A membrane-bound form of glutamate dehydrogenase possesses an ATP-dependent high-affinity microtubule-binding activity

Fabienne RAJAS and Bernard ROUSSET

Institut National de la Santé et de la Recherche Médicale, Unité 369, Faculté de Médecine Alexis Carrel, 69372 Lyon Cédex 08, France

We previously identified a 50 kDa membrane protein which bound to *in vitro* assembled microtubules [Mithieux and Rousset (1989) *J. Biol. Chem.* **264**, 4664–4668]. This protein exhibited the expected properties for mediating the ATP-dependent association of vesicles with microtubules [Mithieux, Audebet and Rousset (1988) *Biochim. Biophys. Acta* **969**, 121–130]. The 50 kDa membrane protein (MP50), initially extracted in very low amount from isolated pig thyroid lysosomes/endosomes, has now been purified from membrane preparations of crude vesicle fractions from pig liver and brain. MP50 was isolated from detergent-solubilized membrane protein by affinity chromatography on immobilized ATP; 3–5 mg of MP50 was obtained from 100 g of liver tissue. Phase partitioning in Triton X-114 indicated that MP50 is a peripheral membrane protein. Radioiodinated liver MP50 bound to microtubules assembled *in vitro*. The binding was inhibited by ATP ($K_i = 0.76$ mM) and displaced by unlabelled liver or brain MP50. Equilibrium binding studies yielded

K_D values of 1.8×10^{-7} M. By N-terminal amino acid sequence analysis, MP50 was identified as glutamate dehydrogenase (GDH), by comparison of V8 protease peptide maps of MP50 with purified liver GDH. Liver MP50 exhibited a low GDH activity: 4–5 units/mg compared with 18 and 34 units/mg for purified bovine and rat liver GDH respectively. Bovine and rat liver GDH yielded six spots from pI 5.7 to 7.2 when analysed by two-dimensional electrophoresis; in contrast, MP50 gave one main spot (corresponding to spot 2 of liver GDH) with a pI of approx. 6.5. Soluble liver GDH from commercial sources exhibited a very low or no microtubule-binding activity. In conclusion, we have found a membrane-bound form of GDH capable of specific and nucleotide-sensitive interaction with microtubules. Our data suggest that GDH isoproteins, the number of which has been undervalued up to now, could have cellular functions other than that of an enzyme.

INTRODUCTION

There is a great deal of evidence for a role of interphase microtubules in the intracellular localization and transport of organelles. The Golgi apparatus, endoplasmic reticulum, mitochondria, secretory granules, endosomes and lysosomes interact with microtubules [1] and the organization of the cytoplasm is determined by the microtubule network [2]. The movement of organelles along microtubules allows delivery and/or concentration of cell components at specific locations. Non-motile or 'stable' interactions of vesicles with microtubules would contribute to the maintenance of given organelles at a determined position in the cell [3–5].

The assembly, organization and functions of microtubules are modulated by interactions with many different proteins. One class of proteins, the microtubule-associated proteins (MAPs), which copurify with microtubules along cycles of polymerization-depolymerization and control microtubule assembly *in vitro*, were found to be involved in the organization of the microtubule network in the cell [6,7]. A second class of proteins is composed of (a) cytosolic motors which mediate the directional (centrifugal or centripetal) transport of organelles [8] and (b) proteins which are potentially involved in microtubule-membrane interactions. Proteins belonging to the second class have recently been defined as microtubule-binding proteins by Rickard and Kreis [9].

The cytosolic motors, kinesin [10], cytosolic dynein or MAP 1C [11] and dynamin [12,13], display transient interactions with microtubules [14]. These mechanochemical enzymes induce the movement of organelles in one and/or the other direction along microtubules [15]. Kinesin is a microtubule plus end-directed motor and dynein is a microtubule minus end-directed motor

[16]. Recently a novel protein, pp170, which binds to microtubules *in vitro* in a nucleotide-sensitive manner, has been characterized by Rickard and Kreis [9]. This cytoplasmic protein, named CLIP-170, mediates the interaction of endocytic vesicles with microtubules [17]. The cytosolic motors are characterized by their nucleotide-dependent microtubule-binding activity and their microtubule-activated ATPase activity which provides the force to move large intracellular organelles [8]. If the microtubule-based motors are expected to bind to a variety of organelles, little is known about the mode of interaction of these molecules with the membrane of the transported organelles [18–20].

The second group of microtubule-binding proteins consists of the organelle-associated proteins. These membrane proteins might be involved in the association of vesicles with microtubules and thus could be considered as anchoring factors. A 58 kDa protein mainly associated with the peripheral membrane of Golgi apparatus has been identified in rat liver cells. The 58 kDa protein binds to preassembled tubulin and can stimulate tubulin polymerization. The proposed function of this protein is to provide attachment sites for the Golgi elements to microtubules [21]. Another membrane protein, the 110 kDa protein, immunologically related to MAP-2 and associated with the cytoplasmic face of the Golgi complex, has also been proposed as a linker element between the Golgi and microtubules [22]. The interaction of the 110 kDa protein with the Golgi apparatus appears to be regulated by factors such as ATP [23] and guanine nucleotides [24]. *In vitro* reconstitution assays of the association between microtubules and organelles have been developed to identify and characterize the molecular species involved in the interaction [5,25]. Van Der Sluijs et al. [26] showed that *N*-ethylmaleimide-sensitive factors and trypsin-sensitive vesicle-associated proteins

are involved in the binding of exocytic vesicles to microtubules. In our laboratory, effort has been made to investigate the mode of association of lysosomal vesicles (prelysosomes or late endosomes and lysosomes) with microtubules. We developed a reconstitution assay [27] based on the use of preassembled microtubules and highly purified lysosomes/endosomes [28]. This assay was used to show that the microtubule-lysosome/endosome interaction is activated by free Mg^{2+} and inhibited by ATP [29], the effect of ATP being independent of ATP hydrolysis. As the vesicle-microtubule interaction required neither the addition of cytosolic factors nor the presence of MAPs, we postulated the existence of vesicle membrane protein(s) capable of binding to microtubules. A membrane protein exhibiting a microtubule-binding activity has been identified [30]. It is a 50 kDa species extracted from purified thyroid lysosomal membranes using CHAPS as a detergent and purified in minute amounts by affinity chromatography on immobilized ATP.

To pursue the molecular characterization of this 'membrane protein of 50 kDa', termed MP50, we have tried to find a way to purify it in sufficient amount to perform biochemical analyses. Assuming that MP50 could be present in different tissues and possibly on different cell organelles, attempts were made to extract MP50 from larger organs such as liver and brain starting from crude vesicle fractions.

Here, we report that MP50 purified from thyroid, liver or brain is a form of a ubiquitous enzyme, glutamate dehydrogenase (GDH). MP50 possesses GDH enzyme activity and the ability to bind to a limited number of high-affinity sites on microtubules.

EXPERIMENTAL

Preparation and solubilization of membrane proteins

Pig tissues were obtained from the local slaughterhouse, transported on ice to the laboratory and processed within 2 h of the death of the animals. All further purification steps were carried out at 0–4 °C. Brain or liver fragments (about 100 g of tissue) in 300 ml of 0.25 M sucrose/10 mM Tris/HCl, pH 7.4, were passed through a metal sieve (pore size 0.4 mm) to disrupt the tissue. The tissue suspension was homogenized in a glass/Teflon Potter homogenizer, rotated at 1000 rev./min with six slow up-and-down strokes. The resulting material was centrifuged at 800 *g* for 20 min. The supernatant was collected and the pellet was rehomogenized and centrifuged under the same conditions. The two supernatants were pooled and centrifuged at 100 000 *g* for 1 h to obtain a crude vesicle fraction. Thyroid lysosomes were purified from a crude vesicle fraction (16 000–520 000 *g*·min) by isopycnic centrifugation on a 30% (v/v) Percoll gradient [28] in an hyperosmotic medium (0.5 M sucrose, 10 mM Tris/HCl, pH 7.4) [27].

The vesicle fractions (the crude fractions from liver or brain or purified thyroid lysosomes) were submitted to a hypo-osmotic pressure-dependent lysis in 10 mM Tris/HCl, pH 7.4, supplemented with protease inhibitors (5 mg/l phenylmethanesulphonyl fluoride, 5 mg/l leupeptin, 5 mg/l aprotinin and 0.8 mg/ml benzamidine) (TPI buffer). The vesicle disruption was completed by a freeze-thaw cycle. The vesicle membranes were pelleted by centrifugation at 100 000 *g* for 30 min, washed (by resuspension and centrifugation) in TPI buffer and then suspended in TPI buffer containing 0.6 M NaCl. The high-salt-treated membrane fractions were pelleted by centrifugation at 100 000 *g* for 30 min and washed again in TPI buffer. The final membrane pellet was resuspended in TPI buffer containing 0.5% (w/v) CHAPS, the membrane protein concentration ranging from 3 to 5 mg/ml. The mixture was maintained at room temperature for 30 min and the insoluble material was removed

by centrifugation at 100 000 *g* for 30 min at 25 °C. Solubilized membrane proteins were stored at –20 °C.

Purification and labelling of MP50

MP50 was purified from CHAPS-solubilized membrane proteins by affinity chromatography on immobilized ATP (ATP-agarose gel, A 9264 from Sigma, St. Louis, MO, U.S.A.) as described by Mithieux and Rousset [30]. About 50 mg of CHAPS-solubilized membrane protein was applied to 1 ml of ATP-agarose gel (packed gel volume). Protein bound to the ATP-agarose gel was eluted by 1 mM ATP in 10 mM Tris/HCl, pH 7.4, after extensive washing of the column with 10 mM Tris/HCl/100 mM NaCl, pH 7.4. The ATP-eluted affinity-purified protein fraction was filtered on a Sephadex G-25 column (PD-10 column from Pharmacia, Uppsala, Sweden) to eliminate ATP. Protein eluted from the column was concentrated up to 10 times using a SpeedVac concentrator from Savant.

Purified MP50 was labelled with $Na^{125}I$ using lactoperoxidase and the glucose-glucose oxidase system to generate H_2O_2 . Some 10–30 μ g of MP50 in 50 μ l of 10 mM Tris/HCl, pH 7.4, was mixed with 450 μ l of 80 mM phosphate, pH 7.0, containing 100 μ Ci of $Na^{125}I$ (Amersham, Les Ulis, France), 0.5 nmol of NaI, 2 μ g of lactoperoxidase (Boehringer-Mannheim), 2 μ g of glucose oxidase (type V from *Aspergillus niger*; Sigma) and 3 μ mol of glucose. After 40 min at room temperature, the reaction was stopped by addition of 1 mM methimazole. To separate residual $Na^{125}I$ from the labelled protein, the mixture was filtered on a PD-10 Sephadex column pre-equilibrated with buffer containing 10 mM sodium phosphate, 154 mM NaCl and 1 mg/ml BSA, pH 7.4 (PBS/BSA). The specific radioactivity of the labelled protein was 2–5 μ Ci/ μ g of protein. Labelled MP50 in PBS/BSA was stored at –20 °C after mixing with an equal volume of glycerol.

Microtubule binding assays

Microtubule proteins were prepared from pig brain by two cycles of temperature-dependent polymerization-depolymerization [31] in buffer A (100 mM Mes, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM GTP, pH 6.4) supplemented with 4 M glycerol and 1 mM GTP in polymerizing steps. Microtubules (2.5 mg of microtubule protein/ml) were assembled in buffer A for 30 min at 37 °C, sheared by five passages through the needle (25 G) of a syringe and kept for 5 min at 25 °C. Preassembled microtubules (250 μ g of protein) were mixed with ^{125}I -labelled MP50 in a final volume of 400 μ l of buffer A containing 10 mg/ml BSA. When present, ATP was added to the mixture just before microtubules. After 30 min of incubation at 25 °C, the mixture was layered on to 1 ml of 1 M sucrose in buffer A and centrifuged at 100 000 *g* for 1 h at 25 °C. Pellets and supernatants were counted for radioactivity in a Packard γ counter and analysed for protein by SDS/PAGE and autoradiography.

One- and two-dimensional PAGE

Analysis of proteins was performed on acrylamide slab minigels in the presence of SDS as described by Laemmli [32]. Two-dimensional electrophoresis was performed as described by O'Farrell [33]. Isoelectric focusing cylindrical gels (first dimension) consisted of 4% (w/v) acrylamide, 0.1% methylene-bisacrylamide, 9.2 M urea, 2% (w/v) CHAPS and 2% (v/v) ampholine (a mixture composed of 1 part of Servalyt 2-11, 2 parts of Servalyt 5-7 and 2 parts of Servalyt 6-9 from Serva, Heidelberg, Germany). Protein samples (10–50 μ g) were dialysed against 1 M acetic acid, dry-concentrated using a SpeedVac and

dissolved in a solution consisting of 9.3 M urea, 2% (w/v) CHAPS and 0.1 M dithioerythritol. After isoelectric focusing, the cylindrical gels were transferred to 9% polyacrylamide slab gels prepared in 375 mM Tris/HCl, pH 8.8. The buffer used for the second dimension consisted of 25 mM Tris, 192 mM glycine and 0.2% (w/v) SDS, pH 8.3. Protein spots were visualized by Coomassie Blue R250 staining. The isoelectric points and molecular masses were determined by comparison with the mobility of two-dimensional SDS/PAGE standards (from Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Protein microsequencing

Proteins separated by one- or two-dimensional electrophoresis were electroblotted on to Immobilon-P membrane [poly(vinylidene difluoride) membrane from Millipore, Bedford, MA, U.S.A.] using 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid/20% (v/v) methanol, pH 11.0, as transfer buffer [34]. Immobilon-P membranes were stained with 0.1% (w/v) Coomassie Blue R250 in 50% (v/v) methanol for 5 min and then destained in 50% (v/v) methanol/10% (v/v) acetic acid for 10 min at 4°C. The membrane was finally rinsed in deionized water for 10 min, air-dried and the band or spot of protein excised and stored at -20°C. The N-terminal amino acid sequence analysis was performed by the Edman degradation method using a gas-phase sequencer from Applied Biosystems. The phenylthiohydantoin derivatives of amino acids were identified by h.p.l.c. Microsequencing was carried out by Dr. L. Denoroy at the Service Central d'Analyse du CNRS, Solaize, France.

GDH assay

GDH (EC 1.4.1.3) was assayed by measurement of NADH oxidation using 2-oxoglutarate as substrate. NADH oxidation was determined by absorbance measurements at 340 nm using a Beckman DU 7400 spectrophotometer. The assay mixture (total volume 0.5 ml) contained 10 mM 2-oxoglutarate, 100 mM ammonium acetate, 400 mM NADH in 10 mM Tris/HCl/2.5 mM EDTA buffer, pH 7.3. One unit of enzyme is defined as the amount of enzyme required to reduce 1 μ mol of 2-oxoglutarate to L-glutamate/min at pH 7.3 and 25°C and in the presence of NH_4^+ ions. Purified preparations of GDH from bovine and rat liver (Sigma) were used for comparison.

Other methods

Peptide mapping was performed by the method of Cleveland et al. [35]. Protein (5–10 μ g) was treated with 200 ng of *Staphylococcus aureus* V8 protease (type XVII) from Sigma for 30 min at 37°C and pH 6.8. The proteolytic fragments were separated by SDS/PAGE on 12% (w/v) acrylamide gels.

Protein was assayed by the method of Lowry et al. [36] with BSA as standard. Samples containing particulate material were pretreated with 1% (w/v) sodium deoxycholate to solubilize membrane protein.

RESULTS

Extraction and purification of MP50 from liver and brain

Attempts to purify MP50 from the membrane fractions in which it was initially identified [30], i.e. membranes of purified thyroid lysosomes, gave very low yields. It was only possible to obtain about 20–30 μ g of MP50 from 100 mg of lysosomal membrane protein, the preparation of which required about 2000 g of thyroid tissue, i.e. more than 200 pig thyroid glands. Assuming

that MP50 could be present in different tissues and on different organelles, we applied the purification procedure to larger organs (liver and brain) starting from crude (post-nuclear) vesicle fractions. The purification scheme included five main steps: (1) the preparation of crude vesicle fractions in 0.25 M sucrose by differential centrifugation, (2) an osmotic-pressure-dependent lysis of vesicles followed by a freeze–thaw cycle to release intravesicular soluble proteins, (3) treatment of the resulting membrane fraction with high-ionic-strength solution (0.6 M NaCl) to eliminate proteins loosely bound to cellular membranes, (4) solubilization of membrane proteins with a zwitterionic detergent, CHAPS, and (5) affinity-chromatography purification of CHAPS-solubilized membrane proteins on immobilized ATP, using specific elution with 1 mM ATP. The polypeptide composition of the protein fractions extracted from pig liver and pig brain by this purification procedure is shown in Figure 1(b) (lanes 2 and 3). The affinity-purified proteins had an apparent

Figure 1 Purification of MP50 from different pig tissues: analysis by one- and two-dimensional electrophoresis

(a) Analysis by SDS/PAGE of CHAPS-solubilized membrane protein fractions from thyroid lysosomes/endosomes (lane 1), crude liver vesicles (lane 2) and crude brain vesicles (lane 3) subjected to affinity chromatography on immobilized ATP; 30 μ g of protein was loaded on the gel. (b) Analysis by SDS/PAGE of the corresponding protein fractions eluted from the ATP column using 1 mM ATP (3–5 μ g of purified protein loaded). Lanes 1–3, MP50 purified from thyroid, liver and brain membrane extracts respectively. The electrophoretic mobility of MP50 (indicated by the arrow) was compared with that of tubulin (lane T). Numbers on the right refer to the migration of protein markers (molecular mass given in kDa). (c) Two-dimensional electrophoretic analysis of MP50. Protein (5–10 μ g) purified by affinity chromatography on immobilized ATP from thyroid lysosomal membranes (1) or liver vesicle membrane (2) was subjected to isoelectric focusing and then SDS/PAGE. Protein was stained with Coomassie Blue. The pI scale was determined from the mobility of two-dimensional markers from Bio-Rad Laboratories.

Figure 2 Equilibrium binding studies of liver ^{125}I -labelled MP50 to preassembled microtubules

^{125}I -labelled MP50 (0.25 μCi , about 100 ng of protein) was incubated with preassembled microtubules (250 μg of microtubule protein) in a total volume of 400 μl of buffer A containing BSA (10 mg/ml) for 30 min at 25 $^{\circ}\text{C}$ in the absence or presence of increasing amounts of unlabelled MP50. Microtubules were then sedimented by centrifugation at 100 000 g for 60 min at 25 $^{\circ}\text{C}$ through a sucrose cushion and the pellets and supernatants were counted for radioactivity and assayed for protein. (a) Percentage of ^{125}I -labelled MP50 found in the microtubule pellet as a function of the amount of unlabelled MP50 added to the incubation mixture. (b) Scatchard plot of the binding data of two separate experiments carried out with two microtubule protein preparations exhibiting different efficiency of microtubule assembly, about 80% (●) and 55% (▲). The microtubule-bound MP50 (B) expressed as μg per 0.4 ml (volume of the incubation mixture) was calculated from the radioactivity measurements and the specific radioactivity of MP50.

molecular mass of 50 kDa, migrating slightly ahead of the β -tubulin subunit. MP50 extracted from liver and brain had the same electrophoretic mobility as MP50 purified from the thyroid (compare lane 1 and lanes 2 and 3 of Figure 1b). Some very minor contaminants of either higher or lower molecular mass depending on the tissue and on the preparation were sometimes observed. The 50 kDa species accounted for at least 90% of the purified protein. MP50 represented a minor component of the different CHAPS-solubilized membrane protein fraction (compare Figures 1a and 1b). A yield of 300–500 μg of MP50 was obtained from 50 mg of CHAPS-solubilized liver vesicle membrane protein. The same amount of detergent-solubilized protein from brain yielded only 80–100 μg of MP50. The overall MP50 purification recovery was between 3 and 5 mg per 100 g of liver tissue and between 0.2 and 0.3 mg per 100 g of brain tissue. To ensure that the 50 kDa protein extracted from the liver, brain and thyroid actually corresponded to the same molecular species, the purified proteins were analysed by two-dimensional electrophoresis. Figure 1(c) shows that thyroid MP50 and liver MP50 produced similar spots at a pI close to 6.5. The same result was obtained with the affinity-purified protein from brain (not shown). On the acidic side of the liver MP50 spot, one can see an

Figure 3 PAGE and autoradiographic analyses of the competitive binding between labelled and unlabelled MP50 to microtubules

The experiment was carried out under the same conditions as those described in the legend of Figure 2. Preassembled microtubules were incubated with ^{125}I -labelled MP50 without or with increasing concentrations of unlabelled MP50 in a total volume of 400 μl . After 30 min at 25 $^{\circ}\text{C}$, the incubation mixtures were centrifuged at 100 000 g for 60 min. The microtubule pellets were resuspended in 300 μl of buffer A. Portions (20 μl) of the microtubule suspension and 20 μl of supernatant, i.e. the incubation volume + sucrose cushion = 1.4 ml, were subjected to SDS/PAGE and autoradiography. (a) and (c) Coomassie Blue-stained gels of the microtubule pellets and incubation supernatants respectively. (b) and (d) Autoradiographs of the gels shown in (a) and (c). Lanes 2–8 correspond to the samples obtained at different unlabelled MP50 concentrations: 0, 1, 3, 5, 10, 20 and 30 $\mu\text{g}/400\text{ }\mu\text{l}$. Samples analysed in lanes 1 were the same as those analysed in lanes 2 except that microtubule protein was omitted. Numbers on the left of the figure indicate the molecular mass of markers in kDa. The arrowheads identify α - and β -tubulin subunits. The arrows indicate the mobility of MP50.

additional component appearing as a faint band. To determine whether MP50 is an integral membrane protein, we analysed its membrane association by using phase partitioning in Triton X-114 [37]. CHAPS was replaced by Triton X-114 (final concentration 1%, v/v) in the vesicle membrane protein solubilization. After separation from the detergent phase, the aqueous phase was subjected to the affinity chromatography on immobilized ATP. The ATP eluate of the column contained MP50 in a purified form (result not shown). This finding indicated that MP50 is hydrophilic and therefore a peripheral membrane protein.

Microtubule-binding activity of liver and brain MP50

MP50 purified from liver and brain have been studied for their microtubule binding activity. Preassembled microtubules were incubated with ^{125}I -labelled liver MP50 with or without unlabelled MP50. Results in Figure 2 show that labelled liver MP50 bound to microtubules; under the conditions used, about 45% of the labelled ligand was found associated with pelleted microtubules. Unlabelled MP50 induced a concentration-dependent displacement of the binding of ^{125}I -labelled MP50 to microtubules (Figure 2a). The electrophoretic analysis reported in Figure 3 demonstrates that unlabelled MP50 caused inhibition of the binding of ^{125}I -labelled MP50 to microtubules without affecting the degree of assembly of microtubules. The amount of tubulin recovered in the different microtubule pellets remained constant whatever the unlabelled MP50 concentration (Figure 3a). The protein band with a mobility of a 66 kDa component corresponds to BSA which was added in large amounts to the incubation mixture to prevent non-specific interaction; the bulk of the BSA was found in the assembly supernatants (Figure 3c). The auto-

Table 2 GDH activity of purified liver MP50

The enzyme activity of liver MP50 was compared with that of commercial preparations of bovine and rat liver GDH. Enzyme activity was measured in the absence or presence of 1 mM ADP, ATP or GTP. Results are expressed as means $\pm$ S.D. for the number of determinations indicated. Values in parentheses indicate the percentage increase or decrease in the activity in the presence of a given effector as compared with control.

Protein	Enzyme activity (units/mg)			
	Without effector	+ ADP	+ ATP	+ GTP
Bovine liver GDH	34.0 $\pm$ 3.1 <i>n</i> = 7	47.3 $\pm$ 4.0 <i>n</i> = 4 (139%)	48.7 $\pm$ 2.2 <i>n</i> = 6 (143%)	1.6 $\pm$ 0.1 <i>n</i> = 5 (4.7%)
Rat liver GDH	17.8 $\pm$ 1.1 <i>n</i> = 7	28.6 $\pm$ 1.0 <i>n</i> = 5 (161%)	28.1 $\pm$ 3.1 <i>n</i> = 5 (158%)	1.2 $\pm$ 0.2 <i>n</i> = 5 (6.7%)
Pig liver MP50	4.6 $\pm$ 0.3 <i>n</i> = 7	9.9 $\pm$ 0.9 <i>n</i> = 7 (215%)	7.1 $\pm$ 0.3 <i>n</i> = 7 (154%)	0.14 $\pm$ 0.02 <i>n</i> = 7 (3.0%)

Figure 5 Sedimentation properties of MP50

Liver MP50 (100 μ g) was layered on 8–25% sucrose gradients and centrifuged in a SW41 Beckman rotor for 16 h at 100 000 *g* (30 000 rev./min) and 4 °C. Fractions (0.5 ml) were collected from the top to the bottom of the tube and assayed for protein by A_{280} measurements. The dotted line indicates the sedimentation of BSA analysed in a separate tube. The arrow localizes the top of the 12 S thyroglobulin peak obtained under the same centrifugation conditions.

identity between GDH and MP50, we compared the V8 protease peptide maps of liver MP50 and bovine liver GDH (results not shown). The two proteins gave three main cleavage fragments of apparent molecular mass 35, 21 and 14 kDa; the 21 kDa band seemed to contain at least two species. The N-terminal sequence of the 35 kDa peptide (Table 1) corresponded with the N-terminal sequence of the intact protein. The sequence obtained for the 21 kDa band derived from MP50 cleavage is reported in Table 1; this sequence could be aligned with an internal consensus sequence for all GDHs starting at Gly-315.

GDH enzyme activity and quaternary structure of MP50

The enzyme activity of liver MP50 (reduction of 2-oxoglutarate to L-glutamate) was compared with that of commercially available GDH preparations from bovine and rat liver (Table 2). The mean specific activity of different preparations of MP50 was

Figure 6 Comparative analysis of the isoprotein pattern of MP50 and GDH

Liver MP50 (about 30 μ g) (a), bovine liver GDH (30 μ g) (b) and a mixture of MP50 (30 μ g) and GDH (30 μ g) (c) were subjected to two-dimensional PAGE. The gels were stained with Coomassie Blue. Arrows indicate the main polypeptide of the MP50 preparation and the corresponding spot in GDH. Numbers in (b) and (c) identify the GDH isoproteins. Spot 3 has been divided into two parts (3a and 3b).

4.6 units/mg, a value 4–8-fold lower than that of purified preparations of liver GDH. The enzyme activity of MP50 was, like that of the GDH preparations used as reference, increased 1.5–2-fold in the presence of 1 mM ATP or ADP and completely inhibited in the presence of 1 mM GTP.

MP50 fractionated by velocity sedimentation on sucrose gradients (Figure 5) gave two main peaks, one sedimenting more slowly than BSA and the other having the same sedimentation behaviour as the 12 S thyroglobulin subunit (molecular mass 330 kDa). The latter peak accounted for 80–90% of the total MP50 protein. Under the same conditions, liver GDH yielded a different sedimentation pattern. About 20% of the protein was found in a 12 S sedimenting peak, whereas most GDH appeared polydispersed and distributed throughout the bottom part of the gradient. Formation of large oligomers is a well-documented property of GDH [43].

Differences between MP50 and GDH

We have analysed the molecular composition of liver GDH by two-dimensional electrophoresis (Figure 6b). Surprisingly, purified preparations of bovine or rat liver GDH consisted of at least six isoproteins with pI ranging from 7.2 (isoprotein 1) to 5.7

Figure 7 Lack of microtubule-binding activity of soluble GDH

¹²⁵I-labelled MP50 was incubated with preassembled microtubules without or with increasing amounts of MP50 (●) or bovine liver GDH (○). The experimental conditions were the same as in Figure 2. Microtubule-bound ¹²⁵I-labelled MP50 was expressed as percentage of total radioactivity.

(isoprotein 6). Three components (isoproteins 1, 2 and 3) were in about the same proportion. Isoproteins 4–6 were less abundant and in decreasing quantities. Isoprotein 2 had the same pI (approx. 6.5) as MP50. In Figure 6, we have compared the GDH isoprotein pattern with that of MP50 using a protein load two or three times higher than that used in the analysis reported in Figure 1. The spot with a pI of 6.5 or isoprotein 2 was the main component, but definite spots probably corresponding to isoproteins 1, 3 and 4 could be reproducibly observed in different MP50 preparations. To demonstrate that the main component of MP50 actually corresponds to isoprotein 2 of GDH, we analysed a mixture of GDH and MP50. As illustrated in Figure 6(c), the MP50 spot coincided with isoprotein 2 of GDH (compare Figures 6a, 6b and 6c). The minor component of MP50, with a pI slightly lower than that of the major spot, co-migrated with the acidic 'tail' of GDH isoprotein 3 (identified as spot 3b in Figures 6b and 6c).

Given the numerous similarities but also the few marked differences between GDH and MP50, it was important to test whether liver GDH, like liver MP50, was capable of interacting with microtubules. Two approaches were used. We looked for a microtubule-binding activity of radioiodinated GDH and we tested the ability of unlabelled GDH to compete with ¹²⁵I-labelled MP50 for binding to microtubules. Neither of these approaches revealed any significant microtubule-binding activity of GDH. Only a very small proportion of ¹²⁵I-labelled GDH associated with preassembled microtubules and this fraction cannot be displaced by excess unlabelled GDH (result not shown). As reported in Figure 7, GDH did not markedly inhibit the binding of tracer amounts of ¹²⁵I-labelled MP50 to microtubules.

DISCUSSION

In this study, we demonstrate that the 50 kDa membrane protein (MP50) initially identified for its ATP-dependent microtubule-binding activity corresponds to a membrane-bound form of an isoprotein of GDH, an enzyme known to be a hexamer composed of six identical subunits of 56 kDa.

The identity between MP50 and GDH is substantiated by the sequence analyses of the first 20 amino acids of the protein as

well as that of the N-terminus of V8 protease-cleavage products. MP50 from pig brain or liver and bovine or rat liver GDH exhibited the same mobility on SDS/PAGE and gave the same peptide maps after V8 protease treatment. MP50 possesses a GDH enzyme activity which, although lower than that of the reference preparations, remains sensitive to nucleotide modifiers [44]. The velocity sedimentation analysis indicated that purified MP50 is in an oligomeric form probably corresponding to a hexamer. Interestingly, MP50 differs from GDH when analysed by two-dimensional electrophoresis. MP50 preparations contained one main component with a pI of 6.5. In contrast, purified GDH from bovine or rat liver was reproducibly composed of at least six components which we designated isoprotein 1 to isoprotein 6. By careful comparison using pI markers, we have been able to determine that liver MP50 had the same pI as isoprotein 2 of liver GDH. The three minor species found in MP50 preparations probably correspond to isoproteins 1, 3 and 4.

The existence of GDH isoforms or isoproteins has been only relatively recently recognized [45] for brain GDH. Four different forms were found. To our knowledge, the most complex pattern found is the one we report here for liver GDH. The GDH isoproteins seem to have almost the same (if not the same) molecular mass. Indeed, purified GDH always gave a homogeneous and narrow band on SDS/PAGE, and no clear difference in mobility (in the second dimension) was detected on two-dimensional electrophoretic fractionation. The origin of the GDH polymorphism is not known. These isoproteins could be the products of different genes [41] and/or different mRNA species generated by alternative splicing. They could also arise by post-translational modifications. The brain GDH isoprotein pattern was reported to be unaltered by treatment with alkaline phosphatase [45]. This indicates that GDH phosphorylation should not account for the observed polymorphism. Another post-translational modification process, i.e. proteolysis, has been discussed [45]. McCarthy et al. [39] found that the commercial preparations of GDH have often undergone a partial N-terminal proteolysis. To test whether the GDH polymorphism we observed could be related to such a degradation, we submitted isoproteins 1–4 of bovine liver GDH to N-terminal microsequencing. The same N-terminal sequence (ADREDDPN) was found for the four spots. As reported by McCarthy et al. [39], the first four amino acids are missing. This type of N-terminal proteolysis has never been found for purified MP50.

GDH is known to be a widely distributed mitochondrial enzyme. Its exact location within the mitochondrial matrix has been debated. By electron microscope immunogold labelling of rat liver ultrathin sections [46], it has been found that most GDH is associated with the mitochondrial inner membrane. Nonetheless, mitochondrial GDH must be loosely bound to the membrane, as it is readily solubilized [47]. Studies mainly carried out on brain [48] but also on liver [47] gave evidence for the existence of a fraction of GDH tightly bound to non-mitochondrial membranes. This particulate GDH found in microsomal fractions had the same molecular mass, chromatographic behaviour and enzyme activity as mitochondrial GDH. However, the two GDH activities differed in their thermal stability and allosteric properties [48]. Attempts to detect differences between GDH activities associated with the mitochondrial matrix and membrane fractions using different polyclonal antibodies have been unsuccessful [49]. It has been postulated that particle liver GDH could correspond to neosynthesized GDH temporarily bound to microsomes [47]. Indeed, GDH is synthesized in the cytoplasm and then translocated to the mitochondrial matrix with concomitant loss of a 6 kDa fragment, presumed to be a signal peptide [41,50].

A role in the synthesis and/or oxidation of glutamate as a neurotransmitter has been attributed to brain particulate GDH.

The analyses of the amino acid sequences of brain and liver GDH of different species obtained by protein sequencing or derived from cDNA cloning show that GDH is not an integral membrane protein. Therefore the GDH association with the inner mitochondrial membrane, which is easily disrupted, could involve interactions of the protein with component(s) of the lipid bilayer. Indeed, it was reported that GDH has a tendency to interact with phospholipids [51], the interaction being decreased by increasing the ionic strength. In contrast, a tight binding of GDH to membranes could involve the membrane insertion of a lipid moiety covalently attached to the protein. MP50 behaves as a protein tightly bound to membranes. Like GDH, MP50 should be devoid of a hydrophobic segment. Indeed, MP50 was found in the aqueous phase after partition in Triton X-114. MP50 remained membrane-bound after high-salt treatment, up to 1.5 M NaCl (results not shown). Its extraction or solubilization from membranes required the use of a detergent.

What is the source of the extracted MP50? As the vesicle fractions from which we prepared the membranes and extracted the protein contained different cell organelles including mitochondria, we cannot completely exclude the possibility of a mitochondrial origin for MP50. However, two separate observations argue against this view. First, the MP50 isoprotein pattern is different from that of purified bovine or rat liver GDH preparations thought to be of mitochondrial origin. Secondly, these GDH preparations did not exhibit the fundamental property of MP50, i.e. the ability to bind to microtubules. From these arguments and taking into account data in the literature, it is reasonable to think that MP50 could be extracted from a membrane GDH 'pool' different from that of the mitochondrial matrix.

As postulated at the beginning of this work, MP50 present on the membrane of a cell organelle could confer on this organelle the capacity to interact with microtubules in a way regulated by ATP. MP50 would play the role of an anchor. On one side, MP50 would be attached to the organelle membrane (as discussed above for tightly membrane-bound GDH) via a lipid (long-chain fatty acid, isoprenoids) covalently bound to the protein, a widespread mode of association of proteins with biological membranes. If MP50 is oligomeric (in the form of hexamers), its membrane association would not require the presence of a lipid-protein adduct on each monomer. On the other side, MP50 would bind to specific sites on microtubules. Indeed, the binding of MP50 to microtubules is characterized by a high affinity constant. If we consider that MP50 is in the form of a hexamer, as supported by the data of Figure 5, the apparent K_a would be close to $0.4 \times 10^8 \text{ M}^{-1}$. Microtubules would possess a very low number of MP50 hexamer-binding sites, about 1 for 200 assembled tubulin molecules. According to the microtubule model of Amos [52], one should find one binding site for an MP50 hexamer per 100 nm microtubule length if the sites are uniformly distributed along the microtubules. These considerations agree with the results of our *in vitro* reconstitution studies based on the use of isolated lysosomes/endosomes and microtubules assembled *in vitro* [29].

Electron-microscopic observations on negatively stained samples have mainly shown one vesicle bound to a microtubule, the length of which varied from 0.5 to 2 μm , or one vesicle interacting with several microtubules (two to six). This latter image would suggest that a vesicle could present several anchoring factors on its surface. Quantitative data obtained in this previous study [27] also fit very well with the existence of a limited number of binding sites on the microtubules. Using a fixed amount of

lysosomes, the formation of microtubule-vesicle complexes increased linearly with the concentration of microtubules and then reached a plateau. In contrast, at a given concentration of microtubules, the amount of complex formed did not depend on the concentration of lysosomes. The association of lysosomal vesicles with microtubules was found to be inhibited by ATP, the concentration inducing a half-maximal effect being 0.83 mM. Interestingly, the concentration of ATP that reduced the binding of MP50, the candidate for the role of anchor to microtubules, was 0.76 mM. It is worth noting that the ATP concentration range active in the binding of MP50 to microtubules and in the vesicle-microtubule interaction, i.e. 0.1–3 mM, corresponds to the expected range of physiological cytoplasmic ATP concentrations in different cell types.

These findings all support our hypothesis that MP50, now identified as GDH (mainly isoprotein 2), mediates the association of vesicles or organelles with microtubules. According to our previous tentative model [29], lysosomes could be subjected to two different types of interaction with microtubules: stable association and motile association bringing into play MP50 and cytoplasmic motor proteins respectively. Lysosome-microtubule interactions mediated by kinesin [53,54] or cytoplasmic dynein [55] have been well documented. Hollenbeck and Swanson [53] elegantly demonstrated that kinesin is responsible for extension of lysosomes as tubulovesicular structures along microtubules. There is now a need for a direct demonstration of the role of MP50 in the stable association of lysosomes with microtubules. Efforts need to be made to generate antibodies directed against MP50 and to analyse their effects on the microtubule-vesicle interaction in the reconstitution assay.

Finally, the present report assigns a possible new function to a well-known enzyme, GDH, which thus could be classified in the group of the multifunctional proteins. In recent years, there have been a number of reports describing new function(s) for known proteins. Protein disulphide isomerase and prolyl hydroxylase [56], the cation-independent mannose 6-phosphate receptor and the insulin-like growth factor II receptor [57], α -enolase and plasminogen receptor [58] have all been shown to reside in the same polypeptide. Another example is given by the crystallin protein family, the major structural protein of the lens [59]. Crystallins were found to share high sequence similarity, or be identical, with various enzymes. Indeed, tests of enzyme activity showed that ϵ -crystallin has lactate dehydrogenase activity, τ -crystallin α -enolase activity and δ -crystallin argininosuccinate lyase activity. Very recently, glyceraldehyde-3-phosphate dehydrogenase was found to be a tRNA-binding protein [60]. Similarly, GDH could have cellular function(s) other than that of an enzyme.

We express our gratitude to Dr. L. Denoroy for his valuable contribution in obtaining the sequence data.

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