Purification and Amino Acid Sequence of Fructose-1,6-bisphosphate Aldolase from the Electric Organ of *Electrophorus electricus* (L.)

Salvatore G. De-Simone[a,b,*], Christiane M. Cardoso de Salles[a], Celia M. Batista e Silva[c], and Aida Hassón-Voloch[c]

[a] Laboratório de Bioquímica de Proteínas e Peptídeos, Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, 21045-900, Rio de Janeiro, RJ, Brasil. Fax: +55 21 2590 3495. E-mail: dsimone@ioc.fiocruz.br

[b] Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brasil

[c] Laboratório de Físico-Química Biológica, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, RJ, Brasil

* Author for correspondence and reprint requests

Z. Naturforsch. **61c**, 884–888 (2006); received February 20/May 26, 2006

A soluble fructose-1,6-bisphosphate aldolase enzyme has been purified 50.2-fold (2.36%) at the homogeneity from the electric organ of *Electrophorus electricus* by one step of DEAE-52 anion exchange chromatography followed by Superose-12 gel filtration-FPLC. Like other aldolase enzymes the *E. electricus* protein is a dimer with two identical subunits of 45 kDa. The *N*-terminal (20 residues) revealed a high homology with *S. aurata* (75%, goldfish), *R. ratus* and *M. musculus* (mouse, 80%) enzymes.

Key words: Fructose-1,6-bisphosphate Aldolase, *Electrophorus electricus* (L.), Purification

Introduction

Fructose-1,6-bisphosphate aldolases (FBPAs; EC 4.1.2.13) are ubiquitous enzymes that catalyze the reversible cleavage of fructose-1,6-bisphosphate (Fru-1,6-P2) and fructose-1-phosphate (Fru-1-P) to dihydroxyacetone phosphate (DHAP) or glyceraldehyde-3-phosphate (G3P). The formation of G3P and DHAP by FBPA constitutes an important step in the Embden-Meyerhof-Parnas pathway (Bessman and Geiger, 1981). Two different classes of FBPAs, which share no significant sequence identity, have so far been characterized (Lorentzen *et al*., 2003). Class I aldolases use covalent catalysis through a Schiff-base intermediate with ketose sugar substrates. The classical FBPA I is mainly found in animals and higher plants and is known to be homotetrameric from several crystal structures (Cooper *et al*., 1996). Class II aldolases of most bacteria and fungi require a divalent metal cation as a cofactor and form a homodimer (Kim *et al*., 1998). Among the class I enzymes found in mammals, there are three tissue-specific isozymes of aldolase that have similar molecular masses and catalytic mechanisms: aldolase A (expressed in muscle and red blood cells), aldolase B (expressed in liver, kidney, and small intestine), and aldolase C (expressed in brain, smooth muscle, and neuronal tissue) (Lebherz and Rutter, 1969). The aldolase isozymes are similar in sequence (Rottmann *et al*., 1987) and they have strictly conserved residues in the active site region consisting of Asp33, Arg42, Lys107, Lys146, Glu187, Ser271, Arg303, and Lys229 (which forms the Schiff-base intermediate) (Choi *et al*., 2001). Aldolases A and B have well-defined roles in glycolysis and gluconeogenesis, but the physiological role of aldolase C remains elusive.

Aldolase not only plays a key role in glycolysis, but also binds to macromolecules unrelated to glycolysis such as F-actin, vacuolar H^+-ATPase, GLUT4, cell surface adhesins, S100A1, thrombospondin-related anonymous protein (TRAP),

Abbreviations: BME, beta-mercaptoethanol; BSA, bovine serum albumin; DHAP, dihydroxy-acetone phosphate; DEAE, diethylamino ethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fru-1,6-P2, fructose-1,6-bisphosphate; FBPA, fructose-1,6-bisphosphate aldolase; Fru-1-P, fructose 1-phosphate; G3P, glyceraldehyde-3-phosphate; G6PDH, glyceraldehyde-6-phosphate dehydrogenase; HK, hexokinase; PCk, phosphocreatine kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TRAP, thrombospondin-related anonymous protein; Tris, tris(hydroxymethyl)aminomethane.

0939–5075/2006/1100–0884 $ 06.00

brain dynein light chain LC8, RNA and tubulin (Arakaki *et al*., 2004). These interactions may be specific to each isozyme. Aldolase B binds to the liver cytoskeleton with higher affinity than to the muscle or brain cytoskeleton. Aldolase C binds less tightly to the cytoskeleton compared with the other two isozymes and this has been attributed to its more acidic pI (Kusakabe *et al*., 1997).

The electric organ of *Electrophorus electricus* (L.) appears to be derived from an atrophied muscle with developed capacity to generate energy for the electric discharge (Nachmansohn *et al*., 1946). The capacity of the electric organ of *E. electricus* for aerobic metabolism is low and the metabolic energy is principally provided by conversion of glycogen to lactate (Williamson *et al*., 1967). Few works have reported characteristics of glycolytic enzymes of the electric organ. In this paper we report the results of the purification and partial characterization of the aldolase from the electric organ of *E. electricus.*

Materials and Methods

Materials

Specimens of *Electrophorus electricus* (L.) were received from the Goeldi Museum (Belém do Pará, Brazil). Phosphocreatine kinase (PCk), triethanolamine, ADP, phenylmethylsulfonyl fluoride (PMSF), hexokinase (HK), glyceraldehyde-6-phosphate dehydrogenase (G6PDH), dithiothreitol (DTT), AMP, NADP, mercaptoethanol, bovine serum albumin (BSA) and diethylamino ethyl (DEAE) cellulose-52 were purchased from Sigma Chemical Company (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) and electrophoresis reagents were from Bio-Rad (Richmond, USA) while sequencing reagents were from Shimadzu (Kyoto, Japan). All other chemicals were from Merck (Darmstadt, Germany).

Tissue extracts

Specimens of *Electrophorus electricus* (L.) were decapitated and a piece of the main organ was excised and homogenized [1 part of tissue to 1.5 parts of solution containing 1 mM Na-EDTA (adjusted to pH 7.6)] in a Sorvall Omni-Mixer. The extractions as well as all subsequent steps were performed at 4 °C. The homogenate was gently stirred for 5 h and the mixture centrifuged ($4{,}300 \times g$, 20 min, 4 °C). The supernatant was spun down ($100{,}000 \times g$, 2 h) and 40% $(NH_4)_2SO_4$ was added. After stand for 2 h at room temperature, the tubes were centrifuged ($20{,}000 \times g$, 30 min), the pellet discarded and $(NH_4)_2SO_4$ was added until 80% saturation. After a new step of centrifugation ($20{,}000 \times g$, 1 h), the aldolase activity was recovered in the pellet.

Ion exchange chromatography

The $(NH_4)_2SO_4$ pelleted material after dissolution was dialyzed exhaustively against Tris-phosphate buffer (10 mM, pH 7.2) containing $MgSO_4$ and 1 mM PMSF. The precipitated dialyzed material was removed by centrifugation and the supernatant applied onto a DEAE-52 column (42×1 cm I.D.), previously equilibrated with the same Tris-phosphate buffer. After washing with the equilibrating buffer the adsorbed proteins were eluted using a linear gradient of NaCl (0 to 1.5 M) in Tris-phosphate buffer. Fractions of 3 ml were collected and the absorbance was measured at 280 nm.

Gel filtration chromatography

The DEAE-52 aldolase containing peak was concentrated (10-fold) and loaded onto a Superose-12 column (30×1 cm I.D.) coupled in a FPLC system (Pharmacia Fine Chemicals, Uppsala, Sweden). Proteins were eluted using 50 mM Tris-phosphate buffer, pH 7.6, containing EDTA and PMSF (1 mM) at a flow rate of 0.3 ml/min.

Enzymological assay

The aldolase activity assay was based on the spectrophotometric method of Oliver (1955) for the reverse reaction (hydrolysis of phosphocreatine). The substrate mixture (100 mM triethanolamine buffer; 25 mM glucose; 12.5 mM magnesium acetate; 1.25 mM ADP; 1300 U/l HK/G6PDH; 1.25 mM DTT; 12.5 mM AMP; 0.75 NADP and 100 mM phosphocreatine) was prepared shortly before use and the pH value adjusted to 7.0. The reaction was initiated by addition of phosphocreatine and recorded for 3 min at 340 nm (Hitachi Spectrophotometer, Model U-330). The activity was expressed as μmol of phosphocreatine kinase (PCk) hydrolyzed per min per milligram protein at 25 °C. For each μmol of PCk consumed, 1 mmol of NADP was produced. Protein was estimated using the Folin-phenol method (Lowry *et al*., 1951).

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide gels in Laemmli buffers (Laemmli, 1970) under reduction conditions. The gels were Coomassie blue stained.

NH_2-terminal sequence

NH_2-terminal amino acid sequence analysis of the purified aldolase was carried out by automated sequential Edman degradation on a gas-phase protein sequencer (Shimadzu, Kyoto, Japan, Model PSQ-1) using the purified protein electroblotted onto a PVDF membrane (De-Simone *et al.*, 2005).

Results

Purification of aldolase

Two steps were used to purify to apparent homogeneity the aldolase enzyme from the electric organ of *E. electricus* with high recovery. The purification procedure described here differs from those reported previously. The aldolase activity was found in the soluble extract (0.71 μmol PCk min^{-1} mg^{-1} protein) obtained from 4.1 g of electric organ and was precipitated with 80% ammonium sulfate.

By passing the dialyzed material (5.08 μmol PCk min^{-1} mg^{-1} protein) through the DEAE column, at least 5 peaks were obtained (Fig. 1A). Peak 2 contained the enzymatic activity 13.3 μmol PCk min^{-1} mg^{-1} protein) and the SDS-PAGE analysis showed three bands with 43kDa–45 kDa. The protein was purified in a further subsequent step by gel filtration FPLC (Fig. 1B). The pooled peak presented only a single band with apparent 45 kDa and an activity of 25.5 μmol PCk min^{-1} mg^{-1} protein. This method affords aldolase to be purified 50 times and the enzyme was apparently homogenous as assessed by SDS-PAGE and *N*-terminal amino acid sequence analysis.

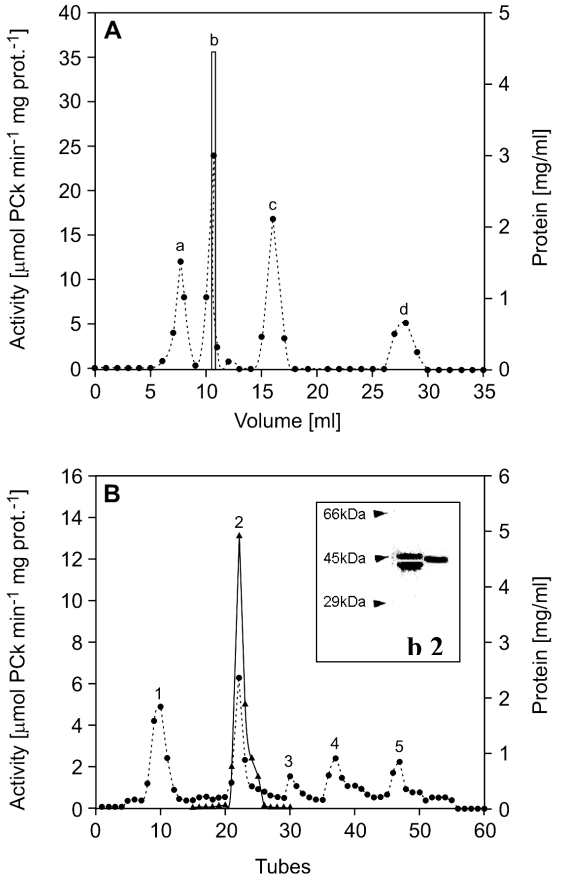

Fig. 1. Purification of the electric organ aldolase activity. (A) The 80% $(NH_4)_2SO_4$ precipitated material was solubilized with Tris-phosphate buffer (10 mM, pH 7.2) and loaded onto a pre-equilibrated DEAE-52 column. The proteins were eluted with a linear NaCl gradient (0–1.5 M) in the same buffer. (B) Fractions with aldolase activity (dark line) were fractioned on a Superose-12 column. Elution was performed with Tris-phosphate buffer (50 mM, pH 7.6). SDS-PAGE (12%) gel shows the analysis of the two peaks (b and 2) with maximal enzymatic activity.

NH_2-terminal sequence

The sequence of a 20 residue-log *N*-terminal sequence obtained by direct amino acid sequencing is shown in Table I. This sequence was aligned and compared with 5 other aldolase sequences. The high degree of similarity of the *E. electricus* (L.) sequence to the other aldolase sequences was evident on first sight and permitted an almost unambiguous alignment. At positions present in all sequences included in the analysis of *E. electricus* aldolase showed 75%–80% identity to most of the aldolase molecules analyzed (Table I) except for related *S. cerevisiae* enzyme (32.7%). No appreciable sequence identity was also found with torpedo, kidney (Friedman and Perryman, 1991), and non-sarcomeric or sarcomeric mitochondrial aldolase proteins (Haas and Strauss, 1990).

Table I. Comparison of the *E. electricus* fructose-1,6-bisphosphate aldolase sequence protein determined by Edman degradation with data bank described sequences.

Source (type)	Sequence	Acession no.	Similarity (%)
Electrophorus electricus	PHAYPALTPE OKKELSQIAQ	this work	
Xenopus laevis (ovary)	PHQYPALTPE OKKELHDIAK	AB2267	80
Oryctolagus coniculus (A)	PHSHPALTPE OKKELSDIAH	K02300	80
Ratus norvegicus (A)	PHPYPALTPE OKKELADIAH	X04261	80
Ratus norvegicus (B)	AHRF PALTSE QKKELSEIAQ	X02284	75
Sparus aurata (goldfish)	THQYPALTTE OKKELQDIAQ	U36777	75
Homo sapiens (B)	AHRFPALTSE QKKELSEIAQ	D00176	75
Mus musculus (A)	PHP YPALTPE OKKELSDIAH	Y00516	80
Ratus ratus	PHP YPALTPE OKKELADIAH	M14420	80
Euonymus japonicus	AALYPALTPE QKKELAEIAQ	D38619	75

Discussion

The *E. electricus* aldolase enzyme was obtained from an aqueous extract after a 100,000 × *g*-centrifugation which showed that the enzyme is well soluble. This result is similar to those found for aldolase of lamprey liver and muscle (Zhang *et al*., 1997).

Using the two procedure steps described in this work the aldolase enzyme was purified 50-fold. By SDS-PAGE analysis it presented a molecular weight of 45 kDa and by gel filtration analysis it was eluted with a retention time corresponding to 80 kDa. These results show that the enzyme is formed by two identical subunits. Lamprey muscle and non-muscle aldolases had molecular masses estimated both to be 160 kDa while those of their subunits estimated by SDS-PAGE were 40 kDa (Chappel *et al*., 1978). As the structural differences of the active site between the aldolase isoforms are not large it is possible that the hybrid form represents a specialization of the assemblage muscle-electric organ during the stage of *E. electricus* evolution.

In summary, we have used a two-step procedure to purify the aldolase enzyme of the electric organ of *E. electricus*. The enzyme is a water-soluble protein with two identical subunits and *N*-terminal amino acid residues similar to other aldolase enzymes. As the organ requires a low glycolytic demand (Torres da Matta *et al*., 1985), it is possible that this electric organ may require the aldolase enzyme to serve in the coordination of energy production and utilization in the region of the electric organ. The regulation of cellular energy metabolism occurs, in part, through compartmentation of metabolic pathways within specific organelles, but also through the function coupling of specific energy-generating pathways with specific energy-utilization pathways. This last hypothesis may include the principal function of the aldolase *E. electricus* enzyme. Thus further studies will be necessary to clearly determine in which cellular activities the aldolase enzyme is involved.

Acknowledgements

This study was supported by CNPq, FAPERJ and FIOCRUZ.

Arakaki T. L., Pezza J. A., Cronin M. A., Hopkins C. E., Zimmer D. B., Tolan D. R., and Allen K. N. (2004), Structure of human brain fructose 1,6-(bis)phosphate aldolase: lysozyme structure with function. Prot. Sci. **13**, 3077–3084.

Bessman S. P. and Geiger P. J. (1981), Transport of energy in muscle: The phosphocreatine shuttle. Science **211**, 448–452.

Chappel A., Hoogenraad N. J., and Holmes R. S. (1978), Purification and properties of the native form of rabbit liver aldolase. Evidence for proteolytic modification after tissue extraction. Biochem. J. **175**, 377–382.

Choi K. H., Shi J., Hopkins C. E., Tolan D. R., and Allen K. N. (2001), Snapshots of catalysis: The structure of fructose-1,6-(bis)phosphate aldolase covalently bound to the substrate dihydroxyacetone phosphate. Biochemistry **40**, 13868–13875.

Cooper S. J., Leonard G. A., McSweeney S. M., Thompson A. W., Naismith J. H., Qamar S., Plater A., Berry A., and Hunter W. N. (1996), The crystal structure of a class II fructose-1,6-bisphosphate aldolase shows a novel binuclear metal-binding active site embedded in a familiar fold. Structure **4**, 1303–1315.

De-Simone S. G., Correa-Netto C., Antunes O. A. C., De-Alencastro R. B., and Silva Jr. F. P. (2005), Bio-

chemical and molecular modeling analysis of the ability of two *p*-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms. J. Chromatogr. B **822**, 1–9.

Friedman D. L. and Perryman M. B. (1991), Compartmentation of multiple forms of creatine kinase in the distal nephron of the rat kidney. J. Biol. Chem. **266**, 22404–22410.

Haas R. C. and Strauss A. W. (1990), Separate nuclear genes encode sarcomere-specific and ubiquitous human mitochondrial creatine kinase isoenzymes. J. Biol. Chem. **265**, 6921–6927.

Kim H., Certa U., Dobeli H., Jakob P., and Hol W. G. (1998), Crystal structure of fructose-1,6-bisphosphate aldolase from the human malaria parasite *Plasmodium falciparum*. Biochemistry **37**, 4388–4396.

Kusakabe T., Motoki K., and Hori K. (1997), Mode of interactions of human aldolase isozymes with cytoskeletons. Arch. Biochem. Biophys. **344**, 184–193.

Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature **227**, 680–685.

Lebherz H. G. and Rutter W. J. (1969), Distribution of fructose diphosphate aldolase variants in biological systems. Biochemistry **8**, 109–121.

Lorentzen E., Pohl E., Zwart P., Stark A., Russell R. B., Knura T., Hensel R., and Siebers B. (2003), Crystal structure of an archaeal class I aldolase and the evolution of (beta alpha)$_8$ barrel proteins. J. Biol. Chem. **278**, 47253–47260.

Lowry O. H., Rosebrough N. J., Farr L. A., and Randall R. J. (1951), Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275.

Nachmansohn D., Cox R. T., Coates C. W., and Machado A. L. (1946), Phosphocreatine as energy source of the action potential. Proc. Soc. Exp. Biol. Med. **52**, 97–99.

Oliver I. T. (1955), A spectrophotometric method for determination of creatine phosphokinase and myokinase. Biochem. J. **61**, 116–122.

Rottmann W. H., Deselms K. R., Niclas J., Camerato T., Holman P. S., Green C. J., and Tolan D. R. (1987), The complete amino acid sequence of the human aldolase C isozyme derived from genomic clones. Biochimie **69**, 137–145.

Torres da Matta J., Voloch A. H., and Hargreaves A. B. (1975), Lactate dehydrogenase from the electric organ of *Electrophorus electricus* (L.): Isozyme analysis. Comp. Biochem. Physiol. **52B**, 351–354.

Williamson J. R., Herczeg B. E., Coles H. S., and Cheung W. Y. (1967), Glycolytic control mechanisms. V. Kinetics of high energy phosphate intermediate changes during electrical discharge and recovery in the main organ of *Electrophorus electricus*. J. Biol. Chem. **242**, 5119–5124.

Zhang R., Kusakabe T., Iwanaga N., Sugimoto Y., Kondo K. Takasaki Y., Imai T., Yoshida M., and Hori K. (1997), Lamprey fructose-1,6-bisphosphate aldolase: Characterization of the muscle-type and non-muscle-type isozymes. Arch. Biochem. Biophys. **341**, 170–176.