Biochimica et Biophysica Acta, 429 (1976) 694—704

BBA 67805

MITOCHONDRIAL AND NUCLEAR GLUTAMATE DEHYDROGENASES IN CHINESE HAMSTER OVARY CELLS IN CULTURE

GIANFRANCO DI MATTEO, GUIDO DI PRISCO
and GIOVANNI ROMEO

International Institute of Genetics and Biophysics, CNR, 80125 Naples (Italy)

(Received November 24th, 1975)

Summary

Nuclear glutamate dehydrogenase (EC 1.4.1.3) activity has been demonstrated in Chinese hamster ovary cells. Some characteristics of this enzyme have been examined and compared with those of the mitochondrial glutamate dehydrogenase from the same source. Differences were detected in the extent of the activation by inorganic phosphate, in the pH versus activity curves, in the affinity of the two enzymes for the cofactor NAD^+ and in the electrophoretic mobility. A different rate of decay of the two enzymes has been observed in cells grown in the presence of chloramphenicol. Immunological studies show that, as in ox liver, the nuclear enzyme has specific antigenic determinants besides those in common with mitochondrial glutamate dehydrogenase.

Finally, experiments of thermal inactivation indicate a higher stability of the mitochondrial enzyme.

Introduction

Glutamate dehydrogenase (EC 1.4.1.3), an enzyme which occupies a key role in metabolism, commonly believed to be localized exclusively in mitochondria, has been found associated with purified nuclear fractions from rat [1—3], pig [4] and ox liver [5,6], Chang's liver cells [7,8], single dorsal root ganglion of rabbit [9] and yeast [10].

Recently, nuclear glutamate dehydrogenase from ox liver has been obtained in homogeneous crystalline form [5,6]. Its structural and kinetic properties have been studied in comparison with those of the mitochondrial enzyme, and several differences have been detected (refs. 5,6,11—13, di Prisco, G. and Zito, R., unpublished observation) thus leading to the hypothesis of the existence of two different proteins with similar biological activity.

The study of the two activities in a controllable and reproducible environment, such as that provided, for example, by animal cells grown in culture media, in an effort to understand the metabolic significance of the nuclear activity, has been further pursued using Chinese hamster ovary cells as starting material, in which we have demonstrated the existence of activities associated with both nuclear and extra-nuclear fractions.

The experiments reported in this paper have shown that the two enzymes differ in several properties. Nuclear glutamate dehydrogenase was activated by inorganic phosphate to a much greater extent and was more temperature-sensitive than mitochondrial glutamate dehydrogenase, showing also a higher anodic electrophoretic mobility. The two enzymes showed similar affinity for glutamate, and non-linear double reciprocal plots of velocity as a function of NAD^+ concentration; however, different apparent Michaelis constants were calculated from these plots. The pH activity curves indicated a maximum at pH 7.4 for mitochondrial glutamate dehydrogenase, either in phosphate or Tris · HCl buffer, nuclear glutamate dehydrogenase showed the same maximum in phosphate, but exhibited it at pH 8.0 in the latter buffer. Moreover, a different rate of decay of the two enzymes was noticed when chloramphenicol was added to the culture medium for at least 48 h; such an effect was not detected with actinomycin D or cycloheximide.

Finally, the results of an immunological study demonstrated that the two enzymes prepared from chinese hamster ovary (CHO) cells behave, as antigens, similarly to those purified from ox liver [12,13].

Materials and Methods

Preparation of subcellular fractions

CHO cells, purchased from the American Type Culture Collection, were grown in Eagle's minimal essential medium containing 10% foetal calf serum as described for other animal cells [14] in Roux bottles (165 cm^2 of surface area). Nuclear and extra-nuclear components were fractionated according to a modification of a published procedure [15]: after trypsinization, the cells were suspended in potassium phosphate buffer (pH 7.4, 1 mM) containing 0.32 M sucrose, 2 mM $MgCl_2$, to reach a cell concentration of $2 \cdot 10^6$/ml. Following centrifugation, the cell pellet was resuspended in 1 mM potassium phosphate buffer pH 7.4 containing 0.01 M NaCl, left for 20 min at 0—4° C, recentrifuged and then disrupted by homogenization in a Dounce all-glass unit in the same phosphate buffer containing 0.32 M sucrose, 1 mM $MgCl_2$ and 0.5% Triton (buffer A). Microscopic examination indicated that no intact cells remained. The nuclei were sedimented by centrifugation for 2 min at $1000 \times g$, washed twice with the same buffer used for the homogenization and then resuspended in 0.1 M potassium phosphate buffer pH 7.4 (one tenth of the original volume). In a control experiment, the RNA/DNA ratio of the isolated nuclei following the second and third washing remained constant, indicating the absence of significant cytoplasmic contamination. Enough potassium phosphate, pH 7.4, was added to the supernatant to reach a final concentration of 0.1 M.

Both nuclear and extra-nuclear fractions were disrupted by sonication for 2 min with a Brownhill Biosonik IV ultrasonic probe and centrifuged for 15 min

at 4000 × *g*. The supernatants contained all activity. The specific activities (forward reaction) were 1.00 and 1.04 nmol NAD^+ reduced per mg protein for mitochondrial and nuclear preparations, respectively.

Assay conditions

The enzymatic activity was assayed fluorometrically at 27° C by following the formation of NADH in a Turner fluorimeter (Model 111) coupled to a recorder. The reaction velocity is expressed as nanomoles of NAD^+ reduced per min per ml of enzyme solution, one nanomole representing one activity unit. Specific activity was defined as units of activity per mg of protein. The standard incubation mixture contained, unless otherwise indicated, 60 μM NAD^+, 10 mM sodium glutamate and 100 mM potassium phosphate buffer, pH 7.4 in a final volume of 1.2 ml. Protein was measured according to Lowry et al. [16]; DNA and RNA were determined according to published procedures [17,18].

Electrophoresis on cellulose acetate strips and cellogel

Electrophoresis was performed at 4° C, in 50 mM Tris · HCl buffer, pH 8.1, at 200 V for 150 min. Strips of cellulose acetate (Gelman Instruments, Ann Arbor, U.S.A.) or sheets of Cellogel (Chemetron, Milan, Italy) were stained for enzymatic activity [19].

Metabolic inhibitors

Cells were grown in medium containing chloramphenicol (40 μg/ml), cycloheximide (200 μg/ml) or actinomycin D (0.5 μg/ml), in order to inhibit specifically the protein synthesis in mitochondria, in the cytoplasm, or the mRNA synthesis, respectively; the medium was changed every other day.

Preparation of antisera

The γ-globulin fraction of the antisera against crystalline ox liver mitochondrial and nuclear glutamate dehydrogenase, prepared as previously described [12], was obtained by ammonium sulphate precipitation [20]. The sera of the same rabbits before immunization were used in all experiments as controls.

Preparation of immunoadsorbents of mitochondrial and nuclear glutamate dehydrogenase

2 g of CNBr-activated Sepharose were suspended in 100 ml of 1 mM HCl. After approximately 30 min, the Sepharose was poured on a Buchner funnel and washed [21], under mild pressure, with 200 ml of 1 mM HCl, followed by 200 ml of 0.1 M sodium bicarbonate, pH 9.0, containing 0.5 M NaCl. The washed Sepharose was then suspended in 20 ml of 0.1 M bicarbonate, pH 9.0/0.5 M NaCl, which contained approximately 40 mg of mitochondrial glutamate dehydrogenase, previously dialyzed against 0.1 M bicarbonate/0.5 M NaCl. Coupling was allowed to proceed for 4 h at room temperature and overnight at 4° C. The Sepharose was filtered and the absorbance and enzyme activity of the filtrate were measured, together with the activity associated to the Sepharose, to assess the amount of enzyme adsorbed. This was usually higher than 90% of the added protein. The Sepharose was then washed with 0.1 M bicarbonate/0.5 M NaCl and then treated with 100 ml of 1 M ethanolamine, pH 8.0, during 1 h at

room temperature. The coupled Sepharose was repeatedly washed alternatively with 0.1 M bicarbonate/0.5 M NaCl and with 0.1 M acetate buffer, pH 4.5/1.0 M NaCl. A last washing was performed with the equilibration buffer (0.1 M phosphate buffer, pH 7.4/0.145 M NaCl).

The coupling of nuclear glutamate dehydrogenase to activated Sepharose was performed essentially according to the same procedure, except that 10 mg of the nuclear enzyme were coupled to 1 g of Sepharose.

Chromatography of antisera

An aliquot of 0.25—0.5 ml of the antiserum of γ-globulin fraction was passed through the antigen-Sepharose column (0.5 × 4 or 0.5 × 8 cm), which was then washed with 0.1 M phosphate buffer pH 7.4/0.145 M NaCl, at a flow rate of 10—20 ml/h. The fractions containing the unadsorbed protein were assayed for adsorbance at 280 nm and for immunoprecipitation (see below). Elution of adsorbed antibodies was carried out with 0.145 M NaCl, adjusted at pH 3.0 with acetic acid or 11.0 with ammonia. The fractions containing these antibodies were immediately neutralized, pooled and concentrated by ultra-filtration.

Immunoprecipitation

The two enzymatically active subcellular fractions were diluted in phosphate-saline buffer, pH 7.4. 50 μl of each were incubated at 37° C with the indicated volumes of antiserum or γ-globulin fraction (at the indicated concentration) for 20 min and overnight at 0° C. After incubation, the reaction mixtures were centrifuged at 25 000 × *g* for 1 h. Supernatants were assayed for enzyme activity.

Results

Kinetics of the forward reaction

When the reaction velocity was followed as a function of concentration of glutamate, essentially the same apparent K_m was found with the two enzymes (Table I). Double reciprocal plots of velocity as a function of concentration of the cofactor NAD^+ were non-linear, similar to the findings with ox and rat liver and Chang's liver cells [1,6,7,22]. From these plots (Fig. 1), two apparent K_m values were calculated for each enzyme, one at low and the other at high NAD^+ concentration. These values (Table I) indicate that nuclear and mitochondrial glutamate dehydrogenase have similar apparent K_m values in the low concentration range; at concentrations of cofactor higher than 0.3 mM, the nuclear enzyme shows a higher affinity for NAD^+.

Effect of pH on activity

The response of activity to pH variations is illustrated in Fig. 2. Measurements were carried out in phosphate buffer up to pH 8.0, then Tris · HCl buffer was used for determinations at higher values. Although ox liver glutamate dehydrogenase is unstable in the latter buffer [23], it can be safely used in the assay mixture in initial velocity measurements, since the reaction substrates afford a 100% protection. Both enzymes showed higher activity in phosphate, with an

TABLE I

APPARENT MICHAELIS CONSTANTS OF NUCLEAR AND MITOCHONDRIAL GLUTAMATE DEHYDROGENASE FOR GLUTAMATE AND NAD^+

When used at fixed concentration, glutamate was 10 mM and NAD^+ 50 μM. Potassium phosphate buffer 0.1 M pH 7.4.

Substrate	Glutamate dehydrogenase (mM)	
	Nuclear	Mitochondrial
Glutamate	0.59	0.61
NAD^+	0.027	0.021
	0.49	1.25

optimum at pH 7.4. The measurements carried out in Tris · HCl buffer, however, indicated that the activity of mitochondrial glutamate dehydrogenase decreased while increasing the pH, whereas that of the nuclear enzyme had another maximum at pH 8.0.

Effect of phosphate on activity

Inorganic phosphate is known to activate mitochondrial glutamate dehydrogenase [24] at a pH higher than 8.0. This effect was shown to occur with nuclear glutamate dehydrogenase, to a much higher extent [1,6,7]. Experiments carried out with the enzyme fractions from CHO cells showed a similar situation (Fig. 3). A 3—4 fold activation of the extra-nuclear activity was brought about by 0.2 M phosphate pH 8.3; the same concentration activated nuclear glutamate dehydrogenase almost 10-fold. The phosphate concentrations re-

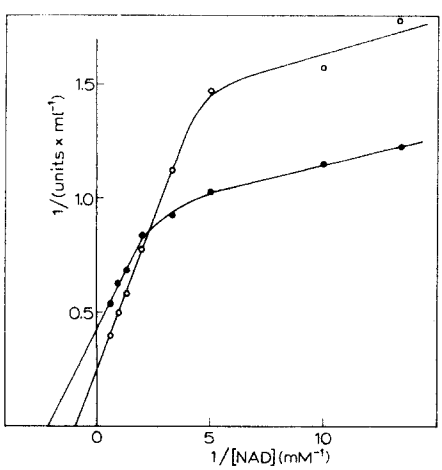


Fig. 1. Double reciprocal plots of velocity as a function of NAD^+ concentration, at pH 7.4, 100 mM potassium phosphate buffer, 25 mM glutamate. (○), mitochondrial and (●), nuclear glutamate dehydrogenase.

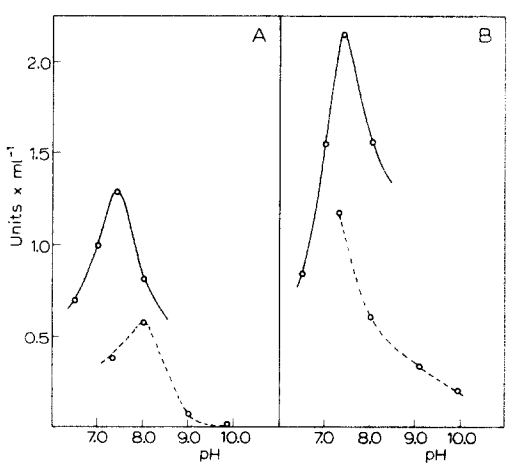
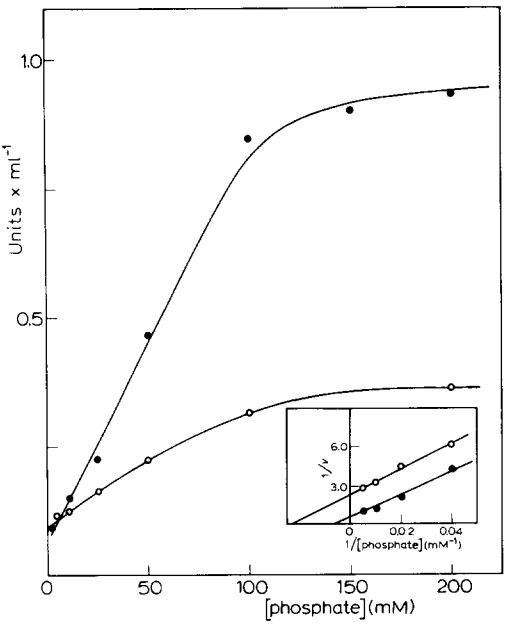


Fig. 2. Effect of pH on activities of nuclear (A) and mitochondrial (B) glutamate dehydrogenase. 10 mM glutamate, 0.4 mM NAD^+ and 100 mM potassium phosphate buffer (solid curve) or Tris · Cl buffer (dotted curve).



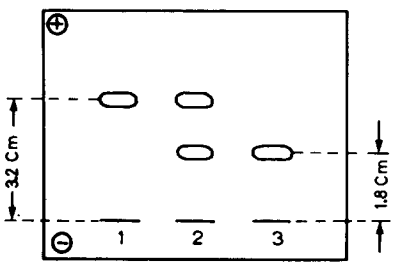


Fig. 3. Effect of phosphate on activities of nuclear (•) and mitochondrial (○) glutamate dehydrogenase. 25 mM glutamate, 50 μM NAD, pH 8.3. The insert shows these results, replotted in double reciprocal form.

Fig. 4. Cellogel electrophoresis of fractions containing: 1, nuclear glutamate dehydrogenase; 2, a mixture of nuclear and mitochondrial glutamate dehydrogenase; 3, mitochondrial glutamate dehydrogenase. 2 μl were applied to the plate, which was then stained for activity [19].

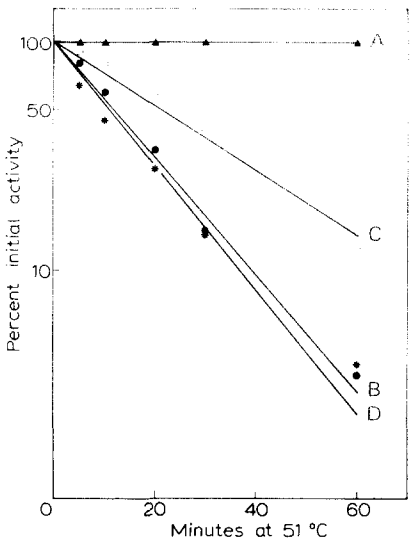
quired for half-maximal velocity were 43 mM and 140 mM, respectively, as calculated from the corresponding double reciprocal plots (see insert in Fig. 3).

Electrophoretic mobility

The electrophoretic mobility of each enzyme was investigated on Cellogel and cellulose acetate, in 50 mM Tris · HCl buffer, pH 8.1. Under these conditions, both proteins appear to be negatively charged, nuclear glutamate dehydrogenase showing a higher anodic mobility (Fig. 4), so that a mixture of the two fractions was resolved into two enzymatically active bands.

Thermal inactivation

Experiments performed with homogeneous preparations of the two enzymes purified from ox liver consistently showed greater thermostability of the mitochondrial enzyme at 40, 45 and 51°C (Di Matteo, G., Romeo, G. and di Prisco, G., unpublished observation). The two enzymes prepared from the subcellular fractionation of CHO cells showed a pattern of thermal inactivation at 51°C comparable to that of the ox liver enzymes (Fig. 5). The mitochondrial enzyme (curve A), in the presence of the buffer containing sucrose (buffer A), was more thermostable than after dialysis against 0.1 M phosphate buffer pH 7.4 (curve C), whereas such protection effect was not observed for the nuclear enzyme (curve B in phosphate buffer; curve D in buffer A, see Methods).



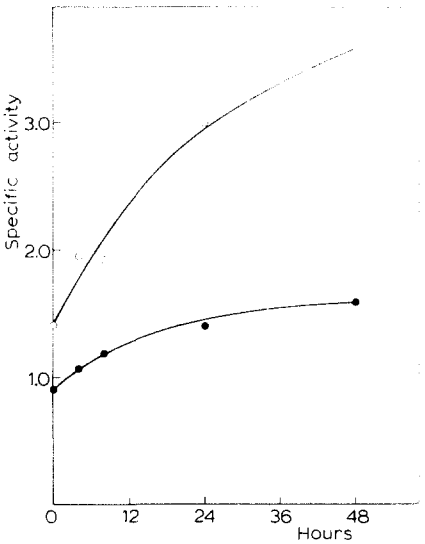


Fig. 5. Kinetics of heat inactivation of glutamate dehydrogenase from the two subcellular fractions of CHO cells. (A) mitochondrial (85 units for 10^6 cells) and (B) nuclear (16 units per 10^6 cells) enzyme, (C) mitochondrial enzyme dialyzed against 0.1 M phosphate buffer pH 7.4 and (D) nuclear enzyme, added with buffer A. The protein concentration of the nuclear and mitochondrial preparations incubated at the indicated temperatures was 0.6 and 0.7 mg/ml, respectively.

Fig. 6. Time course of mitochondrial (●) and nuclear (○) glutamate dehydrogenase, expressed as specific activity during the exponential phase of growth in CHO cells.

The dialyzed mitochondrial enzyme plus buffer A showed a curve of inactivation superimposable to that of the enzyme before dialysis. Sucrose, a component of buffer A, reproduced this protective effect only partially.

The time course of heat inactivation of mixtures of the two enzymes, prepared as described under Methods, was the arithmetic mean of those of the two separate enzyme preparations.

Effect of metabolic inhibitors

Under the conditions used in these experiments, an exponential rate of growth took place up to a concentration of 10^8 cells/bottle. The specific activity of mitochondrial glutamate dehydrogenase increased to about 75% above the initial value during a 48 h period, starting from an inoculum of 10^7 cells; after 48 h, the specific activity of nuclear glutamate dehydrogenase was 250% of the initial value, as shown in Fig. 6. During this period, the percentage of nuclear glutamate dehydrogenase showed slight variations, ranging between 15 and 25% of the total glutamate dehydrogenase activity.

Experiments were performed with several metabolic inhibitors. In cells grown with actinomycin D (inhibitor of mRNA synthesis) and with cycloheximide (inhibitor of protein synthesis in the cytoplasm), the two enzymes maintained similar activity levels, keeping fairly close to the control values; it should be pointed out that the toxicity of these two drugs on CHO cells is high, so that treatment for periods of time longer than 48 and 72 h, respectively, was not feasible. On the contrary, in experiments performed with chloramphenicol

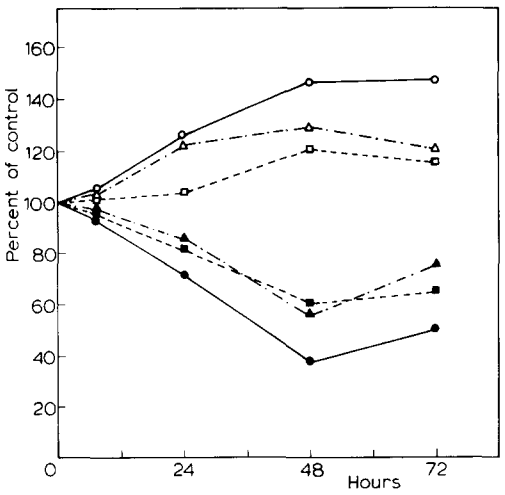


Fig. 7. Time course of the effect of chloramphenicol. Mitochondrial glutamate dehydrogenase: (○) activity/10^6 cells, (□) specific activity; nuclear glutamate dehydrogenase: (●) activity/10^6 cell, (■) specific activity. Cytoplasmic (△) and nuclear (▲) protein are also represented. Each point is expressed as the percentage of its control.

(inhibitor of protein synthesis in mitochondria) the growth of cells resembled that of controls. In the latter experiments, a consistent difference of 55% in specific activities between the levels of mitochondrial and nuclear glutamate dehydrogenase after 48 and 72 h of treatment was obtained (Fig. 7), probably as a consequence of the effect of the antimetabolite on protein biosynthesis in mitochondria rather than on the enzymes themselves. The nuclear activity (both in terms of specific activity and per 10^6 cells) steadily decreased during

TABLE II

EFFECT OF ANTI-NUCLEAR (AND ANTI-MITOCHONDRIAL) GLUTAMATE DEHYDROGENASE SERA, BEFORE AND AFTER CHROMATOGRAPHY ON MITOCHONDRIAL (AND NUCLEAR) GLUTAMATE DEHYDROGENASE SEPHAROSE

Assay conditions as in Materials and Methods. 50 μl of enzyme solution were incubated with 25 and 50 μl serum ($A^{1cm}_{280\,nm}$ = 5.5), as described in Materials and Methods. 25 μl were used for the assay.

Serum present during incubation		Percent of control activity in supernatants	
		Mitochondrial glutamate dehydrogenase	Nuclear glutamate dehydrogenase
Anti-nuclear glutamate dehydrogenase	(25 μl)	43	0
	(50 μl)	0	0
Anti-nuclear glutamate dehydrogenase, after mitochondrial glutamate dehydrogenase-Sepharose	(25 μl)	100	46
	(50 μl)	89	12
Anti-mitochondrial glutamate dehydrogenase	(25 μl)	0	11
	(50 μl)	0	0
Anti-mitochondrial glutamate dehydrogenase after nuclear glutamate dehydrogenase-Sepharose	(25 μl)	95	98
	(50 μl)	92	96

the 72 h period, whereas the mitochondrial one was increased. Protein from the two subcellular fractions closely followed this course.

In control experiments, it was shown that the concentration of chloramphenicol used inhibited the amino acid incorporating activity insensitive to inhibitors of the cytoplasmic protein synthesis [25]. Similarly, the concentration of cycloheximide used inhibited more than 98% of the protein synthesis and that of actinomycin D inhibited 99% of the incorporation of [^{14}C]uridine into RNA.

Immunological studies

The immunological properties of the two enzymes from CHO cells were comparable to those of the purified ox liver enzymes [12,13]. A typical set of results is shown in Table II. These data show that the antisera against crystalline ox liver nuclear and mitochondrial glutamate dehydrogenase precipitated both CHO enzymes. After passage on a Sepharose column coupled to ox liver mitochondrial enzyme,the anti-nuclear glutamate dehydrogenase serum precipitated only the nuclear glutamate dehydrogenase, indicating that the latter enzyme had some specific antigenic determinants. On the contrary, the antimitochondrial glutamate dehydrogenase serum passed through Sepharose, coupled to the ox liver nuclear antigen, was no longer able to precipitate the enzymes in either subcellular fractions. These data suggest that the mitochondrial antigen contains only determinants which are also present in nuclear glutamate dehydrogenase, and no determinants of its own.

Discussion

The existence of structural differences between nuclear and mitochondrial glutamate dehydrogenase has been confirmed by the present study on CHO cells. The higher anodic electrophoretic mobility of the nuclear enzyme constitutes a clear indication of a difference in the net charge of the two proteins. Such characteristic confirms findings with glutamate dehydrogenase from ox liver and yeast [6,10]: in both these cases, nuclear glutamate dehydrogenase showed higher anodic mobility than the mitochondrial enzyme. Moreover, the experiments on the kinetics of thermal inactivation showed that nuclear glutamate dehydrogenase is less thermostable than the mitochondrial enzyme; these results are also indicative of some differences in structure between the two enzymes.

Further indications that also in these cells glutamate dehydrogenase activity is associated to two different proteins come from the experiments on the response of the reaction velocity to variations of pH, NAD^+ and phosphate concentration. In particular, the higher activation brought about by phosphate at pH 8.3 on nuclear glutamate dehydrogenase closely parallels previous findings with the enzyme extracted from nuclei of other tissues [1,6,7,10].

The results of this work were obtained with rather unpure enzyme preparations. However, experiments performed with ox liver and yeast nuclear and mitochondrial preparations, at comparable stages of purity, showed that subsequent purification affected neither the electrophoretic mobility nor the kinetic properties, among which the activity response to phosphate concentra-

tion. Nuclear and mitochondrial glutamate dehydrogenase, extracted from several sources, could always be differentiated with respect to these two characteristics [1,6,7]. Heat inactivation experiments, to be reported elsewhere (di Metteo, G., Romeo, G. and di Prisco, G., unpublished observation), carried out on ox liver preparations, have shown that the presence of contaminant protein can increase the enzyme thermal stability only slightly (not over 10%), probably as a result of protein-protein interaction. Nuclear glutamate dehydrogenase has consistently been found to be less resistant to heat inactivation; the present evidence confirms this peculiarity, and it is therefore safe to conclude that, in CHO cells also, nuclear glutamate dehydrogenase is less thermostable than the mitochondrial enzyme.

The immunological data suggest that the nuclear enzyme possesses some specific antigenic determinants besides those in common with the mitochondrial enzyme, which, in its turn, does not seem to possess determinants other than the common ones. The structural relationship between the two enzymes, based on the immunological evidence, appears therefore to parallel that recently demonstrated for the ox liver enzymes [12,13]. These data clearly indicate that the antisera prepared against the ox liver enzyme react with the antigens present in a phylogenetically distant mammal like the hamster. The same kind of interspecies cross reactivity has been noticed with the enzymes from rat, pig, sheep and pigeon liver [26]. The occurrence of two distinct structural genetic loci codifying nuclear and mitochondrial glutamate dehydrogenase can now be hypothesized in different mammalian organisms; it would thus be interesting to investigate the possible origin of one of these enzymes from the other by gene duplication in some common ancestor. Unfortunately, the immunological assay developed in this study cannot be applied as a routine screening of a mixture of the two enzymes, as it would be desirable, since the antiserum which is non cross-reactive after affinity chromatography is that against the nuclear enzyme, and this component has constantly been found to represent only about 15% of the total glutamate dehydrogenase activity.

The differential rates of increase of the nuclear and mitochondrial glutamate dehydrogenase in relation to cell growth have already been noticed in human Chang's liver cells [7] and constitute a further proof of the different localization of these two enzymes in the cell. With regard to the response to antimetabolites the resistance of CHO cells to chloramphenicol treatment, which contrasts with the higher sensitivity to this drug reported for HeLa cells grown in suspension [25] must be underlined.

The increase in specific activity and activity per cell of mitochondrial glutamate dehydrogenase is similar to that reported for mitochondrial malate dehydrogenase in HeLa cells following a three-day exposure to chloramphenicol [25], as well as that of two enzymes of bacterial specificity involved in mitochondrial protein synthesis in *Neurospora crassa* [27]. In accord with the latter findings, we have also shown, in the presence of chloramphenicol, an increase in the total mitochondrial protein. Our results are therefore in keeping with the hypothesis of synthesis of most mitochondrial enzymes on cytoplasmic ribosomes, followed by incorporation in the mitochondria. This process seems to be controlled by a repressor-like mitochondrial gene product [27]. On the other hand, the enhanced decay of nuclear glutamate dehydrogenase, as well as

the decrease in total nuclear protein in chloramphenicol-treated cells, has not been previously observed. Although this effect remains unexplained at this stage, a possible involvement of signals of protein nature of mitochondrial origin in the control of the level (or the stability) of the nuclear enzyme seems a reasonable working hypothesis.

Acknowledgements

We wish to thank Dr. A.M. Guerrini for fruitful discussions. We also thank Mr. Antonio De Falco for skillful technical assistance and Mrs. Carmela Salzano for her help in this work.

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