lane b) or disappeared from the gel when the loaded fraction was treated with 0.5 unit/ml RNase (Fig. 2, lane c), indicating that RNA is the main component in maintaining the integrity of the β and γ structures. When the nucleoid fraction was treated with DNase (2 units/ml) only the high molecular weight, band α, disappeared (Fig. 2, lane b). All bands were degraded when the loading fraction was treated simultaneously with DNase and RNase (Fig. 2, lane d). Interesting enough, these two bands after treatment with proteinase K moved faster presenting smaller molecular weights complexes (Fig. 3, lane c).

3. A 0.56 kb RNA is bound to ODC

After extensive extraction of nucleoids with phenol, only the RNA band of 0.56 kb RNA was obtained (Fig. 4A, lane e, band γ). The other two bands on lane e (α and δ), correspond to DNA that was co-extracted (band α) and the degradation product (band δ) that appears sometimes. When the two ODC-associated bands β and γ were electroeluted separately from the gel, extracted with phenol/chloroform/isoamyl alcohol and electrophoresed on 1% agarose gel, one band of 0.56 kb was obtained (Fig. 4B, lanes c,d). This experiment indicates that the two ODC-associated RNA fragments are integrated with

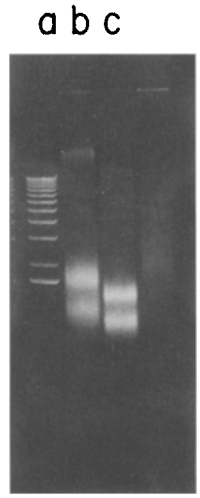

Fig. 3. Electrophoresis of ODC-RNA complexed fragments on 1% agarose gel after treatment with proteinase K. **a** Molecular weight markers of 1 kb ladder: 12, 10,982, 9,964, 8,946, 7,928, 6,910, 5,892, 4,874, 3,816, 2,838, 1,820, 1,636, 0.802, 0.517, 0.506, 0.396, 0.344, 0.298, 0.220, 0.201, 0.154, 0.134 and 0.075; **b** control, as in Fig. 2; **c** nucleoid fractions treated with 0.2 mg proteinase K at 37°C for 30 min

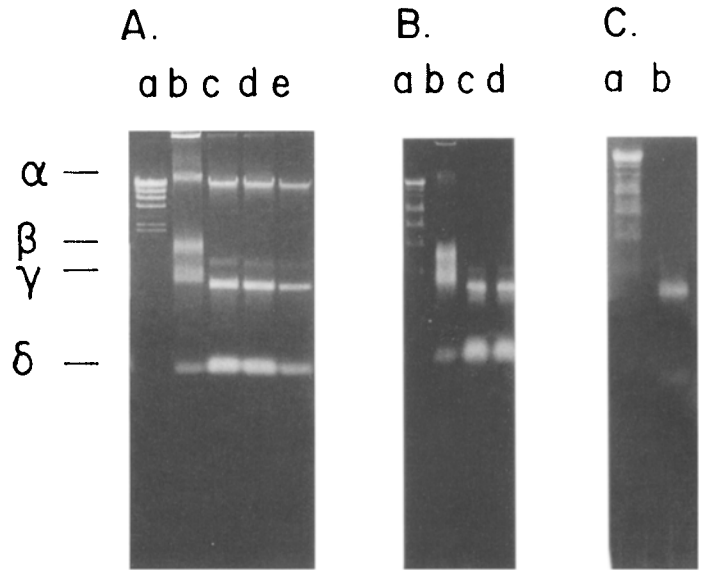

Fig. 4. Electrophoresis on 1% agarose gel of the nucleoid fraction after phenol extractions. **A**, **a** Molecular weight markers; 1 kb ladder; **b** control, not extracted nucleoid fraction; **c** nucleoid fraction extracted once with phenol/chloroform/isoamyl alcohol; **d** extracted twice as in c; **e** extracted three times as in c. **B**, **a** Molecular weight markers as in Aa; **b** control, not extracted nucleoid fraction; **c** The β band was electroeluted from a preparative gel and extracted once with phenol; **d** the γ band was electroeluted from a preparative gel and extracted once with phenol. Electroelution was performed for 1 h at 100 Volts as described (Wienand, 1978) in the presence of 1 mM vanadyl ribonucleotide complex or 1% SDS. **C**, **a** Molecular weight markers as in Aa. **b** RNA was isolated from the nucleoid fraction after DNase treatment as described in Materials and methods

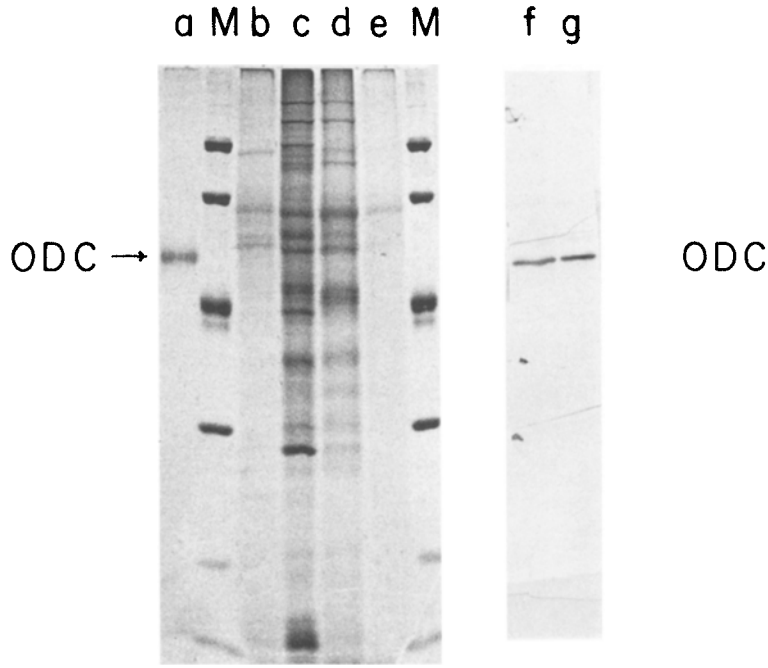

Fig. 5. Separation of the nucleoid proteins on 10% polyacrylamide-SDS gel. Each of the nucleoid band, separated as in Fig. 4 (α, β, γ and δ), was electroeluted, suspended in SDS loading buffer and electrophoresed in 10% polyacrylamide gel. Lanes a to f were stained with $AgNO_3$, whereas lanes f and g were transferred to nitrocellulose membrane and immunostained with ODC-antibody as described in Methods. **a** Purified ODC to homogeneity of *T. thermophilus*; *M* molecular weight markers: 94, 67, 43, 30, 20 and 14 kDa, **b** proteins from the α band of Fig. 4; **c** proteins from the β band of Fig. 4; **d** proteins from the γ band of Fig. 4; **e** proteins of the δ band of Fig. 4; **f** and **g** immunostaining with ODC-antibody of the transferred to nitrocellulose membrane of proteins of β and γ bands, respectively

the same size of RNA but with different proteins attached to it. Following the established procedure for RNA isolation, after treatment of the nucleoid fraction with DNase, only a band of 0.56 kb RNA appeared (Fig. 4C, lane b).

4. Electrophoresis of nucleoid proteins

The four bands of agarose gel (α, β, γ and δ) were electroeluted and electrophoresed separately on a 10% SDS-polyacrylamide gel (Fig. 5). As we can see bands α, β and γ contained a large number of proteins (lanes b, c and d), whereas band δ has only one protein (lane e). The two bands β and γ of the agarose gel which are associated to ODC present different protein patterns. They differ in at least six proteins (lanes c and d), but both are positive to ODC-antibody immunostaining (lanes f and g). Taking into account the apparent similarity in the relative protein composition of the two bands β and γ, we can conclude that the same RNA components can be associated with different proteins.

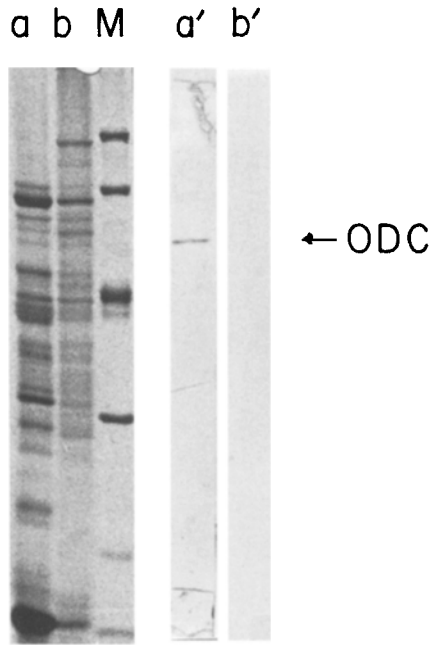

Fig. 6. SDS gel electrophoresis of nucleoid proteins extracted by H_2SO_4. Acid-soluble and acid-insoluble proteins were obtained by treatment of the nucleoid fraction with 0.4 M H_2SO_4 as described in Materials and methods. The extracted materials were electrophoresed on a 10% SDS gel in polyacrylamide. One part of the gel was stained with $AgNO_3$ (lanes a and b) while the rest was immunostained with ODC-antibody (lanes a′ and b′)

5. *Extraction of the non histone proteins*

From the purified nucleoids the acid-soluble and acid-insoluble proteins were obtained with H_2SO_4 as described under Materials and methods. Both fractions were electrophoresed on SDS gels (Fig. 6). One half of the gel was stained with $AgNO_3$ (lane a and b), while the other half was transferred to nitrocellulose paper and stained with ODC-antibodies (Fig. 6, lanes a′, b′). ODC appeared only in the acid soluble fraction indicating that in this microorganism, ODC is an non-histone protein tightly bound to the nucleoid RNA.

6. *Immunoprecipitation of ODC-RNA complex*

The ODC-RNA complex was immunoprecipitated from the nucleoid fraction with ODC antibody bound to protein A-Sepharose beads (Fig. 7). The RNA was eluted from the Sepharose beads with TEL buffer and shows one band of 0.56 kb (Fig. 7, lane c), whereas the non-bound material to the immunoaffinity column does not contain this RNA band (Fig. 7, lane b). When the beads were extracted with SDS and the proteins were run on 10% polyacrylamide gel a major band corresponding to ODC was revealed (data obtained but not shown).

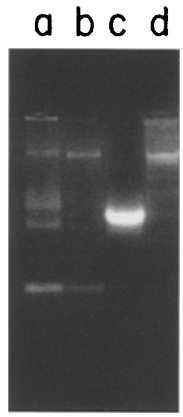

Fig. 7. Electrophoresis on 1% agarose gel of the immunoprecipitated ODC-RNA complex from the nucleoid fraction. The ODC-nucleic acid complex was immunoprecipitated from the nucleoid fraction with ODC-antibody as described Materials and methods. **a** Control, nucleoid fraction; **b** washes of the protein A-Sepharose-ODC-antibody beads precipitated with ethanol; **c** eluted materials with TEL buffer from the protein A-Sepharose-ODC-antibody beads, precipitated with ethanol; **d** molecular weight markers of the λ DNA/Hind III fragments: 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 0.564 and 0.120 kb

Table 1. Summary of the purification procedure of *T. thermophilus* ODC

Steps	Total protein (mg)	Total activity (units)	Spec. act. (units/mg)	Purification (fold)	Yield (%)
Crude extract	3150.0	6482.0	2.0	1.0	100.0
Acidification	1845.0	5925.0	3.2	1.6	91.4
Ammonium-Sulphate (25%–60%)	1018.0	4530.0	4.4	2.1	69.8
DEAE-Biogel A	228.6	3845.0	16.8	8.4	59.3
Heparin-Sepharose	69.7	2100.0	30.1	15.0	32.4
Sephadex-G 150	25.8	935.0	36.2	18.1	14.4
Phenyl-Sepharose	2.2	620.0	282.3	141.1	9.5
Lysine-Sepharose	0.1	414.0	4140.0	2070.0	6.4
Pyridoxamine-Affi-Gel-10	0.06	280.0	4666.0	2333.0	4.3

7. Copurification of ODC with the RNA

ODC was purified from *T. thermophilus*, grown to late log phase, almost to homogeneity with the following purification protocol: a) acidification at pH 6, b) ammonium sulphate, 25%–60% saturation, c) DEAE-cellulose column, d) Heparin-Sepharose column, e) Sephadex G-150, and f) Lysine-Sepharose column. The purification scheme developed for ODC is summarised in Table 1. The specific activity of ODC obtained by this procedure was 283.3 units per mg protein with 137-fold purification. This prepara-

tion was almost homogeneous and a protein band of 55 kDa appeared in SDS-polyacrylamide gel electrophoresis. When this preparation was further run on an agarose gel, it was found that ODC still contains the 0.56 kb of RNA (data not shown).

Discussion

Eventhough it was reported many years ago that ODC is strongly bound to chromatin in many tissues, little is still known about the physiological role of this enzyme to chromatin. That ODC from eucaryotes to archaebacteria is chromatin associated is interesting and it is most probable that this enzyme can bind to a specific nucleotide sequence. Therefore, further studies will be required to elucidate the exact role of ODC in relation to chromatin and the sequential effect of polyamine biosynthesis to the cellular growth.

The findings presented here describe the isolation of ODC, in a complex with RNA in two sets of proteins in the nucleoid fraction of *T. thermophilus*. This RNA, of 0.56 kb in size, was isolated from the nucleoid fraction of gently disrupted cells with lysozyme either by following the ODC activity in sucrose density gradient or by immunoprecipitation with ODC-antibodies. A purification procedure of ODC developed by us, revealed that even after six steps of purification, this enzyme still carries the 0.56 kb RNA on it. Analysis of the complex demonstrates that it is complexed with two major groups of proteins.

The RNA digestion experiments provide some information about the organisation of the RNA and the proteins in the two complexes. That RNA-protein complexes β and γ disappeared by the RNase treatment, together with shifting in small molecular weight forms after proteinase K, indicates that these two complexes are particularly sensitive to ribonuclease. Therefore, the RNA in the complex is not protected by the proteins.

Since this ODC-RNA as a complex with other proteins exists in the nucleoid fraction, it is likely that ODC must play a major role in the RNA processing by providing polyamines in situ. Further clarification of the biochemical properties of the proteins that constitute the nucleoid structure is important for the better understanding of their biological role.

References

Bradford M (1976) A rapid and sensitive method for the regulation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Bartholeyns J (1983) Subcellular distibution of ornithine decarboxylase in rat liver and accessibility of the enzyme to alpha-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase. Life Sci 32: 1305–1312

Choi YD, Dreyfuss G (1984) Isolation of the heterogeneous nuclear RNA-ribonucleoprotein complex (hnRNP): a unique supromolecular assemply. Proc Natl Acad Sci (USA) 81: 7471–7475

Foudouli AC, Kyriakidis DA (1989) Chromatin-associated ornithine decarboxylase at early stages of growth and differentiation of etiolated mono- and dicotyledonous plants. Plant Growth Reg 8: 233–242

Hayashi SI, Canellakis ES (1989) Ornithine decarboxylase antizymes. In: Hayashi SI (ed) International encyclopedia of pharmacology and therapeutics, 129. Pergamon Press, New York

Huang SC, Panagiotidis CA, Canellakis ES (1990) Transcriptional effects of polyamines on polyamine-synthesizing enzymes and ribosomal proteins in *Escherichia coli*. Proc Natl Acad Sci (USA) 87: 3464–3468

Kyriakidis DA, Heller JS, Canellakis ES (1978) Modulation of ornithine decarboxylase activity in *Escherichia coli* by positive and negative effectors. Proc Natl Acad Sci (USA) 75: 4699–4703

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680–685

Morris DR, Boeker EA (1983) Biosynthetic and biodegradative ornithine and arginine decarboxylases from *Escherichia coli*. Methods Enzymol 94: 125–134

Murphy BJ, Brosman ME (1976) Subcellular localization of ornithine decarboxylase in liver of control and growth-hormone-treated rats. Biochem Int 157: 33–39

Oshima T (1983) Novel polyamines in *Thermus thermophilus*: isolation, identification and chemical synthesis. Methods Enzymol 94: 401–411

Panagiotidis CA, Georgatsos JG, Kyriakidis DA (1982) Super induction of cytosolic and chromatin-bound ornithine decarboxylase activities of germinated barley seeds by actinomycin D. FEBS Lett 146: 193–196

Paulin L, Poso H (1983) Ornithine decarboxylase activity from an extremely thermophilic bacterium, *Clostridium thermohydrosulfuricum*. Biochim Biophys Acta 742: 197–205

Poso H, Paulin L (1983) Ornithine decarboxylase activity from an extremely thermophilic bacteria, *Clostridium thermohydrosulfuricum*. Adv Polyamine Res 4: 489–494

Sklaviadis TK, Georgatsos JG, Kyriakidis DA (1985) Purification and properties of ornithine decarboxylase from *Tetrahymena pyriformis*. Biochim Biophys Acta 831: 288–296

Snyder SS, Kreuz DS, Medina VJ, Russel DH (1970) Polyamine synthesis and turnover in rapidly growing tissues. Ann NY Acad Sci 171: 749–771

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci (USA) 76: 4350–4354

Yamazaki K, Nagata A, Kano Y, Imamoto F (1984) Isolation and characterization of nucleoid proteins from *Escherichia coli*. Mol Gen Genet 196: 217–224

Wienand U, Schwarz Z, Felix G (1978) Electrophoretic elution of nucleic acids from gels adapted for subsequent biological tests. FEBS Lett 98: 319–323

Authors' address: A. A. Pantazaki, Laboratory of Biochemistry, Faculty of Chemistry, Aristotle University of Thessaloniki, GR-54006-Thessaloniki, Greece.

Received February 16, 1997