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ORIGINAL ARTICLE

Further characterization of agmatine binding to mitochondrial membranes: involvement of imidazoline I_2 receptor

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Abstract Agmatine, a divalent diamine with two positive charges at physiological pH, is transported into the matrix of liver mitochondria by an energy-dependent mechanism, the driving force of which is the electrical membrane potential. Its binding to mitochondrial membranes is studied by applying a thermodynamic treatment of ligand–receptor interactions on the analyses of Scatchard and Hill. The presence of two mono-coordinated binding sites S_1 and S_2, with a negative influence of S_2 on S_1, has been demonstrated. The calculated binding energy is characteristic for weak interactions. S_1 exhibits a lower binding capacity and higher binding affinity both of about two orders of magnitude than S_2. Experiments with idazoxan, a ligand of the mitochondrial imidazoline receptor I_2, demonstrate that S_1 site is localized on this receptor while S_2 is localized on the transport system. S_1 would act as a sensor of exogenous agmatine concentration, thus modulating the transport of the amine by its binding to S_2.

Keywords Agmatine · Binding · Mitochondria · Imidazoline

Abbreviations

ADC	Arginine decarboxylase
AGM	Agmatine
$\Delta\Psi$	Electrical membrane potential
I_2	Imidazoline receptor type two
MAO	Monoamine oxidase
MPT	Mitochondrial permeability transition
NOS	Nitric oxide synthase
PTP	Permeability transition pore
RLM	Rat liver mitochondria
ROS	Reactive oxygen species

Introduction

Agmatine [1-(4-aminobutyl)guanidine] is a biogenic diamine with two positive charges at physiological pH, discovered for the first time by Kossel (1910). This amine, formed along the polyamines biosynthetic pathway, derives from arginine decarboxylation, through arginine decarboxylase (ADC) activity. For many years this compound was believed to be present only in plants, bacteria and invertebrates, since its recent discovery in rat and bovine brain and then in other mammal organs and cell types. In

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these animals agmatine is both synthesized in situ and assimilated by external sources, e.g. nutrition, and then transported into different organs including the liver.

Agmatine exhibits several biological effects: it acts as a ligand of α-adrenergic and imidazoline I_2 receptors, has neuroprotective and neuromodulating properties and it is involved in the synaptic signal transduction pathway (Halaris and Plietz 2007). Moreover, agmatine often exhibits different effects in dependence of the concentration used: it can act as a nitric oxide synthase (NOS) inhibitor, but also it can enhance the in vivo nitric oxide synthesis in endothelial cells. It can suppress cell growth, decreasing the polyamine content (Agostinelli et al. 2010) and activating the apoptotic process in hepatocytes (Gardini et al. 2001).

In mammalian mitochondria, it behaves as a constitutive component, being present in the matrix together with its metabolic enzymes, ADC and agmatinase, and considering the presence of I_2 receptor.

Previous studies, analyzing its interactions with isolated rat liver mitochondria (RLM), demonstrated a double dose-dependent effect: in fact, at high concentrations (1 mM) it exhibits protective properties, acting as a reactive oxygen species (ROS) scavenger and inhibiting the mitochondrial permeability transition (MPT), while at low concentrations (10–100 μM) it exhibits the opposite effects, by inducing the transition pore (PTP) opening (Battaglia et al. 2007).

In addition, agmatine is transported into the mitochondrial matrix by a specific energy-dependent mechanism whose driving force is the membrane potential ($\Delta\Psi$) (Salvi et al. 2006). This transport system is characterized by different features depending on the organs (Battaglia et al. 2010).

Thus, there is a strictly relationship between agmatine and mitochondria, supported by the notorious interaction between biogenic amines and these organelles (Battaglia et al. 2007), in which, as stated before, the imidazoline receptors I_2 are constitutively present. These receptors are 60-kDa proteins localized on mitochondrial membranes and, in particular, by constituting a monoamine oxidase (MAO) domain. This suggests the receptor involvement in the enzymatic activity control. In addition, agmatine can inhibit MAO activity and this effect could reasonably be mediated by its binding on I_2 receptors. (Bousquet 1997; Head et al. 1998; Toninello et al. 2009).

As above mentioned, a previous study demonstrates the presence of a specific agmatine transport system on mitochondrial membranes (Salvi et al. 2006). This system is characterized by an electrophoretic behaviour with an apparent exponential force/flux relationship suggesting the involvement of a channel. The effect of agmatine binding on I_2 receptor is totally unknown, as well as its physiological role on mitochondria.

In this regard, for better characterizing agmatine interaction with mitochondrial membranes and in particular with I_2 receptor, it was applied a thermodynamic treatment of ligand–receptor interactions (Di Noto et al. 1996, 2002) with a new computational procedure developed in Prof. Di Noto's laboratory.

In this study, by applying the treatment of Scatchard and Hill, it was found that mitochondrial membranes possess two agmatine binding sites S_1 and S_2, both with mono-coordination. These studies allow delineating a preliminary hypothesis concerning agmatine binding sites, the first of which could be identified with the imidazoline receptor I_2 while the second one could be the transport site. These studies were performed in the presence of idazoxan, which is considered a ligand of I_2 receptor.

Materials and methods

Materials

All reagents were purchased from Sigma, except where indicated, and were of the highest purity commercially available. [^{14}C]agmatine was prepared as previously reported (Cabella et al. 2001) and its specific activity was 0.5–0.7 mCi mmol^{-1}.

Isolation of RLM

RLM were isolated by conventional differential centrifugation in a standard medium containing 250 mM sucrose, 5 mM Hepes (pH 7.4) and 1 mM EGTA; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard.

These studies were performed in accordance with the guiding principles in the care and use of animals and were approved by the Italian Ministry of Health.

Standard incubation procedure for RLM

RLM (1 mg prot/ml) were incubated in a water-jacketed cell at 20°C. The standard medium contained 200 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM succinate, 1.25 μM rotenone, 1 mM phosphate. Variations and/or other additions are described with individual experiments presented.

Agmatine uptake and binding analyses

Uptake of [^{14}C]agmatine was determined by a centrifugal filtration method, as previously described (Toninello et al. 1985).

Binding parameters were obtained by applying thermodynamic treatment of ligand–receptor interactions (Di Noto et al. 1996; Dalla Via et al. 1996; Di Noto et al.

2002). For the simulation of agmatine binding (B) as a function of the free agmatine amount in solution (F) it was applied the following equation, characteristic of a system with two binding sites:

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

The binding constants and the consequent energies of interaction were determined using the following Eq. 2 for Scatchard analyses and Eq. 3 for Hill analyses. The rationale of these equations, both obtained from the same general equation (Di Noto et al. 1996, 2002) considers receptors with different groups of binding sites (s) where each one could have multiple coordination (n_i). The subsequent algebric elaborations were previously developed (Dalla Via et al. 1996)

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

$$\ln\left\{ \frac{[B]}{[B_{\max,i}] - [B]} \right\} = \ln\left\{ \sum_{i=1}^{S} \chi_i(F) \left[\frac{1}{K_{i,1}(t)} + \varepsilon_i(F) \right] \right\} + \ln[F] \quad (3)$$

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 ith sites. $[B_{\max,i}]$ is the maximum concentration of the ith sites that may be bound by the ligand, $[B_i]$ is the concentration of ith sites bound by the ligand, $[B_{\max}]$ is the maximum receptor-bound ligand concentration and $[B]$ is the concentration bound to the receptor. $[F]$ is the free ligand concentration, $K_{i,j}(t)$ is the affinity constant of the ligand for the ith site, j is the occupancy number, and t is the time. Equations 4 and 5 were calculated substituting $s = 2$ and $n_i = 1$ in Eqs. 2 and 3 and by considering $t = 0$.

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

where

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

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

Fitting was performed through the computational method described below (see next section).

The distribution of total bound agmatine on its binding sites was calculated by the parameter $X_i(F)$. This parameter is the mole fraction of the ith site that may bound in the receptor and has been calculated by means of Eq. 6:

$$X_i(F) = ([B_{\max,i}] - [B_i])/([B_{\max}] - [B]) = 1/(1 + \beta_i[F]) \quad (6)$$

where β_i is a parameter that describes the influence of the parallel filling of the other kth sites in comparison to filling of the ith site.

The functional dependence of the overall free energy changes (ΔG) on the concentration of free agmatine in solution was calculated from the general Eq. 7:

$$\Delta G = -RT\ln \frac{B}{(B_{\max} - B) \cdot [F]} \quad (7)$$

These data are fitted satisfactorily using Eq. 8 characteristic for two sites both with mono-coordination:

$$\Delta G = -RT\ln\left\{ \frac{\Delta K}{1 + \beta_1[F]} + \frac{1}{K_{2,1}} \right\} \quad (8)$$

The Hill factor $n_H(F)$ has been calculated by means of Eq. 9:

$$n_H(F) = 1 + \frac{-\beta_1 \left[\frac{[F]}{1+\beta_1[F]^2} \right] \Delta K(t)}{\frac{1}{K_{2,1}(t)} + \left[\frac{1}{1+\beta_1[F]} \right] \Delta K(t)} \quad (9)$$

characteristic, again, for a system where two binding sites are present, both with mono-coordination.

Data analyses and fitting methods

Binding parameters and consequent energies were determined using Eq. 2 for Scatchard and Eq. 3 for Hill analysis. After that, $[B_{\max,i}]$, the maximum ith sites concentrations that may be bound by ligand, were determined by fitting Eq. 1 on the $[B]$ versus $[F]$ plots. The fits were performed using a home written package of Igor procedures working in the Igor Pro ver. 6.0.4.0 of WaveMetrix Inc. (Oregon, USA, 2007). The fits are performed using the Igor minimization subroutines which are called from the main package. The distribution of total bound ligands on their respective binding sites has been determined by parameter $x_i(F_i)$. This parameter was calculated using Eq. 6. The reported error deviations of determined parameters were obtained by propagating the experimental and fitting errors by suitable error propagation formulas.

Results

The results reported in Fig. 1 show the agmatine amount, in the absence or in the presence of idazoxan, which binds to mitochondrial membranes at zero time (B), as a function

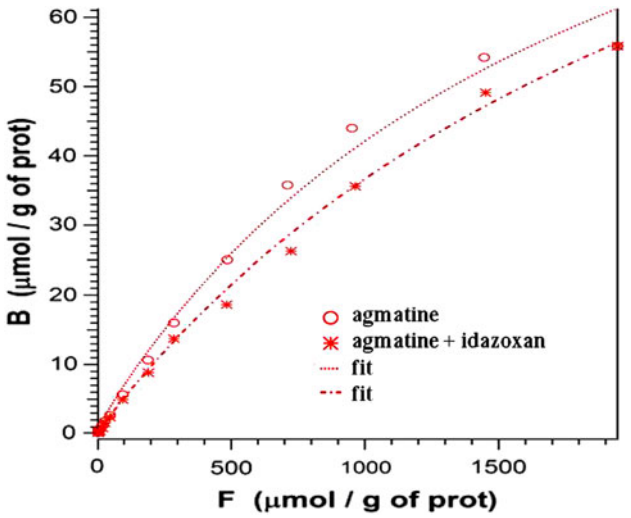

Fig. 1 Zero-time bound agmatine, plotted as a function of the free agmatine concentration. RLM were incubated in standard medium as indicated in "Materials and methods" in the presence of different [^{14}C]agmatine concentrations, in the range of 2–2,000 µM (50 µCi/mmol) and 200 µM idazoxan where indicated. Bound agmatine values (*B*) were obtained by extrapolating at zero-time the concentration-dependent agmatine uptake by energized mitochondria. Free agmatine concentration value (*F*) was obtained by subtracting *B* value from the total agmatine concentration

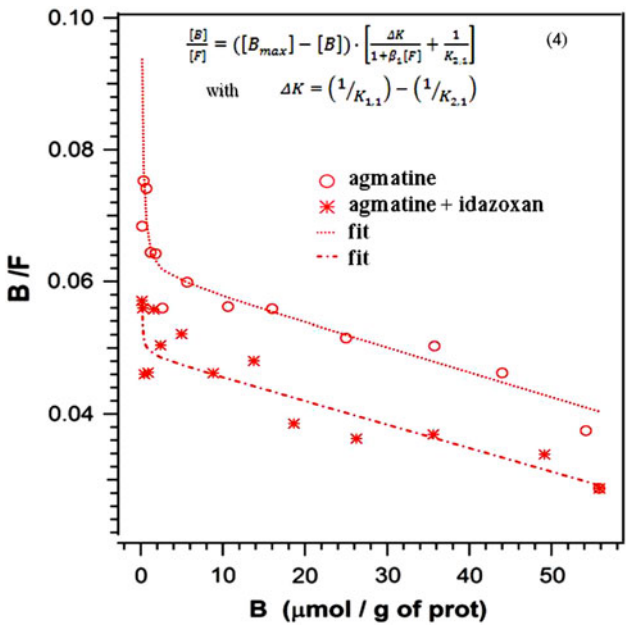

Fig. 2 Agmatine binding analyses with the thermodynamic treatment of Scatchard. Experimental data and procedures as in Fig. 1. The reported *lines* are the theoretical curves, of which is also reported the corresponding Eq. 2

of the free amine concentration present in the medium (*F*). These data were obtained by extrapolating at zero time the linear concentration-dependent agmatine uptake by energized mitochondria, on the *y* axis of an uptake versus time diagram (results not reported, an experimental demonstration is given in Dalla Via et al. 1996). As observable in this figure the inhibition by idazoxan on agmatine binding results are evident.

Figures 2 and 3 report agmatine binding analyses using the thermodynamic treatment of Scatchard and Hill, respectively. Binding data, showed in Fig. 1, plotted as dependence of $[B]/[F]$ on $[B]$ (Fig. 2) and $\ln\{[B]/([B_{max}]-[B])\}$ on $\ln[F]$ (Fig. 3), were simulated with two series of curve profiles belonging to Eqs. 2 and 3 present in "Materials and methods", obtained via computer simulations for several range of parameters *s* (representing the groups of different binding sites of the receptor) and n_i (their multiple occupancies). The curves that satisfactorily simulated the experimental data (Figs. 2, 3) are typical for two binding sites, S_1 and S_2, both with mono-coordination. Scatchard analysis gives us information about the receptor population homogeneity. The obtained function exhibit a typical hyperbolic trend, indicative for the presence of two different binding sites with a single coordination (Fig. 2). The Hill profiles is in accordance with Scatchard representation of binding data. Indeed, a linear behaviour is observed in Fig. 3 which, as expected, deviates slightly from linearity at low *F* values.

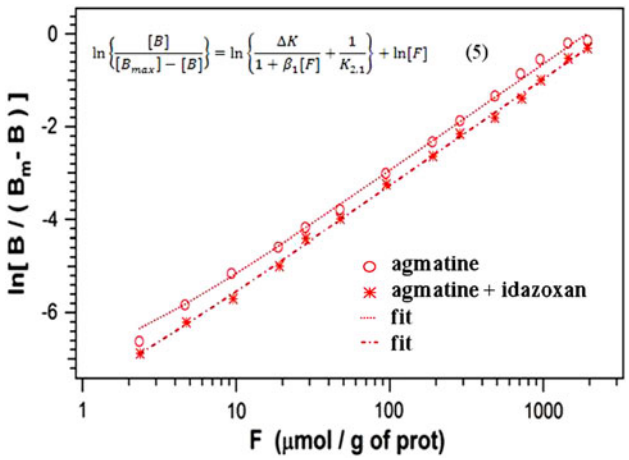

Fig. 3 Agmatine binding analyses with the thermodynamic treatment of Hill. Experimental data and procedures as in Fig. 1. The reported *lines* are the theoretical curves, of which is also reported the corresponding Eq. 3

In Table 1 are reported the agmatine binding parametres (in the absence or in the presence of idazoxan) obtained using either the Scatchard or the Hill data representation method. These data give us some preliminary and qualitative information about the type of process examined. Obviously the fitting of Eqs. 4 and 5 to the Scatchard and Hill representations gives the same parameters. Indeed, as expected Eqs. 2 and 3 were derived from an general equation describing in an unified way the physical–chemistry processes of the investigated binding events (Di Noto

Table 1 Agmatine 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)	$K_{1,1}$ (mg prot/nmol)	$K_{2,1}$ (mg prot/nmol)	β_1 (mg prot/nmol)
AGMATINE	120.22 (3)[a]	1.34 (4)	118.88 (4)	4.9 (2) $\times 10^{-2}$	5.2 (4) $\times 10^{-4}$	93.53 (3)
AGMATINE + IDAZOXAN	132.09 (4)	0.56 (2)	131.52 (5)	2.3 (3) $\times 10^{-2}$	3.7 (6) $\times 10^{-4}$	186.53 (2)

200 μM idazoxan was present in the medium

[a] Standard deviations in the least significant digits are given in parentheses and were determined by the propagation formula on the basis of experimental and fitting error analyses

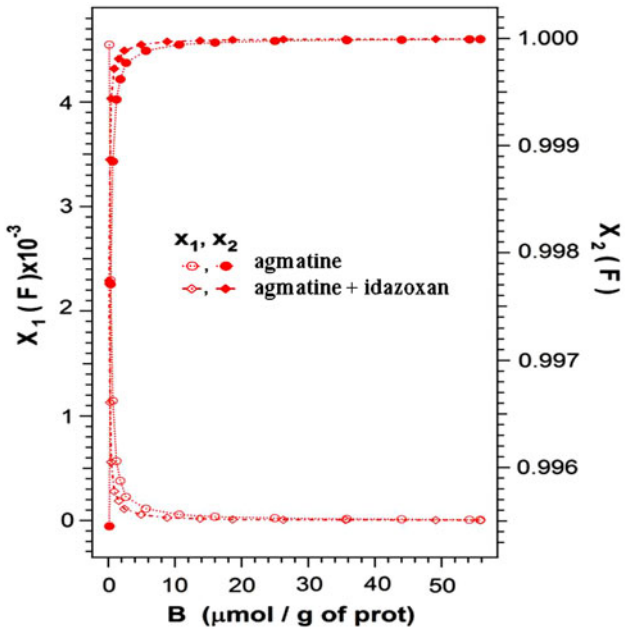

Fig. 4 Molar fraction ratio of agmatine binding. The calculations refer to the amount of agmatine lacking to fill the binding sites. $X_1(F)$ and $X_2(F)$ are the molar fraction ratio to fill the first (S_1) and the second (S_2) agmatine binding sites, respectively. Experimental procedures as in Fig. 1

et al. 1996, 2002). These analyses demonstrate the presence of two binding sites on mitochondrial membranes. Concerning agmatine alone, the total binding site concentration is 120.22 nmol/mg protein distributed between S_1 and S_2 sites in the percentages of 1 and 99, respectively. The association 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. β_1 describes the possible influence of the parallel filling of S_2 on that of S_1 site. This parameter, determined by Eq. 4, results to be very high, indicating that agmatine binding on S_2 can influence its binding on S_1. In the presence of idazoxan it is clear its inhibition on agmatine binding, which is emphasized by the reduced percentage of bound agmatine, is of about 0.4%. This effect is not present on the second site on which idazoxan seems to exhibit an unexpected effect: the total binding site concentration (B_{max}) and the one referring to the second site (B_{max2}) are increased, although the association constant is reduced. Moreover, S_2 influence on S_1 is enhanced (Table 1).

Figure 4 shows the calculation of the molar fraction ratios $X_1(F)$ and $X_2(F)$ for the aliquot of free agmatine that can bind to S_1 and S_2, respectively. These ratios are also named "filling molar fractions" (Di Noto et al. 1996, 2002). The values are obtained using parameter β_1 reported in Table 1. These calculations show that, by enhancing the amount of bound agmatine, $X_1(F)$ diminishes and $X_2(F)$ increases. This means that the higher affinity S_1 site is saturated before S_2 site.

Figure 5 clearly shows that 1 nmol/mg protein of agmatine is sufficient for S_1 saturation, while S_2 requires higher amounts of agmatine. In the presence of idazoxan, again, there is a decrease of agmatine binding to the first site, while the S_2 has even a higher amount of bound agmatine.

The functional dependence of the overall free energy changes (ΔG) on the concentration of free agmatine in solution is showed in Fig. 6. ΔG have been calculated by means of the general Eq. 7 and these data are fitted satisfactorily using Eq. 8, characteristic for two sites both with mono-coordination. As we can suppose from the previous data, ΔG increases in the presence of idazoxan that has an inhibitory effect on agmatine binding.

In Figure 7 the value of the Hill factor, $n_H(F)$, as a function of the amount of free agmatine bound to mitochondrial membrane is reported. This factor has been calculated by means of Eq. 9, characteristic for a system where two binding sites are present, both with mono-coordination. $n_H(F)$ is nearly 1 for all the concentrations tested (2.5–2,000 μM). This result indicates that S_2 can significantly influence S_1 and the two sites act almost like one site in the ligand–substrate interactions. In fact, when this factor is nearly 1, according to the analyses, it means that there is a system where one site is predominant.

Discussion

As previously demonstrated, the natural occurring polyamines are transported into the mitochondrial matrix by a specific electrophoretic mechanism, depending on $\Delta\Psi$,

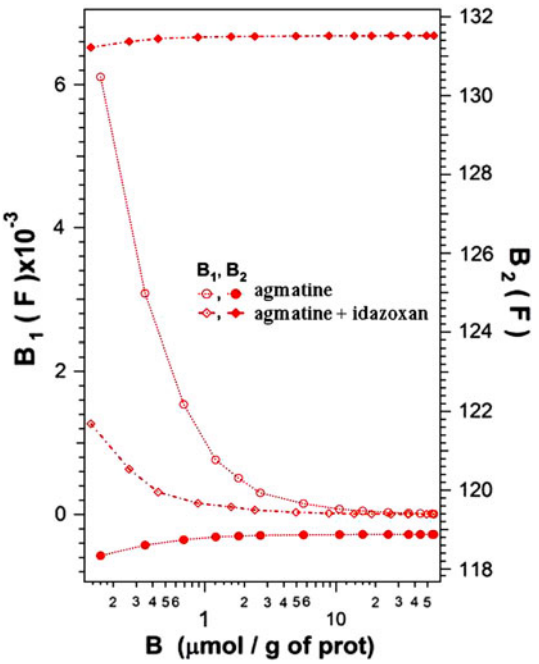

Fig. 5 Subdivision of total bound agmatine, B, in two aliquots, B_1 and B_2, bound to S_1 and S_2, respectively. Experimental procedures as in Fig. 1

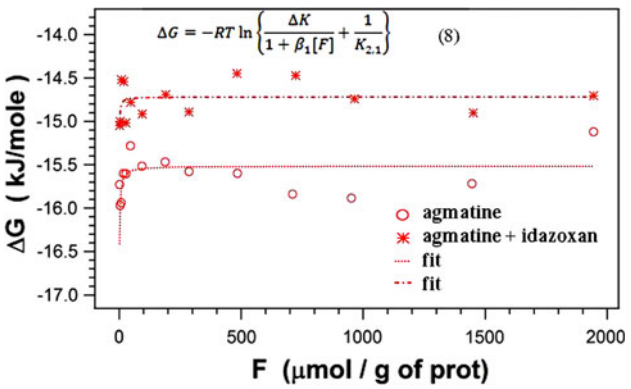

Fig. 6 Free energy changes in agmatine binding. The points are obtained by inserting the experimental values of B, F and B_{max} in general Eq. 2. B and F are the same values of Figs. 2 and 3, while B_{max} is 120.22 nmol/mg protein in the absence of idazoxan and 132.09 nmol/mg protein in its presence (see Table 1). The reported *lines* are the theoretical curves (see Eq. 8) which fit the experimental points. The parameters of this equation have been obtained from Table 1. For ΔK value see Eq. 5 and Fig. 2. Experimental procedures as in Fig. 1

with an apparent exponential force/flux relationship (Toninello et al. 1988, 1992a, b).

This transport system has been identified as a protein channel having two asymmetric energy barriers, with a binding site, located in the energy well between the two barriers, responsible for the transport (Toninello et al. 2000). Another binding site for polyamines, having

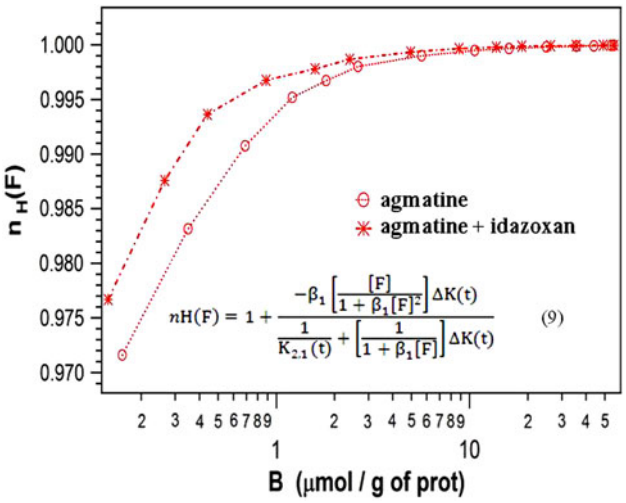

Fig. 7 Hill factor $[n_H(F)]$ for agmatine binding. B and F, reported in Eq. 9, are the same as in Figs. 2 and 3. Other parameters are obtained from Table 1. For ΔK value see Eq. 5 and Fig. 2. Experimental procedures as in Fig. 1

different functions, was also found (Dalla Via et al. 1996, 1999).

Agmatine that can be considered as another polyamine is also transported by a similar electrophoretic mechanism, having several identical properties, but it utilizes a specific transporter identifiable as a "single-binding centre-gated pore" (Salvi et al. 2006).

The present study, performed in the aim to further characterize the above mechanism, confirms, as previously proposed (Salvi et al. 2006), the presence of two binding sites on mitochondrial membranes, but it also evidences the involvement of the imidazoline receptor I_2 in this mechanism.

The presence of two binding sites for agmatine in RLM is demonstrated in Figs. 2 and 3 showing the analyses of Scatchard and Hill, respectively, by utilizing the experimental binding data of Fig. 1.

These analyses, performed using a thermodynamic treatment previously developed (Di Noto et al. 1996, 2002), whose rationale is reported in "Materials and methods", show that the experimental data of Fig. 1 fit satisfactorily the theoretical curves typical for two binding sites, S_1 and S_2, both with mono-coordination and with different features.

As a matter of fact, from the parameters presented in Table 1, agmatine has a major affinity for S_1, if compared with S_2, which has a much higher binding capacity ($B_{max1} = 1.34$ nmol/mg protein while $B_{max2} = 118.88$ nmol/mg protein).

Since bound agmatine was calculated by zero-time extrapolation, the evidenced binding sites have to be located on the 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, that are the contact sites of the two membranes, has to be considered, that is where the transporter could be localized to effectively facilitate agmatine uptake into the mitochondrial matrix.

Considering that agmatine is a ligand of the I_2 receptor, the possibility that this receptor could influence agmatine uptake would represent a significant goal for this investigation. Previous studies, conducted in our lab, demonstrated that the net amount of transported agmatine, in the presence of idazoxan, is not altered, while its initial binding to mitochondrial membranes is significantly modified (Salvi et al. 2006). The analyses of Figs. 1, 2, 3 have been performed in the aim to obtain more precise information about the inhibition of idazoxan, a ligand of I_2 receptor, on the initial agmatine binding observed in the above study. The results of these analyses (see the binding constants reported in Table 1) clearly confirm that idazoxan exhibits an inhibitory effect on agmatine binding, but this inhibition takes place only at the level of S_1 site, while the binding to S_2 increases. The total free energy value, calculated in Fig. 6, is almost similar as that of the other polyamines (Dalla Via et al. 1996, 1999) and suggests that agmatine should bind to mitochondrial sites by conventional weak interactions. Strongly covalent binding have to be excluded. The observation that idazoxan significantly increases this value further confirms its effect.

The thermodynamic model utilized in this study, allows to obtain other new parameters, to better characterize agmatine transport: (1) The coordination number of binding sites. Both sites are mono-coordinated. The binding data fitted very well with both curves (see Eqs. 5 and 6) characteristic for mono-coordination. (2) The molar fraction ratio $X_i(F)$ and the consequent evaluation of the two site filling. As demonstrated by the analyses of Figs. 4 and 5, S_1 is filled at low concentrations of ligand, if compared with S_2. (3) β_1 value (see Eq. 4). This parameter indicates the possible effect induced on agmatine binding to S_1 site by agmatine binding to S_2 site. In principle β_1 values could range from 0 and infinite. Very high values, as in our case, indicate the preponderance of S_2 site on S_1 site in binding phenomena.

These data are supported also by the Hill factor (see Fig. 7), which is almost 1 for all the concentrations tested, demonstrating the predominance of one of the two sites. The preponderance of S_2 site, its high binding capacity and the lack of a clear saturation (Fig. 1) suggest that S_2 site could be the transport site. This hypothesis is supported also by the association constant value, lower than the one referred to S_1, which indicates the low affinity of S_2 site for agmatine (Table 1). Thus, this amine is able to dissociate easily from S_2 site, allowing its subsequent transport.

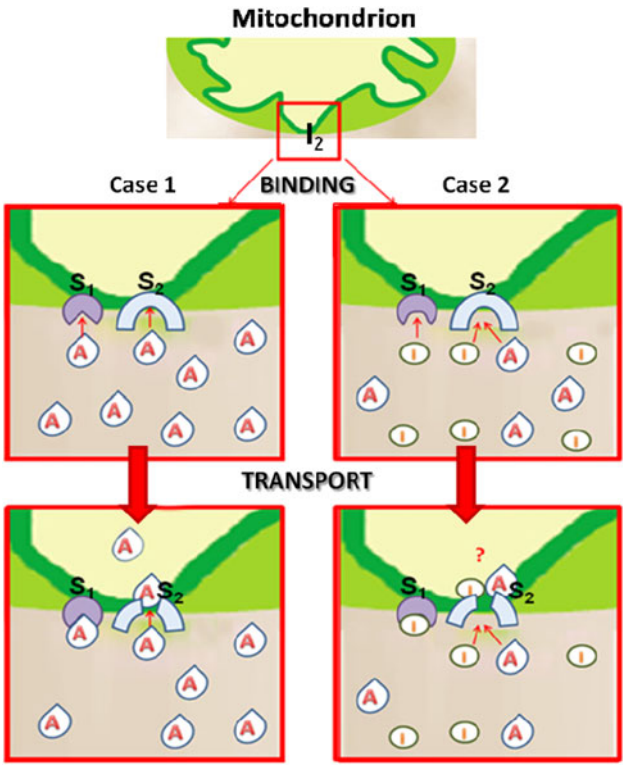

Fig. 8 Schematic representation of the hypothesis on the function of different binding sites for agmatine on mitochondrial membranes. Case 1 refers to a situation/context in which only agmatine (A) is present in the medium while in Case 2 there is also idazoxan (I). When only agmatine is present, this amine can bind both to S_1 and S_2 sites and then be transported into the matrix. The most interesting case is the second where, if idazoxan binds to S_1 site, a lower amount of agmatine could be detected and then the transport properties of S_2 site could be modified, allowing an increased transport of agmatine. At present, it is not clear if also idazoxan could be transported into the matrix

Moreover, the observation that S_2 site binds agmatine before the complete saturation of the first one, supports this possibility.

As regards S_1 site, considering its higher affinity constant in comparison with S_2 and idazoxan inhibition on agmatine binding, it could be identified with the imidazoline receptor I_2. In addition, from our data, it is possible to make some observations about the peculiar behaviour of this ligand. First of all, idazoxan could be considered as an inhibitor of agmatine binding to S_1 site, because its presence seems to exclude the amine occupancy of this site (as we can see from the decrease of the binding and affinity constants for S_1 showed in Table 1). This event does not occur for S_2 site, apparently the transport site. As the two sites are strictly correlated, as evidenced before, it is possible to formulate the following hypothesis, arising from the schematic representation of the results of Fig. 8.

The first site, present on imidazoline receptor I_2, could act as a sensor of agmatine concentration in the medium

and regulate the second site activity concerning agmatine transport into the mitochondria. If idazoxan binds to S_1 site, agmatine concentration detected is lower and this can cause a modification of S_2 site binding and transport properties (Case 2). This phenomenon is evidenced by the increasing binding capacity, allowing S_2 site to bind a major amount of agmatine, and by a decrease of the association costant, facilitating the amine dissociation from the binding site and its consequent transport.

These results underline a remarkable difference of agmatine binding constants from the previous constant detected for the other polyamines, confirming the dissimilarity between the two transport systems in RLM (Salvi et al. 2006). The peculiarity of agmatine binding sites allows us to hypothesize that at least one site can be located on the imidazoline receptor I_2 and can act as a regulator of the transport function of the other site. Thus, these findings could delineate a first physiological function of the I_2 receptor about which there is not any information at mitochondrial level. Anyway the peculiar features of agmatine binding sites and their different behaviours in the presence of idazoxan suggest that agmatine mitochondrial transport could be linked to particular pathophysiological roles of this amine. For example, it is possible that, in vivo, in liver, the I_2 receptor, depending on cytosolic agmatine concentration, and both the pathophysiological conditions and the specific functional requirements of the cell, could address the amine towards MPT induction or prevention.

Experiments are in progress in our laboratory to obtain further information about agmatine interactions with mitochondrial membranes and its correlation with the imidazoline I_2 receptor role on mitochondrial function.

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Conflict of interest The authors declare that they have no conflict of interest.

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