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Submitochondrial localization and membrane topography of Ehrlich ascitic tumour cell glutaminase

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Abstract

The intramitocondrial localization of the phosphate-activated glutaminase from Ehrlich cells has been examined by a combination of techniques, including: mitochondria subfractionation studies, chemical modification with sulfhydryl group reagents of different permeability, enzymatic digestion in both sides of the inner mitochondrial membrane, and immunological studies. Using alkaline extraction at high ionic strength, hypoosmotic shock and freezing–thawing cycle techniques, the enzyme was found in the particulate fraction. On the contrary, glutaminase activity was labile when subfractionation was carried out by digitonin/lubrol method; Western blot analysis localized the inactive enzyme in the matrix fraction. In addition, glutaminase was fully inactivated when mitoplasts were incubated with phospholipase A_2 and phospholipase C. The enzyme also showed a non-linear Arrhenius plot with a break at 24°C. The membrane-impermeant thiol reagents mersalyl and *p*-chloromercuriphenylsulfonic acid do not inhibit glutaminase activity in freeze–thawed mitochondria and mitoplasts, but *N*-ethylmaleimide, which is membrane permeant, strongly inhibited the enzyme. However, mersalyl and *p*-chloromercuriphenylsulfonic acid were effective inhibitors when the alkylation was performed on the matrix side of mitoplasts or using detergent-solubilized enzyme. Furthermore, trypsin digestion of mitoplasts was only effective inactivating glutaminase when the proteolysis was carried out on the matrix side of the vesicles. Enzyme-linked immunosorbent assay of the soluble and membrane fractions obtained in the preparation of submitochondrial particles, revealed that most of the enzyme was solubilized, but in the inactive form. Phase separation with Triton X-114 rendered most of the protein in the aqueous phase. These results taken together discard a transmembrane localization for the protein, whereas they are consistent with anchorage of glutaminase on the matrix side of the inner mitochondrial membrane, the matrix portion of the enzyme being relevant for its function.

Keywords: Tumor glutaminase; Ehrlich cell; Inner mitochondrial membrane; Membrane protein; Topography

Abbreviations: BAEE, *N*-benzoyl-L-arginine ethyl ester; GDH, glutamate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; IMM, inner mitochondrial membrane; NEM, *N*-ethylmaleimide; PAG, phosphate-activated glutaminase; PCMPS, *p*-chloromercuriphenylsulfonic acid; POEE, polyoxyethylene ether W-1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SMP, submitochondrial particles; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TX-100, Triton X-100; TX-114, Triton X-114

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1. Introduction

Mitochondrial phosphate-activated glutaminase (PAG; EC 3.5.1.2) catalyses the first step in the degradation of glutamine, an essential substrate for energy and nitrogen metabolism of rapidly dividing cells and tumours [1,2]. PAG activity has been correlated with malignancy and growth rate in tumours and proliferating tissues [3–5].

In higher organisms, PAG appears as two isoenzymes, named liver and kidney types. The liver isoenzyme is only expressed in adult liver whereas the kidney isoenzyme is present in all other tissues with glutaminase activity, including kidney, brain, small intestine and fetal liver [6]. The complete cDNA for the rat kidney enzyme and about half of the rat liver isoenzyme have been recently cloned [7,8]. The only tumour PAG isolated in a highly purified form, the PAG from Ehrlich ascitic tumour cells [9], has kinetic and immunological characteristics similar to the kidney-type isoenzyme, although it also showed quite different molecular properties [9,10].

Even though mammalian PAG isoenzymes have been purified from several tissues [11–13] and characterized with regard to kinetic and molecular properties [6], the submitochondrial localization of PAG has been a matter of controversy. Previous studies on kidney PAG have reported that the enzyme is located in the matrix region [14], matrix side of the inner mitochondrial membrane (IMM) [15] and in both halves of the IMM, existing two populations of PAG: one oriented in the intermembrane space (c-side); the other in the matrix space (m-side) [16]. More recently, Kvamme et al. [17] have suggested a predominant c-side localization for the pig kidney isoenzyme, based on the inactivation by non-permeant SH-reagents and the lack of mixing between the glutamine-derived glutamate and the endogenous matrix glutamate. The studies conducted on the liver isoenzyme localization are scarce, but the lack of enzyme release after repeated freezing–thawing cycles, the observation of a break in the Arrhenius plot of the native enzyme, and solubilization of the PAG activity after sonication of mitochondria, led to the hypothesis of a loose association between the enzyme and the IMM [18].

The assignment of submitochondrial enzyme locations has also been hampered by the peculiar environment of the mitochondrial matrix, where high protein concentrations can be achieved [19]. So, recent studies have demonstrated that proteins traditionally considered as soluble matrix enzymes, are instead loosely associated with the inner face of the IMM; this group includes tricarboxylic acid cycle, fatty acid oxidation and urea cycle enzymes [20–22]. We approached this problem by a combination of different techniques: physical and chemical disruption of the mitochondria, alkylation with SH-reagents, protein and lipid enzymatic digestions and immunological studies. Convergent arguments from these independent topography experiments indicate that Ehrlich ascites cell PAG does not behave as a typical integral membrane protein, but is anchored in the m-side of the IMM; the matrix portion of the enzyme seems to be a functionally sensitive domain. The involvement of the native lipid environment on the PAG activity was also investigated.

2. Materials and methods

2.1. Ehrlich ascites cells

A hyperdiploid Lettré strain of Ehrlich ascites cells was maintained in 2-month-old female albino Swiss mice OF1 (SPF Ico), as described elsewhere [23]. The life span of the animals after inoculation with $5 \cdot 10^6$ tumour cells was 16 ± 1 days.

2.2. Subfractionation of mitochondria and PAG solubilization

Ascites-tumour-cell mitochondria were isolated essentially as described [5] and suspended at 30 mg/ml in 20 mM Tris, 210 mM mannitol, 70 mM sucrose, 1 mM EGTA (pH 8.0) (buffer A). Mitochondria subfractionation was performed using the original method of Greenawalt [24] modified for Ehrlich cell mitochondria as described elsewhere [25]. Submitochondrial particles were prepared as previously described [26]. PAG solubilization with TX-100 was performed as previously reported [9]. The TX-100 mitochondria-free extract was used immediately and is referred to as soluble PAG.

2.3. Phospholipase treatment of mitoplasts

Mitoplasts (inner membrane plus matrix) at 5 mg/ml protein concentration were treated with different amounts of phospholipase A_2 or phospholipase C for 30 min at 30°C. The assay buffer for phospholipase A_2 contained 20 mM Tris, 210 mM mannitol, 70 mM sucrose, 0.32 M $(NH_4)_2SO_4$, 1 mM $CaCl_2$ (pH 8.0). Phospholipase C digestions were done at similar conditions but with final concentrations of 0.16 M $(NH_4)_2SO_4$ and 7 mM $CaCl_2$. Both reactions were stopped by the addition of 10 mM EDTA. PAG activity was measured immediately in both suspensions, as well as in parallel control samples not treated with phospholipases.

2.4. Trypsin proteolysis

Trypsin digestions were carried out on both sides of the IMM. For the c-side digestion, mitoplasts were resuspended (2 mg/ml) in buffer A, and incubated at 25°C with TPCK-treated trypsin (Worthington). Trypsin was added externally up to a total of 1:50 (w/w) enzyme/mitoplasts ratio, during the 18-h incubation period with gentle shaking. The reaction was stopped by adding trypsin inhibitor in a 1:100 (w/w) trypsin/inhibitor ratio. For the m-side digestion, trypsin was trapped inside the mitoplasts following the general methodology outlined before [27]. Mitoplasts vesicles (2 mg) were loaded with trypsin by rapidly freezing a sample in liquid nitrogen containing 100 μl of mitoplasts and solid trypsin (1 mg) in buffer A and slowly thawing the sample on an ice bath. Two more freezing–thawing cycles were practised. Extravesicular trypsin was removed by a 40-fold dilution of the suspension with ice-cold buffer A and centrifuging at 50 000 rpm in a Beckman type SW-60 rotor for 1 min at 0°C. The pellet was resuspended and centrifuged as above two more times. The final pellet was resuspended in 500 μl of buffer A and incubated at 25°C for 18 h. Parallel control vesicles were treated without trypsin. After incubation, the c-side and m-side digests were immediately assayed for PAG activity.

2.5. Alkylation with SH-reagents

Freeze–thawed mitochondria, mitoplasts or soluble PAG were incubated with different concentrations of thiol reagents (added from concentrated aqueous stock solutions) for 5 min at 25°C. Reactions were stopped with a 200-fold molar excess of β-mercaptoethanol and immediately assayed for PAG activity. Alkylation on the m-side of mitoplasts was achieved by trapping mersalyl and PCMPS at 0.1 and 0.5 mM, respectively, by freezing–thawing cycles as described above. These concentrations had no effect on the PAG activity of mitoplasts when c-side alkylation was performed. The vesicles were incubated for 12 min at 25°C, quenched with a 200-fold molar excess of β-mercaptoethanol and assayed for PAG activity.

2.6. Antibody production and purification

PAG was purified and polyclonal antibodies obtained as described elsewhere [9]. An IgG fraction was also affinity purified against the 72 kDa glutaminase band; these antibodies specifically recognize the Ehrlich carcinoma glutaminase protein by immunoblot analyses in both purified preparations and frozen mitochondria [9]. Western blotting was carried out essentially as described previously [28]. Samples were subjected to SDS-PAGE and then transferred to nitrocellulose. Protein standards were located on nitrocellulose by staining with amido black. Immunodetection with affinity purified anti-glutaminase antibodies was carried out with a biotin-avidin peroxidase kit (Vectastain ABC kit; Vector Labs, Burlingame, CA) as reported previously [9].

2.7. Antibody assay by enzyme-linked immunosorbent assay (ELISA)

Submitochondrial particles and the soluble fraction obtained in the preparation of these particles were diluted with a buffer consisting of 10 mM Tris, 0.12 M KCl, and 1 mM EDTA (pH 7.3), to obtain the optimal working dilutions; 200 μl of the samples were added to each well of the ELISA plates (Immulon-2). After coating by overnight incubation at 4°C, the wells were washed with PBS and blocked with 1% BSA solution in PBS for 1 h at room temperature. After washing with PBS supplemented with 0.05% (w/v) Tween-20 (PBS-T), the plate was incubated at room temperature for 2 h with 200 μl of affinity purified IgG diluted in PBS-T. The plate was washed with PBS-T, and 200 μl of a solution of

peroxidase-conjugated anti-rabbit IgG diluted 1000-fold with PBS-T was added to each well and incubated for 2 h at room temperature. Then, they were washed and filled with a solution of 5 mg/ml *o*-dianisidine in 20 mM potassium phosphate (pH 6) supplemented with 15 mM H_2O_2. After 1 h in the dark at room temperature, the peroxidase reaction was stopped by addition of 20 μl of 2 M H_2SO_4. The absorbance of the samples was followed at 450 nm with a 2001 microplate reader (Whittaker).

2.8. Phase separation with TX-114

The detergent TX-114 was precondensed essentially as described [29]. Freeze–thawed mitochondria or mitoplasts were resuspended at 4 mg/ml in 10 mM Tris, 150 mM NaCl (pH 7.4). Then, 1 ml of this suspension was cooled at 0°C and supplemented with 10 μM pepstatin, 10 μM leupeptin and 0.9% (w/v) TX-114. After centrifugation at $13\,000 \times g$ for 5 min at 4°C, the clear supernatant was transferred to a cold microfuge tube and centrifuged again at $13\,000 \times g$ for 25 min at 4°C. The supernatant was employed for phase separation according to [29], including the reextraction of both phases (aqueous and detergent). The final detergent and aqueous phases were incubated with 10% trichloroacetic acid (TCA) at 0°C for 1 h. The protein pellets were washed with 10% TCA and with cold acetone before being submitted for SDS-PAGE and Western blotting. PAG activity and protein content were also measured in an aliquot of both phases before TCA precipitation.

2.9. Enzyme and protein assays

PAG activity was measured by a stopped assay as described elsewhere [10]. For subfractionation studies, the following marker activities were measured as previously reported [25]: adenylate kinase (EC 2.7.4.3) for the outer membrane plus intermembrane space; citrate synthase (EC 4.1.3.7) for the matrix fraction; and cytochrome *c* oxidase (EC 1.9.3.1) for the inner mitochondrial membrane. To assess the relative membrane permeability of the thiol reagents employed, the matrix content of glutathione was determined in the absence or presence of the indicated amounts of alkylating reagents, following the method of Griffith [30].

For evaluation of the trypsin trapped inside the mitoplasts by freezing–thawing cycles, trypsin activity was measured using *N*-benzoyl-L-arginine ethyl ester (BAEE) as substrate (one BAEE unit = ΔA_{253} of 10^{-3} per min at pH 7.6 and 25°C). Mitoplasts were supplemented with 37 500 BAEE units of trypsin. After freezing–thawing cycles, washing and incubation, the mitoplasts were sonicated and trypsin activity determined. About 1.5% of the initial trypsin was trapped inside the mitoplasts. Protein concentrations were determined by a method recommended for membrane proteins [31], using BSA as a standard. SDS-PAGE was performed as described elsewhere [9]. Apparent molecular masses of proteins were determined by using the Low-Molecular-Weight Calibration Kit of Pharmacia, containing: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

3. Results

3.1. Effect of alkylating SH-reagents on PAG activity

Initial experiments for PAG localization seemed to indicate that the enzyme is somehow attached to the inner mitochondrial membrane, since a different number of treatments for washing and stripping peripheral proteins, including alkaline extraction at pH 9.5 in the presence of high ionic strength (0.5 M KCl), hypoosmotic shock and freezing–thawing cycles, failed to release PAG from mitoplasts; the bulk of the activity was always found in the pellet fraction after high-speed centrifugation (results not shown). Furthermore, a markedly different inhibition pattern of PAG activity was observed with the SH-reagents NEM, mersalyl and PCMPS (Table 1), when soluble or particulate enzyme was used. In the particulate fractions, mitochondria and mitoplasts, NEM strongly inhibited PAG activity but almost no effect was detected with mersalyl and PCMPS. The inhibition by NEM was concentration dependent in the range of 0–1 mM (results not shown). The concentration range used for these reagents was similar to those normally employed in enzyme inhibition studies [17,32]. Moreover, the differences found cannot be ascribed to the different concentrations of thiol-reagents used, be-

Table 1
Effect of thiol reagents on PAG activity in membrane-bound and soluble forms

Reagent	Percentage of PAG activity versus control		
	mitochondria	mitoplasts	soluble
None	100.0	100.0	100.0
NEM 1 mM	24.9 ± 7.9	5.1 ± 1.4	2.0 ± 1.2
Mersalyl 0.1 mM	95.1 ± 3.1	87.7 ± 1.2	1.0 ± 0.7
PCMPS 0.5 mM	88.0 ± 3.0	97.8 ± 1.7	2.5 ± 1.2

Samples were incubated in the presence of the indicated concentrations of reagents for 5 min at 25°C. Reactions were quenched with β-mercaptoethanol and immediately assayed for PAG activity. Values are expressed as means ± S.E. of at least three different experiments. Hundred per cent values in nmol/mg protein/min were 136 ± 9, 126 ± 11, and 111 ± 13, for mitochondria, mitoplasts and solubilized enzyme, respectively.

cause similar results were obtained by increasing the concentrations of mersalyl and PCMPS to 1 mM (not shown). However, at the same concentrations, mersalyl and PCMPS, as well as NEM, were very effective inhibitors of the soluble PAG (Table 1).

Even though NEM is generally referred to as a membrane-permeant reagent whereas mersalyl and PCMPS have been reported to be essentially membrane-impermeant [33], it was necessary to establish the permeability of these reagents in our system (Table 2). Mitochondrial glutathione content (GSH plus GSSG) was chosen as an indicator of the sulfydryl reagent permeability. NEM drastically decreased the matrix glutathione levels, which seems to confirm its permeability through the inner mitochondrial membrane of Ehrlich mitochondria. On the other hand, the matrix glutathione content slightly decreased with mersalyl and PCMPS at the concentrations employed in the chemical modification experiments, providing further evidence that they are membrane-impermeable reagents. However, when the permeability barrier was suppressed by trapping mersalyl and PCMPS inside the mitoplasts by freezing–thawing cycles, both reagents clearly inhibited PAG activity (Table 3). This inhibition actually reflected alkylation of the PAG protein, as judged by the fact that reversal of the inhibition was not observed after solubilization of the mitoplasts with TX-100 (not shown).

Table 2
Glutathione content (reduced and oxidized forms) of the Ehrlich mitocondrial matrix after treatment with thiol reagents

Reagent	[GSH + GSSG] (nmol/mg protein)	%
None	6.0	100
NEM 0.2 mM	3.9	65
NEM 1.0 mM	1.2	20
Mersalyl 0.1 mM	5.5	92
PCMPS 0.5 mM	5.3	88

After incubation with the SH-reagents, mitochondria were quenched with 20 mM β-mercaptoethanol and washed twice with buffer A. Samples were then processed for glutathione content as described by Griffith [30]. Values are means of two different determinations in duplicate.

Table 3
Effect of the permeability barrier afforded by the inner membrane on the PAG inactivation by thiol reagents

Addition	PAG Activity [a]	
	c-side	m-side
None	146 ± 15	130 ± 14
Mersalyl 0.1 mM	130 ± 14	80 ± 15
PCMPS 0.5 mM	116 ± 2	68 ± 16

Thiol reagents were trapped inside the mitoplasts (m-side) or added exogenously (c-side), as outlined in Section 2. Values are means ± S.E. of at least three different experiments.
[a] Units of specific activity: nmol/mg protein/min.

3.2. Subfractionation of Ehrlich mitochondria

To remove the outer membrane and to obtain the mitoplast fraction, Ehrlich mitochondria needed a significant lower concentration of digitonin compared with rat liver mitochondria [24], due to its high cholesterol concentration [34]. After subfractionation of mitoplasts with the detergent POEE, the recovery of various marker enzymatic activities assayed to assess the purity of the different mitochondrial fractions was very satisfactory, with values always higher than 90% [25]. However, most of the PAG activity (88.6% of the initial) was labile under the conditions used for subfractionation. Although the PAG activity was lost, it was possible to detect the protein with affinity purified antibodies against PAG [9]. Western blot of the different fractions obtained during mitochondrial subfractionation revealed that the inactive PAG protein mostly appeared in the matrix fraction (Fig. 1, lane 3); a slight immunoreactivity is observed

Fig. 1. Immunoblot analysis of PAG after submitochondrial fractionation with detergents. Ehrlich mitochondria were fractionated with digitonin/lubrol and the various fractions subjected to SDS-PAGE and Western blotting. The blot was revealed using immunopurified anti-Ehrlich PAG antibodies. Lane 1, inner mitochondrial membrane; lane 2, outer membrane plus intermembrane space; lane 3, mitochondrial matrix. The upper band corresponds to the 72 kDa glutaminase protein; other degradation products appear as low molecular mass bands (65–61 kDa). Equal amounts of protein were applied to each lane.

in the outer membrane plus intermembrane space fraction (Fig. 1, lane 2). PAG appears as the 72 kDa protein band; lower peptide bands of 65 kDa and 61 kDa also arose, due to the proteolysis of the 72 kDa band [9].

3.3. Phase separation by TX-114

When Ehrlich mitochondria were analysed by phase separation using the detergent TX-114, PAG did not retain biological activity; only a residual activity was found in the aqueous phase. The phase separation of hydrophilic and amphiphilic proteins achieved is shown in Fig. 2, which represents a typical Coomassie-stained SDS-gel of the detergent and aqueous phases. Western blotting of both phases with purified anti-PAG antibodies detected the 72 kDa glutaminase band on the detergent-poor aqueous phase (Fig. 3); several low molecular mass bands were also detected, corresponding to proteolytic degradation products, an unavoidable effect when using particulate fractions or initial purification steps of PAG, due to the strong susceptibility to proteolytic attack shown by this enzyme [9].

3.4. ELISA of submitochondrial particles

We also studied the distribution of PAG in submitochondrial fractions obtained in the absence of detergents by a standard sonication procedure. Under these conditions, most of the citrate synthase activity (85–90%) was released to the soluble fraction obtained after high-speed centrifugation of the sonicated sample. Data of ELISA test of submitochondrial particles (SMP) are presented in Fig. 4, and show that the binding of immunopurified anti-PAG antibodies was preferentially found in the soluble fraction and not in the particulate fraction (SMP). These results rule out a transmembrane orientation of the protein, but would seem to indicate that PAG behaves as a matrix enzyme. Interestingly, about 25% of the signal is still found on the SMP fraction (Fig. 4). The specificity of the immunoreaction was demonstrated

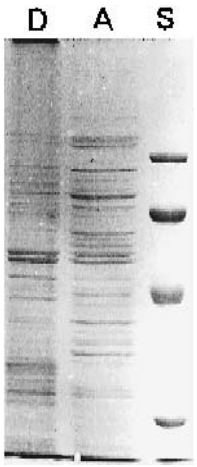

Fig. 2. SDS-PAGE pattern of mitochondrial proteins after phase-separation with TX-114. Ehrlich mitochondria were solubilized with the detergent TX-114 and subjected to phase-separation at 30°C as described in Section 2. Aliquots of both phases in SDS sample buffer with β-mercaptoethanol were analysed by SDS-PAGE and the gel stained with Coomassie blue R-250. Lane D, detergent phase; lane A, aqueous phase; lane S, standard molecular mass markers, from top to bottom: 94, 67, 43, and 30 kDa.

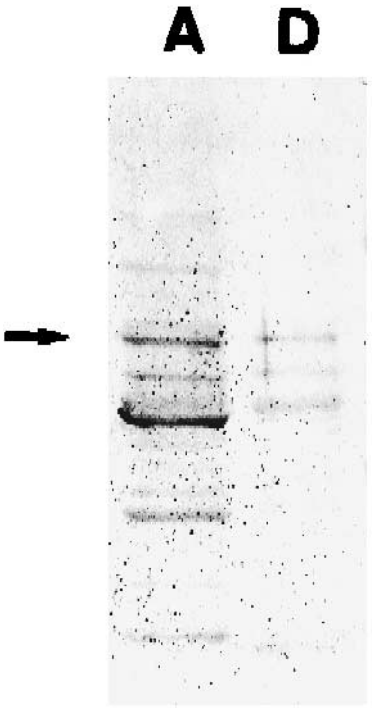

Fig. 3. Western blot of PAG after TX-114 phase-separation of Ehrlich mitochondria. The detergent and aqueous phases obtained were subjected to SDS-PAGE, transferred to nitrocellulose, and immunostained. The blot was revealed with immunopurified anti-Ehrlich PAG antibodies. Lane A, aqueous phase; lane D, detergent phase. The arrow indicates the position of the 72 kDa glutaminase band.

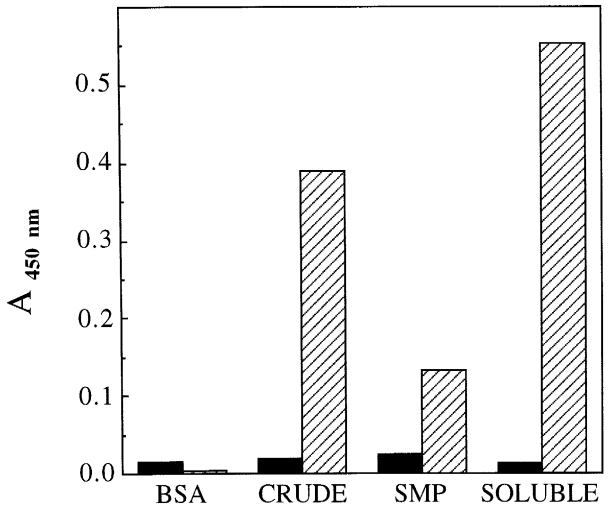

Fig. 4. Distribution of the PAG protein after submitochondrial particles formation assessed by ELISA. A mitochondrial suspension was sonicated and this crude sample was separated by centrifugation in a pellet fraction (submitochondrial particles) and a soluble fraction. Microtitre plates were coated with working dilutions of these samples (2–5 μg protein/ml). Then, they were incubated with diluted immunopurified anti-PAG antibodies. Binding of antibodies was detected by a chromogenic reaction (cf. Section 2). A 100 μg/ml BSA solution was used as a control. Plotted data in ordinate represent values of absorbance at 450 nm. Black boxes, nonimmune control serum. Hatched boxes, anti-PAG antibodies. Four different assays were performed; a typical experiment is depicted.

by using non-immune serum, BSA and plasma membrane vesicles from Ehrlich cells (not shown) as controls. The PAG enzymatic activity was completely lost after the sonication procedure needed to generate SMP particles; it was already undetectable in the crude sample before centrifugation and after separation of the SMP and soluble fractions.

3.5. Phospholipase digestions

The effect of phospholipase treatment on mitoplast PAG activity was also investigated. Although the molecular masses of phospholipase A_2 and C prevent them from penetrating the bilayer, the incubations necessary to carry out these experiments may lead to degradation of lipids exposed at both sides of the membrane. In any case, the data shown in Fig. 5 clearly suggest that complete inactivation of PAG can be achieved upon treatment of the inner mitochon-

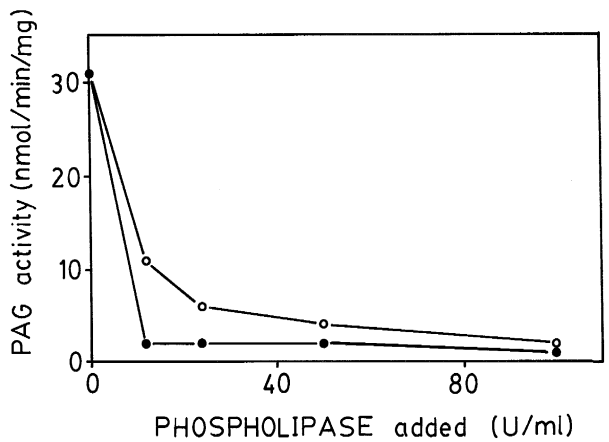

Fig. 5. PAG inactivation by phospholipases. Mitoplasts were incubated for 30 min at pH 8.0 with different concentrations of phospholipase A_2 (●) or phospholipase C (○), in the presence of optimal Ca^{2+} concentration. The reactions were stopped with EDTA and PAG activity immediately assayed.

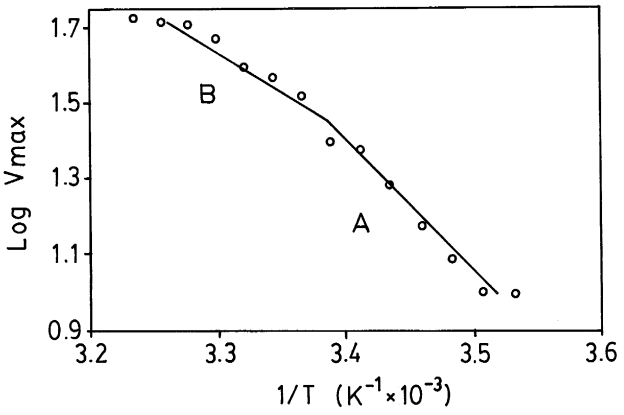

Fig. 6. Arrhenius plot of PAG activity in freeze–thawed mitochondria. Thermal equilibration of the mitoplasts with the media was achieved by 5-min preincubation period at the experimental temperature. The incubation time for measuring PAG activity was shortened to 5 min, to assure that a linear reaction rate was maintained over the whole temperature interval. The lines and break point were determined by linear regression least-squares analyses of the experimental data. The apparent activation energies obtained are 72.1 kJ/mol below 24°C and 32.7 kJ/mol above 24°C.

drial membrane with phospholipase A_2 and C; these results seem difficult to reconcile with a truly matrix localization of the enzyme in the mitochondria.

3.6. Arrhenius plot of the enzyme

Another approach used to examine the relationship of PAG activity to membrane environment was to determine the effects of temperature perturbation of the inner membrane on PAG activity. Arrhenius plot for mitoplasts could be resolved into two linear segments, with heat-inactivation occurring above 40°C (Fig. 6). The plot showed a break point at 24°C, which allows us to calculate two activation energies for the glutaminase reaction: 72.1 kJ/mol for temperatures between 10°C and 24°C, 32.7 kJ/mol for the 24–37°C temperature range. It is noteworthy that a linear Arrhenius plot was obtained when TX-100 was added to the mitoplasts (results not shown); moreover, the Arrhenius plot for the soluble and highly purified form of the enzyme showed no such discontinuity [35].

Table 4
Effect of trypsin proteolysis on the membrane-bound PAG activity

Addition	PAG Activity [a]	
	c-side	m-side
(−)Trypsin	103 ± 7	103 ± 2
(+)Trypsin	104 ± 6	45 ± 8
Inactivation (%)	0	56.3

Trypsin was added externally to the mitoplasts (c-side) or trapped inside (m-side), as described in Section 2. About 700 ± 194 BAEE trypsin units were trapped inside the vesicles. Results are expressed as means ± S.E. of at least three different determinations.

[a] Units of specific activity: nmol/mg protein/min.

3.7. Trypsin proteolysis

Further investigation of the topography of PAG within the inner mitochondrial membrane was carried out by means of enzymatic digestions of the protein with trypsin. The experimental design was based on a selective attack of PAG from each half of the inner membrane; proteolysis from the outer leaflet (c-side) was achieved by incubating sealed mitoplasts with externally added trypsin, whereas proteolysis from the inner leaflet (m-side) was performed by internally trapping the trypsin. It was carefully checked that no trypsin leakage occurred during the digestion. As can be seen in Table 4, PAG was not inactivated at all when mitoplasts were treated with externally added trypsin. In contrast, more than half of the enzyme activity was lost when proteolysis was performed on the m-side of the vesicles. Therefore, it appears that PAG does not have a c-side domain or, at least, it is not functionally sensitive, whereas it seems clear that the protein has a m-side domain and proteolysis of this region has a deleterious effect on activity.

4. Discussion

The topography of PAG in the mitochondria is an important issue which has profound physiological implications in tumour cells, since they have a high glutaminolysis related to the operativity of glutamine, glutamate and phosphate carriers.

The experiments of chemical modification showed that the enzyme was only inactivated by NEM in

membrane fractions (mitochondria and mitoplasts), whereas mersalyl and PCMPS failed to do so; however, they inactivated PAG in a soluble form or when the reagent was trapped inside the vesicles. The inner membrane acts as a permeability barrier so the membrane-impermeant reagents (mersalyl and PCMPS) cannot reach PAG. Thus, it can be deduced that PAG has functionally-sensitive cysteine residues in the m-side of the IMM. Although NEM is widely accepted as membrane-permeant and mersalyl and PCMPS as membrane-impermeant reagents [33], exceptions have also been found [17]. Given the aim of the present work, it is advisable to thoroughly ascertain their degree of permeability; this was correlated with the ability to decrease the matrix glutathione content. While mersalyl and PCMPS had a negligible effect, NEM almost exhausted the mitochondrial glutathione. The conclusion is that the inner membrane becomes a barrier to access to essential residue(s) in PAG.

With regard to glutaminases, only partial inhibition (40–60%) by 1 mM NEM was detected in pig renal mitochondrial and rat brain synaptosomal glutaminases [17,36]. In contrast, the rat liver enzyme was completely inactivated by NEM [37]. Recently, a counteraction of TX-100 on the partial NEM inhibition of pig kidney PAG has been reported [17]. A detergent-promoted conformational change, turning the protein insensitive to the bound sulfhydryl reagent, was suggested to occur. However, our results are not consistent with any counteraction of TX-100 on the NEM inactivation. Moreover, after membrane solubilization with TX-100 the inhibition was maintained and soluble PAG was also effectively inhibited by NEM. On the other hand, the data support that impairment of PAG activity caused by NEM is not due to an indirect effect on mitochondrial carriers, since the glutamine carrier was insensitive to NEM in Ehrlich cells [25] and preincubation of mitochondria with an excess of phosphate did not prevent the inhibition of glutamine hydrolysis.

Submitochondrial fractionation under conditions which yields highly pure mitochondrial fractions, inactivated the enzyme [25]; however, immunoblot analysis clearly demonstrated that most of the enzyme partitioned in the matrix fraction. This result discards an association of the enzyme with the outer membrane or the intermembrane space; in addition, it strongly indicates that PAG does not behave as an integral protein (under our subfractionation conditions, 96.7% of the cytochrome *c* oxidase activity was found in the IMM fraction). However, two possibilities remain: PAG can be a peripheral protein associated with the IMM or, alternatively, a truly matrix-soluble enzyme. The method of subfractionation (digitonin/lubrol) could result in desorption of a peripheral protein, and then inactivation after loosing its anchor to the IMM. This hypothesis is in good agreement with the results reported for β-hydroxybutyrate dehydrogenase, a peripheral enzyme anchored to the m-side of the IMM [38] and also inactivated by the detergent lubrol [39].

Integral and peripheral proteins can be easily separated by phase-separation using the detergent TX-114 [29]. When mitochondria or mitoplasts were fractionated, a markedly different pattern of peptide bands was detected in each phase, but PAG activity was lost again; after analysis by Western blot the PAG polypeptide mostly appeared on the detergent-poor aqueous phase, where peripheral and soluble species are almost always found [40]. Peripheral proteins are anchored to membranes mainly by ionic interactions with the polar groups of lipids and/or proteins; however, a few of them, like β-hydroxybutyrate dehydrogenase, also displays many features of hydrophobic interactions [38]. It is important to keep in mind that stripping of the IMM with alkaline extraction at high ionic strength failed to release PAG. Not all the proteins that are anchored to bilayers via ionic interactions can be removed with salt. Cations do not promote desorption of spectrin [41], myelin basic protein [42] and polymyxin [43]; these may be an example of proteins that interact with membranes in two ways: electrostatically and hydrophobically [38]. In any case, the extreme instability of the enzyme after fractionation with TX-114 points out toward some kind of association with the inner membrane, which is essential for activity.

In submitochondrial particles (SMP) prepared by controlled sonication, the PAG activity was undetectable in the two fractions obtained (soluble and SMP), but immunological assays by ELISA yielded clear-cut results: most of the protein was specifically found in the soluble fraction. Sonication has been reported to solubilize the rat liver enzyme without significantly affecting its activity [13,44]; on the con-

trary, in Ehrlich tumour cell mitochondria sonication inactivates PAG and treatment with detergents was the most effective procedure for enzyme solubilization, although prolonged exposure to TX-100 had a deleterious effect on the enzyme stability [9]. This result might indicate that the detergent strips away the annular lipids of the enzyme, since TX-100 is a strong delipidating detergent [45]. In rat kidney mitochondria, solubilization of PAG also requires 10-fold greater concentrations of various detergents than needed to release soluble matrix activities [12].

If PAG is associated to the inner membrane, structural perturbation or fluidity changes of the bilayer should elicit alterations in PAG activity. Enzymatic digestions of lipids with either phospholipase A_2 or C, completely abolished PAG activity. Phospholipid hydrolysis could result in the disruption of functionally important lipid–protein interactions necessary for proper enzyme function. The same result was observed with two phospholipases yielding different products (lysophospholipids and fatty acids on one hand, diglycerides on the other), which strongly supports that the ultimate reasons for enzyme inactivation are the loss of intact phospholipid and the lipid rearrangement caused by the lipolytic products mentioned [46]. It is noteworthy that rat liver PAG can be effectively solubilized from mitochondria by treatment with phospholipase A_2, with 88% of the total activity recovered in soluble form [47]; the same authors concluded that enzyme kinetics are modulated by interactions with membrane phospholipids.

A break of linearity in the Arrhenius plot of an enzyme can be attributed to several causes, one of them being lipid phase separation occurring within the bilayer [45]. Not only integral proteins show this behavior; peripheral enzymes, like β-hydroxybutyrate dehydrogenase, also presented a break at around 20°C which was abolished after mitochondria solubilization [39]. For Ehrlich PAG the discontinuity also disappeared when detergent was added or when a highly purified form of the enzyme was used; so, the discontinuity in the Arrhenius plot may indicate a phase change in the lipids affecting PAG activity. A similar break for the rat kidney enzyme was attributed to a conformational change of the membrane-bound protein; it was related to alterations in the phospholipid layer and not to diffusional limitations of the supply of substrate or effectors, because the same break was obtained in mitochondria disrupted with 0.04% TX-100, a concentration which did not detach PAG from the membrane [48]. Furthermore, a discontinuity at 23°C for the rat liver enzyme was also argued to support attachment of the enzyme to the inner membrane [18].

By hydropathy analysis of the rat kidney enzyme, it was shown that the protein lacks a membrane-spanning domain [7]; however, mammary glutaminases also showed a notorious hydrophobic character. Thus, attempts to electrophorese these enzymes in standard native PAGE systems failed because they irreversibly aggregated and precipitated [44,49]; only after inclusion of CHAPS or TX-100 in the PAGE systems were these proteins satisfactorily resolved [49]. In addition, purified Ehrlich PAG was strongly bound to phenyl-Superose, which is used to retain highly hydrophobic proteins [50]; moreover, elution was only achieved after complete removal of the salt gradient [9]. Recently, a hydrophobic association of rat kidney PAG with other inner membrane enzymes has been postulated, based on the presence of two contiguous tryptophan residues in the protein sequence [51]. A very attractive hypothesis would be the association of PAG with the mitochondrial glutamine carrier as a channelling mechanism; some early experimental data suggested that glutamine is not released into the matrix but rather delivered directly to glutaminase [52]. Alternatively, PAG might be also part of the mitochondrial transport system for glutamine [53]. However, the IMM vesicles obtained by digitonin/lubrol showed a very active L-glutamine transport activity [25] although they were devoid of PAG protein (this work). So, in Ehrlich mitochondria we conclude that both activities belong to different proteins, although we cannot rule out the possibility of a physical interaction between them [54].

Apart from the submitochondrial location, another physiologically relevant issue is the orientation of the enzyme in the membrane. Proteolytic digestions with trypsin on both sides of the inner membrane strongly suggest a m-side location for Ehrlich PAG, discarding a transmembrane topography of the enzyme. Our results strongly support that most of the functionally-sensitive domain of Ehrlich PAG is located on the m-side of the membrane, and do not agree with the orientation reported for the pig kidney enzyme, where a main external location (c-side) was

postulated [17]. Although species- or tissue-specific differences cannot be discarded, this location will pose several questions, the main one would be the necessity for very active mitochondrial glutamine carriers, as those reported for rat kidney and tumour cells [55,25]. On the other hand, PAG is involved in different key functions in each tissue; in kidney, a predominant c-side location on the IMM might have physiological sense, because it would facilitate the release of ammonium ions on the cytosol during increased renal ammoniagenesis avoiding toxic effects of ammonia on respiratory chain function [17].

5. Concluding remarks

The above exposed experimental evidence supports several issues concerning PAG topography on the inner mitochondrial membrane. (1) The Ehrlich ascites tumour cell PAG is not an integral membrane protein. (2) The enzyme is peripheral and located in the inner leaflet (m-side) of the IMM, in the membrane–water interface. (3) Not only ionic interactions seem to hold the enzyme anchored to the membrane, but hydrophobic interactions with not yet identified lipid or proteins of the IMM are also likely to be involved. (4) The mitochondrial glutamine carrier and PAG belong to different proteins. (5) The lipid environment of the protein may be important in regulating PAG activity, perturbations of its native membrane domain inactivated the enzyme. (6) The protein has its functionally relevant domain in the matrix space, and some thiol groups that would be essential for PAG activity are located in the matrix and/or buried into the IMM. The question of which are the components of the mitochondrial membrane interacting with PAG remains unanswered at present and will require further investigation.

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