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GLUTAMATE TRANSPORT IN PIG HEART MITOCHONDRIA

BINDING AND STRUCTURAL PROPERTIES OF HIGH-GLUTAMATE AFFINITY PROTEOLIPID: RECONSTITUTION STUDIES

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Summary

Previously, a proteolipid that can bind glutamate with high affinity has been isolated from pig heart mitochondrial membranes. A final affinity chromatography on γ-methylglutamate-albumin coreticulated on glass fiber was necessary. This procedure includes long dialysis steps which tend to denature the high-glutamate affinity proteolipid.

Here is described a new method of isolation which avoids long dialysis steps and yields greater amounts of the high-glutamate affinity proteolipid.

The binding of glutamate or aspartate on high-glutamate affinity proteolipid has been studied by gel filtration, by equilibrium dialysis or by a new procedure of rapid centrifugation based on the insolubility of high-glutamate affinity proteolipid in water. The latter method permits the detection of low and high affinity sites for glutamate with a K_d 60 mM and 55 μM, respectively. Among a series of analogues, aspartate appeared to be the best competitor: K_d = 30 μM and two K_i values, 0.37 mM (at high glutamate concentration) and 3.8 μM (at low glutamate concentration). High-glutamate affinity proteolipid binds 0.4 nmol of glutamate but only 0.1 nmol of aspartate per mg protein. The sites for glutamate and aspartate appear to be different but interdependent.

In the presence of high-glutamate affinity proteolipid, externally added glutamate stimulated the efflux of aspartate from preloaded liposomes.

High-glutamate affinity proteolipid contains cardiolipin, phosphatidyl choline and phosphatidyl ethanolamine the distribution of which is different from that of the inner membrane.

The effects of various phospholipases, trypsin, and thiol reagents were studied on the binding of glutamate. High-glutamate affinity proteolipid

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Abbreviations: HGAP, high-glutamate affinity proteolipid.

binds 9 nmol *N*-ethylmaleimide per mg protein but only 6.1 nmol in the presence of glutamate. The dissociation of high-glutamate affinity proteolipid caused by thiol reagents yielded a soluble protein fraction with higher affinity for glutamate.

Electrophoresis and an immunological approach allowed the detection and titration of the glutamate dehydrogenase and aspartate aminotransferase present in high-glutamate affinity proteolipid in inhibited forms, the latter being 26-fold more concentrated than the former.

Introduction

In a previous work the isolation from pig heart mitochondria of a detergent-soluble proteolipid able to bind glutamate with high affinity (HGAP) has been described and a glutamate-transport system was reconstituted by association of HGAP to liposomes entrapping glutamate dehydrogenase and NAD^+ [1]. The dissociation constant of HGAP for [^{14}C]glutamate, 62 μM, is similar to the apparent K_m of entry of glutamate into pig heart mitochondria. The binding of glutamate to HGAP and its transport into liposomes, as well as its entry into mitochondria, were strongly diminished by *N*-ethylmaleimide and partly by Avenaciolide, two known inhibitors of glutamate transport [2,3]; 2-mercaptoethanol and cysteine prevented but did not release the inhibitions, which thus were related to the presence of thiols in HGAP. While HGAP did not affect aspartate aminotransferase or malate dehydrogenase activities, it strongly inhibited glutamate dehydrogenase and gave an homogeneous association with it. These results were developed and discussed further [4]; they could be related to phospholipid-glutamate dehydrogenase interactions first described by us [5] and studied in detail by Godinot [6]. Additionally, urea-dodecyl sulphate gel electrophoresis patterns suggested that HGAP might contain inhibited forms of glutamate dehydrogenase or aspartate aminotransferase, the two main enzymes oxidizing glutamate in pig heart mitochondria [7] since it does not exhibit any of these activities. Nobody knows as yet whether these two enzymes, which can be strongly associated with the inner mitochondrial membrane [6,8,9] might have a role in glutamate transport through this membrane. Thus it appeared important to prove by more accurate methods the participation of these enzymes in the structure and activity of HGAP.

The present paper reports these attempts. Structural and binding studies on HGAP made compulsory a drastic improvement of the isolation process which is described here. The molecular properties were studied by an immunoglobulin approach, electrophoresis and chemical dissociation. Since our first reconstituted transport could suggest that HGAP catalysed a glutamate-OH^- exchange, it appeared interesting to define the effects of other aminoacids or dicarboxylates on the binding and transport of glutamate. Experimental results obtained with a new reconstituted system and during the aspartate binding studies suggest a possible role of HGAP in mediating glutamate-aspartate exchange accros the mitochondrial membrane, as for the antiporter postulated by Azzi et al. [10]. A brief summary of parts of these results has been presented at the FEBS Adv. Course No. 38 [11].

Materials and Methods

Pig heart mitochondria were prepared according to Gautheron et al. [7]. Only preparations with high respiratory control ratios greater than 5 were used.

HGAP was prepared either by the method previously described [1] or by the improved procedure reported in this paper (see results).

Proteins were estimated according to Lowry et al. [12] or Gornall et al. [13]. Aspartate aminotransferase activity was measured according to Pfleiderer [14]. Glutamate dehydrogenase activity was estimated in the forward reaction (oxidation of glutamate) in standard conditions: 20 mM potassium phosphate, pH 7.2 or 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.5 mM NAD^+, 10 mM potassium glutamate, 28°C. Absorbance at 340 nm was monitored. In the reverse reaction, the activity was tested according to Arnold and Maier [15]. Polyacrylamide gel electrophoresis were performed according to Weber and Osborn [16] as modified by Catteral and Pedersen [17]. Binding curves were mathematically interpreted by the iterative computation method of Rosenthal [18].

Pig heart mitochondrial glutamate dehydrogenase was prepared by affinity chromatography as previously described [19]. Mitochondrial isozymes of aspartate aminotransferase were prepared by the method of Ryan and Fottrell [20] modified as follows: after sonication of mitochondria, the supernatant was treated according to Furbisch et al. [21] to obtain apoenzymes free from pyridoxal phosphate; the apoenzymes were then purified by affinity chromatography on Sepharose-pyridoxal phosphate. After reconstitution of holoenzymes by incubation with pyridoxal phosphate they were separated by a second affinity chromatography on Sepharose-hexamethylene diamine and by differential elution. Before loading the column with the mixture, aspartate aminotransferase was converted to its pyridoxamine form in the presence of 20 mM aspartate, while alanine amino transferase was fully transformed to its pyridoxal form by addition of 20 mM pyruvate. Aspartate aminotransferase was not retained on the column and alanine amino transferase was later eluted with 0.2 M potassium phosphate.

Antibodies were obtained from rabbit according to Balinsky et al. [22] with glutamate dehydrogenase and, according to Vaitukaitis et al. [23], with mitochondrial aspartate aminotransferase. Double immunodiffusions were performed by the method of Ouchterlony [24] and microagglutination of activated latex by the method of Herzog et al. [25].

Standard proteins for molecular weight calibration were from Sigma Chemical Co. (myoglobin, ovalbumin), Boehringer (cytochrome *c*) and Koch Light (bovine serum albumin). Beef liver glutamate dehydrogenase (EC 1.4.1.3) and pig heart cytoplasmic aspartate aminotransferase (EC 2.6.1.1) were purchased from Boehringer, Mannheim. Phospholipases (*Crotalus adamanteus* venom, phospholipase C from *Clostridium welchii* and phospholipase D from cabbage) were purchased from Sigma Chemical Co. Trypsin was from DIFCO and soybean trypsin inhibitor from Sigma.

Isotopically labelled compounds were obtained from C.E.A., Saclay, France, as [U-^{14}C]glutamate or *N*-ethylmaleimide and [2,3-^{3}H]aspartate. All reagents were of the best available analytical grade.

Results

1. Improvement of the preparation of HGAP

The method described previously [1] included long dialysis steps which tend to denature HGAP. These steps were replaced by ammonium sulphate precipitation (Table I, B); to increase the final yield an ammonium sulfate fractionation (1.43—1.84 M ammonium sulfate cut) was applied (Table I, D) instead of the affinity chromatography (Table I, C). All attempts to purify further the material recovered after ammonium sulfate fractionation by affinity chromatography were unsuccesful. Table I describes all the purification steps with recovery and yields of each fraction, estimated after the freezing, thawing and sonication step of mitochondrial pellet (Table I, A1) since the glutamate retained by functional mitochondria includes binding and uptake. The final fractions of HGAP did not exhibit any aspartate aminotransferase or glutamate dehydrogenase activity and gave sufficient amounts of material for further detailed studies.

TABLE I

IMPROVED PROCEDURE FOR THE PREPARATION OF HGAP: RECOVERY AND YIELD

For steps A, B and C detailed procedures have been previously described [1]. Final fractions were recovered as pellets. Proteins were estimated according to Lowry et al. [10] after 2 h digestion in 0.5 N NaOH, 80°C, and three ethyl ether extractions. Glutamate binding was assayed under standard conditions: 0.25 M sucrose, 10 mM Tris/acetic buffer, pH 7.2, 30°C, 10 min, with 1.9 μM [^{14}C]glutamate (340 000 dpm/nmol). Separation and washing of insoluble materials were performed by rapid centrifugation (Eppendorf microcentrifuge 3200).

Step	Volume (ml)	Protein (mg)	Specific binding of glutamate [a]	Recovery (%)	Purification factor
Starting material					
Functional mitochondria	275	11 000	11 230		
A. Elimination of soluble or loosely bound proteins					
1. Freezing, thawing, sonication, phosphate washing of mitochondrial pellet	375	9 375	7 692	100	—
followed by:					
2. High ionic strength M KCl	245	6 125	10 434	88.6	1.35
B. Solubilization of membrane residue in 2% cholate [c]	200	1 000	b		
C. Affinity chromatography (batch or column) alternative 1 or 2:					
1. *N*-glutamyl adsorbent elutates		33 [d]	153 680	7	19.9
2. *N*-γ-methylglutamyl adsorbent eluates		20 [d]	116 260	3.25	15.1
Alternative step to C:					
D. Ammonium sulfate fractionation of 2% cholate extract					
1.43—1.84 M ammonium sulfate cut		275 [d]	121 600	46.5	15.8

[a] With functional mitochondria the results includes specific binding and uptake of glutamate. Results expressed in dpm/mg protein.

[b] Cholate interferes with binding determination.

[c] Long dialysis steps previously described [1] were replaced here by 1.84 M ammonium sulphate precipitation. The final pellet was again dissolved in 2% cholate.

[d] No detectable enzymatic activity aspartate aminotransferase or glutamate dehydrogenase.

2. Comparative saturation curves of HGAP and functional mitochondria by glutamate

We see in Fig. 1 that the kinetics of glutamate uptake by functional mitodria (respiratory control ratio over 6) as measured according to the method of Robinson and Chappell [29], are of the Michaelis and Menten type (Fig. 1B) as well as the binding of labelled glutamate to HGAP (Fig. 1A) measured by equilibrium dialysis. These curves allow to calculate an apparent K_m for glutamate entry into pig heart mitochondria of 76.6 ± 15.4 μM and a K_d for binding of glutamate on HGAP of the same order of magnitude: 62 μM [1,4]. It has been checked that the maximal rate of glutamate uptake (0.29 ± 0.02 nmol/min per mg protein [1]) was proportional to the protein concentration.

Estimations of glutamate binding by equilibrium dialysis or gel filtration were long and tedious. A new method of rapid centrifugation was developed, based on the insolubility of HGAP in water. For comparison, the method was applied to all fractions. The fraction (mitochondria, intermediate fractions of the purification procedure, HGAP, 0.2—1 mg protein) was incubated in 1 ml 0.25 M saccharose, 10 mM Tris/acetate, pH, 7.8, for 10 min at 30°C in the presence of [^{14}C]glutamate of various specific radioactivities. Then the suspension was spun down rapidly in a desk-top Eppendorf model 3200 microcentrifuge (10 000 × g, 1—2 min). The supernatant was carefully vacuum-sucked with a Pasteur pipette. The pellet was washed in 1 ml of the same

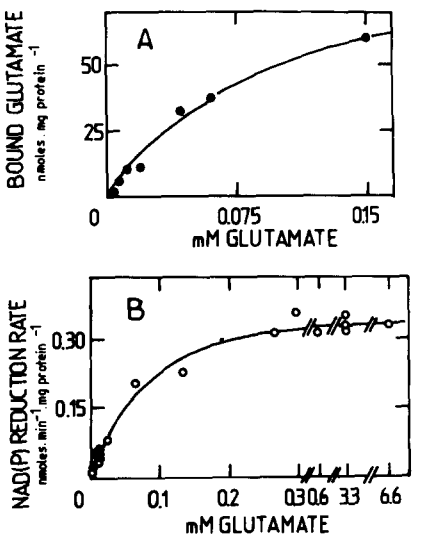

Fig. 1. Comparative influence of added glutamate concentration on glutamate binding by HGAP(A) and endogenous NAD(P)-reduction rate in pig heart mitochondria (glutamate transport). A. Glutamate concentration range 2—500 μM. Equilibrium dialysis: 18 h, 4°C, 10 mM potassium phosphate; 1 mM mercaptoethanol, pH 7.2; Union Carbide Visking tubes 24-Å pores, 50 μm thick. B. Mitochondrial protein (0.5 to 2.5 mg/ml) suspended in 2.9 ml 16 mM Tris · HCl, 112 mM KCl, 5 mM potassium phosphate (K), pH 7.4, 1.6 μg carbonyl cyanide *m*-chlorophenyl hydrazone per mg protein. After complete oxidation of endogenous NAD(P)H, Antimycin A (0.3 μg/mg protein) was added and then glutamate (0—6.6 mM); eight different mitochondrial preparations were tested. Initial rates were recorded at 28°C with an Aminco-Chance dual wavelength spectrophotometer, dual mode: 340 nm/373 nm (reference).

saccharose/Tris/acetate buffer by rapid centrifugation, once or several times. It was dissolved in 0.2 ml formic acid and mixed to 10 ml water-miscible scintillating mixture and counted. Controls were carried out in the presence of 24 mM glutamate.

This method allowed us to study a wide concentration range and to compare the profile of the uptake of glutamate by mitochondria (Fig. 2A) with the binding profile of glutamate to HGAP (Fig. 2B); it revealed two kinds of binding site, with low and high affinity for glutamate, which could not be detected by equilibrium dialysis or gel filtration [1]. The Scatchard plots were analysed according to Rosenthal [18] and were very similar for whole mitochondria and for HGAP. The low affinity sites are probably nonspecific. The high affinity sites gave dissociation constants of the same order of magnitude: 60 μM for mitochondria and 55 μM for HGAP. The average number of sites was 0.45 nmol and 0.4 nmol/mg protein, respectively, for mitochondria and HGAP.

This might be due to a denaturation of a number of sites during HGAP isolation; additionally, in experiments with whole mitochondria, data include binding and uptake of glutamate.

3. Binding properties of HGAP

Influence of pH and protein concentration on glutamate binding. At concentrations between 0.2 and 0.9 mg protein/ml, the binding of glutamate was proportional to protein concentration.

The binding exhibited a narrow range of optimal pH near 8.

Time dependence of glutamate binding and dissociation. Fig. 3 shows the binding of glutamate on HGAP as a function of the time of incubation. If unlabelled glutamate is added after 15 min of incubation, the labelled bound

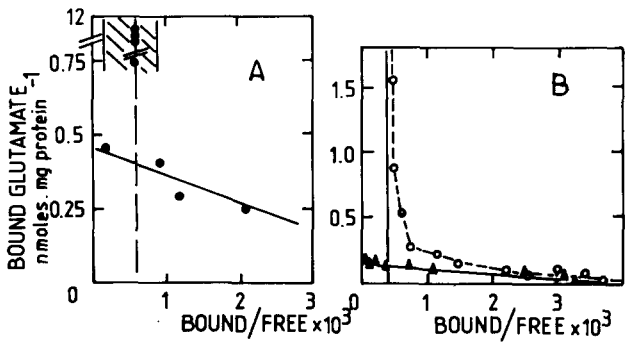

Fig. 2. Comparative uptake of glutamate by pig heart mitochondria (A) and glutamate binding by HGAP(B). A. Mitochondria (0.9 mg protein) were incubated for 10 min at 30°C in 1 ml of 0.25 M sucrose, 10 mM Tris/acetic acid, pH 7.8, with ^{14}C-labelled glutamate isotopically diluted with unlabelled glutamate. Mitochondria were recovered by centrifugation, washed , dissolved in 0.2 ml of formic acid and counted for radioactivity. Glutamate concentration range: $1.9—6600 \cdot 10^{-6}$ M. The hatched part represents 12 points. B. HGAP (0.6 mg protein) was incubated 10 min at 30°C with [^{14}C]glutamate with unlabelled glutamate. Other conditions of binding measurements as described in Table I. Computation on each experimental point (○) allowed determination of the straight line parallel to the ordinate (low affinity sites) calculated in order to obtain best straight line with computed points (△) [18]. Eight different preparations were used. B represents one experiment.

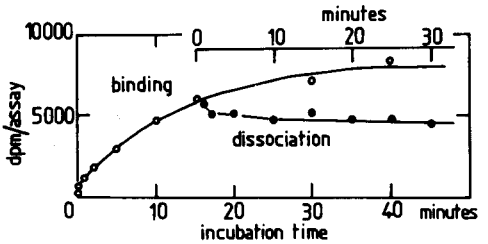

Fig. 3. HGAP-glutamate interaction: binding and dissociation kinetics. HGAP (0.6 mg protein) was pre-incubated 2 min in 1 ml sucrose/Tris buffer, pH 7.8, 30°C to adjust temperature. At "zero" time, 1.9 nmol of [^{14}C]glutamate were added and after increasing incubation times, assays were spun down and washed and pellets counted as in Fig. 1. Dissociation was followed by addition of 1 ml unlabelled glutamate after 15 min incubation. All other operations were carried out as for association kinetics. Results are expressed as bound dpm as a function of time. Dissociation time scale is represented in the upper part of the figure. Backgrounds obtained with 24 mM glutamate are always subtracted.

glutamate is very slowly and partly chased. The binding process is 700 times more rapid than that of the dissociation.

The aboslute K_d calculated from these isotopic chase experiments is 86 μM. The curves allowed the determination of half-times of association and dissociation of glutamate and the corresponding kinetic constants, respectively $k_{+1} = 0.154\ s^{-1}$, $k_{-1} = 0.01326\ \mu mol \cdot ml^{-1} \cdot s^{-1}$; K_d is given by k_{-1}/k_{+1}.

Influence of analogues on glutamate binding. The influence of a series of substrates or analogues on the binding of glutamate on HGAP was studied (Table II); [^{14}C]glutamate was added at a low concentration, 1.9 μM to minimize the interference with low affinity sites. Analogues were added at the con-

TABLE II

EFFECT OF DICARBOXYLATES AND STRUCTURAL ANALOGUES ON GLUTAMATE BINDING BY HGAP

Results are expressed as percentages of 100% control (1.9 μM [^{14}C]glutamate alone). Incubations and binding estimations as in Table I.

Addition	Residual glutamate binding (%)
None	100
24 mM glutamate (control)	0
0.133 mM:	
L-Glutamate	3.5
L-Aspartate	7.4
L-Asparagine	23.7
L-*allo*-OH-Glutamate	27.4
Fumarate	29.7
D-Aspartate	32.4
L-Glutamine	45
L-Glutamic acid-γ-Methyl ester	46.5
α-Keto glutarate	56
D-Glutamate	57
Glutarate	57.5
Oxaloacetate	59
Pyruvate	61.5
Aminooxyacetate	63
Malate	64.8

centration 0.133 mM, i.e., 70 times more concentrated than [^{14}C]glutamate. At this concentration, cold L-glutamate prevented the binding of labeled glutamate by 96%; only L-aspartate appeared a strong competitor. In the same conditions L-asparagine, L-*allo*-OH-glutamate, fumarate, D-aspartate, L-glutamine and the γ-methyl ester of L-glutamate inhibited by more than 50%, while the following molecules inhibited the binding of L-glutamate by less than 50%: α-ketoglutarate > D-glutamate > glutarate > oxaloacetate > pyruvate > amino oxyacetate > malate.

Since L-aspartate appeared to be a strong inhibitor, its binding was studied in the concentration range 1.9—240 μM. Only high affinity sites could be detected with the Scatchard plot, with K_d = 30 μM. Glutamate could prevent the binding of aspartate or could displace bound aspartate.

Comparative binding properties of HGAP for glutamate and aspartate. Whatever the technique used to estimate the binding of glutamate to HGAP, the obtained K_d for high affinity sites was very similar, 62 μM by equilibrium dialysis [1], 86 μM by the association-dissociation procedure, 55 μM by rapid centrifugation; the latter technique also revealed low affinity sites for glutamate (K_d = 60 mM) and only high affinity sites for aspartate (K_d = 30 μM). When aspartate inhibited glutamate binding, two apparent K_i values could be calculated: 0.37 mM (at high concentration of glutamate) and 3.8 μM (at low concentration of glutamate) which were different from the K_d of aspartate; inversely glutamate inhibited aspartate binding. HGAP can bind 0.4 nmol glutamate/mg protein (high affinity) but, curiously, only 0.1 nmol aspartate.

4. New reconstituted system of glutamate transport

In the previous reconstitued systems [1], including HGAP, the influx of glutamate was studied either in *N*-ethylmaleimide inhibited mitochondria or in liposomes entrapping glutamate dehydrogenase + NAD^+, using the method of Robinson and Chappell [29]. In the present study liposomes entrapping aspartate were made. Two different methods were used to measure aspartate efflux (Table III). It can be seen that the presence of HGAP catalyzed some aspartate efflux from liposomes and that the addition of glutamate to the incubation medium of liposomes, slightly stimulated this efflux, the stimulation being proportional to glutamate concentration.

5. Structural properties of HGAP

Lipid composition. In Table IV the lipid composition of HGAP is compared to that of pig heart mitochondria and inner membrane; HGAP contains cardiolipin, the specific marker of the inner membrane, and the two other major phospholipids, phosphatidyl choline and phosphatidyl ethanolamine, the distribution of which is different from that of inner membrane.

Effects of lytic enzymes on glutamate binding. The treatment of HGAP by various phospholipases (A, *C. adamenteus* venom, C from *C. welchii* and D from cabbage) or by trypsin, resulted in a loss of glutamate binding capacity of HGAP, the residual binding being respectively 38%, 29%, 38% and 17% of controls. The digestion products of phospholipases were controled by thin-layer chromatography; phosphatidylcholine was almost completely hydrolzyed by phospholipase C, while phospholipase D transformed cardiolipin to phos-

TABLE III

EFFECT OF GLUTAMATE ON THE ASPARTATE COMPARTMENTATION WITH LIPOSOMES ENTRAPPING [^{3}H]ASPARTATE AND HGAP

Averages of three different experiments with the same starting preparation of liposomes, ±S.E. Other preparations of liposomes gave similar results. Results were corrected for controls with liposomes alone in the absence of HGAP. No effect of glutamate was measured in this latter case. Liposomes were prepared as follows: 200 mg of pig heart mitochondrial phospholipids (Folch's extract [28]) were suspended in 10 ml of 0.25 M sucrose, 10 mM Tris/acetic acid, pH 7.8 containing $220 \cdot 10^6$ dpm of [^{3}H]aspartate and sonicated 6 times 30 s, 0°C. The temperature of the mixture is monitored and maintained below 10°C. The resulting opalescent suspension is immediately passed through a 1 × 25 cm Sephadex G-50 column equilibrated with sucrose/Tris buffer to eliminate any external aspartate; the liposomes are excluded from the gel; then HGAP (3 mg protein) is added to 0.6 ml liposomes (about 0.72 mg lipids) in 0.25 M sucrose, 10 mM Tris/acetic acid pH 7.8 (final volume 0.9 ml). The mixture is incubated for 10 min at room temperature with or without glutamate as indicated. HGAP · liposomes complex is separated and washed from the medium either on Millipore filter (HAWP 0.25) or by rapid microcentrifugation. Radioactivity is estimated on the filters, filtrates, supernatant fluids and pellets (after formic acid digestion) with an Intertechnique SL 40-4K scintillation spectrometer. Filtrate and supernatant fluid correspond to labeled aspartate released in the medium while filter and pellet the aspartate which remained associated with liposomes.

Technique:	Filtration *		Centrifugation **	
Additions to incubation medium	Filtrate	Filter	Supernatant	Pellet
None	2300 ± 540	8650 ± 230	11 900 ± 480	21 000 ± 240
Glutamate 0.9 mM	4460 ± 280	6500 ± 175	13 200 ± 540	20 000 ± 230
Glutamate 4.5 mM	—	—	18 200 ± 210	16 300 ± 580

* Technique described above; cpm.

** Rapid centrifugation technique; dpm.

phatidic acids. The effects of trypsin were only partially prevented in the presence of soybean trypsin inhibitor.

In all these experiments, glutamate binding was measured by Sephadex G-50 gel filtration. HGAP (3.5 mg protein) was incubated in 0.4 ml of 10 mM Tris · HCl, 1 mM $CaCl_2$, pH 7.0, with or without 1 mg of any lytic enzyme, 30 min at 35°C. Then 0.7 ml of 20 mM Tris · HCl, 120 mM KCl, pH 7.0, and 1.9 μM [^{14}C]glutamate were added. After 10 min further incubation at 28°C, 0.56 ml of the mixture were passed through a 1 × 25 cm column of Sephadex G-50.

TABLE IV

LIPID COMPOSITION OF PIG HEART MITOCHONDRIA, INNER MEMBRANE AND HGAP

Phospholipid contents are expressed as percentages of total lipid-phosphorus and were determined as previously described [26].

	Mitochondria [26]	Inner membrane [26]	HGAP
mg lipid/mg protein	0.30	0.48	0.43
Cardiolipin	18	25.4	18.9
Phosphatidylcholine	42.4	26.5	41.7
Phosphatidylethanolamine	30.5	37.9	23
Minor	8	10	16

TABLE V

EFFECTS OF VARIOUS THIOL REAGENTS ON HGAP GLUTAMATE BINDING CAPACITY

After treatment with *N*-ethylmaleimide or Avenaciolide, HGAP was diluted in sucrose/Tris buffer and incubated 10 min, 28°C, with [^{14}C]glutamate (1.9 μM) prior to Sephadex G-50 gel filtration of one-third of the mixture. Bound radioactivity was excluded from the gel with protein and counted. After treatment by potassium tetrathionate or dimethylmaleic acid anhydride, HGAP was recovered after incubation by centrifugation and binding estimated as described in the legend of Fig. 2. Results are expressed as percentages of the controls treated in the same way without thiol reagents. Incubations: *N*-Ethylmaleimide or Avenaciolide (5 min, 28°C), tetrathionate (10 min, 0°C), dimethylmaleic anhydride (2.5 h, 0°C), in the presence respectively, per mg protein, of 0.4 μmol, 94 nmol, 40 μg, 4 mg of the reagent.

Reagent	*N*-Ethylmaleimide	Avenaciolide	Potassium tetrathionate	Dimethylmaleic acid anhydride
Buffer	Sucrose/Tris + 1 mM EDTA, pH 7.8		0.1 M sodium borate, pH 8.5	
Residual glutamate binding (%)	20	67	57	18

Effects of thiol reagents on HGAP. Table V summarizes the action of various agents on glutamate binding on HGAP. *N*-Ethylmaleimide, at the concentration which inhibits glutamate entry into mitochondria [2], and dimethylmaleic anhydride at a concentration which dissociates oligomeric proteins, severely inhibited glutamate binding. In contrast, Avenaciolide at the concentration which inhibits glutamate entry into mitochondria [3], and potassium tetrathionate at a concentration used to protect thiols in enzymes [30], were rather poor inhibitors.

When HGAP (3.5 mg protein/0.5 ml) was incubated with *N*-ethyl[^{14}C]-maleimide (0.4 μmol/mg protein), 9 nmol *N*-ethylmaleimide were bound per mg protein HGAP; the addition of 9.9 mM glutamate to the incubation medium lowered this binding to 6.1 nmol *N*-ethylmaleimide per mg protein HGAP.

Dissociation of HGAP by mild chemical treatment. Previously it has been shown that HGAP contains several different protein subunits [4]. Attempts were made to dissociate HGAP into the glutamate binding component(s). Table VI gives the procedure and results. In a first step, potassium tetrathionate was used to protect -SH groups [30] against dimethylmaleimide anhydride which reacts irreversibly with thiol groups. In the second step, dimethylmaleic anhydride was added to dissociate the proteolipidic HGAP. Table VI shows that tetrathionate alone hardly affected glutamate binding, but that the further treatment by dimethylmaleic anhydride strongly decreased the binding measured after reversal of tetrathionate action by addition of diluted sulphuric acid. However a final soluble protein fraction could be recovered by ammonium sulphate precipitation at pH 6.5 of the dimethylmaleic anhydride/HGAP mixture after elimination of the undissociated material either by high speed centrifugation (60 000 rev./min for 10 min, Beckman rotor type 65) or by gel filtration on acrylamide-Agarose ACA 54 (LKB). The decrease in pH during ammonium sulfate precipitation reversed both the action of tetrathionate and of dimethylmaleic anhydride. The final water soluble fraction thus obtained exhibited a higher binding affinity for glutamate than the starting material HGAP, although the yield was poor.

TABLE VI

DISSOCIATION AND SOLUBILISATION OF HGAP BY CHEMICAL MODIFICATION

HGAP suspensions in sucrose/Tris buffer were recovered by centrifugation (6000 × *g*, for 15 min) and resuspended in 0.1 M sodium borate, pH 8.5, and incubated with potassium tetrathionate (50 μg/mg of HGAP protein), 30 min, 0°C. Excess potassium tetrathionate was washed out after two centrifugations (6000 × *g* for 15 min). The tetrathionate-treated pellet was incubated 2.5 h at 0°C with an excess of dimethyl maleic acid anhydride (4 mg/mg of HGAP protein). The solubilized proteins were recovered alternatively by molecular sieving on acrylamide-Agarose ACA 54 (LKB) where the excluded protein material was considered as the undissociated form, or precipitation with 1.84 M ammonium sulfate after high speed centrifugation. Glutamate binding was tested on the different fractions as described in Table I after reversal of tetrathionate or , after dimethylmaleic acid anhydride action at pH 6.5 for 2.5 h, 0°C. The binding activity of the soluble fraction was measured according to Zaskin et al. [27] modified as follows: 0.325 mg protein are incubated for 10 min, 30°C, in 0.5 ml of sucrose/Tris buffer, pH 7.2, with 1.9 μM [^{14}C]glutamate (340 000 dpm/nmol). A control under the same conditions is obtained by addition of 24 mM unlabelled glutamate. Proteins are then precipitated by addition of an equal volume of 4.1 M ammonium sulfate. The 10 000 × *g*, 2.5 min pellet is carefully separated from the corresponding supernatant and washed with 1 ml of 2.05 M ammonium sulfate in sucrose/Tris buffer. The final pellet is recovered and washed within 5 min and solubilized in 0.2 ml formic acid and transfered in a scintillation vial for estimation of radioactivity.

Fraction	Protein (mg)	Volume (ml)	Glutamate binding (dpm/mg protein)	Recovery (%)		Purification factor
				Protein	Binding	
HGAP	52.2	30	176 000	100	100	1
Tetrathionate-treated HGAP	50	30	154 400	95.8	83	0.87
Tetrathionate + dimethylmaleic anhydride-treated HGAP	50	30	30 480	95.8	16	0.17
Soluble fraction (after centrifugation or gel filtration)	9.75	1.5	345 600	18.6	36.6	2

Polypeptide analysis of the soluble fraction of HGAP. Fig. 4 shows the electrophoretic pattern obtained with the soluble fraction. The relative migration of subunits from the main enzymes oxidizing glutamate, glutamate-

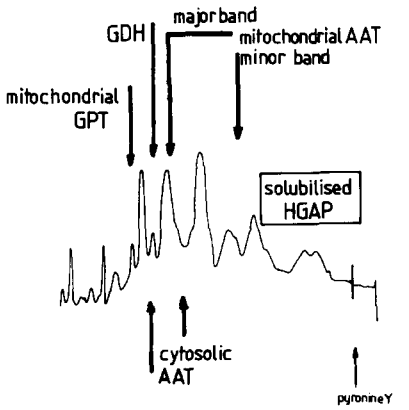

Fig. 4. Polyacrylamide gel electrophoresis of the solubilized fraction from HGAP. Conditions as in Materials and Methods. Relative mobilities have been determined with Pyronine Y as tracking dye. Microdensitometric traces have been obtained with an ISCO gel scanner after Coomassie Brillant Blue staining of proteins. Solubilized fraction as described in the legend of Table VI.

TABLE VII

QUANTITATIVE IMMUNOLOGICAL ANALYSIS OF HGAP SOLUBLE FRACTION

The method used as described by Herzog et al. [25]. Taking, for pig mitochondrial aspartate aminotransferase and glutamate dehydrogenase subunits, molecular weights 50 000 and 55 000, respectively, and assuming that aspartate aminotransferase is a dimer and glutamate dehydrogenase an hexamer.

	μg/mg protein	moles of enzyme/mg protein	Subunit ratio
Aspartate aminotransferase	337	$3.3 \cdot 10^{-9}$	26
Glutamate dehydrogenase	14	$0.042 \cdot 10^{-9}$	

pyruvate transaminase, mitochondrial and cytosolic glutamate-aspartate transaminase and glutamate dehydrogenase are indicated. The comparison of the various bands does not allow a final conclusion about the homology between enzyme subunits and HGAP components. Therefore an immunological analysis was undertaken.

Double immunodiffusion was performed using the HGAP solubilized fraction, anti-glutamate dehydrogenase and antimitochondrial aspartate aminotransferase immunesera (see Materials and Methods). The experiments were not sensitive enough to detect glutamate dehydrogenase subunits in HGAP but allowed us to detect the presence of mitochondrial aspartate aminotransferase subunits as shown by the presence of a light precipitation band between HGAP and mitochondrial aspartate aminotransferase antiserum.

The study was completed by titration of both glutamate dehydrogenase and mitochondrial aspartate aminotransferase using microagglutination of activated latex (see Materials and Methods) [25]. Table VII shows that HGAP contains 26 times less glutamate dehydrogenase subunits than aspartate aminotransferase, the level of which is approx. one-third of total protein in HGAP.

Discussion and Conclusion

The use of a rapid centrifugation technique made possible the detection of low affinity sites for glutamate besides high affinity sites and made faster and easier the estimation of glutamate binding either on purified HGAP or mitochondrial fractions, thus allowing great improvements of the isolation process of HGAP. The new perfected preparation avoids any long dialysis steps, which resulted in a shortening of the procedure and protected HGAP against uncontroled denaturation. Although the affinity-batch step used in our previous procedure [1] was efficient and also could be successfully applied by Michaelis [31] to the partial purification of a glutamate-binding synaptic membrane glycoprotein, however it did not allow to prepare sufficient amounts of HGAP for further studies; the ammonium sulphate fractionation described in Table I resulted in increased amounts of a glutamate-binding fraction which could no longer be purified by affinity chromatography. Moreover the dissociation of the latter fraction by mild chemical treatment led to a soluble fraction with a specific glutamate-binding activity two-fold higher.

As could be expected, the saturation curves for glutamate entry into mitochondria and glutamate binding on HGAP were very similar. Whatever the

technique used for glutamate binding on HGAP, the high affinity K_d values were identical and very similar to the apparent K_m value for glutamate entry into mitochondria. Among various analogues of glutamate, aspartate and asparagine appeared the best competitors of glutamate binding but, unexpectedly, the high affinity binding capacity of HGAP for aspartate was one-quarter of that of glutamate (0.1 nmol instead of 0.4 nmol glutamate per mg protein). This result seems to indicate that aspartate and glutamate binding sites are not identical; however all the results are in favour of an interdependence of both types of site. If the binding of aspartate was measured in the absence of glutamate, a K_d of 30 μM was obtained; but when the effects of aspartate were studied on glutamate binding, the inhibition appeared somewhat competitive and two apparent K_i values could be calculated (3.8 μM and 0.37 mM) when glutamate concentration was low or high, respectively.

These latter effects of aspartate, the fact that additions of external glutamate stimulated the efflux of aspartate from aspartate-loaded liposomes in the presence of HGAP and the fact that aspartate could increase the isotopic chase of labeled glutamate from HGAP [11], suggest a comparison between HGAP and the glutamate/aspartate antiporter postulated by Azzi et al. [10]. However, in our previous reconstituted system [1] with mitochondria or liposomes, HGAP seemed to catalyze a glutamate/OH^- exchange, since no other anion added. Both our types of reconstituted transport seemed not to require energy and thus were estimated in conditions similar to those of whole mitochondria [10]; it is therefore difficult to compare them to the electrogenic transport described by Lanoue et al. [32].

HGAP appeared to contain the three major phospholipids or pig heart mitochondria with a distribution somewhat different from that of whole mitochodria or inner membrane [26]. Additionally, several polypeptides were detected. Treatments by lytic enzymes (various phospholipases or trypsin) strongly decreased glutamate binding on HGAP, indicating the importance of the whole architecture of the complex.

Nine thiol groups/mg protein of HGAP were accessible to *N*-ethyl[^{14}C]-maleimide titration; in the presence of glutamate three among the nine -SH groups were no longer titrated by *N*-ethylmaleimide. These results are in favour of the role of thiol groups in the activity of HGAP and are in agreement with the observed inhibitions of glutamate transport to mitochondria and glutamate binding to HGAP (see also Table V). Isotopic chase experiments suggest that glutamate either may be very tightly bound to the polypeptide part of HGAP of may undergo some transient chemical modification in the polypeptide site. Although it was proved that HGAP exhibited neither glutamate dehydrogenase nor aspartate aminotransferase activity, immunological studies clearly demonstrated that HGAP contained significant amounts of subunits of both enzymes. Aspartate aminotransferase subunits appeared to be 26-fold more abundant than those of glutamate-dehydrogenase and constituted approx. one-third of HGAP polypeptides. These results do not exclude a possible role of these two enzymes acting in inhibited form in the transport of glutamate, as suggested earlier [1]. If this is the case, it would mean that the affinity of these enzymes for glutamate would be strongly increased in HGAP, since their K_m values are 1—3 orders of magnitude higher than the

glutamate K_d of HGAP or apparent K_m value of glutamate entry into mitochondria. To our knowledge, the direct binding of radioactive glutamate on aspartate aminotransferase has not been reported so far. In another work it has been shown by Thévenot et al. [33] that in the presence of an analogue of NAD, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide (H_4-NAD) the affinity of glutamate dehydrogenase for glutamate could be strongly increased and one high affinity site per hexamer was revealed with a K_d of 90 μM, which is of the same order of magnitude as that of HGAP. All other indirect methods gave K_d values in the range 1—100 mM. Cardiolipin, the most specific phospholipid of inner mitochondrial membrane, and a constituent of HGAP, strongly inhibits glutamate dehydrogenase activity [5] (i_{50} = 24 nM) and aggregates with it [6]; however, glutamate binding experiments with aggregates of cardiolipin + glutamate dehydrogenase were difficult to interpret. All our results suggest the importance of the lipids as well as of the polypeptide components in glutamate binding and transport by HGAP but the mechanism of action remains to be proved.

References

1 Julliard, J.H. and Gautheron, D.C. (1973) FEBS Lett. 37, 10—16
2 Meijer, A.J., Brouwer, A., Reijngoud, D.J., Hoek, J.B. and Tager, J.M. (1972) Biochim. Biophys. Acta 283, 421—429
3 Mc Givan, J.D. and Chappell, J.B. (1970) Biochem. J. 116, 37P—38P
4 Gautheron, D.C., Julliard, J.H. and Godinot, C. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G.F., Klingenberg, M. and Siliprandi, N.) pp. 91—96, North-Holland, Amsterdam
5 Julliard, J.H. and Gautheron, D.C. (1972) FEBS Lett. 26, 343—345
6 Godinot, C. (1973) Biochemistry 12, 4029—4034
7 Gautheron, D.C., Durand, R., Pialoux, N. and Gaudener, Y. (1964) Bull. Soc. Chim. Biol. 56, 645—660
8 Hirschberg, E., Snider, D. and Osnos, M. (1964) in Advances in Enzyme Regulation (Weber, G., ed.), Vol. 2, 301—310, Pergamon Press, Oxford
9 Waksman, A., Rendon, A., Crémel, G., Pellicone, C. and Goubault de Brugière, J.F. (1977) Biochemistry 21, 4703—4707
10 Azzi, A., Chappell, J.B. and Robinson, B.H. (1967) Biochem. Biophys. Res. Commun. 29, 148—152
11 Julliard, J.H. and Gautheron, D.C. (1976) in Use of Isolated liver cells and Kidney Tubules in Metabolic Studies. Proc. FEBS Adv. Course No. 38, Luzarches, France, July 27—28, 1975 and Sym. Mitochondrial-Cytosolic Interrelat. Cell Metabol., Paris, France, July 26, 1975, (Tager, J.M., Söling, H.D. and Williamson, J.R., eds.), pp. 98—101, North-Holland, Amsterdam
12 Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
14 Pfleiderer, G. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 381—383, Academic Press, New York
15 Arnold, H. and Maier, K.D. (1971) Biochim. Biophys. Acta 251, 133—138
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Catterall, W.A. and Pedersen, P.L. (1971) J. Biol. Chem. 246, 4987—4994
18 Rosenthal, H.E. (1967) Anal. Biochem. 20, 525—532
19 Godinot, C., Julliard, J.H. and Gautheron, D.C. (1974) Anal. Biochem. 61, 264—270
20 Ryan, E. and Fottrell, P.F. (1972) FEBS Lett. 23, 73—76
21 Furbisch, F.S., Fonda, M.L. and Metzler, D.E. (1969) Biochemistry 8, 5169—5180
22 Balinsky, J.B., Shambaugh III, G.E. and Cohen, P.P. (1970) J. Biol. Chem. 245, 128—137
23 Vaitukaitis, J., Robbins, J.B., Nieschlag, F. and Ross, G.T. (1971) J. Clin, Endocrinol. 33, 988—991
24 Ouchterlony, O. (1949) Acta Pathol. Microbiol. Scand. 26, 507—515
25 Herzog, F., Gaiffe, M., Turpin, A. and Bizzini, B. (1972) Nouv. Presse Med. 1, 935—938
26 Comte, J., Maïsterrena, B. and Gautheron, D.C. (1976) Biochim. Biophys. Acta 419, 271—284
27 Zalkin, H., Pullman, M.E. and Racker, E. (1965) J. Biol. Chem. 240, 4011—4016
28 Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
29 Robinson, B.H. and Chappell, J.B. (1967) Biochem. Biophys. Res. Commun. 28, 249--255

30 Kassab, R., Roustan, C. and Pradel, L.A. (1968) Biochim. Biophys. Acta 167, 308—316
31 Michaelis, E.K. (1975) Biochem. Biophys. Res. Commun. 65, 1004—1012
32 Lanoue, K.F., Bryla, J. and Basser, D.J.P. (1974) J. Biol. Chem. 249, 7514—7521
33 Thévenot, D.R., Godinot, C., Gautheron, D.C., Branlant, G. and Biellman, J.F. (1975) FEBS Lett. 54, 206—211