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Full Length Research Paper

NADP$^+$- dependent isocritrate dehydrogenase from human kidney mitochondria

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NADP-IDH was purified from the mitochondria of human kidney to homogeneity to about 1953-fold and a yield of 19.0%. The enzyme is a dimer with a molecular mass of about 220 to 240 kDa and dependent on Mn^{+2} or Mg^{+2} but other divalent cations do activate it at various degrees. Antibodies that were raised in the rabbit against the enzyme completely inhibited the activity of the enzyme, even when the enzyme could be protected partially by pre-incubating it with isocitrate or ADP. It can be protected fully by pre-incubating the enzyme with a mixture of isocitrate and ADP in the presence of Mg^{+2} or Mn^{+2}. The K_m values of the protected enzyme had the same K_m values for the substrates as those for the purified enzyme. Mononucleotides phosphatases have no effect on NAD-IDH, but all trinucleotides phosphatases especially ATP inhibit the enzyme. The inhibition by ATP (or NADH) cannot be counteracted by ADP in the presence of isocitrate, so ADP cannot enhance NAD-IDH activity nor reverse inhibition by ATP (or NADH), while isocitrate will bind to the enzyme and prevent it from interacting with ADP. The activity of NADP-IDH in mitochondria is probably controlled in a complex way by NADPH, ATP and divalent ions.

Key words: NADP$^+$-IDH, isocitrate, *P* cibacron blue, mitochondria, antibody interactions.

INTRODUCTION

Mitochondrial metabolism is an important source of carbon skeletons, via the tricarboxylic acid (TCA) cycle, which are used in anabolic processes such as porphoryin and amino acid synthesis. The tricarboxylic acid (TCA) cycle is a carbon dioxide fixative pathway where four molecules of CO_2 are fixed to produce one molecule of oxaloacetate in one cycle. The pathway has five enzymes: Isocitrate dehydrogenase, 2-oxoglutarate synthase, phosphoenol pyruvate carboxylase, pyruvate carboxylase and ATP-citrate lyase (Zera et al., 2011).

In humans, three different isocitrate dehydrogenases (IDHs) isoforms catalyze the decarboxylation of isocitrate into 2-oxoglutarate. NAD$^+$-dependent IDH3 (EC 1.1.1.41) is present in mitochondria, while two isoforms of NADP$^+$-dependent IDHs (EC.1.1.1.42) are found in mitochondria (IDH1) and in the cytosol or peroxisomes (IDH2) (Fedøy et al., 2007). The physiological roles of IDH1 and IDH2 are not clearly understood.

IDH1 and IDH2 play several important physiological roles that have been studied most intensively in mammals, plants and fungi (Koh et al., 2004; Contreras-Shannon and McAlister-Henn, 2004; Liu et al., 2006) and both enzymes have been purified and characterized from several invertebrates, vertebrates, plants and fungi with their cDNA sequences determined (Plaut and Gabriel, 1983; Gonzalez-Villaseñor and Powers, 1985; Huh et al., 1993; Loftus et al., 1994; Nekrutenko et al., 1998; Szewczyk et al., 2001; Haraguchi et al., 2003; Contreras-Shannon and McAlister-Henn, 2004) and both are homodimers encoded in the nuclear genome (Loftus et al., 1994).

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Abbreviations: NAD$^+$-IDH, Nicotinamide adenine dinucleotide-isocitrare dehydrogenase; **NADP$^+$-IDH,** nicotinamide adenine dinucleotide phosphate- isocitrare dehydrogenase; **ATP,** adenosine triphosphate; **ADP,** adenosine diphosphate.

This present study deal with the purification and properties of IDH1 purified from the mitochondria that was isolated from a human kidney using immuno-cytochemical techniques and biochemical methods.

MATERIALS AND METHODS

50 g of human kidney from a 20 year old assassinated female was obtained from Department of Forensic Medicine and Pathology, Faculty of Medicine, University of Jordan, Amman, Jordan with the signed consent of the deceased family.

Metabolites and molecular mass standards were purchased from Sigma. DEAE-cellulose, Sephadex G-200 and Sepharose CL-6B were obtained from Pharmacia Biotech. Chemicals for electrophoresis were purchased from Bio-Rad, while all other chemicals were supplied by Merck (Darmstadt, Germany). The reactive dye (Cibacron Blue F3GA) and Hybond-N^+were obtained from Polysciences Europe GmbH, Germany. Motor driven tightly fitting glass/Telfon Potter Elvehjem homogenizer (30 ml).

All glassware was washed three times with distilled water to avoid contamination. Ca^{2+} overload is the most common cause for the dysfunction of isolated mitochondria. All buffers were prepared in the same day of the experiment to avoid bacterial/yeast growth in stored buffers, and since pH depended on temperature, the pH must be measured of all solutions at 25°C.

Preparation of cibacron blue-Sepharose

Cibacron blue F3GA) was coupled directly to CL-Sepharose by the standard procedure of Bohme et al. (1972) as modified by Travis et al. (1976). The procedure is as follows: 1 L of washed Sepharose was mixed at room temperature with 1 L of 1 M NaOH containing 5 g of $NaBH_4$. The mixture was stirred gently and 20 ml of epichlorohydrin (cross-linking agent) was added before it was heated to 60°C for 1 h. The cross-linked gel was stable at high temperature, and washed on a coarse Buchner funnel with hot water until the washing was neutral.

The Sepharose-dye conjugate was prepared by suspending 500 ml of cross-linked gel in an equal volume of water and the mixture warmed to 60°C. The dye (500 mg in 50 ml of water) was added drop wise with vigorous shaking and after 15 min, 50 g of NaCl was added. The mixture was heated to 80°C and 10 g of Na_2CO_3 was added to ensure the chemical bonding of the dye. After 30 min, the dye gel was washed with hot water followed by 0.05 M tris-HCl buffer, pH 8.0 containing 0.05 M NaCl until the washing was colour free. Traces of blue dye were still eluted from freshly coupled gels during the first passage of the enzyme through the column, but subsequent usage however indicated no further dye leakage.

Purification of the mitochondria

The mitochondria were purified according to the method of Frezza et al. (2007) as modified by Gregg et al. (2009) from a human kidney which was rinsed free of blood by using ice-cold buffer A (0.05 M Tris/MOPS and 12.1 g of 1 M sucrose) and then was minced into small pieces using scissors at 4°C. The suspension was transferred into a Teflon pestle with a fresh buffer and homogenized at 1,600 g at 4°C to minimize activation of damaging phospholipases and proteases for few minutes. All other harsher techniques, including glass pestle in a glass potter, could easily damage the mitochondria. The optimal ratio between tissue and isolation buffer ranged between 1:5 and 1:10 (w:v).

The homogenate was transferred into a polypropylene Falcon tube and centrifuged at 600 g for 10 min at 4°C. The pellet (contained the cell debris and nuclei) was discarded and the supernatant (contained all the lighter cellular fractions) was centrifuged at 5,000 g for 20 min at 4°C in chilled centrifuge tubes. The supernatant (contained microsomes, membrane fragments, ribosomes, cytoplasmic enzymes) was decanted and the pellet (contained crude mitochondria) was resuspended in 12 ml of ice-cold buffer A. The re-suspension was enriched in the mitochondria, that was mixed with other organelles such as golgi apparatus, endoplasmic reticulum and vacuoles. In order to obtain pure mitochondria, this re-suspension was loaded onto a 4 ml each of 15, 23, 32 and 60 wt/v step sucrose density gradient prepared in the above buffer and centrifuged at 100,000 g in a SW60Ti rotor (Beckman Ultra centrifuge tube) for 5 h at 4°C. Fractions were then collected from the bottom of the gradient.

The intact mitochondria formed a brown band at the 60 to 32% sucrose interface and removed gently by using a pipette with a cut tip and placed into a separate Beckman centrifuge tube which was filled with buffer. The pure mitochondria was centrifuged for 30 min at 10,000 g at 4°C and the supernatant was decanted, while the precipitate consisted of pure mitochondria was used for the next step. The mitochondria should not be diluted with buffer in order to retain their functionality for a longer time. The concentration of mitochondria in this preparation was about 80 mg ml^{-1}.

Assays for the mitochondria

To identify the mitochondria, slides were prepared from all samples and a drop of methyl green pyronin was added and all slides observed at 400X with bright field: nuclei should stain green, cytoplasm red or pink, and mitochondria could be seen as small dots and protein concentration of each fraction was measured (Kurnick and Mirsky, 1950). Another method for identification of the mitochondria was to use the blue dye 2, 6 Dichlorophenol indolphenol (DCPIP). A mixture of 1 ml of 0.1 M succinate, 1 ml of 5mM KCN, 1 ml of mitochondrial preparation and 4ml 0.05M phosphate buffer (pH 7.5) were mixed and left for 5 min at 25°C. 1 ml of 70 uM DCPIP, 0.3% (w/v) was added to the mixture and read after 5 min at 600 nm. DCPIP will accept an electron from NADH (inside mitochondria) and becomes a reduced colourless form (Boyer, 2000).

Isolation and purification of $NADP^+$-IDH

Mitochondrial preparation (10 ml) was thawed at room temperature and loaded into Sepharose-dye conjugate column (1.5 × 20 cm) washed and equilibrated with buffer B (DEAE-cellulose column (1.5 × 30 cm), which had been equilibrated and washed with buffer B. The column was washed with this buffer until the eluent was free from protein, and the enzyme was eluted with 0 to 2M sodium chloride.

The peaked DEAE-cellulose fractions were pooled, desalted by dialysis and concentrated by colloidion bag and then loaded into a Sephadex G-200 (1.5 × 20 cm) and the bound proteins were collected. The fractions were assayed for enzyme activity and protein content and the peaked fractions were used as the purified enzyme. The peaked fractions from Sephadex G-200 column were pooled together and desalted by dialysis to remove any traces of salt and used as “The purified enzyme”.

Enzyme assay and protein determination

The $NADP^+$-IDH activity was determined by following the rate of $NADP^+$ reduction at 340 nm. The standard assay mixture contained

Table 1. Purification of NADP+-IDH from the mitochondria of a human kidney.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity(unit.mg^{-1})	Purification (-fold)	Yield (%)
Mitochondria preparation	10	2889	82.0	35.2	1	100
Sepharose-cibacron column	26	1393	10.9	127.7	3.62	48.2
DEAE-cellulose	32	965	1.2	804.1	22.8	33.4
Concentrated fraction	10	700	0.04	17500	497.1	24.2
Sephadex G-200	6	550	0.008	68750	1953.1	19.03

0.05 M buffer B, 1.0 mM $MnCl_2$ (or $MgSO_4$), 0.25 mM NADP, 4 mM DL-isocitrate, and an appropriate amount of the enzyme preparation in a final volume of 2.5 ml.

The enzyme activity measurements were made at the ambient temperature of 30°C in a final volume of 2.5 ml. The reaction was initiated by the addition of DL-isocitrate and followed by the increase in A_{340}.

The initial velocities were determined by measuring the change in absorbance at 340 nm uv-vis spectrophotometer. All assays were performed in triplicate. One unit of activity was defined as the amount of enzyme that catalyzed the production of 1 µmole of NADPH per ml of reaction mixture per min under the standard conditions.

Specific activity is defined as units (mg protein^{-1}). Protein was estimated according to Bradford (1976) using Bovine serum albumin (BSA) as a standard.

Estimation of native molecular mass

The molecular mass of $NADP^+$-IDH was estimated by gel filtration using Sephadex G-200 column (1.5 × 20 cm) previously calibrated with protein markers of known size: Glutamate dehydrogenase (350 kD), catalase (240 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), cytochrome C (12.5 kD).

The subunit(s) of NADP-IDH was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) and modified by Maizel (1971) using 12% polyacrylamide gel. Proteins were located in the gel by staining with 0.1% (w/v) Coomassie brilliant blue R-250 in 25% (v/v) ethanol and 10% (v/v) acetic acid. The very faint bands of $NADP^+$-IDH activity were located by running a separate lane which was cut out from the gel before staining. Furthermore, the lane was cut horizontally into 0.25 mm pieces and submerged in buffer B and assayed for enzyme activity as shown in this study's 'methods'. Protein markers earlier mentioned were used as standards.

Immunochemical procedures

Preimmune serum was collected before immunizing a white male rabbit (albinos from local market). Immunization was initiated by injecting 300 µg purified NADP-IDH protein emulsified in Freund's complete adjuvant. A second injection of 300 µg protein mixed with Freund's incomplete adjuvant was given a week later followed by two more injections (100 µg protein each time) at weekly intervals. Blood was collected from the marginal ear vein after 40 day.

The γ-globulin fraction isolated by ammonium sulphate fractionation of the antiserum was used in this study. The Ouchterlony plates were prepared with 1·5% agar in 0·01 M buffer A containing 0·14 M sodium chloride and 0·02% sodium azide. After placing the antigen and antibody in the wells cut into agar plates, the plates were developed in a humid chamber at 0 to 5°C for 24 h. Immunoreactive bands were visualized according to the method of Canton et al. (1996) with some modifications.

Determination of NADP-IDH kinetics

The kinetic properties of the enzyme were determined by using the purified enzyme. In all the kinetic measurements, sufficient amount of the enzyme was added to give a change in A_{340} of 0.1 to 0.2 min^{-1}. The reaction was carried out for 2 to 3 min and the rates were obtained from the initial linear portion of the curves. To overcome variations in reading, all enzyme kinetic assays were initiated by the addition of NADPH. In substrate saturation experiments, standard assay conditions were employed except for the concentration of the substrate being varied. These changes are mentioned at appropriate places.

The various potential inhibitors were incubated with the enzyme and the reaction was subsequently initiated by the addition of NADPH.

Statistical treatment and analysis of data

Enzyme kinetic data presented are typical reproductions of at least three independent experiments and the data were analyzed and plotted using the enzyme kinetics software Sigma Plot (version 8.0). A correlation coefficient (r) of 0·95 or higher was always obtained for these data.

Experimentally determined values are presented as points, while the lines represent best-fits of these data.

RESULTS

Purification

The purification of the IDH1 enzyme from the mitochondria of a human kidney is shown in Table I. The purification protocol rendered a homogenous preparation of $NADP^+$-dependent IDH (about 1953-fold) with specific activity of 68750 units.mg^{-1} and a recovery of 19.03%. Sephadex G-200 removed most of the contaminating proteins with an increase in the specific activity (Table 1). The active fractions of the column were preserved at -20°C, so that a little loss of the activity occurred.

The purity of the enzyme preparation was confirmed, also, by the Ouchterlony double diffusion test. A double antigen-antibody precipitin bands were observed when the purified enzyme was used. It was so faint that it was impossible to photograph them properly.

Table 2. Effect of divalent metal ions on $NADP^+$-IDH activity from the pooled fractions from Sephadex G-200.

Ion (mM)	Effect (100%)
Control	0
Manganese (0.5)	100
Manganese (1.0)	100
Manganese (2.0)	100
Magnesium (0.5)	100
Magnesium (1.0)	100
Magnesium (2.0)	100
Barium (1.0)	82
Barium (2.0)	80
Cadmium (1.0)	87
Cadmium (2.0)	90
Cobalt (1.0)	90
Cobalt (2.0)	86
Calcium (1.0)	70
Calcium (2.0)	77
Copper (1.0)	20
Copper (2.0)	23
Zinc (1.0)	20
Zinc (2.0)	17

Optimum pH and thermostability

The enzyme had an optimum activity at pH 8.0, and 30% of its activity was lost at pH 9.0 or pH 7.0. Its optimum temperature was at 30°C. Upon the incubation of the enzyme for 15 min at 35, 40, 45 and 50°C, the enzyme lost 60, 70, 90 and 98% of its activity, respectively. The addition of 20% glycerol, 5 mM B-mercaptoethanol, Mg^{2+} or any other divalent ion, and DL-succinate (separately or together) did not stop the reduction of the activity. All signs of activity disappeared at 55°C. The enzyme remained stable at -20°C for one month.

Divalent metals effects

The enzyme was dependent on Mn^{+2} or Mg^{+2} but other divalent cations (such as barium, cadmium, cobalt and calcium) would activate it at various degrees. Copper and Zinc would inhibit the enzyme (Table 2).

Estimation of molecular mass

The enzyme is a dimer with a molecular mass of 220 kD as was suggested by Sephadex G-200 column (protein markers were loaded on the column separately). The purified enzyme was electrophoresed on SDS-PAGE and several bands were extending from top to bottom. The $NADP^+$-IDH activity was identified by running a separate lane with pooled active fractions from Sephadex G-200 (Figure 2) as described in this study's 'methods'. By comparing the positions, it became clear that very faint double bands were representing the enzyme with a molecular mass of 240kD.

K_m values

The calculated K_m values for ADP and isocitrate from the linear Lineweaver-Burk plots (Lineweaver and Burk, 1934) were 1 and 2·5 mM, respectively (data not shown).

The enzyme was strictly specific for NADP(H) and no activity was detected with NAD(H). No reaction was observed with D-glutamate, L-glutamine or DL-2-hydroxyglutarate in the biosynthetic reaction.

Inhibition of the enzyme by its antibody

The inhibition of the enzyme activity increased with increasing concentration of the antibody (Figure 3A). Amount of antibodies, which were greater than 300 µg, almost completely inhibited the enzyme activity. A value of 0·01 was obtained for the intercept on the α-axis in the plot of reciprocal of percent inhibition against

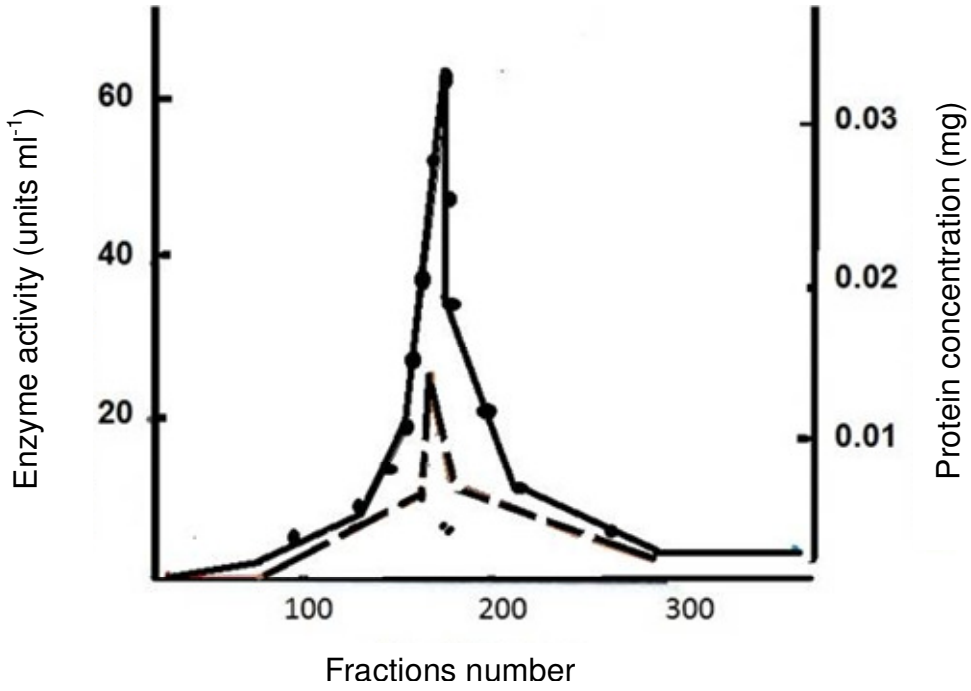

Figure 1. The elution profile of NADP-IDH activity from Sephadex G-200. Enzyme activity (units ml^{-1} (—), protein concentration (mg) (- - - -) as described in this study's methods.

reciprocal of 10 antibody concentration (Figure 3B), indicating complete inhibition. Values greater than 0·01 indicate partial inhibition.

Protection of the enzyme activity against inhibition by antibody

When isocitrate (or ADP) was pre-incubated separately with the enzyme, it was then incubated with the antibody; none of them could provide enough protection against inhibition.

Isocitrate (in the presence of ADP) could provide a partial protection against inhibition by antibody (Table 3), and 90% protection, in the presence of Mg^{+2} or Mn^{+2}, was achieved. The enzyme pre-treated with isocitrate and ADP (in the presence of Mg^{+2} or Mn^{+2}) was designated as the "protected enzyme ".

The protected enzyme was pre-incubated with varying amounts of antibody (Figure 3B). The maximum amount of inhibition of the protected enzyme was only 70%. The minimum concentration of isocitrate and ADP was required for 90% protection of the enzyme. If the concentrations of ADP (0·2 mM) and isocitrate (0.5 mM) were less than their Km values (1 and 2·5 mM, respectively), they were be able to maximally protect the enzyme against inhibition by the antibody. The protection could be due to a conformational change in the enzyme resulting in decreased ability of the antibody to interact with the enzyme or due to the possibility that antigen-antibody complex might be partially active. It was therefore of interest to determine the kinetic parameters for the three forms of the enzyme: the purified enzyme, the protected enzyme and be inhibited enzyme.

Comparison of kinetic parameters of the native, protected and the inhibited enzyme

The K_m values of the three forms of the enzyme were determined. The concentrations of the antibody were chosen such that the "protected" and the inhibited enzymes had identical Vmax values.

The Lineweaver-Burk plots for isocitrate and ADP are shown in Figures 4 and 5, respectively. The K_m values of the protected enzyme had the same K_m values for the substrates as those for the purified enzyme. The specificity of the antiserum was assayed by its ability to decrease the NADP-IDH activity present in Sephadex G-200 fractions (Figure 1). The activity decreased when the extract was incubated with increasing amount of antiserum obtained. The antiserum had the ability to recognize the enzyme.

Effect of mono- and trinucleotides phosphates

Mononucleotides phosphates had no effect on the enzyme but all trinucleotides phosphates (especially ATP) inhibited the enzyme.

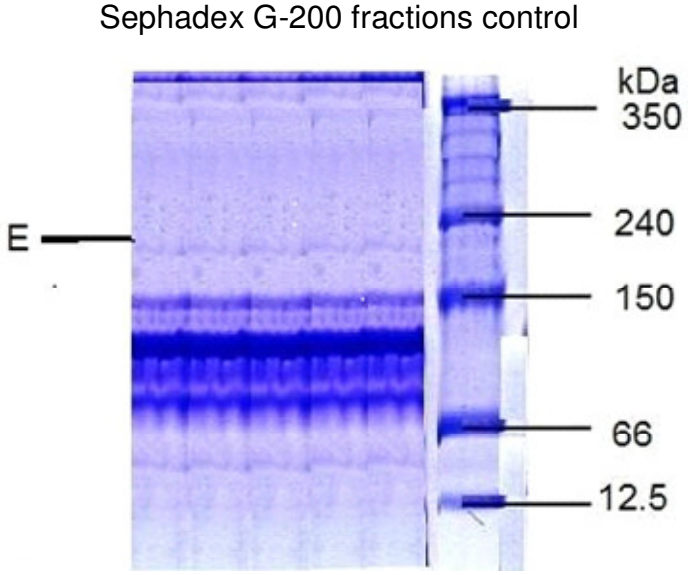

Figure 2. SDS-polyacrylamide gel with the pooled peaked fractions from Sephadex G-200. The bands show NADP-IDH activity.

DISCUSSION

The purification methods allowed the obtaining of a preparation of a high purity, since most contaminated proteins were removed. The enzyme had an optimum pH of 8.0. and 30% of the activity was lost at pH 9.0 or pH 7.0 which in contrast with several researches that claimed that the enzyme had a wide range of pH (6.0 to 9.0), but the thermostability of the purified enzyme agreed with what other researchers found to a great extent (Plaut and Gabriel, 1983; Loftus et al., 1994; Nekrutenko et al., 1998; Contreras-Shannon and McAlister-Henn, 2004).

The shape of the peak from Sephadex G-200 suggested that the enzyme consisted of two subunits which were confirmed by SDS-PAGE that showed two very faint bands of protein with $NADP^+$-IDH activities. It was judged that the enzyme has a molecular mass of 220 kDa by using gel filtration and 240kDa by using SDS-PAGE, respectively. This deviation is expected since SDS-PAGE had its limitations and most proteins would give estimates within a few percentage of their actual weight (Sallantin et al., 1990). Therefore, the molecular mass was within the narrow range of 220 to 240 kDa. These results agreed with several results obtained by the previous researchers, though a little bit higher. Tobacco's NADP-IDH had two forms that have molecular mass of 117,000 and 136,000 kDa, respectively (Gálvez et al., 1996), while $NADP^+$-IDH from cauliflower and yeast had native molecular masses of approximately 100,000 **(**Gallardo et al., 1995) and 80 KDa (Cupp and McAlister-Henn, 1991), respectively.

Several purified $NADP^+$-IDHs had higher molecular masses such as angiosperm which was a dimer of a native molecular mass of 410 kDa **(**Nichols et al., 1995), tetramer with molecular weight of 320 kDa in red kidney beans (Shikara, 2011), and octamer with 320 kDa in Chlamydomona (Martínez-Rivas and Vega, 1998).

Only two $NADP^+$-IDHs were suggested as monomers: The first was from *Crithidia fasciculate* with a molecular weight of 105 kDa (Morris and Weber, 1975) and the second was from pea mitochondria with a molecular mass of 470 kDa (McIntosh, 1997).

The enzyme activity was completely inhibited by its antibody, even when it could be protected partially by pre-incubating it with isocitrate or ADP, and protected completely by pre-incubating it with a mixture of isocitrate and ADP in the presence of Mg^{2+} and Mn^{2+}.

The concentration of antibody required for 50% inhibition of enzyme activity was 0.17 uM for the purified enzyme and 0.78 uM for the protected enzyme. In the presence of infinite concentrations of antibody, the purified enzyme became inactive, while the protected enzyme retained some of its activity (Figure 3b).

The activity of the enzyme might be due to its decreased affinity for the antibody or the stabilization of the active site of the protected enzyme due to the altered conformation of the antigenic determinant due to the binding of isocitrate and ADP to the enzyme. This protection of the activity of the enzyme by substrates against inhibition by antibody was noticed by several researchers (Smyth, 2004; Diaz and Casali, 2002; Mian et al., 1991; Picton et al., 2005).

The heterogeneous population of antibodies contained in the enzyme's antiserum have differential affinities for their binding sites on the enzyme, and until all the antigenic determinants were masked, the enzyme activity persisted which might explain the change in Km values of

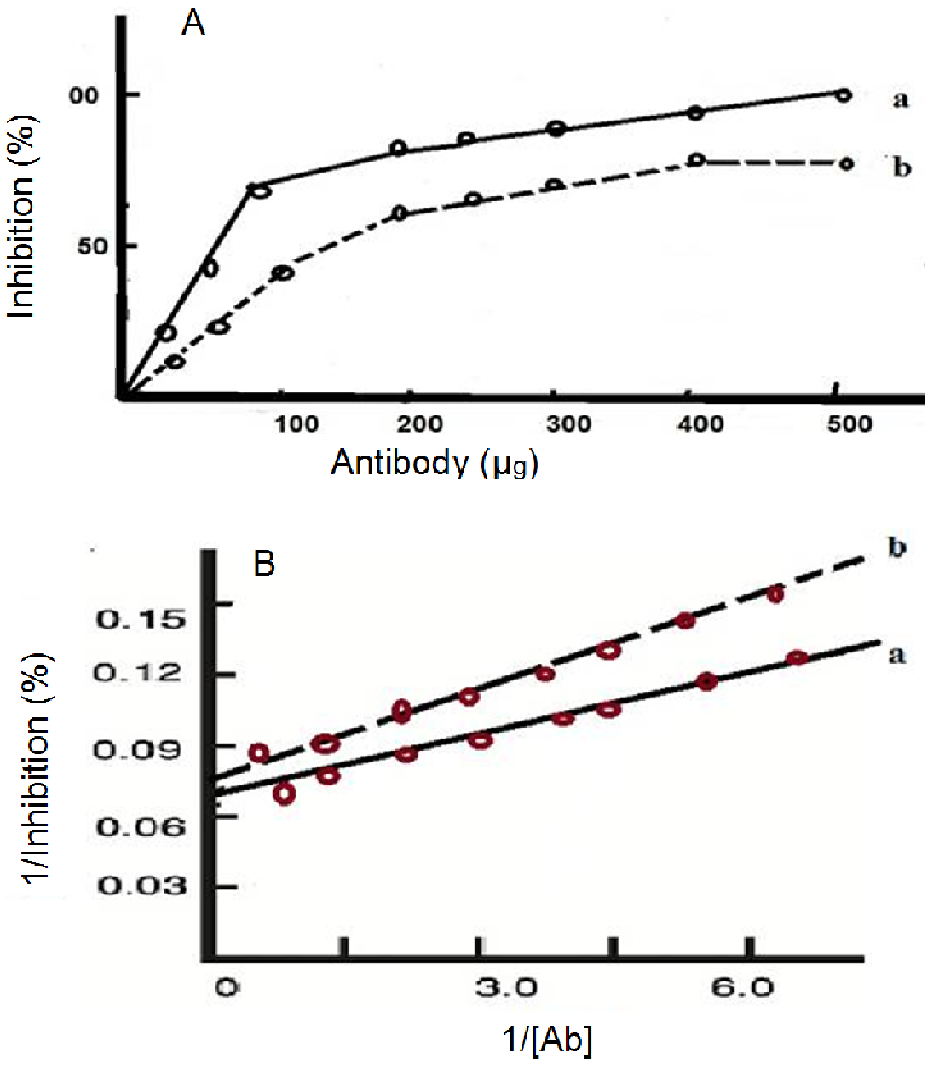

Figure 3. (A)Effect of antibody on the activity of the purified enzyme (-) and the protected enzyme (---). (a) The enzyme (10 ug) was preincubated with varying amounts of globulin fraction for 5 min at 30℃ and the reaction was started by the addition of the saturating concentrations of the substrates (Isocitrate,100 mM; and ADP, 10 mM). The velocity was determined by estimating the antibody formed in the reaction mixture. (b) The enzyme (10 µg) was preincubated with saturating concentrations of isocitrate(100 mM) and ADP(10 mM) for 5 min at 30℃ which was followed by the second preincubation with varying amounts of globulin fraction for 5 min at 30℃. The velocity was determined by estimating the antibody formed in the reaction mixture. **B.** Double reciprocal plot of percent inhibition of the enzyme activity versus the concentration of antibody.

the inhibited enzyme for the substrates. The binding of the antibody at an antigenic determinant close to the active site prevented the substrate interaction, causing inhibition. Alternatively, when some of the binding sites were not occupied, the resulting enzyme-antibody complexes might be active with altered affinities for the substrates. When sub-saturating concentrations of the antibody were added to the enzyme, prior to the addition of the substrates, the mixture consisted of completely inactive enzyme-antibody complex, partially active enzyme-antibody complex and the unreacted native enzyme (Abiko et al., 2005; Lancien et al., 1998)

The partially active enzyme-antibody complex might be responsible for the altered Km values due to its decreased affinities for the substrates. The absence of any change in the Km value of the protected enzyme for the substrates might be due to a substrate-induced conformational change in the enzyme affecting the enzyme-antibody complex. The resulting enzyme-antibody complex could be partially active with the Km

Table 3. Minimum concentration of isocitrate (or ADP) required for protection of the enzyme against inhibition by antibody.

Preincubation with	% inhibition
None*	0
Antibody**	80
Isocitrate (1 mM) + antibody***	60
Isocitrate (1 mM) + Mg + antibody	60
Isocitrate (0.05 mM) + ADP (0.2 mM) + Mg + antibody	15
Isocitrate (0.1 mM) + ADP-Mg (0.5 mM) + antibody	40
Isocitrate (0.5 mM) + ADP-Mg (0.5 mM) + antibody	20
Isocitrate (1 mM)+ ADP-Mg (0.5 mM) + antibody	15
Isocitrate (2 mM) + ADP-Mg (0.5 mM) + antibody	15
ADP + (0.05 mM) + antibody	65
ADP + (0.05 mM) + Mg + antibody	63
Isocitrate (0.05 mM) + ADP-Mg (0.05 mM) + antibody	30
Isocitrate (0.05 mM) + ADP-Mg (0.1 mM) + antibody	30
Isocitrate (0.05 mM) + ADP-Mg (0.2 mM) + antibody	15
Isocitrate (0.05 mM) + ADP-Mg (0.5 mM) + antibody	15

*Enzyme activity in the absence of antibody, normalized to 100. **The enzyme preincubated with the antibody (100 µg) for 2 min at 30°C prior to a second preincubation with the substrates.***The enzyme was preincubated witheither isocitrate (or ADP) for 2 min at 30°C, followed by a second preincubation with the antibody (50 µg). The enzyme activity was measured at saturating concentrations of isocitrate (or ADP).

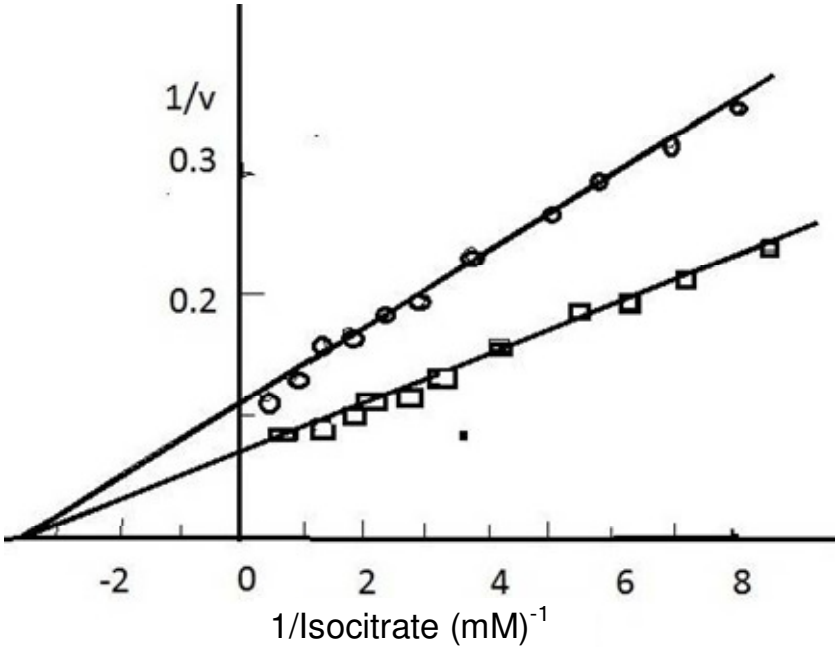

Figure 4. Double reciprocal plot of velocity versus isocitrate concentration. The purified enzyme (- - -) and the protected enzyme (-). The enzyme was pre-incubated with varied amount of isocitrate and 10 mM ADP prior to the addition of antibody (50 µg). The enzyme was pre-incubated with the antibody for 2 min at 30°C followed by a second pre-incubation with isocitrate concentrations.

value similar to the native enzyme. Alternatively, one of antibody species could interact with the altered enzyme conformation to yield a completely active enzyme-antibody complex, while the rest of the reactions led to

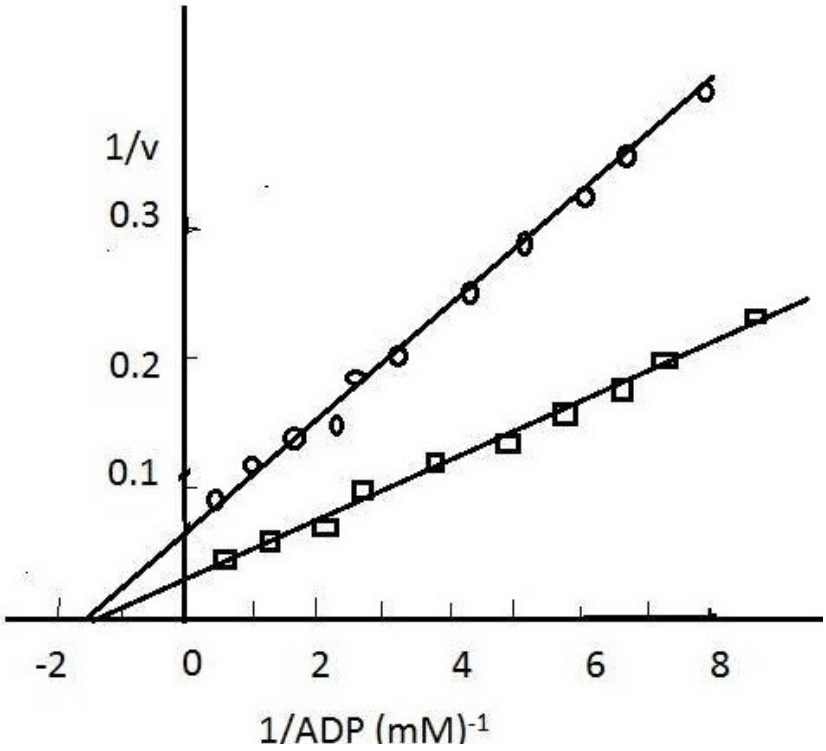

Figure 5. Double reciprocal plot of velocity versus ADP concentration. The purified enzyme (- - -) and the protected enzyme (-). The enzyme was pre-incubated with varied amount of ADP and 5 mM isocitrate prior to the addition of antibody (50 µg). The enzyme was pre-incubated with the antibody for 2 min at 30°C followed by a second pre-incubation with ADP concentrations.

inactive complexes. It is clear that the binding of isocitrate and ADP to the enzyme induced conformational changes and the antigen-antibody reactions can be employed to probe these changes as was done by other researchers (Weiss et al., 2000; Nekrutenko et al., 1998), so further studies are needed to understand the mechanism fully. Further studies are required to clarify the decrease in Vmax that was observed without a change in Km.

ATP and other trinucleotide phosphates inhibited the activity of NADP-IDH competitively; even though some researchers found that it activated the enzyme (Qi et al., 2008).

The effect of AMP was questioned. Several researchers (Gabriel and Gersten, 1992; Plaut and Gabriel, 1983; Hathaway and Atkinson, 1983; Lin and McAlister-Henn, 2003) claimed that the AMP and other mononucleotides phosphates activate the enzyme, but the authors could not prove such claim.

Mg^{2+} and Mn^{2+} (but not other cations) could act as chelating agents to the enzyme at concentrations from 5 mM. ADP and isocitrate enhanced the activity of the enzyme, while a number of substrates and some intermediates of the TCA cycle (such as glutamate, fumarate, pyruvate, succinate and citrate) showed no stimulating effect on the enzyme. This phenomenon was noticed by the present researchers as well by other researchers (Oliver and McIntosh, 1995; Zhao and McAlister-Henn, 1997; Shikara, 2011; Plaut and Gabriel, 1983; Chen and Gadal, 1990).

AMP could not activated NADP-IDH enzyme, even when some researchers claimed the opposite (Hathaway and Atkinson, 1963; Plaut and Gabriel, 1983). The authors believed that such activation was of doubtful physiological significance since the concentration required differed by several orders of magnitude from that present in tissues.

In terms of physiological role of the kidney, it was significant that isocitrate influenced the value of km of NADP-IDH at limiting substrate and coenzyme concentrations and vice versa. In addition, ADP was lowered both constants (Weiss et al., 2000).

The activity of $NADP^{+}$-IDH was influenced by a number of factors such as interacting substrates isocitrate and NADF, and the divalent activators (Mn^{+2} or Mg^{+2}). ADP influenced the enzyme also, while ATP (and NADH) inhibited it competitively. The intermediates of TCA had no effect which suggests that lack of reversibility of the reaction might be partly due to poor binding of TCA intermediates to the enzyme in addition to the product inhibition by ATP (or NADPH). Future studies are aiming to the cloning of the gene and required to understand the regulatory mechanism of NADP-IDH.

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