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Nuclear localization of aldolase A in pig cardiomyocytes

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Summary. The subcellular localization of the muscle aldolase (aldolase A) in cardiomyocytes was determined immunocytochemically by light and electron microscopy. The enzyme was localized in the cytoplasm and also in cardiomyocyte nuclei. Inside the nuclei it was preferentially localized in the heterochromatin region. The nuclear localization was confirmed by the measurement of aldolase activity in subcellular fractions of a heart muscle, and in isolated nuclei of cardiomyocytes. There was no detectable aldolase activity in isolated cardiomyocyte nuclei fractions if the fraction was not preincubated with a solution containing Triton X-100 and KCl. The calculated concentration of aldolase in the nucleus was about 0.6 μM. This paper is the first report on the localization of aldolase A inside cardiomyocyte nuclei.

Key words: Adolase, Cardiomyocyte, Nucleus, Heterochromatin

Introduction

D-fructose 1,6-bisphosphate aldolase [EC 4.1.2.13] catalyzes the cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. A, B and C aldolase isozymes have been found in mammalian tissues. The tissue distribution study revealed that aldolase A is predominantly located in muscle, aldolase B in the liver and the kidney (Lebherz and Rutter, 1969; Penhoet et al., 1969a). In other tissues two different aldolase isozymes and their hybrid forms have been found as a result of simultaneous expression of two isozyme genes, like in the brain where aldolase A and C and their hybrids are present (Penhoet et al., 1969a).

The subcellular localization of aldolase isozymes has also been investigated. Investigations revealed that muscle aldolase is bound to F-actin (Palgiaro and Taylor, 1988; Wang et al., 1997). In a skeletal muscle as well as in the cardiomyocytes aldolase has been found on the sarcoplasmic reticulum (Xu and Becker, 1998). Aldolase B has been localized in a nuclear position in the liver and renal cells (Saez and Slebe, 2000). Our preliminary experiments indicated that the presence of aldolase A in the nuclei of the cardiomyocytes might also be expected. The primary aim of the present work was to locate aldolase in the nuclei of cardiomyocytes. The results of immucytochemical investigation with light and electron microscopy as well as the determination of aldolase activity in the isolated nuclei of cardiomyocytes confirmed that aldolase is localized within the nuclei of cardiomyocytes.

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Materials and methods

Antibody Diluent with Background Reducing Components and DAB chromogen were purchased from DAKO (USA), Paraformaldehyde, Glutaraldehyde and Coomassie Brilliant Blue R–250 were from Fluka (Switzerland) and polyester wax was from EMS (USA). Anti-Rabbit IgG gold conjugate (5 nm) was from ICN (USA). Anti-mouse IgG (Fc specific) Peroxidase Conjugated, Nitrocellulose Membranes, Fructose 1,6-bisphosphate (F1,6-P2), Bovine Serum Albumin (BSA), Dithiothreitol (DTT), Ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), Ethylene-diaminetetraacetic acid (EDTA), Phenylmethylsulfonyl fluoride (PMSF), N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), ß-Nicotinamide adenine dinucleotide, reduced form (NADH) and other reagents were from Sigma (USA).

All reagents were of the highest purity commercially available.

Enzyme purification and determination of its activity

Rabbit muscle aldolase and pig muscle aldolase were purified according to Penhoet et al. (1969) and Dzugaj et al. (1974), respectively. Aldolase activity was assayed spectrophotometrically at 37 °C in the presence of coupling enzymes: triose 3-phosphate isomerase and glycerol 3-phosphate dehydrogenase following the decrease of NADH absorbance. The assay system of 1 ml contained: 50 mM Tris-HCl buffer, pH 7.5, 5 U

glycerol 3-phosphate dehydrogenase, 5 U triose 3-phosphate isomerase, 1 mM fructose 1,6-bisphosphate and 0.2 mM NADH.

Antibody production and Western blot

Antiserum against the rabbit muscle aldolase was raised in mice by an intracutaneous injection of the electrophoretically pure enzyme and Freund's complete adjuvant. Immunoglobulins were purified by ammonium sulfate precipitation according to Tijssen (1985). The specificity of the immunoglobulins was checked by Western blot (Towbin et al., 1979). SDS-PAGE (Sodium dodecyl sulphate – polyacrylamide gel electrophoresis) was performed according to Laemmli (1970). The final acrylamide monomer concentration was 10% (w/v) for the separating gel and 4% (w/v) for the stacking gel.

Immunocytochemistry

Light microscopy

A fresh porcine heart was provided by a local slaughterhouse. A muscle tissue cut out from the porcine heart was fixed in Bouin's fluid and embedded in polyester wax. Dewaxed tissue sections were treated with 1% H_2O_2 for 30 min, washed in PBS (phosphate-buffered saline), pH 7.4, and incubated with 10% normal goat serum for 30 min at room temperature. Subsequently, the sections were incubated overnight at 4 ºC with polyclonal anti-muscle aldolase immunoglobulins (at 1:30 dilution). Then, sections were rinsed in PBS and incubated with goat anti-mouse IgG (Fc-specific) peroxidase-conjugate (20 min. at 37 ºC; dilution 1:50) and washed in PBS with 0.1% Triton X-100. Finally, the peroxidase activity was developed using 3,3'-diaminobenzidine. In control reactions the primary antibody was omitted, or normal mouse serum was used as a first layer. All immunoglobulins were diluted in Antibody Diluent.

Electron microscopy

Small fragments of the porcine heart muscle tissue were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in phosphate buffer and embedded in Epon. Sections were mounted on nickel grids. Before immunostaining, sections were incubated with 2% normal goat serum, 1% BSA and 50 mM glycine in TBS-Triton X-100 (TBS – Tris-buffered saline: 50 mM Tris, 2.5% NaCl, 0.2% Triton X-100). Then they were incubated with polyclonal antibodies against muscle aldolase (at 1:50 dilution) overnight at 4 °C, washed with TBS and incubated with goat anti-mouse antibody gold-conjugate (1:100) for 1.5 h at room temperature. After that, grids were thoroughly washed with TBS and distilled water to remove unbound antibodies. Before microscopic examination, the sections were counterstained with uranyl acetate.

In controls, the primary antibodies were omitted or the non-immune mouse serum was used as a first layer.

Heart muscle fractionation

5 g of porcine ventricles were placed in 45 ml of buffer A (10 mM HEPES, 1 mM DTT, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM PMSF, 2 mM $MgCl_2$, 0.25 M sucrose, pH 7.4, at 4 °C) and homogenized. The homogenate was subsequently filtered through two and four layers of a cheesecloth and fractionated by the differential centrifugation method (de Duve et al., 1955).

Each pellet was dissolved in a small amount of buffer A and centrifuged again for the maximal removal of cytosol. Fractions were pre-incubated with 1% Triton X-100 and 250 mM KCl for 15 min. at room temperature, and aldolase activity was measured.

Isolation of cardiomyocyte nuclei

Cardiomyocyte nuclei were isolated using a non-enzymatic extraction technique as described by Gizak and Dzugaj (2003). All steps were performed at 4 °C. 5 g of porcine ventricles were placed in 45 ml of buffer A and homogenized for 15 s. The homogenate was filtered as described above and centrifuged at x1000g for 10 min. The pellet was resuspended in 15 vol. of buffer A, filtered through Millipore filter (pore size 41 μm) and centrifuged as before. The pellet was resuspended in 10 ml of buffer A with 0.5% Triton X-100 to further lyse the cells, centrifuged as before, and finally resuspended in 2 volumes of buffer containing 10 mM HEPES, 1 mM DTT, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM PMSF, 2 mM $MgCl_2$ and 2.2 M sucrose (pH 7.4 at 4 °C). Nuclei resuspended in the above-mentioned buffer were layered on two ice-cold layers of sucrose (2.7 M and 2.4 M. sucrose) and centrifuged in a TLS-55 Beckman rotor at 36 000 rpm (x112000g) for 2 hours at 4 °C. After centrifugation, cardiomyocyte nuclei could be isolated at the interface of the 2.4/2.7 M sucrose.

Then the nuclei were suspended in the appropriate volume of buffer with 0.25 M sucrose to achieve a 2.2 M final concentration of sucrose, and layered again on the sucrose gradient and centrifuged as described above. These steps were performed to ensure the high purity of cardiomyocyte nuclei.

The aldolase activity in isolated cardiomyocyte nuclei was determined after pre-incubation of the sample with a solution containing 1% Triton X-100 and 250 mM KCl for 15 min at room temperature.

To determine the number of isolated nuclei, Thom-Zeiss cell was used.

Results

Immunocytochemical localization of aldolase A in cardiomyocytes and Western blot analysis

The subcellular distribution of muscle aldolase was

examined with light microscopy and transmission electron microscopy. The analysis of tissue sections stained with the indirect immunoperoxidase method revealed a strong positive reaction in cardiomyocyte nuclei and a weaker one in the cytoplasm (Fig. 1A). No labelling occured in the absence of the primary antibody, or when the normal serum was used instead of the primary antibody (Fig. 1B).

The specificity of the interaction between antiserum and its antigen was checked by Western blot analysis. The results showed a single band in the homogenate of the pig heart, corresponding to the purified pig muscle aldolase (Fig. 2).

The results obtained from light microscopy were confirmed by using electron microscopy and the indirect immunogold method. Aldolase A was localized within sarcomeres and in nuclei. Staining was observed on Z-lines and the actin-containing filaments (Fig. 3A). In the nuclei, the positive reaction was restricted to the heterochromatin area (Fig. 4A). No labelling occurred in the absence of the primary antibody, or when the normal serum was used as a first layer (Fig. 3B, 4B). The same results were obtained from a rat heart tissue analysis (data not shown).

Pig heart fractionation and isolation of cardiomyocyte nuclei

The activity of aldolase from 7 porcine hearts was investigated in the course of this study. The fractions obtained through differential centrifugation enabled a preliminary estimation of subcellular distribution of aldolase. The aldolase activity was detectable in all fractions (Table 1). Pre-incubation of the fractions with the solution containing Triton X-100 and KCl resulted in the increase of the aldolase activity. Additionally, there was no detectable aldolase activity in cardiomyocyte nuclei fraction if the fraction was not preincubated with the solution containing Triton X-100 and KCl. The control reaction indicated that Triton X-100 and KCl had no effect on aldolase activity determination (data not shown). This indicated that the aldolase activity measured in the cardiomyocyte nuclei fraction was related to the enzyme which was released from the nuclei.

It is well known that noncardiomyocyte nuclei constitute about 70% of all cardiac nuclei (Jackowski and Kun, 1981). A non-enzymatic extraction technique (Gizak and Dzugaj, 2003) was used to isolate a pure fraction of cardiomyocyte nuclei from the total amount. Importantly, this technique ensured that no cytosol remained in the fraction. No residual contamination by myofilaments or membranes was observed.

To determine the size and the volume of cardiomyocytes from paraffin-embedded material, ocular micrometry was used; in addition, a morphometric study of cardiomyocyte nuclei was performed, and nuclear volume was calculated. Assuming that mean nuclear

Table 1. Aldolase activity in homogenate and subcellular fractions.

FRACTION	ALDOLASE ACTIVITY (U/g tissue)		% ACTIVITY
	A	B*	
Homogenate	5.06	18.36	100
Nuclear fraction	0.037	0.26	1.4
Mitochondrial fraction	0.016	0.06	0.32
Microsomal fraction	0.034	0.12	0.65
Cardiomyocyte nuclei	0	0.036	0.19

*: Fractions pre-incubated with the solution containing 1% Triton X-100 and 250 mM KCl.

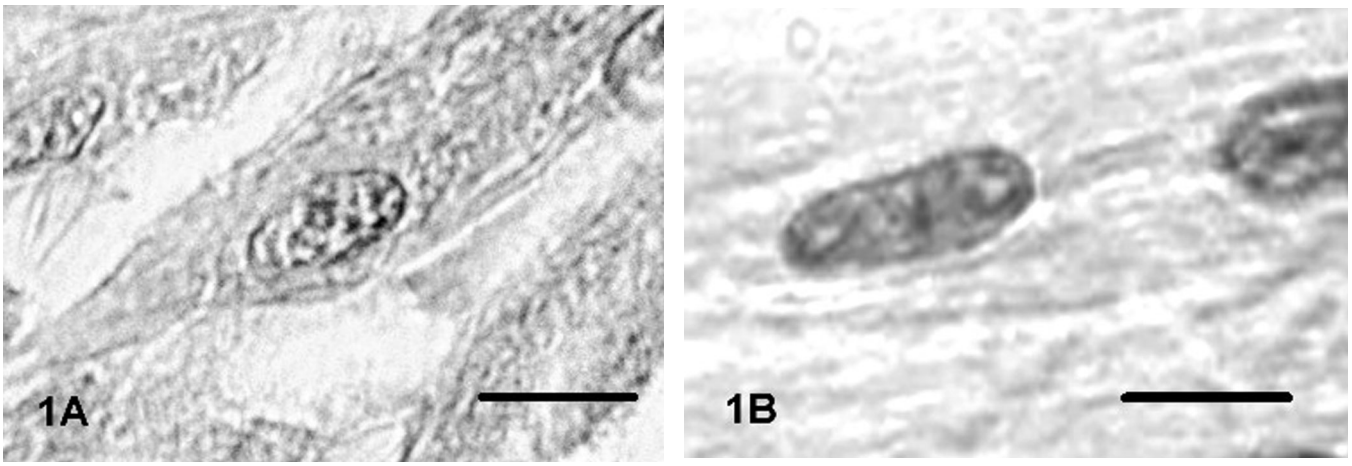

Fig. 1. Analysis of pig heart muscle sections stained with the indirect immunoperoxidase method. **A.** Tissue stained with anti-muscle aldolase immunoglobulins and peroxidase-conjugated secondary antibodies. **B.** Control reaction (with omission of the specific antibodies) counterstained with Mayer's haematoxylin. x 6000. Bar: 12 μm.

volume was 307 μm^3 the concentration of aldolase in the nucleus was about 0.6 μM compared with 5 μM in the whole cardiomyocyte.

Discussion

The reaction catalyzed by aldolase is reversible, therefore this enzyme may participate in glycolysis as well as in gluconeogenesis. It has been postulated that aldolase A is a glycolytic enzyme, and that aldolase B is tailored for gluconeogenic pathway (Penhoet et al., 1969b). Aldolase B is also involved in fructose metabolism. The specific role of aldolase C is unknown. Several papers are available indicating that in muscle cells the synthesis of glycogen from non-carbohydrate precursors takes place (McLane and Holoszy, 1979). Therefore, it may be expected that aldolase A catalyzes cleavage as well as the synthesis of F1,6-P_2. The heart muscle, like the skeletal muscle, is regarded as a striated muscle. Nevertheless, differences in metabolic pathways and in their regulation between these two types of muscles have been presented. Whether the synthesis of glycogen from carbohydrate precursors takes place in the heart muscle is still open to discussion. In the skeletal and heart muscle the same aldolase A isozyme has been found (El-Dorry and Bacila, 1977), but the aldolase concentration in the skeletal muscle is much higher, ca 140 μM, in comparison with ca 5 μM in the heart muscle.

The enzymes of carbohydrate metabolism have been considered as a soluble constituent of the cell but many studies indicate that enzymes of carbohydrate metabolism are reversibly associated with subcellular structures (Clarke and Masters, 1975; Masters et al., 1987; Ovadi and Srere, 2000; Gizak et al., 2003). This association alters regulatory properties and kinetics of the enzymes (Liou and Anderson, 1980), and, additionally, the interactions between metabolically sequential enzymes facilitate channeling of substrates (Ovadi and Srere, 2000). We found that the high percentage of aldolase in the heart muscle is bound to subcellular elements (Table 1). The activity of aldolase determined in homogenate is substantially increased after incubation with Triton X-100 and KCl.

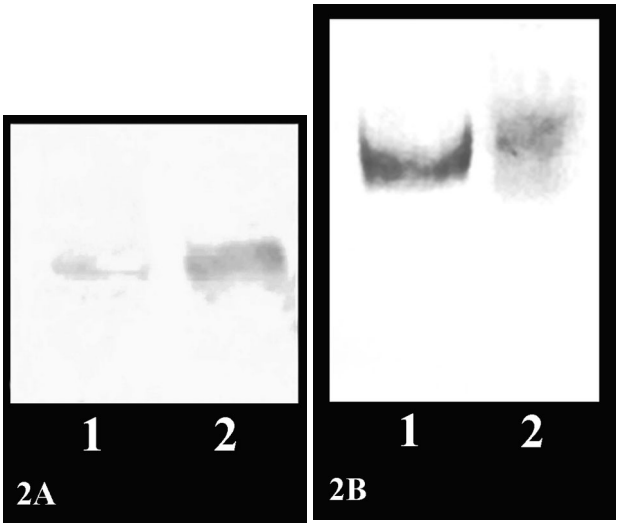

Fig. 2.. Western blot analysis. Blottings were carried out using anti-muscle aldolase immunoglobulins and peroxidase-conjugated secondary antibodies: **A.** Immunoblotting of 5 μl homogenate from the pig heart muscle (lane 1) and 1.5 μg purified pig muscle aldolase A (lane 2). Molecular weight of the pig muscle aldolase monomer: 39 kDa. **B.** Immunoblotting of 1.5 μg purified pig muscle aldolase A (lane 1) and 25 μl cardiomyocyte nuclei fraction (lane 2).

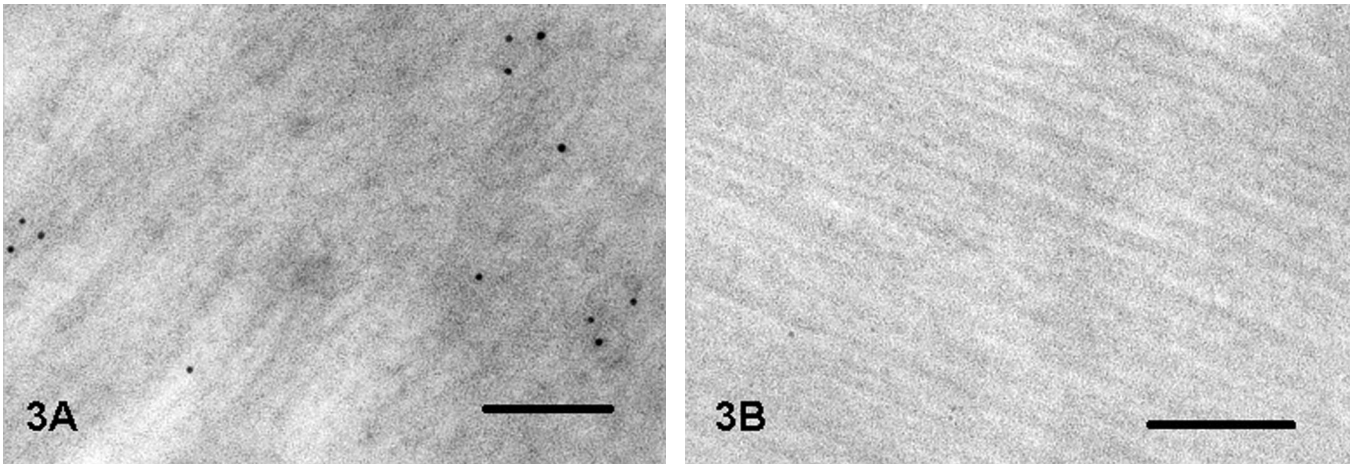

Fig. 3. Analysis of pig heart muscle ultrasections in electron microscopy. **A.** The localization of aldolase A in the cardiomyocyte cytoplasm. The tissue stained with anti-muscle aldolase immunoglobulins and gold-conjugated secondary antibodies. **B.** Control reaction (with omission of the specific antibodies). x 246,500. Bar: 100 nm.

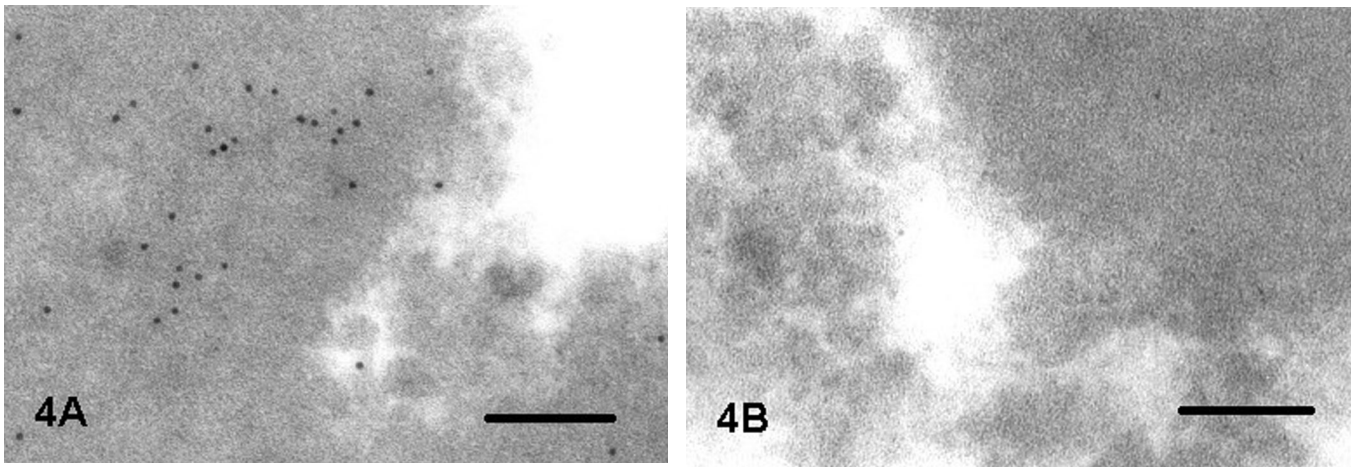

Fig. 4. Analysis of pig heart muscle ultrasections in electron microscopy. **A.** Heterochromatin localization of aldolase A in the cardiomyocyte nucleus. The tissue stained with anti-muscle aldolase immunoglobulins and gold-conjugated secondary antibodies. **B.** Control reaction (with omission of the specific antibodies). x 246,500. Bar: 100 nm.

Several enzymes of carbohydrate metabolism have been found in cell nuclei: glucokinase (Miwa et al., 1990); aldolase B (Saez and Slebe, 2000); glyceraldehyde 3-phosphate dehydrogenase; lactate dehydrogenase; phosphoglycerate kinase (for review see Ronai, 1993); glycogen synthase (Ferrer et al., 1997); and fructose 1,6-bisphosphatase (Gizak and Dzugaj, 2003). The presence of aldolase in the nuclei of cardiomyocytes raises a question concerning its physiological role. Unlike glycogen synthase, whose function is supposedly the same in the cytosol and the nucleus, the other enzymes' physiological roles seem to vary from the cytosol to the nucleus. It has been hypothesized that phosphoglycerate kinase may participate in DNA synthesis and cell-cycle progression (Popanda et al., 1998). Glyceraldehyde 3-phosphate dehydrogenase recognizes the sequence and structural features of the RNA and is involved in transcription (Sirover, 1997), lactate dehydrogenase is a recognized stabilizing factor and it participates in DNA reparation. One may hypothesize that fructose 1,6-bisphosphatase is phosphorylated in close vicinity to NLS and that might be the signal inducing the movement of the enzyme to the nucleus where it could induce the expression of its own gene. Aldolase in the cardiomyocyte nuclei seems to be associated with subnuclear structures. Considering the physiological role of aldolase in the nucleus at least two possibilities may be taken into account. Like nuclear glycogen synthase, aldolase might have the same function in the nucleus and in the cytosol. The other possibility is that aldolase in the nucleus is a signal protein modulating gene expression. The interaction of aldolase with DNA has been reported but no information on the physiological meaning of this phenomenon has been released (Ronai et al., 1992). The localization of aldolase in the heterochromatin region rather supports the latter possibility. Further studies are necessary to elucidate the physiological role of aldolase in the nucleus.

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