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Biochimica et Biophysica Acta 1284 (1996) 247–252

BBA Biochimica et Biophysica Acta

Spermine binding to liver mitochondria

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Received 2 April 1996; revised 16 July 1996; accepted 17 July 1996

Abstract

Non-equilibrium binding of spermine to mitochondrial membranes is studied in rat liver mitochondria by applying a new thermodynamic treatment of ligand–receptor interactions (Di Noto, V., Dalla Via, L., Toninello, A. and Vidali, M. (1996) Macromol. Theory Simul. 5, 165–181). The presence on mitochondrial membranes of two spermine binding sites, both with monocoordination, is demonstrated. The calculated binding energy is characteristic for weak interactions. The treatment allows also to evaluate the variations of the molar fraction ratio of spermine bound to sites 1 and 2 as function of total bound spermine. The possible role of the two sites is discussed.

Keywords: Spermine; Binding site; Ligand–receptor interaction; Mitochondrion; (Liver)

1. Introduction

Due to their high binding capacity, polyamines stabilize various membrane systems including mitochondria [1]. The binding of spermine to mitochondrial membranes may explain the protection exhibited by this compound against aging damages [2,3] and the prevention of permeability transition of liver and heart mitochondria promoted by Ca^{2+} in association with P_i [4,5], or peroxidants [6,7], or acyl-CoA [2].

Polyamines are transported in liver [8–10], heart [2] and brain [11] mitochondria by an electrophoretic mechanism requiring high membrane potential and exhibiting a non-ohmic force–flux relationship.

Due to the existence in mitochondria of a bidirectional transport system for spermine [12], its concentration may undergo variations so modulating the activity of some matrix enzymes such as pyruvate dehydrogenase [13–15] and citrate synthase [16] and the replication of mitochondrial DNA [17]. Furthermore spermine promotes mitochondrial uptake of some cytosolic enzymes such as the precursor of ornithine carbamoyl transferase [18], hexokinase [19], casein kinase II [20] and casein kinase I [21].

In this paper we have studied the binding of spermine to intact liver mitochondria membranes. The experimental results submitted to the thermodynamic treatment of Scatchard and Hill analyses previously described [22] demonstrate the presence on mitochondrial membranes of two specific sites for spermine, both with monocoordination. Furthermore energy values of polyamine binding to mitochondrial membranes are given.

2. Experimental procedures

Rat liver mitochondria were isolated in 0.25 M sucrose and 5 mM Hepes-HCl (pH 7.4) by conventional differential centrifugation. Protein concentration of mitochondria was estimated by a biuret method with bovine serum albumin as standard. Incubations were carried out at 20°C for 5 min with 1 mg of mitochondrial protein/ml in the standard medium used in previous studies for spermine transport [2,8–12]: 200 mM sucrose, 10 mM Hepes-HCl (pH 7.4), 5 mM succinate, 1.25 μM rotenone, 1 mM phosphate. Sodium salts were used.

Additions of [^{14}C]spermine are indicated in the descriptions of specific experiments. Uptake of [^{14}C]spermine was determined by a centrifugal-filtration method as previously described [8,9]. Membrane potential ($\Delta\Psi$) was measured as reported in [9]. All mitochondrial preparations, incubated in the standard medium, exhibited $\Delta\Psi$ values in the range of 175 to 185 mV.

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PII S0005-2736(96)00140-X

Binding constants and consequent interaction energies were calculated by utilizing Eq. (1) for Scatchard and Eq. (2) for Hill analysis. The rationale for these equations, both obtained by the same general equation (see [22]), considers receptors having groups of different binding sites (s) where each may have multiple occupancies (n_i).

$$\frac{[B]}{[F]} = \sum_{i=1}^{s} \{[B_{\max,i}] - [B_i]\} \cdot \left[\frac{1}{K_{i,1}(t)} + \varepsilon_i(F)\right] \quad (1)$$

$$\ln\left\{\frac{[B]}{[B_{\max}] - [B]}\right\} = \ln\left\{\sum_{i=1}^{s} x_i(F)\left[\frac{1}{K_{i,1}(t)} + \varepsilon_i(F)\right]\right\} + \ln[F] \quad (2)$$

where

$$\varepsilon_i(F) = \sum_{k=2}^{n_i} \frac{[F]^{k-1}}{\prod_{j=1}^{k} K_{i,j}(t)}$$

represents the appropriate measure of the extent of multiple coordination on the i-th site. $[B_{\max,i}]$ is the maximum i-th sites concentration that may be bound by the ligand, $[B_i]$ is the concentration of i-th sites bound by the ligand, $B_{\max}$ is the maximum receptor-bonded ligand concentration and $[B]$ is the receptor-bonded ligand concentration. The free ligand concentration is $[F]$ and $K_{i,j}(t)$ the affinity constant of the ligand for the i-th site and j-th occupancy number, t is time.

Fitting was performed using a FORTRAN program developed in our laboratory. This realizes the minimization by using the MINUIT subroutine by using the Simplex algorithm [23] which is called from the main program. The program is written in VAX FORTRAN 5.0 and it works in the operating system VMS version 5.0.

The distribution of total bound spermine on their respective binding sites has been calculated by parameter $X_i(F)$. This parameter is the mole fraction of the i-th site that may be bound in the receptor and has been calculated by means of Eq. (3) [22]

$$X_i(F) = ([B_{\max,i}] - [B_i])/([B_{\max}] - [B]) = 1/(1 + \beta_i[F]) \quad (3)$$

where the quantity β_i can be seen as a parameter that describes the influence of the parallel filling of the other k-th sites in comparison to those of the i-th site.

Spermine binding energy and Hill factor have been calculated by means of algebraic transformations of Eq. (2) as previously developed [22].

3. Results

The results reported in Fig. 1 show that spermine uptake by energized mitochondria during the first 5 minutes of incubation is linear with time at all the concentrations tested. The linear trend of spermine uptake allows to extrapolate on the y-axis (see dotted line segments and open circles in Fig. 1) the different aliquots of polyamine that bind to the mitochondrial membrane at zero-time. These aliquots plotted versus the total external concentration (inset in Fig. 1) show that spermine binding to mitochondrial membrane tends to saturation.

Fig. 2a and b reports spermine binding analysis using the thermodynamic treatment of Scatchard and Hill (see [22]). Binding data (Fig. 1), plotted as dependence of $[B]/[F]$ on $[B]$ and $\ln\{[B]/([B_{\max}] - [B])\}$ on $\ln[F]$, were simulated with two series of curve profiles belonging to Eqs. (1) and (2), obtained via computer simulations for several range of parameters s and n_i. The theoretical curves that satisfactorily fitted the experimental data (solid

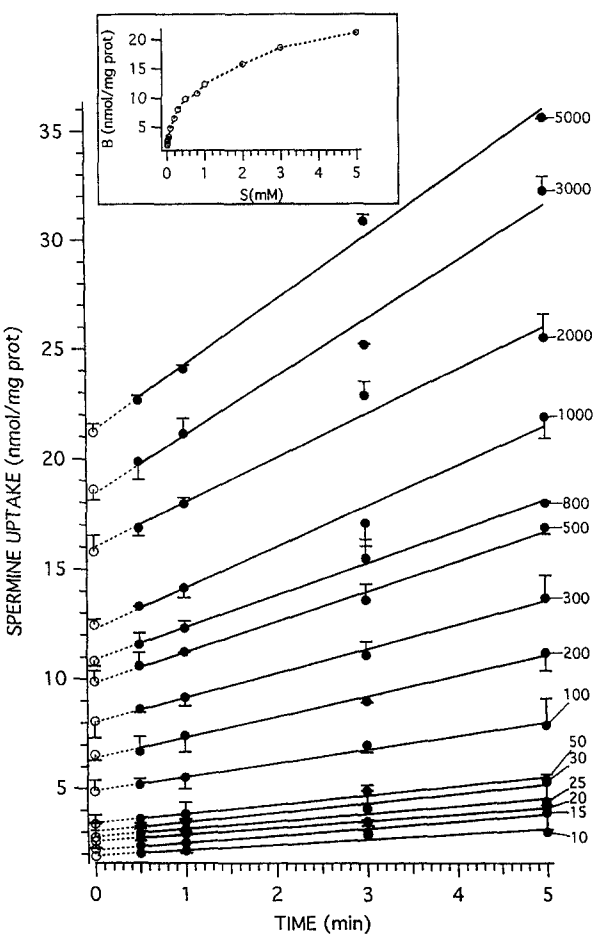

Fig. 1. Concentration-dependent spermine uptake by energized mitochondria. Rat liver mitochondria were incubated in the standard medium as indicated in Section 2 in the presence of different [^{14}C]spermine concentrations in the range of 10–5000 μM (S) (0.05 μCi/ml) as indicated along the curves. Open circles give the amount of spermine that binds instantaneously at zero-time. In the inset zero-time bound spermine (B) has been plotted as a function of the exogenous spermine concentration (S). The results represent the mean value of 12 experiments.

Table 1
Spermine binding parameters determined by curve fitting of Eqs. (4) and (5) in the data of Scatchard and Hill plots, respectively

B_{max} (nmol/mg prot)	B_{max1} (nmol/mg prot)	B_{max2} (nmol/mg prot)	$K_{1,1}$ (mol/l)	$K_{2,1}$ (mol/l)	β_1 (mg prot/nmol)	χ [b]
23.08(4) [a]	8.29(2)	14.79(2)	$42.3(3) \cdot 10^{-6}$	$909(12) \cdot 10^{-6}$	0.161(3)	0.0339

[a] Standard deviations in the least significant digits are given in parentheses.
[b] χ indicates the goodness of fitting.
$\chi = (\Sigma|(N_o) - (N_c)|)/(\Sigma|(N_c)|)$, where (N_o) is the experimental value and (N_c) is the calculated one. For a Scatchard plot, N is given by B/F and for a Hill plot by $\ln[(B)/(B_{max} - B)]$.

lines of Fig. 2a and b) are typical for two binding sites, S_1 and S_2, both with monocoordination. The corresponding Eqs. (4) and (5), obtained by substituting $s = 2$ and $n_i = 1$ in Eqs. (1) and (2) and by considering $t = 0$, are:

$$\frac{[B]}{[F]} = ([B_{max}] - [B]) \cdot \left[\frac{\Delta K}{1 + \beta_1[F]} + \frac{1}{K_{2,1}} \right] \quad (4)$$

where $\Delta K = (1/K_{1,1}) - (1/K_{2,1})$

$$\ln\left\{ \frac{[B]}{[B_{max}] - [B]} \right\} = \ln\left\{ \frac{\Delta K}{1 + \beta_1[F]} + \frac{1}{K_{2,1}} \right\} + \ln[F] \quad (5)$$

In Table 1 are reported spermine binding parameters obtained by using either the Scatchard or the Hill data representation method. Obviously the fitting of Eqs. (4) and (5) to the Scatchard and Hill data representations gives the same parameters owing to the fact that they are obtained from the same binding experiments and that Eqs. (1) and (2) are obtained by the same general equations (see [22]). These analyses demonstrate the presence on mitochondrial membranes of two binding sites, each binding one equivalent of spermine. The total binding site concentration is 23.08 nmol/mg protein distributed between S_1 and S_2 sites in the percentages of 36 and 64, respectively. The dissociation constants $K_{1,1}$ and $K_{2,1}$ of S_1 and S_2 sites, respectively, demonstrate that S_1 has a higher affinity than S_2. In fact $K_{1,1}$ is 21.5 times lower than $K_{2,1}$. β_1 describes the possible influence of the parallel filling of S_2 on that of S_1 site. This parameter is determined by Eq. (3).

In Fig. 3 are reported the molar fraction ratios $X_1(F)$ and $X_2(F)$ for the aliquot of free spermine that can bind to S_1 and S_2 sites, respectively. These ratios are also named 'filling molar fractions' [22]. The values are obtained by using parameter β_1 reported in Table 1. The calculations show that by enhancing the amount of bound spermine, $X_1(F)$ diminishes and, conversely, $X_2(F)$ increases. This means that the higher affinity S_1 site is filled before S_2 site as demonstrated in the inset of Fig. 3.

Fig. 4 shows the functional dependence of the overall free energy changes (ΔG) on the concentration of free

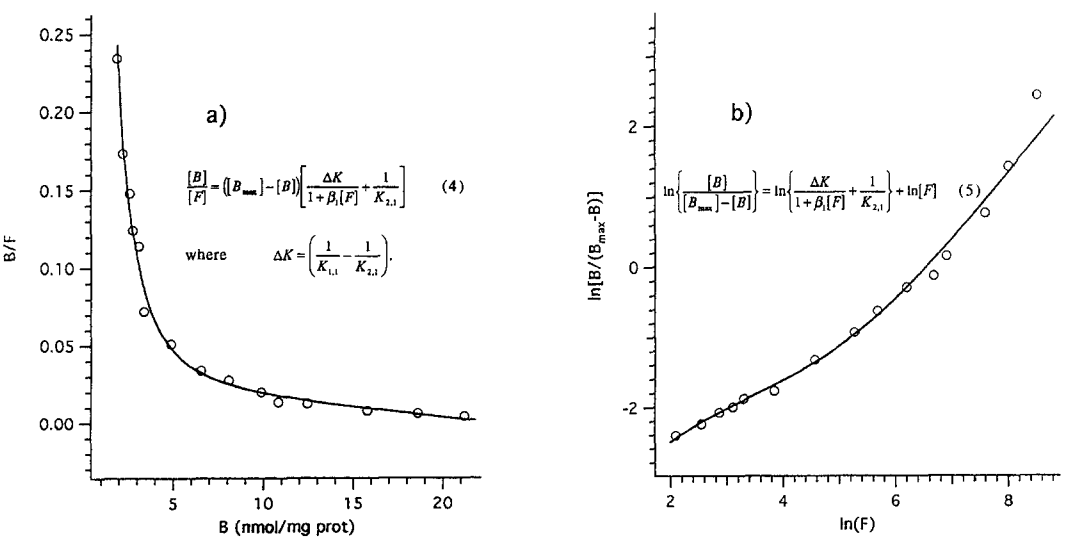

Fig. 2. Spermine binding analysis with the thermodynamic treatment of Scatchard (a) and Hill (b) methods. The experimental data (open circles) have been calculated as follows: bound spermine values (B) as reported in Fig. 1; free spermine concentration (F) by subtracting bound spermine (B) from total spermine concentration (S) as reported in Fig. 1. The continuous lines are the theoretical curves, of which are also reported the corresponding equations, Eq. (4) for Scatchard (a) and Eq. (5) for Hill (b).

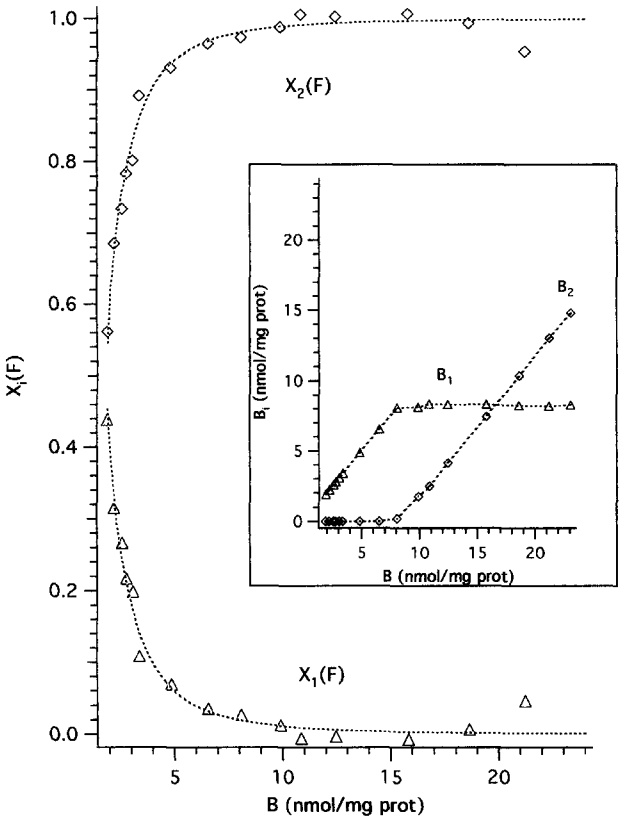

Fig. 3. Molar fraction ratio of spermine binding. The calculations refer to the amount of spermine lacking to fill the binding sites. $X_1(F)$ and $X_2(F)$ are the molar fraction ratio to fill the first (S_1) and the second (S_2) spermine binding site, respectively. For example when mitochondria are incubated in a medium containing 500 μM spermine, the amount of polyamine bound to the membranes is 10 nmol/mg prot. (see results in Fig. 1). This aliquot shows a $X_1(F)$ value of 0.02 and a $X_2(F)$ value of 0.96. By taking into account Eq. (3) and considering $B_{max1} = 8.29$ nmol/mg protein and $B = 10$ nmol/mg protein, is possible to calculate how the aliquot of 10 nmol/mg protein is subdivided between the two sites, that is $B_1 = 8.27$ nmol/mg protein and $B_2 = 1.73$ nmol/mg protein. The inset reports the subdivision of total bound spermine, B, in two aliquots, B_1 and B_2, bound to S_1 and S_2 sites, respectively.

spermine in solution. ΔG have been calculated by means of the general Eq. (6):

$$\Delta G = -RT \ln \frac{B}{(B_{max} - B) \cdot [F]} \qquad (6)$$

The data are fitted satisfactorily by using Eq. (7) characteristic for two sites both with monocoordination:

$$\Delta G = -RT \ln \left\{ \frac{\Delta K}{1 + \beta_1 [F]} + \frac{1}{K_{2,1}} \right\} \qquad (7)$$

In Fig. 5 is reported the value of the Hill factor $n_H(F)$ as a function of the amount of spermine bound to mitochondrial membrane. The Hill factor has been calculated by means of Eq. (8):

$$n_H(F) = 1 + \frac{-\beta_1 \left[\frac{[F]}{1 + \beta_1 [F]^2} \right] \Delta K(t)}{\frac{1}{K_{2,1}(t)} + \left[\frac{1}{1 + \beta_1 [F]} \right] \Delta K(t)} \qquad (8)$$

characteristic for a system where are present two binding sites, both with monocoordination.

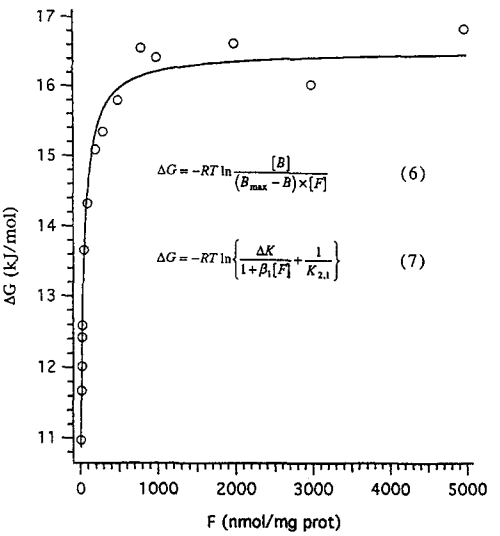

Fig. 4. Free energy changes in spermine binding. The points are obtained by inserting the experimental values of B, F and B_{max} in general Eq. (6). B and F are the same values of Fig. 2a and b respectively, while B_{max} is 8.29 nmol/mg protein (see Table 1). The continuous line is the theoretical curve (see Eq. (7)) which fits the experimental points. The parameters of this equation have been obtained from Table 1. For ΔK value see Eq. (4) and Fig. 2a.

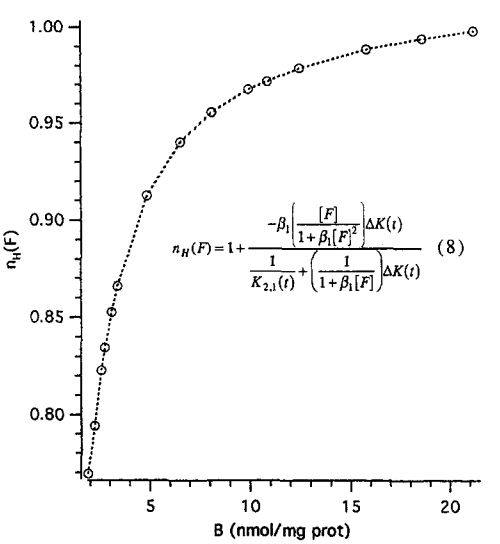

Fig. 5. Hill factor ($n_H(F)$) for spermine binding. B and F, reported in Eq. (8), are the same as in Fig. 2a and b. Other parameters are obtained from Table 1. For ΔK value see Eq. (4) and Fig. 2a.

4. Discussion

Spermine uptake by mitochondria reported in Fig. 1 is the sum of two processes: electrophoretic matrix transport [9,10] and membrane binding. The discrimination between these two processes is difficult. Correct evaluation of spermine binding requires the exclusion of its transport. This is not possible because specific inhibitors of spermine transport are not yet known and mitochondrial deenergization, which similarly blocks the electrophoretic transport, induces changes on binding sites properties (unpublished data). Nevertheless the thermodynamic model previously developed both for equilibrium and far from equilibrium binding processes [22], allows to overcome this problem. In fact it demonstrates that the binding processes could be studied correctly also at zero-time. By taking into account that the amounts of bound spermine at zero-time (Fig. 1) are in a non-equilibrium state, the characteristics of the model are appropriate to study spermine binding sites.

As evidenced by the Scatchard and Hill plots reported in Fig. 2, the experimental data fit perfectly the theoretic curves typical for two monocoordinated sites S_1 and S_2. The two sites S_1 and S_2, that emerge from the curve-fitting, have low-affinity ($K_{1,1} = 42.3$ μM and $K_{2,1} = 909$ μM) and high-capacity binding ($B_{max1} = 8.29$ and $B_{max2} = 14.79$ nmol/mg protein). $K_{1,1}$ and $K_{2,1}$ are non-equilibrium affinity constants (Table 1).

Since bound spermine was calculated by zero-time extrapolation, the evidenced binding sites have to be located on outer membrane and/or on the external surface of the inner membrane. Also the possibility of binding to the external side of the gap junctions has to be considered.

The total free energy value, calculated in Fig. 4, suggests that spermine should bind to mitochondrial sites by conventional weak interactions, probably including cation–π interactions [24]. Strong ion pair [25,26] or covalent bindings have to be excluded.

The thermodynamic model [22] allows to characterize these new parameters: (1) *The coordination number of binding sites.* In our case both sites are monocoordinated. In fact the binding data were fitted very well with both curves (see Eqs. (4) and (5)) characteristic for monocoordination. (2) *The molar fraction ratio $X_i(F)$ and the consequent evaluation of the two sites filling.* As demonstrated by the analysis of Fig. 3, S_1 site is filled at comparatively lower concentrations of the ligand than S_2 site. (3) β_1 *value* (see Eq. (3)). This parameter indicates the possible effect induced on spermine binding to S_1 site by spermine binding to S_2 site. Theoretically β_1 values are in the range between 0 and infinite. Very low values, as in our case (0.161), indicate that the binding on S_2 site has practically no influence on the binding to S_1 site. The independence of the two sites is further supported by the Hill factor (see Fig. 5), which has a value of 1 when the two sites are saturated. However it is possible to note that for very low amount of bound spermine Hill factor has a value less than 1. This could demonstrate the existence of a very weak negative, cooperative effect in these conditions.

The independence of the two binding sites could be related to their different functions. In this regard it should be noted that at the 50 μM concentration spermine completely prevents permeability transition of mitochondria [27] and it is also transported into the mitochondrial matrix [10]. At this concentration, the membrane binds about 3 nmol/mg protein (Fig. 1) and the filling ratio analysis shows that this aliquot binds almost only to S_1 site (see Fig. 3 and also the example reported in the corresponding legend). It is then reasonable to conclude that spermine binding to S_1 site is competent for the prevention of mitochondria permeability transition. The identification of the protein on which is localized S_1 site could be important to individualize the constituents of the transition pore. Furthermore spermine binding to S_1 site also represents the preliminary operation for polyamine transport into the mitochondrial matrix. The existence of a weak membrane binding with low affinity is consistent with this assumption. On the other hand, since only at concentrations higher than 100 μM spermine is able to induce casein kinase II and casein kinase I mitochondrial uptake [20,21], it is quite possible that S_2 site might be involved in the spermine-dependent mitochondrial transport of some proteins [18,21].

By analogy with Ca^{2+} low-affinity binding sites to calsequestrin in the sarcoplasmic reticulum [28] and to calreticulin in the endoplasmic reticulum [29], it can be assumed that the low affinity and high capacity of the two spermine binding sites might play a role in buffering the cytosolic spermine concentration.

Intracellular levels of polyamines are regulated both by biosynthetic and catabolic processes [30] and their transport across the plasma membrane [31]. In particular conditions an increased intracellular requirement for spermine could also be rapidly fulfilled by spermine release from mitochondrial low-affinity binding sites.

Acknowledgements

This research was supported in part by grants from the C.N.R. Progetto Finalizzato Invecchiamento. We are grateful to Mr. Mario Mancon for his skilful technical assistance.

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