# ORNITHINE DECARBOXYLASE

Expression and regulation in rat brain and in transgenic mice


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### *Abstract*

Ornithine decarboxylase (EC 4.1.1. 17) is the first and the rate-controlling enzyme in polyamine biosynthesis. It decarboxylates L-ornithine to form diamine putrescine. ODC activity in cells is strictly regulated and one of the central elements of ODC regulation is an inhibitory protein called antizyme. Antizyme binds to ODC, inhibits its activity and targets ODC for the proteasomal degradation. Essentiality of polyamines for the normal cell growth and proliferation is well known. Recently their roles in the regulation of several classes of cation channels have been discovered. Some of these channels are expressed abundantly in the brain, which has increased interest in the polyamine metabolism in the central nervous system.

In this study guanosine 5'-triphosphate activatable ODC was detected in the rat brain lysates. This activation was more significant after antizyme was separated from ODC. GTP-activatable ODC was more resistant to heat and displayed higher Vmax than kidney ODC. Previously GTP-activatable ODC had been found in mammalian tissues only in some tumors. ODC and antizyme expression in brain was localized by in situ hybridization and immunocytochemistry. Both proteins displayed wide and largely overlapping expression patterns restricted to neurons. The proteins were localized predominantly to cytoplasm at the most brain regions, but antizyme had a main localization in nuclei in some regions of the brain. In addition, the role of one of the most highly conserved regions in eukaryotic ODCs was studied using site-directed mutagenesis. The aspartate-233 to valine mutation was made and detected to increase Km values for the cofactor PLP and the substrate L-ornithine as well as Ki value for the inhibitor DFMO.

In another part of this study a transgenic mouse line expressing ODC under the control of viral promotor was generated. The most significant changes in ODC activity were detected in reproductive organs of male mice. The high number of infertile transgenic males supported earlier reports about the importance of balanced polyamine metabolism for spermatogenesis. Infertility of female mice was increased as well, but the involvement of polyamines remained unproven. Transgenic mice were prone to various pathological conditions such as inflammations and tumour formation, which may be due to deregulated polyamine metabolism.

*Keywords:* polyamines, antizyme, guanosine triphosphate binding protein

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Solna, February 2002 Pekka Kilpeläinen

# Abbreviations

| | |
|---|---|
| AdoMetDC | S-adenosyl methionine decarboxylase |
| AMPA | -amino-3-hydroxy-5-methyl-4-isoxazole-propionate |
| ATP | adenosine 5’ triphosphate |
| cAMP | cyclic adenosine monophosphate |
| CHO | Chinese hamster ovary |
| CNS | central nervous system |
| CREB | cAMP responsive element binding |
| DENSPM | $N^1$-$N^{11}$-diethylnorspermine |
| DFMO | -difluoromethylornithine |
| eIF-4F | eukaryotic translation initiation factor 4F |
| eIF-5A | eukaryotic translation initiation factor 5A |
| GTP | guanosine 5’ triphosphate |
| JNK | c-Jun aminoterminal kinase |
| Kir | inward rectifying potassium channel |
| MGBG | methylglyoxal bis(guanylhydrazone) |
| MTA | 5’ methylthioadenosine |
| NMDA | N-methyl-D-aspartate |
| ODC | ornithine decarboxylase |
| ORF | open reading frame |
| PAO | polyamine oxidase |
| PMF-1 | polyamine modulated factor 1 |
| SSAT | spermidine/spermine $N^1$-acetyltransferase |
| TM2 | transmembrane site 2 |
| TPA | tumor promotor 12-O-tetradecanoylphorbol 13-acetate |
| UTR | untranslated region |
| UVB | ultraviolet radiation B |

# List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Kilpeläinen P & Hietala O (1994) Activation of rat brain ornithine decarboxylase by GTP. Biochem J 300: 577-582.

II Kilpeläinen P & Hietala O (1998) Mutation of aspartate-233 to valine in mouse ornithine decarboxylase reduces enzyme activity. Int J Biochem Cell B 30: 803-809

III Kilpeläinen P, Rybnikova E, Hietala O & Pelto-Huikko M (2000) Expression of ODC and its regulatory protein antizyme in the adult rat brain. J Neurosci Res 62: 675-685.

IV Kilpeläinen P, Saarimies J, Kontusaari S, Järvinen M, Soler A, Kallioinen M & Hietala O (2001) Abnormal ornithine decarboxylase activity in transgenic mice increases tumor formation and infertility. Int J Biochem Cell B 33: 507-520.

# Contents

Acknowledgements
Abbreviations
List of original articles
Contents
1 Introduction ........ 13
2 Review of the literature ........ 14
2.1 Polyamines ........ 14
2.1.1 Structure and properties ........ 14
2.1.2 Physiological roles ........ 15
2.1.2.1 DNA binding ........ 15
2.1.2.2 DNA and protein synthesis ........ 16
2.1.2.3 Cell growth ........ 16
2.1.2.4 Apoptosis ........ 17
2.1.2.5 Hypusine synthesis ........ 18
2.1.2.6 Other roles ........ 19
2.1.3 Polyamine metabolism in mammals ........ 20
2.1.3.1 Biosynthesis ........ 20
2.1.3.2 Catabolism and interconversion ........ 23
2.1.3.3 Transport ........ 25
2.1.4 Polyamines in the central nervous system ........ 26
2.1.5 Polyamines and the regulation of inward rectifying potassium channels ........ 28
2.1.6 Polyamines and cancer ........ 30
2.2 Ornithine decarboxylase ........ 33
2.2.1 Enzyme protein and catalytic activity ........ 33
2.2.2 Gene and mRNA ........ 34
2.2.3 Regulation ........ 35
2.2.3.1 Regulation of transcription ........ 35
2.2.3.2 Regulation of translation ........ 37
2.2.3.3 Post-translational modifications ........ 39
2.2.3.4 Degradation and its regulation ........ 40
2.2.3.5 Guanosine 5'-triphoshate activation ........ 41

2.3 Antizyme ........................................42
2.3.1 Protein and functions ........................................42
2.3.2 Gene family ........................................43
2.3.3 Regulation ........................................44
3 Aims of the present study ........................................47
4 Materials and Methods ........................................49
4.1 Assay of ornithine decarboxylase and antizyme activities (I, II, IV) ........................................49
4.2 Preparation of brain extracts (I) ........................................49
4.3 Construction of plasmids and site directed mutagenesis (II) ........................................50
4.4 Cell culture and DNA transfection (II) ........................................50
4.5 Northern analysis (II) ........................................51
4.6 Generation and analysis of transgenic mice (IV) ........................................51
4.7 *In situ* hybridization (III) ........................................52
4.8 Immunocytochemistry (III) ........................................52
4.9 Other methods ........................................53
5 Results ........................................54
5.1 Detection and characterization of GTP-activatable ornithine decarboxylase in the rat brain (I) ........................................54
5.2 Expression of ornithine decarboxylase and antizyme in the rat brain (III) ........................................55
5.3 Site-directed mutagenesis and analysis of D233V-mutated ornithine decarboxylase (II) ........................................57
5.4 Generation and analysis of transgenic mice (IV) ........................................57
6 Discussion ........................................59
6.1 GTP-activatable ornithine decarboxylase in the rat brain ........................................59
6.2 Expression of ornithine decarboxylase and antizyme in the rat brain ........................................62
6.3 Mutation of aspartate-233 to valine in mouse ornithine decarboxylase ........................................65
6.4 Transgenic mice ........................................66
6.5 Conclusions ........................................68
7 References ........................................69

# 1 Introduction

The diamine putrescine and polyamines spermidine and spermine are ubiquitous physiological cations. They are essential for normal cell growth and differentiation. Their functions at the molecular level are not completely clarified, but are almost certainly mediated by their ability to bind various negatively charged structures and macromolecules in cells. The most important single characteristics of polyamines is likely to be their ability to bind nucleic acids and especially DNA. In addition, polyamines have important role in the regulation of some major classes of cation channels and spermidine is a precursor of an unusual amino acid hypusine needed for the synthesis of translation initiation factor 5A. Polyamine biosynthesis is regulated by two key enzymes, ornithine decarboxylase and S-adenosylmethionine decarboxylase. The activities of these decarboxylases are usually low in resting cells, but increase rapidly and transiently after a wide variety of growth inducing stimuli. Polyamines themselves regulate negatively both enzymes, which emphasizes the significance of maintaining polyamine concentration between appropriate limits within cells. One central component in controlling cellular polyamine levels is antizyme protein, the synthesis of which is induced by polyamines. Antizyme has ability to inhibit ODC activity and polyamine transport to cells. ODC is targeted for proteasomal degradation by binding antizyme.

In this study we wanted to elucidate the role of ornithine decarboxylase and antizyme in the polyamine metabolism of the central nervous system. The guanosine 5'-phosphate activatable ODC was discovered and characterized from the rat brain lysates. Previously GTP-activatable ODC had been found in mammalian tissues only in some tumors. Furthermore, ODC and antizyme expression in brain was localized by *in situ* hybridization and immunocytochemistry. In another part of the study a transgenic mouse line expressing ODC under the control of a MMTV-LTR promotor was generated and used to study the pathological and physiological effects of deregulated ODC expression during the life of transgenic animals. In addition, the role of one of the most highly conserved regions in eukaryotic ODCs was studied using site-directed mutagenesis.

# 2 Review of the literature

## 2.1 Polyamines

### *2.1.1 Structure and properties*

The polyamines, spermidine, spermine and the diamine precursor, putrescine, are positively charged aliphatic amines that have shown in numerous studies to be essential for normal cell growth and differentiation. Putrescine $NH_2(CH_2)_4NH_2$ and spermidine $NH_2(CH_2)_4NH(CH_2)_3NH_2$ occur in almost all living species, spermine $NH_2(CH_2)_3NH$-$(CH_2)_4NH(CH_2)_3NH_2$ is less common in prokaryotes. Since their primary and secondary amino groups are all protonated at physiological pH, putrescine is divalent, spermidine trivalent and spermine tetravalent organic cation. In the cells polyamines interact electrostatically with negatively charged moieties such as DNA, RNA, proteins and phospholipids. The unique feature of polyamine structure compared to inorganic cations like $Mg^{2+}$ or $Ca^{2+}$ is that they have positive charges at defined distances and between them methylene groups that can participate in hydrophobic interactions. Thus polyamines form stronger and more specific interactions than inorganic cations. (for reviews, see Davis *et al*. 1992, Marton & Pegg 1995, Thomas & Thomas 2001).

The importance of polyamines for normal cell growth and functions is emphasized by the complex regulative circuitry of synthesis, degradation, uptake and efflux used to adjust and maintain cellular polyamine concentration at a certain level. When cells are deprived of polyamines they cease to grow and proliferate, but usually they do not die (Balasundaram *et al*. 1991, Balasundaram *et al*. 1993). Trophic stimuli increases intracellular polyamine concentrations that are highest in rapidly proliferating cells. Generally spermidine and spermine are present in millimolar concentrations, whereas putrescine levels are slightly lower (Morgan 1990, Watanabe *et al.* 1991, Igarashi & Kashiwagi 2000). However, most polyamines within cells are bound to nucleic acids and other negatively charged structures. Hence, their free and potentially “reactive” concentration is much lower than total concentration.

### *2.1.2 Physiological roles*

#### *2.1.2.1 DNA binding*

The most important single characteristics of polyamines is likely to be their ability to bind nucleic acids and especially DNA. Polyamines neutralize the charges on the phosphate groups of DNA, interact with nucleic acid bases and dock into the major or minor grooves of the double helix (Feuerstein *et al*. 1990, Feuerstein *et al*. 1991, Tippin & Sundaralingam 1997, Deng *et al*. 2000). They can increase the melting temperature $T_m$ of DNA in a concentration-dependent manner (Thomas & Bloomfield 1984). The increases are of the order of 10 – 20 ˚C at physiological spermine concentration suggesting that polyamines may have a significant role in stabilizing the DNA structure *in vivo*. Another consequence of polyamine binding to DNA is the condensation of DNA occuring both with naked DNA (Gosule & Schellman 1976, Pelta *et al*. 1995) and chromatin (Marquet *et al*. 1986, Sen & Crothers 1986). Immunocytochemical studies of spermidine and spermine have showed that these polyamines are associated with highly compacted mitotic chromosomes (Hougaard *et al*. 1987, Sauve *et al*. 1999) although they may have more stabilizing than regulating effect on the chromatin structure during the cell cycle (Laitinen *et al*. 1998).

Polyamines have also an ability to induce conformational changes to DNA. Their binding has been reported to promote the conversion of the right-handed B-DNA to a left-handed Z-DNA (Thomas *et al*. 1991, Bancroft *et al*. 1994, Hasan *et al*. 1995) or to an alternative form of right-handed helix, A-DNA (Jain *et al*. 1989). However, the polyamines can also bind B-DNA so that no change in the secondary structure is detected by Raman spectroscopy (Deng *et al*. 2000). Z-DNA is induced mainly in blocks of alternating purine-pyrimidine sequences and plays a role in transcriptional control (Herbert & Rich 1999). The other way how polyamines have been suggested to regulate transcription is by inducing bending of DNA after binding to the major groove (Feuerstein *et al*. 1986, Feuerstein *et al*. 1989, Rouzina & Bloomfield 1998). Polyamines promote DNA bending by neutralizing the negative charges on DNA phosphate, reducing the energy requirement for bending, and thus facilitating enchanced protein-DNA interactions. DNA bending itself is a major pathway for transcriptional regulation of gene expression (Kerppola 1998, Coulombe 1999). Many DNA binding proteins exert their action by their ability to bend DNA and, in higher organism, cooperative bending produced by multiple transcription factors produce the required response in transcription. Polyamine concentration has been shown to effect the binding of several transcription factors to DNA (Thomas & Thomas 1993, Panagiotidis *et al*. 1995, Desiderio *et al*. 1999). In the case of the estrogen receptor, polyamines have been demonstrated to effect directly the conformation of the estrogen responsive element in DNA (Thomas *et al*. 1997a, Lewis *et al*. 2000). Polyamines appear also to be involved in signaling pathways that regulate synthesis of a transcription factors or modulate their binding activity via phosphorylation (Wang *et al*. 1993, Wang *et al*. 1999, Pfeffer *et al*. 2000, Wang *et al*. 2001b).

### *2.1.2.2 DNA and protein synthesis*

When cells suffer from the deprivation of polyamines the first detectable effects are observed in DNA and protein synthesis. DNA replication can be impaired within one cell cycle after seeding cells in the presence of polyamine biosynthesis inhibitors (Fredlund & Oredsson 1996a, Fredlund & Oredsson 1996b) or culturing cells deficient in polyamine biosynthesis without exogenous polyamines (Laitinen *et al.* 1998). Nuclei isolated from the polyamine depleted cells are reported to synthesize 70-80 % less DNA than nuclei from control cells (Koza & Herbst 1992). These cells may accumulate short DNA fragments corresponding in size to Okazaki fragments (Pohjanpelto & Hölttä 1996). Chromatin from the cells suffering from the prolonged depletion of polyamines is more sensitive to digestion by nucleases (Snyder 1989, Basu *et al*. 1992, Laitinen *et al.* 1998). In protein synthesis the effect of polyamine deficiency is seen as an impairement of polysome formation (Hölttä & Hovi 1985), that could be a result of decrease in the initiation and/or elongation of protein synthesis (Takemoto *et al.* 1983). These effects may be explained by the ability of polyamines to bind and influence secondary structures of mRNA, tRNA, and rRNA (Kusama-Eguchi *et al.* 1991, Yoshida *et al.* 1999, Igarashi & Kashiwagi 2000).

### *2.1.2.3 Cell growth*

Depletion of polyamines leads to changes in the expression of numerous growth-related genes. However, the molecular mechanisms by which these gene expression patters are regulated are mostly unknown and the regulatory pathways involved are just at the beginning of being revealed. Cell cycle arrest after polyamine depletion appears to be mediated by induction of cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ via p53-dependent mechanisms (Kramer *et al*. 1999, Ray *et al.* 1999, Ray *et al*. 2001), although also p53-independent pathway may exists (Nemoto *et al*. 2001, Ray *et al.* 2001). Polyamine depletion has been reported to stabilize both p53 protein and mRNA (Li *et al*. 2001b). In the cells transformed by the overexpression of polyamine biosynthetic enzymes, $p27^{KIP1}$ expression is greatly decreased confirming the significance of $p27^{KIP1}$ for the polyamine responsive cell cycle control (Ravanko *et al.* 2000). In addition to $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and p53, polyamine depletion has effect on the expression of various cyclins. Changes in cyclin A, B1 and D1 expression have been reported (Thomas & Thomas 1994, Thomas *et al*. 1997b, Marty *et al*. 2000); with the changes in D1 being most apparent. Above listed alterations in gene expression may be mediated by transcription factor NF- B that is rapidly activated in the cells depleted from polyamines (Pfeffer *et al*. 2001).

### *2.1.2.4 Apoptosis*

Programmed cell death, apoptosis, and cell growth are two sides of the coin; pathways regulating them are partially overlapping and a signal inducing cell proliferation in some cell type or physiological state, can in other cells or circumstances lead to apoptotic death. Thus, it is not surprising that in addition to cell growth, polyamines are involved in the regulation of apoptosis. Paradoxically, it appears that they can act as promoting, modulating or protective agents in apoptosis. (For a review, see Schipper *et al.* 2000)

It has been recognised for years that under certain conditions polyamines have toxic effects on cells (Allen *et al.* 1979, Gahl & Pitot 1979). Abnormally high polyamine concentrations are well known to be detrimental to cell growth and able to lead to cell death (He *et al.* 1993, Poulin *et al.* 1993) that has been shown in several cases to be apoptotic by nature (Tome *et al.* 1994, Tobias & Kahana 1995, Poulin *et al.* 1995b, Xie *et al.* 1997). The oxidation of spermidine and spermine either by serum amine oxidase or by the intracellular FAD-dependent polyamine oxidase produces hydrogen peroxide and aminoaldehydes that are strong inducers of apoptosis (Parchment & Pierce 1989, Ha *et al.* 1997). However, polyamines appear to be able to induce apoptosis also without oxidation (Brunton *et al.* 1991, Mitchell *et al.* 1992, Alhonen *et al.* 2000), and it is unclear which mechanism plays a major role in cells.

Various studies with different cell systems have shown that the activity of ornithine decarboxylase is fastly and markedly increased after inducing apoptotic cell death (e.g. Donato *et al.* 1991, Grassilli *et al.* 1991, Manchester *et al.* 1993, Desiderio *et al.* 1995, Penning *et al.* 1998, Lindsay & Wallace 1999). This leads in some cases, as one could expect, to increases in cellular polyamine levels, but at least equally often the polyamine concentrations are actually decreased. The irreversible inhibitor of ODC, -difluoromethylornithine (DFMO), inhibits apoptosis in nearly all cell systems where it has been tested (e.g. Piacentini *et al.* 1991, Manchester *et al.* 1993, Monti *et al.* 1998, Penning *et al.* 1998, Ray *et al.* 2000). However, it is worth noticing that DFMO treatment up-regulates S-adenosylmethionine decarboxylase (AdoMetDC), another of the key enzymes in polyamine biosynthesis, and although putrescine and spermidine levels are decreased by DFMO, spermine levels are often increased. Interestingly, inhibitors of AdoMetDC (Kaneko *et al.* 1998, Penning *et al.* 1998, Satoh *et al.* 1999, Ray *et al.* 2000) and spermidine/spermine analogs (McCloskey *et al.* 1996, Kramer *et al.* 1997, Shah *et al.* 2001) generally induce or increase apoptosis. ODC activity effects mostly putrescine levels whereas AdoMetDC provides decarboxylated S-adenosylmethionine for spermidine and spermine synthesis, suggesting that putrescine and higher polyamines could have different roles in the apoptotic cell death. ODC may in some cases have a very active role in the regulation of apoptosis. It has been suggested to be a direct mediator of apoptosis induced by overexpression of c-*myc* proto-oncogene (Packham & Cleveland 1994). This suggestion was based on the observation that enforced expression of ODC, like c-Myc, was sufficient to induce accelerated cell death following IL-3 withdrawal from murine myeloid cells. In this context, ODC is a mediator of c-Myc-induced apoptosis since c-Myc regulates ODC expression at the level of transcription (Bello-Fernandez *et al.* 1993).

Although accumulation of polyamine levels appears to be able to trigger apoptosis, decrease in polyamine levels, especially of spermidine and spermine, seems to be a more common feature in apoptosis (e.g. Manchester *et al.* 1993, Tome *et al*. 1997, Penning *et al.* 1998, Moffatt *et al*. 2000, Nitta *et al*. 2001). It is highly conceivable that very low levels of polyamines may actually promote apoptosis. Firstly, depletion of polyamines can lead to cell cycle arrest or apoptosis by affecting the p53/p21/p27 cell cycle regulatory pathway (Kramer *et al.* 1999, Ray *et al.* 1999, Li *et al*. 1999b). Secondly, polyamines are important in the regulation of ion transport and the stabilization of important cellular components such as cell membranes and chromatin structures. Therefore depletion of polyamine levels might induce destabilization of important cell structures, leading to loss of cell integrity and finally inducing cell death (Schipper *et al.* 2000). On the other hand, the depletion of polyamines even after cell-cycle arrest may not be sufficient to induce apoptosis alone (Li *et al.* 1999b, Li *et al*. 2001a), but may sensitize cells to apoptosis induced by other factors. The altered susceptibility after polyamine depletion seems to be inducer-specific implicating that polyamines are involved in the regulation of some apoptotic pathways, but not all of them (Stefanelli *et al*. 2001, Li *et al.* 2001a). Not surprisingly, spermidine and/or spermine have protective effects against apoptosis in several cell types ranging from neurons (Harada & Sugimoto 1997), Ehrlich ascites tumor (Moffatt *et al.* 2000) and B cell lymphoma (Nitta *et al.* 2001) cells to paracite *Trypanosoma cruzi* (Piacenza *et al*. 2001). Again the protective effect can be specific to the pathway inducing apoptosis (Hegardt *et al*. 2000) and may be mediated by activation of transcription of genes required for cell proliferation and survival (Shah *et al.* 2001).

Although the working mechanisms of polyamines in apoptosis are still inconclusive, it is obvious that excessive levels as well as extremely low levels of polyamines interfere with their specific cellular interactions and effect on their important physiological activities. It has been suggested, that the real physiological significance of polyamine synthesis may reside in cell cycle control and cell survival (Schipper *et al.* 2000). Therefore, the early induction of ODC often observed during apoptosis may be related to the progression of the cell into a cell cycle phase until a checkpoint is reached from which apoptosis is triggered as a result of cell death-inducing signals. Alternatively, in conditions where apoptosis is inhibited and/or cells have genetic lesions, constitutive ODC expression can lead to cell transformation and deregulated cell growth as observed (Auvinen *et al.* 1992, Moshier *et al*. 1993, Auvinen *et al*. 1997). After the cell cycle check-point polyamine levels may actually decrease during the apoptotic process.

#### *2.1.2.5 Hypusine synthesis*

For a long time the only highly specific and unequivocally established function of polyamines was to provide 4-aminobutyl moiety for a synthesis of an unusual amino acid hypusine (Park *et al*. 1981, Park *et al*. 1982). The aminobutyl group is transferred from spermidine to a highly conserved lysine of eukaryotic translation initiation factor 5A (eIF-5A) that is together with a variant form eIF-5A2 the only protein known to contain

hypysine (Park *et al*. 1993, Jenkins *et al*. 2001). Hypusine is formed in two steps: at first deoxyhypusine synthase (EC 1.1.1.249) catalyzes the transfer of an aminobutyl moiety to a lysine residue to form a deoxyhypusine residue that is subsequently converted to hypusine in a reaction catalyzed by deoxyhypusine hydroxylase (EC 1.14.99.29). Hypusination is required for the biological activity of eIF-5A (Park 1989, Smit-McBride *et al*. 1989). Hypusine formation is tightly coupled to cell proliferation; its formation may increase by 30-fold after growth-stimulus, and it is essential for cell survival (Chen & Chen 1997, Park *et al*. 1997). Distruption of either the eIF-5A or deoxyhypusine synthase gene in yeast leads to a lethal phenotype (Schnier *et al*. 1991, Sasaki *et al*. 1996, Park *et al*. 1998). Inhibition of deoxyhypusine synthase in mammalian cells causes growth arrest (Park *et al*. 1994, Chen *et al*. 1996, Shi *et al*. 1996), cell death (Tome & Gerner 1997), or tumor differentiation (Chen *et al.* 1996). However, hypusine-containing eIF-5A is not required for global protein synthesis. In a conditionally eIF-5A-deficient yeast, the protein synthesis is inhibited only by about 30 % in nonpermissive conditions, and this is accompanied by a slight change in the polysome profile (Kang & Hershey 1994). Rather, activated eIF-5A appears to facilitate the translation of specific subsets of mRNA, maybe the subset of mRNAs required for cell division, and hence the requirement of eIF-5A for cell proliferation would be explained (Park *et al.* 1997). The significance of eIF-5A for cell proliferation is further emphasized by the facts that the recently found tissue-specific eIF-5A2 variant is expressed, in addition to testis and brain, strongly in a colorectal adenocarcinoma cell line (Jenkins *et al.* 2001) and that it has been isolated as a candidate oncogene related to the development of ovarian cancer and certain other solid tumors (Guan *et al*. 2001). Putrescine accumulation in the DFMO resistant cell line after removal of DFMO from the culture induces apoptosis (Tome *et al.* 1997) that have been attributed to the inhibition of hypusine formation. The generality of this phenomenon in other cell types remains to be determined. Interestingly, in the tomato plant eIF-5A may facilitate translation of the mRNAs required for the programmed cell death (Wang *et al*. 2001d).

Although it seems clear that hypusine containing eIF-5A is needed for protein synthesis, the way in which it is, is not fully understood. The protein was initially identified as a putative translation initiation factor based on its ability to stimulate methionyl puromycin synthesis under *in vitro* conditions (Kemper *et al*. 1976). More recent evidence suggests that eIF-5A facilitates protein synthesis by promoting nuclear transport of specific mRNAs (Ruhl *et al*. 1993, Katahira *et al*. 1994). It has also been proposed that eIF-5A may be involved in mRNA turnover, acting downstream of decapping (Zuk & Jacobson 1998). A tentative consensus mRNA sequence for eIF-5A binding has been identified (Xu & Chen 2001). The consensus is present in over 400 human ESTs, but it's not known whether these represent physiological substrates of eIF-5A.

#### *2.1.2.6 Other roles*

During the last ten years polyamines have been shown to function as endogenous activators and/or blockers of several major classes of cation channels. They are able to

activate, inhibite or block NMDA receptors (Ransom & Stec 1988, Williams *et al*. 1989, Williams 1997) and to block AMPA and kainate receptors (Donevan & Rogawski 1995, Kamboj *et al.* 1995). All these three receptor groups belong to the class of glutamate-activated receptor channels. Other cation channels blocked by polyamines are inwardly rectifying $K^+$ channels (Fakler *et al.* 1994, Ficker *et al.* 1994, Lopatin *et al*. 1994), nicotinic acetylcholine receptor channels (Haghighi & Cooper 1998, Haghighi & Cooper 2000), cyclic nucleotide-gated channels (Lu & Ding 1999), and voltage-gated $Ca^{2+}$ (Scott *et al*. 1993) and $Na^+$ (Huang & Moczydlowski 2001) channels. These observations have raised a lot of interest and the role of polyamines in these contexts is discussed in detail in the chapters 2.1.3 and 2.1.4.

In addition to those discussed above, new functions for polyamines are proposed regularly. One of the most exciting ones is the suggestion that polyamines could modulate the synthesis of nitric oxide (Southan *et al*. 1994, Szabo *et al*. 1994, Baydoun & Morgan 1998). This modulation may be mediated at least partly by down-regulation of L-arginine transport (Mössner *et al*. 2000). *Vice versa*, nitric oxide has reported to be able to effect polyamine biosynthesis by inhibiting ornithine decarboxylase enzyme (Bauer *et al*. 2001, Ignarro *et al*. 2001). Thus, the regulation of these versatile small-molecular modulators of cellular functions may be partially interconnected.

### *2.1.3 Polyamine metabolism in mammals*

#### *2.1.3.1 Biosynthesis*

Most living organisms are capable of synthesizing polyamines from the precursor amino acids, arginine and methionine (Davis *et al.* 1992, Marton & Pegg 1995, Morgan 1999). The metabolic pathways for the synthesis and interconversion of polyamines are well known and established. They are shown in Fig. 1. The first steps committed to polyamine biosynthesis are the decarboxylations of ornithine to form putrescine and S-adenosylmethionine to form decarboxylated S-adenosylmethionine. These irreversible reactions are catalysed by ornithine decarboxylase (ODC, EC 4.1.1.17) and S-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50), respectively. Ornithine may be derived from plasma, or intracellular arginine can be converted to ornithine by arginase (EC 3.5.3.1.) in a reaction of the urea cycle pathway. S-adenosylmethionine is a common donor of methyl groups in cells and is formed when an enzyme called ATP:L-methionine S-adenosyltransferase (EC 2.5.2.6) catalyzes activation of L-methionine. Once AdoMet has been decarboxylated, it is no longer available for transmethylation reactions in the cell.

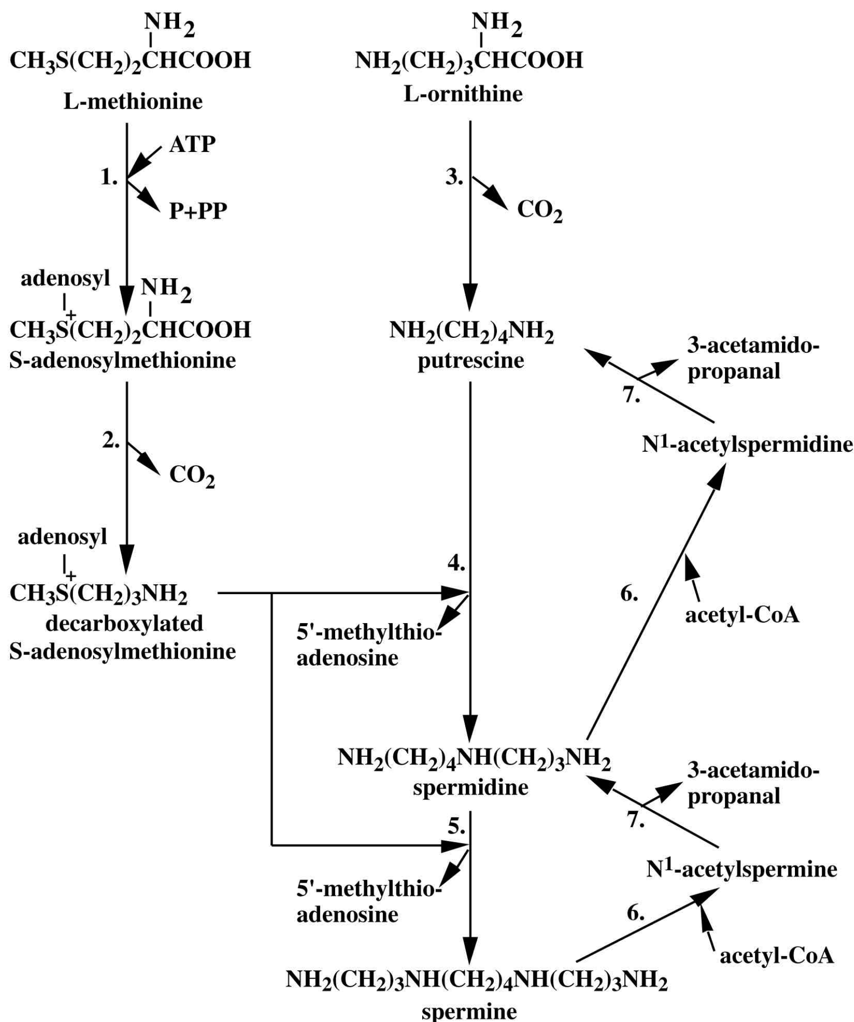


**Figure 1. Biosynthesis and interconversion of polyamines. The enzymes catalyzing reactions are: 1. ATP:L-methionine S-adenosyltransferase (EC 2.5.1.6), 2. S-adenosylmethionine decarboxylase (EC 4.1.1.50), 3. Ornithine decarboxylase (EC 4.1.1.17), 4. Spermidine synthase (EC 2.5.1.16), 5. Spermine synthase (EC 2.5.1.22), 6. Spermidine/spermine $N^1$-acetyltransferase (EC 2.3.1.57), and 7. Polyamine oxidase (EC 1.4.3.4)**

Polyamine biosynthesis is controlled mainly by two key enzymes, ODC and AdoMetDC. Their activities are rapidly increased or decreased as a response to various positive or negative stimuli. This is based on their fast turnover rate, the half-life of

enzyme activity is between 10 and 20 minutes for ODC (Seely *et al*. 1982a, Isomaa *et al*. 1983) and from 30 to 60 for AdoMetDC (Pegg 1979, Shirahata & Pegg 1985). Regulation of ODC is discussed later in the chapter 2.2.3. AdoMetDC is regulated at the level of transcription, translation, post-translational processing and protein degradation. Increased levels of mammalian AdoMetDC mRNA, which presumably are due to changes in transcription, have been seen in response to growth promoting factors and to a decline in spermidine produced by a variety of inhibitors (Shirahata & Pegg 1986, Pajunen *et al*. 1988, Jänne *et al*. 1991, Svensson *et al*. 1997). Insulin also increases AdoMetDC mRNA synthesis and an insulin-responsive element has been found in the rat AdoMetDC promotor (Soininen *et al*. 1996).

In virtually all of the circumstances reported in which enhanced levels of AdoMetDC mRNA have been observed, these increases in mRNA are insufficient to account for the increases in AdoMetDC protein content (see e.g. Pajunen *et al.* 1988, Persson *et al*. 1989, Stjernborg *et al*. 1993, Svensson *et al.* 1997). All mammalian AdoMetDC mRNAs have a long 5'UTR of about 330 nucleotides, the sequence of which is very highly conserved. The leader sequence contains a small internal open reading frame (ORF) that is located close to 5' terminus (11-14 nucleotides from terminus), is identical among all reported mammalian cDNAs, is in perfect context for translation and has been shown to be translated *in vitro* (Hill & Morris 1992, Hill & Morris 1993, Raney *et al*. 2000). ORF codes a peptide of six amino acids with a sequence MAGDIS. High polyamine levels repress the translation of AdoMetDC mRNA efficiently (Kameji & Pegg 1987b) and that repression is not dependent on the secondary structure of 5'UTR of mRNA (Shantz *et al*. 1994), but requires the presence of ORF in the leader sequence (Ruan *et al*. 1996).

AdoMetDC is synthesized as a proenzyme which then undergoes an internal processing reaction forming and subunits and a pyruvate prosthetic group, which is located at the amino terminus of the subunit (Shirahata & Pegg 1986, Stanley *et al.* 1989). The processing and activity of AdoMetDC are increased by putrescine, providing a means by which the increased availability of putrescine raises the formation of decarboxylated AdoMet (Kameji & Pegg 1987a, Pegg *et al*. 1988).

The degradation of AdoMetDC is also regulated, but at present, the mechanisms for the degradation and for the alterations in degradation seen in response to polyamines, inhibitors and other stimuli are unknown (Shirahata & Pegg 1985, Autelli *et al.* 1991, Svensson *et al.* 1997).

Spermidine and spermine synthases are considered to have only a minor role in the regulation of intracellular polyamine levels. They both are constitutively expressed and stable enzymes regulated mainly by the availability of their substrate, decarboxylated adenosylmethionine. Nevertheless, there are several reports about increased spermidine synthase activity after growth promoting stimuli (Hannonen *et al*. 1972, Oka *et al*. 1977, Käpyaho *et al.* 1980, Korpela *et al.* 1981, Kauppinen 1995). Increase is dependent on new protein synthesis and could be caused both by increased transcription of spermidine synthase gene and more efficient translation of spermidine synthase mRNA (Kauppinen 1995). Transforming growth factor 1 that inhibits growth of many cell lines has been suggested to inhibit transcription of the spermidine synthase gene (Nishikawa *et al.* 1997).

Spermine synthase enzyme is found only in eukaryotes and it is not essential for growth in yeast or mammalian cells (Hamasaki-Katagiri *et al.* 1998, Korhonen *et al.*

2001). Gyro (Gy) mice that are deficient in spermine synthase and PHEX gene (regulating phosphate metabolism) show compared to mice deficient only in PHEX gene, reduced viability and fertility, lower body weight and bodily growth, reduced skeletal mineralization and deficiencies in neurological function although analysis of brain tissue revealed no gross or histological abnormalities (Lorenz *et al.* 1998, Meyer *et al.* 1998, Mackintosh & Pegg 2000). Fibroblasts taken from these mice were sensitized to ultraviolet irradiation and alkylating agents suggesting that spermine is needed to protect chromosomal DNA (Mackintosh & Pegg 2000, Nilsson *et al.* 2000b, Stefanelli *et al.* 2001).

The overruling theme in the regulation of polyamine biosynthesis appears to be to keep polyamine levels within certain limits: to avoid too low and, on the other hand, too high polyamine concentrations. This is well manifested in the results obtained from experiments with transgenic mice. Although overexpression of ODC results in the increase in putrescine levels, only minimal changes have been detected in the concentration of spermidine and spermine (Halmekytö *et al.* 1991a, Halmekytö *et al.* 1993, Alhonen *et al.* 1996). Similarly, transgenic mice overexpressing AdoMetDC (Heljasvaara *et al.* 1997) or spermidine synthase (Kauppinen *et al.* 1993) displayed no marked changes in the spermidine or spermine levels, not even after crossbreeding these mice with the mice overexpressing ODC. Indispensability of polyamines has been demostrated also by disruption of the ODC gene in embryonic stem cells and genereating mice harboring a disrupted ODC gene (Pendeville *et al*. 2001). ODC-heterozygous mice were viable, normal, and fertile, whereas the completely ODC-deficient embryos were capable of uterine implantation on the embryonic day 3.5 and induced maternal decidualization, but failed to develop substantially thereafter.

#### *2.1.3.2 Catabolism and interconversion*

In contrast to the extensive studies of polyamine biosynthesis, polyamine degradation has received much less attention untill quite recent years. Mammalian cells have two pathways for polyamine catabolism. An interconversion or recycling pathway converts spermidine and spermine back to putrescine. Terminal catabolic pathway oxidizes polyamines forming aminoaldehydes that cannot be recycled to polyamines. (For reviews, see Casero & Pegg 1993, Seiler 2000)

The interconversion pathway (Fig. 1) is initiated in an acetylation reaction catalyzed by spermidine/spermine $N^1$-acetyltransferase (SSAT, EC 2.3.1.57). The acetyl group is transferred from acetyl-coenzyme A to an aminopropyl moiety of spermine or spermidine. The $N^1$-acetylspermidine or $N^1$-acetylspermine is then oxidized by the constitutive intracellular flavin adenine dinucleotide-dependent polyamine oxidase (PAO, EC 1.4.3.4.), which cleaves the polyamine at a secondary amino nitrogen to release 3-acetamidopropanal and putrescine or spermidine, respectively. Polyamines and their acetylated derivatives are also acted upon at the primary amino groups by diamine oxidases, which are copper-containing amine oxidases (EC 1.4.3.6.). These reactions

belong to terminal catabolism of polyamines. Products of reactions include -aminobutyric acid, 3-acetamidopropanal, hydrogen peroxide ($H_2O_2$) and ammonia.

SSAT acetylates the aminopropyl end of spermidine forming $N^1$-acetylspermidine. However, mammalian cells contain also $N^8$-acetylspermidine (Erwin *et al*. 1984, Pegg *et al*. 1990). Furthermore, $N^1$,$N^8$-diacetylspermidine (Hiramatsu *et al.* 1995) and $N^1$,$N^{12}$-diacetylspermine (van den Berg *et al*. 1988) have been found in urine. Their functions and enzymes participating in their synthesis are unknown. It has been suggested that acetylation at $N^8$ directs spermidine to be transported from nucleus to cytoplasm (Casero & Pegg 1993).

Tissue polyamine oxidase activity is usually so high that intracellular levels of $N^1$-acetylspermidine or $N^1$-acetylspermine are below the limits of detection, thus the rate of polyamine degradation is regulated by the activity of SSAT. The amount and activity of SSAT are low in most cell types, but are efficiently induced by a number of factors including various toxic agents, hormones, growth factors, polyamines and polyamine analogs (Casero & Pegg 1993, Thomas & Thomas 2001). Polyamines induce the synthesis of polyamine modulated factor 1 (PMF-1) that is a putative cotranscription factor binding to polyamine responsive element in SSAT promotor (Wang *et al.* 1999, Wang *et al*. 2001a). Similarly to ODC and AdoMetDC, SSAT has a very short half-life; that of SSAT is about 15 minutes (Matsui & Pegg 1981, Persson & Pegg 1984). Polyamine analogues greatly increase the half-life of SSAT (Parry *et al*. 1995, Fogel-Petrovic *et al*. 1996) apparently by inhibiting ubiquitination of the enzyme and preventing its targeting to proteosomal degradation (Coleman & Pegg 2001). Polyamine oxidase was cloned very recently and although it is considered to be constitutively expressed enzyme, its mRNA and activity were induced in non-small cell lung carcinoma cell line by polyamine analog $N^1$, $N^{11}$-bis(ethyl)norspermine 5- and 3-fold, respectively (Wang *et al*. 2001c).

Effects of SSAT overexpression have been studied extensively using transgenic animals. Tissues of these mice showed markedly distorted polyamine pools, which in most cases were characterized by the appearance of $N^1$-acetylspermidine, a massive accumulation of putrescine, and significant decreases in spermidine and/or spermine pools (Pietilä *et al*. 1997, Suppola *et al*. 1999). The most striking phenotypic change was permanent hair loss likely due to overaccumulation of putrescine (Pietilä *et al.* 2001). Inducible overexpression of SSAT gene in rat under methallothionein promotor led to acute pancreatitis (Alhonen *et al.* 2000). In mice the overexpression protected brain from kainate-induced toxicity (Kaasinen *et al.* 2000), but enhanced general sensitivity to the polyamine analog $N^1$, $N^{11}$-diethylnorspermine (Alhonen *et al.* 1999). Interestingly, a hybrid transgenic mice overexpressing both ODC and SSAT under methallothionein promotor showed further accelerated catabolism of hepatic polyamines manifested in a massive putrescine accumulation and in an extreme reduction of spermidine and spermine pools (Suppola *et al.* 2001). The latter may be partly due to enhanced efflux of polyamines. These results strongly suggest that the catabolisms is the overriding regulatory mechanism in the polyamine metabolism, and that the major aim of the regulation is to prevent an over-accumulation of the higher polyamines.

### *2.1.3.3 Transport*

Most cells have the capacity to synthesise polyamines to a greater or lesser extent and although, historically, it was believed that *de novo* biosynthesis was by far and away the main supply of polyamines in cells, it is now clear that this is not the case. Additional polyamines are required in the cells with high polyamine demand, such as tumors and normal but rapidly proliferating cells. Exogenous polyamines are also transported to cells when synthesis is disturbed or to organisms that do not produce polyamines. They may be obtained from dietary sources, through synthesis by intestinal microorganisms, or by the release from other cells. (for a review, see Seiler *et al.* 1996)

All mammalian cells have an active polyamine transport system. Most cells take up polyamines by carrier-mediated and energy-dependent mechanisms, but the nature of the energy coupling is still unclear. Transport is at least partly driven by membrane potential (Poulin *et al*. 1995a, Poulin *et al*. 1998). Transport of putrescine and spermidine may be sodium-sensitive, but not sodium-dependent, whereas spermine uptake is not effected by changes in extracellular sodium (Morgan 1999). Divalent cations such as $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ are essential at least for putrescine and spermidine transport (Brachet *et al*. 1995, Poulin *et al.* 1998). The number of carriers involved in the uptake is still debated, and it appears that the number can vary according to cell type. Many cells apparently have a single transporter for putrescine, spermidine and spermine (Seiler *et al.* 1996). The affinity for the transporter increases with the number of positive charges from putrescine to spermine. However, multiple carrier types per cell are not uncommon. For example, in human umbilical vein endothelial cells there seem to be two carriers, one capable of transporting all three amines and another shared by spermidine and spermine (Morgan 1992). Furthermore, porcine aortic endothelial cells have been reported to posses three carriers, one for both polyamines and one for putrescine (Bogle *et al.* 1994). Generally the specificity of polyamine transport is not stringent. Derivatives with alkyl substituents on the primary amino groups (Porter *et al*. 1987) or substituted carbon chains (Sarhan *et al*. 1987) are transported by different cell lines, as well as biogenic amine agmatine (Satriano *et al*. 2001). Even compounds with relatively poor structural resemblance to the natural polyamines, such as AdoMetDC inhibitor methylglyoxal bis(guanyl-hydrazone) (Alhonen-Hongisto *et al.* 1984) and widely used herbicide paraquat (Byers *et al*. 1987) may share the same transport system with polyamines.

The proteins and composition of polyamine transport system in mammalian cells are not known. Using photoaffinity labelling methods, several polyamine-binding proteins have been detected in the plasma membrane of mammalian cells, but their identification and participation in polyamine transport are still unconfirmed (Felschow *et al.* 1995, Felschow *et al*. 1997). In addition to depletion of intracellular polyamine levels, growth stimulus enhances transport (Seiler *et al.* 1996). As a rule, factors that increase polyamine formation enhance also their uptake from environment. High intracellular polyamine levels inhibit transport from the environment. This effect is at least partly mediated by the antizyme (He *et al.* 1994, Mitchell *et al.* 1994), a multifunctional protein that became originally known as an inhibitor of ODC.

Intestinal mucosa and alveolar epithelium are two tissues where polyamine transport appears to be of particular importance. In intestinal mucosa enterocytes take up

polyamines from the gut lumena. The transporting capacity of a single enterocyte is not different from that of other cells in the body, but there are an enourmous number of cells in mucosa. In addition, passive diffusion through epithelium without uptake to enterocytes may contribute significantly to the total transport of polyamines (Milovic *et al.* 2001). Polyamines taken up from intestine have been shown to be important for the regeneration and growth of the mucosa (Osborne & Seidel 1989, Wang *et al.* 1991) and contribute to the growth of tumors (Sarhan *et al.* 1989, Sarhan *et al.* 1992). Alveolar epithelial cells are endowed with a very efficient polyamine uptake system (Hoet & Nemery 2000), but the physiological significance of the polyamine transport to epithelium is still obscure. Polyamine levels have been suggested to be linked to pulmonary hypertension induced by chemicals (Olson *et al.* 1989) or hypoxia (Atkinson *et al.* 1987). In a small number of studies it has been observed that polyamines can contribute to the suppression of immunologic reactions in the lung (reviewed in Seiler & Atanassov 1994).

Excretion of polyamines from mammalian cells is much less studied than uptake. Most articles published are about the excretion of polyamines and their acetylated derivatives to urine in healthy and diseased individuals, the individuals being either laboratory animals or human patients (see e.g. Heffner *et al.* 1995, O'Brien *et al.* 1995, Hyltander *et al.* 1998, Langen *et al.* 2000). At the cellular level excretion is avery poorly understood subject. Polyamine uptake-deficient CHO (Byers *et al.* 1994) and COS (Hyvönen *et al.* 1994) cells are able to release polyamines, therefore uptake and export appear to be mediated by different transport systems. Efficient release by diffusion is unlikely in view of the hydrophilic character of polyamines and carrier-mediated export is implied also by studies on excretion from human cancer cell lines (Mackarel & Wallace 1993, Mackarel & Wallace 1994) and erythrocyte cells (Fukumoto & Byus 1996). It has been suggested that antizyme may regulate also excretion (Sakata *et al.* 2000).

### *2.1.4 Polyamines in the central nervous system*

The essential role of polyamines in the development and differentiation of central nervous system is well documented although their exact role in neurogenesis is not known. They might play an important role in neuronal cell division, differentiation, axonogenesis, synaptogenesis and synaptic plasticity. Likewise, a lot of research effort has been directed to investigate the involvement of polyamines in various pathological conditions of the CNS. Enhanced ODC activity is a common response to different pathological stimuli in the brain: physical, thermal, chemical and metabolic stress all induce increase in ODC activity. (for reviews, see Kauppinen & Alhonen 1995, Johnson 1998, Bernstein & Müller 1999, Seiler 2000).

ODC and AdoMetDC are characteristically regulated in the brain during development. In mammalian brain ODC activity as well as the amount of immunoreactive enzyme protein, are highest around the day of birth (Anderson & Schanberg 1972, Laitinen *et al.* 1982, Onoue *et al.* 1988, Morrison *et al.* 1998). During the few first weeks or months brain ODC activity declines to a low adult level. ODC activity in adult mouse brain is

about 70-fold lower than at the time of birth (Suorsa *et al*. 1992). AdoMetDC activity is very low after birth and increases as the brain matures (Suorsa *et al.* 1992, Morrison *et al*. 1993a). This increase is 6-fold in human and 8-fold in mouse brain.

Extracellular polyamines are known to regulate the N-methyl-D-aspartate (NMDA) subtype of glutamate-activated receptor channels in the CNS (Ransom & Stec 1988, Williams *et al.* 1989), which are believed to have a major physiological functions in the induction of long-term potentiation and in the regulation of embryonal neuronal development (Bliss & Collingridge 1993, Schlaggar *et al*. 1993). Long-term potentiation is thought to underlie certain types of learning and memory. Transgenic mice overexpressing ODC exhibit a significantly elevated seizure threshold to chemical and electrical stimuli, and impaired performance in spatial learning and memory tests (Halonen *et al*. 1993). Further analysis *in vitro* using hippocampal slices revealed that the excitatory synaptic transmission was altered, but no alterations in long-term potentiation were detected, although it is possible that extracellular putrescine levels in slices were not comparable to *in vivo* situation (Pussinen *et al*. 1998).

Intracellular polyamines in CNS are responsible for intrinsic gating and rectification of strong inward rectifier $K^+$ (Kir) channels (Fakler *et al.* 1994, Ficker *et al.* 1994, Lopatin *et al.* 1994) and nicotinic acetylcholine receptor ion channels (Haghighi & Cooper 1998, Haghighi & Cooper 2000, Bixel *et al.* 2001). Kir channels stabilise resting membrane potential in both excitable and non-excitable cells, and control the excitability treshold in neurons and muscle cells (Reimann & Ashcroft 1999, Oliver *et al.* 2000). The role of polyamines in their regulation is discussed in the chapter 2.1.5. Nicotinic acetylcholine receptor calcium channels are widespread in the nervous system where they function as postsynaptic receptors to excite neurons or as presynaptic receptors to modulate neurotransmitter release (Sargent 1993, Role & Berg 1996). They have been implicated in a wide variety of cognitive functions, including visual and auditory processing, nociception, and attention and memory mechanism (Picciotto *et al*. 1995, Bannon *et al*. 1998, Xiang *et al.* 1998, Marubio *et al.* 1999, Vetter *et al.* 1999). The location of polyamine binding sites in a receptor have been mapped (Bixel *et al.* 2001), but otherwise little is known about the mechanism and the physiological significance of rectification exerted by polyamines.

Increase in ODC activity is a common response to different pathological stimuli in the brain. AdoMetDC activity has been reported to decrease after traumatic injury (Henley *et al*. 1997) and cerebral ischemia (Rohn *et al*. 1992), increase in epilepsy (Rohn *et al.* 1992, Morrison *et al*. 1994) and in Alzheimer's disease (Morrison *et al.* 1993b), and remain unchanged after restrained stress (Gilad & Gilad 1996). The induction of ODC activity is most pronounced following severe metabolic stress as produced by cerebral ischemia (Paschen *et al*. 1993). Induction of ODC and concomitant decrease in AdoMetDC activity brings about a high increase in the putrescine level. There is a single report on elevated $N^1$-acetylspermidine levels in gerbil and rat brains after CNS injury (Rao *et al.* 2000), which suggests that SSAT activity is increased as well and may contribute to the putrescine levels. The levels of spermidine and spermine do not change significantly or they can decrease temporarily (Paschen *et al.* 1993). The role of increased putrescine concentration is still unclear. In some studies, excessive polyamines have been implicated in neuronal degeneration after CNS injury (Paschen *et al.* 1993, Schmitz *et al*. 1993, Kindy *et al*. 1994, Baskaya *et al*. 1997, Dogan *et al*. 1999a, Dogan *et al*. 1999b). On the

other hand, it has been suggested that the postischemic activation of polyamine metabolism is necessary for the recovery of neurons from metabolic stress because the pretreatment of animals with polyamines reduces the postischemic development of neuronal necrosis (Gilad & Gilad 1991). This was supported by observation that after cerebral ischemia ODC is up-regulated also in regions where no cellular damage usually occurs (Keinänen *et al.* 1997) and studies on transgenic rats overproducing ODC have demostrated that induction of ODC has neuroprotective role in this model of transient focal cerebral ischemia (Lukkarinen *et al.* 1997, Lukkarinen *et al.* 1998).

A few histochemical studies on the expression patterns of ODC and antizyme in CNS have been published. Studies on ODC have focused mainly on the developmental pattern of expression or on the effect of pathological stimuli on expression. Polyamines themselves have also been localized employing immunocytochemical methods. Detectable ODC expression appears to be restricted to neurons (Müller *et al.* 1991, Müller *et al.* 1993, Bernstein & Müller 1995, Ichikawa *et al.* 1997, Ichikawa *et al.* 1998, Gritli-Linde *et al.* 2001), glial expression of ODC has been reported only in few cases (Bernstein & Müller 1999). These reports have clearly stated that ODC is a cytoplasmic protein. Antizyme expression is similarly confined to neurons as demostrated both by immunocytochemistry and *in situ* hybridization (Junttila *et al.* 1995, Gritli-Linde *et al.* 2001). Antizyme is mainly localized to the cytoplasm, but as shown in this thesis (III) and later by others (Gritli-Linde *et al.* 2001) it can be found in certain cell types or developmental stages predominantly or even virtually exclusively in nuclei. Although glial cells do not contain detectable amount of ODC, immunocytochemical studies with antibodies against spermidine and spermine have shown that polyamine levels in these cells are comparable to neurons and even higher in some cases (Laube & Veh 1997, Biedermann *et al.* 1998, Skatchkov *et al.* 2000). It has been suggested that the glial cells could be a polyamine storage (Laube & Veh 1997), but it is not known how their high polyamine concentration is formed and maintained.

### *2.1.5 Polyamines and the regulation of inward rectifying potassium channels*

Inwardly rectifying potassium (Kir) channels have two main physiological roles: they stabilise the resting membrane potential near the $K^+$ equilibrium potential and they mediate $K^+$ transport across membranes (for reviews, see Williams 1997, Reimann & Ashcroft 1999, Oliver *et al.* 2000). Kir channels conduct more current when the membrane potential is hyperpolarized than when it is depolarized from the $K^+$ equilibrium potential by an equivalent amount, i.e. Kir channels favour the inward flow of potassium ions. This property results principally from a voltage-dependent block of outward currents by cytoplasmic $Mg^{2+}$ and polyamines that enter the pore under the influence of the membrane voltage field and impede $K^+$ efflux.

There are seven subclasses of Kir channels of which Kir2 channels (Kir2.1 – Kir2.4) represent typical “strong” rectifiers. The term strong refers to the high voltage-dependence of rectification. Polyamines, mainly spermine, are responsible for the

intrinsic gating and rectification of Kir2 channels (Fakler *et al.* 1994, Ficker *et al.* 1994, Lopatin *et al.* 1994, Fakler *et al.* 1995) and Kir3 channels (Yamada & Kurachi 1995). The latter belong to a subfamily of G-protein-activated intermediate rectifiers. Polyamines have been demonstrated to rectify also ATP-dependent Kir4.1 channel (Fakler *et al.* 1994, Fakler *et al.* 1996) and weak Kir6.2 channels in alkaline pH (Baukrowitz *et al.* 1999). Weak rectifyers of Kir1 subfamily are relatively insensitive to polyamines (Fakler *et al.* 1994, Yamada & Kurachi 1995, Riochet *et al.* 2001).

Members of Kir2 subfamily are typically expressed in heart, skeletal muscle and nervous system. Another subfamily expressed widely in CNS is Kir3, in addition to central neurons they are found in a variety of tissues and cell types, among them cardiac myocytes (Oliver *et al.* 2000). Kir4.1 is expressed in glial cells and may be their predominant Kir channel (Takumi *et al.* 1995, Hibino *et al.* 1999, Kofuji *et al.* 2000). Rather ubiquitous Kir6 channels are expressed also in neurons of the CNS (Liss & Roeper 2001). The significance of Kir channels for the normal function of cells is confirmed by observations that their genes are mutated in three human and one murine hereditary diseases. Mutation in Kir1.1 leads to Bartter's syndrome type III (Simon *et al.* 1996). The Kir2.1 channel is mutated in Andersen's syndrome (Plaster *et al.* 2001) and Kir6.2 in familial persistent hyperinsulinaemic hypoglycaemia of infancy (Thomas *et al.* 1996) whereas knock-out mice lacking Kir6.2 channel are susceptible to generalized seizures after brief hypoxia (Yamada *et al.* 2001). Mutation in the pore region of Kir3.2 results in *weaver* phenotype of mice (Patil *et al.* 1995). Bartter's syndrome is a renal tubular disorder characterised by salt-wasting, hypokalaemia and metabolic acidosis. Andersen's syndrome is a rare disorder characterized by periodic paralysis, cardiac arrhythmias, and relatively severe dysmorphic features. *Weaver* mice have selectively lost brain neurones and they suffer from ataxic gait.

Sequence comparison between the prototypes of strong and weak rectifiers, Kir2.1 and Kir1.1 has led to identification of three residues defining the voltage-dependence of polyamine block: negatively charged glutamate or aspartate residues in the second transmembrane segment (TM2 site) (Fakler *et al.* 1994, Lu & MacKinnon 1994, Stanfield *et al.* 1994, Wible *et al.* 1994) and in two sites of the cytoplasmic C-terminus (Taglialatela *et al.* 1995, Yang *et al.* 1995, Kubo & Murata 2001). Exchange of the neutral TM2 site in the weakly rectifying Kir1.1 to a negatively charged residue converted this channel into a strong inward-rectifier (Wible *et al.* 1994), while neutralizing TM2 site and any of C-terminal sites in Kir2.1 greatly reduced spermine-mediated rectification (Taglialatela *et al.* 1995, Kubo & Murata 2001). The requirement of negatively charged amino acid at the TM2 and C-terminal sites for strong rectification suggests electrostatic interactions between these sites and the positively charged blocker molecules (Oliver *et al.* 2000). This is further supported by findings that a reporter cysteine at the TM2 site of the transmembrane segment can be readily modified with cysteine-reactive reagents indicating exposure of TM2 site to the lumen of pore (Lu *et al.* 1999). Kir6.2 displays polyamine-responsive rectification only in alkaline pH. This pH-dependency has been shown to be confered by C-terminal histidine. Around neutral pH, the histidine is protonated and Kir6.2 channels present weak inward-rectification, while alkalization that deprotonates the residue results in strongly rectifying channels (Baukrowitz *et al.* 1999). This pH-dependent rectification might in metabolic stress inhibit electrical activity and help protect the cell from energy depletion.

It has been shown using inhibitors of polyamine biosynthesis and ODC-deficient CHO cell line that changes in cellular polyamine contents can indeed alter the rectification of Kir channels and the excitability of cells (Bianchi *et al.* 1996, Shyng *et al.* 1996). The effect of elevated polyamine levels on Kir channels has been studied using transgenic mice that overexpress ODC in the heart under control of the cardiac -myosin heavy chain promotor (Lopatin *et al.* 2000). In transgenic hearts putrescine levels were elevated 35-fold, the spermidine level was increased 3.6-fold, but spermine was essentially unchanged. Despite these changes, alterations in Kir currents of trangenic cardiomyocytes were relatively small. Inward rectification as well as voltage dependence of rectification were essentially unchanged although density of current was reduced. In cardiomyocytes isolated from Gyro (Gy) mice that are deficient in spermine synthase (Lorenz *et al.* 1998, Meyer *et al.* 1998) and totally lacking spermine but displaying increased (5.4-fold in the heart) spermidine levels, inward rectification of Kir channels was slighly reduced (Lopatin *et al.* 2000), but the density of Kir currents was unchanged. Direct manipulation of polyamine levels in myocytes by pipette dialysis indicated that spermidine and especially spermine are major controllers of rectification in intact cells. Putrescine plays relatively little role in controlling, but may cause significant weakly voltage-dependent block. The ODC transgenic and Gy mice are apparently normal, and only relatively minor effects on action potential characteristics and heart rates can be expected. Both cardiac hypertrophy (Calderera *et al.* 1974, Bartolome *et al.* 1980) and epilepsy (Laschet *et al.* 1992, Hayashi *et al.* 1993, Mialon *et al.* 1993) are each associated with increased polyamine levels and with enhanced excitability, but it is not known whether these two things are related to each others.

### *2.1.6 Polyamines and cancer*

In cancer cells and tissues polyamine levels and polyamine biosynthesis are highly elevated (Scalabrino & Feriolo 1982, Pegg 1988, Marton & Pegg 1995). ODC has been the main focus of interest, but many human cancers show also increased AdoMetDC activity, although to a lesser extent than ODC activity. ODC becomes activated after treatment with chemical carcinogens and tumor promotors, as well as in cells transformed by various oncogens, such as v-*src*, *neu* and *ras* (Hölttä *et al.* 1988, Pegg 1988, Sistonen *et al.* 1989a, Sistonen *et al.* 1989b, Auvinen *et al.* 1992). Furthermore, the overexpression of normal human ODC in NIH3T3 or RAT-1 fibroblasts induces malignant transformation including the ability to grow as colonies in semi-solid medium and to form rapidly progressing tumors in nude mice (Auvinen *et al.* 1992, Moshier *et al.* 1993, Auvinen *et al.* 1997). In another study overexpression of ODC was not able to transform NIH3T3 cells without co-expression of c-Ha-*ras* oncogene (Hibshoosh *et al.* 1991). Cells overexpressing ODC were more readily transformed by c-Ha-*ras* than wild-type NIH3T3 cells. Also overexpression of AdoMetDC can lead to malignant transformation of NIH3T3 cells (Paasinen-Sohns *et al.* 2000). Interestingly, AdoMetDC appeared to be an even more potent inducer of transformation than ODC.

A possible role for ODC in carcinogenesis was suggested for the first time based on a series of studies carried out using a skin-tumor model initiated by a carcinogen (initiator) and promoted by phorbol ester (O'Brien *et al.* 1975a, O'Brien *et al.* 1975b). These treatments resulted in high induction of ODC activity. Although cells tranformed by overexpression of ODC (Auvinen *et al.* 1992) or AdoMetDC (Paasinen-Sohns *et al.* 2000) were able to form tumors in nude mice, the first transgenic mice overexpressing ODC did not show increased levels of spontaneous tumorigenesis during their entire life-time (Alhonen *et al.* 1995) and were actually able to maintain normal levels of polyamines in their tissues (Halmekytö *et al.* 1991a, Halmekytö *et al.* 1993). However, they displayed enhanced papilloma formation in response to chemical skin tumor promotion (Halmekytö *et al.* 1992). Similar observations were made with K6/ODC transgenic mice that overexpressed ODC in hair follicle keratinocytes (O'Brien *et al.* 1997) and these mice display a slightly increased sensitivity even to spontaneous tumors (Megosh *et al.* 1995). A single low dose of carcinogen was generally enough to initiate tumorigenesis in the K6/ODC mice. They often harboured mutations to c-Ha-*ras* gene after administration of initiating doses of carcinogens (Megosh *et al.* 1998). When the K6/ODC transgenic mice were bred with TG.AC v-Ha-*ras* transgenic mice, double transgenic mice produced spontaneously skin carcinomas (Smith *et al.* 1998). Their spontaneous tumors were reversed by ODC inhibitor DFMO (Lan *et al.* 2000). Similar kinds of results were obtained when cells infected with ODC or *ras* construct or with both were transferred to nude mice (Clifford *et al.* 1995). Only cells infected to overproduce both ODC and c-Ha-*ras* oncoprotein were able to form tumors. The K6/ODC mice – now called K5/ODC mice – have recently been shown to be very sensitive to UVB radiation (Ahmad *et al.* 2001). After 30 weeks of repeated exposure to UVB, 40 % of the K5/ODC mice were found to develop epidermal tumors, whereas SKH-1 hairless mice, the most common and highly sensitive model for photocarcinogenesis (Bickers & Athar 2001), did not develop tumors for up to 50 weeks. The central role of ODC in epidermal carcinogenesis has been even further emphasized by the observation that the targeted expression of ODC inhibitor antizyme in the keratinocytes of transgenic mice decreased sensitivity to chemical carcinogenesis (Feith *et al.* 2001).

Tumor cells may also have a specific ODC form or specific post-translational modification or regulation of ODC may take place, because ODC activity in some tumors is activated by GTP (O'Brien *et al.* 1986, O'Brien *et al.* 1987, Hietala *et al.* 1988), normally a property only of some bacterial ODCs. Tumor specific GTP-activatable ODC is discussed in more detail later in the chapter 2.2.4.

It is not clearly understood how forced overexpression of ODC or AdoMetDC leads to malignant transformation. The involvement of some components of common signal transduction pathways has been shown. Transformation by ODC overexpression resulted in phosphorylation of Ras nucleotide exchange factor Sos-1, Raf-1 kinase and c-Jun that forms a transcription factor AP-1 as a dimer with c-Fos and regulates transcription of target genes (Paasinen-Sohns & Hölttä 1997). Interestingly, Erk1 and Erk2 kinases of the MAP family were not needed to mediate signal. Similarly, c-Jun appeared to be the integral mediator of signaling in transformation induced by AdoMetDC overexpression (Paasinen-Sohns *et al.* 2000). Transformant displayed constitutive activation of the c-Jun $NH_2$-terminal kinase (JNK) pathway. The expression of dominant-negative mutants of JNK1 and SEK1, both kinases upstream from c-Jun, reverted the phenotype of the

AdoMetDC transformants. At the level of cell cycle regulation, the largest common effect in the ODC and AdoMetDC transformants was the constitutive down-regulation of cyclin-dependent kinase inhibitor p27$^{Kip1}$ and its loss from the cyclinE/cyclin-dependent kinase 2 complexes (Ravanko *et al.* 2000). In addition, the level of cyclin D1 and cyclin D1-dependent kinase as well as total cyclin dependent kinase 4 activities were elevated in both transformants suggesting that the expression of ODC or AdoMetDC may effect on cell cycle regulation in many ways.

With high levels of polyamines being so strongly associated with rapid proliferation and growth of tumors, it is understandable that great effort has been placed on designing inhibitors of polyamine synthesis and polyamine analogs and testing their ability to restrict growth of tumor and cancer cells. One of the first inhibitors, and maybe the most studied, is  -difluoromethylornithine, an irreversible inhibitor of ODC (Bey *et al*. 1978, reviewed in Meyskens & Gerner 1999). Treatment of mammalian cell cultures, rodents, or humans with DFMO generally causes a suppression of putrescine and spermidine contents in cells and tissues in which intracellular polyamine pools depend on ODC activity, without affecting spermine levels (Gerner & Mamont 1986, Pegg 1988, Meyskens *et al.* 1998). Inhibition of ODC has been found to suppress tumor formation in experimental models of bladder (Nowels *et al.* 1986), breast (Thompson *et al.* 1986), intestinal (Nigro *et al*. 1986) and skin carcinogenesis (Peralta Soler *et al*. 1998, Arbeit *et al.* 1999).

However, early clinical cancer therapeutic trials with DFMO were disappointing, and at high doses several side effects have occurred including diarrhea, abdominal pain, moderate anemia and temporal loss of hearing (Abeloff *et al*. 1984, Abeloff *et al*. 1986, Talpaz *et al.* 1986, Harari *et al.* 1990). DFMO has been later observed to have a considerable effect on recurrent gliomas (Levin *et al*. 1992). On the other hand, in phase III trial, no benefit of DFMO was seen in the treatment of glioblastoma multiforme when tested together with accelerated hyperfractionation or standard fractionated radiotherapy (Prados *et al.* 2001). Currently DFMO is mainly studied and tested as a chemoprevention agent (see e.g. Love *et al.* 1993, Meyskens *et al.* 1994, Mitchell *et al*. 1998b, Carbone *et al.* 2001, Simoneau *et al*. 2001). At low doses proposed for long-term chemoprevention trials, no systematic side effects have been seen. Trials are at the stage were suitable doses are determined and effect of DFMO on tissue polyamine levels tested.

Other polyamine synthesis inhibitors tested in clinical trials are AdoMetDC inhibitors methylglyoxal bis(guanylhydrazone) (MGBG) (Warrell & Burchenal 1983, Herr *et al.* 1986) and SAM486A (CGP 48664) (Paridaens *et al*. 2000). MGBG is toxic if doses are high, in combination with DFMO it has shown some effect against gliomas (Levin *et al.* 1992). SAM486A has displayed promising antiproliferative activity in cell cultures (Regenass *et al*. 1994, Manni *et al*. 1995, Mi *et al*. 1998) and preclinical animal studies (Paridaens *et al.* 2000).

Various investigators have synthesized structural analogues that can be taken into cells by the polyamine transport system and that can mimic the natural polyamines by down-regulating synthetic or inducing catabolic pathways, but that are unable to substitute for polyamines in terms of supporting cell growth and differentiation (reviewed in Casero & Woster 2001). Most analogues are symmetrically or unsymmetrically alkylated derivatives of polyamines. Alkyl groups are generally added to terminal primary amino groups. Many of analogues have displayed some antitumor activity in cancer cell lines

(Porter *et al.* 1987, Porter *et al*. 1991, Chang *et al*. 1992, Davidson *et al*. 1993) and in xenografts of human tumors (Bernacki *et al*. 1995, Sharma *et al*. 1997). Perhaps the most successful alkylpolyamine to date is $N^1$-$N^{11}$-diethylnorspermine (DENSPM). It has been tested in phase 1 clinical trial for doses (Creaven *et al*. 1997, Streiff & Bender 2001) and is currently undergoing phase II trial (Casero & Woster 2001). It induces highly SSAT activity that leads to decrease in cellular polyamine levels (Gabrielson *et al*. 1999).

## 2.2 Ornithine decarboxylase

### *2.2.1 Enzyme protein and catalytic activity*

Ornithine decarboxylase (ODC, EC 4.1.1.17) catalyzes conversion of L-ornithine to putrescine. The enzyme is specific for the L-isoform of ornithine; $K_m$ value for the substrate is 0.09 mM (Pegg & McGill 1979, Seely *et al*. 1982b, Coleman *et al*. 1993, Osterman *et al*. 1994). Mammalian ODC can act also on lysine and very inefficiently on arginine (Pegg & McGill 1979, Osterman *et al*. 1995a), but these reactions are not of significance for cellular metabolism. All known ODCs require pyridoxal 5´-phosphate (PLP) as a cofactor. The mammalian enzyme binds the cofactor relatively weakly and it can be removed by a rather simple procedure (Poulin *et al*. 1992). $K_m$ value for the PLP is 0.2 - 0.3 µM (Obenrader & Prouty 1977, Seely *et al.* 1982b).

Active mammalian ODC is homodimer with 2-fold symmetry. Monomers are comprised of two domains. Residues 46-283 form an / barrel domain and the remaining carboxy-terminal residues form a -sheet domain including two separate sheets. Two active sites in the dimer are formed at the interface between the / barrel domain of one monomer and the -sheet domain of the other subunit (Tobias & Kahana 1993, Osterman *et al.* 1995a, Osterman *et al*. 1995b, Kern *et al*. 1999, Almrud *et al.* 2000). Subunits have molecular weight of about 51 kDa and the polypeptide chain consists of 461 amino acids, an exception being hamster ODC that has 455 amino acids (Grens *et al*. 1989, Yao *et al*. 1995). Several of those amino acids have been shown to be essential or of importance for catalytic activity. Lysine-69 binds PLP through a Schiff base (Poulin *et al.* 1992) and may play a role in the proper positioning of the substrate for efficient decarboxylation (Osterman *et al*. 1999). The irreversible inhibitor DFMO forms covalent adducts mainly with Cys-360 and to some extent with Lys-69 (Poulin *et al.* 1992) indicating that these amino acid residues are located at or very close to the active site. Cys-360 may function as a proton donor in catalysis (Kern *et al.* 1999, Jackson *et al*. 2000), other potential proton donors are Lys-69 and more unlikely His-197. Cys-360 is needed for the reaction specifity. When it is mutated catalytic rate is diminished and the major reaction is a decarboxylation-dependent transamination resulting in the formation of pyridoxamine 5'-phosphate (PMP) and -aminobutyraldehyde instead of PLP and putrescine (Jackson *et al.* 2000). This may be due to the protonation of C4'-carbon in PLP instead of C -carbon in ornithine. Gly-387 is essential for the formation of dimer (Tobias *et al.* 1993), but the reason for this is not clear (Kern *et al.* 1999). Enzyme

activity is decreased when acidic Asp-88, Glu-94, Asp-233 or most significantly, Glu-274 are mutated (Osterman *et al.* 1995a). Glu-274 interacts with N1-nitrogen of PLP and forms an acidic cluster with Asp-88 and Asp-233 that, with three bound water molecules form a network of hydrogen bonds that probably influences the electron-withdrawing properties of the cofactor (Kern *et al.* 1999). Two other residues shown to have an effect on catalytic activity are Lys-115 and Lys-169 (Lu *et al.* 1991, Tsirka & Coffino 1992). The latter is involved in a salt-bridge stabilizing dimer (Kern *et al.* 1999), but no role for the former is known.

The catalytic mechanism of ODC is typical to all PLP-dependent decarboxylases (Kern *et al.* 1999, Osterman *et al.* 1999, Jackson *et al.* 2000). In the absence of substrate a holoenzyme contains an internal aldimine where the PLP is bound to the active-site lysine-69 by a Schiff-base linkage. The ornithine substrate reacts at first with the cofactor via transaldimination reaction to form an external aldimine. This is followed by the release of $CO_2$ and the formation of a quinonoid intermediate that is protonated to form again external aldimine, now consisting of putrescine and PLP bound to each others via a Schiff-base linkage. Putrescine is then dissolved from the active site and PLP forms a Schiff base with Lys-69. PLP-dependent enzymes achieve reaction specifity by positioning specific residues or molecules (like Cys-360 in ODC) that interact with each groups surrounding the C carbon of the substrate in a geometry that favours a particular bond cleavage (Jansonius 1998). Otherwise, any of three bonds (the fourth one is in a Schiff base) around the C carbon might be cleaved, enabling a broad range of reactions including transamination, racemization, retro-aldo cleavage and deamination in addition to decarboxylation. In decarboxylases transaldimination leads to the orientation of the carboxylate perpendicular to the pyridine ring of PLP leading to cleavage of the bond between the C carbon and carboxylate.

### *2.2.2 Gene and mRNA*

Complete structure and nucleotide sequence of ornithine decarboxylase gene from mammalians is known for mouse (Coffino & Chen 1988, Katz & Kahana 1988), rat (van Steeg *et al*. 1988, Wen *et al*. 1989, van Steeg *et al*. 1990), human (Fitzgerald & Flanagan 1989, van Steeg *et al*. 1989, Hickok *et al*. 1990, Moshier *et al*. 1990), and bovine (Yao *et al*. 1998). In addition, the complete amino acid sequence (Srinivasan *et al*. 1987, Grens *et al.* 1989) and nucleotide sequence of the promotor region (GenBank accession number X53906) is known for hamster ODC. All mammalian ODC genes have 12 exons and 11 introns. Location of exon-intron boundaries is identical in all ODC genes. The transcription unit is relatively short, 6 – 8 kb depending on species. The first intron is considerably longer than others, 2.0 – 3.0 kb, and contains potential or demonstrated regulatory elements. These elements as well as those upstream in the promoter region are discussed in detail in the next chapter. Exons range from 85 bp to about 900 bp in length.

Mammals have several ODC gene-like sequences in their genomes, but apparently only one functional gene, others being pseudogenes. Active mouse ODC gene has been localized to chromosome 12 (Cox *et al.* 1988), functional hamster ODC gene is in the

chromome 7 (Tonin *et al*. 1987), human gene in the chromosome 2 (Winqvist *et al*. 1986, Hsieh *et al*. 1990, Radford *et al*. 1990) and rat gene in the chromosome 6 (Deng *et al.* 1994).

Northern analysis has revealed that rodents express two species of ODC mRNA (Berger *et al*. 1984, Kontula *et al*. 1984, Gilmour *et al*. 1985, van Kranen *et al*. 1987) whereas in human (Hickok *et al*. 1987, Radford *et al.* 1990) and bovine (Yao *et al.* 1995) only single ODC mRNA has been detected. The longer rodent mRNA is 2.6 – 2.7 kb in length, ant the shorter 2.1 – 2.2 kb. They are the result of the use of two separate polyadenylation signals present at the 3' untranslated region. Human ODC mRNA has two polyadenylation signals as well, but apparently only that giving the shorter transcript is used.

The open reading frame encoding mammalian ODC is 1383 nucleotides long the only exception being hamster ODC mRNA with the coding region of 1365 nucleotides. The coding region is highly conserved. Mouse and rat amino acid sequences differ only at 14 sites from each others. Hamster and bovine ODCs are the most divergent mammalian ODCs. They have 56 differences in amino acid sequences. The 5'-untranslated region of ODC mRNA is exceptionally long. Hamster has the ODC 5' leader sequence slightly shorter than 300 nucleotides, those of other mammals exceed 300 nucleotides in length. The leader sequence is GC-rich and has been suggested to form secondary structures with a high free energy and stability (Brabant *et al*. 1988, Katz & Kahana 1988, Wen *et al.* 1989). The region also contains a small open reading frame as does AdoMetDC mRNA. This ORF may have a role in the regulation of translation, although the nucleotides flanking the translational start site do not conform perfectly well to the consensus sequence for the translation initiation (Kozak 1989).

### *2.2.3 Regulation*

#### *2.2.3.1 Regulation of transcription*

ODC is one of the most highly regulated enzymes in eukaryotic organisms. The enzyme activity is induced rapidly up to several hundred fold by a great variety of factors stimulating cell growth and proliferation. These factors can be such as hormones, tumor promoters and growth factors. In almost all cases the increase in activity is accompanied by roughly equivalent changes in the amount of enzyme protein, and thus ODC appears not to be generally regulated by post-translational modifications or by allosteric effectors. The accumulation of ODC protein is controlled in gene transcription, mRNA translation and enzyme degradation. In addition, ODC activity is specifically inhibited by antizyme protein before degradation and negatively feed-back regulated by polyamines. (reviewed in (Davis *et al.* 1992, Shantz & Pegg 1999)

Some increase in the amount of ODC mRNA is detected in virtually all cases when ODC activity is stimulated. This may mostly be due to increased transcription, but in several cases stabilization of ODC mRNA has also been detected (Laitinen *et al*. 1984,

Berger & Porter 1986, Hölttä *et al.* 1988, Chen & Chen 1992). Induction of ODC activity by growth factors is maybe the most apparent case where induction is mainly a result of elevated ODC mRNA levels (Feinstein *et al.* 1985, Greenberg *et al.* 1985). Also application of phorbol ester and tumor promotor 12-O-tetradecanoylphorbol 13-acetate (TPA) leads to rapid increase in ODC mRNA followed slightly later (Gilmour *et al.* 1985, Verma *et al*. 1986a, Verma 1988) or even simultaneously (Verma *et al*. 1986b, Hsieh & Verma 1989) by increase in ODC activity.

Signaling pathways leading to ODC induction are only partly known. Protein kinase C and phospholipase C have been suggested to be involved in mediating TPA stimulated induction of ODC (Verma *et al.* 1986b). Protein kinase C activity is required also for ODC induction by oxidative stress (Otieno & Kensler 2000). Activation of protein kinase A has been shown to lead to a rapid elevation of ODC gene transcription (Abrahamsen *et al*. 1992). In human endothelial cell line ECV304, p44/42 MAPK pathway was required for induction of ODC by any stimulus employed, i.e. serum, histamine and ATP (Flamigni *et al*. 2001).

Whatever the nature of transcription inducing stimulus, its effect has to be mediated to ODC promotor and responsive elements. The ODC promotor region is typical to ubiquitously expressed genes. It contains TATA and CAAT boxes, although the latter is relatively poorly conserved and several GC-rich areas that are potential binding sites for the members of Sp/Krüppel-like transcription factor family regulating expression of various house-keeping genes, but also tissue-specific and growth- or differentiation related expression of target genes (Black *et al.* 2001). From this family, Sp1 (Li *et al.* 1994, al-Asadi *et al*. 1995, Kumar *et al*. 1995) and inhibitory Sp3 (Kumar & Butler 1997) as well as two Krüppel-like factors ZBP-89 (Law *et al.* 1998) and ZBP-99 (Law *et al.* 1999) have been shown to bind to the ODC promotor and to regulate expression of reporter or endogenous ODC gene. Other transcription factors demonstrated to bind and regulate ODC promotor are c-Myc/Max dimers (Bello-Fernandez *et al.* 1993, Pena *et al*. 1993, Tobias *et al*. 1995, Walhout *et al*. 1997), and WT1 tumor suppressor (Moshier *et al*. 1996, Li *et al.* 1999a). The ODC promotor has even been used in viral gene transfer vectors to achieve c-Myc- and N-Myc-regulated protein expression in tumor cells (Pawlik *et al*. 2000, Iyengar *et al*. 2001). Also cAMP-responsive element mediates regulation of the ODC promotor and binds proteins from nuclear extracts, but the identity of these proteins is not clear and there are contradictory reports on involvement of CREB-binding protein family (Palvimo *et al*. 1991, Abrahamsen *et al.* 1992, Palvimo *et al*. 1996). In addition, a negative regulative element has been localized to the ODC promotor (Zhao *et al*. 2000). This element is a putative Ets-binding site, but a protein of 55-60 kDa specifically bound to it was not antigenically related to c-Ets-1. Members of Ets family mediate responsiveness to extracellular signals, including mitogens and phorbol esters (Wasylyk *et al*. 1998). It is known that phorbol esters do not regulate the ODC promoter via AP-1 transcription factor as they do to many promotors (Kim *et al*. 1994, Mar *et al*. 1995), but regulation may be mediated at least partially by the general transcription initiation complex organising at the TATA box (Reddig *et al*. 1996). And finally, although the ODC promotor does not contain a consensus androgen response element, androgen receptor has been suggested in one study to bind on the promotor region (Bai *et al*. 1998).

### *2.2.3.2 Regulation of translation*

The many cases cellular increase in ODC activity and protein content can not be explained merely by the increased transcription or stabilization of ODC mRNA, and not by stabilization of ODC protein, indicating that regulation may take place also at translational level. Translational regulation appears to have relatively the most significant role when ODC activity is induced by amino acids (Kanamoto *et al*. 1987, Chen & Chen 1991, Chabanon *et al*. 2000), insulin (Blackshear *et al*. 1987, Manzella *et al*. 1991) or, at least in some experimental systems, by hypotonic shock (Poulin & Pegg 1990, Lövkvist-Wallström *et al.* 1995, Lövkvist-Wallström *et al.* 2001). When it comes to negative regulation, inhibition of ODC mRNA translation by polyamines has been observed in several studies (e.g. Kameji & Pegg 1987b, Persson *et al*. 1988, Kanamoto *et al*. 1991, Lövkvist *et al.* 1993).

In the study of translational regulation of ODC, much of the work has focused on the long 5'-untranslated region (5'UTR) of its mRNA. The 5'UTR is composed of two distinct segments (Brabant *et al.* 1988, Katz & Kahana 1988, Wen *et al.* 1989). There is a 5' proximal GC-rich segment, spanning the first 180 nucleotides, which is predicted to form a very stable hairpin structure and which contains a small upstream open reading frame close to it 3' end. The second segment covers the rest of 5'UTR and is relatively unstructured. Although most genes encode mRNAs with short unstructured 5'UTRs, oncogenes and genes involved in cellular proliferation often encode mRNAs with longer 5'UTRs that may form secondary structures (Kozak 1989, Gray & Henze 1994).

In rabbit reticulocyte lysates, translation of both mouse and rat ODC containing the full length 5'UTR was reduced by 95% compared to mRNA containing very short 5' leader sequence (Ito *et al.* 1990, Manzella & Blackshear 1990, Kashiwagi *et al.* 1991, Van Steeg *et al.* 1991). The conserved GC-rich region in the 5' end of 5'UTR repressed translation to the same extent as entire 5'UTR (Manzella & Blackshear 1990, Van Steeg *et al.* 1991). Similarly, in cultured cells, the expression of reporter genes was inhibited by up to 99% when the full length ODC 5'UTR was inserted immediately before the initiation codon (Grens & Scheffler 1990, Manzella & Blackshear 1990, Shantz *et al.* 1994). The GC-rich 5' end of the leader sequence was again almost as effective as the full length 5'UTR in suppressing protein synthesis (Grens & Scheffler 1990, Manzella & Blackshear 1990, Shantz *et al.* 1996b).

The 3'UTR of ODC mRNA is also relatively long (300 nucleotides), but has less potential to form stable secondary structures than 5'UTR. However, it has been demonstrated in various expression systems that the 3'UTR may interact with the 5'UTR of ODC mRNA in such a way that the repressive effect of the 5'UTR on translation is relieved (Grens & Scheffler 1990, Lorenzini & Scheffler 1997). Interestingly, according to a recent study, the hypotonic induction of ODC is highly dependent on the presence of 3'UTR, but not on the presence of 5'UTR in ODC mRNA (Lövkvist-Wallström *et al.* 2001). In the same study it was pointed out that ODC 3'UTR contains region corresponding to AU-rich elements (AREs). These elements are located in the 3'UTR of a number of growth-related mRNAs, including mRNAs coding for a number of proto-oncogenes and cytokines (Chen & Shyu 1995).

All mammalian ODCs have a small internal open reading frame in their 5'UTR. The sequence around the AUG codon lacks the –3 purine but does contain a G residue in position +4 thought to be necessary for efficient translation (Kozak 1991, Kozak 1992). The predicted peptide sequence is not as conserved as that of the AdoMetDC internal ORF, but contains 10 amino acids in the most mammalian ODC mRNAs. The internal ORF is located approximately 150 nucleotides from the 5' end of mRNA. Its translation has not been demonstrated, but the mutation of its initiation codon appears to increase the translation of major reading frame of reporter construct (Grens & Scheffler 1990, Manzella & Blackshear 1990, Shantz *et al.* 1996b). In contrast, no difference in translational efficiency between mRNAs containing wild-type or mutated AUG in 5'UTR was detected in *in vitro* translation reactions (Van Steeg *et al.* 1991). This may just indicate that some cellular factor responsible for the release of translation inhibition is limiting in reticulocyte lysates.

Eukaryotic initiation factor 4F (eIF4F) mediates cap-dependent translation. It is thought to be able to unwind secondary structures in the 5'UTRs, thereby facilitating ribosome binding to the 5' end of mRNA (Gingras *et al*. 1999, Pestova *et al*. 2001). The recruitment of ribosome to mRNA is the rate-limiting step of translation under most circumstances and a primary target for translational control. One subunit of eIF4F is eIF4E. It functions directly in the recognition of the mRNA 5' cap structure. A cell line overexpressing eIF4E is transformed (Lazaris-Karatzas *et al*. 1990) and ODC activity is increased by over 30-fold compared to wild-type cell line (Shantz & Pegg 1994, Shantz *et al.* 1996b). DFMO (Shantz & Pegg 1994) and expression of dominant negative ODC mutant (Shantz *et al*. 1996a) are able to partly revert the transformed phenotype, but the formation of phenotype is likely contributed also by the enchanced expression of various oncogenes. Overexpression of eIF4E has been suggested to promote translation of all mRNAs that contain a long and structured 5'UTR (Lazaris-Karatzas *et al.* 1990). For example c-*sis*, c-*lck* and c-*myc* proto-oncogenes have this kind of mRNAs. Very interestingly, it has been reported that ODC mRNA is translated also using cap-independent mechanism (Pyronnet *et al.* 2000). In the a cap-independent translation ribosome binds to an internal ribosome entry sire (IRES) in 5'UTR. This mechanism appears to be functioning in ODC mRNA translation during the G2/M transition of cell cycle maybe providing sufficient levels of polyamines before mitosis.

Low levels of polyamines are necessary for general protein synthesis but excessive polyamine levels inhibit the translation of most mRNAs. Translation of ODC mRNA is both stimulated and inhibited at lower concentrations of polyamines than translation generally (Kameji & Pegg 1987b, Persson *et al.* 1988, Ito *et al.* 1990). The reducing effect of increased polyamine content on ODC mRNA translation has been observed both in reticulocyte lysates (Kameji & Pegg 1987b, Persson *et al.* 1988, Ito *et al.* 1990) and in cells in culture (Kanamoto *et al.* 1991, Kameji *et al.* 1993, Lövkvist *et al.* 1993). Removing or truncating the 5'UTR from ODC mRNA abolishes the polyamine effect according to several studies (Ito *et al.* 1990, Kashiwagi *et al.* 1991, Lövkvist *et al.* 1993). However, the exact location of any polyamine responsive element in the 5'UTR has yet to be defined. Furthermore, two studies have failed to detect any effects of polyamines on translation of ODC mRNA *in vitro* (Van Steeg *et al.* 1991) or reporter construct containing the ODC 5'UTR *in vivo* (Grens & Scheffler 1990). In addition, it has been reported that the polyamine mediated regulation of ODC expression is independent on

5'UTR (van Daalen Wetters *et al*. 1989b) or both 5' and 3'UTR (Lövkvist-Wallström *et al.* 2001). It has been speculated that in these cases polyamines may induce rapid cotranslational degradation of ODC. Taken together, it is likely that the regulation of ODC has cell specific characteristics, and that intracellular levels of other factors combine with changes in polyamines to influence translation.

### *2.2.3.3 Post-translational modifications*

ODC induction is nearly always accompanied by a similar increase in the amount of ODC protein. According to the general concept, post-translational modification and allosteric effectors do not have a significant role in ODC regulation. However, these might be of importance in specific tissues or physiological conditions. For example, in several tumor lysates ODC activity can be stimulated by GTP suggesting either allosteric regulation or GTP-dependent post-translational modification or involvement of GTP-binding regulatory protein (O'Brien *et al.* 1986, O'Brien *et al.* 1987, Hietala *et al.* 1988, Hietala *et al*. 1990). So far the only demonstrated post-translational modification of mammalian ODC is phosphorylation (Rosenberg-Hasson *et al*. 1991, Worth *et al*. 1994).

The first step in recognition of ODC as a phosphoprotein was successful *in vitro* phosphorylation by casein kinase II (Meggio *et al*. 1984). The phosphorylation site was later identified to be Ser-303 (Rosenberg-Hasson *et al.* 1991). Phosphorylation of Ser-303 in COS-cells or *in vitro* did not have an effect on enzyme activity or stability (Meggio *et al.* 1984, Rosenberg-Hasson *et al.* 1991, Kanamoto *et al.* 1993). Ser-303 was the only phoshorylation site detected in COS cells, but in a transformed macrophage cell line RAW264 ODC was phosphorylated at, at least two serines and two threonines (Worth *et al.* 1994, Reddy *et al.* 1996). Another of serines was at the position 303. Purified phosphorylated ODC was more stable than unphosphorylated and its $V_{max}$ was 1.5-fold higher (Reddy *et al.* 1996).

In human keratinocytes phosphorylated ODC has been observed to be preferentially associated with insoluble cellular proteins (Pomidor *et al*. 1999). The reason for this localization is not known. A fraction of ODC protein has been reported to translocate to the cell surface membrane during cell activation and tranformation (Heiskala *et al*. 1999). This translocation was dependent on $p47^{phox}$-related membrane targeting sequence (Nauseef *et al*. 1993) comprising amino acids 165-172 in ODC. When Ser-167 of ODC was mutated to alanine, the mutant ODC was unable to move to the cell surface. It is not clear why ODC translocates to the membrane and it is unclear whether the Ser-167 is actually phosphorylated. In the $p47^{pbox}$ protein phosphorylation of the corresponding serine supports translocation (Huang & Kleinberg 1999), but is not strictly essential (DeLeo *et al*. 1995). In any case, the function of postranslational modifications in ODC are likely to be like this, to be needed for some specific property in some specific situation.

### *2.2.3.4 Degradation and its regulation*

ODC is a very labile protein. The half-life of enzyme activity is one of the shortest known for mammalian enzymes, it can be as short as 10 – 20 minutes (Seely *et al.* 1982a, Isomaa *et al.* 1983) and at its longest only from one hour to two hours (Hayashi *et al*. 1996). The short half-life of activity is due to rapid degradation of enzyme protein, because the half-life of immunoreactive ODC is only slightly longer than that of enzyme activity (Isomaa *et al.* 1983, Laitinen *et al.* 1984). Rapid turnover is essential for sensitive regulation and rapid induction of enzyme activity. When general protein synthesis in cells is increased, the amount of labile proteins increases much more rapidly than that of stable proteins (Tabor & Tabor 1984).

ODC is degraded via an exceptional pathway (reviewed in Hayashi 1995, Murakami *et al*. 2000, Coffino 2001b). Inhibitor protein antizyme, synthesis of which is greatly induced by polyamines (Fong *et al*. 1976, Heller *et al*. 1976), binds to the ODC monomer, inhibits its activity and targets the protein for rapid degradation by the 26S proteasome complex (Murakami *et al*. 1992a). Proteasome 26S is the main neutral protease of the cell and it was for a long time thought to degrade only proteins tagged with the poly-ubiquitin chain (Verma & Deshaies 2000). ODC was the first exception to this rule and to date only the cyclin-dependent kinase inhibitor $p21^{cip1}$ (Sheaff *et al*. 2000) and the NS2 protein of parvovirus minute virus (Miller & Pintel 2001) have been found to be degraded by the 26S proteasome in ubiquitin-independent manner. Immunoremoval of the proteasome from cell extracts or use of a proteasome inhibitor *clasto*-lactacystin  -lactone in cell cultures almost completely inhibited the degradation of ODC showing that the proteasome 26S pathway is in deed the very major pathway for ODC degradation (Murakami *et al*. 1999).

The 26S proteasome is a multisubunit complex, consisting of a central proteinase called the 20S proteasome, and two terminal regulatory subcomplexes, termed PA700 (also called 19S complex) and PA28 (11S regulator) (Murakami *et al.* 2000). The 20S unit is a 700 kDa cylinder-shaped particle having multiple catalytic centers located within a hollow cavity of the cylinder. The 26S complex contains one regulatory subunit at its ends, these can both be PA700s or PA28s or also heterocomplexes exist containing both PA700 and PA28. However, homo-PA28 proteasome is incapable of degrading ODC (Tanahashi *et al*. 2000). Other variants of the 26S, but not 20S, proteasome degrade ODC *in vitro* in the presence of ATP and antizyme. Most likely, degradable proteins are at first recognised by the regulatory subunits of the proteasome complex and then ulfolded and fed to inner cavities of the 20S core particle (Hershko & Ciechanover 1998). The 26S proteasome irreversibly inactivates ODC prior to its degradation (Murakami *et al.* 1999). The inactivation, possibly due to unfolding, is coupled to sequestration of ODC within the 26S proteasome. This process requires antizyme and ATP, but not proteolytic activity of the proteasome. Antizyme is generally recycled and is only seldomly degraded (Tokunaga *et al*. 1994). The purified 26S proteasome can slowly degrade ODC even in the absence of antizyme, but the physiological significance of this degradation is unclear (Murakami *et al.* 2000).

Mutagenesis and structural studies have revealed the elements of ODC that are responsible for the interaction with antizyme and for ODC degradation. The carboxy-

terminal region encompassing amino acids 423 – 462 has been shown to be important for both unstimulated and polyamine-stimulated degradation of ODC, although other areas also may be involved (Li & Coffino 1992, Li & Coffino 1993, Mamroud-Kidron *et al.* 1994). PEST regions, i.e. areas rich in proline, glutamate, serine and threonine, are typical to many rapidly degraded proteins (Rogers *et al*. 1986). The carboxy-terminal region of ODC contains one of the two PEST regions, but removal of the last five amino acids outside of the PEST region (Ghoda *et al.* 1992) or a single amino acid exchange in the PEST region (Cys441 to TRp) (Miyazaki *et al.* 1993) also stabilizes ODC, suggesting that this PEST sequence is not the only determinant of ODC instability. Another internal PEST region (amino acids 298 – 333) does not appear to be associated with ODC instability (Ghoda *et al.* 1992). In addition, an internal region of ODC (amino acids 117 – 140) is required for antizyme-binding and thus for antizyme-dependent degradation (Li & Coffino 1992). This region is highly conserved in mammals, avians, and amphibians whose ODC are rapidly degraded by an antizyme-dependent process (Hayashi *et al.* 1996).

The most likely interpretation of the observations presented above is that the putative "degradation signal" of ODC is located at the carboxy-terminal region which is normally masked, but exposed by attachment of antizyme and finally recognised in some way by the 26S proteasome (Li & Coffino 1993). On the other hand, according to the crystal structure of the human full-length ODC this model may be too simplified (Almrud *et al.* 2000). It appears that the carboxy-terminus from residue 401 is not buried by the structural core of homodimeric ODC. However, the binding of antizyme to the ODC monomer may yet induce other conformational changes to make ODC more susceptible to proteolysis or make the carboxy-terminus accessible still further to aminoterminus.

ODC degradation is regulated equally strictly as synthesis and provides another means to control polyamine levels in cells. There are several observations about increased stability of ODC during induction of enzyme activity. Androgen elevates ODC activity in kidneys dramatically and this is partly explained by the 4 – 10 –fold increase in half-life of ODC (Seely *et al.* 1982a, Isomaa *et al.* 1983) whereas hypotonic shock results in a 3 – 6 fold increase in half-life (Poulin & Pegg 1990, Tohyama *et al.* 1991). The most important factors enhancing ODC degradation are polyamines (Glass & Gerner 1986, Hölttä & Pohjanpelto 1986, Kanamoto *et al.* 1986) that, as mentioned earlier, induce synthesis of antizyme (Fong *et al.* 1976, Heller *et al.* 1976).

#### *2.2.3.5 Guanosine 5'-triphoshate activation*

Mammalian ODC was thought to be lacking any allosteric effectors untill it was obseved that the enzyme in mammalian epidermal papillomas can be activated by GTP (O'Brien *et al.* 1986). Activation was detected in raw cell lysates and could be a result of a real allosteric interaction or post-translational modification requiring GTP, or involvement of GTP-binding regulatory protein. Later GTP activation has been detected in human squamous cell carcinoma (Hietala *et al.* 1988), colorectal adenocarcinoma (Hietala *et al.* 1990), gastric cancer (Okuzumi *et al.* 1991) and colorectal carcinoma (Matsubara *et al.*

1995). It was possible to separate GTP-activatable ODC from non-activatable enzyme by gel-filtration chromatography (O'Brien *et al.* 1987) suggesting co-purifying regulatory or modifying protein(s) or allosteric interaction that has considerable effect on the conformation of enzyme. GTP-activatable ODC displays a higher $K_m$ value than “normal” ODC and GTP activates the enzyme by decreasing $K_m$ close to or below normal level. In some cases GTP effects also $V_{max}$ or, in some rare cases, GTP activates ODC effecting only $V_{max}$. GTP-activatable enzyme was more heat-stabile and more resistant to DFMO inhibition. It has been suggested that GTP-activatable ODC could be advantageous to tumor cell growth (Hietala *et al.* 1988), because it appears to be more stabile and its activity may be more readily deregulated. However, according to experimental data, it seems that the detection of GTP-activatable ODC in tumors may indicate more favourable patient prognosis (Matsubara *et al.* 1995).

## 2.3 Antizyme

### *2.3.1 Protein and functions*

Antizyme was originally identified from rat hepatoma cells cultures as a protein that inhibits ODC activity and is induced after addition of putrescine to cells (Fong *et al.* 1976). Soon it was demonstrated to be present in several other cell lines and to be induced also by spermidine and spermine (Heller *et al.* 1976) – which are actually more efficient inducers (Matsufuji *et al.* 1995). Since that antizyme has been observed to exists in various organisms from yeast and fungi to nematodes, insects and vertebrates. Its presence in bacteria has been suggested (Canellakis *et al.* 1993, Pantazaki *et al.* 1999), but also called into question (Ivanov *et al*. 1998a). In addition to inhibiting ODC activity, antizyme was demostrated to target ODC for degradation (Murakami *et al.* 1992a), to regulate polyamine uptake to cells (He *et al.* 1994, Mitchell *et al.* 1994) and potentially excretion out from the cells (Sakata *et al.* 2000). Within vertebrate species antizyme has multiple isoforms which are discussed in the next chapter. This chapter focuses on antizyme 1 that was the first observed and is the most studied and most ubiquitous. (For reviews, see Ivanov *et al*. 2000a, Coffino 2001a, Coffino 2001b).

Antizyme 1 has a molecular weight of 26 kDa. Its polypeptide chain consists of 227 amino acids in mouse (Kankare *et al.* 1997) and rat (Miyazaki *et al.* 1992), and 228 amino acids in human (Tewari *et al*. 1994, Hayashi *et al*. 1997). It has very high affinity to ODC monomer, dissociation constant $K_d$ being $10^{-11}$ $M^{-1}$ (Kitani & Fujisawa 1984). The functional domains of antizyme 1 responsible for binding to ODC, tagging ODC for degradation and inhibition of polyamine transport have been defined. The carboxyterminal half (amino acids 121 - 227) is sufficient for binding to ODC (Ichiba *et al*. 1994, Li & Coffino 1994) and is essential for inhibition of the polyamine transport function (Sakata *et al*. 1997). Amino acids 69 – 112 are necessary for antizyme-mediated destabilization of ODC and are sufficient to confer accelerated degradation to unstable heterologous proteins when linked to them covalently (Li *et al*. 1996).

Antizyme 1 plays an important role in the regulation of cellular polyamine levels. Alterations in antizyme expression can have an effect on cellular functions and be of physiological significance. In addition to ODC and AdoMetDC, antizyme expression can be altered in cancer, either decreased (Tsuji *et al*. 1998) or increased (Saverio *et al*. 2000). The latter is likely to be the normal response to elevated polyamine concentrations and may, together with increased SSAT activity, prevent accumulation of polyamines to toxic levels. On the other hand, high concentrations of polyamines in primary cultures of prostate carcinoma cells suppressed cell growth of poorly metastatic cells that were able to induce antizyme expression, but not growth of highly metastatic cells unable to induce antizyme (Koike *et al.* 1999). Furthermore, forced antizyme overexpression in hamster malignant oral keratinocyte cell line resulted in the reversion of malignat phenotype and induction of epithelial differentiation and DNA demethylation (Tsuji *et al*. 2001) whereas targeted antizyme expression in the skin of transgenic mice reduced tumor promotor induction of ODC and decreased sensitivity to chemical carcinogenesis (Feith *et al.* 2001). When antizyme was overexpressed in immortalized human prostatic epithelial cells spermidine and spermine levels decreased only slightly, but putrescine levels decreased by 3-fold (Scorcioni *et al*. 2001). This led to basicly similar accumulation of cells in the S phase of the cell cycle as detected when cells suffer from polyamine deficiency (Fredlund & Oredsson 1996a, Fredlund & Oredsson 1996b). However, expression of antizyme 1 is not required for cell viability. According to unpublished observations (Matsufuji *et al.* unpublished data), antizyme knock-out mice are viable, morphologically normal and fertile, but they have high perinatal mortality with about one-third dying in the days before and after full term (Coffino 2001a, Coffino 2001b).

There are some very interesting unanswered questions about antizymes and their functions. Unpublished results suggesting that antizyme 1 targets cyclins and cyclin-dependent kinases for degradation have already been reviewed (Coffino 2001b) and it is possible that antizymes are not involved only in the regulation of polyamine metabolism, but more largely regulating various growth or cell cycle-related processes. This is further supported by the observation that Smad1 protein is bound and targeted for degradation by antizyme 1 (Gruendler *et al.* 2001). Smad proteins mediate signaling induced by the members of transforming growth factor superfamily and related to cell proliferation, differentiation and apoptosis (Itoh *et al*. 2000). The more exact picture about the roles of antizymes will certainly emerge in the near future. It would also be interesting to find out: how antizyme controls polyamine transport? Is it the degradation of some transport system component that antizyme is initiating? Could it be possible to find still elusive mammalian polyamine transport proteins by searching proteins interacting with antizyme?

### *2.3.2 Gene family*

The antizymes form an ancient gene family. Within vertebrate species, multiple isoforms are found; humans have at least four antizymes encoded by different genes (Coffino 2001a). These antizymes share three common features. They display structural homology,

that is strongest in the carboxy-terminal half required for the binding of ODC. They appear to be able to associate with ODC, reducing its activity and, depending on isoform, potentially enhancing its degradation. Finally, their synthesis is stimulated by polyamines via ribosomal frameshifting that requires conserved motifs in the mRNA near the site of frameshifting.

Antizyme 1 was the first described and it has wide tissue distribution (Matsufuji *et al*. 1990). Antizyme 2 has a similar wide tissue distribution, but it is expressed less abundantly (Ivanov *et al*. 1998b). Antizyme 3 is expressed only in male germ cells in a post-meiotic stage of their differentiation to mature sperm (Tosaka *et al*. 2000, Ivanov *et al*. 2000c). Antizyme 4 is presently known only as an EST (Coffino 2001a). Human antizyme 2 is 54 % identical to human antizyme 1, but 99.5 % identical to mouse antizyme 2 indicating unexpectedly high selection pressure against changes in antizyme 2 proteins (Ivanov *et al.* 1998b). Antizyme 3 is more divergent. Human and mouse antizyme 3s show 29 – 38 % identity with antizymes 1 and 2 (Tosaka *et al.* 2000, Ivanov *et al.* 2000c).

Antizymes 1 and 2 both bind and inhibit ODC, and they are approximately equipotent as inhibitors of polyamine uptake (Zhu *et al.* 1999). Antizyme 2 enhanced ODC degradation in insect cells strongly overexpressing antizyme, but it had little or no ability to drive proteosomal degradation of ODC *in vitro*. Antizyme 2 could be suitable to act as a reversible inhibitor of ODC activity (Zhu *et al.* 1999). In this way, it might store the enzyme for future use. Antizyme 3 binds and inactivates ODC (Ivanov *et al.* 2000c), but no further information about its biochemical properties has yet emerged. It is apparently needed in late spermatogenesis for temporarily restricted control of polyamine production.

### *2.3.3 Regulation*

The most important factor regulating antizyme synthesis is cellular polyamine concentration. Polyamines induce antizyme by a rare mechanism; by programmed ribosomal frameshifting (Matsufuji *et al.* 1995). The antizyme mRNA contains two overlapping open reading frames. The second of these encodes most of the protein, but lacks an initiation codon. Translation initiates in reading frame 1, must shift to reading frame 2 for production of functionally active antizyme. Polyamines increase the efficiency of antizyme mRNA frameshifting just before translation would otherwise terminate in reading frame 1. The site of frameshifting in mammalian antizyme 1, as well as antizyme 2, is UCCUGA, where quadruplet translocation occurs at UCCU to shift reading to +1 frame before the UGA stop codon (Matsufuji *et al.* 1995, Ivanov *et al.* 1998b). For the frameshifting to occur efficiently, it is important that the 3'base of the quadruplet is the first base of the stop codon. Other important features are a pseudoknot just 3' of the shift site and a specific sequence 5' of the shift site (Matsufuji *et al.* 1995, Ivanov *et al.* 1998b, Ivanov *et al.* 2000a). A pseudoknot 3' of shift site is a common stimulator for eukaryotic –1 frameshifting, but the synthesis of antizyme is the only known case utilizing it in +1 frameshifting (Ivanov *et al*. 2000b). None of these *cis*-acting

sequences appears to mediate polyamine-specific induction. Instead, the polyamine induction of frameshifting seems to be mediated directly by the translational machinery itself, i.e. the ribosome or some component of it (Matsufuji *et al.* 1995, Ivanov *et al.* 2000a).

Frameshifting in antizyme mRNA is remarkably conserved during evolution in yeasts, molds, fungi, nematodes, insects and vertebrates (Ivanov *et al.* 2000a). The frameshifting sequence is also relatively well conserved. When putative antizyme sequence was analysed from 26 organism, in three somewhat distantly related nematodes the frameshifting sequence was UUU UGA instead of the usual UCC UGA. In the two molds and one fungus the shift site was CCC UGA. Putrescine, spermidine and spermine are all able to induce frameshift when rat antizyme mRNA was translated *in vitro* in rabbit reticulocyte lysate (Matsufuji *et al.* 1995). The optimal concentration was lowest for spermine, 0.12 mM, spermidine induced frameshifting optimally at concetration 0.8 mM and putrescine at 4 mM. The optimal concentrations of spermidine and spermine are in physiological ranges. Polyamines can bring the efficiency of frameshifting up to about 30 % and stimulate it over 10-fold compared to the level with no added exogenous polyamines in the reticulocyte lysate.

Otherwise not so much is known about the regulation of antizyme. Interleukin-1 induced up-regulation of antizyme mRNA has been detected in a human melanoma cell line and was shown to be due to elevated antizyme gene transcription (Yang *et al*. 1997). Polyamine depletion has been observed to markedly decrease transcription of antizyme 1 gene, but high polyamine concentrations did not have an effect on the steady-state levels of antizyme mRNA (Nilsson *et al.* 1997). Osmotic stress has been reported to induce changes in cellular levels of antizyme, hypotonic growth medium decreased and hypertonic increased the amount of antizyme (Mitchell *et al.* 1998a). This regulation appears to be mediated via alterations in antizyme stability. In one study, antizyme expression was reported to be controlled according to cell cycle (Bettuzzi *et al*. 1999).

A very interesting, but still poorly known, phenomenon in the regulation of antizyme is the antizyme inhibitor protein. It was detected at first from rat liver extracts as a protein that re-activated antizyme-inactivated ODC by replacing ODC in an ODC-antizyme complex (Fujita *et al*. 1982). It has higher affinity for ODC than antizyme (Kitani & Fujisawa 1989, Murakami *et al*. 1989). Its molecular weight is similar to ODC and both rat (Murakami *et al.* 1996) and human (Nilsson *et al*. 2000a) antizyme inhibitors are nearly 50 % identical to corresponding ODC, but display no enzyme activity. Expression of antizyme inhibitor seems to be growth-related, since inhibitor was rapidly induced in growth-stimulated mouse fibroblasts (Nilsson *et al.* 2000a) and its gene was identified by differential display as one of those genes showing enhanced expression in gastric tumors compared to normal gastric tissue (Jung *et al*. 2000).

Very recently a cloning of a novel ODC-like protein expressed in brain and testis was reported (Pitkänen *et al*. 2001). This human protein is 54 % identical to ODC and 45 % identical to antizyme inhibitor. Its putative antizyme-binding region is more similar to the antizyme-binding region of antizyme inhibitor than to that of ODC. This together with the facts that its expression in CHO moderately but clearly increases ODC activity in cell lysates, but not in reticulocyte lysates when expressed *in vitro*, suggests that the novel protein either is able to release ODC from the ODC-antizyme complex or possess weak

ODC activity itself. It might be another antizyme inhibitor or possess some other still unknown activities or functions, which are regulated by antizyme.

# 3 Aims of the present study

The functions that have been attributed to polyamines are many, varied and, in some cases, controversial but it is indisputable that polyamines are a requirement for cells to either grow or function in an optimal manner. Since the polyamine levels are increased in cancer cells and tissues as well as in all rapidly proliferating cells, polyamine research has traditionally placed a lot of emphasis on a role of polyamines in normal and malignant cell growth. However, attempts to use inhibitors of biosynthetic enzymes as chemotherapeutic agents have been mostly disappointing. This is apparently attributable to the polyamine transport system and dietary polyamines, but the contribution of abnormally regulated polyamine metabolism is equally obvious. The finding that polyamines are involved in the regulation of specific receptors such as the NMDA receptors and ion channels such as inwardly rectifying $K^+$ channels has renewed interest in polyamine mediated regulation of cellular functions. It has also increased interest in polyamine metabolism in the central nervous system where NMDA receptors and inwardly rectifying $K^+$ channels are both expressed abundantly.

In this study we wanted to elucidate the role of the first and crucial enzyme of polyamine biosynthesis, i.e. ornithine decarboxylase, and its protein inhibitor, antizyme, in the polyamine metabolism of CNS. In particular we were interested in whether the guanosine 5'-phosphate activatable ODC activity could be found in CNS. In another part of this study, we investigated the pathological consequences of lifelong deregulated ODC expression.

Aims of the present study are summarised as follows:

1. To investigate whether GTP-activatable ODC can be found in the CNS, where the basal activity of ODC has been suggested by others to be resistant to DFMO, which is a characteristic feature of the GTP-activatable ODC activity in tumours and if found, to characterize properties of the GTP-activatable brain ODC and compare them to the properties of tumour enzyme.

2. To localize and compare ODC and antizyme expression in adult rat brain using *in situ* hybridization and immunocytochemistry.

3. To study employing site-directed mutagenesis of aspartate-233, the role of one of the most highly conserved regions in eukaryotic ODCs and at the same time test whether the GTP-activation of ODC could be affected by this mutation.

4. To generate a transgenic mouse line expressing ODC cDNA under the control of an MMTV-LTR promotor and use it to study the pathological and physiological effects of deregulated ODC expression during the life of transgenic animals.

# 4 Materials and Methods

## 4.1 Assay of ornithine decarboxylase and antizyme activities (I, II, IV)

ODC activity was assayed by measuring the release of $CO_2$ from [1-$^{14}$C]-ornithine (Amersham International, Buckinghamshire, UK) essentially as described (Jänne & Williams-Ashman 1971). The reaction mixture contained 12.5 mM Tris-HCl buffer, pH 7.3, 1 mM EDTA, 2.5 mM DTT, 0.2 mM PLP and 15 μM L-ornithine (1.25 nmol of [1-$^{14}$C]-ornithine). Kinetic parameters were determined from Lineweaver-Burk reciprocal plots (I, II). In the kinetic analysis the concentration of L-ornithine varied from 0.05 mM to 1 mM. To determine the sensitivity to heat inactivation, tissue lysates were preincubated at 55 ˚C (I). To measure the effect of DFMO inhibition the samples were incubated with either L-ornithine (1.25 mM) or DFMO (2.5 mM) for one hour at 37 ˚C, then dialysed overnight against a 2000-fold excess of the reaction mixture buffer without L-ornithine and assayed for ODC activity (I, II).

Antizyme activities (I) were determined as described (Fong *et al.* 1976). The principle of the assay was to mix the antizyme sample with an ODC preparation of known activity, and to compare the observed enzyme activity in the mixture with the expected value.

## 4.2 Preparation of brain extracts (I)

Sprague-Dawley male rats were killed by decapitation. The brains were carefully removed and frozen at –70 ˚C for one hour. Dissections were performed on an ice-cooled glass plate by the method of Glawinski and Iversen (Glowinski & Iversen 1966). The brain region were stored at –70 ˚C until used and then homogenised in 3 vol. of cold 25 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA, 5 mM dithiothreitol (DTT) and 0.1 mM pyridoxal 5'-phosphate (PLP). The homogenates were centrifuged at 105 000 x g for one hour at 4 ˚C. After double $(NH_4)_2SO_4$ precipitation (20 % - 60 %) the pellets were

dissolved in 0.1 M Tris-HCl buffer, pH 7.1, containing 4 mM EDTA, 4 mM DTT and 0.4 mM PLP.

The dissolved pellets were treated with 250 mM NaCl and applied to a Sephadex G-75 Superfine column (Pharmacia Biotech AB, Uppsala, Sweden) to separate ODC and antizyme. The column was eluated with 0.1 M Tris-HCl buffer, pH 7.1, containing 4 mM EDTA, 4 mM DTT, 0.4 mM PLP and 250 NaCl. After gelfiltration NaCl, which decreases ODC activity in assay mixture (Kallio *et al*. 1979), was removed from fractions using Sephadex G-25 columns (PD-10 columns, Pharmacia Biotech AB, Uppsala, Sweden). Desalted fractions were used for ODC and antizyme activity assays.

## 4.3 Construction of plasmids and site directed mutagenesis (II)

Mouse ODC cDNA was a kind gift from Dr. S. Gilmour (The Lankenau Medical Research Center, Philadelphia, PA, USA). The plasmid pODC1.7 was obtained by cloning a 1691 bp *Sal*I - *Pvu*II fragment of the mouse ODC cDNA into a pMSG eukaryotic expression vector (Pharmacia Biotech AB, Uppsala, Sweden) containing the dexamethasone-inducible mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter. The plasmid pODC1.7(D233V) contained the *Sal*I - *Pvu*II fragment of the ODC cDNA in which the codon GAT, encoding the aspartate residue 233, was changed to GTT, encoding valine. Oligonucleotide-directed mutagenesis was carried out by subcloning the *Sal*I - *Pvu*II fragment into an M13 mp9 phage and using the “gapped duplex” method (Kramer *et al*. 1984) according to the manufacturer's instructions (site directed mutagenesis kit, Boehringer Mannheim, Mannheim, Germany). The mutagenic oligonucleotide used was 5'-CTGCTTG*T*TATTGGT GG-3', mismatch being in italics. The sequences of DNA constructs were verified using the manual Sanger dideoxynucleotide method (Sanger *et al*. 1977).

## 4.4 Cell culture and DNA transfection (II)

The ODC-deficient Chinese hamster ovary cell line, C55.7, was generously provided by Dr. I. Scheffler (University of California San Diego, La Jolla, CA, USA). C55.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Paisley, UK) with low glucose and 10% fetal calf serum (FCS) supplemented with 0.5 mM putrescine, nonessential amino acids mixture and antibiotics. The cell lines derived by transfection of the C55.7 cells were grown in the above medium but without added putrescine. The cells expressing mutated mouse ODC were grown in a medium containing 0.1 μM dexamethasone in order to induce ODC-activity for maintaining constant growth.

The C55.7 cells were transfected using the standard calcium phosphate coprecipitation method with a glycerol shock (Ausubel *et al*. 1993). Two days after transfection, the cells were transferred to a medium lacking putrescine and containing dialyzed FCS for selection of the stable transfectants. The selected cultures were grown either in the

presence or absence of 0.1 μM dexamethasone and selection was carried out for 17 - 21 days before isolating colonies. The integration of transfected constructs was confirmed both by the polymerase chain reaction and Southern analysis.

For ODC activity assays cells were fed with fresh medium containing 0.1 μM dexamethasone 7 hours before harvesting. Cells were homogenised by repeated freezing and thawing on a dry-ice bath and dissolved in 25 mM Tris-HCl buffer, pH 7.5, containing 2.5 mM DTT, 0.1 mM EDTA and 0.5 mM phenylmethylsulfonylfluoride (PMSF). For kinetic assays cell lysates were fractionated with a two-step $(NH_4)_2SO_4$ precipitation (20 % - 60 %). The protein precipitate was dissolved in homogenisation buffer and dialysed before ODC activity assays.

## 4.5 Northern blot analysis (II)

Expression levels of wild-type and D233V-ODC mRNAs in C55.7 cells were measured using Northern blot analysis. Total RNA from cultured cells was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987). RNA gel electrophoresis and transfer onto nitrocellulose were performed by standard techniques (Sambrook *et al*. 1989). Blots were probed with a labelled 1691 bp *Sal*I-*Pvu*II fragment of the mouse ODC cDNA and autoradiographed. The autoradiography films were scanned using a laser densitometer (Computing Densitometer Model 300A, Molecular Dynamics, Sunnyvale, CA, USA). ODC mRNA levels in different samples were normalized by comparing them to the levels of 28S rRNA determined from the same blots.

## 4.6 Generation and analysis of transgenic mice (IV)

The transgenic mice were produced by the pronuclear microinjection technique (Hogan *et al*. 1986). Prior to microinjection, the pODC1.7 plasmid (II) was digested with Tth *111*I$^-$ and *Bam*HI to release the MMTV LTR promotor and ODC cDNA from the vector. The Tth*111*I - *Bam*HI fragment was isolated by preparative agarose gel electrophoresis and purified by CsCl centrifugation. The purified DNA was injected into the pronucleus of fertilized oocytes from superovulated C57Bl/6 x C3H/Hej mice mated with males of the same hybrid strain. The microinjected zygotes were transferred into oviducts of pseudopregnant foster females.

The transgenic animals were identified by extracting high molecular weight DNA from 2 - 3 cm sections of mice tails. The primary detection of the transgene was carried out with the polymerase chain reaction and confirmed by Southern hybridization. The genotyped mice were analysed by determining ODC activity in tissue lysates and by histological examination of tissue sections. For ODC activity assays frozen tissues (kept at –70˚C) were homogenised in 1 – 1.5 ml of ice-cold 25 mM Tris-HCl, pH 7.4, containing 2.5 mM DTT, 0.1 mM PLP, 0.1 mM EDTA and 0.5 mM PMSF. The

homogenates were clarified by centrifuging at 105 000 x g for 60 min at 4 °C. The supernatants were assayed for ODC enzyme activity. For histological examination fresh tissue samples were fixed in 4% formaldehyde in 10 mM sodium phosphate, 0.148 M NaCl, pH 7.4, and embedded in paraffin. Sections of 5 μm were cut, deparaffinized and stained with hematoxylin and eosin.

## 4.7 *In situ* hybridization (III)

Male adult Sprague-Dawley rats were used for *in situ* hybridization. The frozen tissues were sectioned with Microm HM 500 cryostat at 14 μm and thaw-mounted onto polysine glass slides (Menzel, Germany). The oligonucleotides used in hybridization corresponded to nucleotides 1474-1518 (antisense) of rat ODC mRNA (Wen *et al.* 1989) and to nucleotides 71-109 (antisense) of rat antizyme mRNA (Matsufuji *et al.* 1995) and were labeled at the 3'end with [$^{33}$P]dATP (New England Nuclear Research Products, Boston, MA, USA). Several control probes with the same length and similar GC content and specific activity were used to determine the specificity of the hybridization. The specificity of probes was also verified by Northern analysis.

*In situ* hybridization was carried out as described previously (Kononen & Pelto-Huikko 1997). After hybrization the sections were either covered with Kodak Biomax MR autoradiofilm (Kodak, Rochester, NY, USA) for 30-60 days or dipped in Kodak NTB2 nuclear track emulsion and exposed 90 days at 4°C. The hybridization signals were quantified using an image analysis system consisting of IBM-PC, Sensi-Cam digital camera (PCO Computer Optics GmbH, Kelheim, Germany), Nikon 55-mm lens and Northern Light precision illuminator (Imaging Research, St. Catharine's, Ontario, Canada). The measurements were carried out using Image-Pro Plus programme (Media Cybernetics, Silver Spring, MD, USA). The grey levels corresponding to $^{14}$C-plastic standards (Amersham International, Buckingham-shire, UK) lying within the exposure range of the film were determined and used in a Lagrange approximation to construct the grey level to activity transfer function. The borders of the measuring fields were interactively defined, and the average activity in the tissue fields was calculated. At least five sections were measured, and the mean value was used.

## 4.8 Immunocytochemistry (III)

Rats were anesthetized with pentobarbital (30 mg/kg, i.p.) and perfused transcardially first with 100 ml of saline followed by a mixture of 4 % paraformaldehyde and 0.1 % picric acid in 0.1 M phosphate buffered saline (PBS), pH 7.3, for 4 – 5 min. After perfusion the brains were excised and further fixed by immersion in the same solution for 60 min. The samples were cryoprotected with 15 % sucrose in PBS before sectioning in the cryostat. Immunocytochemistry was carried out using ABC-method. Primary antibodies were rabbit antiserum against mouse ODC (Laitinen *et al.* 1986) and rabbit

antiserum against rat antizyme (Matsufuji *et al.* 1990). Diaminobenzidine was used as a chromogen. After staining sections were dehydrated, mounted and examined with Nikon Microphot-FXA microscope. Controls included omission of the primary or secondary antibodies and preabsorption of the primary antibodies with respective proteins.

## 4.9 Other methods

Protein concentrations were measured by the method of Lowry (Lowry *et al*. 1951) with bovine serum albumin as the standard (I, II, IV). Polymerase chain reaction and Southern analysis were accomplished (II, IV) according to standard procedures (Ausubel *et al.* 1993). For statistical analysis, the Student's t-test was used (I).

# 5 Results

## 5.1 Detection and characterization of GTP-activatable ornithine decarboxylase in the rat brain (I)

The brains of adult male rats were dissected to five regions: cerebellum, cortex, hippocampus, hypothalamus and "midbrain". When crude cytosol fractions or ammonium sulphate-precipitated dialysates were assayed for ODC activity, the hypothalamus and midbrain showed highest activity and the cerebellum lowest. However, the difference was less than 10-fold. The presence of 0.1 mM GTP in the enzyme assay increased ODC activity in the cerebellum lysate nearly two-fold, increases in other lysates were minor and activity even decreased in the dialysed $(NH_4)_2SO_4$-precipitate of hippocampus lysate (0.82-fold change).

In order to further characterize the ODC activities of brain regions, gel-permeation chromatography in the presence of 0.25 M NaCl was performed to dissociate the ODC-antizyme complex and to separate the ODC and the antizyme from each other. The GTP-activatable ODC activity was clearly found from each brain regions in the eluted fractions. In the cerebellum ODC activity was increased 4.2-fold in the presence of 0.1 M GTP, in all other regions increases were between 2.0- and 2.7 fold. The specificity of activation was tested by incubating the brain and kidney enzymes with various nucleotide triphosphates. As expected, kidney ODC was not affected by any of nucleotides tested. However, the ODCs of midbrain and hypothalamus that were chosen to represent brain ODC, were stimulated 1.5 – 2-fold by GTP, dGTP and ATP, and slightly by dATP. The pyrimidine nucleotides were ineffective. The $K_{1/2}$ values for GTP and ATP were approximately 2 μM and 40 μM, respectively, indicating that GTP was favoured over ATP and could activate brain ODC in lower concentration. The GTP-induced activation of ODC was essentially irreversible since activity was elevated even after GTP was removed from the preincubated lysates by extensive dialysis. GTP appeared to effect on ODC mainly by increasing $V_{max}$ value of the enzyme.

Because the GTP-activation of ODC could be detected clearly only when the enzyme was dissociated and separated from the antizyme, we studied the sensitivity of brain and

kidney ODCs for the inhibition by antizyme. A constant amount of ODC activity was mixed with increasing amount of partially purified brain antizyme and the ODC activity remaining was assayed. There was no difference in the inhibition of the kidney ODC in the presence or absence of GTP, whereas the GTP-activatable brain ODC activity from the gel filtration fractions of the cerebellum appeared to be more sensitive to antizyme inhibition than activity assayed in the absence of GTP. When sensitivity to heat inactivation was studied, brain ODC activity was more stable than activity in kidneys. However, GTP did not have any significant effect on the heat sensitivity of brain ODC.

Since it has been suggested that the basal ODC activity in the neocortex and the cerebellum is resistant to DFMO (Zawia *et al*. 1991), we determined the effect of DFMO on the ODC activity in the different brain regions. DFMO inhibited around 60 % of the ODC activity from all brain regions. However, the activities detected after extensive dialysis were very low both in the controls and in the DFMO inhibited samples. Kidney ODC was inhibited virtually completely.

## 5.2 Expression of ornithine decarboxylase and antizyme in the rat brain (III)

In order to obtain detailed expression patterns for ODC and antizyme in the rat brain and to compare their expression we employed both *in situ* hybridization and immunocytochemistry. *In situ* hybridization showed that ODC and antizyme mRNAs are widely expressed in the brain. The hybridization signal for ODC mRNA was clearly lower than that of antizyme mRNA in all areas investigated, therefore twice as long exposure time was used for ODC mRNA than for antizyme mRNA. The highest expression for both genes was detected in the cerebellar cortex, hippocampus, hypothalamic paraventricular and supraoptic nuclei, locus coeruleus, olfactory bulb, piriform cortex and pontine nuclei (Table I). Considering larger brain regions, thalamus showed lowest expression for both mRNAs. The ODC and antizyme mRNA-expressing cells were large indicating that they were very likely neurons.

Immunocytochemistry confirmed the results of *in situ* hybridization. Immunoreactivity for both ODC and antizyme was detected widely. ODC antibody displayed mainly cytoplasmic staining in all areas, whereas antizyme protein had two distinct patterns of subcellular localization. In most regions of brain, the antizyme staining was localized in the cytoplasm, as expected. However, antizyme was predominantly detected in the nerve cell nuclei in some areas including the cerebellar cortex, anterior olfactory nucleus, frontal cortex, cingulate cortex, the central nucleus of amygdala, ventromedial hypothalamic nucleus, anterior hypathalamic area, ventroposterior and ventrolateral thalamic nucleus, cochlear nuclei and trapezoid body.

*Table 1. Semiquantitative evaluation of ODC and antizyme mRNA distribution in rat brain.*

| Brain region | ODC mRNA | Antizyme mRNA | Brain region | ODC mRNA | Antizyme mRNA |
|---|---|---|---|---|---|
| Telencephalon | | | Subthalamic nucleus | ++++ | ++++ |
| Olfactory bulb: | | | Hypothalamus: | | |
| Internal granular layer | ++++ | ++++ | Suprachiasmatic nucleus | ++ | +++ |
| Mitral cell layer | ++++ | ++++ | Anterior hypothalamic area | ++ | +++ |
| Anterior olfactory nucleus | +++ | ++++ | Paraventricular nucleus | ++++ | ++++ |
| Piriform cortex | ++++ | ++++ | Supraoptic nucleus | ++++ | ++++ |
| Olfactory tubercle | +++ | ++++ | Ventromedial nucleus | +++ | +++ |
| Frontal cortex | +++ | +++ | Arcuate nucleus | +++ | +++ |
| Cingulate cortex | +++ | +++ | Posterior nucleus | +++ | ++ |
| Insular cortex | +++ | +++ | Mammillary nuclei | + | +++ |
| Frontoparietal cortex | +++ | +++ | Mesencephalon | | |
| Endopiriform nucleus | ++ | ++ | Superior colliculus | + | ++ |
| Caudate putamen | + | ++ | Inferior colliculus | ++ | +++ |
| Hippocampus: | | | Subst. nigra (pars compacta) | +++ | +++ |
| CA1/4 | ++++ | ++++ | Red nucleus | +++ | ++++ |
| Dentate gyrus | ++++ | ++++ | Retrorubal nucleus | ++ | +++ |
| Entorhinal cortex | + | +++ | Ventral tegmental area | + | ++ |
| Amygdala: | | | Pons/medulla | | |
| Basolateral nucleus | ++ | ++ | Pontine nuclei | +++ | ++++ |
| Central nucleus | ++ | ++ | Dorsal tegmental nucleus | ++ | +++ |
| Medial nucleus | + | ++ | Ventral tegmental nucleus | +++ | +++ |
| Cortical nucleus | ++ | ++ | Reticular tegmental nucleus | + | +++ |
| Diencephalon | | | Trapezoid body | ++ | +++ |
| Epithalamus: | | | Superior olive | + | + |
| Medial habenula | +++ | ++++ | Inferior olive | + | ++ |
| Nucleus of Darkschewitz | +++ | +++ | Raphe nuclei | + | ++ |
| Thalamus: | | | Locus coeruleus | ++++ | ++++ |
| Anterodorsal nucleus | ++ | +++ | Cochlear nuclei | ++ | +++ |
| Paratenial nucleus | + | ++ | Motor trigeminal nucleus | ++ | ++ |
| Reticular nucleus | + | ++ | Pr. sensory trigem. nucleus | +++ | +++ |
| Paraventricular nucleus | ++ | +++ | Principal oculomotor nucleus | +++ | +++ |
| Rhomboid nucleus | ++ | ++ | Facial nucleus | ++ | +++ |
| Ventroposterior nucleus | + | ++ | Prepositus hypogl. nucleus | ++ | +++ |
| Centromedial nucleus | ++ | +++ | Vestibular nuclei | ++ | +++ |
| Subparafascicular nucleus | + | +++ | Nucl. spinal tr. trigem. nerve | ++ | ++ |
| Medial geniculate nucleus | + | ++ | Nucleus solitary tractus | + | ++ |
| Zona incerta | ++ | ++++ | Cerebellar cortex | ++++ | ++++ |

Abundance of mRNAs was determined from optical density measurement: ++++ > 100 % above the backround, +++ > 75 % above the backround, ++ > 50 above the backround, + >25 % above the backround.

## 5.3 Site-directed mutagenesis and analysis of D233V-mutated ornithine decarboxylase (II)

A point-mutation changing aspartate-233 to valine was introduced into mouse ODC cDNA and ligated to the pMSG expression vector under MMTV-LTR promotor. The effects of the mutation on the enzymatic activity and catalytic properties of ODC were studied by establishing C55.7 cell lines expressing either wild-type or mutated ODC. Colonies transfected with the pODC1.7 plasmid encoding wild-type ODC grew faster and were larger than those transfected with pODC1.7(D233V). Twenty-two of pODC1.7 transfectants and eight of pODC1.7(D233V) transfectants were assayed for ODC activity. Clone K2 of pODC1.7 and clone B7 of pODC1.7(D233V) transfectants were selected for further studies. The integration of the transfected constructs into genomes of K2 and B7 cells was confirmed both by the polymerase chain reaction and Southern analysis. K2 and B7 clones expressed ODC at relatively high level, but were still typical representative of their groups. ODC activity in B7 cells was 4.6-fold lower compared to that in K2 cells, although the level of ODC mRNA was 2.3-fold higher in the B7 cells. The D233V-mutation resulted in an increase in the $K_m$ values for the substrate L-ornithine and the cofactor PLP by about 20-fold. Similarly, the mutated enzyme had 15-fold higher $K_i$ value for DFMO.

## 5.4 Generation and analysis of ornithine decarboxylase expressing transgenic mice (IV)

A transgenic mouse line expressing ODC cDNA under the control of an MMTV-LTR promotor was generated and used to study the pathological and physiological effects of deregulated ODC expression during the life of transgenic animals. After the transgenic founder animal was generated, the transgenic mice were followed in seven generations. The average litter size, including both live and dead pups, was not significantly different from that of non-transgenic. However, the breeding of homozygous transgenic mice proved to be difficult due to the high number of infertile mice. In later generations, the ratio of males to females decreased, resulting in low availability of transgenic males for reproduction. At birth, the offspring appeared normal. At about 4 weeks of age, some of transgenic pups were smaller than non-transgenic controls, but that did not prevent them from reaching normal size at adult stage.

When ODC activities were systematically assayed in tissues of transgenic and control animals, the most significant difference was detected in some of the reproductive organs of male mice. The ODC activity was two times higher in testis, about 20 times higher in seminal vesicle and fat pads and about 400 times higher in preputial gland of transgenic males when compared to age-matched non-transgenic animals. A moderate increase in ODC activity was assayed also in the heart and lung of transgenic males. In contrast, the ODC activity was significantly lower in the prostate and kidney tissues. The lower ODC activity in kidneys was observed also in the female transgenic mice, but there were no

significant changes in the enzyme activity in the reproductive organs of females. In addition to kidneys, the transgenic females had lower ODC activity in the heart, but increased activity in the brain and liver. Changes were statisticly significant but moderate.

The low fertility rate of transgenic mice can be explained by abnormalities found in the histological examination of sexual organs. The testes of the transgenic mice were smaller than those of control animals. Although the testicular epithelium of most fertile transgenic males was normal and produced normal-appearing spermatozoa, the testicular tissues of several infertile transgenic mice showed a reduced number of mature spermatozoa and immature spermatogenic cells were seen in the testicular lumen. The number of preputial glands in the infertile transgenic mice was reduced and the epithelium in the lumen was occasionally stratified and keratinized. The seminal vesicles and prostates of the transgenic males appeared to be normal. The ovaries of infertile female transgenic mice were smaller than those control females. In addition, the number of follicles was low and chronic inflammatory infiltrates were frequently seen.

The physiological and pathological state of heterozygous transgenic mice was followed for a two-year period. No abnormalities were observed before the age of six months. However, as the mice aged, the number of diseased animals increased and by the age of two years pathological changes were found in 25% of the transgenic mice. Histological examination of tissues from necropsies revealed that most common pathological alterations were inflammatory processes including pancreatis, hepatitis, sialoadenitis and pyelonephritis. Spontaneous tumours were found in eight transgenic mice, including a benign fibroid skin tumour and vascular tumour in the liver, three mammary carcinomas with one lung metastasis, one intestinal adenocarcinoma and a lymphoma. Age-matched non-transgenic mice had no overt histological abnormalities.

# 6 Discussion

## 6.1 GTP-activatable ornithine decarboxylase in the rat brain

The present study was the first to report activation of mammalian ODC in normal tissues by GTP or by any other potential allosteric effector. Some prokaryotic ODCs such as biosynthetic ODC of *E. coli* (Applebaum *et al*. 1977, Anagnostopoulos & Kyriakidis 1996) and *Lactobacillus 30a* ODC (Oliveira *et al*. 1997), and ODCs from some lower eukaryotes such as *S. cerevisiae* (Tyagi *et al*. 1981) are activated allosterically by GTP. GTP-activatable ODC has been detected also in tumor cell lysates prepared from mouse epidermal papillomas (O'Brien *et al.* 1986, O'Brien *et al.* 1987), human squamous cell carcinoma (Hietala *et al.* 1988), colorectal adenocarcinoma (Hietala *et al.* 1990), gastric cancer (Okuzumi *et al.* 1991) or colorectal carcinoma (Matsubara *et al.* 1995). In these cases, as well as in the present work, it is not known whether GTP is a real allosteric effector of ODC or if it activates ODC indirectly via other regulatory protein(s).

The ability of GTP to activate ODC varied in lysates prepared from different parts of brain, but the activation was always higher when antizyme was removed from the lysate by gel-filtration chromatography. The activation was highest in the cerebellum lysates both before and after gel-filtration (2- and 4.2-fold). ODC from the cortex and hippocampus displayed lowest activation, but in the latter the total enzyme activity was highest. In ammonium sulphate precipitated tumour lysates GTP activation was at highest 8-fold, but generally less than 2-fold (Hietala *et al.* 1988, Hietala *et al.* 1990).

The heat sensitivity of brain ODC was comparable with that of mouse epidermal tumour GTP-activatable ODC, which is more stable than the normal epidermal ODC (O'Brien *et al.* 1986, O'Brien *et al.* 1987) or kidney ODC that was used as a control in this study. However, GTP has a substantial protective effect against heat inactivation of the GTP-activatable tumour ODC, but this effect was not observed with the brain enzyme which does not necessarily indicate differences between brain and tumour ODC, but may

imply differences in the utilization or regulation of degradative machinery in different tissue lysates.

Tumour ODC was activated only by GTP and dGTP (O'Brien *et al.* 1986, O'Brien *et al.* 1987) whereas brain ODC could be activated by both purine nucleotide triphoshates, and their deoxyforms. GTP was highly favoured in activation over ATP, the $K_{1/2}$ value for GTP was 2 µM and for ATP 40 µM. Tumour ODC has a lower $K_{1/2}$ for GTP, 0.1 µM, although the order of magnitude was similar. These GTP concentrations are significantly lower than typical GTP concentrations in cells which range from ≈ 50 µM to 200 - 300 µM (Otero 1990, Jinnah *et al.* 1993). In that case ODC may always be maximally activated by GTP in cells if activation is not prevented e.g. by antizyme. We detected activation clearly in all brain lysates when antizyme was dissociated from ODC and removed by gel-filtration chromatography. Agreeing with previous data, it appeared that GTP-activatable ODC is inhibited more readily by antizyme. On the other hand, ammonium sulphate precipitation and gel-filtration chromatography decreased GTP concentration in the lysates and might have made the activation possible, i.e. most of the enzyme may have been "activated" in intact cells and raw lysates, and this activation could have been at first reversed by the removal of GTP, which would have made the enzyme again more clearly GTP-activatable. However, GTP-activation was apparently irreversible, since even after extensive dialysis activity in a sample preincubated with GTP was higher than in a sample preincubated without GTP. These samples were not activated by GTP after dialysis and the possibility remains that GTP only stabilized the enzyme in preincubation, although this may not be likely because there was no stabilizing effect by GTP on brain ODC when heat stability was tested. Furthermore, also tumour enzyme has been shown to be irreversible activated by GTP and in this case the enzyme sample preincubated without GTP was activated after dialysis (O'Brien *et al.* 1987). GTP activated brain ODC mainly by increasing the $V_{max}$ value indicating enhanced catalytic efficiency. In contrast, GTP effected mainly $K_m$ values of tumour ODC (O'Brien *et al.* 1986, O'Brien *et al.* 1987, Hietala *et al.* 1988).

There are several explanations how GTP-activatable ODC is brought about. At the very beginning of this work it was known that human and mouse genomes contain several ODC-like sequences and it was considered whether a normally silent ODC gene could be expressed in tumour cells. This was later shown to be virtually impossible. The human genome contains two highly ODC–like loci and another of them is a pseudogene, that can not be activated by simple mutation (Hickok *et al.* 1990, Radford *et al.* 1990). Recently cloned ODC-like protein is moderately identical to ODC (54 %) and might even posses some ODC activity (Pitkänen *et al.* 2001). However, it has a valine residue in a position corresponding to Cys-360, which means that its ODC-like catalytic efficiency would almost certainly be greatly diminished from that of ODC (Coleman *et al.* 1993), and that catalysis would probably not be specific for the L-ornithine or for decarboxylation (Jackson *et al.* 2000). Nevertheless, lack of Cys-360 may do ODC activity resistant to DFMO, because Cys-360 is the major binding residue of DFMO (Poulin *et al.* 1992). This could explain the low residual ODC activity we detected in the brain lysates after DFMO inhibition. In tumour cells ability to be activated by GTP could be a consequence of mutation in ODC gene, but GTP-activatable ODC in brain can not be explained by mutation and it is not plausible that two basicly different mechanisms could results in the formation of GTP-activatable ODC. It seems more apparent that GTP

activation is a result of tissue specific posttranslational modification or tissue specific function of regularoty protein(s).

In principle, GTP could bind to ODC directly affecting its catalytic function, or GTP could activate a GTP-binding protein which mediates the activation of ODC, or it could be a substrate for a kinase that phosphorylates ODC. The first alternative is not likely. Structures and amino acid sequences of GTP-binding sites from several proteins are well known (Geyer & Wittinghofer 1997, Takai *et al*. 2001). ODC does not contain obvious putative binding sites for GTP, and although point mutations could create areas resembling the binding sites, they can not explain the presence of GTP-activatable enzyme in the brain as noted above. It is not plausible either that a posttranslational modification could create a binding site. The alternative suggesting that GTP acts as a substrate for a kinase appears to be more realistic. There are kinases that accept both ATP and GTP as a substrate (Richert *et al*. 1979, Payne & Dahmus 1993), and also kinases that accept deoxynucleotides as a substrate (Levy-Favatier *et al*. 1987, Payne & Dahmus 1993). The kinase using GTP as a substrate need not phosphorylate ODC directly. It could be involved in a signaling cascade leading finally to the phosphorylation of ODC. On the other hand, the non-hydrolyzable GTP analog GTP[ -S] can also activate tumour ODC (O'Brien *et al.* 1987) although it certainly can not be used as a substrate for a kinase. The effect of GTP[ -S] on brain ODC was not tested in this work. The third and also a very plausible, alternative is that GTP activates at first GTP-binding regulatory protein that regulates ODC directly or activates a signaling pathway leading to ODC phosphorylation.

Properties of GTP-activatable brain ODC agree well with those reported for the phosphorylated ODC from macrophage cell line (Reddy *et al.* 1996). The phosphorylated enzyme was more stable and displayed a higher $V_{max}$ value. Phosphorylation would also explain irreversibility, or reversibility in some cases, depending whether phosphatases and kinases are present. e.g. in the brain, fractions of ODC could be unphosphorylated and bound to antizyme and it could be phosphorylated when cells are lysed and antizyme removed. Alternatively, cell lysis could expose ODC to phosphatases that dephosphorylate the enzyme, which then again could be phosphorylated and activated. This scheme requires that kinase co-purify with ODC in the gel-filtration chromatography. On the other hand, if GTP activation is a result of direct interaction with regulatory protein, the regulatory protein should as well co-purify with ODC. It should be pointed out, that the different properties of tumour and brain ODC are understandable and to a certain extent expected. The enzyme may be phosphorylated at different residues or could be regulated by different regulatory proteins in the brain and tumors which have very different characteristics to tissues. The phosphorylation pattern in the tumours may even be aberrant.

Why is there GTP-activatable ODC in the brain and in some tumours? CNF is a particular tissue when it comes to polyamine metabolism. Polyamines have there specific functions in the regulation of ion channels and glutamine-activated receptor channels (Haghighi & Cooper 1998, Oliver *et al.* 2000). Polyamines have an essential role in the development and differentiation of CNS and their metabolism is altered in various pathological conditions (Bernstein & Müller 1999). Antizyme concentration in CNS is higher than elsewhere (Laitinen *et al.* 1985, Gritli-Linde *et al.* 2001). Brain is together with testis the tissues where transgenic mice overexpressing ODC have the most significant changes in putrescine and polyamine levels (Halmekytö *et al.* 1991a,

Halmekytö *et al.* 1993). GTP-activatable ODC could be used to modulate some of the CNS-specific functions of polyamines or it could be important during restricted time in the development of neurons. In tumours ODC regulation may be working aberrantly and in the corresponding non-malignant cells GTP-activatable ODC could most likely exists, if it existed at all in this cell type, only during development and differentiation, since tumour cells are generally poorly differentiated.

Clarifying of the origin and function of GTP-activatable ODC would be aided by the purification of the enzyme. If the ability to be activated by GTP is lost during purification, it is likely that effector proteins or kinases are required. Potential roles of phosphorylation in ODC activation could be studied using [ -$^{32}$P]-GTP and/or [ -$^{32}$P]-ATP and immunoprecipitating ODC from labelling reactions. One way to address these questions could be to study whether recombinant ODC is activated in brain lysate, which even could be depleted from endogenous ODC activity. If exogenous ODC were activated, site-directed mutagenesis could be employed to elucidate the mechanism of GTP-activation. Dominant negative mutant forms of signaling proteins could be tested for the ability to prevent GTP-activation. An example of signaling proteins that might be involved in the phosphorylation and/or GTP-activation of ODC are members of MAP kinase pathway. MAP kinases themselves phosphorylate serine and threonine residues in many growth-related – but also in other type of – proteins (Pearson *et al.* 2001).

## 6.2 Expression of ornithine decarboxylase and antizyme in the rat brain

We carried out this study in order to localize and compare ODC and antizyme expression in different brain regions. Polyamines have a plethora of demonstrated or suggested functions in CNS and polyamine metabolisms by CNS has characteristic features of its own, which is discussed in the previous chapter and in the chapters 1.1.3 and 1.1.4. Considering all interesting observations surprisingly little is known about the regulation of polyamine metabolism and the roles of ODC and antizyme in the normal adult brain. The amount of ODC protein in various tissues usually correlates well with enzyme activity, but in brains a noticeably high amount of immunoreactive ODC is present although enzyme activity is relatively low (Laitinen 1985). This may be due to the antizyme, which is present in the brain and could form a complex with the enzyme. The complex is formed efficiently at least when cells are lysed for ODC purification (Laitinen *et al.* 1986). However, in cell lysates and in cell lines of non-neural origin ODC in ODC-antizyme complex is degraded extremely rapidly (Murakami *et al*. 1992b, Murakami *et al*. 1992c, Murakami *et al.* 1993). Brain might be exception to this rule or ODC and antizyme could be expressed in different locations in the CNS. Correlation of expression patterns of ODC and antizyme to known function of polyamines in CNS could as well be interesting and potentially informative. Actually we considered that the localization of ODC and antizyme expression is a definite prerequisite for the comprehensive understanding of the balanced regulation of polyamine metabolism in CNS.

Both immunohistochemistry and *in situ* hybridization experiments demonstrated that ODC and antizyme are widely expressed in the rat brain. Their expression was clearly confined to neurons, agreeing with earlier observations about ODC expression in rodent and human brains (Müller *et al.* 1991, Müller *et al.* 1993, Bernstein & Müller 1995, Ichikawa *et al.* 1997, Ichikawa *et al.* 1998, Gritli-Linde *et al.* 2001). Glial cells have reported to display ODC activity (Laube & Veh 1997) as expected due to the ubiquitous presence and essentiality of polyamines, but immunoreactivity for ODC is on the detectable level only in certain rare cases (Bernstein & Müller 1999).

The highest expression for both genes was detected in the cerebellar cortex, hippocampus, hypothalamic paraventricular and supraoptic nuclei, locus coeruleus, olfactory bulb, piriform cortex and pontine nuclei. Overall expression pattern matched well with those reported later by others (Gritli-Linde *et al.* 2001). There was no obvious correlation between ODC mRNA expression and enzyme activities determined in our previous study (I) apparently because the brains were dissected to five relatively roughly defined regions and because antizyme mRNA was nearly always expressed strongly concomitantly with ODC mRNA leaving the final controlling of protein expression levels to depend on translational and post-tranlational regulation.

Polyamines are responsible for the intrinsic gating and rectification of those subgroups of inward rectifying Kir channels which are strong or intermediate rectifyers such as Kir2 and Kir3 channels (Ficker *et al.* 1994, Lopatin *et al.* 1994). Distribution of Kir2 and Kir3 mRNAs in the adult rodent brain has been studied in details (Karschin *et al.* 1994, Kobayashi *et al.* 1995, Dissmann *et al.* 1996, Horio *et al.* 1996, Karschin *et al.* 1996, Töpert *et al.* 1998) and exhibits interesting similarities with the expression of ODC and antizyme mRNAs reported in the present study. We detected a very high expression of both ODC and antizyme mRNAs in nearly all those regions that have been reported to express Kir2 or Kir3 channels at the high level. The only major exception with Kir2 channels was thalamus, where relatively high levels of antizyme mRNA were observed, but ODC mRNA was expressed only moderately. Furthermore, expression of all Kir2 mRNAs, like ODC and antizyme mRNAs, is restricted to neurons, no signals for them have been detected in glial cells (Horio *et al.* 1996, Karschin *et al.* 1996). Except Kir3.4 mRNA which is expressed only at the low levels in the brain, the overall distribution of other Kir3 mRNAs is rather widespread and overlapping in many CNS neurons. There is some controversial data, but generally olfactory bulb, hippocampus, cortex, thalamus and cerebellum all exhibit a strong hybridization signal for Kir3.1, Kir3.2 and Kir3.3 mRNAs (Karschin *et al.* 1994, Kobayashi *et al.* 1995, Dissmann *et al.* 1996, Karschin *et al.* 1996). Again all these regions with the exception of thalamus showed high ODC and antizyme expression in our study. High and simultaneous ODC and antizyme mRNA levels suggest that polyamine concentrations are regulated strictly and rapidly and maybe in a wider range in these regions. This might imply that the controlling of polyamine levels is indeed used to regulate Kir channels *in vivo*. In the thalamus hybridization signal for antizyme mRNA concentration is strikingly higher than for ODC mRNA. Could this mean that in the thalamus a lower polyamine concentration and weaker inward rectifying is required or sufficient?

Expression levels for ODC mRNA were clearly lower than ones for antizyme mRNA in all the areas investigated, and the appropriate signal for ODC mRNA was obtained by using twice as long exposure time as for antizyme. Although ODC is regulated at the

levels of transcription, translation and the degradation of enzyme protein (Davis *et al.* 1992, Shantz & Pegg 1999), the regulation of transcription plays nearly always a predominant role. When ODC expression is not stimulated the levels of ODC mRNA could be expected to be low. This is supported by the fact that ODC mRNA is relatively rapidly degraded with the half-life of 2.5 to 5 hours (Berger & Porter 1986, Weiner & Dias 1992). The principal regulation of antizyme expression takes place at the translational level where polyamines induce a programmed ribosomal frameshifting in antizyme mRNA (Coffino 2001a, Coffino 2001b). The synthesis of antizyme as a rapid response to increasing polyamine concentration can occur only if cells maintain a certain level of antizyme mRNA continuously and this has been shown to be the case, a large amount of antizyme mRNA is constitutively expressed in rat tissues and its half-life after actinomycin D treatment is as long as 12 hours (Matsufuji *et al.* 1990).

Consistently with others (Karschin *et al.* 1994, Kobayashi *et al.* 1995, Dissmann *et al.* 1996, Karschin *et al.* 1996) we detected immunoreactivity for ODC mainly in the cytoplasm. In most areas of brain antizyme staining was also localized in the cytoplasm, but interestingly in other areas including the cerebellar cortex, frontal cortex, cingulate cortex, cohlear nucleus and some nuclei in the thalamus, hypothalamus, and amygdala; antizyme protein was predominantly expressed in the nerve cell nuclei. Nuclear localization of antizyme was later confirmed by others employing different antibody (Gritli-Linde *et al.* 2001). They reported nuclear staining for antizyme in Purkinje cells of cerebellar cortex and, disagreeing with our observations, in the CA1-3 fields of hippocampus, but did not present a detailed report of overall expression pattern.

It is possible that in the regions where antizyme was localized to nuclei, it is rapidly degraded in the cytoplasm and the nuclear staining is predominant only because antizyme in the nuclei is not degraded with similar efficiency. Another possibility is that antizyme might have some unknown special function in the nucleus. It has appeared that the regulation and functions of antizyme are more complicated than previously thought. Antizyme has been shown to interact with signaling protein Smad1 and to target it together with, or potentially independently on ubiquitination for proteasomal degradation (Gruendler *et al.* 2001). Smad proteins mediate signaling induced by transforming growth factor superfamily from cell mebrane receptors to nucleus where they translocate (Itoh *et al.* 2000). Furthermore, unpublished, but reviewed, results suggest that antizyme interacts also with the components regulating cell cycling, i.e. with cyclins and cyclin-dependent kinases (Coffino 2001b), which are nuclear proteins (Jordan *et al.* 2000, Ino & Chiba 2001, Small *et al.* 2001) Antizyme is devoid of a defined nuclear localization signal, but in the computerized analysis the similarity of antizyme amino acid composition to that of nuclear proteins gave evidence for a first choice location of antizyme to the nucleus with an expected prediction accuracy of 82.5 % (Gritli-Linde *et al.* 2001).

The recent studies have demonstrated that antizymes form a gene family. In the CNS in addition to antizyme 1, also antizyme 2 is expressed (Ivanov *et al.* 1998b). The oligonucleotide we used as a probe in *in situ* hybridization experiments corresponded to nucleotides 71 – 109 of rat antizyme mRNA (GenBank accession number D10706). This area is not homologous between mouse antizyme 1 and 2, and generally the nucleotide sequence of antizyme 1 is significantly different from that of antizyme 2. Apparently a probe specific for one antizyme mRNA would not hybridize with those of the other

family members under stringent conditions. We cannot rule out the possibility that antibody against antizyme 1 recognises also antizyme 2, although similarity between mammalian antizymes 1 and 2 appears to be less than 70 %. Moreover, the fact that antizyme mRNA was found to be about 16 times more abundant in human tissues than that of antizyme 2 (Ivanov *et al.* 1998b), may suggest that antizyme 2 protein is unlikely to significantly contribute to the signal observed with the anti-antizyme 1 antibody.

## 6.3 Mutation of aspartate-233 to valine in mouse ornithine decarboxylase

All eukaryotic ODCs contain several highly conserved regions and the amino acid residues 232-238 form one of the most highly conserved sequences. We mutated aspartete-233 which is the only acidic residue in the -LD(I/V)GGGF- motif to valine. The mutation resulted in increases in the $K_m$ values for the substrate L-ornithine and the cofactor PLP by about 20-fold. $K_i$ value for the DFMO increased 15-fold. Glycine-rich sequences like this have been implicated in the formation of the cofactor binding region in the PLP-dependent enzymes (Marceau *et al*. 1988a, Marceau *et al*. 1988b).

According to the crystal structure of mouse ODC (Kern *et al.* 1999), the position and orientation of the PLP in the active site is governed primarily by protein interaction with the 5'-phosphate and the pyrimidine nitrogen, N1. The phosphate binds in a pocket formed by two loops (235-241, 274 – 276) and the aminoterminus of 10'th helix that follows immediately the second loop. The first loop contains three consecutive glycines (235 – 237) that are conserved in all group IV PLP-dependent decarboxylases (Kern *et al.* 1999). Glycine-237 and –276, arginine-277 and tyrosine-389 form hydrogen bonds to the phosphate of PLP. The primary interaction of N1 is with glutamate-274. Glutamate-274 forms together with aspartate-88, glutamate-274 and aspartate-233 a cluster of acidic residues that, with three bound water molecules form a network of hydrogen bonds that probably influences the electron-withdrawing properties of the cofactor. It is understandable that the mutation of aspartate-233 can effect PLP-binding, or the orientation or position of the cofactor and thus also the substrate binding.

Other glycine-rich regions which interact with phosphate groups of nucleotides are found in the guanine nucleotide binding domains. The PM1 region in c-H-*ras* protein (de Vos *et al*. 1988, Valencia *et al*. 1991) has similarities in amino acid sequence with mammalian ODC, especially if conservative amino acid substitutions are allowed. The most apparent of similarities is between amino acids 230 – 240 of ODC and 5 – 15 of c-H-*ras.* The amino acid sequence of residues 230 - 240 in ODC is -*HLLDIGGGØØG*- and that of residues 5 - 15 of c-H-*ras* is *KLVVVGAGØØG*-. Homologous, either identical or similar, amino acids are written in italics, amino acids designated Ø are not thought to be indispensable for GTP-binding (Möller & Amons 1985, de Vos *et al.* 1988, Valencia *et al.* 1991). However, the aspartate-233 to valine-233 mutation did not, at least alone, cause the activation of ODC by GTP.

## 6.4 Ornithine decarboxylase expressing transgenic mice

A transgenic mouse line expressing ODC cDNA under the control of a MMTV-LTR promotor was generated and used to study the pathological and physiological effects of deregulated ODC expression during the life of transgenic animals. When ODC activities were systematically assayed in tissues of transgenic and control animals, the most significant difference was detected in some of the reproductive organs of male mice. Testis displayed two times higher, seminal vesicle about 20 times higher and fat pads in preputial glands about 400 times higher ODC activity when compared to age-matched non-transgenic animals. In contrast, there were no significant changes in the enzyme activity in the reproductive organs of females. Interestingly, the ODC activity was significantly lower in the prostate and kidney tissues of transgenic males. The lower ODC activity in kidneys was observed also in the female transgenic mice. Changes in ODC activity in other tissues (lung, liver, heart, brain displayed altered activity) were statisticly significant but moderate.

The first reported transgenic mouse lines were produced by using the human ODC gene containing its own promoter (Halmekytö *et al.* 1991a, Halmekytö *et al*. 1991b, Halmekytö *et al.* 1991c). Those transgenic mice overexpressed ODC in all tissues studied, with the exception of small intestine and male kidney. Later ODC overexpression has been targeted successfully to keratinocytes (Megosh *et al.* 1995) or to heart (Shantz *et al*. 2001) by using tissue-specific promotors and metallothionein I promotor has been used to control inducible overexpression of ODC (Alhonen *et al.* 1996). In these cases ODC expression patterns were as expected. Most transgenic mouse lines carrying MMTV LTR fusiongenes express transgenes at variable levels in different tissues, but the levels are particularly high in the mammary glands (Sinn *et al.* 1987, Müller *et al.* 1988, Tsukamoto *et al*. 1988, Bouchard *et al*. 1989, Matsui *et al*. 1990, Müller *et al*. 1990, Suda *et al*. 1990, Berard *et al*. 1994). In our study, however, mice carrying the MMTV/ODC cDNA fusiongene showed no elevation in ODC activity in the mammary gland. It is apparent that out transgene was integrated to genomic locus that could be transcribed significantly only in certain tissues where the responsiveness to glucocorticodes or/and post-translation regulation of ODC determined the level of ODC overexpression. It is possible that the transciptional activity of the genomic area around the integration site depends also on the age of mice. The single transgenic pup we analysed displayed increased ODC activity compared to non-trangenic pup in all tissues examined except in pancreas – although this kind of analysis is of course not statistically valid. Our study showed both increases and decreases in enzyme activity in tissues of transgenic mice. This might imply that the integration of transgene has had in some tissues effect on the expression of endogenous ODC genes either directly or indirectly. Decreases in ODC activity were relatively moderate and could be explained by the changes in general metabolism, signaling pathways or “well being” of tissue which have decreased the growth potential of tissue.

In addition to the integration site, there are other factors that could have contributed to the moderate only overexpression of ODC. The high ODC activity found in other ODC transgenic mice (Halmekytö *et al.* 1991a, Halmekytö *et al.* 1991b, Halmekytö *et al.* 1991c) might have been caused by the human construct used, which contains ODC's own

strong promoter (Brabant *et al.* 1988, Palvimo *et al.* 1991), or by the lack of putative silencer elements on the 5’ flanking region further upstream (Halmekytö *et al.* 1991a, Halmekytö *et al.* 1991c). It has been suggested earlier that the responsive elements in the codin region of ODC cDNA are involved in the regulation of the ODC expression (van Daalen Wetters *et al*. 1989a). Human elements might not be functional in mice, but our transgene was of murine origin and may have contained active elements.

Transgenic mice with high polyamine biosynthesis activity have regulatory mechanisms preventing accumulation of polyamines (Halmekytö *et al.* 1993, Heljasvaara *et al.* 1997). In contrast, in transgenic mice that constitutively and strongly overexpress deregulated ODC in the skin (Megosh *et al.* 1995, O'Brien *et al.* 1997), the polyamine homeostasis is disturbed and the mice develop spontaneous skin tumors. These observations are in agreement with the suggestion that the effects of ODC overexpression are dependent on the cell type and on the level of overexpression. We carried out a histological analysis of our mice in order to study whether the life-long deregulated ODC expression effected on physiology and pathology of tissues. We found several abnormalities in transgenic mice ranging from inflammatory processes to spontaneous tumors. These could be caused by deregulated ODC expression since increased occurrence of spontaneous tumors has been reported in other transgenic mice overexpressing ODC (Megosh *et al.* 1995). Similarly, pancreatis we found in several mice has been detected in rats overexpressing SSAT (Alhonen *et al.* 2000). Overexpression of SSAT increases putrescine levels as does ODC overexpression. Another alternative is that the integration of transgene has changed expression of some endogenous gene(s) leading to pathological alterations in tissues.

The number of infertile transgenic mice was high. Histological examination suggested that alterations in maturation of gonadal cells might have been responsible for the high rate of infertility. Previously, reduced spermatogenesis in infertile transgenic mice has been reported (Halmekytö *et al.* 1991a). At the same study, also a dramatic increase in the glandular tissue of the preputial gland was described. In our material, the structure of the preputial gland was highly variable both in the control and transgenic animals and we speculate that the reduction of glands observed in our infertile transgenic male mice may have been caused by excessive secretion and consequent tissue depletion. The increased infertility of transgenic males in our study is very likely due to overexpression of ODC, since we detected significant increase in ODC activity in testis and polyamines are postulated to be of great importance in spermatogenesis (reviewed in Coffino 2000). The role of polyamines is spermatogenesis is further emphasized by the existence of testis-specific antizyme form, antizyme 3. Furthermore, tissue-specific variant of hypusinated eIF-5A, eIF-5A2 (Jenkins *et al.* 2001), and a recently discovered ODC-like protein (Pitkänen *et al*. 2001) are expressed strongly only in brain and testis. In contrast to most male sexual organs, the ovaries of the transgenic ODC mice did not overexpress ODC, suggesting that the observed degeneration of ovaries of infertile transgenic mice were not directly related to ODC gene expression.

## 6.5 Conclusions

In this study we wanted to elucidate the role of the first and crucial enzyme of polyamine biosynthesis, i.e. ornithine decarboxylase, and its protein inhibitor, antizyme, in the polyamine metabolism of CNS. In particular we were interested in whether the guanosine 5'-phosphate activatable ODC activity previously detected in mammalians only in some tumours, could be found in CNS as we hypothesized. We were able to show that ODC in mammalian brain lysates is activated by GTP, and that this activation is more significant after antizyme is separated from ODC in the gel filtration chromatography. GTP-activatable ODC was more resistant to heat denaturation and displayed higher $V_{max}$ than kidney ODC. We continued our studies by localizing ODC and antizyme expression in the adult rat brain using both *in situ* hybridization and immunocytochemistry. We detected wide and mostly overlapping expression patterns. Interestingly, we observed that in some areas of brains antizyme had mainly nuclear localization. In addition, we mutated aspartate-233 to valine in mouse ODC. The mutation increased $K_m$ values for the cofactor PLP and the substrate L-ornithine as well as $K_i$ value for the inhibitor DFMO.

Another major aim of this study was to generate transgenic mouse line expressing ODC cDNA under the control of a MMTV-LTR promotor, and to use it to study the pathological and physiological effects of deregulated ODC expression during the life of transgenic animals. We detected the most significant changes in ODC expression in reproductive organs of male mice. The number of infertile mice was high supporting earlier reports about the importance of balanced polyamine metabolism for normal spermatogenesis. The involvement of polyamines in the fertility of females remained unproven. Transgenic mice were prone to various pathological conditions, which may be due to deregulated polyamine methabolism.

The most interesting results of this study were the detection of GTP-activatable ODC in normal mammalian tissue and the detection of the mainly nuclear localization for antizyme in some areas of brain. These both observations were reported for the first time in our study. The latter has already confirmed by others and may imply together with some other recent observations that the antizyme is much more versatile and multifunctional protein than recognised still few years ago.

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