Acta Biochimica Polonica

Vol. 51 No. 4/2004

953–962

QUARTERLY

Benzodiazepine binding to mitochondrial membranes of the amoeba *Acanthamoeba castellanii* and the yeast *Saccharomyces cerevisiae*★✪

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Received: 09 March, 2004; revised: 01 July, 2004; 28 October, 2004

Key words: Ro5-4864, mitochondrial benzodiazepine receptor, *Acathamoeba castellanii*, *Saccharomyces cerevisiae*

Benzodiazepine binding sites were studied in mitochondria of unicellular eukaryotes, the amoeba *Acathamoeba castellanii* and the yeast *Saccharomyces cerevisiae*, and also in rat liver mitochondria as a control. For that purpose we applied Ro5-4864, a well-known ligand of the mitochondrial benzodiazepine receptor (MBR) present in mammalian mitochondria. The levels of specific [^{3}H]Ro5-4864 binding, the dissociation constant (K_D) and the number of [^{3}H]Ro5-4864 binding sites (B_{max}) determined for fractions of the studied mitochondria indicate the presence of specific [^{3}H]Ro5-4864 binding sites in the outer membrane of yeast and amoeba mitochondria as well as in yeast mitoplasts. Thus, *A. castellanii* and *S. cerevisiae* mitochondria, like rat liver mitochondria, contain proteins able to bind specifically [^{3}H]Ro5-4864. Labeling of amoeba, yeast and rat liver mitochondria with [^{3}H]Ro5-4864 revealed proteins identified as the voltage dependent anion selective channel (VDAC) in the outer membrane and adenine nucleotide translocase (ANT) in

★Part of the results were presented at the11th European Bioenergetics Conference, Brighton, Great Britain, 2000.

✪This work was supported in part by a grant from the State Committee for Scientific Research (KBN-0437/P04/01/20).

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Abbreviations: ANT, adenine nucleotide translocase; B_{max}, total number of ligand binding sites; K_D, dissociation constant; MBR, mitochondrial benzodiazepine receptor; Ro5-4864, 7-chloro-1,3-dihydro-1-methyl-*p*-(chlorophenyl)-2*H*-1,4-benzodiazepine-2-one; VDAC, voltage dependent anion selective channel.

the inner membrane. Therefore, the specific MBR ligand binding is not confined only to mammalian mitochondria and is more widespread within the eukaryotic world. However, it can not be excluded that MBR ligand binding sites are exploited efficiently only by higher multicellular eukaryotes. Nevertheless, the MBR ligand binding sites in mitochondria of lower eukaryotes can be applied as useful models in studies on mammalian MBR.

Benzodiazepines are commonly used therapeutic agents because of their sedative and anxiolytic effects mediated by $GABA_A$ receptors located in the central nervous system (Braestrup & Squires, 1977). Another recognition site for these drugs, termed the peripheral benzodiazepine receptor (PBR), has been found in mammalian peripheral tissues, i.e. outside the nervous system (Anholt *et al.*, 1985; Antkiewicz-Michaluk *et al.*, 1988) and glial cells in the brain (Basile & Skolnick, 1986). Since subcellular localization and fractionation studies indicate that the receptor is associated mainly with mitochondria it is also termed the mitochondrial benzodiazepine receptor (MBR) (Krueger, 1995). The tissue distribution of MBR in mammalian cells shows a distinct cell-specific pattern and its level is determined in a tissue dependent manner by hormonal regulation and stress (Bar Ami *et al.*, 1994; Dar *et al.*, 1991). MBR density is high in steroidogenic tissues, in particular in the adrenal gland, while in the kidney, heart, testis, ovary and uterus it is approximately five times lower (Anholt *et al.*, 1985; Antkiewicz-Michaluk *et al.*, 1988; Gavish *et al.*, 1999). A definitive role has not been elucidated for MBR but strong evidence indicates that it is important in steroid biosynthesis (Stocco & Clark, 1997; Weizman *et al.*, 1997), control of mitochondrial respiration (Hirsch *et al.*, 1989; Kinnally *et al.*, 1993), cell proliferation (Black *et al.*, 1994), flow of calcium ions (Python *et al.*, 1993), cellular immunity (Lenfant *et al.*, 1986), malignancy (Hardwick *et al.*, 1999; Ikezaki & Black, 1990), and apoptosis (Kroemer *et al.*, 1997; Hirsch *et al.*, 1998; Tatton & Olanow, 1999).

The structure of MBR is still not well defined. According to some data MBR is formed by an 18 kDa protein (pK18) present in the mitochondrial outer membrane (Zoratti & Szabo, 1995; Szewczyk & Wojtczak, 2002). The protein is probably associated with the voltage dependent anion selective channel (VDAC) and adenine nucleotide translocase (ANT), located in the outer and the inner membrane, respectively. On the other hand, there are also data indicating that MBR is a complex of three distinct proteins, namely pK18, VDAC and ANT, located within the contact sites between the outer and the inner membranes (McEnery *et al.*, 1992). Since MBR ligands, e.g. Ro5-4864 (7-chloro-1,3-dihydro-1-methyl-*p*-(chlorophenyl)-2*H*-1,4-benzodiazepine-2-one), bind specifically to VDAC and ANT (Pellow & File, 1984) the second hypothesis seems to be more reasonable and constitutes a starting point in our studies.

Unicellular eukaryotes constitute very useful models in studies on cell physiology of higher *Eucaryota,* including human. Thus, finding functional orthologues of MBR in lower eukaryotes could simplify studies on the receptor function and organization. Moreover, it could provide some important data concerning the role of MBR in mitochondria evolution. Therefore, we studied the presence of Ro5-4864 binding sites in mitochondria of the amoeba *Acanthamoeba castellanii* and the yeast *Saccharomyces cerevisiae,* which are often applied as model organisms in studies on plant and animal mitochondria, respectively. In the case of the latter we used two strains, namely a wild type and a VDAC knock-out one, which enabled us to verify the role of VDAC in the binding of Ro5-4864 observed for mammalian cells (McEnery *et al.*, 1992; Garnier *et al.*, 1994). Moreover, it has been reported that pK18 is not present in *S. cerevisiae* mitochondria (Riond *et al.*, 1991) which allowed us to check the requirement for

this protein for formation of Ro5-4864 binding sites in mitochondria.

Here we report that mitochondria of *A. castellanii* and the studied *S. cerevisiae* strains specifically bind Ro5-4864. Specific [^{3}H]Ro5- 4864 binding sites were found in the mitochondrial outer membrane and mitoplasts of the yeast and only in the mitochondrial outer membrane of the amoeba. The proteins responsible for the binding were identified to be VDAC and ANT. Thus, mitochondria of lower eukaryotes display at least some of the properties that are necessary for the formation of functional MBR in mammalian cells.

MATERIALS AND METHODS

Materials. The following organisms were studied: the soil amoeba *Acanthamoeba castellanii*, strain Neff (Neff, 1957), two *Saccharomyces cerevisiae* strains – the parental *POR1* strain M3 (*MAT***a**, *lys2 his4 trp1 ade2 leu2 ura3*), and M22-2 (Δ*por1*) containing a deletion of the most of the *POR1* gene (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998), as well as male Wistar rats.

[^{3}H]Ro5-4864 of a specific activity of 84.5 Ci/mmol was purchased from Du Pont NEN (Germany). Ro5-4864 was from Sigma. All other chemicals were of the highest purity commercially available.

Isolation of mitochondria, mitoplasts and the mitochondrial outer membrane. Yeast cells were grown at 28°C in YPG medium (1% yeast extract, 2% peptone, 3% glycerol) at pH 5.5 and mitochondria were isolated according to a published procedure (Daum *et al.*, 1982). Amoeba mitochondria were isolated as described in (Hryniewiecka *et al.*, 1979). Rat liver mitochondria were prepared according to the method described in (Brdiczka *et al.*, 1968). Yeast mitoplasts were obtained by the swelling-shrinking procedure as described (Daum *et al.*, 1982) while mitoplasts of rat liver and amoeba were obtained by swelling of mitochondria in 5 mM phosphate buffer at 4°C for 30 min. The mitochondrial outer membrane and mitoplasts were separated on a step 15–30–60% sucrose gradient buffered with 10 mM Tris/Cl, pH 7.4, in the case of yeast mitochondria and with Mops/KOH, pH 7.2, in the case of amoeba and rat liver mitochondria. To estimate the degree of purity of the mitoplasts we measured the residual activity of the mitochondrial intermembrane space enzyme adenylate kinase and compare it with that determined for mitochondria (Stobienia *et al.*, 1999). The results obtained indicated effective removal of the mitochondrial outer membrane.

Binding assays. The binding of [^{3}H]Ro5-4864 to mitochondrial membranes was assayed as follows. Mitoplasts and the mitochondrial outer membrane (500 μg of mitochondrial protein) were incubated in 50 mM Hepes, pH 7.4, for 60 min at 4°C with 3 nM [^{3}H]Ro5-4864. After the incubation samples were filtered under reduced pressure through Whatman GF/B filters pretreated with 0.5% polyethylenimine in 50 mM Hepes (pH 7.4) for at least 30 min and then washed three times with 30 ml of 100 mM NaCl, 20 mM Tris/Cl (pH 7.4) at 4°C. The filters were incubated in scintillation cocktail (Formula 989, Du Pont Germany) for 24 h and counted for radioactivity. Since the results of preliminary experiments indicated that [^{3}H]Ro5-4864 binding was not saturated even at 10 nM Ro5-4864, to determine the dissociation constant (K_D) we carried out competitive binding experiments using Ro5-4864 in the concentration range from 3×10^{-9} to 3×10^{-5} M. The total number of binding sites (B_{max}) was determined by Scatchard plot analysis (Weiland & Molinoff, 1981). Nonspecific binding was measured in the presence of a saturating concentration of non-radioactive Ro5-4864 (10 μM) and 3 nM [^{3}H]Ro5-4864. Under these conditions all specific binding sites but only few unspecific sites bind the unlabeled ligand and the labeled ligand binds with unspecific sites. Specific binding was calculated by sub-

tracting the nonspecific binding from total binding.

Labeling of mitochondrial proteins. Labeling of mitochondria with [^{3}H]Ro5-4864 was performed as described in (McEnery *et al.*, 1992). Purified mitochondria (50 μg of protein) were incubated with 100 nM [^{3}H]Ro5-4864 for 1 h at 4°C and 5× Laemmli buffer was added afterwards. The samples were resolved on 12% SDS/PAGE and transferred to nitrocellulose. After spraying the nitrocellulose membranes with En^{3}Hance (Du Pont NEN, Germany), fluorographic detection was performed by a six month exposure at –80°C against Fuji Medical X-ray film.

RESULTS AND DISCUSSION

The use of radiolabeled ligands in receptor binding experiments is a well-documented experimental approach allowing identification, localization and pharmacological characterization of the receptors. Taking into account that mitochondrial benzodiazepine receptor (MBR) is probably a protein complex involving the voltage dependent anion selective channel (VDAC) and adenine nucleotide translocase (ANT), we measured [^{3}H]Ro5-4864 binding to mitochondria of two species of lower eukaryotes, the amoeba *A. castellanii* and the yeast *S. cerevisiae* – wild type and a VDAC mutant. As a control we used mitochondria of rat liver displaying low level of MBR (Antkiewicz- Michaluk *et al.*, 1988). Table 1 shows the levels of specific [^{3}H]Ro5-4864 binding calculated for mitoplasts and the outer membrane isolated from the studied mitochondria. The highest level of specific [^{3}H]Ro5-4864 binding was obtained for the mitochondrial outer membrane and mitoplasts of rat liver, 47.78 ± 2.57 and 20.75 ± 3.2 fmoles per mg of mitochondrial protein, respectively. In the case of amoeba and yeast the calculated levels of specific binding were approximately one order of magnitude lower. For the outer membrane of wild type yeast and mitoplasts of wild type and mutant yeast the levels of [^{3}H]Ro5-4864 specific binding turned out to be similar, 1.84 ± 0.35, 3.0 ± 0.28 and 4.5 ± 0.32 fmoles per mg of mitochondrial protein, respectively. Thus, the absence of VDAC does not change the expression of the [^{3}H]Ro5-4864 binding sites located in the inner membrane of mitochondria. In contrast, VDAC seems to be important for [^{3}H]Ro5-4864 binding to the outer membrane of yeast mitochondria since in the absence of VDAC the specific binding of [^{3}H]Ro5-4864 decreased to 0.04 ± 0.01 fmoles per mg of mitochondrial protein. In the case of the outer membrane and mitoplasts obtained from amoeba mitochondria, the specific binding of [^{3}H]Ro5-4864 was estimated

Table 1. Specific binding of Ro5-4864 to the mitochondrial outer membrane and mitoplasts.

Nonspecific binding was assessed by measuring [^{3}H]Ro5-4864 binding in the presence of 10 μM unlabeled Ro5-4864 and specific binding was calculated by subtracting the nonspecific binding from total binding of [^{3}H]Ro5-4864. The data are mean values of two independent experiments.

	Specific binding [fmole/mg mito protein]	
	the outer membrane	mitoplasts
Rat liver	47.78 ± 2.57	20.75 ± 3.2
Amoeba	2.48 ± 0.18	0.06 ± 0.02
Wild type yeast	1.84 ± 0.35	3.0 ± 0.28
Mutant yeast	0.04 ± 0.01	4.5 ± 0.32

to be 2.48 ± 0.18 and 0.06 ± 0.02 fmoles per mg of mitochondrial protein, respectively. Thus, amoeba and yeast mitochondria display similar levels of specific [^{3}H]Ro5-4864 binding to the outer membrane but differ distinctly in the capability of the ligand binding to the inner membrane.

To calculate the dissociation constant (K_D), which measures the affinity of a ligand for its binding site, we carried out competitive binding experiments using Ro5-4864 in the concentration range from 3×10^{-9} to 3×10^{-5} M and 3 nM [^{3}H]Ro5-4864 (Fig. 1). As we expected from the specific binding levels, [^{3}H]Ro5-4864 displayed comparable affinity to the mitochondrial outer membrane of wild type yeast and amoeba (K_D = 70 ± 6 nM and 20 ± 2 nM, respectively) but the obtained affinity was one order of magnitude lower than that for the outer membrane of rat liver mitochondria (K_D= 2.4 ± 0.5 nM). Since the outer membrane of rat liver mitochondria contains the pK18 protein, which is absent from the membrane of yeast mitochondria (Riond *et al.*, 1991), it might be concluded that pK18 is an important factor influencing Ro5-4864 binding to the outer membrane, which is in agreement with published data (Garnier *et al.*, 1994; Krueger, 1995). The presence of pK18 in amoeba mitochondria has not been detected yet, however, the same order of magnitude of the K_D calculated for amoeba and yeast mitochondrial outer membranes suggests the absence of the protein. On the other hand, the affinity of [^{3}H]Ro5-4864 for its binding sites in mitoplasts of rat liver as well as of mutant and wild type yeast was nearly the same (K_D = 9 ± 1 nM, 17 ± 5 nM and 23 ± 2 nM, respectively) and distinctly higher than that in the case of amoeba mitoplasts (K_D = 566 ± 43 nM). The lowest affinity of [^{3}H]Ro5-4864 for its binding sites was obtained in the case of the mitochondrial outer membrane of mutant yeast (K_D = 927 ± 83 nM). Taking into account the data concerning the levels of specific [^{3}H]Ro5-4864 binding and the determined affinity (K_D) one may conclude that specific Ro5-4864 binding sites are present in the mitochondrial inner membrane of wild type and mutant yeast as well as in the mitochondrial outer membrane of wild type yeast and amoeba. In the case of the mitochondrial outer membrane of mutant yeast the lack of specific Ro5-4864 binding sites could be explained by the absence of VDAC. Thus, also in the case of lower eukaryotes mitochondria, VDAC seems to be involved in specific binding of Ro5-4864, which has been previously reported for mammalian mitochondria (McEnery *et al.*, 1992; Pellow & File, 1984).

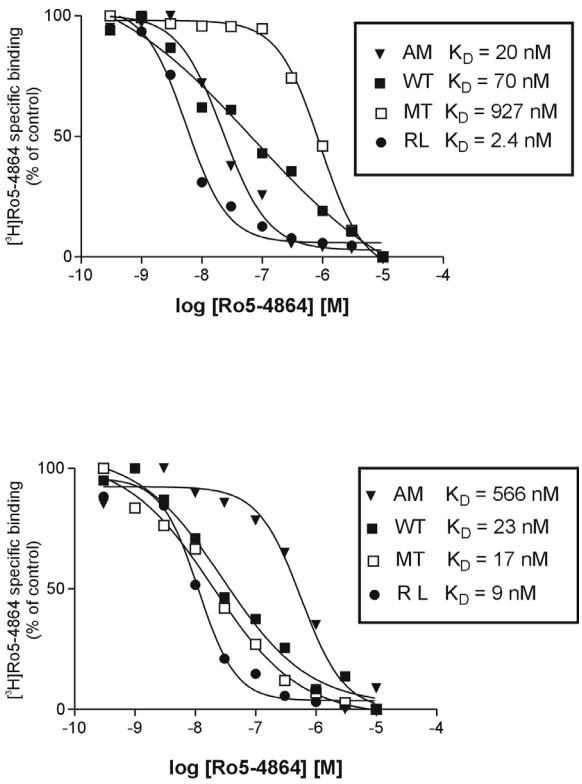

Figure 1. Specific binding of [^{3}H]Ro5-4864 to the mitochondrial outer membrane (A) and mitoplasts (B).

The figure shows concentration-dependent displacement of [^{3}H]Ro5-4864 by unlabeled drug, estimated as described in Materials and Methods. [^{3}H]Ro5-4864 concentration was 3 nM. Representative results from two independent experiments are shown. The calculated values of K_D varied by not more than 13% in these two experiments. AM, amoeba; WT, wild type yeast; MT, mutant yeast; RL, rat liver.

Further, depletion of VDAC does not change the properties of the inner membrane binding sites since there is no significant difference between the levels of specific binding and K_D calculated for mitoplasts of wild type and mutant yeast. On the other hand, the absence of specific Ro5-4864 binding sites demonstrated for amoeba mitoplasts could be explained by the position of the organism in molecular phylogenetic tree where it appears on a branch basal to the divergence points of plants, animals and fungi (Wainright *et al.*, 1993; Gray *et al.*, 1999). It might be speculated that the binding sites simply have not evolved in the mitochondrial inner membrane of amoeba, similar to plant mitochondria which share many common features with amoeba mitochondria (Hryniewiecka *et al.*, 1978; Hryniewiecka, 1986). However, the mitochondrial outer membrane of amoeba, like the membrane of wild type yeast, contains specific Ro5-4864 binding sites. Thus, this feature seems to be more widespread within the eukaryotic world.

An equally important parameter in receptor characteristics is B_{max}, i.e. the total number of ligand binding sites (Fig. 2). The highest values of the total number of Ro5-4864 binding sites were obtained for the outer membrane and mitoplasts isolated from rat liver mitochondria (86 ± 4 and 83 ± 5 fmoles per mg of mitochondrial protein, respectively), while the lowest ones for the mitochondrial outer membrane of mutant yeast and amoeba mitoplasts (12 ± 6 and 11 ± 3 fmoles per mg of mitochondrial protein, respectively). In the case of wild type and mutant yeast mitoplasts as well as in the case of the mitochondrial outer membrane of wild type yeast the calculated values of B_{max} were comparable (26 ± 5, 30 ± 2 and 35 ± 6 fmoles per mg of mitochondrial protein, respectively). The value of B_{max} obtained for the mitochondrial outer membrane of amoeba was slightly lower, namely 22 ± 3 fmoles per mg of mitochondrial protein. Thus, the values of B_{max}, like the values of K_D (Fig. 1) and the levels of specific binding (Table 1), suggest similarity in the distribution of Ro5-4864 binding sites in rat liver and yeast mitochondria. The very low value of B_{max} obtained in the case of the mitochondrial outer membrane of mutant yeast indicates again that VDAC seems to be necessary for Ro5-4864 binding sites in the mitochondrial outer membrane. However, depletion of VDAC does not influence B_{max} of the inner membrane as there is no difference in the value of the parameter calculated for wild type and mutant yeast mitoplasts. On the other hand, the results obtained for fractions of amoeba mitochondria indicate a dominant role of the outer membrane in the formation of Ro5-4864 binding sites. Since Ro5-4864 binds specifically to mammalian ANT (Garnier *et al.*, 1994), it might be speculated that ANT is also responsible for the observed specific binding of Ro5-4864 by rat liver and yeast mitoplasts. In this case the lack of an involvement of ANT in Ro5-4864 binding in amoeba mitochondria might result from differences in ANT properties between amoeba and yeast.

The possibility of Ro5-4864 binding to VDAC and ANT was confirmed by fluorography of proteins derived from the studied mitochondria incubated with [^{3}H]Ro5-4864 (Fig. 3). In the case of wild type yeast mitochondria [^{3}H]Ro5-4864 bound to VDAC and ANT while in the case of mutant yeast mitochondria the ligand bound to ANT and Tom40. The latter is the main component of the TOM complex channel, which translocates imported proteins across or incorporates into the mitochondrial outer membrane (Neupert & Paschen, 2001). It has been reported recently that in the case of the studied mutant yeast, Tom40 is upregulated (Kmita & Budzinska, 2000; Antos *et al.*, 2001). Thus, the binding of [^{3}H]Ro5-4864 to Tom40 is probably only detectable in the case of yeast mitochondria devoid of VDAC. On the other hand, since the TOM complex channel substitutes for VDAC in the absence of the latter (Kmita & Budzinska, 2000; Antos *et al.*,

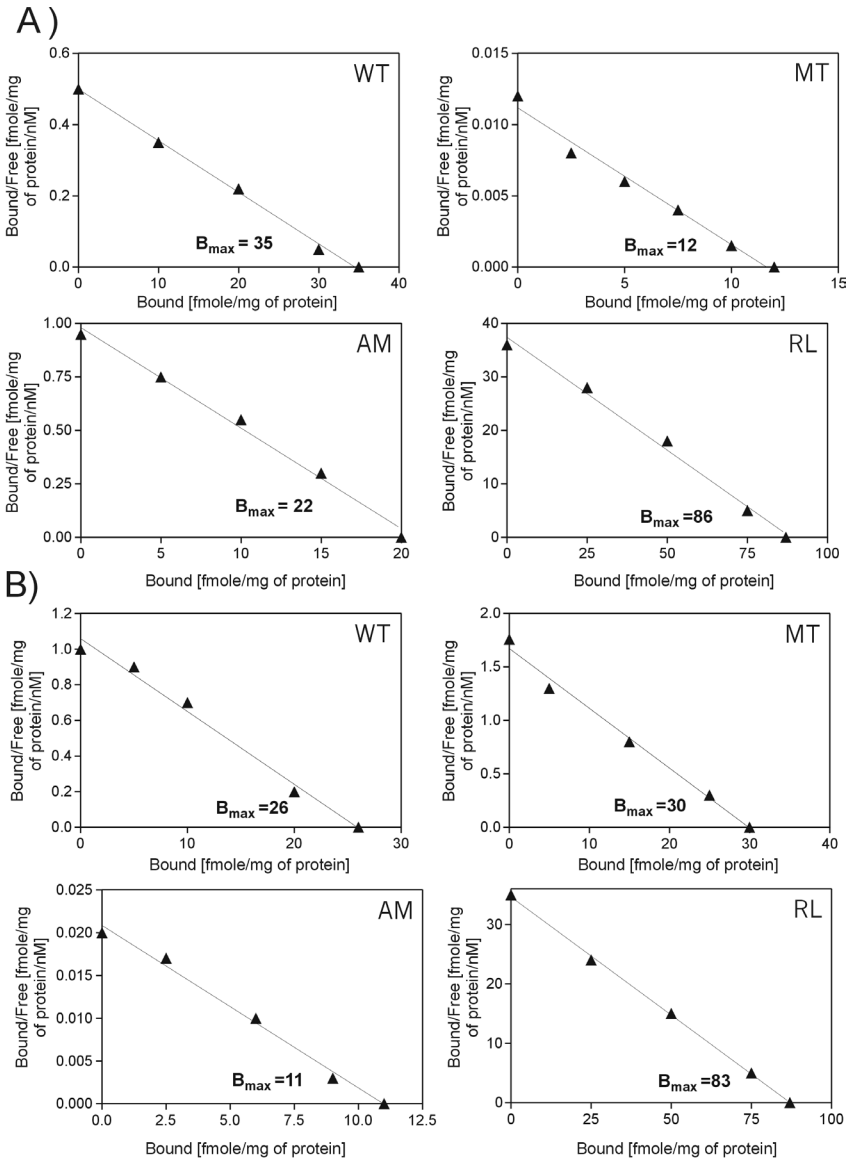

Figure 2. Scatchard plot analysis of [^{3}H]Ro5-4864 binding for the mitochondrial outer membrane (A) and mitoplasts (B).

The calculated values of B_{max} varied by not more than 12% in two independent experiments. AM, amoeba; WT, wild type yeast; MT, mutant yeast; RL, rat liver.

2001), the binding of [^{3}H]Ro5-4864 to Tom40 seems to confirm indirectly the involvement of VDAC in forming the [^{3}H]Ro5-4864 binding sites in yeast mitochondria. In the case of amoeba mitochondria the only protein able to bind [^{3}H]Ro5-4864 was VDAC. It should be stressed here that the identity of the proteins revealed by fluorography was confirmed by immunodetection (not shown).

In conclusion, the obtained results indicate that mitochondria of the amoeba *A. castellanii* and the yeast *S. cerevisiae* contain proteins able to bind a specific ligand of MBR, Ro5-4864, with affinity comparable to that calculated for rat liver mitochondria. However, the total number of Ro5-4864 binding sites (B_{max}) in amoeba and yeast mitochondria is lower than in rat liver mitochondria. This, in turn, probably hinders the explanation of the function of the Ro5-4864 binding sites in mitochondria of lower eukaryotes. We have observed that in the case of reconstituted VDAC isolated from amoeba, yeast and rat liver mitochondria, addition of Ro5-4864

causes an increase of the channel lower conductances (data not shown), which suggests an involvement of the ligand binding sites in the regulation of the mitochondrial outer membrane permeability. However, Ro5-4864 has no effect on the energetic coupling of amoeba and yeast mitochondria (not shown), as has been reported for mitochondria of

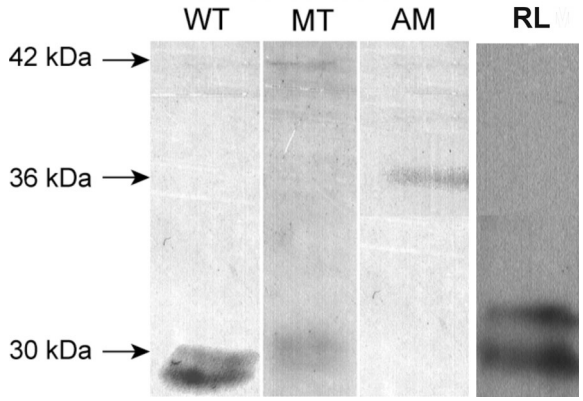

Figure 3. Binding of [^{3}H]Ro5-4864 to mitochondrial proteins.

Mitochondria (50 μg protein) were incubated in the presence of 100 nM [^{3}H]Ro5-4864. Proteins were resolved on 12% SDS/PAGE gels and protein-ligand complexes detected by fluorography. WT, wild type yeast mitochondria; MT, VDAC mutant yeast mitochondria; AM, amoeba mitochondria; RL, rat liver mitochondria.

mammalian tissues displaying high activity of MBR, e.g. adrenal glands (B_{max}= 1240 fmoles per mg of mitochondrial protein), as well as low activity of MBR, e.g. rat liver (B_{max} = 80 fmoles per mg of mitochondrial protein) (Anholt *et al.*, 1985; Antkiewicz-Michaluk *et al.*, 1988). Moreover, the obtained data do not allow one to determine whether the Ro5-4864 binding proteins form independent receptors or collaborate in complexes. Nevertheless, one may conclude that these proteins might bind Ro5-4864 independently, since depletion of VDAC did not affect Ro5-4864 binding to the inner membrane sites in yeast mitochondria, and amoeba mitochondria contain the Ro5-4864 binding sites only in the outer membrane. Although it can not be excluded that the MBR ligand binding sites in mitochondria are exploited efficiently only by higher multicellular eukaryotes, the sites in unicellular eukaryotes' mitochondria can be applied as useful models in studies on mammalian MBR. On the other hand, the differences in distribution of Ro5-4864 binding sites between amoeba and yeast constitute a very interesting finding from the evolutionary point of view.

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