Multisite Phosphorylation of Ornithine Decarboxylase in Transformed Macrophages Results in Increased Intracellular Enzyme Stability and Catalytic Efficiency*

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Ornithine decarboxylase (ODC) is the initial inducible enzyme in the polyamine biosynthetic pathway. In the transformed macrophage-derived RAW264 cell line, ODC was overproduced and existed in both unphosphorylated and phosphorylated forms. To date, the only protein kinase known to phosphorylate mammalian ODC is casein kinase II (CKII). ODC was phosphorylated in vitro by CKII and subjected to exhaustive sequential proteolysis with trypsin and V8 protease. Two-dimensional peptide mapping showed only one phosphopeptide; two-dimensional phosphoamino acid analysis of the phosphopeptide revealed only 32P-labeled serine. ODC was metabolically radiolabeled with 32P in RAW264 cells and also subjected to proteolysis, two-dimensional peptide mapping, and phosphoamino acid analysis. Two phosphopeptides were generated from the metabolically radiolabeled ODC, including one that migrated similarly to the peptide phosphorylated by CKII in vitro. Each of the in situ radiolabeled ODC peptides contained both 32P-labeled serine and threonine residues. Thus, in RAW264 cells, ODC is phosphorylated on at least one serine residue in addition to that phosphorylated by CKII and on at least two threonine residues. Phosphorylated ODC had an increased stability to intracellular proteolysis compared with unphosphorylated ODC, their half-lives being 49.2 ± 3.78 and 23.9 ± 2.6 min (p = 0.001), respectively. The phosphorylated and unphosphorylated forms of ODC were independently purified to homogeneity. Kinetic analysis revealed that the catalytic efficiency of the phosphorylated form of ODC was 50% greater than that of the unphosphorylated form; the unphosphorylated ODC had a V max of 20.54 ± 1.65 μmol/min/mg, whereas the phosphorylated form had a V max of 30.61 ± 2.6 μmol/min/mg (p = 0.005). Phosphorylation of ODC by CKII has no effect on enzyme activity. Taken together, these findings demonstrate that regulation of ODC activity is governed by as yet unidentified protein kinases.

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the initial rate-limiting enzyme in polyamine biosynthesis. Putrescine, the product of ODC catalysis, and the subsequent metabolic pathway products spermidine and spermine are small aliphatic nitrogenous bases that fulfill structural and regulatory roles in protein and nucleic acid biosynthesis and function (1). Cellular ODC activity increases rapidly and transiently in response to trophic, proliferative, or toxic stimuli (2). Both somatic cell genetic and pharmacologic experimental approaches have demonstrated that expression of ODC is essential for cell growth. Regulation of enzyme expression at the levels of DNA transcription, mRNA translation, and protein turnover have been described (for reviews, see Refs. 3 and 4). However, little is known about potential posttranslational regulation of ODC.

Multiple forms of ODC protein occur in a variety of cells and tissues. Anion exchange chromatography resolves several peaks of ODC activity in extracts prepared from calf liver (5), rat thymus and kidney (6), rat heart (7), and HT29 cells (8). IEF analysis of ODC isolated from HTC cells (9), kidney (10), skin (11), and RAW264 cells (12) shows that multiple isolectric forms of ODC protein are present. ODC is phosphorylated in situ in murine macrophage-derived, Abelson virus-transformed RAW264 cells (13), Friend erythroleukemia cells (14), 651-3 myeloma and monkey COS 7 cells (15), a murine mammary tumor variant cell line, EXOD-1 (16), and a variant rat hematoma HT29 cell line, DH23b (17). A consensus sequence for casein kinase II (CKII)-catalyzed phosphorylation is conserved in the eucaryotic ODC amino acid sequence around serine 303 (18–20). CK II phosphorylates ODC on serine in vitro (21–23), with a K m similar to that of its other cellular substrates (22); however, this phosphorylation has no effect on ODC activity. Only radiolabeled phosphoserine residues were detected in ODC isolated from metabolically radiolabeled 653-1 (15), EXOD-1 (16), and DH23b cells (17). Rosenberg-Hasson et al. (15) reported that in COS-7 cells transiently transfected with wild type and Ser303 → Ala mutant ODC expression vectors, serine 303 is the major (if not the only) amino acid residue phosphorylated on the enzyme protein. Furthermore, mutation of ODC serine 303 to alanine had no effect on the cellular ODC protein half-life or enzyme activity.

In contrast, our preliminary findings indicated that RAW264 cell ODC phosphorylated in situ has a greater catalytic capacity than the native form of the enzyme (24). We have previously shown that ODC is phosphorylated in RAW264 cells on both serine and threonine residues (25). These findings led us to

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1 The abbreviations used are: ODC, ornithine decarboxylase; IEF, isoelectric focusing; CKII, casein kinase II; PMSP, phenyl methylsulfonyl fluoride; DTT, dithiothreitol; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PYP, pyridoxal 5’-phosphate.
question whether ODC was subject to phosphorylation in RAW264 cells at additional sites to that phosphorylated by CKII. In this report we demonstrate by comparative two-dimensional phosphopeptide mapping and phosphoamino acid analysis that in the RAW264 cell line ODC is phosphorylated on at least three amino acid residues in addition to that phosphorylated by CKII. We also conducted studies to investigate the significance of intracellular phosphorylation of ODC in RAW264 cells. The results demonstrate two functional consequences of the in situ phosphorylation of ODC. The phosphorylated form of the enzyme was more resistant to proteolysis in situ than the unphosphorylated form. In addition, kinetic analysis demonstrated that the purified phosphorylated form of ODC has a higher catalytic capacity than the purified unphosphorylated form of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and fetal bovine serum were obtained from Life Technologies, Inc. Nitrocellulose was obtained from Schleicher & Schuell. Urea was from Polyscience (Warrington, PA). Triton X-100, phenylmethylsulfonyl fluoride (PMSF), diithiothreitol (DTT), and sodium dodecyl sulfate were from Boehringer Mannheim. [32P]Orthophosphate, [35S]ATP, and [32P]methylene were purchased from ICN (Irvine, CA). Panofsin was obtained from Calbiochem. Molecular weight markers for silver staining, pH 4–6 ampholines, goat anti-rabbit IgG-horseradish peroxidase, and Affi-Gel 10 were obtained from Bio-Rad. Molecular weight standards for autoradiography, pH 3–10 ampholines, DEAE-Sepharose CL-6B, and Mono-Q-Sepharose were obtained from Pharmacia Biotech Inc. DL-3-10-amino-iso-butylxanthine, Brij 35, sodium orthovanadate, ATP, trypsin, soybean trypsin inhibitor, o-phospho-1-serine, o-phospho-1-threonine, and o-phospho-1-tyrosine were purchased from Sigma. The cellulose thin layer chromatography (TLC) plates were from Eastman Kodak Co. Hunter Thin Layer Peptide Mapping System model HTLE-7000 was obtained from C.B.S. Scientific Co. (Del. Mar., CA). The Betscape 603 blot analyzer was purchased from Betagen Corp. (Waltham, MA). All other reagents were of analytical grade.

Cell Culture—Abelson virus-transformed, murine macrophage-derived RAW264 cells (26) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum, 2.2 mM sodium bicarbonate, 15 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells for ODC or radiolabeling assays were plated in six-well cluster plates at 2 × 10^5 cells/well in 2 mL of DMEM and were serum starved overnight. These conditions have previously been shown to lead to maximal ODC induction (27). Cells were stimulated by the addition of 10% fetal bovine serum, 1 mM Ca^2+, 1 mM isobutylmethylxanthine, and 1 μg/ml lipopolysaccharide.

Electrophoresis, Isoelectric Focusing, and Immunoblotting—SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described (28) using 8% gels. Gels were stained with silver (29) or transferred to nitrocellulose for 4 h at 36 V in 200 mM glycine buffer, pH 9.1, and 20% methanol. Apparent molecular weight values were determined by interpolation on a plot of log M_r versus mobilities of standard proteins.

Pure ODC and cell extracts were analyzed by a high resolution slab gel IEF technique as described previously (12). Briefly, samples solubilized in 4.5 ml urea, 1% Nonidet P-40, 2.5% 2-mercaptoethanol, 25% glycerol, and 0.8% pH 4–6 ampholines were separated on a 4.5% polyacrylamide slab gel containing 8% urea, 1.5% Nonidet P-40, 1.2% pH 3–10 ampholines, and 4.8% pH 4–6 ampholines for 16 h at 200 V. Quantitative transfer of cellular proteins from IEF gels to nitrocellulose required a wash sequence and a two step electrophoretic transfer as described (12) to disrupt amylate-protein binding, which prevented the mobilization of the proteins.

Nitrocellulose membranes containing proteins electrophoretically transferred from SDS-PAGE and IEF gels were stained with 5% bovine serum albumin in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 200 mM NaCl) and then incubated with a nonimmune rabbit anti-ODC antisemum (30) at a 1:1000 dilution for 16 h at 4°C. To detect the bound antibodies, the washed membrane was incubated for 2 h at room temperature with a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase followed by development with the substrate 4-chloro-1-naphthol.

Metabolic Radi labeling—RAW264 cells were maintained in DMEM supplemented with 10% fetal bovine serum and grown to confluency on a 100-mm tissue culture dish (5 × 10^7 cells/dish). After overnight incubation in serum-free DMEM, cells were stimulated for 4 h, washed three times with serum-free, phosphate-free DMEM, and then metabolically radiolabeled by incubation in the presence of 10 μCi of [32P]orthophosphate in phosphate-free DMEM for 3 h. After washing five times with DMEM, the cells were scraped into 3 mL of pH 7.5, 10 mM Na2HPO4, 10 mM NaH2PO4, pH 7.5. The cells were sonicated, and the supernatant was collected after centrifuging the sample at 1800 × g for 10 min.

In vitro Phosphorylation of ODC by CKII—CKII was purified from calf kidney (31, 32). The kinase was preincubated for 10 min in a reaction mixture containing a final concentration of 50 μM Tris-HCl, pH 7.5, 10 mM MgCl2, and 50 μM unlabeled ATP, prior to the addition of ODC. Routinely, 35 ng of ODC was incubated with 5 ng of CKII in a total volume of 95 μL containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 10 mM NaCl, and 50 μM [α-32P]ATP (200 μCi/mL). After incubation for 30 min at 30°C, the reaction was terminated by addition of reaction mixture to nondenatured, stimulated RAW264 cell supernatant in buffer A containing 5 mM EDTA.

Peptide Mapping and Phospho amino Acid Analysis—The reaction mixtures prepared by the in situ and in vitro phosphorylation protocols were incubated at 4°C for 1 h with the ODC antiserum at a 1:100 dilution, and the resultant antigen-antibody complexes were precipitated with phenol slurry. The precipitates were washed once with buffer A containing 2 mM NaCl and 1% Brij 35, centrifuged, and washed once with buffer A alone. The pellets were subjected to SDS-PAGE on an 8% acrylamide gel, and the separated proteins were electrophoretically transferred to nitrocellulose. After washing with 50 mM Tris-HCl, pH 7.4, and drying, adjacent lanes from the nitrocellulose were subjected to autoradiography or to immunoblot analysis for ODC. The radiolabeled ODC bands that migrated coincidently with the immunoreactive ODC band were excised and processed for peptide mapping.

Two-dimensional peptide mapping was performed as described previously (33, 34) with the following modifications. The ODC samples on the nitrocellulose membrane were sprayed with 3×300 μL of freshly prepared 50 mM NH4HCO3, pH 8.1, and initially proteolyzed by incubation in serum-free DMEM, cell were stimulated for 4 h, washed, and the supernatant containing the peptides was removed from the nitrocellulose, and the trypsin activity was neutralized by the addition of 40 μL of soybean trypsin inhibitor. After incubating for 1 h at 37°C, the contents were dried by centrifugation under vacuum in a SpeedVac and redissolved in 300 μL of freshly prepared 50 mM NH4HCO3, pH 7.7. V8 protease (20 μL of a 1 mg/ml stock in 100 μL HCl) was added to the peptide mixture and incubated for 5 h at 37°C. After SpeedVac drying, the peptides were subjected to performic acid oxidation as described (33). The samples were then analyzed by two-dimensional peptide mapping. The first dimension separation was accomplished at 1000 V for 1 h in a buffer containing 5% acetic acid and 0.5% pyridine on the Hunter Thin Layer Peptide Mapping System (model HTLE 7000). The second dimension was ascending chromatography for 4 h in a buffer containing 37.5% 1-butanol, 25% pyridine, and 7.5% acetic acid. The TLC plates were dried, and the radiolabeled peptide spots were located by autoradiography.

Radiolabeled ODC peptides were eluted from TLC plates by scraping the cellulose material off the plastic plates and rinsing the samples in 300 μL of first dimension phosphoamino acid analysis buffer. The supernatants containing the radiolabeled ODC peptides were separated from the cellulose material by centrifugation, lyophilized, and then subjected to acid hydrolysis for 4 h in the presence of 6 N HCl at 110°C (33). Phosphoamino acid standards (3 μL of a solution containing 10 mM phosphoserine, 10 mM phosphothreonine, 10 mM phosphotyrosine) were added to the acid hydrolysates, and the contents were dried in a SpeedVac. The samples were resuspended in 5 μL of the first dimension buffer (7.8% ethanolic acid and 2.5% formic acid), applied to TLC plates, and electrophoresed for 1 h at 1100 V. The second dimension separation was achieved in 5% acetic acid and 0.5% pyridine at 1300 V for 25–45 min. The phosphorylated amino acid standards were located by ninhydrin staining of the TLC plates, and the radiolabeled amino acids were located by using the Betscope 603 blot analyzer.

Quantitation of ODC Half-life—Four hours after stimulation of se-
rum-starved cells, 25 μg/ml of cycloheximide was added to the culture medium. At 10-min intervals, the cells were harvested, and the cellular extracts were separated by either SDS-PAGE or IEF. ODC was quantitated by immunoblotting with anti-ODC antibody and soft laser scanning densitometry to determine band densities. The band density at maximum ODC expression just prior to cycloheximide addition was assigned the value 100%. Band densities obtained after cycloheximide addition were expressed as a percentage of the maximum ODC band density. A linear relationship exists between band density and 0.5–50 ng ODC protein (data not shown).

Alternatively, after an initial 3-h stimulation, methionine-free medium containing fresh stimulants and 500 μCi/ml [35S]methionine was added to the cells, and incubation continued for 60 min. To quantitate the rate of decline of newly synthesized 35S-labeled ODC, the cultures were washed and refed with fresh, prewarmed, complete medium containing 10 mM methionine, and the cells were harvested at 10-min intervals. The ODC was immunoprecipitated, fractionated by SDS-PAGE, and transferred to nitrocellulose. After localization by immunoblotting and autoradiography, the radiolabeled ODC was quantitated by cutting out the reactive bands, dissolving them in dimethyl sulfoxide, and quantitating the radioactivity by liquid scintillation spectrometry.

### Purification of Unphosphorylated and Phosphorylated ODC

The unphosphorylated and phosphorylated ODC isoelectric forms were purified to homogeneity by the following procedure. Seven liters of RAW264 cells, grown in suspension culture to a density of 1 × 10^6 cells/ml, were harvested and washed with serum-free medium DMEM, the cells were resuspended in 60 ml of buffer containing 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 1 mM PMSF, 0.025 mM vanadate, 0.02% Brij 35, 10 mM pyridoxal 5′-5-phosphate (PYP) containing 0.5 mM L-ornithine plus 0.5 mM NaF, 0.1% Brij 35, 10 mM NaCl, 2.5 mM DTT, 1 mM PMSF, 0.025 μM vanadate. After washing with 2 column volume of buffer B, the column was eluted at a flow rate of 20 ml/h with a linear gradient of 0.125–0.25 M NaCl in a total volume of 500 ml of buffer B. Two peaks of ODC activity were eluted, pooled individually, and purified independently through the next stages.

The pooled ODC peaks 1 and 2 were each diluted with buffer C (25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM DTT, 1 mM PMSF, 0.025 μM vanadate) to a conductivity of 3.8 millisiemens or lower. The diluted sample was applied to a Mono-Q column (2 × 30 cm) pre-equilibrated with buffer D (buffer C containing 0.15 M NaCl) and washed with 2 column volume of buffer D. The column was eluted at a flow rate of 20 ml/h with a linear salt gradient of 0.15–0.35 M NaCl in a total volume of 500 ml of buffer D. Purification of a pyridoxamine 5-phosphate Affi-Gel 10 affinity column was as described (36), except that the buffer contained 25 mM Tris-HCl, pH 7.3, 0.02% Brij 35, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM DTT, 1 mM PMSF, 0.025 μM vanadate, and 50 mM NaCl. The ODC was eluted using the same buffer containing 10 mM pyridoxal 5′-5-phosphate (PYP) at a flow rate of 8 ml/h.

### ODC Assay and Determination of Enzyme Kinetic Parameters

ODC activity was routinely measured in 200 μl of assay buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM DTT, 5 mM NaF, 0.1% Brij 35, 1 mM PMSF, 60 μM PYP) containing 0.5 mM L-ornithine plus 0.5 μCi of L-(1-14C)ornithine. The reaction was stopped after 15–50 min with 1 ml of 10% TCA, and the 14CO2 evolved was trapped on a Whatman No. 1 paper disk filter saturated with 20 μl of 2% NaOH. The radioactivity on the disks was determined by liquid scintillation spectrometry. Protein was determined by the method of Bradford (37) using bovine serum albumin as the standard. Active ODC protein content was determined by reaction with [3H]fluoromethylornithine (38).

Kinetic parameters were determined using assay conditions similar to those described above. To determine the K_m of ODC for ornithine, 0.5 μCi of [3H]ornithine was used in each assay, and the final concentration of L-ornithine was varied from 0.01 to 0.8 mM. Before testing with varying concentrations of DTT or PYP, the ODC samples were extensively dialyzed using a Centricon 30 filtration unit. After the last filtration step, active ODC protein content was quantitated by reaction with [3H]fluoromethylornithine. The ODC samples were assayed at DTT concentrations of 0–2 mM or PYP concentrations of 0–1 μM. Kinetic parameters were determined by Lineweaver-Burke reciprocal plot analysis. p values were determined using Student’s t test.

### Comparative Phosphopeptide Mapping and Phosphoamino Acid Analysis of ODC Phosphorylated in Vivo by Casein Kinase II and in Situ in RAW264 Cells

ODC was phosphorylated in
in a similar manner from metabolically labeled RAW264 cells and transferred to an nitrocellulose membrane. ODC was isolated in a similar manner from metabolically labeled RAW264 cells (\textit{in vitro}) and subjected to two-dimensional peptide mapping. The TLC plates were autoradiographed for 24 h to visualize the 32P-labeled peptides. Tip of arrowhead, site of application of the protease-digested ODC samples. The first dimension separation (electrophoresis) was conducted in the horizontal axis, and the second dimension (chromatography) was conducted in the vertical axis.

\textit{In vitro} by reaction with CKII and \textit{in situ} by metabolic radiolabeling of stimulated RAW264 cells. To control for cytosolic cellular factors, after the \textit{in vitro} reaction was terminated it was added to nonradiolabeled, stimulated RAW264 cell supernatant, and subsequently the radiolabeled ODC was immunoprecipitated from the \textit{in vitro} and \textit{in situ} reactions. The immunoprecipitates were subjected to SDS-PAGE, the separated proteins were transferred to nitrocellulose membranes, and adjacent lanes were immunoblotted with the ODC antisera or autoradiographed. The radiolabeled ODC bands were excised and analyzed by two-dimensional phosphopeptide mapping after exhaustive sequential digestion with trypsin and V8 protease to determine the number of peptides containing 32P-labeled amino acid residues. A representative two-dimensional phosphopeptide analysis of ODC radiolabeled in \textit{vitro} by CKII is shown in Fig. 2A. Only a single radiolabeled peptide was observed, which migrated toward the anode in the first dimension but migrated slowly in the second dimension, consistent with its being negatively charged and hydrophilic. By contrast, the two-dimensional phosphopeptide map of ODC phosphorylated in \textit{in situ} in RAW264 cells (Fig. 2B) revealed at least two major 32P-labeled peptides, including one that migrated similarly to the ODC peptide seen after \textit{in vitro} phosphorylation by CKII. The other \textit{in situ} phosphorylated ODC peptide was also hydrophilic but migrated toward the cathode, \textit{i.e.} was positively charged.

The proteolytic cleavage peptides of ODC phosphorylated \textit{in vitro} and \textit{in situ} were eluted and subjected to two-dimensional phosphoamino acid analysis. As shown in Fig. 3A, the ODC phosphopeptide generated \textit{in vitro} by CKII-mediated phosphorylation contained only 32P-labeled serine. However, both the anionic and cationic phosphopeptides from the \textit{in situ} labeled ODC contained both 32P-labeled serine and threonine residues (Fig. 3, B and C).

**Turnover of Unphosphorylated and Phosphorylated ODC in \textit{In Vitro}**—The use of cycloheximide to study protein turnover rates has been shown to produce artifactual results in some instances because of drug effects on cellular processes other than protein synthesis. Therefore, the ODC turnover rate in RAW264 cells was determined initially by two independent techniques, one of which did not require the addition of cycloheximide. First, the rate of decline of immunodetectable enzyme activity (fractions 85–94) were pooled separately, and the enzyme affected its stability to intracellular proteolysis. Secondly, the rate of ODC degradation was determined after the addition of excess unlabeled methionine to [35S]methionine metabolically radiolabeled cells by immunoprecipitation of 35S-ODC. As shown in Fig. 4A, the time-dependent decrease of immunodetectable ODC protein after cycloheximide addition (t1/2, 43.8 min) was the same as the decrease of 35S pulse-labeled immunoprecipitable ODC protein (t1/2, 41.3 min). Thus, in RAW264 cells cycloheximide does not appear to interfere with cellular processes other than protein synthesis, allowing its use in the study of intracellular proteolysis of ODC. However, the decay of ODC was not a first order process over the entire course of the experiment. With either method, a discontinuity in the rate of degradation was always seen between 70 and 80 min, suggesting that there may be multiple components of ODC that have different turnover rates.

Since the ODC present in stimulated RAW264 cells exists in unphosphorylated and phosphorylated states (Fig. 1B), it was possible to determine whether the \textit{in situ} phosphorylation of the enzyme affected its stability to intracellular proteolysis. Cycloheximide was added to cells stimulated for 4 h, and the cell samples were harvested at 15-min intervals and subjected to IEF separation and immunoblot detection. A representative experiment showing the decay of the unphosphorylated and phosphorylated ODC forms is shown in Fig. 4B. Phosphorylated ODC had an intracellular half-life of 46.8 min, approximately 2-fold greater than the 26.7-min half-life of the unphosphorylated enzyme. In four separate experiments determining the half-life of the two forms of ODC, the phosphorylated form of ODC clearly had a statistically significant (p = 0.001) longer half-life (49.2 ± 3.78 min) than the unphosphorylated enzyme protein (t1/2, 23.9 ± 2.6 min).

**Kinetic Characterization of Purified Unphosphorylated and Phosphorylated ODC**—To study their kinetic characteristics, the phosphorylated and unphosphorylated forms of ODC from RAW264 cells were separated and purified to homogeneity. The elution profile of ODC activity from a typical DEAE-Sepharose CL-6B column is shown in Fig. 5A. The more basic peak 1 of ODC activity (fractions 54–64) and the more acidic peak 2 of ODC activity (fractions 85–94) were pooled separately, and each was further purified as described under “Experimental Procedures.” The silver-stained SDS-PAGE of the two ODC preparations showed that both forms of ODC were purified to apparent homogeneity and had identical apparent molecular weights of 54,000 (Fig. 5B). The two ODC preparations were also resolved on IEF gels, transferred to nitrocellulose, and subjected to immunoblot analysis (Fig. 5C). Stimulated RAW264 cell extract (lane 1) was used as a reference for the
migration of the two major isoelectric forms of ODC. Pure, peak 1 ODC was applied to lane 2. This preparation of the enzyme contained only one isoelectric form, which migrated coincident with the more basic, unphosphorylated form of ODC seen in the cell extract. Pure peak 2 ODC (lane 3) contained one major and one minor isoelectric form. The major enzyme form migrated coincident with the major acidic phosphorylated form of ODC detected in the cell extract, whereas the minor enzyme component migrated to an even more acidic position. Lane 4 contains a mixture of the purified peak 1 and 2 ODC preparations and reconstitutes the migration pattern of the unphosphorylated and phosphorylated ODC observed in the cell extracts. Thus, Fig. 5, B and C, documents the homogeneity of the unphosphorylated and phosphorylated preparations of ODC. It also demonstrates that peak 1 contains the major, more basic, unphosphorylated ODC form, whereas peak 2 contains a major and a minor more acidic, phosphorylated form of ODC.

The activity of the unphosphorylated and the phosphorylated forms of ODC was assayed in the presence of increasing concentrations of the substrate ornithine. The amounts of each isoelectric form of purified ODC in the assays were equivalent, based on quantitation of active enzyme protein reacting with
ODC Phosphorylation Increases Enzyme Activity and Stability

![Graph showing ODC activity](image)

**Fig. 5. Purification of phosphorylated and unphosphorylated ODC.** A, stimulated RAW264 cell supernatant was applied to a DEAE-Sepharose CL-6B column, washed, and eluted with a 0.125–0.25 M NaCl gradient and assayed for ODC activity. B, Silver-stained SDS-PAGE of purified peaks 1 (lane 1) and 2 (lane 2). C, Immunoblot of ODC preparations separated by high resolution IEF. Lane 1, stimulated RAW264 cell supernatant; lane 2, purified peak 1; lane 3, purified peak 2; lane 4, sample of peaks 1 and 2 combined.

([3H]difluoromethylornithine. This method of quantitation rigorously confirmed that any differences observed in the activity of the in situ unphosphorylated compared with the phosphorylated form of ODC were not artifacts resulting from the presence of inactive enzyme protein in either preparation of ODC. The results of nine separate experiments are shown in Fig. 6A. The two enzyme preparations were measured in each experiment in triplicate at each concentration of ornithine tested. Lineweaver-Burke analysis revealed that the $K_m$ for ornithine for both the in situ unphosphorylated and phosphorylated forms of ODC was 0.11 mM. However, the $V_{max}$ of the phosphorylated form was 50% greater than that of the unphosphorylated form ($n = 9; p = 0.005$); the unphosphorylated form had a $V_{max}$ of $20.54 \pm 1.65 \mu$mol/min/mg, whereas the phosphorylated form had a $V_{max}$ of $30.61 \pm 2.6 \mu$mol/min/mg.

As shown by the experiment in Fig. 6B, the unphosphorylated and phosphorylated preparations of ODC exhibited similar $K_m$ values of 0.14 ± 0.02 mM for the cofactor PYP but differing $V_{max}$ values of 39.1 and 62.9 μmol/min/mg, respectively. Full activity of ODC in in vitro assays requires a reduced environment, as optimally attained by the addition of the sulfhydryl reducing agent DTT (2, 3). Structure-activity analysis of the enzyme molecule indicates this is probably due to the role of the conserved cysteine 390 in the formation of the active site.

To determine whether in situ phosphorylation of ODC altered this requirement, the activity of the two forms of the enzyme was determined in the absence and presence of increasing concentrations of DTT. Both forms of ODC had similar EC$_{50}$ values of 0.109 ± 0.14 mM for the reductant; however, as shown in Fig. 6C, phosphorylated ODC was more active than unphosphorylated ODC, with $V_{max}$ values of 30.3 and 18.8 μmol/min/mg, respectively.

**DISCUSSION**

ODC has been demonstrated to be phosphorylated in situ (19–17, 25), but the relevance of this posttranslational modification of the enzyme has remained unclear. The present results demonstrate that intracellular phosphorylation of ODC can lead to an increase in both the intracellular stability and the catalytic capacity of the phosphorylated enzyme compared with the unphosphorylated enzyme.

Different ionic forms of ODC protein have been shown to exist. Multiple peaks of ODC activity elute from anion exchange chromatography columns loaded with extracts of various tissue and cultured cell types (5–8, 35, 40–42). It has been demonstrated in several experimental systems that phosphatase treatment and rechromatography of the second, more acidic peak of ODC shifts the enzyme’s elution position to that of the first peak (35, 42). Furthermore, the initial, more basic peak of ODC can be phosphorylated by CKII, whereas the second, more acidic peak of ODC is not a substrate for this kinase (41). Taken together, these results suggested that the basis of the multiple forms of ODC was enzyme phosphorylation. Fig. 5 demonstrates that the two major ionic peaks of ODC isolated by anion exchange chromatography of RAW264 cell extract are identical to the unphosphorylated and phosphorylated forms of ODC present in these cells.

We have previously observed that ODC from RAW264 cells is phosphorylated on both serine and threonine residues (25). The consensus phosphorylation sequence for CKII is a serine or threonine residue followed by acidic amino acids. The most critical acidic residue is the third amino acid at the carboxyl-terminal position to the serine or threonine, with additional acidic residues improving the affinity of the serine or threonine for CKII-catalyzed phosphorylation (43). The native amino acid sequence of murine ODC (18–20) contains 10 potential CKII phosphorylation consensus sequences, 6 containing serine (residues 32, 33, 91, 135, 303, and 445) and 4 containing threonine (residues 5, 19, 85, and 203). Therefore, we analyzed RAW264 cell ODC to identify the amino acid(s) phosphorylated in vitro by CKII. In agreement with the report of Rosenberg-Hasson et al., (15), we found that serine was the only ODC amino acid residue modified by CKII in vitro. Since we can identify phosphothreonine residues in ODC phosphorylated in RAW264 cells, we conclude that the in situ phosphorylation of RAW264 ODC threonine residues is catalyzed by a protein kinase(s) distinct from CKII.

Proteolytic digestion of ODC phosphorylated in situ generated anionic and cationic phosphopeptides, both of which contained phosphoserine and phosphothreonine, whereas digestion of ODC phosphorylated in vitro by CKII generated only an anionic phosphopeptide, which contained only phosphoserine. Since the trypsin and V8 protease treatment and two-dimensional peptide mapping of ODC phosphorylated in vitro by CKII and in situ in metabolically [32P]-radiolabeled RAW264 cells was carried out in parallel and under the exact same experimental conditions, it is unlikely that the cationic phosphopeptide detected in the in situ phosphorylated ODC is an incomplete proteolytic cleavage product of the anionic phosphopeptide detected in both the in vitro and in situ phosphorylated ODC. If it were, the peptide would have also been
detected in the ODC preparation phosphorylated in vitro. Thus, our results demonstrate that there is at least one serine residue, in addition to that residue phosphorylated by CKII, and at least two threonine residues in the ODC molecule that are phosphorylated in RAW264 cells. The finding that ODC is subject to multisite phosphorylation in this cell line is consistent with the accompanying observation that a homogeneous preparation of phosphorylated ODC from RAW264 cells has a greater catalytic capacity than a similar preparation of unphosphorylated ODC. This finding is in contrast to data we and others have reported showing that in vitro phosphorylation of ODC by CKII has no effect on the decarboxylating activity (21, 22).

Rosenberg-Hasson, et al. (15) found that only phosphoserine was present in ODC phosphorylated in mouse myeloma 653-1 cells. In addition, phosphopeptide analysis of this ODC showed only phosphopeptides analogous to those observed in ODC phosphorylated by CKII. These findings led them to conclude that in cells, ODC is phosphorylated at a single location (serine 303) and that CKII, or a protein kinase with similar characteristics, is responsible for this phosphorylation. However, our findings in the mouse Abelson virus-transformed,
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macrophage-derived RAW264 cell line clearly demonstrate that such a conclusion about intracellular phosphorylation of ODC cannot be generalized to all cell types. ODC in RAW264 cells is phosphorylated on both serine and threonine residues, which are present at at least four distinct sites in the enzyme amino acid sequence, only one of which is consistent with the site phosphorylated by CKII.

There are at least two potential explanations for the reported differences: (i) different cell lines and tissues may inherently express unique phosphorylation patterns of ODC or may differentially acquire unique patterns after transformation with different oncogenes; with regard to the latter, we did assess the ability of bcr-abl to phosphorylate ODC but found that no phosphorylation occurred; and (ii) the peptide-mapping technique used in the study of 653-1 cells used only a single protease and by design generated only incomplete digestion products (15). This experimental approach could obscure the detection of unique phosphopeptides present in cellulyrly phosphorylated ODC versus ODC phosphorylated by CKII. In addition, the phosphoamino acid analysis of metabolically radiolabeled ODC was conducted for just a single time, 2 h. We have previously shown that the detection of radiolabeled ODC phosphothreonine residues is optimal after longer times of acid hydrolysis (25), a finding consistent with the fact that the stability to acid hydrolysis of both the peptide bond and the phosphoester bond is significantly greater for phosphothreonine than for phosphoserine (44). Thus, the selection of only a single, relatively short acid hydrolysis time may not have been sufficient for the detection of phosphothreonine.

Having available homogeneous preparations of the unphosphorylated and phosphorylated ODC from RAW264 cells has allowed us to conduct independent kinetic characterizations of the two forms of the enzyme. Any potential interference from enzyme-modifying cellular factors such as free polyamines or ODC antizyme (a small ODC-binding protein that reversibly inactivates the enzyme and mediates polyamine-induced ODC degradation (45–47)) has been circumvented by purification of the two forms of ODC. With regard to all variables tested, the phosphorylated ODC from RAW264 cells has a higher catalytic capacity than the unphosphorylated form. An analysis of this rigor would not be possible using crude cell extracts prepared from cells transiently transfected with mutant or wild type ODC expression vectors.

Recently, the results of in vitro transcription and translation studies using plasmids with ODC mutated at active site residues have led to the conclusion that the active form of ODC is a dimer (48) containing two active sites at the subunit interface (49). Under these experimental conditions, it has been shown that mixing of purified mutant ODC enzymes with the wild type results in rapid exchange of the subunits under physiologic conditions (50). The fact that we were able to purify unphosphorylated and phosphorylated ODC to homogeneity in an active state indicates that each form is able to assemble into a functional homodimer. We have assessed whether mixing of the RAW264 cell unphosphorylated ODC with the phosphorylated ODC would result in either an increase or decrease in the V_{max} of the mixed population, indicative of heterodimerization of the two enzyme forms with one enzyme state being dominant over the other. Our results showed that if the unphosphorylated and phosphorylated ODC enzyme molecules did form heterodimers, the dimers had an average maximal activity of the two enzyme forms.

Intracellular proteolysis of ODC is a regulated process. The half-life of the enzyme, which is extremely short (15–30 min) in normal tissues (51), is altered by growth stimulation, transformation, toxic injury, alterations in specific extracellular amino acids, or alterations in cellular polyamine content (for reviews, see Refs. 2 and 3). The complete molecular mechanism(s) by which all these alterations in ODC half-life occur is unknown, although it is known that the polyamine-stimulated degradation is facilitated by antizyme (45–47) and occurs in complex with the 26S proteasome (52, 53).

A role for posttranslational modification of ODC in regulating the turnover of the enzyme has been previously suggested by two different experimental approaches. First, it was observed that a reticulocyte lysate system would degrade in vitro synthesized ODC but not cell-derived ODC, leading the authors to speculate that an intracellular posttranslational modification of ODC may regulate its degradation (54). A second group of studies demonstrated a difference in the intracellular half-life of the two major ionic forms of ODC eluted from anion exchange columns. In all the experimental systems used, including serum-stimulated HTC cells (55), livers from chloroform-treated rats (56) or kidneys from rats treated with dexmethasone, prolactin, or growth hormone (42), the second, more acidic form of ODC had a longer half-life than the first, more basic form. The increase in half-life of the more acidic ODC isoelectric form compared with the more basic form ranged from 2- to 100-fold in the various experimental systems studied.

Phosphorylation has been shown to regulate protein stability to intracellular degradation. For example the stability of c-Fos is decreased by the phosphorylation state of c-Jun when heterodimers of c-Fos and c-Jun are formed (57). Also, Rag-1 and Rag-2, proteins that mediate the assembly of antigen receptor genes, degrade rapidly once they are phosphorylated (58). The monospecific antiserum to ODC and the high resolution slab gel IEF and immunoblotting method we have developed allows us to separate and directly quantitate the amount of the unphosphorylated and phosphorylated forms of ODC in intact RAW264 cells. Thus, we have directly demonstrated that in RAW264 cells the phosphorylated form of ODC has a longer half-life in situ than the unphosphorylated form. The in situ kinetic data presented in Fig. 4 permit further interpretation. The significant differences in the cellular half-life of phosphorylated and unphosphorylated ODC strongly suggest that the two forms of ODC are not in rapid equilibrium with each other within the environment of the intact cell. However, the data do not permit us to discern whether the phosphorylated form of ODC is a direct substrate for the degradative machinery of the cell or whether phosphorylated ODC is first dephosphorylated and then degraded.

In conclusion, we have demonstrated that ODC in stimulated RAW264 cells exists in both unphosphorylated and phosphorylated states and that ODC is phosphorylated at multiple sites, in addition to that phosphorylated by CK II. The consequences of the posttranslational modification are 2-fold: (i) the phosphorylated enzyme is more stable to proteolytic degradation; and (ii) its catalytic efficiency is significantly increased. To date, the only protein kinase documented to phosphorylate ODC is CKII; however, this phosphorylation has no effect on ODC activity. Thus, it appears that regulation of ODC activity and stability by phosphorylation is mediated by as yet unidentified protein kinases. The identity of the phosphorylated amino acid residues of ODC that regulate its intracellular stability and catalytic capacity is under investigation.

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Multisite Phosphorylation of Ornithine Decarboxylase in Transformed Macrophages Results in Increased Intracellular Enzyme Stability and Catalytic Efficiency
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