Purification and Partial Characterization of Glyceraldehyde-Phosphate Dehydrogenase from Electric Organ of *Electrophorus electricus* (L.)

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The glyceraldehyde-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) was purified to homogeneity from electric organ of *Electrophorus electricus* (L.) by a hydrophobic chromatography method on deacetylcolchicine-Sepharose. The purification resulted in a 162 fold increase in specific activity of the GAPDH and final yield was approximately 37%. The purified enzyme showed a single band in SDS-PAGE, with an apparent molecular mass of 36 kDa. The purity of the colchicine-Sepharose isolated material was analysed by isoelectrophocusing and immunoblotting using a heterologous rabbit serum anti-GAPDH. Sequence analysis of the 40-N-terminal amino acids, determined by Edman degradation, revealed its identity to other GAPDHs proteins being the largest number of identical amino acids to lobster (92.5%), rabbit muscle (85%) and human liver (80%) GAPDH.

Introduction

Electrophorus electricus (L) is a teleosteo distributed in South America through the Orinico bay and Amazonian basin from Brazil. This animal is the single live representant of its genera and species. Histological observation made in its electric tissue, has shown specific characteristics and, therefore, it was suggested that the organ was derived from an atrophied muscle with developed capacity to generate energy (Falcato-Ribeiro *et al.,* 1977). This fact was established by metabolic studies from different laboratories showing that the electric organ is a tissue with anaerobic capacity (Pearse and Almeida, 1963; Hargreaves and Wanderley, 1969; Torres da Matta *et al.,* 1983). Lactic acid has been found in substantial quantity (Torres da Matta *et al.,* 1975) and creatine phosphate and ATP at sufficient high concentration providing energy for the electric discharge (Nachmansohn *et al.,* 1946).

The enzyme glyceraldehyde-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is present in the cytosol of most organisms so far studied (Fothergill-Gilmore and Michels, 1993) and occupies a position of central importance in generating energy from the metabolism of carbohydrates. It has been remarkable conserved during evolutionary process having an homotetrameric structure with subunits of 35–37 kDa (Fothergill-Gilmore and Michels, 1993).

This paper reports the results of the purification and partial characterization of the GAPDH from the electric organ of *E. electricus* at very high factor (162 fold) after ammonium sulfate precipitation followed by a single chromatographic step

Abbreviations: ATP, adenosine triphosphate; DAC-Sepharose, deacetylcolchicine coupled to carboxyhexyl Sepharose; EDTA, ethylenediamino tetracetic acid; GAPDH, glyceraldehyde-phosphate dehydrogenase; KPE buffer, 0.05 M potassium phosphate buffer pH 7.2 containing 1 mM EDTA; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PBS-NP40, phosphate buffer pH 7.3 containing 150 mM NaCl and 0.1% nonidet P-40; PVDF, poly(vinylidene difluoride).

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on colchicine-CH-Sepharose, and discusses its similarities with other GAPDH enzymes.

Materials and Methods

Preparation of the electric organ extract

Electric organ (5 g) was washed three times in PBS and submitted to three cycles of freezing-thawing in 0.05 M potassium phosphate buffer pH 7.2 containing 1 mM EDTA (KPE buffer). Insoluble material was removed by centrifugation (12,000×*g*, 30 min, 4 °C), and the supernatant fractionated with 70% saturated ammonium sulfate. The pellet was dissolved in KPE buffer and dialysed overnight against 2.5 M NaCl-KPE. The insoluble material during the dialysis was removed by centrifugation (105,000×*g*, 30 min, 4 °C) and the supernatant immediately used or stored at 10 °C until use.

Hydrophobic chromatography on colchicine-Sepharose

Dialysed extract made 2.5 M NaCl/KPE was applied to 5 ml columns of deacetylcolchicine (DAC) Sepharose, to which DAC had been previously coupled (Kocha *et al.*, 1989). The amount of bounded colchicine was 1.5 μmol g^{-1} of wet carboxyhexyl (CH)-Sepharose 4B. The DAC-CH Sepharose columns were washed through with 2.5 M NaCl/KPE buffer and the proteins eluted with KPE buffer. Fractions of 2 ml, having 280 nm of absorbing material, were collected and concentrated using centrifugal ultrafiltration microconcentrators (Centricon 10, Amicon) with nominal molecular mass cut off of 10,000. Solutions of purified GAPDH always were maintained in PBS containing NAD^+, EDTA, dithiothreitol (1 mM each) and 0.5 M ammonium sulfate.

Polyacrylamide gel electrophoresis and Western-blot

SDS polyacrylamide-gel electrophoresis was performed using 12% polyacrylamide gels (Laemmli, 1970) under reduction conditions. The gels were Coomassie blue-R 250 or silver stained (Bio-Rad kit). For immunoblotting, samples (30–40 μg of proteins) were electroblotted in a Bio-Rad transblot system onto nitrocellulose paper for 2 h. The nitrocellulose was saturated with 3% (w/v) non-fat powdered milk in PBS, washed three times with PBS-NP40 (0.1%) and incubated for 2 h with 1:150 dilution rabbit anti-*Trypanosoma cruzi* GAPDH serum. The sheets were washed three times with PBS-NP40, and incubated for 2 h with anti-rabbit Ig peroxidase conjugated. After washing, the immune complexes were revealed with diaminobenzidine (Towbin and Gordon, 1979).

Isoelectrophocusing (IEF)

IEF was performed as described by O' Farrel (O'Farrel *et. al.*, 1979) using 2.5% ampholine pH 4–10 or a mixture of ampholine pH 7–9/9–11 (Pharmacia Fine Chemicals, U. S. A.).

NH_2-terminal sequence

NH_2-terminal amino acid sequence of purified protein was carried out by automatic sequential Edman degradation in a gas-phase protein sequencer (Shimadzu, Kyoto, Japan, Model PSQ-1) with subsequent identification of the phenylthiohydantoin derivatives of amino acids by reversed-phase HPLC (Giovanni De Simone *et al.*, 1994). The sequencing was performed with an initial yield of 60% and a repetitivity of 96%.

Protein estimation and enzymological assay

Protein was estimated using the Lowry's method (Lowry *et al.*, 1951), whilst GAPDH activity was monitored by the oxidation of NAD^+ to NAD, recording the absorbance change at 340 nm within 60 s in a Beckman DU-C5 spectrophotometer. Assays contained 1.0 mM ATP, 300 μM NAD^+, 5.6 mM 3-phosphoglycerate, 5.0 mM $MgSO_4$, 1.0 mM EDTA, 1.0 mM dithiothreitol and 50 ng of rabbit glyceraldehyde-phosphate dehydrogenase or 50 μg of the enzyme sample to be assayed in a total volume of 1 ml. The reaction was initiated by addition of phosphoglycerate.

Results

Purification of GAPDH

GAPDH was solubilized from total supernatant extract of *E. electricus* organ with three cycles of freeze-thawing and then purified by hydrophobic chromatography on DAC- Sepharose. When a 2.5 ml sample (395 μg protein) of $(NH_4)_2SO_4$ precipitated and dialysed *E. electricus* extract is

loaded on a colchicine-Sepharose column (1.5 μmol g^{-1}) most proteins (291 μg) passed through unbound whereas the GAPDH was adsorbed by the DAC-column and subsequently eluted by KPE (104 μg, Fig. 1). This method affords GAPDH to be purified 162 fold with about 37% yield (Table I).

The purified enzyme was apparently homogeneous as assessed by SDS-PAGE (Fig. 1) and isotachophoresis (Fig. 2), migrating as a single band with an apparent M_r of 36,000 and pI of 7.3. Heterologous antibodies anti-*T. cruzi* GAPDH recognised the 36 kDa protein in Western blot analysis (Fig. 3) confirming that this protein represents subunits of the dehydrogenase enzyme and that some antigenic segments were conserved. These results suggested that this protein had a tetrameric homopolymer structure in the native state, like all other GAPDHs.

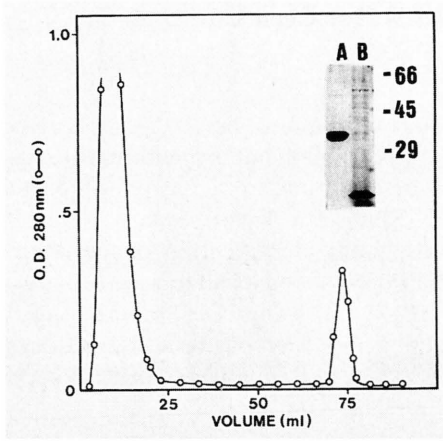

Fig. 1. Purification of *Electrophorus electricus* GAPDH on colchicine-Sepharose hydrophobic chromatography. About 395 μg of $(NH_4)_2SO_4$ precipitated protein of electric organ was applied onto a 5 ml colchicine-Sepharose column equilibrated with KPE buffer containing 2.5 M NaCl. The column was washed with the same buffer and then the GAPDH was eluted with KPE. Flow rate was 90 ml h^{-1} (2 ml/tube) and the absorbance measured at 280 nm. Insect is shown a silver stained SDS-polyacrylamide gel electrophoresis (10%) of DAC-Sepharose KPE eluted (A) and total extract (B). The molecular weight of marker proteins are indicated.

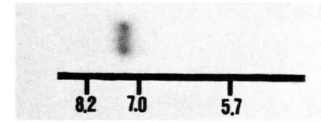

Fig. 2. Isoelectrophocusing analysis of purified GAPDH. The numbers on the horizontal axis refer to the pH gradient.

Fig. 3. Immunological characterization of purified glyceraldehyde phosphate dehydrogenase by immunoblotting. Lane A, crude extract of *E. electricus* electric organ; lane B colchicine-Sepharose purified GAPDH reacting with rabbit immune serum anti-*Trypanosoma cruzi* GAPDH.

NH_2-terminal sequence

The sequence of a 40-residue-long N-terminal sequence obtained by direct amino acid sequencing is shown on Table II. This sequence was aligned and compared with 10 other GAPDH se-

Table I. Purification of *E. electricus* glyceraldehyde-phosphate dehydrogenase.

Fraction[1]	Total protein[2]	Enzyme activity[3]	Specific activity[4]	Purific. (-fold)[5]	Yield (%)
Total extract	43.00	1.303	0.030	–	100
$(NH_4)_2SO_4$ pellet	0.39	0.910	2.333	77.7	68
Recovered from DAC-Sepharose column	0.10	0.487	4.870	162.3	37

[1] From 5 g of organ
[2] The total protein (mg) measured by Lowry's method.
[3] Total number of U.
[4] The enzyme activity divided by the protein concentration (U mg^{-1}).
[5] The increase in specific activity.

Table II. Comparison of the NH_2-terminal amino acid sequence of the 36 kDa *E. electricus* protein with the amino acid sequences of the known GAPDH molecules obtained from the translated GenBank database.

Source	Amino acid sequence						Acession n°
E. electricus		VKVGIQHFGR	IGRLVTRAAF	NSGKVGVVAI	NDPFIDYMVY		This work
H. americanus (lobster)		SKVGIDHFGR	IGRLVLRAAL	NSGKVGVVAI	NDPFIDYMVY		
O. caniculus (rabbit)		VKVGVNGFGR	IGRLVIRAAF	NSGKVGVVAI	NDPFIDPHYM		
T. vaginalis	RIGRL	VFRACRKLYP	KDIQVVAIHD	LGDIKTNVYL	LK I DTAHRA		L11394
H. sapiens (muscle)	MGK	VKVGVNGFGR	IGRLVIRAAF	NSGKVDIVA I	NDPFI I DLNY		J04038
H. sapiens (liver)	MGK	VKVGVNGFGR	IGRLVTRAAF	NSGKVDIVA I	NDPFIDLNYM		X01677
E. coli	MTI	VKVGINGFGR	IGRIVFRAAQ	KRSD I EIVA I	NDLLDADYMA		X02662
T. aquaticus	M	VKVGINGFGR	IGRQVFRILH	SRGVEVA L I N	DLTDNKTLAH		X16595
D. melanogaster	MS	VK I GINGFGR	IGRLVLRAAI	DKGANVVAVN	DPFIDVKYMV		M11255

quences. These were selected to include most aligned and available aquatic animal sequences and representative samples of mammalian muscle sequences and major groups of multicellular eukaryotes. The high degree of similarity of the *E. electricus* sequence to other GAPDH sequences was evident on first sight and permitted an almost unambiguous alignment. At positions present in all sequences included in the analysis, *E. electricus* GAPDH showed <92.5% identity to most of GAPDH molecules analysed (Table II). Residues strictly conserved among all eukaryotic GAPDHs were also conserved in the *E. electricus* molecules, including the **FGRIGR** sequence (residues 8–13) in the AMP site of the NAD^+ binding N-terminal domain.

Discussion

Biochemical modification of the glycolytic metabolism of electric organ of *E. electricus* has been studied as a function of denervation with substantial alteration of the activities of the enzymes lactate dehydrogenase (LDH), malate dehydrogenease (MDH) and also the concentration of ATP, lactic and pyruvic acids (Torres da Matta *et al.*, 1985). The GAPDH is a glycolytic allosteric enzyme that could be subjected to metabolic regulation and has been purified from various sources, but no work has been carried out on the *E. electricus* enzyme. Experiments on the homogeneity and various physicochemical properties of the purified enzyme are described in the present work. The GAPDH from *E. electricus* organ was purified by ammonium sulfate fractionation followed by DAC-Sepharose column. The last step, which corresponds to a 37.3% fraction (4.870 U mg^{-1}), yielded the enzyme (Table I). It is interesting to note that all other reported GAPDHs have been purified from precipitates obtained above 50% ammonium sulfate concentration, some being above 70%. This observation suggests a similarity in the overall surface structure of these enzymes. The purified GAPDH was found to be homogeneous, as judged by SDS-PAGE and IEF being its isoletric point similar to most mammalian enzymes (pI 7.2–7.4). The enzyme was also found to be very similar to protein from lobster (92.5%), rabbit muscle (85%), sequence obtained in our laboratory from a commercial source (Sigma Chemical Co, U. S. A.) and human liver (80%) enzyme with respect to N-terminal amino acid (Table II) and specific activity (Vieira *et al.*, 1983; Kuzminskaya *et al.*, 1991; Soukri *et al.*, 1995; Bourguignon *et al.*, 1997). No sequence similarity was found with the corresponding enzyme from *Trichomonas vaginalis.*

In conclusion, the use of a one-step procedure for its purification, has lead to an improved access for partially sequencing the protein and some enzymological studies and may be important to understand its role in the carbohydrate metabolism and muscular origin of the electric organ of *E. electricus.*

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