FEBS Letters 435 (1998) 6–10 FEBS 20769

Rat liver mitochondria can hydrolyse thiamine pyrophosphate to thiamine monophosphate which can cross the mitochondrial membrane in a carrier-mediated process

Maria Barile[a,*], Daniela Valenti[a], Carmen Brizio[a], Ernesto Quagliariello[a], Salvatore Passarella[b]

[a] *Dipartimento di Biochimica e Biologia Molecolare, Università di Bari, and Centro di Studio sui Mitocondri e Metabolismo Energetico, C.N.R., Via Orabona 4, 70126 Bari, Italy*
[b] *Dipartimento di Scienze Animali, Vegetali e dell'Ambiente, Università del Molise, Campobasso, Italy*

Received 27 July 1998

Abstract We show here that TPP → TMP conversion can take place in rat liver mitochondria. This occurs via the novel, putative TPP pyrophosphatase localised in the mitochondrial matrix, as shown both by digitonin titration and by an HPLC enzyme assay carried out on the mitochondrial matrix fraction. Certain features of the reaction, including the substrate and pH dependence, are reported. Additional evidence is given that externally added TMP can cross the mitochondrial membrane in a manner consistent with the occurrence of a carrier-mediated process. This can occur both via the TPP translocator and via a novel translocator, inhibited by CAT but different from the ADP/ATP carrier.

Key words: Thiamine monophosphate; Thiamine pyrophosphate; Mitochondrial transport

1. Introduction

Although the structural and functional biology of mitochondrial energy metabolism in mammals has been deeply investigated in the last 40 years, the mechanisms by which the vitamin-derived cofactors involved in energy metabolism are synthesised and degraded in the mitochondrial compartments are still rather obscure. Some cofactor synthesis occurs in mitochondria from the vitamins and/or precursors taken up. Indeed, this uptake of certain vitamins and the synthesis of certain cofactors in isolated mammalian mitochondria has already been reported, as has the contrasting fact that several cytosolically synthesised cofactors enter isolated mitochondria (for references see [1]). By contrast, the knowledge of mitochondrial cofactor degradation is rather poor. The existence of a specific mitochondrial pyridoxal 5′-phosphate phosphatase has been suggested [2] and more recently, NAD and flavin cofactor hydrolysis have been shown to occur, catalysed by enzymes localised in the outer mitochondrial compartments [3,1].

The above described state of the art requires further investigation regarding the occurrence of mitochondrial enzymes involved in vitamin and vitamin cofactor metabolism. In particular, it would be worthwhile to gain a better insight into the mitochondrial metabolism of TPP, which represents 30% of the total cellular cofactor in hepatocytes [4]. As far as TPP synthesis and localisation are concerned, the possibility of TPP synthesis in RLM has long since been ruled out [5,6]. Consistently, in hepatocytes, externally added thiamine is converted to TPP via the cytosolic thiamine pyrophosphokinase (EC 2.7.6.2) [7], with TPP being taken up by RLM in a carrier-mediated process [8]. However, no information is available concerning TPP catabolism and the aim of the current investigation was to ascertain whether and how mitochondria can hydrolyse TPP and what the fate of the hydrolysis product(s) is. The occurrence of TPP → TMP conversion is shown in RLM as catalysed by the putative TPPase with additional evidence that TMP can cross the mitochondrial membrane in exchange for TPP.

2. Materials and methods

All reagents and enzymes were from Sigma (St. Louis, MO, USA). Mitochondrial substrates were used as Tris salts at pH 7.0–7.3. Digitonin was from Merck. H_2O and CH_3CN used for HPLC were from J.T. Baker (Deventer, The Netherlands).

RLM and pRLM were obtained from male Wistar rats (150–200 g) [1], and resuspended up to 50–60 mg protein in the standard medium consisting of 0.25 M sucrose, 20 mM Tris-HCl, pH 7.25, 1 mM EDTA. Mitochondrial protein was determined according to Waddel and Hill [9].

The TPP loading procedure was carried out as described [8], by adding RLM with 450 μM TPP.

The mitochondrial matrix fraction was obtained from pRLM [10], with the fraction purity checked by measuring the activities of MAO (EC 1.4.3.4), ADK (EC 2.7.4.3) and GDH (EC 1.4.1.3), as marker enzymes for the outer membrane, intermembrane space and matrix, respectively [11–13].

Digitonin titration was carried out according to Boyer et al. [3]. Briefly, RLM (5 mg) were incubated at 0–2°C for 15 min in a medium (0.5 ml) consisting of 0.22 M mannitol, 0.07 M sucrose, 2 mM HEPES-Tris, pH 7.4, in the presence of varying amounts of digitonin and then centrifuged at 20 000 × *g* for 2 min. The lysosomal and microsomal contamination was checked by measuring the acid phosphatase (EC 3.1.3.2) and the NADPH-cytochrome *c* reductase (EC 1.6.2.4) activities [14,15].

The TPP and TMP mitochondrial metabolism was investigated as follows: either pRLM (solubilised with Triton X-100) or mitochondrial matrix fraction was incubated at 25°C in 1.4 mL of 50 mM sodium acetate buffer pH 5.0 for 1 min and then either TPP or TMP was added. At various points in time, aliquots (200 μl) were taken from the reaction mixture and rapidly added with SDS (0.5%)

*Corresponding author. Fax: (39) (80) 5443317.
E-mail: m.barile@biologia.uniba.it

Abbreviations: ADK, adenylate kinase; CAT, carboxyatractyloside; GDH, glutamate dehydrogenase; MAO, monoamine oxidase; pRLM, purified rat liver mitochondria; RLM, rat liver mitochondria; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate; TPPase, TPP pyrophoshatase

PII: S0014-5793(98)01007-2

to stop any enzymatic reaction. TPP and TMP hydrolysis products were checked by photometric measurements and HPLC analysis. In the former case, the rate of Pi appearance was measured [16], with correction made for the mitochondrial endogenous Pi. In the case of HPLC measurements, neutralised perchloric extracts (5–40 μl) [1] were analysed for thiamine, TMP and TPP amount using a Kontron Instrument HPLC system (including a model 420 pump and model 425 gradient former equipped with a 450 MT2 data system). Use was made of a RP-8 Lichrosorb-NH_2 column (12.5 cm × 3 mm) equipped with a guard column (4 cm × 4 mm). The solvent was of 70 mM potassium phosphate buffer (pH 7.4) with 60% CH_3CN. Elution was carried out at room temperature with a flow rate of 1.5 ml/min. Eluted thiamine derivatives were converted to thiochrome [17] and detected fluorimetrically by means of a Kontron Instruments SFM 25 fluorimeter (excitation and emission wavelengths at 375 nm and 435 nm, respectively). Thiamine, TMP and TPP retention times (about 2, 4.5 and 5.5 min, respectively) were measured in each experiment, with a quantitative determination carried out by means of a calibration curve, obtained using standard solutions, the concentrations of which were determined photometrically ($\varepsilon_{266nm} = 8.5\ mM^{-1}\ cm^{-1}$ for TMP and TPP). Under the conditions used, TMP and TPP fluorescence did not differ significantly. Since the rates of Pi appearance and TMP formation showed no significant difference, either photometric or HPLC assay was used.

TMP and TPP uptake assays were carried out as follows: RLM (6–9 mg protein) were incubated for 1 min at 25°C in 0.35 ml of the standard medium in the presence of [^{14}C]sucrose and ^3H_2O (0.1 μCi each). Uptake reaction was started by adding the substrate and stopped when appropriate by rapidly centrifuging at 14 000 × *g* in a refrigerated Ole Dich microcentrifuge equipped with an Eppendorf rotor. Mitochondrial pellets were carefully separated from supernatants, washed in 1 ml of ice-cold standard medium and centrifuged to obtain perchloric extracts, and the amount of thiamine derivative actually taken up by RLM was calculated [8]. The content of TMP and TPP in neutralised extracts was measured via HPLC, whereas the content of TMP plus TPP was measured by directly following the fluorescence of the thiochrome, using a LS50 Perkin Elmer spectrophotofluorimeter. When only TMP content was found to increase (as checked via HPLC), the thiochrome fluorescence was used to measure TMP uptake. In another experimental approach, the amount of TPP in the neutralised extracts was estimated enzymatically by measuring the pyruvate decarboxylase (EC 4.1.1.1) activity, which is restored when TPP is added to the partially inactive apoenzyme [8].

Pi (via Pi carrier), succinate-malate exchange (via dicarboxylate carrier), *cis*-aconitate transport (via tricarboxylate carrier), oxaloacetate uptake (via oxodicarboxylate carrier) and ADP/ATP exchange (via adenine nucleotide carrier) were determined as described elsewhere [18].

3. Results

3.1. TPP hydrolysis by RLM

In order to determine whether RLM can accomplish thiamine derivative metabolism, either TPP or TMP (1 mM each) was added to pRLM ruptured with Triton X-100 (0.5%) and Pi appearance in the reaction medium was followed. With TPP, the amount of Pi in the reaction mixture was found to linearly increase with time up to 80 min, at a rate of Pi appearance of 0.7 ± 0.3 nmol Pi/min/mg protein in three different experiments. Pi appearance was prevented by SDS (0.1%), which denatures the proteins, and by NaF (10 mM), an inhibitor of cell phosphatases and phosphodiesterases. By contrast, no Pi formation was found when TMP was added to ruptured pRLM. Taken together, these results show that RLM can hydrolyse externally added TPP yielding TMP as the product.

In order to identify the mitochondrial compartment in which TPP metabolism takes place, the release of TPP dephosphorylating activity from RLM was measured as a function of the digitonin/protein ratio (Fig. 1). First, as a control,

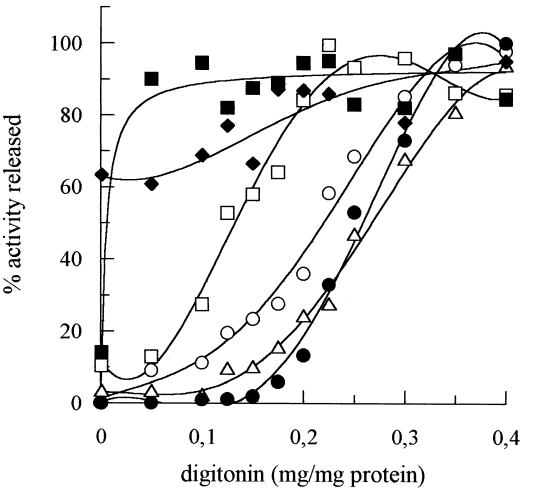

Fig. 1. Submitochondrial localisation of TPPase activity: digitonin titration. Digitonin titration was carried out as described in Section 2. The enzymes measured were acid phosphatase (■), NADPH-cytochrome *c* reductase (♦), ADK (□), MAO (○), GDH (△) and TPPase (●). Values are given as the mean of four experiments carried out with different mitochondrial preparations. The standard errors, not reported for clarity's sake, ranged between 2 and 20% of the mean values.

the release profiles of the lysosomal acid phosphatase and the microsomal NADPH-cytochrome *c* reductase from the mitochondrial pellet were measured. Both enzymes were released almost completely at 0.1 mg digitonin/mg protein ratios (90% and 70% respectively). By contrast, the release of MAO, ADK and GDH, used as mitochondrial marker enzymes, required digitonin/protein ratios higher than 0.1. The profile of the TPP dephosphorylating activity followed that of GDH, thus showing that the enzyme that hydrolyses TPP is localised in the matrix fraction.

In order to identify the product(s) of TPP hydrolysis catalysed by a mitochondrial matrix protein, in a first set of experiments TPP (0.2 mM) was added to the mitochondrial matrix fraction and neutralised perchloric extracts, obtained at different incubation times, were analysed by HPLC (Fig. 2). The matrix contents of TPP and TMP were found to be about 500 and 70 pmol/mg matrix protein, respectively, whereas no thiamine was detectable. Upon TPP addition, TMP was accumulated in the mitochondrial matrix, at a rate of 0.23 nmol/min/mg protein. Interestingly, both TPP and TMP failed to cause thiamine formation within the observation time. These results show that TPP → TMP+Pi conversion occurs in the mitochondrial matrix catalysed by the novel mitochondrial TPPase (EC 3.6.1.-).

Some features of TPP hydrolysis catalysed by a matrix protein were then investigated (Fig. 3). The rate of TPP → TMP conversion was studied as a function of the amount of mitochondrial protein in the 0–0.8 mg range and a linear dependence was found, with the apparent rate constant being about 0.92 nmol/min/mg. The rate of TPP hydrolysis showed a broad maximum of activity in the 5–7 pH range and was very low at alkaline pH (Fig. 3A). Finally, the dependence of the rate of TPP hydrolysis was studied as a function of the substrate concentration: hyperbolic characteristics were found with K_m and V_{max} values equal to 0.50 ± 0.07 mM and 1.07 ± 0.07 nmol/min/mg, respectively (Fig. 3B).

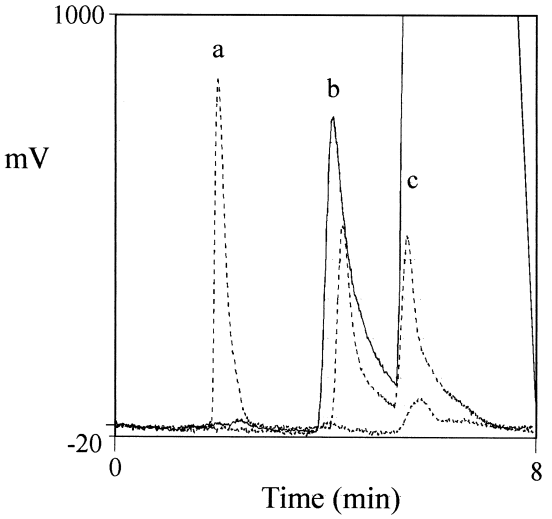

Fig. 2. HPLC evidence of TMP appearance following TPP addition to the mitochondrial matrix fraction. Mitochondrial matrix fraction (0.8 mg protein) was incubated for 1 min under the experimental conditions described in Section 2 and then TPP (0.2 mM) was added. The figure reports the chromatograms of the mitochondrial matrix fraction before (dotted line) and after 80 min incubation with TPP (solid line) and the chromatogram of standard solutions of thiamine (30 pmol, a), TMP (35 pmol, b) and TPP (25 pmol, c) (dashed line).

3.2. TMP transport in isolated mitochondria

In order to determine whether RLM are permeable to TMP, a series of experiments was carried out in which TMP was added to mitochondria and fluorimetric measurements made of the intramitochondrial TMP content (Fig. 4), thus measuring the net increase of the thiamine derivative content in RLM. In Fig. 4A the time course of TMP uptake by RLM is shown. The TMP taken up was found to increase with time, with an initial rate equal to about 220 pmol/min/mg protein and a maximum uptake, reached after about 3 min incubation, of about 390 pmol/mg protein. The dependence of TMP uptake rate on temperature was investigated at temperatures ranging between 1 and 26°C and reported as an Arrhenius plot (Fig. 4B). An activation energy value of about 7 kcal/mol was calculated, in fairly good agreement with the value obtained for other transport processes (for references see [19]). The dependence of the uptake rate on extramitochondrial TMP concentration was also investigated (Fig. 4C). Satura-

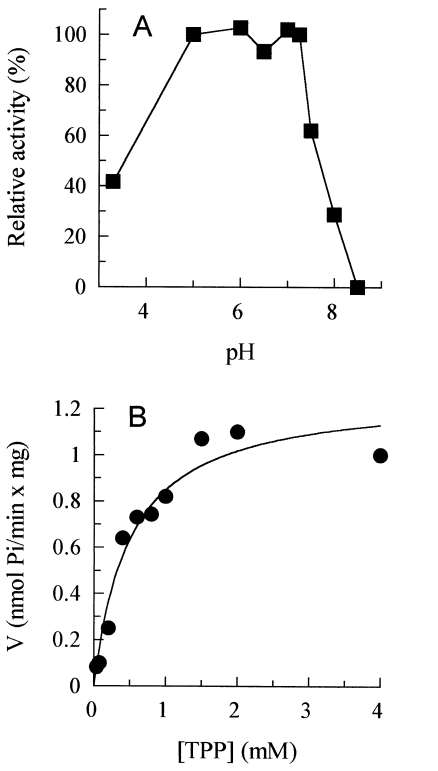

Fig. 3. Some features of TPP hydrolysis catalysed by the mitochondrial matrix fraction. TPP (1 mM) hydrolysis was assayed by photometrically measuring the rate of Pi appearance (V) as described in Section 2. A: pH profile of the rate of TPP hydrolysis. The rate of TPP hydrolysis measured at each pH is reported as a percentage of that measured at pH 5.0. B: The dependence of the rate of TPP hydrolysis on the substrate concentration.

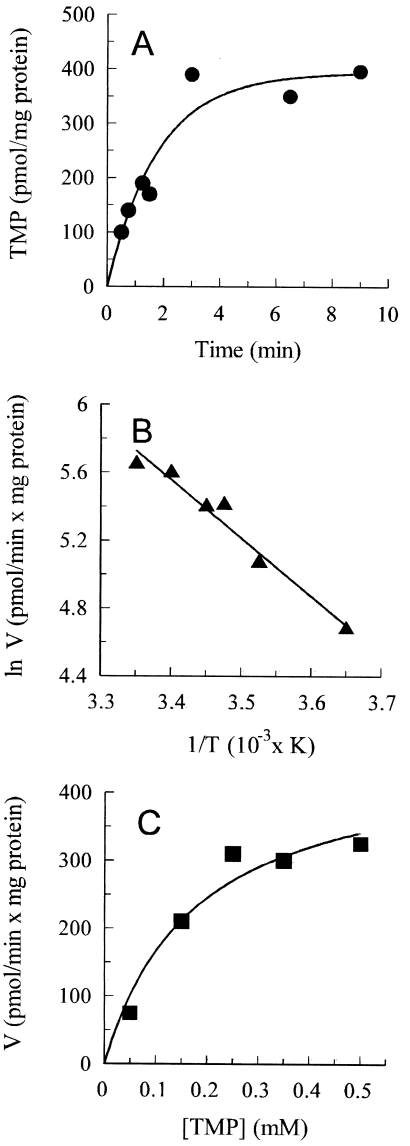

Fig. 4. Some features of TMP uptake by isolated RLM. TMP (200 μM) uptake reaction was studied as described in Section 2. A: The time course of TMP uptake by isolated RLM. B: The temperature dependence of the rate of TMP uptake (measured in 45 s) reported as the Arrhenius plot. C: The dependence of the rate of TMP uptake on the extramitochondrial concentration of TMP.

tion characteristics were found, thus strongly suggesting the existence of a translocator which can mediate TMP transport into mitochondria. K_m and V_{max} values were equal to 180 ± 78 μM and 462 ± 78 pmol/min/mg protein, respectively.

In order to gain a degree of insight into TMP transport, both the capability of certain inhibitors of mitochondrial carriers to affect TMP uptake and TMP capability to affect the activity of certain mitochondrial translocators were investigated. Mersalyl, butylmalonate, phenylsuccinate and 1,2,3-benzenetricarboxylate, which are powerful inhibitors of Pi, dicarboxylate, oxodicarboxylate and tricarboxylate carriers, respectively [19], failed to inhibit the rate of TMP (60 μM) uptake into mitochondria. Consistently, when the capability of 0.5 mM TMP to inhibit the Pi, dicarboxylate, oxodicarboxylate or tricarboxylate translocators was checked as in [18], no inhibition was found (data not shown). It should be noted that, in each case, the concentration of the substrate used was close to its K_m value as determined under the same experimental conditions. These results show that TMP uptake does not involve the Pi, the dicarboxylate, the oxodicarboxylate or the tricarboxylate translocators.

Interestingly, 50% inhibition of TMP uptake was found in the presence of CAT (2 μM). It should be noted that CAT has been previously found to inhibit TPP uptake by RLM [8]. Thus, given that CAT is a powerful specific inhibitor of the ADP/ATP translocator [20], the capability of TMP to use this antiporter was checked by measuring the rate of ADP/ATP exchange. Externally added ADP (5 μM) causes efflux of the newly synthesised ATP with a rate in fairly good agreement with that expected by considering the K_m and V_{max} values previously reported [20]. Both CAT and oligomycin were found to strongly inhibit ATP appearance outside mitochondria. Conversely, no inhibition was found due to TMP (up to 0.5 mM) addition.

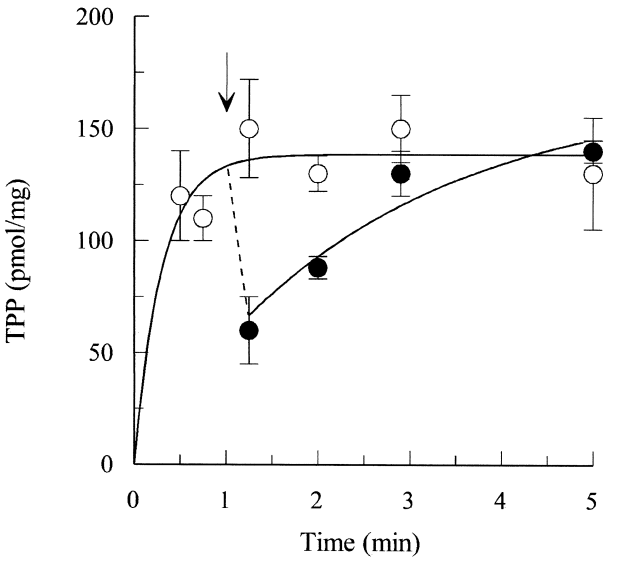

Fig. 5. Time course of TPP uptake: effect of TMP addition. RLM (7 mg protein) were incubated for 1 min at 25°C under the experimental conditions described in Section 2. The uptake reaction was started by the addition of TPP (190 μM) and stopped at the indicated points in time, with the amount of TPP actually taken up (○) enzymatically measured as described in Section 2. At the arrow, either medium (○) or TMP (125 μM) (●) was added to the mitochondrial suspension and the amount of TPP measured at the indicated points in time. Values are given as the mean of three repeats (± S.E.M.) performed using the same mitochondrial preparation.

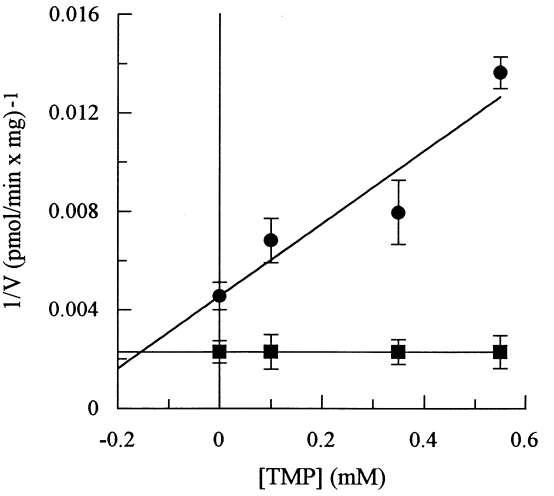

Fig. 6. Dixon plot of the TMP inhibition on the rate of TPP uptake. The TPP uptake reaction was started by the addition of either 22 (●) or 220 μM TPP (■) either in the presence or in the absence of TMP at the indicated concentrations. The rate of TPP uptake (V) was enzymatically measured as described in Section 2. Values are given as the mean of three experiments and reported with their standard errors.

Finally, experiments were carried out to determine whether TMP can use the carrier which catalyses TPP transport into mitochondria [8]. To achieve this, first the capability of externally added TMP to cause TPP efflux from the matrix was checked with enzymatic measurement of TPP mitochondrial content. In a set of experiments either RLM or TPP-loaded RLM were added with TMP and the possible exchange reaction stopped 1 min later. While a 45% decrease of the intramitochondrial TPP content in TPP-loaded RLM was found, there was no significant decrease in RLM not loaded with TPP (data not shown). In another experiment the time course of TPP uptake by RLM was investigated (Fig. 5). The uptake reaction was started by the addition of TPP (190 μM) to isolated RLM and stopped at different incubation times, with measurement made of the amount of TPP taken up by mitochondria. When TPP uptake was complete (about 1 min), TMP (125 μM) was added. This was found to cause a decrease in the TPP content of the mitochondria, consistent with the efflux of the TPP already taken up. As expected in the light of the existence of a single carrier shared by both TPP and TMP, the rate of TPP uptake proved to be inhibited by externally added TMP. The nature of TMP inhibition was investigated by means of a Dixon plot (Fig. 6). TMP proved to be a competitive inhibitor of TPP uptake rate with a K_i value equal to 150 μM.

4. Discussion

This work shows a novel property of RLM: they can metabolise TPP. This occurs in virtue of the presence of an enzyme exhibiting TPPase activity, localised in the matrix space of mitochondria. The mitochondrial localisation is shown both because RLM is essentially free of lysosomes and microsomes, and more importantly, in the light of both the digitonin titration and direct TPPase assay in the matrix fraction of RLM. Our experimental findings successfully ap-

ply to certain criteria used to show the occurrence of enzyme catalysed reactions: the linear interrelationship between the rate of reaction and amount of the enzyme (mitochondrial matrix protein in this case), the occurrence of hyperbolic dependence on the substrate concentration, the pH profile and, finally, its inhibition by NaF.

The physiological fate of TMP formed during mitochondrial TPP degradation remains quite obscure. Since TMP can cross the mitochondrial membrane, we assume that, in the route of its degradation, TMP is converted to thiamine in the cytoplasm, where TMP phosphohydrolase (EC 3.1.3.-) is located, thus allowing for new TPP synthesis via the cytosolic thiamine pyrophosphokinase [7]. We show here that TMP can permeate the mitochondrial membrane in a carrier mediated process, as suggested both by the occurrence of the hyperbolic dependence on substrate concentration and by the inhibition of TMP uptake due to CAT. The experimental data reported in this paper are consistent with the conclusion that TMP and TPP share the same carrier to cross mitochondrial membrane. This is substantiated by their mutual inhibition and by TPP efflux from the mitochondria caused by TMP added to TPP-loaded RLM. The finding that the K_i value is close to the K_m measured for TMP uptake further suggests that the same translocator could be involved in the transport of both TPP and TMP. Nonetheless, TMP uptake measured as an increase in the thiochrome fluorescence, i.e. without efflux of thiamine/thiamine derivatives, supports the existence of a novel translocator able to mediate TMP uptake in uniport and/or in exchange with another anion, perhaps Pi, even though the possibility that the TPP translocator might exert the two modes of transport, as reported for the carnitine carrier (see [21]), cannot be ruled out.

In conclusion, the picture emerging for TPP mitochondrial metabolism in liver, as obtained by this and our previous paper [8] is the following: RLM are permeable to TPP and TMP which is formed in the matrix from TPP imported in a carrier mediated transport, perhaps in exchange with TMP. Thus, mitochondria contain carriers and enzymes which can provide transport for and degradation of their own thiamine derivatives.

Acknowledgements: The linguistic review of this manuscript by Dr Steve Reshkin is gratefully acknowledged. The authors would like to thank Dr S. Delfine who participated as a student in the initial stages of this work, and Dr Caterina De Virgilio who made the HPLC measurements. The skilful technical assistance of Mr Vito Giannoccaro is gratefully acknowledged. This work was partially financed by a C.N.R. (97.04000.CT04) grant to Maria Barile and by a M.U.R.S.T. (Programmi di Ricerca Scientifica di Interesse Nazionale) grant to Salvatore Passarella.

References

[1] Barile, M., Brizio, C., De Virgilio, C., Delfine, S., Quagliariello, E. and Passarella, S. (1997) Eur. J. Biochem. 249, 777–785.
[2] Lui, A., Lumeng, L. and Li, T.-K. (1981) J. Biol. Chem. 256, 6041–6046.
[3] Boyer, C.S., Moore, G.A. and Moldéus, P. (1993) J. Biol. Chem. 268, 4016–4020.
[4] Dianzani, M.U. and Dianzani Mor, M.A. (1957) Biochim. Biophys. Acta 24, 564–568.
[5] Voskoboev, A.I. and Averin, V.A. (1981) Vopr. Med. Khim. 27, 239–243.
[6] Barile, M., Passarella, S. and Quagliariello, E. (1986) Biochem. Biophys. Res. Commun. 141, 466–473.
[7] Deus, B. and Blum, H. (1970) Biochim. Biophys. Acta 219, 489–492.
[8] Barile, M., Passarella, S. and Quagliariello, E. (1990) Arch. Biochem. Biophys. 280, 352–357.
[9] Waddel, W.J. and Hill, C. (1956) J. Lab. Clin. Med. 48, 311–314.
[10] Pedersen, P.L., Greenawalt, J.W., Reynafarje, B., Hullihen, J., Decker, G.L., Soper, J.W. and Bustamente, E. (1978) Methods Cell Biol. 20, 411–476.
[11] Schnaitman, C., Erwin, G. and Greenawalt, J.W. (1967) J. Cell. Biol. 32, 719–735.
[12] Feldhaus, P., Frohlich, T., Goody, R.S., Isacov, M. and Schirmer, R.H. (1975) Eur. J. Biochem. 57, 197–204.
[13] Schmidt, E. (1963) in: Methods of Enzymatic Analysis (Bergmeyer, H.U., Ed.), pp. 752–755, Academic Press, New York.
[14] Shibko, S. and Tappel, A.L. (1963) Biochim. Biophys. Acta 73, 76–86.
[15] Phillips, A.H. and Langdon, R.G. (1962) J. Biol. Chem. 237, 2652–2660.
[16] Saheki, S., Takeda, A. and Shimazu, T. (1985) Anal. Biochem. 148, 277–281.
[17] Bettendorf, L., Grandfils, C., De Rycker, C. and Schoffeniels, P. (1986) J. Chromatogr. 382, 297–302.
[18] Barile, M., Valenti, D., Passarella, S. and Quagliariello, E. (1997) Biochem. Pharmacol. 53, 913–920.
[19] LaNoue, F.K. and Schoolwerth, A.C. (1984) in: Bioenergetics, pp. 221–261, Elsevier, Amsterdam.
[20] Passarella, S., Ostuni, A., Atlante, A. and Quagliariello, E. (1988) Biochem. Biophys. Res. Commun. 156, 978–986.
[21] Indiveri, C., Tonazzi, A. and Palmieri, F. (1991) Biochim. Biophys. Acta 1069, 110–116.