HIGH GLUTAMATE AFFINITY PROTEOLIPID FROM PIG-HEART MITOCHONDRIA.

Is it a component of a glutamate translocator?†

Jacques H. JULLIARD and Danièle C. GAUTHERON

Laboratoire de Biochimie Dynamique, E.R.A. no. 266 du C.N.R.S., Université Claude Bernard de LYON, 43 Bd du 11 Novembre 1918, 69621 Villeurbanne, France

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1. Introduction

In 1965 Chappell and Crofts [1] concluded that there exist anion translocators in inner mitochondrial membranes, glutamate having a specific one. To our knowledge no such anion translocator has been isolated up to now and their true nature and mechanism of action remain unknown. Nobody knows as yet if the main glutamate oxidizing enzymes, GDH* and AAT, play a role in its translocation through the inner membrane. Both enzymes are located in the inner membrane-matrix compartment and it has been shown that GDH exists in two different forms [2] one of which is tightly bound to the inner membrane. The role of this membrane-bound GDH is not yet understood but the great affinity of GDH for membrane cardiolipin can account for its tight binding [3–5].

Besides we have shown [6] that AAT coupled on both sides of an artificial membrane of collagen [7] stimulates glutamate diffusion across the membrane.

In the present paper we report the isolation procedure for HGAP, a very insoluble proteolipid extracted from pig-heart mitochondria which exhibits all the properties expected from a glutamate translocator while it presents neither GDH nor AAT activities.

2. Materials and methods

Pig-heart mitochondria were prepared in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4, according to the method of Crane et al. [8] modified as previously reported [9].

Fig. 1 describes the purification scheme of HGAP starting from frozen mitochondria; the first steps (part 1) lead to the P_A pellet which exhibits neither GDH nor AAT activity. P_A is dissolved in 2% cholate and treated by affinity-batch separation (part 2) on human serum albumin reticulated on glass fibre and loaded with glutamate or with the γ-methylester of glutamate to separate proteins exhibiting glutamate affinity. This system was prepared as follows: 84 ml of 10 mM phosphate (K^+), pH 7.0, 34 g finely divided Pyrex glass fibre, 25 ml human serum albumin (22%), 3.2 ml of 25% glutaraldehyde, are well homogenized at −30°C; the frozen cake is kept overnight at −30°C, then thawed, manually divided and washed at room temperature with 10 mM phosphate (K^+), pH 7.0. The residue is incubated 24 hr in the same phosphate buffer with 50 mM glutamate or the γ-methylester of glutamate and 3% glutaraldehyde, then extensively washed successively in phosphate buffer, phosphate buffer + 2% cholate, phosphate buffer + 2% cholate + M KCl. The final residue, after equilibration with

* *Abbreviations* used: HGAP, high glutamate affinity proteolipid; GDH, glutamate dehydrogenase (EC 1.4.1.3); AAT, aspartate aminotransferase (EC 2.6.1.1); NEM, *n*-ethyl maleimide; CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone.

† Preliminary results were the subject of a communication at the 8th FEBS Meeting, 1972 [6] and at the XI Jornadas Bioquimicas Latinas, 1973, Salamanca.

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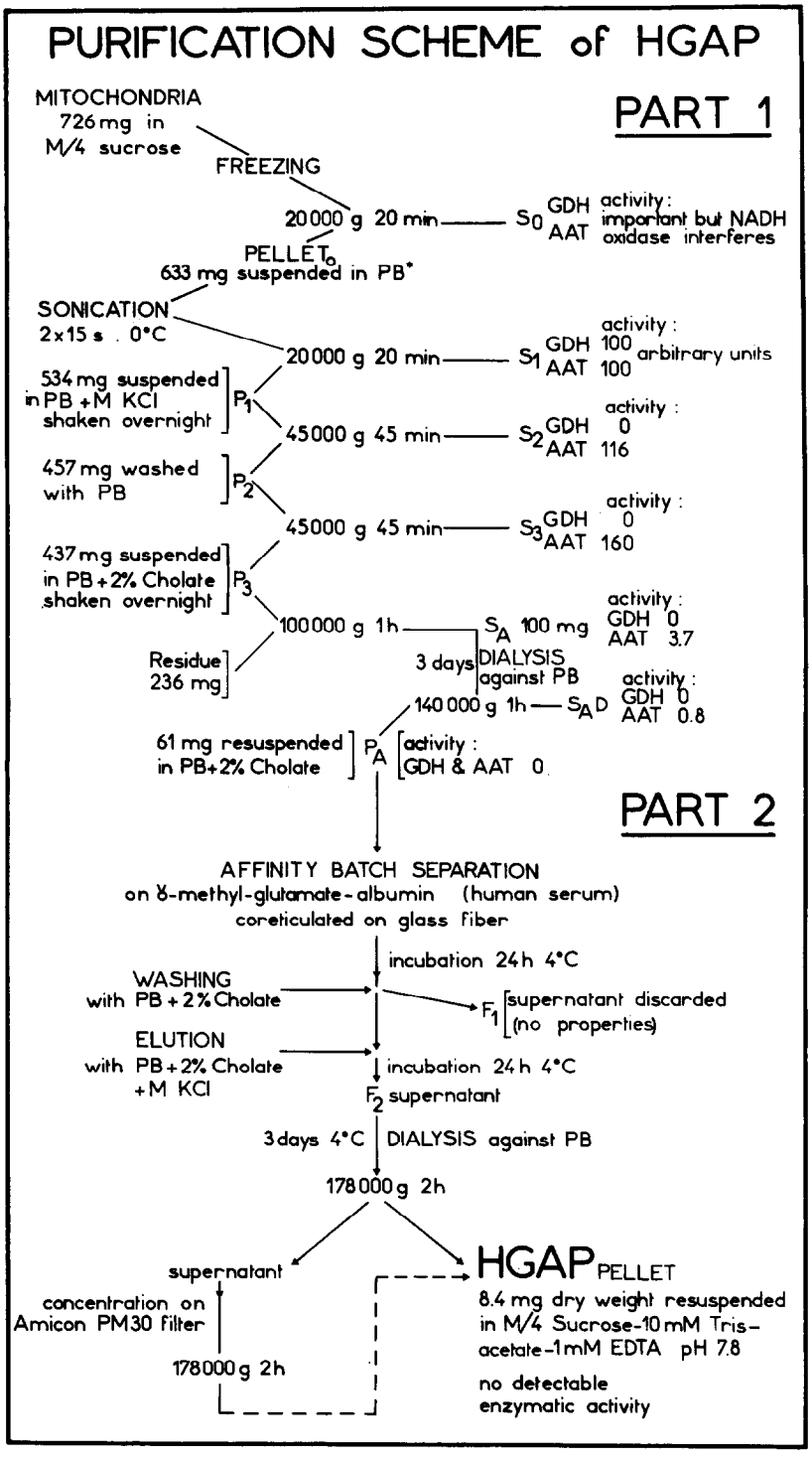

Fig. 1. PB*: 10 mM phosphate (K^+), 1 mM 2-mercaptoethanol, pH 7.2. For procedure see Materials and methods.

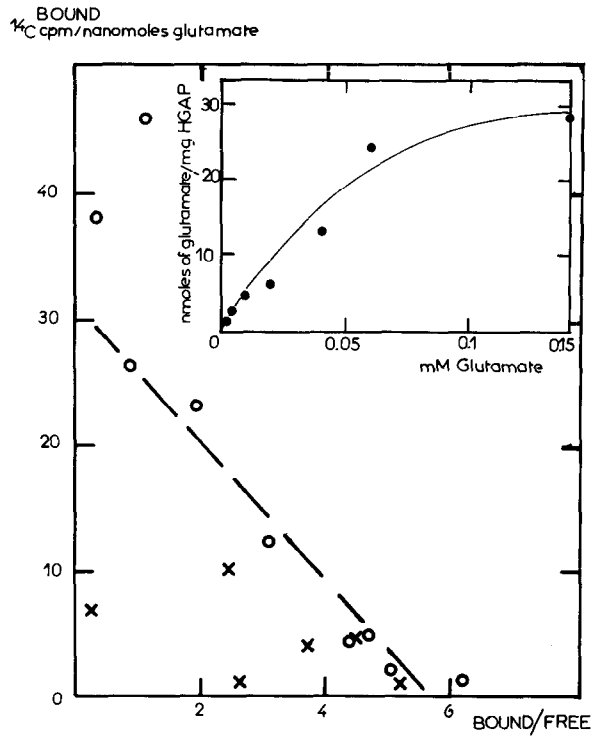

Fig. 2. High affinity binding of glutamate by HGAP. Glutamate concentration range 2–500 μM. Equilibrium dialysis: 18 hr, 4°C, in 10 mM phosphate (K^+) (Union Carbide Visking tubes, 24 Å pores, 50 μ thick). 1 mM 2-mercaptoethanol, pH = 7.2. (X) Control: material recovered on albumin alone; (O) HGAP. Insert – Saturation curve of HGAP by glutamate.

Table 1
Effects of NEM and Avenaciolide on glutamate binding by HGAP.

	Control	+ Avenaciolide	+ NEM
Total cpm in excluded volume	600	400	125
Percent residual binding		64	18
Percent inhibition		36	82

5 min incubation of 0.378 mg HGAP in 0.2 ml 0.25 M sucrose, 10 mM Tris–acetate, 1 mM EDTA, pH 7.8, 28°C; when present: 0.4 μmole NEM or 94 nmoles Avenaciolide per mg protein. Then 0.3 μCi [2, 3-^{3}H]glutamate was added (final concn. 70 mM). After 10 more minutes incubation, the mixture was passed through a 1× 25 cm Sephadex G-50 (coarse) column. Counts were made on equal fractions of the excluded volume. Averages of two experiments.

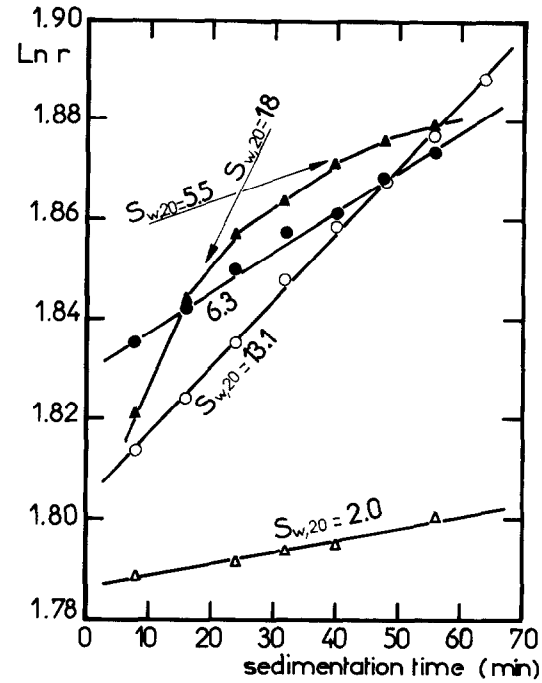

Fig. 3. Sedimentation velocities of HGAP supernatant: (▲—▲—▲) heavy peak; (△—△—△) light peak; (○—○—○) beef liver GDH (Boehringer, Mannheim); (●—●—●) HGAP + GDH. Centrifugation run in 10 mM (K^+) phosphate buffer, 1 mM 2-mercaptoethanol, pH = 7.2, 20°C. Rotor AND, Beckman analytical centrifuge model E.

10 mM phosphate (K^+), 1 mM 2-mercaptoethanol, 2% cholate, pH 7.2, is ready for affinity-batch separation. P_A separates into two fractions absorbing at 280 nm: F_1 is not retained by the batch; the M KCl required to elute F_2 indicates that F_2 is tightly retained by the glutamate or by the γ-methylester of glutamate which strongly inhibits glutamate entry into brain tissue [10] and into pig-heart mitochondria. If the separation is conducted on a column, F_1 gives a sharp peak which includes 86% of P_A. Dialysis and centrifugation of F_2 yield HGAP (pellet or soluble form).

3. Results and discussion

3.1. *Physicochemical properties of HGAP*

3.1.1. Glutamate binding

HGAP exhibits high affinity for glutamate which permits its purification. Fig. 2 shows that [^{14}C]gluta-

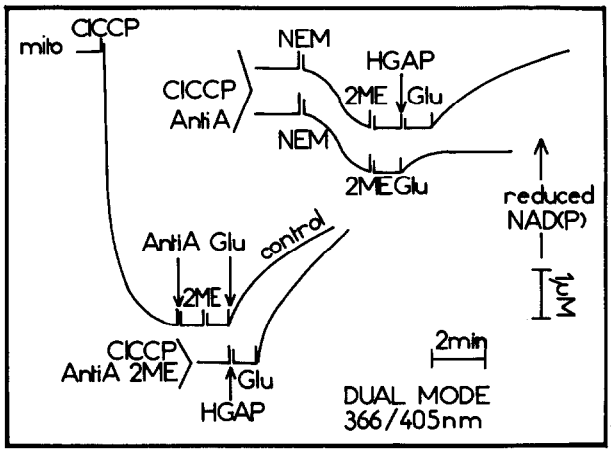

Fig. 4. Mitochondrial oxidation of glutamate and effect of HGAP. 1.15 mg/ml of pig-heart mitochondrial proteins were incubated in: 3 ml of 20 mM Tris–HCl, 125 mM KCl, pH = 7.2, 28°C with CCCP (1.6 μg/mg) until complete oxidation of endogenous NAD(P). Then 0.16 μg/ml antimycin A (anti A) was added to prevent any reoxidation followed by 0.66 mM 2-mercaptoethanol to both control or NEM inhibited assays (0.4 μmoles NEM/mg protein). HGAP: 0.05 mg/ml i.e. 43 μg/mg mitochondrial protein; if HGAP was added before 2-mercaptoethanol to inhibited assays no release of inhibition occurred since HGAP reacts with NEM.

mate binding gives a saturation curve (insert); the Scatchard plot reveals only high affinity sites: K_d = 62 μM.

Avenaciolide [11] and NEM [12, 13], which inhibit glutamate entry in mitochondria or in other tissues [10], prevent glutamate binding to HGAP (table 1).

3.1.2. Lipid composition

HGAP is a proteolipid containing about 0.6 μmoles phospholipids/mg protein, i.e. 45% of the dry weight. The lipid moiety is made mainly of phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin with traces of minor components. Lipid analysis was performed as described elsewhere [14].

3.1.3. Molecular properties

The characterization of the protein moiety is not easy owing to the strong tendency of HGAP to give very insoluble aggregates which are difficult to analyse by electrophoresis. Gel electrophoresis of HGAP on 6% polyacrylamide according to Ray and Marinetti [15] gives two bands while a significant proportion of the material is excluded. The soluble form of HGAP (178 000 *g* supernatant, fig. 1) exhibits the same biological properties and gives two distinct peaks on analytical ultracentrifugation (fig. 3), a light

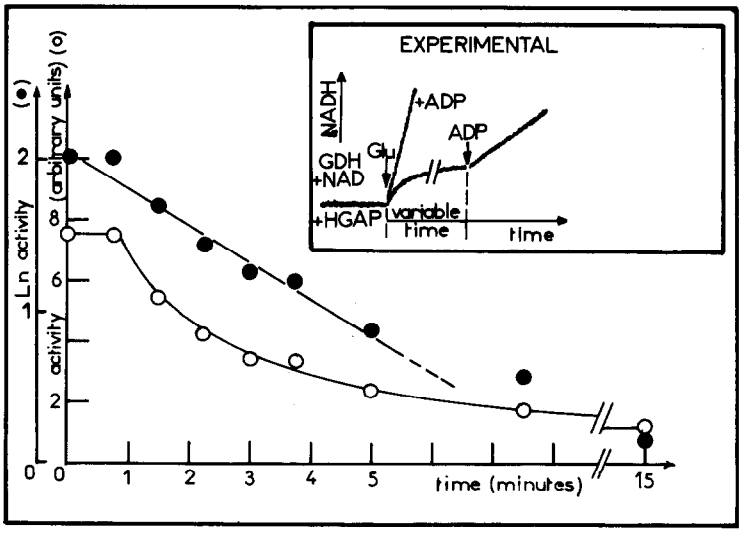

Fig. 5. Time-dependent inhibition of beef liver GDH activity by HGAP. Effect of ADP: 0.3 μg GDH was incubated with 0.16 mM NAD^+ in 3 ml 10 mM phosphate (K^+), 1 mM 2-mercaptoethanol, 0.5 mM EDTA, 28°C, with 3.33 μg HGAP/ml; 1.66 mM glutamate was added at time = 0; 0.033 mM ADP, a positive effector of GDH, was added either together with glutamate (0 time) or after a variable time. GDH activity is measured by the reduction of NAD^+.

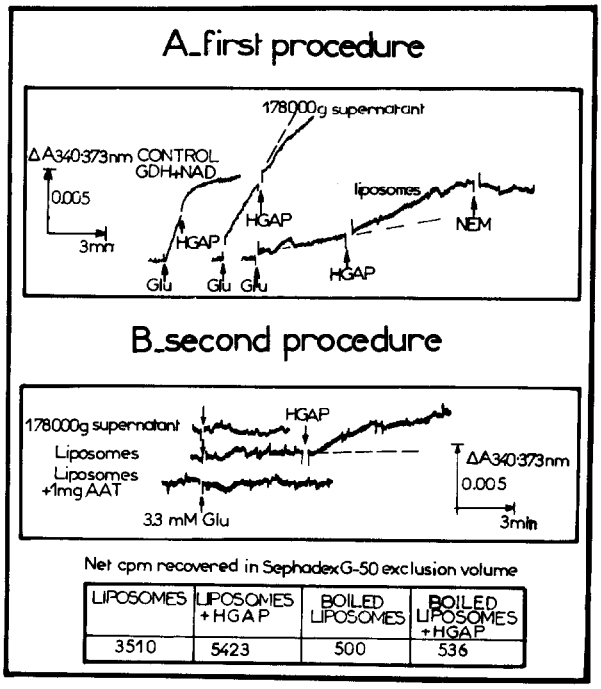

Fig. 6. Effects of HGAP on glutamate entry into liposomes containing GDH: A) first procedure. Liposomes were prepared in 10 ml, 10 mM Tris–acetate, 1 mM EDTA, 0.25 M sucrose, 0.1 M ammonium sulfate, 0.25 M KCl, 1% Lubrol WX, pH 7.8, from 500 mg of pig-heart mitochondrial lipids Folch extract [17]; B) second procedure, identical, except that the lipids were sonicated at 0°C, 5 min at maximal power (MSE disintegrator 60 W). *Then* in both cases 0.1 mg GDH/ml and 15 mM NAD^+ were added. The mixture was dialysed 3 days against 0.25 M sucrose, 10 mM Tris–acetate, 1 mM EDTA, pH 7.8 and finally spun down for 2 hr. Liposomes were the 178 000 *g* pellet. GDH activity was measured by following NAD reduction in the same buffer as for the dialysis: Glutamate, 3.3 mM; NEM, 0.4 μmole/mg protein; Control: GDH, 0.3 μg; NAD^+, 0.16 mM. Labelled glutamate incorporation was conducted using the technique described in the legend to table 1.

($S^0_{20,w}$= 2.0) and a heavy one exhibiting a disaggregation phenomenon as shown by the variation of $S^0_{20,w}$ from 18 at the beginning of the run to 5.5, at the end, as the fraction is diluted; this soluble HGAP, concentrated on an Amicon ultrafilter PM 30, yields insoluble HGAP pellets.

Soluble HGAP and GDH give an homogeneous association with $S^0_{20,w}$= 6.3 while GDH alone shows $S^0_{20,w}$= 13.1.

3.1.4. Other properties

HGAP exhibits great thermal susceptibility and should be frozen quickly in liquid nitrogen to retain its activity when stored at −20°C.

3.2. *Biological properties of HGAP*

3.2.1. Effects of HGAP on whole pig-heart mitochondria

HGAP added to whole mitochondria stimulates glutamate entry (fig. 4) as measured by the endogenous NAD(P) reduction according to Chappell [16] and partly restores glutamate entry in NEM-inhibited mitochondria. Fuscin [17] and avenaciolide [11] produce atypical inhibition of glutamate entry in pig-heart mitochondria but HGAP restores state 3 respiratory stimulation.

3.2.2. Effects of HGAP on GDH

Fig. 5 shows that HGAP strongly inhibits GDH activity while it does not affect malate dehydrogenase or AAT activities; the cardiolipin content of HGAP could account for this strong inhibition but the inhibition pattern is very different to that previously described with pure cardiolipin [3]; here the inhibition increases during the time course of either glutamate oxidation or α-ketoglutarate reduction, suggesting that HGAP acts as an irreversible inhibitor. ADP, a well-known activator of GDH prevents HGAP inhibition and partially releases it.

3.2.3. Effects of HGAP on liposomes

Fig. 6 shows that liposomes made of crude pig-heart mitochondrial lipids (Folch's extract [18]) containing small amounts of GDH and NAD^+, offer a good test for the translocating properties of HGAP. According to Chappell's technique [16] if glutamate enters, intraliposomal NAD^+ will be reduced. When the lipids were not sonicated (fig. 6A) GDH was distributed mainly in the supernatant where the NAD^+ reduction is inhibited by HGAP, as observed in the control (pure GDH + NAD^+). On the contrary the addition of HGAP to liposomes stimulates internal NAD^+ reduction which proves firstly that some GDH is really enclosed in the liposomes, otherwise HGAP would inhibit it and secondly that HGAP catalyses glutamate entry, otherwise, NAD would not be reduced; NEM

inhibits the entry as is observed with whole pig-heart mitochondria. In the second procedure (fig. 6B) where lipids are sonicated, no GDH activity could be detected either in the supernatant or in the pellet in the absence of HGAP; HGAP makes glutamate accessible to GDH inside the liposomes as shown by the NAD^+ reduction, which proves that GDH cannot interact directly with HGAP. A further proof is that HGAP increases [^{3}H]glutamate incorporation into liposomes by 50% indicating some external binding of glutamate; boiled liposomes show a low binding capacity and no HGAP effects.

3.2.4. Apparent K_m for glutamate entry into pig-heart mitochondria and K_d of HGAP

Glutamate entry into pig-heart mitochondria was measured using the method of Chappell [16] by following the reduction of endogenous NAD^+(P) either in the dual wavelengths (340–373 nm) or in the split beam mode (340 nm, two cuvettes) in 16 mM Tris–HCl, 112 mM KCl, 5 mM P_i (K^+), CCCP (90 nmoles/mg protein), Antimycin A (0.3 μg/mg protein), pH 7.4 (3–4.8 mg protein/3 ml). Only mitochondria with respiratory control ratios over 5 give reproducible results. Perfect Michaelis kinetics are obtained versus external glutamate; averages of 8 experiments give: approx. K_m (glutamate entry): 76.6 ± 15.4 μM: this value is very close to the K_d (62 μM) of glutamate binding to HGAP; approx. V_{max} (glutamate entry): 0.29 ± 0.02 nmoles NADH/min/mg protein Standard error of the means calculated according to Wilkinson [19] by a computational procedure.

4. Conclusions

Starting from 726 mg pig-heart mitochondrial protein (quick biuret method) 8.4 mg dry weight HGAP were obtained; because of the number of purification steps and the use of 2% cholate, this amount cannot reflect the true mitochondrial concentration of HGAP.

HGAP exhibits all the properties expected for a glutamate translocator: it is a proteolipid very insoluble in water, this being an intrinsic property of membrane proteins; it contains large amounts of the phospholipids found in mitochondrial inner membranes especially cardiolipin. This agrees with the inner membrane location of anion translocators [1]; it binds glutamate with high affinity with a K_d = 62 μM very close to the K_m = 76.6 μM, for glutamate entry in well-coupled pig-heart mitochondria. According to Oxender [20] this is what should be expected from a translocator; NEM and avenaciolide inhibit labelled glutamate binding to HGAP at concentrations which inhibit glutamate translocation into pig-heart mitochondria; it restores glutamate translocation into mitochondria preinhibited by NEM, avenaciolide or fuscin; it provokes glutamate entry into liposomes made of pig-heart mitochondrial lipids; this HGAP-dependent glutamate entry is inhibited by NEM; it exhibited no enzymic properties towards glutamate (neither GDH, nor AAT activities); one can expect that a translocator does not consume the substrate it carries.

However, the following experimental results may reflect the involvement of an inhibited form of GDH as well as of HGAP in the glutamate translocator: the association of GDH and HGAP (which contains cardiolipin) inhibits GDH and gives a single peak on ultracentrifugation; cardiolipin inhibits GDH with a strong affinity: K_i = 24 nM [3]; GDH aggregates with cardiolipin in specific conditions depending upon cardiolipin concentration, glutamate, coenzyme and nucleotide concentration [21].

As a concluding remark, if HGAP stimulation of glutamate entry into liposomes and pig-heart mitochondria was only a general detergent-like effect, upon HGAP addition, NAD^+ would leak out of both particles; NAD^+ concentration would become lower and no GDH activity could be enhanced. Besides HGAP could inhibit GDH. However this does not exclude the possibility that HGAP provokes a rearrangement of lipid–protein interactions.

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References

[1] Chappell, J.B. and Crofts, A.R. (1965) Biochem. J. 95, 707–716.
[2] Hirschberg, E., Snider, D. and Osnos, M. (1964) in: Avances in Enzyme Regulation (Weber, G., ed.), Vol. 2, pp. 301–310
[3] Julliard, J.H. and Gautheron, D.C. (1972) FEBS Letters 25, 343–345.
[4] Godinot, C. and Lardy, H.A. (1973) Biochemistry 12, 2051–2060.
[5] Dodd, G.H. (1973) European. J. Biochem. 33, 418–438.
[6] Julliard, J.H., Coulet, P. and Gautheron, D.C. 8th FEBS Meet. Abstr., Amsterdam, 1972, no. 168.
[7] Julliard, J.H., Godinot, C. and Gautheron, D.C. (1971) FEBS Letters 14, 185–188.
[8] Crane, F.L., Glenn, J.F. and Green, D.E. (1956) Biochim. Biophys. Acta 22, 475–487.
[9] Godinot, C., Vial, C., Font, B. and Gautheron, D.C. (1969) Eur. J. Biochem. 8, 385–394.
[10] Balar, V.J. and Johnston, G.A.R. (1972) J. Neurochem. 19, 2657–2666.
[11] McGivan, J.D. and Chappell, J.B. (1970) Biochem. J. 116, 37 P.
[12] Briand, Y., Younès, A. and Durand, R., 8th FEBS Meet. Abstr., Amsterdam, 1972, No. 1127.
[13] Meijer, A.J., Brouwer, A., Reijngoud, D.J., Hoek, J.B. and Tager, J.M. (1972) Biochim. Biophys. Acta 283, 421–429.
[14] Comte, J., Gautheron, D., Peypoux, F. and Michel, G. (1971) Lipids 6, 882–888.
[15] Ray, T.K. and Marinetti, G.V. (1971) Biochim. Biophys. Acta 233, 787–791.
[16] Robinson, B.H. and Chappell, J.B. (1967) Biochem. Biophys. Res. Commun. 28, 249–255.
[17] Vignais, P.M., Vignais, P.V., Sato, N. and Wilson, D.F. (1971) in: Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C.S., eds.), pp. 1011–1021, Adriatica Editrice, Bari
[18] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
[19] Wilkinson, G.N. (1961) Biochem. J. 80, 324–332.
[20] Oxender, D.L. (1972) in: Ann. Rev. Biochem. (Snell, E.E., ed.), p. 801, Annual Reviews Inc.
[21] Godinot, C. (1973) Biochemistry, in press.