Properties and submitochondrial localization of pig and rat renal phosphate-activated glutaminase

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Roberg, Björg, Ingeborg A. Torgner, Jon Laake, Yutaka Takumi, Ole P. Ottersen, and Elling Kvamme. Properties and submitochondrial localization of pig and rat renal phosphate-activated glutaminase. Am J Physiol Cell Physiol 279: C648–C657, 2000.—Two pools of phosphate-activated glutaminase (PAG) were separated from pig and rat renal mitochondria. The partition of enzyme activity corresponded with that of the immunoreactivity and also with the postembedding immunogold labeling of PAG, which was associated partly with the inner membrane and partly with the matrix. The outer membrane was not labeled. PAG in intact mitochondria showed enzymatic characteristics that were similar to that of the membrane fraction and also mimicked that of the polymerized form of purified pig renal PAG. PAG in the soluble fraction showed properties similar to that of the monomeric form of purified enzyme. It is indicated that the pool of PAG localized inside the inner mitochondrial membrane is dormant due to the presence of high concentrations of the inhibitor glutamate. Thus the enzymatically active PAG is assumed to be localized on the outer face of the inner mitochondrial membrane. The activity of this pool of PAG appears to be regulated by compounds in the cytosol, of which glutamate may be most important.

mitochondria; mitochondrial inner membrane; immunoelectron microscopy

PHOSPHATE-ACTIVATED GLUTAMINASE (PAG; EC 3.5.1.2) catalyzes the conversion of L-glutamine (Gln) to L-glutamate (Glu) and ammonia. It is a highly regulated mitochondrial enzyme playing an important part for the kidney ammonia production. Purified PAG from pig kidney has earlier been shown to exist in at least two interconvertible kinetic forms. These kinetic forms correspond with different conformations of the purified kidney enzyme, as seen in electron micrographs (19).

Furthermore, evidence has been obtained for the existence of a soluble and membrane-associated form of PAG with distinct enzymatic properties in isolated mitochondria from pig and rat kidney (21). We have also reported that PAG in intact pig kidney (12) and brain (23) mitochondria has kinetic properties consistent with only one species of PAG, and evidence has been presented to show that the enzyme has a functional localization on the outer face of the inner membrane. However, this would not exclude that PAG anatomically is also localized within the inner mitochondrial membrane. Curthoys and Watford (3) proposed that the apparently different submitochondrial localization of PAG in pig and rat mitochondria was due to species differences. Thus the subunit sizes of rat and pig PAG are different. Rat renal PAG consists of two immunologically and structurally related peptides of molecular mass of 66 and 68 kDa, the abundance of the larger subunit being about one-fourth of the smaller one (3). Pig renal PAG has also previously been found to contain two subunits of 64 and 57 kDa, but later the 57-kDa subunit had been shown to be an artifact probably due to partial proteolysis (11). However, the deduced amino acid sequence of a cDNA from pig kidney cultured cells is 99% identical to the COOH-terminal 176 amino acids of the rat renal PAG (20).

The aims of the present study are to clarify the submitochondrial localization of soluble (Sol-PAG) and membrane (Mb-PAG) fractions of PAG in pig and rat renal mitochondria and to clarify which of these forms is active in the intact mitochondria.

Here we report that Mb-PAG and Sol-PAG have different enzymatic properties. PAG localized inside the inner mitochondrial membrane most likely is dormant, and PAG activity in intact mitochondria shows the properties of Mb-PAG.

MATERIALS AND METHODS

Materials. L-[U-14C]Gln was obtained from DuPont-NEN, and L-Gln as well as most other reagents were obtained from Sigma Chemical (St. Louis, MO). The peptides used for antibody production, corresponding to the NH2 and COOH termini (Cys-SEILQELGKGG, amino acids 77–87, and Cys-TVHKNLDDL, amino acids 665–674, respectively) of the published sequence of the rat kidney glutaminase (25), were
possessed at the Biotechnology Center, University of Oslo. A low-molecular-weight calibration kit (protein standards for electrophoresis), protein A-Sepharose, and Sephadex G-25 were from Pharmacia Biotech, Upsala, Sweden. Surface-Amps X-100 (containing 10% peroxide-free Triton X-100), Sulfolink, Immunopure gentle Ag/Ab elution buffer, Immunopure IgG purification kit, and m-maleido-benzoyl-N-hydroxysuccinimide ester were from Pierce, Rockford, IL. Horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin was from Dakopatts Glostrup, Denmark, and Lowicryl HM20 resin was from Lowi, Waldkraiburg, Germany. Colloid gold particles were from Amersham, Arlington Heights, IL. Rats (Wistar) were obtained from the local animal facilities at the Premedical Building of the University of Oslo, and pig kidneys were from Fellesslakteriet Gilde, Oslo, Norway.

Isolation of mitochondria from renal tissue. The mitochondria were isolated from fresh pig kidney cortex or whole rat kidneys by differential centrifugation as previously described (12) in a buffer containing 250 mM d-mannitol, 70 mM sucrose, 1 mM EDTA, 0.1 mM EGTA, 10 mM maleate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF; an inhibitor of serine proteases), and 10 mM HEPES, pH 7.4, with washing at least five times by consecutive resuspension of the mitochondrial pellets and centrifugation.

Because we have measured Glu formed in the PAG assay (see Assay of PAG), contamination by brush-border membranes, which contain phosphate-independent glucaminase (PIG), would constitute a source of error, especially in the cases where low phosphate concentration was used. PIG (also called maleate-activated glucaminase) that is found to be identical to γ-glutamyl transferase (EC 2.3.2.1) (3, 30) is a marker for the brush-border membranes. To minimize contamination by PIG, the fluffy, grayish layer was carefully removed from the surface of the mitochondrial pellet (12). The mitochondria were thereafter resuspended to contain about 25 mg of protein/ml in 250 mM d-mannitol, 70 mM sucrose, and 10 mM HEPES at pH 7.4. PIG activity is heat stable; thus its activity was assayed after heat inactivation of PAG in the mitochondrial fractions (55°C for 10 min) (10, 30) that did not inactivate γ-glutamyl transferase. Otherwise, the same conditions were used as in the PAG assays (see Assay of PAG). After the pig or rat mitochondrial were washed five times, the contamination of PAG by PIG was <1%.

Fractionation and permeabilization of the isolated mitochondria. Soluble and membrane fractions were separated from frozen (−70°C) and thawed mitochondria. The mitochondrial suspension was diluted with 50 mM Tris·HCl and 1 mM EDTA, pH 8.0, to 10 mg of protein/ml. After ultracentrifugation for 40 min at 158,000 g in a Beckman L5-65 ultracentrifuge, as described by Roberg et al. (21), the membranes were reconstituted to the original volume in the dilution buffer described above.

β-Hydroxybutyrate dehydrogenase (BDH; EC 1.1.1.30), which is a marker enzyme for the inner face of the inner mitochondrial membrane, was strongly inhibited by mersaly and p-hydroxymercuribenzoate in the disrupted mitochondria, whereas these sulfhydryl-reactive (SH) reagents, being impermeable to the inner membrane, had no effect on BDH in intact mitochondria (12). Thus a major fraction of the mitochondria was disrupted. Further sonication, as well as repeated cycles of freeze/thawing and washing, a more gentle procedure than sonication, led to losses of the PAG activity. Therefore, further purification of the membrane fraction was unsuccessful and also unnecessary because the properties of Mb-PAG after one cycle of freeze/thawing were distinct from that of Sol-PAG (see Results).

To render the mitochondrial inner membrane permeable to small molecules but not to proteins (12), intact mitochondria were treated with a low concentration of peroxide-free Triton X-100 (0.03%) immediately before PAG assay. Triton X-100 itself, in this low concentration, did not inhibit PAG activity (not shown). Protein concentration was measured by the bicinchoninic acid method (27).

Assay of PAG. Glu hydrolysis was assayed at 25°C with two methods designated 1) optimal assay and 2) normal assay (12). 1) The incubation medium contained 30 mM L-Gln, 10 mM Tris·HCl, and 150 mM potassium phosphate at pH 6.6. The PAG activity of intact mitochondria was in the range of 140–160 nmol·min−1·mg−1 of total mitochondrial protein. The optimal assay employed for comparison with earlier studies of polymerized and nonpolymerized purified PAG (13, 29) differed slightly from the above mentioned one, but this did not affect the results. 2) The incubation medium contained 5 mM L-Gln, 20 mM HEPES, 80 mM KCl, 0.5 mM MgSO4, and 20 mM potassium phosphate at pH 7.4. Mannitol was added to obtain isotonic conditions. The concentrations of Gln and phosphate used, which were higher than the physiological ones, were necessary to measure the low activities of Sol-PAG. The incubation time for both assays was usually 1-4 min, but Sol-PAG normally had to be incubated up to 15 min.

The Glu concentration was measured fluorophotometrically as in Ref. 12, but the effects of Glu were examined, [14C]Glu was added to the substrate. In the latter case, the Glu was separated from the Gln by ion exchange chromatography (26) and counted in a Packard Tricarb liquid scintillation counter. Less than 10% of the substrate Gln was consumed during the assay, and the initial rate was generally constant under the conditions used. Because of the mild contamination by PIG activity, the Glu hydrolysis was assumed to be due to PAG alone.

Antibodies. Rabbit antiserum was raised against the synthetic peptides (see Materials) coupled via the cysteine to keyhole limpet hemocyanine using m-maleido-benzoyl-N-hydroxysuccinimide ester as a coupling agent. The antiserum against the glutaminase C-peptide was purified by passing it through a Sulfolink C-peptide column, eluted with Immunopure Gentle Ag/Ab elution buffer, and further purified using an Immunopure kit involving a protein A column. The NH2-terminal antiserum was used unpurified because of loss of affinity during purification.

Immunoblotting. After SDS-PAGE, the proteins were electrotransferred to nitrocellulose and incubated with the rabbit antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. The antibody-antigen complexes were visualized by 0.05% (wt/vol) diaminobenzidine, 0.03% (wt/vol) NiCl2, and 0.03% (wt/vol) H2O2 in 50 mM NH4CO3. Densitometry of immunoblots. The developed blots were scanned in a desktop scanner (Hewlett-Packard ScanJet 3c) at 200–300 dpi and analyzed in Adobe Photoshop. The densities of the spots were multiplied with the number of pixels to calculate the integrated value. For whole mitochondria, the integrated values showed a linear relationship with the amount of protein applied to the gel, up to ~20 μg mitochondrial protein.

Fixation and crosslinking for immunoelectron microscopy. For immunoelectron microscopic experiments, freshly isolated mitochondria (from pig kidney) were resuspended in a slightly hypotonic buffer, consisting of 100 mM d-mannitol, 0.1 mM PMSF, and 10 mM HEPES, pH 7.4, in an attempt to increase the distance between the mitochondrial membranes. The mitochondria were incubated for 10 min at room temperature, centrifuged for 10 min at 27,000 g (4°C), and
resuspended to a protein concentration of about 10 mg/ml. Next, 0.5 ml of the mitochondrial suspension was added to 0.5 ml of fix solution, which contained 100 mM t-mannitol, 0.1 mM PMSF, and 10 mM HEPES, pH 7.4, in addition to 8% p-formaldehyde and 1% glutaraldehyde, and it was mixed by careful vortexing. The mitochondria were then spun down, 10 min at 15,000 g, after 10 min incubation on ice. The tubes were incubated for one additional hour with the same fixative solution, then the supernatant was replaced by the mannitol-HEPES-PMSF solution. The pellet was further processed for freeze substitution and postembedding electron microscopy as described in Immunoincubations.

Freeze substitution. The pellets were cryoprotected in graded concentrations of phosphate-buffered glycerol and rapidly frozen in liquid propane (−170°C) in a cryofixation unit (Reichert KF80, Vienna, Austria). The specimens were transferred to 0.5% uranyl acetate dissolved in anhydrous methanol (−90°C) in a cryosubstitution unit (AFS; Reichert). The temperature was raised stepwise to −45°C. The samples were infiltrated with Lowicryl HM20 resin, and polymerization was induced by ultraviolet light for 48 h. A detailed description of the procedure has been published (15).

Immunoincubations. Ultrathin sections were mounted on nickel grids or gold-coated grids and processed for immunogold cytochemistry as described by Matsubara et al. (15). Briefly, the sections were treated with a saturated solution of NaOH in absolute ethanol for 2–3 s, rinsed, and incubated sequentially in 1) a Tris (50 mM)-buffered solution of NaCl (50 mM) with 0.1% Triton X-100 at pH 7.4 (TBNT); 2) 2% human serum albumin in TBNT; 3) antiserum to PAG (0.1–1.0 µg/ml) in TBNT and 2% human serum albumin; 4) 2% human serum albumin in TBNT; and 5) goat anti-rabbit immunoglobulins coupled to 15-nm colloidal gold particles and diluted 1:20 in TBNT with 2% human serum albumin or with 2% human serum albumin and 5 mg/ml polyethylene glycol. Finally, the sections were counterstained and examined in a Philips CM10 transmission electron microscope.

Control experiments. The antisera (1 µg/ml) were substituted with 1 µg/ml nonimmune IgG or preadsorbed with 1 µg/ml of the peptides used for immunization. This completely removed labeling from the sections as reported by Laake et al. (14).

Analysis of gold particle distribution. Under experimental conditions similar to those used here, the maximum distance between the epitopes and the respective gold particles was about 30 nm, as determined in a model system (15). Hence, gold particles with centers that were localized within two gold particle diameters of the inner mitochondrial membrane were considered to signal epitopes associated with this. Particles that were situated further away from the membrane (in the interior of the mitochondrion) were attributed to epitopes in the mitochondrial matrix. Obviously, the values obtained by this approach represent a conservative estimate of the epitope fraction in the matrix compartment.

Computer assays and calculations for the kinetic experiments. The maximal reaction velocity (Vmax) was determined by nonlinear regression (data program Prism from GraphPad) using primary plots of reaction velocity v vs. the concentration of phosphate. For Michaelis-Menten-type plots, the one-site hyperbola approximation was used, and for sigmoid plots, a user-defined equation was inserted into the program, based on the Michaelis-Menten-Hill modification (Eq. 1) (24) of the Michaelis-Menten equation

\[ v = \frac{V_{\text{max}} [A]^{\text{app}}}{K' + [A]^{\text{app}}} \]  

where [A] = the concentration of the activator phosphate. The apparent Hill coefficient (napp), which is equal to or usually less than the apparent number of activator binding sites per molecule of enzyme, was determined by nonlinear regression of primary plots as well. K′ = a constant comprising interaction factors and the intrinsic dissociation constant. K′ is not equal to the activator concentration that yields half-maximal velocity (the constant usually designated Km) but has the relation (Eq. 2)

When \( v = 0.5 \cdot V_{\text{max}} \), then \( [A]^{\text{app}} = K' \)  

When \( n_{\text{app}} = 1 \), Eq. 1 reduces into a Michaelis-Menten-type plot, and the K’ corresponds to Km.

Unless indicated otherwise, data from an average of three to six experiments are presented with SE, n = number of experiments. The activity of PAG (nmol·min⁻¹·mg⁻¹ total mitochondrial protein⁻¹) was for the sake of simplicity presented as percent of the respective control value. Statistical significance was determined by unpaired, two-tailed t-tests, either by Student’s test or by Welch’s alternate test for different SDs.

RESULTS

Immunoblotting. Figure 1A shows that SDS-PAGE and subsequent immunoblotting of isolated pig kidney mitochondria revealed only one band with molecular mass of ~64 kDa, using either the COOH-terminal (C) or the NH₂-terminal (N) antiserum. Both Mb- and Sol-PAG contained the same immunoreactive protein as the isolated mitochondria (M; Fig. 1B). Isolated rat mitochondria, as well as Mb- and Sol-PAG, produced similar results using both antibodies, except that a very faint additional band of slightly higher molecular mass could
be seen at high protein concentrations, corresponding to the two bands previously observed (3). 

Enzyme activity and immunoreactivity of Sol-PAG and Mb-PAG. Table 1 shows that PAG is present both in the membrane fraction and the soluble fraction. The distribution of PAG immunoreactivity as determined on blots of both of these fractions corresponds to that of the PAG optimal enzyme activity. Most of the PAG enzyme activity, as measured with the optimal assay, appears to be in Mb-PAG: 66% and 79% for pig and rat, respectively.

To determine if the distribution of PAG immunoreactivity is independent of the antibody used, parallel immunoblots from four different pig kidney mitochondrial preparations were developed with either the COOH-terminal or NH2-terminal antiserum. No significant difference was found between the two groups of immunoblots, and they were therefore pooled.

Intramitochondrial localization of PAG protein by postembedding immunogold electron microscopy of pelleted mitochondria. Sections of the mitochondrial pellet show that the slightly swelled pig renal mitochondria are labeled with the antibodies against the COOH-terminal end of PAG (Fig. 2A). Gold particles were associated with the cristae and matrix but not with the outer mitochondrial membrane. Similar results were obtained using the NH2-terminal antibody (Fig. 2B). With the COOH-terminal antibody, 44% of the gold particles were located over the mitochondrial matrix and farther than 30 nm away from the inner mitochondrial membrane (Table 2). (These particles cannot represent epitopes in the latter membrane; see MATERIALS AND METHODS.) The corresponding value for the NH2-terminal antibody was 29%.

The phosphate activation curves of PAG in the intact mitochondria and of Sol-PAG and Mb-PAG. Figure 3 shows that the activation curves of Sol-PAG for phosphate in pig and rat are sigmoidal. The corresponding curves are nearly hyperbolic for Mb-PAG (Fig. 2), but slightly sigmoid in rat (Fig. 3B), in keeping with a previous report (26), and this is also the case for PAG in the intact mitochondria. The apparent Hill coefficients are estimated by nonlinear regression analyses of the primary plots to be 2.0 ± 0.1 for pig (n = 3) and 2.3 ± 0.1 for rat (n = 6) Sol-PAG, and for intact and Mb-PAG 1.0 ± 0.1 and 1.0 ± 0.1 for the pig (n = 3) and 1.5 ± 0.1 and 1.6 ± 0.1 for the rat (n = 6), respectively. To further underscore the similarities of the PAG in intact mitochondria and Mb-PAG phosphate activation curves, in contrast to that of Sol-PAG, secondary plots are also shown (Fig. 3, insets): double inverse plots of the data for intact as well as Mb-PAG from pig can be fit to straight lines by nonlinear regression (Fig. 3A) and are only slightly curved upward for the corresponding rat enzyme fractions (Fig. 3B), whereas the data for Sol-PAG from both species are clearly much more curved, showing positive cooperativity. Thus the PAG phosphate activation curves of both the intact mitochondria and Mb-PAG are similar but clearly different from that of the Sol-PAG.

Patterns of inhibition by Glu of PAG in the intact mitochondria and of Sol-PAG and Mb-PAG. Figure 4 shows that the inhibition pattern of Glu is different for the two forms of PAG. The Mb-PAG from both pig (Fig. 4, A and B) and rat (Fig. 4, C and D) is significantly less inhibited by Glu than the Sol-PAG when assayed using [14C]Glu with both the normal assay (Fig. 4, A and C) and the optimal assay (Fig. 4, B and D). The Glu inhibition pattern of PAG in intact mitochondria is similar to that of Mb-PAG. Therefore, it appears as if the expressed PAG activity in the intact mitochondria is of the Mb-PAG type, and as shown for pig mitochondria (Fig. 4B), similar to that of the polymerized form of purified PAG, but different from that of Sol-PAG and of the monomeric form of the purified enzyme.

The well-known counteraction of Glu inhibition by phosphate (3, 11) can be seen by comparing the two types of assay used. A low concentration of Glu inhibited more using the normal assay containing a low-phosphate concentration (Fig. 4, A and C) than assays containing a high-phosphate concentration (Fig. 4, B and D).

Mitochondrial glutamate concentration. To estimate the endogenous concentration of Glu in the mitochondrial matrix, Glu was measured fluorophotometrically (as in Assay of PAG) in ethanol extracts of thoroughly washed intact pig kidney mitochondria and calculated to be 17 ± 2 mM (n = 41), assuming the matrix volume to be 0.4 μl/mg mitochondrial protein (16).

The pH dependence of PAG in the intact mitochondria and of Sol-PAG and Mb-PAG. As shown in Fig. 5, the Sol-PAG from both pig (A and B) and rat (C and D) kidney is more pH dependent than the Mb-PAG.

For intact pig kidney mitochondria, the pH profile of PAG activity is very similar to that of the Mb-PAG and the polymerized form of purified PAG, which are significantly less inhibited at pH 7.5 and 7.0 than the Sol-PAG. For the intact rat kidney mitochondria, the optimal PAG activity is almost unaffected by pH in the lower range. The activity of rat Mb-PAG, as measured by the optimal assay, is inhibited somewhat more by low pH than that of intact mitochondria but significantly less than that of
Sol-PAG. The pH profile of pig Sol-PAG is similar to that of the monomeric form of purified PAG.

Activation of PAG by disruption of intact mitochondria. Figure 6 shows that after disruption of the mitochondrial inner membrane by freeze and thaw treatment, the PAG optimal activity is stimulated, and by separating the Mb-PAG from Sol-PAG, the sum of the activities in these fractions is higher than that of the intact mitochondria. Triton X-100 in the concentration of 0.03% has been reported to render the inner membrane permeable for small molecules but not for mitochondrial proteins (12). Treatment with this concentration of Triton X-100 added to intact mitochondria increases PAG activity, compared with that of untreated, intact mitochondria. Frozen and thawed mitochondria with and without added Triton X-100 (Fig. 6, c and d) showed no significant difference. No PAG enzyme activity could be detected in the extramitochondrial incubation medium, also after the addition of Triton X-100 (not shown).

Table 2. The mitochondrial subcompartmentalization of PAG immunogold labeling

<table>
<thead>
<tr>
<th>Mitochondrial Compartment (Pool)</th>
<th>COOH-terminal %</th>
<th>NH₂-terminal %</th>
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<tbody>
<tr>
<td>Inner mitochondrial membrane, IMM</td>
<td>55 ± 2</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>Matrix</td>
<td>44 ± 2*</td>
<td>29 ± 2*</td>
</tr>
<tr>
<td>Other (including outer membrane)</td>
<td>1 ± 0.4**</td>
<td>1 ± 0.5**</td>
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Data are number of gold particles expressed as percent ± SE (100% = total number of particles associated with each mitochondrion). Forty mitochondria were analyzed for each antibody. Particles were attributed to the inner mitochondrial membrane if their centers of gravity were situated within 30 nm (2 particle diameters) of this (see MATERIALS AND METHODS). *P < 0.01 vs. IMM; **P < 0.001 vs. IMM and matrix.

DISCUSSION

Localization and properties of Mb-PAG and Sol-PAG. In previous works, different mitochondrial localizations of pig (9, 11) and rat (3) kidney and rat brain (8) PAG were reported. Thus PAG was found to be
membrane associated, confined to the matrix region, or localized to both of these compartments. Here we report that in isolated frozen mitochondria, roughly two-thirds of both the enzyme activity and the immunoreactivity of PAG are found in the membrane fraction, and one-third in the soluble one.

In our previous analysis of perfusion-fixed rat brain (14), we found that immunogold particles representing PAG were aligned with the cristae. However, due to the limited resolution of the immunogold technique, the existence of a PAG pool in the mitochondrial matrix could not be excluded. In the present study, the mitochondria were subjected to gentle swelling before fixation and immunolabeling to help differentiate between the matrix and membrane compartments. Data based on this approach suggest that a sizeable fraction of PAG resides in the matrix rather than being associated with the inner mitochondrial membrane. The gold particle counts indicate that the matrix fraction amounts to 29–44%. As pointed out in MATERIALS AND METHODS, these values should be considered as conservative estimates because some PAG molecules in the matrix (a subpopulation of those situated within 60 nm of the inner mitochondrial membrane) will inevitably be recorded as membrane associated.

Why the two different PAG antibodies gave slightly different results in terms of matrix/membrane ratios using the immunogold technique is not known. However, because both antibodies gave the same distribution of PAG immunoreactivity among supernatant and precipitate after SDS treatment of the two separate fractions, this discrepancy does not reflect a real difference in antigen distributions. Because SDS treatment destroys all protein-protein interactions, one possibility is that the COOH terminus is engaged in the anchoring of PAG to the membrane, or in PAG polymerization, and that this interferes with antibody binding to the COOH-terminal epitope.

Taken together, the three techniques indicate that there are two pools of PAG (inner membrane associated and soluble, localized in the matrix) and that this is true for both rat and pig renal mitochondria. Furthermore, the Mb-PAG and Sol-PAG also show distinct
enzymatic properties. We have not been able to detect any major differences between pig and rat mitochondria, except for the divergence in phosphate kinetics and subunit composition.

The membrane-associated PAG and the soluble matrix-PAG could be isoenzymes. However, because both forms show corresponding immunoreactive bands of PAG in intact mitochondria, these forms are apparently not distinct protein species but different conformations of the same enzyme protein.

**PAG in intact mitochondria has properties similar to that of Mb-PAG.** We have compared various properties of PAG in intact mitochondria with those of Mb-PAG and Sol-PAG. We have previously reported (12) that the Gln activation curve of intact pig mitochondria showed Michaelis-Menten kinetics, in contrast to that of disrupted mitochondria, demonstrating that only one enzyme form is predominantly active.

The phosphate activation curve for PAG in intact mitochondria is rather similar to that of Mb-PAG and very different from that of the Sol-PAG, which has more cooperative profiles both in the pig and rat mitochondria. Similar to the Mb-PAG, PAG in intact mitochondria is far less sensitive to inhibition by the reaction product Glu than the Sol-PAG. This is independent of the assay condition used, but the extent of this inhibition is known to be counteracted by phosphate (3, 11). The pH profile of PAG activity in intact mitochondria is also very similar to that of Mb-PAG and distinct from that of Sol-PAG. Thus the enzymatically active fraction of PAG in the intact mitochondria has properties similar to that of Mb-PAG.

**PAG in intact mitochondria has properties similar to that of the polymerized form of purified enzyme.** Puriﬁed PAG from pig kidney has been shown to consist mainly of two interconvertible enzyme forms, a polymerized, insoluble form (PB-form) and a monomeric, soluble form (T-form) (13) with distinct enzymatic properties (29) and electron microscopic appearances (19). In addition, a soluble P-form that represents a dimerization of the T-form with almost similar catalytic properties as those of the PB-form, has been characterized in the pig kidney (19). This is similar to the different enzyme forms described by Souri et al. (28) for mitochondrial very-long acyl-CoA dehydrogenase subunits, for which association to the mitochondrial inner membrane seems necessary for dimeriza-
tion. In rat kidney mitochondria, a dimeric enzyme form (5) is described that corresponds with the P-form. We found that the Glu inhibition curves and pH curves of PAG in the intact pig renal mitochondria correspond with those of the PB-form but not those of the T-form. Therefore, the PAG that is active in intact mitochondria may be dimerized, polymerized, or have other linkages (e.g., to proteins) to give the enzyme this conformation and these properties. The clustering of particles, as observed by immunogold labeling of PAG in cerebellar mitochondria, could represent polymerization or a close association of PAG monomers (14). The Sol-PAG, which most likely is localized in the matrix region, has properties similar to the monomeric purified PAG (T-form) and has probably the same subunit composition.

Evidence for a dormant internal and an active external pool of PAG. We investigated the effect of partially disrupting the membranes of intact pig renal mitochondria by treatments that render the inner mitochondrial membrane permeable to small molecules but not to proteins. Thus the enzyme activity of PAG is considerably stimulated by treatment of intact mitochondria with Triton X-100 and also by freezing and thawing, whereas Triton X-100 showed no significant effect in addition to that of freezing and thawing. This is at variance with previous findings for pig kidney and rat brain mitochondria using Triton X-100 (12, 23), and may be caused by loss of activity in the earlier work due to instability of the enzyme. However, the pig renal mitochondrial PAG activity is also increased by swelling the mitochondria in a hypotonic medium whereby the membrane may be damaged and the matrix volume greatly increased (10). This is also supported by the finding that the sum of the enzyme activities of the isolated Mb-PAG and Sol-PAG exceeds that of intact mitochondria. Thus it is indicated that PAG in intact mitochondria can be activated by a partial disruption of the inner membrane, which suggests that at least a fraction of PAG resides inside this membrane. In addition, results from immunolabeling of PAG in intact mitochondria support this suggestion.

We have estimated the matrix Glu concentration in pig kidney mitochondria to be about 17 mM, which is a conservative estimate due to possible leakage of Glu. Similarly, in brain mitochondria we have found a matrix Glu concentration of about 10 mM (22). This is lower than that of synaptic vesicles, being about 100 mM (4), which is assumed to be of the same order of magnitude as that of mitochondria in nerve terminals (6). In addition, the Gln/Glu ratio in the mitochondria is expected to be very low (see below). A high Glu concentration in the mitochondria is in accordance with quantitative immunogold data that have revealed an enrichment of Glu immunoreactivity in these organelles in brain (6). Because, in addition, under physiological conditions, phosphate in the matrix is largely bound as calcium phosphate (18) and Glu counteracts the activation by phosphate, PAG is likely to be inhibited. After partial disruption of the inner mitochondrial membrane, the activity of PAG increased as discussed above. By this treatment, the internal Glu concentration is likely to be reduced due to leakage, whereby the dormant PAG is activated, as discussed above.

For rat kidney mitochondria, Curthoys and Shapiro (2) have reported that the ratio of labeled Gln/Glu in the presence of phosphate is close to zero in the matrix and that Glu is a competitive inhibitor with Gln (26). This indicates that PAG inside the inner mitochondrial membrane is dormant also in rat kidney mitochondria.

Previous reports have demonstrated that PAG in intact rat kidney and brain mitochondria, as well as in intact pig kidney mitochondria, is inhibited by nonpenetrable SH reagents in the presence of low phosphate and Gln concentrations at pH 7.4 (7, 12, 23). These findings suggest the assumption that external, functional localization of PAG in the inner mitochondrial membrane also applies to rat mitochondria. However, Curthoys and Watford (3) report no such inhibition of rat kidney mitochondria, but the assay was performed with 150 mM phosphate at pH 8.6. We have shown that under these conditions (optimal assay), the inhibition by nonpenetrable SH reagents disappears in intact pig kidney mitochondria (12). We assume that this is due to conformational change of the enzyme protein.

The inhibition of PAG by low Glu concentrations in intact mitochondria (Fig. 4, A and C) strongly suggests the presence of a pool of PAG that is not in contact with the high concentration of Glu in the mitochondria. Thus when intact pig and rat mitochondria are assayed for PAG by the normal assay in the presence of 4 mM glutamate, there is a significant inhibition of about 24%. However, externally added 4 mM Glu in addition to the high concentration of Glu already present would not be expected to increase the inhibition significantly if all PAG molecules were localized inside the inner membrane. These findings provide additional support that the functionally active PAG is indicated to be localized at the outer face of the inner mitochondrial membrane.

Physiological implications. Because the polymerized and monomeric forms of purified PAG are interconvertible, an equilibrium may exist between matrix-PAG and the membrane-associated PAG. Thus electron microscopic studies suggested that purified pig kidney PAG polymerizes by end-to-end aggregation of P-form dimers (19). On this basis we propose the following model to explain our findings: in the matrix of intact mitochondria, there is a monomeric or dimeric, dormant form of PAG, resembling the soluble T- or P-forms of purified enzyme, which is in equilibrium with a membrane-associated dimeric form, resembling the P-form, that again is in equilibrium with the PB-form. However, we assume that due to the high concentration of glutamate inside the inner mitochondrial membrane and matrix, only PAG molecules that have their active site accessible to the outer face of this membrane are enzymically active. Factors that lower the internal Glu concentration or increase the activation by phosphate, such as certain fatty-acyl CoA de-
and fatty acids in low concentrations and calcium (10, 11), may convert the dormant PAG into a functionally active one. Hence, dormant PAG may constitute a reserve that can be drawn on when needed.

As discussed above, PAG facing the cytosolic compartment may be regulated by extramitochondrial activators, as well as by inhibitors such as Glu. This has been demonstrated for pig kidney mitochondria (9, 11) and may be important for the regulation of the cytosolic concentrations of Gln and Glu. The effectors act by regulating the $K_a$ value for activation by phosphate, the concentration of which is rather constant, about 10 mM in the cytosol. Because the functional PAG under physiological conditions is dependent on the Glu/Gln-ratio that normally is $\sim$3–4 (1), the activity of PAG facing the cytosol is normally repressed. However, e.g., under metabolic acidosis, the cytosolic Glu concentration drops markedly, whereupon the PAG flux increases as shown in two closely related porcine kidney cell lines (LLC-PK$_1$ and LLC-PK$_1$–F363–F373) (17). Thus the cytosolic Glu concentration may be the most important regulator of the PAG activity in vivo. The PAG activity is controlled by extracellular Glu and by factors that modulate the cytosolic Glu concentration (1).

In conclusion, with the use of enzymatic, immunochromical, and immunogold electron microscopic studies, it has been demonstrated that pig and rat renal mitochondria contain two pools of PAG with distinct enzymatic properties. Intact mitochondria appear to express only PAG accessible to the outer face of the inner mitochondrial membrane. This PAG has properties similar to that of the polymerized form of purified pig kidney PAG and is most likely regulated by cytosolic Glu. The fraction of PAG that is localized inside the inner mitochondrial membrane is dormant, and evidence is presented that its activity is depressed by the rather high matrix concentration of Glu.

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