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# ABSENCE OF INACTIVATION OR PHOSPHORYLATION OF ORNITHINE DECARBOXYLASE BY NUCLEAR PROTEIN KINASE NII AND OF IMMUNOLOGICAL CROSS-REACTIVITY BETWEEN RNA POLYMERASE I AND ORNITHINE DECARBOXYLASE

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SUMMARY. Incubation with protein kinase NII did not result in phosphorylation or inactivation of mouse kidney ornithine decarboxylase. Partially purified ornithine decarboxylase preparations contained a protein kinase activity and stimulated the activity of RNA polymerase I. However, these properties were due to contaminating protein(s) since further purification reduced the kinase activity and removal of the ornithine decarboxylase with a specific antiserum did not abolish the ability to stimulate RNA polymerase I. Antibodies to RNA polymerase I did not interact with ornithine decarboxylase and antibodies to ornithine decarboxylase did not interact with RNA polymerase I. These results indicate that: a) mammalian ornithine decarboxylase activity is not regulated by phosphorylation by protein kinase NII or the contaminating kinase, and b) the ability of impure preparations of ornithine decarboxylase to stimulate RNA polymerase I is due to a contaminating unrelated protein.

Ornithine decarboxylase (ODC) is a key enzyme in polyamine synthesis by mammalian cells since it provides the only route for de novo putrescine production (1). It has been suggested that ODC protein may also play a role in the synthesis of RNA by acting as an initiation factor for RNA polymerase I (2-10). An affinity between RNA polymerase I and ODC was demonstrated by the ability of ODC to bind to an RNA polymerase I affinity column (2). Although relatively impure preparations were used in these studies, RNA synthesis by RNA polymerase I in vitro was enhanced when ODC was added (2,3) and ribosomal RNA synthesis by Xenopus oocytes was increased by micro-injection of ODC (4). These results have been interpreted as indicating that ODC may be synonymous with a regulatory subunit of RNA polymerase I (5). Kuehn and colleagues have reported that in the slime mold, Physarum polycephalum, ODC is phosphorylated

Abbreviations used: ODC; ornithine decarboxylase; RIA, radioimmunoassay; DFMO, α-difluoromethylornithine; PAGE, polyacrylamide gel electrophoresis.

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by a polyamine dependent protein kinase and that this phosphorylated ODC protein (which had a M.W. of 70,000) stimulates transcription of ribosomal genes by RNA polymerase I in chromatin preparations (6,7,10). The phosphorylation of ODC also led to the loss of its decarboxylase activity and it was suggested that the phosphorylation provided a feed-back control of polyamine biosynthesis since the kinase was activated by polyamines (7,10). Despite apparent differences in the subunit compositions some similarities exist between polyamine dependent kinase from P. polycephalum and the well known protein kinase NII (11-15). Further, a similar kinase and substrate protein of M.W. 70,000 was reported to be present in bovine spermatozoa (8) Ehrlich ascites tumor cells (9) and rat liver (10). Since it has been shown that purified RNA polymerase I contains the two polypeptides of the protein kinase NII and that the activity of the polymerase is increased by the polyamine dependent phosphorylation carried out by this kinase (14,16,17), it appeared possible that ODC might be a substrate for the NII kinase and be present in the RNA polymerase I complex. Antibodies to ODC (18) and to RNA polymerase I (16) and highly purified enzyme preparations of known specific activity and purity were used to investigate these possibilities.

## MATERIALS AND METHODS

Materials.

ODC purification, assay and antibody production. ODC was assayed and purified starting from kidneys of mice treated with androgens (19). The purity of the preparation was determined by both RIA (18) and by comparison of the specific activity with that predicted for the homogeneous enzyme on the basis of titration with the suicide substrate DFMO (19). Some experiments were carried out with material purified through the pyridoxamine-affinity column stage (specific activity 11 μmol $CO_2$ released/ min/mg) and some with material further purified by gel filtration to a specific activity of 50 μmol $CO_2$ released/min/mg. Antiserum was raised in rabbits immunized with the homogeneous mouse kidney ODC (18). Immunoprecipitation of ODC protein (18) and PAGE (19) were carried out as previously described. A marker labeled preparation of ODC was labeled by reaction with [5-$^3$H]DFMO (19).

Purification of RNA polymerase I and protein kinase NII and production of antibodies to RNA polymerase I. Purification of RNA polymerase I and production of antibodies were achieved by the previously published protocol (16). One unit of RNA polymerase I corresponds to 1 nmol UMP incorporated/30 min at 30°C. Protein kinase NII was purified through the phosvitin-Sepharose chromatography stage as described previously (13). One unit of kinase corresponds to 1 pmol of [$\gamma^{32}$P]ATP transferred to protein substrate/30 min at 30°C.

## RESULTS

ODC was purified about 2,500-fold from kidneys of androgen-treated mice. This preparation was about 20% pure based on the titration with DFMO (19) and RIA (18). It was then incubated with protein kinase NII isolated from Morris hepatoma (Table 1). As indicated in Table 1, no loss of ODC activity occurred even when the medium contained 2.5 mM spermine which maximally activates the NII kinase. The phosphorylation of protein under these conditions was also studied using [$\gamma$-$^{32}$P]ATP. A substantial incorporation of $^{32}$P into protein occurred in the presence or absence of the NII kinase; addition of exogenous protein kinase NII did not increase the phosphorylation of proteins in the ODC preparation beyond that observed with the endogenous kinase(s) present (data not shown).

Next, the effect of the same partially purified ODC on RNA polymerase I activity was determined (Table 2). The polymerase activity was stimulated by 2-3 fold. These results indicate that the protein kinase or other factors present in the ODC preparation stimulated RNA polymerase I activity (Table 2).

TABLE 1
EFFECT OF PROTEIN KINASE NII ON ODC ACTIVITY

| Additions to incubation medium | ODC activity remaining (nmol $CO_2$ released/min/aliquot) |
|---|---|
| Experiment A | |
| None | 0.79 ± 0.06 |
| 5 mM $MgCl_2$, 1 mM ATP | 0.82 |
| 46 units protein kinase NII | 0.80 |
| 5 mM $MgCl_2$, 1 mM ATP, 46 units protein kinase NII | 0.77 |
| Experiment B | |
| None | 1.00 |
| 5 mM $MgCl_2$, 1 mM ATP | 1.00 |
| 5 mM $MgCl_2$, 1 mM ATP, 2.5 mM spermine | 0.93 |
| 5 mM $MgCl_2$, 1 mM ATP, 2.5 spermine + 46 units protein kinase NII | 1.03 |

The incubation mixture contained 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA 2.5 mM dithiothreitol, 0.03% Brij 35, ODC (20% pure; 0.8 µg protein in experiment A and 0.9 µg in experiment B) and the additions shown in a total volume of 0.1 ml. After incubation at 37° C for 30 min, 0.01 ml aliquots were removed and assayed for ODC activity. The indicated protein kinase NII activity refers to the enzyme activity under standard conditions with casein as acceptor.

TABLE 2
EFFECT OF ODC ON RNA POLYMERASE I ACTIVITY

| Enzyme preparation added | RNA polymerase activity (units) |
|---|---|
| Experiment A | |
| RNA polymerase I | 7.7 |
| ODC | 0 |
| RNA polymerase I + ODC | 22.3 |
| Experiment B | |
| RNA polymerase I | 7.7 |
| RNA polymerase I + supernatant from ODC + control serum | 16.6 |
| RNA polymerase I + supernatant from ODC + anti-ODC serum | 15.2 |

In both experiments, 275 ng (0.55 pmol) of RNA polymerase I was added as shown. In experiment A, 80 ng of ODC protein (c20% pure) was added. This contained 4.6 units of protein kinase. In experiment B, two samples of the same ODC protein (180 ng in 0.2 ml) were incubated with control serum or anti-ODC followed by goat-anti-rabbit serum and removal of the precipitate (19). Both supernatants contained 150 units/ml of protein kinase activity, but the ODC activity was 0 in samples treated with anti-ODC and 25 nmol $CO_2$/min/ml in the sample treated with control serum. Aliquots equivalent to 3 units of protein kinase and 45 ng of the original protein were added into the RNA polymerase assays.

However, the ability to stimulate RNA synthesis and the kinase are unlikely to be an integral part of the ODC protein for two reasons. First, further purification by gel filtration increased the ODC specific activity 5-fold from 11 μmol $CO_2$/min/mg to 50 μmol/min/mg but decreased the protein kinase activity from 58 units/μg to 11 units/μg. Second, the supernatant obtained after immunoprecipitation of the partially purified ODC with a specific antiserum to ODC retained its capacity to stimulate RNA polymerase I (Table 2, Experiment 2). Furthermore, the protein phosphorylated was not ODC since less than 4% of the incorporated radioactivity was precipitated by antibodies to ODC (Table 3) and the radioactive bands detected after separation of the protein by PAGE did not correspond to an authentic marker of [$^3$H]DFMO-labeled-ODC (Figure 1).

The possibility that RNA polymerase I and ODC have common subunits or protein sequence in common was tested by solid phase radioimmunoassay using monospecific antibodies to ODC and to RNA polymerase I. As shown in Table 4 no cross-reactivity was detected. Also, in other experiments, the antibodies

TABLE 3
IMMUNOPRECIPITATION OF PHOSPHORYLATED PROTEIN USING ANTIBODIES TO ODC

| Addition | Total radioactivity precipitated (cpm) |
|---|---|
| Antiserum to ODC | 586 |
| Control antiserum | 339 |
| 5% trichloracetic acid | 6125 |

ODC (0.3 μg) was incubated as in Table 1 in the presence of 5 mM $MgCl_2$ and 50 μCi (0.17 μM) [$\gamma$-$^{32}$P]ATP. The sample was diluted to 2 ml with 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2.5 mM dithiothreitol, 0.03% Brij 35, and dialyzed against 4 changes of 200 volumes of this buffer. The final dialysate contained 490,000 cpm in 2 ml and 0.025 aliquots were used for immunoprecipitation (19). All of this radioactivity was insoluble in 5% trichloroacetic acid.

to RNA polymerase I did not precipitate any radioactivity when incubated with DFMO-labeled-ODC under conditions in which all of the label was precipitable by antibodies to ODC (results not shown).

## DISCUSSION

Our experiments do not support the concepts that mammalian ODC is inactivated by mammalian nuclear protein kinase NII or that ODC plays any role in the action of RNA polymerase I. The results show definitively that mouse kidney ODC is not inactivated or phosphorylated by nuclear protein kinase NII.

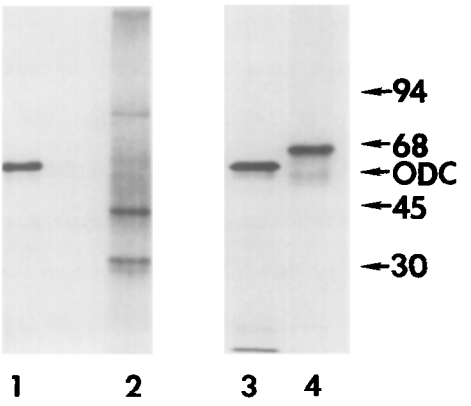


Figure 1. Separation between ODC and phosphorylated protein. After incubation with [$\gamma$-$^{32}$P]ATP as in Table 3, ODC preparations were separated by PAGE under denaturing conditions. Lanes 1 and 3 show marker preparations of ODC labeled by reaction with $^{3}$H-DFMO. Lanes 2 and 4 show the proteins into which $^{32}$P was incorporated. In Lane 2, the ODC used was the material purified by pyridoxamine-affinity chromatography and in Lane 4, the ODC was further purified through the gel filtration step (19).

TABLE 4
RADIOIMMUNOASSAY OF RNA POLYMERASE I AND ODC

| Purified enzyme added | [$^{125}$I]-protein A bound in presence of serum shown (cpm ± S.E.M.) | | |
|---|---|---|---|
| | Control Serum | Anti-RNA Polymerase I | Anti-ODC |
| RNA polymerase I | 214 ± 21 | 5147 ± 223 | 234 ± 57 |
| ODC | 191 ± 35 | 202 ± 48 | 5139 ± 117 |

Electrophoretically homogenous preparations (16,19) of RNA polymerase I (2 μg) or ODC (50 ng) were adsorbed to polystyrene microtiter wells and incubated with the antiserum shown followed by [$^{125}$I]-protein A to quantitate the immunocomplex formation as previously described (20).

The subunit M.W. of rodent ODC is 50-55,000 as determined by labeling with DFMO (19,21), by immunoblotting techniques using extracts processed to avoid proteolytic degradation (22) or by immunoprecipitation of [$^{35}$S]methionine labeled ODC (23). The protein of M.W. 68-70,000 detected as the substrate of the protein kinase isolated by Kuehn and colleagues (8-10) is, therefore, not the correct size to be ODC. On the other hand, recent studies have suggested that cytoplasmic casein kinase II (casein kinase G) can phosphorylate ODC, but that such phosphorylation did not alter the decarboxylase activity (E. M. Chambaz, personal communication). These results indicate that casein kinase G, nuclear protein kinase NII and the slime mold kinase have different substrate specificities.

The absence of any antigenic cross-reactivity between purified RNA polymerase I and ODC indicates that it is unlikely that ODC protein is a regulatory subunit of RNA polymerase. The antiserum to RNA polymerase I has been shown to react with all of the subunits of this enzyme except the two smallest which have M.W. of 19,500 and 17,500 (16). Our experiments could be criticized on the grounds that the ODC used was obtained from mice and the RNA polymerase I from rat tumors. However, it is known that the antiserum to mouse kidney ODC reacts almost equally well with rat liver ODC and that the proteins from the two species are very similar (18,24). The ODC antiserum reacts not only with the active enzyme but also with ODC inactivated by either addition of DFMO, complexation with "antizyme" or aggregation in the absence

of thiols (18,24). It is, therefore, unlikely that the protein modified by phosphorylation or transglutamination (5) would not interact with the antibody. In the experiments in which ODC protein was shown to act as a kinase or transglutaminase substrate and to stimulate ribosomal RNA synthesis (reviewed in 5 and 10) no convincing evidence of the homogeneity of the protein was given and the ODC activity was much less than that of the partially purified preparations which we have used. It is, therefore, possible that the effects observed were due to the presence of contaminating proteins and this interpretation would be consistent with our results.

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