A structure–effect study of the induction by polyamines of the transport *in vitro* of the precursor of ornithine transcarbamylase

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Polyamines induce the transport *in vitro* of the precursor of ornithine transcarbamylase (pOTC) into isolated rat liver mitochondria by facilitating its functional binding to these organelles. Comparative studies of the effect on the *in vitro* transport of pOTC of polyamine derivatives and related compounds have allowed us to establish that: (i) at least two protonated amino

groups per molecule are necessary to induce the pOTC transport; (ii) a distance of three $-CH_2$ -groups between the amino groups in diamines is enough to induce this effect, although no differences were observed with diamines having distances of three to eight $-CH_2$ -groups. Longer distances resulted in a marked decrease of the effect.

INTRODUCTION

Like the vast majority of mitochondrial proteins (reviewed in [1]), ornithine transcarbamylase (OTC) is encoded in the nucleus and translated on cytoplasmic polysomes as a larger precursor (pOTC) containing an N-terminal leader peptide [2]. The precursor is recognized by mitochondria and translocated across both mitochondrial membranes [3], after which the leader peptide is proteolytically cleaved in two steps to produce mature OTC [4]. In the first step, pOTC is processed by a matrix protease to a product, designated iOTC, intermediate in size between pOTC and mature OTC. In a second step, iOTC is converted to mature OTC [5].

Previous studies have reported that pOTC transport requires soluble cytosolic component(s) present in the rabbit reticulocyte lysate [6,7] or in rat liver cytosol [8–10]. More recently, we have reported that naturally occurring polyamines (putrescine, spermidine or spermine) alone, at physiological concentrations, induce the transport *in vitro* of rat liver pOTC into rat liver mitochondria as efficiently as the other more complex media used [11]. Studies on the role played by polyamines in this process revealed that these metabolites are involved in the functional binding of pOTC to mitochondria, probably by inducing a conformation of this precursor that favours the specific recognition on the mitochondrial surface [12]. We report in this paper experiments carried out to gain insight into the molecular mechanism underlying the action of polyamines in the induction of pOTC transport into isolated rat liver mitochondria. We tested different monoamines, non-biogenic polyamines and polyamine derivatives in order to correlate structural and functional aspects of these compounds with their effect on the transport of pOTC *in vitro*.

EXPERIMENTAL

Materials

Restriction enzymes and RNA polymerase were obtained from Boehringer (Mannheim, Germany). [³⁵S]Methionine (1000 Ci/mmol, 10 mCi/ml) was from New England Nuclear. Nuclease-treated rabbit reticulocyte lysate was from Promega. Methylamine, ethylamine, *n*-propylamine, *n*-butylamine, 1,2-

diaminoethane, 1,3-diaminopropane, putrescine, cadaverine, 1,6-diaminohexane, 1,7-diaminoheptane, 1,8-diaminooctane, 1,10-diaminodecane, L-ornithine, L-lysine, spermidine, *N*¹-acetylspermidine, *N*⁸-acetylspermidine and *N*-acetylputrescine were purchased from Sigma Chemical Co. Ammonia and 1,9-diaminononane were from Merck. α -Difluoromethylornithine (DFMO), (2*R*,5*R*)- δ -methyl acetylenic putrescine (MAP) and *N*-butyl-1,3-diaminopropane (*N*-butyl-1,3-DAP), were generously provided by the Merrell Dow Research Institute (Strasbourg, France).

In vitro transcription and translation

pOTC mRNA of rat liver was obtained by using the specific rat liver cDNA [11]. This cDNA was cloned in the transcription SP6 system. The specific RNA obtained [13] was translated in a nuclease-treated reticulocyte system as described by Pelham and Jackson [14], for 60 min at 30 °C, after which the reaction mixture was adjusted to 12 μ g/ml cycloheximide.

pOTC import into isolated mitochondria

Rat liver mitochondria were isolated and incubated with radio-labelled pOTC as previously described [15]. The incubation mixture (total volume 100 μ l) usually contained 400 μ g of mitochondrial protein, transport medium (12.5 mM Hepes, pH 7.4; 0.25 M sucrose; 5 mM succinate; 75 μ M ADP; 1.25 mM P_i), 2 μ l of the cell-free translated mixture (40000 c.p.m./ μ l pOTC) and additions as indicated. After 30 min at 30 °C (with shaking), mitochondria were re-isolated by centrifugation in an Eppendorf centrifuge (10000 *g*, 5 min) and washed with 1 ml of 200 mM D-mannitol/70 mM sucrose/1 mM EDTA/2 mM Hepes, pH 7.6. Finally, mitochondria were suspended in 50 μ l of SDS sample buffer.

Published procedures were used to analyse labelled translation products by SDS/PAGE [12% (w/v) gel] and fluorography [16].

RESULTS

It has been reported that putrescine, spermine or spermidine alone, at physiological concentrations (1–4 mM), induces the

Abbreviations used: DAP, diaminopropane; DFMO, α -difluoromethylornithine; iOTC, intermediate size OTC; MAP (2*R*,5*R*)- δ -methyl acetylenic putrescine; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase; pOTC, precursor OTC.

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Figure 1 Effect of monoamines on the transport *in vitro* of pOTC

An aliquot (2 μ l) of the translation mixture ($\sim 40\,000$ c.p.m./ μ l pOTC) was incubated with isolated rat liver mitochondria in the transport medium, in the presence of: no additions (lane 1), 1 mM spermidine (lane 2), 4 mM spermidine (lane 3), 4 mM ammonia (lane 4), 4 mM methylamine (lane 5), 4 mM ethylamine (lane 6), 4 mM *n*-propylamine (lane 7), 1 mM *n*-butylamine (lane 8), or 4 mM *n*-butylamine (lane 9). Mitochondria were analysed by SDS/PAGE and subsequent fluorography.

Figure 2 Effect of α,ω -diamines on the transport *in vitro* of pOTC

An aliquot (2 μ l) of the translation mixture ($\sim 40\,000$ c.p.m./ μ l pOTC) was incubated with isolated rat liver mitochondria in the transport medium, in the presence of: (a) no additions (lane 1), 4 mM 1,2-diaminoethane (lane 2), 4 mM 1,3-DAP (lane 3), 4 mM putrescine (lane 4), 4 mM cadaverine (lane 5), (b) 1,6-diaminohexane, 1 mM (lane 1) and 4 mM (lane 2); 1,7-diaminoheptane, 1 mM (lane 3) and 4 mM (lane 4); 1,8-diaminooctane, 1 mM (lane 5) and 4 mM (lane 6); 1,9-diaminononane, 1 mM (lane 7) and 4 mM (lane 8); 1,10-diaminodecane, 1 mM (lane 9) and 4 mM (lane 10); and spermidine, 1 mM (lane 11) and 4 mM (lane 12). Mitochondria were analysed by SDS/PAGE and subsequent fluorography.

Figure 3 Effect of *N*-acylation of polyamines on the transport *in vitro* of pOTC

An aliquot (2 μ l) of the translation mixture ($\sim 40\,000$ c.p.m./ μ l pOTC) was incubated with isolated rat liver mitochondria in the transport medium, in the presence of: no additions (lane 1); spermidine, 1 mM (lane 2) and 4 mM (lane 3); *N*¹-acetylspermidine, 1 mM (lane 4) and 4 mM (lane 5); *N*⁸-acetylspermidine, 1 mM (lane 6) and 4 mM (lane 7); *N*-acetylputrescine, 1 mM (lane 8) and 4 mM (lane 9); and *N*-butyl-1,3-DAP, 1 mM (lane 10) and 4 mM (lane 11). Mitochondria were analysed by SDS/PAGE and subsequent fluorography.

transport of rat liver pOTC into isolated rat liver mitochondria, and studies have been done to establish which steps of the transport pathway require the presence of polyamines. To understand better the structural and chemical features that make polyamines competent to induce the transport of pOTC into isolated mitochondria, *in vitro* transport experiments have been carried out in which biogenic polyamines were substituted in the incubation mixture by related compounds at the same concentration, differing from them in a series of parameters including the number of amino groups per molecule, the distance(s) between them and degree of protonation (basicity) of the nitrogen atoms.

Concerning the number of amino groups per molecule required to induce the transport of pOTC, Figure 1 shows that ammonia and the monoamines methylamine, ethylamine, *n*-propylamine and *n*-butylamine do not induce pOTC transport (lanes 4–8). When α,ω -diamines with methylene chain lengths of 2–10 were tested, however, they were found to be effective in inducing

pOTC transport for a wide range of chain lengths. As shown in Figure 2(a), diamines such as 1,3-DAP (lane 3) and 1,5-diaminopentane (cadaverine) (lane 5), at 4 mM, induced the transport of pOTC as efficiently as the biogenic polyamine putrescine (1,4-diaminobutane) (lane 4). The shortest diamine, 1,2-diaminoethane (lane 2), had a very small effect on the pOTC transport. Figure 2(b) shows the effect on this process of diamines having 6–10 methylene groups between the two amino groups. Induction of the transport of pOTC by 1,6-diaminohexane, 1,7-diaminoheptane or 1,8-diaminooctane (lanes 1–6) is similar to that obtained with putrescine, but an increase of the chain length (1,9-diaminononane) results in a decrease of the amount of pOTC transported into mitochondria. No transport was observed with 1,10-diaminodecane (lanes 9 and 10).

These results suggest that induction of pOTC transport requires at least two amino groups per molecule with a distance between them equivalent to 3–8 methylene groups.

To study whether protonation of the amino groups is one of the key requirements in the action of polyamines on pOTC transport, we tested the effect of polyamines and related compounds in which basicity at the level of one of the nitrogen atoms was modified by *N*-acylation or *N*-alkylation. Figure 3 shows the effect on the transport of pOTC into isolated mitochondria of *N*-acetylputrescine, *N*¹-acetylspermidine, *N*⁸-acetylspermidine and *N*-butyl-1,3-DAP. The results of this experiment clearly show that both *N*¹-acetylspermidine (lanes 4 and 5) and *N*⁸-acetylspermidine (lanes 6 and 7) induced pOTC transport. Furthermore, *N*-acetylation of one of the amino groups of putrescine abolished the capacity of this diamine to induce the transport of pOTC (lanes 8 and 9) although *N*-butyl-1,3-DAP did induce this process (lanes 10 and 11).

Finally, we tested the effect on pOTC transport of electron-withdrawing groups that affect basicity of amino groups but without involving the lone pair of electrons of the nitrogen atom in a resonant process. For this purpose, we chose ornithine, lysine, DFMO and MAP, which can be considered as polyamine derivative compounds (Table 1). Ornithine is a non-proteogenic amino acid and is the substrate of OTC and ornithine decarboxylase (ODC); lysine is a proteogenic amino acid which differs from ornithine in only a methylene group, and DMFO and MAP are irreversible inhibitors of ODC [17]. Putrescine and cadaverine were included as controls. All of these molecules can be considered as α,ω -diamines containing 4 or 5 methylene groups which differ in the position or nature of the electron-withdrawing group affecting the basicity of one of two amino groups (Table 1). As shown in Figure 4, the amino acids ornithine and lysine (lanes 2 and 4 respectively) as well as DMFO (lane 7) do not induce pOTC transport at all, and the MAP (lane 6) does so only slightly, compared with the transport induced by putrescine and cadaverine.

DISCUSSION

We have studied structural and chemical features that make biogenic polyamines competent to induce the transport of pOTC into isolated rat liver mitochondria. From the results obtained with mono- and diamines, it seems clear that at least two amino groups per molecule are necessary to induce pOTC transport and that α,ω -diamines with hydrocarbon chains of 3–8 methylene groups induce pOTC transport as efficiently as putrescine, spermidine and spermine, but that this effect decreases dramatically as chain length increases. These results agree with those reported by Romano et al. [18] concerning the effect of diamines on the *N*-methyl-D-aspartate receptor complex, showing that the length of the methylene chain is a critical determinant of the

Table 1 Structure and effect on pOTC transport of tested compounds

Compounds have been represented by predominant (~ 90%) species present at pH 7.4, according to the pK values.

Compound		pOTC transport
Ammonia	NH_4^+	—
Methylamine	$\text{CH}_3\text{-NH}_3^+$	—
	$\text{CH}_3\text{-(CH}_2)_n\text{-NH}_3^+$	
Ethylamine	$n = 1$	—
<i>n</i> -Propylamine	$n = 2$	—
<i>n</i> -Butylamine	$n = 3$	—
	$^+\text{H}_3\text{N-(CH}_2)_n\text{-NH}_3^+$	
1,2-Diaminoethane	$n = 2$	+
1,3-Diaminopropane	$n = 3$	++
1,4-Diaminobutane (putrescine)	$n = 4$	++
1,5-Diaminopentane (cadaverine)	$n = 5$	++
1,6-Diaminohexane	$n = 6$	++
1,7-Diaminoheptane	$n = 7$	++
1,8-Diaminooctane	$n = 8$	++
1,9-Diaminononane	$n = 9$	+
1,10-Diaminodecane	$n = 10$	—
	$^+\text{H}_3\text{N-(CH}_2)_n\text{-CH(NH}_3^+\text{)-COO}^-$	
Ornithine	$n = 3$	—
Lysine	$n = 4$	—
<i>N</i> -acetylputrescine		—
Spermidine		++
<i>N</i> ¹ -acetylspermidine		++
<i>N</i> ⁸ -acetylspermidine		++
<i>N</i> -butyl-1,3-DAP		++
MAP		+
DFMO		—

activity of diamines at the polyamine recognition site, although a rather wide range of the number of methylene groups of the diamine chain gives the same effect. The explanation for this could be that these modules are flexible and long diamines may easily adopt conformations with inter-nitrogen distances mimicking those of short diamines, although this is less likely as chain length increases.

The results obtained with polyamine-related compounds in which basicity at the level of one of the nitrogen atoms was modified by *N*-acylation or *N*-alkylation support the idea that diamines must be protonated to induce pOTC transport. Given that *N*-acylation abolishes the basicity of the amino nitrogen, because of the presence of an adjacent carbonyl group that involves the lone pair of electrons of nitrogen in a resonance

Figure 4 Effect of the electron-withdrawing groups affecting basicity of amino groups of polyamines on the transport *in vitro* of pOTC

An aliquot (2 μ l) of the translation mixture ($\sim 40\,000$ c.p.m./ μ l pOTC) was incubated with isolated rat liver mitochondria in the transport medium, in the presence of: no additions (lane 1), 4 mM ornithine (lane 2), 4 mM putrescine (lane 3), 4 mM lysine (lane 4), 4 mM cadaverine (lane 5), 4 mM MAP (lane 6), and 4 mM DFMO (lane 7). Mitochondria were analysed by SDS/PAGE and subsequent fluorography.

process, and given that *N*-alkylation increases the basicity of the amino group by providing a greater effective electron density at nitrogen [19], the results obtained with *N*-acetylputrescine and *N*-butyl-1,3-DAP strongly suggest that diamines must be protonated to induce pOTC transport. The possibility that the lack of induction of pOTC transport by *N*-acetylputrescine might be caused by a steric disturbance caused by the *N*-acetyl group seems unlikely because no loss of induction was observed with *N*-alkylation in the case of *N*-1,3-butyl-DAP. In addition, it should be noted that both *N*¹-acetylspermidine and *N*⁸-acetylspermidine induced pOTC transport. These two compounds differ from spermidine in that one of the three amino groups of spermidine (the first or the last) has lost its basicity by *N*-acylation, although the two intact amino groups are separated by four or three methylene groups respectively as in spermidine (Table 1). These results are consistent with the explanation given above concerning the effect of *N*-acetylputrescine and *N*-butyl-1,3-DAP, and provide additional evidence that two protonated amino groups per molecule are sufficient to induce this process. It seems irrelevant whether the amino groups are primary or secondary.

Interestingly, protonated diamines such as ornithine and lysine do not induce pOTC transport. It is well known that the α -COOH group of α -amino acids results in a decrease of the basicity of the α -NH₂ group, as in the case of ornithine and lysine. But this decrease is unlikely to be responsible for the inability of these molecules to induce pOTC transport, because the α -amino groups (pK ~ 9) are protonated at physiological pH [19]. The incompetence of ornithine and lysine in induction of pOTC transport is most probably caused by the negative charge of the -COO⁻ group which interferes with the positive charge of -NH₃⁺, preventing the efficient interaction of this molecule with the polyamine recognition sites of a possible receptor in mitochondrial membranes [1], or pOTC itself [12], etc.

In addition to the α -COOH group, DFMO has an α -CF₂ group with an electron-withdrawing effect that dramatically decreases the basicity of the α -NH₂ (pK 6.53) (Marion Merrell Dow Research Institute, Strasbourg, France, personal communication) and, therefore, protonation of this group at pH 7.4. Induction of pOTC transport by MAP is much lower than that of putrescine. This is probably attributable to the electron-withdrawing effect of the acetylene group (Table 1), as reflected by the pK value of the amino group located at the adjacent carbon (pK 7.7) (Marion Merrell Dow Research Institute,

Strasbourg, France, personal communication). This means that only some 66% of this compound has protonated the two amino groups.

In conclusion, these results suggest two of the chemical and structural features that make polyamine compounds competent to induce pOTC transport into isolated mitochondria: these molecules must be, at least, di-cations at physiological pH; and a distance equivalent to three -CH₂-groups (~ 5 Å) [18] between the two protonated groups is enough to induce pOTC transport. It has been reported that polyamines favour the efficient binding of pOTC to mitochondria [12] but the structure or molecule with which polyamines interact remains to be determined. Given that the monoamines are unable to induce pOTC transport and given that the diamine 1,3-DAP induces transport as efficiently as putrescine and other longer diamines, it seems likely that polyamines interact with a structure having two negatively charged groups, or electronegative atoms—with a distance between them of ~ 5 Å (0.5 nm). It is therefore apparent that induction of the *in vitro* transport of pOTC does not demand that the polyamine molecule have more than two protonated amino groups (i.e. spermidine and spermine), although this characteristic can increase the probability of an efficient interaction between the polyamine and the interaction sites.

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