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MULTIPLE IONIC FORMS OF ORNITHINE DECARBOXYLASE DIFFER IN DEGREE OF PHOSPHORYLATION

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Two major ionic forms of ornithine decarboxylase were separated by column chromatography of extracts of kidneys from androgen-treated male CD-1 mice on DEAE-Sepharose CL-6B, and purified individually to apparent homogeneity. On SDS-PAGE, a single major protein band of M_r 50000 was present in each. When incubated with casein kinase II, purified from rat liver cytosol, only one form of the enzyme, which represented 20% of the total ornithine decarboxylase in the tissue, became phosphorylated. The major form, which was eluted later from the column, could be phosphorylated only after treatment with alkaline phosphatase, indicating that the phosphatase removed enzyme-bound phosphate already attached at the casein kinase II phosphorylation site. Evidence for the occurrence of a phosphorylated form of the enzyme in kidneys of dexamethasone-treated rats is also presented.

Ornithine decarboxylase (ODC, E.C. 4.1.1.17) is a major site of regulation in the biosynthesis of polyamines in mammalian cells. Because of the importance of these components in controlling cell growth, proliferation, differentiation and function (1-3), the properties and regulation of the enzyme have been studied in a variety of cells. The enzyme is highly inducible, and following treatment of appropriate cells with agents such as steroid or peptide hormones, growth factors, mitogens, carcinogens, drugs, tumour promoters or viruses, increases in ODC activity of 5-500 fold have been reported (4-6). In stimulated systems, the increased ODC activity is due to an increase in the amount of ODC protein. The accumulation of ODC seems to depend on an increased rate of synthesis, resulting from stimulation of transcription and/or translation (2,3,7), and on an increase in stability of ODC, as shown by an increase in half-life (8). The occurrence of multiple ionic forms of ODC can be demonstrated by column chromatography of crude tissue extracts on DEAE-Sepharose CL-6B. This was observed in thymus and kidney of control rats, and in the kidney of dexamethasone-treated animals (9). The same distribution of activity has been observed in HTC cells (10), in liver of control and chloroform-treated rats (11), in heart of control and

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isoproterenol-treated rats (12,13), and in kidney of control and androgen-treated mice (14). The distribution of activity between the two forms can vary in different cell systems. These separate forms of the enzyme have a M_r of 50000-54000, and do not differ in kinetic properties (10,13), but may differ in their half lives *in vivo* (11,15). It has also been demonstrated that these multiple ionic forms are not due to artifactual interactions with cofactors or with other proteins during preparation, since the charge difference is maintained even in the presence of 8 M urea (16). The origin of the difference in ionic properties responsible for the separation of the two main forms is not yet known. It is possible that different proportions of acidic amino acids could account for the charge difference, or addition of a negative charge by a posttranslational modification could result in separation of the native and modified molecules.

Several recent studies show that at least part of the ODC in several tissues may exist as a phosphoprotein (17). It has also been demonstrated that highly purified ODC from heart of isoproterenol-treated rats can be phosphorylated *in vitro* by casein kinase II from liver cytosol (18). Phosphorylation of ODC from mouse kidney by casein kinase II from rabbit reticulocytes has also been demonstrated (17), although the mouse enzyme was not phosphorylated by a nuclear protein kinase (19).

In this paper, we describe experiments designed to investigate the possible relationship between the phosphorylation of ODC and the occurrence of multiple ionic forms of the enzyme. In our experiments, we purified the two forms from kidney of androgen-treated mice, and investigated conditions necessary for their phosphorylation by casein kinase II. We found that form A, the peak eluted first from the ionic exchange column, could be phosphorylated as purified. Phosphorylation of form B, the more acidic species, required prior treatment with a phosphatase before phosphorylation would occur. The same was true for form B purified from kidney of dexamethasone-treated rats.

MATERIALS AND METHODS

Animals: Adult male mice of CD-I strain were obtained from the Animal Care Centre at the University of British Columbia. The animals received testosterone propionate in saline (100 mg/kg) by subcutaneous injection, and were sacrificed 24 hours later. Female Wistar rats, body weight 60-80 g, were also obtained from the Animal Care Centre. The rats received 200 µg dexamethasone in ethanol-saline by intraperitoneal injection, and were sacrificed 5 hours after treatment. New Zealand rabbits, 2-3 Kg body weight, were used for production of antiserum.

Preparation of Enzymes and ODC Antiserum: ODC was purified from extracts of kidney prepared by homogenizing the tissue in buffer A (50 mM Tris, 3 mM dithiothreitol, 0.1 mM EDTA, pH 7.3), and centrifuging the homogenate at 20000 g for 20 minutes at 4°. The supernatant was used as the source of ODC. The

enzyme was purified by chromatography in 3 steps - DEAE-Sepharose CL-6B, as described previously (9), but with a change in the limits of the NaCl gradient from 0.125-0.3 M to 0.125-0.250 M, an AffiGel-Pyridoxamine phosphate affinity column (20), and a Heparin-Sepharose column (21).

The two peaks of activity eluted from the DEAE-column were designated A and B, and were purified individually through the next stages when preparing ODC for phosphorylation studies. In preparing ODC for the production of antiserum, the activity in peaks A and B was pooled, and purified as a single sample. Antiserum to mouse kidney ODC was developed by injecting purified enzyme into a rabbit by the schedule used in other laboratories (22). ODC was assayed by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine HCl as previously described (9). Casein kinase II was purified from rat liver by a published procedure (23).

Phosophorylation of ODC: Aliquots of purified ODC (containing up to 14 µg of enzyme) were incubated in 210 µl (final volume) with 3 units of casein kinase II (1 unit - catalyzes incorporation of 1 nmol P into casein in 15 minutes) and γ[^{32}P]ATP (2.25 µCi/nmol) in the presence of 10 mM $MgCl_2$ and 114 mM KCl in 50 mM Tris-HCl, pH 7.0, at 30° for 20 minutes. After precipitation with 10% TCA and washing the precipitate, the proteins were dissolved in buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, and 10% glycerol) by boiling, and analyzed on a 12.5% SDS-PAGE (24). Where indicated, some samples of ODC were incubated with Agarose-linked alkaline phosphatase before phosphorylating as above. Pretreatment with 10 units of phosphatase was carried out in 200 µl of 50 mM Hepes, pH 7.5, 3 mM dithiothreitol, 0.1 mM EDTA and 1 mM $MgCl_2$ at 30° for 10 minutes. The phosphatase was removed by centrifugation.

Immunoprecipitation of ODC: After incubation of ODC with casein kinase II and labelled ATP, EDTA was added to the sample (final concentration 11 mM). ODC was immunoprecipitated by incubation with rabbit antiserum to mouse ODC, followed by incubation with protein A-Sepharose. The protein A-bound immunocomplex was washed extensively with 50 mM Hepes, pH 7.3 containing 3 mM dithiothreitol and 0.1 mM EDTA. The proteins were solubilized by boiling the precipitate with SDS-containing buffer as above, and analyzed on 12.5% SDS-PAGE.

Materials: L-[1-^{14}C]ornithine HCl (55 mCi/mmol) was received from Amersham, and γ[^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham or New Enland Nuclear. Protein A-Sepharose and Agarose-linked alkaline phosphatase were obtained from Sigma. Other supplies were of reagent grade.

RESULTS

Preparation of Individual Species of ODC: The separation of the two major species of ODC in preparation of kidney from testosterone-treated mice by chromatography on a DEAE-Sepharose CL-6B column shown in figure 1 is very similar to that reported by others (14). Each of the two forms, labelled A and B in order of elution was further purified as described in methods. Two forms of ODC were also prepared from kidneys of dexamethasone-treated rats, and purified by the same procedure. The purity of the various species of ODC has been assessed by staining SDS gels with Coomassie blue, and in each case, only one stained protein was evident, M_r 50000-52000 Da, which could be immunoprecipitated with antiserum to mouse ODC (data not shown). The

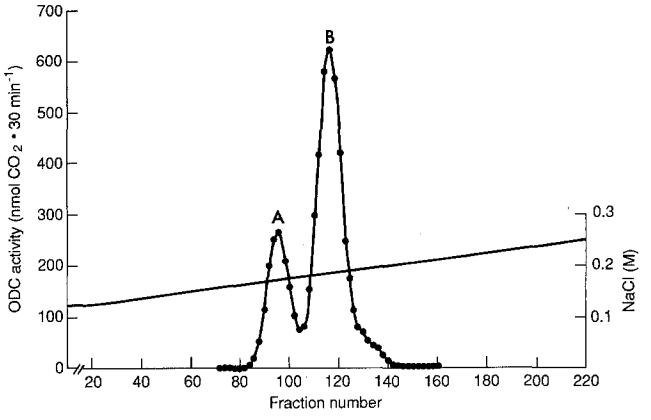

Figure 1. Separation of mouse kidney ODC activity into ionic species. A cytoplasmic fraction of kidney homogenate prepared from tissue of androgen-treated mice was applied to a (1.5 X 24 cm) column of DEAE-Sepharose CL-6B equilibrated with buffer (50 mM Hepes, 3 mM dithiothreitol, 0.1 mM EDTA, pH 8.0) containing 0.125 M NaCl. After washing with the same buffer, a 400 ml gradient of NaCl (0.125-0.250 M) in buffer was used to elute ODC activity.

preparations were not homogeneous, since a trace contaminant appeared when the enzyme was phosphorylated (see below). In spite of the extensive purification obtained, we have not achieved the very highest specific activity of the enzyme which has been reported (21,25), although our results have been in the range reported by other groups for the mouse system (26). In numerous preparations of ODC for the phosphorylation experiments and for antibody production the problem seems to be loss of activity rather than dilution by contaminants. The specific activity of the two forms of rat ODC after 60000 fold purification was in the range of 1-3 μmole CO_2/min/mg, similar to that reported for the rat heart (12).

Phosphorylation of Separate Species of ODC: Autoradiograms of the gels developed with samples of mouse kidney ODC species A and B following incubation with casein kinase II and $\gamma[^{32}P]$-ATP are shown in figure 2. The subunit of casein kinase II is autophosphorylated (M_r 27000 Da), and the kinase preparation also contains a phosphorylatable contaminant (M_r 36000 Da). Comparison of lanes A-1 and B-1 shows that form A of the mouse ODC was phosphorylated (M_r 49000 Da) directly by the kinase, but form B was not. Both A and B were phosphorylated if treated with alkaline phosphatase prior to incubation with the casein kinase II (lanes A2 and B2). Treatment with the phosphatase did not obviously affect the extent of phosphorylation of form A, but significant phosphorylation of form B was apparent only when the ODC was first treated with the phosphatase.

Results in figure 3 show that phosphorylated A and B from mouse kidney can be immunoprecipitated by antiserum to mouse ODC. Lane 1, figure 3A

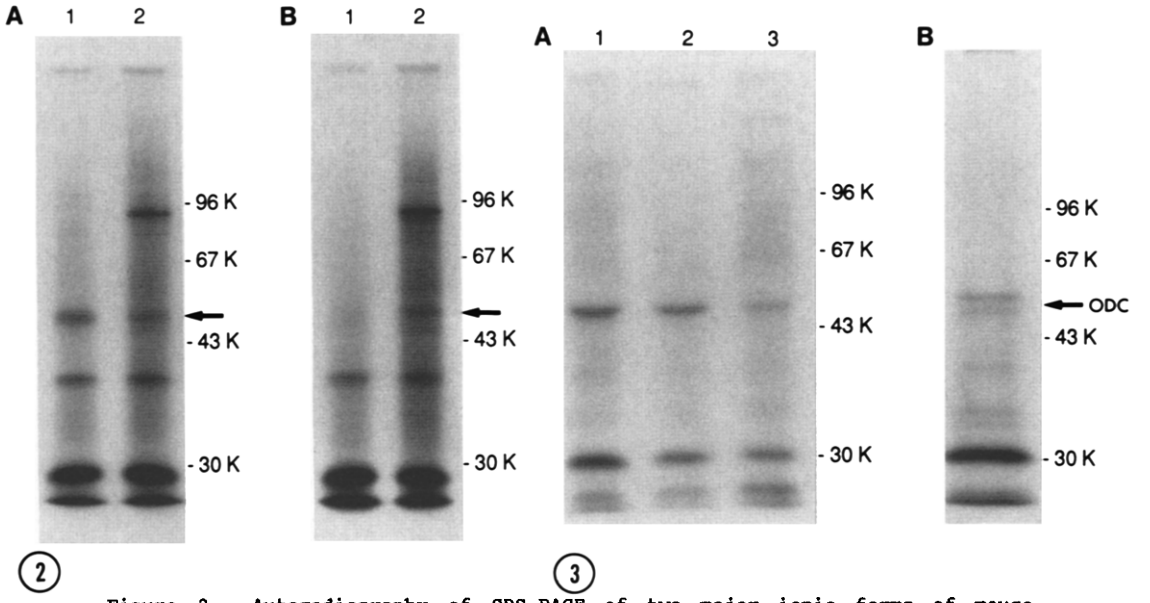

Figure 2. Autoradiography of SDS-PAGE of two major ionic forms of mouse kidney ODC phosphorylated by casein kinase II - effect of prior treatment with alkaline phosphatase.

Samples of each of purified A and B were incubated with casein kinase II and $\gamma[^{32}P]ATP$ as described in Methods, and the proteins analyzed in lanes A1 and B1 respectively. Identical samples were treated with alkaline phosphatase (see Methods), and after removal of the phosphatase, were then incubated with casein kinase II and labelled ATP. The proteins were analyzed on gels shown in lanes A2 (for species A) and B2 (for form B).

Figure 3. Immunoprecipitation of ODC preparations phosphorylated by casein kinase II. Samples of different forms of ODC were treated with rabbit antiserum to mouse ODC and with protein A-Sepharose after incubation with casein kinase II and $\gamma[^{32}P]ATP$, and the proteins analyzed on SDS-PAGE as described in Methods.

A - Lane 1 - mouse A form phosphorylated directly with casein kinase II;
Lane 2 - mouse A form phosphorylated with casein kinase II after treatment with alkaline phosphatase;
Lane 3 - mouse B form phosphorylated after treatment with alkaline phosphatase.

B - Rat B form phosphorylated with casein kinase II after treatment with alkaline phosphatase.

contains form A which was phosphorylated directly, while lanes 2 and 3 contain, respectively, form A and B which were treated with phosphatase before incubation with the kinase. These data also show that the contaminant in the mouse ODC preparations, M_r 88000 Da, is not precipitated by antiserum to ODC. Figure 3B shows that form B from rat kidney, after treatment with phosphatase, can be phosphorylated by casein kinase II, and the main phosphorylated protein was immunoprecipitated with the antiserum. No phosphorylation of the B form of the rat enzyme could be demonstrated unless the enzyme was first treated with the phosphatase.

DISCUSSION

Our studies support the suggestion that phosphorylation of ODC by casein kinase II is biologically significant. The occurrence of phosphorylated ODC

in mouse kidney was indicated by studies with a monoclonal antibody 3B9, which would not recognize ODC phosphorylated by casein kinase in vitro, and would precipitate only 60-80% of ODC in the tissue extracts while a polyclonal would immunoprecipitate all of the ODC activity in tissue preparations from rats, hamsters or humans (17).

While several groups have reported the existence of multiple charged forms of ODC, separable by DEAE-anion exchange chromatography (9-12,14) or by isoelectric focussing (27), the reason for their separation has not been identified. It has recently been demonstrated that the two isoforms of ODC maintain their charge difference even in the presence of 8 M urea, which should disrupt interaction of ODC with other proteins (16). This shows clearly that the multiple forms of ODC do not result from non-specific or specific protein associations. Our results show a difference in phosphate content between A and B. Since A can be phosphorylated directly by casein kinase II, this monomer appears to represent unphosphorylated, or native ODC. On the other hand, B seems to be phosphorylated in vivo, since casein kinase II failed to phosphorylate the species unless it was first treated with the phosphatase. The previous report that mouse kidney ODC could not be phosphorylated in vitro may be explained by a difference in specificity of the nuclear kinase used, or by the use of naturally occurring phosphorylated ODC, as substrate (19), since this form of ODC predominates in androgen-stimulated kidney.

The physiological role of the phosphorylation is uncertain. It has been termed a "silent" phosphorylation, since the kinetic properties of rat heart ODC were not altered by phosphorylation with casein kinase II (18). However, it may affect the stability of ODC. In several stimulated systems, the half life of ODC was significantly increased. In those experiments where the multiple forms of ODC were studied separately, the half-life of the more acidic form, corresponding to form B in our studies, or form II of Mitchell (15), was much longer than in control tissues. The half-life of the first ionic species was similar in control and stimulated tissues (11,15). We have observed that the ODC activity in a rat kidney preparation was decreased by 50-70% by treatment with alkaline phosphatase for 30 min, and at the end of that time, only form A was present in the extract as shown by DEAE-anion exchange chromatography (unpublished observation). Form B could have been inactivated or degraded following dephosphorylation. In any case, the importance of the phosphorylation is also suggested by the conservation of the phosphorylation site, ser-303 (28), in mouse (29), rat (30), hamster (31) and human (32) enzyme.

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