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Mitochondrial import of a yeast cytoplasmic $tRNA^{Lys}$: possible roles of aminoacylation and modified nucleosides in subcellular partitioning

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Abstract The yeast $tRNA^{Lys}_{CUU}$ is transcribed from a nuclear gene and then unequally redistributed between the cytosol (97–98%) and mitochondria (2–3%). We have optimized the conditions for its specific import into isolated mitochondria. However, only a minor fraction (about 0.5%) of the added tRNA was translocated into the organelles. An in vitro transcript, once aminoacylated, appeared to be a better import substrate than the natural tRNA which carries modified nucleosides. The tRNA is translocated across mitochondrial membranes in its aminoacylated form and remains relatively stable inside the organelle. Possible roles of aminoacylation, tRNA-protein interactions and nucleoside modification in subcellular partitioning of the tRNA are discussed.

Key words: Mitochondrial import; $tRNA^{Lys}_{CUU}$; Aminoacylation; (*Saccharomyces cerevisiae*)

1. Introduction

Mitochondrial import of tRNA is now considered to be a widely spread process, having been reported in the yeast *Saccharomyces cerevisiae*, in higher plants, in *Tetrahymena* and in trypanosomatids (for a review, see [1]). Various sets of cytosolic tRNAs are imported in different organisms; they range from a single tRNA in yeast [2,3] to the all of mitochondria-associated tRNAs in trypanosomatids [4,5]. The recent development of in vitro and in vivo tRNA import test systems provided direct evidence for the existence of a mitochondrial tRNA import pathway [6–10]. However, the mechanisms of tRNA targeting and translocation across mitochondrial membranes remain largely unknown. To study these mechanisms, we have set up a specific in vitro import assay using isolated mitochondria of *S. cerevisiae* [7]. In this organism, a single nuclear-coded tRNA, the $tRNA^{Lys}_{CUU}$ (tRK1) is in part associated with the mitochondrial matrix [2,3]. The mitochondrial concentration of tRK1 is comparable to that of minor native mitochondrial tRNAs. Yeast cells contain two other lysine isoacceptors, the nuclear-coded $tRNA^{Lys}_{U*UU}$, (tRK2) which is restricted to the cytosol and the mitochondrial DNA-coded $tRNA^{Lys}cmnm5_{UUU}$ (tRK3) which is only found within mitochondria. In our import assay, tRK1 is specifically taken up by isolated mitochondria in an energy- and membrane potential-dependent manner, requiring the mitochondrial pre-protein translocation machinery, including a mitochondrial outer membrane receptor, MOM19, and an essential inner membrane translocation factor, MIM44 [11]. In addition, soluble cytoplasmic proteins are also required [7,8]. We have recently identified two of these factors as the cytoplasmic lysyl-tRNA synthetase (KRS) and the precursor of the mitochondrial lysyl-tRNA synthetase (MSK) [12]. The main role of KRS appears to be aminoacylation of tRK1, which is a prerequisite for its mitochondrial targeting. The precursor of MSK is also essential for the import process. Since MSK is able to form stable complexes with the aminoacylated form of tRK1, we favor the hypothesis of its carrier function for the tRNA [12].

Despite the high selectivity of the in vitro import reaction of tRK1, only a minor fraction of the tRNA added to the import mixture is in fact transported into the organelles. This contrasts with the high efficiency of in vitro import of mitochondrial pre-proteins [13]. On the other hand, in vivo tRK1 is unequally distributed between the cytosol and mitochondria, only 2–3% being associated with the mitochondrial matrix [2,3]. Such an unequal compartmentalization is not unique for tRK1. The MRP RNA is mostly present in the nucleus, only a minor portion being associated with mitochondria [14]. Some proteins, for example, isoforms of tRNA modification enzymes, are also unequally distributed between different cellular compartments [15,16]. It is plausible that the reasons for unequal compartmentalization of tRK1 in vivo and for its low import efficiency in vitro might be similar.

Here we attempted to increase the import efficiency of tRK1 by optimizing different parameters of the in vitro import reaction. Based on our results, we propose two possible mechanisms governing intracellular compartmentalization of tRK1.

2. Materials and methods

The *S. cerevisiae* strain YPH500 [17] was used for isolation of mitochondria as described elsewhere [7,11]. In some experiments (see section 3), mitochondria were preincubated with various amounts of *E. coli* 16S and 23S rRNA (Boheringer Mannheim) at 0°C for 10 min and repelleted before the import assay. The in vitro import was directed by crude extracts (IDP) from $\Delta MOM19_{IRV}$ cells [18] carrying plasmid pG11/T6 [19] to overexpress in the cytoplasm the precursor form of MSK [12]. Immunodepletion of KRS was performed using anti-KRS antibodies (kindly provided by M. Mirande) as described previously [12]. To obtain an in vitro tRK1 transcript, the tRK1 gene (from plasmid pY109 kindly provided by H. Feldmann) was cloned using PCR under control of T7 promoter with a *Bst*NI site at the 3'-terminus of the coding sequence. The resulting plasmid, ptRK1-T7, was *Bst*NI-digested and transcribed using T7 RNA polymerase. Transcripts were gel-purified and subjected to heat denaturation followed by renaturation in the presence of 5 mM $MgCl_2$ and 1 mM spermidine. Aminoacylation was carried out with purified KRS (kindly provided by M. Mirande) as described previously [12]. *E. coli* $tRNA^{Phe}$ was purchased from Boehringer Mannheim. Yeast $tRNA^{Asp}$ was an in

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Abbreviations: tRK1, yeast $tRNA^{Lys}_{CUU}$; tRK2, yeast cytoplasmic $tRNA^{Lys}_{U*UU}$; KRS, yeast cytoplasmic lysyl-tRNA-synthetase; MSK, yeast mitochondrial lysyl-tRNA-synthetase; IDP, import directing proteins; MP, mitochondrial proteins

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vitro transcript aminoacylated with purified yeast aspartyl-tRNA synthetase (both kindly provided by A. Wolfson). After aminoacylation the enzyme was removed by phenol treatment. The standard import reaction (200 μl) contained freshly isolated mitochondria (100 μg of mitochondrial protein), 3 pmol of (5′-^{32}P)-labelled tRNA and 5–150 μg of IDP in 0.44 M mannitol, 20 mM HEPES-KOH (pH 6.8), 20 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM diisopropyl fluorophosphate, 0.1 mM L-lysine, 0.5 mM phospho*enol*pyruvate and 4 units of pyruvate kinase. The import was carried out at 25–30°C for 20 min. Mitochondria were then treated with a mixture of RNases [3]. Mitoplasts were generated by hypotonic shock (10 mM HEPES-KOH, pH 6.8, 10 min at 0°C) and purified after addition of sorbitol to 0.44 M. Mitoplasts were lysed in 1% SDS, 0.1 M sodium acetate (pH 4.8) and 0.05% diethyl pyrocarbonate at 60°C and mitochondrial RNA was phenol-extracted at the same temperature. Imported tRNAs were analyzed by denaturing 15% polyacrylamide gel electrophoresis and autoradiography. Quantitation of tRNA import was performed by scanning the gels in a Bio-Image Analyzer (Fuji). For import of [^{14}C]aminoacylated tRNAs, 1 μg of tRNA was aminoacylated with [^{14}C]lysine or [^{14}C]aspartic acid (Amersham, >200 mCi/mmol) and $0.2 \times x10^6$ cpm of labelled tRNA (trichloroacetic acid-precipitable material) were taken for each import assay. Import of [^{14}C]aminoacyl-tRNAs was measured as the label present in trichloroacetic acid precipitates of total mitochondrial RNA. All the values are the average of three or four independent experiments.

3. Results and discussion

3.1. Optimization of the in vitro import reaction of tRK1

Specific uptake of tRK1 by isolated mitochondria is energy-dependent and requires both the integrity of the mitochondrial pre-protein translocation machinery and the presence of cytoplasmic soluble proteins [7,8,11]. We show here that the import efficiency of tRK1 depends upon the concentrations of tRK1 and IDPs in the import mixture and upon the presence of ATP, and varies as a function of pH and temperature (Fig. 1). Optimal import conditions (100 μg of mitochondrial proteins, 3 pmol tRK1, 50 μg of IDPs in 0.2 ml, 1 mM ATP and the presence of an ATP regeneration system, pH 6.8, 25°C) yielded 0.05 pmol of imported tRK1 per mg of mitochondrial proteins (MP). This amount represents only 0.16% of the tRK1 molecules added to the import assay. The in vivo mitochondrial content of tRK1 can be estimated to be around 2.5 pmol per mg MP ([2,3], I.A. Tarassov). This means that the efficiency of the in vitro import is only about 2% of that in vivo.

Quantitation of tRK1 degradation in the import mixture and in repurified mitochondria [7] indicated degradation of about 20% of the added tRK1 after a 30 min incubation with all components of the assay. Therefore, the low amount of tRK1 detected within mitochondria cannot be explained solely by its degradation in the import mixture and/or inside the organelle. The efficiency of tRK1 import may, however, be decreased by non-specific binding of the tRNA to the mitochondrial surface. In fact, isolated mitochondria bind tRK1 as well as *E. coli* tRNA^{Phe} (which is not imported) non-specifically (Fig. 2A). Mitochondria-bound tRNA can be removed, although not entirely, by treatment of mitochondria with a mixture of RNases. In fact, total elimination of bound tRNA can only be achieved by removal of the outer mitochondrial membrane and RNAse treatment of the mitoplasts. The fraction of mitochondria-bound tRK1 may be inaccessible for import, which in turn would affect its import efficiency. To test this hypothesis, we performed competition experiments by adding non-labelled tRNAs to the import mixture (Fig. 2B). Import assays were conducted in the simultaneous presence of labelled tRK1 and of increasing amounts of non-labelled tRK1 or of *E. coli* tRNA^{Phe}. A 10-fold excess of non-labelled tRK1 completely blocked the import of labelled tRK1, due to specific competition. In contrast, addition of increasing amounts of non-labelled tRNA^{Phe} did not inhibit tRK1 import but rather had a slight enhancement effect (at 5–10 μg of competitor tRNA/mg MP). In order to neutralize the non-specific RNA-binding sites at the mitochondrial surface, we introduced a step of pre-incubation of mitochondria with *E. coli* rRNA prior to the tRK1 import reaction. As shown in Fig. 2C, rRNA-treated and repurified mitochondria were in fact more efficient in the import assay. Under optimal conditions, the import efficiency reached on average 0.1 pmol tRK1 per mg MP. Nevertheless, this value corresponds to only 0.3% of the tRK1 added to the in vitro assay and to 4% of its in vivo mitochondrial pool. We are therefore forced to admit that the low in vitro tRK1 import efficiency must rely on reasons other than non-specific binding of the tRNA to mitochondria.

As a possible mechanism leading to unequal distribution of tRK1 among the cytosol and mitochondria, we suggest that in the cytoplasm binding of tRK1 is subjected to competition between IDP and factors of the cytoplasmic translation apparatus. Indeed, our previous results indicate that aminoacylation of tRK1 by the KRS (or, at least, formation of a tRK1-KRS complex) is required for its interaction with pre-MSK

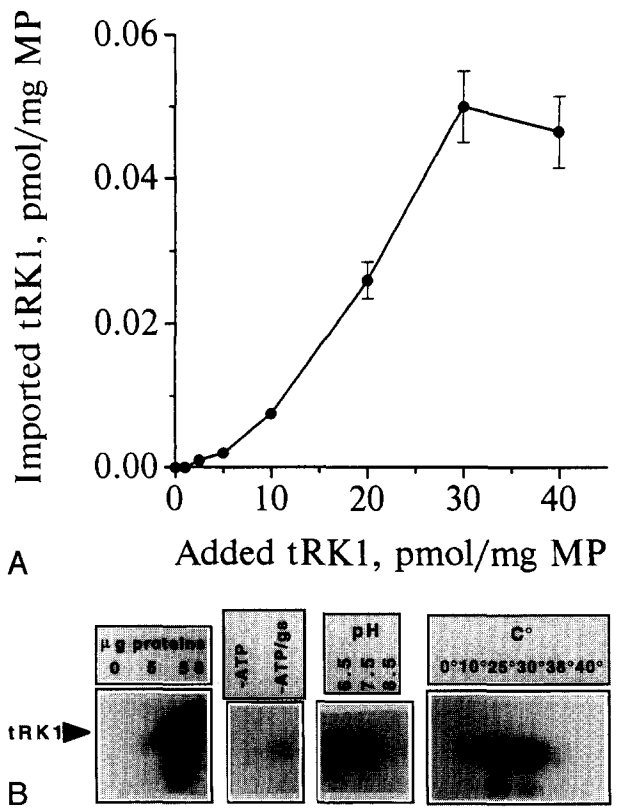

Fig. 1. Optimization of tRK1 in vitro import conditions. Each assay was performed with the same amount of mitochondria corresponding to 100 μg of mitochondrial protein. (A) Dependence of in vitro tRK1 import efficiency upon the amount of added tRK1. (B) Autoradiographic detection of the imported tRK1 after electrophoretic separation of RNA extracted from repurified mitochondria. 'Proteins', amount of IDP; '−ATP', without ATP; '−ATP/gs', without ATP-generation system.

and, in turn, for its translocation across mitochondrial membranes [12]. Formation of ternary complexes which include tRK1, KRS and pre-MSK may reflect an intermediate step leading to the mitochondrial targeting of tRK1 [12]. We therefore suggest that the competitive binding of tRK1-KRS complexes by either pre-MSK (alone or in association with other

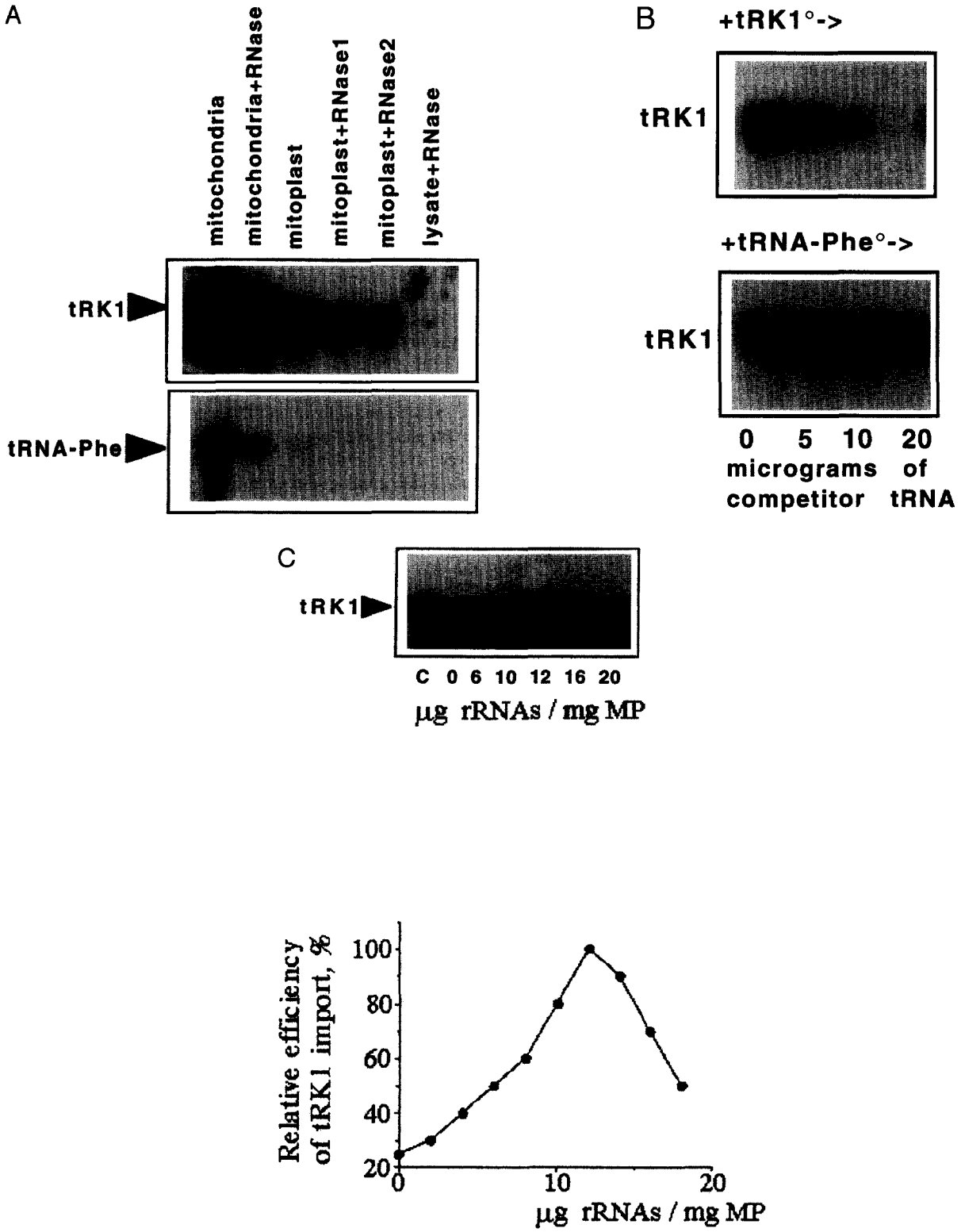

Fig. 2. (A) Effect of non-specific binding of tRNA to mitochondria. 'Mitoplasts+RNAse 1' and 'Mitoplasts+RNAse 2' differed in the duration of RNase treatment after mitoplast isolation (10 and 20 min, respectively); 'Lysate+RNAse', after the import assay mitochondria were lysed with 0.5% Triton X-100 in the presence of RNAses. (B) Competition effect of non-labelled tRNAs onto tRK1 in vitro import. '+tRK1°->' and '+tRNA^{Phe}->', addition of increasing amounts of non-labelled tRK1 and *E. coli* tRNA^{Phe}, respectively (μg competitor tRNA per mg MP). (C) Effect of pretreatment of mitochondria with non-labelled *E. coli* ribosomal RNAs. Autoradiographic detection of the imported tRK1 ('C', ^{32}P-labelled tRK1 using in the import assay) and dependence of the relative tRK1 import efficiency upon rRNA concentration.

IDP) or cytoplasmic translation factors might be an essential step in the intracellular partitioning of tRK1. Such a hypothesis is in agreement with the fact that in the cytoplasm of higher eucaryotes, tRNAs (either deacylated or aminoacylated) are tightly integrated in RNA-protein interactions of the protein synthesis pathway ('channelled tRNA cycle'), which implies that no 'free' tRNAs exist in the cytosol [20,21].

3.2. *An in vitro transcript of the tRK1 gene is a better import substrate than the natural tRNA*

An alternative explanation for unequal compartmentalization of tRK1 would be the existence of a minor subpopulation of 'import-active' tRNA molecules differing from 'import-inactive' molecules by structural features. All the eight chromosomal tRK1 gene copies we have identified in strain YPH499, have identical coding sequences (I.A. Tarassov, unpublished). It is therefore not plausible that import-active and import-inactive tRK1 molecules differ by their primary sequence. However, it is not known whether mitochondrial and cytoplasmic pools of tRK1 differ in the nature or extent of nucleoside modification [3,22]. This opens the possibility that some specific nucleoside modification (or its absence) could play a role as an import discriminator. To investigate this possibility we compared the mitochondrial import efficiency of an in vitro-synthesized tRK1 transcript (which is expected not to carry modified nucleosides) with that of the natural fully modified tRK1. Taking into account that aminoacylation of tRK1 is a prerequisite for its mitochondrial import [12], we tested the import of the in vitro transcript and of the natural tRNA in their deacylated forms and after aminoacylation with KRS prior to the import. The dependence of import efficiencies upon the concentration of IDP is shown in Fig. 3. The deacylated transcript proved to be a poor import substrate, while the aminoacylated transcript showed a higher import efficiency than both the deacylated and aminoacylated forms of natural tRK1. The poor import efficiency of the deacylated transcript can be explained by its low level of aminoacylation during the incubation in import mixture. Indeed, the ratio between the K_{cat}/K_m values of aminoacylation reaction with KRS for tRK1 and for the in vitro transcript was found to be 16,7. The aminoacylated transcript is imported with maximal

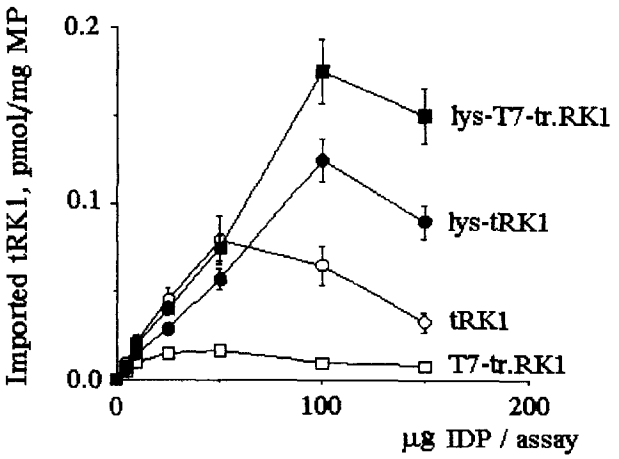

Fig. 3. Dependence of import efficiency upon IDP concentration. 'T7-tr.RK1', in vitro T7-transcript of the tRK1 gene; 'lys-', RNA was aminoacylated prior to the import assay, then KRS was removed by phenol treatment; IDP immuno-depleted with anti-KRS antibodies [12] were used in import assay.

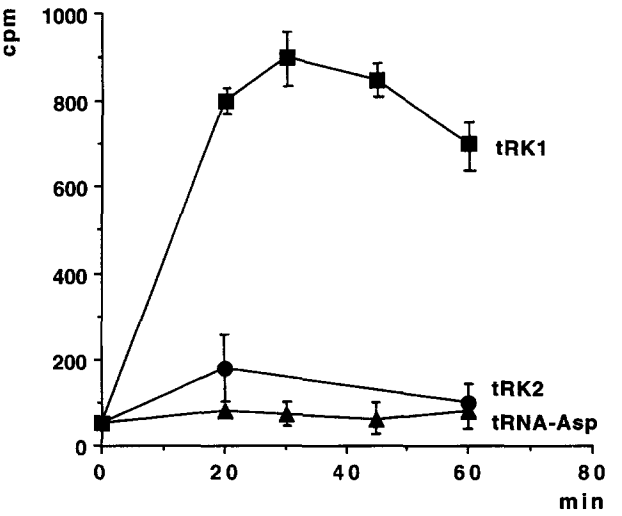

Fig. 4. In vitro import of [^{14}C]aminoacyl-tRNAs. *y*-axis values correspond to ^{14}C label associated with mitochondrial tRNA as a function of time (*x*-axis values) of incubation of mitochondria with the [^{14}C]aminoacyl-tRNAs. tRNA-Asp was an in vitro synthetized transcript of the yeast tRNA^{Asp} gene.

efficiency of 0.15–0.2 pmol per mg MP, which corresponds to 0.45–0.6% of the transcript added to the in vitro assay (i.e., 6–8% of the in vivo mitochondrial pool of tRK1). Taken together, these results indicate that modified nucleosides are not necessary for recognition of tRK1 by import factors but do not eliminate the possibility that some specific nucleoside modification(s) have a role of import discriminator(s). Alternatively, hypomodification of tRK1 could lead to greater flexibility of the tRNA structure [23,24] which, in turn, could facilitate its transmembrane transfer.

3.3. *tRK1 is imported in its aminoacylated form which remains relatively stable within mitochondria*

As already stressed, aminoacylation of tRK1 by KRS (or at least formation of a tRK1-KRS complex) is a prerequisite for its mitochondrial targeting [12]. This opens the possibility that the tRNA might be imported in its aminoacylated form. To provide direct evidence for this hypothesis, we studied the fate of [^{14}C]lysyl-tRK1 during the course of its import into isolated mitochondria (Fig. 4). As controls, the behaviour of aminoacylated tRK2 and of an aminoacylated tRNA^{Asp} transcript, both of which are not imported, has also been studied. With these two tRNAs, as expected, no trichloroacetic acid-precipitable ^{14}C label was detected in mitochondrial RNA. In contrast, when aminoacylated tRK1 was used, increasing amounts of ^{14}C label were found in mitochondrial RNA, at least during the first 30 min of the import reaction. This result clearly shows that tRK1 is translocated across mitochondrial membranes in its aminoacylated form. The amount of [^{14}C]lysyl-tRK1 detected in mitochondrial RNA after a 30 min import reaction corresponds to 0.2–0.3% of the aminoacylated tRNA added to the reaction, which is very similar to the value obtained using ^{32}P end-labelled tRK1 (see above).

The fact that the mitochondrial pool of aminoacylated tRK1 was maximal after 30 min incubation (see Fig. 4) is consistent with our previous results showing that the in vitro import system is saturated within this period [7]. Since this amount only slightly decreased during the following 30 min, the aminoacylated form of tRK1 is relatively stable within

mitochondria, at least in vitro. The fact that tRK1 enters mitochondria in its aminoacylated form and remains aminoacylated, at least for some time, within the organelle, might be relevant to its mitochondrial function, possibly indicative of an involvement in mitochondrial protein synthesis.

4. Conclusion

Taken together, our results show that only a small fraction of the cytoplasmic tRK1 is translocated into mitochondria and suggest two possible mechanisms leading to its unequal intracellular distribution. The first one supposes the existence of only a minor fraction of hypomodified tRK1 competent for import. The second one proposes that intracellular partitioning of tRK1 results from competition between IDP and other proteins (most probably cytosolic translation factors) for binding to the aminoacylated tRNA. To distinguish between these two possible mechanisms, characterization of new IDP and mechanisms of their interactions with tRK1 and KRS, as well as identification of 'import determinants' within the tRK1 sequence will be helpful.

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