Chapter 2

Early developmental profile of ornithine decarboxylase in the frog *Microhyla ornata* and its regulation by polyamines

Introduction:

Ornithine decarboxylase (ODC), the key enzyme of the polyamine biosynthetic pathway in animal tissues, is rapidly induced with the onset of growth and development (Bachrach, 1980; Russell, 1985). Mammalian ODC has been shown to turn over rapidly with a half-life of less than 0.5 hr (Russell and Snyder, 1969; Obenrader and Prouty, 1977). The enzyme is also under feedback regulation by polyamines and shows marked decline in activity in response to exogenous putrescine and spermidine (Pegg et al., 1978; Kallio et al., 1979; Seely and Pegg, 1983b; Glass and Gerner, 1986; Holtta and Pohjanpelto, 1986; Kanamoto et al., 1986).

Most of the studies on ODC and its regulation have been carried out on mammals using *in vitro* systems (reviewed by Hayashi, 1989). Recently two reports appeared on the induction and regulation of ODC in adult frog tissues (Baby and Hayashi, 1991 a,b). Early developmental profile of ODC is reported in *Xenopus* embryos (Russell, 1971; Osborne et al., 1991). The embryonic development in *M. ornata* is much faster (Padhye and Ghate, 1989) than in *Xenopus laevis* (Neiuwkoop and Faber, 1975). The activity pattern of ODC during early development of the frog...
M. ornata and its regulation by exogenous polyamines are reported in this chapter.

Early embryonic development of all vertebrates has been shown to be associated with gradual increase in ODC and S-adenosylmethionine decarboxylase. S-Adenosylmethionine decarboxylase (EC 4.1.1.50) catalyzes the conversion of S-adenosylmethionine to S-5'-deoxyadenosyl-(5')-3-methylthiopropylamine (decarboxylated S-adenosylmethionine) and CO₂. This is a rate-limiting enzyme in the synthesis of spermidine (Pegg, 1986). In order to examine whether the early activation of ODC in M. ornata is also accompanied by an activation of S-adenosylmethionine decarboxylase, the activity of this enzyme was measured in early developmental stages of this frog.

Materials and Methods:

Chemicals:

Putrescine, spermidine, spermine, L-ornithine, S-adenosyl-L-methionine, pyridoxal-5-phosphate, methylglyoxal bis(guanylhydrazone), α-methylornithine, dithiothreitol, methylbenzethonium hydroxide, bovine serum albumin and 2,5-diphenyl oxazole were obtained from Sigma Chemical Co. (St. Louis, USA). DL-(1-14C)ornithine monohydrochloride (61 mCi/mmol; 50 μCi/ml) and S-adenosyl-L-(carboxy-14C)methionine (60 mCi/mmol; 25 μCi/ml) were obtained from Radiochemical Center, Amersham, UK.

Live material:

Freshly fertilized eggs of M. ornata were collected and maintained in the laboratory as described in chapter 1. Feeding
in *M. ornata* tadpoles begins only after 100 hr of development (Padhye and Ghate, 1989). Since experiments were restricted to the first 100 hr of development, they were not fed during the experimental period.

**Treatment:**

Ornithine, putrescine, spermidine, spermine, α-methylornithine (all 2 mM) and methylglyoxal bis(guanylhydrazone) (100 μM) were dissolved in filtered sterile pond water. 2 mM ornithine, putrescine and spermidine used in this experiment was the lowest effective concentration in suppressing ODC activity. The pH of ornithine, α-methylornithine and polyamine solutions was adjusted to that of pond water (7.5-7.8) before use. Jelly and vitelline membranes surrounding the embryos were removed with fine forceps before treatment. All treatments were carried out in Corning glass petri dishes with an equal number of embryos in the control and experimental dishes. Sufficient pond water was provided in order to avoid overcrowding (1 ml/embryo). Whenever the treatment was continued for more than 24 hr, tadpoles were transferred to fresh medium. Embryos were withdrawn from experimental and control dishes at different time intervals, washed with three changes of filtered sterile pond water, quickly rinsed in homogenizing medium and chilled immediately.

**ODC assay:**

ODC was assayed by measuring the release of ¹⁴CO₂ from DL-(¹⁴C)ornithine by the method of Janne and Williams-Ashman (1971) with minor modifications. Extracts were prepared by homogenizing 20 embryos (or oocytes) in 2 ml of ice-cold 50 mM Tris-HCl (pH 7.2) containing 0.1 mM EDTA, 1 mM dithiothreitol and
50 μM pyridoxal-5-phosphate, followed by centrifugation at 15,000g for 15 min at 4°C. The supernatant was used for enzyme assay. No activity was detectable in the pellet. In all experiments involving treatments, both the control and experimental supernatants were dialyzed against 100 volumes of the solution used for homogenization.

The standard incubation mixture contained 10 μmoles of Tris-HCl buffer (pH 7.2), 0.25 μmole of L-ornithine, 0.2 μCi of DL-(1-14C) ornithine (specific activity 61 mCi per mmole), 1.2 μmoles of dithiothreitol, 0.06 μmole of pyridoxal-5-phosphate and tissue extract (0.1 to 0.2 ml) in a total volume of 0.25 ml. The reaction vessel consisted of a glass tube closed with a rubber stopper which carried a polypropylene center well. The CO2 produced in the reaction was trapped in 0.1 ml of methyl benzethonium hydroxide present in the polypropylene center well. In all the experiments two nonenzymic controls were used in which the tissue extract was replaced by homogenization medium. Incubation time was 1 hr at 37°C. The reaction was stopped by injecting 0.5 ml of 10% trichloroacetic acid into the reaction mixture carefully through the rubber stopper. The tubes were incubated for a further period of 20 min at room temperature (26 ± 1°C) to ensure that all the dissolved CO2 was released from the acidified medium and trapped in the methyl benzethonium hydroxide present in the center well. The polypropylene center well was then carefully removed from the rubber stopper and dropped into a scintillation counting vial. After adding 4.0 ml of toluene scintillator (0.4% 2,5-diphenyloxazole; Conroy et al., 1977) the radioactivity was counted in a Beckman Liquid Scintillation Spectrometer at an efficiency of 88%. ODC activity
was expressed as nmoles CO₂ formed from ornithine per hr at 37°C.

**S-Adenosylmethionine decarboxylase assay:**

The activity of S-adenosylmethionine decarboxylase was assayed by measuring ¹⁴CO₂ released from S-adenosyl-L-(carboxy-¹⁴C)methionine according to the method of Pegg and Poso (1983). Extracts were prepared by homogenizing 100 embryos or tadpoles in 2.0 ml ice-cold 25 mM sodium phosphate, pH 7.5, 2.5 mM dithiothreitol, 2.5 mM putrescine and 0.1 mM EDTA. The homogenate was centrifuged at 15,000g for 15 min at 4°C and the supernatant was used for the assay.

The standard reaction mixture contained 5 μmoles of sodium phosphate buffer (pH 7.5), 1.25 μmoles of putrescine; 0.05 μmole of S-adenosylmethionine, 5 μl of S-adenosyl-L-(carboxy-¹⁴C)methionine (0.125 μCi) and tissue extract (0.1 to 0.2 ml) in a total volume of 0.25 ml. In the reagent blank, 0.2 ml of trichloroacetic acid was added before addition of tissue homogenate. The reaction vessel and other details of the assay were exactly as given for ODC.

Methods for polyamine estimation and protein estimation are described in Chapter 1.

**Results:**

CO₂ formation was linear with respect to volume of embryo extract (up to 200 μl) and incubation time (for at least 60 min) (Fig. 9). When the tissue extract was incubated in the presence of 1 mM α-difluoromethylornithine for 30 min at room temperature, evolution of CO₂ was completely (>99%) inhibited.

The 1 mM concentration of L-ornithine used was adequate to saturate the enzyme under the assay conditions employed here.
Figure 9

Effect of enzyme concentration (a) and incubation time (b) on the evolution of CO$_2$ from ornithine in the ornithine decarboxylase assay.
The apparent Km value of the enzyme for L-ornithine calculated from the double reciprocal plots (Fig. 10) in two separate experiments were 0.06 and 0.08 mM. These values are well within the range of Km reported for ODC from various eukaryotic sources (Kaye, 1984; Pegg, 1989). ODC from embryonic tissue of *M. ornata* appears to have a greater affinity for L-ornithine as compared to frog liver ODC (Baby and Hayashi, 1991a).

ODC activity increased dramatically on initiation of development and was almost 50-fold higher at four to eight cell stage (1 hr) as compared to the activity in mature ovarian oocytes (Fig. 11). This high level of ODC decreased rapidly as development progressed to neurulation (10 hr). The enzyme activity again increased reaching high levels in newly hatched tadpoles (32 to 34 hr). Thereafter ODC activity decreased at 40 hr of development before reaching the third and highest peak at 50 to 52 hr of development. After this peak ODC levels decreased rapidly to low levels at 60 hr. Thus during the first 60 hr of development in *M. ornata*, three prominent peaks of ODC activity were observed (Fig. 11). Measurement of polyamine levels during this period of development showed more or less a continuous increase in putrescine and spermidine content without any significant change in spermine content (Fig. 12).

Early activation of ODC is not accompanied by an activation of S-adenosylmethionine decarboxylase in *M. ornata*. In contrast to the pattern of ODC, S-adenosylmethionine decarboxylase activity was low during early embryonic development, it started increasing in early tadpoles and maximum activity was obtained in free swimming tadpoles (Fig. 13). The activity levels and the pattern of S-Adenosylmethionine decarboxylase were comparable to
Figure 10

Effect of substrate concentration on ornithine decarboxylase activity

ODC activity (V) was assayed in the presence of different concentrations of L-ornithine (S). DL-(1-14C) ornithine concentration was kept constant. S versus V plots are shown in Figure (a) and Lineweaver-Burk double reciprocal plots in Figure (b).
Figure 11

ODC activity during early development of *M. ornata*

0, Protein based values; ●, per-specimen (embryo or tadpole)-based values. * Activity level in mature ovarian oocyte; 1 = 4 to 8 cell stage; 2 = 16 to 32 cell stage; 3 = 64 cell stage; 4 = blastula; 5 = early gastrula; 6 = early neurula; 7 = initiation of tail bud formation and differentiation of head, trunk and tail; 8 = pre-hatched tadpole; 9 = post-hatched tadpole; 10 & 11 = swimming tadpole. Values are means ± standard deviation of at least three different samples.
Figure 12
Polyamine changes during early development of *M. ornata*.

- O, putrescine; •, spermidine; Δ, spermine; mean ± standard deviation. Each time point represents mean of at least four observations and for each estimation 100 embryos/tadpoles were pooled; samples were collected and polyamine levels were estimated as described in Chapter 1. 1 = blastula; 2 = gastrula; 3 = neurula; 4 = pre-hatched tadpole; 5 = post-hatched tadpole; 6 & 7 = swimming tadpole.
Figure 13

S-Adenosylmethionine decarboxylase activity during early development of M. ornata

Each value represents mean of two different estimations. Details of assay are given in the text.
those reported in Xenopus (Russell, 1971) and chick (Lowkvist et al., 1985).

Treatment with ornithine and polyamines was carried out in late neurula stage (15 hr of development), when ODC activity was low (Fig. 14). Putrescine (2 mM) and spermidine (2 mM) prevented the normal increase in ODC activity. Addition of ornithine (2 mM) also prevented the increase in ODC activity (Fig. 14) although it was less effective than putrescine and spermidine.

At the concentrations used in the present study, ornithine and putrescine did not show any toxic effect during the entire period of treatment, while spermidine and spermine were found to be toxic. Due to this toxic effect treatment with spermidine and spermine could not be continued beyond 55 and 35 hr of development, respectively. Spermine did not suppress ODC during the 20 hr of treatment.

To determine whether the suppression of ODC by ornithine was due to its conversion to putrescine and/or spermidine, developing tadpoles (26 hr) were treated with α-methylornithine. α-Methylornithine, an analog of ornithine, binds to the enzyme, but is decarboxylated at a much slower rate and is a competitive inhibitor of ODC (Abdel-Monem et al., 1974). ODC activity was similar in controls and embryos treated with α-methylornithine, indicating that enzymatic conversion of ornithine to putrescine and/or spermidine is responsible for the suppression of ODC activity in ornithine treated embryos (Fig. 15).

The levels of putrescine and spermidine were also measured in ornithine-treated and control embryos. There was a marginal increase (4-10%) in putrescine and spermidine content in ornithine-treated tadpoles as compared to controls (Table 4).
**Figure 14**

Effect of ornithine (2 mM), putrescine (2 mM), spermidine (2 mM), and spermine (2 mM) treatment on ODC activity in early developmental stages of *M. ornata*.

Treatment started after 15 hr of development at late neurula stage. ●, control; ○, ornithine treated; △, putrescine treated; ▲, spermidine treated; ■, spermine treated. Each time point represents the mean of two different experiments. Details of treatment and ODC assay are given in the text.
Figure 15

Effect of ornithine (2 mM) and $\alpha$-methylornithine (2 mM) on ODC activity in early tadpoles of *M. ornata*

Treatment started after 26 hr of development. Tadpoles were freed of their vitelline membrane before commencement of treatment. ●, control; O, ornithine treated; Δ, $\alpha$-methylornithine treated. Each time point represents the mean of two different experiments. Details of treatment and ODC assay are given in the text.
Table 4
Polyamine changes in response to ornithine treatment in tadpoles of *M. ornata*

Treatment duration is given in parentheses. Each value represents mean of two different experiments and for each estimation 100 tadpoles were pooled. Putrescine and spermidine levels were estimated as described in chapter 1.

<table>
<thead>
<tr>
<th>Total age in hours</th>
<th>Putrescine (nmole/specimen)</th>
<th>Spermidine (nmole/specimen)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>treated</td>
</tr>
<tr>
<td>30 (4)</td>
<td>1.57</td>
<td>1.72</td>
</tr>
<tr>
<td>36 (10)</td>
<td>1.78</td>
<td>1.86</td>
</tr>
<tr>
<td>56 (30)</td>
<td>1.99</td>
<td>2.07</td>
</tr>
</tbody>
</table>
In order to assess whether it is putrescine itself or spermidine that is responsible for bringing about this suppression in ODC activity, the conversion of putrescine to spermidine was blocked by addition of 100 μM methylglyoxal bis(guanylhydrazone) (MGBG). Addition of MGBG alone resulted in increased ODC activity and putrescine accumulation (Fig. 16) as reported for mammalian systems (Holtta, et al., 1973; Heby et al., 1977). Addition of MGBG along with putrescine (2 mM) led to a decrease in ODC activity after an initial elevation (Fig. 16). The above results suggested that putrescine alone, independent of its conversion to spermidine, is capable of bringing about suppression of ODC. Measurement of putrescine and spermidine content in MGBG-treated tadpoles showed accumulation of putrescine and depletion of spermidine content (Table 5) confirming that MGBG blocked S-adenosylmethionine decarboxylase in *M. ornata* tadpoles.

**Discussion:**

ODC levels in *M. ornata* embryos were higher than those reported in *Xenopus* (Russell, 1971; Osborne et al., 1991) and chick (Lowkvist et al., 1985) embryos. Increased levels of ODC activity and polyamine accumulation are correlated with rapid growth and development (Russell and Snyder, 1968; Heby 1981; Joseph and Baby, 1988). Therefore, high levels of ODC activity in *M. ornata* as compared to *Xenopus* (Russell, 1971; Osborne et al., 1991) may be attributed to the rapid rate of development in the former (Padhye and Ghate, 1989) as compared to the latter (Nieuwkoop and Faber, 1975). In addition, the low osmolarity of pond water (0.2 to 0.4 mM Na⁺ and K⁺; Padhye and Ghate, 1992) as
Figure 16

Effect of MGBG (100 μM) and MGBG (100 μM) + putrescine (2 mM) on ODC activity in early tadpoles of *M. ornata*

●, control; ○, MGBG treated; △, MGBG + putrescine treated. Treatment started after 26 hr of development. Tadpoles were freed of their vitelline membrane before commencement of treatment. Each time point represents the mean of two different experiments. Details of treatment and ODC assay are given in the text.
Table 5

Putrescine accumulation and inhibition of spermidine synthesis in response to methylglyoxal bis-(guanylhydrazone) treatment (100 μM) in developing tadpoles of the frog *M. ornata*

Treatment duration is given in parentheses. Each value represents mean ± standard deviation of at least 3 different samples and for each estimation 100 tadpoles were pooled. Putrescine and spermidine levels were estimated as described in Chapter 1.

<table>
<thead>
<tr>
<th>Total age in hours</th>
<th>Putrescine (nmoles/specimen)</th>
<th>Spermidine (nmoles/specimen)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control treated</td>
<td>control treated</td>
</tr>
<tr>
<td>30 (26)</td>
<td>1.67 ± 0.28 1.82 ± 0.09</td>
<td>0.56 ± 0.08 0.50 ± 0.15</td>
</tr>
<tr>
<td>60 (56)</td>
<td>1.84 ± 0.03 2.48 ± 0.16</td>
<td>0.93 ± 0.02 0.74 ± 0.07</td>
</tr>
<tr>
<td>84 (80)</td>
<td>2.31 ± 0.07 3.18 ± 0.39</td>
<td>1.43 ± 0.15 0.91 ± 0.16</td>
</tr>
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compared to the salt containing medium (30 mM NaCl) used for Xenopus development (Paris et al., 1988) may be another important factor responsible for higher ODC activity in M. ornata than in Xenopus (see Chapter 3). ODC activity in Xenopus increases gradually after fertilization and reaches its highest level in the swimming tadpole; there is only one peak of ODC activity in Xenopus with its maximum at the swimming tadpole stage (Russell, 1971). However, a recent report on the expression and regulation of ODC during embryonic development of Xenopus showed that ODC activity increased in the embryos after fertilization, reaching maximum levels before mid-blastula transition and thereafter the activity decreased (Osborne et al., 1991). Embryonic development in other vertebrates like chick (Lowkvist et al., 1985), rat, mouse and rabbit (Fozard et al., 1980b) has been shown to be associated with gradual increase in ODC reaching peak levels during gastrulation. Therefore, the unusually high levels of ODC and the different activity pattern of this enzyme during early development of M. ornata, as compared to other vertebrates (Heby, 1981), may be caused by the low osmolarity of the pond water in which they develop.

Addition of exogenous putrescine and spermidine prevented the increase in ODC activity observed in untreated M. ornata embryos. Cultured mammalian cells also showed that ODC is under negative feedback regulation by putrescine and other polyamines (Kay and Lindsay, 1973; Clark and Fuller, 1975; Dirks et al., 1986; Holtta and Pohjanpelto, 1986; Kanamoto et al., 1986, 1991; Persson et al., 1989; Tohyama et al., 1991). Similar results were obtained in Neurospora (Davis et al., 1985; Barnett et al., 1988). In yeast, however, spermidine and spermine caused ODC
suppression, while putrescine was without any significant effect (Tyagi et al., 1981). A more recent study, however, contradicted the above report by showing that the decay rate of ODC remained unchanged in yeast cells after polyamine treatment and polyamines regulate ODC expression at a post-translational step prior to assembly of active form of the enzyme (Fonzi, 1989). Thus there seems to be differences in the metabolic signals that regulate ODC in different groups of eukaryotes.

Addition of ornithine also led to suppression of ODC activity. The need to convert ornithine to putrescine for the suppression to occur is indicated by the experiments using the competitive inhibitor, α-methylornithine. At comparable concentration, α-methylornithine was not effective in suppressing ODC activity.

Rapid elevation of ODC is always followed by a rapid decline, suggesting that increased putrescine synthesis leads to suppression of ODC activity. Furthermore ornithine treatment at the tadpole stage (Fig. 15), when ODC activity is rather high, led to suppression of activity faster as compared to ornithine treatment at the neurula stage (Fig. 14), again suggesting a role for putrescine in ODC regulation.

The lack of correlation between the ODC activity pattern and polyamine pattern in M. ornata embryos (Figs. 11 & 12), is seemingly contradictory to the role of these amines in the regulation of ODC. ODC and polyamine changes which are in apparent contradiction to the accepted role of putrescine and spermidine in the regulation of ODC have also been reported in Neurospora (Davis et al., 1985) and cultured mammalian cells (Porter et al., 1987; Holm et al., 1989). This may be explained
by assuming that intracellular polyamines are bound or compartmentalized and are unavailable as pathway intermediates and ODC responds to variations in the free cytosolic polyamines, rather than to the total cellular polyamine content. In case of Neurospora only 10-20% of the cellular putrescine and spermidine are freely accessible as pathway intermediates, the rest being sequestered in vacuoles or membrane bound (Paulus and Davis, 1981, 1982; Paulus et al., 1983). In the light of these observations, ODC activity profile during M. ornata development would be expected to show correlation only with the small free cytosolic polyamine levels and not with the total cellular polyamines.

The cyclic pattern of ODC during early development of M. ornata may be explained by the assumption that an increase in ODC activity leads to increased putrescine production, which brings about a suppression of ODC synthesis and acceleration of its decay (see Chapter 4). Such cessation of polyamine synthesis probably leads to rapid depletion of the freely accessible polyamines as pathway intermediates which in turn triggers another ODC peak. Thus developing embryos of M. ornata have high fluctuating levels of ODC which is under negative feedback regulation by putrescine and spermidine.