Amino Acids (2004) 27: 367–372
DOI 10.1007/s00726-004-0116-2

Amino Acids
Printed in Austria

Polyamines biosynthesis and oxidation in free-living amoebae

Review Article

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Received April 26, 2004
Accepted July 15, 2004
Published online October 15, 2004;

Summary. In this paper we describe the polyamine biosynthesis and oxidation processes, giving an overview about recent results in free-living Amoebae.

The protozoa polyamine levels are different in comparison with mammalian cells. Also, the polyamine levels in protozoa cells change if these species are pathological or not for the human beings. All the amoeba strains show high concentrations of 1,3-diaminopropane (DAP), spermidine and acetylspermidine while spermine is absent. In these amoeba a considerable polyamine oxidase activity has been found, which acts on N^8-acetylspermidine, but not on free polyamines. This enzyme is responsible, together with polyamine acetylase, of DAP synthesis whose function is not well known.

Keywords: Polyamines – Acetylpolyamines – Ornithine decarboxylase – S-adenosylmethionine decarboxylase – Polyamine oxydase – Free-living amoebae

Abbreviations: DAP, 1,3-diaminopropane; DFMO, Difluoromethyl ornithine; ODC, ornithine decarboxylase; SAMDC, S-adenosylmethionine decarboxylase; DAO, diamine oxidase; PAO, polyamine oxidase; MGBG, methylglyoxal bis (guanylhydrazone); PLP, pyridoxal phosphate; DTT, dithiothreitol; Dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; PAM, primary amoebic meningoencephalitis; GAE, granulomatous amoebic encephalitis; CNS, central nervous system

Introduction

Aliphatic polyamines putrescine, spermidine and spermine, occur ubiquitously in organisms and have important functions in the stabilization of cell membranes, biosynthesis of informing molecules, cell growth and differentiation, as well as adaptation to osmotic, ionic, pH and thermal stress (Slocum et al., 1984; Tabor and Tabor, 1984, 1985, 1989; Pegg, 1986).

These cationic substances are implicated in multiple functions, therefore it is not surprising that intracellular levels of polyamines are regulated by different mechanisms (Igarashi and Kashiwagi, 1999, 2000).

The inhibition of polyamine metabolism has important pharmacological and therapeutic implications for the control of physiological processes, reproduction, cancer and parasitic diseases (Sjoerdsma and Schechter, 1984; Williams-Ashman and Seidenfeld, 1986; Shukla, 1990).

Difluoromethyl ornithine (DFMO), the irreversible inhibitor of ornithine decarboxylase, has proved to be curative in trypanosomiasis, coccidiosis, and certain other protozoan infections (Bacchi, 1981; Sjoerdsma and Schechter, 1984; Schechter and Sjoerdsma, 1986). Polyamine metabolism also appears to be a target for the action of berenil, pentamidine and other cationic antiprotozoan drugs (Bacchi, 1981; McCann et al., 1983; Pegg, 1986). Studies on Ornithine Decarboxilase (ODC) and polyamine metabolism in amoebae are rather limited and commonly refer to the effect of ODC inhibitors such as DFMO and other substrate analogs on cell proliferation (Kim et al., 1987a; Gillin et al., 1984; Chayen et al., 1984). Some amoeba species, such as *E. invadens* and *E. histolitica*, have displayed remarkable resistance to a variety of drugs including DFMO (Ferrante et al., 1984; Gupta et al., 1987a; Kim et al., 1987a). Recent reports have suggested the idea that parasites with an high turnover of ODC are resistant to DFMO because they always contain a fraction of newly synthesized and active enzyme, therefore not DFMO inhibited, (Heby and Persson, 1990) sufficient to produce small amounts of putrescine rapidly converted into spermidine, which can support protozoan proliferation (Carrillo et al., 2000).

To gain further insight into polyamine metabolism in parasitic amoebae, it has been considered important to extend our studies to different species of free-living amoeba, e.g. *Naegleria* and *Acanthamoeba* spp., which are clinically important because they occasionally infect human beings.

These species are widely distributed and, when infecting humans via the mucosal surface, spread through the haematic system causing skin, ear, eye or chronic central nervous system (CNS) infections. CNS disease due to *Naegleria* spp., called primary amoebic meningoencephalitis (PAM), occurs in healthy people after exposure to polluted waters in heated swimming pools, rivers and lakes. The clinical course is acute, often fulminant and characterized pathologically by necrotizing haemorrhagic meningoencephalitis, involving mainly the base of brain, brainstem and cerebellum. In contrast, some *Acanthamoeba* spp. cause an opportunistic CNS disease, ''granulomatous amoebic encephalitis'' (GAE), in chronically and pathologically immunocompromised subjects, like in AIDS.

Polyamine distribution and synthesis

In many organisms, polyamines originate from L-ornithine and methionine. Ornithine decarboxylase (EC 4.1.1.17), a key enzyme in polyamine metabolism, decarboxylates L-ornithine to yield putrescine which is then converted to higher polyamines spermidine and spermine by successive addition of aminopropyl groups derived from decarboxylated S-adenosylmethionine (Pegg, 1986). Results reported from studies with some *Acanthamoeba* species seem to support the view that these protozoa possess a biosynthetic pathway for spermidine via putrescine (Kim et al., 1987b). Nevertheless, the major polyamine component is the diamine 1,3-diaminopropane (DAP), then spermidine and putrescine (Table 1), while spermine is detected in cells grown in complex but not in chemically defined media (Kim et al., 1987b).

The small amounts of norspermidine detected occasionally in amoebae by other Authors (Kim et al., 1987b) may result from the interaction with a large pool of DAP in cells with enzymes for spermidine biosynthesis. The very low pool of putrescine reported for amoeba may be linked to very high stimulation of S-adenosylmethionine decarboxylase (SAMDC) by putrescine and in consequence of the use of the putrescine for the synthesis of spermidine. The maintenance of a low pool of putrescine in the cell may also be due to the fact that, normally, higher concentrations of putrescine can cause lysis of the cell like has been reported in *A. culbertsoni* (Srivastava and Shukla, 1982).

Ornithine decarboxilase (ODC) activity has been detected in all species of free-living amoebae considered (Table 2). ODC activity is regulated by inactivation of the enzyme (Holtta and Pohjanpelto, 1986). *In vitro* studies have demonstrated that ODC from lower eukaryotes, such as fungi (Calvo-Mendez et al., 1987; Lapointe and Cohen, 1983), is strongly inhibited by putrescine. The ODC activity studied here was inactivated by the three polyamines in a mode essentially similar to that previously described (Calvo-Mendez et al., 1993), that is, in an increasing order of effectiveness of spermine > spermidine > putrescine (Table 3). It is worth noting that these polyamines bear

Table 1. Polyamine contents in free-living amoeba species

Polyamines	Acantham. Russ	Acantham. Polyphaga	Acantham. Castellani	Acantham. Culbertsoni	Naegleria Lovaniensis	Naegleria Australiensis
DAP	57.7 ± 6.3	18.6 ± 2.7	61.5 ± 5.4	61.9 ± 6.2	38.6 ± 2.1	55.6 ± 6.1
PUT	4.2 ± 0.6	1.9 ± 0.4	5.2 ± 1.3	5.6 ± 0.7	1.7 ± 1.0	2.0 ± 0.3
SPD	12.4 ± 1.6	7.6 ± 1.2	15.3 ± 1.9	16.8 ± 1.4	2.8 ± 0.5	5.1 ± 1.3
SP	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
N^8-AcSPD	6.1 ± 0.5	4.4 ± 0.5	10.2 ± 1.9	10.5 ± 1.7	4.1 ± 0.4	5.9 ± 0.6
AcSP	2.1 ± 0.3	1.5 ± 0.1	4.9 ± 0.3	5.7 ± 0.3	1.5 ± 0.2	2.0 ± 0.2
AcPUT	2.9 ± 0.2	1.3 ± 0.1	3.1 ± 0.3	4.0 ± 0.8	1.1 ± 0.2	2.4 ± 0.3
N^1-AcSPD	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.

DAP, 1,3-diamonipropane; *PUT*, putrescine; *SPD*, spermidine; *SP*, spermine; *N^8-AcSPD*, N^8-AcetylSpermidine; *AcSP*, AcetylSpermine; *AcPut*, AcetylPutrescine; *N^1-AcSPD*, N^1-AcetylSpermidine; *N. D*, not detected

Polyamines in the perchloric acid (PCA) homogenate were converted to their dansyl derivatives and analysed according to Wittich et al. (1987)

Values are expressed as nmoles/10^6 cells and represent the mean ± s.d. for four different determinations

Homogenates of 72 h-old cultures was used for the assay

Table 2. Ornithine decarboxylase activity in amoeba species

Amoebae strains	Enzyme activity (nmoles CO_2 liberated/h/mg protein)
Acanthamoeba Culbertsoni	2.14 ± 0.54
Acanthamoeba Russ	1.89 ± 0.73
Acanthamoeba Polyphaga	0.76 ± 0.09
Acanthamoeba Castellani	1.19 ± 0.15
Naegleria Lovaniensis	0.79 ± 0.10
Naegleria Australiensis	0.98 ± 0.07

Homogenate of 72 h-old culture was used as source of the enzyme. Values represent the mean ± s.d. of four different determinations
ODC activity was assayed by measuring the liberation of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described earlier (Calvo-Mendez et al., 1993), except that the assay buffer was 50 mM Tris-HCl pH 8.5, the reaction mixture contained 0.2 mM pyridoxal phosphate (PLP) and 2 mM dithiothreitol (DTT)
Protein was estimated by method of Lowry et al. (1951) or that of Bradford (1976)

2, 3 and 4 net positive charges, respectively. Whether the ability to inhibit ODC is affected by the cationic strength of these molecules remains an interesting possibility that is worthy of consideration for further investigations. These studies support the view that Acanthamoebae employs a biosynthetic pathway for spermidine with putrescine as an intermediate (Kim et al., 1987b; Gupta et al., 1987a).

It has been shown that the multiplication of *A. culbertsoni* is inhibited by Methylglyoxal bis(guanylhydrazone) (MGBG), a cytotoxic drug that inhibits cell proliferation (Kay and Pegg, 1973) and is also a potent competitive inhibitor of SAMDC activity. Moreover, it contains an MGBG-sensitive S-adenosylmethionine decarboxylase enzyme stimulated by putrescine (Gupta et al., 1987a, b).

MGBG, as well as numerous of its analogs, possesses significant curative properties against most infections caused by *Trypanosoma* and *Leishmania* (Bitonti et al., 1986). MGBG has also been shown to have antileishmanial activity (Bachrach et al., 1979). The antileishmania effect of MGBG was reversed by treatment with spermidine or spermine. It is unlikely that the spermine reversal of MGBG effect is due to an increased degradation of spermine into spermidine by spermine acetyltransferase or polyamine oxidase, two enzymes involved in the conversion of spermine to spermidine in eukaryotic system (Mukhopadhyay and Madhubala, 1995).

Polyamine oxidation

In mammalian cells, however, spermine and spermidine are converted back into putrescine by the combined actions of the spermidine/spermine N^1-acetyltransferase and polyamine oxidase and rechanneled into the polyamine biosynthetic process (Pegg and McCann, 1982, 1988; Seiler and Heby, 1988). In *Acanthamoeba culbertsoni*, a cytosolic polyamine N-acetyltransferase that preferentially catalyses the acetylation of spermidine in the N^8-position was identified (Shukla et al., 1996). In addition to spermidine, the enzyme also catalysed the acetylation of spermine and putrescine.

It is obvious from the ratio of the products that the preferred acetylation site was the N^8-position (Shukla et al., 1996), which shows a clear preference for the aminobutyl end of polyamine. In this respect, the enzyme is different from the mammalian cytosolic polyamine N-acetyltransferase, which has been shown to have selectivity for the aminopropyl moieties of polyamines and, therefore, to form exclusively N^1-acetylspermidine from spermidine when incubated with acetyl-Co A (Della Ragione and Pegg, 1983). On the other hand, it resembles the calf-liver nuclear N-acetyltransferase, which, in addition to

Table 3. Effects of polyamines treatment on ODC activity in amoeba species

Amoeba species	ODC activity after putrescine	ODC activity after spermidine	ODC activity after spermine
Acanthamoeba Culbertsoni	90.8 ± 0.6	70.0 ± 0.5	49.7 ± 0.3
Acanthamoeba Russ	92.8 ± 0.3	72.8 ± 0.4	55.8 ± 0.5
Acanthamoeba Polyphaga	96.6 ± 0.1	74.6 ± 0.6	59.3 ± 0.4
Acanthamoeba Castellani	91.3 ± 0.3	71.3 ± 0.4	52.9 ± 0.2
Naegleria Lovaniensis	94.9 ± 0.2	73.9 ± 0.2	56.5 ± 0.4
Naegleria Australiensis	93.8 ± 0.1	72.9 ± 0.3	54.6 ± 0.3

Data represent the % of the enzyme activity after polyamine addition in growth medium respect to the enzyme activity without polyamines
Putrescine, spermidine and spermine were added to the culture medium at concentration of 0.1 mM respectively

catalysing histone acetylation, is also known to acetylate spermidine in the N^8-position (Libby, 1978). In mammalian cells, the role of N^8-acetylspermidine in polyamine metabolism remains unclear. Apart from being an excretory product found in human, mouse and rat urine (Seiler et al., 1981), N^8-acetylspermidine is known only to be a substrate for a deacetylating enzyme that converts this acetylated spermidine derivative back into spermidine (Blankenship and Marchant, 1984), suggesting a reversible sequestration of excessive spermidine. However, the metabolism of N^8-acetylspermidine in *A. culbertsoni* is totally different from that of the mammalian host (Shukla et al., 1992).

The presence of a potent polyamine oxidase (PAO) activity has been demonstrated in the cell-free extracts of *A. culbertsoni* (Kishore et al., 1993). The enzyme utilised N^8-acetylspermidine as the preferred substrate while the N^1-acetylspermidine was a very poor substrate for the enzyme. A very low activity was also detected with N-acetylputrescine. Free polyamines, such as DAP, putrescine, spermidine and spermine did not serve as substrates for the enzyme (Kishore et al., 1993).

The polyamine oxidase present in *A. culbertsoni* has been observed to have specificity for N^8-acetylspermidine as a substrate converting it into 1,3-diaminopropane (DAP) (Shukla et al., 1996), whereas the flavine adenine dinucleotide (FAD) dependent polyamine oxidase of rat liver cytosol has virtually no activity on this diamine (Bolkenius and Seiler, 1981).

It can therefore be assumed that the acetylation of spermidine in the N^8-position followed by the oxidative deamination of N^8-acetylspermidine to DAP by the polyamine oxidase, as previously reported (Shukla et al., 1996), explains the origin of the large amounts of this diamine detected in all the amoeba species here considered.

Conclusions

The present results demonstrate that DAP, acetylspermidine and spermidine are the principal polyamines in mentioned amoeba species in which putrescine, acetylputrescine and acetylspermine were also detected at low concentration and spermine was absent, according to the observations of Zhu et al. (1989). These Authors found high concentrations of DAP, low concentration of putrescine and absence of spermine in growing cells of *A. castellani*. On the other hand, Kim et al. (1987b) reported appreciable amounts of spermine in *A. castellani* grown in complex medium (Kishore et al., 1993), but was not present in cells grown in chemically defined medium (Shukla et al., 1990a). These observations have also shown that these protozoa contain relatively high concentrations of acetylated polyamines, in contrast to mammalian cells in which acetylated polyamine concentrations are normally very low or not detectable, and acetylspermidine or acetylspermine are not present or found in very low concentration (Tabor and Tabor, 1984). It is possible that this is a reflection of the greater metabolic flexibility required by the protozoa, in order to rapidly respond to the sudden metabolic stresses imposed by frequently experienced environmental perturbations. The pattern for the free-living Amoebae spp. (see Table 1) is, however, in agreement with reports on mammalian cells and bacterium, concluding that acetylation of polyamines is a physiological deactivation process, which converts the polyamines to a metabolically inert form (Wallace, 1987; Seiler, 1987). The consequence of polyamine acetylation is to decrease the positive charge, which helps to displace them from anionic binding sites as well as to increase their lipophilic properties that may aid transport processes (Seiler, 1987). Polyamine acetylation appears to be a component of a cellular mechanism involved in polyamine turnover and excretion (Seiler, 1987), which could function as a means of reducing the polyamine intracellular concentration. In addition, storage of the acetylated (metabolically inactive) form of polyamines in the dormant cell (cysts) could provide the newly activated cell with a readily available source of polyamines for growth purposes. In fact, in mammalian tissue putrescine is formed from acetylputrescine and spermidine from N^8-acetylspermidine by direct hydrolysis, and putrescine is formed from N^1-acetylspermidine and spermidine from N^1-acetyl spermine by transformation (Seiler, 1987).

The major polyamine component of *Acanthamoeba* species, including *A. culbertsoni*, is the diamine 1,3-diaminopropane (Zhu et al., 1989; Shukla et al., 1990b; Kishore et al., 1993). The formation of DAP from spermidine and spermine by the action of diamine oxidase (DAO) and polyamine oxidase occurs in plants (Smith, 1985). Previous work (Shukla et al., 1992) revealed that this diamine was the product of the oxidative deamination of N^8-acetylspermidine by a novel type of polyamine oxidase in *A. culbertsoni*. Furthermore, the same authors have identified and characterized a polyamine N-acetyltransferase that catalyses the acetylation of spermidine in both the N^1- and N^8-positions (Shukla et al., 1996).

The presence of a polyamine oxidase which utilises N^8-acetylspermidine as the preferred substrate (Kishore et al., 1993), indicates a role for this enzyme in the

production of DAP, the major polyamine found in *Acanthamoeba*. However, the significance of DAP biosynthesis, regulation, and response to environmental parameters as well as its function in Amoebae remains to be elucidated.

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