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Detection of Structural Differences between Nuclear and Mitochondrial Glutamate Dehydrogenases by the Use of Immunoadsorbents†

Guido di Prisco* and Luigi Casola

ABSTRACT: Structural differences between crystalline mitochondrial and nuclear glutamate dehydrogenases from ox liver have been detected by immunological techniques. Antisera prepared against each enzyme precipitate both glutamate dehydrogenases; upon immunodiffusion, the antiserum against the nuclear enzyme gives a line of incomplete identity with the two antigens, whereas the antiserum against the mitochondrial enzyme gives a line of complete identity. Fractionation of the antibodies contained in each antiserum by means of an immunoadsorbent, to which the nuclear or the mitochondrial enzyme has been covalently linked, shows that nuclear glutamate dehydrogenase (GDH) contains specific antigenic determinants as well as determinants common to the mitochondrial enzyme, whereas the latter appears to have no antigenic portions which are not present in the nuclear antigen, in accord with the results of immunodiffusion. The antibodies against determinants common to both enzymes precipitate and inhibit them, whereas the specific anti-nuclear GDH antibodies precipitate but do not inhibit the nuclear antigen.

Nuclear fractions from different tissues have been shown to contain glutamate dehydrogenase (di Prisco et al., 1968, 1970; Zbarsky et al., 1968; di Prisco and Strecker, 1970; Franke et al., 1970; Kato and Lowry, 1972; Herzfeld et al., 1973; Camardella et al., 1975). This enzyme was formerly believed to be of mitochondrial localization exclusively.

GDH[1] from ox liver nuclei has been extracted, purified, and crystallized, and the study of its characteristics has revealed a number of kinetic and structural differences in comparison with the mitochondrial enzyme (di Prisco et al., 1972; di Prisco and Garofano, 1974, 1975).

Recently, an investigation of the immunological properties of the two dehydrogenases has been undertaken. Our initial results (Casola et al., 1974) indicated that nuclear GDH contains specific antigenic determinants as well as determinants common to the mitochondrial enzyme. Such conclusions were attained by performing an affinity chromatography of the anti-nuclear GDH antiserum on a mitochondrial GDH immunoadsorbent column. This work, however, did not yet provide an answer to the question whether

† From the International Institute of Genetics and Biophysics, C.N.R., 80125 Naples, Italy. *Received March 19, 1975.*
A preliminary account of these findings has been presented (di Prisco et al., 1974).
[1] Abbreviation used is: GDH, glutamate dehydrogenase.

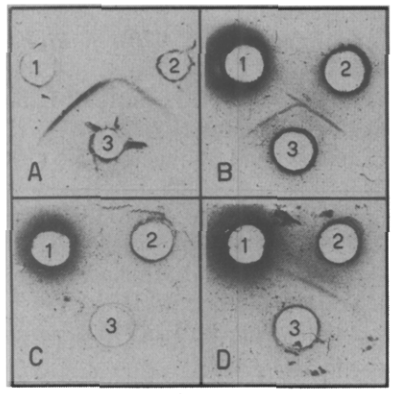

FIGURE 1: Agar gel precipitin patterns with sera against mitochondrial and nuclear GDH: (1) mitochondrial GDH; (2) nuclear GDH; (3) γ-globulin fraction of (A) anti-mitochondrial GDH serum, (B) anti-nuclear GDH serum, (C) anti-mitochondrial GDH serum, passed through nuclear GDH-Sepharose, and (D) anti-nuclear GDH serum, passed through mitochondrial GDH-Sepharose. Precipitin lines were stained to show enzyme activity (Talal et al., 1964) or protein. All γ-globulin fractions had been adjusted to the same optical density at 280 nm.

mitochondrial GDH also had its own specific antigenic determinants. The experiments reported in this paper logically extend this investigation of the immunological characterization of the two enzymes. The availability of a discrete amount of crystalline nuclear GDH (di Prisco and Garofano, 1974, 1975) had made possible the preparation of a nuclear GDH-Sepharose immunoadsorbent, on which an antiserum to the mitochondrial enzyme could be fractionated.

The results of this study demonstrate the existence in the mitochondrial antigen of determinants common to nuclear GDH; however, unlike nuclear GDH, the mitochondrial enzyme does not appear to have determinants other than these common ones.

The results of experiments on antibody inhibition suggest that the two enzymes, as could be expected in view of the similarity of their function, have a closely resemblant structure in the portion of the molecule containing the active region, or essential in maintaining it in the active conformation.

Experimental Section

Preparation of Antisera. Two rabbits were immunized with mitochondrial and two with nuclear GDH by multiple intramuscular injections of 1–2 mg of the antigen in 2 ml of complete Freund's adjuvant (Difco) emulsion (1:1 volume ratio) at weekly intervals, for 5 consecutive weeks. The animals were then bled by cardiac puncture, the antisera prepared, and the γ-globulin fraction of each antiserum was obtained by ammonium sulfate precipitation (Campbell et al., 1970). The sera of the same animals before immunization were used in all experiments as controls.

Preparation of Immunoadsorbents of Mitochondrial and Nuclear GDH. Two grams of CNBr-activated Sepharose was suspended in 100 ml of 1 m*M* HCl. After approximately 30 min, the Sepharose was poured on a Buchner funnel and washed (Porath et al., 1967), under mild vacuum, with 200 ml of 1 m*M* 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–0.5 *M* NaCl, which contained approximately 40 mg of mitochondrial GDH, previously dialyzed against 0.1 *M* bicarbonate–0.5 *M* NaCl. Coupling was allowed to proceed for 4 hr at room temperature and overnight at 4°. The Sepharose was filtered and the optical density and enzyme activity of the filtrate were measured, together with the activity bound to the Sepharose, in order to assess the amount of enzyme covalently attached. This was usually greater 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 hr at room temperature. The coupled Sepharose was washed repeatedly and alternately 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 0.1 *M* phosphate buffer (pH 7.4)–0.145 *M* NaCl.

Table I: Effect of Anti-Mitochondrial and Anti-Nuclear GDH Sera on Enzyme Activity.[a]

Serum Present during Incubation	Enzyme Activity in Supernatants	
	Mitochondrial GDH	Nuclear GDH
Control[b]	11,250	11,250
Anti-mitochondrial GDH	120	95
Anti-nuclear GDH	270	220

[a] Activity is expressed as picomoles of NADH formed/minute per milliliter; 50 μl of enzyme solution (0.4 mg/ml) was incubated with 50 μl of serum (A_{280nm}^{1cm} = 6.5) as indicated in the Experimental Section; 50 μl was used for the assay. [b] Serum from the same animals before immunization.

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

Chromatography of Antisera. An aliquot of 0.25–0.5 ml of the antiserum or γ-globulin fraction was passed through the antigen–Sepharose column (0.5 × 4 or 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/hr. The fractions containing the unadsorbed protein were assayed for absorbance 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 ultrafiltration.

Immunoprecipitation. Mitochondrial and nuclear GDH were diluted in phosphate-saline buffer (pH 7.4). Each antigen solution (50 μl) was incubated at 37° with an equal volume of antiserum or γ-globulin fraction (both antigen and antisera at the indicated concentration) for 20 min and overnight at 0°. After incubation, the reaction mixtures were centrifuged at 25,000*g* for 1 hr. Supernatants and precipitates were assayed for enzyme activity.

Enzyme Assay. The activity was measured fluorometrically as previously described (di Prisco and Garofano, 1975). The assay mixture contained 10 m*M* sodium glutamate, 1.2 m*M* NAD^+, and 50 m*M* phosphate buffer (pH 7.6), in a final volume of 1.2 ml.

Gel Diffusion and Staining. Immunodiffusion in agar gel was carried out at room temperature (Ouchterlony, 1949). After 30 hr at room temperature, gels were washed for 1 day at 2–4° in 0.1 *M* phosphate buffer–0.145 *M* NaCl (pH 7.4) and stained for GDH activity (Talal et al., 1964). Gels were also stained for proteins by the following method: they were washed in distilled water for a few hours, dried, and

Table II: Removal of Antibodies to Mitochondrial and Nuclear GDH from Complex Antisera (γ-Globulin Fraction) by the Use of Homologous Immunoadsorbents.[a]

Serum Present during Incubation	Immunoadsorbent Used	Enzyme Act. in Supernatants: Mitochondrial GDH	Enzyme Act. in Supernatants: Nuclear GDH
Control[b]	None	12,500	11,850
Anti-mitochondrial GDH	None	3,250	2,700
Anti-mitochondrial GDH	Mitochondrial GDH–Sepharose	12,750	12,100
Anti-nuclear GDH	None	5,100	3,950
Anti-nuclear GDH	Nuclear GDH–Sepharose	12,250	10,700

[a] Experimental conditions as in Table I. The γ-globulin fractions, in the immunoprecipitation incubation mixtures, were adjusted to $A_{280nm}^{1cm} = 1.1$. [b] As in Table I.

Table III: Effect of γ-Globulin Fraction of Each Anti-GDH Serum on Enzyme Activities following Chromatography on the Heterologous GDH–Sepharose.[a]

Serum Present during Incubation	Enzyme Act. in Supernatants: Mitochondrial GDH 1	Mitochondrial GDH 2	Nuclear GDH 1	Nuclear GDH 2
Control[b]	5760	2280	3640	1370
Anti-mitochondrial GDH	0	32	0	0
After nuclear GDH–Sepharose	5260	2250	3660	1350
Anti-nuclear GDH	6	21	15	17
After mitochondrial GDH–Sepharose	5480	2190	39	36

[a] A_{280nm}^{1cm} of γ-globulin fractions = 1.250. Incubation and assays carried out as in the previous tables, except that the concentrations of GDH solutions prior to mixing were: (1) 0.02 mg/ml; (2) 0.008 mg/ml. [b] As in Table I.

stained with 0.2% Ponceau S Red in 3% trichloroacetic acid or 0.25% Coomassie Brilliant Blue in water–methanol–acetic acid (5:5:1, v/v), and destained in 3% acetic acid.

Materials. Nuclear GDH was crystallized as previously described (di Prisco and Garofano, 1974, 1975). Mitochondrial GDH was purchased from Boehringer, Mannheim, Germany. CNBr-activated Sepharose was from Pharmacia, Uppsala, Sweden. All other reagents were of the highest purity commercially available.

Results

The antisera against the two enzymes were allowed to react with both antigens, by immunodiffusion and immunoprecipitation. When the two antigens, placed in adjacent wells of the agar gel, were diffused against the anti-mitochondrial GDH serum, a line of complete identity was observed (Figure 1A). In contrast, the formation of a spur, indicative of incomplete identity, was observed on the side containing the well of nuclear GDH when the anti-nuclear GDH serum was allowed to react in agar gel with the two antigens (Figure 1B). These results confirmed our conclusions that nuclear GDH possesses specific determinants as well as determinants in common with the mitochondrial enzyme (Casola et al., 1974); it appeared, on the other hand, that all determinants of mitochondrial GDH were contained in the nuclear antigen molecule, as tested by immunoprecipitation procedures.

Table I illustrates the results of immunoprecipitation. After incubation of the anti-mitochondrial or anti-nuclear GDH sera with either enzyme, the supernatants obtained after centrifuging the reaction mixtures retained virtually no enzyme activity as compared to control experiments carried out with normal serum.

It has been reported (Borek et al., 1967; Mannik and Stage, 1971) that specific antibodies can be isolated from antisera with the aid of immunoadsorbents. Immunoadsorbents of mitochondrial and nuclear GDH were prepared by binding either protein to CNBr-activated Sepharose. The effectiveness of the antigen–Sepharose columns to isolate specific antibodies was tested by chromatographing each antiserum, or γ-globulin fraction, on the immunoadsorbent column of the homologous antigen. Once run through the column, each serum completely lost the ability to inhibit or precipitate either enzyme (Table II). These results thus proved the usefulness of this technique; the antigen, bound to Sepharose, complexed all the corresponding antibodies present in the aliquot of serum or γ-globulin fraction run through the column.

By coupling one antigen to Sepharose and subsequently chromatographing the antiserum against the other enzyme, it is therefore possible to establish, from the immunological properties of the effluent, the degree of antigenic similarity of the two proteins. The results reported previously (Casola et al., 1974) with the antiserum to nuclear GDH, after chromatography on mitochondrial GDH–Sepharose, as well as the line of precipitation still observed in agar gel with nuclear GDH (Figure 1D), strongly suggested the existence of specific anti-nuclear GDH antibodies.

Complementary reciprocal experiments were carried out in order to establish the possible presence of specific anti-mitochondrial GDH antibodies. An immunoadsorbent of nuclear GDH was prepared by binding the crystalline enzyme to Sepharose. When the antiserum, or γ-globulin fraction, to mitochondrial GDH was passed through this column, the unadsorbed protein was no longer able to precipitate (or inhibit) either enzyme (Table III). For comparison, results obtained with the anti-nuclear GDH γ-globulin fraction before and after passage through mitochondrial GDH–Sepharose column are included in Table III; these data are consistent with our previous findings (Casola et al., 1974).

These results were confirmed by gel diffusion analysis. After passing through the nuclear GDH–Sepharose column, the anti-mitochondrial GDH serum did not form any precipitin band with either antigen (Figure 1C, compared with Figure 1A, referring to the same antiserum before chromatography), as could be expected from the lack of inhibition illustrated in Table III.

The immunoprecipitation and gel diffusion experiments outlined in Table III and Figures 1A and 1C indicate the absence of determinants in the mitochondrial antigen other than those present also in the nuclear enzyme. Another implication of these findings is that the immunoadsorbent coupled to either antigen retained antibodies corresponding to the same antigenic determinants present in the two proteins and having identical immunological properties. This hypothesis was indeed supported by the experimental results. The adsorbed antibodies were eluted from each column at alkaline pH (Figure 2 shows a representative chromato-

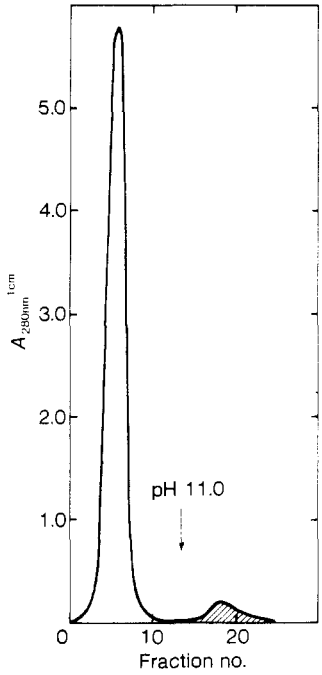

FIGURE 2: Chromatography of 0.5 ml of γ-globulin fraction (A_{280nm}^{1cm} = 50) of anti-nuclear GDH serum on a mitochondrial GDH-Sepharose column; fraction volume, 1.5 ml. The fractions corresponding to the hatched area were pooled, neutralized, and concentrated to A_{280nm}^{1cm} = 1.5.

Table IV: Effect of the Antibodies Retained by Mitochondrial and Nuclear GDH–Sepharose on Enzyme Activity, after Elution at pH 11.0.[a]

Antibodies Present during Incubation	Mitochondrial GDH		Nuclear GDH	
	1	2	1	2
Control[b]	2100	970	1620	860
Eluted from mitochondrial GDH–Sepharose	650	60	490	15
Eluted from nuclear GDH–Sepharose	510	40	310	15

[a] A_{280nm}^{1cm} of γ-globulin fractions = 1.20. Incubations and assays carried out as in the previous tables, except that the concentrations of GDH solution prior to mixing were: (1) 0.008 mg/ml; (2) 0.004 mg/ml. [b] As in Table I.

graphic pattern, obtained by passing an anti-nuclear GDH γ-globulin fraction on a mitochondrial GDH-Sepharose column) and assayed for their ability to inactivate the two enzymes. After incubation with two different concentrations of mitochondrial and nuclear GDH and centrifugation, little enzyme activity was present in the supernatants (Table IV). Results virtually identical with those outlined in Figure 2 and Table IV were obtained when the adsorbed antibodies were eluted at pH 3.0.

The antisera to both enzymes, as indicated by the immunodiffusion and immunoprecipitation experiments, contained precipitating antibodies. To check for the existence of inhibiting antibodies (Cinader and Lafferty, 1963), aliquots of the two antisera were incubated for 15 min at 37° with each GDH, and the activity was then measured. This experiment differed from immunoprecipitation in that no centrifugation was carried out to remove the antigen–antibody complex, so that any inhibition observed under these

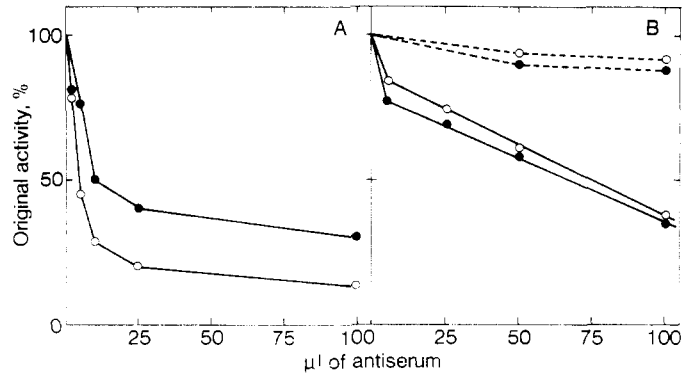

FIGURE 3: Inhibition of nuclear (●) and mitochondrial (○) GDH by anti-mitochondrial (A) and anti-nuclear (B) GDH serum. The dotted curves of B refer to anti-nuclear GDH serum after mitochondrial GDH-Sepharose chromatography; 0.2 μg of either GDH was incubated in the fluorimeter cuvette in the presence of the indicated volume of antiserum and control serum (A_{280nm}^{1cm} = 6.0) for 15 min at 37°, in the assay mixture without glutamate, which was then added to start the reaction.

conditions would reflect the presence of inhibiting antibodies. In other words, if all antibodies present were precipitating, but not inhibiting, levels of activity similar to those of the control would be obtained, since the complex would be enzymatically active. As shown in Figure 3, both antisera were effective in inhibiting the two enzyme activities. Similar inhibition curves were obtained with the antibodies of each serum retained by the immunoadsorbent (not reported in the figure). In contrast (dotted curves), the anti-nuclear GDH serum after mitochondrial GDH-Sepharose chromatography, containing the specific anti-nuclear GDH antibodies, had a negligible inhibiting effect on the two activities, although it was effective in precipitating the nuclear antigen (Casola et al., 1974; also Figure 1D and Table III). Thus, it can be concluded that the inhibition brought about by the two antisera was almost entirely due to the antibodies to the antigenic determinants common to the two enzymes.

Discussion

The results outlined in this paper, together with previously reported data (Casola et al., 1974), clearly demonstrate that mitochondrial and nuclear GDH are immunologically distinguishable proteins. The GDH-Sepharose immunoadsorbents used in this work have shown that antibodies to either enzyme can be effectively purified from complex antisera. By coupling either one of the two enzymes to Sepharose, it was possible to adsorb to this immunoadsorbent a small but detectable amount of antibodies present in the antiserum against the other enzyme. Following elution, these antibodies were equally effective in inhibiting both enzymes; they correspond therefore to antigenic determinants common to the two proteins.

The results with anti-nuclear GDH serum, or γ-globulin fraction, passed through a mitochondrial GDH-Sepharose column (Casola et al., 1974; also, Figure 1D and Table III), clearly indicate that nuclear GDH, beside antigenic determinants common to the mitochondrial enzyme, also has its own specific determinants. This conclusion is strengthened by the gel immunodiffusion results, which showed a line of incomplete identity when the two antigens, placed in adjacent wells, were diffused against anti-nuclear GDH serum; the shape of the line indicates the presence of specific determinants in the nuclear antigen.

The same situation does not present itself with mitochondrial GDH. The antiserum against this enzyme appears to contain only antibodies to determinants in common with the nuclear antigen, and none to determinants specific to the mitochondrial enzyme. This conclusion, drawn from the observed line of complete identity, shown in Figure 1A, received full support from the affinity chromatography experiments.

The two antisera contain both precipitating and inhibiting antibodies. Those specific to the nuclear antigen, contained in the anti-nuclear GDH serum, are effective in precipitating, but not in inhibiting, the two activities. The higher inhibiting effect shown by antibodies against determinants common to the two enzymes is hardly surprising, in view of the similarity of function of the two proteins, presumably reflected in a similar structural conformation in and around the active sites, or in an area essential to maintain these in the active configuration. The two enzymes would thus have a high degree of structural similarity in a portion of the molecule, presumably quite large, containing the active sites, but also a certain degree of dissimilarity, leading to differences in kinetic and structural properties (di Prisco et al., 1972; di Prisco and Garofano, 1974, 1975) and accounting for the existence of specific antigenic determinants in nuclear GDH.

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