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POLYAMINE-MEDIATED DEGRADATION OF ORNITHINE DECARBOXYLASE IN CHINESE HAMSTER OVARY CELLS

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POLYAMINE-MEDIATED DEGRADATION OF ORNITHINE DECARBOXYLASE IN CHINESE HAMSTER OVARY CELLS

by

James Russell Glass

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

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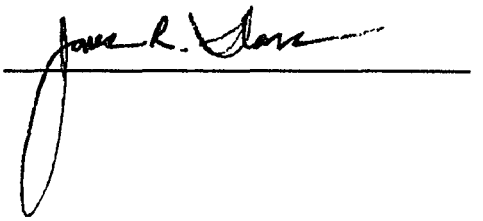
SIGNED: 

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ABSTRACT

The objective of this research was to identify specific mechanisms involved in the regulation of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway. Immunochemical techniques were used to study post-translational modifications of the ODC protein in relation to activity alterations.

Initial experimentation showed that Chinese hamster cells maintained in a defined medium express an ODC protein stable to intracellular degradation. Treatment of these cells with exogenous ornithine or polyamines resulted in a rapid loss of enzyme activity, without detectable changes in the enzyme specific activity. The loss of enzyme activity was a result of accelerated ODC degradation, as determined by immunoprecipitation of pre-labeled protein. In addition, spermidine, but not ornithine, totally inhibited new ODC synthesis.

The mechanism of accelerated ODC degradation was investigated and found to occur by an apparent novel mechanism. Degradation of ODC was both ubiquitin-independent and non-lysosomal, and there was also no detectable accumulation of a modified form of ODC protein. In addition, it was found that a component of protein synthesis is required for this process, as inhibitors (cycloheximide, emetine, puromycin) blocked polyamine-accelerated degradation.

ODC cDNA was used to synthesize both ODC specific mRNA and protein using in vitro synthesis. These systems may allow the generation of sufficient quantities of material which can be used to recreate in vitro the specific components involved in polyamine inhibition of ODC synthesis and the protease(s) responsible for degradation.

The major finding of this work is the direct demonstration that ODC is a stable intracellular protein in the absence of putrescine and spermidine depleted cells (Chapter 2). In addition, that degradation occurs by a novel mechanism, with a requirement for some component of protein synthesis (Chapter 3). Finally, these studies describe the in vitro production of ODC protein and mRNA which should facilitate further studies of polyamine regulation of ODC degradation and synthesis (Chapter 4).

CHAPTER 1

INTRODUCTION

The polyamines, spermidine and spermine, and their precursor, putrescine, are polycationic molecules found in virtually all mammalian cells. Interest in these compounds was first initiated by the description of spermine phosphate well over 300 years ago by Antoine von Leevwenhoek (Bachrach, 1973). The high concentrations found in cells and their ubiquitous distribution indicate potentially important functions and indeed many have been ascribed to the polyamines based on both in vitro and in vivo studies (Tabor and Tabor, 1976; Tabor and Tabor, 1984). Clearly the most obvious characteristic of these molecules is their polycationic character, the charged groups being separated by distinct carbon chains for each (Figure 1), leading to potential specificity for ionic interactions with acidic constituents. The polyamines have been described to interact in many specific fashions with both DNA and RNA (Bachrach, 1973; Cohen, 1971; Tabor and Tabor, 1976), specific examples being: the stabilization of DNA conformation (Behe and Felsenfeld, 1981) and stochiometric binding to sites on tRNA molecules (Pochon and Cohen, 1972; Quugley, Teeter and Rich, 1978).

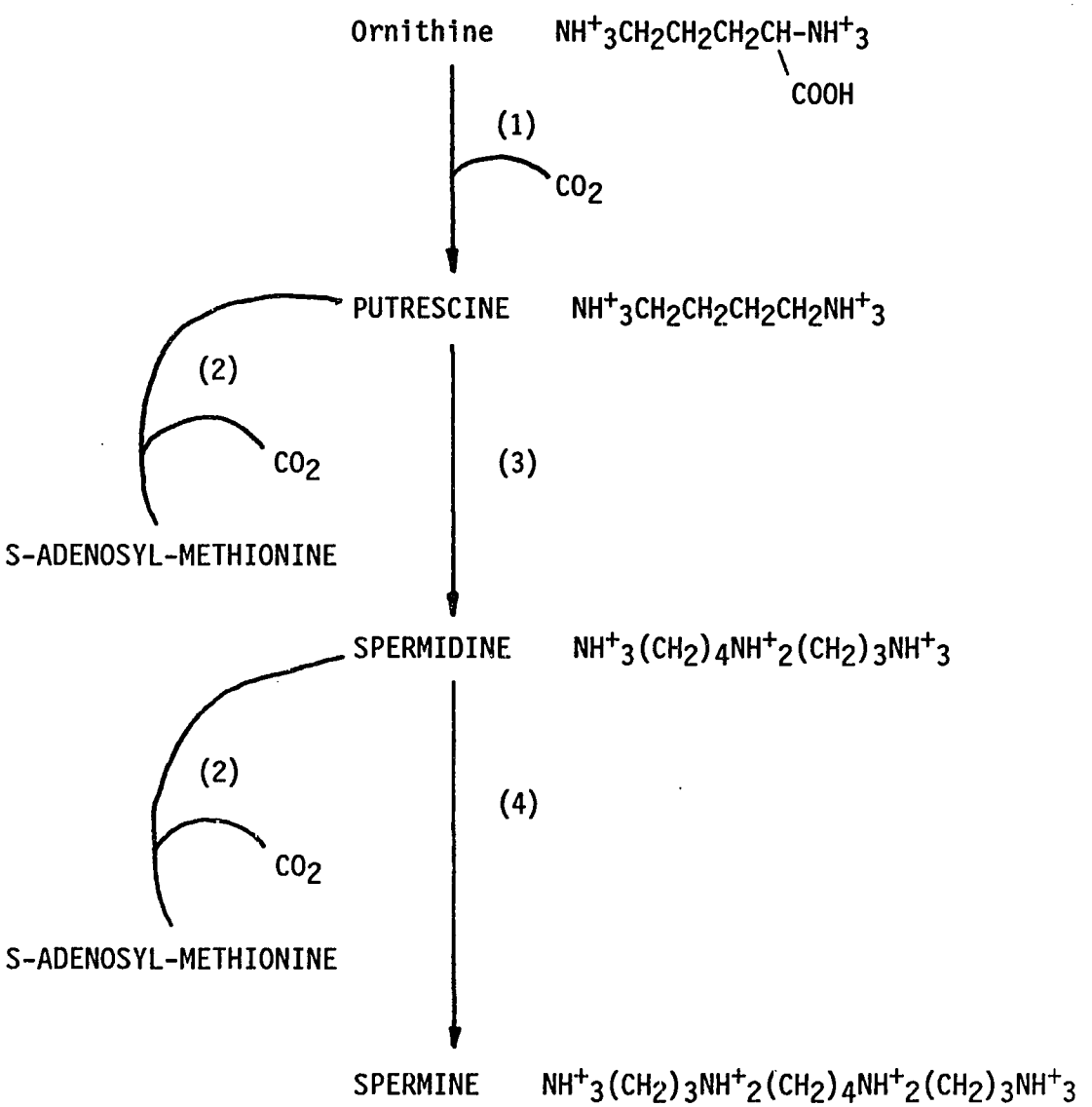

Figure 1. Polyamine biosynthesis pathway in mammalian cells.

The enzymes involved are: 1, ornithine decarboxylase ODC (E.C.4.1.1.17); 2, S-adenosyl-methionine decarboxylase SAM DC (E.C.4.1.1.50); 3, spermidine synthase (E.C.2.5.1.16); 4, spermine synthase (E.C.1.5.1.22). Adapted from Pegg, 1986.

Other functional roles described for polyamines include stimulation of protein synthesis; possibly interacting at the level of fidelity of translation (Jelenc and Kurlano, 1979; Gorini, 1969), chain initiation and elongation (Takemoto, Nagamatsu and Oka, 1983) or stimulation of aminoacyl-tRNA synthesis (Tabor and Tabor, 1976). Stimulation of protein kinases has been reported (Bachrach, Kaye and Chayen, 1983). These selected descriptions of polyamine function indicate that they could play major roles in cellular processes, yet assigning a specific function in vivo is a difficult task due to their simple chemical nature. Indeed, arguments have been raised that other ionic alterations and pH effects could result in functions previously delegated to polyamines from in vitro experiments (Tabor and Tabor, 1976).

Possibly, the most clearly defined role in cellular functions is that cells absolutely require polyamines to maintain optimal growth. Ham (1964, 1965) first found that putrescine and related amines were essential as growth factors for mammalian cells in tissue culture. This work was followed by the descriptions by Russell and Snyder (1968, 1969) that polyamine synthesis in regenerating tissues was greatly accelerated, as represented by the induction of ornithine decarboxylase (ODC), the first enzyme in polyamine biosynthesis. A large number of growth stimuli have since been reported to induce polyamine biosynthesis, including hepatectomy (Russell and Snyder, 1968), hormones (growth hormone, corticosteroids and testosterone) (Janne, Poso and Raina, 1978),

epidermal growth factor (Stastny and Cohen, 1972) transformation (Holtta, et al., 1981) the addition of fresh serum-containing media to quiescent cells (Hogan, McElhinney and Murden, 1974), and treatment with tumor promoters (O'Brien, 1976).

The development of specific inhibitors for polyamine synthetic enzymes has allowed a direct determination of the polyamine requirement for proliferation. Treatment of cells in culture with α-difluoro-methylornithine (DFMO), an enzyme activated suicide inhibitor for ODC (Metcalf et al., 1978) leads to depletion of intracellular putrescine and spermidine content and cessation of proliferation (Mamont et al., 1978). Restoration of growth can be achieved by supplying putrescine in the medium. These initial descriptions have since been repeated using many different cell types (Pegg, 1986). These results along with the descriptions of ODC activity and polyamine levels increases after growth stimuli implicate the polyamines as being essential for optimal cellular proliferation. The specific mechanism responsible for cessation of growth during polyamine depletion is not known. Inhibition of any of the putative functions assigned to polyamines (i.e., translation, enzyme activation) could be involved in the process.

Polyamine Biosynthesis and Interconversion

A portion of the mammalian polyamine biosynthetic pathway is shown in Figure 1. Ornithine is converted into putrescine, liberating CO_2, by the action of ODC. Putrescine is modified by the addition of a propylamine moiety to form spermidine, by an

aminopropyltransferase called spermidine synthase. A second aminopropyltransferase, spermine synthase, catalyzes the addition of another propylamine group to spermidine to form spermine. The donor molecule of these proplyamine groups is S-adenosyl-methionine, which is decarboxylated by the enzyme S-adenosyl-methionine decarboxylase (SAMDC) with the liberation of a propylamine group. The rate of polyamine biosynthesis is controlled by alterations in the two decarboxylases in the pathway, ODC and SAMDC. Spermidine synthase and spermine synthase activities appear constitutive, and no major induction of these enzymes has been measured (Pegg, 1986).

In addition to forward synthesis, a retroconversion pathway also exists for the metabolism of polyamines. Spermidine/spermine N^1-acetyltransferase (SAT) catalyzes the conversion of those polyamines to N^1-acetylated derivatives. Polyamine oxidase action then results in formation of putrescine (from N'-acetylspermidine) or spermidine (from N'-acetylspermine) and the generation of 3-acetamidopropanal and H_2O_2. Polyamine retroconversion is controlled by alterations in SAT activity. While this pathway is clearly demonstrable in mammalian cells, the significance of these reactions in regulating intracellular polyamine content is still poorly understood (Pegg, 1986).

Regulation of Polyamine Biosynthesis

Ornithine decarboxylase, the first and rate-limiting enzyme in polyamine biosynthesis, has been the subject of many investigations. In addition to the striking alterations in enzyme

activity in response to stimuli, the enzyme turnover rate has been described as the fastest for any mammalian enzyme, with a biological half-life of 10-30 minutes (Tabor and Tabor, 1984). These findings are based on measurements of ODC activity, and only recently has a direct assay co-relating ODC protein and activity been performed. Because of various interpretations of correlative findings, research addressing specific mechanisms by which ODC activity is regulated has been the most active and controversial in the field of studies concerning polyamine metabolism. Many avenues for enzyme activity control have been suggested including post-translational modification, induction of macromolecular inhibitors, and alterations of both synthesis and degradation rates.

Treatment of cells with exogenous diamines (1,3 diamino-propane and putrescine) and polyamines lead to a rapid loss of ODC activity. This is an apparently universal effect in mammalian cells, being reported in 3T3 cells (Clark and Fuller, 1975), KB cells (Pett and Ginsberg, 1968), human lymphocytes (Kay and Lindsay, 1973), regenerating rat liver (Janne and Holtta, 1974), Chinese hamster ovary cells (Gerner et al., 1985; Glass and Gerner, 1986a), and androgen-stimulated mouse kidney (Seely and Pegg, 1983b). The administration of polyamines and measurement of ODC activity changes have been widely used to analyze regulatory pathways for ODC. While the decrease in enzyme activity is dramatic and reproducible, the mechanism by which this occurs has, until recently, been unknown.

It has been suggested that polyamines post-translationally modify ODC protein resulting in enzyme inactivation. The utilization of polyamines as substrates for transglutaminase (Folk et al, 1980) prompted Russell (1981) to hypothesize that ODC is directly modified by polyamines *in vivo*. Modification of partially purified calf-liver ornithine decarboxylase *in vitro* by this mechanism has been described (Russell, 1931), but any significance *in vivo* is still uncertain. Inverse correlations between transglutaminase activity and ornithine decarboxylase levels have been reported in tissues (Scott, Meyskens and Russell, 1982) but a direct interaction *in vivo* has not been demonstrated.

Polyamine activation of protein kinases and modification of ODC by phosphorylation has also been reported. Atmar and Kuehn (1981) have described a polyamine-activated kinase in *Physarum polycephalum* which co-purified with ODC from nucleoli. This kinase inactivated ODC by phosphorylation *in vitro* (Daniels, Atmar and Kuehn, 1981). Mammalian ODC can also serve as a substrate for phosphorylation *in vitro* (Meggio et al., 1984), and the presence of multiple forms has been detected in mouse kidney based on isoelectric mobility (Isomaa et al., 1983; Seely et al., 1985). Mitchell et al. (1985a) have reported that charge alterations in ODC result in increased susceptibility to proteolysis. The nature of this charge shift was not determined, but was consistant with phosphorylation. However, a direct functional role for phosphorylation of this enzyme *in vivo* has not been characterized.

The production of a macromolecular inhibitor of ODC mediated by polyamines has also been reported as being a major regulatory mechanism of ODC activity (Canellakis, et al. 1979). This protein, termed the "antizyme" with a molecular weight of approximately 26,500, is induced in cells after stimulation with high concentrations (i.e., millimolar) of exogenous polyamine. Mixture of cell homogenates treated with polyamine and non-polyamine treated samples leads to inactivation of ODC activity. The antizyme has a high affinity for ODC protein, although the interaction is non-covalent, as the proteins can be dissociated by treatment with high salt. It has been postulated that antizyme induction is mediated by specific binding sites for polyamines on the cell surface (Canellakis et al., 1978). The production of antizyme only in response to non-physiological concentrations of polyamine has led some investigators to question the relevance of antizyme regulation of ODC under normal physiological conditions. The inability to recover active ODC from cells treated to induce antizyme indicates that this pathway is irreversible. It has been suggested that antizyme interactions with ODC could target both proteins for rapid degradation (Persson, Seely and Pegg, 1984).

Alterations in both synthetic and degradation rates are another mechanism of regulation of ODC activity. In their classic paper, Berlin and Schimke (1965) indicated that major increases in protein content can occur after an increase in protein synthesis rate if that enzyme has a rapid turnover time. That ODC has the

shortest half-life of any mammalian enzyme suggests that small fluctuations in synthesis and degradation can lead to rapid changes in enzyme activity. This is supported by observations that ODC increases in response to stimuli could be blocked by inhibitors of both protein (Russell and Synder, 1969b) and RNA synthesis (Byus and Russell, 1975). Also, fluctuations in the half-life (i.e., loss of activity after cycloheximide treatment) occur during cell growth, with turnover rates ranging anywhere from 15 to 400 minutes (Chen and Canellakis, 1977). Studies have demonstrated that the loss of peptide bound radiolabeled DFMO parallels that of the half-life of ODC measured by activity (Seely, Poso and Pegg, 1982b), indicating that the protein is degraded, rather than being inactivated leading to a change in enzyme specific activity.

Definitive studies of the many regulatory mechanisms described for ODC have been hindered due to the extremely low level of the protein in most tissues. In tissues and cells ODC protein represents as little as one part in 6,000,000 of the soluble protein (Pegg et al., 1982), and in maximally induced tissue can only reach one part in 350,000 (Kameji et al, 1982). Pegg and workers have found, however, that in testosterone-stimulated mouse kidney, the protein content can reach one part in 8,000 (Seely, Poso and Pegg, 1982c; Isomaa et al., 1983). ODC was purified to homogenity from these tissues and used to create both polyclonal (Seely and Pegg, 1983a; Isomaa, et al., 1983) and monoclonal (Pegg et al., 1984) antibodies. The availability of antibodies and the use of a

radiolabeled emzyme-activated inhibitor, DFMO, has allowed direct determination of changes in protein content and specific activity. Upon decarboxylation, DFMO forms an active intermediate which covalently binds to the ODC protein. The binding of DFMO to ODC occurs in a 1:1 ratio (Pritchard et al., 1981), therefore a determination of radioactivity bound to protein gives a determination of the amount of active ODC protein present in the system. Initial studies using mouse kidney (Seely and Pegg, 1983a) and serum-stimulated 3T3 cells (Erwin, Persson and Pegg, 1983) showed that ODC activity alteration results from an increase in protein molecule number and not by an activation of pre-existing enzyme.

Another strategy used to circumvent the problems of lack of material has been the development of mutant cell lines and manipulation of culture conditions leading to overexpression of ODC. The initial isolation of mutants was described by Mamont and co-workers (1978), who selected for rat hepatoma cells which could proliferate in the presence of high concentrations of α-methylornithine, a competitive inhibitor of ODC. This cell line, termed HMO_A has an altered half-life for ODC, but not as a result of mutations in the enzyme protein (Pritchard et al., 1982). Mutant strains of Chinese hamster cells selected for growth in high concentrations of DFMO have also been isolated. These cells overproduce ODC protein, due to gene amplification and stabilization. Another overproducing mutant is a mouse lymphoma

cell line in which 15% of the total protein synthesized is ODC (McConlogue and Coffino, 1983). ODC cDNA has recently been prepared from this cell line (McConlogue et al., 1984), and from another mouse lymphoma mutant (Kahana and Nathans, 1984).

An alternative to selection of mutant cell lines has been the description of cell culture conditions which result in alterations in polyamine metabolism. Specifically, the characterization of serum-free medium conditions which allow the maintenance of cells at growth rates comparable to serum containing medium thus eliminating undefined serum components. Sertich and co-workers (Sertich et al., 1986) have described such a medium capable of maintaining Chinese hamster cells and characterized polyamine metabolism in these cells. ODC activity is dramatically increased (10-100 fold) in comparison to cells maintained in serum-containing medium. The increased activity is due to stabilization of ODC protein, leading to increased molecule number, and resulting in an ODC activity increase. This stabilization is thought to be brought about by decreased intracellular contents of putrescine and spermidine, as ornithine is not supplied by an extracellular source (i.e., serum). Spermine content remains constant in comparison to the cells maintained in serum-containing medium. Ponjanpelto et al. (1985) have also described serum-free maintenance of CHO cells and these cells also display altered polyamine metabolism.

This Investigation

The goal in this study was to determine the role of post-translational modifications involved in the regulation of ODC in mammalian cells. Specific questions addressed were to determine potential modifications responsible for influencing enzyme activity and biological half-life of the enzyme. The approach used to clarify this area of ODC regulation was to follow specific changes in ODC protein structure during activity alterations. This was accomplished using immunochemical techniques to monitor radiolabeled peptides corresponding to ODC, and to quantitate active ODC protein molecules by the binding assay using radiolabeled inhibitor DFMO, initially described by Pritchard et al. (1981). The cell system used in this study was CHO cells maintained in serum-free medium described by Sertich (1986). These cells display altered polyamine metabolism, characterized by a stable ODC protein. This tissue culture cell system is useful for the study of ODC regulation, due to the ease of manipulation of the experimental conditions (radiolabeling proteins, inhibitor treatment) and the ability to generate sufficient quantities of cells. The use of a defined-medium also eliminates unknown extracellular factors present in serum (i.e., ornithine, polyamines) which may influence ODC regulation. This system represents an extreme derepression of ODC regulation by the polyamines, due to the low levels intracellularly. Treating these cells with extracellular polyamines and measuring changes in ODC protein and activity represents a model system which

can be related to other mammalian cells which normally have high intracellular levels of polyamines. In addition, the work presented here describes the production of ODC protein in vitro from mouse cDNA isolated by Kahana and Nathans (1984).

The dissertation consists of chapters addressing specific aspects of ODC regulation and metabolism. Chapter 2 describes the mechanism by which polyamines regulate ODC activity in CHO cells. Specific modification of ODC structure and mechanisms of ODC degradation are addressed in Chapter 3. Chapter 4 includes the description of techniques to produce large quantities of ODC protein from cDNA in test tube reactions. Finally, Chapter 5 summarizes the major new findings of this research and includes a discussion of potential directions for future research suggested by these discoveries.

CHAPTER 2

POLYAMINE-MEDIATED TURNOVER OF ORNITHINE DECARBOXYLASE IN CHINESE-HAMSTER OVARY CELLS

Introduction

The precise study of the regulation of ODC by polyamines has been hampered in cell-culture systems, since growth media characteristically contain undefined components. A defined medium, lacking serum, has been developed which contains known growth factors (transferrin, $FeSO_4$, insulin) capable of maintaining Chinese-hamster ovary (CHO) cell growth rates and viability at values near those of cells growing in serum supplemented medium. A detailed comparison of the polyamine contents, growth rates and ODC activity between CHO cells maintained in this defined medium and in medium containing serum has been described (Sertich et al., 1986). The chemically defined medium lacks an exogenous ornithine supply, thus limiting polyamine accumulation.

In this Chapter the use of these conditions to study the regulation of ODC activity in exponentially growing cells after restoration of intracellular polyamine content is described. Both [^{3}H]DFMO binding and immunochemical techniques were used to measure changes in ODC enzyme activity and protein content after the addition of exogenous substrate and end product.

Materials and Methods

Materials

L-[1-^{14}C]Ornithine (60 mCi/mmol), L-[^{35}S]methionine (1120 Ci/mmol), α-DL-difluoromethyl[3,4-^{3}H]ornithine ([^{3}H]DFMO; 26.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). A bacterial absorbant, Staphylococcus A (IGSORB) was purchased from The Enzyme Center (Malden, MA, U.S.A.). Tissue-culture plastic was purchased from Falcon (Oxnard, CA, U.S.A.), and culture medium was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). All other chemical were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture Techniques

CHO cells were maintained in exponential growth at 37°C in McCoy's 5A medium supplemented with insulin (10 μl/ml), transferrin (5 μg/ml), $FeSO_4$ (1.1 μg/ml), 100 units of penicillin/ml and 100 μg of streptomycin/ml, at 37°C in air/CO_2 (9:1). Cells maintained under these conditions have a colony-forming efficiency of 60-80% and a doubling time of 16-18 hours (Sertich et al., 1986).

Measurement of ODC Activity

Cells were scraped from culture dishes, centrifuged at 900 g for 6 min, and disrupted by sonication in buffer containing 0.05 M-sodium/potassium phosphate, pH 7.2, 0.1 mM-EDTA, 1.0 mM-dithiothreitol and 20 μM-pyridoxal phosphate (ODC buffer), at a concentration of 10^7 cells/ml. A soluble fraction was obtained by

centrifugation at 10,000 x g for 5 min in a Beckman Minifuge. Then 200 μl of the soluble fraction was incubated at 37°C for 30 min in the presence of 0.15 μCi of L-[1-^{14}C]ornithine (final concn. 500 μM) and the reaction was terminated by addition of 0.5 ml of 1 M-citric acid. ODC activity was determined by the liberation of $^{14}CO_2$ during the reaction period. One unit of activity corresponds to 1 nmol of substrate decarboxylated in 30 minutes.

Polyamine Analysis

Acid-soluble polyamine contents were determined by using the reverse-phase ion-pair h.p.l.c. procedure originally described by Seiler and Knodgen (1980). Cells were collected from exponentially growing cultures by first removing the culture medium and washing the cell monolayer twice with ice-cold phosphate-buffered saline (70 mM-NaCl, 3 mM-KCl, 8 mM-Na_2HPO_4, 1 mM-KH_2PO_4, pH 7.3). The cells were harvested into phosphate-buffered saline with a rubber policeman, and cell numbers were determined with an electronic particle counter (Coulter Electronics, Hialeah, FL, U.S.A.). The cells were disrupted by sonication, and the soluble proteins precipitated with 0.2 M-$HClO_4$. The acid-soluble fractions were obtained by centrifugation at 10,000 x g for 5 minutes. Polyamine contents were then determined from samples of these cells, using diaminohepatane as an internal standard, by comparing the relative retention times of endogenous polyamines with those of standard preparations of putrescine, spermidine and spermine.

Radiolabeled DFMO Binding

To prepare radiolabeled ODC protein, soluble cell homogenates (at least 5 mg of soluble protein per reaction) were incubated with 0.28 μM-[^{3}H]DFMO in ODC buffer (500 μl) for 2 hours at 37°C. This concentration of drug and incubation time produced over 95% inactivation of activity. Primary antiserum (the characterization of this antiserum has been previously described; Seely and Pegg, 1983a) was then added (1:500 final dilution). This dilution yields 100% removal of ODC activity from supernatants after immune precipitation. The mixture was incubated for 1 hour at 20°C, and immunoreactive proteins were collected by addition of 20 μl of 10% (v/v) suspension of formalin-fixed Staphylococcus A by centrifugation after 30 minutes at 20°C. The pellet was washed once with 750 μl of Buffer A (1% Triton X-100, 0.5% sodium deoxycholate, 5 mM-EDTA, 250 mM-NaCl, 25mM-Tris/HCl, pH 7.5) and proteins were solubilized into 40 μl of SDS solubilization buffer at 100°C. Specificity of binding and quantification were determined by fractionation of labeled proteins by SDS/polyacrylamide-gel electrophoresis, by a slight modification of the method of Laemmli (1970). Bisacrylamide was replaced with N N'-diallytartardiamide (Bio-Rad), so that gel slices were dissolved by the addition of 2% periodic acid. Samples for SDS/polyacrylamide-gel electrophoresis were heated at 100°C for 5 minutes in 2% SDS/5% β-mercaptoethanol/3% sucrose in 300 mM-Tris/HCl, pH 7.0 (SDS solubilization buffer).

Immunoprecipitation

Exponential-phase cultures (1.5 x 10^7 cells/reaction) were labeled with 100 μCi of [^{35}S]methionine/ml in methionine-free medium. At appropriate times after incubation, cells were harvested by scraping with a rubber policeman, disrupted in ODC buffer at 2 x 10^7 cells/ml, and then 50 μl of a 1:500 dilution of primary antiserum was added to 500 μl samples of the cell homogenate. Controls were incubated with non-immune serum. After 1 hour at 20°C, 3 μl of 10% suspension of formalin-fixed Staphylococcus A was added, and 30 minutes later the complexes were collected by centrifugation at 10,000 x g for 5 minutes. Non-specific binding was decreased by washing the pellet with 10 x 750 μl Buffer A. Proteins were eluted from the Staphylococcus A at 100 °C in 40 μl of SDS solubilization buffer and separated by SDS/polyacrylamide-gel electrophoresis.

Protein Determination

Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Results

To identify proteins corresponding to ODC, [^{35}S]methionine-labeled immunoreactive peptides were detected by co-migration with radiolabeled [^{3}H]DFMO-bound proteins on polyacrylamide gels. It had been shown that this enzyme-activated irreversible inhibitor serves as a useful marker for ODC protein, binding specifically in a 1:1

molar ratio with the enzyme (Erwin et al., 1983; Persson et al., 1984). These data are shown in Figure 2. Labeling cells in methionine-deficient medium plus 100 μCi of [^{35}S]methionine/ml, followed by immunoprecipitation with anti-ODC serum, yielded one band which was present in the homogenates treated with anti-ODC serum (lane c), which co-migrated with the marker band seen in lane (a). This protein was not present in homogenates treated without anti-ODC serum (lane b) and had a subunit M_r of approximately 54,000, a value consistent with that of ODC isolated from another Chinese hamster cell line (Choi and Scheffler, 1983).

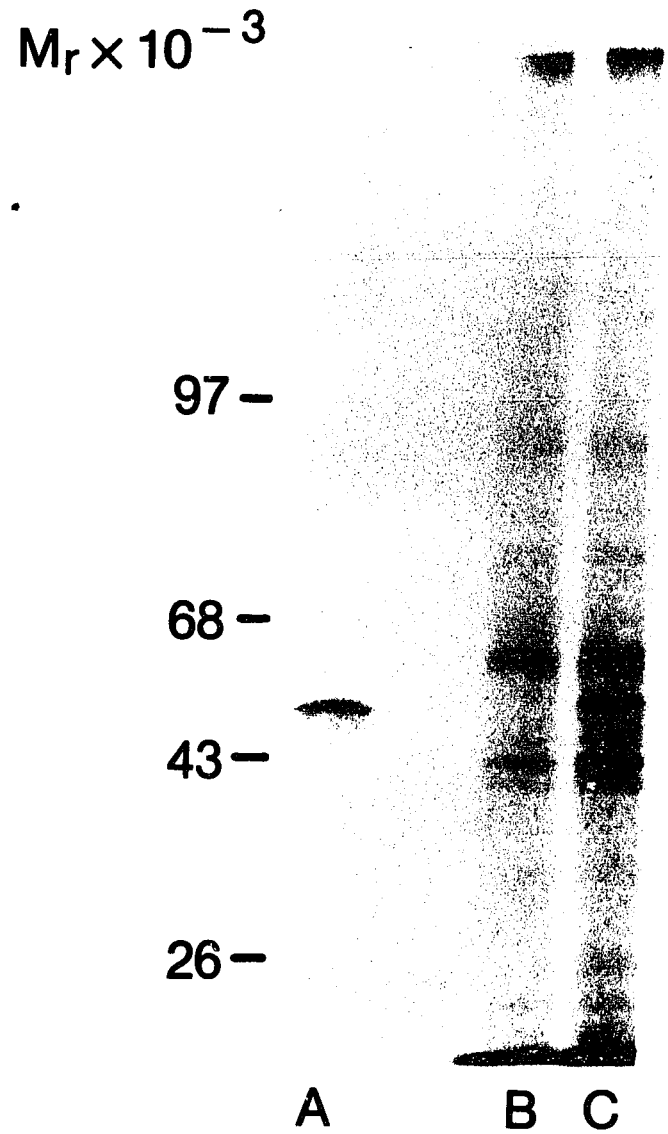

Figure 2. Identification of radiolabeled peptides corresponding to ODC from Chinese hamster ovary cells by immunoprecipitation.

Exponentially growing CHO cells were labeled with 100 μCi of [^{35}S]methionine/ml for 1 hour in methionine-deficient medium. Cells were harvested by scraping, sonicated into ODC buffer and immunoprecipitated as described in Materials and Methods. Lanes: (A) [^{3}H]DFMO-binding proteins immunoprecipitated from whole cell homogenates; (B) control, incubated without the primary antiserum; (C) proteins precipitated by the anti-ODC serum.

Since diamines and polyamines were known to influence ODC activity, and CHO cells growing in the chemically defined medium had substantially depressed intracellular putrescine and spermidine pools (Sertich et al., 1985; see also the next section), ODC activity and [^{3}H]DFMO binding to anti-ODC-antibody-immunoprecipitable protein were determined after restoration of intracellular polyamine pools. Restoration was accomplished by addition of 100 μM-ornithine or -putrescine to the culture medium. This concentration was chosen for study, as previous work had shown that 100 μM-ornithine led to maximal inhibition of ODC activity after a 24 hour incubation interval. The data shown in Figure 3(A) demonstrated that treatment with exogenous ornithine or putrescine led to a rapid decay in enzyme activity, with putrescine having a greater effect than ornithine. The results are plotted as a percentage of control, where the control activity represents untreated samples. Total inhibition of activity was observed after 8 hours of treatment with ornithine or 6 hours of treatment with putrescine. The decline in activity paralleled a decrease in [^{3}H]DFMO binding (Fig. 3B), implying that polyamines were not altering the enzyme catalytic activity, but that the number of active molecules was decreasing. The ratio of drug bound to activity was about 13 fmol/unit, and was consistent with that described for ODC from mouse kidney (Seely, Poso and Pegg et al., 1982a).

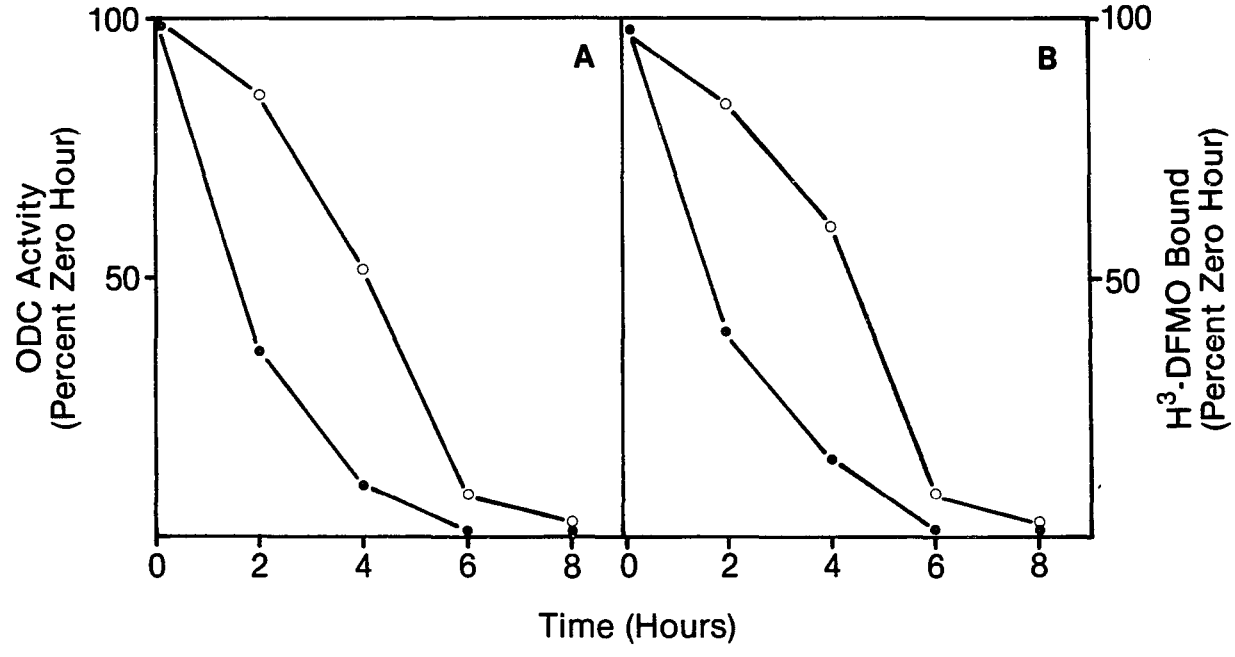

Figure 3. ODC activity and [^{3}H]DFMO-binding protein contents in Chinese hamster cells after treatment with exogenous 100 μM-ornithine or putrescine.
(A) ODC activity of exponentially growing CHO cells, maintained in defined medium, after various times of treatment with either 100 μM-ornithine (O) or 100 μM-putrescine (●).
(B) Immunoprecipitable [^{3}H]DFMO-binding proteins in samples treated in a manner identical with those in (A).

The data in Figure 4 document changes in the polyamine content of cells after treatment with the exogenous amines. Cells treated with 100 μM-ornithine (Fig. 4A) exhibited a steady increase in putrescine content, reaching a maximum of approximately 0.67 nmol/10^6 cells after 6 hours of exposure. Spermidine content increased linearly as a function of treatment time. At 8 hours, the content of intracellular spermidine was approximately 1.25 nmol/10^6 cells. Addition of exogenous putrescine (Fig. 4B) led to rapid intracellular accumulation of this diamine, reaching a maximum value of 1.25 nmol/10^6 cells after 2 hours, and the rate of formation of spermidine was identical with that of cells treated with ornithine.

The data presented in Figure 3 indicate that the inhibition of ODC by polyamines is mediated by an overall change in active protein content, with subsequent activity and protein amounts decreased to zero. One possible mechanism responsible would be a polyamine effect to inhibit the synthesis of ODC totally, thereby leading to a decline in ODC content as the protein is degraded. To determine whether ODC synthesis was being inhibited by polyamine restoration, cells were pulse-labeled for 30 minutes with [^{35}S]methionine at various times after addition of 100 μM-ornithine to the culture medium. Determination of labeling kinetics of ODC under these conditions revealed that 30 minutes is the minimal labeling time required to recover sufficient incorporation of radiolabel into ODC protein to yield a significant autoradiographic signal.

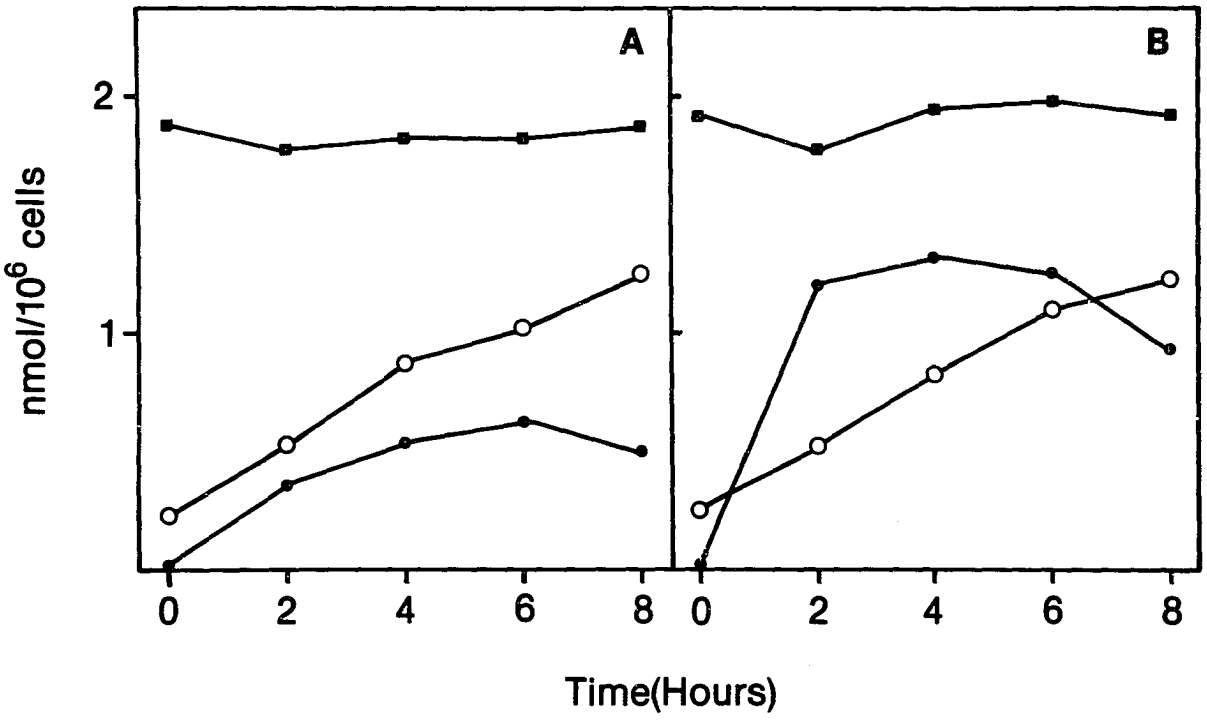

Figure 4. Polyamine content after addition of 100 µM-ornithine or -putrescine.

Cells were treated for various times with 100 µM-ornithine or -putrescine, harvested by scraping, and processed for polyamine analyses as described in Materials and Methods. Putrescine (●), spermidine (○) and spermine (■) contents are shown after ornithine addition (A) and after putrescine treatment (B).

Anti-ODC-antibody-immunoprecipitable protein was collected immediately after each labeling period and analyzed by SDS/polyacrylamide-gel electrophoresis. Figure 5 shows that incorporation of [^{35}S]methionine into the unique immunoreactive peptide was not totally inhibited at any time of treatment, even though ODC activity was substantially depressed after 2 hours of treatment. Lane (a) represented the untreated control sample (100% ODC activity), lane (b) 2 hours of ornithine treatment, representing 82% of control enzyme activity, lane (c) 4 hours treatment representing 24% of control enzyme activity, lane (d) 6 hours treatment, representing 20% activity, and lane (e) 8 hours treatment representing 15% of control ODC activity. Lanes (f)-(j) were equivalent amounts of cell protein treated with non-immune serum. Although there is some variation in labeled protein recovered under these conditions, this is a result of differences in total radiolabeled protein recovered for each time point. Densitometric analysis of this autoradiograph reveals less than 10% variance in the unique immunoprecipitated peptide with respect to absolute protein recovered. These data showed that addition of exogenous ornithine did not substantially alter the rate of ODC protein synthesis, although enzyme activity was decreased by up to 85%, indicating that synthesis is not being totally inhibited during the treatment period.

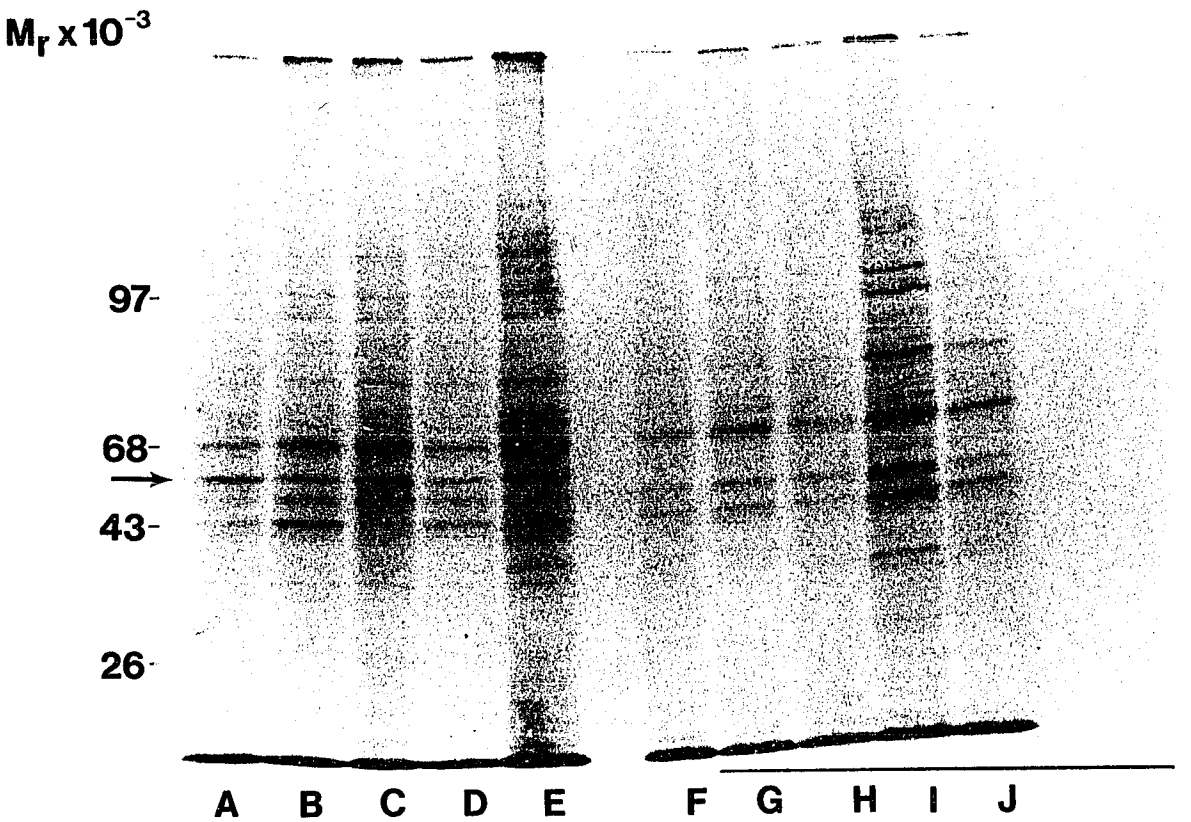

Figure 5. Immunoprecipitation of newly synthesized proteins in the presence of exogenous 100 μM-ornithine.

Ornithine (100 μM) was added to the medium of replicate CHO cultures, and cells were incubated for periods up to 8 hours. At various times after ornithine addition, cultures were pulse-labeled with 100 μCi of [^{35}S]methionine/ml for 30 minutes in methionine-deficient medium containing 100 μM-ornithine. Samples were harvested and protein was immunoprecipitated with anti-ODC serum. Lanes (A)-(E) represent newly labeled proteins immunoprecipitated from cells treated for 0, 2, 4, 6 or 8 hours with ornithine. Lanes (F)-(J) are control lanes treated identically with those in lanes (A)-(E), but without the addition of the primary antiserum. Arrow points to band corresponding to ODC.

It had previously been shown that the 1,3-diaminopropane-induced inhibition of ODC activity in androgen-stimulated mouse kidney was due to enhanced degradation of protein (Persson et al., 1984). To determine whether our observed changes in activity and DFMO binding were a result of increased degradation rates, cells were labeled with [^{35}S]methionine and immunoprecipitable protein was analyzed from cells chased for 5 hours with medium containing 100 μM-ornithine, -putrescine or -spermidine. These data are shown in Figure 6. The exposure of pulse-labeled ODC protein to subsequent exogenous amine led to a measurable decrease in immunoprecipitable protein, as determined by autoradiography. Treatment with no amine addition (lane B) showed no change in radiolabeled peptide during the 5 hour chase interval. Addition of 100 μM-ornithine (lane C), -putrescine (lane D) or -spermidine (lane E) led to a dramatic decrease in the amount of labeled protein of M_r approximately 54,000,, even though equivalent total protein contents were applied to each gel lane. These data represent one example of this type of experiment, with replicate experiments all showing very little change in radiolabeled ODC in the absence of exogenous polyamine addition. These data indicated that ODC activity was mediated by intracellular polyamine content through mechanisms enhancing the turnover of the protein.

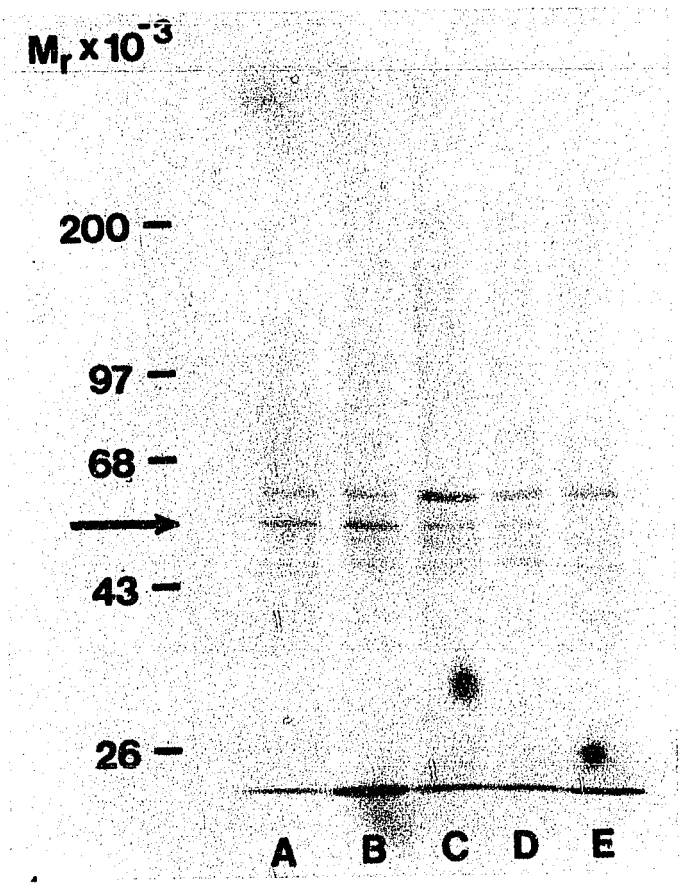

Figure 6. Effects of 100 μM-ornithine, -putrescine or -spermidine on the turnover of ODC.

Cells were labeled with 100 μCi of [^{35}S]methionine/ml for 30 minutes. Cultures were washed once, and medium without label, but containing 100 μM-ornithine, -putrescine or -spermidine, was added. After a 5 hour incubation, cells were harvested and proteins immunoprecipitated with anti-ODC serum. Lanes: (A) proteins precipitated from cells immediately after the 30 minutes labeling period; (B) proteins precipitated from cells not treated with any exogenous amine during the 5 hour time period; (C), (D) and (E) proteins precipitated from cells treated with ornithine, putrescine or spermidine respectively. Arrow points to band corresponding to ODC.

Discussion

Immunochemical techniques were used to analyze the regulation of ODC under conditions of substrate limitation in exponentially growing Chinese-hamster cells. The maintenance of cells in a chemically defined medium allows the elimination of an external ornithine supply (serum ornithine concentration is approximately 4.5 μM; Baetz et al., 1975), thus limiting polyamine accumulation. The cells grown in the defined medium have no detectable putrescine and lowered spermidine contents, and ODC activity is 1-3 units/10^6 cells during exponential-phase growth. The chemically defined medium was used to study the effects of dramatic changes in intracellular putrescine and spermidine concentrations on ODC, without using inhibitors, and should facilitate studies on polyamine regulation of ODC. Restoration of intracellular polyamine pools by addition of exogenous ornithine or putrescine leads to a rapid decay in ODC activity and [^{3}H]DFMO binding (Fig. 3). These data are similar to those reported by Persson et al (1984) who have shown that total ODC protein in androgen-stimulated mouse kidney rapidly declines after treatment with 1,3-diaminopropane. The measured decline in activity after restoration of polyamine pools by ornithine treatment is not due to a total inhibition of synthesis of of the protein, as had been postulated (Clark and Fuller, 1975; McCann et al., 1979; Murakami et al., 1985), as detection of newly synthesized ODC protein by immunoprecipitation is not decreased after treatments with exogenous ornithine, which diminishes activity to less than 15%

of that of untreated controls (Fig. 5). The observed effects appear to be a result of enhanced turnover of the protein, as measured by decreased recovery of immunoprecipitated prelabeled ODC (Fig. 6) after treatment with the various naturally occurring amines. There is no detectable turnover of the protein in the absence of polyamine (compare Fig. 5, lanes a and b), and this could explain the increased ODC activity in the cells grown under these conditions (Sertich et al, 1986). The determination of basal rates of ODC turnover, and their relation to the polyamine-mediated turnover in these cells, remain to be determined. These data support the contention that ODC degradation is mediated by intracellular polyamines, yet specific digestion of the protein to individual amino acids has not been demonstrated.

The mechanism responsible for enhanced turnover of ODC protein under these conditions is not yet known. The appearance of an ODC-inhibitory protein in response to exogenous amine exposure has been well documented (Canellakis et al., 1979, 1985). It has been proposed that this protein could participate in the turnover of ODC by targeting the protein to specific degradative pathways (Persson et al., 1984), similar to the ubiquitin conjugation mechanism described by Ciechanover et al (1984). It is unknown whether the antizyme is induced under these experimental conditions, and if this protein is modulating the turnover of ODC. Kyriakidis et al. (1984a) have reported that DFMO will bind to ODC-antizyme complexes, thus predicting an alteration in binding/unit activity

after addition of exogenous polyamines. The data presented here show that the amount of ODC-antibody-immunoreactive protein capable of binding DFMO decreases in parallel with enzyme activity (Fig. 3), such that catalytic activity is not changed after restoration of intracellular polyamine content. It is possible that the complexes do not accumulate, are labile intermediates in the turnover, and therefore are not detectable under these experimental conditions. Studies presented in the next sections of this thesis address this possibility.

Polyamine-mediated post-translational modifications of ODC have been reported, including phosphorylation (Atmar & Kuehn, 1981), transglutaminase-mediated conjugation of polyamine (Russell, 1981), and the appearance of multiple forms of the protein (Loeb et al, 1984; Mitchell et al, 1985a). It has been postulated that the modified ODC could be an intermediate in a pathway involved in the turnover of the protein (Mitchell et al., 1985). The incorporation of neither radiolabeled phosphate nor radiolabeled putrescine into anti-ODC antibody immunoreactive proteins under conditions of polyamine treatment which decreased ODC enzyme activity could be detected in these studies (data not shown). Whether those modifications are specific to ODC from other cell types remains to be determined.

The ability of intracellular polyamine content to regulate ODC protein could explain measured alterations in the half-life of the enzyme under various growth states, culture conditions and

stimuli of activity (Clark, 1974; Hogan and Murden, 1974; Chen and Canellakis, 1977). The utilization of polyamines for functions related to proliferation could release this control of ODC turnover, leading to an increase in the measured half-life of enzyme activity. The system described here shows a dramatic stabilization of ODC protein (i.e. half-life is greater than 5 hr), and probably represents an extreme example of polyamine control of ODC degradation, a situation where the cells have a total depletion of putrescine and greatly decreased levels of spermidine. Similar stabilization of ODC has been shown where polyamine leels are decreased by inhibitors of ODC (α-methyl ornithine, DFMO)(McCann et al., 1977; Mamont et al., 1978a; Karvonen and Poso, 1984), and the results presented here for a cell line which has a normally low intracellular polyamine content, further support these observations. The subsequent re-accumulation of polyamines then accelerates the degradation rate of ODC protein.

CHAPTER 3

SPERMIDINE MEDIATES DEGRADATION OF ORNITHINE DECARBOXYLASE BY A NON-LYSOSOMAL, UBIQUITIN-INDEPENDENT MECHANISM

Introduction

Recently, it has been shown in mammalian cells that ornithine decarboxylase activity is regulated by changes in the intracellular amount of enzyme protein (Seely and Pegg, 1983a; Isomaa et al,. 1983; Erwin et al.,1983). This is accomplished by alterations in both rates of synthesis and degradation (Seely and Pegg, 1983b; Persson et al., 1984; Gilmour et al., 1986). Polyamines act to regulate both of those processes by a feedback-like regulation process (Seely and Pegg, 1983b; Kahana and Nathans, 1985a; Dircks et al., 1986; Glass and Gerner, 1986b; Kanamoto et al., 1986).

The mechanism by which cells selectively degrade intracellular proteins is not well understood, though many studies suggest multiple pathways for protein catabolism (Hershko and Ciechanover, 1982; Ciechanover et al., 1984a). Lysosomes are clearly involved in protein degradation under nutritional deprivation conditions and have been postulated to control the turnover of long-lived proteins (Ciechanover et al., 1984a). The turnover of the majority of short-lived proteins is non-lysosomal.

Recently, an ATP-dependent proteolytic system has been reconstituted from reticulocytes, and involves the conjugation of ubiquitin to substrates for degradation. The use of a murine cell line, ts85, containing a temperature sensitive mutant in the ubiquitin conjugation pathway has suggested that greater than 90% of short-lived proteins are catabolized by this mechanism (Ciechanover et al., 1984b). How ODC protein is degraded is unknown. It has been postulated that post-translational modification is an intermediary step prior to degradation (Mitchell et al., 1985a). Alternatively, the interaction of the polyamine-induced inhibitor of ODC, the antizyme (Canellakis et al., 1979) has been shown to closely parallel the degradation rate of ODC (Murakami and Hayashi, 1985). This implies a targeting system similar to that of ubiquitin conjugation, though a direct determination of antizyme-ODC interaction which then results in degradation has not been shown.

Manipulation of intracellular polyamine content using both inhibitors (Karvonen and Poso, 1984) and medium conditions (Sertich et al., 1986) has allowed studies on the mechanism by which polyamines regulate ODC synthesis and degradation. The rapid turnover of ODC protein (see Tabor and Tabor, 1984) can be induced by treatment with exogenous ornithine and the polyamines (Chapter 2). The studies described here use CHO cells maintained in serum-free medium to investigate the mechanism by which polyamines, and in particular, spermidine, mediate ODC degradation. The roles of ODC protein modification, lysosomes and ubiquitin-mediated proteolysis

in this process are addressed. These results show that ODC degradation occurs by a mechanism independent of those pathways, while active protein synthesis is needed for accelerated turnover.

Materials and Methods

Materials

L-[1-^{14}C]Ornithine (60 mCi/mmol), L-[^{35}S]methionine (1120 Ci/mmol), α-DL-difluoromethyl [3,4-^{3}H]ornithine ([^{3}H]DFMO; 26.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA). A bacterial absorbant, Staphylococcus A (IGSORB) was purchased from the Enzyme Center (Malden, MA). Tissue-culture plastic was purchased from Falcon (Oxnard, CA) and culture medium and serum were obtained from Gibco Laboratories (Grand Island, NY). All other chemicals were purchased from Sigma Chemical Co St. Louis, MO).

Cell Culture Techniques

Chinese hamster ovary cells were maintained in McCoys 5A medium (Gibco) supplemented with insulin (10 μg/ml), transferrin (5μg/ml),$FeSO_4$ (2.5μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37`C in 90% air/10% CO_2 atmosphere. As indicated earlier, Sertich et al. (1986) have recently detailed the growth rates, viability and polyamine metabolism of the cells maintained under these conditions. The ts85 mouse cells were maintained in Ham's F-12 medium (Gibco) supplemented with 5% calf serum. The cells were routinely grown in plastic culture flasks at 32°C in a 95% air/5% CO_2 atmosphere. Cell numbers were determined using an electronic particle counter (Coulter Electronics, Hialeah, FL).

ODC Activity Measurements

ODC activity was determined as previously described (Glass and Gerner, 1986a). Cells were harvested from the dishes by scraping, collected by centrifugation, and disrupted by sonication in a buffer containing 0.05 M sodium/potasium phosphate, pH 7.2, 0.1 mM EDTA, 1.0 mM dithiothreitol and 20μM pyridoxal phosphate (ODC buffer). The enzyme activity was determined by the liberation of $^{14}CO_2$ during a 30 minute reaction period at 37°C.

Polyamine Analysis

The acid-soluble polyamine content of the cells was determined using the reverse phase ion-pair high pressure liquid chromatographic procedure described by Seiler and Knodgen (1980). Cells were harvested into phosphate-buffered saline (70 mM NaCl, 3 mM KCl, 8 mM Na_2 HPO_4, 1 mM KH_2PO_4, pH 7.2) using a rubber policeman, collected by centrifugation and disrupted by sonication into 0.2 M $HClO_4$ at a concentration of 5 x 10^6 cells/ml. The acid-soluble fraction was obtained by centrifugation at 8,900 x g for 5 minutes in a Beckman microfuge. Polyamine contents were then determined in 200 μl aliquots from these samples, and comparing the relative retention times and quantity with those of standard preparations of putrescine, spermidine and spermine.

Immunoprecipitation

Cells (2 x 10^7) were radiolabeled with 100 μCi/ml ^{35}S-methionine at 37°C for 30 minutes in methionine deficient medium. The labeling conditions yielded sufficient incorporation of radioactivity into ODC protein to allow visualization using autoradiography (Glass and Gerner, 1986a). The dishes were washed extensively with Puck's Saline A, and fresh medium containing excess unlabeled methionine was added to these cultures. At appropriate times, cells were harvested by scraping, and solubilized by sonication into ODC buffer. Primary anti-serum (1:5000 final dilution; previously characterized by Seely and Pegg, 1983a) was added, incubated for 18 hours at 4°C, followed by addition of 3 μl of a 10% solution of formalin-fixed Staphylococcus A (IGSORB). After 30 minutes at 20°C, immune complexes were collected by centrifugation at 8,900 x g for 3 minutes. Non-specific binding was reduced by washing the pellet 6 times with buffer A (1% Triton X-100, 0.5% sodium deoxycholate, 3 mM EDTA, 250 mM NaCl, 25 mM Tris/HCl pH 7.5). The proteins were eluted from the Staph A by addition of lysis buffer (9.5 m urea, 4% β-mercaptoethanol, NP040, 2% ampholytes) and separated by two-dimensional electrophoresis as previously described (O'Farrell, 1975). The ampholyte range consisted of a 1:1 mixture (2% v/v final concentration) of 4-6.5 and 3.5-9.5, respectively.

Radiolabeled DFMO Binding

To prepare [^{3}H]DFMO labeled ODC protein, 3 x 10^7 cells were solubilized in ODC buffer and [^{3}H]DFMO was added to yield a final concentration of 0.289 μM. After 5 hours at 37°C, ODC was immunoprecipitated as previously described and the proteins separated by two-dimensional electrophoresis. After drying, radiolabeled proteins were determined by fluorography.

Results

Changes in ODC Protein Mediated by Spermidine

Monolayer cultures of CHO cells maintained in defined medium were radiolabeled for 30 min with 100 μCi/ml [^{35}S] methionine, and then chased with unlabeled medium containing 10 μM spermidine. The cells were collected at varying times and ODC protein was immunoprecipitated and separated by two-dimensional electrophoresis. These data are shown in Figure 7. Panel a represents [^{3}H]-DFMO bound immunoprecipitable protein, to serve as an independent standard showing the expected migration of ODC on the two-dimensional gels (Persson et al., 1984). A comparison of a silver-stained gel containing the DFMO-bound ODC with the autoradiographs shows identical migration of the immunoprecipitated proteins (data not shown). ODC from untreated cells has a M_r of approximately 54,000 and an isoelectric point between 5.0 and 6.0, consistent with other descriptions (Seely et al., 1985).

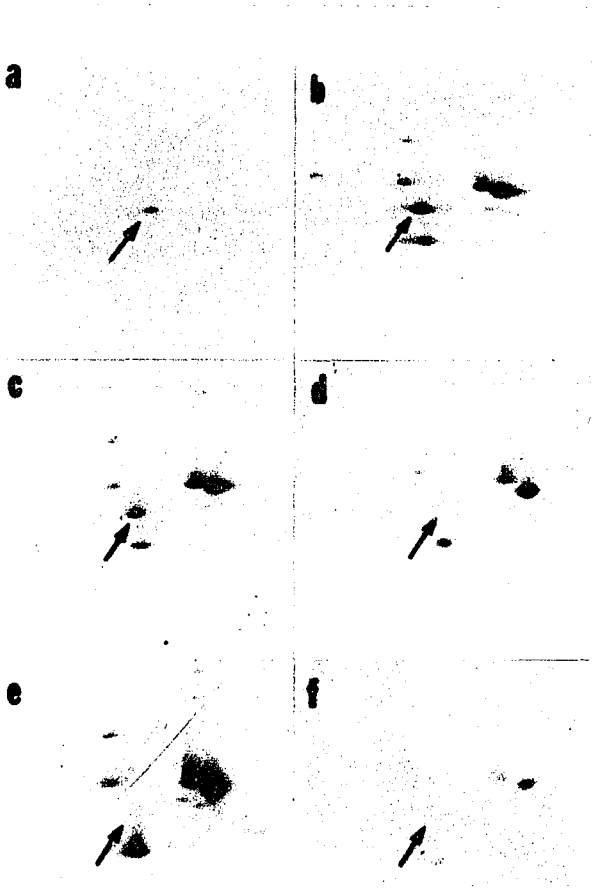

Figure 7. Two-dimensional electrophoresis of ODC after spermidine treatment.

Monolayer cultures of CHO cells were radiolabeled with 100 μCi/ml [^{35}S]methionine for 30 min in methionine deficient media. The cells were then washed, and unlabeled medium containing 10 μM spermidine was added. At varying times cells were harvested, ODC immunoprecipitated as described in Methods and separated by two-dimensional electrophoresis. Panel a represents radiolabeled DFMO-bound protein as a marker for ODC; Panel b (0 min), c (90 min), d (150 min), e (210 min) after the addition of 10 μM spermidine. Panel f shows proteins from a sample identical to that in panel b precipitated in the absence of the primary antisera. Each panel shows molecular weight range of 35,000-70,000 and pI range of approximately 5-7, with the acidic end to the right.

In untreated samples, at least five proteins are immunoprecipitated but only one with identical molecular weight and isoelectric point as the marker protein (Panel b). The addition of 10 μM spermidine to the cultures leads to a progressive loss of radiolabeled ODC by 90 min (Panel c), 150 min (Panel d), and 210 min (Panel e) after addition of exogenous spermidine. All of the samples contained the same amount of protein. Panel f shows a sample, identical to that shown in panel b, but prepared in the absence of primary antiserum. No protein corresponding to ODC, as seen in panel a, is apparent, while other proteins (corresponding to similar peptides seen in panels b-e) are seen. Thus, these proteins are not altered forms of ODC, but rather are peptides non-specifically precipitated by the adsorbant. During the rapid turnover of ODC seen in Figure 7, there is no detection of a post-translationally modified protein resulting in alterations in charge-state (compare Panels b and c). In addition, there are no changes in molecular weight, indicating that modified forms of the protein do not accumulate during degradation. In the absence of added spermidine there was no measurable decay of ODC activity or protein content for up to 6 hours (Glass and Gerner, 1986a).

Spermidine Inhibition of ODC Synthesis

In addition to accelerating the degradation rate, spermidine also acts at the translational level to inhibit new ODC synthesis. These data are shown in Figure 8.

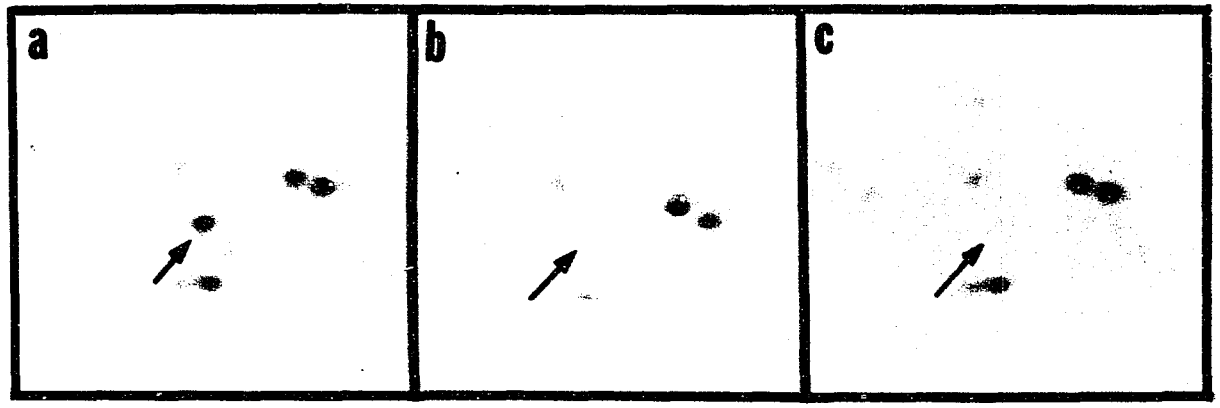

Figure 8. Effect of spermidine on ODC synthesis.

Log-phase cultures of CHO cells were treated with 100 μM spermidine and at varying times after the newly synthesized cell proteins were radiolabeled with 100 μCi/ml [35S]methionine 30 minutes prior to harvesting, in methionine deficient medium supplemented with spermidine. After 30 (Panel A), 90 (Panel B), and 180 (Panel C) minutes of treatment the cells were harvested, ODC protein immunoprecipitated and separated by two-dimensional electrophoresis.

Cells were treated with 10 μM spermidine and at varying times were labeled with [^{35}S]methionine for 30 minutes, in methionine-free media containing 10 μM spermidine. The cells were harvested after a labeling period of 0-30 minutes (Panel A), 60-90 minutes (Panel B), or 150-180 minutes (Panel C) of spermidine treatment; ODC was then isolated by immunoprecipitation and separated by two-dimensional electrophoresis as described in Methods. By 60 minutes of treatment (Panel B) the recovery of label into ODC protein is nearly undetectable, and at 180 minutes (Panel C), no labeled protein is detected.

With the acceleration of degradation rate, the ability to recover labeled ODC protein may be compromised and therefore below the limits of detection. This is probably not occurring as the maximal half life measured is 15 minutes (see below; Figure 9), and assuming immediate degradation, this would still yield an approximate 25% recovery of labeled protein, within the limits of detection by this methodology. These data are consistent with recently published results using Erlich Ascites cells (Persson, Holm and Heby,, 1986), and Chinese hamster cells maintained in serum-free conditions (hollta and Pohjanpelto, 1986). Those workers have also measured ODC mRNA content and show that polyamines have nc effect on stability, and thus argue that polyamines influence ODC mRNA translation and not transcription. While this data may appear to contradict that shown in Figure 5 (Chapter 2) for ornithine addition, it may be a spermidine-specific effect, and mah reflect

varying stages of polyamine regulation of ODC. By this model, as intracellular polyamine content is increased, the degradation rate is initially accelerated, and as subsequent spermidine content is increased, new synthesis of ODC protein would be depressed. These data could also be explained by potential intracellular polyamine compartmentalization. That is, the spermidine accumulation from extracellular sources is accessible to compartments not available to that made from ornithine decarboxylation to form putrescine, which then serves as the substrate for spermidine formation. Polyamine compartmentalization has been described, yet specific localization is difficult to interpret due to potential redistribution during the fractionation techniques (McCormick, 1978).

Effect of Temperature on Polyamine-Mediated ODC Turnover

Temperature manipulations have been used to analyze pathways of intracellular protein turnover (Neff et al., 1979; Bates et al., 1982) and to determine activation energies for the degradation of known protein substrates (Hough and Rechsteiner, 1984). To characterize the mechanism by which spermidine-mediated ODC degradation occurs, the effect of temperature on this process was analyzed. Figure 9 (panel A) shows the decay of ODC activity with time when monolayer cultures of CHO cells were treated with 10 μM exogenous spermidine at various temperatures. This concentration of spermidine yielded maximal rates of inactivation and was an apparent saturating dose, as 100 μM and 1 mM concentrations gave identical activity decay kinetics. The data in Figure 9 (panel A) show an

initial lag time, followed by a rapid decline in enzyme activity after spermidine is added to cultures. The linear activity decrease occurs with apparent first order kinetics and is temperature dependent. The temperature effect was measured over the range from 22° to 37°C, as polyamine uptake was greatly diminished at temperatures lower than 20°C (data not shown); this is probably a reflection of membrane changes at lower temperatures (Dunn, Hubbard and Aronson, 1980; Hough and Rechsteiner, 1984).

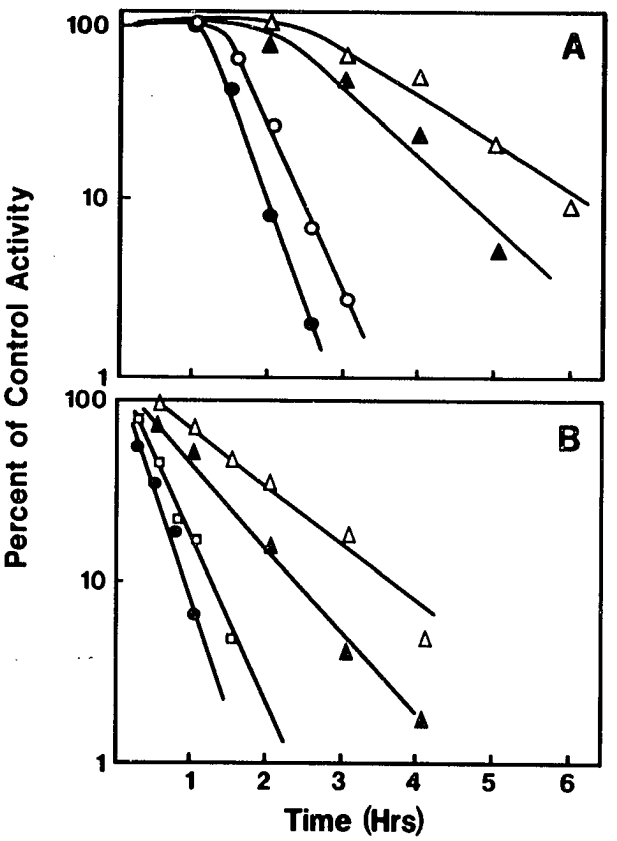

Figure 9. The effect of temperature on spermidine-mediated inactivation of ODC.

Panel A shows the rate of decay of ODC activity in cells treated continuously with 10 μM spermidine at the various temperatures. (●) 37°, (o) 31°, (▲) 26.5°, (Δ) 22°. Panel B; ODC activity at various temperatures following pre-treatment with 10 μM spermidine for 3 hours at 22°C. After this loading period, cells were washed extensively with Puck's Saline A, and fresh medium containing no exogenous spermidine was added. The dishes were then shifted to the indicated temperatures and ODC activity was determined. (●) 37°, (□) 31.5°, (▲) 26.5°, (Δ) 22°. Control activities represent approximately 2500 pmol/10^6 cells/30 min. Each data point represents the mean of triplicate determinations.

The observed lag could have been a result of a temperature-dependent effect on the accumulation of a degradative intermediate or a decrease in rate of polyamine uptake, since polyamine uptake occurs by an active transport mechanism (Porter, Bergeron and Stolowich, 1982). Spermidine accumulation was measured at both 22° and 37°C and this data is shown in Table 1. These results demonstrate that cells maintained in serum-free medium have no detectable putrescine (the limit of detection is approximately 20 pmoles) and low spermidine content (Sertich et al., 1986). In addition, these data show that accumulation of spermidine is temperature dependent. Cells incubated at 37°C have a total spermidine content of 1.03 nmol/10^6 cells after a 1 hour incubation with 10 μM exogenous spermidine, while those at 22°C have only accumulated 0.67 nmol/10^6 cells. Pulse-chase experiments, similar to those shown in Figure 7 have been carried out, and no accumulation of a modified ODC at 22°C after titration with spermidine for up to 3 hours was observed (Figure 10).

Table 1. Polyamine Content After Treatment with 10 μM Spermidine at Either 37° or 22°.

Treatment		Polyamine content (nmol/10^6 cells)		
Time	Temperature	Putrescine	Spermidine	Spermine
0		N.D.	.21	.94
1	37	--	1.34	1.09
	22	--	.88	1.05
2	37	--	1.67	1.06
	22	--	1.21	.88
3	37	--	1.88	1.12
	22	--	1.59	.91
4	37	--	1.60	1.09
	22	--	1.85	.87

Polyamine content of cells treated with 10 μM spermidine at varying temperatures. The cells were treated with 10 μM spermidine at either37° or 22°, harvested at 0, 1, 2, 3, 4 hrs after treatment and polyamine content determined. N.D. = none detected.

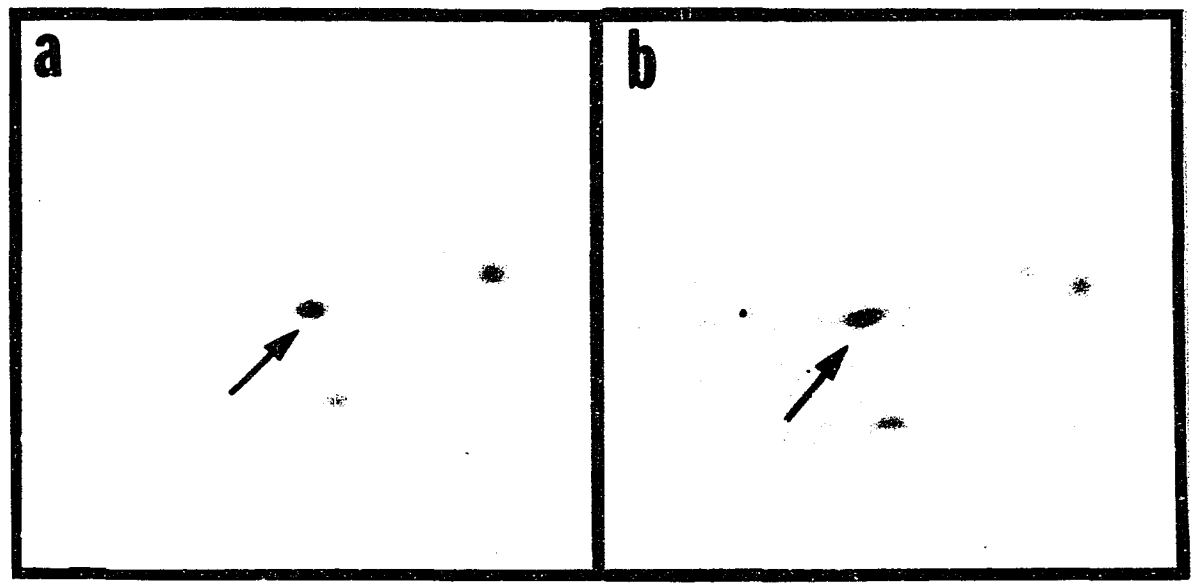

Figure 10. Two-dimensional electrophoresis of ODC after treatment at 22°C with 10 μM spermidine.

Cells were labeled with 100 μCi/ml [^{35}S]methionine for 30 minutes in methionine deficient medium. The dishes were washed with Puck's Saline A, placed either at 37°C in unlabeled medium or at 22°C in medium containing 10 μM spermidine. After 3 hours the cells were harvested, ODC immunoprecipitated and separated by two-dimensional electrophoresis.

The fact that polyamines accumulate intracellularly at 22°C, without a rapid ODC activity decay, allowed us to ask whether active polyamine transport was necessary for enzyme inactivation. Cells were treated with 10 μM spermidine for 3 hours at 22°C to accumulate endogenous spermidine contents in excess of 1.5 nmol/10^6 cells. The dishes were washed extensively to remove exogenous spermidine, and then shifted to varying temperatures. ODC activity was then determined at times after the temperature shift. These data are shown in Figure 9,panel B. The rate of inactivation of ODC is again temperature dependent, and is identical to that of cultures treated continuously with spermidine (panel A). The initial lag period is initially not seen in cultures treated in this manner, which have a high intracellular spermidine content. The exact concentration of spermidine dictating an acceleration of ODC degradation is unknown, and may reflect a wide variety of parameters (i.e. intracellular localization, growth rate). These data indicate that active polyamine transport is not directly linked to the ODC inactivation rate, as the same rates of enzyme activity decay are measured during continuous spermidine treatment or when cells are loaded with the same spermidine content at 22°C, and are then shifted to some higher temperature in the absence of exogenous spermidine. Polyamine content does not appreciably change after spermidine loading at 22°C after a shift to higher temperature indicating that polyamine efflux does not occur at lower temperatures leading to depression of enzyme activity (data not shown).

Recent studies by Hough and Rechsteiner (1984) have analyzed the effect of temperature on the degradation of proteins, known to be degraded by specific mechanisms. Therefore it was of interest to calculate the Arrhenius activation energy (E_a) for ODC turnover from the data in Figure 2, and compare that with known E_as for protein degradation. These results are shown in Figure 11. Linear relationships between log k (rate constants for the exponential decrease in enzyme activity) and T^{-1} were obtained for both conditions of inactivation shown in Figure 9 and yielded E_as of approximately 16 kcal/mole. Hough and Rechsteiner have calculated E_as of degradation to be 27 kcal/mol for both microinjected proteins, and those degraded by reticulocyte-lysate ATP-dependent proteolysis. In contrast, the E_as calculated for rapidly degraded endogenous proteins, or substrates of known proteases (i.e. chymotrysin), are approximately 13-16 kcal/mol (Hough and Rechsteiner, 1984). The E_a for ODC degradation, being approxiamtely 16 kcal/mol, suggests that the mechanism of ODC degradation is ubiquitin-independent, and is presumably mediated by a specific protease(s).

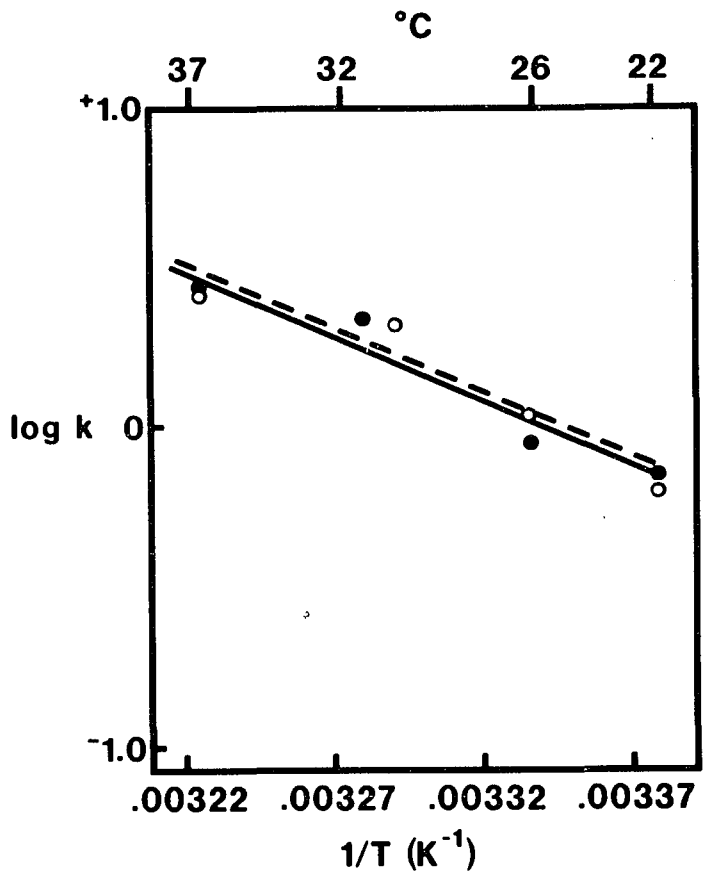

Figure 11. Arrhenius analysis of spermidine-mediated ODC turnover.

Lines were determined by linear regression of data obtained in Figs. 1 and 2. (●) (——) continuous treatment with 10 μM spermidine, r=0.93, (o) (----) cells pre-treated with spermidine at 22° and then shifted to varying temperatures, r=0.95.

Ubiquitin Involvement in ODC Turnover

To test directly whether ubiquitin was directly involved in ODC turnover, ODC activity was measured in ts85 cells (a mouse mamarry carcinoma cell line), a temperature sensitive cell cycle mutant (Mita et al., 1980), whose major lesion is in the E_1 activating enzyme of the ubiquitin conjugation pathway (Finley et al., 1984). Ciechanover et al. (1984a, 1984b) have described the use of this cell line to determine the involvement of ubiquitin conjugation in short-lived protein degradation (i.e. half-life $<$ 2 hrs) and have concluded that greater than 90% of this class of proteins is degraded by this pathway. ts85 cells were shifted from 32° (permissive) to 39° (nonpermissive) and ODC activity was assayed as a function of time after the shift. At 3 hours and 6 hours after the temperature shift, 10 μM spermidine was added to the dishes. If the ubiquitin conjugation system were involved in ODC turnover, the predicted result would be an increase in activity after temperature shift (as the protein is stabilized), and no effect of spermidine to decrease ODC activity. The data presented in Figure 12 show that ODC activity decreases as a function of time at 39°, and this decline is accelerated by the addition of spermidine. While a comparison of the ODC activity decay rate between the wild-type is not shown, the ODC activity still declines at a rapid rate (with a halflife of approximately 30-40 minutes) in the absence of ubiquitin conjugation. Pulse-chase studies similar to those described in Figure 1 have confirmed that this decline in activity is due to degradation and not the accumulation of an inactive intermediate.

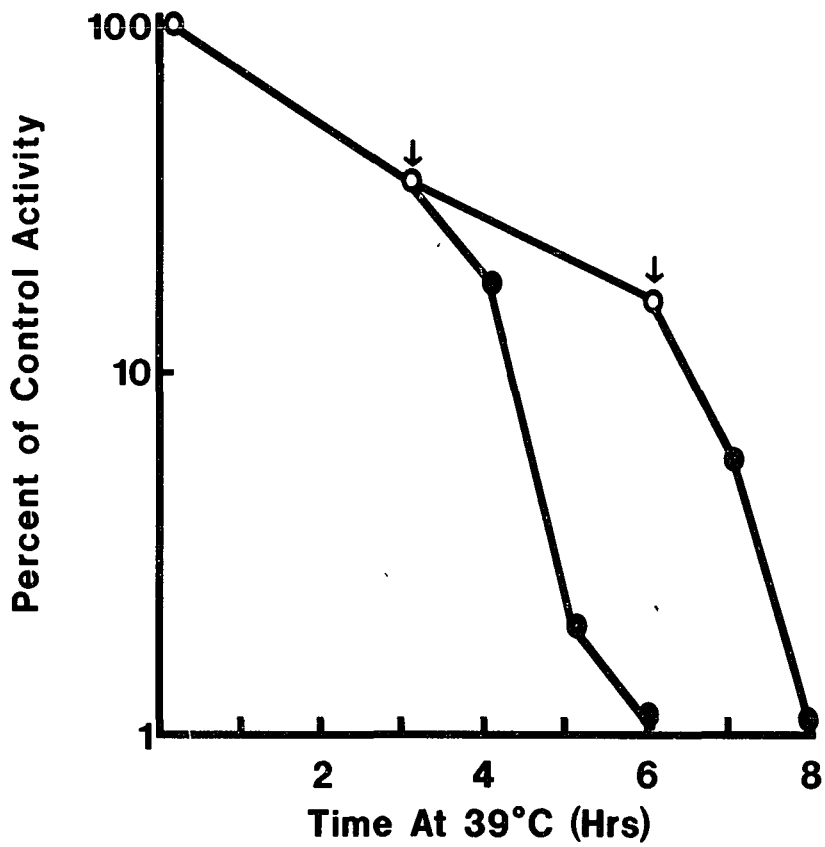

Figure 12. ODC inactivation in ts85 cells at the non-permissive temperature.

ts85 cells maintained in exponential growth at 32° were shifted to 39°, and ODC activity was measured at varying times after the shift (o). At 3 hrs and 6 hrs, 10 μM spermidine was added (arrows) and enzyme activity determined (•). Control activity represents 270 pmol/10^6 cells/30 min.

Inhibitors and Polyamine Mediated ODC Degradation

In studies analyzing the role of lysosomes in degradation, various inhibitors of lysosomal function have been shown to block catabolism of proteins known to be degraded by this pathway (Poole and Ohkuma, 1981; Rote and Rechsteiner, 1983). The effects of these agents on spermidine mediated ODC turnover were determined. Chloroquine and ammonium chloride, two agents which alter intralysosomal pH, did not inhibit the ODC activity decrease. In addition, antipain, leupeptin, and chymostatin, specific inhibitors of lysosomal proteases, (Neff et al., 1979; Katznelson and Kulka, 1982) also had no effect (Table 2). Decay of radiolabeled ODC was also measured under these conditions (data not shown), and the lysosomatrophic agents did not inhibit the degradation. The lack of effect of lysosomatrophic agents on spermidine induced ODC degradation indicated that lysosomes are not involved in this process.

The effects of protein synthesis inhibition were also analyzed in an attempt to further characterize the findings of Mitchell et al. (1985b). They have recently shown that dicyclohexylamine, an inhibitor of spermidine synthase, leads to a decrease in intracellular spermidine content. In addition, ODC activity induced by serum stimulation was greater than 4-fold above untreated cells, and that ODC can be inactivated by addition of exogenous spermidine. This effect was shown to be inhibited by cycloheximide. These effects of protein synthesis inhibition on this system are shown in Table 3.

Table 2. The Effect of Inhibitors on 10 μM Spermidine Mediated Inactivation of Ornithine Decarboxylase

Additions	Percent of Control Activity
None	100
SPD	< 1
SPD + NH_4Cl 20 mM	< 1
SPD + Chloroquine 0.25 mM	< 1
SPD + Antipain (200 μg/ml)	< 1
SPD + Leupeptin (200 μg/ml)	< 1
SPD + Chymostatin (200 μg/ml)	< 1

Cells were treated as indicated, incubated at 37°C for 3 hours and enzyme activity was determined. Values represent the percent of activity of identical cultures treated with the inhibitors alone. Control activity represents 2650 $pmol \cdot 10^6 \cdot 30\ min^{-1}$.

Table 3. The Effect of Protein Synthesis Inhibition on ODC Inactivation

Additions	Percent of Untreated Activity	Percent of Control Activity
Untreated	100 (1212 pmol/10^6 cells/30 min)	
+ SPD	<2	
Cycloheximide 20 μg/ml	48	
+ SPD	59	122
Emetine 100 μg/ml	76	
+ SPD	71	93
Puromycin 100 μg/ml	104	
+ SPD	51	48

Monolayer cultures of CHO cells were shifted to 22°C and treated either with 10 μM spermidine (+ SPD) or left untreated for 3 hrs. The cells were then washed extensively with saline and shifted to 37°C in the presence of the inhibitors. ODC activity was determined 2 hours later. Control activity represents cultures treated only with inhibitors.

Treatment of cells with cycloheximide, emetine and puromycin led to inhibition of the spermidine-mediated degradation of ODC, consistent with and extending previous observations (Chen and Canellakis, 1977; Mitchell et al., 1985b). Cells treated with spermidine at 22°C, and then shifted to 37°C for 2 hours have less than 2% of control (no spermidine added) ODC activity. Inhibition of protein synthesis during this interval blocks the spermidine-mediated loss of ODC activity by 50-100%, depending upon the inhibitor used. This is not due to a rate decrease in ODC activity, as similar results were found after a 4 hour treatment interval with spermidine. Under the conditions used, protein synthesis, as measured by [^{3}H]leucine incorporation into TCA insoluble fractions, was inhibited by greater than 95% for each of the inhibitor concentrations used.

Discussion

The ability to manipulate intracellular putrescine and spermidine content in CHO cells without affecting cell growth rates and without the use of inhibitors (Sertich et al., 1986; Glass and Gerner, 1986a) has facilitated the investigation of the mechanism by which these polyamines regulate ODC activity. ODC protein is stabilized in these cells, and the degradation can be accelerated by treatment with exogenous ornithine and polyamines (Sertich et al., 1986; Glass and Gerner, 1986a). These data indicated that intracellular polyamines directly regulate the well described rapid turnover of ODC protein (see references in Tabor and Tabor, 1984).

These cells have been used to analyze the pathway by which polyamines, specifically spermidine, mediate the accelerated degradation of ODC.

Others have reported that ODC can be post-translationally modified, resulting in a charge shift of the protein (i.e. phosphorylation), and that this modification makes the protein more labile to degradation (Mitchell et al., 1985a). This effect has been investigated directly by determining changes in isoelectric point and molecular weight during spermidine-enhanced degradation. ODC protein was pulse-labeled with [^{35}S]methionine, and then chased in medium containing spermidine. Separation of immunoprecipitable ODC by two-dimensional electrophoresis showed no shift in isoelectric mobility or molecular weight (Figure 7).

Analysis of the effects of temperature on spermidine-enhanced ODC degradation has yielded the answer to two questions: 1) What is the activation energy for ODC degradation and how does it relate to known activation energies for degradation of other proteins?, and 2) Is polyamine transport involved in ODC degradation? The Arrhenius analysis (Figure 11) shows that the E_a for ODC degradation is approximately 16 kcal/mole. This activation energy is consistent with that for known protease action as described by Hough and Rechsteiner (1984) being approximately 15-16 kcal/mole. This value contrasts with the higher E_a (27 kcal/mole) measured for the degradation of both microinjected proteins and those degraded by a known ubiquitin-dependent pathway. These data support the hypothesis

that ODC degradation occurs by a ubiquitin-independent mechanism, possibly involving a specific protease(s). Treating the cells at 22° with spermidine, allowing intracellular accumulation and then shifting to higher temperatures also showed that the active transport of polyamine is not critical for ODC inactivation (Fig. 9, panel B). The identical E_a for ODC degradation obtained for continuous, or pre-treatment with spermidine indicate that the intracellular polyamine content correlates with the regulation of ODC activity. Decay of ODC activity at 22°C is first seen when the spermidine content is greater than 1.50 nmol/10^6 cells, a value which is within physiological limits for CHO cells (Sertich et al., 1986). These data indicate that the initial period in which ODC activity is not decreasing exponentially is due to a difference in polyamine uptake, and it is possible that a critical intracellular concentration is required for spermidine-enhanced ODC degradation.

ODC degradation does not occur via a lysosome pathway, as suggested by the inability of lysosomotrophic agents to block the decay of activity (Table 2). The data using the inhibitors are also supported by the activation energy required for turnover. This conclusion is not surprising, as lysosomes are thought to catabolize long-lived proteins (Hershko and Ciechanover, 1982).

As previously described by Mitchell et al. (1985b), cycloheximide was found to block the spermidine enhanced ODC turnover. In addition, emetine and puromycin also block the effect (Table 3). Interestingly though, puromycin inhibits inactivation by

50%, as compared to cycloheximide and emetine which totally inhibit degradation. Puromycin, in contrast to emetine and cycloheximide, induces nascent peptide chain release, and truncated peptides have been shown to be rapidly degraded (Ciechanover et al., 1984a). Whether nascent peptides can interact with ODC to enhance degradation is unknown. These data are interesting in that they indicate that active protein synthesis is required for ODC degradation, and suggest that an inducible protein(s), like the antizyme (Canellakis et al., 1979), is (are) involved in this process. Indeed, Hayashi and coworkers have shown a direct correlation between antizyme accumulation and ODC degradation (Murakami and Hayashi, 1985). The antizyme interaction with ODC could act to alter the conformation of the protein allowing recognition by a protease which then degrades both proteins. Alternatively, Pegg and others have suggested the interaction could be a targeting mechanism similar to the ubiquitin system. The estimations of the activation energy (Figure 11) and the ATP-independence of ODC degradation suggest that the ODC protease is different from the recently purified protease complex which degrades ubiquitin conjugates (Hough et al., 1986).

Another possibility is that a labile protease is required and protein synthesis inhibition leads to depletion of this protein. This idea is not supported by the many descriptions of ODC half-life in cells where the intracellular polyamine content is high (see Tabor and Tabor, 1976, 1984). Those studies measured a decay of ODC

activity after protein synthesis inhibition, indicating a constitutive level of an accumulation of some polyamine induced co-factor. It is probable that reduction of polyamine content leads to a depletion of this pool in the cell system used here, and restoration of normal ODC turnover mediated by polyamines requires continued protein synthesis. Clearly, detailed kinetics of antizyme induction by polyamines will be required to determine the significance of this protein in ODC degradation.

The possibility that ODC turnover was a ubiquitin-dependent mechanism has been further analyzed by using the ts85 cell line, a temperature sensitive mutant for ubiquitin conjugation. Treatment of these cells with spermidine at the nonpermissive temperature results in rapid ODC activity loss (Figure 12). These observations support those of Dircks et al. (1986) who have shown that ODC turnover occurs in the absence of ATP, and concluded that ubiquitin conjugation is not necessary for degradation.

The data presented support the conclusion that ODC degradation occurs by a mechanism which is non-lysosomal and ubiquitin-independent. Active polyamine transport is not necessary for accelerated ODC turnover and there is no major accumulation of a particular modified ODC protein. It is possible that spermidine could be interacting directly to activate a short-lived protease, and this is then recognizing ODC sequences for degradation. The lack of a general stimulus of protein catabolism by polyamines (Kanamoto et al, 1986) seems to rule out the possibility that

polyamines generally activate proteases, yet whether a specific protease for ODC exists remains to be determined.

The polyamines could also be interacting directly with the ODC protein, in a non-covalent manner, to alter the conformation and allow recognition by a protease(s). Studies which have utilized DFMO to inactivate the protein show that active-site binding does not alter the decay rate (Seely and Pegg, 1983b). This point was addressed here by determining ODC protein turnover after enzyme inactivation by 10 mM DFMO and no evidence for stabilization was found. Treatment with DFMO alone does not lead to the accelerated turnover. This indicates that direct interactions between polyamines and the active site are not crucial for turnover.

The data presented define components critical for the degradation of ODC in vivo and should prove useful in creating an in vitro system to study this process. Decay of ODC protein is ATP independent, has an activation energy of approximately 16 kcal/mol and is dependent upon a polyamine-induced component requiring protein synthesis. Whether that co-factor is the antizyme or some other protein remains to be determined.

CHAPTER 4

ORNITHINE DECARBOXYLASE PRODUCTION *IN VITRO* USING MOUSE cDNA

Introduction

Studies on ODC activity regulation have been hampered by the small amount of enzyme protein present in most tissues and cell cultures (Kameji et. al., 1982, Seely et al., 1982a). The availability of high titer anti-sera (Kameji et al., 1982; Seely and Pegg, 1983a; Isomaa et al., 1983) and production of mutant cell lines (McConlogue and Coffino, 1983; Kahana and Nathans, 1984; Choi and Scheffler, 1983) which greatly overproduce ODC protein have facilitated the investigation of this enzyme, and have been used to isolate cDNA sequences specific for ODC protein (Kontula et al., 1984, Kahana and Nathans, 1984, McConlogue et al., 1984). Though recent work has detailed the effects of polyamines in regulating ODC in a feedback type mechanism at the translational level (Kahana and Nathans, 1985b; Persson et al., 1986) and by influencing the stability of ODC protein(Persson et al., 1984; Glass and Gerner, 1986a, 1986b; Hollta and Pohjanpelto, 1986), the detailed mechanism by which these effects occur is unknown.

In an attempt to recreate an *in vitro* system for ODC degradation, one goal was to acquire sufficient quantity of

ODC protein for study. Advantage has been taken of recent techniques of *in vitro* transcription (Melton et al., 1984) and *in vitro* translation of cloned cDNA to produce ODC specific mRNA and protein from a previously characterized ODC cDNA (Kahana and Nathans, 1984, 1985a). This chapter describes this methodology, which enables the production of sufficient quantities of easily obtainable ODC mRNA and protein. This methodology is preliminary for a more detailed analysis of potential polyamine interactions with these molecules.

Materials and Methods

Materials

L-[^{35}S]methionine (1120 Ci/mmol) was purchased from New England Nuclear (Boston, MA). A bacterial absorbant, Staphyloccus A (IGSORB) was purchased from the Enzyme Center (Malden, MD U.S.A.). Ampholytes (pH 4-6.5, 3.5-9.5) were obtained from LKB (Gathersburg, MD). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

In Vitro Transcription and Translation

Plasmid pmODC-2 was the gift of Dr. Daniel Nathans (Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD). The 1691 bp fragment of ODC cDNA was derived from the mouse ODC cDNA clone, pmODC-1, initially described by Kahana and Nathans (1985a). This fragment was derived from digestion of pmODC-1 with Sal I and Pvu II and

contains 68 bp of the 5' leader sequence, the complete coding sequence and 240 bp of the 3' non-transcribed sequence. The Sal I and Pvu II recognition sites were converted to an EcoR I site and to a Bam HI site, respectively, and inserted into the multiple cloning site of plasmid pSP65 (ProMega Biotec) 5' to the promoter for the bacteriophage Sp6 RNA polymerase. The plasmid DNA as received was transferred into E. coli DH1, and plasmid DNA was prepared essentially as described (Maniatis et al., 1982).

ODC mRNA was prepared in vitro as described by Melton et al. (1984) using as a template pmODC-2 DNA digested with restriction endonuclease BamH I and Pvu II. Fifteen units of SP6 RNA polymerase was added to a reaction (final volume 100 μl) consisting of 40 mM Tris-Cl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 1 mM DTT, 125 units RNasin, 0.5 mM each of ATP, CTP, UTP and GTP, and 3 μg DNA template. After 2 hrs at 37°C, 2 units of DNase was added to digest the template DNA and the incubation was continued for 15 min. The sample was extracted one time with phenol:chloroform:isoamyl alcohol (50:48:2) which had been equilibrated with 10 mM NaCl, 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA, extracted one time with chloroform:isoamyl alcohol (24;1), precipitated in 70% ethanol at -20° for $>$ 2 hours, collected at 15,000 x g for 15 min and passed over a "spun-column" of Sephadex G-50 (Maniatis et al., 1982), which had been equilibrated with 10 mM Tris-Cl, ph 8.0, 0.1 M NaCl, and 0.1 mM EDTA. The RNA was recovered from the excluded volume and used directly in the translation reaction.

To determine the size of reaction products, aliquots of the ODC RNA made in vitro were electrophoresized through a 1.0% agarose gel in the presence of formaldehyde (Maniatis et al., 1982) using calf liver 28S (4800 b) and 18S (1850 b) and E. coli 23S (2904 b) and 16S (1541 b) ribosomal RNA as markers. The gel was stained with ethidium bromide according to Maniatis et.al.(1982) and photographed.

Cell-free synthesis of ODC in rabbit reticulocytes was performed as recommended by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) using 5 μCi [^{35}S]methionine per reaction. ODC mRNA was added at the indicated final concentrations in a volume of 10 μl and reactions incubated at 30°C for 1 hour. The reactions were terminated by addition of 80 μl of 4 SDS solubilization buffer (2% SDS, 5% β-mercaptoethanol, 3% sucrose in 300 mM Tris/HCl pH 7.0). For solubilization and separation by two-dimensional electrophoresis, 100 μl of isoelectric focusing (IEF) lysis buffer (8 M urea, 4% β-mercaptoethanol, 5% NP-40, 2% ampholytes pH 3.5-9.5) was added after completion of the translation reaction.

Gel Electrophoresis

One dimensional SDS polyacrylamide gel electrophoresis was performed using a slight modification of the method of Laemmli (1970). Bisacrylamide was replaced with N,N'-diallyltartardiamide (Bio Rad), so that gel slices could be dissolved by the addition of 2% periodic acid. The method of O'Farrell (1975) was used for the

separation of proteins by two-dimensional electrophoresis. Ampholytes of pI ranges 3.5-9.5 and 4-6.5 were used in a 1:1 ratio.

Immunoprecipitation

After in vitro translation of ODC mRNA, the samples were diluted with 170 μl of buffer containing 0.05 sodium/potassium phosphate, pH 7.2, 0.1 mM EDTA, 1.0 mM dithiothreitol, 20 μm pyridoxal phosphate and 100 μM phenylmethylsulfonylfluoride (ODC Assay Buffer), followed by addition of 5 μl of a 1:500 dilution of primary antiserum (characterized in Seely and Pegg, 1983). After 1 hour at 20°C, 5 μl of 10% suspension of formalin-fixed staph A was added, and 20 minutes later the complexes were collected by centrifugation at 7,500 x g for 2 minutes. Proteins were eluted from the staph A at 100°C for 5 minutes in 50 μl of SDS solubilization buffer.

ODC Activity

Following <u>in vitro</u> translation of ODC mRNA the samples were diluted with 170 μl ODC assay buffer, 6 μCi of L-[1-^{14}C]ornithine (52.4 mCi/mmol)was added and reactions were incubated at 37°C for 30 minutes. ODC activity was determined by the liberation of $^{14}CO_2$ during the reaction period. One unit of enzyme activity is defined as 1 nmol of ornithine decarboxylation per minute at 37°C.

Results

The plasmid used here contains a full length cDNA coding for mouse ODC and 68 bp of the 5' leader sequence, which is inserted 5'

to the promoter for the bacteriophage Sp6 RNA polymerase. Transcription of the linearized plasmid pmODC-2 *in vitro* using Sp6 RNA polymerase yields a single RNA species. The *in vitro* transcription reaction was performed and the RNA was electrophoresed on an agarose gel as described in Methods. The photograph in Figure 13 shows that a highly homogenous RNA preparation is obtained (Lanes B and C) whose size is 1691 base pairs (bp) consistant with that expected for the full-length transcription of the pmODC-2 sequence. From 3 separate transcription reactions, using 3 micrograms of plasmid DNA, 11, 13, and 17 micrograms of RNA were produced in a 2 hour reaction at 37°. The RNA prepared for *in vitro* translation was not modified by a 5'-cap structure.

The addition of ODC mRNA produced by *in vitro* transcription to a rabbit reticulocyte lysate cell-free translation system yields one predominant peptide of approximately 54,000 M_r (Figure 14A).

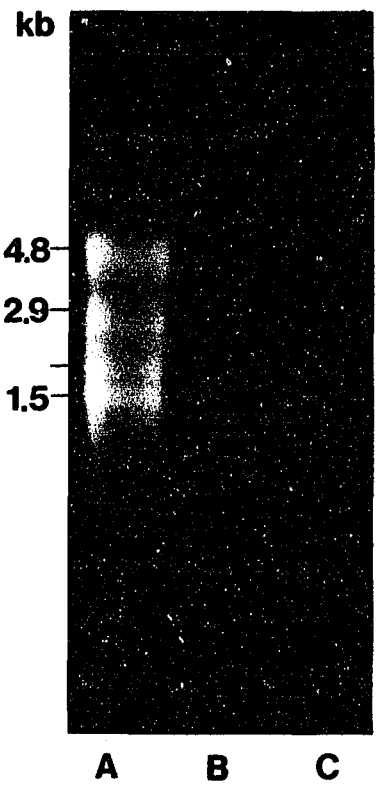

Figure 13. Agarose gel electrophoresis of RNA produced by in vitro transcription of pmODC-2.

Linearized pmODC-2 was transcribed in vitro as described in Methods, and the RNA separated by 1.0% agarose gel electrophoresis and photographed. Lane A represents ribosomal RNA markers, Lane B 10 μg and Lane C 5 μg of in vitro transcribed pmODC-2.

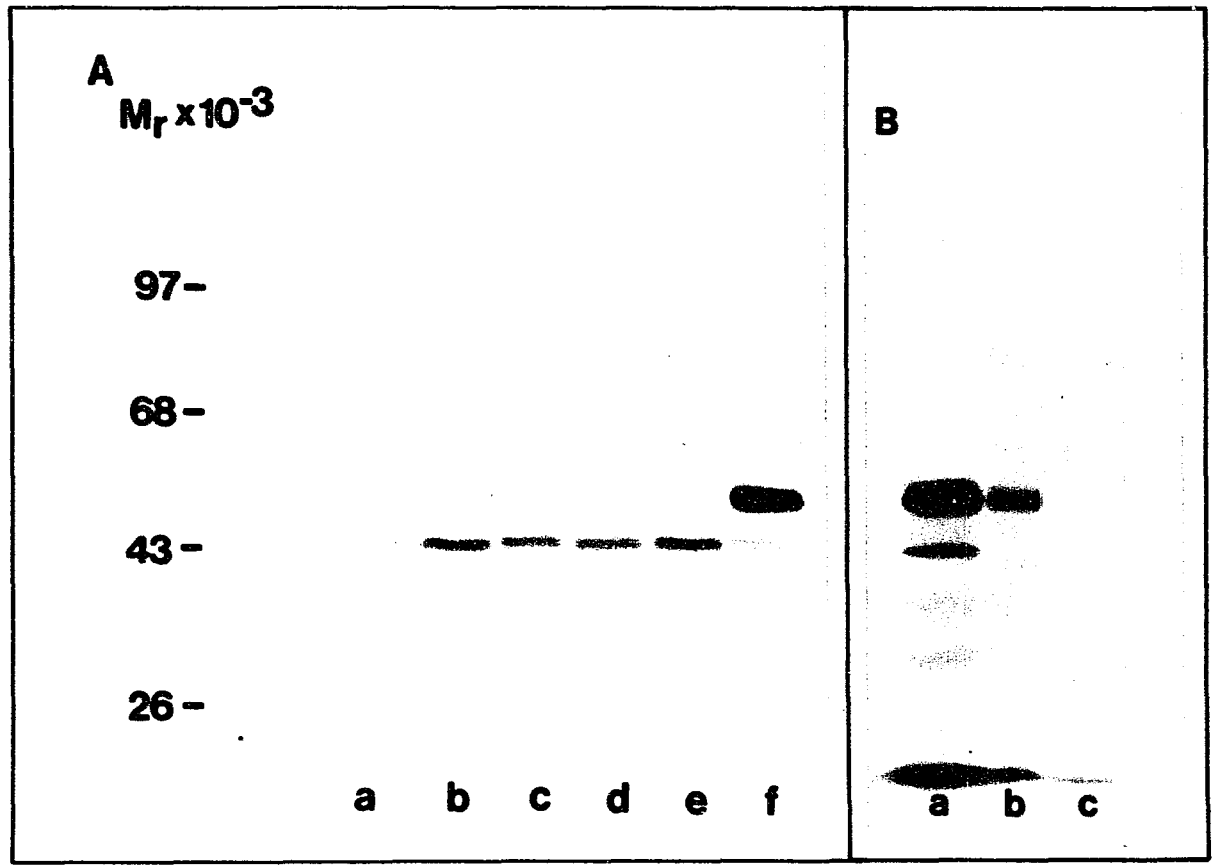

Figure 14. Cell-free synthesis of ODC in rabbit reticulocyte lysates from ODC mRNA produced by in vitro transcription.

(A) Various concentrations of ODC mRNA were added to in vitro translation mix, and incubated for 1 hr at 30°C. Proteins were fractionated by SDS polyacrylamide electrophoresis and visualized by autoradiography: Lane a) no addition, b) 1 ng, c) 10 ng, d) 50 ng, e) 150 ng, f) 250 ng ODC mRNA. (B) Immunoprecipitation of in vitro translated proteins by anti-ODC antiserum. Lane a) in vitro translated proteins after addition of 250 ng ODC mRNA, b) proteins immunoprecipitated by anti-ODC antiserum, c) non-specific proteins precipitated by staph A.

Detectable synthesis of the protein in vitro is not evident until a concentration of 250 ng RNA per reaction reached (Figure 14A, Lane f), and this concentration represents the optimum for in vitro synthesis. Addition of higher concentrations of RNA does not lead to a greater production of this peptide (data not shown). In the absence of exogenously added RNA (Figure 14A, Lane a), a radiolabeled peptide of M_r 43,000 is produced, presumably resulting from non-synthetic labeling of endogenous protein (Jackson and Hunt, 1983). To determine whether the protein synthesized in vitro from ODC mRNA corresponds to actual ODC peptides, immunoreactivity was determined by precipitation with anti-ODC anti-serum. These results are shown in Figure 14B. Lane a shows the protein profile from a standard in vitro translation using 250 ng ODC mRNA. Lane b represents those proteins immunoprecipitated by the specific anti-serum, and Lane c are non-specific proteins absorbed by the staph A. The major protein at $M_r \simeq 54,000$ is specifically precipitated along with two other minor species at $M_r \simeq 38,000$ and 30,000, respectively. The protein pattern observed here is similar to that shown for in vitro synthesis of ODC mRNA isolated from mouse kidney (Kontula et al., 1984).

To further characterize ODC protein synthesized in this system, the distribution of in vitro translated proteins was analyzed by two-dimensional electrophoresis. Mouse ODC has a heterogenous isoelectric point of approximately 5.4 (Seely et al., 1985; McConlogue and Coffino, 1983) and the protein made in vitro has a very similar isoelectric mobility (Figure 15, Panel A).

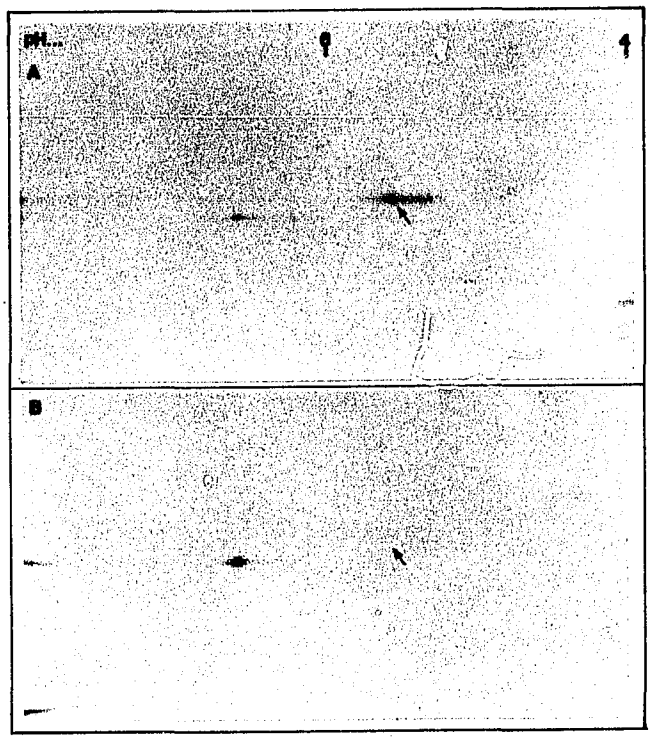

Figure 15. Two-dimensional electrophoresis of <u>in vitro</u> translated proteins after addition of ODC mRNA to rabbit reticulocyte lysates.

ODC mRNA was added at a final concentration of 250 ng to the rabbit reticulocyte lysate translation mixture. After 1 hr at 30°C, the reaction was terminated, the proteins were separated by two-dimensional electrophoresis, and visualized by autoradiography. Panel A) ODC mRNA; panel B) no addition of exogenous RNA.

The non-specific protein labeled in the absence of RNA is shown in Figure 15, Panel B. The two forms of ODC seen in mouse kidney are not apparent under our conditions, but there is evidence of charge heterogeneity. The nature of this heterogeneity is unknown and represents a minor amount of the total ODC protein made in this cell-free system.

To quantitate the amount of ODC protein made in each translation reaction, ODC activity and radioactive incorporation into the 54,000 M_r species were determined from varying RNA preparations. These data are shown in Table 4. ODC activity was determined as described in Methods from 2 separate RNA preparations. Lysates not containing ODC mRNA showed no detectable enzyme activity. These data are shown as experiments 1 and 2 in Table 4. The activities measured represent radioactivities of ~ 30,000 cpm $^{14}CO_2$ liberated per reaction, which were completely inhibited by incubating replicate samples with DFMO. For quantitation of protein content samples were fractionated by SDS polyacrylamide electrophoresis and bands were visualized using autoradiography. The M_r 54,000 band was excised and solubilized in 2% periodic acid and radioactivity was determined. These data are shown as experiments 3 and 4 in Table 4. ODC sequence data predicts 15 methionine per protein (Kahana and Nathans, 1985a), and assuming that all the methionine incorporated are radiolabeled, the specific radioactivity can be used to derive an estimate of the amount synthesized.

Table 4. Determination of the Amount of ODC Protein and Activity Produced by *In Vitro* Translation

	ODC Activity (Units/reaction)	pgrams ODC/ reaction	Specific Activity (Units/mg)
EXPT 1	.012 ± .004 (3)		
EXPT 2	.012 ± .0008 (4)		
EXPT 3		211 ± 82 (2)	
EXPT 4		171 ± 7 (2)	
Average	.012	191	62,827

ODC was translated *in vitro* after addition of 250 ng ODC mRNA, and ODC activity and protein content determined as described in Material and Methods. The numbers in parentheses refer to the number of separate reactions per experiment.

Four independent reactions from two different *in vitro* translations yielded radioactivity in protein corresponding to 171 to 211 picogram ODC per reaction, (~ 85,000 cpm ^{35}S-methionine per M_r 54,000 band) using the assumptions just stated. These values should represent the minimum amount produced, due to the assumption that all the methionine incorporated is radiolabeled. This leads to a calculated specific activity of this protein of ≃ 63,000 units/mg, similar to that of the purified mouse kidney enzyme (47,000 units/mg) (Seely, Poso and Pegg, 1982a). The calculation of specific activity in this study could be an overestimate of the real value due to the possible underestimate of the quantity of protein produced *in vitro*. It is not known whether the smaller molecular weight species have catalytic activity, and since these are not included in the calculations, would contribute to potential variance.

Discussion

ODC protein from cloned cDNA sequences was produced by using techniques of *in vitro* transcription and translation. That the major protein translated *in vitro* maintained the characteristics of ODC isolated from cells was based on the following criteria: a) molecular weight of ≃ 54,000 and immunoprecipitation using anti-ODC specific anti-serum (Figure 14), b) two-dimensional electrophoresis which reveals a pI of approximately 5.4, (Figure 15) consistent with that for the purified enzyme (Seely,Poso and Pegg, 1982a) and c) an enzyme specific activity very similar to that of purified mouse

kidney ODC (Table 4). The procedure described here yields ODC protein in sufficient quantity and radioactive specific activity to allow further investigations concerning ODC structure, and indicate that the protein can be used to study specific aspects of ODC regulation. Also, manipulation of this cDNA into a cell expression system (i.e. insect, E. coli) potentially could generate larger quantities of ODC protein.

In the presence of intracellular polyamines ODC has a half life as short as 10-30 minutes (Tabor and Tabor, 1984; Glass and Gerner, 1986b; Pegg, 1986), one of the fastest turnover rates known for mammalian proteins. The mechanism for ODC degradation is unknown, but appears not to involve ubiquitin conjugation or lysosomes, which are thought to be two major pathways for intracellular protein catabolism (Glass and Gerner, 1986b). ODC protein produced by the in vitro methodologies described here could be used to further characterize the specific mechanism of ODC degradation. Rechsteiner and coworkers have elegantly shown that microinjection of radiolabeled proteins can be used to determine specific in vivo degradation pathways (Rote and Rechsteiner, 1983; Hough and Rechsteiner, 1984). Sufficient radiolabel is recovered in essentially a single protein to allow detection of ODC protein turnover after microinjection (Table 4), and to study potential in vivo degradative pathways. This system allows the use of an endogenous cellular protein and comparisons could be made in a manner similar to that described for other exogenous protein

substrates. In addition, ODC produced *in vitro* could also serve as a substrate for the isolation of a specific protease(s) and co-factors which could be responsible for the rapid degradation rate observed *in vivo*.

Recently, it has been proposed that a specific amino acid sequence may dictate the intracellular stability of proteins. These sequences (termed PEST regions; proline (P), glutamic (E), serine (S), and threonine (T) rich) have been found in 12 proteins with intracellular half lives of less than 2 hours (Rogers et al., 1986). ODC as analyzed by this schema, has optimum PEST site regions, predicting rapid turnover. As suggested by Rogers et al.(1986), a direct test of this hypothesis would be to induce alterations in these sequences and measure the intracellular stability of the protein. ODC produced from *in vitro* methodologies would be suitable for these types of experimental manipulation, specifically by methods of site-directed mutagenesis (Smith, 1985) to modify individual amino acids.

The methods described here also show that *in vitro* transcription can be used to generate large quantities of ODC specific mRNA. Recent results indicate that ODC synthesis *in vivo* is regulated at the translational level by polyamines (Kahana and Nathans 1985b; Persson et al., 1986). The mechanism for polyamine translational inhibition is unknown, but interaction with the unusually long 5' untranslated region of ODC mRNA has been postulated to have an effect on translatibility of the message

(Pegg, 1986). It is evident that RNA secondary structure can affect *in vivo* translation (Kozak, 1986; Pelletier and Sonenburg, 1985), and polyamines could be acting in a similar manner specifically with ODC mRNA. Manipulation of the 5' upstream regions of the ODC cDNA, and production of mRNA *in vitro* using the plasmid construction described here would allow a direct determination of sequences involved in polyamine inhibition of ODC translation.

CHAPTER 5

SUMMARY

The major goal undertaken in this study was to determine the role of post-translational mechanisms involved in ODC regulation. The high titer anti-sera supplied by Dr. A.E. Pegg allowed immunochemical methods to analyze directly post-translational modifications of ODC protein. Potential alterations in structure could then be related to activity changes and would more clearly define the suggested regulatory pathways for enzyme activity, i.e. phosphorylation, transamidation, intracellular stability, and alterations in synthetic rate.

Perhaps the most interesting and novel finding of this work is that ODC is a stable protein in vivo in the absence of substantial intracellular putrescine and spermidine pools. Restoration of these intracellular polyamines leads to rapid turnover, without alterations in absolute enzyme specific activity. These findings are the first to directly demonstrate that polyamines regulate the degradation of ODC. Indeed, these observations dictate that the statement "ODC has a much faster turnover rate than any other mammalian enzyme" (Tabor and Tabor, 1984) be amended to include: "in the presence of intracellular polyamines".

It has also been determined that the degradation pathway for ODC is independent of the well characterized ubiquitin conjugation or lysosomal systems. Based on kinetic evidence, Hough and Rechsteiner (1984) predicted the existence of an alternative degradation pathway for short-lived proteins, and the observations presented here support that hypothesis, and extend this notion to show that a specific endogenous protein (ODC) is degraded with similar kinetics. In addition, the hypotheses that modified forms of ODC accumulate prior to turnover has been tested directly by two-dimensional electrophoresis. No alternative species of ODC protein has been isolated in either CHO or ts85 cells. This indicates that an accumulation of modified enzyme is not required for proteolysis, but does not rule out the existence of an unstable modified intermediate.

That polyamines act to induce the turnover of ODC indicates that the absolute structure of the protein in the absence of polyamines does not suffice to signal rapid intracellular proteolysis. It has been previously suggested that the acidic nature of ODC could result in rapid degradation in vivo (Dice and Goldberg, 1975; Tabor and Tabor, 1984). However, it is possible that ODC protein structure confers an interaction with some polyamine-induced co-factor. The inhibitor studies presented here show that some component of protein synthesis is required for rapid ODC degradation, yet the nature of this co-factor remains to be determined. An initial interpretation of these studies could be

that a protein is necessary, perhaps a labile protease or a targeting protein, though another alternative is that the cofactor involvement could represent an RNA component. Some aspects of intracellular proteolytic targeting have described specific tRNA molecules as being essential co-factors (Ciechanover et al., 1985; Ferber and Ciechanover, 1986). A polyamine interaction could be expected due to the well documented effect of binding to tRNA which alters their structure (Cohen, 1971; Tabor and Tabor, 1976). Perhaps ODC-RNA interactions are involved in targeting for proteolysis. If common sequences are recognized by a specific protease (i.e. the PEST hypothesis; Rogers et al., 1986), it is possible that the ODC co-factor could interact to expose these sites for proteolytic recognition.

The combination of results presented here should allow the characterization of an in vitro degradation system for ODC. The ability to produce ODC in vitro from the cDNA clone supplied by Dr. D. Nathans, greatly diminishes the problems associated with obtaining sufficient quantities of ODC from tissues. Use of the protein as a substrate for degradation, in addition to the knowledge gained from the in vivo studies (i.e., ATP independence, 15-16 kcal/mole activation energy, co-factor requirement), should result in the isolation of components required for ODC degradation. The specificity of the mechanism could then be analyzed for other in vivo short-lived proteins.

Another previously undetermined mechanism of ODC regulation presented here is the direct demonstration that spermidine inhibits ODC mRNA translation. If intracellular polyamine content regulates both the synthesis and degradation rates of ODC protein, an obvious question arises: Are the two mechanistically linked? Based upon the results presented here, a hypothesis has been developed that encompasses these two observations as well as the requirement for protein synthesis in ODC inactivation is as follows: Polyamines interact at the 5' upstream region of ODC mRNA to inhibit initiation at the first AUG codon, re-initiation occurs downstream at internal initiation sites creating truncated ODC protein, which are then capable of interacting with active subunits. These "hybrid" proteins interact with active ODC subunits which are then recognized as foreign (or perhaps result in exposure of masked sequences) and are rapidly degraded. The concepts of RNA secondary structure influencing translation and termination-reinitiation have recently been documented (Pelletier and Sonenburg, 1985; Kozak, 1986; Peabody and Berg, 1986). This hypothesis would predict that alterations in ODC mRNA structure (the 5' untranslated region) would influence the intracellular stability. This hypothesis is directly testable using the techniques of *in vitro* transcription and site-directed mutagenesis to create modified ODC cDNA. Transfection studies using these constructs would then allow direct analysis of the effects of these modifications on ODC stability *in vivo.*

In summary, the major findings of this study and their relation to ODC regulation are outlined in Figure 16. Clearly, the regulation of this growth related enzyme is quite sophisticated, and encompass many points of interaction by the polyamines. Understanding these pathways will benefit our overall knowledge concerning aspects of protein synthesis and degradation.

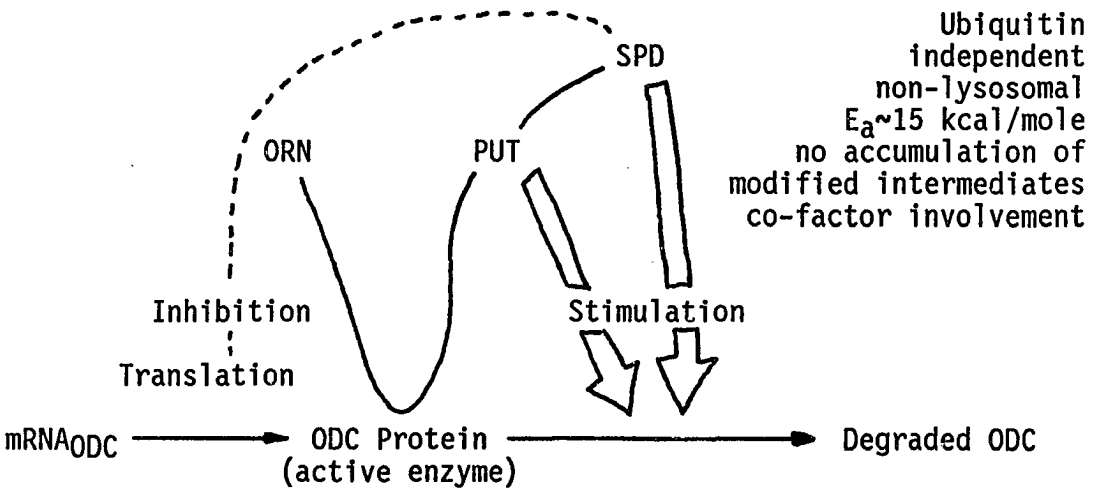

Figure 16. Polyamine regulation of ODC.

This schematic represents the points where putrescine and spermidine act to regulate intracellular ODC activity and includes what is known concerning the mechanism of ODC degradation.

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